



**TWO-STAGE BIOHYDROGEN AND BIOMETHANE PRODUCTION
USING GRANULAR ACTIVATED CARBON IMMOBILIZED BEADS AS
SUPPORT CARRIER**

By

TAN JIA BOH

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Master of Science.**

September 2023

FK 2023 18

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Master of Science.

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September 2023

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Biohydrogen and biomethane gases are popular alternatives to renewable energy as the production of these green gases can be generated using biomass. The first objective of this study was to assess the microbial growth of biofilm formation on granular activated carbon (GAC) in two-stage biohydrogen and biomethane production. The microbial growth of biofilm formation was studied based on 5 different inoculum percentages (v/v) of cow dung (2.5%, 5%, 7.5%, 10%, and 12.5%) and 5 different ratios of glucose concentration (2.5g/L, 5g/L, 7.5g/L, 10g/L, and 12.5g/L) added into the system using batch reactor. Based on the modified Gompertz modeling, it is shown that 15% inoculum has the highest biohydrogen and biomethane production rate at 0.88mmolH₂/L.h and 43.87mmolCH₄/L.d. The 10g/L of glucose addition has also contributed to the maximum specific growth rate of 0.99h⁻¹ for biohydrogen and 0.85h⁻¹ for biomethane based on Monod modeling. The second objective was to optimize and characterize the influence of alginate and chitosan on GAC immobilized beads using response surface methodology (RSM). Three main factors in the composite central design (CCD) were the concentration of GAC, the concentration of alginate-chitosan, and the drying method while the three responses were the bead diameter, stability, and swelling degree. Alginate and chitosan beads with desirability of 90% and 100% each contained 0.50 and 0.52g/L of GAC, 1.23 and 1.52% of alginate-chitosan and freeze-dried. Immobilized alginate and chitosan beads formed using the optimum factors were then added into the batch reactor for biohydrogen and biomethane production. When fitted into the modified Gompertz equation, biohydrogen production was higher when chitosan beads were added into the reactor, where H_m was 76.17mL with a maximum rate (R_m) of 1.92mL/h. Similar to biohydrogen production, biomethane production, M_m is higher by adding chitosan beads into the reactor (195.25mL) at a rate of 7.31mL/d. The third objective was to assess the energy recovery using GAC immobilized beads at different bioreactor operating conditions. Based on the techno-economic assessment, when the inflow rate of cow dung was 55L/day.cow, the working volume of the bioreactor

was 132.00m³. Lab scale bioreactor fermentation was carried out in continuous batch showed the maximum biogas production was 934.54mL/gVS with a rate of 60.55mL/gVS.d and a lagged phase of 1.04 day. When converted to electricity, an estimated 14,681kWh of electricity can be generated daily, contributing to RM154,158.90 of revenue. The payback period of this system is 6 years with an ROI of 47.00%. Overall, this study has successfully evaluated the two-stage biohydrogen and biomethane production from cow dung by optimizing alginate-chitosan immobilized on GAC and estimated the energy recovery of anaerobic digester in the industry.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains.

**PENGHASILAN BIOHIDROGEN DAN BIOMETAN SECARA DUA
PERINGKAT MENGGUNAKAN MANIK TIDAK MOBIL KARBON AKTIF
BERBURAN SEBAGAI PEMBAWA SOKONGAN**

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Gas biohidrogen dan biometana adalah alternatif popular kepada tenaga boleh diperbaharui kerana pengeluaran gas hijau ini boleh dijana menggunakan biojisim. Objektif pertama adalah untuk menilai pertumbuhan mikrob pembentukan biofilm pada karbon teraktif berbutir (GAC) dalam pengeluaran biohidrogen dan biometana secara dua peringkat. Pertumbuhan mikrob pembentukan biofilm dikaji berdasarkan 5 peratusan inokulum berbeza (v/v) tahi lembu (2.5%, 5%, 7.5%, 10%, dan 12.5%) dan 5 nisbah kepekatan glukosa yang berbeza (2.5g/L, 5g/L, 7.5g/L, 10g/L dan 12.5g/L) ditambah ke dalam sistem menggunakan reaktor kelompok. Berdasarkan pemodelan Gompertz yang diubah suai, adalah ditunjukkan bahawa 15% inokulum mempunyai kadar pengeluaran biohidrogen dan biometana tertinggi pada 0.88mmolH₂/L.h dan 43.87mmolCH₄/L.d. Penambahan glukosa 10g/L juga telah menyumbang kepada kadar pertumbuhan spesifik maksimum 0.99j⁻¹ untuk biohidrogen dan 0.85j⁻¹ untuk biometana berdasarkan pemodelan Monod. Objektif kedua adalah untuk mengoptimumkan dan mencirikan pengaruh alginat dan kitosan ke atas manik tak bergerak GAC menggunakan kaedah permukaan tindak balas (RSM). Tiga faktor utama dalam reka bentuk pusat komposit (CCD) ialah kepekatan GAC, kepekatan alginat-kitosan, dan kaedah pengeringan manakala tiga tindak balas adalah diameter manik, kestabilan, dan darjah bengkak. Manik alginat dan kitosan dengan kemahuan 90% dan 100% setiap satu mengandungi 0.50 dan 0.52g/L GAC, 1.23 dan 1.52% alginat-kitosan dan kering beku. Manik alginat dan kitosan tidak bergerak yang dibentuk menggunakan faktor optimum kemudiannya ditambah ke dalam reaktor kelompok untuk penghasilan biohidrogen dan biometana. Apabila dipasang ke dalam persamaan Gompertz yang diubah suai, pengeluaran biohidrogen adalah lebih tinggi apabila penapaian menambahkan manik kitosan ke dalam reaktor di mana Hm ialah 76.17mL dengan kadar maksimum (Rm) 1.92mL/j. Sama seperti pengeluaran biohidrogen, penghasilan biometana, Mm adalah lebih tinggi dengan menambahkan manik kitosan ke dalam reaktor (195.25mL) pada kadar 7.31mL/d. Objektif ketiga adalah untuk menilai pemulihan tenaga menggunakan

manik tidak bergerak GAC pada keadaan operasi bioreaktor yang berbeza. Berdasarkan penilaian tekno-ekonomi, apabila kadar aliran masuk tahi lembu adalah 55L/hari.lembu, isipadu kerja bioreaktor ialah 132.00m³. Penapaian bioreaktor skala makmal dijalankan dalam kelompok berterusan menunjukkan pengeluaran biogas maksimum ialah 934.54mL/gVS dengan kadar 60.55mL/gVS.d dan fasa tertinggal 1.04 hari. Apabila ditukar kepada elektrik, anggaran 14,681kWj tenaga elektrik boleh dijana setiap hari, menyumbang kepada RM154,158.90 hasil. Tempoh bayaran balik sistem ini ialah 6 tahun dengan ROI sebanyak 47.00%. Secara keseluruhannya, kajian ini telah berjaya menilai pengeluaran biohidrogen dan biometana dua peringkat daripada tahi lembu dengan mengoptimumkan alginat-kitosan yang tidak bergerak pada GAC dan menganggarkan pemulihan tenaga penghadam anaerobik dalam industri.



ACKNOWLEDGEMENTS

I would like to give my biggest thanks to dear God as He will be always giving me the strength that I needed to finish this project successfully.

This process would not have been done in another way if it was not for the kindness and guidance of those who has been lending me their helping hands. Especially my supervisor, Dr. Nur Syakina Jamali from Chemical and Environmental Engineering Department, Universiti Putra Malaysia. She has shown me immense patience and wisdom through her constructive criticism and valuable suggestion throughout this final year project. I would also like to thank two of my co-supervisors, Prof. Dr. Zurina Zainal Abedin and Assoc. Prof. Dr. Ir. Hasfalina Che Man for guiding me throughout my time here in UPM with knowledge that has contributed immensely in the project.

I would like to thank all the assistance that I have received from the laboratory assistance staffs who were selflessly sharing the knowledge on conducting lab works and tests.

My family plays a crucial role in supporting me in all ways possible so that I could continue this project. My partner in life has also been very encouraging with words of affirmation.

Last but not least, my sincere thanks to all my friends who have given me support and helps of any kinds along the route to finish this project. Without the help from them, I would not be able to finish this on time and accomplish this undertaking.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

GAC	Granular Activated Carbon
RSM	Response Surface Methodology
VFA	Volatile Fatty Acids
COD	Chemical Oxygen Demand
VS	Volatile Solid
CH ₄	Methane
CO ₂	Carbon Dioxide
H ₂ O	Water
GHG	Greenhouse Gas
HRT	Hydraulic Retention Time
OLR	Organic Loading Rate
CPH	Combined Power and Heat
V _c	Volume of Gas Collecting Chamber
V _{gs}	Volume of Gas Storage Chamber
V _f	Volume of Defermentation Chamber
V _s	Volume of Sludge Layer
NPV	Net Present Value
IRR	Internal Return Rate
PI	Profitability Index
ARR	Average Return Rate
PP	Payback Period
UPM	University Putra Malaysia
°C	degree Celcius
SBR	Sequenceing Batch Reactor

TVS	Total Volatile Solids
TC	Total Carbohydrates
GC	Gas Chromatography
TCD	Thermal Conductivity
CCD	Central Composite Design
HPR	Hydrogen Production Rate
HY	Hydrogen Yield
MPR	Methane Production Rate
MY	Methane Yield
PCE	Purchase Cost of Equipment

CHAPTER 1

INTRODUCTION

1.1 Research Background

Fossil fuel has been the source of energy generation for centuries. The dependency on such usage is so vast in the world that it is almost irreplaceable. Even though fossil fuel brings energy to our daily lives, it has also brought us significant problems as the combusting of fossil fuel has directed to a bigger problem: global warming. The emission of greenhouse gases, carbon dioxide, is released into the atmosphere tremendously. According to The National Oceanic and Atmospheric Administration of The USA, the current global average concentration of CO₂ in the atmosphere is 421 ppm as of May 2022 (0.04%). Hence, researchers from all over the world are looking for solutions to combat the problem. The best alternative would be to use renewable energy. Renewable energy can come from wind, hydro, solar and others. One of the most popular is biomass. Biomass is suitable to be adapted in tropical countries like Malaysia with ongoing big agricultural industries (Norfadilah et al., 2016).

With the abundance of biomass, this seems to be a promising source to produce renewable energy using various methods (Sharma et al., 2018). Biomass contributes approximately 12% of the world's energy supply and is especially significant in developing countries with 40-50% of the energy supply. Researchers have now focused more on the production of biofuels by utilising biomass derived from waste from human activities, animal farming and agricultural (Khan et al., 2018). The biomass waste collected can be used as source of anaerobic fermentation to produce biogases because the organic wastes contained high microorganism activity that could benefit to the biogases production. A wide range of hydrogen and methane producing microorganisms are present in biomass like cow dung (Phowan & Danvirutai, 2014).

The biohydrogen and biomethane production processes can be conducted in a two-stage plant which is more effective. The process of anaerobic digestion takes place through four successive stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In this first stream, hydrogen and carbon dioxide are produced and collected. The resulting digestate is then introduced into the methanogenic reactor for the second production, biomethane. This is a faster and more efficient way of gas production where the concentration of methane is as high as 85%(R. Li et al., 2009). The hydrogen produced in the first stream can be collected and directly used in combustion engines as well as in fuel cells after purification (Bharathiraja et al., 2016). The biomethane can be

used as a natural gas for cooking purpose as well as automotive sector. For biohydrogen and biomethane plants to operate, a wide choice of sources can be used as long as it is high in anaerobic microbes to start up the anaerobic system. Suspended culture is usually the preferred way for anaerobic fermentation to be carried out but researchers are facing issues such as low biogas yield due to the unstable condition in the reactor causing the microorganisms in the reactor to undergo high hydraulic stress. Therefore, immobilization of cells is introduced to the field (Frasconi et al., 2013).

Immobilization cells can be used to enhance the performance of biogas production as compared to suspended cells. There are various methods of immobilization including adsorption, entrapment and encapsulation. For adsorption, there is a physical attachment of bacteria cells onto the surface of the carrier. Biochar is widely used for this purpose as it has non-reactive characteristics. Activated carbon does not undergo any chemical reaction during the fermentation process. With its large surface area, it can hold onto more microorganisms hence increase the population of the bacteria which aids in the production of hydrogen and methane gases (Kumar et al., 2016). Entrapment on the other hand, involving the entrapment of enzyme by a cross-linked polymer within the polymeric material such as sodium alginate. Research reported the stability of immobilized cell could be enhanced via the fusion of microbial cells into rigid network of polymer due to the mechanical firmness and good porosity of carrier thus providing good anaerobic conditions to microorganism during the hydrogen and methane production (Obia et al., 2016).

1.2 Problem Statement

Biohydrogen and biomethane gases have gained attention in the research field as a green energy source (Bharathiraja et al., 2016). During the fermentation process, researchers always face issues such as low biogas production that are related to the inoculum and substrate of the system. The volume of inoculum and glucose concentration in the system could affect the biogas production. These two factors are correlated as the inoculum is the source of microorganisms while glucose acts as the food source to them. Without the right proportion of these two factors, microorganisms could not perform optimally causing a deficit biogas issue.

Most researchers have produced biogases using the anaerobic fermentation with suspended culture. They encountered problems such as low bacterial biomass concentration in the reactor over time when old substrate is removed and replaced with new ones. During the process of feeding new substrate into the reactor, microorganisms often experienced washed-out phenomenon due to the high hydraulic stresses and low hydraulic retention time, causing low bacterial concentration in the system. Therefore, leads to the low molar of hydrogen yield and low conversion of substrate. By introducing attached biofilm on the microbial

support carrier has shown significant increment in biogases production, resulting in a better production yield as the cell density has increased within the system. Granular Activated Carbon (GAC) as a support carrier have been thoroughly documented as a support matrix in anaerobic dark fermentation (Syakina & Jahim, 2016). GAC is said to have inert properties towards any chemical reaction hence the addition of such carrier will not disrupt the reactions inside the system. GAC is also known to have high surface area with a porous structure that is most suitable to facilitate the colonization of cells hence increasing the cell density in the reactor (Che Zuhair et al., 2018).

Even so, the physical attachment that is formed due to adsorption of cells on the surface of GAC has low mechanical strength causing the microorganisms to have a hard time to withstand the harsh conditions such as low hydraulic retention time during the continuous fermentation process. Under long term operation of sequential cycles, the cells could easily detach from the GAC as the physical attachment of the cells are not supported by any chemical linkage. Once the cells slowly detached and died off in the reactor, population of the hydrogen and methane producer decreases and causing a direct impact on the production of biohydrogen and biomethane gases. (Badiei et al., 2011). To overcome the problem of unstable biofilm formation, in-depth knowledge of microbial growth and innovative solutions are required. Thus, the GAC attached biofilm entrapped with alginate and chitosan, respectively. The entrapment process formed a chemical linkage between the cells and the alginate/chitosan materials hence, making the cells condition to be more stable as detachment would not occur easily, thus improving the matrix support's mechanical properties. Strong ionic interaction is formed in the beads between the entrapment material and the cells attached on GAC (P. Sekoai et al., 2016). This entrapment process will improve the microbial density as it forms a protection outer layer over the microorganisms on the GAC hence, results in sustaining high population of microorganisms in the beads which then gives a positive impact on the biohydrogen and biomethane gases production.

For countries that rely on agricultural activities, the ever-abundant cow manure wastewater is usually be seen as animal waste that contributes no benefits to the economy. In fact, this biomass has the potential in generating biogas as renewable energy while treating it (Damayanti et al., 2020). However, for the aims of creating useful commodities from cow manure, there are fewer literature design considerations of an anaerobic digester hence farmers have no interest in converting waste-to-energy. Many factors such as the bioreactor size and profit of the system will need to be taken into consideration when constructing an anaerobic digester especially for those without any background in this field. A Microsoft Excel assessment for biogas production using cow manure wastewater is wished to be outlined as one of the easy approach modelling for the industry aiding not only the farmers but also the economy of the country towards a more sustainable future (Mainardis et al., 2019).

1.3 Research Objectives

The main objective of this research is to study the development of granular activated carbon-alginate and granular activated carbon-chitosan based immobilized beads for two-stage biohydrogen and biomethane production. To achieve this, the following are the objectives of this study along the course of research:

- i. Objective 1: To assess the microbial growth of biofilm formation on granular activated carbon in two stage biohydrogen and biomethane production.
- ii. Objective 2: To optimize and characterize the influence of alginate and chitosan on GAC immobilized beads using response surface methodology.
- iii. Objective 3: To assess the energy recovery using GAC immobilized beads at different bioreactor operating condition.

1.4 Scope and Limitations of Research

In this research, two-stage anaerobic dark fermentation was used for biohydrogen and biomethane production. Anaerobic dark fermentation does not require sunlight and oxygen and usually applicable on many types of biomass such as animal waste, food waste and other organic wastes (Lin et al., 2018). During the first stage of fermentation, biohydrogen gas was produced while the second stage produces biomethane gas.

In this research, cow manure is used as a source of inoculum because it is a cheap and readily available bio-resource. Biohydrogen and biomethane productions using cow manure as feedstock produces a clean and easily accessible biological energy and cleans up the environment simultaneously in a sustainable approach (Perera & Nirmalakhandan, 2011).

The adsorption of GAC attached biofilm is weak in mechanical strength and unstable. Thus, the entrapment method is used for entrapping with the GAC attached biofilm between the carrier materials such as alginate and chitosan. The entrapment is one of the techniques in immobilization methods which is used to enhance the enzyme productivity and microbial cells (P. T. Sekoai et al., 2018).

Alginate is one of the materials used in the entrapment process in this research. Alginate (AL) is a biopolymer extracted from brown seaweeds (Shim et al., 2019). Alginate has many advantages including its availability, non-toxicity and most importantly it can be easily formed into gels which is very suitable to be implemented in this research to entrap the biofilm on GAC. In the presence of divalent cations, such as Ca^{2+} , the forming of a strong interaction between carboxylic groups is present in the structure of the alginic acid (Bt Ibrahim et al.,

2019). The formation of such structure provides a gel like layer which is insoluble in water and irreversible under heat.

On the other hand, another material that is used in this project is chitosan. It is derived from exoskeletons of crustaceans and cell walls of fungi and insects (Mahaninia, 2016). Chitosan can be found in abundance in nature and it has very similar benefits as alginate. Researchers make use of chitosan to process into different products such as gels, membranes, beads and microparticles. The presence of hydroxyl, amino groups or protonated NH_3 makes it easily form a chemical linkage with the cells in the reactor to increase the strength of the cells to sustain in the reactor (Sivagurunathan et al., 2014).

An energy recovering assessment is done by using Microsoft Excel where microbial kinetic studies, bioreactor dimension and a series of economic studies are evaluated. Economic analysis of a small-scale ranch with 2 kW energy generation is done. The economic aspects of the project including converting cow dung into biogas and fertilizer as value-added end products.

CHAPTER 2

LITERATURE REVIEW

2.1 Two-stage Biohydrogen and Biomethane Production

Looking at the current trend nationwide, most of the big industries are looking for a solution to cope with environmental issues especially those involve with reducing emission of greenhouse gases and reliance on fossil fuel to carry out everyday tasks (Khan et al., 2018b). Hence, researchers are working their hands on searching for renewable energy that could replace fossil fuel in the near future. The concern for such issues has been increasing throughout the years and people are starting to focus more on renewable energy. There are different types of natural renewable energy available such as thermal, wind, water and biomass (Reyna-Gómez et al., 2019). From the perspective of socio-economic, biomass stands out among the others. Unlike the wind and water where people need to build huge water dam to generate electricity or big windmill farm to gather wind energy. Biomass is said to be green as it converts agricultural waste into useful products such as biogas (Sarangi & Nanda, 2020).

In the recent years, biohydrogen has emerged out as a clean, carbon neutral and renewable source of energy (A. Singh et al., 2015). This gas has no odour, no taste and bring no harm to all beings as there will be no pollutants being generated when hydrogen undergoes combustion but only produce water as the end product (Khanna & Das, 2013). On the other hand, biomass is known to produce biomethane and to be used for cooking purpose (Ounnar et al., 2012). A better alternative that has gained importance in the production of biogases are biohydrogen and biomethane. Hythane is a combination of hydrogen and methane. Biohydrogen and biomethane gases can be produced through anaerobic fermentation. It seems to be an alternative from single gas production as it has lower free energy and its ability to consume organic substances derived from agricultural waste (Chu & Wang, 2017a). Biohydrogen and biomethane have great potential in producing bioenergy in mass as it is commonly known to produce by utilizing digestible feedstock such as crops and animal waste as long as it provides abundance of microorganisms.

The production of biohydrogen and biomethane can be grouped into two types, single stage and two-stage. In this work, two-stage biohydrogen and biomethane production is more suitable as the inoculum for both reactors are consist of different types of microorganisms in order to produce two types of gases. During the first stage of fermentation where biohydrogen is produced, the inoculum of the first stage reactor undergoes heat-pretreatment to inhibit the pores-forming methane producer. The substrate is fermented to hydrogen and volatile fatty

acids (VFA) (Jomnonkhaow et al., 2021). During this stage of dark fermentation, VFA produced in substrate will then be transferred to another reactor which contains inoculum for methane producer (Z. Liu et al., 2013). The two-stage could increase degradation efficiency and improved negative impacts of inhibitive compounds in feedstock with high yield and purity of products. Other benefits of two-stage production including the increment of net energy balance and stability with better control of acid phase (W. W. Li & Yu, 2011). Two-stage also provides higher organic loading rates while increases specific activity of methanogens leading to an increase in methane production rates and increased overall chemical oxygen demand (COD) and volatile solid (VS) reduction efficiencies compared to one-stage hydrogen or methane fermentation (Ajayi-Banji & Rahman, 2022).

The obvious effects of two different groups of microorganisms, which are acidogens and methanogens result in different physiology, growth kinetics and sensitivity to the environmental condition. Two groups of microorganisms have different requirements to perform optimum in their respective reactors. Hence, acidogens and methanogens are enriched separately in two reactors to optimize their growth based on their proper environmental conditions (Yin et al., 2021).

2.2 Dark Fermentation using Biological Method

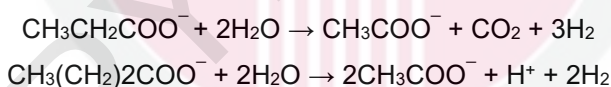
To produce biohydrogen and biomethane based on biomass, there are two approaches which are biological method and thermochemical. For thermochemical approach, it is a process of decomposition by using organic matter, through combustion, pyrolysis and gasification to produce biogases. By using thermochemical method, microorganisms will need to undergo harsh conditions such as high temperature and pressure that cause low energy efficiency because energy consumption for the system to sustain including constant supply of electricity to maintain the high temperature of the reactor is higher than the biogas production (Medina-Morales et al., 2021). Compared to thermochemical methods, the biological approach has researched and verified the biogases production is safer and more economical. Biohydrogen and biomethane that are produced through the biological method such as fermentation not only have lower energy intensive and it can be produced by using renewable sources such as food wastes and other industrial waste (Łukajtis et al., 2018a). Biohydrogen and biomethane productions can be easily carried out as it is normally conducted at ambient temperature and pressure. Different substrates can be used as this method requires substances with high organic carbon source. Many researchers have found out that synthetic wastewater including sucrose, glucose and starch are among the best options to be used as substrates in order to produce biogases (Weiß & Alt, 2017).

For the biological method, hydrogen and methane gases usually being produced by autotrophic and also heterotrophic microorganisms (Hallenbeck & Ghosh, 2009). For autotrophic conversions, the carbon source is inorganic carbon dioxide which is utilized by microalgae. Heterotrophs will form simpler

compounds by converting the organic carbon sources, producing molecular hydrogen. Heterotrophic conversion is simpler and easier to produce hydrogen and methane. Under this conversion, there are mainly two types which are photofermentation and dark fermentation (Ghimire, 2016). Photofermentation requires light energy to initiate the microorganisms and do the reaction, while dark fermentation is carried out in the dark, without light.

Among the technologies for biohydrogen and biomethane production, dark fermentation is one of the best and cost-effective because its conversion efficiency is fast. A vast range of raw materials can be used for the dark fermentation process as the feedstock used can be of any renewable complex waste biomass. From the view of economic interest, the process can produce other valuable platform biochemical (Ghimire et al., 2015). Even so, there are a few obstacles to overcome in using dark fermentation to produce hydrogen and methane gases. Different fermentation pathways will result in different end products (Nasr et al., 2015).

Among the most common pathways for dark fermentation of hydrogen production from glucose are acetate, butyrate, and propionate pathways. Acetate and butyrate produce hydrogen, while propionate consumes hydrogen (Hawkes et al., 2007). The theory of hydrogen yield can be achieved between 2 and 4 moles of hydrogen per mole of glucose is limited. Hence, the metabolism of the culture is directed towards acetate formation with the optimal conditions in order to achieve higher yield of hydrogen (Kongjan et al., 2011). At the same time, the production of propionate needs to be avoided (Hussy et al., 2003). Two thermodynamically unfavourable pathways from butyrate and propionate can occur when the concentration of hydrogen as the product is controlled to its minimum (Stams and Plugge, 2009).



The same pathway can be applied to the acetate pathway as it can be shifted forward when carbon dioxide does not exist in the headspace (Nikolajeva et al., 2015).

The application of such technology still does not have a scaled-up system. The hydrogen produced is still in low amounts, and the feedstock could be in high cost, which causes the production cost to increase proportionally (Ren et al., 2011). Besides that, there are other barriers, including the process conversion efficiencies need to be maximized, and utilization of the by-products. From the perspective of environmental, reducing the water and energy in the input of the process is necessary to reduce the wastage and be more environmentally friendly (Ghimire, 2016). Therefore, immediate attention is focused on the yield of hydrogen and methane by changing the feedstock to low-cost materials, which is waste biomass such as agricultural residues, and organic waste that can be found in municipalities and also industries (Kapdan & Kargi, 2006).

After all, the dark fermentation process does not produce hydrogen and methane only; there are some other by-products as well. These by-products including volatile fatty acids (VFAs), lactic acids, alcohols and un-hydrolyzed residues. These by-products have a lot of uses as they can undergo energy recovery and be used as feedstock for other chemical purposes (Xing et al., 2010).

2.3 Anaerobic Digestion

In anaerobic digestion, diverse bacterial, archaeal microorganisms perform an essential function in the process. There are four stages engaged in anaerobic digestion: hydrolysis, acidogenesis, acetogenesis, and methanogenesis as shown in Figure 2.1 (Nour & Nour, 2017).

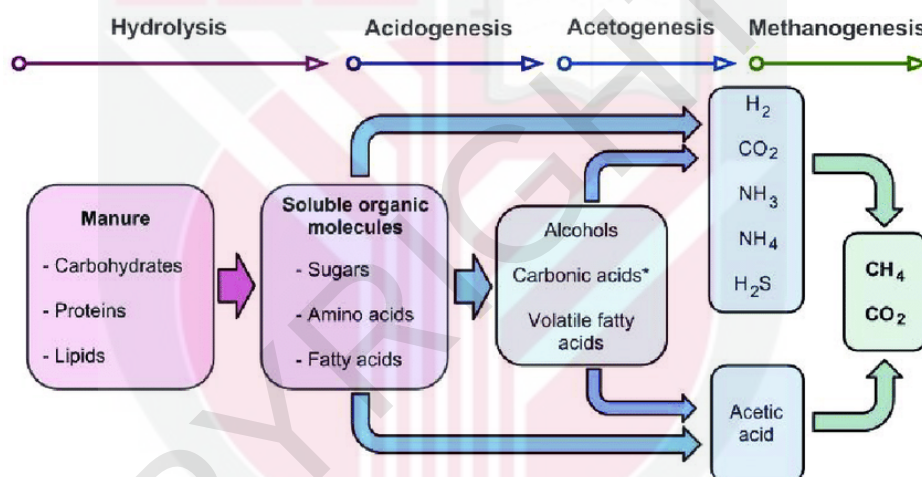


Figure 2.1 : Metabolic pathways of anaerobic digestion
(Source : Nour & Nour, 2017)

Biomass would undergo hydrolysis as the first stage where the biomass containing carbohydrates, proteins and lipids will then be digested into soluble organic molecules such as sugars, amino acids and fatty acids. Then, during acidogenesis, the soluble organic molecules will be further digested into alcohols and volatile fatty acids. At this point, acid content in the digester is high and will further be digested during acetogenesis. The products of acetogenesis process including hydrogen gas which is the main product to be collected. Further process, methanogenesis will produce methane gas and carbon dioxide (Nour & Nour, 2017).

Different types of microbes have a specific role to play and contribute to multiple stages, helping to degrade these complex organic compounds into the final

common goal: biogas, in this case, will be biohydrogen and biomethane. It is possible to generate a few types of biogases, but it relies on those aspects such as the types of substrates used, the conditions of the reactor, and the microbes that are already present in the system itself. Commonly, methane (CH₄), carbon dioxide (CO₂), and water vapor (H₂O) are what the biogas is made up of, while there will be traces of some other gases too. Acidogenesis is the step where hydrogen is produced. Acidogenesis is a fermentation process whereby anaerobic microorganisms utilize soluble compounds as a carbon energy source. These are processed into volatile fatty acids (VFAs), alcohols and biogas (Khan et al., 2018a).

The different microbes' reactions and balances are very complex for anaerobic digestion and affect the end product. Even a minor variation in the environment of fermentation will also cause the microbe population to vary accordingly. Also, each of them has an ideal range of conditions for them to grow. Identifying the factors that will affect the life and growth cycle of the microbial population is essential. Several parameters that could affect anaerobic digestion, such as the form of feedstock used, the inoculum source, the moisture ratio, the temperature of the digestate, the time taken of reaction, the concentration of solids and others where influence the life cycle of the microbial growth. The maximum volume of biogas will be generated using this element to be balanced to the most optimal condition for digestion (Albini et al., 2019).

For dark anaerobic fermentation, two types of culture could be used, which are pure culture and also mixed culture. Examples of some widely used species for hydrogen production are *Clostridium* species, strict anaerobes, gram-positive, rod-shaped, and endospore formers (Wang & Yin, 2017). There are some differences between pure culture and mixed cultures and the main difference will be the end product. In mixed cultures, the type of species within the culture will decide the end product whereas for pure culture the end product can be predicted because it depends on the species type (El-Sayed Nasr et al., 2017). For example, by using only *Clostridium* species as pure culture in anaerobic digestion, *Clostridium* species undergoes non-butyrate pathways during dark fermentation, hence it is predictable to have acetate and ethanol as the by-products (Mao et al., 2015).

In most of the recent studies, mixed culture using anaerobic sludge, composts and municipal sewage sludge is gaining more attention. They are often the preferred choices of inoculum in the fermentation process to produce biohydrogen and biomethane. Compared to pure culture, mixed culture is easier in terms of the operation as it is carried out in non-sterile environments because it can be hardly maintain for pure cultures (Sivagurunathan et al., 2016). Even so, sometimes the hydrogen produced by the bacteria might consist of hydrogen-consuming bacteria that will consume the hydrogen gas again. Hence, pre-treatment is needed to overcome this problem as it can suppress bacteria that consume hydrogen (Albini et al., 2019). During the second stage of biomethane production, pre-treatment is not necessarily needed.

2.4 Cow Manure as Inoculum

In India, a vast variety of materials is used to generate biogas, for example, cow manure, poultry waste, water hyacinth, straw, weeds, leaf, animal excrement, domestic rubbish and industrial solid and liquid waste (Edwin & Sekhar, 2015). Animal manure, such as cow manure, contains a high percentage of manure fibers due to their herbivore diet. Cow manure has enormous potential to become a feedstock for producing value-added chemicals such as the mono-sugars from the fibers (Yu Li, Zhao, et al., 2020). Cow manure is a complicated substance consisting of dissolved and undissolved organic components such as polysaccharides, proteins, lipids and inorganic matter essential for the chemical composition. The characteristics of cow manure is as shown in Table 2.1 (Alkhrissat, 2024).

Table 2.1: Characteristics of cow manure

Parameter	Unit	Cow Manure
Total Solids	gTS/kg wet weight	143.8
Volatile Solids	gVS/kg wet weight	120.7
Total Kjeldahl Nitrogen	gN/kg VS	2.42
Total Phosphorus	gP/kg VS	0.670
Total Ammonia Nitrogen	gN/kg VS	0.233
Total Organic Carbon	%DM	50.2
Chloride Ion	Mg/kg DM	6990
Electrical Conductivity	$\mu\text{S}/\text{cm}$	1226.5
C/N	%	20.74
pH	-	7.16
Elemental Analysis	-	
Carbon	%DM	54.31
Hydrogen	%DM	5.85
Oxygen	%DM	37.04
Nitrogen	%DM	2.8

(Source : Alkhrissat, 2024)

Other than that, using animal manure as feed to produce biogas also contributes to sustainable development, which was promoted in recent years by reducing greenhouse gas (GHG) emissions (Argun et al., 2017). Besides, while reducing the emission of GHG to the environment, manure processing technologies could be helpful to close nutrient cycles while practicing appropriate manure management in intensive livestock production areas (Nazari et al., 2018). This waste emits methane and other toxic gaseous, mainly sulphur, nitrogen, and phosphorus derivatives to the environment. The conventional animal manure used is dairy manure (cows and goats), poultry manure, and swine manure. Lack of land for manure disposal and increasingly stringent requirements demand more effective manure management strategies and alternatives (Rehl & Müller, 2011). Biogas production using animal manure has already been widely installed in many countries across the globe (Foged et al., 2011).

In order to enhance the biohydrogen and biomethane production, a good source of inoculum plays a crucial role. Inoculum provides extra hydrogen and methane-producing microbial consortium which enhances anaerobic digestion. Inoculum also helps in reducing lag time, provides important micronutrients and makes digestion more stable (Bella & Venkateswara Rao, 2022). Relationships between substrates and inoculum based on different aspects such as the amount of inoculum used and rate of biogas production, have always been under investigation. The amount of inoculum in a system can directly cause the inhibitions created due to the presence of organic and inorganic toxicants (Chen et al., 2014). Some studies suggested that lower ratios prevent the induction of enzyme essential while higher ratios could be toxic (Lauzurique et al., 2022). Hence, it is essential to study the inoculum.

In previous studies, it is found that a substrate to inoculum ration of 1.0 $S_{\text{substrate}}/gV_{\text{inoculum}}$ is sufficient to prevent the rapid acidification problem (Elbeshbishy et al., 2012). Another study also reported that methane content increased in biogas with an inoculum-substrate ratio ranging from 1 to 4 (Owamah et al., 2021).

2.5 Immobilized Cell Culture

Two different cell cultures that can produce biohydrogen and biomethane gases through anaerobic dark fermentation are suspended cell and immobilized cell. The suspended cell is more widely used due to its better mass transfer rate between microorganisms and substrate (Chu et al., 2011). But there's a slight problem in maintaining sufficient amount of bacterial population in the bioreactor at low hydraulic retention time (HRT). Therefore, many researches started to come out with another alternative.

Under comparison with suspended cells, it seems like immobilized cells could

enhance the density of cells, and it can be operated stably at low HRT. This is also preferable for big industries to be applied when operated without sterilization (Aminah et al., 2016). One study conducted by Rai et al. has shown that hydrogen production from cheese whey wastewater using dark fermentation with both suspended and immobilized cell cultures have produced different amount of hydrogen yield. By using immobilized cell, it has shown a slightly higher yield in the production of biogas. The immobilized co-culture within the MNP-doped polymers exhibited much higher cumulative methanol of up to 70.74 mmol/L than the production of 22.34 mmol/L by free cells after ten cycles of reuse (Patel et al., 2022).

Many immobilization techniques are available such as surface-attached biofilms, self-flocculated granules and entrapment within porous matrix. Immobilization techniques are illustrated in Figure 2.2 (Lu et al., 2020). The immobilized cell system provides many advantages, such as enhancing biohydrogen and biomethane yield, resistance to cell washout phenomenon and toxic metals. Moreover, the microbial cells are protected against the shear stress by stirring, and it even got possible to recycle back the microorganism in the immobilization cell system (P. T. Sekoai et al., 2018). Mass transfer limitation was more substantial in entrapped calls as reported by Wu & Chang in their research. This is due to the effect on the operation condition and biohydrogen and biomethane production (Wu et al., 2006). Additionally, entrapment of these microorganisms increases the stability of the cells by providing an appropriate growth environment (Gokfiliz & Karapinar, 2017).

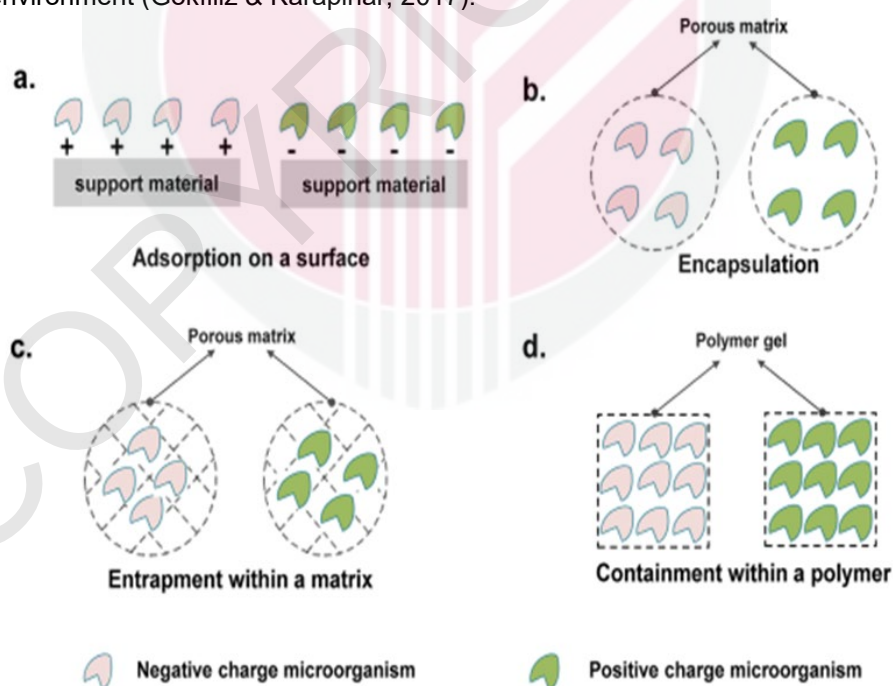


Figure 2.2: Methods of immobilization in biohydrogen production

(Source : Lu et al., 2020)

A suitable carrier for immobilized cells should have both chemical and physical

properties that are optimum in terms of mechanical stability, physical form, porosity and reactivity. To select the best support material for immobilized cell in biohydrogen and biomethane production, several studies have been conducted, including the operation conditions such as substrate concentration, pH and temperature. The porosity characteristic of the carrier materials is emphasized in this matter as it will help prevent excess cell leakage and mass transfer limitations for biohydrogen and biomethane production to be carried out effectively (Hassan et al., 2014). Particle size and particle number density affect the hydrogen production rate as well. High particle number density will cause excessive biofilm growth, causing an interruption in the mass transfer limitation and also troublesome in collecting the gas produced (B. Hu et al., 2007).

Granular Activated Carbon (GAC) is a usual term used to relate materials based on carbon with a high specific surface area, a large permeable design that has been established and many functional groups oxygenated (Quesada et al., 2020). Immobilization technologies are focused primarily on the method of granulation or biofilm attachment (Jamali et al., 2016). It is also well documented as a support matrix to form a biofilm layer on the surface. GAC has good mechanical properties, low toxicity, and inertness sustaining cell viability and enhancing cell density (Damayanti et al., 2020). It is grown freely in the internal porous structure and on the carrier material's circumstances to develop the attached biofilm (C. Zhang et al., 2017).

2.6 Alginate

Alginate (Al) has been frequently used in many industries, including food, biomedicine and textiles. It is originated from salts of alginic acid in brown algae. It consists of a 1,4-linked D-mannuronic and L-guluronic acid of linear heteropolysaccharide isolated from different algae species, which is a good polymer material for the immobilization method (Quesada et al., 2020). Alginate is commonly used with sodium and calcium ions because they can easily create a gelatin capsule that are versatile for encapsulation and entrapment. Figure 2.3 represents alginate schematic diagram, commonly obtained by Na_2CO_3 , encouraging sodium alginate dissolution (Nascimento Júnior et al., 2020).

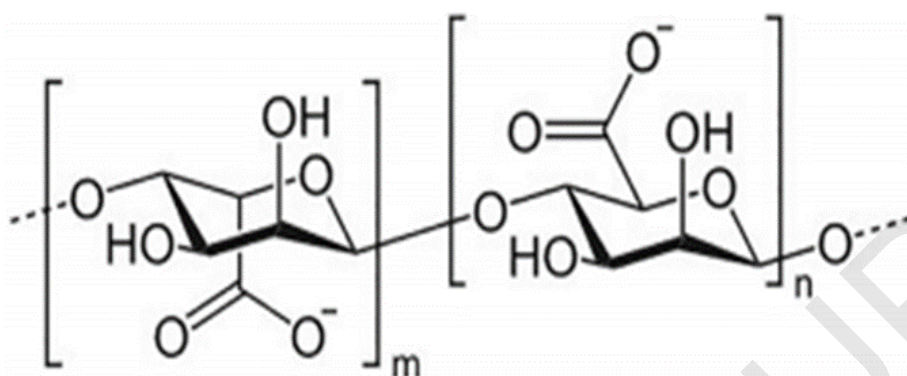


Figure 2.3: Schematic visualization of the primary alginate structure
(Source : Nascimento Júnior et al., 2020)

Among the advantages of using alginate are the availability of this compound, good biocompatibility, non-toxicity, affordable and the capacity to quickly shape gels (Benhouria et al., 2015). The most favorable formation of alginate is usually in the form of beads. In such manner, alginate beads can be further improved by mixing with other hybrid substances. Adding foreign compound increases the porosity, mechanical strength and properties (Jung, Choi, et al., 2016). In the alginate gel matrix, microorganisms are well regulated and are determined to have a pore size of less than 17nm. The small pore size aids in retaining microbial cell at low HRT and harsh anaerobic environment (Jung, Jeong, et al., 2016).

2.7 Chitosan

Chitosan (CH) is a derivative from chitin, a biopolymer mainly from the exoskeleton of crustaceans and cell walls of fungi and insects. It is composed of β (1,4)-linked 2-acetamido-2-deoxy- β -D-glucopyranose and 2-amino-2-deoxy- β -D-glucopyranose as shown in Figure 2.4 (Hamedi et al., 2018).

Scientists have widely used chitosan thanks to its natural abundance, non-toxicity and biodegradability. The versatile usage of chitosan can be seen in both medical and water remediation fields. Like alginate, chitosan are easily processed into gels, membranes, nanofibres, beads, microparticles, nanoparticles, scaffolds, and sponges, allowing a wide range of applications. Chitosan is often known to be one of the best matrix chromatography because it has amino ($-\text{NH}_2$) groups and hydroxyl ($-\text{OH}$) which it provides active reaction for links the enzymes efficiently (Bouyahmed et al., 2018).

Despite having good adsorption ability, chitosan is commonly used as hydrogel beads. This can be justified because the raw flake or powder has a crystallized structure, limiting the adsorption in the amorphous region of the crystals. The hydrogel beads are simple to prepare, and the method enables the expansion of polymer chains, decreases crystallinity and improves the access to internal active sites, all fundamental characteristics for adsorption purposes (Qu & Luo, 2020).

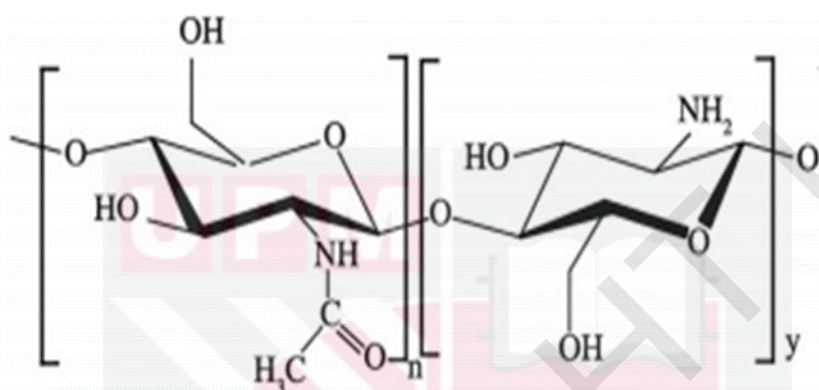


Figure 2.4: Schematic visualization of the primary chitosan structure
(Source : Hamed et al., 2018)

2.8 Kinetic Modelling of Biohydrogen and Biomethane Production

Kinetic modelling is very significant in biohydrogen and biomethane production as a growth correlated product. To determine the effect, relationship, the function of the variable and predict the biohydrogen and biomethane production during fermentation, different models can be tested. The model and role of biohydrogen and biomethane production are shown in Table 2.2 (L. Singh & Wahid, 2015).

Several factors can influence biohydrogen and biomethane performance during fermentation, like substrate and concentrations limit, temperature, pH and type of reactor. In order to enhance, evaluate and simulate hydrogen productivity during fermentation, modelling of hydrogen production is essential. The kinetics of cell growth and formation of products, utilization of substrate and inhibition are statistical models (A. Singh et al., 2015).

Table 2.2: Kinetics analysis in biohydrogen and biomethane production

Models	Functions
Arrhenius	Temperature effect on hydrogen and methane production
Monod or Michaelis-Menten	Growth of microbes on hydrogen and methane production
Logistic	Description of bacterial cell growth
Haldane-Andrew and Hans-Levenspiel	Inhibition of substrate or dependency of the substrate based on particular growth
Leudeking-Piret	Correlation between the growth of cell and hydrogen and methane production rate
Andrew	Relationship between pH and utilisation of substrate
Gompertz	Progression of cumulative of hydrogen and methane production

(Source : L. Singh & Wahid, 2015)

The Zwietering modification of Gompertz model, also known as the “modified Gompertz” is typically applied to bacterial growth data. Modified Gompertz model involves the absolute growth rate and lag time, which is interpreted as the time between a microbial population is transferred to a new habitat recovers and when a considerable cell division occurs. When this model is fitted to microbial growth data, the dependent variable is typically transformed into the logarithm of the relative population size. The advantage of applying this model to the study of biohydrogen and biomethane production is that the growth coefficient constitutes the absolute growth rate at inflection, and that A (the upper asymptote) does not affect this parameter (Tjørve & Tjørve, 2017).

2.9 Bioreactor Design Parameter

Anaerobic bioreactor, also called anaerobic digester, plays a significant role in producing biogas. It acts as a physical structure that provides anaerobic

conditions known as biogas plants where the microbiological reaction occurs. The digester should be airtight and watertight. Organic food source mixed with water enters the digester or a large tank. Biogas is produced and collected within the large tank before exiting the biogas system. Construction material varies for each biogas plant, in different shapes and sizes (Edwin & Sekhar, 2015).

2.9.1 pH

The pH range of 6.5 to 7.5 is ideal as methane bacteria cannot do its work below pH 6.5. The pH value should not drop below 6.2. Too low of a pH inhibits the production of biogas. Also, the optimum pH to obtain the highest amount of biogas during anaerobic fermentation is reported to be pH 7 (Budiyono et al., 2018). Besides that, regarding cow dung as raw material, the pH used in a comparative study of biogas in India is 7.2 (Pandey et al., 2009).

2.9.2 Temperature

Another environmental factor is temperature. The optimum temperatures for global biogas production are 30-38°C and 55 -65°C, mesophilic and thermophilic, respectively (Łukajtis et al., 2018). However, study done by Budiyono stated that with cow manure as a biodegradable food source for the microbes are reportedly in the mesophilic range with 33.5°C, 35°C and 38.5°C (Budiyono et al., 2014). A temperature range from 35°C to 55°C is also used to develop the modified Gompertz model (Ghatak & Mahanta, 2017). Although the higher temperature will give out a higher yield of biogas, an external energy source is needed to maintain the temperature of the content in the bio-digester. On the other hand, mesophilic is preferred because it is closer to our room temperature (Imam et al., 2013). Estimating gas production rate per ton of manure at different temperatures is carried out. Based on the results shown in Table 2.3, higher temperature yields higher gas production per day where 35°C has not only the highest production (2.0m³ per day) but also the shortest digestion period of 1 month (Ezekoye & Ezekoye, 2014).

Table 2.3: Biogas production rate at different temperatures

Temperature (°C)	Gas production (m ³ per day)	Digestion period (months)
15	0.150	12
20	0.300	6
25	0.600	3
30	1.000	2
35	2.000	1

(Source : Ezekoye & Ezekoye, 2014)

2.9.3 Hydraulic Retention Time (HRT)

Hydraulic retention time (or hydraulic loading) is defined as an average number of days a feedstock stays in a digester, influencing the amount of biogas produced. Hydraulic loading is calculated by dividing the reactor's volume by daily feed input (waste/cow manure). The range of HRT should be 60 to 80 days. HRT is a measure of time for the methanogens to convert organic acids into methane (S. Liu et al., 2020).

Microorganisms in the anaerobic reactor take a certain time to complete a process cycle. Hence, a too short of time may lead to incomplete conversion of waste to biogas and low biogas production. Long HRT on the other hand is not doing any good to the biogas plant as the microbes can be starved due to the lack of substrate available in the system ((Yu Li, Zhao, et al., 2020).

The minimum hydraulic loading varies based on the type of digester, the operating temperature, and the type and amount of solids going into the digester (Gould, 2015). Edwin suggests the HRT be greater than 20 days to utilize the full potential of the microbes (Edwin & Sekhar, 2015). The HRT used by researchers is 60 days (Budiyo et al., 2014), 70 days (Agrahari & Tiwari, 2014), and 40 days (Imam et al., 2013). The biogas rate tends to obey a sigmoid S curve as generally occurs in a microbial growth curve. The sigmoid S curve behavior of the biogas rate is because biogas is one of the metabolites produced by the microbes in the inoculum. Based on the S curve trend, the study of biogas production can be interpreted as the biogas starts to emerge from day 11 to day 15 and slowly increases in gas production. Around week 3 of the process, the microbial activity is at its maximum with the highest production rate. This phase

is also known as the exponential growth phase. Decrease of biogas production starts after 35 to 50 days of anaerobic treatment in the bioreactor.

2.9.4 Organic Loading Rate (OLR)

The organic loading rate is the rate of the organic feed to be fed into the biogas system, and it is measured by mass/day. In this study, the organic loading source is cow manure. High fiber content results in a favorable gas generation rate. A continuous feed of organic waste should be fed to the bioreactor and ensure it is always sufficient. The daily input of cow manure should conform to reactor size. High daily input of cow manure rate will cause the biogas production to decrease (Edwin & Sekhar, 2015).

Cow manure, by nature is in a semi-solid state. In cow farms or milk farms, cow manure is collected by shovel or washed away using tap water. The washed-away cow manure effluent is then collected. Before fermentation or digestion occurs, it must be mixed with water if the cow manure is solid or semi-solid. In a lab-based experiment, the waste is mixed with rumen with a ratio of 1:1 to obtain a better biogas yield (Budiyo et al., 2014). However, it is less likely to do so in a large-scale milk factory, nevertheless a continuous process. The fresh manure to water weight ratio chosen is 1:1, or for diluting cow manure waste, the weight ratio of water to relative dry waste is 2.5:1, according to Forhad (Imam et al., 2013). Assuming 1L of water is equivalent to 1kg. From other literature sources, the OLR of 660kg of slurry is digested in a 0.971m³ bio-digester. Cow manure slurry is obtained from fresh waste to water with a weight ratio of 1:2 (Ezekoye & Ezekoye, 2014).

For a typical milk factory with 700kg foreign milk cow, up to 56kg of manure is produced per day based on the manure production (Wresta et al., 2015). Based on Wresta, the rate of cow manure to be fed to the digester to generate an amount of electrical energy, m_{cm} (kg/day) is calculated by:

$$m_{cm} = \frac{E}{0.008973} \times 0.1 \quad (2.1)$$

Where E is the electrical energy to be generated (kWh/day).

The rate of water to be added to the digester to produce biogas, m_w (kg/day) is calculated by:

$$m_w = m_{cm} \quad (2.2)$$

The feeding rate of slurry to the digester is the summation of manure rate and water rate, as follow:

$$m_s = m_{cm} + m_w \quad (2.3)$$

The volumetric rate of feeding slurry, F (m^3/day) is calculated by mass of slurry with the density of the slurry, ρ (mixture of slurry and water, kg/m^3):

$$F = \frac{m_s}{\rho} \quad (2.4)$$

2.9.5 Biogas Generation Rate

From a metric ton of organic waste, usually, a biogas yield of 50 to 70 m^3 can be obtained per digestion cycle. Around 50% of the carbon in biomass is theoretically available for gas generation. Biogas generation rate varies according to several factors such as temperature, pH and others as mentioned in the literature above. The potential production of biogas is also directly related to volatile solids content. The methane productivity of dairy cattle manure is 1481 L/kg VS (Møller et al., 2004).

According to Sarkar, the biogas generation yield from cow manure is 0.037 m^3/kg . Other than that, in other research, 0.002 m^3/kg (Wresta et al., 2015) and 0.018 m^3/kg (Agrahari & Tiwari, 2014) of biogas generation rate are obtained. Based on previous research, from the perspective of energy generation in the form of electricity, biogas produced from 100 grams or 0.1 kg of cow manure can potentially generate 0.008973 kWh of power (Wresta et al., 2015).

In the view of the mathematic model, the biogas production rate is predicted to obey Gompertz equation.

$$P = A \cdot \exp \left\{ - \exp \left[\frac{U_e}{A} (\lambda - t) + 1 \right] \right\} \quad (2.5)$$

Where,

P = cumulative of the specific biogas production ($\text{mL}/\text{g VS}$)

A = biogas production potential ($\text{mL}/\text{g VS}$)

U = maximum biogas production rate ($\text{mL}/\text{g VS}/\text{day}$)

λ = lag phase period or minimum time required to produce biogas (day)

e = mathematical constant value 2.718282

t = time period of biogas production (day)

In previous research, the expression of kinetic parameters, A , U , and λ shown below, is fitted into the modified Gompertz model shown in Table 2.4.

Table 2.4: Expression of kinetic parameters in modified Gompertz Model

Kinetic Constants	Researcher
$A = 2.449 \times e^{0.0502 \times T}$	
$U = 0.0375 \times e^{0.0674 \times T}$	
$\lambda = 12.943 \times e^{-0.016 \times T}$	
T = mesophilic temperature	(Ghatak & Mahanta, 2017)
HRT = hydraulic retention time, 1 to 50 days	
$A = 73.81 \text{ (mL/g.VS)}$ $U = 1.74 \text{ (mL/g.VS.d)}$	
$\lambda = 14.75 \text{ (days)}$	(Budiyono et al., 2014)

2.9.6 Electricity

One of the benefits of biogas produced from anaerobic digestion is to generate electricity and heat by a combined power and heat (CPH) unit, or just electricity in a generator. The efficiencies of a CPH unit or a generator are 37% (Gebrezgabher et al., 2010) and 39% (Akbulut, 2012). Other than that, a recent study in 2018, it is reported that a CPH unit has 38% net electric efficiency and 48% thermal efficiency (Ruiz et al., 2018). 1 m³ of biogas produces hourly 2.14 kWh of electricity and 2.47 kWh of heat. Hence, the total energy value of the biogas is 5.5 kWh/m³. Biogas can also be compared with other materials, for example 1 m³ of biogas is also equivalent to 6000 calories, 1 liter of alcohol, 0.8 liter of petrol and 0.6 liter of crude oil. On the other hand, Swedish Gas Technology Centre reported that 1m³ of biogas has a value of 6 kWh of electricity, assuming the 60% methane percentage.

Based on the study on the energetic and economic analysis of biogas plant in dairy farm suggested that the investment in the biogas plant would be profitable (payback period of 3.41 years in the best scenario), achieved thanks to operating as demand-driven biogas plant with CHP 499 kW_e working 12 h/day (4250 h/year). The second aspect of profitability is the use of biomass (mainly biowaste) from a farm as a substrate (Pochwatka et al., 2023). Another study of a micro-scale biogas plant for the treatment of organic municipal waste stated that the electrical power produced from the biogas is partially used to satisfy the process

demands, which amount to 0.63 kWe (considering a consumption of 7.2 kWh/t of digester feed) for pumping and stirring, and to 4 kWe for solid–liquid separation where the remaining 9.53 kWe are injected in the national electricity grid. The sale of renewable power and heat produced account for about 43 % of the total incomes (Barbera et al., 2022).

2.10 Economic Analysis

For an energy recovery model, economic analysis is vital for farmers to apply such technology in the industry. Economic analysis done by previous researchers includes economic parameters such as Net Present Value (NPV), Internal Return Rate (IRR), Profitability Index (PI), Average Rate of Return (ARR), and Payback Period (PP) (Wresta et al., 2015). All the costs are referred to year 2019, to avoid the high costs variability introduced due to Covid-19 pandemic and the current geopolitical situation.

The previous research done by Wresta is the study of the economic feasibility of small-scale biogas power generation plants in different farmers' investment conditions. The condition varies in land requirements and operational labor costs to estimate the working capital of the system. Wresta assumes the working capital rotation is 1 month. The average life span of an industrial plant is estimated to be 10-20 years.

While calculating NPV, the discounted rate is assumed to be 10% (Gebrezgabher et al., 2010), 14% (Akbulut, 2012), and 7.398% (Ruiz et al., 2018). The main challenge for estimating the investment costs is that the expenditure varies from case to case. The aspects included as investment cost depends highly on the specific installation needs for certain plant. Therefore, it is difficult to determine the investment cost beforehand. However, from the literature that have been studied, it is necessary to mention that the total investment costs for an anaerobic digester can vary from €2500 (RM12,824.00) to €7500 (RM38,471.99) per kWh/h electricity generation (Akbulut, 2012).

Akbulut also added that for a study of 28,105-ton manure/annual, the total labor cost, transport cost of animal manure, operating and maintenance cost, animal feed cost, taxes, electricity consumptions cost, and heat consumption cost, respectively, were taken as €11,520 (RM59,092.97), €10,620 (RM54,476.34), €4000 (RM20,518.39), €3,372,600 (RM17,300,083.75), €150,960 (RM774,364.18), €59,500 (RM305,211.11), and €6626 (RM33,988.72) per year. The capital cost to be estimated for a chemical process plant are often based on the estimated purchase cost of major equipment items. These are the equipment needed for the process, while other costs are to be estimated as factors of the equipment cost. The factorial cost estimation is widely used during plant design. Economic evaluations are also important as they may help the organization evaluate the economic performance before deciding to invest.

2.11 Summary

Based on the above literature review, this chapter covered that biohydrogen and biomethane have a high potential as an alternative energy source for fossil fuel. Methods of biohydrogen and biomethane production were also discussed in this chapter including the biological method hence more favorable to anaerobic dark fermentation as it has a higher biogas yield. GAC is also discussed as how it is a good carrier material in biofilm attachment as GAC is inert and porous. Several immobilization methods were mentioned. The advantages of alginate and chitosan to be used as gel polymer in forming immobilized beads were also discussed in this chapter. Alginate and chitosan have different ionic groups that are useful in forming gel beads. For the bioreactor design, a few important factors were considered, including pH, temperature, HRT and OLR. These factors significantly affect the biogas yield hence the amount of electricity generated. Not only that, the tank's dimension is also considered. Lastly, the economic analysis of the biogas plant is studied based on economic parameters such as Net Present Value (NPV), Internal Return Rate (IRR), Profitability Index (PI), Average Rate of Return (ARR), and Payback Period (PP).

CHAPTER 3

METHODOLOGY

3.1 Introduction

The research methodology is the planning of the research with appropriate procedures to achieve the objectives of this study. This ensures the research can be carried out smoothly where every stage of the research has its milestone to achieve. This study mainly involved laboratory work. Experiments were carried out to get the data for this study. The procedures used in this work came mainly from the Standard Methods for the Examination of Water and Wastewater™, 24th edition (APHA 2022), the comprehensive reference covers all aspects of wastewater analysis standard methods. The straightforward process of methodology is depicted in Figure 3.1.

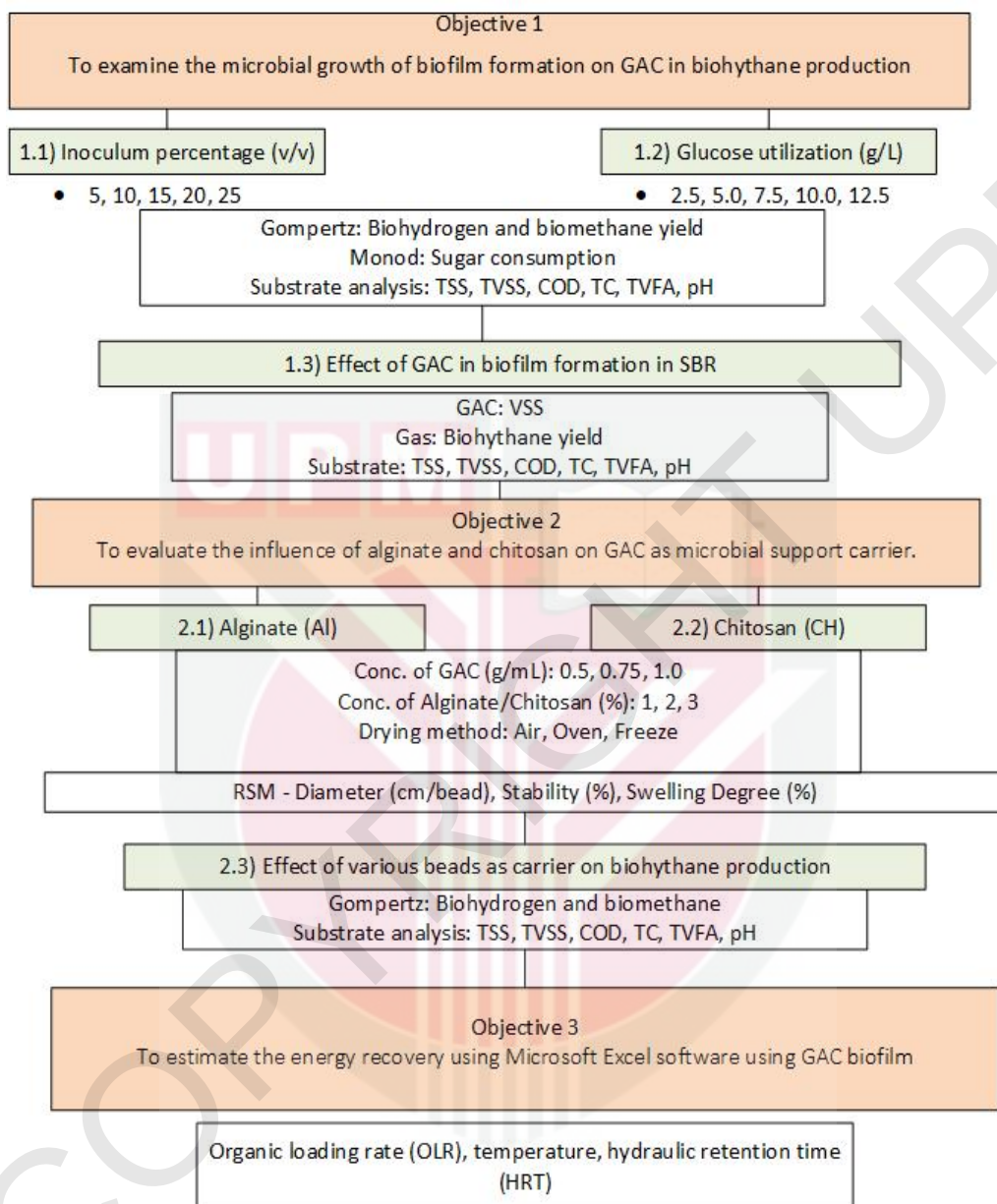


Figure 3.1: Process of methodology

3.2 Materials

3.2.1 Cow Manure as Inoculum

The inoculum feedstock used in this study was raw cow manure obtained from

Dairy farm in Ladang 16, University Putra Malaysia (UPM), Serdang. The cow effluent flows to manhole 1 for collection & separation and the next flow to manhole 2 as holding sump before being fed into the first digester. Raw samples were collected on 09.07.2020 at the man hole using 10L plastic container. Cow dung samples were labelled and stored in refrigerator at 4°C for future use throughout the study.

3.2.2 Synthetic Medium

In this study, synthetic medium was used in order to provide a constant substrate for the microbial growth. The medium composition was taken from (Jamali et al., 2017) with a slight modification.

Table 3.1: Chemical compositions of synthetic medium

Chemical Composition	Quantity (g/L)
R&M Ammonium Chloride, NH_4Cl	1
R&M Sodium Chloride, NaCl	2
R&M Magnesium Chloride, Hexahydrate, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.5
R&M Calcium chloride dehydrate, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.05
R&M Dipotassium hydrogen phosphate trihydrate, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	1.5
R&M Potassium dihydrogen phosphate, KH_2PO_4	0.75
R&M Sodium bicarbonate, NaHCO_3	2.6
Oxoid, UK Yeast extract (Powder)	2
R&M D-Glucose, anhydrous	Varies

3.2.3 GAC as Carrier Material

Granular Activated Carbon (GAC) is used as a support matrix in the development of attached biofilm in this study. GAC is known for its inertness that does not react with any chemicals in the reactor throughout the fermentation period. GAC is also porous and acts as a good carrier for microorganisms to attach to it. The GAC used in this study is made up of coconut shells and supplied by KI Carbon Solutions Sdn. Bhd. Malaysia. GAC were sieved and obtained on an average of 2-3mm.

The GAC was sifted to acquire a particle size of 2-3 mm, as shown in Figure 3.1. Table 3.2 shows the characteristics of GAC that were analyzed in the literature. In biohydrogen and biomethane production, biofilm-based systems have been frequently studied as immobilization cell networks as they increase the rate of production and population dynamics.

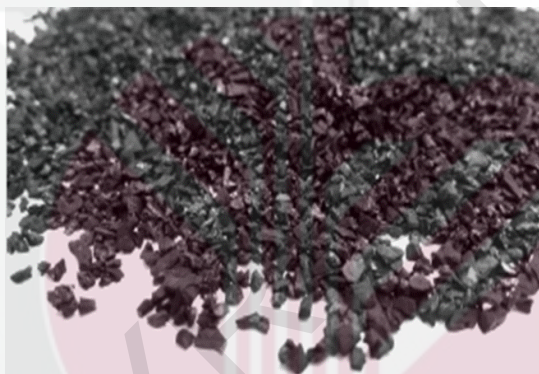


Figure 3.2: GAC as a microbial carrier with 2-3 mm particle sizes
(Source : Jamali et al, 2016)

Table 3.2: Characteristics of GAC

Characteristics	Units	Values
Surface Area	BET-m ² /g	1000-1150
Pore Volume	cm ³ /g	0.44
Moisture Content	%	<5%
Ash Content	%	<3%

3.2.4 Alginate and Chitosan

Alginate and chitosan are common biopolymer materials that are used as gel due to their ionic particles. They can help to improve the support matrix mechanical strength of biofilm attachment in the reactor. By forming immobilized beads, microorganisms are less exposed to harsh environments in the reactor, therefore, able to sustain for a longer period of time. With the aid of these biopolymers also able to prevent the washout phenomenon that is common in biogas reactors. This study purchased the R&M sodium alginate powder and chitosan flakes extracted from crab shells from BT Science Sdn. Bhd. Malaysia.

3.3 Experimental Procedures

3.3.1 Effect of Inoculum Percentage (v/v)

In this study, five different percentages over working volume (80 mL) of inoculum have been chosen to undergo batch fermentation using 100 mL serum bottles. For batch fermentation, the temperature of Daihan Scientific WB-11 Digital Water Bath 11Litre with dome lid from Korea was set at 37°C (Mesophilic) with no agitation. Then each bottle was purged with nitrogen gas before closed with rubber stopper and then clamped with BIOFLOW white PTFE/white silicone septa, 20mm Crimp-top aluminium cap with 10mm center hole, Malaysia to make the environment in the bottles anaerobic. Then, biohydrogen and biomethane production were fitted into the Modified Gompertz Equation to evaluate the effect of these ratios on biohydrogen and biomethane production. No control with 0 inoculum was conducted as inoculum is the source of microorganisms hence without any inoculum meaning only substrate is in the reactor hence no reaction could occur. Based on literature done for anaerobic digestion, 25% inoculum contains too much of microorganisms in the system that will lead to substrate deficiency.

Table 3.3: Samples of inoculum percentage in batch fermentation

Sample Labelling	Inoculum Percentage (v/v)
H5, CH5	5
H10, CH10	10
H15, CH15	15
H20, CH20	20
H25, CH25	25

The modified Gompertz equation has been extensively utilized for the productivity of biohydrogen and biomethane production by fermentation, as shown in Equation (3.1) below. The cumulative production of hydrogen and methane in this study was determined using this analysis.

$$H_t = P_m \exp \left\{ -\exp \left[\frac{R_m \times e}{P_m} (\lambda - t) + 1 \right] \right\} \quad (3.1)$$

Where;

H_t = cumulative hydrogen/methane production (mL)

P_m = maximum hydrogen/methane production (mL),

R_m = maximum hydrogen/methane production rate (mL/h)

e = Euler's number ($e = 2.7182$)

λ = lag phase time (h)

t = incubation time (h)

This equation generated parameter estimates for three leading regression coefficients to describe the lag region, linear region and tailing region of the sigmoid curve, which represent by H_m , R_m and λ , respectively. The Microsoft Excel Software was used to construct the cumulative hydrogen and methane data profile in the Modified Gompertz Equation to determine the parameters.

3.3.2 Utilization of Glucose

Substrate source played an essential role in producing biohydrogen and biomethane gases. Lignocellulosic materials are often selected as substrate materials for biohydrogen and biomethane production due to many reasons such as their abundancy, it is renewable and, most importantly, they contain high cellulose that can be decomposed into glucose (Prakasham et al., 2009). Glucose is the main carbohydrate source for the cells' microorganisms to

function, hence producing biogas.

During the characterization of the cow dung sample obtained, it is found that the total carbohydrate value was 7.25 g/L. To determine the optimum level of glucose provided in the system, batch fermentation with similar method and condition for the effect of inoculum was carried out with five different glucose levels introduced as shown in Table 3.4 below.

Table 3.4: Samples of glucose concentration in batch fermentation

Sample Labelling	Glucose Concentration (g/L)
H2.5, CH2.5	2.5
H5.0, CH5.0	5.0
H7.5, CH7.5	7.5
H10.0, CH10.0	10.0
H12.5, CH12.5	12.5

Monod modelling is used to evaluate the glucose consumption of microorganisms in the system concerning the total volatile solids. The Lineweaver-Burk plot is used in order to correlate with the Monod modelling. The Lineweaver-Burk plot is a graphical representation of enzyme kinetics. The x-axis is the reciprocal of the substrate concentration, or $1 / [S]$, and the y-axis is the reciprocal of the reaction velocity, or $1 / V$. In this way, the Lineweaver-Burk plot is often also called a double reciprocal plot as shown in Equation 3.2 below (Jamaludin et al., 2024).

$$\mu = \mu_{max} \frac{[S]}{k_S + [S]} \quad (3.2)$$

3.3.3 Biofilm Formation in Continuous Reactor

Two types of inoculums were prepared for hydrogen system and methane system. Inoculum undergoes heat pretreatment for hydrogen production where the cow dung was heated at 80°C for 1 hour. The heat pretreatment is to inhibit the growth of the methanogenic population in the inoculum. Before the fermentation, the reactors were purged with nitrogen gas to make them anaerobic. Then throughout the experiment, reactors were put inside WB-11 water bath from Daihan Scientific, Korea, where the temperature was maintained at mesophilic (37°C) in a 1 L Schott Duran bottle with no agitation. HRT and pH

for both reactors were set separately. For hydrogen, HRT was 2 days at pH 6, while methane's HRT was 14 days at adjusted pH 7.5. 1M NaOH and 1M HCl were used to adjust the pH for the respective reactors. Substrate from the biohydrogen reactor was transferred to the Methane reactor accordingly. The gases were collected using the water displacement method, where the measuring cylinder was placed inverted in pH 2 hydrochloric acid. Figure 3.3 shows the schematic diagram of the setup of the experiment continuous batch fermentation while Figure 3.4 shows the schematic diagram of two-stage biohydrogen and biomethane production.

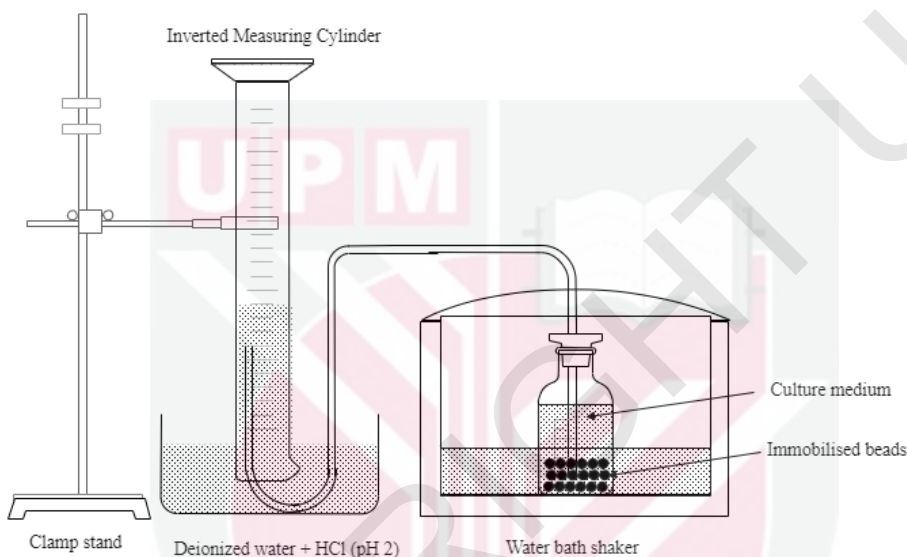


Figure 3.3: Experimental setup for continuous batch fermentation

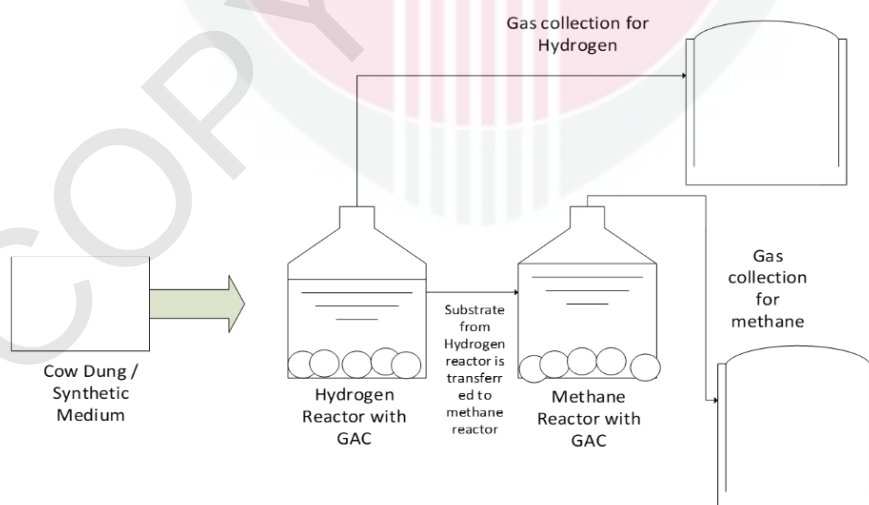


Figure 3.4: Schematic diagram of two-stage biohydrogen and biomethane production

3.3.4 Formation of Immobilized Beads

3.3.4.1 Alginate Beads

Using alginate to produce immobilized beads is simple as it can produce uniform particles and reduces aggregation. With the encapsulation of alginate, it is found that the alginate layer doesn't affect carbon adsorption. Due to its porous structure, it allows the solution to pass through the gel-like alginate.

In the first step, sodium alginate solution is prepared in distilled water at a concentration of 1-3%, at room temperature. Then, the solution is stirred for at least an hour for complete homogenous to avoid the clumping of alginate in it. Then, 0.5, 0.75 and 1.0 g/L of GAC were added into the alginate solution in separate beakers. Instead of using the conventional method of syringe the beads, this study is unsuitable because the carrier material used is GAC. So, the method of scooping-and-drop is used by using spatula to scoop a small portion of the alginate solution with GAC mixed in it, then dropped into Calcium Chloride (CaCl_2) solution with 0.1, 0.2 and 0.3 M concentration, respectively. The beads would form once alginate was dropped and in contact with calcium chloride solution. Once the beads were formed, they would sink and settle to the bottom of the beaker.

After leaving the beads in the solution for a few minutes, the beads were filtered and rinsed thoroughly using distilled water to remove the excess calcium chloride solution. The beads were then distributed equally and placed on a flat plate to be dried separately using different methods including open air drying with room temperature, oven dry in the oven at 50°C and freeze dry in chiller at 4°C for 24 hours. After 24 hours, the beads were collected for analysis (Shu & Zhu, 2002).

3.3.4.2 Chitosan Beads

A similar approach was used in the preparation of chitosan beads. For chitosan, the compound has to be dissolved in organic acids. In this study, the acid used was acetic acid 0.1 M to dissolve chitosan. Then, the chitosan solution is stirred until homogenous. GAC of 0.5, 0.75 and 1.0 g/L were added into the beakers separately and stirred using a magnetic stirrer until reaching a uniform dispersion.

Chitosan beads can be formed by an alkali coagulation bath where chitosan precipitates. Similar to the method mentioned above for alginate beads, chitosan mixed GAC solution was scooped using a spatula and dropped into sodium hydroxide solution of 0.1, 0.2 and 0.3 M concentration. The beads would form once chitosan contained with GAC was dropped and in contact with sodium

hydroxide solution. Once the beads were formed, they would sink and settle to the bottom of the beaker.

After leaving the beads in the solution for a few minutes, the beads were filtered and rinsed thoroughly using distilled water to remove the excess sodium hydroxide solution. The beads were then distributed equally and placed on a flat plate to be dried separately using different methods including open air drying with room temperature, oven dry in the oven at 50°C and freeze dry in chiller at 4°C for 24 hours. After 24 hours, the beads were collected for analysis (Chang & Juang, 2007).

3.4 Analytical Methods

3.4.1 Analysis of Liquid and Gaseous Sample

3.4.1.1 pH

Before the pH meter was used, it had to be calibrated using buffer solution pH 4 and pH 7. The electrode was then rinsed with distilled water before being placed into the samples. It took approximately 1 to 2 minutes for the pH meter to be stable with the reading not fluctuating then the pH value was recorded.

3.4.1.2 Total Volatile Solids (TVS)

Using the method as mentioned in Standard Methods for the Examination of Water and Wastewater™, 24th edition (APHA 2022), firstly, two crucibles were burned in a furnace at 550°C for 30 minutes to ignite clean. Then, each crucible with a fiber disk added was pre-weighed, and the readings were recorded. Next, 3 mL of the samples were pipetted then transferred into the crucibles with fiber disk. Vacuum suction was used to remove the water of the solution, leaving only the dry ones, they were then being placed into the oven to dry at 105°C for 3 hours. After that, the samples were taken out and cooled at room temperature. The post-weight was taken and recorded for each sample. The TVS was calculated by using the Eq. 3.3 below:

$$\text{TVS (g/L)} = \frac{[C_1 - D_1]}{\text{Volume of sample, mL}} \quad (3.3)$$

C_1 = weight of dried residue + fibre disk + crucible (post weight), mg

D_1 = weight of fibre disk + crucible (pre-weight), mg

3.4.1.3 Total Volatile Suspended Solids (TVSS)

Using also the method as mentioned in Standard Methods for the Examination of Water and Wastewater™, 24th edition (APHA 2022), Continuing from TVS, the crucible samples were ignited in the furnace for 550°C for 30 minutes. Then it was being taken out and place to be cooled at room temperature. The post-weight was taken for each sample. The TVSS was calculated by using the Eq. 3.4 below:

$$TVSS \text{ (g/L)} = \frac{[C_2 - D_2]}{\text{Volume of sample, mL}} \quad (3.4)$$

C₂ = weight of dried residue + fibre disk + before ignition (pre-weight), mg

D₂ = weight of dried residue + fibre disk + after ignition (post-weight), mg

3.4.1.4 Total Volatile Fatty Acids (TVFAs)

The measurement of the volatile acidity as the acid content was based on 350°C distillation method in Standard Methods for the Examination of Water and Wastewater™, 24th edition (APHA 2022). The recovery factor of cow dung was determined to about 70% before the experiment of the samples were carried out. The recovery factor was calculated using the Eq. 3.5. 100 mL of well mixed samples was placed in a 500 mL distillation flask with 100 mL distilled water and also 5mL of concentrated sulphuric acid. The samples were mixed thoroughly, connected to the condenser, and distilled at about 5 mL over a minute into a beaker. The first 15 mL of the distillate was discarded and the following 150mL was collected in a 250 mL beaker. This 250 mL was then being used for titration by using 0.1 M sodium hydroxide (NaOH). The volatile acidity of the sample was calculated using Eq. 3.6 below:

$$\text{Recovery Factor, } f = K/L \quad (3.5)$$

K = volatile acid concentration recovered in distillate, mg/L

L = volatile acid concentration in standard solution used, mg/L

$$\text{Volatile acidity, (mg of acetic acid/L)} = \frac{M \times P \times 60000}{\text{Volume of sample} \times f} \quad (3.6)$$

M = mL of titrant to reach endpoint

P = equivalent NaOH per litre titrant

f = recovery factor

3.4.1.5 Chemical Oxygen Demand (COD)

Based on the Standard Methods for the Examination of Water and Wastewater™, 24th edition (APHA 2022), samples were first being diluted by using a dilution factor of 50. Then 2.5 mL of the diluted sample was being added to the vial tubes,

where one of the vial tubes was filled with distilled water only because this will be used as a blank. The tubes were then carefully added with 1.5 mL of potassium dichromate solutions ($K_2Cr_2O_7$) and 3.5 mL of sulphuric acid solutions (H_2SO_4). The tubes were tightly capped and inverted each tube several times to mix thoroughly. After that, the vials were placed in a COD reactor and heated at $150^\circ C$ for 2 hours. After 2 hours, the tubes were taken out and placed to cool at room temperature. Then, transfer the sample to the conical flask, and two drops of ferroin indicator were added. After that, the sample was titrated with standardized 0.10 M ferrous ammonium sulphate (FAS). Lastly, continuous the titration until the change in colour to reddish-brown. The COD concentration can calculate using Eq. 3.8 below:

$$\text{Molarity of FAS solution} = \frac{\text{Volume of } K_2Cr_2O_7, mL}{\text{Volume of FAS, mL}} \quad (3.7)$$

$$COD (mg O_2/L) = \frac{[(A - B) \times M \times 8000]}{\text{Volume of sample, mL}} \quad (3.8)$$

Where;

A= Volume of FAS used for blank, mL

B= Volume of FAS used for sample, mL

M= Molarity of FAS

8000 = mili equivalent weight of oxygen (8) $\times$ 1000 mL/L

3.4.1.6 Total Carbohydrates (TC)

The Phenol-sulphuric acid method was used in order to determine the total carbohydrate. A standard curve was obtained where 0.5 mL phenol and 2.5 mL of 98% concentrated sulphuric acid solution were added into 0.5mL of glucose. The vials needed to be sure that they were clean and dry. Then, they were left to cool down for 30 minutes for digestion to occur. Lastly, the samples reading was then measured using spectrometer at wavelength 490 nm and plotted as standard curve graph as shown in Figure 3.5.

Similar procedures were carried out using cow dung with dilution factor 100, then added into 0.5 mL phenol and 2.5 mL of 98% concentrated sulphuric acid solution, left for 30 minutes digestion and collect the reading using spectrometer at wavelength 490nm to plot against the standard curve graph.

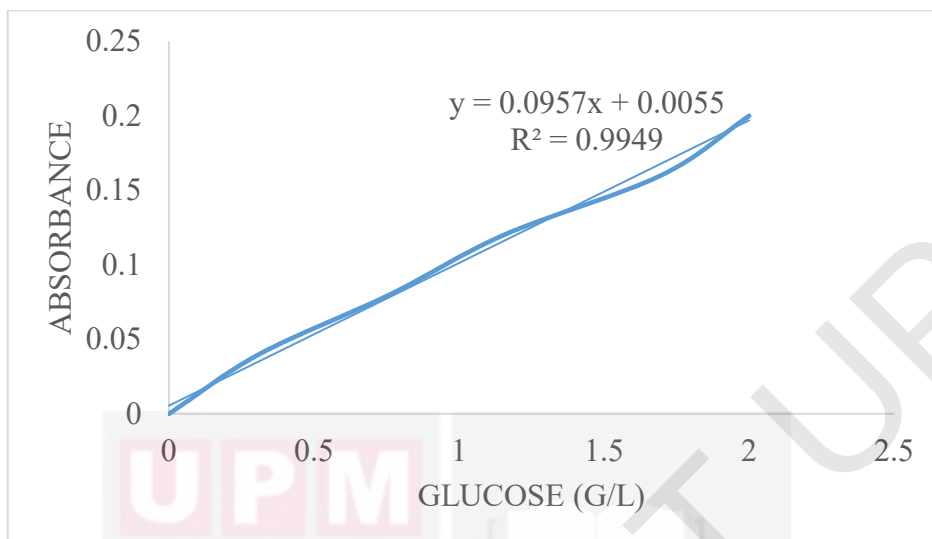


Figure 3.5: Standard curve of glucose

3.4.1.6 Gas Chromatography (GC)

Gas Chromatography (GC) is used to specify the percentage of different gas compositions present in the yielded gas. The hydrogen and methane composition in the biogas was measured using the Gas Chromatograph (GC) model Agilent 6980 N, Agilent Technology, USA. The GC was equipped with a capillary column (Agilent 19095P-Q40, 30 m × 530 μm × 40 μm) and a thermal conductivity detector (Agilent, USA) with an injection temperature of 110 °C. Helium was used as the carrier gas at 50 °C with a 20 ml/min flow rate. A 1 ml gas-tight syringe was used to collect 1 ml of the gas samples and immediately injected into the GC at 55 °C and a pressure of 65 kPa. It was followed by a hold time of 60 °C/min (maintained for 20 min once the temperature reached 220 °C).

3.4.2 Analysis of Immobilized Beads

3.4.2.1 Diameter

The diameter of the beads was measured using a Vernier callipers. The beads were measured accordingly and then divide by the total number of beads in a batch produced.

3.4.2.2 Stability

The stability of the beads depends on its mechanical strength. The beads could burst when the osmotic pressure inside the beads was too high. The bead's stability is estimated by suspending a fixed amount of beads in 500mL of 0.9% (v/v) NaCl solution at 37°C, 50 rpm. After 24 hours, the stability is obtained by dividing the number of burst beads / the number of beads given x 100% (Shu & Zhu, 2002).

3.4.2.3 Swelling Degree

Swelling degree of the beads is measured based on the weight of the beads before and after being submerged in distilled water for 24 hours, 50rpm. The swelling degree is obtained by dividing the weight of beads after 24 hours / the weight of beads initial x 100% (Shu & Zhu, 2002).

3.4.2.4 Response Surface Methodology (RSM)

RSM has been widely used in several fields such as to optimize the effect of process variables. In this study, RSM was used to study the factors affecting the formation of immobilized beads using alginate and chitosan. By using composite central design (CCD), a design of RSM with 27 different models were computed with different concentration of GAC (g/L), concentration of alginate/chitosan (g/L), and drying method. From the models, 3 responses were analysed under this design of experiment which are diameter, stability and swelling degree. The experimental data was then analysed using RSM to confirm the alignment of the proposed model with the experimental results. This indicated a strong correspondence between the model and experimental outcomes. The second-order polynomial function is commonly employed to predict the response, taking into account the interactions between two factors or parameters (Bypour et al., 2024).

3.5 Basis of Calculation for Energy Recovery Model

The location chosen to study for the energy recovery model is Farm Fresh Dairy Farm, located at Ladang 16, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia. The farm is estimated to have 200 number of milking cows with a basis of cow manure effluent of 45-55 L/day.cow. The figures mentioned will be used as a basis for the calculation in this part of the research for the energy recovery model to achieve objective 3 stated earlier.

3.5.1 Material Balance

The fundamentals of material balance will be used in this part of the research while the material balance of the process and calculations were done in Microsoft Excel solver. The process is based on steady-state operation, whereby the values of the variables in the process do not change with time (M. Felder & W. Rousseau, 2005). For a continuous process, the balance is simply given as:

$$\text{Input} + \text{generation} = \text{Output} + \text{consumption} \quad (3.9)$$

3.5.2 Yield of Biogas

The yield of biogas based on lab-scale bioreactor for the energy recovery model is calculated using the kinetic model developed by Ghatak (Ghatak & Mahanta, 2017). The biogas production of cow manure is simulated by using modified Gompertz plots.

Table 3.5: Kinetics parameter at different temperature

Kinetic					
Parameter/ temperatures	35°C	40°C	45°C	50°C	55°C
A (mL/g.VS)	28.990	18.463	21.643	33.951	36.708
U (mL/g.VS.day)	0.939	0.528	0.760	1.344	1.340
λ (days)	8.017	6.654	6.232	6.045	5.129

Table 3.5 shows the kinetic constant at different temperature. By fitting all the parameters at different temperatures obtained experimentally, a new range of expression of the kinetic parameter, A, U and λ is obtained by Ghatak. The following expressions are fitted into the modified Gompertz model.

$$A = 2.449 \times e^{0.0502 \times T}, U = 0.0375 \times e^{0.0674 \times T}, \lambda = 12.943 \times e^{-0.016 \times T} \quad (3.9)$$

And the equation becomes

$$P = 2.4429 \times e^{0.0502 \times T} \exp \left\{ -\exp \left[\frac{0.0375 \times e^{0.0674 \times T} \times e}{2.4429 \times e^{0.0502 \times T}} \times (12.943 \times e^{-0.016 \times T} - \text{HRT}) + 1 \right] \right\} \quad (3.10)$$

Where T = temperature (°C)

HRT = hydraulic retention time, 1 to 50 days

The Electricity produced (kWh) is calculated by using the equation as follow (Centre, 2012):

$$E_{el} = \text{Volume of biogas produced (m}^3\text{)} \times 6 \left(\frac{\text{kWh}}{\text{m}^3} \right) \quad (3.11)$$

3.5.3 Dimension

According to the journal “Design Considerations of An Anaerobic Digester for Producing Biogas Loaded with Cow Manure” written by M.Edwin and S.Joseph, the designs are built on several volumes (Edwin & Sekhar, 2015).

A series of formulas calculate the volume of bio-digester. The various dimensions are the volume of gas collecting chamber (Vc), Volume of gas storage chamber (Vgs), Volume of the fermentation chamber (Vf), and Volume of sludge layer (Vs). The total volume of biodigester is the summation of all volumes stated, V= Vc + Vgs + Vf + Vs. The geometrical dimensions of biogas digester are given in Table 2.5.

Table 3.6: Dimensions of biogas digester

For Volume	For Geometrical Dimensions	
$V_c \leq 5\% V$	$D = 1.3078 \times V^{1/3}$	
$V_s \leq 15\% V$	$V_1 = 0.0827 D^3$	$V_2 = 0.05011 D^3$
$V_{gs} + V_f = 80\% V$	$V_3 = 0.3142 D^3$	
	$R_1 = 0.725 D$	$R_2 = 1.0625 D$
$V_{gs} = 0.5 (V_{gs} + V_f + V_s) K$	$f_1 = D/5$	$f_2 = D/8$
Where K = Gas production rate per digester volume per day. For K= $0.4 \text{ m}^3/\text{m}^3 \cdot \text{day}$	$S_1 = 0.911 D^2$	$S_2 = 0.8345 D^2$

(Source : Edwin & Sekhar, 2015)

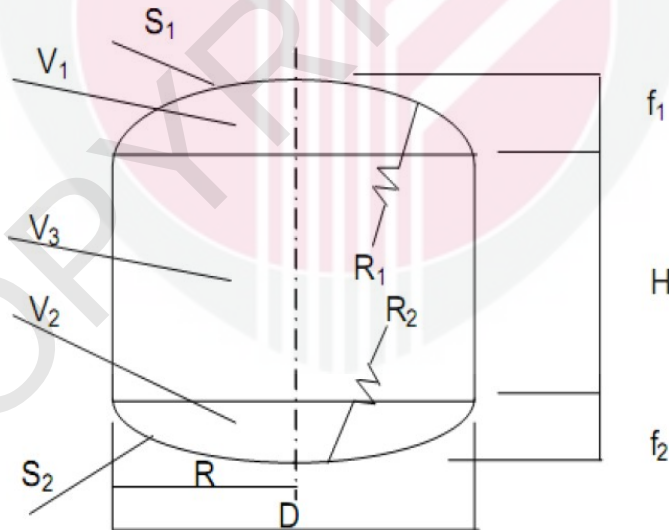


Figure 3.6: Geometrical dimensions of the cylindrical shape biogas digester body

(Source : Edwin & Sekhar, 2015)

3.5.4 Economic Analysis

An economic analysis is conducted for the biogas system. The currency rate has been fluctuating and is declining in recent years. Therefore, it was presumed that the currency exchange rate is RM4.73 per USD in this study to transform the economic value. The life span of this biogas system is estimated to be 20 years. The selling price of electricity is RM 0.35/ kWh according to TNB Malaysia and residential consumption of energy for a typical Malaysia household is 19.973kWh/day (Mohammed et al., 2018).

The economic analysis begins with the calculation of fixed capital cost, total investment cost and operational cost, the value of profit, and cash flow. Several economic parameters, including Net Present Value (NPV), Internal Return Rate (IRR), Profitability Index (PI), and Payback Period (PP), are calculated in this study (Wresta et al., 2015).

Working capital is the capital needed to operate the production process in one period of working capital rotation, from when the raw material is bought until the time when the realization of cash is received. Working capital is calculated as 5% of fixed capital to cover the cost of initial solvent change. The fixed capital is calculated using factorial estimation method, where the factors are listed in Table 3.5 as F1 to F9. F1 to F7 were used in the calculation for the total physical plant cost (PPC), then this PPC obtained will be used in the calculation for fixed capital and times with F8+F9.

Table 3.7: Typical factors for the estimation of project fixed capital cost

Item		Process type: Fluids- solid
1.	Major equipment cost, total purchase cost	PCE
F1	Piping	0.30
F2	Instrumentation	0.15
F3	Electrical	0.10
F4	Buildings, process	0.10
F5	Storages	0.10
F6	Site development	Not applicable
F7	Ancillary buildings	Not applicable
Total physical plant cost (PPC)		
PPC = PCE (1+F1+...+F6)		PPC = PCE X 1.75
F8	Contractor's Fee	0.05 or none (for small plant project)
F9	Contingency	0.05
Fixed capital = PPC (1+F8+F9)		Fixed capital = PPC X 1.1

The equipment cost estimation is done using correlations with on a U.S. Gulf Coast basis with the following equation (Ray Sinnot & Gavin Towler, 2013).

$$C_e = a + bS^n \quad (3.12)$$

Where

C_e = Purchased equipment cost on a U.S. Gulf Coast basis, Jan. 2010
 a, b = Constant from Table 7.2 in Chemical Engineering Design
 S = Size parameter

n = Exponent for that type of equipment

Total Investment value includes the component of working capital and fixed capital.

$$\text{Investment Value} = \sum (\text{Fixed capital, working capital}) \quad (3.13)$$

Operational costs is estimated to judge the viability of a project. The costs are divided into two groups:

- a) Fixed operating costs: Maintenance, operating labour, laboratory costs, supervision, plant overheads, capital charges, rates and taxes, and insurance.
- b) Variable costs: Raw Materials, miscellaneous operating materials, utilities.

$$\text{Operational cost} = \sum (\text{Fixed cost, variable cost}) \quad (3.14)$$

NPV value is the deduction result of the present value of proceeds with the present value of investment cost.

$$NPV = \sum_{t=0}^n \frac{CF_n}{(1+k)^n} \quad (3.15)$$

Where n is the year when the expense or revenue occurs, CF is cash flow at period n , k is the discount rate (7.398%). This discount rate takes into account both opportunity costs and risk premiums. IRR was calculated as the discount rate at which NPV equals zero, where k is the interest rate. The subsidies were not taken into account in the calculation. The formulas of other parameters are as follows:

$$PI = \frac{\sum \text{Present value of proceeds}}{\text{Present value of initial investment}} \quad (3.16)$$

$$PP = A + \frac{B}{C} \quad (3.17)$$

Where A = the last year when the cash accumulation is positive, B = the last positive cash accumulation value, C = the proceeds in the first year when cash accumulation is negative.

The biogas feeds a combined heat and power (CHP) unit of 38% net electric efficiency and 48% thermal efficiency. Power is assumed to sell directly to the grid with no additional losses. The thermal energy is used onsite to heat the pasteurization process (Ruiz et al., 2018).

According to Tenaga Nasional Berhad, a dairy farm is considered under medium voltage general specific agricultural; Air Selangor considers dairy farm under commercial. Therefore, the price of the purchased utilities presumed is listed in Table 3.8.

Table 3.8: Presumed price of utilities purchased, 2019

Utilities	Tariff (RM)
	30.30/kW
Electricity (TNB) ^a	0.35/kWh
	Minimum payment = 600/month
Water (AirSelangor) ^b	2.07/m ³ (for first 35 m ³)
	2.25/m ³ (for >35 m ³)

^a Pricing & Tariffs - Tenaga Nasional Berhad

^b Water Tariff Information » Pengurusan Air Selangor

3.6 Computer Software

The software used in this study is Microsoft Excel 2016. The built-in functions provided by Microsoft Excel are used in modelling, for example, solver, logical functions, statistical functions, mathematics, trigonometry functions, and economic functions.

The parameters to monitor in Microsoft Excel are the microbial kinetic studies, bioreactor dimension and various economic studies which are listed in Table 3.9. The design criteria are simple and straightforward, emphasizing biogas yield, bioreactor size and cost of the biogas production model.

Table 3.9: Parameters to monitor in Microsoft Excel

Output to be monitor	
Output 1 - Biodigester Dimension	- Working volume of bioreactor
	- Inlet flowrate of bioreactor
	- Volume (V_c, V_{gs}, V_f, V_s)
Output 2 - Microbial Kinetic	- Height (f_1, H, f_2)
	- Diameter (D, R_1, R_2)
	- Biogas Yield
Output 3 - Economics	- The volume of biogas produced
	- The electricity that can be generated
	- Revenue
	- Total Capital Investment
	- Annual Operating Cost
	- Annual Profit
	- NPV
	- IRR
	- Payback Period
	- PI
	- ROI
	- Cash Flow diagram

The Microsoft Excel Kit aims to create a biogas production model. The parameters listed in Table 3.9 can be generated automatically when the user key in several required inputs. It gives users the convenience of coming up with a biogas production model and economics studies that users may find useful in their initial project development phase.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents the results of this study based on the objectives and discusses the results obtained from experimental work. This chapter will be divided into 9 sections where the results are discussed accordingly in terms of the characterization of cow dung, the effect of inoculum percentage, glucose utilization and effect of GAC on biofilm formation. Then, the optimization of alginate and chitosan on GAC beads were also analyzed using central composite design (CCD) in Design Expert software. The effect of the two types of beads in biohydrogen and biomethane production was also observed and discussed in this chapter of the thesis. Lastly, an energy recovery model is also evaluated based on the bioreactor design, microbial kinetic studies and economic studies.

4.2 Characterization of Cow Dung

Cow dung has been recognized as a valuable resource for anaerobic fermentation as it contains high value of total volatile suspended solids (TVSS), indicating high concentration of active microorganisms. Samples of cow dung were collected and characterized as shown in Table 4.1.

Table 4.1: Characteristics of cow dung sample

Parameter	Value
pH	7.49 ±0.01
COD (g/L)	58.53 ±0.64
TVFA (g CH ₃ COOH/L)	12.32 ±0.85
TC (g/L)	6.27 ±1.01
TS (g/L)	10.84 ±0.91
TVS (g/L)	8.64 ±0.75
TVSS (g/L)	4.47 ±0.35

Table 4.1 it is shown that cow dung has a neutral pH at 7 hence with a low alkalinity (1.37 g/L) and also relatively low total volatile fatty acids, TVFA (12.32 gCH₃COOH/L). The result shows that the total solids, TS of cow dung were 10.84 g/L, total volatile solid, TVS was slightly lower than TS at 8.64 g/L because during TVS, cow dung has been filtered using microfiber paper. Even after burning the solids and volatile solids, the leftover of total volatile suspended solids, TVSS was 4.47 g/L, which is approximately half of its total solids. The total volatile suspended solids (TVSS) method is a rough measure of organic-solids concentration in samples of activated sludge derived by measuring volatile solids. Because bacteria are mostly organic, the TVSS test is a better indicator of organic-solids concentrations and therefore, TVSS is widely used as an indicator for the content of bacteria in a sample. Also, from the observation of chemical oxygen demand, COD also shows a high demand of oxygen in cow dung sample at 58.53 g/L. Hence, cow dung has a big potential to be used as an inoculum source for anaerobic digestion. Even so, total carbohydrate TC in cow dung is low (6.27 g/L) since cow undergoes rumination hence most of the glucose in their food is consumed by the cows, resulting in low carbohydrate in cow dung (Budiyo et al., 2014).

4.3 Effect of Inoculum Percentage (v/v) of Cow Dung Sludge

Inoculum, as the source of microorganisms in any anaerobic fermentation affects the production of biohydrogen and biomethane in many ways. This section studied 5 different percentage of inoculum (5%, 10%, 15%, 20% and 25%) using batch fermentation to investigate the effect on biohydrogen and biomethane production based on Gompertz analysis.

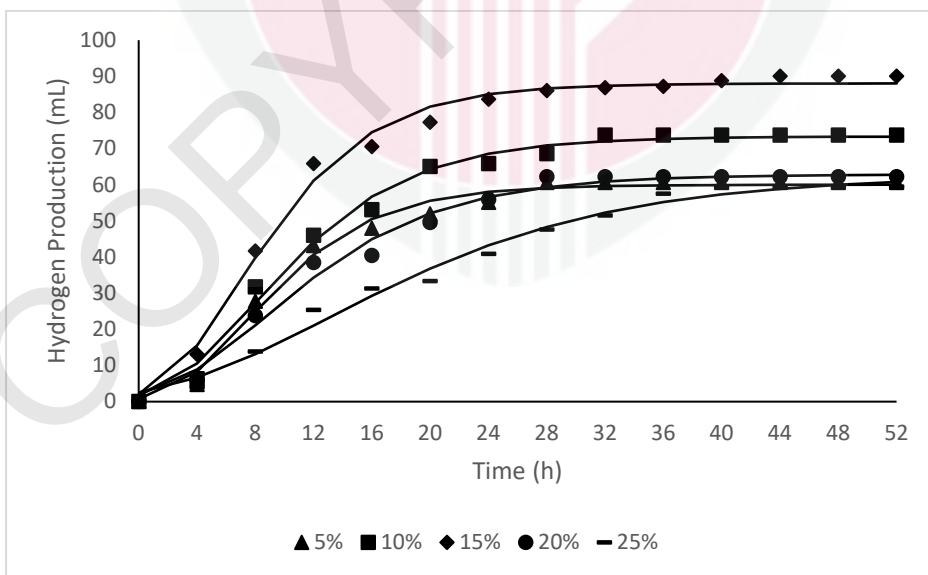


Figure 4.1: Gompertz analysis of different inoculum percentages for hydrogen gas

Based on Figure 4.1, it is shown that the highest production of hydrogen gas was at 15% of inoculum, followed by 10%. The maximum hydrogen produced was 89.99 mL for 15% and 73.74 mL for 10%. The experimental production did not defer far from the Gompertz prediction as the maximum hydrogen gas produced would be 88.06 mL and 73.34 mL. However, the remaining 3 percentages of inoculum have very similar maximum production of hydrogen gas, where 20% produced 62.24 mL, 5% produced 60.66mL and 25% produced 59.47 mL. The difference between the lowest and highest gas production was 30.52 mL, a big gap. 25% of inoculum was not optimum for anaerobic fermentation as cow dung has high cellulose content, causing the bacteria to take more time and energy to undergo hydrolysis, causing the low gas production (Chu & Wang, 2017). 5% was on the opposite where too little inoculum was available in the system, causing low bacteria count, leading to low gas production. Not only was the final product affected, but the time required for the fermentation to begin also took longer. Gompertz analyzed that for 5% of inoculum, the lagged phase was 2.44 hours, comparing to the optimum 15% which only required about 1.70 hours of lagged time.

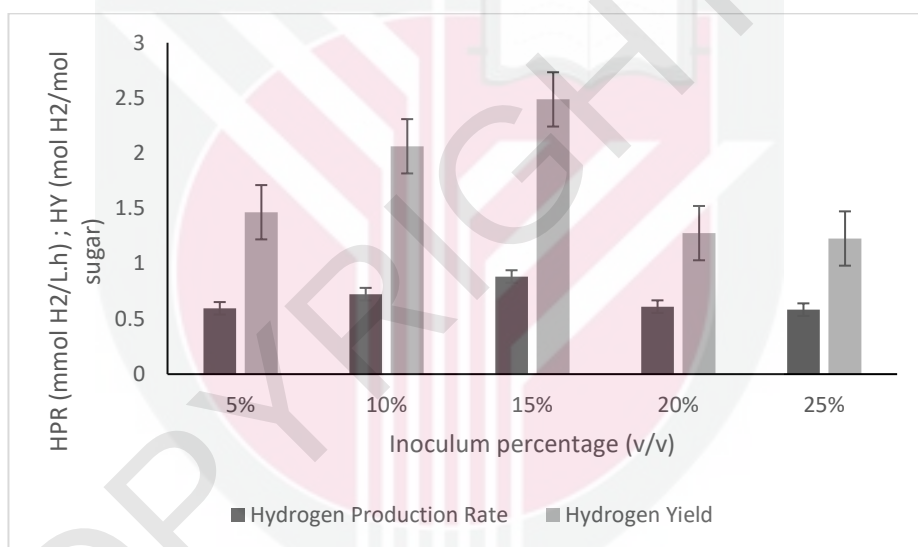


Figure 4.2: HPR and HY based on different inoculum percentage

From the production rate, it is observed that 15% of inoculum has the fastest rate of 0.88 mmolH₂/L.h, they were followed by 10% (0.72 mmolH₂/L.h). Similarly to the gas production, 20% (0.61 mmolH₂/L.h), 5% (0.60 mmolH₂/L.h), and 25% (0.58 mmol.H₂/L.h) did not defer too much from each other. Another aspect to be taken into consideration of gas production is the yield. In this case, 15% also has the highest hydrogen yield (2.49 molH₂/mol sugar), while 25% (1.23 molH₂/mol sugar) is the lowest among all. When the experimental data was calculated using the Modified Gompertz Equation to analyze, it is shown that 15%, with its highest hydrogen, also contributes to the fastest maximum rate at 6.32 mL/h, showing that using 15% of inoculum, the hydrogen gas produced could go up to 6.32 mL in an hour. Then, the rate slows down from 10% (4.54

mL/h) to the slowest at 25%, which could only produce at a maximum rate of 2.07 mL/h.

Table 4.2: HPR, HY and Gompertz analysis based on different inoculum percentage

Sam ples	Hydrogen		Modified Gompertz Equation			Total Volatile Fatty Acids
	Producti on Rate, HPR (mmolH ₂ /L.h)	Yield, HY (mol H ₂ /mol sugar)	Maximum Hydrogen , Hm (mL)	Maximu m Rate, Rm (mL/h)	Lagged time, Lamda (h)	TVFA (gCH ₃ C OOH/L)
5%	0.60	1.47	60.03	4.48	2.44	59.80
10%	0.72	2.06	73.34	4.54	1.99	75.60
15%	0.88	2.49	88.06	6.32	1.70	80.10
20%	0.61	1.28	62.87	3.40	1.77	72.90
25%	0.58	1.23	62.61	2.07	1.83	58.40

The hydrogen yield (HY) in this study is calculated based on the mol of hydrogen in 1 mol of sugar. Based on Table 4.2, the highest hydrogen yield was 2.49 mol H₂/mol sugar with the inoculum percentage of 15%. If acetate is the end product, a stoichiometric yield of 4 mol of hydrogen per mol glucose is obtained. If butyrate is the end product, then 2 mol of hydrogen is produced per mol of glucose. However, the formation of propionate requires the utilization of 2 mol of hydrogen. In contrast, the production of ethanol produces no hydrogen (Sukphun et al., 2023). When acid pre-treated POME was used in anaerobic system, the batch fermentation maximum hydrogen production rate and yield of 0.208 mmol/L/h and 1.69 mol H₂/molTC_{consumed} were achieved at optimum pre-treatment conditions of pH 5.5 and thermophilic temperature (60 °C) (Arisht et al., 2022). Another study done using agricultural waste such as corn and wheat

straw, showed the hydrogen yield to be at 80.09 mL/gTS and 62.49 mL/gTS (Yameng Li et al., 2018). From other study was found that under mesophilic fermentation, the maximum hydrogen yield was 228.01 mL/glucose (Y. Zhang et al., 2023).

Since this was a two-stage biohydrogen and biomethane production, TVFA from the hydrogen reactor was the feedstock to the second stage of the process, which was the methane reactor. Therefore, TVFA for hydrogen reactor was also analyzed and found out that it was relative to hydrogen production. The higher the production of hydrogen gas, the higher the VFA produced. So, the highest production of 15% also has the highest TVFA (80.10 gCH₃COOH/L), and 10% was the second highest (75.60 gCH₃COOH/L), followed by 20% (72.90 gCH₃COOH/L). The lowest two, 5% and 25% had TVFA that were close (59.80 and 58.40 gCH₃COOH/L). The initial TVFA in cow dung was only about 12 gCH₃COOH/L, so even at 25% could also produce about 5 times more of VFA than its initial. Hence, cow dung fermentation is very much suitable for a two-stage biohydrogen and biomethane production because it does produce not only high hydrogen yield for the first part of the reaction but also high TVFA as the end result that will then be carried on to the second part of the reaction (Yin et al., 2021).

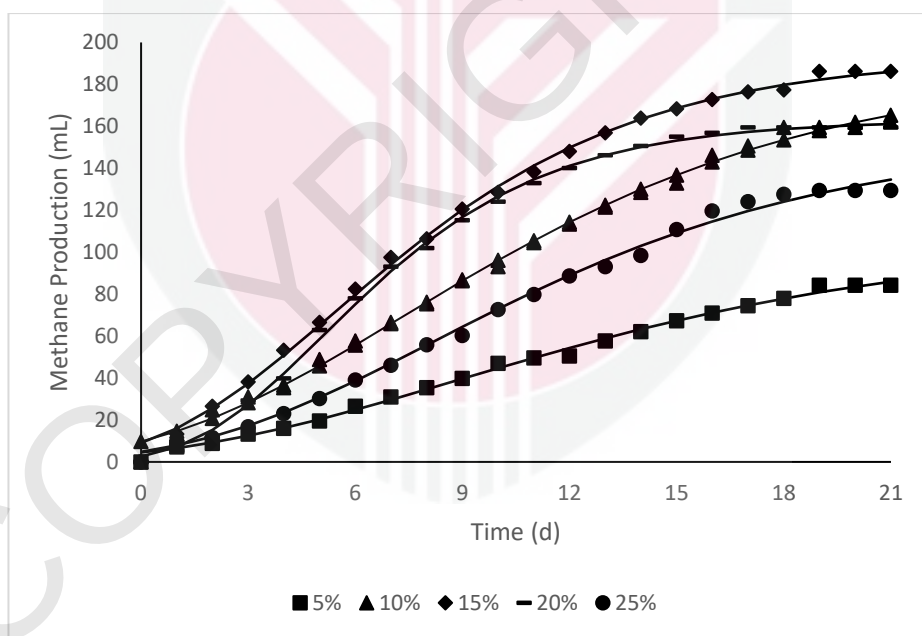


Figure 4.3: Gompertz analysis on different inoculum percentages for methane gas

For methane production, the process took a longer time hence the time unit was recorded in days instead of hours. Figure 4.3 shows that methane production slowed down after 14 days and eventually stopped after day 18. Similar to hydrogen production, the highest cumulative methane production was also 15%

of inoculum (186.14 mL) where it has a steady inclination as compared to the other 4 samples. However, 20% was close to 15% entering the second week of fermentation but the process slowed down and reached to a constant at 159.55 mL. Following by 10% inoculum, which had the third highest methane production based on the graph trend but it was competitive with 20% towards the ending with a slightly higher methane gas at 162.21 mL. 5% has the lowest methane gas production (84.21 mL) towards the end of the batch fermentation.

However, the experimental data did not differ much after being analyzed using the modified Gompertz equation. Based on Table 4.3, for 15% inoculum, the maximum methane produced was 193.55mL with a maximum rate of 16.80 mL/d, indicating that in a day, by using 15% of inoculum, the methane gas could produce up to 17 mL in a day. Of course, methane is a much slower process when compared to hydrogen production. The second highest was 10% (188.0 mL), with a maximum rate of 14.72 mL/d. There was an obvious difference between the highest (10%) and the lowest (5%) as the slowest maximum rate was only 5.04 mL/d with low maximum methane at 105.60 mL. Even so, the lagged phase for 5% was not the slowest at 27.36 hours but then it quickly came to a constant where it could not produce more methane gas and stayed stationary for a long period of time. From the graph, it was also observed that 15% has the highest gas production and the shortest lagged time (13.68 hours). Usually, for methane fermentation, the lagged phase could go up for a few days before the microorganisms could start to work (Aryal et al., 2018). But using substrate from stage 1 to feed into the methane reactor has contributed to the ready TVFA; hence the lagged time was also significantly shortened (Hallenbeck & Ghosh, 2009). This could solve the longer lagged time of methane production as the whole process was already sped up.

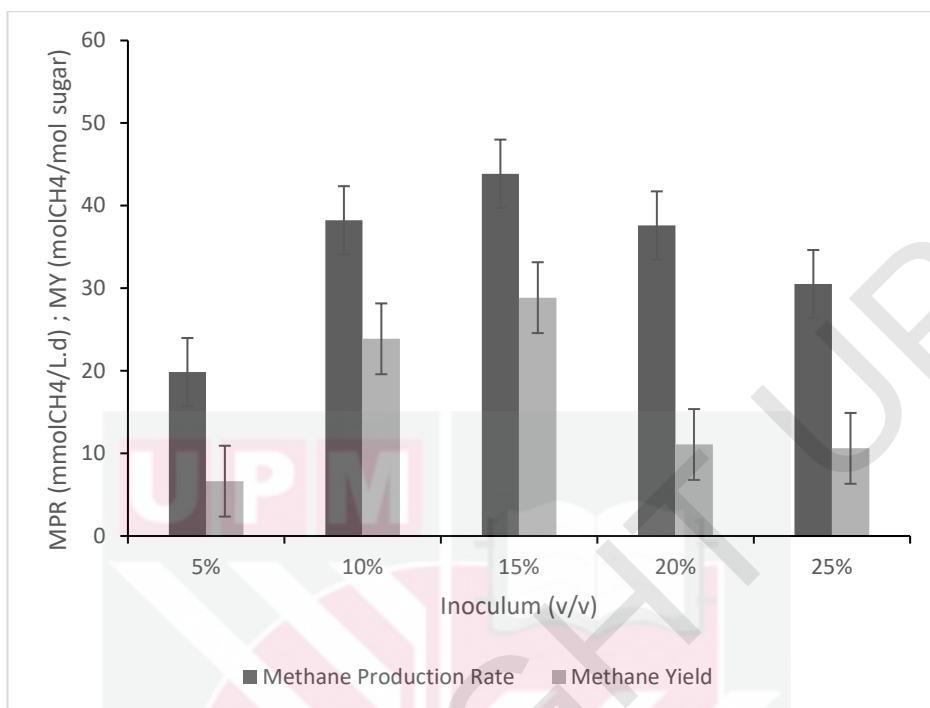


Figure 4.4: MPR and MY based on different inoculum percentage

Based on Figure 4.4, it is shown that with 15% of inoculum, the methane production rate was the highest (43.87 mmolCH₄/L.d), and the lowest was 5% of inoculum (19.85 mmolCH₄/L.d). 15% also has the highest methane yield (28.86 molCH₄/mol sugar) followed by 10% (23.88 molCH₄/mol sugar). Both 25% and 20% have very close values for methane yield where they only have a slight difference (11.08 and 10.61 molCH₄/mol sugar). With its lowest methane production rate, 5% also has the lowest methane yield, only 6.64 molCH₄/mol sugar. The yield for 5% is 4 times lesser when it was using 15% of inoculum. Other studies showed that using municipal solid waste as substrate under mesophilic fermentation, 263 L/kg-tCOD_{removal} of methane yield was obtained (Nguyen et al., 2021). Other research such as using corn steep liquor also obtained 11.8 L/L of methane yield during the anaerobic fermentation using 35°C temperature (Chorukova et al., 2022). Another study suggested methane production using molasses using pH 7 and 35°C temperature, yielded 0.35 L/g-COD of methane (Vilela et al., 2021).

Table 4.3: MPR, MY and Gompertz analysis based on different inoculum percentage

Samples	Modified Gompertz Equation					Total Volatile Fatty Acids
	Methane					TVFA
	Production Rate, MPR	Yield, MY (mmol CH ₄ /L.d)	Maximum Methane, Mm (mL)	Maximum Rate, Rm (mL/d)	Lagged time, λ (hr)	
5%	19.85	6.64	105.60	5.04	27.36	80.00
10%	38.23	23.88	188.00	14.72	14.16	72.50
15%	43.87	28.86	193.55	16.80	13.68	68.80
20%	37.60	11.08	162.70	10.28	38.16	78.00
25%	30.50	10.61	154.82	8.71	38.40	90.20

Based on Table 4.3, the lowest value for TVFA was 15%, with only 68.80gCH₃COOH/L and the second lowest was 10% with 72.50 gCH₃COOH/L. From 80.10 gCH₃COOH/L in the hydrogen reactor, TVFA has been consumed in the methane reaction causing the value to decrease. It was observed that a similar situation occurred for 10% inoculum. For the rest, the TVFA value did not decrease but went to the opposite trend, showing that in the hydrogen reactor, the process might not be thoroughly done hence in the methane reactor, some of the hydrogen microbes were still undergoing hydrolysis phase in order to produce more TVFA for methane reaction to occur (Albini et al., 2019). However,

even in the methane reaction, some acids were also produced, contributing to the high TVFA value.

In conclusion, it is shown that the highest hydrogen production was using 15% inoculum percentage at 0.88 mmolH₂/L.h, then contributing to the highest methane production also when using 15% of inoculum at 43.87 mmolCH₄/L.d. At 15% also it was observed to have the highest yield for both hydrogen and methane at 2.49 molH₂/mol sugar and 28.86 molCH₄/mol sugar each. Hence, 15% of inoculum percentage is the optimum for two-stage biohydrogen and biomethane production and this percentage was then used throughout the rest of the experiment.

4.4 Microbial Growth Rate on Glucose Utilization (g/L)

Carbohydrates are the source of energy provider for the microorganisms involved in the chemical reaction to produce biohydrogen and biomethane gases. From the characterization of cow dung, it was found that cow dung itself contained 6.27 ± 1.01 g/L of total carbohydrates. From the literature that have studied, it was found that while optimum hydrogen yield was determined at a glucose level of 10 g/L in granular sludge or biofilm reactor, a sharp drop in hydrogen yield occurred at glucose content larger than 40 g/L (Raketh et al., 2023). Therefore, this part of the project utilised a range of glucose from 2.5g/L to 12.5 g/L to determine the microbial growth rate based on Monod Modelling. The method of TVSS is used to determine the biomass attached because bacteria was mostly organic hence after deriving from TVS, the remains could indicate the organic-solids concentration. Hence, higher the biomass attached indicating more microbes were attached on the samples during the fermentation process (Jamali et al., 2019).

Table 4.4: Specific growth rate, hydrogen productivity, and maximum biomass obtained from each of the different sugar loading evaluations

Sugar Loading (g/L)	Sugar Consumption (g/L)	Lag Phase (h)	Maximum Biomass (gVSS/L)	Specific Growth Rate, μ (h ⁻¹)	Hydrogen Production Rate (mmol H ₂ /L.h)
2.5	2.07	5.18	12.67	0.69	0.54
5	4.02	4.61	13.33	0.71	0.75
7.5	6.41	4.03	15.33	0.84	0.77
10	8.94	2.28	18.67	0.96	0.88
12.5	10.37	2.51	18.33	0.94	0.87

As tabulated in Table 4.4, the lowest sugar loading was 2.5 g/L with a sugar consumption of 2.07 g/L. The maximum biomass produced was 12.67 gVSS/L. The HPR obtained was also the slowest among all samples which was 0.54 mmolH₂/L.h. When the sugar loading increased from 2.5 g/L to 5.0 g/L, it was observed that the specific growth rate, $\mu_{\max}$ has a slight increment from 0.69 h⁻¹ to 0.71 h⁻¹, and resulting in higher biomass (13.33 gVSS/L). Then, when sugar loading was added to 7.5 g/L, sugar consumed increased to 6.41 g/L, where the maximum biomass produced grew more at 15.33 gVSS/L. Another trend to observe was that the lag phase of hydrogen production has also decreased following the faster specific growth rate of microbes. At 10 g/L, microbial growth rate has reached its peak at 0.96h⁻¹ and also the highest HPR of 0.88 mmolH₂/L.h. At this point, the maximum biomass was the highest (18.67 gVSS/L) and also lowest lag phase of 2.28 hours. When sugar was continued to add at a consistency of 2.5 g/L, the maximum biomass produced showed a slight decrease (18.33 gVSS/L) hence affecting the specific growth rate to drop slightly as well, causing the HPR to maintain almost equal to 10 g/L sample.

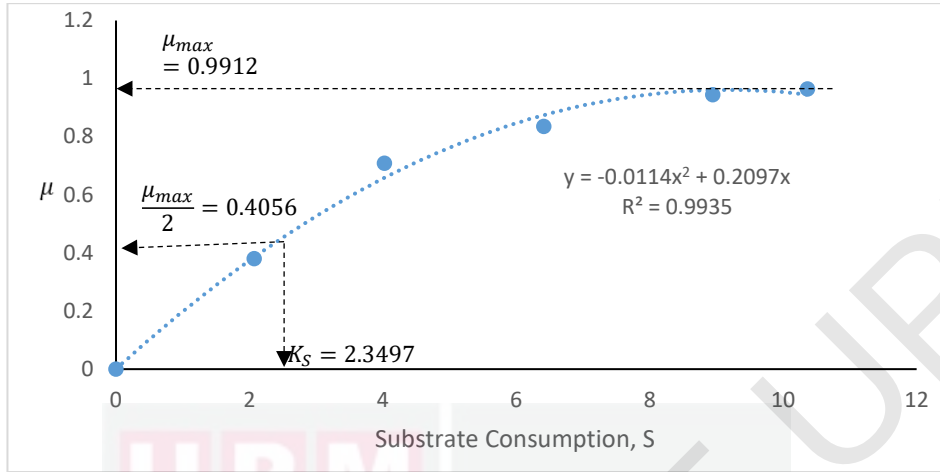


Figure 4.5: Graph of specific growth rate, μ (h^{-1}) versus substrate consumption, S (g/L) for all five different sugars loading studied for hydrogen production.

The correlation between specific growth rate and sugar consumed was confirmed using the Monod kinetic model, as shown in Figure 4.5. From the above figure, the maximum specific growth rate ($\mu_{\max}$), obtained was 0.99 h^{-1} , and the specific growth rate (μ), was 0.49 h^{-1} ($\mu_{\max}/2$) which led to the cell growth saturation coefficient (K_s) of 2.35. Compared to the results of the specific growth rate in Table 4.4, when sugar loading was 10 g/L to 12.5 g/L, the specific growth rate was consistent to 0.99 h^{-1} as deduced from the Monod kinetic model. Based on this observation, it indicates that 10 g/L was the optimum sugar utilisation level for hydrogen production. As more sugar concentration was added to the reactor, the bacteria growth was shown to be stationary hence causing more wastage of sugar that is not fully utilized by the microbes. In this study, the cell growth saturation coefficient, K_s obtained was 2.35, lower than the optimum sugar consumed of 10 g/L. In Monod model equation, the value of K_s , the substrate affinity of the hydrogen producing culture was found depending on the carbohydrate content of the substrate. Therefore, in this system, the specific growth of attached-biofilm was measured based on kinetic growth rate as attained:

$$\mu = \frac{0.991}{S + 2.35} \quad (4.1)$$

Table 4.5: Specific growth rate, methane productivity, and maximum biomass obtained from each of different sugar loading evaluation

Sugar Loading (g/L)	Sugar Consumption (g/L)	Lag Phase (h)	Maximum Biomass (gVSS/L)	Specific Growth Rate, μ (h ⁻¹)	Methane Production Rate (mmol CH ₄ /L.day)
2.5	2.37	19.31	12.00	0.39	24.39
5	4.66	14.75	14.33	0.69	27.88
7.5	7.05	13.90	19.67	0.70	28.40
10	9.58	8.56	22.00	0.74	32.93
12.5	11.50	9.88	20.33	0.91	30.84

Similar to hydrogen production, in the second stage of methane production, the range of sugar loading in the reactor was also from 2.5 g/L to 12.5 g/L. Starting from the lowest, almost all of the sugar content was consumed. Even so, the maximum biomass was the lowest of all (12.00 gVSS/L) and has the longest lag phase of 19.31 hours. The specific growth rate was also low (0.39 h⁻¹) and has the lowest methane productivity at 24.39 mmolCH₄/L.d. When the sugar loading increased from 5.0 to 7.5 g/L, the lag phase showed a decrement from 14.75 hours to 13.90 hours. The biomass produced in the system has also increased from 14.33 gVSS/L to 19.67 gVSS/L. The specific growth rate for these two sugar contents did not show much difference, 0.69 and 0.70 h⁻¹ each. So, the methane production rate did not differ much for these two samples as well, 27.88 and 28.40 mmolCH₄/L.d each. From Table 4.5 it was observed that methane productivity peaked when sugar was added to 10g/L at 32.93 mmolCH₄/L.d. This also led to a slight increase in specific growth rate (0.74 h⁻¹), and biomass was then increased to 22.00 gVSS/L. When using 10 g/L of sugar, methane production also has the shortest lag phase which was 8.56 hours. However, based on Figure 4.6, it was shown that when sugar loading was 12.5 g/L, the specific growth rate was beyond the maximum rate. Even so, methane productivity has decreased to 30.84 mmolCH₄/L.d, and maximum biomass has also decreased to 20.33 gVSS/L.

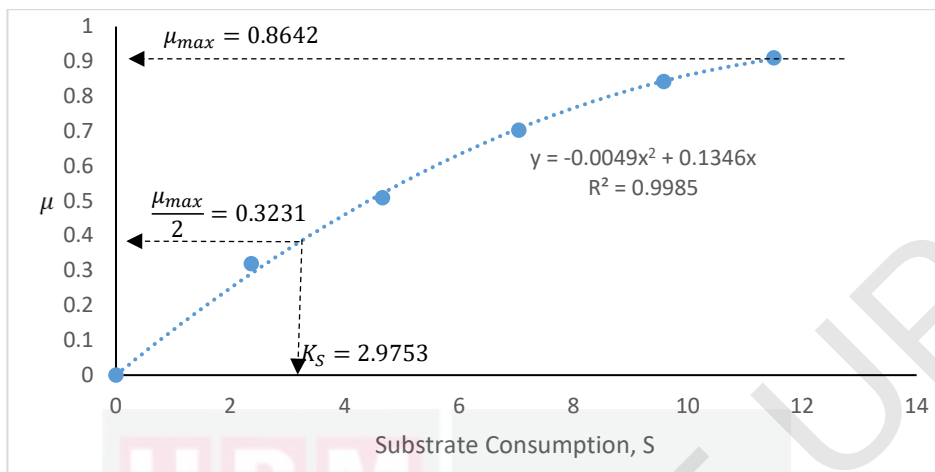


Figure 4.6: Graph of specific growth rate, μ (h^{-1}) versus substrate consumption, $S(\text{g/L})$ for all five different sugars loading studied for methane production

From Figure 4.6, the maximum specific growth rate ($\mu_{\max}$), obtained was 0.86 h^{-1} , and the specific growth rate (μ), was 0.32 h^{-1} ($\mu_{\max}/2$), which lead to the cell growth saturation coefficient (K_s) of 2.98. Sugar loading of 10 g/L has the closest specific growth rate to the maximum one which was 0.74 h^{-1} . When 12.5 g/L of sugar was added, the growth rate was an outlier showing that excessive sugar was added only causing wastage as methane productivity decreased along with the maximum biomass produced. In methane production, the cell growth saturation coefficient, K_s obtained was 2.98, higher than the lowest sugar loading in this study but lower than the optimum sugar consumed of 10 g/L . Therefore, in this system, the specific growth of attached biofilm was measured based on the kinetic growth rate as attained:

$$\mu = \frac{0.8642}{S + 2.9753} \quad (4.2)$$

4.5 Effect of GAC on Biofilm Formation in Sequencing Batch Reactor

The fermentation for this part of the study was done in a sequencing batch reactor where acclimatization of the cells was required. The volume of biogas produced was recorded daily. Hydraulic retention time (HRT) for hydrogen reactor was 48 hours while methane reactor took longer, 14 days. HRT was selected based on the batch profiling performance done in Sections 4.3 and 4.4, showing the production of biohydrogen 4.3 and 4.4, and biohydrogen production stayed constant after 48 hours while methane was after 14 days. The optimum inoculum percentage was 15% with 10g/L of glucose added for

both hydrogen and methane reactors.

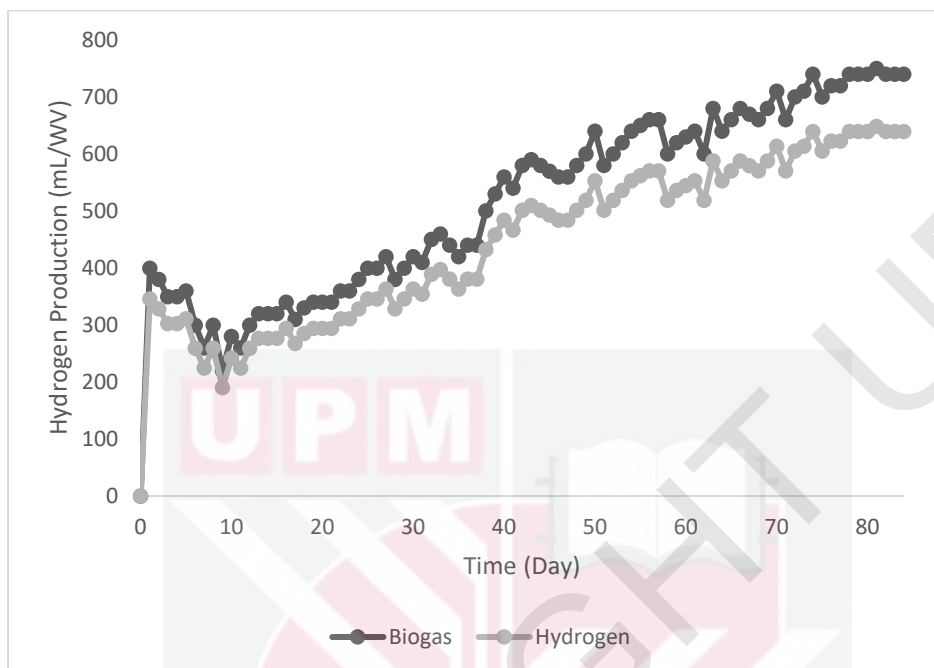


Figure 4.7: Graph of biogas and hydrogen production recorded daily

An increasing trend was observed based on Figure 4.7, where the hydrogen production started with 0mL at the very beginning and with rather fast pace has increased up to 400mL using 800mL of working volume on the next day. Within the same week, hydrogen gas produced was inconsistent with high fluctuation as it dropped to the lowest at 200 mL/WV. The microbes in the reactor were adapting to the new environment introduced; hence some time was needed before the production could be consistent. In the following 10 to 20 days, hydrogen production was showing an exponential rise, from 190 mL/WV to 580 mL/WV. During the exponential phase, the microbes already adapted to the environment and started to feed on the carbohydrate in the reactor, producing almost triple of its lowest gas production. From day 40 to 50, a steady rise of hydrogen production was still observed. After day 50, hydrogen production has slowly settled down at 640 mL/WV to 710 mL/WV. There was still little fluctuation observed at this stage. The high and low in between was mainly caused by the short HRT where the substrate in the reactor was replaced, hence there was a sudden spike when the microbes were fed to new medium (Barca et al., 2016). The microbes then entered the steady state phase where the hydrogen production could not go any further and only has little changes for the last 10 days. Hydrogen production was maintained at a volume between 740 to 750 mL/WV in this fermentation phase, concluding the final hydrogen production was at 750 mL/WV per day after being stable.

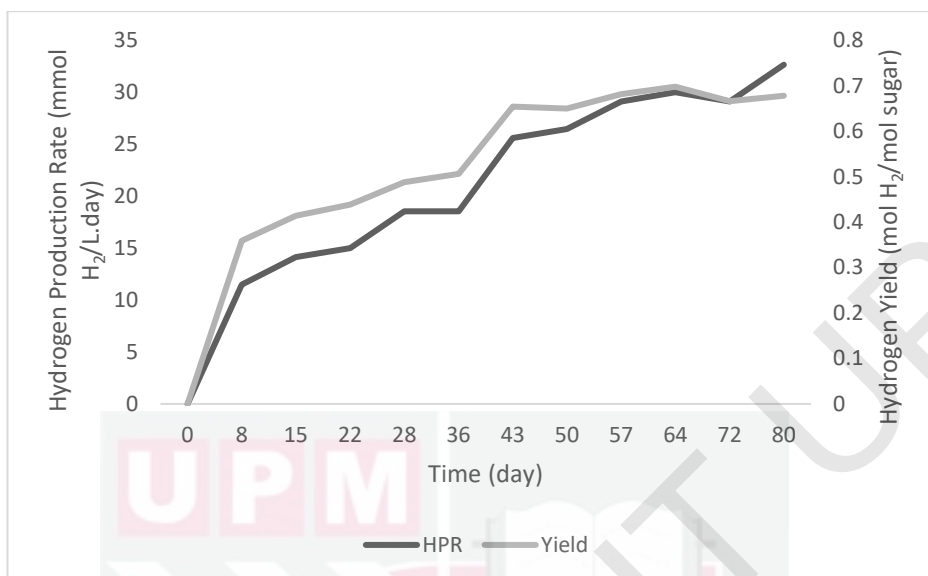


Figure 4.8: Hydrogen production rate and hydrogen yield

Figure 4.8 also shows the hydrogen production rate (HPR) and hydrogen yield (HY) graph. HPR represents the concentration of hydrogen produced in a day in 1 liter. The trend of the graph has clearly portrayed that as time passed by, the productivity of hydrogen increased. When productivity increases, HY should be increasing as well as yield was showing the mol of hydrogen gas produced for 1mol of sugar. According to the chemical equation of dark fermentation, 1mol of sugar could produce up to 4 mol of hydrogen gas. Therefore, the microorganisms were getting more active in the reactor, hence consuming more glucose, leading to increased hydrogen production (Albini et al., 2019). The graph in Figure 4.8 shows a steep rise for both HPR and HY, similar to the biogas production in Figure 4.7, indicating that the microbes in the reactor were consuming available carbohydrate, and glucose hence producing a lot of gases. On day 8, the highest HPR was already 11.48 mmolH₂/L.d and HY was 0.36 molH₂/mol sugar. Then, entering the following weeks, HPR and HY were rising at a slower pace fluctuating around 14 to 20 mmolH₂/L.d for HPR and 0.4 to 0.5 molH₂/mol sugar for HY. On the 6th week of fermentation, HPR and HY reached a peak before entering the constant phase, where HPR was 25.61 mmolH₂/L.d and HY was 0.65 molH₂/mol sugar. The productivity of hydrogen gas did not stop there but only had small increment, finally stopping at 32.67 mmolH₂/L.d. For HY, there were so few changes as seen on the graph, finally stopped at 0.68 molH₂/mol sugar.

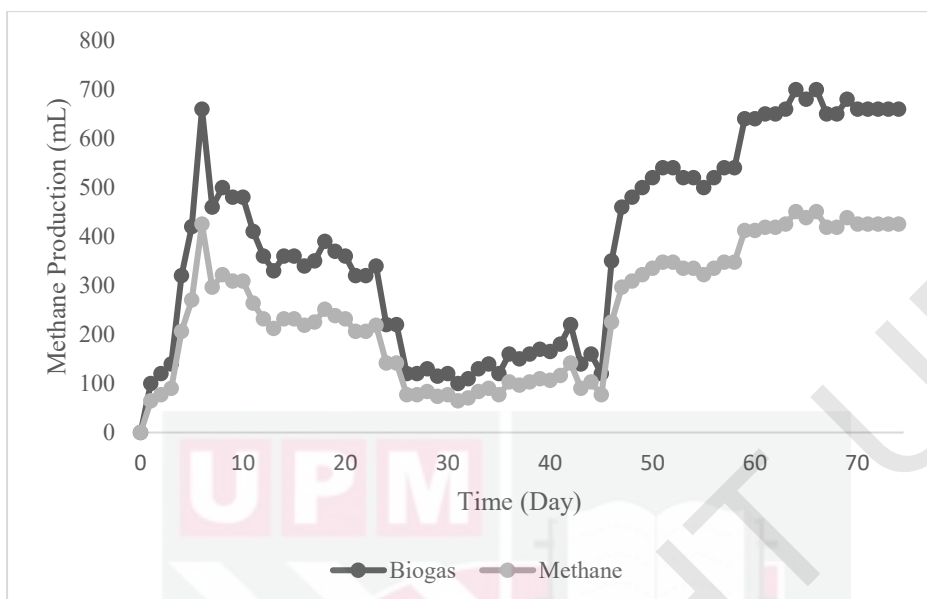


Figure 4.9: Graph of biogas and methane production recorded daily

On the other hand, for methane reactor has an overall bigger fluctuation throughout the 75 days of fermentation. At the beginning, methane production started at 0 mL/WV and then within a week, the gas production was increased up to the highest production of 660 mL in 800 mL of working volume. This showed a promising result, but as soon as entering the second week of fermentation, gas production dropped drastically to 500 mL/WV and then further down to 220 mL/WV. After day 25, the production rate has stayed at its lowest, ranging from 120 to 160 mL/WV. Then, on day 45, methane production showed a positive side, increasing tremendously to 460 mL/WV within a week. The addition of substrate from hydrogen reactor provided TVFA readily for methanogens to kick start the fermentation process, hence the increasing trend. But once consumed, the methanogens required a longer time to adapt to the environment and to digest the substrate, therefore it experienced a drop in methane production and required longer period of downtime compared to hydrogen as it has longer HRT (Bergland et al., 2015). After that, methane production continued to grow higher until 650 mL/WV on day 60. After the last HRT cycle, methane production has finally reached its constant phase and remained in the range of 640 to 660 mL/WV of production.

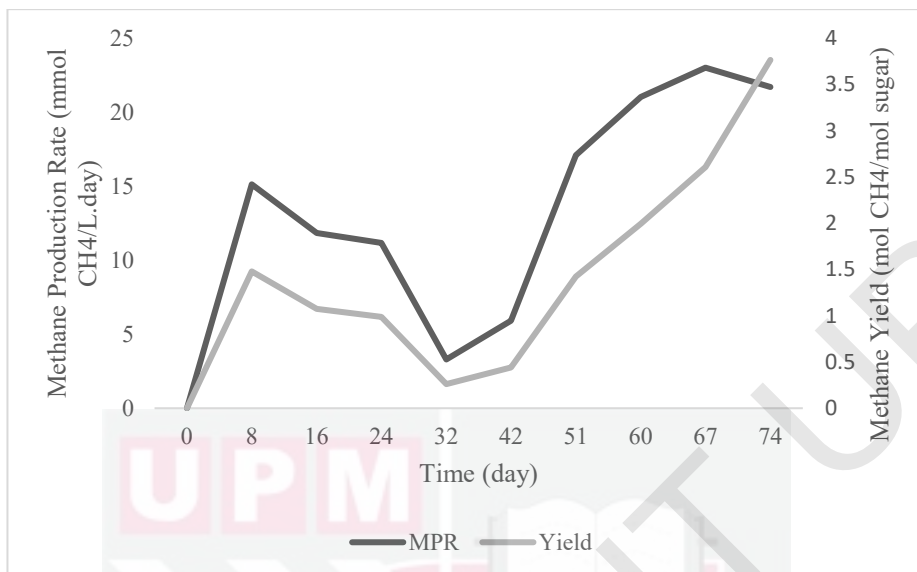


Figure 4.10: Methane production rate and methane yield

Methane production rate (MPR) and methane yield (MY) have similar trends as the biogas production as shown in Figures 4.9 and 4.10. MPR and MY also experienced excitement within the first week of fermentation, where the productivity and yield increased from 0 to 15.13 mmolCH₄/L.d and 1.48 molCH₄/mol sugar each. Then, methane microorganisms started to slow down and experienced a downfall especially after day 24 where MPR dropped from 11.18 mmolCH₄/L.d to its lowest low (3.29 mmolCH₄/L.d). Similarly, MY has also dropped from 0.99 molCH₄/mol sugar to only 0.26 molCH₄/mol sugar. After another cycle of HRT, when a new substrate was added, there was a sudden rise for both MPR and MY, as seen in Figure 4.10. The continuous increment showed good methane productivity as it reached to the last cycle of HRT, showing steady productivity of 21.71 mmolCH₄/L.d and 3.77 molCH₄/mol sugar of methane yield.

Table 4.6: Productivity, yield and analysis of hydrogen and methane in SBR

Sample	Productivity		Yield		COD Removal		TVFA	Energy Product ion Rate	Biomass attached
	%	mmol gas/L.d	L gas/L.d	mol gas/mol sugar	%		gCH ₃ COOH/L	kJ gas/L.d	gVSS/mL
Hydrogen	86.45	32.67	0.80	0.68	82.86		27.43	5.20E-06	1.07
Methane	64.42	21.71	0.53	3.77	57.14		17.48	3.45E-06	1.37

Based on the summary in Table 4.6, hydrogen has a higher productivity than methane as hydrogen gas has higher gas purity and higher mol of gas based on the metabolic pathway. Gas sample from both reactors were tested using gas chromatography (GC) and it was found that in the biogas from hydrogen reactor, it contained 86.45% of hydrogen while the biogas from methane reactor was 64.42%. Both had more than half of its gas content made up of the gas wanted from the reaction. In terms of productivity, hydrogen has 32.67 mmolH₂/L.d and methane has 21.27 mmolCH₄/L.d only. In terms of gas yield, hydrogen has 0.68molH₂/mol sugar while methane has higher yield which was 3.77molCH₄/mol sugar. Similar to research done by Raketh also showing that methane yield is higher than hydrogen in a two-stage bioreactor where the highest methane production yield of 558.01 mLCH₄/g-VS was achieved from hydrogen effluent and the highest hydrogen yield of 115.57 mLH₂/g-VS was obtained (Raketh et al., 2023). As discussed earlier, substrate from the hydrogen reactor has high TVFA (27.53 gCH₃COOH/L) compared to methane (17.48 gCH₃COOH/L), showing that substrate from the hydrogen reactor can be substrate for the second stage of the reaction for methane production. Chemical oxygen demand (COD) removal for the hydrogen reactor was 82.86% which was very promising and 57.14% for the methane reactor which was very good. Biogas production not only produces green energy for the industry, it could also

be an alternative for wastewater treatment as it shows significant drop in COD level for both reactors (Mohammadi et al., 2014).

The weight of microbial cell (VSS) on the GAC was analyzed for hydrogen and methane reactor representing the accumulated biomass. The weight ratio of biofilm attached cell on GAC over to clean GAC as a blank was determined to be 1.07 ± 0.01 gVSS/g GAC and 1.37 ± 0.01 gVSS/g GAC, respectively as shown in Table 4.6. The development of biofilm on the surface of the GAC was effectively formed as proven by the above result. The biofilm developed by the attachment helped to maintain cell viability and prevented the cells from washing out of the system, resulting in increased culture density. The surface porosity of the GAC made it easier for the sludge to attach to the carrier surface and form a stable biofilm (Jamali et al., 2016). Thus, steady biohydrogen and biomethane productions were also achieved towards the end of the process.

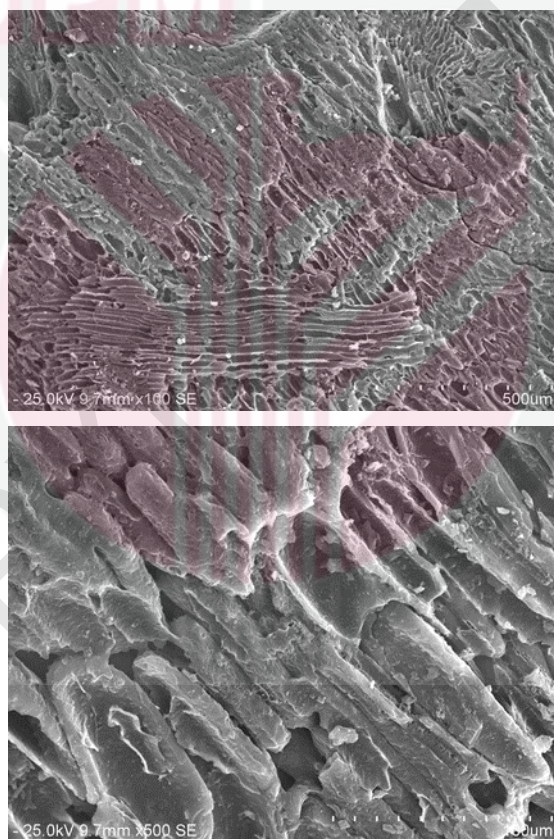


Figure 4.11: SEM Image of cell immobilization under magnification of 100 and 500

The findings also demonstrated that the acclimatization process encouraged cell density to attach on the GAC surface layer, resulting in steady and consistent biohydrogen and biomethane production under mesophilic conditions.

Therefore, it leads to having a high propensity in binding capability, primarily for organic matter, and thus provide a nutrient-rich environment that promotes microbial adhesion due to the mechanical stability of biofilms formed on activated carbon (Chu et al., 2011). The immobilization methods are primarily founded on the granulation or biofilm attachment approaches, with the biofilm attachment method being used in this experiment (Hawkes et al., 2007). Biofilm-based methods have been extensively studied as immobilization cell systems in hydrogen and methane production as they improve production rate and population dynamics (Kerčmar & Pintar, 2017).

In a nutshell, GAC is a good carrier for biofilm formation as it helps sustain the cultural density in a system, enhancing biohydrogen and biomethane production. Hydrogen and methane are also well produced under the addition of GAC as its inert property did not disturb the chemical content inside the reactor. To improve the sustainability of GAC, alginate and chitosan were added to form GAC beads.

4.6 Optimization of Alginate on GAC Beads

In order to provide an immobilized carrier that could increase the productivity of biohydrogen and biomethane production, in this study, GAC was coated with alginate and chitosan. In the alginate gel matrix, microorganisms are well regulated and are determined to have a pore size of less than 17nm. The small pore size aids in retaining microbial cell at low HRT and harsh anaerobic environment (Jung, Jeong, et al., 2016) while chitosan is often known to be one of the best matrix chromatography because it has amino ($-NH_2$) groups and hydroxyl ($-OH$) which it provides active reaction for links the enzymes efficiently (Bouyahmed et al., 2018).

2 parameters that could affect the synthesis of such beads were studied, which are the concentration of GAC added and the concentration of alginate/chitosan. For the optimization of the beads, these two parameters were considered and the concentration for GAC was selected in the range of 0.50 g/mL, 0.75 g/mL and 1.00 g/mL. Alongside with it was the concentration of alginate 1, 2 and 3% were selected. Another parameter that could affect the sizes of the beads was the drying method. Three methods were used in this optimization: air dry, oven dry and freeze dry. Corresponding to the 3 factors, the beads were analyzed based on 3 responses, which were diameter (cm/bead), stability (%) and swelling degree (%). The stability of the beads depends on its mechanical strength. The beads could burst when the osmotic pressure inside the beads was too high especially under the condition where the beads were to submerged in the substrate for a long period of time. Beads with a higher stability meaning the beads could sustain for longer time in the reactor (Huang & Lin, 2017). On the other hand, swelling in polymers refers to the absorption of a solvent or liquid, causing an increase in the polymer's volume. Swelling of the beads can induce mechanical stress on the polymer structure, causing internal strain or tension. This stress can weaken the polymer chains and make them more susceptible to destruction (Stachowiak et al., 2023).

27 experiments were run based on the factors and are tabulated as shown in Table 4.7. The diameter of the beads was not more than 2 cm in average. The most oversized diameter was 1.59cm/bead while the most minor diameter was 1.01 cm/bead when dried with air. The big diameter was due to the high concentration for both GAC and alginate as the one with 1.59 cm has 1.00 g/mL of GAC using 3% of alginate concentration. Contrary to the smallest bead, it has the lowest concentration for GAC and alginate, which were 0.50 g/mL and 1.00%. These factors could significantly affect the sizes of the beads.

The alginate beads have a fairly high percentage of stability as well. Some of the beads has a 100% stability, indicating that the beads did not burst after submerging in sodium chloride solution for 24 hours. A hundred per cent stability was observed when the alginate concentration was either 2 or 3% with the lower GAC concentration. The lower the GAC content, the beads have more spaces which was made up of alginate hence providing more stable beads, causing the beads not to burst easily in the solution. On the other hand, at the lowest stability of 46.67%, the GAC concentration was reasonably high at 1.00 g/mL and also the lowest alginate concentration of 1%. Hence, the beads would burst easily, resulting in low stability.

For swelling degree, which determines the mass of the beads before and after being immersed in water for 24 hours, the lowest was 34.28% while the highest could go up to 93.46%. At concentration alginate of 1.0% and concentration of GAC 0.5 g/mL, the swelling degree was at its lowest, but when the concentration of GAC was increased to 1.00 g/mL, the swelling degree was the highest, even though they have the same drying method. Therefore, the swelling degree is not a significant response in optimizing alginate beads.

Table 4.7: Numeric factors of experimental designs with three independent variables and stability and swelling degree for alginate beads

Run	Factors			Experimental Response		
	Conc.	Conc.	Drying	Diameter	Stability	Swelling
	GAC					
	(g/mL)	Al (%)	method	(cm/bead)	y (%)	degree (%)
	<i>x</i>	<i>y</i>	<i>z</i>	<i>d</i>		
1	0.75	2.00	Oven	1.48	100.00	54.64
2	1.00	1.00	Oven	1.29	46.67	93.62
3	0.75	2.00	Air	1.53	96.55	45.54

Table 4.7: Continued

4	0.75	3.00	Freeze	1.50	96.77	76.32
5	0.50	2.00	Oven	1.40	100.00	62.90
6	0.75	3.00	Oven	1.56	85.29	84.90
7	0.50	3.00	Oven	1.46	100.00	60.74
8	0.75	1.00	Air	1.37	88.88	72.72
9	0.75	1.00	Oven	1.32	57.14	57.06
10	0.75	1.00	Freeze	1.26	91.30	60.34
11	1.00	2.00	Air	1.46	80.00	70.08
12	0.50	3.00	Air	1.45	100.00	56.96
13	0.50	1.00	Freeze	1.10	95.83	34.28
14	1.00	1.00	Freeze	1.23	66.00	93.46
15	0.50	1.00	Oven	1.06	75.00	64.02
16	0.50	3.00	Freeze	1.41	91.30	57.32
17	1.00	2.00	Freeze	1.38	97.96	76.92
18	1.00	1.00	Air	1.37	65.00	83.04
19	0.50	1.00	Air	1.01	89.00	87.70
20	0.50	2.00	Air	1.37	95.83	44.28
21	1.00	2.00	Oven	1.52	97.96	70.82
22	1.00	3.00	Air	1.59	97.96	69.30
23	0.75	3.00	Air	1.44	100.00	73.86
24	0.75	2.00	Freeze	1.50	100.00	71.06
25	0.50	2.00	Freeze	1.33	100.00	73.28
26	1.00	3.00	Oven	1.54	95.83	49.68
27	1.00	3.00	Freeze	1.55	100.00	87.90

By applying the multiple regression analysis to the test results using CCD, the quadratic polynomial relating the factors and the responses was developed as shown in Equation 4.3, 4.4, 4.5.

$$\text{Diameter (cm/bead)} = -0.180741 + 2.32x + 0.552778y - 0.12xy - 1.2444x^2 - 0.079444y^2 \quad (4.3)$$

$$\text{Stability (\%)} = 66.90519 + 21.48x + 34.43972y + 69.88333xy - 144.46222x^2 - 17.75722y^2 \quad (4.4)$$

$$\text{Swelling Degree (\%)} = -3.88556 + 83.77333x + 13.64167y - 10.75333xy \quad (4.5)$$

Where x is concentration of GAC (g/mL) and y is concentration of alginate (%).

An analysis of variance (ANOVA) was conducted to investigate the significance of fit for the quadratic model based on experimental data Table 4.8, 4.9 and 4.10. The p-value was used to investigate the significance of each coefficient and the degree of interaction between each of the independent variables studied. The independent variables of selected factors are more significant with greater F-value and small p-value.

Table 4.8: ANOVA for the response diameter (cm/bead) for alginate beads

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	p-value	Remark
Model	0.55	11.00	0.05	13.61	< 0.0001	significant
A-Conc. GAC	0.10	1.00	0.10	27.31	0.00	
B-Conc. Alginate	0.34	1.00	0.34	94.29	< 0.0001	
C-Drying method	0.01	2.00	0.00	1.25	0.31	
AB	0.01	1.00	0.01	2.96	0.11	

Table 4.8: Continued

AC	0.01	2.00	0.00	0.84	0.45
BC	0.00	2.00	0.00	0.35	0.71
A ²	0.04	1.00	0.04	9.94	0.01
B ²	0.04	1.00	0.04	10.37	0.01
Residual	0.05	15.00	0.00		
Cor Total	0.60	26.00			
Coefficient of determination, R ² = 0.909; adjusted R ² = 0.842; predicted R ² = 0.676					

From analysis of ANOVA for response data of diameter (cm/bead) as summarized in Table 4.8, the model F-value of 13.61 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.050 indicate model terms are significant. In this case, A, B, A², B² are significant model terms. Values greater than 0.10 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The predicted R² of 0.676 is in reasonable agreement with the adjusted R² of 0.842 as the difference is less than 0.2. Adequate precision measures the signal-to-noise ratio. A ratio that is greater than 4 is desirable. In this case, the ratio based on ANOVA is 12.30, which indicates an adequate signal. Therefore, this model can be used to navigate the design space.

Table 4.9: ANOVA for the response stability (%) for alginate beads

Source	Sum of Squares	Degree Freedom	Mean Square	F-value	p-value	
Model	15004.74	11.00	1364.07	5.29	0.00	significant
A-Conc.	2802.01	1.00	2802.01	10.87	0.00	
GAC						
B-Conc.	5594.35	1.00	5594.35	21.70	0.00	
Alginate						

Table 4.9: Continued

C-Drying method	55.67	2.00	27.84	0.11	0.90
AB	3662.76	1.00	3662.76	14.21	0.00
AC	453.23	2.00	226.62	0.88	0.44
BC	55.67	2.00	27.84	0.11	0.90
A ²	489.13	1.00	489.13	1.90	0.19
B ²	1891.91	1.00	1891.91	7.34	0.02
Residual	3866.33	15.00	257.76		
Cor	18871.07	26.00			
Total					
Coefficient of determination, $R^2 = 0.795$; adjusted $R^2 = 0.645$; predicted $R^2 = 0.845$					

For stability, statistical ANOVA was shown that the model Fvalue of 5.29 implies the model is significant. There is only 0.18% chance that an F-value this large could occur due to noise. P-values less than 0.05 indicate model terms are significant. A, B, AB, and B2 are significant model terms in this case. The predicted R^2 of 0.245 is not as close to the adjusted R^2 of 0.645 as one might normally expect as the difference is more than 0.2. This may indicate a large block effect that is affected by the outliers such as the 100% stability found in Table 4.9. Besides, the ratio of adequate precision of 8.490 which is greater than 4 indicates an adequate signal.

Table 4.10: ANOVA for the response swelling degree (%) for alginate beads

Source	Sum of Squares	Degree Freedom	Mean Square	F-value	p-value
Model	2669.96	9.00	296.66	1.10	0.41
A-Conc.	987.75	1.00	987.75	3.66	0.07
GAC					
B-Conc.	134.81	1.00	134.81	0.50	0.49
Alginate					
C-Drying method	64.48	2.00	32.24	0.12	0.89
AB	86.73	1.00	86.73	0.32	0.58
AC	613.15	2.00	306.57	1.13	0.34
BC	783.04	2.00	391.52	1.45	0.26
Residual	4591.89	17.00	270.11		
Cor Total	7261.85	26.00			
Coefficient of determination, $R^2 = 0.368$; adjusted $R^2 = 0.033$; predicted $R^2 = -0.861$					

Based on ANOVA for the third response, swelling degree, the model F-value of 1.10 implies the model is not significant relative to noise. There is a 41.34% chance that an F-value this large could occur due to noise. Besides, P-values less than 0.05 indicate model terms are significant; hence in this case, there are no significant model terms as all the values are more than 0.05 as shown in Table 4.10. From the table also showed a negative predicted R^2 . This negative value of predicted R^2 implies that the overall mean may better predict swelling degree. The adequate precision of 4.228 indicates an adequate signal.

In order to understand the effects of variables studied on the responses, three-dimensional (3D) response surface graphs were plotted where the response on

Z-axis against two independent variables. When the graphs are presented in this manner, it could show the effect of different variables at an optimum level while having the third variable maintained as constant value. The shape of the 3D graphs could show the significance of the mutual interaction between the variables.

Based on Figure 4.11, it is shown that effect between two variables at which the third variable was constant. From the 3D plots indicated that the interaction between the variables for diameters and stability was significant but swelling degree was not. For the diameter of the beads, as the alginate concentration increases, the diameter of the beads increased to a certain extent until it reached the peak and then starts to go downhill along with the increment of the alginate concentration. Similarly, for the concentration of GAC. When the amount of GAC added to the beads were more, diameter of the beads increased as well and then decreased towards the maximum. Even though more GAC was added to the formation of the beads but excessive of beads caused the GAC hard to bind together to form beads. Hence, after drying, GAC started to fall off the beads, causing the beads to be smaller compared to the optimum. The falling off of GAC from the beads also indicated that the beads' stability decreased when the concentration of GAC increased. A similar trend can be observed from the 3D plots. On the other hand, the swelling degree of the beads showed no significance to the factors.

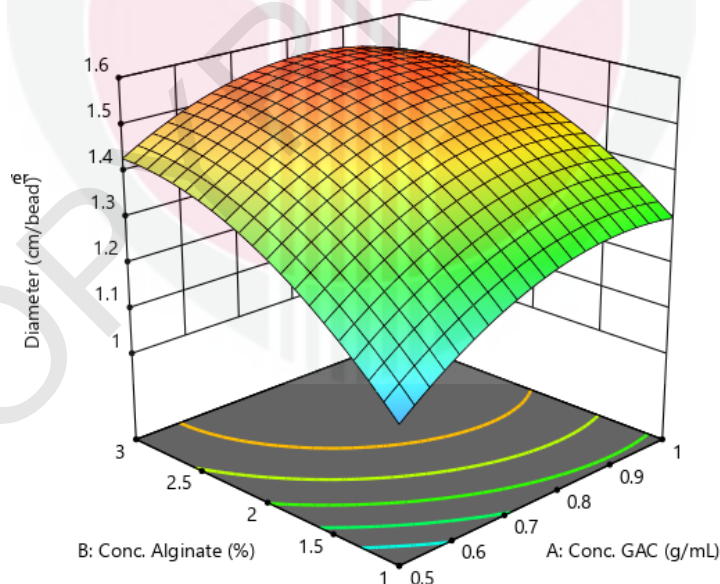


Figure 4.12A: 3D plots of alginate beads for diameter

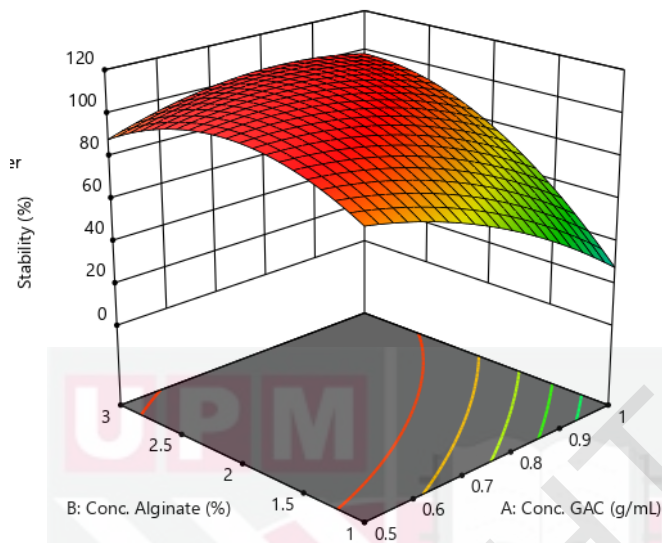


Figure 4.12B: 3D plots of alginate beads for stability

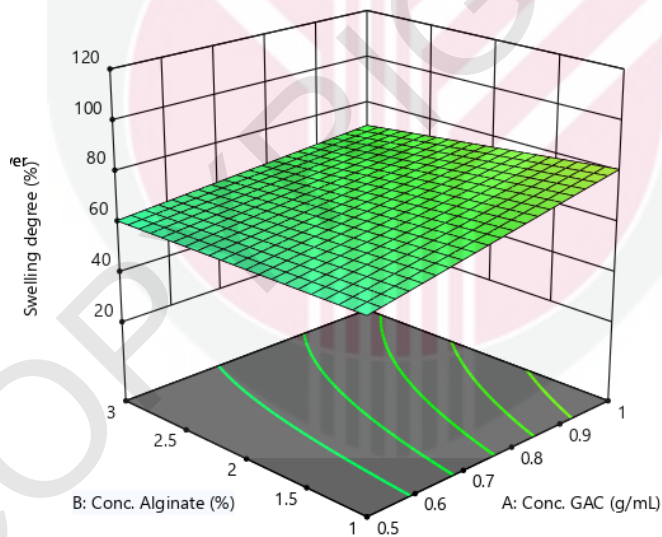
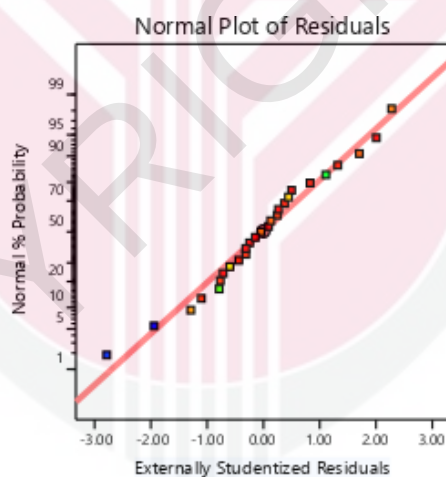
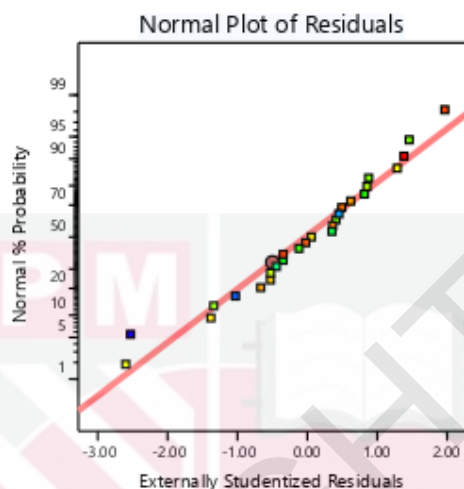


Figure 4.12C: 3D plots of alginate beads for swelling degree

Figure 4.12 verifies that the predicted data of the response surface by the regression model have corresponded to those observed in the range of the process parameters tested. It is essential to determine the adequacy of the fitted model in order to make sure that this model provides an approximation to the

actual conditions. The regression model can be described as inaccurate when there is insufficient fit to the data. From Figure 4.12, the normal assumption was acceptable for both diameter and stability. But for the swelling degree, the points were plotted away from the normal line showing that it is inadequate to describe the formation of alginate beads.



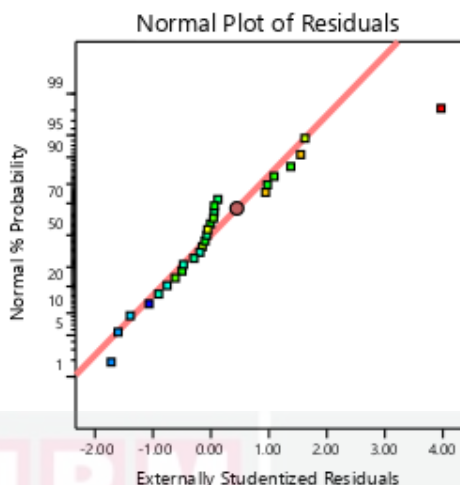


Figure 4.13: Normal probability of studentized residuals for alginate beads

Table 4.11: Confirmation of model validity based from optimum condition predicted by RSM

Model experiment	Diameter	Stability	Swelling Degree
	cm/bead	%	%
Predicted by RSM	1.15	100.00	48.17
Experimental Obtained	1.24	97.60	45.15
Error (%)	6.63	2.40	6.26

The model then analyzed the optimum condition to produce alginate beads, and it concluded that the optimum conditions included using a concentration of GAC 0.500g/mL, a concentration of Alginate 1.230% and then drying the beads using the freezing method. The model also predicted the diameter of the beads to be 1.15 cm/bead with a stability of 100% and a swelling degree of 48.17%. Alginate beads were produced using the optimum conditions provided by the model and then analyzed again based on the 3 responses. It was found that the diameter was 1.24 cm/bead on average. The percentage of error was 6.63%, which is considerably low. For the stability, experimentally found out that it was 97.60% with an error of only 2.40%, which has a small error only. Lastly, the swelling degree based on the experiment was 45.15%, showing an error of 6.26%, which is also lower than 10%.

In conclusion, the optimum factors in producing alginate beads are 0.50 g/mL of GAC, 1.23% of alginate, and then freezing as the drying method. The model showed that these factors affect the diameter and stability of alginate beads. However, it shows no significance towards the swelling degree of alginate beads. The model's accuracy was confirmed through the error of prediction by RMS and experimental, showing all of the responses resulted in a small margin of error under the optimum conditions.

4.7 Optimization of Chitosan on GAC Beads

Another material added to the beads to enhance the beads performance was chitosan, a natural polymer substance made from shell crab. The three factors selected for optimizing GAC beads were similar to alginate beads, including the concentration of GAC (mg/L), the concentration of chitosan (%) and drying method. To justify the responses of the beads, the range of concentration for both GAC and chitosan selected were also the same as alginate beads. For GAC, the range of concentration was from 0.50 g/mL, 0.75 g/mL and 1.00 g/mL, while the concentration of chitosan selected were 1%, 2% and 3%. The three drying methods were also same with alginate beads: air dry, freeze dry and oven dry.

A total of 27 runs of experiment were carried out with different concentration of GAC, chitosan and drying method. Based on Table 4.12, the smallest size of the beads was 1.08cm/bead. Beads with this size also have the lowest stability, at only 57.14% but with a high swelling degree of 96.93%, which was the second highest. From this data set, we could say that the smaller beads size is not optimum in terms of stability and swelling degree as the beads are easily dissolved in the solution indicating the high percentage of swelling degree, causing it to have low stability. Even though this bead is made of 1.00 g/mL of GAC and 2% chitosan, the beads' outcome was relatively small. On the other hand, the biggest bead in size is 1.67 cm/bead. Using the similar concentration of GAC and chitosan, the beads in this experiment were dried up in an oven. The bigger in size did not contribute to a higher stability as the beads only have 75% stability but relatively low swelling degree of 60.74%.

There were several runs of experiments that achieved 100% stability, showing none of the beads dissolved in the solution after 24 hours. These beads include those with all three concentrations of GAC and also mostly 2-3% of chitosan. Due to the high percentage including all three concentrations, it was shown none of the factors affect the responses. There was no significant finding in such similar trend can be observed for a swelling degree as well. A lower swelling degree is desired as the beads should be able to retain its original shape and density. The lowest percentage was 38.96% by using 0.75 g/mL of GAC and 1% of chitosan, while the highest percentage (100%) consists of 0.50 g/mL of GAC and 1% chitosan.

Table 4.12: Numeric factors of experimental designs with three independent variables and stability and swelling degree for chitosan beads

Run	Factors			Experimental Response		
	Conc. GAC (g/mL)	Conc. Chitosan (%)	Drying method	Diameter (cm/bead)	Stabilit y (%)	Swelling degree (%)
	x	y	z			
1	0.75	2.00	Freeze	1.36	100.00	68.92
2	0.75	1.00	Oven	1.20	93.62	93.68
3	0.75	1.00	Freeze	1.23	61.11	58.20
4	0.50	2.00	Freeze	1.46	91.30	66.62
5	0.75	3.00	Freeze	1.38	95.83	65.42
6	0.50	1.00	Air	1.24	58.00	94.60
7	0.75	3.00	Oven	1.40	100.00	69.84
8	0.50	1.00	Oven	1.25	83.33	84.08
9	1.00	2.00	Oven	1.67	75.00	60.74
10	1.00	2.00	Air	1.31	61.54	84.73
11	0.50	3.00	Air	1.41	100.00	97.19
12	0.75	3.00	Air	1.37	95.83	75.10
13	1.00	3.00	Freeze	1.23	100.00	65.26
14	0.50	1.00	Freeze	1.28	78.57	100.00
15	1.00	1.00	Air	1.46	97.96	71.38
16	1.00	2.00	Freeze	1.08	57.14	96.93
17	1.00	1.00	Freeze	1.49	100.00	63.52
18	0.75	2.00	Air	1.35	100.00	73.83

Table 4.12: Continued

19	1.00	3.00	Oven	1.29	91.30	55.26
20	0.50	2.00	Oven	1.43	100.00	79.98
21	1.00	3.00	Air	1.22	100.00	59.97
22	0.75	2.00	Oven	1.34	97.96	72.60
23	0.50	3.00	Oven	1.35	96.55	72.34
24	0.75	1.00	Air	1.25	64.81	38.96
25	1.00	1.00	Oven	1.43	100.00	60.76
26	0.50	3.00	Freeze	1.43	100.00	86.94
27	0.50	2.00	Air	1.41	91.30	78.22

By applying the multiple regression analysis to the test results using CCD, the linear polynomial relating the factors and the responses was developed as shown in Equation 4.6, 4.7, 4.8.

$$\text{Diameter (cm/bead)} = 0.968333 + 0.460000x + 0.271667y - 0.35333xy \quad (4.6)$$

$$\text{Stability (\%)} = 74.00056 - 3.58000x + 7.89500y \quad (4.7)$$

$$\text{Swelling Degree (\%)} = 100.20000 - 31.42667x - 0.992222y \quad (4.8)$$

Where x is concentration of GAC (g/mL) and y is concentration of alginate (%).

An analysis of variance (ANOVA) was conducted to investigate the significance of fit for the quadratic model based on experimental data Table 4.13, 4.14 and 4.15. The p-value was used to investigate the significance of each coefficient and the degree of interaction between each of the independent variables studied. The independent variables of selected factors are more significant with greater F-value and small p-value.

Table 4.13: ANOVA for the response Diameter (cm/bead) for chitosan beads

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remark
Model	0.15	9.00	0.02	1.43	0.25	not significant
A-Conc.	0.00	1.00	0.00	0.03	0.87	
GAC						
B-Conc.	0.00	1.00	0.00	0.29	0.60	
Chitosan						
C-Drying method	0.01	2.00	0.01	0.46	0.64	
AB	0.09	1.00	0.09	7.79	0.01	
AC	0.04	2.00	0.02	1.87	0.19	
BC	0.00	2.00	0.00	0.06	0.94	
Residual	0.20	17.00	0.01			
Cor Total	0.36	26.00				
Coefficient of determination, $R^2 = 0.431$; adjusted $R^2 = 0.130$; predicted $R^2 = -0.447$						

The model F-value of 1.43 implies the model is insignificant relative to noise. There is a 25.05% chance that an F-value this large could occur due to noise. Hence, the diameter response is not significant in optimizing chitosan beads. P-value less than 0.050 indicate model terms are significant. In this case, only AB is a significant model term. The other model terms which are larger than 0.10 are not significant. Hence, it is to be said that in order to improve this model, some of the model terms can be reduced. A negative predicted R^2 (-0.447) implies that the overall mean may better predict the response. A ratio of adequate precision greater than 4 is desirable. In this case, the adequate precision is 4.6704 showing it is adequate, hence this model can be used to navigate the design space.

Table 4.14: ANOVA for the response stability (%) for chitosan beads

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remark
Model	1424.29	4.00	356.07	1.72	0.18	not significant
A-Conc. GAC	14.42	1.00	14.42	0.07	0.79	
B-Conc. Chitosan	1121.96	1.00	1121.96	5.41	0.03	
C-Drying method	287.91	2.00	143.96	0.69	0.51	
Residual	4558.64	22.00	207.21			
Cor Total	5982.93	26.00				
Coefficient of determination, $R^2 = 0.238$; adjusted $R^2 = 0.010$; predicted $R_2 = -0.1512$						

For the stability of the beads, the model F-value of 1.72 implies the model is also not significant relative to the noise. There is a 18.18% chance that an F-value this large could occur due to noise. Also, B (0.03) is a significant model term in this case as the P-value is less than 0.05. Similar with diameter, other model terms can be reduced to improve the model. Stability also has a negative predicted R^2 , implying that the overall mean may be a better predictor, while the adequate precision has a ratio of 4.063, showing that is adequate and this model can be used to navigate the design space.

Table 4.15: ANOVA for the response swelling degree (%) for chitosan beads

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remark
Model	1170.38	4.00	292.60	1.41	0.26	not significant
A-Conc.	1111.09	1.00	1111.09	5.35	0.03	
GAC						
B-Conc.	17.72	1.00	17.72	0.09	0.77	
Chitosan						
C-Drying method	41.57	2.00	20.79	0.10	0.91	
Residual	4565.10	22.00	207.50			
Cor Total	5735.48	26.00				
Coefficient of determination, $R^2 = 0.204$; adjusted $R^2 = 0.059$; predicted $R^2 = -0.177$						

The model F-value for a swelling degree of the chitosan beads is not significant relative to the noise as there is a 26.35% chance that the F-value this large could occur due to noise. In this case, A is a significant model term with P-value of 0.03, lesser than 0.05, while the others are greater than 0.10, showing that they are not significant, and hence can be reduced to improve the model. For swelling degree, a negative R^2 is found as well. Also, the adequate precision of the swelling degree is 3.30, less than the desired 4, hence it should not be used to navigate the design space. A swelling degree is not significant to the design of the experiment.

Three-dimensional (3D) response surface graphs were also plotted for the chitosan beads analysis where the response on Z-axis against two independent variables. When the graphs are presented in this manner, it could show the effect of different variables at an optimum level while having the third variable maintained as a constant value.

From Figure 4.13, the 3D plot shows the relationship between all the 3 different responses towards the 2 factors in an overall drying method. In the case of diameter, the size of the beads increased along with the concentration of chitosan and also GAC. More GAC added in the forming of the beads, the beads will eventually become bigger in size, and higher percentage of chitosan added would ease the process of binding the GAC together in a bead shape. Even after drying, GAC would not easily fall off, forming these beads in increasing size.

Besides that, for the stability of the beads, stability also increases when concentration of chitosan increases. As mentioned earlier, the higher concentration of chitosan helps the beads to hold together firmly, resulting a firmer beads formation. But when the concentration of GAC increases, there were no significant changes in the graph, showing a relatively straight line in the range of 75-80%, hence there is an effect on the stability towards concentration of GAC.

These chitosan beads have the most negligible effect on swelling degree. When the concentration of chitosan increases, the swelling degree remained the same in the range of 80%. This could mean that chitosan did not have the absorption characteristic; hence the beads would not swell up or dissolve in the solution. Anyway, swelling degree is slightly affected by the concentration of GAC; as more GAC is added, it could affect the size of the beads, hence causing the swelling degree to increase.

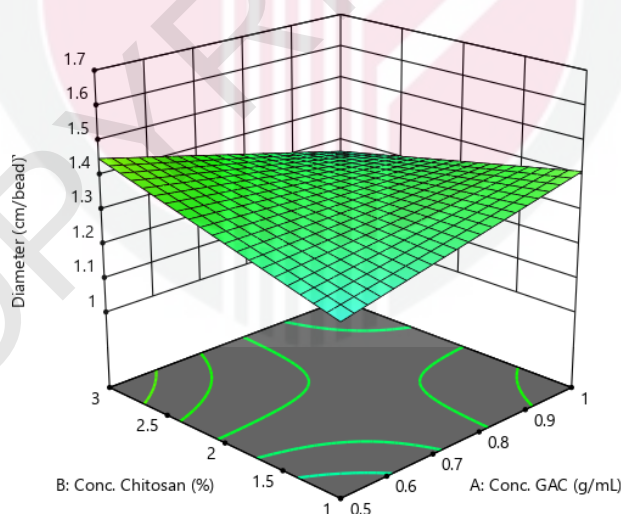


Figure 4.14A: 3D plots of chitosan beads for diameter

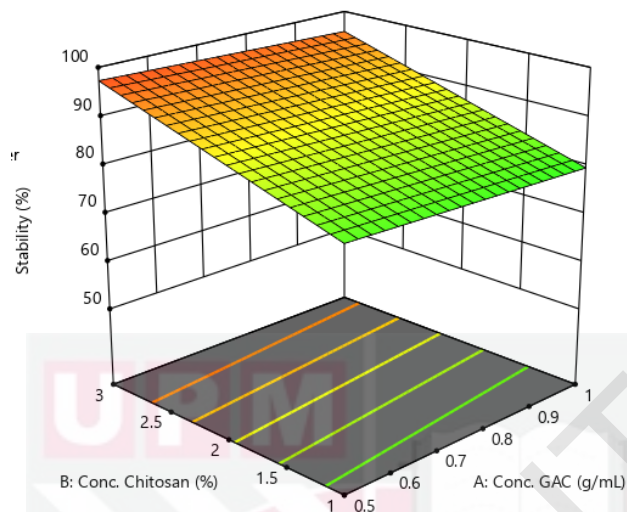


Figure 4.14B: 3D plots of chitosan beads for stability

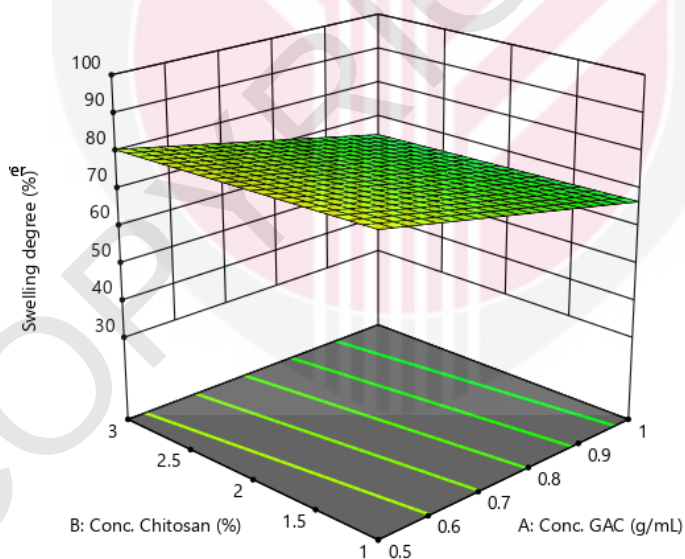
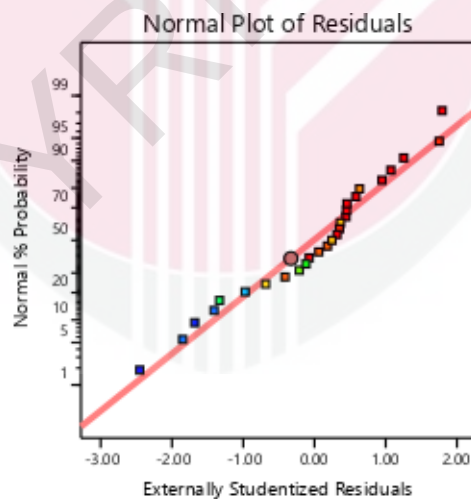
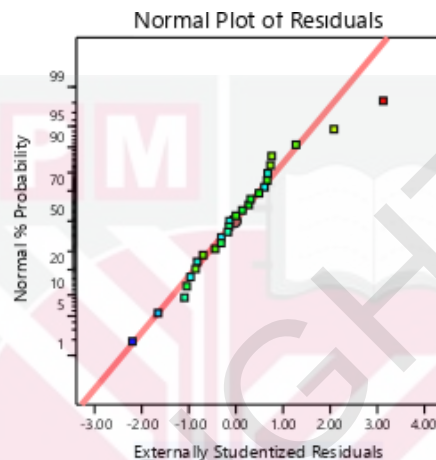


Figure 4.14C: 3D plots of chitosan beads for swelling degree

Figure 4.14 verifies that the predicted data of the response surface by the regression model corresponded to those observed in the range of the process parameters tested. It is crucial to determine the adequacy of the fitted model in order to make sure that this model provides an approximation to the actual

conditions. The regression model can be described as inaccurate when there is insufficient poor fit to the data.

The typical assumption for diameter was acceptable as the points were closed to each other and also towards the regular line. However, a few outliers showed the beads' extreme conditions. For stability of the beads, the points were far from the central and normal line, showing that the data was insignificant. Same goes to the swelling degree of the beads, where the points were moving away from the normal line and also consists of a few outliers.



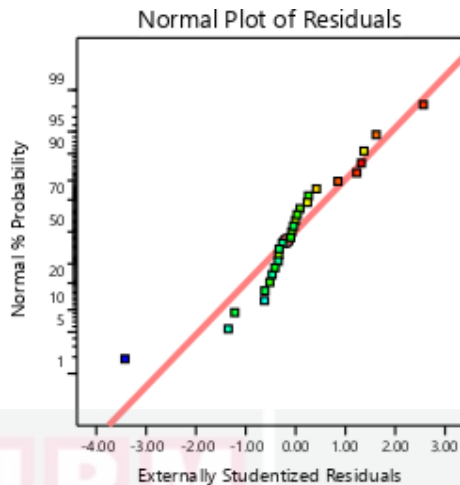


Figure 4.15: Normal probability of studentized residuals for chitosan beads

The RSM analysis calculated the optimum solution and stated that the chitosan beads with a 100% desirability are made of 0.517 g/mL of GAC, 1.546% of chitosan and freeze dry. Another batch of beads were fabricated using the optimum conditions provided by the system and was found out as tabulated in Table 4.16.

Table 4.16: Confirmation of model validity based from optimum condition predicted by RSM for chitosan beads

Model experiment	Diameter	Stability	Swelling Degree
	cm/bead	%	%
Predicted by RSM	1.40	92.11	78.93
Experimental Obtained	1.43	91.30	79.56
Error (%)	2.17	0.89	0.79

Based on RSM prediction, the size of the beads should be 1.40cm/bead with stability of 92.11% and swelling degree 78.93%. From the experimental batch, a very similar size was produced at 1.43cm/bead, with an error of 2.17%. For the stability of the beads, it was found out to be 91.30%, with a minor 0.89% error. Lastly, the swelling degree determined was 79.56%, with only 0.79% the difference. The small difference in error indicated that the optimum beads were confirmed and verified.

To summarize, the optimum condition of chitosan beads is obtained with a diameter of 1.40 cm/bead and high stability of 92%. However, there was no significant effect of the factors towards the responses on the optimization of chitosan beads based on the analysis above. The model's accuracy was confirmed through the error of prediction by RMS and experimental, showing all of the responses resulted in small margin of error under the optimum conditions.

4.8 Effect of Alginate-Chitosan Beads as Carrier on Biohydrogen and Biomethane Production

Based on the optimum conditions obtained from the previous objectives, batch fermentation of biohydrogen and biomethane was carried out using three different carriers in the system: GAC, GAC-Al and GAC-C. The conditions of fermentation used in this part of the experiment were 15% inoculum and 10 g/L of glucose with working volume of 80 mL. Alginate beads and chitosan beads were also fabricated again using the optimum selection suggested by RSM with the highest desirability. The volume of these carriers as immobilized beads added into the reactor for batch fermentation were the same which was 1:1 ratio to the inoculum (15% of WV). Even though GAC-Al and GAC-C coated with different material but the GAC used for the coating is the same as the one used as control which is GAC of the same size and same type.

The batch profile was fermented for 52 hours, where the gas production started to reach a stationary profile. In the first quarter of the experiment, it was observed that all three of the systems performed rather slowly with close production among the three. After the 12th hours, hydrogen production increased exponentially. However, hydrogen production for GAC-Al was still considered to be low compared to GAC and GAC-C. GAC-C had the best performance among all as observed in Figure 4.18. The maximum hydrogen production during the exponential phase was up to 46.0 mL, while GAC was 38.5 mL and the least, GAC-Al, only produced 35.7 mL of hydrogen gases. After entering the 36th hour, hydrogen production started to slow down and entering their respective stationary phase. There was still minor fluctuation but then remained constant for the last 3 points of the profile. GAC-C ended with the highest hydrogen production, with a total of 63.8 mL, followed by GAC 60.3 mL, only a slight difference of 3.5 mL with the highest amount. The lowest production was GAC-Al, only 48.4 mL of hydrogen gas was collected.

The RSM analysis concluded that chitosan beads did not have significant effects on the stability and swelling degree of the beads. The experiment showed that chitosan beads could sustain the rough environments of an anaerobic digester, hence providing a stable carrier for the microorganisms to adhere to the beads. Consequently, producing more hydrogen gas. Microorganisms also preferred chitosan, which has a cation group that is able to bind them together and enhance the gas production.

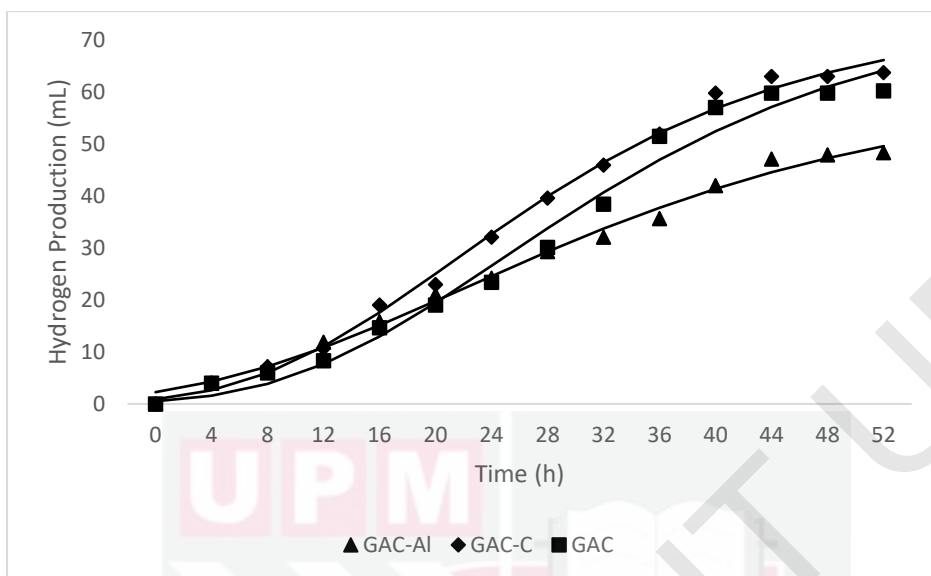


Figure 4.16: Hydrogen production based on modified Gompertz equation for GAC, GAC-AI and GAC-C

When comparing the hydrogen production rate (HPR) of these three batches, it was found that GAC-C has the highest HPR of $0.63 \text{ mmolH}_2/\text{L.h} \pm 0.02$, which was slightly higher than the GAC batch of $0.59 \text{ mmolH}_2/\text{L.h} \pm 0.03$. GAC-AI had the lowest among all which was $0.47 \text{ mmolH}_2/\text{L.h} \pm 0.02$. The same as well for the hydrogen yield (HY), GAC-C had the highest yield of $1.12 \text{ molH}_2/\text{mol sugar}$, followed by GAC ($1.02 \text{ molH}_2/\text{mol sugar}$) and the lowest was GAC-AI ($0.70 \text{ molH}_2/\text{mol sugar}$).

Both HPR and HY show that GAC-C had the best performance with the highest value of HPR and HY, respectively. For each mol of sugar, 1.12 mol of hydrogen gas can be produced. Even so, the result did not differ much from that of GAC because GAC itself is a very strong carrier and widely used material for anaerobic fermentation. Previous studies showed that chitosan inhibits methanogenesis by reducing the activity of methanogens. There is a consent that flocculation of humic substances by chitosan alleviates its interference with electron transport, leading to enhanced the enzymes activity presence within the reactor. Even though some researchers stated that chitosan limits the hydrolysis and acidification of the digestion system, but the main pathways of hydrogen production in the fermentation process are ethanol- and butyrate-type fermentations, and the amount of hydrogen production fluctuates only when the acid-fermentative pathways associated with hydrogen production change. (Fan et al., 2023).

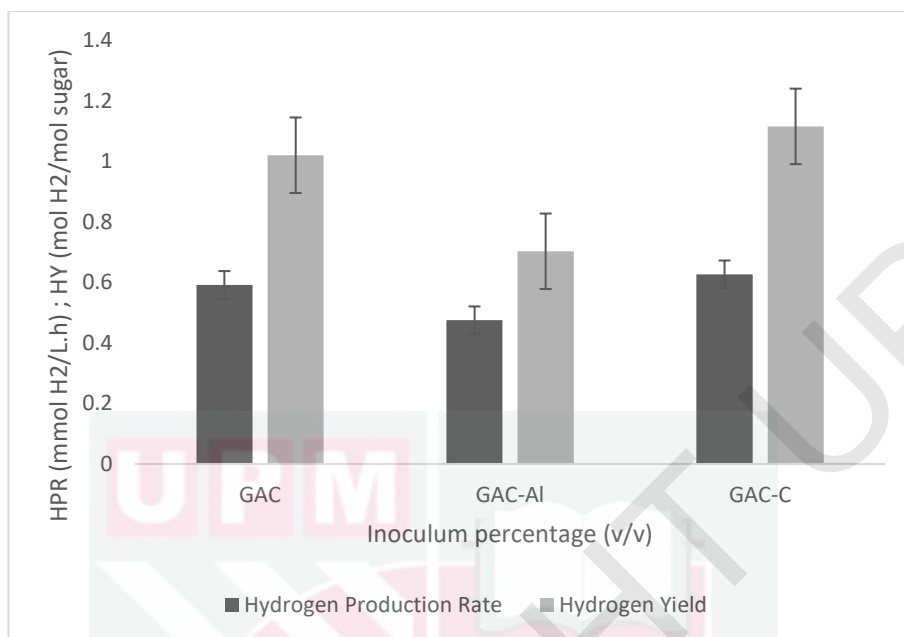


Figure 4.17: Hydrogen production rate and hydrogen yield for GAC, GAC-Al and GAC-C

When fitted into the modified Gompertz equation, GAC-C has the highest maximum hydrogen (76.17 mL) and maximum rate (1.92 mL/h). The maximum rate indicates that GAC-C could produce 1.92 mL of hydrogen gas in one hour, while GAC-Al could only produce 1.20 mL, which is 0.72 mL lesser. Not only that, the maximum hydrogen production for GAC-Al was the lowest (60.60 mL) with the longest lagged time. 9.39 hours was required for the microorganisms to start being active to enter the exponential phase. Considering GAC-C only required 3.51 hours, GAC-Al definitely used longer hours. For GAC, the lagged time calculated by modified Gompertz equation was 6.93 hour, considerably long as well for the microorganisms to start producing hydrogen gases. Chitosan has high hydrophilicity due to having a large number of hydroxyl groups and functional groups in the structure such as amine groups. These were some of the reasons why chitosan has high adsorption potential. Not only that, the functional groups' presence also helped to increase chemical reactivity between the cells and chitosan hence the microorganisms were more likely to be adsorbed on GAC-C, which contributed to the shorter lagged phase (Hsu et al., 2024).

Table 4.17: Hydrogen productivity and maximum biomass obtained from GAC, GAC-AI and GAC-C

Sampl es	Modified Gompertz					Total Volatile Fatty Acids,TV FA	Bio mas s atta che d (gV SS/ mL)
	Hydrogen		Equation				
	Producti	Yield,	Maxi	Maxim	Lagg		
	on Rate,	HY	mum	um	ed		
	HPR		Hydr	Rate,	time,		
(mmolH	(mol		Rm	Lamd			
2/L.h)	H2/mol	(mL)	(mL/h)	(h)	(gCH3CO		
		sugar)			OH/L)		
GAC	0.59	1.02	74.29	1.82	6.93	71.38	0.63
GAC- AI	0.47	0.70	60.60	1.20	9.39	65.33	0.35
GAC- C	0.63	1.12	76.17	1.92	3.51	74.18	0.80

After the fermentation, beads sample from each batch was removed to determine their biomasses attached. Based on the data in Table 4.17, GAC-C has the highest biomass attached. There was 0.80 gVSS/mL of biomass found and 0.63 gVSS/mL of biomass attached on the GAC batch of samples. However, for GAC-AI, biomass attached was significantly lower, with only 0.35 gVSS/mL. Alginate is not stable when submerged in the acidic solution as fermentation produced TVFA for more than 24 hours, causing some of the beads to disperse, hence microbes in the system could not attach to the beads for a longer period. When exposed to the harsh environment, the microbes could not perform well, resulting in the low production of hydrogen gas. In another word, chitosan does not easily disintegrate in acidic solution, hence the ability to allow the microbes to bind on the surface (Segale et al., 2016). The method of TVSS is used to determine the biomass attached on the beads because bacteria was mostly

organic hence after deriving from TVS, the remains could indicate the organic-solids concentration. Hence, higher the biomass attached indicating more microbes were attached on the samples during the fermentation process (Jamali et al., 2019).

Then, the substrate from the hydrogen reactor was used in the methane reactors for methane gas production. Similar to the previous system, GAC, GAC-Al and GAC-C beads were used in three different batches of fermentation. The fermentative conditions were based on the optimum results obtained from previous objectives, where 10g/L of glucose was used with 15% of inoculum in an 80mL working volume.

The methane gas production is shown in Figure 4.17. Methane production requires a longer period of time hence 21 days of fermentation was selected. In the first week of the fermentation process, lesser gas was produced as the microbes in the digester would require more time to acclimatize. Three carriers have produced very close levels of methane gases, in the range of 40mL to 50 mL. Then, the production started to grow gradually, until the stationary phase. Entering week 2 of fermentation, it was apparent that GAC-C has higher methane gas production compared to the other two. By the end of week 2, methane gas production for GAC-C had achieved 81mL, while GAC and GAC-Al only produced 69.5 mL and 69.3 mL respectively. The stationary phase of the methane fermentation started around day 18, when the gas production for these three systems has reached a constant value. The maximum methane gas produced by GAC-C was up to 116.6 mL, while GAC produced 89.2 mL and the least would be GAC-Al, with only 85.1 mL.

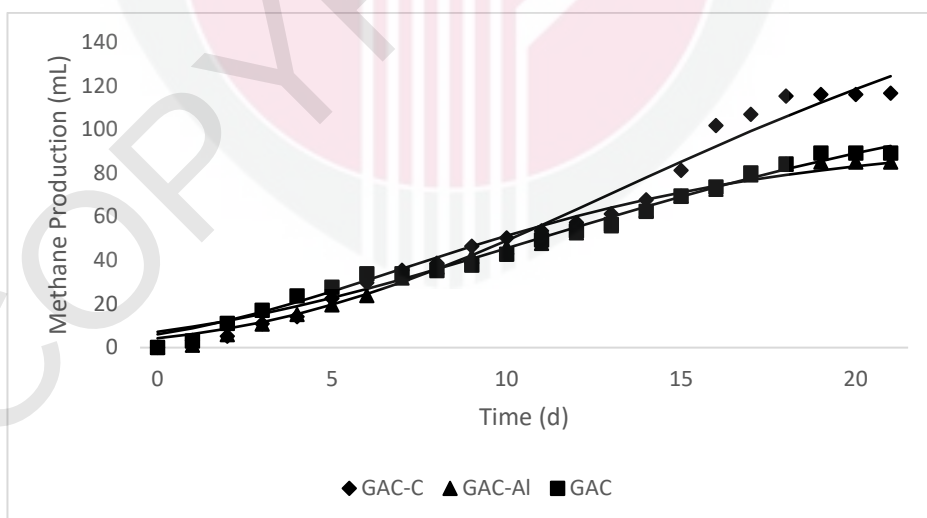


Figure 4.18: Methane production based on modified Gompertz equation for GAC, GAC-Al and GAC-C

Based on the gas production, methane productivity and yield could also be determined as shown in Figure 4.18. GAC-C also has the highest production rate of 27.48 mmolCH₄/L.d for methane. GAC had a production rate of 21.02 mmolCH₄/L.d while GAC-AI was 20.05 mmolCH₄/L.d. Considering GAC and GAC-AI had very close value of methane production; therefore they also have very similar production rate, with a slight difference of 0.97 mmolCH₄/L.d. From the graph also showed that GAC-C had the highest methane yield, where one mole of sugar could produce 9.33 mol of methane gas. While GAC also showed good productivity with methane yield of 7.26 molCH₄/mol sugar and GAC-AI had a yield of 4.95 molCH₄/mol sugar. Previously, researcher stated that chitosan can change ruminal fermentation by shifting volatile fatty acids (VFAs) profile and increasing the propionate concentration. The propionate formation determined the amount of hydrogen available to methanogenic bacteria. Therefore, the maximization of hydrogen metabolic pathway away from methanogenesis and towards VFAs has the potential to increase ruminant production (TONG et al., 2020). From the increment of VFAs from the hydrogen reactor that inhibits methanogenic bacteria has lead to increment of methane yield because substrate of hydrogen reactor contained high value of VFAs, which is favorable to methanogenesis.

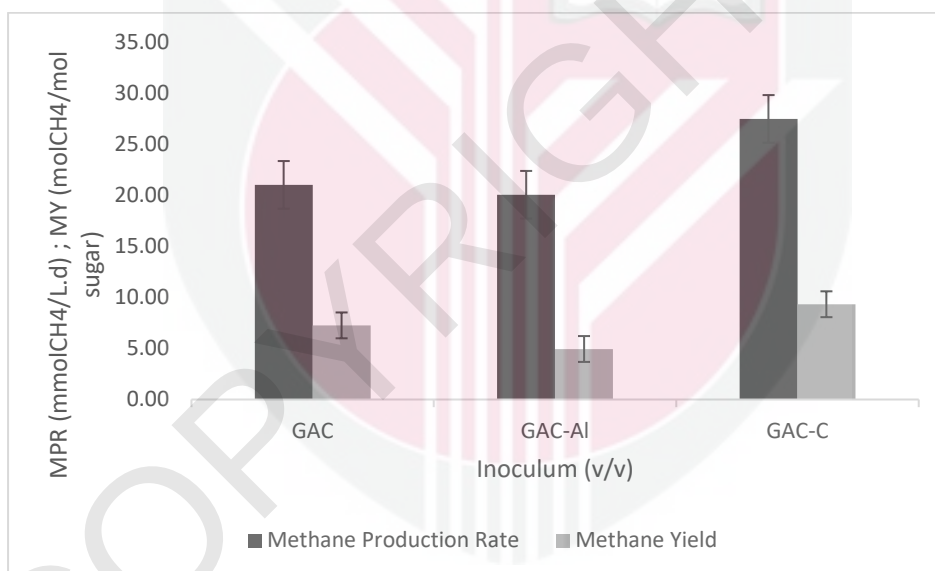


Figure 4.19: Methane production rate and methane yield for GAC, GAC-AI and GAC-C

When fitted into the modified Gompertz equation, the maximum methane production, maximum rate and lagged time was tabulated in Table 4.18. GAC-C has the highest maximum methane production (195.25 mL) with a maximum rate of 7.31 mL/d and also the shortest lagged time (0.59 d). On the other hand, GAC-AI still has the lowest productivity, with only 95.82 mL to be its maximum production and a maximum rate of 4.86mL/d. GAC-AI also has the longest lagged phase of 3.34 days.

Table 4.18: Methane productivity and maximum biomass obtained from GAC, GAC-AI and GAC-C

Sampl es	Modified Gompertz Equation					Total	Bioma ss Attach ed
	Methane		Maxim	Maximum Rate, Rm	Lagg	Volatil	
	Producti	Yield,	um		ed	e	
	on Rate, MPR	MY	Metha ne, Mm		time, Lam da	Fatty Acids, TVFA	
	(mmolC H ₄ /L.d)	(mol CH ₄ /m ol sugar)	(mL)	(mL/d)	(d)	(gCH ₃ COOH /L)	(gVSS/ mL)
GAC	21.02	7.26	131.01	5.23	0.63	68.80	0.10
GAC- AI	20.05	4.95	95.82	4.86	3.34	63.58	0.07
GAC-C	27.48	9.33	195.25	7.31	0.59	61.32	0.11

Furthermore, the beads were taken out and undergo VSS analysis to determine their attached biomass. It was then found out that all three of them had low attachment because methane process would need a longer time for the microbes to acclimatize and attach to the carrier, but some was still able to be found. GAC-C, with its highest productivity, has 0.11 gVSS/mL of biomass attached on the beads. There was not much of a difference for GAC as the biomass attached analyzed was 0.10 gVSS/mL. Even though GAC-AI has the lowest methane production, the biomass attached was only 0.04 gVSS/mL lesser than of GAC-C.

In conclusion, when three different carriers were used in biohydrogen and biomethane production, GAC-C beads had the highest productivity for both hydrogen and methane gases. Chitosan beads was able to produce more gases and also collected the most biomass attached on it. GAC-C bead is a better host

for the microorganisms in the system during anaerobic digestion.

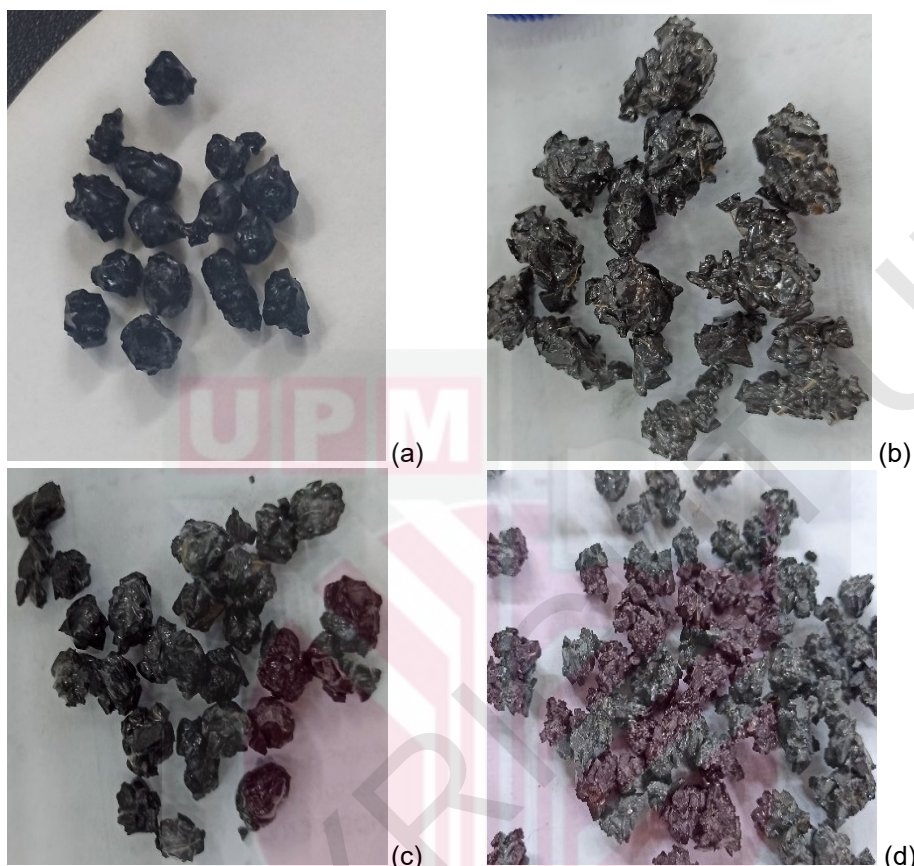


Figure 4.20: Images of the GAC-AI beads before fermentation (a), GAC-C beads before fermentation (b), GAC-AI beads after fermentation (c) and GAC-C beads after fermentation (d)

4.9 Energy Recovery

An energy recovery modelling was then developed using Microsoft Excel in order to assess biogas production. This model was based on a cow farm here in Malaysia, where the cow manure wastewater collected was used as the inoculum and substrate for the anaerobic digester. Several aspects including the bioreactor design, microbial kinetic studies and economic studies were carried out and presented in the modelling kit.

4.9.1 Bioreactor Design

The design of a bioreactor involved several factors such as the flow rate of cow manure and hydraulic retention time (HRT). The volume of cow manure

effluent in the unit of L/day.cow produced from the factory ranged from 45 to 60 L per day per cow, with an approximation of 200 cows in the factory. Working volume (WV) was first calculated using Equation (4.9):

$$WV (m^3) = \frac{0.6 \times \text{Vol of cow manure effluent} \times \text{num of cow} \times \text{COD}}{OLR \times 1000}, \quad (4.9)$$

Where COD is chemical oxygen demand (g/L), and OLR is the organic loading rate (kg COD/m³.day).

When the cow manure wastewater was collected raw from the farm, it was stored in a 10L bottle and left to settle down for a week. The wastewater was then separated by gravity into two distinct layers, where the upper part of the bottle was termed as the manure slurry supernatant, which was then used in the bioreactor for fermentation. The supernatant obtained was roughly 6L hence only 60% of the total cow manure was considered in the calculation for the working volume of the bioreactor. The remaining 40% was mainly made up of the digested cellulose and lignocellulose by the cows in the farm such as grass and grains. They could be used as wastewater substrate in anaerobic digester with the suitable condition for the fermentation process.

The minimum working volume of the bioreactor would be 96 m³ using the minimum rate of cow manure produced at 40 L/day.cow as shown in Table 4.19. When the rate of cow manure increased from 45 to 55 L/day.cow, the working volume of the biogas increased from 108 m³ to 132 m³ as well. The maximum working volume in this study was 144 m³ with a rate of 60 L/day.cow based on the maximum rate provided by Ladang 16, UPM.

Table 4.19: Flow rate of cow manure with its respective bioreactor volume and hydraulic retention time (HRT)

Rate of Cow Manure Produced (L/day.cow)	60%		HRT (Days)/Cow			
	Supernatant of The Rate of Cow Manure Produced (L/day.cow)	Working Volume of Bioreactor (m ³)	The Volume of Bioreactor (m ³)	Manure Flow Rate (m ³ /Day)		
				20	40	60
40	24	96.00	120.00	4.80	2.40	1.60
45	27	108.00	135.00	5.40	2.70	1.80
50	30	120.00	150.00	6.00	3.00	2.00
55	33	132.00	165.00	6.60	3.30	2.20
60	36	144.00	180.00	7.20	3.60	2.40

With the increment of rate of cow manure produced, eventually more biogas shall be produced. But with a faster flow rate of cow manure, indicating larger working volume hence bigger bioreactor size. Economically wise, it was not encouraged to have a biogas plant size which is too big because the construction of the plant would not only require more land and space, as well as more funding would be needed to build the plant, which could directly affect the ROI of the farm. Not only that, more maintenance would be involved and if the supply of cow manure is low at a certain point, the big headspace available in the reactor would directly affect the biogas production as well. Therefore, 55 L/day.cow of cow manure flow rate was selected in this study as the plant size was sufficient in terms of electricity generation and also wise in terms of capital. The working volume of the bioreactor was evaluated at 132 m³, and the total bioreactor volume was 165 m³.

Another important factor to consider in bioreactor design is hydraulic retention time (HRT). HRT has a direct effect on the supply of energy to the microorganisms in the reactor. A sufficient concentration of sugar content could help to boost the anaerobic system. On the other hand, if the sugar supply is low, microbes undergo deficiency of energy supply, thus causing low production of biogas.

The shorter the HRT, the higher the flow rate of cow manure per day, which then leads to a higher biogas yield too. When it was set at 20 days for a full cycle of biogas production, the value of cow manure flow rate was ranging from 4.8 to 7.2 m³/day for 40 to 60 L/day.cow of cow manure flow rate based on Table 4.19 above. Then, when HRT was set to 60 days, cow manure flow rate was then reduced to a range of 1.60 to 2.40 m³/day, which was considered to have a more minor variation when compared to 20 days HRT. As shorter HRT contributes to higher biogas production, the lowest HRT was selected at 20 days with the flow rate of cow manure of 55 L/day.cow. It was tabulated in Table 4.19 that the flow rate of cow manure was 6.6 m³/day.

The detailed geometrical dimension of the bioreactor could be calculated and tabulated in Table 4.20 based on the flow rate of cow manure and HRT.

Table 4.20: Dimensions of the bioreactor

Parameter	Unit	Value
The total volume of the digester (V)	m ³	165.00
Diameter (D)	m	7.17
The sectional volume of digester		
V1	m ³	30.52
V2	m ³	18.49
V3	m ³	115.96
Radius of digester		
R1	m	5.20
R2	m	7.62
R	m	3.59
Height of digester		
f1	m	1.43
H	m	28.71
f2	m	0.90
Curved surface		

Table 4.20: Continued

S1	m	46.87
S2	m	42.94
Heat transfer area	m ²	646.98

A 20% of headspace was reserved for the design of this bioreactor. Headspace plays an important role in dark fermentation. The volume of headspace needed to be sufficient to reduce the risk of high gas pressure inside the reactor itself, causing stress for the microorganisms where the gas might be consumed back by them, causing low biogas production.

4.9.2 Biogas Kinetic Studies

In order to determine the biogas yield, a lab scale bioreactor was made to imitate the fermentation of the real biogas plant by using a 1L Schott Duran bottle with a working volume of 800 mL. The biogas production was then fitted into modified Gompertz equation and evaluated the biogas production potential, maximum biogas production rate and lagged phase.

From the graph shown in Figure 4.19, the microorganisms in the system was activated shortly after 1 to 2 days of fermentation. Then, the biogas production continued to increase and entered its exponential phase after the first week. Biogas production was seen to increase from approximately 200 mL to 700mL within 5th to 15th day of the batch profiling. After the second week, the biogas production rate started to slow down and eventually came to a constant after 20 days. There were only small fluctuations for the biogas production towards the end of the experiment, reaching a stationary phase with volume of roughly 900 mL. Based on the Gompertz fitting also it was concluded that fermentation process started to slow down after 20 days, hence the selected HRT of 20 days for the design of the bioreactor was suitable because the prolonged period of time did not contribute much to the gas production anymore and new food needed to be fed into the system in order to sustain the microbes.

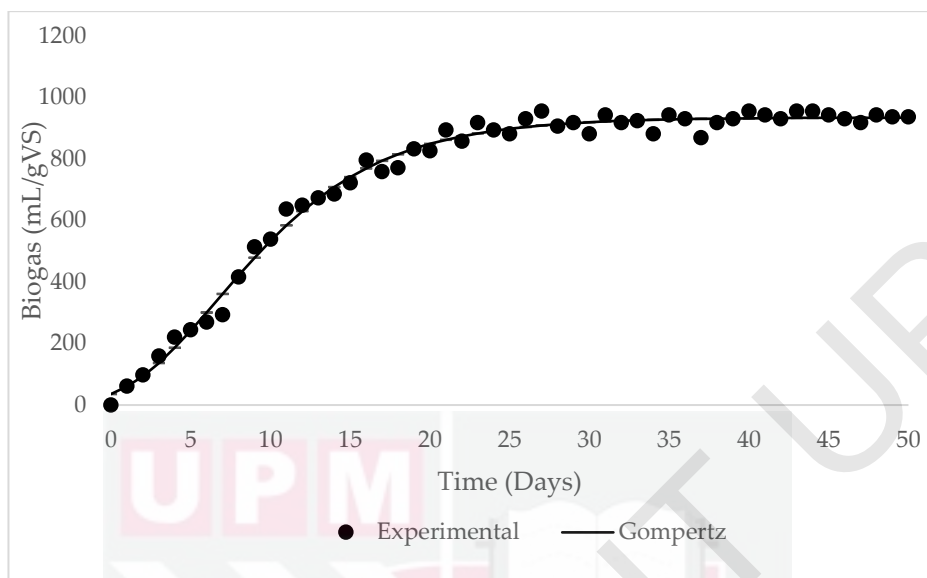


Figure 4.21: Biogas production (mL/gVS) in an 800 mL of a 1 L modified lab-scale bioreactor in batch fermentation

The maximum biogas production, A , was calculated based on the modified Gompertz equation and found that the highest prediction was 934.54 mL/gVS with a maximum biogas production rate, U of 60.55 mL/gVS.day and a lagged phase of 1.04 days. A similar experiment was done by another researcher who found that the optimum daily methane yield was 102 mL/gVS within 50 days. When the experiment was prolonged for another 50 days, the daily methane yield was 146 mL/gVS (Yu Li, Achinas, et al., 2020). Another research also showed that by using cow manure alone as the substrate in anaerobic digestion, 144 mL/gVS of methane was obtained daily (Sun et al., 2015).

Table 4.21: Microbial kinetic studies of biogas production

Parameters	
Biogas production potential, A (mL/gVS)	934.54
Maximum biogas production rate, U (mL/gVS.day)	60.55
Lag phase (days)	1.04
Biogas yield, P (mL biogas/gVS)	848.66
The volume of biogas produced (m^3 /day)	7,340.90
The volume of biogas produced/HRT cycle (m^3)	146,818.00

Based on the prediction based on modified Gompertz equation, the data was then substituted back to the Ghatak's remodified Gompertz. By using HRT = 20 days, the biogas yield (P) was obtained at 848.66 mL/gVS, and predicted to have total volume of biogas produced in a day is 11.28 m³ and 225.6m³ in a full cycle. Other researchers stated that a longer HRT provided an extended period of time for cellulose degradation in cow manure to produce higher biogas yield (Mostafa Imeni et al., 2019). According to the experimental data, there was no need for a prolonged period as the fermentation has entered its constant phase after day 20.

Based on another research done using food waste and cow dung as co-digestion anaerobic plant, the amount of food waste available will be 0.35–0.45 kg per capita per day (UNEP Food Waste Index Report, 2021) by the families under the assumption that there is a constant supply of food waste, with 30-35 days of acclimatization for the inoculum in the digester to produce constant rate of biogas. At the OLR of 2 kg Vs/m³/day, the digester will tend to produce nearly 5.25 L/day of biogas that will be sufficient to obtain uninterrupted supply of biogas for the household (Kesharwani & Bajpai, 2021).

4.9.3 Economic Studies

One of the main products of this anaerobic digester was the generation of electricity using the biogas produced from the system. Based on Swedish Gas Technology Centre essential data on biogas in 2012, it was estimated that 1m³ of biogas could produce up to 6kwh or energy but only about 2kwh was the power yield after all the energy losses were considered. Therefore, as tabulated in Table 4.22, the volume of biogas produced in a day was 7340.90 m³, resulted in 14681.80 kwh of electricity produced in a day. This result in a very high and efficient system, if there is no major issue with the fermentation, such as the ample supply of substrate and also under optimum operation conditions.

According to the TNB Pricing and Tariffs 2019, the selling price of electricity is 33.40 sen/kWh. Within a month, the revenue from selling electricity from the biogas reactor was estimated to be RM154,158.90 and RM1,695,747.90 in a year. The amount of electricity produced can then be supplied back to the dairy farm and helped to save about RM5138 on the monthly electricity bill. The utilization of cow manure wastewater as a useful resource has brought benefits to the environment and the owner's spending on the farm.

Other economic parameters such as total purchase cost of equipment (PCE), fixed capital, investment, revenue, and profit were evaluated based on the calculations shown in chapter 3 and tabulated in Table 4.22 below. The total purchase cost of equipment (PCE) including estimated price for bioreactor with carbon steel as material (RM500,000), water pump installed on the reactor (RM35,000), gas scrubber and gen-set facilities (RM180,000) and activated carbon as the carrier material (RM40,000) (Ruiz et al., 2018). Then, estimated

fixed capital was also calculated using Table 3.6, where the total investment required for the project was RM1,426,950.00. Since the raw material (cow dung) was obtained from waste, there was no cost for the raw material but other costs such as utilities (water and electricity) were estimated for a small-scale dairy farm to be RM1,833.35/day. The annual operating costs were estimated and tabulated as shown in Table 4.22 below. Variable cost consists of any miscellaneous materials and utilities (given rate of RM2.07/m³ water used). Since miscellaneous materials are not considered in this research, hence variable cost only involves utilities. Fixed costs consist of maintenance charges, capital charges, insurance and local taxes. However, in this study, only maintenance cost is taken into account.

Table 4.22: Annual operating cost

Variable costs		
Raw materials	-	RM/year
Miscellaneous materials	5,000.00	RM/year
Utilities	605,005.09	RM/year
Total Variable costs	610,005.09	RM/year
Fixed costs		
Maintenance	71,347.50	RM/year
Capital charges		RM/year
Insurance	5,000.00	RM/year
Local taxes	10,000.00	RM/year
Total Fixed costs	86,347.50	RM/year
Direct production costs =Total Variable		
Cost + Total Fixed Cost	696,352.59	RM/year
Annual operating cost	696,352.59	RM/year

Table 4.23: Economics output on biogas production in a Microsoft Excel modeling system.

No.	Parameters	Unit	Value
1	Electricity produced	kWh/day	14681.80
		kWh/month	440454.00
2	Electricity Selling Price	RM/month	154158.90
		RM/year	1695747.90
3	Total Equipment Purchase Cost, PCE	RM	755000.00
4	Fixed Capital	RM	1426950.00
5	Total Capital Investment	RM	1426950.00
6	Revenue	RM/year	1695747.90
7	Annual Operating Cost	RM/year	696352.59
8	Annual Profit	RM/year	999395.31
9	Payback Period	year	6
10	Return on Investment, ROI		0.47
11	Net Present Value, NPV	RM	1613820.28
12	Internal Rate of Return, IRR		21%

The bioreactor was desirable to be located near the feedstock source, which is the farm's collection point of cow manure, to avoid any excessive handling and transportation of feedstock to the reactor, as well as to reduce the cost on piping required. Variable consisted of any miscellaneous materials and utilities such as water and electricity to run the plant were calculated as well. Some other fixed costs such as maintenance charges and insurance were also considered. The total capital investment was calculated to be RM1.4million.

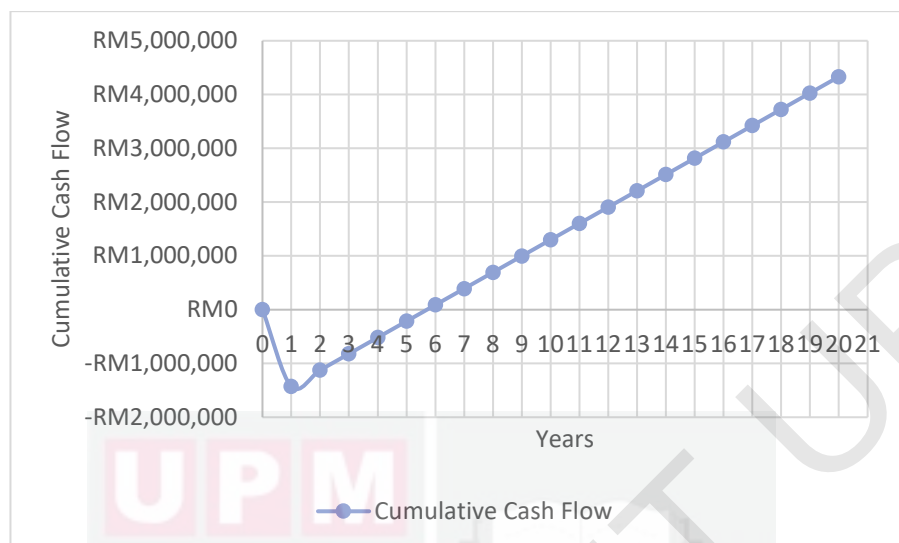


Figure 4.22: Cash flow diagram of biogas plant in 20 years

The biogas plant could earn up to RM1695747.90 with an annual operating cost of RM696,352.59 including labor and other utilities. The profit of this biogas plant was estimated around RM999,395.31 in a year. In Figure 4.22 shows the cumulative cash flow of the plant. Due to the new construction, it was assumed to have zero revenue by the end of the first year. When it entered the second year, there was an increment in the profit, and then keep growing for the continuous year. In about 6 years' time, the investment would have been covered by the annual profit, stating that this biogas plant has a payback period of 6 years. This project also seemed to have a ROI of 47% and an internal rate of return at 21%.

Another researcher which has analyzed the economic studies of anaerobic digester suggested that the generated electricity was sold to the grid at price of 0.125 \$/kWh while the price of electricity purchased was 0.081 \$/kWh. With the selling of electricity back to the grid has contributed greatly to the income of the plant that the payback was reduced to 2.4 years than that for the case with no-selling electricity to the grid (5.6 years). (Sanaye & Yazdani, 2022) A higher IRR shows an attractive project proposal where another study using also anaerobic digester with cow dung as feedstock has an IRR of 11.2% under treated conditions and also payback period of 8.2 years (Paritosh et al., 2021). Based on Li (2018), the results of batch scale anaerobic digester having 5 L volume and 20% TS showed payback period of 10.9 years with 11.7% of IRR for a full scale 300 m³ solid state anaerobic digester (Yangyang Li et al., 2018).

To conclude, the relationship between HRT, cow manure inflow rate and the economics were successfully evaluated. An energy recovering model was assessed based on a lab-scale biogas plant and found out that the biogas potential was 934.54 mL/gVS with a maximum rate of 60.55 mL/gVS.day, and a

lagged phase of 1.04 days. Hence, lead to a biogas production of 146818m³, by using HRT 20 days. Based on this amount of biogas produced, the electricity generated was 14681 kwh per day.



CHAPTER 5

CONCLUSION AND RECOMMENDATION

The production of biohydrogen and biomethane gases using cow manure wastewater has a big potential as it is a green alternative for wastewater treatment at the same time producing renewable energy. This chapter summarizes this study's findings and proposes recommendations for future research.

5.1 Conclusion

For the inoculum percentage, it was found that the one with the highest hydrogen production was using 15%, where the hydrogen productivity was 0.88 mmolH₂/L.h. Similarly for methane production, 15% also contributed the highest productivity at 43.87 mmolCH₄/L.d. Both hydrogen and methane were observed to have the highest yield when 15% of inoculum, 2.49 molH₂/mol sugar and 28.86 molCH₄/mol sugar, respectively. Therefore, 15% inoculum percentage is the optimum for two stage biohydrogen and biomethane production.

For the glucose utilization, at 10 g/L, microbial growth rate for hydrogen production was 0.96 h⁻¹ with the highest HPR, 0.88 mmolH₂/L.h and maximum biomass 18.67 gVSS/L, with the shortest lag phase of 2.28 hour. Based on Monod modelling, the maximum specific growth rate for hydrogen production ($\mu_{\max}$), obtained was 0.99 h⁻¹, and the specific growth rate (μ), was 0.49 h⁻¹ ($\mu_{\max}/2$) which lead to the cell growth saturation coefficient (K_s) of 2.35. 10 g/L of glucose resulted in a microbial growth rate that is the close to the maximum; hence it is the optimum concentration of glucose used in hydrogen production. For methane, productivity peaked at 10g/L glucose as well at 32.93 mmolCH₄/L.d, a maximum growth rate of 0.74h⁻¹ and biomass of 22.00 gVSS/L, with the shortest lagged phase of 8.56 hours. From Monod modelling, the maximum specific growth rate ($\mu_{\max}$) obtained was 0.85 h⁻¹, and the specific growth rate (μ), was 0.42 h⁻¹ ($\mu_{\max}/2$), which lead to the cell growth saturation coefficient (K_s) of 2.98. Sugar loading of 10 g/L has the closest specific growth rate to the maximum one. Hence, 10 g/L of glucose is the optimum glucose utilization for two stage biohydrogen and biomethane production.

Using the optimum conditions from the above findings, two-stage biohydrogen and biomethane production was fermented using sequencing batch profiling. The final hydrogen production was 750 mL with hydrogen productivity of 32.67 mmolH₂/L.d and hydrogen yield of 0.68 molH₂/mol sugar. On the other hand, the final methane production was at a constant of 660 mL, methane productivity of 21.71 mmolCH₄/L.d and 3.77 molCH₄/mol sugar of methane yield.

The factors with highest desirability in producing alginate beads are 0.50 g/mL of GAC, 1.23% of alginate, and then freezing as the drying method. The model showed that these factors affect the diameter and stability of alginate beads. However, it shows no significance towards the swelling degree of alginate beads. Besides, the chitosan beads with a 100% desirability was made of 0.52 g/mL of GAC, 1.55% of chitosan and freeze dry. The accuracy of the model was confirmed through the error of prediction by RMS and experimental, showing all of the responses resulted in small margin of error under the suggested conditions.

After reproducing the beads using the conditions suggested by RSM, they were then used in two stage biohydrogen production. The results showed that GAC-C beads had the highest productivity for both hydrogen and methane gases. During hydrogen production, after fitted into the modified Gompertz equation, GAC-C has the highest maximum hydrogen (76.17 mL) and maximum rate (1.92 mL/h) with the shortest lagged phase 3.51 hours. Then, during methane production, GAC-C also has the highest maximum methane production (195.25 mL) with a maximum rate of 7.31 mL/d and the shortest lagged time (0.59 d).

Using a fabricated lab-scale biogas plant, an energy recovering model was assessed based on the relationship between HRT, cow manure inflow rate and the economics. 55 L/day.cow of cow manure flow rate was selected in this study as the plant size was sufficient in terms of electricity generation and also wise in terms of capital. The working volume of the bioreactor was evaluated at 132 m³, and the total bioreactor volume was 165 m³. The microbial kinetic studies found that the biogas potential was 934.54 mL/gVS with a maximum rate of 60.55 mL/gVS.day, and a lagged phase of 1.04 days. Biogas production also showed a constant in gas production after 20 days hence the HRT of this model was also set to be 20 days. When data was evaluated in the model, an approximation of 14681kwh of electricity could be produced daily, leading to RM154,158.90 generated from selling of the biogas. The biogas plant has a payback period of 6 years. This project also seemed to have a ROI of 47% and an internal rate of return at 21%.

5.2 Recommendation

There are a few recommendations that could help the research projects in the future to bring some improvements to the renewable energy field.

In order to increase the gases production, a support carrier is introduced into the system. Instead of using GAC-C beads that is clear, researchers can first produce the biofilm attachment on the GAC, then only coat with a layer of chitosan to not only protect the biofilm, but also to fasten the production of gases because microbes are already in the active state to carry out fermentation process.

The usage of cow manure as inoculum is not as productive due to the high amount of lignocellulose. Cow rumen can be used as the inoculum instead and cultivate the microbes for cell attachment. Microorganisms in the rumen is more active and readily available, then only use cow manure as the substrate for the feeding.

The characteristic of the cow manure effluent must be done by the users to ensure the input to the Microsoft Excel energy recovery model is most accurate and targeted to their project. The characteristics of cow manure effluent include TS, TVS, and COD as the mentioned parameters might affect the result of the following output.



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Tan, J. B., Jamali, N. S., Tan, W. E., Man, H. C., & Abidin, Z. Z. (2021). Techno-economic assessment of on-farm anaerobic digestion system using attached-biofilm reactor in the dairy industry. *Sustainability (Switzerland)*, 13(4), 1–15. <https://doi.org/10.3390/su13042063>

