



**NI-MAGNETITE NANOPARTICLE AS ADDITIVE IN
CELL IMMOBILIZATION IN BIOHYDROGEN PRODUCTION**

By

NINA FARHANA BINTI MOHD JAMALUDIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

July 2024

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July 2024

Chairman : Nur Syakina binti Jamali, PhD
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Previously, the cell immobilization technique using granular activated carbon was implemented due to its porous nature and large surface area, which promote microbial activity and attachment. However, low hydrogen productivity was still widely reported. So, this study aims to utilize nickel (Ni) and magnetite (Fe_3O_4) as additives in granular activated carbon (GAC), namely, GAC- NiFe_3O_4 , to study its ability as microbial support carriers in thermophilic biohydrogen production. From the study's first objective, this work has successfully synthesized GAC- NiFe_3O_4 originated from coconut shells embedded with the additives using the co-precipitation method. GAC- NiFe_3O_4 demonstrated a higher hydrogen production rate (HPR) of 65.49% than control (GAC). The analysis of variance showed a reliable optimization model with an R-squared of 0.93 and the predicted HPR of 21.21 mL H_2 /L.h. The result was further validated at 20.33 ± 0.32 mL H_2 /L.h with a 4.16% error from the predicted value. For the second objective, GAC- NiFe_3O_4 was successfully immobilized with mixed culture in an attached biohydrogen production system. The immobilization utilizing GAC- NiFe_3O_4 shortened biohydrogen production from 66-d to 26-d,

incrementing hydrogen yield (HY) by 57.30%. Subsequently, the effect of sludge on the GAC-NiFe₃O₄ ratio, which ranged from 1:0.5-4, was studied. The optimum HY of 1.64 ± 0.04 mol H₂/mol sugar consumed was achieved at a ratio of 1:1. In addition, the optimum initial sugar (ranges 10-30 g/L) concluded from the MONOD model was at 20 g/L, with maximum specific growth rate, specific growth rate, and cell growth saturation coefficient of 2.05 h⁻¹, 1.98 h⁻¹, and 6.96 g/L, respectively. The sequencing analysis revealed that the dominant species was *Thermoanaerobacterium sp.* at 99% abundance. In the third objective, this work has successfully optimized operational parameters in terms of pH (6-7), temperature (60-80°C), and agitation (60-180 rpm) utilizing the optimum immobilized GAC-NiFe₃O₄ obtained previously. The optimal pH obtained was at pH 6.0 with HY of 2.66 ± 0.09 mol H₂/mol sugar consumed. At 60°C, the highest HY was obtained at 2.75 ± 0.11 mol H₂/mol sugar consumed. The optimal agitation obtained was at 120 rpm, attaining the highest HY of 3.44 ± 0.54 mol H₂/mol sugar consumed. Furthermore, in the solid loading rate (SLR) study, which ranges from 5-25%, 10% of SLR was found to be optimum, with 66.67% total suspended solids degradation. The optimum hydraulic retention time was 3 h from 3-48 h with an energy production rate of 29.19 kJ/L. Following all the previous optimum conditions obtained, the final objectives were successfully achieved. This work has characterized the collected POME in terms of physiochemical, heavy metal, and sugar analysis. The total carbohydrates (TC) study ranges from 10-100%, showing 50% of POME POME (TC= 14.64 ± 0.11 g/L) can be used to obtain maximum HY with 94.12% sugar utilization. *Thermoanaerobacterium sp.* was identified as the dominant species at 67% abundance. GAC-NiFe₃O₄ showed potential for heavy metal removal in POME from 29.71-99.86%. Overall, applying GAC-NiFe₃O₄ showed high potential

in biohydrogen production while providing a sustainable alternative to the bioremediation of POME.

Keywords: Biohydrogen Production, Thermophilic Dark Fermentation, Cell Immobilization, Nanoparticle Addition, Nickel and Magnetite

SDG: GOAL 7: Affordable and Clean Energy, GOAL 13: Climate Action



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Doktor Falsafah

NANOZARAH NI-MAGNETIT SEBAGAI BAHAN TAMBAHAN DALAM IMOBALISASI SEL DALAM PENGHASILAN BIOHIDROGEN

Oleh

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Sebelum ini, teknik imobilisasi sel menggunakan karbon teraktif berbutir telah dilaksanakan kerana sifat berliang dan kawasan permukaan yang besar, yang menggalakkan aktiviti dan lampiran mikrob. Walau bagaimanapun, productiviti hydrogen yang rendah masih dilaporkan secara meluas. Jadi, kajian ini bertujuan untuk menggunakan nikel (Ni) dan magnetit (Fe_3O_4) sebagai bahan tambahan dalam karbon teraktif berbutir (GAC), dinamakan GAC- NiFe_3O_4 , untuk mengkaji keupayaannya sebagai pembawa sokongan mikrob dalam pengeluaran biohidrogen termofilik. Daripada objektif pertama kajian, kerja ini telah berjaya mensintesis GAC- NiFe_3O_4 yang berasal daripada tempurung kelapa yang dibenamkan dengan bahan tambahan menggunakan kaedah pemendakan bersama. GAC- NiFe_3O_4 menunjukkan kadar pengeluaran hidrogen (HPR) yang lebih tinggi sebanyak 65.49% daripada kawalan (GAC). Analisis varians menunjukkan model pengoptimuman yang boleh dipercayai dengan R-kuadrat sebanyak 0.93 dan HPR yang diramalkan sebanyak 21.21 mL $\text{H}_2/\text{L.h}$. Hasilnya telah disahkan selanjutnya pada 20.33 ± 0.32 mL $\text{H}_2/\text{L.h}$ dengan ralat 4.16% daripada nilai yang diramalkan. Untuk objektif kedua, GAC-

NiFe₃O₄ telah berjaya diimobalisasi dengan kultur campuran menggunakan sistem lampiran dalam penghasilan biohidrogen. Imobilisasi yang menggunakan GAC-NiFe₃O₄ memendekkan pengeluaran biohidrogen daripada 66 hari kepada 26 hari, meningkatkan hasil hidrogen (HY) sebanyak 57.30%. Selepas itu, kesan enapcemar pada nisbah GAC-NiFe₃O₄, yang berjulat antara 1:0.5-4, telah dikaji. HY optimum pada nisbah GAC-NiFe₃O₄, yang berjulat antara 1:0.5-4, telah dikaji. HY optimum 1.64 ± 0.04 mol H₂/mol gula yang digunakan dicapai pada nisbah 1:1. Di samping itu, gula awal optimum (julat 10-30 g/L) yang disimpulkan daripada model MONOD adalah pada 20 g/L, dengan kadar maksimum pertumbuhan spesifik, kadar pertumbuhan spesifik, dan pekali tepu pertumbuhan sel pada 2.05 h^{-1} , 1.98 h^{-1} , dan 6.96 g/L, masing-masing. Analisis penjujukan menunjukkan bahawa spesies yang dominan ialah spesies *Thermoanaerobacterium* pada kelimpahan 99%. Dalam objektif ketiga, kerja ini telah berjaya mengoptimumkan parameter operasi dari segi pH (6-7), suhu (60-80°C), dan pergolakan (60-180 rpm) menggunakan GAC-NiFe₃O₄ yang diimobalisasi sebelumnya. pH optimum yang diperolehi adalah pada pH 6.0 dengan HY sebanyak 2.66 ± 0.09 mol H₂/mol gula yang digunakan. Pada suhu 60°C, HY tertinggi diperolehi pada 2.75 ± 0.11 mol H₂/mol gula yang digunakan. Pergolakan optimum yang diperolehi adalah pada 120 rpm, mencapai HY tertinggi sebanyak 3.44 ± 0.54 mol H₂/mol gula yang digunakan. Tambahan pula, dalam kajian kadar muatan pepejal (SLR), yang berjulat antara 5-25%, 10% daripada SLR didapati optimum, dengan 66.67% jumlah degradasi pepejal terampai. Masa pengekal hidraulik optimum ialah 3 jam dari 3-48 jam dengan kadar pengeluaran tenaga 29.19 kJ/L. Mengikut semua keadaan optimum sebelumnya yang diperolehi, objektif akhir berjaya dicapai. Kerja ini telah mencirikan POME yang dikumpul dari segi analisis fisiokimia, logam berat, dan gula. Kajian jumlah karbohidrat (TC) berkisar antara 10-100%, menunjukkan 50% POME (TC= 14.64 ± 0.11 g/L) boleh digunakan untuk

mendapatkan HY maksimum dengan 94.12% penggunaan gula. Spesies *Thermoanaerobacterium* dikenal pasti sebagai spesies dominan pada kelimpahan 67%. GAC-NiFe₃O₄ menunjukkan potensi penyingkiran logam berat dalam POME daripada 29.71-99.86%. Secara keseluruhan, penggunaan GAC-NiFe₃O₄ menunjukkan potensi tinggi dalam pengeluaran biohidrogen sambil menyediakan alternatif yang mampan kepada bioremediasi POME.

Kata Kunci: Penghasilan Biohydrogen, Penapaian Gelap Termofilik, Imobilisasi Sel, Penambahan Nanozarah, Nikel dan Magnetit

SDG: MATLAMAT 7: Tenaga yang berpatutan serta bersih, MATLAMAT 13: Tindakan iklim

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LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal ribonucleic acid
AC	Activated carbon
Ag	Silver
Al	Aluminium
AN	Ammoniacal nitrogen
ANOVA	Analysis of variance
ASEAN	Association of Southeast Asian Nations
ATR	Attenuated total reflectance
BET	Brunauer-Emmet-Teller
BLAST	Basic local alignment search tools
BOD	Biological oxygen demand
BSA	Bovine serum albumin
C	Carbon
Ca	Calcium
CCD	Central composite design
Cd	Cadmium
Co	Cobalt
CoO	Cobalt oxide
COD	Chemical oxygen demand
CPD	Critical point dryer
Cr	Chromium
Cu	Copper
DIET	Direct interspecies electron transfer
DOE	Design of experiment
EDX	Energy dispersive x-ray

EPR	Energy production rate
EPS	Extra polymeric substance
EtOH	Ethanol
Fe	Iron
FESEM	Field emission scanning electron microscopy
FPP	Four-point probe
F-tests	Fisher statistical test
FTIR	Fourier transform infrared
FWER	Family-wise error rate
FWHM	Full width at half maximum
GAC	Granular activated carbon
GAC-C	Commercial granular activated carbon
GAC-O	Synthesized granular activated carbon
GAC-NiFe ₃ O ₄	Ni-Magnetite granular activated carbon
GC	Gas chromatography
gDNA	Genomic deoxyribonucleic acid
Gr	Graphene
H ₂	Hydrogen
H ₂ SO ₄	Sulphuric acid
HAc	Acetic acid
HBu	Butyric acid
HCl	Hydrochloric acid
HLa	Lactic acid
HPLC	High-performance liquid chromatography
HPa	Propionic acid
HPR	Hydrogen production rate

HRT	Hydraulic retention time
HT	Hydrotalcite
HY	Hydrogen yield
ICP-OEP	Inductively coupled plasma optical emission spectroscopy
K	Potassium
Mg	Magnesium
Mn	Manganese
N	Nitrogen
N/A	Not available
NADH	Reduced nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
NEEAP	National Energy Efficiency Action Plan
Ni	Nickel
NP/NPs	Nanoparticle/Nanoparticles
OFAT	One-factor-at-a-time
O&G	Oil and grease
P	Phosphorus
PAC	Powder activated carbon
PCR	Polymerase chain reaction
POME	Palm oil mill effluent
R ²	Coefficient of determination
RID	Refractive index detector
RM	Ringgit Malaysia
RSM	Response surface methodology
SEB	Sarawak Energy Sdn Bhd

SLR	Solid loading rate
SMP	Soluble microbial products
SMS	Soluble monomeric sugar
TAE	Tris-acetate- ethylenediaminetetraacetic acid
TC	Total carbohydrates
TCD	Thermal conductivity detector
TMS	Total monomeric sugar
TP	Total phosphorus
TVFA	Total volatile fatty acid
TSS	Total suspended solids
VAB	Volatile attached biomass
VSM	Vibrating-sample magnetometry
VSS	Volatile suspended solids
XRD	X-ray diffraction
Zn	Zinc

CHAPTER 1

INTRODUCTION

1.1 Background

The 1980s marked the start of the development of biohydrogen production processes. Steam reforming and gasification are the most industrially feasible biohydrogen production techniques (Martino et al., 2021). However, dark fermentation has been the most researched biochemical process (Hidalgo et al., 2022). Biohydrogen generation processes, including photofermentation, dark fermentation, and biophotolysis, are being developed most actively in countries like China, Japan, and India (Azbar & Levin, 2012). Still, most laboratory-based techniques for producing biohydrogen have not yet been translated to a commercial setting. Many elements, including the temperature and pH of the process, the type of microorganisms, the energy supply, the presence of light and oxygen, and the enzymes in the metabolism, determine high biohydrogen productivity (Saravanan et al., 2021).

As one of the countries with an active agricultural sector, Malaysia has abundant biomass resources. Malaysia generates a minimum of 168 million tonnes of biomass waste annually (Ozturk et al., 2017). Among the ASEAN nations, Malaysia is in a good position to encourage using biomass as a renewable energy source. In 2019, Sarawak Energy Sdn Bhd (SEB) invested RM 15 million in a pilot hydrogen production plant from hydro sources (Sarawak Energy, 2019). SEB even set up a refueling station project to evaluate the viability of hydrogen and fuel cells in powering Sarawak's transportation sector. The plant is expected to produce 130 kg of hydrogen daily, powering five buses and 10 cars daily (Sarawak Energy, 2019). With

zero-emission capability, these vehicles represent the future of the automobile. Since Malaysia has the advantage of producing hydrogen at the cheapest cost (from the abundance of biomass produced) compared to hydro electrolysis, with the advancement of technology, Malaysia should soon be able to realize the export of hydrogen to other countries. It will become the country's next revenue source rather than just exporting liquefied natural gas.

Adopting biomass in the green technology industry needed further intervention despite the established market incentive and government policies. The government's commitment to the Renewable Energy Policy and Action Plan 2019 also supports the feasibility of green energy and population growth (Government of Malaysia, 2013). The efforts are to ensure sustainability and resilient socio-economic development by achieving 4000 megawatts of installed renewable energy capacity by 2030 (*National Biomass Strategy*, 2020).

In light of this concern, biohydrogen production is then considered. Biohydrogens are well-known as cleaner energy sources than fossil fuels because they are green, clean, and high-energy fuels that do not harm the environment (Ayodele et al., 2023). Malaysia aspired to move the hydrogen economy towards net zero by 2050 (*MOSTI*, 2023). Countries such as Japan, South Korea, China, Australia, and other developing countries in ASEAN have begun to adopt hydrogen into mobility and energy-intensive sectors to meet the environmentally driven regulations and standards adopted by different sectors (Handayani et al., 2022).

Biohydrogen can be applied to supply high-density, adaptable power and propulsion to various industries (Ahmad et al., 2011). However, the challenges lie in the cost of generating hydrogen from renewable sources and end-use applications due to low technology maturity across Malaysia's hydrogen value chain (Veza et al., 2023). Since Malaysia is among the world's leading exporters of palm oil, exporting 13.75 million tonnes of palm oil in 2023 (Malaysian Palm Oil Board, 2016), the waste generation from the industry is also abundant. Palm oil waste accounts for 91% of the biomass feedstock (Salleh et al., 2020). Given the ongoing expansion of the world economy on renewable energy, utilizing palm oil mill effluent (POME) from the palm oil industry has become feasible for biohydrogen production.

1.2 Problem Statement

Previously, biohydrogen production studies focused on suspended systems, which are typically inefficient for a continuous process because of poor cell density or bacterial washout (Ahn et al., 2005; Zhao, L. et al., 2017). Therefore, cell immobilization enhanced and sustained the biomass without cell washout from the reactor (Jamali, Dzul Rashidi, et al., 2019; Osuna et al., 2015). Several prior research studies have used activated carbon as a support material for cell immobilization in biohydrogen production (Jamali et al., 2016, 2021; Park et al., 2019). This is attributed to its porous nature and large surface area, which promote microbial activity and attachment (Jamali, Dzul Rashidi, et al., 2019). However, recovering activated carbon can be difficult, increasing the process costs. In addition, low hydrogen productivity was still widely reported (Liu Q. et al., 2011; Lutpi et al., 2016).

To overcome low hydrogen productivity, many approaches have been utilized, such as the addition of nanoparticles into the fermentative medium, enhancing the concentration of enzyme involved in biohydrogen generation, improvement of the cell immobilization techniques, and optimization of the operating parameters (Abdul Aziz et al., 2022; Khan et al., 2016; Pomdaeng et al., 2024; Rambabu, Show, et al., 2020). For the addition of nanoparticles, previous research obtained a 44% increase in biohydrogen production upon adding ferric oxide (Fe_2O_3) nanoparticles compared to without any addition (Sinharoy & Pakshirajan, 2020). Moreover, immobilization of enzymes has also been reported to be beneficial (Vaghari et al., 2016). It has been used to improve substrate selectivity, activity, and stability for successful application in industrial processes (Kotwal et al., 2024). In general, the commercialization of biohydrogen production is aided by advancing and adjusting numerous aspects through technical techniques. Then, producing biohydrogen has been proven to run more smoothly when operating parameters are optimized (Preethi et al., 2019).

Among the approaches, the area of nanoparticles (NPs) addition is expanding with numerous systems applied to enhance hydrogen production (Mohanraj et al., 2014; Mullai et al., 2013; Yang & Wang, 2018). Previous studies reported the integration of NPs such as nickel, iron, copper, and zinc as trace elements acting as micronutrients to the bacteria (Abdul Aziz et al., 2022; Beckers et al., 2013; Mullai et al., 2013). These studies used the NPs due to their remarkable physical and chemical properties, such as the large surface area and high reactivity (Srivastava et al., 2020).

Generally, hydrogen production from biological processes is mostly catalyzed by hydrogenase enzymes in dark fermentation (Sun Y. et al., 2019). There are three active

sites of hydrogenase: [NiFe], [FeFe], and [Fe] (Abdul Aziz et al., 2022). Based on prior research, biohydrogen production can be heavily impacted by the existence of iron (Fe) or nickel (Ni) metal ions, as they play a crucial role in boosting the effectiveness of hydrogenase enzymes (Patel et al., 2018). Thus, adding Ni and Fe NPs will impact biohydrogen production and increase hydrogen productivity. The additive can be a co-factor on the active sites of hydrogenase, greatly increasing biohydrogen synthesis yield (Taherdanak et al., 2015). However, low conductivity, limited nutrient availability, and slower biodegradability can hinder the process and lead to low biohydrogen productivity (Sun Y. et al., 2019).

Many strategies have been developed to increase biohydrogen productivity from NP additives. However, they are still far from being used alone in a sustainable and economic approach (Cipolatti et al., 2016). The incomplete or one-time use of the nanoparticle materials may become more expensive and labor-intensive for the biohydrogen process (Srivastava et al., 2017; Yang B. et al., 2011). Thus, immobilizing the nanoparticles on a suitable support carrier will likely enhance the stability for real-world applications, even in hostile settings, and allow for the potential for material reuse (Bai et al., 2009; Basile et al., 2012). When the NPs are integrated into activated carbon structures, the capability can be enhanced to catalyze specific chemical reactions for biohydrogen production. It is expected that the stability and activity of the biohydrogen production process will increase when immobilized on such materials. Besides, the environmental concern regarding releasing NPs into the environment can also be prevented by implementing the technique (Shinde et al., 2023; Yusuf et al., 2024).

Nevertheless, the performance of porous media of activated carbon integrated with nanoparticles as additives must be further investigated. This is due to the limitation of extensive study, especially when using POME as the substrate in biohydrogen production. Several factors, such as synthesis approach, immobilization techniques, operating parameters, and structure of microbial diversity leading to different reaction pathways, are among the required analyses for further application. In addition, complying with standard discharge limits poses a notable challenge for palm oil mills, prompting the exploration of various bioremediation methods (Aziz et al., 2020; Soffian et al., 2022). Thus, simultaneous bioremediation and biohydrogen production from POME were suggested in this study.

Overall, this study aims to utilize nickel (Ni) and magnetite (Fe_3O_4) NPs as additives in granular activated carbon (GAC) to study their ability as microbial support carriers in thermophilic biohydrogen production from POME. The techniques will provide a more eco-conscious and sustainable approach to the biohydrogen production process to minimize the environmental impact. The current development could accelerate the enhancement of hydrogen production's biological processes. The application of NPs integrated products is one of the key components in the future high-technology market.

1.3 Objectives

The objectives of this research are:

- i. To synthesize nickel-magnetite granular activated carbon and optimize the molar ratio of nickel to iron, molar ratio of granular activated carbon to additives, and type of granular activated carbon.

- ii. To develop immobilization of the mixed culture bacteria on nickel-magnetite granular activated carbon under thermophilic dark fermentation conditions and evaluate the initial sugar loading and microbial community analysis.
- iii. To evaluate the pH, temperature, agitation, solid loading rate, and hydraulic retention time to determine the optimum process condition utilizing immobilized nickel-magnetite granular activated carbon for biohydrogen production.
- iv. To assess the effect of total carbohydrates by applying nickel-magnetite granular activated carbon to produce biohydrogen from POME while obtaining the microbial community analysis and heavy metal removal potential.

1.4 Scope of Study

The scope of the study was segmented into four main sections that covered all four objectives. Initially, a preliminary study was conducted to prepare granular activated carbon (GAC). Next, a seed-mixed culture obtained from a previous study was cultivated until constant performance was achieved. The same cultivated seed was maintained throughout the study.

Next, the scope of the study covers the synthesis optimization of nickel-magnetite granular activated carbon (GAC-NiFe₃O₄) for the study's first objective. The optimum conditions were investigated regarding the molar ratio of nickel to iron, the molar ratio of GAC to additive, and the type of granular activated carbon. The biohydrogen production rate measures the overall process performance. The optimum synthesized GAC-NiFe₃O₄ was later characterized in terms of electrical properties, morphology, composition, magnetic properties, structure, and chemical composition.

After the optimum GAC-NiFe₃O₄ was obtained from the synthesis, the next scope of cell immobilization optimization was conducted. Firstly, the effect of the ratio (1: 0.5-4) of GAC-NiFe₃O₄ needed to the amount of sludge used towards biohydrogen production was studied. Subsequently, the optimum ratio was utilized to develop the immobilized cell using GAC-NiFe₃O₄ as a microbial support carrier in thermophilic dark fermentation. A comparative analysis of the attached and suspended systems was later conducted. Next, implementing the Monod model, the immobilized- GAC-NiFe₃O₄ was utilized to study the effect of sugar utilization at various initial sugar concentrations (10-30 g/L). The microbial community involved in the process was further analyzed by performing a microbial attachment and identification study.

Subsequently, the scope continued with the optimization of process parameters utilizing the established immobilized- GAC-NiFe₃O₄. Optimization of pH (5.0-7.0), temperature (50-70°C), and agitation (60-180 rpm) was performed. Next, the optimum pH, temperature, and agitation were utilized to study the effect of solid loading rate (SLR) and hydraulic retention time (HRT). Five variables (5%, 10%, 15%, 20%, and 25% w/v) were chosen in the SLR study. In contrast, five variables (48 h, 24 h, 12 h, 6 h, and 3 h) were chosen in the HRT study. All experimental responses were toward the productivity of biohydrogen.

The final scope was the utilization of POME for biohydrogen production. The POME was first characterized in physiochemical analysis and heavy metal quantification. Then, the effect of total carbohydrates (TC) was studied using six variables (10%, 30%, 50%, 70%, 90% and 100% v/v). All optimum conditions obtained were further validated. The biohydrogen production from POME utilizing GAC-NiFe₃O₄ was

performed with the optimum conditions obtained from the start. The microbial community involved in the process was also determined by microbial identification. Finally, the potential of GAC-NiFe₃O₄ in heavy metal removal was also evaluated in ten different types of heavy metals (K, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, and Zn).

1.5 Organization of Thesis

The thesis comprises five chapters. The first chapter, Chapter 1, describes the background of the research study. Chapter 1 includes the problem statement of the research, which leads to the study's objectives. The scope of the study concerning the objectives was presented before the organization of the thesis was explained.

Chapter 2 consists of the literature review section. In this section of the thesis, the literature review concerning the study was presented after information on the related topic was collected and summarized. The existing field summary was presented to indicate the study's context further. Reviews on biohydrogen production were made under several subheadings, including feedstock, inoculum, and soluble microbial products. An insight review on nanoparticle addition for biohydrogen enhancement was next presented. Important factors affecting biohydrogen production were reviewed and summarized accordingly. The utilization of optimization tools was reviewed as a part of the study.

Chapter 3 outlines the materials and methods used in the study. The flow of the methodology concerning the objectives was outlined before being presented. Sample collection, preparation, and characterization were among the preliminary studies. Culturing a mixed seed culture and general analysis of liquid and gas samples were

explained further. The modified Gompertz equation was included in this section as it will be used throughout the study for validation. A detailed methodology for each objective was presented in the subsequent subchapters.

Chapter 4 documented the results and discussion of the study. The first subchapter explained the synthesis optimization of the GAC-NiFe₃O₄. The performance of the optimum GAC-NiFe₃O₄ towards biohydrogen production was examined and later characterized. The second subchapter explained the optimization of cell immobilization on GAC-NiFe₃O₄. The effect of the inoculum ratio on GAC-NiFe₃O₄ was presented before the comparison between the suspended and attached system was studied. The initial sugar loading was studied using the Monod model, followed by microbial community analysis. The third subchapter explained the optimization of process parameters in biohydrogen production. The process parameter was optimized regarding pH, temperature, and agitation. The effect of SRT and HRT was also documented in this chapter. The final subchapter explained biohydrogen production from POME. The effect of TC on biohydrogen production was evaluated and validated, followed by microbial community analysis. The potential of GAC-NiFe₃O₄ in heavy metal removal from POME was presented and discussed at the end of the chapter.

The final chapter, Chapter 5, consists of the overall conclusion of this study. Some recommendations are also included for any future work related to the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Considering fossil fuels have limited sources and cannot be renewable, using them over an extended period is no longer sustainable. In this situation, biofuels' regenerative nature and adaptability to various biological sources can lower our reliance on fossil fuels while overcoming energy security issues (He et al., 2012; Mao et al., 2015). Positively, the rapid increase in biofuel production could contribute to reducing the carbon footprint resulting from charring fossil fuels (He et al., 2012). Currently, the most promising biofuels utilized worldwide are biogas (Mao et al., 2015). Hydrolysis is the initial step in biogas production, then comes acidogenesis and methanogenesis (Zainal et al., 2018). Hydrolysis is the process by which bacteria that produce fermentative cultures break down complex chemical substances into simpler monomers like amino acids, fatty acids, and monosaccharides (Zainal et al., 2018). These simple monomers, which include sugars, will then undergo additional degradation to produce acetate, hydrogen, and carbon dioxide during the acidogenesis process (Zainal et al., 2018). In the methanogenesis process, acetate, hydrogen, and carbon dioxide will serve as the precursors for the creation of methane (Zainal et al., 2018).

Biogas can be segregated into a few components of interest, such as biomethane, biodiesel, bioethanol, and biohydrogen (Daniel et al., 2023; Devi et al., 2023; Feng et al., 2023; Monika et al., 2023; Novia et al., 2023). The most environmentally friendly option is biohydrogen, with a maximum energy content of 142.0 KJ/g (Zilouei &

Taherdanak, 2015). Many approaches can produce biohydrogen, including dark and photo fermentation from organic substrate. In photofermentation, photophosphorylation transforms light energy into chemical energy (Bisaria, 2014). The possibility of producing hydrogen from free solar light and organic wastes makes photo fermentation as a method of producing hydrogen appear viable. Its low light conversion efficiency, low rate of hydrogen synthesis, and expensive photo bioreactors make its application far from practical (Sang et al., 2006). A thorough investigation is required to improve the rate of hydrogen synthesis and light absorption efficiency (Sang et al., 2006).

Dark fermentation, on the other hand, is a thoroughly proven and encouraging approach to producing biohydrogen. Dark fermentation is a biological process that converts organic materials into hydrogen gas without the presence of light. It is carried out by various anaerobic bacteria under anaerobic conditions. Dark fermentation for biohydrogen production has several benefits, including low cost, no need for light, versatility, and lower carbon emission (Ayodele et al., 2023). **Equation 2.1** shows a chemical process that occurs during dark fermentation that produces biohydrogen (Martino et al., 2021):



The benefits of dark fermentation make it a desirable method for producing biohydrogen, which has caused the method to evolve and improve over time. Dark fermentation is a rapid hydrogen production method, often adapted to large-scale fermentation techniques (Kumar et al., 2019). Hence, this method was chosen for the study. Besides biohydrogen generation, fermentation is extensively employed in

wastewater treatment due to its ability to produce minimal sludge compared to conventional aerobic processes. Additionally, it has the potential for energy recovery and proves to be more cost-efficient due to its simplicity in operation (Ansari et al., 2017). The broad adoption and successful performance of technologies designed to produce biohydrogen can benefit users and the wider community. Biohydrogen derived from various biological sources offers a solution to reduce heavy reliance on depleting natural resources, thus addressing concerns regarding energy insecurity. This is due to its renewable nature, wide applicability, and abundant availability (He et al., 2012; Mao et al., 2015). In an optimistic outlook, the swift advancement of biohydrogen production can lessen the world's dependence on fossil fuels, meet global energy demands, and diminish the carbon footprint associated with fossil fuel utilization. Biohydrogen production, therefore, decelerates global warming and climate change (He et al., 2012).

2.1.1 Feedstock for Biohydrogen Production

Biohydrogen was able to be produced from a variety of substrates, including simple sugars like glucose and sucrose (Sang et al., 2006). However, many studies utilized industrial (such as the food industry and brewery industry) and agricultural waste (such as dairy waste and palm oil mill effluent) that comprises organic compounds (Chu C.Y. et al., 2013; Liu I.C. et al., 2011; Stavropoulos et al., 2016). In addition, agronomic waste like Napier grass, cassava, and brewery spent grains are among the employed substrates for biohydrogen production (Balakrishnan et al., 2023; Martinez-Burgos et al., 2020; O. Sarkar et al., 2021). Utilizing the mentioned waste can mostly treat the waste while producing biohydrogen in return. As the global economy expands, biohydrogen production is increasingly utilized to repurpose POME from the

oil palm industry. POME is valued as a renewable energy source due to its carbohydrate content, mainly glucose and xylose, at 20 g/L and 11-12 g/L, respectively (Rosli et al., 2015). The abundance of organic acids, proteins, and lipids in POME renders it an excellent source of sugar feedstock and holds promise as a potential substrate for biohydrogen production (Zainal et al., 2018). Many former reports have successfully utilized POME as the substrate for hydrogen production (Abdullah et al., 2020; Arisht et al., 2022; Ismail I. et al., 2010; Maaroff et al., 2019; Mahmud et al., 2019; Rosa et al., 2020). The feedstock potential comparison is summarized in **Table 2.1**. From the table, POME has a higher (16.3-44.2%) potential in biohydrogen production than others.

Table 2.1 : Feedstock potential comparison

Feedstock	Biogas yield (L/ kg VS)	Reference
Food waste	600	(Zhang R. et al., 2007)
Cattle manure	400-450	(Sidik et al., 2013) (Pöschl et al., 2010)
POME	502-717	(O-Thong et al., 2012) (Sidik et al., 2013)
Swine manure	400-450	(Chae et al., 2008)
Vegetable waste	450	(Ramanujam, 2015)

2.1.2 Inoculum for Biohydrogen Production

There are two types of microorganisms in a biological hydrogen generation system: hydrogen producers and non-hydrogen producers (refer to **Figure 2.1**). Microorganisms that generate hydrogen comprise photosynthetic, photo-fermentative, and dark fermentative species (Bisaria, 2014). Green algae and cyanobacteria are examples of photosynthetic microorganisms that split water into hydrogen and oxygen using light as an energy source (Ahmed et al., 2021). In the presence of light, photo fermentative bacteria were responsible for breaking down organic materials into

hydrogen (Srivastava et al., 2020). In contrast, dark fermentative microorganisms convert organic substrate into hydrogen through a sequence of metabolic processes. The hydrogenase enzyme system oxidates electrons and reduces protons to produce hydrogen (Martino et al., 2021). The dark fermentation can occur in the absence of light, in contrast to photo fermentation.

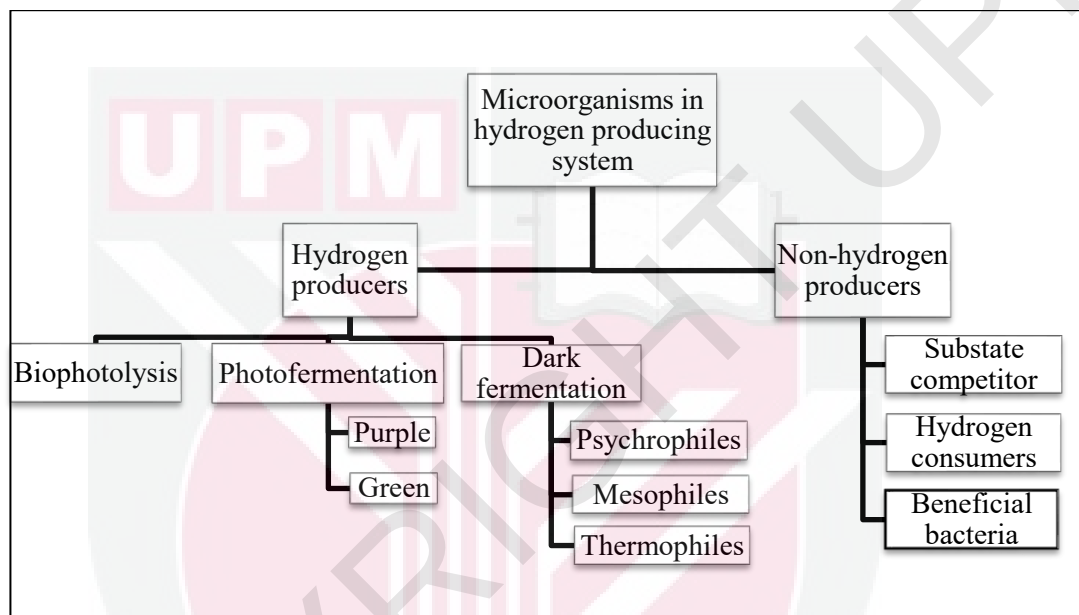


Figure 2.1 : Diversity of hydrogen-producing microorganisms (Bisaria, 2014)

Under dark fermentation, the bacteria can be segregated into different temperature tolerances of psychrophiles, mesophiles, and thermophiles bacteria (Bisaria, 2014). Psychrophiles are microorganisms that can thrive in cold environments (-20-10°C), while mesophiles and thermophiles are in moderate (10-45°C) and hot (45-80°C) environments, respectively (Bisaria, 2014). In comparison, the thermophiles anaerobic bacteria contributed more significantly to biohydrogen production (Sun et al., 2021; Toledo-Cervantes et al., 2020). Thermophiles generate a robust hydrogenase, enhancing the rate of hydrogen production rate (HPR) and hydrogen yield (HY) (Jamali et al., 2016; Prasertsan et al., 2009). Thermophiles operate at higher

temperatures that will likely increase the HPR and HY due to the reduced presence of hydrogen-consuming bacteria (Kargi et al., 2012). In addition to this, thermophiles contain a significant source of resilient enzymes. Hot wastewater, such as POME, was an attractive substrate for hydrogen production as the production was released at 70-80°C (Khongkliang et al., 2019).

Table 2.2 compares the performance in terms of hydrogen yield by different bacteria populations. The bacteria population was highly varied among the reactors based on the substrate type and operational conditions (Jamali, Md Jahim, et al., 2019). A previous study demonstrated the biohydrogen process could withstand the toxicity effect of furfural due to the presence of hydrogen-producing bacteria such as *Bacilli* (8.9%) and *Clostridia* (5.4%) of the *Firmicutes* species, as well as phyla *Proteobacteria* (22.4%), *Actinobacteria* (9.1%), *Bacteroidetes* (2.6%), and *Chloroflexi* (10.1%). (Tawfik et al., 2024) As previously mentioned, a number of significant populations of bacteria, including those belonging to the phylum *Firmicutes* and *Proteobacteria*, including *Bacilli*, *Clostridium*, *Klebsiella*, and *Enterobacter*, are engaged in the steady and effective synthesis of biohydrogen (Sivagurunathan et al., 2016).

One of the major concerns in using mixed cultures is the hydrogen consuming pathway by homoacetogenesis and methanogenesis (Mahmod et al., 2019). However, Kraemer & Bagley (2008) reported that hydrogen consumption was of minor concern in continuous fermentative biohydrogen systems because it could only account for 2-11% of the HY decrease, and this could be further avoided by sparging nitrogen gas to the continuous bioreactor. In addition, The inoculum pre-treatment by thermal pre-

treatment was reported to be one of the ways to create extreme conditions where spore-forming hydrogen producers will survive, whereas non- spore-forming methanogens are killed or inhibited (Mahmod et al., 2019). A previous study revealed that the bacteria population depends highly on the fermentation conditions (Tawfik et al., 2024), hence the need for the study.

Table 2.2 : Performance of different bacteria populations

Bacteria population	Culture type	Bacteria type	Hydrogen yield	Reference
<i>Bacilli Clostridia</i>	Mixed culture	Mesophiles	2.0 mol H ₂ /mol sugar	(Tawfik et al., 2024)
<i>Clostridium</i>	Mixed culture	Thermophiles	2.5 mol H ₂ /mol sugar	(Mahmod et al., 2019)
<i>Clostridium beijerinckii RZF-1108</i>	Pure culture	Mesophiles	1.9 mol H ₂ /mol sugar	(Zhao X. et al., 2012)
<i>Clostridium beijerinckii NCIMB8052</i>	Pure culture	Mesophiles	2.1 mol H ₂ /mol sugar	(Seelert et al., 2015)
<i>Clostridium butyricum</i>	Pure culture	Mesophiles	2.2 mol H ₂ / mol sugar	(Beckers et al., 2013)
<i>Clostridium butyricum</i>	Mixed culture	Mesophiles	2.5 mol H ₂ / mol sugar	(Zhao W. et al., 2013)
<i>Thermoanaerobacterium thermosaccharolyticum</i>	Mixed culture	Thermophiles	2.2 mol H ₂ / mol sugar	(Jamali, Md Jahim, et al., 2019)
<i>Thermoanaerobacterium thermosaccharolyticum</i>	Mixed culture	Thermophiles	1.0 mol H ₂ / mol sugar	(Jamali et al., 2017)
<i>Thermoanaerobacterium thermosaccharolyticum</i>	Mixed culture	Thermophiles	1.2 mol H ₂ / mol sugar	(Jamali, Dzul Rashidi, et al., 2019)
<i>Trichoderma asperellum BPLMBT1</i>	Pure culture	Mesophiles	2.8 mol H ₂ /mol sugar	(Shanmugam et al., 2020)

2.1.3 Soluble Microbial Products

There are two metabolic pathways in the production of hydrogen from glucose (**Figure 2.2**) (Kumar et al., 2019). The first pathway is the formate hydrogen production pathway (refer to **Equation 2.2**). In this pathway, formate is broken down by formate hydrogen lyase enzyme. The second pathway is the nicotinamide adenine dinucleotide

(NADH)-dependent hydrogen production pathway (refer to **Equation 2.3**). In the NADH-dependent hydrogen production pathway, NAD re-oxidation occurs with the aid of the hydrogenase enzyme. The chemical reaction is as follows:

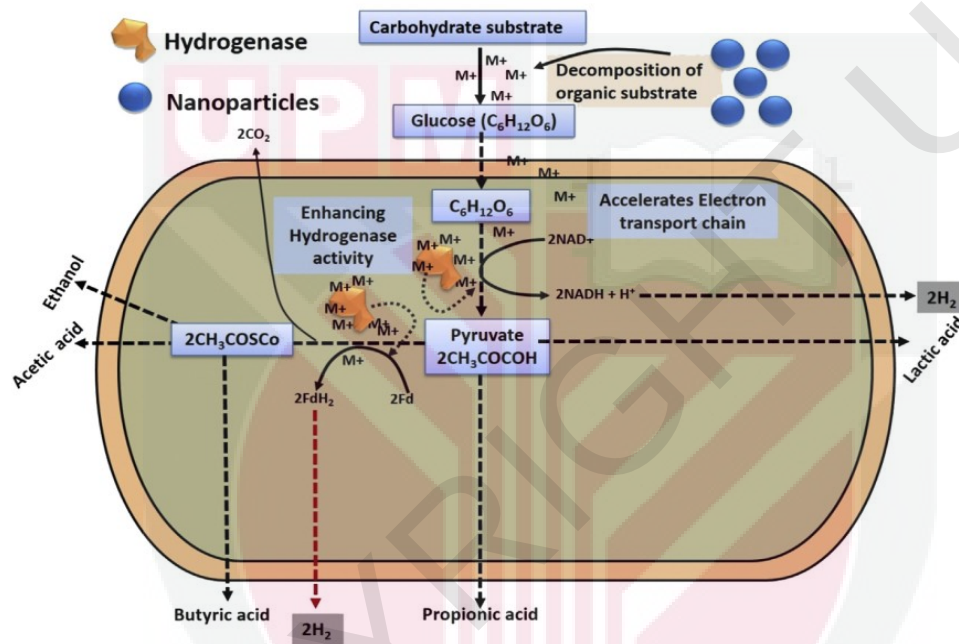
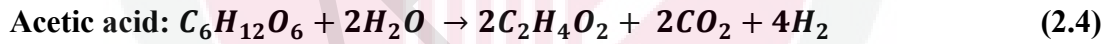


Figure 2.2 : Biohydrogen production pathways utilizing nanoparticles
(Kumar et al., 2019)

Soluble microbial products (SMP) are the end product of numerous microbial metabolic pathways, including solvents and total volatile fatty acids (TVFA).

Equations 2.4-2.8 show the overall chemical reaction in the metabolic pathways (Emisha et al., 2024). The SMP quantification is considered the most accurate way to assess biohydrogen generation performance. Lutpi et al. (2016) and Jamali et al. (2019) stated in their work that the generation of TVFA as a liquid product from several metabolic pathways is directly related to the synthesis of biohydrogen. The

formation of acetic acid (**Equation 2.4**) and butyric acid (**Equation 2.5**) favors the biohydrogen production as 2 mol of acetic acid will concurrently produce 4 mol of hydrogen, whereas 2 mol of hydrogen will be produced via the butyric acid pathway (Maaroff et al., 2019). Despite the most frequent reason for biohydrogen inhibition through lactic acid metabolic pathways (**Equation 2.6**), few reported that lactic acid production assisted hydrogen generation and the discussion is ongoing (García-Depraet et al., 2021). A previous study reported that propionic acid production (**Equation 2.7**) does not favor biohydrogen production since 2 mol of hydrogen will be consumed instead of produced (Lutpi et al., 2016). Ethanol production (**Equation 2.8**) also does not favor hydrogen production since the pathways are shifted toward solvent formation (Maaroff et al., 2019). Based on the equations provided, more than 70% of the total SMP are butyrate and acetate, resulting in greater biohydrogen output (Lutpi et al., 2016). It is also well-known that the biohydrogen yield will be better if the TVFA/SMP is higher (Narasaiah & Mandal, 2019).



2.2 Immobilization for Biohydrogen Enhancement

The dark fermentation process is an efficient method to transform organic waste into biohydrogen. However, the low settling velocity in a conventional reactor makes it hard to separate solids from liquids, leading to larger reactor volume, lower effluent quality, and unstable fermentation (Montañés Alonso et al., 2014). In light of this

concern, immobilization technology has been innovated to enhance productivity while overcoming the demerits of the conventional process (Kumar et al., 2016). Nearly all hydrogen-producing bacteria can be confined within the biosystem using immobilization technology, providing protection against inhibitory substances (Singh et al., 2013). Immobilized cells in dark fermentation offer continuous reactor operation, better removal of organic material from the aqueous phase (with adsorptive properties of immobilizing agents), and effortless separation of the carrier from treated effluent (Srikanth et al., 2017). So, in this study, using immobilized cells in dark fermentation was proposed for biohydrogen production, considering all the advantages mentioned.

2.2.1 Additives for Cell Immobilization

Recently, there have been approaches to address the challenge of low biohydrogen production in dark fermentation, mainly cell and enzyme immobilization (Woo et al., 2022). To put it briefly, there are two types of cell immobilization: induced and spontaneous (Junter & Jouenne, 2004). Induced immobilization involves intentionally entrapping or linking microbial cells to matrices or supports, sometimes using chemical methods to form covalent bonds, although this can compromise cell viability (Woo et al., 2022). Spontaneous immobilization occurs when microbial cells naturally adsorb to carriers via weak non-covalent bonds and may form biofilms, enhancing attachment and immobilization (Woo et al., 2022).

Based on previous studies, GAC is known to be a support material in cell immobilization in thermophilic biohydrogen production (Jamali, Dzul Rashidi, et al., 2019; Lutpi et al., 2015; Park et al., 2019; Song et al., 2019). The large surface area,

inertness, low toxicity, and exceptional mechanical properties characterize GAC. Additionally, its highly porous structure encourages cell viability and allows fermentative bacteria to grow freely within the porous structure as well on the surface of the material (Barca et al., 2015). It has been shown that using nanoparticles on GAC enhances bacterial community growth (up to 57.91%), leading to increased biohydrogen production (by 46.9-60%) even under mesophilic conditions (Zhao et al., 2020). Hydrogenase-catalyzed biohydrogen production will function better in such immobilization techniques at thermophilic temperatures (60°C), as used in this study. Previous studies have shown that thermophilic anaerobic bacteria produced more biohydrogen at high temperatures than mesophilic bacteria (Asada et al., 2000; Wenqian Zhao et al., 2020). In a study on the effect of immobilized cells on thermophilic biohydrogen production in a fluidized bed reactor, 1.24 mol H₂/mol glucose was obtained (Jamali, Dzul Rashid, et al., 2019). Compared to different sugar utilization, up to 5.60 mol H₂/mol sucrose was attained (Lutpi et al., 2015). Despite the enhancement (28-69.60%) in biohydrogen production previously reported, a suitable strategy for supporting cell growth was further required (Moura et al., 2020; Reddy et al., 2017). As such, enzyme immobilization was widely implemented (Moustafa et al., 2023; Shanmugam et al., 2020).

Enzyme immobilization involves attaching or entrapping enzymes to support matrices, similar to cell immobilization. Techniques include adsorption, covalent binding, cross-linking, and encapsulation (Meryam Sardar, 2015). Previous researchers have reviewed the differences between each immobilization technique (Kotwal et al., 2024; Meryam Sardar, 2015; Woo et al., 2022). Among the immobilization techniques, covalent binding is widely used in industry to immobilize

the enzyme due to its strong bonding and resistivity. Immobilizing the enzyme on an appropriate matrix should increase its stability for practical application, even in unfavorable circumstances, lowering its cost with the possibility of reusing the immobilized enzyme (Basile et al., 2012). Enzyme immobilization on a basic matrix such as activated carbon could delay the desired outcome and cause the loss of enzyme structure and activity (Chu et al., 2011). However, immobilizing enzymes on nanomaterials is anticipated to address these shortcomings (Chu et al., 2011). The stability of the enzyme or protein is increased through enzyme immobilization by NPs. For example, immobilization of cellulases on chitosan-coated magnetite (Fe_3O_4) NPs has been reported to improve thermal and pH stability while increasing the catalytic characteristics of cellulases (exhibited optimal temperature of 60°C) by 1.5 times when compared to free cellulase, which demonstrated an optimal temperature of 50°C (Sánchez-Ramírez et al., 2017). Previously, the addition of NPs without immobilization improved enzyme production (Srivastava et al., 2015). All can be achieved at a low concentration of NPs (Srivastava et al., 2020). The synthesis of biohydrogen is catalyzed by combining NPs large surface area and small size, which enhances the reaction sites available for enzyme interaction with the substrate (Sekoai et al., 2019; Vaghari et al., 2016). Nevertheless, the NPs may significantly reduce catalytic activity during catalytic transformations because of the effects of magnetic and nanometer-scale on the active surface area. (Kangari & Yavari, 2016). Therefore, the NPs must be loaded onto a biocompatible porous carrier (as suggested in this study) to inhibit nanoparticle aggregation while dispersing the particles across the pores and surface to enhance the number of available catalytic sites. The electron transport rate amongst the microorganisms is accelerated when NPs are loaded over porous GAC because it increases the material's conductivity and offers many electron

transfer sites (Chen, Rotaru, Liu et al., 2014). For example, a previous study on enzyme immobilization on silica nanoparticles obtained 1.3 times higher biohydrogen production (3.62 mmol H₂/L.h) than the control (free enzyme) (Kotwal et al., 2024). The techniques also increased biohydrogen production activity by 75% from 35% on free enzymes using chitin and chitosan as support material (Verma et al., 2020).

Additionally, other conductive materials like metal oxides and biochar have the potential to facilitate direct electrical connections for direct interspecies electron transfer (DIET). Microorganisms can generate conductive biofilms or aggregates on the surfaces of conductive materials or vice versa, depending on the particle size. DIET can enhance biohydrogen production in a setting with additional conductive materials. The microorganisms can adhere to the surface of the conductive materials instead of necessarily congregating for direct physical contact (Chen, Rotaru, Shrestha, et al., 2014). The adhered will lead to improvements in the lag time and production rate. For example, a study discovered that addition with Fe₃O₄ was more successful at promoting a DIET than naturally occurring biological electrical connections. (Kato & Watanabe, 2010). Furthermore, recent evidence indicates that materials such as activated carbon can enhance DIET by improving electron exchange (Chen, Rotaru, Shrestha et al., 2014). As a result, using GAC-NiFe₃O₄ in this study may already show the further potential to stimulate microbial attachment in biohydrogen production.

Presently, enzymatic hydrolysis represents a significant expenditure within a biohydrogen production process. Its disadvantages include partial substrate conversion into the intended product, slower reaction rate, and enzyme deactivation at

high temperatures (Srivastava et al., 2019). Furthermore, the process becomes more expensive and time-consuming when a highly concentrated commercial enzyme is used partially or simultaneously (Yang et al., 2011). Appropriate NPs matrix type, concentration, and ideal immobilization conditions should be investigated to make the immobilization of enzymes on NPs feasible. Due to the ease of collecting and recycling NPs, enzymatic immobilization on NPs has received much attention lately.

Therefore, this study suggests that this method facilitates and improves biohydrogen production. By supporting the hydrogen-producing [NiFe]-, [FeFe]-, and [Fe]-hydrogenase enzymes and their antimicrobial qualities, the approach of nanoparticle addition has been proposed as a means of enriching the hydrogen producers in the mixed culture (Kim & Kim, 2011). Nanoparticle addition has a strong potential to improve biohydrogen production due to its specific structure and physiochemical features (Srivastava et al., 2019). Adding nanoparticles could enhance the anaerobic digestion system by enabling intracellular electron transport and providing essential nutrients for the growth of microorganisms (Di et al., 2022).

Adding nanoparticles in this study is anticipated to expedite while improving biohydrogen production. All the literature reviews regarding the utilization of additives to enhance biohydrogen production are summarized in **Table 2.3**.

Table 2.3 : Summary of recent research on additives to enhance biohydrogen production

Enhancement Approach	Feedstock	Culture Source	Process Condition	Carbon Source	Biohydrogen Productivity	Reference
A) Addition of nanoparticles						
Ag	Synthetic media	Mixed culture	Mesophilic	Sucrose	2.5 mol H ₂ /mol sugar	(Zhao W. et al., 2013)
Al, Cu, and Fe	Synthetic media	Mixed culture	Mesophilic	Hexose	4.3 mol H ₂ /mol sugar	(Zhu et al., 2014)
CoO and NiO	POME	Single culture	Mesophilic	N/A	0.56 L H ₂ /g COD _{removed}	(Mishra, Thakur, et al., 2018)
CoO and NiO	Rice mill wastewater	Single culture	Mesophilic	Glucose	181.1 mL H ₂ /L	(Rambabu, Bharath, Thanigaivelan, et al., 2021)
Fe and Ni	Starch	Mixed culture	Mesophilic	N/A	149.8 mL H ₂ /g.VS	(Taherdanak et al., 2015)
Fe and Ni	Synthetic media	Mixed culture	Mesophilic	Glucose	551 mL H ₂ /g VS	(Taherdanak et al., 2016)
Fe and Mg	Synthetic media	Single culture	Mesophilic	Glucose	1.9 mol H ₂ /mol sugar	(Zhao X. et al., 2012)
Fe and Mg	Pre-treated starch	Mixed culture	Mesophilic	Glucose	1.9 mol H ₂ /mol sugar	(Bao et al., 2013)
Fe, N and P	POME	Mixed culture	Thermophilic	N/A	16.9 mmol H ₂ /L.h	(Prasertsan et al., 2009)
Fe ₂ O ₃	Carbon monoxide	Mixed culture	Mesophilic	N/A	1.6 mmol H ₂ /L	(Sinharoy & Pakshirajan, 2020)
Fe ₂ O ₃ and FeSO ₄	Synthetic media	Single culture	Mesophilic	Glucose	1.7 mol H ₂ /mol sugar	(Mohanraj et al., 2014)
				Sucrose	5.2 mol H ₂ /mol sugar	
Fe ₂ O ₃ and Ni	Dairy wastewater	Mixed culture	Mesophilic	N/A	47.7 mmol H ₂ /g VSS.d	(Gadhe et al., 2015a)
Fe ₂ O ₃ and Ni	Distillery wastewater	Mixed culture	Mesophilic	N/A	18.1 mmol H ₂ /g VSS.d	(Gadhe et al., 2015b)
Ni	Synthetic media	Mixed culture	Mesophilic	Sucrose	2.1 mol H ₂ /mol sugar	(Gou et al., 2015)
Ni	Synthetic media	Mixed culture	Mesophilic	Glucose	2.5 mol H ₂ /mol sugar	(Mullai et al., 2013)
Ni-Gr	Synthetic media	Mixed culture	Thermophilic	N/A	41.3 mL H ₂ /g COD	(Elreedy et al., 2017)
SO ₄	Synthetic media	Mixed culture	Mesophilic	Glucose	2.8 L H ₂ /d	(Hwang et al., 2009)
B) Immobilization using nanoparticles						
γ-Fe ₂ O ₃	Pre-treated starch	Mixed culture	Mesophilic	Glucose	104.8 mL H ₂ /g COD _{removed}	(Nasr et al., 2015)
Fe and Fe ₃ O ₄	Sugarcane bagasse	Single culture	Mesophilic	Glucose	0.9 mol H ₂ /mol sugar	(Reddy et al., 2017)
Fe ₃ O ₄	Cotton stalk	Single culture	Mesophilic	Glucose	0.4 mol H ₂ /mol sugar	(Zhang, Qin et al., 2021)
				Xylose		

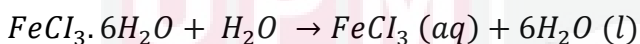
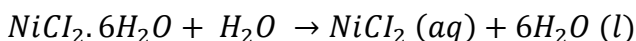
Table 2.3 : Continued

C) Immobilization using GAC						
GAC	Synthetic POME	Mixed culture	Thermophilic	Sucrose	4.3 mmol H ₂ /L.h	(Lutpi et al., 2016)
GAC	Synthetic POME	Mixed culture	Thermophilic	Glucose Xylose	7.8 mmol H ₂ /L.h	(Jamali, Md Jahim, et al., 2019)
GAC	Synthetic POME POME	Mixed culture	Thermophilic	Glucose Xylose	5.2 mmol H ₂ /L.h	(Jamali, Dzul Rashidi, et al., 2019)
D) Immobilization using activated carbon embedded with nanoparticles						
PAC-NiFe ₂ O ₄	Citric acid wastewater	Mixed culture	Mesophilic Thermophilic	Glucose	260 mL/g sugar 192 mL/g sugar	(Zhao W. et al., 2020)
GAC-Fe ₃ O ₄	Pre-treated dates fruit waste	Single culture	Mesophilic	N/A	238.7 mL H ₂ /g	(Rambabu, Bharath, et al., 2020)
GAC-ZnNiHT	Synthetic media	Mixed culture	Mesophilic	Sucrose	2.62 mol H ₂ /mol sugar	(Sybounya & Nitisoravut, 2021)
GAC-NiFe ₃ O ₄	Synthetic media POME	Mixed culture	Thermophilic	Glucose Xylose	3.74 mol H ₂ /mol sugar 1.77 mol H ₂ /mol sugar	This study

PAC: Powder activated carbon; GAC: Granular activated carbon; POME: Palm oil mill effluent.

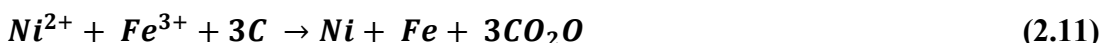
2.2.2 Reaction Mechanism of the Synthesis

In the synthesis of GAC-NiFe₃O₄, when NiCl₂·6H₂O and FeCl₃·6H₂O are combined with water, dissolution or hydration occurs, causing the complex to disintegrate into its nickel, iron, and chloride ions components in an aqueous solution. This reaction's chemical equation can be expressed as follows:



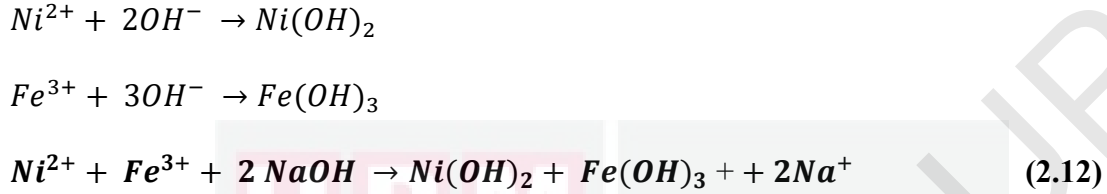
As a result, based on **Equations 2.9** and **2.10**, six molecules of water were discharged into the solution throughout the dissolving process because nickel and iron chloride both possess six waters of hydration. This exothermic reaction means heat and energy are emitted when the ions dissociate.

GAC, a highly porous structure with a large surface area, interacts effectively with molecules and ions in solution. Through adsorption, the GAC, Ni²⁺, and Fe³⁺ interacted. The GAC will attract and capture molecules and ions on its surface through electrostatic interactions, effectively retaining them. Alternatively, by donating electrons to the Ni²⁺ and Fe³⁺ ions, the GAC can function as a reducing agent as follows:



Based on **Equation 2.11**, the GAC converts the Ni²⁺ and Fe³⁺ ions into their elemental states, while the carbon also reacts with oxygen to produce carbon dioxide, which is

released as a gas. Subsequently, a co-precipitation reaction occurs when Ni^{2+} and Fe^{3+} ions interact with NaOH at high temperatures (110°C). Co-precipitation involves a doubled displacement reaction that creates insoluble metal hydroxides by the NaOH hydroxide ions (OH^-) switching places with the Ni^{2+} and Fe^{3+} ions. This reaction's chemical equation is as follows:



In **Equation 2.12**, $Fe(OH)_3$ and precipitates were formed when the Ni^{2+} and Fe^{3+} ions reacted with the hydroxide ions (OH^-) from NaOH. The Cl^- and Na^+ ions remain in the solution until rinsed out with deionized water after the reaction is completed. In addition to accelerating the reaction, the high temperature makes the reactants more soluble. The synthesis is completed when the reaction forming precipitates, attached to the surface of GAC, as shown in **Equation 2.13**:



2.2.3 Characterization Analysis

Coconut shells have been widely used as activated carbon. The product can be modified to meet certain aims. This study synthesized the coconut shells into producing granular activated carbon embedded with nanoparticles ($GAC-NiFe_3O_4$) as microbial support carriers for thermophilic biohydrogen production. With the addition of Ni and Fe_3O_4 , the material should become conductive, which can be tested using a four-point probe (FPP), as previously mentioned (Zhao W. et al., 2020). The structure, composition, and morphology of the synthesized material ($GAC-NiFe_3O_4$) can be

characterized by the Fourier transform infrared spectroscopy (FTIR), the X-ray diffraction (XRD) and the field-emission scanning electron microscopy (FESEM) following (Petcharoen & Sirivat, 2012). Moreover, the material (GAC-NiFe₃O₄) should also exhibit magnetic characteristics due to the addition of Fe₃O₄. The lag between an applied magnetic field and a ferromagnetic material's change in magnetization is known as magnetic hysteresis. A loop appears on a magnetization vs magnetic field graph when the material retains some magnetization after the external magnetic field is eliminated or reversed. This loop's important characteristics are saturation magnetization (maximum magnetization) and coercivity (the field required to demagnetize the material) (Rambabu, Bharath, Banat, et al., 2021). On the other hand, porosity was determined using a Brunauer-Emmet-Teller (BET) method (Kacan, 2016). Based on the literature review, the BET surface area was reported to be between 1.74 and 28.04 m²/g (Kacan, 2016; Kusurini et al., 2018; Venault et al., 2008; Yang et al., 2016). Further significance of the characterization analysis of GAC-NiFe₃O₄ is explained in the discussion section of the thesis.

2.2.4 Potential of Heavy Metal Removal

Heavy metals are highly toxic and cannot degrade through conventional biological reactions (Nyirenda et al., 2022). The discharge of heavy metals into the water system can cause serious health issues for living organisms (Liu et al., 2023). In the present study, the source of the granular activated carbon is coconut shell. Coconut shells are an excellent source of activated carbon because they contain cellulose, hemicellulose, and lignin. In addition, materials containing lignocellulosic properties are excellent adsorbent materials for activating carbonaceous materials (Oil et al., 2019).

Adsorbents are materials that adsorb wastewater pollutants, while adsorbates are the substances to be adsorbed. The type of adsorbents, the amount of adsorbate in the mixture, and the effectiveness of the adsorption system all influence the rate of adsorption (Oil et al., 2019). However, in this study, the conventional method of using activated carbon as an adsorbent was further enhanced with NPs additives (Nickel and Magnetite), creating a carbon composite. Nowadays, the composite adsorbent is rigorously studied for the treatment of POME (Ismail et al., 2022; Liu et al., 2022; Mandal et al., 2021; Shahrokhi-Shahraki et al., 2021;). Implementing activated carbon composites has shown (refer to **Table 2.4**) the significant potential to be heavy metal adsorbent.

Table 2.4 : Potential of heavy metal removal by different carbon composites

Absorbent	Wastewater	Absorbate	Removal (%)	Reference
AC- SiO ₂	Aqueous solution	Pb, Cd, Ni, Cr, Zn	24.0-90.0	(Karnib et al., 2014)
AC-SiO ₂	Aqueous solution	Cu, Pb, Cd, Zn	62.6-98.1	(Nyirenda et al., 2022)
AC-FeO	Aqueous solution	Pb, Cu, Zn, Cd	40.0- 95.0	(Liu X. et al., 2022)
AC-Fe ₃ O ₄	Aqueous solution	Cd, Pb	92.7- 97.9	(Wang N. et al., 2024)
AC- SiCin	Aqueous solution	Cu, As	44.2-92.0	(Mubarak et al., 2022)
AC-ZnO	Aqueous solution	Pb, Cr, Cu	38.3-88.2	(Zhang, Qiyun et al., 2020)
AC-NiFe ₂ O ₄	Aqueous solution	Cu, Zn	58.8-92.8	(Moussa et al., 2021)
AC-Fe ₃ O ₄	Aqueous solution	Pb, F	25.0-90.0	(Alagarasan et al., 2022)

The conventional method of activated carbon utilization compared to carbon composites as adsorbent is summarised in **Table 2.5**. Regarding porosity, both activated carbon and carbon composites were reported to have high surface area and

porosity. For example, the previous researcher reported a high surface area of 801.11-941.23 m²/g of activated carbon from coconut shells (Jamali et al., 2017). Carbon composites, on the other hand, were reported to have a lower surface area of 1.74-28.04 m²/g (Kacan, 2016; Kusrini et al., 2018; Venault et al., 2008; Yang et al., 2016). However, the pore size distribution for carbon composites can be altered for the specific heavy metal according to the ionic size (Choi et al., 2023). By adjusting the pore structure, the selectivity could be further enhanced.

In terms of forces in activated carbon, there's an attractive interaction between the metal ions and the carbon surface named Van der Waals forces (Zhao et al., 2024). Compared to activated carbon, the carbon composite contains ion-exchange properties that enable ion exchange between the ions in the composite and heavy metal. The ion exchange is possible due to the functional group on the composite surface. Functional groups can be introduced onto the composite's surface to form chemical bonds with metal ions, thus increasing adsorption capacity (Kim et al., 2021). Carbon composites can be chemically modified or functioned to improve their affinity for certain heavy metals.

On the other hand, activated carbon can chemically bind heavy metal ions through ion exchange and physical adsorption. Like activated carbon, carbon composite areas can develop electrostatic charges on the surfaces, attracting oppositely charged heavy metal ions (Zhang et al., 2021).

Furthermore, activated carbon may contain organic molecules that form complexes with heavy metal ions (Zhang et al., 2021). The complexes can enhance the adsorption

capacity of the carbon for specific metals. In comparison, carbon composites can contain organic molecules or functional groups that form complexes with heavy metal ions. The complexes can be more stable than the individual ions and enhance adsorption.

Comprehensively, different activated carbon/ carbon composites may have a greater affinity for certain metals based on their composition and surface modifications. However, both activated carbon and carbon composites have the advantage of regenerated and reused by desorbing the adsorbed heavy metals (Nguyen et al., 2023). These advantages make them sustainable for heavy metal remediation in specific applications. Overall, implementing carbon composites in this study proved to have the potential to remove heavy metals from POME. However, further study needs to be done on carbon composite materials to target specific heavy metals and optimize the performance for heavy metal remediation. Carbon composites provide a versatile tool for heavy metal remediation in water treatment, environmental cleanup, and industrial processes while producing biohydrogen in return.

Table 2.5 : Comparison of activated carbon and carbon composites as absorbent

Term	Activated carbon	Carbon composites
Structure	High surface area and porosity	
Pore size distribution	Activated carbon contains micropores and mesopores of various sizes. These different pore sizes allow for the adsorption of heavy metal ions of varying sizes.	The pore size distribution in carbon composites can be tailored to target specific heavy metals. Different metals have different ionic sizes, so adjusting the composite's pore structure can enhance selectivity.
Forces	Activated carbon attracts heavy metal ions through weak Van der Waals forces. These forces arise due to the proximity of electrons between the metal ions and the carbon surface, creating an attractive interaction that holds the metal ions in place.	Carbon composites possess ion-exchange properties. In this mechanism, the composites exchange ions for heavy metal ions in the solution. Functional groups on the composite surface can facilitate this ion exchange process.
Chemical binding	Activated carbon can chemically bind heavy metal ions through ion exchange and physical adsorption.	Carbon composites can be chemically modified or functionalized to improve their affinity for certain heavy metals. Functional groups can be introduced onto the composite's surface to form chemical bonds with metal ions, enhancing adsorption capacity.
Electrostatic interactions	Like activated carbon, carbon composites can develop electrostatic charges on their surfaces, attracting oppositely charged heavy metal ions.	
Complexation	Activated carbon can contain organic molecules that form complexes with heavy metal ions. The complexes can enhance the adsorption capacity of the carbon for specific metals.	Carbon composites can contain organic molecules or functional groups that form complexes with heavy metal ions. The complexes can be more stable than the individual ions and enhance adsorption.
Specificity	Different activated carbon/ carbon composites may have a greater affinity for certain metals based on their composition and surface modifications.	
Regeneration	Activated carbon and carbon composites can be regenerated and reused by desorbing the adsorbed heavy metals. This makes them a sustainable choice for heavy metal remediation in specific applications.	

2.3 Important Factors Affecting Biohydrogen Production

In recent years, abundant studies have highlighted the importance of recognizing the various factors influencing biohydrogen production. Factors such as initial pH, temperature, agitation, SLR, HRT, and substrate composition were more likely to

impact biohydrogen production. All of these factors were reviewed in the following section.

2.3.1 pH

One of the crucial parameters in biohydrogen production is the pH. The accumulation of biohydrogen is entirely pH-dependent and inversely correlates with hydrogen yield (Silva et al., 2019; Czitrom, 2016). So, any alterations in pH can impact the growth and metabolic pathway of bacteria. The soluble metabolites indicate that the changes influenced the metabolic pathway of the hydrogen-producing bacteria in pH (Khongkliang et al., 2019). This emphasizes the significance of controlling pH during reactor operations to ensure optimal conditions for biohydrogen production

The pH between 4.5-9 is optimal for biohydrogen production (Stavropoulos et al., 2016). Sivagurunathan et al. (2015) found the best biohydrogen production when the pH exceeds 5.5 and less effective at pH 5. Lowering pH affects hydrogenase enzymes and reduces the growth of hydrogen-producing bacteria (Liu et al., 2011). The sporulating hydrogen-producing bacteria can thrive in environments with extreme pH levels, in which methanogens may not be able to survive and be eliminated (Shida et al., 2009). Overall, pH is a key factor in determining the growth of bacteria. **Table 2.6** summarizes the optimum pH for biohydrogen production, which was found between

4-9.

Table 2.6 : Comparison of optimum pH for biohydrogen production

Substrate	Range	Optimum	Reference
Dairy wastewater	4.0-5.3	4.0	(Silva et al., 2019)
End-of-life dairy products	4.0-5.7	5.0	(Stavropoulos et al., 2016)
Napier grass	4.5-7.0	5.5	(Balakrishnan et al., 2023)
Food industry wastewater	5.0-7.0	5.5	(Chu et al., 2013)
Synthetic POME	5.0-6.5	5.5	(Lutpi et al., 2016)
Synthetic POME	5.0-7.5	5.5	(Lutpi et al., 2015)
Cassava processing wastewater	4.0-9.0	6.0	(Martinez-Burgos et al., 2020)
Brewery yeast waste	4.0-7.0	6.0	(Liu et al., 2011)
Sewage sludge	5.0-8.0	6.0	(Xiao et al., 2013)
POME	5.0-7.0	6.0	(Jamali, Dzul Rashidi, et al., 2019)
Lactate wastewater	6.5-8.5	8.5	(Ziara et al., 2019)
Brewery spent grains	4.0-10.0	9.0	(Sarkar et al., 2021)

2.3.2 Temperature

Temperature is important because it affects the generation of biohydrogen and byproducts, the degradation of substrates, and the growth of microorganisms (Chozhavendhan et al., 2020). Dark fermentation can occur in the following temperature ranges: ambient (15-30°C), mesophilic (30-49°C), thermophilic (50-64°C), hyperthermophilic (65-80°C), and extreme thermophilic (>80°C) (Preethi et al., 2019). Thermodynamically, higher temperatures encourage hydrogen production because they increase the system's entropy (Akaçin et al., 2024). For example, previous researchers examined the optimum temperature for dark fermentation of POME, which ranges from 30-50°C (Zainal et al., 2018). They reported the lowest desirability for biohydrogen under 50°C. Furthermore, tests conducted in the thermophilic temperature range demonstrated the highest yield for hydrogen production. In contrast, tests conducted in the mesophilic range produced very low values for hydrogen production, indicating that these temperatures are not favorable to hydrogen production (Sillero et al., 2023). Overall, high temperatures can simplify

microbial diversity and encourage hydrolysis in a way that benefits biohydrogen production. However, due to complex microbial communities and the constituent component in the dark fermentation process, the optimal temperature is required to obtain high HPR and HY. **Table 2.7** summarizes the optimum temperature for biohydrogen production between 31°C and 60°C.

Table 2.7 : Comparison of optimum temperature for biohydrogen production

Substrate	Range (°C)	Optimum (°C)	Reference
Sago wastewater	25-35	31	(Ulhiza et al., 2018)
Synthetic media	30-42	36	(Martinez-Burgos et al., 2020)
Synthetic media	30-40	37	(Xiao et al., 2013)
Lactate wastewater	35-55	45	(Ziara et al., 2019)
Synthetic	20-50	50	(Dogmaz & Cavas, 2023)
POME	30-50	50	(Zainal et al., 2018)
Tequila vinasses	50-60	55	(Toledo-Cervantes et al., 2020)
Sewage sludge	35-70	55	(Sillero et al., 2023)
POME	37-70	57	(Zainal et al., 2022)
Synthetic media	55-65	60	(Akaçin et al., 2024)

2.3.3 Agitation

Agitation significantly impacts biohydrogen production. It affects the mass transfer of gases, substrate availability, and the process's overall performance. In biohydrogen production, agitation helps the mass transfer (Trad et al., 2016; Younesi et al., 2008). Efficient agitation will enhance the contact between the microbial cells and the substrate, leading to improved gas transfer. By mixing, a homogenous environment could be maintained, preventing the formation of a floating layer and enhancing high biogas production, thus improving the energy efficiency of anaerobic digestion (Kaparaju et al., 2008; Tian et al., 2015). Mixing can ensure uniform nutrient distribution and, thus, favorable microbial growth (Trad et al., 2016). Other than that, agitation can assist in removing oxygen, creating anaerobic conditions necessary for

hydrogen-producing bacteria (Yetilmezsoy & Sakar, 2008). By applying agitation, the settling of microbial cells can be prevented, ensuring continuous contact between cells and substrate, which is crucial in sustaining biohydrogen production (Chezeau & Vial, 2019). However, excessive agitation might cause higher energy usage and mechanical stress on the microorganism, impacting their productivity and viability (Zhang Y. et al., 2020). Therefore, optimum agitation must be obtained to ensure the right balance of agitation intensity to optimize the biohydrogen production process. **Table 2.8** summarizes the optimum agitation for biohydrogen production between 25-500 rpm.

Table 2.8 : Comparison of optimum agitation for biohydrogen production

Substrate	Reactor (L)	Range (rpm)	Optimum (rpm)	Reference
Municipal solid waste	1	25-150	25	(Zhang Y. et al., 2020)
Molasses	18	40-120	80	(Brindhadevi et al., 2021)
POME	6.5	10-150	100	(Srirugsa et al., 2019)
Basal medium	1	110-130	120	(Susilaningsih et al., 2020)
POME	0.121	100-250	200	(Jamil et al., 2009)
Wastewater treatment plant	2	40-200	200	(Chezeau & Vial, 2019)
Dark fermented POME	2	100-300	200	(Mishra, Singh, et al., 2018)
Wheat straw	0.188	0-200	200	(Stabel et al., 2022)
Synthetic media	2	150-500	500	(Younesi et al., 2008)

2.3.4 Solid Loading Rate

The SLR differs from the organic loading rate (OLR). OLR refers to the rate at which organic material is applied to the system, which measures the quantity of material that microorganisms need to digest within a specified time frame (Venkata Mohan et al., 2007). Conversely, the SLR encompasses all solids in the influent, providing insight into total solids loading on the system (Abdulgader et al., 2023). The SLR can

significantly impact biohydrogen production's hydraulic performance and efficiency in dark fermentation.

The effect of SLR on biohydrogen production can vary on several factors. For example, a high SLR can initially increase the hydrogen yield because the available substrate will also increase. However, when further increased, excessively high SLR can lead to inhibition or imbalance in microbial activity, reducing the overall hydrogen yield (Singh et al., 2013). High SLR may also result in mass transfer limitations, making it difficult for microorganisms to access the substrate (Chu et al., 2012). The mass transfer limitations can hinder the efficiency of the process, thus contributing to lower hydrogen production. Extremely high SLR might cause process instability due to increased acidification, accumulation of inhibitory compounds, or excessive production of volatile fatty acids, which can inhibit hydrogen production (Singh et al., 2013). In this case, optimal SLR can enhance fermentation performance by providing sufficient organic matter for microbial activity.

Overall, SLR may affect the microbial community composition, metabolic pathways, and substrate utilization efficiency. Balancing SLR is then crucial for maintaining the energy balance in the process. A SLR that is too low might result in the underutilization of the available substrate, while a SLR that is too high might cause issues with process stability. Thus, optimization of SLR is essential for maximizing biohydrogen production. Hence, the parameter was studied in the biohydrogen production utilizing GAC-NiFe₃O₄ to obtain the optimum SLR. **Table 2.9** summarizes the optimum SLR for biohydrogen production between 4-62.5%.

Table 2.9 : Comparison of optimum SLR for biohydrogen production

Substrate	Range (%)	Optimum (%)	Reference
Green alga	1-5	4	(Maswanna et al., 2018)
Municipal solid waste	5-15	7.5	(Chu C. et al., 2012)
POME	4-16	10	(Singh et al., 2013)
Waste fish oil	4-24	13.2	(Balraj et al., 2023)
Food waste hydrolysate	10-20	15	(Han et al., 2015)
Food waste hydrolysate	10-40	20	(Kuffour et al., 2013)
Sago liquid waste	5-30	25	(Yanti & Ahmad, 2018)
Synthetic media	25-100	62.5	(Sa et al., 2018)

2.3.5 Hydraulic Retention Time

The HRT is crucial in biohydrogen production as it directly affects cost-effective operation. The HRT indicates the amount of time the substrate remains in the bioreactor. The impact of HRT is often studied in the design and optimization of bioreactors due to its significant effects. Microbial activity, substrate utilization, and other environmental factors are among the general factors that could affect HRT toward hydrogen productivity. For example, different microorganisms have varying growth rates and metabolic activities. It has been established that methanogens grow slower than acidogens (Khan et al., 2016). So, a low solid retention time (SRT) could prevent the methanogens from having adequate time to break down volatile fatty acids (VFA) and create carbon dioxide and methane (Lee et al., 2014). The acidogens, however, only need a minimal HRT to hydrolyze the substrates (Khan et al., 2016). Thus, often lower HRT favors the growth of certain hydrogen-producing bacteria, enhancing biohydrogen production. However, an HRT that is too short might not allow for complete substrate utilization.

On the contrary, longer HRT causes a metabolic change that is thought to be detrimental to the synthesis of hydrogen, moving from an acidogenic to a

methanogenic pathway (Mohan et al., 2011). There's often an optimal range of HRT for biohydrogen production. **Table 2.10** summarizes the optimum HRT for biohydrogen production between 3-48 h.

Table 2.10 : Comparison of optimum HRT for biohydrogen production

Substrate	Range (h)	Optimum (h)	Reference
Hydrolyzed waste wheat	1-8	3	(Gorgec & Karapinar, 2018)
Paper mill effluent	1.2-24	4	(Namdarimonfared et al., 2023)
Anaerobic sludge	2-6	6	(Karapinar et al., 2019)
Synthetic media	3-48	6	(Jamali, Md Jahim, et al., 2019)
Cornstalk hydrolysate	4-24	6	(Zhao et al., 2017)
POME	3-9	7	(Syrat et al., 2022)
Crude glycerol	0.76-9.24	7.72	(Gomes et al., 2020)
Low strength wastewater	6-48	8	(Atiqueuzzaman et al., 2019)
POME	8-24	8	(Zainal et al., 2018)
De-oiled Jatropha waste	8-48	12	(Kumar, Sen, et al., 2016)
Organic solid waste	4.6-27	16	(Santiago et al., 2020)
Synthetic media	12-48	24	(Lu et al., 2019)
POME	24-48	48	(Mamimin et al., 2012)

2.3.6 Total Carbohydrates

The primary components of the substrate that produce biohydrogen are carbohydrates, proteins, and lipids (Chozhavendhan et al., 2020). However, the major component contributing to biohydrogen production would be carbohydrates. Carbohydrates, mainly consisting of glucose (20-40%), are the primary substrate for hydrogen-producing bacteria (Vardar-Schara et al., 2008). The highest theoretical yield that could be obtained in biohydrogen production is 4 mol of hydrogen per mol of glucose. However, hydrogen yields can vary depending on the paths of the microbes and end products.

Previous research investigating the effects of total carbohydrate content on dark fermentation has shown a linear correlation between biohydrogen production and the total carbohydrates present in the substrate (Alibardi & Cossu, 2016). Another study concluded that higher carbohydrate content increases biohydrogen production efficiency (Aslan et al., 2023). The maximum observed biohydrogen efficiency was 3.51 mL/g at a substrate loading of 40 g/L and a total carbohydrate concentration of 5.95 mg/L (Aslan et al., 2023). However, an optimization study obtained the optimum carbohydrates at 56% (15 g/L) with the highest hydrogen production of 188 mL H₂/g VS (Tarazona et al., 2022). The biohydrogen production pathways showed that POME's TC (such as sugars and other organic compounds) can significantly impact biohydrogen production (Mahmod et al., 2022). Because some carbohydrates are more readily fermentable than others, the concentration of carbohydrates in POME can influence the hydrogen production rate and yield (Mahmod et al., 2022). Hence, the effect of TC on POME was further investigated in the study.

2.4 Optimization Tools

2.4.1 One-factor-at-a-time

One-factor-at-a-time (OFAT) is a well-known conventional method used in optimization in which only one factor is tested at a time. The optimum conditions obtained from OFAT would be based on the independent parameter that yields the highest outcome, assuming that each variable has no interactions (Czitrom, 2016).

2.4.1.1 Holm-Bonferroni

The Holm-Bonferroni approach was used to attain this study's statistical analysis from the OFAT data obtained. The one-way analysis of variance (ANOVA) was used to express the data by post hoc tests from the Holm-Bonferroni method following Sarmast Sh et al. (2022). For the three independent replicates, $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ are the means $\pm$ standard deviation. This technique was initially designed to control FWER or family-wise error rate and to avoid false positive conclusions (Sarkar et al., 2012). However, it is necessary to manage the risk of making Type 1 errors when testing several hypotheses simultaneously (De & Baron, 2012; Sarkar et al., 2012).

2.4.2 Response Surface Methodology

Response Surface Methodology (RSM) is a widely used modeling technique that developed, improved, and optimized the response variable in the statistical design of experiments (Calfee & Piontkowski, 2016).

In RSM, various design types exist, such as Central Composite Design, Box-Behnken, d-optimal, Plackett-Burman, and Two-level Factorial.

A previous study utilized Box-Behnken Design in parameter optimization (Akaçin et al., 2024). The Box-Behnken Design specializes in 2 to 31 factors that vary more than 2 levels. On the other hand, the assumption that there are no two-factor interactions can be made using this design. It works well for ruggedness testing when users want to ensure that none of the conditions significantly impact the response. **Table 2.11** presents a correlation strength for assessing the coefficient of determination (R^2) (Z-

12: *Correlation and Simple Least Squares Regression - Westgard, n.d.*). A previous researcher carried out the optimization process by comparing central composite design (CCD) and two-level factorial design (Salleh et al., 2011). The R^2 obtained was 0.998 and 0.960 for CCD and two-level factorial design, respectively. The higher R^2 implies that CCD has a higher accuracy than others, hence selected for this study. **Figure 2.3** shows RSM's Central Composites Design (CCD) flow (Bhattacharya, 2021).

Table 2.11 : Correlation strength

Ranges of R^2	Correlation
0.90-1.00	Very high
0.70-0.89	High
0.50-0.69	Moderate
0.30-0.49	Low
0.00-0.29	Little, if any

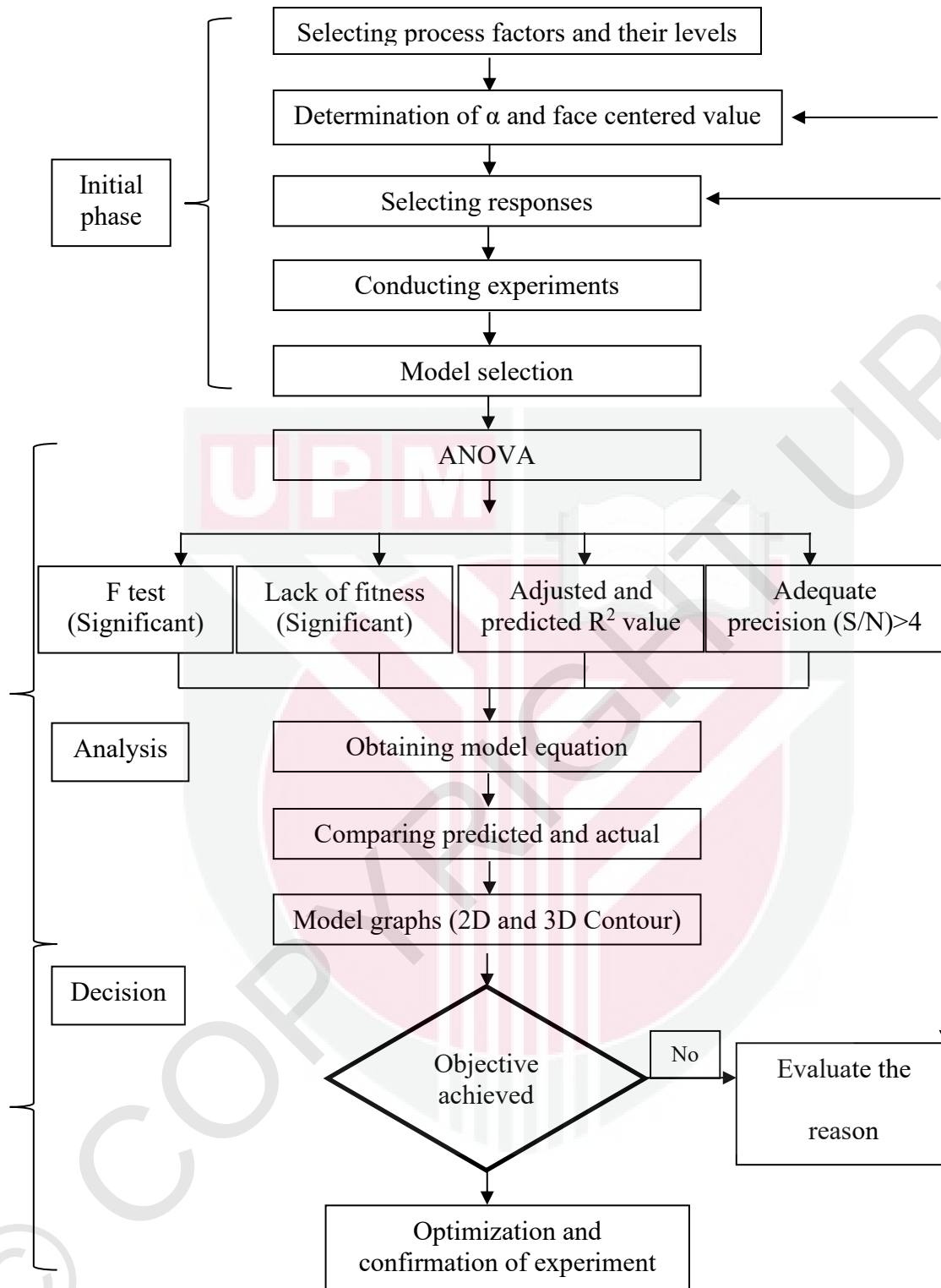


Figure 2.3 : Central Composites Design in Response Surface Methodology
(Bhattacharya, 2021)

2.4.2.1 Analysis of Variance

Analysis of Variance (ANOVA) was utilized to analyze RSM's response. Using the ANOVA, the goodness of fit was determined by R^2 . At the same time, the Fisher statistical test (F-test) in ANOVA was used to determine the statistical significance of the regression experimental design (Chauhan et al., 2013). To demonstrate the dependability of the study's findings, only effects with a confidence level greater than 95% (p-value less than 0.05) were accepted (Azami et al., 2011).

The tools will suggest the best-fitted model from the ANOVA statistical analysis and generate a response graph for the evaluated response. A three-dimensional (3D) surface graph for each response will be modelled using RSM, making it convenient to find the optimal point (Bas and Boyaci, 2007). The corresponding independent variable value can represent the best response based on the 3D-Surface Model Graph (Bradley, 2007). The optimum response region for the design will be identified when the topography of the response surface in RSM is thoroughly analyzed, displaying the ridge lines, local minimum, and local maximum (Olayiwola et al., 2011; Montgomery, 2001). RSM is generally utilized in this study to optimize the conditions of tested variables and maximize the research response. Thus, it is anticipated that simpler and faster experimental designs would be adequate for this research optimization.

2.5 Chapter Summary

Based on the literature review presented, this chapter reviewed the past and current literature concerning the study. As for the introduction, various feedstocks and inoculum sources were presented, emphasizing the biological conversion of dark fermentation into biohydrogen production and other soluble microbial products. Next,

immobilization for biohydrogen enhancement was reviewed in this chapter, which includes the additives for cell immobilization, the reaction mechanisms, characterization analysis, and the potential of heavy metal removal. Important factors that affect biohydrogen production, such as pH, temperature, agitation, SLR, HRT, and TC, were also presented in this chapter. The final part of this chapter includes the optimization tools available for parameter optimization, such as OFAT and RSM, as part of the engineering aspect of the study.



CHAPTER 3

MATERIALS AND METHODS

3.1 Flow of Methodology

The methodology was divided into two general contents. The first one (subchapters 3.2 and 3.3) focused on the preliminary study and general methodology. The subchapters consist of the methodology for preparing granular activated carbon, preparation of synthetic media, cultivation of inoculum, and general analysis of samples. The critical part of the methodology was the second content (subchapter 3.4-3.7), which described the methodology used to achieve the four objectives of this study. The methodology for synthesizing GAC-NiFe₃O₄ was initially described to achieve the first objective. The next part describes the methodology for optimizing cell immobilization using the GAC-NiFe₃O₄ to achieve the second objective. After that, the methodology for process optimization for biohydrogen production was described in detail to achieve the third objective. Finally, all the optimum conditions obtained from the first to third objectives were implemented using POME as a substrate to achieve the final objective. In this part, the method to determine the heavy metal removal potential was also described in detail. The overall process flow chart of the study is further illustrated in **Figure 3.1**.

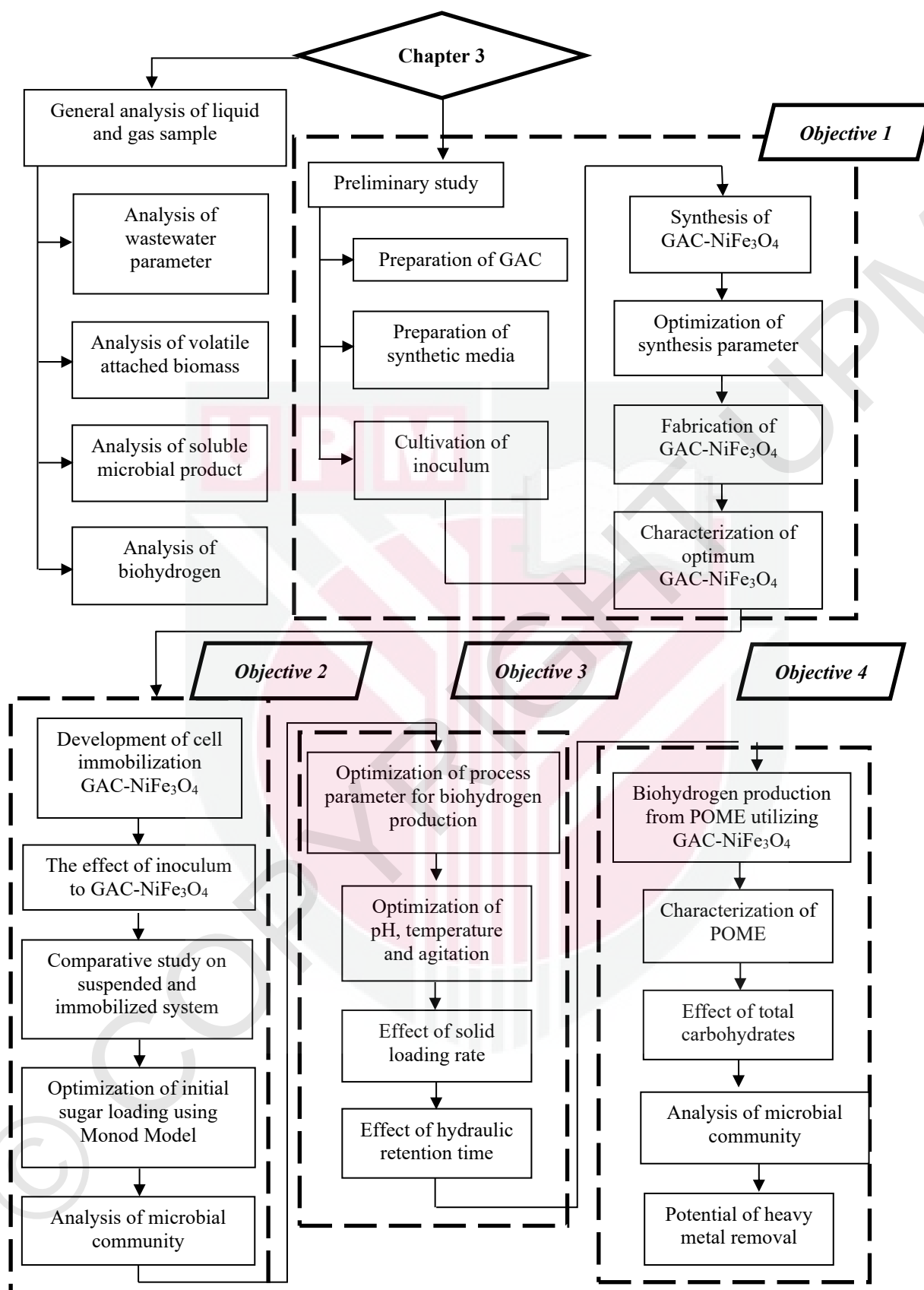


Figure 3.1 : Schematic diagram of the method developed for the study

3.2 Preliminary Study

3.2.1 Preparation of Granular Activated Carbon

Firstly, coconut shell was collected from a market located in Sepang, Selangor, Malaysia. Originating from the coconut shells, the granular activated carbon (GAC) carrier was synthesized according to Chen, Rotaru, Shrestha, et al. (2014), with modifications. The coconut shell was first thoroughly cleaned. Then, before cooling to room temperature (27-30°C), the coconut shell was carbonized for 1 hour at 400°C. A heavy-duty blender was used to refine the char produced before the char meshed to 2-3 mm in size (**Figure 3.2**). After that, the char was activated at room temperature (27-30°C) for 12-18 hours using 1 M NaOH in a 3:1 (NaOH: char) ratio. The activated carbon was washed using deionized water until it achieved a neutral pH (7.0). Subsequently, the activated carbon was dried for three hours at 110°C, producing the end product of granular activated carbon called GAC-O. For comparison, commercialized granular activated carbon, namely GAC-C, made from coconut shells, was bought from Concepts Ecotech Sdn Bhd, Malaysia, with the same meshed size (2-3 mm). The GAC-C and GAC-O were enclosed tightly until further use.

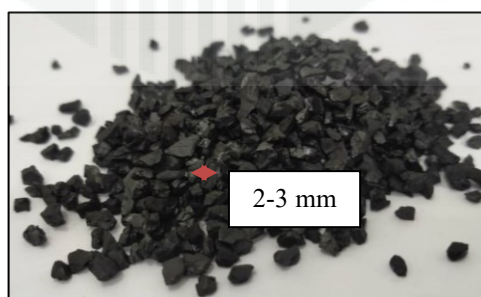


Figure 3.2 : GAC at mesh size 2-3mm

3.2.2 Preparation of Synthetic Media

The synthetic media was prepared by mixing a liter of deionized water with 1.00 g of ammonium chloride (NH_4Cl), 2.00 g of sodium chloride (NaCl), 0.50 g of magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), 0.05 g of calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 1.50 g of potassium hydrogen phosphate ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$), 0.75 g of monopotassium phosphate (KH_2PO_4), 2.60 g of sodium bicarbonate (NaHCO_3), and 2.00 g of yeast extract. 5 g/L of each glucose and xylose was added as carbon sources to produce biohydrogen, replicating the sugar content of palm oil mill effluent following Jamali & Jahim (2016).

3.2.3 Cultivation of Inoculum

The inoculum utilized in the study was a seed-mixed culture derived from POME sludge. It was obtained from an acclimated bioreactor from Green Technology Laboratory, Universiti Putra Malaysia, collected from a previous study by Jamali, Dzul Rashidi, et al. (2019). The seed mixed culture was initially heated to 80°C for 60 minutes to inhibit the growth of the methanogenic population (Jamali et al., 2016). The cultivation was performed with a working volume of 200 mL from a 250 mL Schott Duran bottle. A 90% (v/v) of synthetic media was added as a substrate mixed with 10% (v/v) of seed mixed culture. The bottles were sealed with a rubber stopper and then purged with nitrogen gas (± 2 minutes) to create anaerobic conditions. The cultivation was developed over 2 days of HRT at an initial pH of 6.0 in a water bath shaker (120 rpm). Upon utilization, each inoculum will be extracted only at the exponential phase to ensure optimum performance.

3.3 General Analysis of Samples

3.3.1 Analysis of Wastewater Parameter

The test methods (details in **Appendix B**) utilized in this study for the liquid sample were summarized in **Table 3.1**.

Table 3.1 : Summary of the test method for wastewater parameter

Parameter	Unit	Test method	Reference
pH	-	APHA Standard Method (4500-H ⁺)	(APHA et al., 2005)
Biological oxygen demand (BOD)	mg/L	APHA Standard Method (5210B)	(Montes, 2016)
Chemical oxygen demand (COD)	mg/L	HACH Spectrophotometer Method (8000)	(HACH, 2015)
Total Suspended Solid (TSS)	mg/L	APHA Standard Methods (2540 D)	(Federation, 1999)
Volatile Suspended Solid (VSS)	mg/L	APHA Standard Methods (2540 E)	(Federation, 1999)
Oil and Grease (O&G)	mg/L	HACH Spectrophotometer Method (10056)	(HACH, 2015)
Total Volatile Fatty Acid (TVFA)	mg/L	APHA Standard Method (5560 C)	(APHA et al., 2005)
Ammoniacal nitrogen (AN)	mg/L	APHA Standard Methods (4500-NH ₃)	(APHA et al., 2005)
Total phosphorus (TP)	mg/L	HACH Spectrophotometer Method (10127)	(HACH, 2015)
Total Carbohydrates (TC)	mg/L	Colorimetric Method Determination of Sugars	(APHA et al., 2005)

3.3.2 Analysis of the Attached Biomass

The attached biomass at the carrier (GAC-NiFe₃O₄) was analyzed in terms of the extra polymeric substance (EPS) and volatile attached biomass (VAB). For the EPS analysis, 1.5 g of sample (immobilized GAC-NiFe₃O₄) was weighted and placed inside a centrifuge tube. The sample was then mashed with a glass rod to detach the EPS. Then, 10 mL of distilled water was added to the centrifuge tube and mixed well into EPS suspension. Extraction was performed by adding 0.06 mL of 37% (w/w)

formaldehyde to the EPS suspension and stored at 4°C for 1 hour. Next, 5 mL of 1 M sodium hydroxide (NaOH) was used to treat the suspension at 4°C for 3 hours. The mixture was centrifuged at 10,000 G for 15 minutes to obtain soluble EPS as the supernatant. The soluble EPS was then subjected to TC and protein content measurements. The phenol-sulphuric acid technique (refer to **Appendix B**) was used to determine the TC composition in the extracted EPS regarding glucose concentration. The protein concentration was identified according to the Bradford assay protocol, which applied bovine serum albumin (BSA) as the standard. Initially, a few concentrations (0 g/L, 0.2 g/L, 0.4 g/L, 0.6 g/L, 0.8 g/L, 1.0 g/L, and 1.2 g/L) of the BSA solution were prepared by adding distilled water. After that, 2.5 mL of Bradford reagent was added to 0.25 mL of each sample in a clean test tube and gently stirred. The mixture was incubated for 10 minutes before pipettes into the cuvette. The absorbance of each solution was measured using a UV-Vis Spectrophotometer (Shimadzu UV-1800) at the wavelength (λ) range 465-595 nm (for protein determination) and 480 nm (for TC determination) with distilled water as blank. The standard curve was prepared by plotting the absorbance value against the corresponding protein and TC concentration. Similar steps were performed to determine the protein and TC concentration of the extracted EPS by replacing the BSA standard with the sample.

The method for evaluating VAB followed previous studies by Jamali, Md Jahim et al. (2019), and Jalali et al. (2023). After a stable biohydrogen production, the immobilized-GAC-NiFe₃O₄ sample was collected from the fermentation and dried for an hour at 100°C in the oven. After cooling to room temperature (27-30°C), the sample was weighed before being burned at 550°C in the furnace. The sample was then

cleaned thoroughly once it had cooled down. Next, the earlier procedure was repeated to determine the weight of the clean carrier. The VAB concentration was calculated using **Equation (3.1)**, considering the attached biomass, density of clean carrier, sample, and working volume.

$$VAB \text{ (g/L)} = \frac{(\text{Attached biomass } (\frac{g}{L}) - \text{Density of clean carrier } (\frac{g}{L})) \times \text{Sample volume (L)}}{\text{Working volume (L)}} \quad (3.1)$$

3.3.3 Analysis of Soluble Microbial Product

The soluble microbial products (SMPs) in this study indicate the combined concentration of acetic acid (HAc), butyric acid (HBu), lactic acid (HLa), propionic acid (HPa), and ethanol (EtOH). After centrifuging the effluent samples for 20 minutes at 6000 rpm, the supernatant was filtered using a 0.22 µm syringe filter. Next, Perkin Elmer high-performance liquid chromatography (HPLC) was used to quantify the individual concentration of (SMPs) in the supernatant. The HPLC (Model: JASCO CO-2065 Plus) consists of Rezex ROA-Organic Acid H⁺ (8%), 300 mm x 7.8 mm column with refractive index detector (RID). The isocratic elution and mobile phase (5 mM H₂SO₄) were utilized at room temperature (27-30°C) with a constant flow rate of 0.6 mL/min. A 20 µL injection volume was used with a fixed column temperature of 60°C. The substance concentration could be obtained by generating a pure components standard curve at different concentrations.

3.3.4 Analysis of Biohydrogen

Biogas volume readings were taken every 4 hours until the production reached the stationary phase in the system. The composition of biohydrogen in biogas produced was measured using a Gas Chromatograph (GC) of Agilent Technologies, Model:

6890N. The GC had a thermal conductivity detector (TCD) with a SUPELCO column (Model: Carboxen-1010 PLOT, Dimension: 30m x 0.53mm) at an injection temperature of 110°C. Helium was the carrier gas with a 20 mL/min flow rate at 50°C. Throughout the study, the sugar consumed was quantified based on TC analysis (details test method in **Appendix B**). As a response, the hydrogen yield (HY) was calculated based on the hydrogen production to sugar consumption, as shown in the **Equation (3.2)**:

$$HY \left(\frac{\text{mol } H_2}{\text{mol sugar consumed}} \right) = \frac{\text{Mol of hydrogen produced (mol)}}{\text{Mol of sugar consumed (mol)}} \quad (3.2)$$

In addition, a response in terms of hydrogen production rate (HPR) was determined based on the hydrogen gas generated per liter working volume over fermentation time following **Equation (3.3)**:

$$HPR \left(\frac{\text{mL } H_2}{\text{L.h}} \right) = \frac{\text{Amount of hydrogen gas produced (mL)}}{\text{Working volume (L)} \times \text{Fermentation time (h)}} \quad (3.3)$$

The HPR will be fitted to a modified Gompertz equation to represent the capacity of biohydrogen production (Jamali, Md Jahim et al., 2019). The model considers maximum biohydrogen production rate (R_m), lag phase (λ), fermentation time (t), and Euler number (e) as shown in **Equation (3.4)**:

$$H_t (\text{mL}) = H_m (\text{mL}) \times \exp \left\{ -\exp \left[\frac{R_m \left(\frac{\text{mL}}{\text{h}} \right) e}{H_m (\text{mL})} (\lambda - t)(h) + 1 \right] \right\} \quad (3.4)$$

Where,

H_t = cumulative hydrogen production (mL)

H_m = maximum hydrogen production (mL)

R_m = maximum hydrogen production rate (mL/h)

e = Euler number

λ = lag phase time (h)

t = incubation time (h)

3.4 Synthesis of GAC-NiFe₃O₄

3.4.1 Optimization of Synthesis Parameter

The design of experiment (DOE) was developed using CCD under RSM from Design Expert Software Version 8.0 (Stat-Ease Inc., Minneapolis, USA). One categorical factor (type of GAC) and two numerical factors (Ni: Fe and NPs: GAC) were developed with a total of 26 experiments. **Table 3.2** shows the DOE developed. The DOE responses were analyzed using the obtained from this software. The R^2 was utilized in evaluating the goodness of fit. At the same time, the F-test in the ANOVA was used to assess the statistical significance of the experimental regression experimental design. The reliability of the study's finding is indicated by the effect with a confidence level of greater than 95% (p-value less than 0.05) (Azami et al., 2011).. Additional model validation was conducted using suggested optimum conditions obtained to predict optimum biogas production from CCD. The model's validity was supported by comparing the predicted and experimental values. The reliability of the findings was represented by the percentage error calculated.

Table 3.2 : Independent variable in CCD

Factor	Symbol	Level				
		(-α)	(-1)	(0)	(+1)	(+α)
Ni: Fe	A_1	0.136	0.250	0.525	0.800	0.914
NPs: GAC	B_2	0.006	0.010	0.020	0.030	0.0341
Type of GAC	C_3	GAC-O		GAC-C		

*NPs= Ni: Fe

3.4.2 Synthesis of GAC-NiFe₃O₄

The GAC-NiFe₃O₄ was fabricated in an alkaline solution using the co-precipitation method following Zhao et al. (2020) with modifications. The preparation was quantitatively determined at 5.41 g of iron (III) chloride hexahydrate (FeCl₃.6H₂O) and 2.38 g of nickel (II) chloride hexahydrate (NiCl₂.6H₂O), dissolved to make a Fe³⁺ and Ni²⁺ mixed solution in 100 mL of distilled water. To generate AC, Fe³⁺, and Ni²⁺ mixed suspension, 12 g of GAC was combined into the solution at room temperature (27-30°C) with vigorous mixing (using electromagnetic stirring) applied for 1 hour. After that, the mixed solution was heated to ± 100°C and maintained in a liquid boiling state. After that, 150 mL of an alkaline solution containing 3.40 g of NaOH was rapidly added into the boiling suspension to complete the reaction. The solution was boiled for 2 hours before cooling down. Eventually, the carbon was dried at 70°C for 24 hours after being washed with deionized water (until it reached a neutral pH of 7.0), producing GAC-NiFe₃O₄ (**Figure 3.3**). The GAC-NiFe₃O₄ was enclosed tightly until further use.

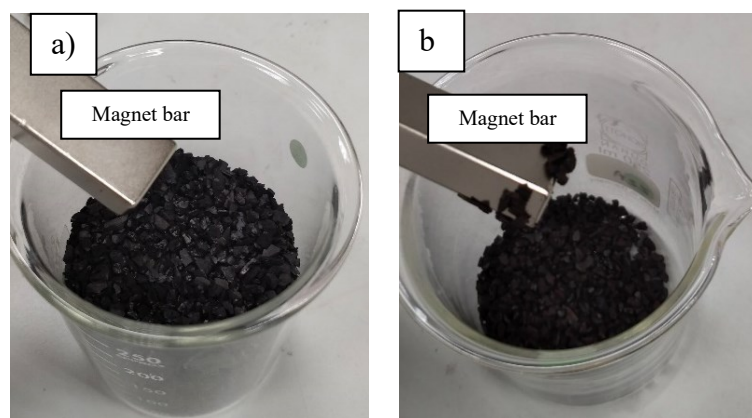


Figure 3.3 : a) Before, GAC and b) After, GAC-NiFe₃O₄

3.4.3 Characterization of Optimum GAC-NiFe₃O₄

3.4.3.1 Electrical Properties

The conductivity was quantified employing a four-point probe (FPP) by Mitsubishi Chemical Analytech (Model: Loresta-GX, MCP-T700) at 0.1 μ A of constant current. The conductivity of each sample was directly measured using the FPP.

3.4.3.2 Physical Structure

The X-ray diffraction (XRD) peaks were obtained employing Shimadzu Lab (Model: XRD-6000) to describe the crystalline characteristics. The sample was tightly packed into the sample holder after being ground into a powder form (<40 μ m in size). The samples were scanned at a scanning speed of 0.02°/min in the nickel-filtered Cu-K _{α} radiation mode (λ = 1.5406 Å) with a 2-theta (Θ) scale from 10-70° (30 kV, 30 mA).

3.4.3.3 Chemical Composition

The functional group was determined employing Fourier-transform infrared spectroscopy (FTIR) (Model: Perkin Elmer Spectrum 100) with a wave-number range

of 4000- 600 cm^{-1} and resolution of 0.01 cm^{-1} . Upon analysis, the material was initially ground into a powder form ($<40\ \mu\text{m}$ in size). The absorption mode is in percentage (%) transition with an attenuated total reflectance (ATR) detector.

3.4.3.4 Elemental Composition

The surface elemental analysis was examined employing energy dispersive X-ray (EDX) (Model: Nova NanoSEM 230). The samples were mounted on carbon tape attached to an aluminum stub, and then gold coating (1-3% w/v) was applied. Then, it was inserted into the mounting holes. A focus knob was used to point the surface of the sample, and the EDX will provide the specific element detected on the surface of the analyzed sample.

3.4.3.5 Magnetic Properties

The magnetic hysteresis of the sample at room temperature ($\sim 27^\circ\text{C}$) was measured up to a maximum field of 14 kOe employing a vibrating sample magnetometer (VSM) (Model: LakeShore 7404 Series). This analysis applies an external magnetic field to the sample, causing its magnetization to align with the field. Next, the sample is shifted up and down at a predetermined frequency, and the sample's magnetic moment is identified.

3.4.3.6 Porosity

The porosity was determined using Brunauer-Emmet-Teller (BET) Micromeritics (Model: TriStar II Plus). The sample was first degassed using Micromeritics degassed (Model: VacPrep 061). The sample was degassed at a stage ramp of 30°C at $10^\circ\text{C}/\text{min}$

and held for 10 minutes. Then, a BET analysis was conducted using a nitrogen adsorption-desorption isotherm at 77K.

3.4.3.7 Morphology

The morphology was examined using field emission scanning electron microscopy (FESEM) (Model: Nova NanoSEM 230). The samples were mounted on carbon tape attached to an aluminum stub, and then gold sputter coating (1-3% w/v) was applied. It was then placed into the mounting holes. The working distance was adjusted using a focus knob, and selected images were captured in a magnification range of 1,000x-100,000x.

3.5 Development of Cell Immobilization using GAC-NiFe₃O₄

3.5.1 The Effect of Inoculum on GAC-NiFe₃O₄ Ratio

Initially, this study compares the effect of inoculum to GAC-NiFe₃O₄ ratio. The ratio (v/w) of sludge to GAC-NiFe₃O₄ varied from 1:0.5 to 1:4, with control (1:0). Batch fermentation was performed with a working volume of 200 mL from a 250 mL Schott Duran bottle. An 80% (v/v) synthetic media was added as a substrate mixed with inoculum (established from the previous section at the exponential phase) and GAC-NiFe₃O₄ (at different ratios). The bottles were sealed with a rubber stopper and then purged with nitrogen gas ($\pm$ 2 minutes) to create anaerobic conditions. The fermentation was carried out for 48 hours at pH 6, 120 rpm, and 60°C. Five repeated batches were conducted to assess the productivity of the biohydrogen production. These tests were each run in three duplicates.

3.5.2 Comparative Study on Suspended and Attached System

Following the findings from the previous sections, a comparative study was conducted on suspended and attached systems in fed-batch fermentation at 2 days HRT. The experiment was conducted with a 450 mL working volume of a 500 mL Schott Duran bottle. For the suspended system, 90% (v/v) synthetic media was added as a substrate, with 10% (v/v) of inoculum (extracted at the exponential phase). In the attached system, an 80% (v/v) synthetic media was added as a substrate with 10% (v/v) of inoculum (extracted at the exponential phase) and 10% (v/v) of GAC-NiFe₃O₄. The bottles were sealed with a rubber stopper and then purged with nitrogen gas (± 2 minutes) to create anaerobic conditions. Until stable biogas readings were observed, both suspended and attached system readings were taken every 2 days. The HY was determined based on the hydrogen produced over the sugar consumed (refer to **Equation 3.2**) as the response to the fermentation outcomes. Next, to represent the capacity of biohydrogen production, the HY was fitted to a modified Gompertz (refer to **Equation 3.3**) (Jamali, Md Jahim et al., 2019).

3.5.3 Optimization of Initial Sugar Loading using Monod Model

Following the establishment of cell immobilization in an attached system, the Monod model was used to investigate the utilization of ideal sugar (xylose and glucose combined). Experimentation and data analysis are required to estimate the rate coefficient constant and attain the ideal sugar utilization (Abdul Aziz et al., 2022). Five initial sugar concentrations (10 g/L, 15 g/L, 20 g/L, 25 g/L, and 30 g/L) were studied using the OFAT approach in batch fermentation, employing a 1:1 xylose to

glucose ratio. OFAT approach tests one factor at a time, assuming that each variable has no interactions (Czitrom, 2016).

All experiments were triplicated with a working volume of 200 mL from a 250 mL Schott Duran bottle. A 90% (v/v) of the synthetic media was added as a substrate with 10% (w/v) of immobilized-GAC-NiFe₃O₄ (extracted at the exponential phase established from the previous section). The bottles were sealed with a rubber stopper and then purged with nitrogen gas (± 2 minutes) to create anaerobic conditions.

For cell growth, the Monod equation was given in **Equation (3.5)**, where μ is the specific growth rate of the cell (h^{-1}), μ_m is the maximum specific growth rate of the cell (h^{-1}), S is the concentration of limiting sugars (g/L), and k_s is the saturation constant (g/L).

$$\mu (h^{-1}) = \frac{\mu_m(h^{-1}) \cdot S(\frac{g}{L})}{S(\frac{g}{L}) + k_s(\frac{g}{L})} \quad (3.5)$$

3.5.4 Analysis of Microbial Community

3.5.4.1 Microbial Attachment

The surface of GAC-NiFe₃O₄ was observed using FESEM (Model: Nova NanoSEM 230) to validate microbial attachment. Initially, the immobilized-GAC-NiFe₃O₄ was washed for three changes with 0.1 M sodium cacodylate buffer for 30 minutes each. Next, the sample was post-fixed for 2 hours at 4°C with 1% osmium tetroxide before washing again for three changes with 0.1 M sodium cacodylate buffer for 30 minutes each. After washing, acetone dehydration was carried out at 35%, 50%, 75%, and 95% (w/w) for 30-45 minutes. This was subsequently by final dehydration at 100% (w/w)

for three changes for 1 hour each. The dehydrated sample was then dried using a critical point dryer (CPD) (Model BAL-TEC SCD 005) for about 1 ½ hours. Upon using FESEM, the sample was gold-coated (1-3% w/v) using a sputter coater.

3.5.4.2 Microbial Identification

The microbial identification was conducted by performing microbial community analysis to identify the microorganisms involved in the fermentation. The cells were collected through centrifugation at 7000 rpm for 10 minutes (Jamali et al., 2017). A commercial kit by MP Biomedicals (FastDNATM Spin Soil Kit) extracted genomic deoxyribonucleic acid (gDNA). The quality of the gDNA was tested using 1% Tris-acetate- ethylenediaminetetraacetic acid (TAE) agarose gel. The locus-specific sequence primers were used to amplify the purified gDNA within bacterial 16S V3-V4 (5' to 3') region:

Forward: CCTACGGGNGGCWGCAG

Reverse: GACTACHVGGGTATCTAATC

All the polymerase chain reactions (PCR) were carried out using the 1st BASE REDiant 2X PCR Master Mix. Then, the bacterial 16S rRNA genes were re-amplified using locus-specific sequence primers with overhang adapters as follows:

Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-

Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-

According to the manufacturer's guidelines, the dual indices were attached to the amplicon PCR using Illumina Nextera XT Index Kit v2. The library quality was quantified employing the Agilent Bioanalyzer 2100 System by Agilent DNA 1000

Kit. The sequencing was then proceeded using the MiSeq platform using 300 PE. The gene sequences obtained were matched against SILVA nr database V138.1 for identification. The sequences obtained were further compared with existing sequences available in GenBank using the National Centre for Biotechnology Information (NCBI) using the basic local alignment search tools (BLAST) program (Jamali et al., 2016).

3.6 Optimization of Process Parameter for the Biohydrogen Production

3.6.1 Optimization of pH, Temperature, and Agitation

Following the findings from previous sections, the OFAT approach was utilized in this study. The effect of pH, temperature, and agitation on biohydrogen production utilizing the immobilized-GAC-NiFe₃O₄ was examined. The effect of pH was investigated by controlling the initial pH at 5.0 to 7.0 by 0.5 increments, using either 1 M hydrochloric acid (HCl) or 1 M NaOH. Other factors were kept constant at 120 rpm and 60°C. The effect of temperature was investigated by using five operational temperatures of 50°C, 55°C, 60°C, 65°C, and 70°C. Other factors were kept constant at 120 rpm and an initial pH of 6.0. The effect of agitation was investigated by manipulating the agitation at five different rpm with control (no agitation). The effect of rpm was tested at 60 rpm, 90 rpm, 120 rpm, 150 rpm, and 180 rpm. Other factors were kept constant at 60°C and an initial pH of 6.0. The experimental design was summarized as tabulated in **Table 3.3**.

All experiments were triplicated with a working volume of 200 mL of a 250 mL Schott Duran bottle. A 90% (v/v) synthetic media was added as a substrate with 10% (w/v) of immobilized-GAC-NiFe₃O₄ (extracted at the exponential phase). The bottles were

sealed with a rubber stopper and then purged with nitrogen gas (± 2 minutes) to create anaerobic conditions.

The fermentation was run in a fed-batch system until stable performance. Every 2 days, the effluent was withdrawn (for further analysis), and then the reactor was fed. The biogas was collected using the water displacement method (pH 2.0, adjusted using the 2 M HCl). The volume of water displaced equals the volume of gas. Every 4 hours, a reading of biogas volume was taken until no significant changes ($\pm 5\%$) were observed. The airtight gas syringe was used to capture the biogas for gas composition analysis. The effluent supernatant was analyzed for VSS, VAB, TVFA, and TC.

Table 3.3 : Independent variable for pH, temperature, and agitation

Run	pH (Constant 120 rpm and 60°C)	Temperature (°C) (Constant 120 rpm and pH 6.0)	Agitation (rpm) (Constant 60°C and pH 6.0)
1	5.0	50.0	60.0
2	5.5	55.0	90.0
3	6.0	60.0	120.0
4	6.5	65.0	150.0
5	7.0	70.0	180.0

3.6.2 Effect of SLR on Biohydrogen Production

Following the findings from previous sections, the OFAT approach was utilized in this study to examine the effect of SLR on biohydrogen production. The SLR varied from 5% to 25% with a 5% (w/v) increment. The fed-batch fermentation system was performed with a working volume of 200 mL from a 250 mL Schott Duran bottle. A 90% (v/v) of the synthetic media was added as a substrate with 10% (w/v) of immobilized-GAC-NiFe₃O₄ (extracted at the exponential phase). The bottles were sealed with a rubber stopper and then purged with nitrogen gas (± 2 minutes) to create

anaerobic conditions. The fermentation was performed at pH 6, 120 rpm, and 60°C for 48 hours. Five repeated fed-batches were conducted to assess the productivity of the biohydrogen production at different SRTs. These tests were each run in three duplicates.

3.6.3 Effect of HRT on Biohydrogen Production

Following the findings from previous sections, the OFAT approach was utilized in this study to examine the effect of HRT on biohydrogen production. There are five HRT tests, which are 48, 24, 12, 6, and 3 hours (h). A fed-batch fermentation system was performed with a working volume of 200 mL from a 250 mL Schott Duran bottle. A 90% (v/v) of the synthetic media was added as a substrate with 10% (w/v) of immobilized-GAC-NiFe₃O₄ (extracted at the exponential phase). The bottles were sealed with a rubber stopper and then purged with nitrogen gas (± 2 minutes) to create anaerobic conditions. The fermentation was performed at pH 6, 120 rpm, and 60°C for 48 h. Five repeated fed-batches were conducted to assess the productivity of the biohydrogen production at different HRTs. These tests were each run in three duplicates. Further analysis of the energy production rate (EPR) by the dark fermentation process can be calculated using hydrogen combustion energy (286 J/mmol) (Sivagurunathan et al., 2015) following **Equation (3.6)**:

$$EPR \left(\frac{kJ}{L.d} \right) = HPR \left(\frac{mmol H_2}{L.d} \right) \times 286 \left(\frac{J}{mmol} \right) \times \left(\frac{1 kJ}{1000 J} \right) \quad (3.6)$$

3.7 Biohydrogen Production from POME Utilizing GAC-NiFe₃O₄

3.7.1 Characterization of POME

Initially, raw POME was collected in a palm oil mill based in Labu, Negeri Sembilan, Malaysia. The fresh POME was collected from the primary collection point. On-site pH and temperature were measured upon collection. The BOD, COD, TSS, VSS, O&G, AN, and TP were measured according to APHA Standards Methods for Examination of Water and Wastewater (Federation, 1954). Upon storage, the POME was kept cooled at 4°C to prevent further degradation.

The sugar content (oligosaccharides) at different POME concentrations was analyzed using the method previously described (Jamali, Dzul Rashidi, et al., 2019). The method utilizes 72% sulphuric acid (H₂SO₄) hydrolysis to degrade the complex sugar into monomeric sugar. The volume of H₂SO₄ was calculated based on **Equation (3.7)**, where the $V_{72\%}$ is the volume of H₂SO₄ (mL), V_s is the initial volume of sample (mL), $C_{4\%}$ is the concentration of 4% w/w H₂SO₄ (41.0 g/L), $C_{72\%}$ is the concentration of 72% w/w H₂SO₄ (1176.3 g/L), and $[H^+]$ is the concentration of hydrogen ions (moles/L).

$$V_{72\%} = \frac{[(C_{4\%} \times V_s) - (V_s \times [H^+] \times \frac{98.08 \text{ g H}_2\text{SO}_4}{2 \text{ moles [H}^+]})]}{C_{72\%}} \quad (3.7)$$

3.7.2 Effect of TC in POME on Biohydrogen Production

Various POME concentrations of 10%, 30%, 50%, 70%, 90%, and 100% (v/v) were used to study TC's effect on biohydrogen production. Different POME concentrations were prepared by mixing the POME with distilled water (e.g., 10% v/v of POME, 90%

v/v of distilled water). The experimental design was developed utilizing the OFAT approach. The TC was tested at five different concentrations while other factors were kept constant. The fermentation was operated in batch fermentation with a working volume of 200 mL from a 250 mL Schott Duran bottle. Each bottle was filled with 90% (v/v) of POME (at different concentrations each) and 10% (w/v) of immobilized-GAC-NiFe₃O₄ (extracted at the exponential phase). The pH of each sample was adjusted to 6.0 with either 1 M HCl or 1 M NaOH. The bottles were sealed with a rubber stopper and then purged with nitrogen gas ($\pm$ 2 minutes) to create anaerobic conditions. The fermentation was performed at pH 6, 120 rpm, and 60°C for 48 h.

The Holm-Bonferroni method was utilized in all OFAT studies to avoid false positive conclusions when testing several hypotheses simultaneously (De & Baron, 2012; Sarkar et al., 2012).

3.7.3 Analysis of Microbial Community

The microbial community analysis was conducted following the materials and methods described in Subchapter 3.5.4.2: Microbial Identification.

3.7.4 Potential of Heavy Metal Removal

The potential for heavy metal remediation in POME was analyzed, and the percentage of heavy metals before and after biohydrogen production from POME was analyzed utilizing GAC-NiFe₃O₄. The heavy metal elements that were analyzed are potassium (K), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn) and zinc (Zn). These elements were analyzed using a Perkin Elmer Inductively Coupled Plasma Optical Emission Spectroscopy

(ICP-OES) (Model: Optima 7300 DV). Upon preparation, 100 mL of raw sample was degraded using 5 mL of 65% nitric acid (HNO_3). The mixed solution was then boiled until 15-20 mL remained. The remaining was then filtered using a filter paper (101 Qualitative 12.5 cm), and the filtrate was collected in a 100 mL volumetric flask. Deionized water was added to the flask up to a final volume of 100 mL. Dilution was then made when necessary. The concentration of the heavy metal is obtained by generating a standard curve for pure components at different concentrations.

3.8 Chapter Summary

Based on the materials and methods, this chapter presented all the research methodologies to achieve the study objectives. This chapter was divided into two general contents. The first contents (subchapters 3.2 and 3.3) would include the preliminary and general methodology utilized throughout the research. This part includes the methodology for preparing granular activated carbon, preparing synthetic media, cultivating inoculum, and general analysis for samples. The next part of the chapter (subchapter 3.4- 3.7) includes the detailed methodology for executing each research objective. This subchapter includes the methodology for synthesis optimization, the development of cell immobilization, optimization of process parameters, and simultaneous biohydrogen production and heavy metal removal potential from POME utilizing the GAC- NiFe_3O_4 .

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Optimization of GAC-NiFe₃O₄

The results of the control experiment and synthesis optimization of GAC-NiFe₃O₄ from RSM are presented in **Tables (4.1) and (4.2)**, respectively. The interaction of three variables (Ni: Fe, NPs: GAC, and type of GAC) at the center point (0) level was illustrated using the 3D-surface model graph (**Figure 4.1**). The maximum HPR attained at the center point corresponded to the specific optimum peak on each response surface. From **Figure 4.1**, a significant interaction was observed between the independent variables. The suggested optimum condition of GAC-NiFe₃O₄ synthesis was determined at Ni: Fe (0.53) and NPs: GAC (0.02), with GAC-O being the most favorable type of GAC. The HPR obtained under these optimal conditions was 20.33 ± 0.32 mL H₂/L.h, 2.78 times greater than the control (GAC without NPs addition), which was just 7.32 ± 0.93 mL H₂/L.h (**Table 4.1**). The biogas produced had a maximum hydrogen concentration of 64.71% and a minimum hydrogen content of 22.25%. Previous studies supported the resulting percentage, ranging from 14-69% (Jamali, Md Jahim et al., 2019; Reddy et al., 2017; Zhang et al., 2021).

Table 4.1 : Result of the control experiment

Control experiment	Percentage hydrogen production	Hydrogen production rate
	(%)	(mL H ₂ /L.h)
GAC-O	15.17 ± 0.32	7.32 ± 0.07
GAC-C	20.18 ± 0.27	7.26 ± 0.07

Table 4.2 : Result of synthesis optimization of GAC-NiFe₃O₄ using RSM

Std	<i>A</i>	<i>B</i>	<i>C</i>	HPR
	Ni: Fe	NPs: GAC	Type of GAC	mL H ₂ /L.h
1	0.25	0.01	GAC-O	12.58 ± 2.99
2	0.80	0.01	GAC-O	15.46 ± 2.54
3	0.25	0.03	GAC-O	12.60 ± 2.77
4	0.80	0.03	GAC-O	14.90 ± 3.18
5	0.14	0.02	GAC-O	12.84 ± 3.12
6	0.91	0.02	GAC-O	12.79 ± 2.24
7	0.53	0.01	GAC-O	06.77 ± 2.58
8	0.53	0.03	GAC-O	13.57 ± 3.58
9	0.53	0.02	GAC-O	22.78 ± 1.04
10	0.53	0.02	GAC-O	24.23 ± 1.42
11	0.53	0.02	GAC-O	23.87 ± 3.36
12	0.53	0.02	GAC-O	20.39 ± 2.09
13	0.53	0.02	GAC-O	14.80 ± 2.39
14	0.25	0.01	GAC-C	02.42 ± 0.31
15	0.80	0.01	GAC-C	15.87 ± 0.47
16	0.25	0.03	GAC-C	08.84 ± 0.66
17	0.80	0.03	GAC-C	04.36 ± 0.55
18	0.14	0.02	GAC-C	15.10 ± 1.68
19	0.91	0.02	GAC-C	16.72 ± 1.42
20	0.53	0.01	GAC-C	18.05 ± 2.34
21	0.53	0.03	GAC-C	04.31 ± 0.49
22	0.53	0.02	GAC-C	19.99 ± 2.33
23	0.53	0.02	GAC-C	22.90 ± 3.46
24	0.53	0.02	GAC-C	20.71 ± 2.76
25	0.53	0.02	GAC-C	19.62 ± 3.42
26	0.53	0.02	GAC-C	19.38 ± 1.39

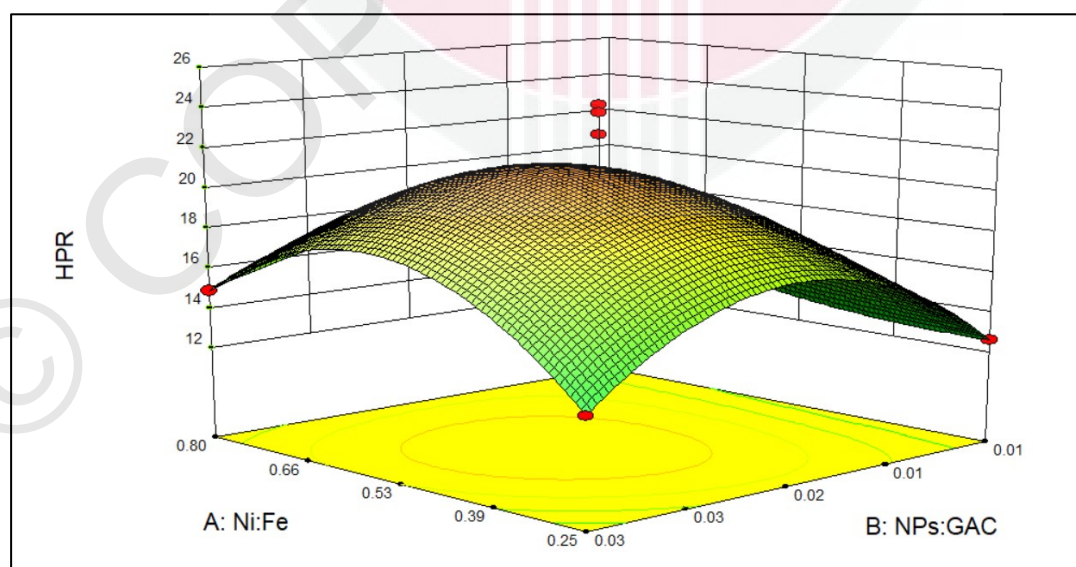


Figure 4.1 : 3D-surface model graph of the optimization of GAC-NiFe₃O₄

According to the result, using GAC-O instead of GAC-C to synthesize GAC-NiFe₃O₄ obtained slightly higher (3.26%) biohydrogen production. The expected HPR from GAC-O and GAC-C from RSM modeling are 21.21 mL H₂/L.h and 20.52 mL H₂/L.h, respectively. Despite this, GAC-O was chosen in light of attaining the optimum response.

Previous research was found to synthesize the GAC from raw materials (coconut shell) instead of direct utilization of the commercialized GAC considering the support material, activation surface, activation condition, and the retention of the pre-cursors on the support material (Engliman et al., 2020; Yahya et al., 2015). Hence, controlling the process of turning coconut shells into activated carbon (GAC-O, for example, in this study) increases the material's economic worth by lowering disposal costs and supplying inexpensive raw materials for manufacturing due to the utilization of the agriculture waste (Prahas et al., 2008). Comparatively, GAC-C was directly obtained from the manufacturer; hence, along with the higher all-inclusive cost to obtain the material, there is no control over the efficient method to fabricate according to the specific aim of biohydrogen production.

4.1.1 Statistical Analysis

In the DOE by RSM, five replicates at the center point of CCD were simultaneously applied to investigate the effect of the variables (Ni: Fe, NPs: GAC, and type of GAC). A total of 26 experiments were conducted involving two numerical factors, Ni: Fe (ranging 0.25 to 0.80) and NPs: GAC (ranging 0.01 to 0.03), along with one categorical factor, type of GAC (either GAC-O or GAC-C). The response towards the DOE was HPR in mL H₂/L.h. The R² obtained from the CCD can determine the

goodness of fit of the experimental model. The F-test, on the other hand, determines the significance of the statistical regression. The reliability of the result can be represented by the confidence level (p-value). The analytical statistics from ANOVA were reliable to be fit with experimental data and summarized in **Table 4.3**. Then, a quadratic model was obtained as in **Equation (4.1)**:

Hydrogen productivity rate (HPR)

$$\begin{aligned}
 = & 20.87 + 0.28(A) - 1.23(B) - 0.34(C) - 2.31(AB) \\
 & + 0.30(AC) - 3.63(BC) - 3.25(A^2) - 5.10(B^2) - 2.17(ABC) \\
 & + 0.52(A^2B) + 0.94(A^2C) + 1.49(AB^2) + 0.43(B^2C) - 1.64(A^2B^2) \\
 & + 3.06(A^2BC) - 0.18(AB^2C) - 4.03(A^2B^2C)
 \end{aligned}$$

Where: A-Ni: Fe, B-NPs: GAC, C-type of GAC **(4.1)**

Based on **Table 4.3**, the F-value (5.99) of the model indicates that the model is significant. Noise has a mere 0.73% probability of resulting in the F-value of a model this high. The Sum of Squares (871.82) for the quadratic model represented the Regression Sum of Squares. Each regression source's degree of freedom (DF) was one, totaling 17 DF for the model source. The Mean Square, obtained at 51.28, signifies the division of the Sum of Squares by the corresponding DF. The Prob>F less than 0.05 implies the significance of the model terms. Based on the result, the significant model terms were BC , A^2 , B^2 , A^2B^2C . Certain significant effects were disregarded, with the effect being influenced by the factors already present in the model. To indicate the reliability of a result, effects with a confidence level exceeding 95 % (p-value less than 0.05) were preferred (Azami et al., 2011). Thus, a small parameter alteration can impact the entire model analysis. Overall, the model proved

adequate for navigating the design space, as implied by the satisfactory R^2 value obtained at 0.93.

Further residual analysis is required to validate the ANOVA's reliability. A linear straight line indicates a good normal probability plot, while an S-shaped line indicates a poor normal plot. A good plot should correlate with a random scatter in the residuals versus the projected response, and any megaphone shape should point to a bad plot. **Figure 4.2 (a)** illustrates the normal probability plot for HPR where the errors were normally distributed, as indicated by the straight line by the residuals. Nonetheless, based on **Figure 4.2 (b)**, the point is randomly distributed without any distinctive structure or noticeable pattern on the residuals versus the predicted response. Essentially, the suggested model was appropriate, and any deviation from the independence or constant variance assumptions may be refuted.

Table 4.3 : The quadratic regression model of ANOVA

Terms	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value
Model	871.82	17	51.28	5.99	0.0073
<i>A</i> -Ni: Fe	0.62	1	0.62	0.07	0.7947
<i>B</i> -NPs:GAC	12.03	1	12.03	1.41	0.2700
<i>C</i> -type of GAC	1.19	1	1.19	0.14	0.7196
<i>AB</i>	42.80	1	42.80	5.00	0.0558
<i>AC</i>	0.70	1	0.700	0.08	0.7824
<i>BC</i>	105.48	1	105.48	12.32	0.0080
<i>A</i> ²	120.90	1	120.90	14.12	0.0056
<i>B</i> ²	296.81	1	296.81	34.65	0.0004
<i>ABC</i>	37.66	1	37.66	4.40	0.0693
<i>A</i> ² <i>B</i>	1.09	1	1.09	0.13	0.7302
<i>A</i> ² <i>C</i>	10.20	1	10.20	1.19	0.3070
<i>AB</i> ²	8.87	1	8.87	1.04	0.3385
<i>B</i> ² <i>C</i>	2.07	1	2.07	0.24	0.6361
<i>A</i> ² <i>B</i> ²	10.74	1	10.74	1.25	0.2953
<i>A</i> ² <i>BC</i>	37.52	1	37.52	4.38	0.0697
<i>AB</i> ² <i>C</i>	0.13	1	0.13	0.02	0.9062
<i>A</i> ² <i>B</i> ² <i>C</i>	65.01	1	65.01	7.59	0.0249
Pure Error	68.52	8	8.57	-	-
Cor Total	940.34	25	-	-	-

$$R^2 = 0.93$$

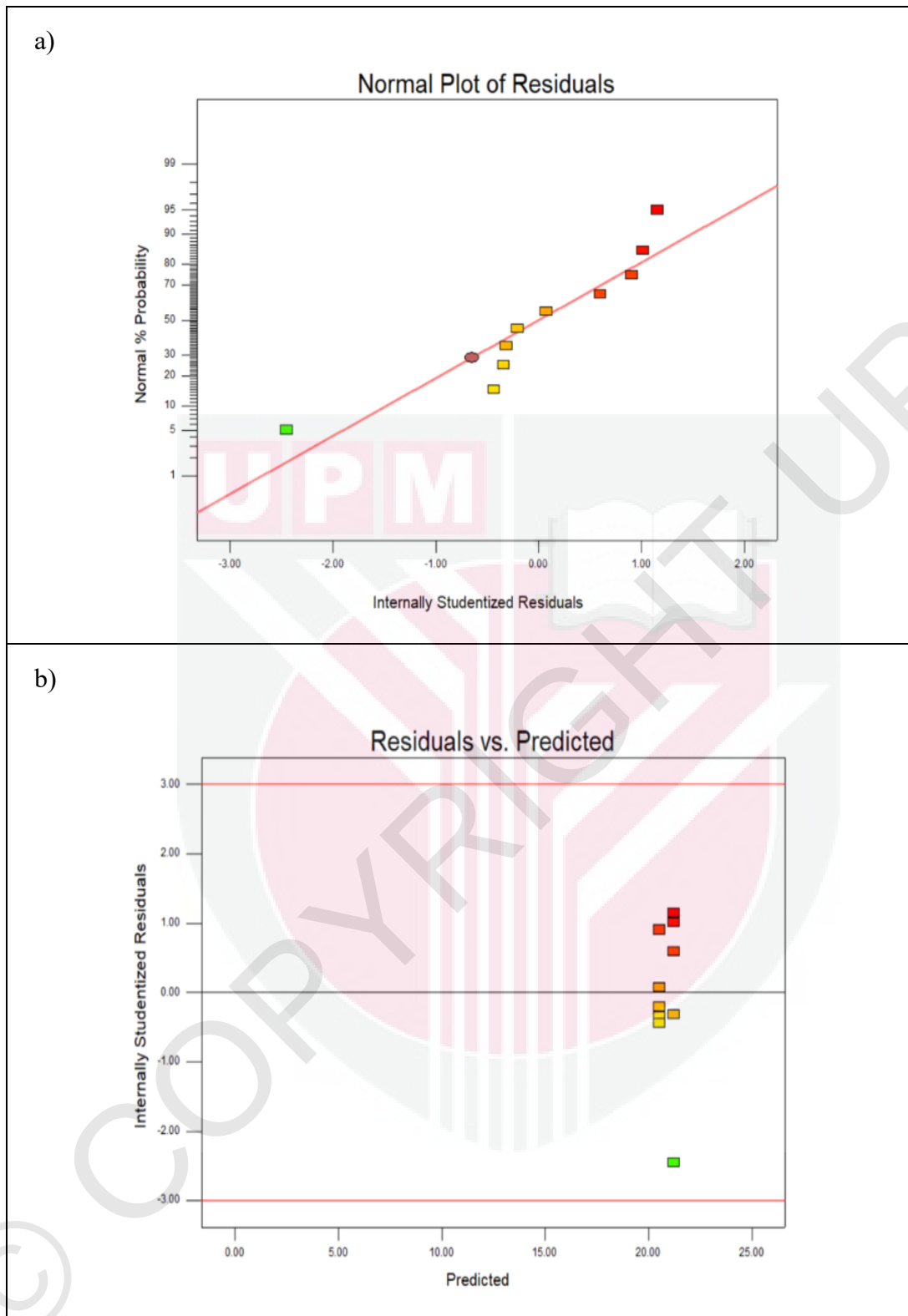


Figure 4.2 : a) Normal plot of residuals and b) Residuals versus predicted plot

4.1.2 Model Validation and Kinetic Modeling

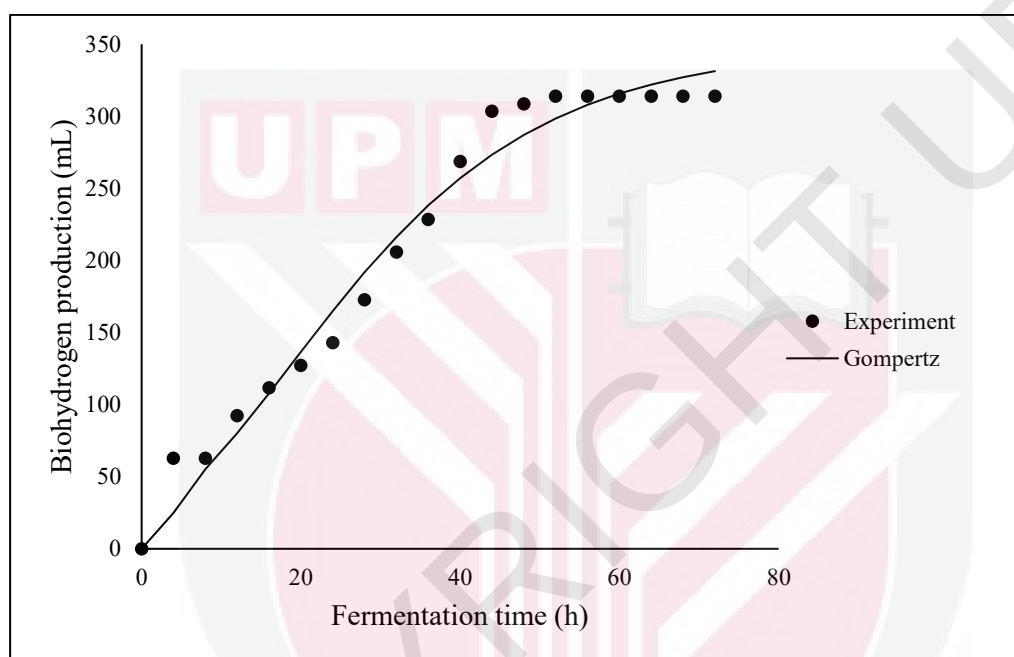
The HPR was predicted at 21.21 mL H₂/L.h utilizing the suggested optimum condition in the obtained quadratic model (**Equation 4.1**). The prediction was further validated through experimentation in three duplicates. The experiment utilizes the optimum condition suggested in the model at 0.53 of Ni: Fe, 0.20 of NPs: GAC, and GAC-O for the type of GAC. The HPR obtained from the validation experiment was 20.33 ± 0.32 mL H₂/L. This represents a 4.15% error from the predicted value.

In addition to model validation, kinetic modeling was studied using the modified Gompertz equation. The result from the kinetic modeling was tabulated in **Table 4.4**. According to the kinetic modeling, the maximum biohydrogen production (H_m) was 343.00 ± 7.34 mL H₂, the maximum biohydrogen production rate (R_m) was 6.51 ± 1.00 mL H₂/h, and the lag phase (λ) was 0.86 ± 0.27 h. The results have shown that a high biohydrogen production rate could be acquired using the suggested optimum condition.

Figure 4.3 illustrates the graph plotted to fit the experiment to Gompertz of the cumulative biohydrogen production over the fermentation time (h). The biohydrogen production from experimental data ($R^2 = 0.93$) showed a strong correlation, as seen in the figure (**Figure 4.3**), with the expected outcome derived from the modified Gompertz equation.

Table 4.4 : Model validation through the predicted optimum condition

Experiment	Hydrogen production rate	Hydrogen yield	Parameter values for H ₂ production using the modified Gompertz equation		
	HPR (mL H ₂ /L.h)	HY (mmol/L.h)	H _m (mL)	R _m (mL/h)	λ (h)
Predicted	21.21	0.946	-	-	-
Validation	20.33 ± 0.32	0.907 ± 0.01	343.00 ± 7.34	6.51 ± 1.00	0.86 ± 0.27
Error (%)	4.15		-	-	-

**Figure 4.3 : Cumulative biohydrogen production fitted to the Gompertz model**

4.1.3 Characterization of the Optimum GAC-NiFe₃O₄

Based on the FPP result, the conductivity for the GAC-NiFe₃O₄ was $2.49 \times 10^7 \pm 0.22 \Omega$. The result counts for higher (95.64%) conductivity than control (GAC-O), at $0.11 \times 10^7 \pm 0.36 \Omega$. It has been demonstrated that the increased conductivity speeds up the rate at which the microorganisms break down the substrate by facilitating electron transmission (Aulenta et al., 2013).

The synthesized GAC-NiFe₃O₄ were also examined for their crystalline and elemental structure using the test of XRD. **Figure 4.4** illustrates the diffractograms obtained for the GAC-NiFe₃O₄. The NPs (NiFe₃O₄) resulted in the peaks of diffraction with their respective miller indices at 25.0° (3,3,1), 30.7° (2,2,0), 35.0° (3,1,1), 43.3° (4,0,0), 57.6° (5,1,1), and 64.2° (4,4,0). The outcomes were in agreement with the Standard X-ray Diffusion Powder Patterns (Bldg, 1955), reflecting the cubic spinel structure crystal formation of NiFe₃O₄ (Petcharoen & Sirivat, 2012; Rambabu, Bharath, et al., 2020; Shanmugam et al., 2020; Zhao W. et al., 2020). Furthermore, the carbon's crystalline structure was described by peaks of diffraction at 2 θ values of 20.4° (0,0,2) and 37.9° (1,0,0). The Debye-Scherrer equation ($D = 0.93 \lambda / \beta \cos \theta$) was utilized to determine the average crystalline size (D) of the Fe₃O₄ nanoparticles, where λ represent the incident X-ray wavelength, β denotes the full width at half maximum (FWHM) and θ is the diffraction angle (Nakate et al., 2019). The calculated D was 16.05-35.55 nm. In contrast, other studies found that the value of D ranged 10.0-69.3 nm, confirming the interaction between the GAC and the NPs (Petcharoen & Sirivat, 2012; Rambabu, Bharath, et al., 2020).

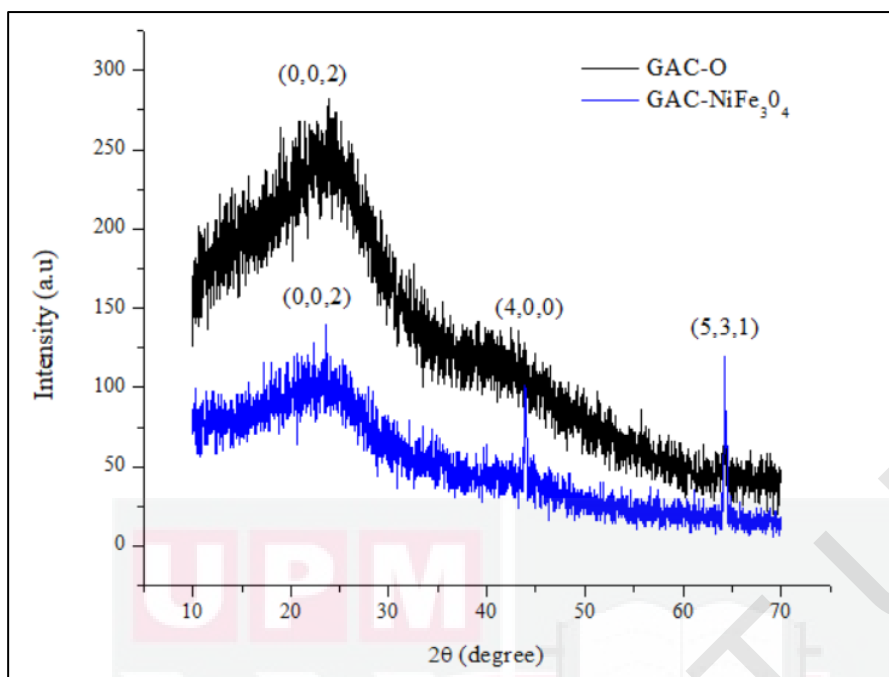


Figure 4.4 : XRD diffractograms of GAC-NiFe₃O₄

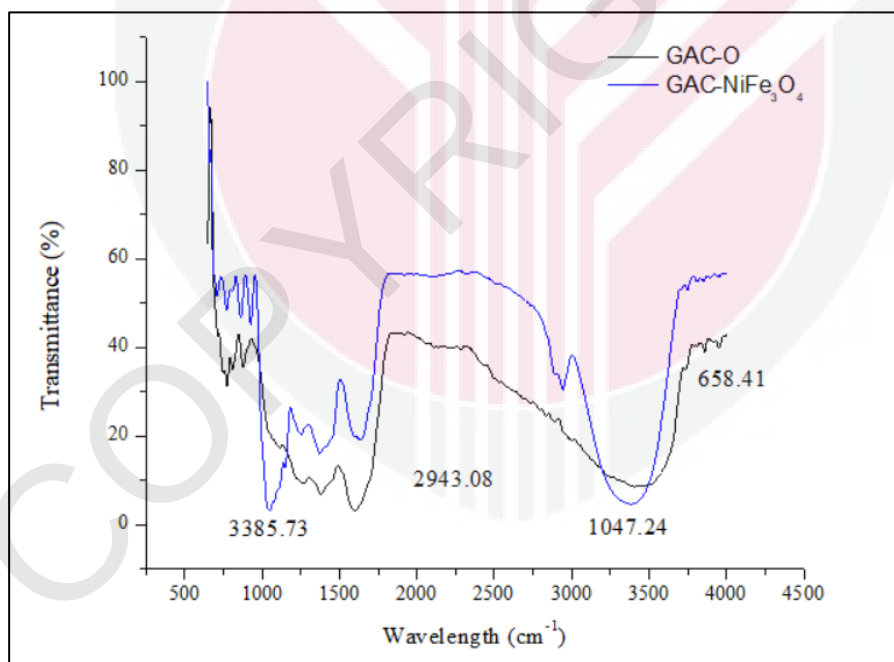


Figure 4.5 : FTIR spectra of GAC-NiFe₃O₄

The FTIR spectra range 400-4000 cm^{-1} of the GAC-NiFe₃O₄, as illustrated in **Figure 4.5**. The large peak at 1638.69 cm^{-1} and 3385.73 cm^{-1} was identified as the vibrational of the -OH group. One possible explanation could be that the hydroxyl group bonded to the sample surfaces matches the hydrogen bonding in the coconut shell (Anyika et al., 2017). Furthermore, the existence of the C-O bond is indicated by a prominent peak centered at 1047.24 cm^{-1} , while the existence of the M-O bond is indicated by an intense peak centered at 658.41 cm^{-1} . These two peaks, taken together, can be attributed to the NPs on the surface (Mohan et al., 2011). In addition, some peaks in the FTIR spectra centered at 1373.29 cm^{-1} and 2943.08 cm^{-1} corresponded to C-O functional groups and OH bending, respectively. The various functional groups of GAC-NiFe₃O₄ demonstrated the sample's stability in temperature tolerant of up to 1000°C, rendering it dependable for thermophilic dark fermentation (Anyika et al., 2017).

EDX was performed to confirm that the chemical binding was successful. The summary of the elemental composition and the EDX spectra of the samples is shown in **Figure 4.6**. The existence of the four major elements was confirmed by the spectra as carbon (C), oxygen (O), iron (Fe), and nickel (Ni). Based on the spectra, the highest components were C at 49.07%, O at 41.10%, Fe at 6.09%, and Ni at 2.74%. The presence of Fe and Ni was altered due to the co-precipitation process on the surface of granular activated carbon during synthesis. The EDX spectra confirmed the success of chemical binding.

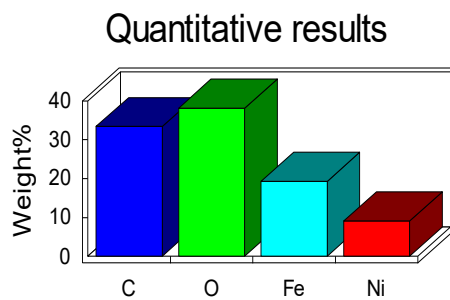


Figure 4.6 : Quantitative EDX result of GAC-NiFe₃O₄.

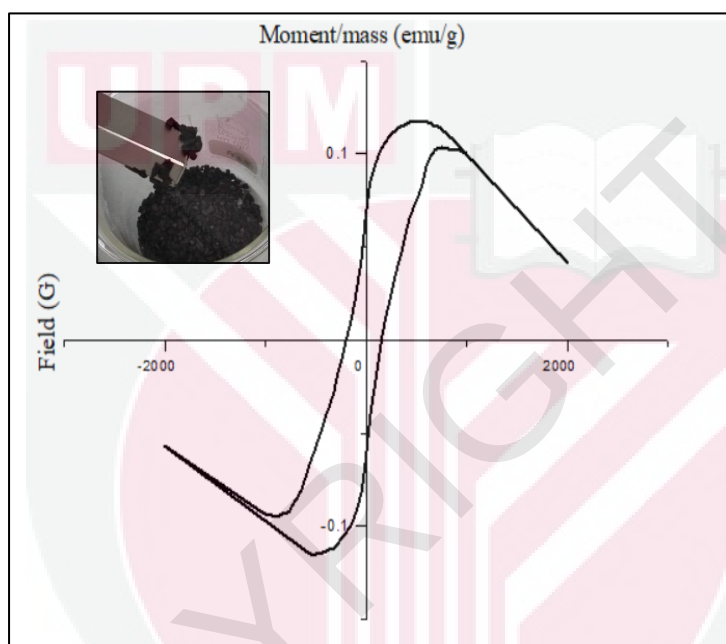


Figure 4.7 : Magnetic hysteresis curves of GAC-NiFe₃O₄

Table 4.5 : BET characteristics of GAC-NiFe₃O₄

Characteristics	Unit	GAC-O	GAC-NiFe ₃ O ₄
Surface area	m ² /g	15.53	1.07-1.68
Pore volume	cm ³ /g	0.0062	0.0002
Pore size	nm	1.59	0.51-0.73
Pore width	nm	1.7-300	1.7-300

Besides EDX, the magnetization of GAC-NiFe₃O₄ was examined with VSM to confirm the magnetic effect brought on by the presence of Fe₃O₄. **Figure 4.7** shows the generated magnetic hysteresis curve. Saturation magnetization values ranged from

0.74-1.03 emu/g, while the coercivity ranged from 780.70-1442.4 G. This suggests that ferrimagnetic behaviors are exhibited by GAC-NiFe₃O₄ (Lee et al., 2019).

The BET and Barret, Joyner, and Halenda (BJH) techniques interpreted the sample's surface area and pore size. The GAC-NiFe₃O₄ BET surface area was discovered at $1.37 \pm 0.43 \text{ m}^2/\text{g}$ (refer to **Table 4.5**), accounting for $6.26 \text{ m}^2/\text{g}$ of the pore's total area. The GAC-NiFe₃O₄ has pore width ranges of 1.7-300 nm with a pore size diameter of $0.62 \pm 0.16 \text{ nm}$. The adsorption-desorption (isotherms) demonstrate that GAC-NiFe₃O₄ belongs to the Type IV macroporous category (Pandey et al., 2021).

The acquired FESEM picture aligned with the BET results. The heterogeneous morphological structure of GAC-NiFe₃O₄ was shown by the FESEM morphology (**Figure 4.8**) at several magnifications (3,500x-25,000x). **Figure 4.8 (a)** shows the porous structure of a clean GAC without any NPs addition at several magnifications (3,500x and 10,000x). **Figure 4.8 (b)** shows the clean GAC-NiFe₃O₄ at 20,000x and 25,000x magnifications. Nanoparticles can exist in multiple crystal shapes, such as cubic, dodecahedron, octahedral, octahedron, and opaque (Arakaki et al., 2010). Therefore, the micrograph of **Figure 4.8 (b)** suggested that the existence of crystal form with opaque transparency could be attributed to NiFe₃O₄.

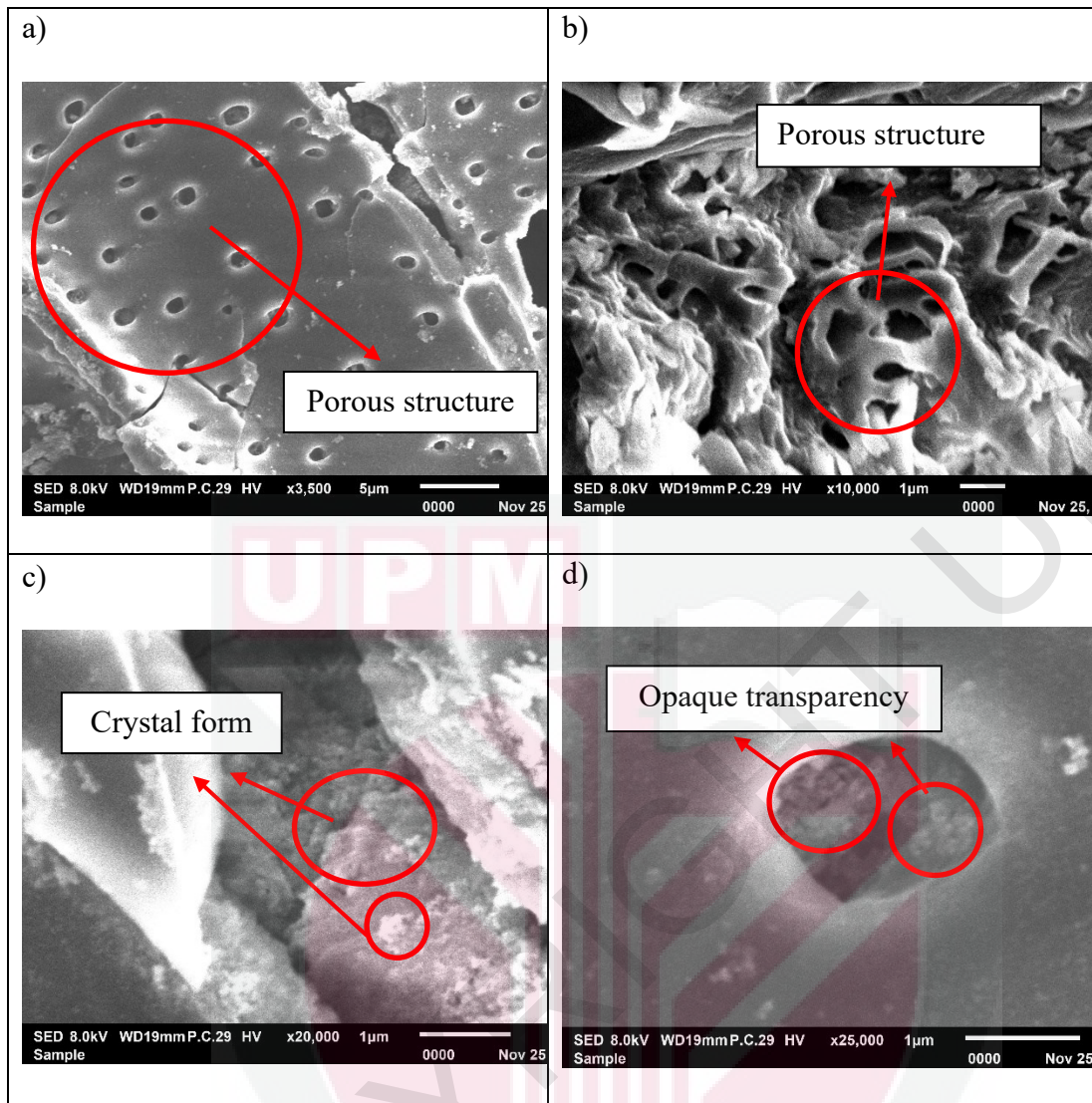


Figure 4.8 : Clean GAC without additives at magnification a) 3,500x, b) 10,000x, Clean GAC-NiFe₃O₄ at magnification c) 20,000x, d) 25,000x

4.2 Optimization of Cell Immobilization using GAC-NiFe₃O₄

4.2.1 Effect of Inoculum to GAC-NiFe₃O₄ Ratio

The effect of five different inoculum to GAC-NiFe₃O₄ ratios on biohydrogen production is illustrated in **Figure 4.9**. Based on **Table 4.6**, the lowest HY of 0.54 ± 0.02 mol H₂/mol sugar consumed was attained at a 1:4 ratio. The higher Ni and Fe concentration (>600 mg/L) present during the fermentation process may have an inhibiting effect, resulting in the lowest hydrogen output. A modest (200-600 mg/L)

concentration of NPs was shown in a prior study to increase the synthesis of biohydrogen, and an amount greater than that impedes the process (Zhao W. et al., 2020). Compared to a greater ratio of 1:4, a higher HY of 0.62 ± 0.03 mol H₂/mol sugar consumed could be achieved even at control (1:0). Every trace element, such as Ni, Fe, Mo, and Zn, serves a specific function on the metabolic pathways that a mixed culture uses to produce hydrogen. For instance, most hydrogenase active sites comprise essential trace metals of Ni²⁺ and Fe³⁺ (Wenqian Zhao et al., 2020). Previous studies stated that [Ni-Fe] hydrogenases are found in a large population of bacteria, while the [Fe-Fe] hydrogenases are found only in a small number of bacteria (Wang J. & Wan, 2008). Therefore, the optimal concentration of the elements must be obtained since biohydrogen production requires the appropriate quantity of Ni²⁺ and Fe³⁺ for enzyme growth and activity.

In this study, the optimal ratio was obtained in Run B at a 1:1 (v/w) ratio, which yielded the highest hydrogen at 1.64 ± 0.04 mol H₂/mol sugar consumed. The result corresponded to the Gompertz constant of H_m, R_m, λ at 453.38 mL, 18.88 mL/h, and 6.65 h, respectively (refer to **Table 4.6**). Compared with another study of thermophilic biohydrogen production by Jamali et al. (2016), the maximum yield recorded was competitive with this study at 1.77 mol H₂/mol sugar consumed at a 1:2 ratio (v/w). However, the λ (13.3 h) obtained in the study is significantly greater (50%) than the present work (λ = 6.65 h). Compared to a system of non-thermophilic biohydrogen production, a study attained H_m of 438 mL H₂ at the lowest ratio of 1:5 (v/v) (Carrillo-Verástegui et al., 2022). The reported maximum hydrogen production was slightly lower (3.39%) than in this study. The optimum concentration of Ni and Fe trace elements may accelerate enzyme catalysis, participate in the acid-base balance, and

encourage microbial activity during biohydrogen production (Keskin et al., 2018).

Thus, one may argue that the ratio should not exceed a certain threshold (in this study, not more than a 1:1 ratio) in a system to prohibit the microbial population from generating hydrogen.

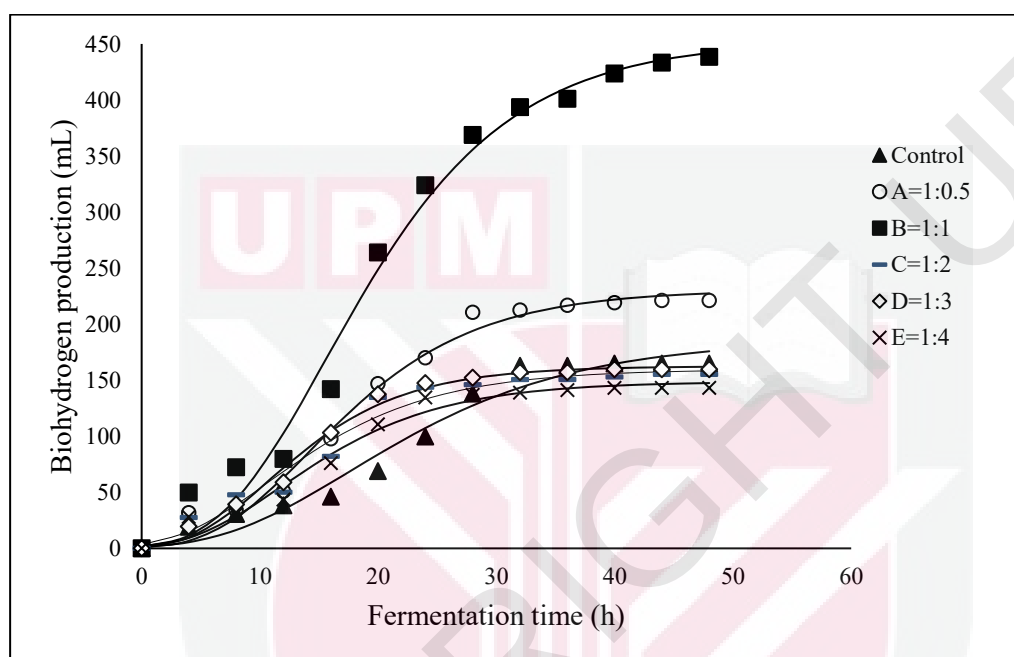


Figure 4.9 : Biohydrogen production at different inoculum to GAC-NiFe₃O₄ ratios

Table 4.6 : Result of H₂ productivity at different inoculum to GAC-NiFe₃O₄ ratios

Run (Sludge: GAC-NiFe ₃ O ₄) (v/w)	H ₂ productivity		Parameter values for H ₂ production using the modified Gompertz equation		
	HY (mol H ₂ /mol sugar consumed)	HPR (mL H ₂ /L.h)	H _m (mL)	R _m (mL/h)	λ (h)
Control (1:0)	0.62 ± 0.03	17.31 ± 0.83	186.68	6.36	6.80
A (1:0.5)	0.82 ± 0.05	22.97 ± 1.25	230.65	10.66	6.08
B (1:1)	1.64 ± 0.04	45.67 ± 1.00	453.38	18.88	6.65
C (1:2)	0.58 ± 0.05	16.08 ± 1.31	159.74	7.51	2.86
D (1:3)	0.59 ± 0.14	16.55 ± 3.93	162.51	9.22	4.20
E (1:4)	0.54 ± 0.02	15.01 ± 0.57	148.74	7.18	4.15

Dark fermentation is usually produced with the SMP (HAc, HBu, HLa, HPa, and EtOH). The SMPs are important indicators for monitoring the effectiveness of hydrogen production. The rise in SMP over the whole ratio range was associated with the formation of hydrogen and was indicative of acidogenic metabolic processes. During the process of producing biohydrogen, metal ions will enter the bacterial cell through the cell wall, and this mechanism will alter the rate of electron transfer (Kumar et al., 2019).

Based on **Table 4.7**, the TVFA to SMP in this study ranges significantly from 0.68 to 0.89. The ratio of 1:1 yielded the best results regarding hydrogen production efficiency, at 0.89 of TVFA over SMP ratio. At a ratio of 1:05, the lowest TVFA/SMP ratio of 0.74 indicated the hydrogen production process was similar to the acidogenesis pathway (Lutpi et al., 2016). In this study, HBu and HLa, which dominated the process, accounted for 89.8–94.8% of the total end products. Various factors can influence the dominance of the lactic acid metabolic pathway during the cell immobilization process. When previously regarded as one of the most common causes that leads to the inhibition of hydrogen production, several studies show otherwise (Detman et al., 2019, 2021; García-Depraect et al., 2021). In this study, lactate-producing bacteria have a more outstanding role in inducing stable microbial associations that produce hydrogen. As a result of their widespread distribution and adaptable metabolic technology, lactate-producing hydrogen can excel in dark fermentation processes (García-Depraect et al., 2021).

Other than that, it is recognized that neither EtOH nor HPa promotes hydrogen generation. The increasing of EtOH and HPa concentration from 0-300 mmol/L

declines the substrate degradation efficiency, hydrogen generation potential, hydrogen yield, and hydrogen production rate (Wang et al., 2008). However, H₂ and methane gas were not found throughout the study.

To validate the significance of the inoculum to GAC-NiFe₃O₄ ratio study, the Holm Bonferroni method was implemented and presented in **Figure 4.10**. Based on **Figure 4.10**, the p-values are all ≤ 0.05 . The smaller p-value (0.05 or less) indicates the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study.

Table 4.7 : Result of TVFA/SMP at different inoculum to GAC-NiFe₃O₄ ratios

Run	HAc	HBu	HLa	EtOH	SMP	TVFA	TVFA/SMP
	(mM)						
Control	0.0088	0.0415	0.0365	0.0415	0.1284	0.0869	0.68
A	0.0096	0.0434	0.0725	0.0434	0.1689	0.1255	0.74
B	0.0200	0.0464	0.3235	0.0464	0.4364	0.3900	0.89
C	0.0067	0.0435	0.0592	0.0435	0.1529	0.1094	0.72
D	0.0086	0.0399	0.0888	0.0399	0.1771	0.1372	0.77
E	0.0086	0.0379	0.0574	0.0379	0.1418	0.1039	0.73

TVFA = HAc + HBu + HLa; SMP = TVFA + EtOH.

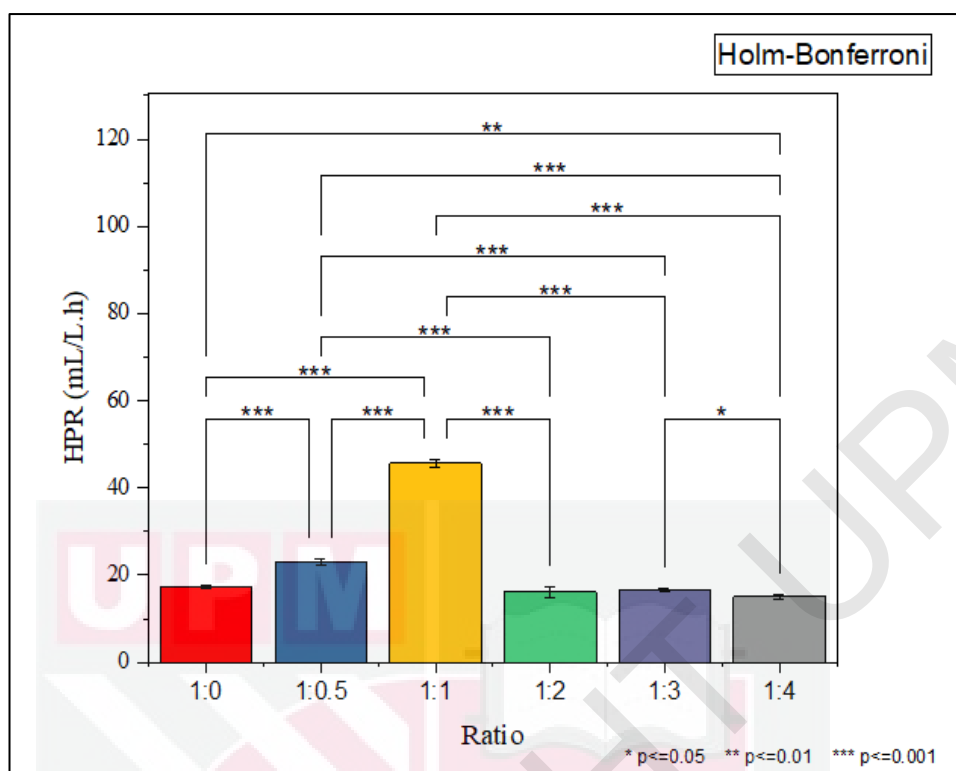


Figure 4.10 : Statistical analysis on the effect of inoculum to GAC-NiFe₃O₄ ratio

4.2.2 Comparative Study on Suspended and Immobilized Cell

The performance of the suspended and attached systems was investigated in a comparative study, and the result is illustrated in **Figure 4.11 (a) & (b)**. Stable hydrogen production was obtained from a 66-day cycle corresponding to the suspended system. It was shortened to a 26-day cycle corresponding to the attached system using GAC-NiFe₃O₄ (refer to **Figure 4.11 a & b**).

The suspended system's performance is illustrated in **Figure 4.11 (a)**. The figure shows that the biohydrogen production reached stable biogas readings (readings were taken every 2 days) above the 66-day cycle. The average HY obtained (upon reaching stable biogas production) from the suspended system is 0.16 ± 0.03 mol H₂/mol sugar consumed. In comparison, based on **Figure 4.11 (b)**, at the beginning of the cycle for

the immobilized system, the production produces 1300 mL of biogas, or 62.3% of the total, over four times the working volume. With an average of 2.35 ± 0.84 mol H₂/mol sugar consumed, overall, the maximum HY achieved 2.94 ± 0.16 mol H₂/mol sugar consumed.

The biogas output varied during the fermentation process, but upon reaching 26 days towards the 44-day operation, the hydrogen gas production appears consistent based on **Figure 4.11 (b)**. Through the sequence feeding system (2 days of HRT) during the acclimatization process, consistent hydrogen gas output was achieved, averaging $50.77 \pm 1.11\%$. A batch profile was then conducted after 26 days of the immobilization development and was fitted with the Gompertz equation. **Figure 4.12** shows that the biohydrogen production profile fits well with the Gompertz model.

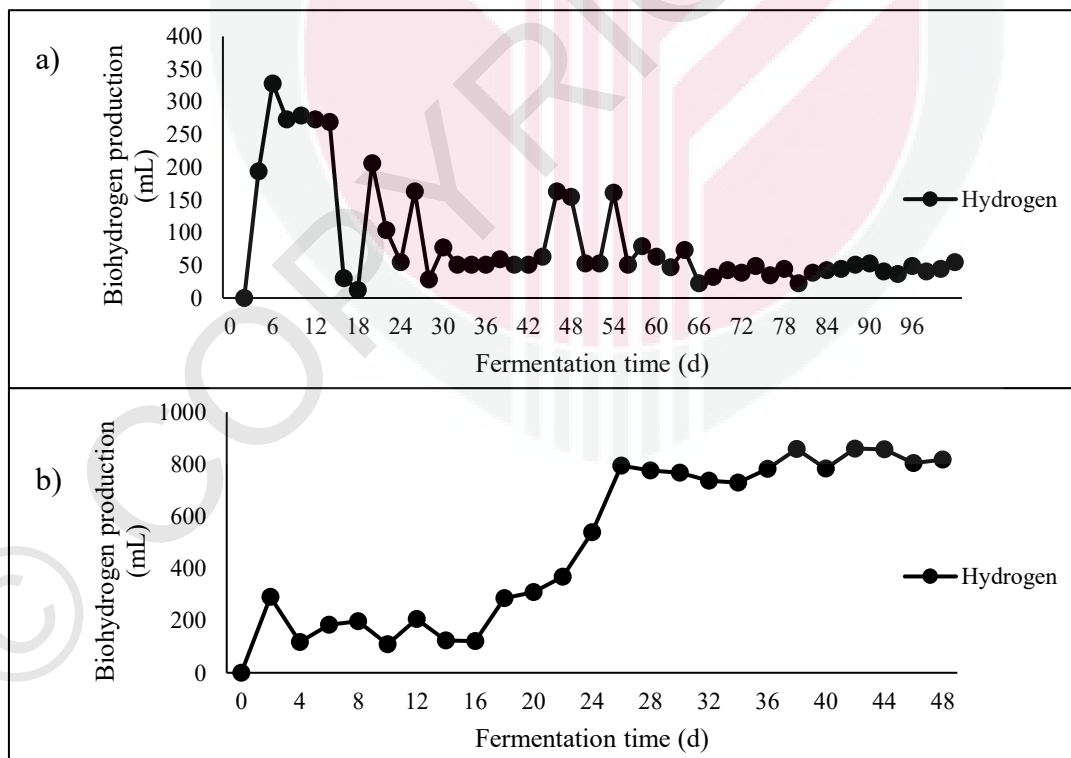


Figure 4.11 : Performance of biohydrogen production in a) suspended and b) immobilized system

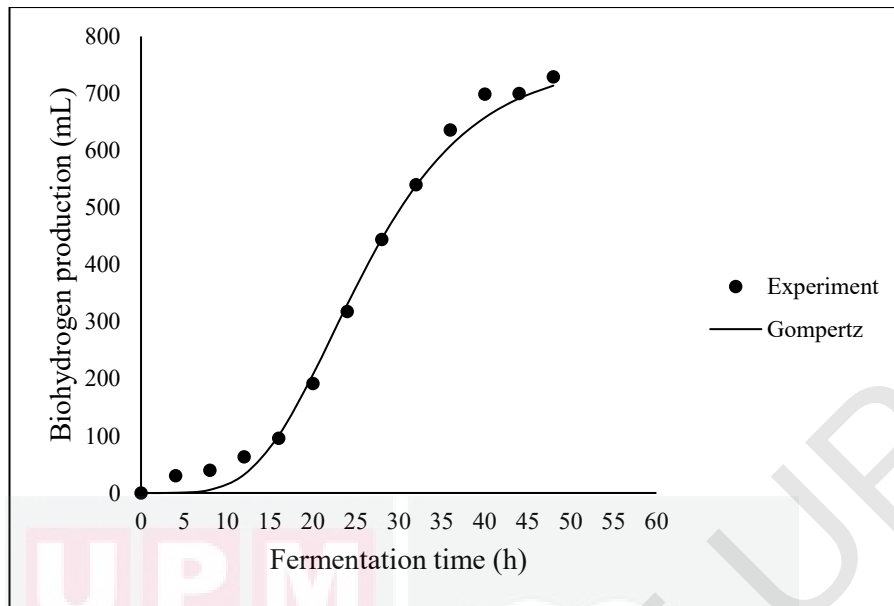


Figure 4.12 : Biohydrogen production validation on the immobilized system

4.2.3 Optimization of Initial Sugar Loading using Monod Model

This study employed the Monod model to describe the growth kinetics of microorganisms, specifically the correlation between the concentration of a limiting substrate and the growth rate of the microbial population. The Monod model can establish the ideal sugar utilization rate to optimize microbial growth and biohydrogen generation productivity. In the initial sugar loading study, $\ln$ VSS and time (t) can be plotted to illustrate the exponential phase of batch fermentation.

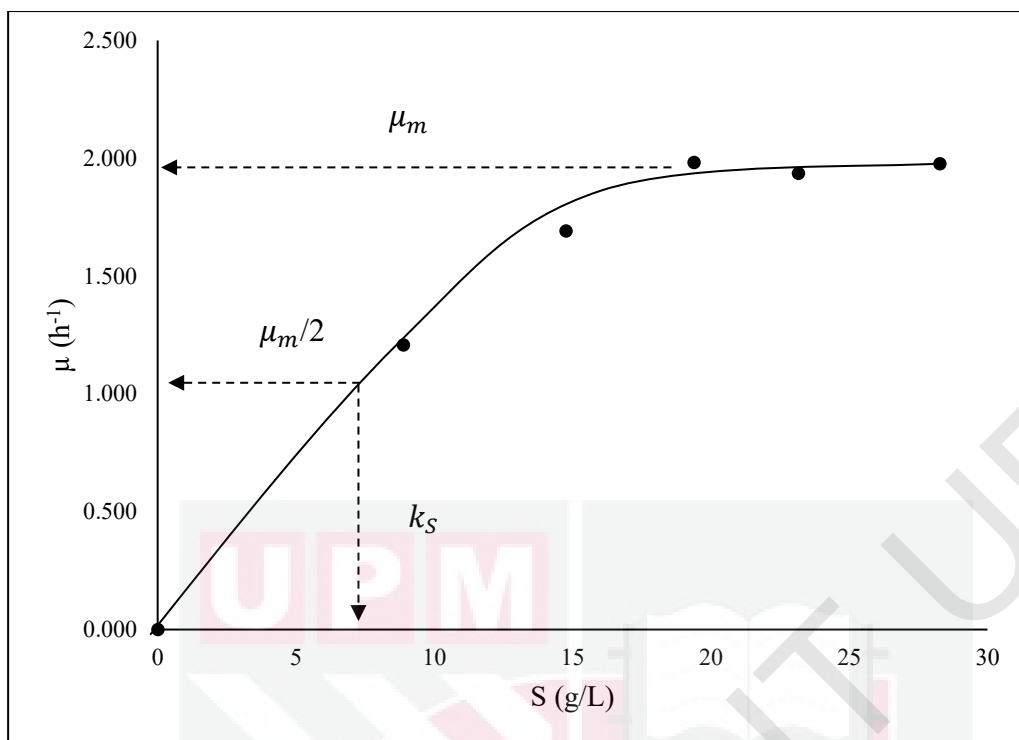


Figure 4.13 : Graph of Monod model obtained from the sugar utilization study

From the result of this study, a graph of $\ln VSS$ versus t was plotted, as illustrated in **Figure 4.13**. The figure allows for determining the specific growth rate (μ) corresponding to various initial sugar loading. The increase in initial sugar loading up to 15 g/L enhanced the specific growth rate. When initial sugar loading was 20 g/L, the bacteria consumed 19.79 g/L of sugar, resulting in a specific growth rate of 1.98 h^{-1} . The growth rate was derived from the steepest gradient obtained from the $\ln VSS$ versus t graph. The specific growth rate remained constant with 0.03 variation as the initial sugar loading was further raised (>20 g/L). Moreover, it was found that 23.17 g/L of sugar was consumed from an initial sugar loading of 25 g/L, resulting in a specific growth rate of 1.94 h^{-1} . Sugar consumption was 28.28 g/L (specific growth rate of 1.98 h^{-1}) when 30 g/L of initial sugar loading was applied. Based on the sugar consumption results, it can be concluded that the growth rate remained constant within the range of 20–30 g/L. Supporting results were further tabulated in **Table 4.8**.

Table 4.8 : Result of H₂ productivity at different initial sugar loadings

Sugar loading	Sugar consumed		Lag phase (λ)	Attached biomass (VAB)	Specific growth rate (μ)	Hydrogen production rate (HPR)	Hydrogen yield (HY)
g/L	g/L	%	h	g/L	h ⁻¹	mL H ₂ /L.h	mol H ₂ /mol sugar consumed
10	8.88	88.79	8.5	8.33	1.208	24.12	0.88
15	14.76	98.43	8.0	9.67	1.691	38.94	0.93
20	19.79	98.96	6.8	10.67	1.981	68.98	1.03
25	23.17	92.66	7.5	10.33	1.935	37.43	0.52
30	28.28	94.27	7.0	12.67	1.976	40.97	0.55

According to **Table 4.8**, at the lowest initial sugar loading (10 g/L), 8.88 g/L of sugar consumption was recorded, and the lowest VAB was produced at 8.33 g/L. Moreover, of all the initial sugar loading evaluated, when the initial sugar loading of 10 g/L was applied, the lowest HPR was obtained at 24.14 mL H₂/L.h. With the increase of initial sugar loading to 20 g/L, the HPR also increased. The highest HPR of 68.98 mL H₂/L.h were obtained at 20 g/L and VAB of 10.67 g/L. When increased to 30 g/L, the HPR decreased to 40.97 mL H₂/L.h, with a VAB of 12.67 g/L.

As shown in **Figure 4.14**, the Monod kinetic model validated the interaction of the specific growth rate of microorganisms to the quantity of sugar consumed. According to the figure, the maximum specific growth rate (μ_m) was obtained at 2.05 h⁻¹ with a specific growth rate (μ) of 1.98 h⁻¹ and cell growth saturation coefficient (k_s) of 6.96 g/L. The maximum specific growth rate attained remained consistent at 2.05 h⁻¹ at the application of initial sugar loading from 20 g/L to 30 g/L. The consistency implies that initial sugar loading of 20 g/L yielded the optimal growth rate. This indicates that the bacterial growth rate will remain constant beyond an initial sugar loading of 20 g/L, leading to more unused or wasted sugar (Jamali, Dzul Rashidi, et al., 2019).

According to the Monod model, the substrate affinity of the immobilized cells is dependent upon the value of k_s (Jamali, Dzul Rashidi, et al., 2019). This parameter reflects how readily the cells can utilize the substrate in the environment (Abdul Aziz et al., 2022). Therefore, k_s plays a crucial role in determining the efficiency of substrate utilization by the immobilized cells. In this study, **Equation (4.2)** represents the specific growth rate of the immobilized cells, obtained from application of the Monod model.

$$\mu = \frac{2.05}{S+6.96} \quad (4.2)$$

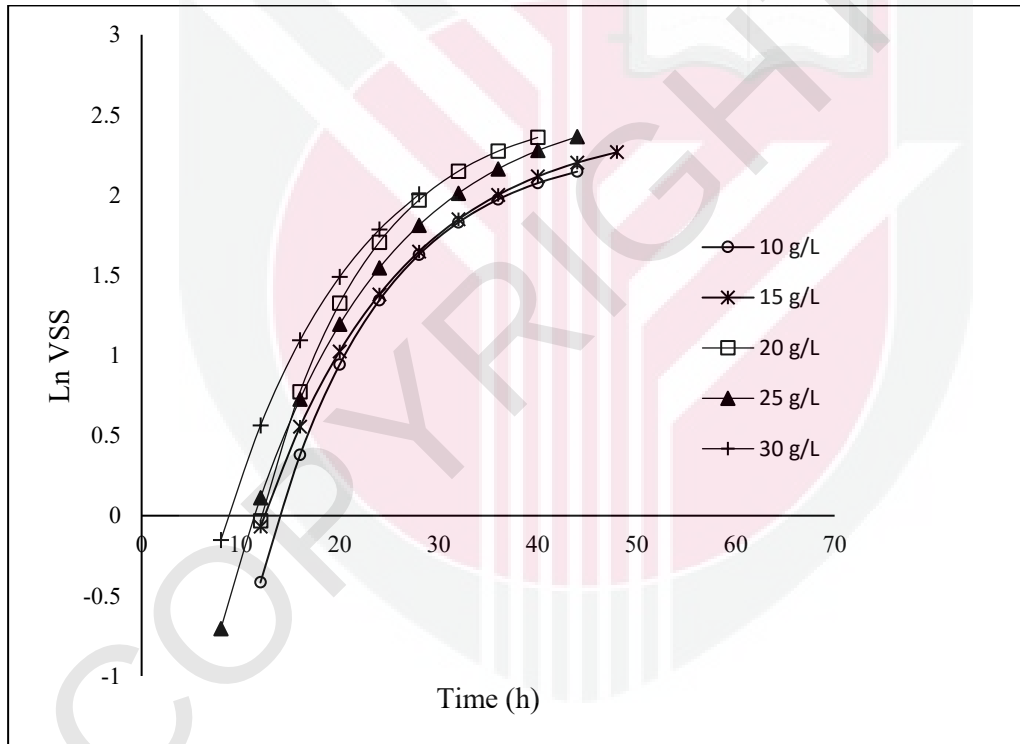


Figure 4.14 : Graph of ln VSS and time

The nonlinear regression analysis of the equation yielded an R^2 greater than 0.90 for all initial sugar loading applied. To validate the significance of the initial sugar loading study, the Holm Bonferroni method was implemented and presented in **Figure 4.15**. From the figure, the p-values are all ≤ 0.05 . The smaller p-value (0.05 or less) indicates

the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study.

Overall, the Monod model can facilitate determining one of the crucial factors limiting microbial growth in cell immobilization. The optimal initial sugar loading will be utilized throughout the subsequent objectives utilizing synthetic media.

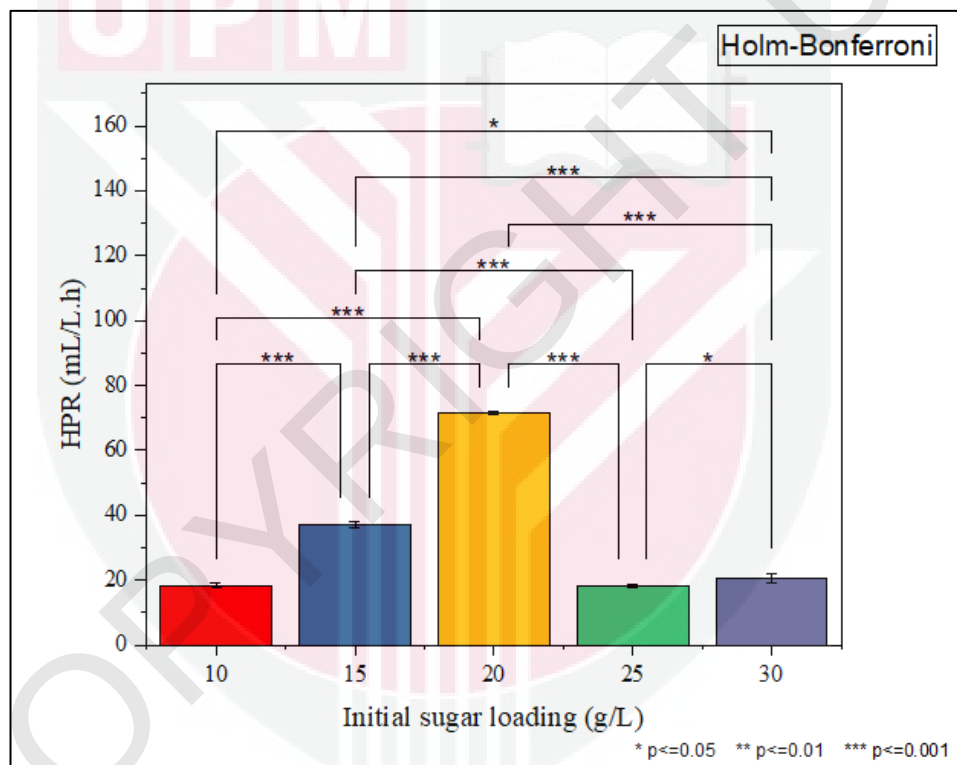


Figure 4.15 : Statistical analysis of the effect of initial sugar loading on HPR

4.2.4 Analysis of Microbial Community

4.2.4.1 Microbial Attachment

The microbial cell attachment on the surface of GAC-NiFe₃O₄ was observed under a FESEM after 100 days of cultivation. The images of before (**Figure 4.16**) and after (**Figure 4.17**) cell immobilization proved GAC-NiFe₃O₄ to act as a support carrier for the microbial community. An immobilized community of microorganisms was clearly visible in the images, firmly adhered within the cavities and on the surface of GAC-NiFe₃O₄. The attachment showed excellent and stable immobility at VAB and EPS of 8.46 ± 0.87 and 0.26 ± 0.00 g/L, respectively. The rod-shaped bacterial cells appeared to be the dominant consortia on and within the GAC-NiFe₃O₄ based on their morphology characteristics. Bacteria were attached to GAC-NiFe₃O₄ owing to its surface porosity, even with NPs binding. Therefore, it has been demonstrated that NPs facilitate and stabilize the hydrogen-producing bacteria. GAC-NiFe₃O₄ can then be utilized to improve biohydrogen productivity.

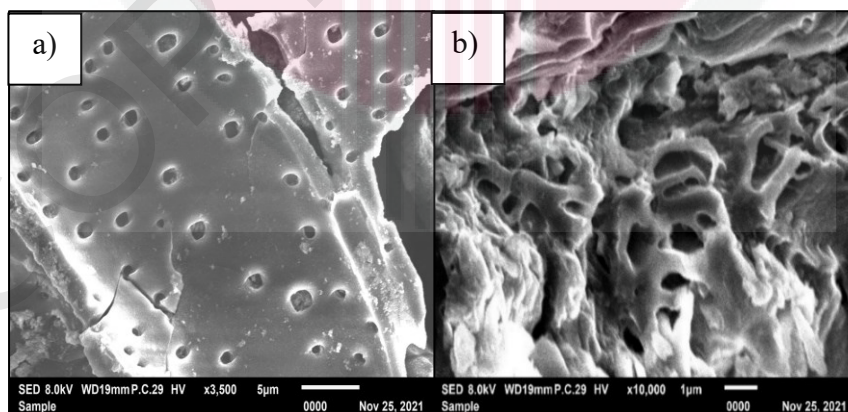


Figure 4.16 : Images of GAC-NiFe₃O₄ surface before microbial attachment at magnification a) 3,500x and b) 10,000x

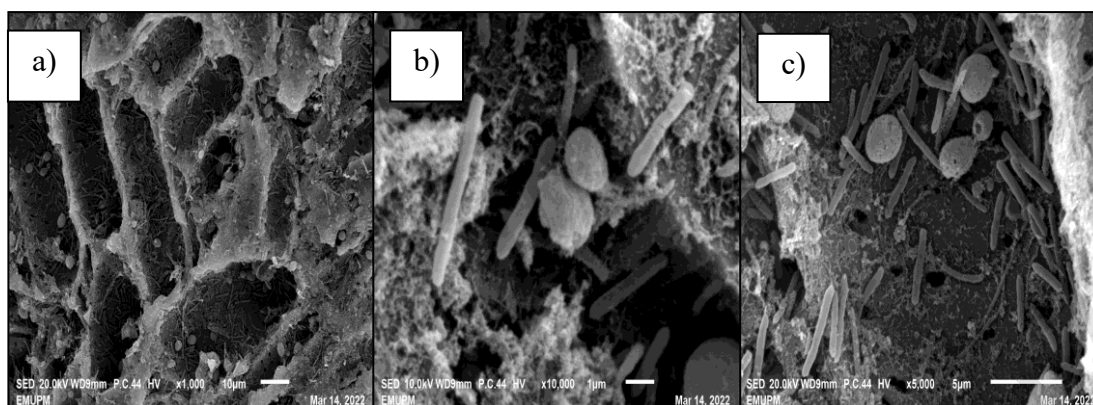


Figure 4.17 : Images of GAC-NiFe₃O₄ surface after microbial attachment at magnification a) 1,000x, b) 5,000x and c) 10,000x

4.2.4.2 Microbial Identification

The abundance of microorganisms based on phylogenetic hierarchy on multiple levels is illustrated in **Figure 4.18**. At the phylum level, 100% of the dominant bacterial community were *Firmicutes*. Previous reports show that most bacteria that produce biohydrogen are *Firmicutes*, which could interact with a variety of substrates (Yang & Wang, 2019). Previous studies revealed that the most common phylum was *Firmicutes* when producing biohydrogen using biochar, ranging from 95.1% to 97.6% (Deng et al., 2022). At the genus level, *Thermoanaerobacterium* was the most dominant species in the study. These species have been investigated for heterotrophic biohydrogen production in various operating conditions, including pH and temperature (Li et al., 2011). This study shows a considerable enrichment of *Thermoanaerobacterium*. *Thermoanaerobacterium* contains hydrolase useful for fermentative biohydrogen production and can grow significantly at temperatures ranging from 55 °C to 70 °C. Based on previous research, *Thermoanaerobacterium* species were more effective in generating biohydrogen than *Clostridium* and *Enterobacter* species (Zhang K. et al., 2019). Moreover, a previous study indicated

that a high abundance of *Thermoanaerobacterium* species accelerated biohydrogen production (Jia et al., 2019).

Furthermore, **Table 4.9** shows that *Thermoanaerobacterium* species were dominant in this study, totaling 99% abundance percentage. The isolated sequences were then subjected to basic local alignment search tools (BLAST) and analyses against GenBank sequences. Subsequently, it was shown that *Thermoanaerobacterium xylandolyticum*, *Thermohydrogenium kirishiense*, *Thermoanaerobacterium calidifontis*, and *Thermoanaerobacterium aciditolerans* *Thermoanaerobacterium* were 98.25% identical to dominant species of *Thermoanaerobacterium* in this study (refer to **Table 4.10**).

Thermoanaerobacterium species were discovered to be obligately anaerobic, spore-forming, gram-positive, saccharolytic, and thermophilic bacteria (Mcclure, 2006). When it was initially discovered, *Thermoanaerobacterium* was classified under the genus *Clostridium* (McCoy & McClung, 1935). *Thermoanaerobacterium* can generate CO₂ and H₂ from various carbohydrates, including glucose, lactose, and starch (Mcclure, 2006). Compared to mesophilic hydrogen fermentation, this specific *Thermoanaerobacterium* species was found to have higher hydrogen output and productivity in thermophilic hydrogen fermentative (Toledo-Cervantes et al., 2020).

A comparison of the biohydrogen productivity of different bacteria strains was tabulated in **Table 4.11**. Despite the presence of an indigenous microbe in wastewater, Litti et al. (2022) achieved a high hydrogen productivity rate (at 2.47 mmol H₂/L/h and 13.3 mL H₂/g COD/h) using *Thermoanaerobacterium* species, with *Thermoanaerobacterium thermosaccharolyticum*, as the dominating microbial

community. The dark fermentation of xylose and cellobiose with optimum growth rate was observed at 60°C and pH 7.5 (Litti et al., 2022). In addition, a previous study also revealed that using mixed culture dominated by *Thermoanaerobacterium* species increased the hydrogen productivity rate by 60% to 25.9 mmol H₂/L.d with a total carbohydrate conversion of 92 ± 2.7% (O-Thong, Prasertsan, Intrasingha, et al., 2008). All the prior studies can strongly support the *Thermoanaerobacterium* species' ability to enhance hydrogen production.

Other than producing hydrogen, Saripan et al. (2021) also found that a *Thermoanaerobacterium* species could co-produce ethanol and hydrogen from lignocellulosic material, namely *Thermoanaerobacterium thermosaccharolyticum* (KKU-ED1). The optimal conditions were a pH of 7.0, a temperature of 55°C, and a cellulose content of 20 g/L (Saripan et al., 2021). Remarkably, research has also shown that sludge rich in *Thermoanaerobacterium* can effectively break down phenol (up to 65% phenol removal efficiency) and produce a large amount of hydrogen at 4.2 L H₂/L-POME (Mamimin et al., 2012). Hence, *Thermoanaerobacterium* species was not only reported as a super producer of hydrogen (established high yield of hydrogen at 3-4 mol H₂/mol sugar consumed) but also showed potential in bioremediation of wastewater.

Table 4.9 : Abundance percentage of the dominant bacteria community

No	Taxonomy	Score	Abundance %
1	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacteriales;Family III;Thermoanaerobacterium	85905	59.3
2	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacteriales;Family III;Thermoanaerobacterium;thermosaccharolyticum	54674	37.7
3	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacteriales;Family III;Thermoanaerobacterium;aotearoense	1133	0.8
4	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae	865	0.6
5	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacteriales;Family III;Thermoanaerobacterium;thermosaccharolyticum	681	0.5
6	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Geobacillus;stearothermophilus	274	0.2
7	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacteriales;Family III;Thermoanaerobacterium	179	0.1
8	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacteriales;Family III;Thermoanaerobacterium;thermosaccharolyticum	146	0.1
		Total	99.3

Table 4.10 : Sequences comparison against BLAST

No	Description	Total score	Similarity (%)	Accession
1	Thermoanaerobacterium xylanolyticum strain LX-11 16S ribosomal RNA	701	98.25	NR_102771.1
2	Thermohydrogenium kirishiense strain DSM 11055 16S ribosomal RNA	701	98.25	NR_117161.1
3	Thermoanaerobacterium calidifontis strain Rx1 16S ribosomal RNA	701	98.25	NR_113051.1
4	Thermoanaerobacterium aciditolerans strain 761-119 16S ribosomal RNA	701	98.25	NR_042856.1
5	Thermoanaerobacterium aotearoense strain JW/SL-NZ613 16S ribosomal RNA	695	98.00	NR_026296.1
6	Thermoanaerobacterium xylanolyticum strain LXII 16S ribosomal RNA	691	97.75	NR_044623.1
7	Thermoanaerobacterium thermosulfurigenes strain 4B 16S ribosomal RNA	691	97.75	NR_044622.1
8	Thermoanaerobacterium thermostercoris strain Buff 16S ribosomal RNA	691	97.77	NR_122103.1

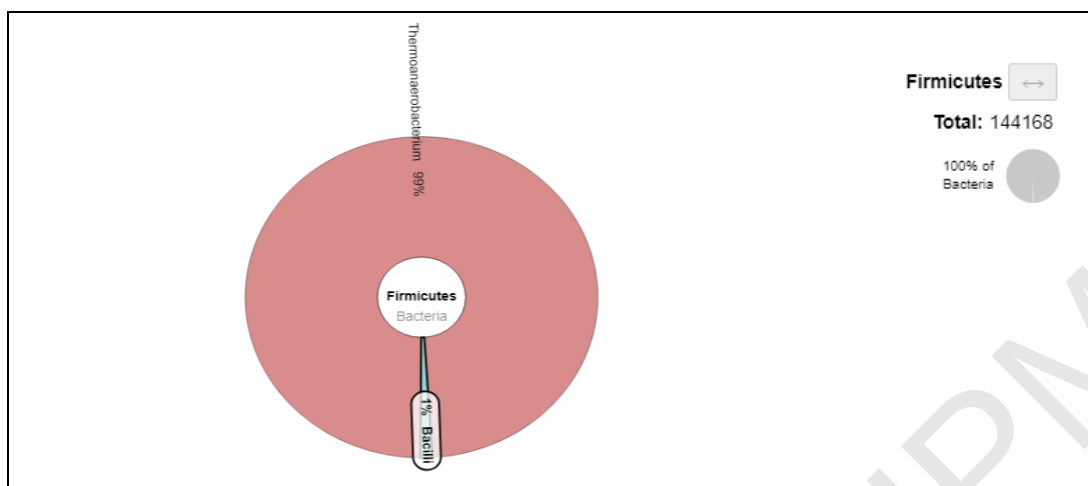


Figure 4.18 : Abundance of microorganisms of phylogenetic hierarchy

Table 4.11 : Comparison of biohydrogen productivity of different bacteria strain

Bacteria name	Carbon source	HPR	HY	Reference
<i>Bacilli</i>	Glucose	1.48 L H ₂ /L.d	2.06 mol H ₂ /mol glucose	(Tawfik et al., 2024)
<i>Clostridia</i>	Wastewater	11.75 L H ₂ /L.POME.d	2.45 mol H ₂ /mol sugar	(Mahmod et al., 2019)
<i>Clostridium beijerinckii</i> RZF-1108	Glucose	106.00 mL H ₂ /L.h	1.96 mol H ₂ /mol glucose	(Zhao X. et al., 2012)
<i>Clostridium beijerinckii</i> NCIMB8052	Glucose	77.60 mL H ₂ /L.h	2.10 mol H ₂ /mol glucose	(Seelert et al., 2015)
<i>Clostridium butyricum</i>	Glucose	86.00 mL H ₂ /L.h	2.2 mol H ₂ /mol glucose	(Beckers et al., 2013)
<i>Clostridium</i> LS2	Wastewater	365.00 mL H ₂ /L.h	0.38 L H ₂ /g COD _{added}	(Singh et al., 2013)
<i>Thermoanaerobacterium thermosaccharolyticum</i>	Glucose Xylose	7.80 mmol H ₂ /L.h	2.20 mol H ₂ /mol of sugar	(Jamali, Md Jahim, et al., 2019)
<i>Thermoanaerobacterium thermosaccharolyticum</i>	Glucose Xylose	100.80 mmol H ₂ /L.d	1.01 mol H ₂ /mol sugar	(Jamali et al., 2017)
<i>Thermoanaerobacterium thermosaccharolyticum</i> <i>Thermoanaerobacterium aciditolerans</i> <i>Thermoanaerobacter brockii</i>	Sucrose	7.81 L H ₂ /L.d	236.00 mL H ₂ /g COD	(Khongkhiang et al., 2019)
<i>Thermoanaerobacterium thermosaccharolyticum</i> <i>Thermoanaerobacterium bryantii</i>	Wastewater	9.10 L H ₂ /L.d	0.27 l H ₂ /g COD	(Prasertsan et al., 2009)
<i>Thermoanaerobacterium thermosaccharolyticum</i>	Glucose Xylose Arabinose	5.20 mmol H ₂ /L.h	1.24 mol H ₂ /mol sugar	(Jamali, Dzul Rashidi, et al., 2019)
<i>Trichoderma asperellum</i> BPLMBT1	Wastewater	25.00 L H ₂ /L.d	2.80 mol H ₂ /mol sugar	(Shanmugam et al., 2020)
<i>Thermoanaerobacterium</i>	Glucose Xylose	104.10 ± 13.01 mL H ₂ /L.h	3.74 ± 0.47 mol H ₂ /mol sugar	This study

4.3 Optimization of Process Parameter for the Biohydrogen Production

4.3.1 Optimization of pH, Temperature, and Agitation

4.3.1.1 Effect of pH

The effect of different initial pH on biohydrogen production utilizing GAC-NiFe₃O₄ was shown in **Figure 4.19**. From the figure, pH 6.0 illustrates optimum hydrogen production with the highest HY at 2.66 ± 0.09 mol H₂/mol sugar consumed (refer to **Table 4.12**). The optimum pH of 6.0 met several previously reported values (Jamali, Dzul Rashidi, et al., 2019; Martinez-Burgos et al., 2020; Xiao et al., 2013). Based on **Table 4.12**, the pH of the fermentative medium decreased to around 3.95-4.74 after 48 h. The pH ranges from 5.0-7.5 and is effective for producing biohydrogens (Jamali et al., 2017; Lutpi et al., 2015, 2016; Preethi et al., 2019). Compared to this study, the hydrogen production runs efficiently above pH 5.5 and poorly below pH 5.0. According to a prior study, the hydrogenase enzyme's activity was hindered at lower pH values(<5.5), which resulted in minimal hydrogen production (Khongkliang et al., 2019).

The lowest HY in this study was 0.66 ± 0.04 mol H₂/mol sugar consumed from an initial pH of 5.0 and a low final pH of 3.95 ± 0.36 . These findings demonstrate how acid secretion affects a cell's capacity to keep its internal pH stable, which limits microbial growth and substrate uptake (Chong et al., 2013). At higher initial pH (>6.0), based on **Table 4.11** shows a decreasing pattern of hydrogen yield by 45.5%-69.9% from 2.66 ± 0.09 and 0.80 ± 0.04 mol H₂/mol of sugar consumed. However, an initial pH of 7.0 has been shown to have a higher hydrogen yield than an initial pH range of 5.0 to 6.5 (Lutpi et al., 2015). The pH has a more substantial impact that may

change the toxicity of some harmful substances and influence the hydrogenase activity (Abdul et al., 2013).

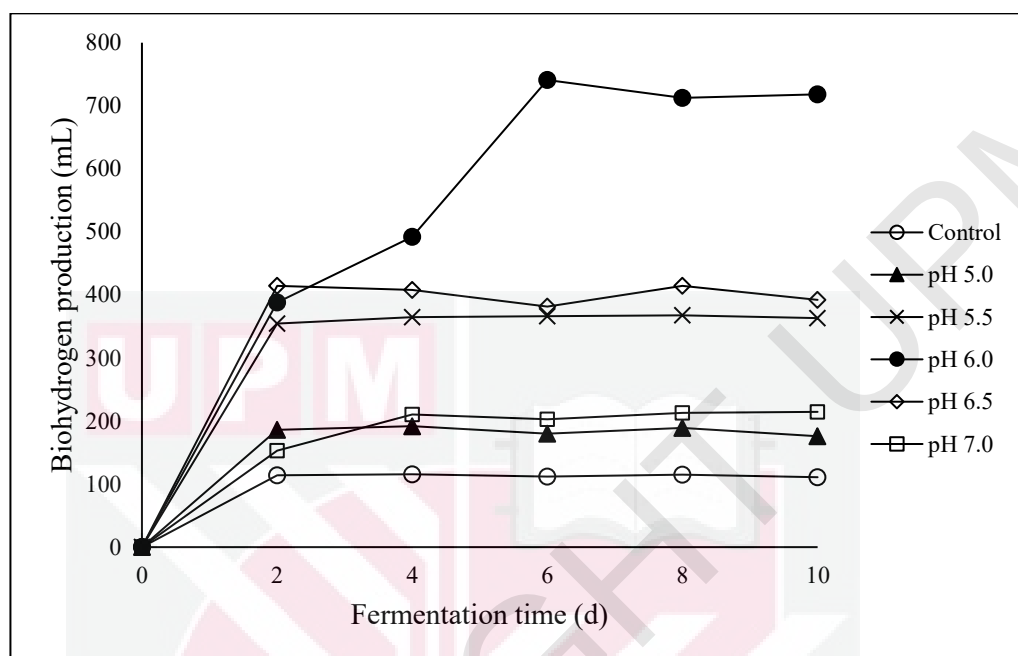


Figure 4.19 : Biohydrogen production at different pH

According to **Table 4.12**, the maximum VAB is produced at an initial pH of 6.0 (8.80 g/L), with a declining trend observed at lower and higher pH values. A prior study by Ibrahim et al. (2013) reported restricted microbial growth at initial pH values higher or lower than 6.0, which validated the results of this study. At an initial pH of 5.0, the lowest VAB was measured at 4.53 g/L. Reducing the initial pH harms microbial growth and metabolism. Therefore, lower microbial growth was linked to HY dropping below pH 5.0. Microbial growth at higher initial pH values (>6.0) is restricted but significant, varying between 14.66% and 22.50% from VAB at 6.0 (8.80 g/L). Subsequently, the ability of hydrogen-producing bacteria to live at extremely high pH levels is limited (Liu et al., 2011).

The TVFA accumulation in this study was recorded highest at 5702.86 mg/L when initial pH 6.0 was implemented (refer to **Table 4.12**). The TVFA at the optimal initial pH of 6.0 was 33.47% higher than the pH at a slightly acidic microenvironment (initial pH<6.0). Similarly, at a higher initial pH (> 6.0), the TVFA dropped by 25.65%. Applying a pH higher than 6.5 may cause fatty acid synthesis, which can impede the efficient production of biohydrogen. Thus, forming those fatty acids will constrain long-term hydrogen production, lowering the system buffering capacity. Conversely, the hydrogen-producing bacteria will adapt to the environment slower at lower pH values (<4.0), which will affect the HY (Ghimire et al., 2017).

The result obtained from the modified Gompertz equation is presented in **Table 4.12**. At the optimum pH of 6.0, the Gompertz constant was found to be $H_m = 974.99$ mL, $R_m = 17.90$ mL/h, and $\lambda = 1.27$ h. Employing the modified Gompertz equation, **Figure 4.20** validated the biohydrogen production potential with maximum HPR and lag phase. This equation was fitted using the cumulative hydrogen production data. With $R^2 > 0.99$, the modified Gompertz equation best fits all experimental data.

The hydrogenase activity may generally rise or fall depending on the pH influence. According to earlier research, the pH range of 5.5-6.5 contributed to the high HY, consistent with this study (Wang & Wan, 2009).

Table 4.12 : Result of H₂ productivity at different pH

pH		HY (mol H ₂ / mol sugar consumed)	Parameter values for H ₂ production using the modified Gompertz equation			VAB (g/L)	TVFA (mg/L)
Initial	Final		H _m (mL)	R _m (mL/h)	λ (h)		
Control	3.21 ± 0.19	0.42 ± 0.03	103.34	8.20	4.19	4.32	2784.92
5.0	3.95 ± 0.36	0.66 ± 0.04	257.29	4.21	2.78	4.53	3794.29
5.5	4.13 ± 0.37	1.35 ± 0.07	422.40	10.54	2.73	6.43	4382.86
6.0	4.31 ± 0.07	2.66 ± 0.09	974.99	17.90	1.27	8.80	5702.86
6.5	4.59 ± 0.54	1.45 ± 0.05	452.69	11.30	2.73	7.51	4371.43
7.0	4.74 ± 0.53	0.80 ± 0.04	220.54	7.90	2.89	6.82	4240.00

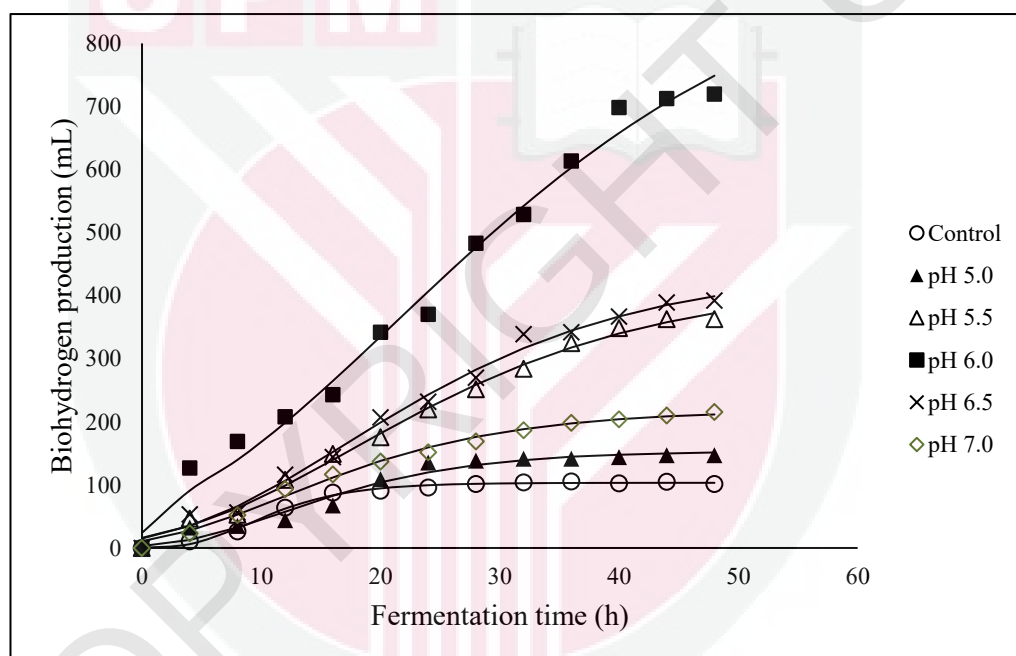


Figure 4.20 : Biohydrogen production at different pH fitted to the Gompertz model

To validate the significance of the effect of the pH study, the Holm Bonferroni method was implemented and presented in **Figure 4.21**. From the figure, the p-values are all ≤ 0.05 . The smaller p-value (0.05 or less) indicates the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study.

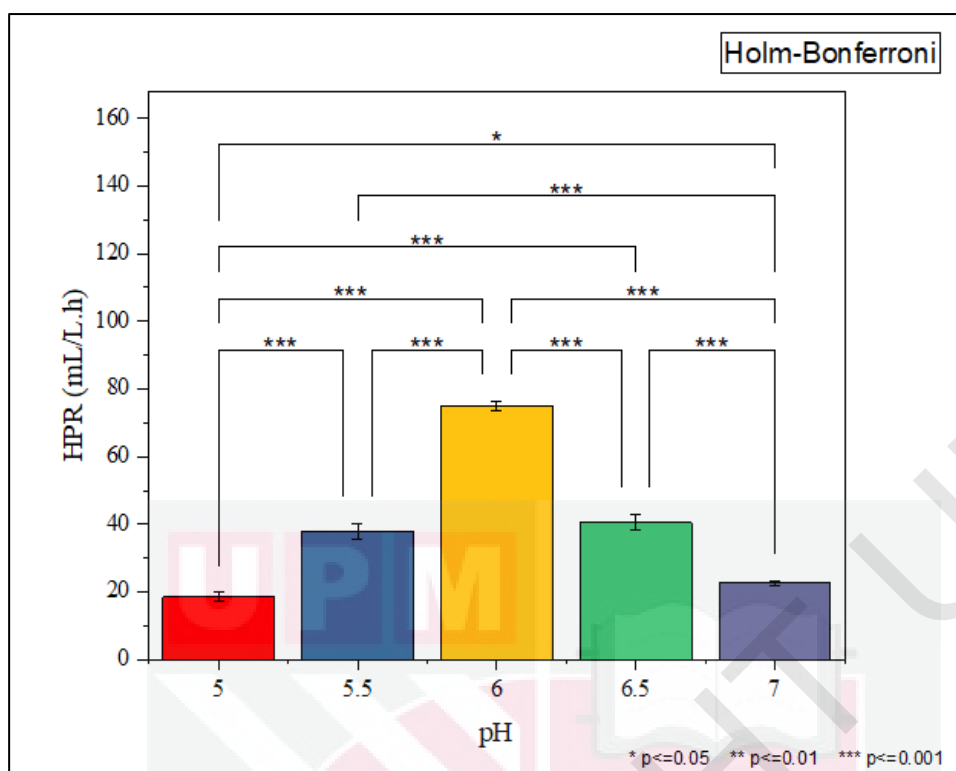


Figure 4.21 : Statistical analysis of the effect of pH on HPR

4.3.1.2 Effect of Temperature

The effect of temperature on biohydrogen production is shown in **Figure 4.22**. From the figure, the optimal temperature (60°C) obtained gives the highest HY of 2.75 ± 0.11 mol H₂/mol sugar consumed (refer to **Table 4.13**). On the other hand, the lowest yield of 0.58 ± 0.01 mol H₂/mol sugar consumed is obtained at 50°C, which is 78.9% lower than the HY at optimal temperature. At 1.28 ± 0.03 mol H₂/mol sugar consumed and 1.32 ± 0.11 mol H₂/mol sugar, respectively, the HY varied by only 3.12% at 55°C and 70°C. Although it is 37.5% less than the optimal temperature (60°C), the HY at 65°C (1.72 ± 0.14 mol H₂/mol sugar consumed) is noticeably higher than the yield at 50°C, 55°C, and 70°C.

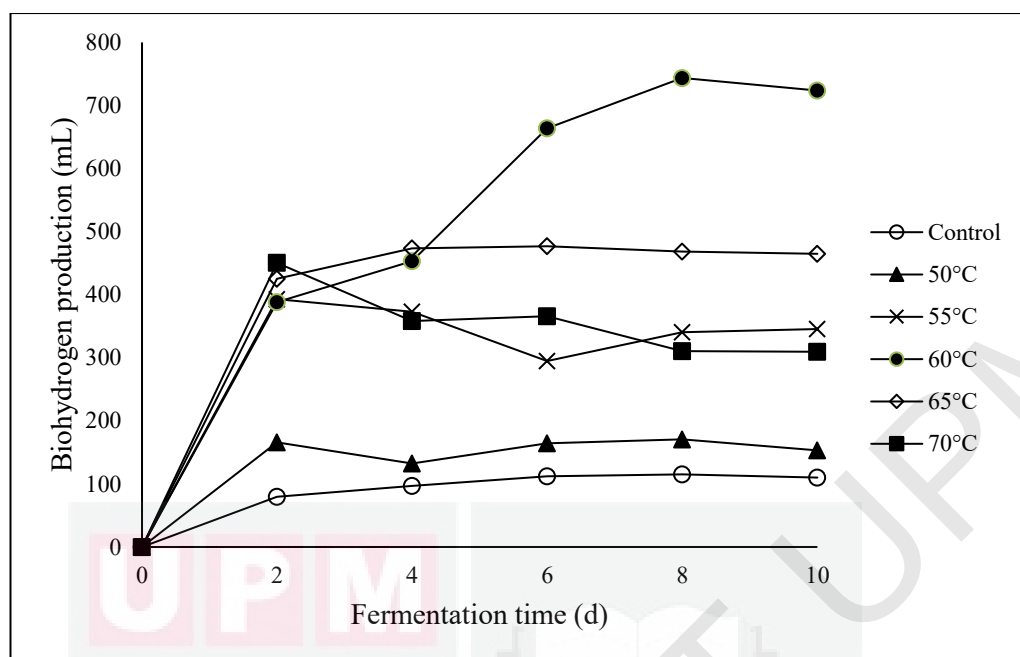


Figure 4.22 : Biohydrogen production at different temperatures

Table 4.13 : Result of H₂ productivity at different temperatures

Temperature (°C)	HY (mol H ₂ /mol sugar consumed)	Parameter values for H ₂ production using the modified Gompertz equation			VAB (g/L)	TVFA (mg/L)
		H _m (mL)	R _m (mL/h)	λ (h)		
Control	0.37 ± 0.04	127.37	0.10	3.23	3.86	2744.32
50	0.58 ± 0.01	160.73	6.13	2.11	4.20	2840.00
55	1.28 ± 0.03	363.35	13.21	5.08	4.42	5017.14
60	2.75 ± 0.11	934.58	18.78	1.89	7.48	5322.86
65	1.72 ± 0.14	501.92	16.62	4.64	7.18	3785.71
70	1.32 ± 0.11	402.73	8.22	3.56	4.53	2988.57

Table 4.13 indicates that the maximum VAB obtained at 60°C yields the highest VAB (7.48 g/L), with a declining trend observed at lower and higher temperatures. At 50°C, the lowest VAB of 4.20 g/L was measured. Due to slower microbial growth, operating at 50°C resulted in a drop in biohydrogen production. On the contrary, as the temperature increased to 55°C, more biohydrogen was produced. Microbial growth

was restricted but considerable at higher temperatures ($>60^{\circ}\text{C}$), with a difference of 4.01%-39.44% from VAB at 60°C (7.48 g/L).

The TVFA accumulation in this study was recorded highest at 5322.86 mg/L when optimal temperature (60°C) was implemented (refer to **Table 4.13**). The TVFA dropped 46.65% at the lowest temperature (50°C) compared to the TVFA at the optimal temperature (60°C), resulting in the lowest HY of 0.58 mol H_2 /mol sugar consumed. Likewise, at the highest temperature of 70°C , there was a 43.85% drop in TVFA with HY of 1.32 mol H_2 /mol sugar consumed. The TVFA varied from 5.74% to 28.88% when operating at a temperature $\pm 5^{\circ}\text{C}$ from optimal. The temperature influences microbial growth and substrate degradation, impacting hydrogen production (Perna et al., 2013). According to a prior study, hydrogenase activity and consumption rise with temperature (Xiao et al., 2013). Furthermore, it has been suggested that conducting the fermentation process at a higher temperature could increase its efficiency by promoting hydrolysis and lowering microbial diversity to promote hydrogen production (Jung et al., 2011). However, during hydrogen production, a temperature higher than optimal could lead to enzyme degradation (Hernández-Mendoza et al., 2014)

The result obtained from the modified Gompertz equation is presented in **Table 4.13**. At the optimum temperature of 60°C , the Gompertz constant was found to be $H_m = 934.58$ mL, $R_m = 18.78$ mL/h and $\lambda = 1.89$ h. Employing the modified Gompertz equation, **Figure 4.23** validated the biohydrogen production potential with maximum HPR and lag phase. This equation was fitted using the cumulative hydrogen

production data. With $R^2 > 0.99$, the modified Gompertz equation best fits all experimental data.

To validate the significance of the effect of the temperature study, the Holm Bonferroni method was implemented and presented in **Figure 4.24**. From the figure, the p-values are all ≤ 0.001 . The smaller p-value (0.05 or less) indicates the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study.

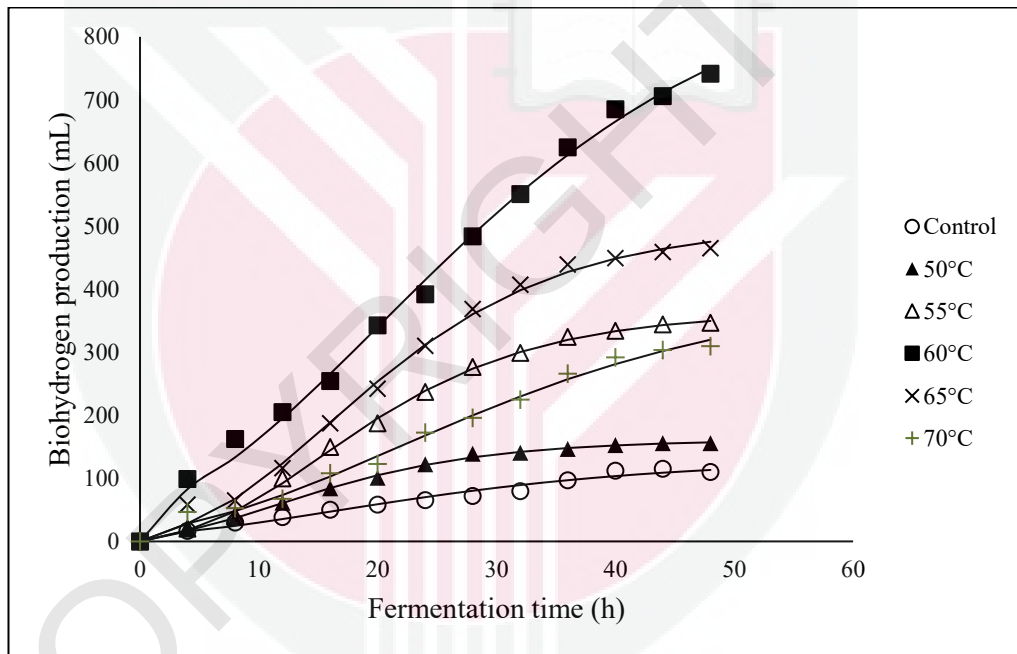


Figure 4.23 : Biohydrogen production at different temperatures fitted to the Gompertz model

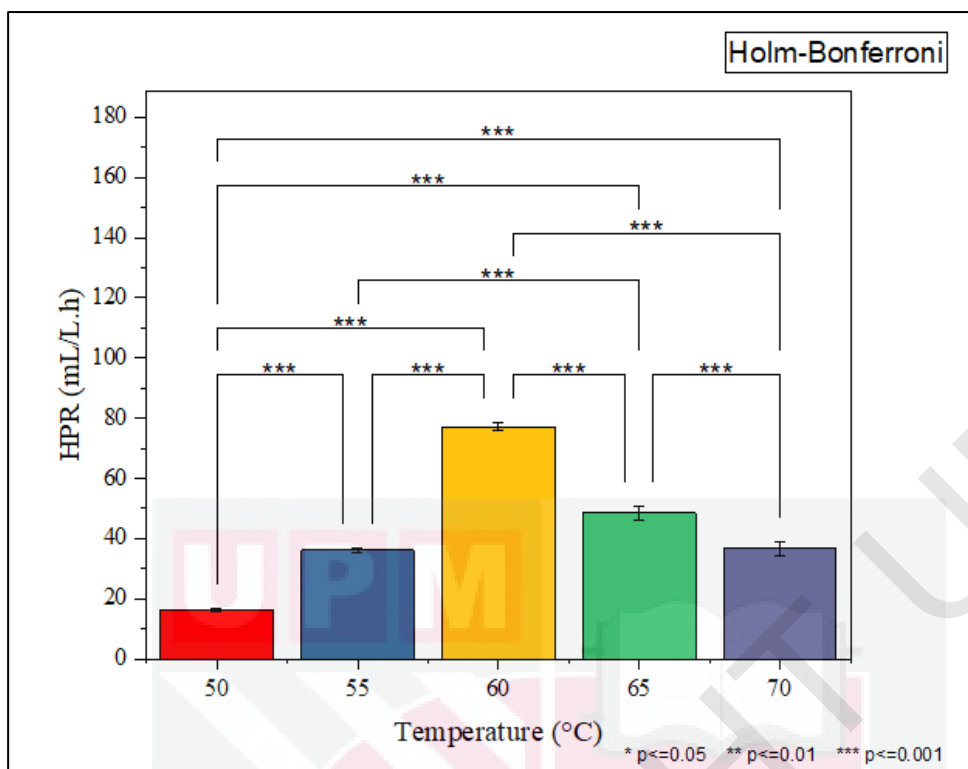


Figure 4.24 : Statistical analysis of the effect of temperature on HPR

4.3.1.3 Effect of Agitation

The effect of agitation on biohydrogen production is shown in **Figure 4.25**. From the figure, the optimal agitation (120 rpm) obtained gives the highest HY of 3.44 ± 0.54 mol H₂/mol sugar consumed (refer to **Table 4.14**). While HY of 0.44 ± 0.10 mol H₂/mol sugar consumed was achieved at 150 rpm, the lowest HY of 0.39 ± 0.04 mol H₂/mol sugar consumed was obtained at 180 rpm, representing an 11.36% difference. The HY was 1.21 ± 0.01 mol H₂/mol sugar consumed and 1.86 ± 0.39 mol H₂/mol sugar consumed at 60 and 90 rpm, respectively. $0.74 \text{ mol} \pm 0.01$ H₂/mol sugar consumed was obtained when no agitation (control) was used, much higher than the HY obtained at 180 rpm (0.39 ± 0.04 mol H₂/mol sugar consumed).

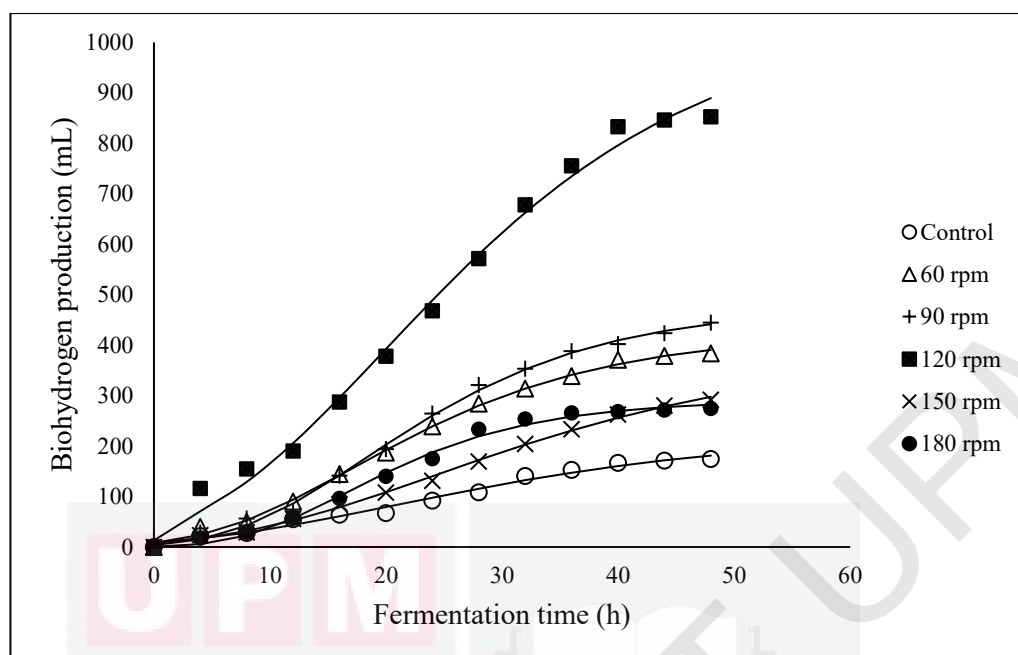


Figure 4.25 : Biohydrogen production at different agitations

Table 4.14 : Result of H₂ productivity at different agitations

Agitation	HY (mol H ₂ / mol sugar consumed)	Parameter values for H ₂ production using the modified Gompertz equation			VAB (g/L)	TVFAs (mg/L)
		H _m (mL)	R _m (mL/h)	λ (h)		
Control	0.74 ± 0.01	224.03	4.68	3.12	7.70	4200.00
60	1.21 ± 0.01	424.14	12.59	4.70	7.75	4948.57
90	1.86 ± 0.39	475.93	15.22	6.67	8.78	5640.00
120	3.44 ± 0.54	1054.86	24.37	3.90	9.11	6234.29
150	0.44 ± 0.10	387.16	8.02	6.49	6.00	4822.86
180	0.39 ± 0.04	291.17	11.76	7.40	5.89	4628.57

Table 4.14 indicates that the maximum VAB (9.11 g/L) was obtained at 60 rpm, and a declining trend was observed at lower and higher agitation. At 50 rpm, the lowest VAB of 5.89 g/L was measured. The obtained value was 24% less than the control. The TVFA accumulation in this study was recorded highest at 6234.29 mg/L when optimal agitation was implemented. When the agitation increased or decreased from 120 rpm, a reduced pattern in TVFA accumulation was observed.

According to a prior study, 100 rpm was the optimal agitation that support microbial growth and enzyme production (Ibrahim et al., 2013). There have been reports of possible cell damage if agitation speed is increased (>200 rpm) due to extreme shear force (Zhou et al., 2018). The findings were aligned with this study, which obtained optimal agitation of less than 200 rpm at 120 rpm. Agitation must be implemented to maintain consistency in the medium's chemical and physical composition. The consistency ensures the mass and oxygen transfer between phases (Mustafa et al., 2019). Furthermore, agitation can affect microbial growth through morphological changes and growth variances brought on by the shear pressures (Mustafa et al., 2019).

The result obtained from the modified Gompertz equation is presented in **Table 4.14**. At the optimum agitation of 120 rpm, the Gompertz constant was found to be $H_m = 1054.86$ mL, $R_m = 24.37$ mL/h, and $\lambda = 3.90$ h. Employing the modified Gompertz equation, **Figure 4.26** validated the biohydrogen production potential with maximum HPR and lag phase. This equation was fitted using the cumulative hydrogen production data. With $R^2 > 0.97$, the modified Gompertz equation best fits all experimental data.

To validate the significance of the effect of the agitation study, the Holm Bonferroni method was implemented and presented in **Figure 4.27**. From the figure, the p-values are all ≤ 0.001 . The smaller p-value (0.05 or less) indicates the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study.

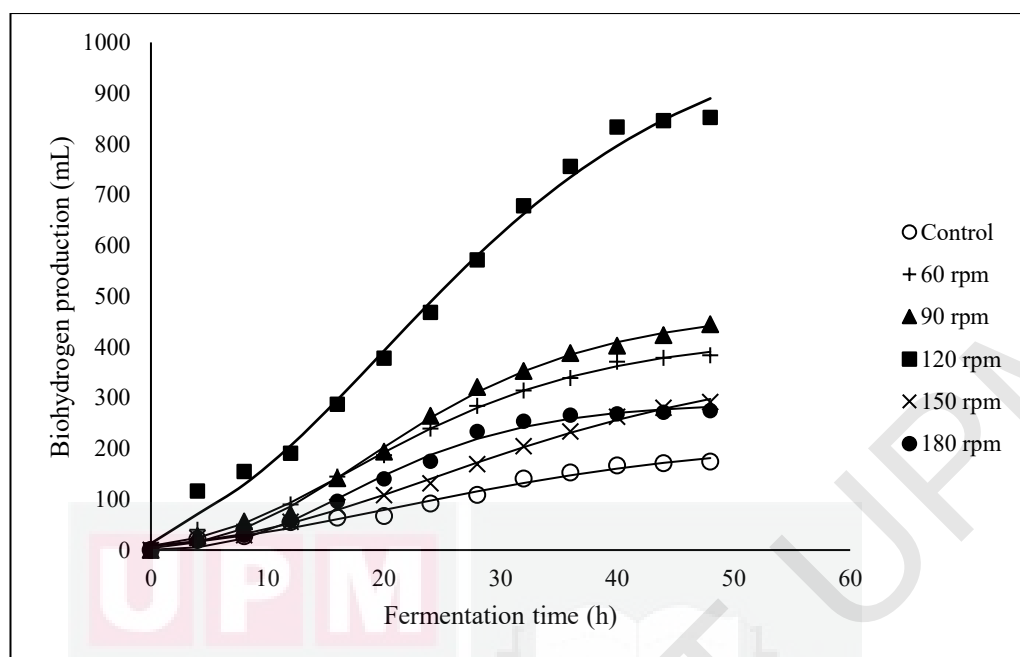


Figure 4.26 : Biohydrogen production at different agitations fitted to the Gompertz model

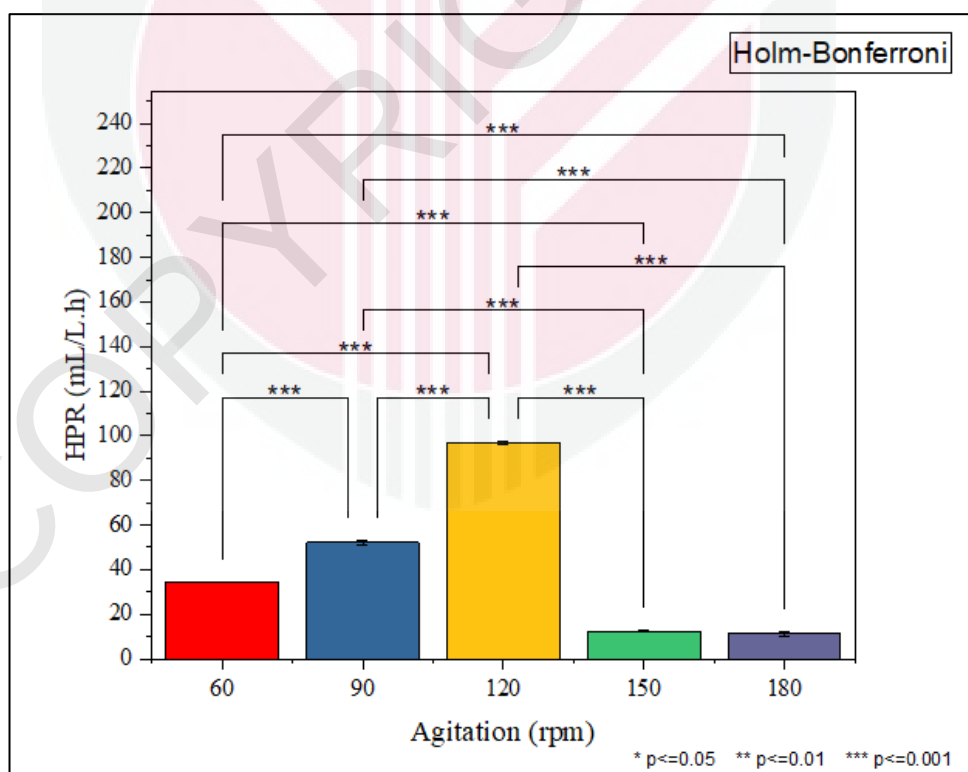


Figure 4.27 : Statistical analysis of the effect of agitation on HPR

4.3.2 Effect of SLR on Biohydrogen Production

The solid substrate concentration during fermentation is called the SLR when producing biohydrogen. This parameter can greatly influence the yield and efficiency of biohydrogen production in dark fermentation. Five different SLRs were studied at 5%, 10%, 15%, 20%, and 25% (w/v), which responded to biohydrogen productivity as tabulated and summarized in **Table 4.15** and **Figure 4.30**, respectively. From the table and figure, 10% SLR resulted in the highest HY and HPR at 2.37 ± 0.07 mol H₂/mol sugar consumed and 67.20 ± 2.02 mL H₂/L.h, respectively. The 10% SLR also led to the highest TSS (g/L) degradation of 66.67% and sugar utilization (g/L) of 99.90%.

Table 4.15 : Result of H₂ productivity at different SLRs

SLR	TSS degradation	Sugar utilization	HPR	HY	Parameter values for H ₂ production using the modified Gompertz equation		
	% (w/v)		(mL H ₂ /L.h)	(mol H ₂ / mol sugar consumed)	H _m (mL H ₂)	R _m (mL H ₂ /h)	λ (h)
5	25.88	99.60	49.07 ± 1.04	1.74 ± 0.04	579.56	13.74	5.78
10	66.67	99.90	67.20 ± 2.02	2.37 ± 0.07	834.30	15.94	0.76
15	18.52	99.70	57.25 ± 0.94	2.03 ± 0.03	629.56	16.82	3.71
20	12.04	99.50	49.95 ± 2.32	1.77 ± 0.08	569.78	13.62	4.72
25	10.49	99.89	46.88 ± 1.72	1.67 ± 0.06	485.52	15.30	6.65

When the SLR was lowered to 5% (from 10%), the HY and HPR decreased to 1.74 ± 0.04 mol H₂/mol sugar consumed and 49.07 ± 1.04 mL H₂/L.h, correspondingly. The decreased HY and HPR may have resulted from the lower nutrient supplementation to support microbial growth and metabolic activities. The low supply causes a nutrient imbalance, which can affect the composition of the microbial community and, consequently, hydrogen production (Singh et al., 2013).

The metabolic activities tabulated in **Table 4.16** supported the statement. The VFA/SMP value is lowest at 0.72 (5% SLR) compared to the other SLRs. On the other hand, when the SLR was further increased from 10% to 25%, the HY and HPR showed a decreasing pattern. High SLR was reported to cause the accumulation of inhibitory compounds, such as organic acids and other metabolic byproducts (Singh et al., 2013). These inhibitory substances can negatively impact the hydrogen-producing bacteria's activity, resulting in lower hydrogen productivity (Pomdaeng et al., 2024).

However, at 25% SLR, the highest VFA/SMP was recorded at 0.87 (see **Table 4.16**). While VFA is essential for hydrogen production, excessive concentration inhibits the microbial community (Chu et al., 2012). Since the microbial community is complex, any imbalance with the dominance of acid-forming bacteria over hydrogen-producing bacteria can lead to elevated VFA production without increasing hydrogen production (Chu et al., 2012). High VFA production can cause the fermentation process to favor organic acid production over hydrogen (Han et al., 2015).

Table 4.16 : Result of TVFA/SMP at different SLRs

POME % (v/v)	HAc	HBu	HLa (mM)	HPa	EtOH	TVFA/SMP
5	0.0155	0.0181	0.0000	0.0000	0.0130	0.72
10	0.0322	0.0314	0.0092	0.0000	0.0118	0.86
15	0.0308	0.0321	0.0000	0.0000	0.0120	0.84
20	0.0347	0.0348	0.0109	0.0000	0.0151	0.84
25	0.0307	0.0322	0.0109	0.0000	0.0220	0.87

TVFAs = HAc + HBu+ HLa; SMPs = TVFAs + EtOH.

Figure 4.28 validated the biohydrogen production potential with maximum HPR and lag phase (refer **Table 4.15**) using the modified Gompertz equation model. The cumulative hydrogen production data was obtained to fit this equation. The R^2 ranged from 0.98-0.99, validating the biohydrogen production.

To validate the significance of the SLR study, the Holm Bonferroni method was implemented and presented in **Figure 4.29**. From the figure, the p-values are all ≤ 0.001 . The smaller p-value (0.05 or less) indicates the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study. The optimal SLR can vary depending on the microbial community activity and fermentation conditions used in a particular fermentation system. Thus, optimizing the SLR was a critical aspect of biohydrogen production utilizing GAC-NiFe₃O₄. It requires a balanced substrate utilization and hydrogen yield while minimizing inhibitory effects and process complexity.

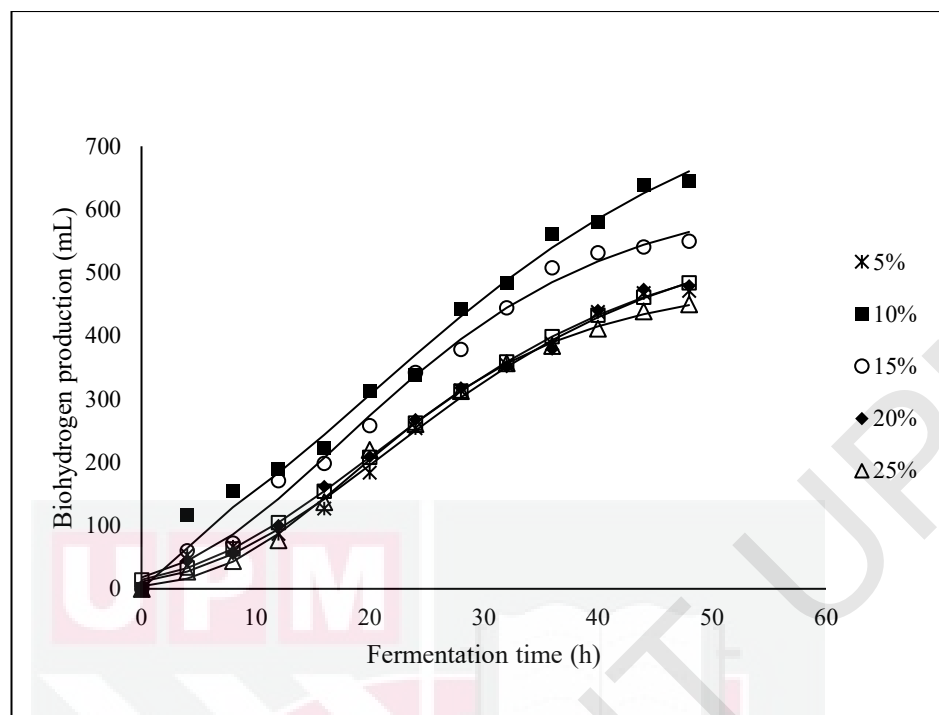


Figure 4.28 : Biohydrogen production at different SLRs fitted to the Gompertz model

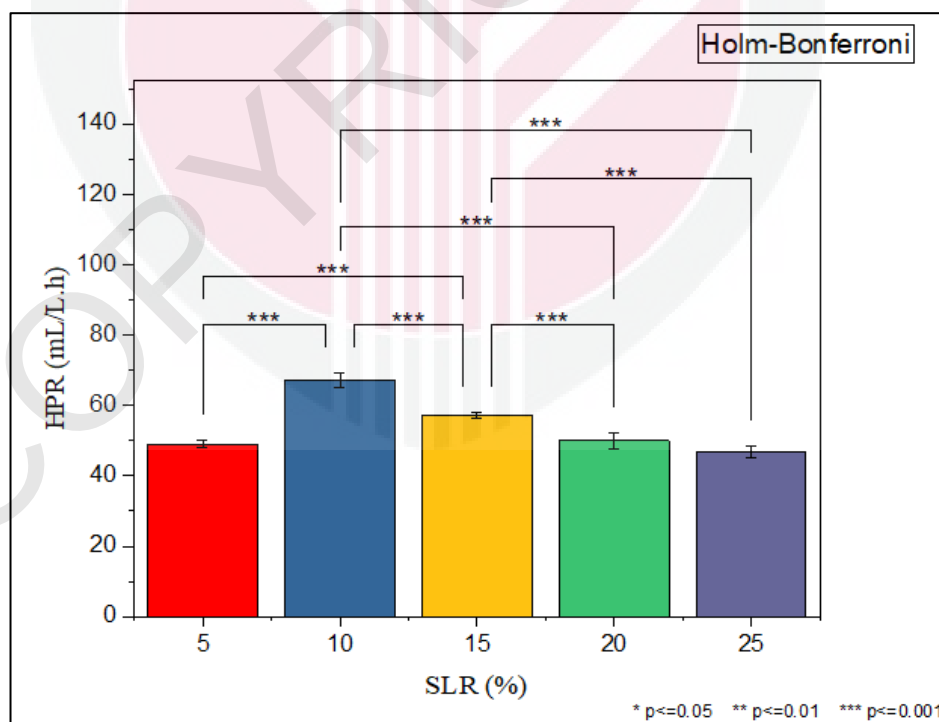


Figure 4.29 : Statistical analysis of the effect of SLR on HPR

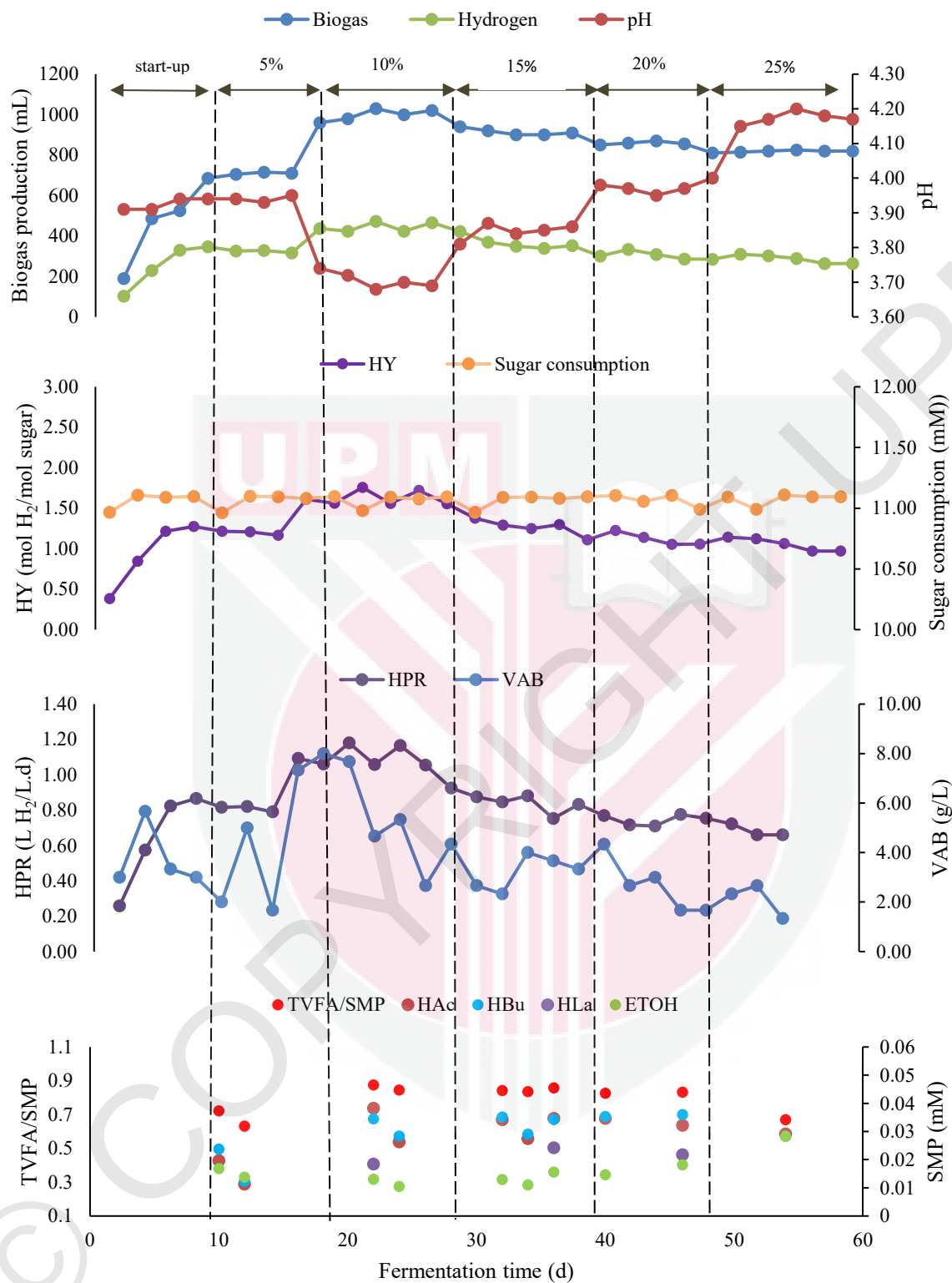


Figure 4.30 : The overall effect of SLR on biohydrogen productivity

4.3.3 Effect of HRT on Biohydrogen Production

One of the crucial process parameters for continuous biohydrogen production is HRT. A very low HRT can cause cell washout. Increasing HRT, on the other hand, might cause the pattern of hydrogen fermentation to change to methanogenic fermentation (Syirat et al., 2022). Thus, optimization of HRT involves finding a balance between maximizing hydrogen production and maintaining a stable bioreactor performance (Kumar, Sen, et al., 2016).

Five different HRTs were studied at 48 h, 24 h, 12 h, 6 h, and 3 h, which responded to biohydrogen productivity as tabulated and summarized in **Table 4.16** and **Figure 4.33**, respectively. Based on **Table 4.16**, the highest HRT (48 h) obtained the lowest HPR and HY at 26.81 ± 0.55 mL H₂/L.h and 0.96 ± 0.02 mol H₂/ mol sugar consumed, respectively. The lowest VFA/SMP (0.81 at 48 h) from **Table 4.17** indicates that the excessively long HRT led to the accumulation of inhibitor compounds, affecting microbial activity and thus reducing hydrogen productivity.

When the HRT was further shortened from 48 h to 3 h, the hydrogen productivity could show an increasing pattern. At the lowest HRT of 3 h, the highest HPR (104.10 ± 13.01 mL H₂/L.h) and HY (3.74 ± 0.47 mol H₂/ mol sugar consumed) was acquired. The hydrogen concentration also improved from 65.99% to 75.71% with an increase in energy production rate (EPR) up to 29.19 ± 3.65 kJ/L. d. The optimal HRT of 3 h obtained from this study is comparably lower (50-57.14%) than the previous study, which obtained optimal HRT ranges of 6-7 h (Karapinar et al., 2019; Syirat et al., 2022; Zhao et al., 2017). However, the optimal HRT obtained in this study aligned with the optimal HRT found in a previous study at 3 h (Gorgec & Karapinar, 2018).

It is apparent that for the microbes to consume a substrate completely, they require an appropriate contact time. The amount of time required to use substrate efficiently in this study is decreased by lowering the HRT. The optimal HRT can vary depending on the microbial community activity and fermentation conditions used in a particular fermentation system. Thus, optimizing the HRT was a critical aspect of biohydrogen production utilizing GAC-NiFe₃O₄. Maintaining steady reactor performance and optimizing biohydrogen production must be balanced.

Table 4.17 : Result of H₂ productivity at different HRTs

HRT	HPR	HY	EPR	Parameter values for H ₂ production using the modified Gompertz equation		
(h)	(mL H ₂ /L.h)	(mol H ₂ / mol sugar consumed)	(kJ/L. d)	H _m (mL H ₂)	R _m (mL H ₂ /h)	λ (h)
48	26.81 ± 0.55	0.96 ± 0.02	7.52 ± 0.15	257.81	14.82	7.33
24	34.03 ± 4.34	1.21 ± 0.15	9.54 ± 1.22	332.23	15.20	6.16
12	48.19 ± 5.84	1.72 ± 0.21	13.51 ± 1.64	493.36	17.14	5.72
6	69.98 ± 2.12	2.51 ± 0.08	19.62 ± 0.59	829.36	19.82	5.33
3	104.10 ± 13.01	3.74 ± 0.47	29.19 ± 3.65	1228.07	28.78	3.93

Table 4.18 : Result of TVFA/SMP at different HRTs

HRT (h)	HAc	HBu	HLa (mM)	HPa	EtOH	TVFA/SMP
48	0.0130	0.0531	0.0639	0.0031	0.0305	0.81
24	0.0134	0.0487	0.0862	0.0026	0.0228	0.87
12	0.0134	0.0425	0.1019	0.0016	0.0118	0.93
6	0.0123	0.0412	0.0937	0.0009	0.0097	0.94
3	0.0058	0.0390	0.0558	0.0058	0.0058	0.95

Figure 4.31 validated the biohydrogen production potential with maximum HPR and lag phase (refer **Table 4.17**) using the modified Gompertz equation model. The

cumulative hydrogen production data was obtained to fit this equation. The R^2 ranged from 0.97-0.99, validating the biohydrogen production.

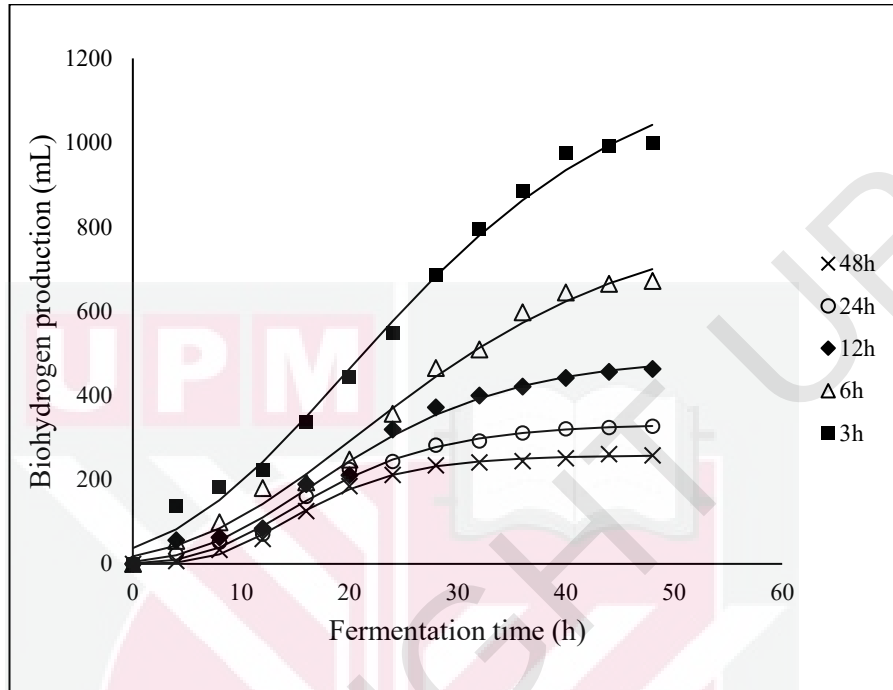


Figure 4.31 : Biohydrogen production at different HRTs fitted to the Gompertz model

To validate the significance of the HRT study, the Holm Bonferroni method was implemented and presented in **Figure 4.32**. From the figure, the p-values are all ≤ 0.05 . The smaller p-value (0.05 or less) indicates the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study.

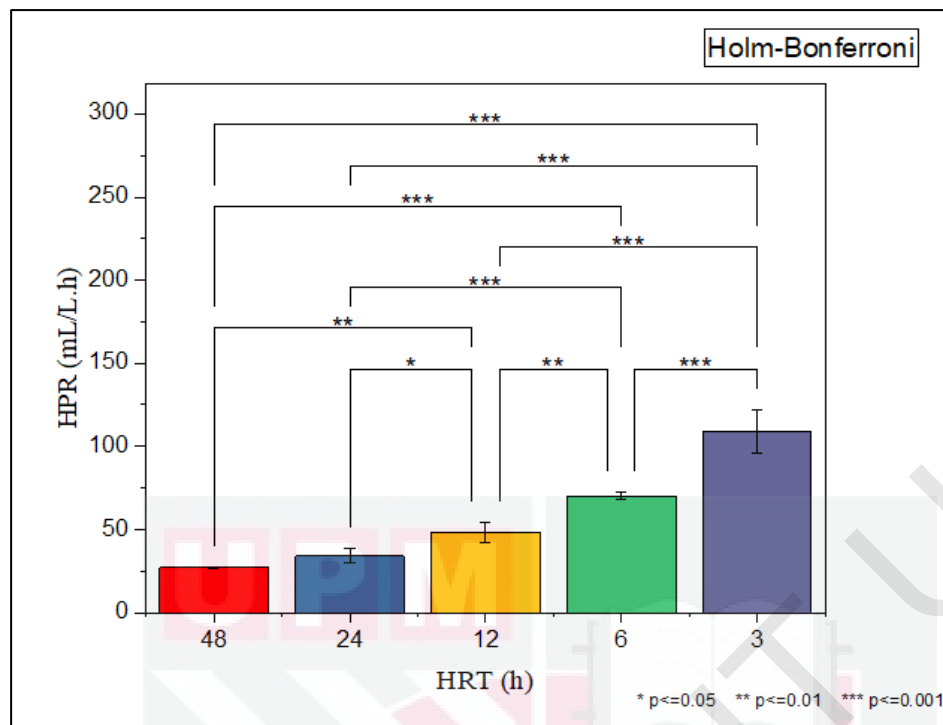


Figure 4.32 : Statistical analysis of the effect of HRT on HPR

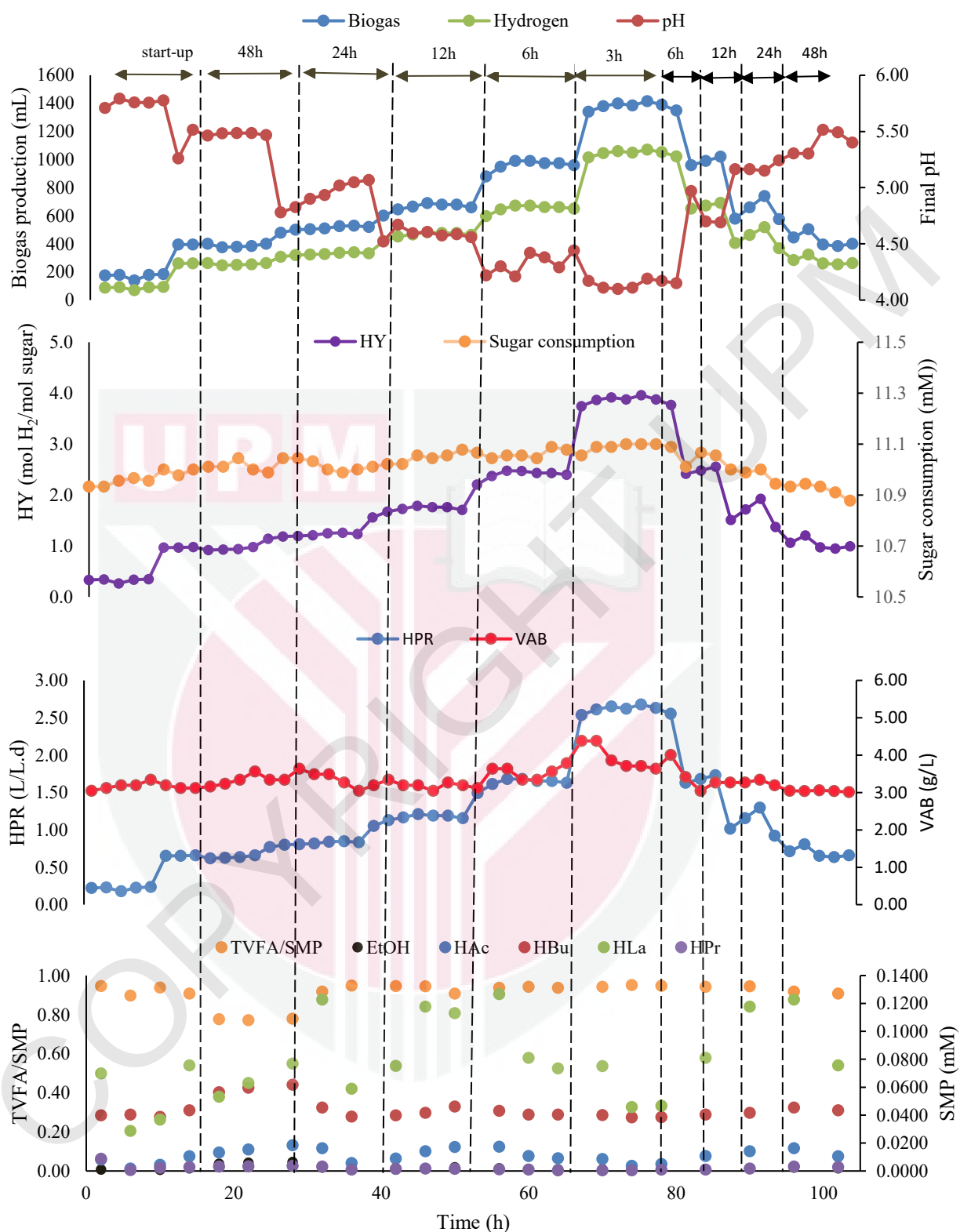


Figure 4.33 : The overall effect of HRT on biohydrogen productivity

4.4 Biohydrogen Production from POME Utilizing GAC-NiFe₃O₄

4.4.1 Characterization of POME

The collected POME was brownish with thick sludge containing oil and fine cellulosic fruit residues. The POME was further characterized in three duplicates to determine the composition of its components. The results are presented in **Table 4.19** and were compared to previous findings by (Abdullah et al., 2020; Ismail et al., 2010; Jamali et al., 2021; Maaroff et al., 2019; Mahmod et al., 2019).

Table 4.19 shows that the pH of raw POME upon collection is slightly higher than the previous finding ranges of 4.00-4.35 (Ismail et al., 2010; Maaroff et al., 2019; Mahmod et al., 2019). The temperature of the collected POME was slightly lower than the review made by (Mahmod et al., 2022) at 80-90°C but slightly (6.43%) higher than that of Mahmod et al. (2019). The POME contains high concentration of COD (22908.33 ± 300.35 mg/L), BOD (12450.00 ± 396.86 mg/L), TSS (12333.33 ± 333.33 mg/L), and VSS (11555.56 ± 1170.63 mg/L), which reflects the high contents of organic matter.

The high content of COD and BOD was consistent with previous findings by (Mahmod et al., 2022). The environmental challenges posed by POME are significant due to its high level of COD and BOD, which can lead to water pollution (Wang et al., 2023). However, the value of COD (**Table 4.19**) is comparatively lower by 48.02%-73.42% compared to (Abdullah et al. 2020; Jamali et al., 2021; Maaroff et al., 2019; Mahmod et al., 2019). Except for TP, the TSS, VSS, O&G, and AN values were also comparatively low but within the range (Mahmod et al., 2022). Even though the POME was extracted at the same point, the chemical and physical characteristics

would vary each time. The characteristics depend on different factors, such as harvest season and the efficiency of the palm oil extraction process (Rosa et al., 2020).

Table 4.19 : Characteristics of raw POME

Parameter	Unit	Raw POME	Reference	Standard Discharge Limit*
pH		4.88 ± 0.02	4.1-5.2	5-9
Temperature	°C	67.33 ± 6.11	47-90	40°C
COD	mg/L	22908.33 ± 300.35	15,000-100,000	200
BOD	mg/L	12450.00 ± 396.86	10,250-43,000	50
TSS	mg/L	12333.33 ± 333.33	5000-54,000	100
VSS	mg/L	11555.56 ± 1170.63	9000-72,000	N/A
O&G	mg/L	1666.67 ± 141.42	130-18,000	10
AN	mg/L	39.67 ± 8.99	35-90	50
TP	mg/L	68.57 ± 1.63	94-131	N/A
SMS	mg/L	3.31 ± 0.37	1.6-6.8	N/A
TMS	mg/L	7.43 ± 0.34	4.0-17.6	N/A
K	mg/L	1714.50 ± 10.70	1281-1928	N/A
Ca	mg/L	274.65 ± 1.39	276-405	N/A
Cd	mg/L	0.02 ± 0.00	0.01-0.02	0.02
Co	mg/L	0.07 ± 0.00	0.04-0.06	N/A
Cr	mg/L	28.50 ± 0.20	0.05-0.43	0.05
Cu	mg/L	0.46 ± 0.00	0.8-1.6	1.0
Fe	mg/L	184.60 ± 1.30	75-164	5.0
Mg	mg/L	259.00 ± 3.90	254-344	N/A
Mn	mg/L	37.10 ± 0.21	2.1-4.4	1.0
Zn	mg/L	1.04 ± 0.01	1.2-1.8	2.0

*Environmental Quality (Industrial Effluent) Regulations 2009

4.4.2 Effect of TC in POME on Biohydrogen Production

Total carbohydrates are the primary substrate for hydrogen-producing bacteria. Therefore, the concentration of carbohydrates can affect hydrogen productivity. The TC was optimized to enhance this study's biohydrogen production from POME.

By referring to **Table 4.20**, the TC of the raw POME (100% POME) was 19.12 ± 0.41 g/L, which is slightly higher (3.92%) than prior research at 18.37 ± 0.21 g/L (Mahmod et al., 2019). In comparison, the previous study reported the TC in raw POME at 36.6

± 1.4 g/L (Bai et al., 2009), which was 47.8% higher than this study. The highest HPR and HY were obtained at 50% POME (TC of 14.64 ± 0.11 g/L) at 73.23 ± 3.46 mL H_2 /L.d and 1.77 ± 0.08 mol H_2 /mol sugar consumed.

On the other hand, at the highest TC (19.12 ± 0.41 g/L at 100% POME), the HPR was 28.56 ± 0.29 mL H_2 /L.d, obtaining the lowest HY of 0.53 ± 0.01 mol H_2 /mol sugar consumed. Higher TC concentration in POME often reflected higher substrate concentration for bacteria to produce hydrogen (Srivastava et al., 2021). The higher substrate concentration should increase hydrogen production because faster digestion may occur, leading to a higher production rate. Even so, in this case, at the highest TC, POME may contain inhibitory substances (such as fatty acids and phenolic compounds) from palm oil extraction (Mahmod et al., 2022). High concentrations of these inhibitory substances certainly negatively affect the hydrogen-producing microorganism and impede biohydrogen production, thus explaining the lowest HY obtained. Moreover, previously reported high substrate concentration leads to high hydrogen partial pressure accumulation due to biohydrogen production inhibition by solventogenesis (Fan et al., 2004; Mahmod et al., 2022).

When the POME percentage was lowered to 90-50%, the TC decreased from 18.75 ± 0.19 to 14.64 ± 0.11 g/L (see **Table 4.20**). However, towards the decreasing of TC (from 90-50% POME), the HPR and HY showed increasing pattern from 42.91 ± 5.20 - 73.23 ± 3.46 mL H_2 /L.d and 0.81 ± 0.10 - 1.77 ± 0.08 mol H_2 /mol sugar consumed, respectively.

Subsequently, when the POME percentage was lowered (<50%), the TC achieved the lowest point at 7.67 ± 0.09 g/L. The lowest HPR was obtained at 17.98 ± 1.38 mL H₂/L.d. The finding was expected at low TC, as higher substrate concentrations are required to facilitate hydrogen production by the hydrogen-producing bacteria (Srivastava et al., 2021).

A previous optimization study supported the findings obtained. The particular study concluded that the most hydrogen production (188 mL H₂/g VS) was attained at the optimum total carbohydrates of 56% (15 g/L) (Tarazona et al., 2022). In contrast, a study found that a higher carbohydrate content increases the efficiency of producing biohydrogen (Aslan et al., 2023). When 40 g/L of the substrate was loaded, and 5.95 mg/L of total carbohydrates were present, the greatest biohydrogen efficiency measured was 3.51 mL/g (Aslan et al., 2023). The biohydrogen production and the total amount of carbohydrates in the substrate are linearly correlated, according to earlier studies examining the impact of total carbohydrate content on dark fermentation (Aslan et al., 2023), without any further optimization study as reported in this study.

Table 4.20 : Result of H₂ productivity at different TCs

POME v/v (%)	TC		HPR		HY		Parameter values for H ₂ production using the modified Gompertz equation		
	Initial (g/L)	Consumed (g/L)	(mL H ₂ /L. d)	(mL H ₂ /g COD.d)	(mol H ₂ /mol sugar consumed)	(mL H ₂ /g VSS)	H _m (mL H ₂)	R _m (mL H ₂ /h)	λ (h)
10	7.67 ± 0.09	4.23 ± 0.10	17.98 ± 1.38	41.76 ± 11.06	0.83 ± 0.06	287.14 ± 22.13	206.50	8.98	6.82
30	11.08 ± 0.13	9.14 ± 0.09	43.81 ± 0.32	101.74 ± 2.52	1.40 ± 0.01	699.64 ± 5.04	438.46	18.91	6.08
50	14.64 ± 0.11	13.78 ± 0.01	73.23 ± 3.46	170.07 ± 27.66	1.77 ± 0.08	1169.51 ± 55.33	746.55	23.14	1.33
70	15.39 ± 0.26	13.52 ± 0.09	52.21 ± 5.35	121.26 ± 42.84	1.20 ± 0.12	833.86 ± 85.68	539.45	16.78	5.72
90	18.75 ± 0.19	13.72 ± 0.09	42.91 ± 5.20	99.65 ± 41.61	0.81 ± 0.10	685.28 ± 83.21	481.48	14.23	7.92
100	19.12 ± 0.41	12.10 ± 0.21	28.56 ± 0.29	66.34 ± 2.28	0.53 ± 0.01	456.16 ± 4.57	291.60	13.25	10.20

As previously mentioned, higher TC often resulted in the enhancement of biohydrogen production. However, in this study, lowering the TC leads to higher biohydrogen production. Specific microbial and environmental factors can explain these cases. For example, some hydrogen-producing microorganisms can adapt to low carbohydrate concentrations and more efficiently utilize the available substrates (Fan et al., 2004). These microorganisms may have evolved mechanisms to extract energy from other compounds in POME, such as organic acids or even residual lignocellulosic materials, leading to biohydrogen production (Ong et al., 2021).

Likewise, certain microbial species were interconverted, which can undergo a metabolic shift, favoring pathways that produce more hydrogen (Mahmod et al., 2022). Some microbial communities have complex syntrophic interactions. In these interactions, different microorganisms can work together with one microbe, breaking down complex substrates onto simpler compounds that another microbe can use to produce hydrogen (Kouzuma et al., 2015). These syntrophic relationships can enhance hydrogen production even in low carbohydrate concentrations. The most critical explanation would be remedying inhibitory substances at low carbohydrate concentrations. When the inhibitors are reduced, hydrogen-producing microorganism activity will undoubtedly increase (B. Wang et al., 2008).

Co-substrates in POME, such as volatile fatty acids, can stimulate biohydrogen production even in low carbohydrate conditions. The consumption of carbohydrates, considering glucose the primary soluble carbohydrate, was related to forming soluble microbial products (SMP). **Table 4.21** shows the production of SMP in this TC study, which consists of EtOH and VFA of HAc, HBu, HLa, and HPa. The table shows the

highest VFA/SMP obtained at 70% POME (TC of 15.39 ± 0.26 g/L) at 0.85. Various factors influence the production of VFA and SMP in anaerobic digestion. This study shows high VFA and SMP production but lower hydrogen production at 70% POME. The low production might be due to the composition of the microbial community, which is responsible for producing VFA and SMP while producing hydrogen (Arisht et al., 2022). If the dominant microorganisms in the system are more inclined towards VFA and SMP production, hydrogen production will be limited. In other words, the effect of TC can significantly impact the metabolic pathways of the microorganisms. Specific substrates may favor VFA and SMP production over hydrogen production. Thus, the HY might be higher if the substrate favors hydrogen production over VFA and SMP production. These explained the reason for the slightly lower VFA/SMP at 0.74 able to obtain the highest HY (1.77 ± 0.08 mol H₂/mol sugar consumed) compared to the highest VFA/SMP of 0.85 with only 1.20 ± 0.12 mol H₂/mol sugar consumed.

Table 4.21 : Result of TVFA/SMP at different TCs

POME % (v/v)	HAc	HBu	HLa (mM)	HPa	EtOH	TVFA/SMP
10	0.02	0.26	0.01	0.00	0.09	0.76
30	0.03	0.00	0.15	0.00	0.11	0.63
50	0.05	0.00	0.26	0.03	0.12	0.74
70	0.09	0.00	0.44	0.03	0.10	0.85
90	0.10	0.00	0.43	0.01	0.28	0.66
100	0.11	0.00	0.52	0.00	0.26	0.71

Figure 4.34 validated the biohydrogen production potential with maximum HPR and lag phase (refer **Table 4.20**) using the modified Gompertz equation model. The cumulative hydrogen production data was obtained to fit this equation. The R² ranged from 0.97-0.99, validating the biohydrogen production.

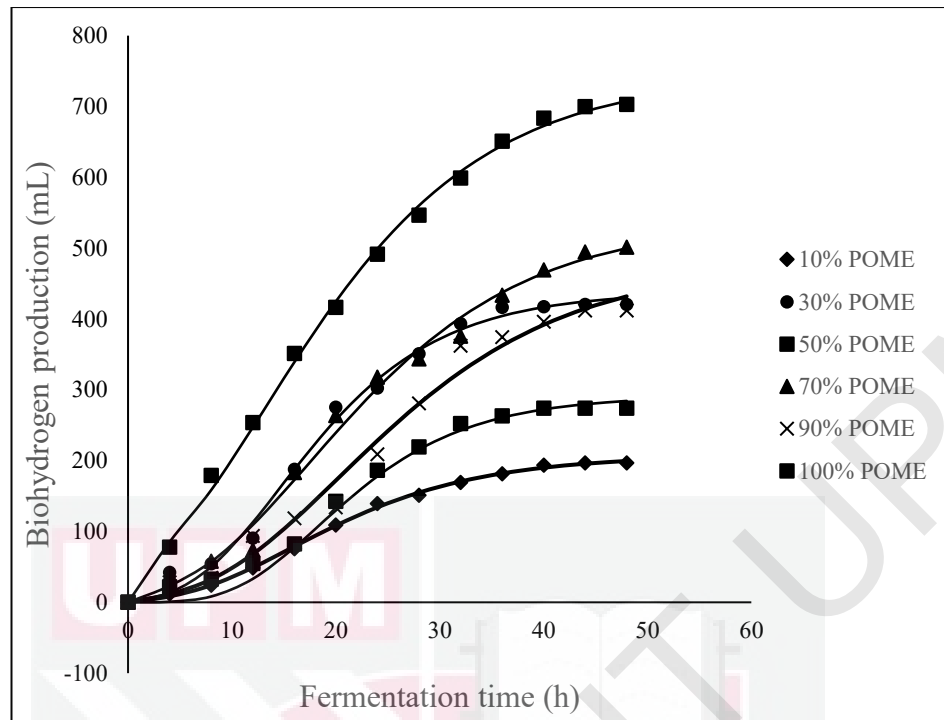


Figure 4.34 : Biohydrogen production at different POME percentages fitted to the Gompertz model

To validate the significance of the TC study, the Holm Bonferroni method was implemented and presented in **Figure 4.35**. From the figure, the p-values are all ≤ 0.01 . The smaller p-value (0.05 or less) indicates the statistical analysis's greater significance (Azami et al., 2011). Hence, the p-values indicated the reliability of the result and its significance to several hypotheses in the study.

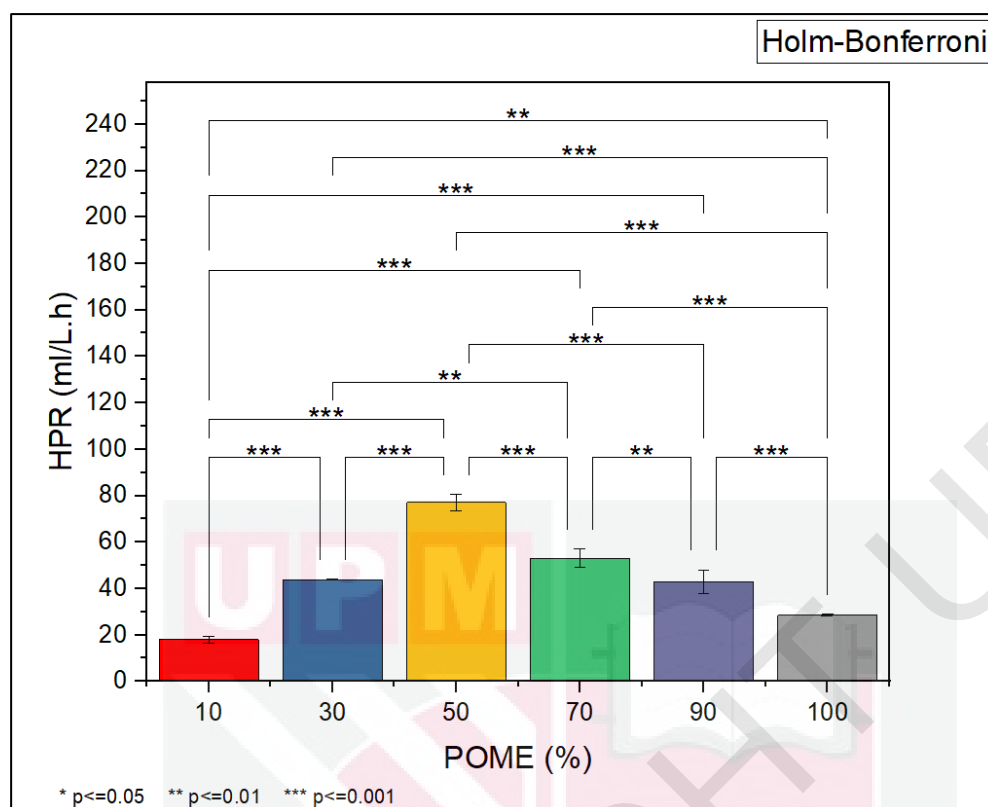


Figure 4.35 : Statistical analysis of the effect of TC on HPR

4.4.3 Analysis of Microbial Community

The abundance of microorganisms on the multiple levels of the phylogenetic hierarchy is illustrated in **Figure 4.36**. The dominant bacteria community consisted of *Firmicutes* (98%) at the phylum level. Nonetheless, the dominant genera in this study were *Thermoanaerobacterium* at the genus level at 67%, followed by *Bacili* (22%) and *Clostridia* (10%). Based on the relative abundance of the dominant microbial community tabulated in **Table 4.22**, *Thermoanaerobacterium* species were identified as dominant (63.9%) in this study. The isolated sequences were compared and analyzed through GenBank sequences using basic local alignment search tools (BLAST).

From the BLAST, it was found (refer to **Table 4.23**) that the dominant species of *Thermoanaerobacterium* were identical to *Thermohydrogenium thermosaccharolyticum* (100%), *Thermoanaerobacterium bryantii* (98.75%), *Thermoanaerobacterium xylanolyticum* (98.50%), and *Thermoanaerobacterium calidifontis* (98.50%).

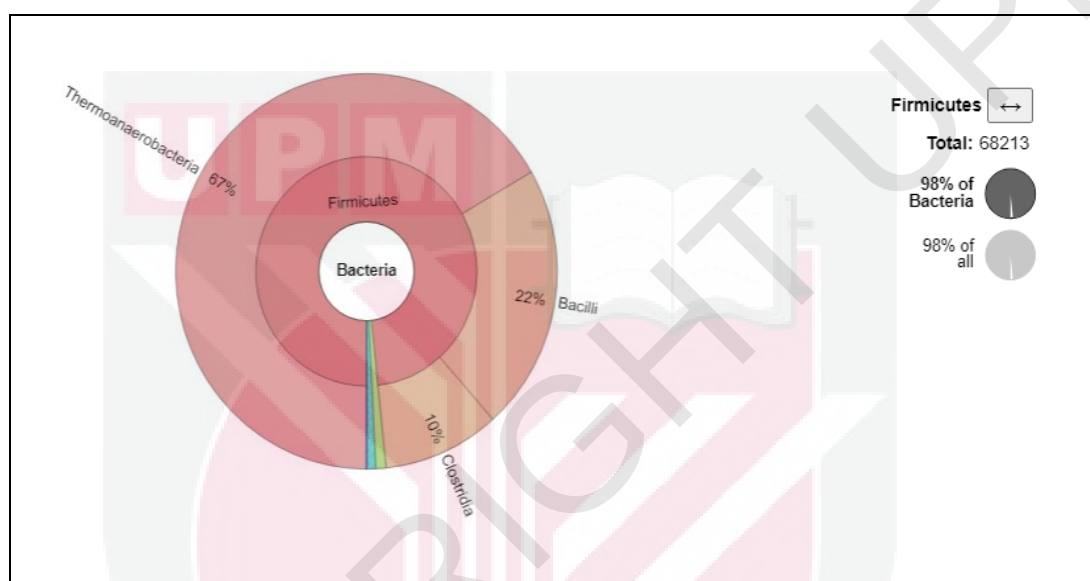


Figure 4.36 : Abundance of microorganisms of phylogenetic hierarchy

This subchapter utilized complex wastewater (POME) for biohydrogen production by implementing the GAC-NiFe₃O₄. The *Thermoanaerobacterium* is considered among the most promising microorganisms in hydrogen production. Previously, it has been reported that using isolated *Thermoanaerobacterium Thermosaccharolyticum* PSU-2 in hydrogen production can produce a maximum hydrogen production yield of 2.53 mol H₂/ mol hexose (O-Thong, Prasertsan, Karakashev, et al., 2008). Besides enhancing hydrogen production, the *Thermoanaerobacterium* also shows the ability to reduce COD in POME.

As reported by the previous researcher, *Thermoanaerobacterium*-rich sludge can produce 6.33 ± 0.142 H₂/L POME with $55 \pm 1.5\%$ of COD removal efficiency and $92 \pm 2.7\%$ of total carbohydrate conversion (O-Thong, Prasertsan, Intrasingkha, et al., 2008). The combination of *Thermoanaerobacterium* *Thermosaccharolyticum* with biochar was previously reported to promote metabolic pathways related to substrate degradation and microbial metabolism (Zhang K. et al., 2019). Compared to previous studies, the dominant (67%) bacteria in this study of *Thermoanaerobacterium* species was proved to have a high capability for hydrogen production.

Table 4.22 : Abundance percentage of the dominant bacteria community

No	Taxonomy	Score	Abundance %
1	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacterales;Family III;Thermoanaerobacterium;thermosaccharolyticum	21899	31.6
2	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacterales;Family III;Thermoanaerobacterium;thermosaccharolyticum	9441	13.6
3	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacterales;Family III;Thermoanaerobacterium	6786	9.8
4	Bacteria;Firmicutes;Thermoanaerobacteria;Thermoanaerobacterales;Family III;Thermoanaerobacterium	6145	8.9
5	Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae; Lactobacillus	3185	4.6
6	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Geobacillus; stearothermophilus	3158	4.6
7	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus; coagulans	3064	4.4
8	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Geobacillus	1947	2.8
	Total		80.2

Table 4.23 : Sequences comparison against BLAST

No	Description	Total score	Similarity (%)	Accession
1	Thermoanaerobacterium thermosaccharolyticum strain CT72 16S ribosomal RNA gene	739	100.00	JX_984968
2	Thermoanaerobacterium thermosaccharolyticum strain D120-70 16S ribosomal RNA gene	739	100.00	AF_247003
3	Thermoanaerobacterium thermosaccharolyticum strain CT6 16S ribosomal RNA gene	734	99.75	JX_984971
4	Thermoanaerobacterium thermosaccharolyticum strain DJA2 16S ribosomal RNA gene	728	99.50	KJ_831072
5	Thermoanaerobacterium bryantii 16S ribosomal RNA gene	712	98.75	AY_140670
6	Thermoanaerobacterium xylanolyticum strain LX-11 16S ribosomal RNA	706	98.50	NR_102771
7	Thermoanaerobacterium calidifontis strain Rx1 16S ribosomal RNA	706	98.50	NR_113051
8	Thermoanaerobacterium xylanolyticum LX-11	3532	98.50	CP_002739

4.4.4 Potential of Heavy Metal Removal

Heavy metals are highly toxic and cannot degrade through conventional biological reactions. The discharge of heavy metals into the water system can cause serious health issues to living organisms. POME contains significant plant components like K, Ca, and Mg. Still, the toxicity of these metals from effluent application in soil reduces the growth and development of the plant itself (Anwar Hossain et al., 2015).

Previous studies implementing coconut shells into activated carbon have shown the potential as a heavy metal absorbent (Cazetta et al., 2011). Coconut shell has been reported as an excellent source of activated carbon because it contains cellulose, hemicellulose, and lignin (Gunawan et al., 2022). In addition, materials containing lignocellulosic properties are excellent adsorbent materials for activating carbonaceous materials (Oil et al., 2019). Adsorbents are materials that adsorb wastewater pollutants, while adsorbates are the substances to be adsorbed. The

adsorption rate is affected by the adsorbents' nature, the adsorbate concentration, and the adsorption system's efficiency (Oil et al., 2019). However, in this study, the conventional method of using just activated carbon as an adsorbent was further enhanced to carbon composites.

In this study, the potential of heavy metal remediation in POME was simultaneously investigated, along with biohydrogen production, using GAC-composites. Firstly, the content of heavy metals (K, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, and Zn) in raw POME was quantified and listed in **Table 4.24**. All the heavy metal content on raw POME was consistent with (Mahmod et al., 2022), except for the value of Cr and Mn (66.28 and 8.43 times higher, respectively). Heavy metal remediation could be conducted at 29.71-99.86% removal. The removal of Cr was highest at 99.86%, followed by Co and Mn at 97.12% and 96.90%, respectively. The high removal showed that nanoparticle-enhanced granular activated carbon can potentially remove heavy metals in POME.

Table 4.24 : Result of heavy metal removal using GAC-NiFe₃O₄

Parameter	Unit	Raw POME	After treatment	% Removal
K	mg/L	1714.50 ± 10.70	908.40 ± 8.25	47.02
Ca	mg/L	274.65 ± 1.39	113.00 ± 1.84	58.86
Cd	mg/L	0.02 ± 0.00	0.00 ± 0.00	80.00
Co	mg/L	0.07 ± 0.00	0.00 ± 0.00	97.12
Cr	mg/L	28.50 ± 0.20	0.04 ± 0.00	99.86
Cu	mg/L	0.46 ± 0.00	0.25 ± 0.00	44.98
Fe	mg/L	184.60 ± 1.30	29.33 ± 0.50	84.11
Mg	mg/L	259.00 ± 3.90	94.42 ± 0.84	63.93
Mn	mg/L	37.10 ± 0.21	1.15 ± 0.02	96.90
Zn	mg/L	1.04 ± 0.01	0.73 ± 0.00	29.71

Comparatively, in **Table 4.25**, the present study shows a higher potential (up to 99.9%) of heavy metal removal efficiency compared to the previous studies

(Alagarasan et al., 2022; Karnib et al., 2014; Liu B. et al., 2023; Liu X. et al., 2022; Manirethan & Balakrishnan, 2020; Moussa et al., 2021; Mubarak et al., 2022; Nyirenda et al., 2022; Wang N. et al., 2024; Zhang, Qiyun, et al., 2020). Different activated carbon composites may have a greater affinity for certain metals based on their composition and surface modifications (Nguyen et al., 2023). The adsorption rate is affected by the adsorbents' nature, the adsorbate concentration, and the adsorption system's efficiency (Oil et al., 2019).

The conventional method was activated carbon utilization as an adsorbent. Regarding porosity, both activated carbon and carbon composites were reported to have high surface area and porosity. For example, the previous researcher reported a high surface area of 801.11-941.23 m²/g of activated carbon from coconut shells (Jamali et al., 2017). Carbon composites, on the other hand, were reported to have a lower surface area of 1.74-28.04 m²/g (Kacan, 2016b; Kusrini et al., 2018; Venault et al., 2008; Yang L. et al., 2016). However, the pore size distribution for carbon composites can be altered for the specific heavy metal according to the ionic size (Choi et al., 2023). By adjusting the pore structure, the selectivity could be further enhanced.

Overall, implementing carbon composites in this study proved to have the potential to remove heavy metals from POME. However, further study needs to be done on carbon composite materials to target specific heavy metals and optimize the performance for heavy metal remediation. Carbon composites provide a versatile tool for heavy metal remediation in water treatment, environmental cleanup, and industrial processes while producing hydrogen.

Table 4.25 : Comparison of heavy metal removal with previous studies

Absorbent	Wastewater	Absorbate	Removal (%)	Reference
AC-melanin	Aqueous solution	Hg, Cr, Pb, Cu	84.6-93.8	(Manirethan & Balakrishnan, 2020)
AC-silica	Aqueous solution	Pb, Cd, Ni, Cr, Zn	24.0-90.0	(Karnib et al., 2014)
AC-silica	Aqueous solution	Cu, Pb, Cd, Zn	62.6-98.1	(Nyirenda et al., 2022)
AC-FeO	Aqueous solution	Pb, Cu, Zn, Cd	40.0- 95.0	(Liu X. et al., 2022)
AC-Fe ₃ O ₄	Aqueous solution	Cd, Pb	92.7- 97.9	(Wang N. et al., 2024)
AC- Carborundum	Aqueous solution	Cu, As	44.2-92.0	(Mubarak et al., 2022)
AC-ZnO	Aqueous solution	Pb, Cr, Cu	38.3-88.2	(Zhang, Qiyun et al., 2020)
AC-NiFe ₂ O ₄	Aqueous solution	Cu, Zn	58.8-92.8	(Moussa et al., 2021)
AC-Fe ₃ O ₄	Aqueous solution	Pb, F	25.0-90.0	(Alagarsan et al., 2022)
AC-MgO	Tap water	Pb, Cd	82.1-97.3	(Liu B. et al., 2023)
	Lake water			
	Industrial wastewater			
GAC-NiFe ₃ O ₄	POME	K, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Zn	29.7- 99.9	This study

*AC= Activated carbon; GAC=Granular activated carbon

4.5 Chapter Summary

Overall, based on results and discussion, all the findings of this study were significantly discussed. Initially, this chapter presented the optimum condition obtained for the synthesis and further discussed the model validation through kinetic modeling followed by the characteristics of the optimum GAC-NiFe₃O₄. The following subchapter includes the results and discussion of the second objective of the study regarding the development of cell immobilization using GAC-NiFe₃O₄. The effect of inoculum ratio to GAC-NiFe₃O₄, comparative study of suspended and attached systems, optimization of initial sugar loading using the Monod Model, and microbial community analysis were included in this subchapter. The next subchapter presented the results and discussion on optimizing process parameters for biohydrogen production. This subchapter includes optimizing process parameters of pH, temperature and agitation, SLR, and HRT for biohydrogen production. Consequently, the final subchapter presented the results and discussion on the biohydrogen from POME utilizing the GAC-NiFe₃O₄. Under this subchapter, the characterization of POME, the effect of TC in biohydrogen production, microbial community analysis, and heavy metal removal potential were further presented and discussed.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The first objective was successfully achieved. NPs embedded into GAC derived from coconut shells were effectively synthesized to function as microbial support carriers for biohydrogen production. The results showed that GAC-NiFe₃O₄ has a 65.49% higher HPR under a thermophilic dark fermentation system. From the optimization of the synthesized condition, GAC-O was identified as the preferred GAC with Ni: Fe of 0.53 and NPs: GAC of 0.02, with ANOVA predicted HPR of 21.21 mL H₂/L.h. Upon experimental validation, 20.33 mL H₂/L.h was obtained with a 4.16% deviation from the predicted value. The maximum HY attained in the study was 0.91 mol H₂/mol sugar consumed. Incorporating NPs as an addition in biohydrogen production proved to have significant advantages, providing a more environmentally sustainable approach.

GAC-NiFe₃O₄ has been successfully utilized to enhance biohydrogen production, significantly reducing production time from 66 to 26 days and increasing HY by 57.30%. The highest HY of 1.64 ± 0.04 mol H₂/mol sugar consumed was achieved with a 1:1 inoculum to GAC-NiFe₃O₄ ratio. The MONOD model identified an optimal initial sugar concentration of 20 g/L, with a maximum specific growth rate of 2.05 h⁻¹, a specific growth rate of 1.98 h⁻¹, and a cell growth saturation coefficient of 6.96 g/L. The dominant microbial species was *Thermoanaerobacterium* (98.5%). These findings confirm that GAC-NiFe₃O₄ enhances biohydrogen production by reducing

production time and increasing yield, with optimal conditions identified for further process optimization.

The study also successfully identified optimal conditions for maximizing biohydrogen production. An initial pH of 6.0 yielded the highest HY at 2.66 ± 0.09 mol H₂/mol sugar consumed, 84.21% higher than control. The optimal temperature of 60°C resulted in an HY of 2.75 ± 0.11 mol H₂/mol sugar consumed, 86.55% higher than the control. Optimal agitation at 120 rpm led to an HY of 3.44 ± 0.54 mol H₂/mol sugar consumed, 78.49% higher than control. The SLR study revealed that a 10% SLR achieved a maximum HY of 2.37 ± 0.07 mol H₂/mol sugar consumed, with 99.9% sugar utilization and 66.67% TSS degradation. An HRT of 3 hours also resulted in a maximum HY of 3.74 ± 0.47 mol H₂/mol sugar consumed, achieving a TVFA/SMP ratio of 0.96 and EPR of 29.19 kJ/L. These findings demonstrate that optimizing key operational parameters significantly enhances biohydrogen production efficiency. The optimized conditions identified in this study can serve as valuable guidelines for future biohydrogen production systems, leading to improved energy recovery and process sustainability.

The characterization of POME was accomplished. The highest HY of 1.77 ± 0.08 mol H₂/mol sugar consumed was achieved at 50% POME, with an optimal TC concentration of 14.64 ± 0.11 g/L and 94.12% sugar utilization. The dominant microbial species identified was *Thermoanaerobacterium* (67.00%). The use of GAC-NiFe₃O₄ in this study also demonstrated a significant potential for heavy metal removal from POME, with removal efficiencies ranging from 29.71% to 99.86%.

These findings highlight the effectiveness of GAC-NiFe₃O₄ in advancing sustainable and efficient wastewater treatment processes.

5.2 Recommendation

The weaknesses and limitations of each approach and technique developed in the research study have indicated the following areas as recommendations for further work. Based on the bacterial identification in this study, the *Thermoanaerobacterium* species was dominant. The isolation and identification of fermentative hydrogen producers with a high yield and high hydrogen production rate are important for developing commercial sustainable biohydrogen production processes. Hence, a single strain of *Thermoanaerobacterium* in this study can enhance hydrogen production, and isolation was therefore recommended. The application of the single strain can be further examined to check the potential in terms of economic and technical feasibility compared to the current study.

This study revealed 3 h as the optimum HRT for biohydrogen production utilizing GAC-NiFe₃O₄. For industrial applications, further improvement in the processes is still required. Control and operation strategies are typically enhanced by physical and chemical elements, which only offer temporary fixes and have a negative impact on the characteristics of microorganisms. Moreover, it is unclear if alterations in the metabolism of a specific microbial population, the microflora population as a whole, or both can be responsible for a shift in the production of products by anaerobic microflora. It was suggested that the next step be to optimize the structure and properties of the microbial community to overcome such instability and achieve high hydrogen-production efficiency.

Applying the GAC-NiFe₃O₄ in biohydrogen production at larger or industrial scales, at which the performance may differ from the laboratory scale, was recommended for further study. Metal nanoparticles' intriguing and occasionally surprising characteristics can be partially attributed to the material's surface features taking precedence over its bulk characteristics. Better production and product control are expected with the implementation, and increased control typically translates into increased safety. Wealth will increase due to nanotechnology, and people will appreciate wealth in the form of safety. This procedure often includes safety rules, public debate, and product testing. Optimistically, nanotechnology will enable the construction of new factories at the same low cost and quick speed, as well as the production of high-quality items at extremely low cost.

Based on this study on the heavy metal removal potential utilizing the GAC-NiFe₃O₄ revealed its high capability. However, further study on the effect of adsorption variables can be examined. Variables such as pH, temperature, contact time, and adsorbent dosage are critical parameters worth studying as rate-controlling steps in removing heavy metals from wastewater. Metal ion adsorption onto GAC-NiFe₃O₄ can be further investigated using the adsorption kinetic models of pseudo-first order, pseudo-second order, and intraparticle diffusion equations. Furthermore, the selectivity and reusability of the material as absorbent can also be investigated as a crucial economic indicator for practical application. The individual heavy metal batch study could be performed to understand the regeneration ability of the GAC-NiFe₃O₄ as an absorbent.

Table 5.1 : Summary of conclusion

Objective	Scope of work	HPR		HY mol/mol	TVFA				SMP (mM)	Biofilm	
		mL/L.h	mmol/L.h		HAc (mM)	HBu (mM)	HLA (mM)	HPa (mM)		VAB (g/L)	TC consumed (%)
1.3	Optimization of GAC-NiFe ₃ O ₄	20.33 ± 0.32	0.99 ± 0.10	0.91 ± 0.01	TVFA = 9.63 ± 0.75 g/L					NIL	NIL
2.1	Optimization of sludge ratio	45.67 ± 1.00	1.87 ± 0.04	1.64 ± 0.04	0.0200	0.0464	0.3235	0.0000	0.4364	7.33 ± 0.78	97.8 ± 0.03
2.2	Immobilization using GAC-NiFe ₃ O ₄	83.94 ± 2.95	3.46 ± 0.12	2.97 ± 0.10	TVFA = 3.60 ± 0.75 g/L					7.40 ± 0.16	99.6 ± 0.02
2.3	Sugar utilization using Monod model	68.96 ± 0.78	2.82 ± 0.03	1.03 ± 0.01	TVFA = 5.14 ± 0.13 g/L					10.67 ± 0.34	99.4 ± 0.01
3.1.1	Optimization of pH	74.96 ± 2.58	3.06 ± 0.11	2.66 ± 0.09						8.80 ± 0.46	99.3 ± 0.02
3.1.2	Optimization of temperature	77.22 ± 3.21	3.15 ± 0.13	2.75 ± 0.11	0.0440	0.0492	0.0484	0.0000	0.3421	7.48 ± 0.11	99.2 ± 0.01
3.1.3	Optimization of agitation	97.01 ± 5.25	3.96 ± 0.62	3.44 ± 0.54						8.78 ± 0.09	98.8 ± 0.04
3.2	Optimization of SLR	67.20 ± 2.02	2.75 ± 0.62	2.37 ± 0.07	0.0322	0.0314	0.0092	0.0000	0.1847	7.72 ± 0.14	99.9 ± 0.04
3.3	Optimization of HRT	104.10 ± 13.01	4.25 ± 0.53	3.74 ± 0.47	0.0086	0.0401	0.0750	0.0005	0.1317	8.13 ± 0.69	99.7 ± 0.01
4.2	Optimization of TC in POME	73.23 ± 3.46	2.99 ± 0.14	1.77 ± 0.08	0.0899	0.0000	0.4441	0.0328	0.6636	29.91 ± 5.80	94.1 ± 0.03

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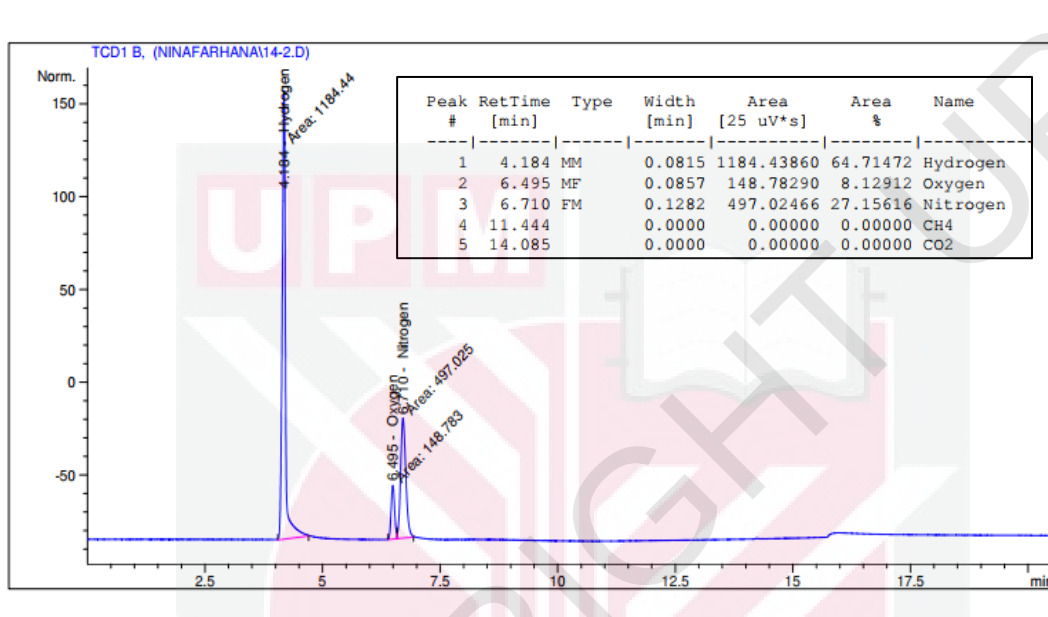
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APPENDICES

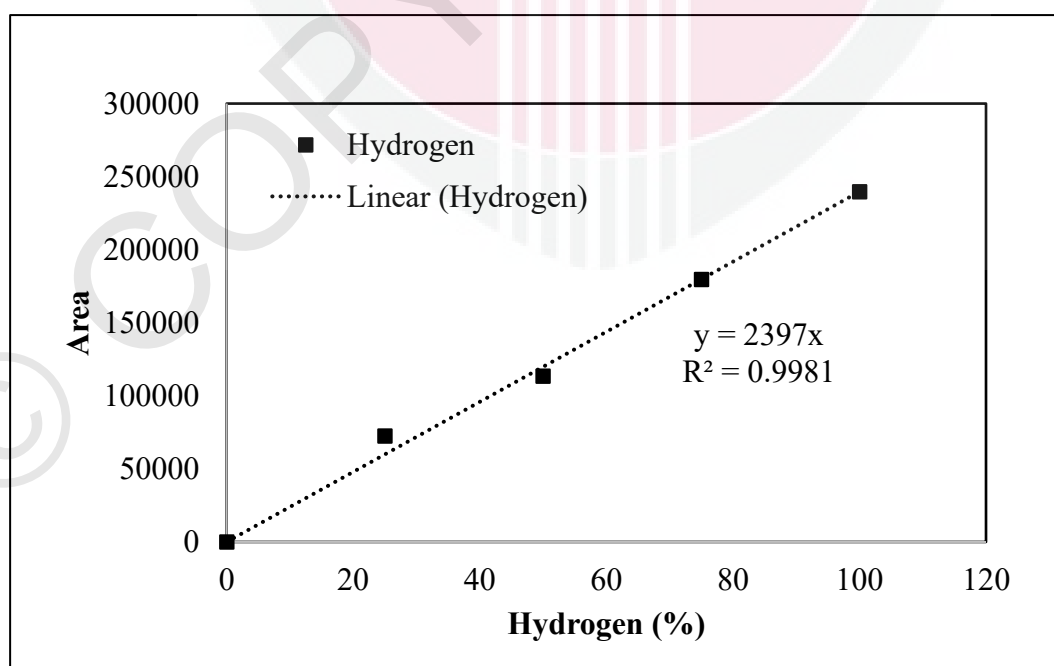
Appendix A

Supporting Details

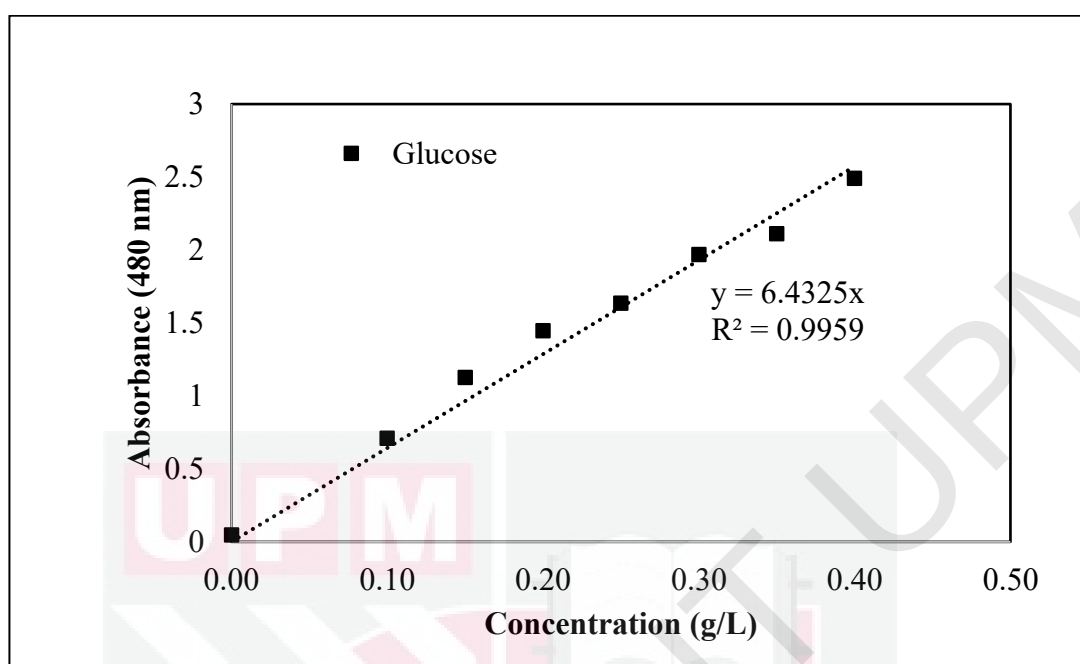
A1 Sample of chromatogram for biogas composition analysis



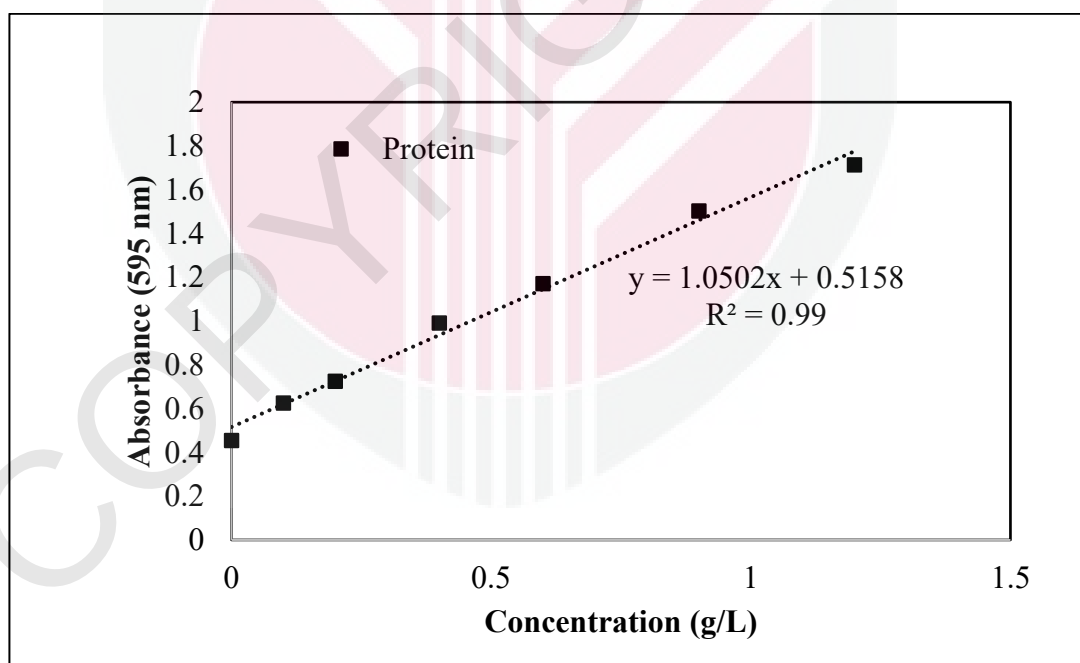
A2 Standard curve hydrogen gas using gas chromatography



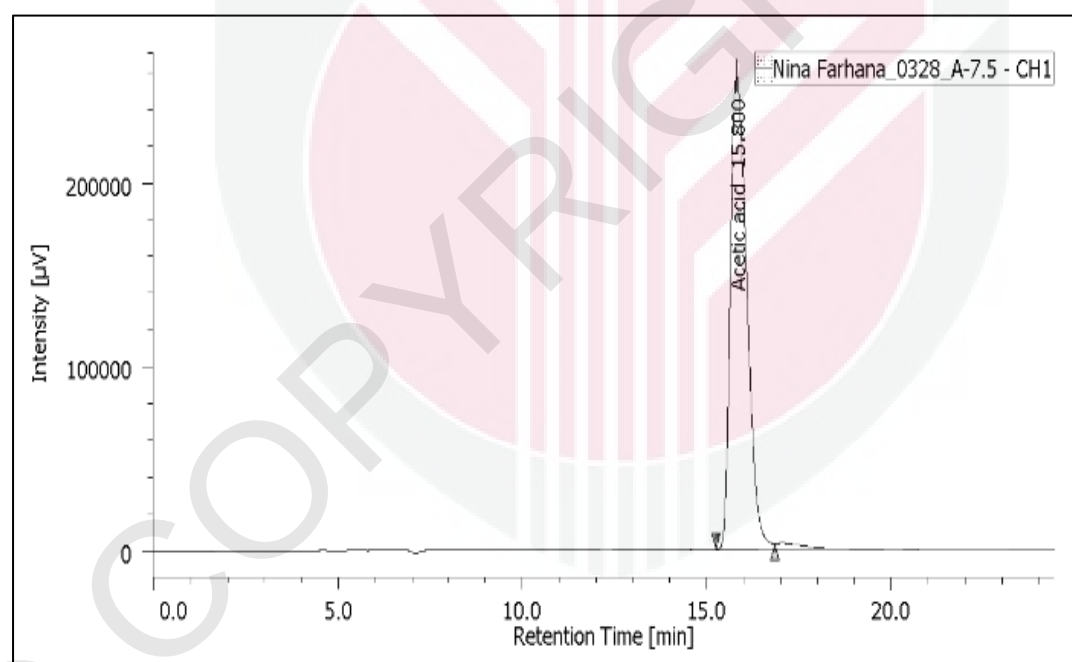
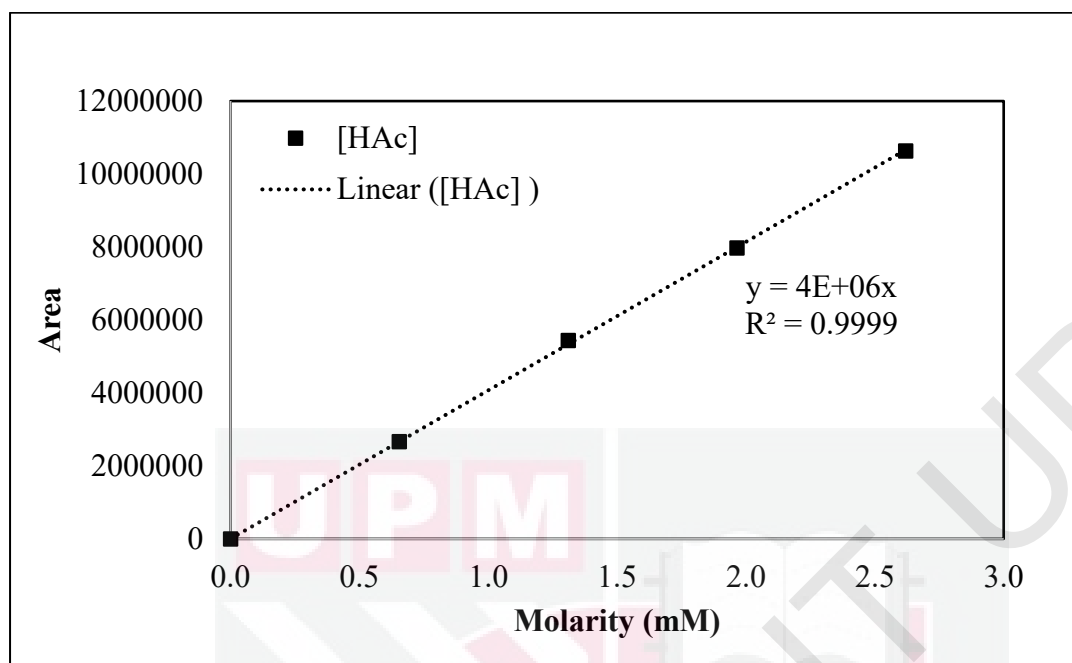
A3 Standard curve total carbohydrate using the phenol-sulphuric method



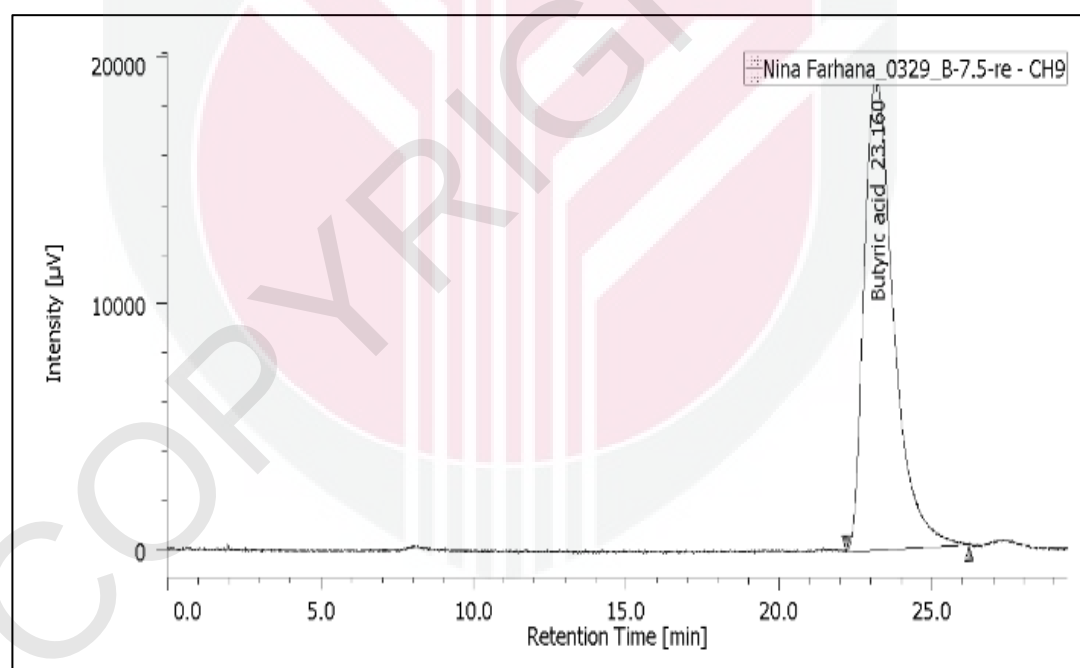
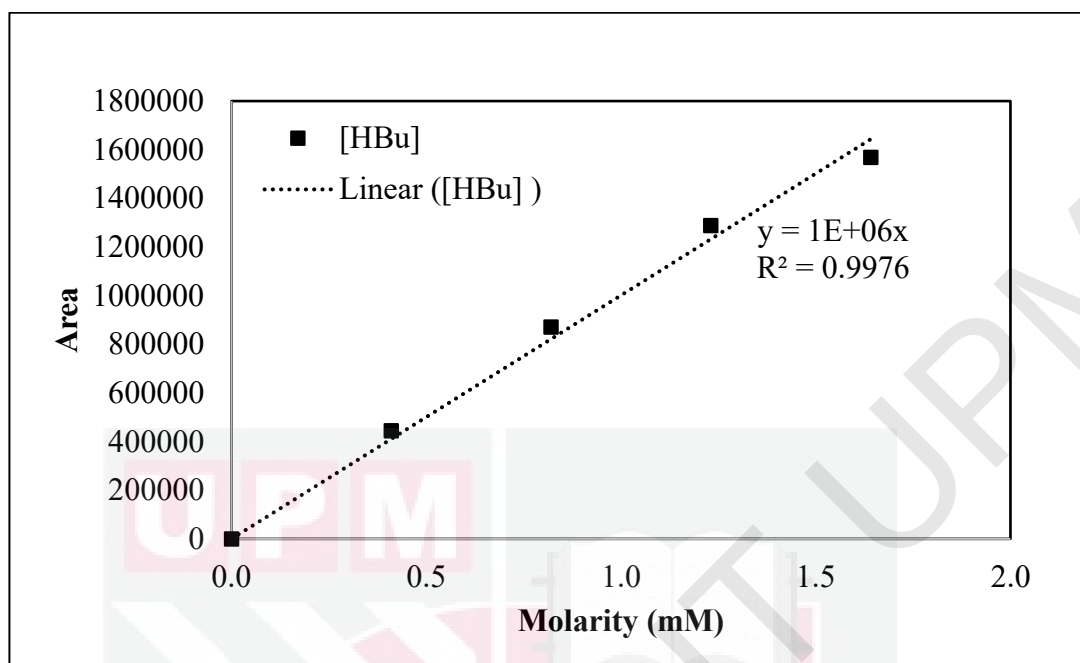
A4 Standard curve protein using the Bradford assay method



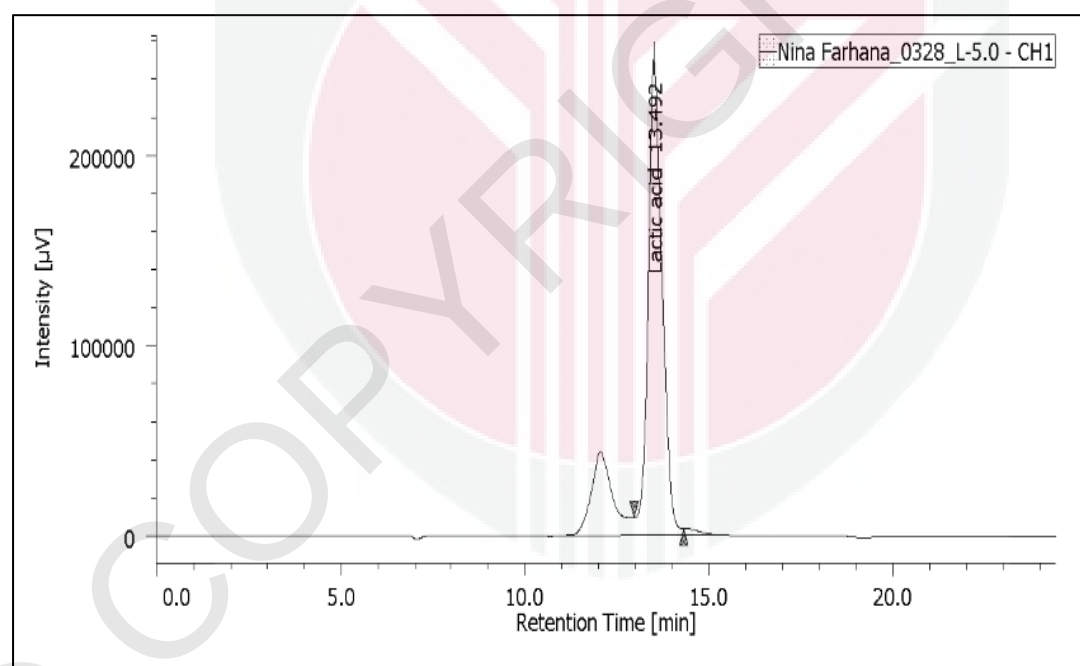
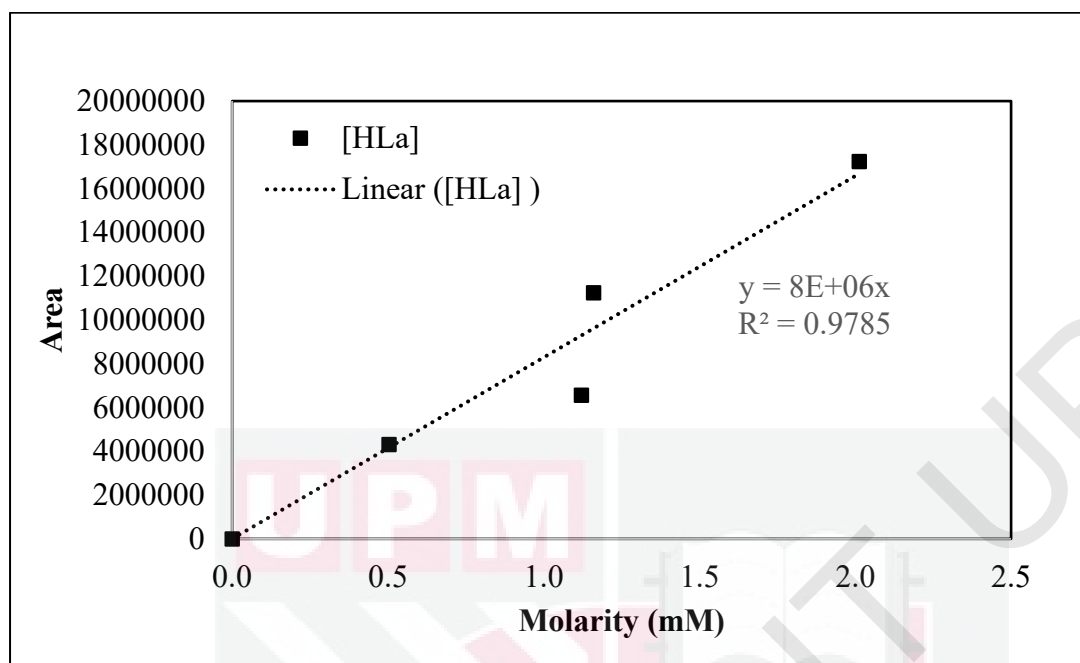
A5 Standard curve and chromatogram for acetic acid using HPLC



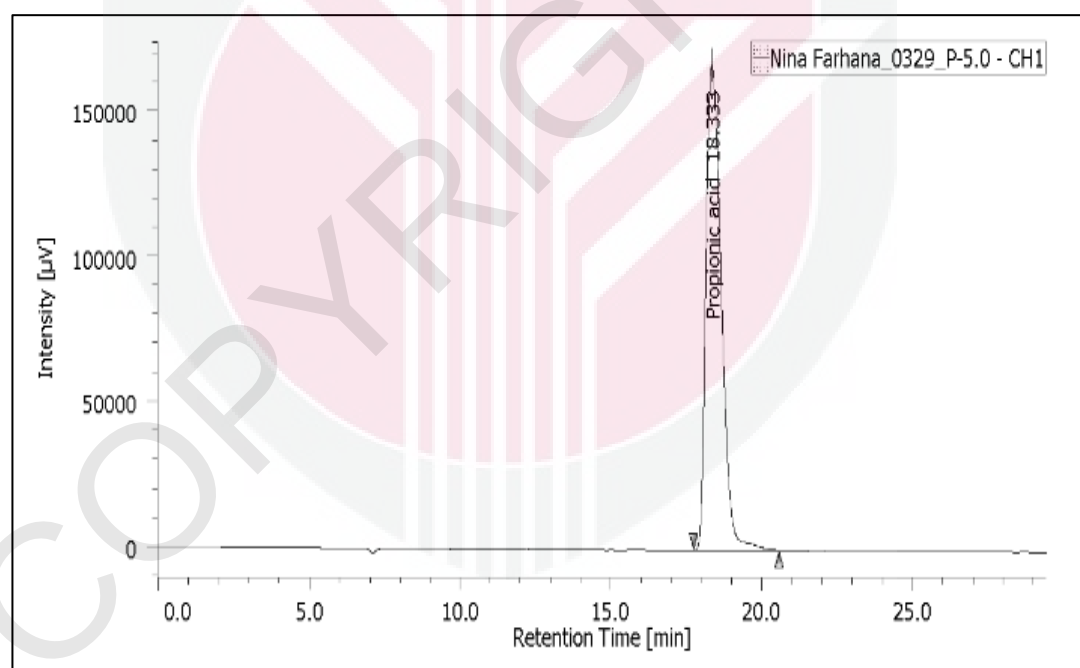
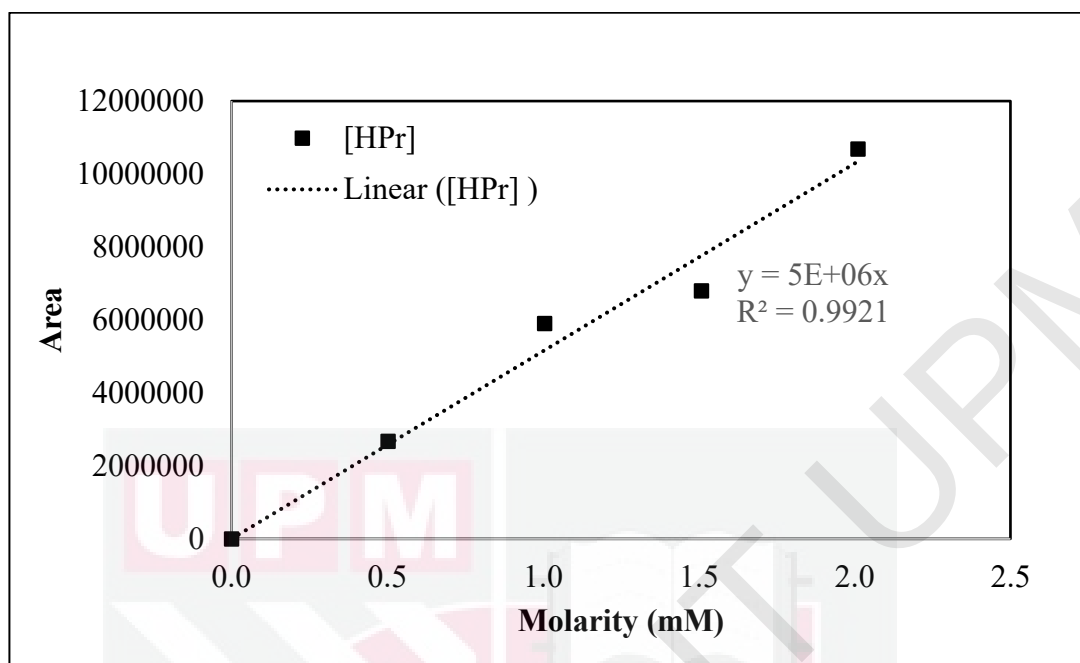
A6 Standard curve and chromatogram for butyric acid using HPLC



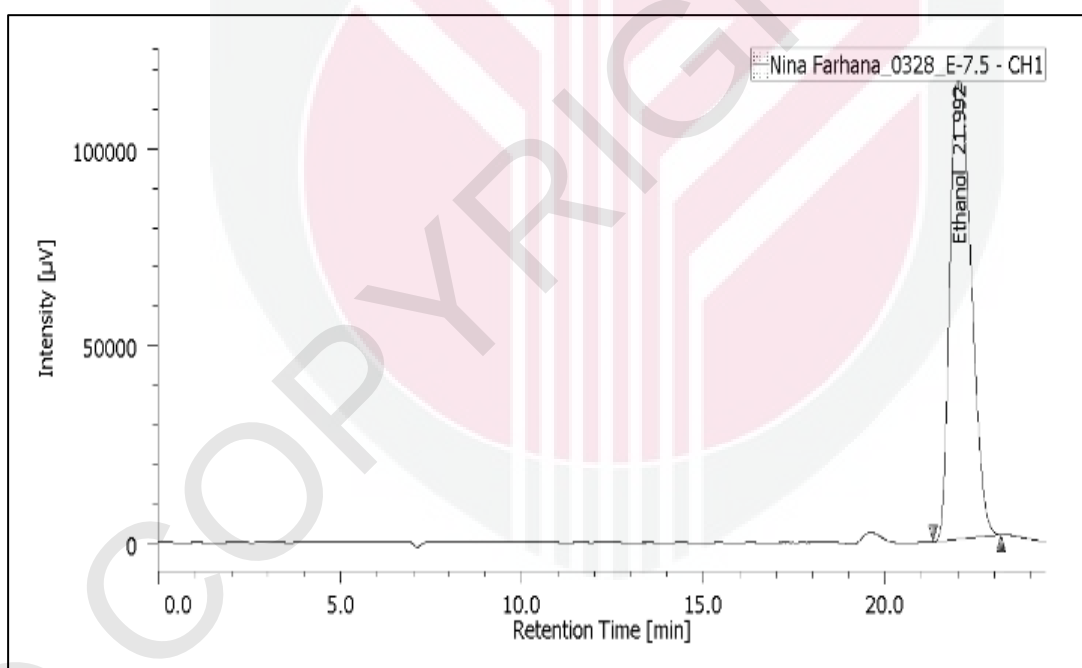
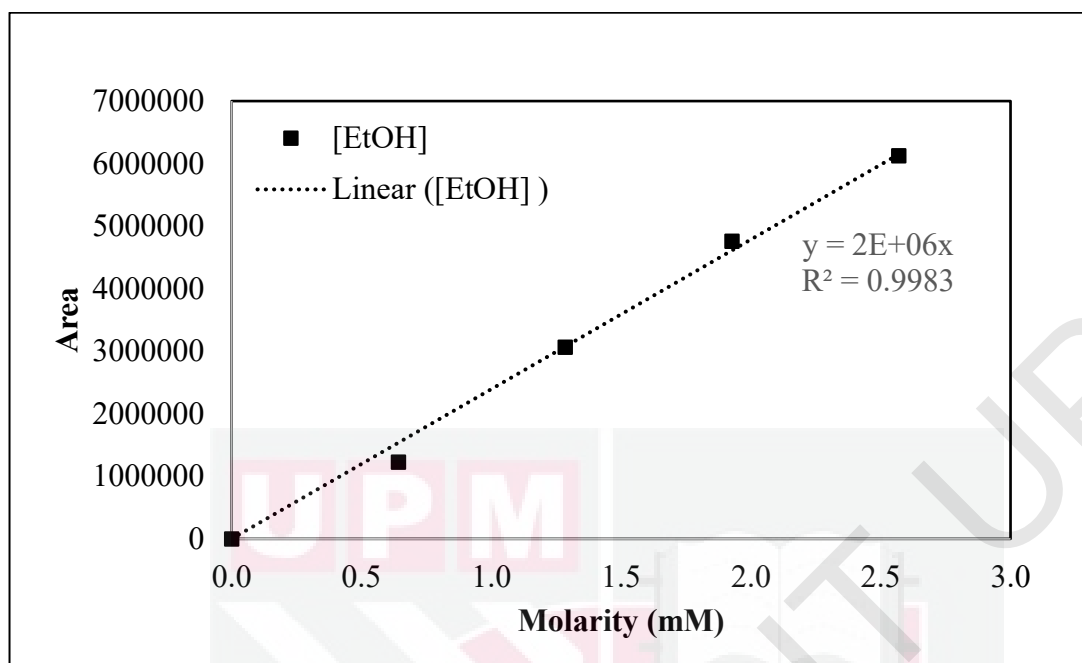
A7 Standard curve and chromatogram for lactic acid using HPLC



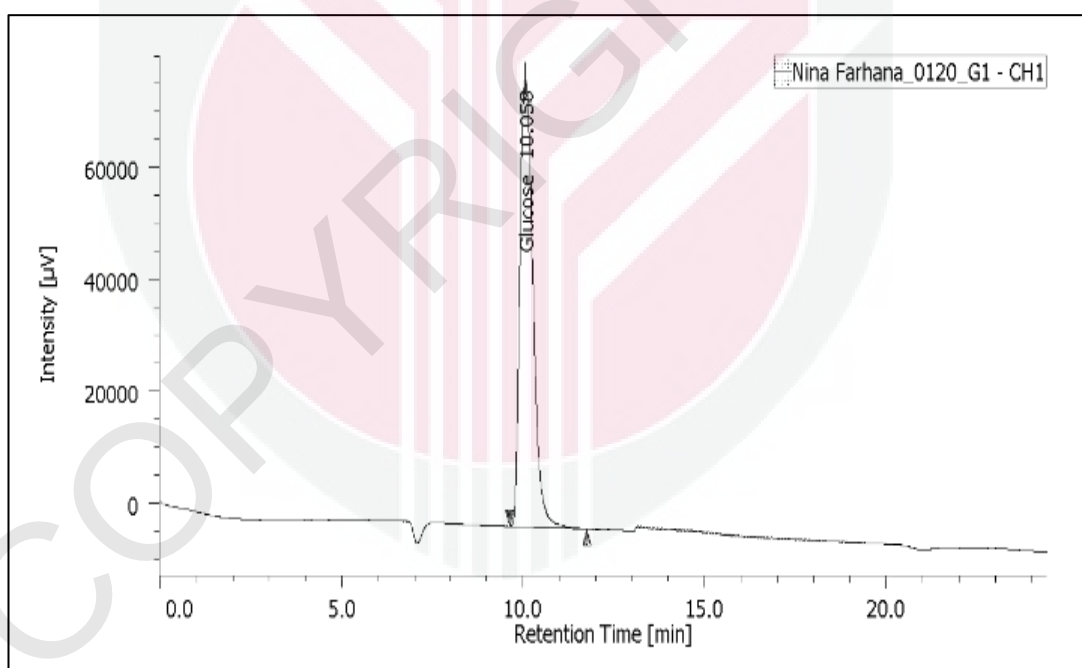
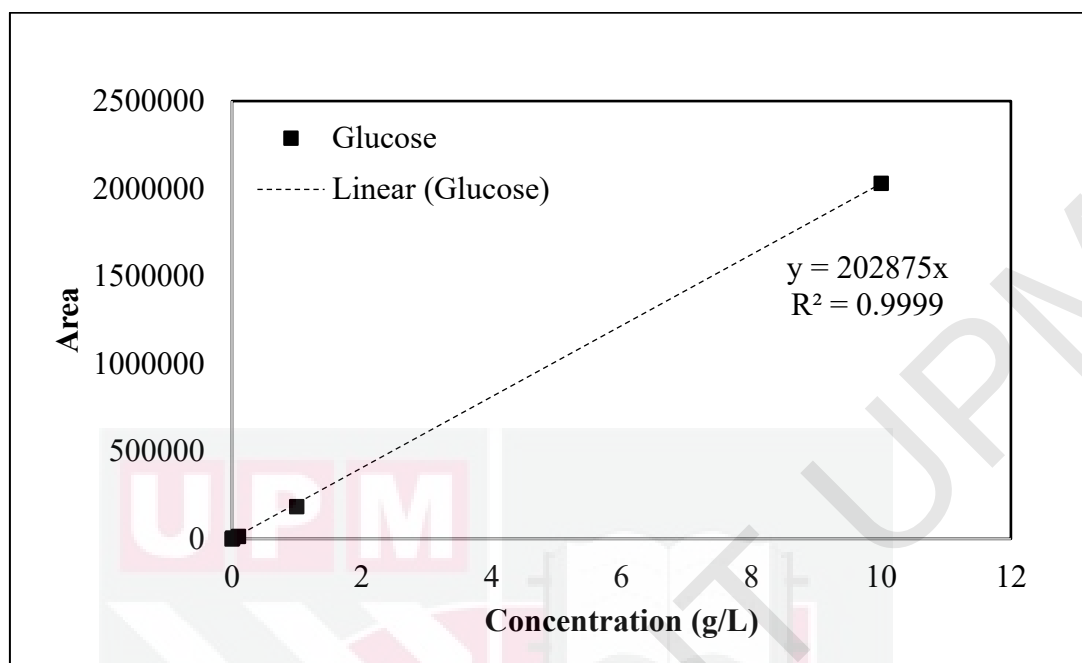
A8 Standard curve and chromatogram for propionic acid using HPLC



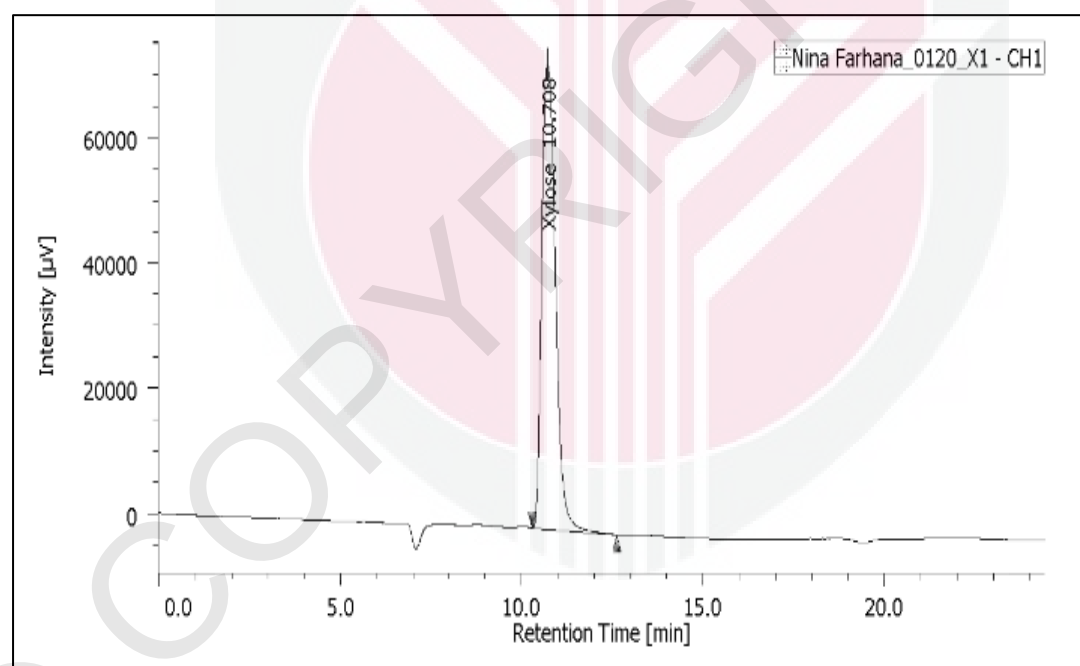
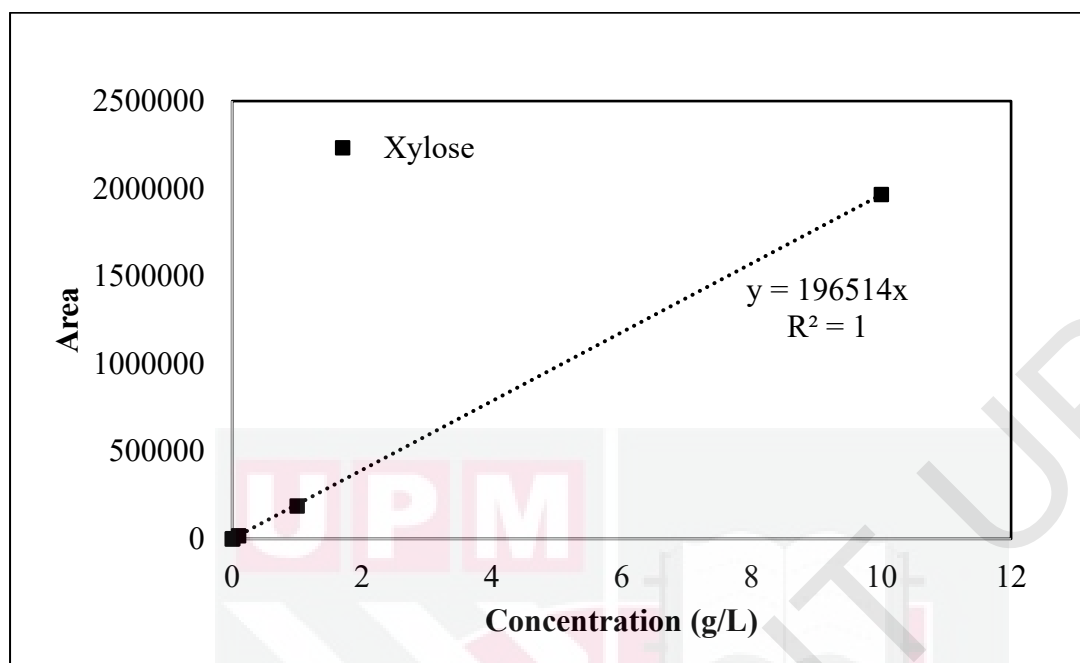
A9 Standard curve and chromatogram for ethanol using HPLC



A10 Standard curve and chromatogram for glucose using HPLC



A11 Standard curve and chromatogram for xylose using HPLC



A12 Statistical analysis of ANOVA

Response 1 HPR					
ANOVA for Response Surface Reduced Fifth Model					
Analysis of variance table [Partial sum of squares - Type III]					
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	871.82	17	51.28	5.99	0.0073 significant
A-Ni:Fe	0.62	1	0.62	0.072	0.7947
B-NPs:GAC	12.03	1	12.03	1.40	0.2700
C-type of GA	1.19	1	1.19	0.14	0.7196
AB	42.80	1	42.80	5.00	0.0558
AC	0.70	1	0.70	0.082	0.7824
BC	105.48	1	105.48	12.32	0.0080
A ²	120.90	1	120.90	14.12	0.0056
B ²	296.81	1	296.81	34.65	0.0004
ABC	37.66	1	37.66	4.40	0.0693
A ² B	1.09	1	1.09	0.13	0.7302
A ² C	10.19	1	10.19	1.19	0.3070
AB ²	8.87	1	8.87	1.04	0.3385
B ² C	2.07	1	2.07	0.24	0.6361
A ² B ²	10.74	1	10.74	1.25	0.2953
A ² BC	37.52	1	37.52	4.38	0.0697
AB ² C	0.13	1	0.13	0.015	0.9062
A ² B ² C	65.01	1	65.01	7.59	0.0249
Pure Error	68.52	8	8.56		
Cor Total	940.34	25			

The Model F-value of 5.99 implies the model is significant. There is only a 0.73% chance that a "Model F-Value" this large could occur due to noise.

Std. Dev.	2.93	R-Squared	0.9271
Mean	15.23	Adj R-Squared	0.7723
C.V. %	19.22	Pred R-Square	N/A
PRESS	N/A	Adeq Precisor	7.716
Case(s) with leverage of 1.0000: Pred R-Squared and PRESS statistic not defined			

Final Equation in Terms of Actual Factors:

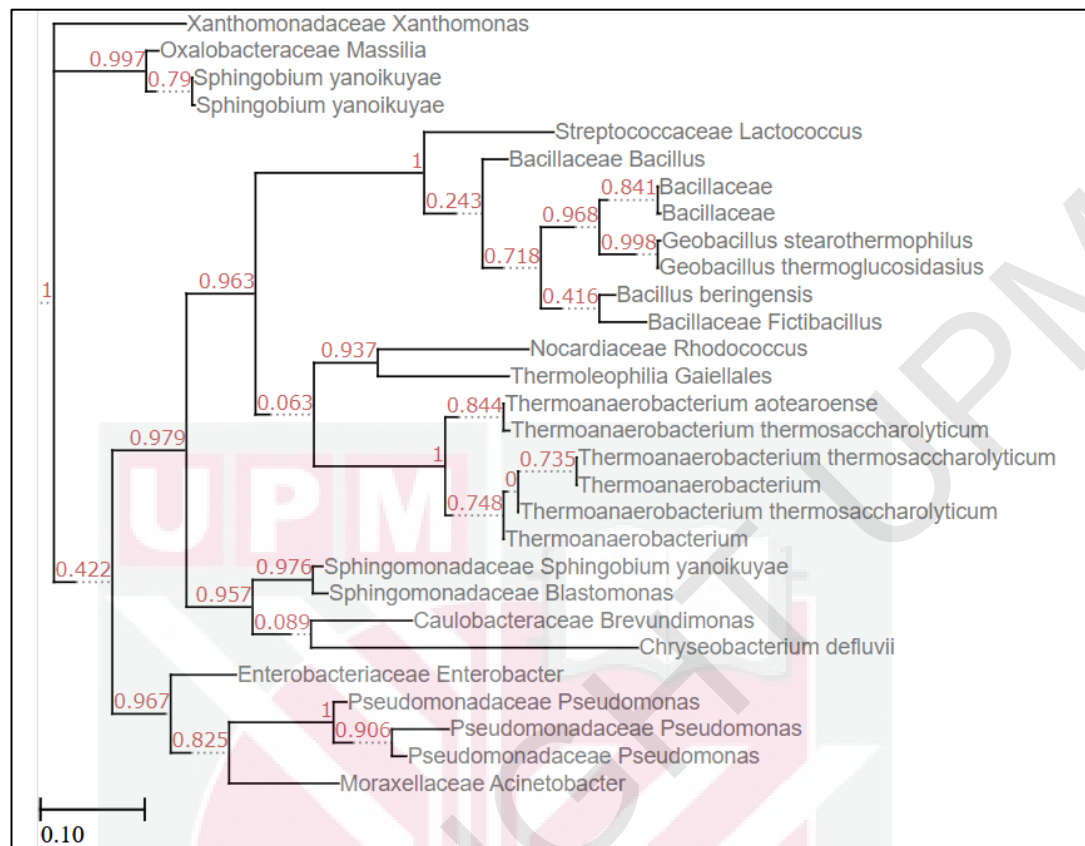
$$\begin{aligned} \text{type of GAC} \quad \text{GAC O} \\ \text{HPR} = & +21.88772 \\ & -125.09686 * \text{Ni:Fe} \\ & -936.08219 * \text{NPs:GAC} \\ & +14856.24503 * \text{Ni:Fe} * \text{NPs:GAC} \\ & +138.24337 * \text{Ni:Fe}^2 \\ & +6955.80010 * \text{NPs:GAC}^2 \\ & -16015.39923 * \text{Ni:Fe}^2 * \text{NPs:GAC} \\ & -2.84547\text{E}+005 * \text{Ni:Fe} * \text{NPs:GAC}^2 \\ & +3.16419\text{E}+005 * \text{Ni:Fe}^2 * \text{NPs:GAC}^2 \end{aligned}$$

$$\begin{aligned} \text{type of GAC} \quad \text{GAC C} \\ \text{HPR} = & -136.59414 \\ & +505.44165 * \text{Ni:Fe} \\ & +13084.25347 * \text{NPs:GAC} \\ & -40522.94564 * \text{Ni:Fe} * \text{NPs:GAC} \\ & -425.23840 * \text{Ni:Fe}^2 \\ & -2.85187\text{E}+005 * \text{NPs:GAC}^2 \\ & +34731.05440 * \text{Ni:Fe}^2 * \text{NPs:GAC} \\ & +8.47873\text{E}+005 * \text{Ni:Fe} * \text{NPs:GAC}^2 \\ & -7.49754\text{E}+005 * \text{Ni:Fe}^2 * \text{NPs:GAC}^2 \end{aligned}$$

Final Equation in Terms of Coded Factors:

$$\begin{aligned} \text{HPR} = & +20.87 \\ & +0.28 * A \\ & -1.23 * B \\ & -0.34 * C \\ & -2.31 * A * B \\ & +0.30 * A * C \\ & -3.63 * B * C \\ & -3.25 * A^2 \\ & -5.10 * B^2 \\ & -2.17 * A * B * C \\ & +0.52 * A^2 * B \\ & +0.94 * A^2 * C \\ & +1.49 * A * B^2 \\ & +0.43 * B^2 * C \\ & -1.64 * A^2 * B^2 \\ & +3.06 * A^2 * B * C \\ & +0.18 * A * B^2 * C \\ & -4.03 * A^2 * B^2 * C \end{aligned}$$

A13 Phylogenetic tree diagram from analysis of microbial community



Appendix B

Procedures

B1 pH

pH was measured directly in the undiluted sample using a combination electrode. Before each measurement, the electrode was rinsed and wiped with soft tissue paper.

B2 Total suspended solid (TSS) and Volatile Suspended Solid (VSS)

Materials

Glass fiber filter disk, 47 mm @ 70 mm – pre-dry in the oven

Measuring cylinder, 100 mL

Pipette, 10 mL

Analytical balance

Oven – preheated to 103°C to 105°C

Desiccator

Buchner flask and funnel

Vacuum pump

Aluminum weighing dishes/Crucible dish

Methodology

1. The filter disk was dried in the oven at 103°C to 105°C for 1 hour, cooled in a desiccator, and weighed.
2. The filtering apparatus was assembled, and suction began. The filter was wet, and a small volume of distilled water was used to seat it.
3. A 10 mL water sample (mixed to ensure homogeneity) was pipette onto the center of the filter disk in a Buchner flask using gentle suction (under vacuum).
4. The filter was washed with three successive 10 mL volumes of distilled water, allowing complete drainage between washings, and suction was continued for about 3 minutes after filtration.
5. The filter was carefully removed from the filtration apparatus and transferred to an aluminum weighing /crucible dish as support.
6. It was dried for at least 1 hour at 103°C to 105°C in an oven, cooled in a desiccator to balance temperature, and weighed.
7. The drying, cooling, desiccating, and weighing cycle was repeated until a constant weight was obtained.
8. The TSS in the water and wastewater samples was calculated using the following equation:

$$mg \frac{TSS}{L} = \frac{((A - B) \times 1000)}{Sample\ volume, mL}$$

Where;

A = weight of filter + dried residue, mg

B = weight of filter, mg

The VSS is the number of organic substances found in the solid portion of liquid samples after being burned in the furnace at 550°C for 30 minutes. After burning, the organic residue on the sample was put in a desiccator for 2 hours. Then, the weight of the sample was recorded, and the VSS was calculated using:

$$VSS \left(\frac{g}{L} \right) = \frac{(A - B)}{\text{volume of sample added}}$$

Where,

A= mass of filter and residue dried at 105°C (g)

B= mass of filter and residue burned at 550°C (g)

B3 Biological Oxygen Demand (BOD)

Materials

Incubation bottles: 300 mL bottles

BOD incubator

Volumetric flask, 1 L

Beaker, 500 mL

Dissolved oxygen meter

Methodology

Reagents were prepared in advance but discarded if there was any sign of precipitation or biological growth in the stock bottles. Use grade or better reagents for all chemicals and distilled or equivalent water.

a. Phosphate buffer solution

Dissolve 8.5 g potassium dihydrogen phosphate, 21.75 g dipotassium phosphate, 33.4 g sodium phosphate dibasic heptahydrate, and 1.7 g ammonium chloride in about 500 mL distilled water and dilute to 1 L. The pH should be 7.2 without further adjustment.

b. Magnesium sulfate solution

Dissolve 22.5 g magnesium sulfate heptahydrate in distilled water and dilute to 1L.

c. Calcium chloride solution

Dissolve 27.5 g calcium chloride in distilled water and dilute to 1 L.

d. Ferric chloride solution

Dissolve 0.25 g ferric chloride hexahydrate in distilled water and dilute to 1 L.

e. Acid and alkali solutions, 1 N for neutralizing caustic or acidic waste samples.

i. Acid- Add 28 mL concentrated sulfuric acid to distilled water slowly and while stirring. Dilute to 1 L.

ii. Alkali- Dissolve 40 g sodium hydroxide in distilled water. Dilute to 1 L.

1. *Preparation of dilution water:* 1 mL of phosphate buffer, magnesium sulfate, calcium chloride, and ferric chloride solution was added into 1 L volumetric flask. Add distilled water to 1 L.
2. A 10 mL wastewater sample was put into a 500 mL beaker.
3. Dilution water up to 300 mL was added into the same beaker.
4. pH value was adjusted to 6.5 to 7.5 by adding acid/alkali.
5. A 300 mL water dilution was prepared as the control in another 500 mL beaker.
6. All prepared samples and control were put in 300 mL incubation bottles each.
7. The concentration of dissolved oxygen (DO) for each sample was measured and recorded using a dissolved oxygen meter.
8. Water was added to the flared mouth of the bottle and covered with aluminum foil.
9. All the bottles were put in a BOD Incubator for five days. The temperature was set at 20°C.
10. The final DO value was measured after five days.
11. BOD₅ was calculated according to the formula:

$$\text{BOD}_5, \text{ mg/L} = (D_1 - D_2) / P$$

Where;

D₁ = DO value in the initial sample

D₂ = DO value in the final sample

P = Decimal volumetric fraction of sample used

Or;

$\text{BOD}_5, \text{ mg/L} = (D_1 - D_2) \times \text{Dilution factor}$

Dilution factor = Bottle volume (300 mL) / Sample volume (mL)

B4 Chemical Oxygen Demand (COD)

Materials

COD Digestion Reactor

Spectrophotometer, HACH DR/5000

COD Digestion Reagent Vial LR @ HR

COD rack

Volumetric pipette, 2 mL

Paper towel/Tissue

Methodology

1. A 100 mL sample was homogenized for 30 seconds in a blender.
*For samples containing large amounts of solids, increase the homogenization time.
2. For the 200-15,000 mg/L range or to improve the accuracy and reproducibility of the other ranges, the homogenized sample was poured into a 250 mL beaker and gently stirred with a magnetic stir plate.
*If the sample does not contain suspended solids, repeat steps 1 and 2.
3. The COD Reactor was turned on and preheated to 150°C. The safety shield was placed in front of the reactor.

4. The caps were removed from two COD Digestion Reagent Vials.
*Be sure to use vials for the appropriate range.
5. One vial was held at a 45-degree angle. A clean volumetric pipette was used to add 2.00 mL of sample to the vial. This was the prepared sample.
6. A second vial was held at a 45-degree angle. A clean volumetric pipette was used to add 2.00 mL de-ionized water to the vial. This was the blank.
7. The vials were capped tightly, rinsed with de-ionized water, and wiped with a clean paper towel.
8. The vials were held by the cap over a sink and inverted gently several times to mix. The vials were placed in the preheated COD Reactor.
*The sample vials will become very hot during mixing.
9. The vials were heated for two hours.
10. The reactor was turned off, and the vials were left to cool to 120°C or less.
11. Each vial was inverted several times while still warm and then placed into a rack to cool to room temperature (~27°C).
12. "Hach Programs" was touched. Program "430 COD LR (Low Range) or 435 COD HR (High Range/High Range Plus)" was touched "Start".
13. The outside of the vials was cleaned with a damp towel followed by a dry one to remove fingerprints or other marks.
14. The 16-mm adapter was installed, and the blank was placed into the adapter.
15. "Zero" was touched, and the display showed 0 mg/L COD.
16. The sample vial was placed into the adapter when the timer beeped. "Read" was touched, and results will appear in mg/L COD.

B5 Oil and Grease

Materials

Oven

Weighing balance

Separating funnel

Crucible dish

Hexane

Methodology

1. A 250 mL sample was prepared and poured into a separating funnel.
2. 20 mL of hexane was added to the separating funnel and was shaken vigorously.
3. The solution was allowed to rest for 10 minutes.
4. The initial weight of the crucible was weighed.
5. The lower layer of water from the separating funnel was discarded using filter paper, and the upper hexane layer was collected and put inside the crucible.
6. The crucible was dried in the oven for 2 hours and then reweighed.
7. The oil and grease were calculated by using the formula given below:

$$\text{Oil and grease } \left(\frac{g}{L} \right) = \frac{(A - B) g \times 1000}{ml \text{ sample}}$$

Where;

A = weight of the initial sample (g)

B = weight of the final sample (g)

B6 Total Volatile Fatty Acid (TVFA)

Materials

Distilled water

98% Sulphuric Acid

0.1 M Sodium Hydroxide

pH meter

Heating mantle

Beaker, 250 mL

Round Bottom flask, 500 mL

Measuring cylinder, 250 mL

Pipette, 5 mL

Buchner flask and funnel

Methodology

1. A 100 mL sample was poured inside a 500 mL round bottom flask.
2. 100 mL of distilled water and 5 mL of concentrated sulphuric acid were added to the same flask.
3. The flask was then connected to a condenser and adapter tube and distilled.
4. Discard the first 15 mL, and then 150 mL distillate was collected in a 250 mL beaker.
5. The solution was titrated with 0.1 M sodium hydroxide solution until the endpoint was pH 8.3.
6. The amount of 0.1 M sodium hydroxide used was recorded, and the TVFA was determined using the following equation:

$$TVFA, \left(\frac{mg}{L} \right) = \frac{Titrant (ml) \times 0.1 M H_2SO_4 \times 60000}{sample\ volume, ml \times f}$$

Where:

Recovery factor, $f = \frac{a}{b}$

a= volatile acid concentration recovered in distillate (mg/L)

b= volatile acid concentration in the standard solution used (mg/L)

B7 Ammoniacal Nitrogen (AN)

Materials

Borate buffer solution

Sulphuric acid/sodium hydroxide

Boric acid solution

Indicator solution

pH meter

Heating mantle

Beaker, 250 mL
 Conical flask, 250 mL
 Measuring cylinder, 50 mL
 Pipette, 50 mL
 Buchner flask and funnel

Methodology

1. After being taken out from the fridge, the sample was left to reach room temperature ($\sim 27^{\circ}\text{C}$).
2. A 250 mL sample was measured and poured inside a beaker before 12.5 mL of borate buffer solutions was added to the sample.
3. The pH of the solution was adjusted to about 9.5 with either sulphuric acid or sodium hydroxide.
4. The solution was transferred into a round bottom flask and placed on a heating mantle.
5. 25 ml of plain boric acid solution was added into a 250 mL flask, followed by 0.25 mL of mixed indicator solution (a mixture of methylene blue and methyl red dissolved in alcohol), and was mixed thoroughly.
6. The condenser connected the round bottom flask and the flask containing the indicator solution. The tap was opened to allow water to flow inside the condenser.
7. The round bottom flask was heated to start the distillation process, and the vapor of the sample was condensed and run into the end receiver tank (flask with indicator solution).
8. The distillation was continued until the condensate (condensed vapor of the sample flowing into the receiving flask) reached a volume of about 100 mL. So, the total volume in the receiving flask should be $(25+100)$ mL = 125 mL.
9. Steps 2-8 were repeated for blank; the sample was replaced with distilled or deionized water.
10. The heating mantle was switched off, and the tap closed before removing the receiving flask from the distillation unit.
11. Distilled or deionized water was added into the receiving flask up to 250 mL, and the titration step was prepared.
12. The solution was titrated with 0.02 M sulphuric acid solution; the endpoint is the appearance of a pale lavender color.
13. The amount of sulphuric acid used was recorded, and the $\text{NH}_3\text{-N}$ was determined using the following equation:

$$\text{NH}_3, \left(\frac{\text{mg}}{\text{L}} \right) = \frac{(A - B) \times 280}{\text{sample volume, mL}}$$

Where:

A = volume of titrated sulphuric acid for sample (mL)

B = volume of titrated sulphuric acid for blank (mL)

B8 Total Phosphorus (TP)

Materials

COD digestion reactor
Spectrophotometer, HACH DR/5000
Total Phosphorus Test 'N Tube Vial HR
Polyethylene dropper
Molybdovanadate reagent
Potassium Persulfate Powder Pillow
1.54 N sodium hydroxide
Deionized water
Test tube rack
Volumetric pipette, 5 mL
Funnel
Paper towel/tissue

Methodology

1. The COD reactor was turned on and heated to 150°C. A safety shield was placed in front of the reactor.
2. "Hach Programs" was touched. Program "541 P Total HR TNT" was selected. "Start" was touched.
3. A volumetric pipette was used to add 5 mL of deionized water to a Total Phosphorus Test 'N Tube Vial (the blank).
4. A volumetric pipette was used to add 5 mL of sample to a Total Phosphorus Test 'N Tube Vial (the sample).
5. A funnel was used to add the contents of one Potassium Persulfate Powder Pillow for each Phosphonate vial. The vials were capped tightly and shaken to dissolve.
6. The vials were placed in the COD reactor.
7. The timer icon was touched, and then "OK" was touched. A 30-minute heating period began.
8. After the timer beeps, hot vials were carefully removed from the reactor and placed in the test tube rack to allow room temperature (~27°C)
9. A volumetric pipette was used to add 2.0 mL of 1.54 N sodium hydroxide to each vial. The cap was inverted to mix.
10. A polyethylene dropper was used to add 0.5 mL of Molybdovanadate Reagent. Vials were capped and inverted to mix.
11. The timer icon was touched "OK". A 7-minute reaction period will begin. The sample was read within seven to nine minutes after adding the Molybdovanadate Reagent.
12. The vials were wiped with a damp towel, followed by a dry one, to remove fingerprints or other marks.
13. The blank was placed in the cell holder once the timer beeps.
14. "Zero" was touched, and the display showed 0.0 mg/L PO_4^{3-} .
15. The prepared sample was placed into the cell holder, and the results appeared in mg/L PO_4^{3-} .

B9 Total Carbohydrates (TC)

Materials

UV-VIS Spectrophotometer

Cuvette

5% Phenol

98% Sulphuric acid

Methodology

1. The highly concentrated liquid sample was initially diluted with distilled water.
2. 0.5 mL of 5% phenol and 2.5 mL of 98% concentrated sulphuric acid were mixed well into 0.5 mL of the sample.
3. The solution was left to cool down for 30 minutes at room temperature ($\sim 27^{\circ}\text{C}$) for digestion.
4. A 2 cm plastic disposable cuvette must be cleaned and dried before the mixture is transferred.
5. The absorbance was measured by using a UV-VIS Spectrophotometer at 490 nm absorbance and compared with the standard curve for the total carbohydrates value.
6. Record all the readings.

Appendix C

Sampling and Experimental Set-Up

C1 Sampling POME



POME collection port

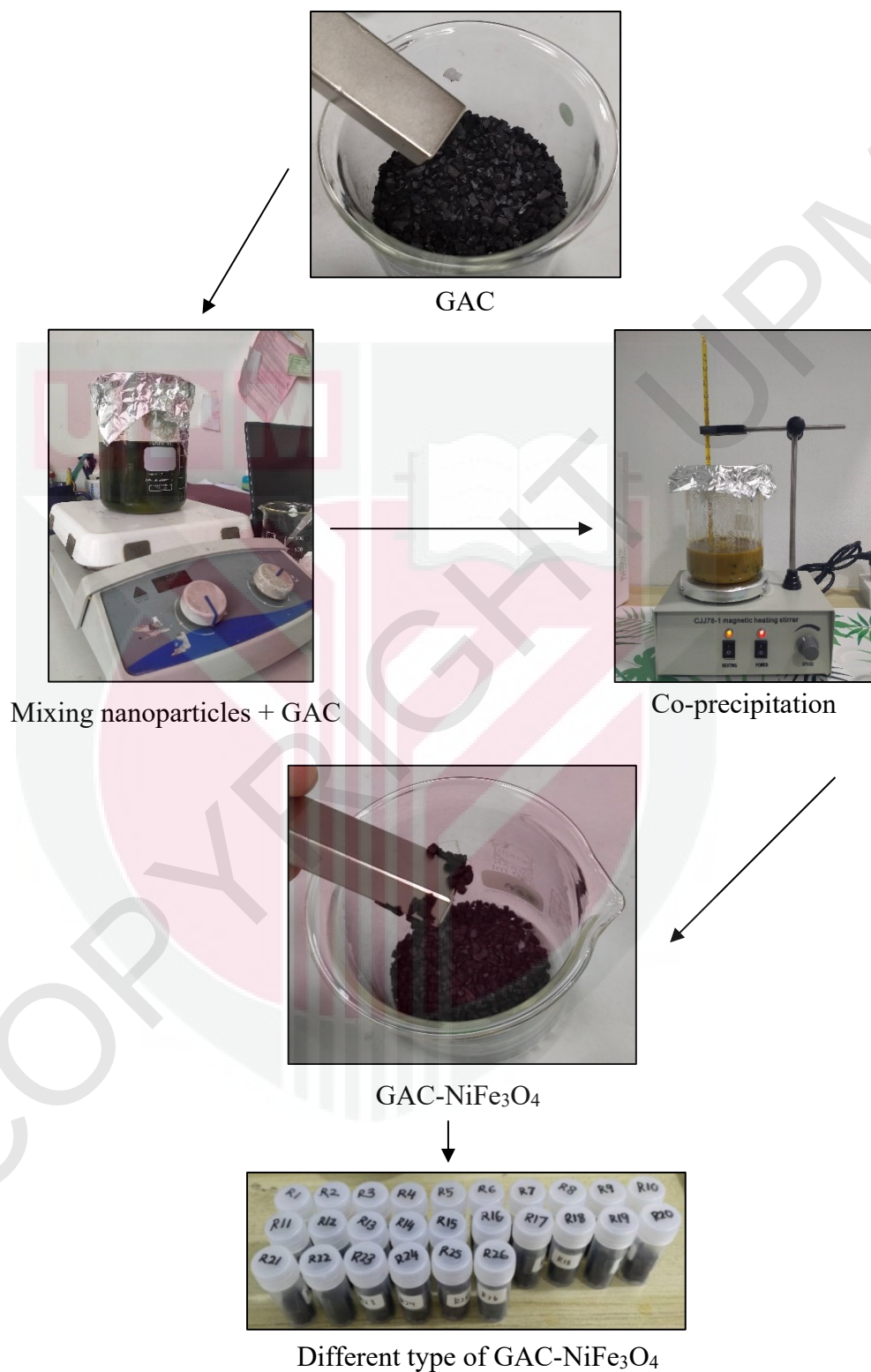
C2 Sampling coconut shell



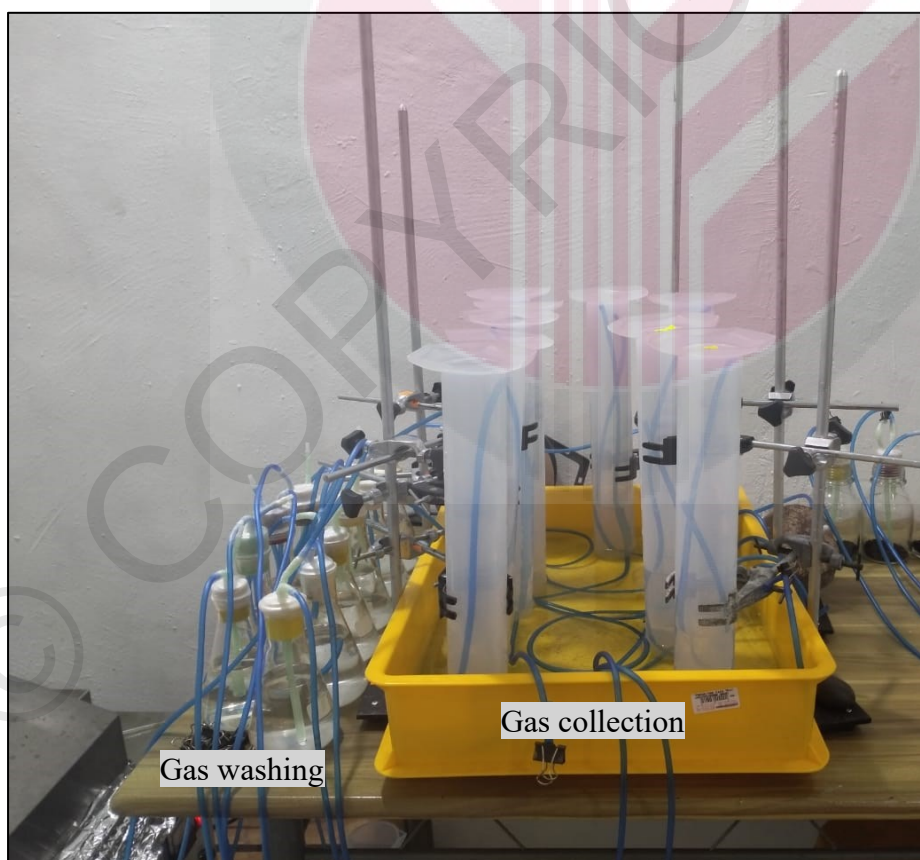
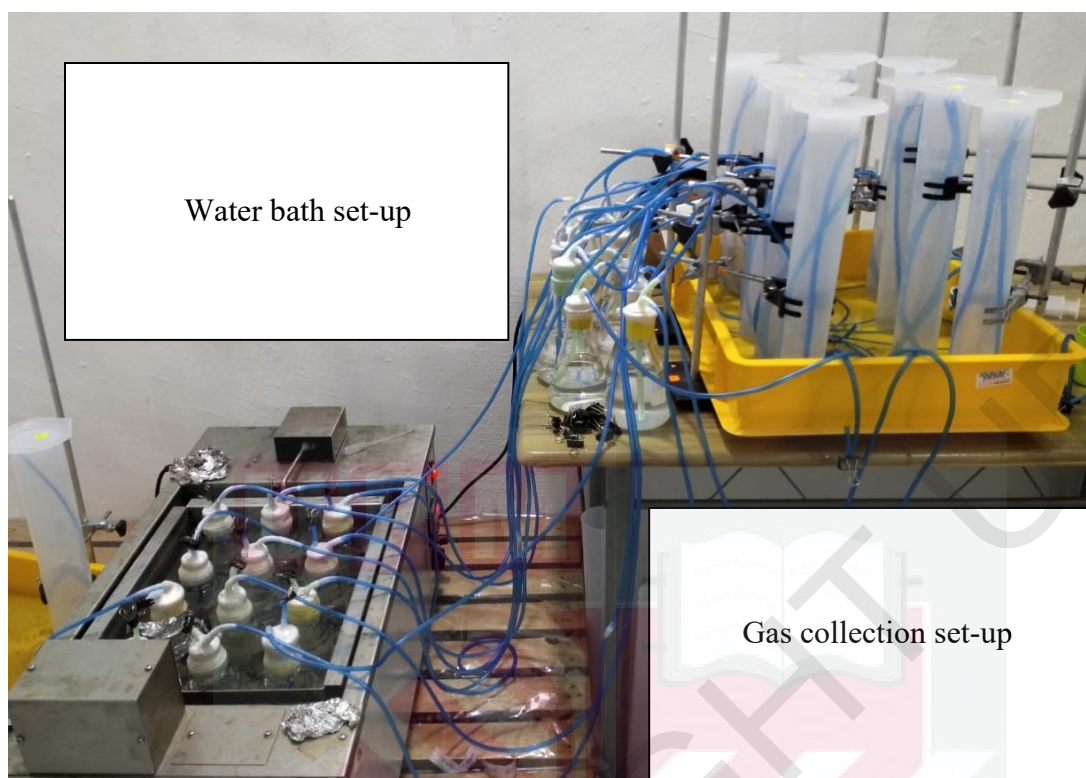
C3 Preparation of granular activated carbon (GAC)



C4 Synthesis of GAC-NiFe₃O₄



C5 Experimental set-up





Different inoculum to GAC-NiFe₃O₄ ratios



Immobilized GAC-NiFe₃O₄

LIST OF PUBLICATIONS

A. Publishes Journals

- N. F. Mohd Jamaludin, N. S. Jamali, L. C. Abdullah, S. Idrus, N. S. Engliman, and P. M. Abdul, "Biohydrogen production with utilisation of magnetite nanoparticles embedded in granular activated carbon from coconut shell," *Int. J. Hydrogen Energy*, vol. 48, no. 31, pp. 11695-11708, 2023.
- N. F. M. Jamaludin, L. C. Abdullah, S. Idrus, N. S. Engliman, J. P. Tan, and N. S. Jamali, "Nickel-iron doped on granular activated carbon for efficient immobilization in biohydrogen production," *Bioresour. Technol.*, vol. 391, p. 129933, 2024.
- N. F. M. Jamaludin, N. S. Engliman, S. F. A. Manaf, S. Idrus, L. C. Abdullah, and N. S. Jamali, "Effect of pH, temperature, and agitation on thermophilic biohydrogen production by using immobilized cells on carbon composites (GAC-NiFe₃O₄)," *Chem. Eng. J.*, vol 485, p. 149980, 2024.

B. Publications in Progress

- N. F. M. Jamaludin, L. C. Abdullah, S. Idrus, N. S. Engliman, and N. S. Jamali, "Enhanced granular activated carbon for simultaneous biohydrogen production and heavy metal remediation utilizing palm oil mill effluent."
- N. F. M. Jamaludin, L. C. Abdullah, S. Idrus, N. S. Engliman, and N. S. Jamali, "Optimization of solid loading rate and hydraulic retention time in thermophilic biohydrogen production utilizing carbon nanocomposites".