
**UNIVERSITI PUTRA MALAYSIA**

# *EVALUATION OF GRANULAR-ACTIVATED CARBON-ATTACHED BIOFILM COATED IN ALGINATE-CHITOSAN FOR BIOHYDROGEN PRODUCTION*

**NUR FARAHANA BT DZUL RASHIDI**

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By

**NUR FARAHANA BT DZUL RASHIDI**

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

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**June 2022**

**Chair : Nur Syakina Bt Jamali, PhD**
**Faculty : Engineering**

Biohydrogen production via an anaerobic dark fermentation process at thermophilic conditions is recognized as an excellent biological method and more cost-effective due to its ability to perform without light energy and oxygen source. At thermophilic, this research aims to investigate the effect of bacterial immobilization on a matrix made of alginate and chitosan co-polymerization towards biohydrogen production. In the first objective, the effect of hydrogen production using granular activated carbon (GAC) as a microbial support carrier in forming GAC-attached biofilm was evaluated based on different amounts of sugar concentration as microbial feedstock. The comparison between initial sugars loading was conducted in a water bath shaker at 120rpm in 800 ml working volume. The acclimatization was operated in a sequencing batch system at a thermophilic temperature of 60ºC and the initial feedstock was set at pH 6. The fermentation process was continuously carried out until a steady state of biogas was obtained and it showed the attached-biofilm system successfully stabilizing hydrogen production after 40 days. The second objective involved the entrapment process in the formation of GAC-attached biofilm using alginate and chitosan as carrier polymers in the form of beads. Bacterial immobilization was done by entrapment of GAC-attached biofilm into 0.5g,1g,2g,3g and 4g of alginate and chitosan respectively (GAC-Alg and GAC-AlgC). The immobilized beads for both alginate and chitosan were conducted in batch fermentation using a synthetic medium at a temperature of 60°C, pH 6.0 and in 200 ml working volume. The entrapment of GAC-attached biofilm provides good support for microorganisms to grow and colonize where high bacterial loads were observed under a scanning electron microscope (SEM). Lastly, in the final objective, this research was conducted to assess the performance of GAC-Alg and GAC-AlgC immobilized beads by using POME as a fermentation medium. It has been observed that the GAC-Alg immobilized beads resulted in stable hydrogen production after 52 hours with a consistent HPR of 1.02 mmol $H_2$/l.h and 1.83 mmol $H_2$/l.h for GAC- AlgC. Overall, this study showed the immobilization of bacteria-entrapped beads promising approach to protect the bacteria colonization during the fermentation, thus retaining and

promoting microbial growth and protecting the microbial from an unfavourable environment**.**

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

**PENILAIAN KARBON BERBUTIR-AKTIF YANG DILAMPIRKAN DENGAN BIOFILM BERSALUT DALAM ALGINATE-CHITOSAN UNTUK PENGHASILAN BIOHIDROGEN**

Oleh

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Pengeluaran biohidrogen melalui proses penapaian gelap anaerobik pada keadaan termofilik diiktiraf sebagai kaedah biologi yang sangat baik dan lebih menjimatkan kos kerana keupayaannya untuk melakukan tanpa tenaga cahaya dan sumber oksigen. Pada termofilik, penyelidikan ini bertujuan untuk menyiasat kesan imobilisasi bakteria pada matriks yang diperbuat daripada pempolimeran bersama alginat dan kitosan terhadap penghasilan biohidrogen. Dalam objektif pertama, kesan penghasilan hidrogen menggunakan karbon teraktif berbutir (GAC) sebagai pembawa sokongan mikrob dalam membentuk biofilem yang dipasangkan GAC dinilai berdasarkan jumlah kepekatan gula yang berbeza sebagai bahan suapan mikrob. Perbandingan antara pemuatan gula telah dijalankan dalam shaker mandi air pada 120rpm dalam 800 ml isipadu kerja. Aklimatisasi dikendalikan dalam kelompok penjujukan – sistem pada suhu termofilik 60 oC dan bahan suapan awal ditetapkan pada pH 6. Proses penapaian dijalankan secara berterusan sehingga keadaan stabil biogas diperolehi. Keputusan menunjukkan sistem biofilm terpasang berjaya menstabilkan pengeluaran hidrogen selepas 40 hari, dengan pengeluaran biogas terkumpul yang lebih tinggi pada pemuatan gula 20 g/L sebanyak 2274.75 ± 411.83 mL. Objektif kedua melibatkan proses pemerangkapan dalam pembentukan biofilm melekat GAC menggunakan alginat (Alg) dan kitosan (C) sebagai polimer pembawa dalam bentuk manik. Imobilisasi bakteria dilakukan dengan memerangkap biofilem GAC yang dipasang ke dalam 0.5g,1g,2g,3g dan 4g alginate (GAC-Alg). Manik tak bergerak GAC-Alg yang dioptimumkan kemudiannya terperangkap dengan 0.5g,1g,2g,3g dan 4g kitosan (GAC-AlgC). Manik tak bergerak untuk kedua-dua alginat dan kitosan telah dijalankan dalam penapaian kelompok menggunakan medium sintetik pada suhu 60°C, pH 6.0 dan dalam isipadu kerja 200 ml. Perangkap biofilem GAC yang dilampirkan memberikan sokongan yang baik untuk mikroorganisma untuk membesar dan menjajah di mana beban bakteria yang tinggi diperhatikan di bawah mikroskop elektron pengimbasan (SEM). Akhir sekali, kajian ini dijalankan untuk menilai prestasi manik tak bergerak GAC-Alg dan GAC-AlgC dengan menggunakan efluen kilang kelapa sawit (POME) sebagai medium penapaian. Telah

diperhatikan bahawa manik tidak bergerak GAC-Alg menghasilkan pengeluaran hidrogen yang stabil selepas 52 jam dengan HPR yang konsisten sebanyak 1.02 mmol $H_2$/l.h dan 1.83 mmol $H_2$/L.h bagi GAC-AlgC. Secara keseluruhan, kajian ini menunjukkan imobilisasi manik yang terperangkap bakteria adalah pendekatan yang menjanjikan untuk melindungi kolonisasi bakteria semasa penapaian, dengan itu mengekalkan dan menggalakkan pertumbuhan mikrob dan melindungi mikrob daripada persekitaran yang tidak baik.

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

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**Declaration by Members of Supervisory Committee**

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AD | Anaerobic digestion |
| ANOVA | Analysis of variance |
| Alg | Alginate |
| AlgC | Alginate-chitosan |
| BOD | Biochemical oxygen demand |
| $CaCl_2$ | Calcium chloride |
| $C_xH_x$ | Hydrocarbon |
| COD | Chemical oxygen demand |
| $CO_x$ | Oxide of carbon |
| EPS | Extracellular polymeric substances |
| FTIR | Fourier transform infrared |
| GAC | Granular activated carbon |
| GC | Gas Chromatography |
| $H_2$ | Hydrogen |
| HID | Helium ionization detection |
| HRT | Hydraulic retention time |
| mg | Miligram |
| mL | mililiter |
| $NO_x$ | Oxide of nitrogen |
| OD | Optical density |
| PAM | Poly acryl amide |
| PGA | Poly glycolic acid |
| PLA | Poly lactic acid |
| POME | Palm oil mill effluent |
| SEM | Scanning electron microscopy |
| $SO_x$ | Oxide of Sulpher |
| TCOD | Total chemical oxygen demand |
| TDC | Thermal detector conductor |
| TS | Total Solid |

| | |
|---|---|
| TSS | Total Suspended Solid |
| TVS | Total Volatile Solids |
| VSS | Volatile suspended solid |

# CHAPTER 1

# INTRODUCTION

## 1.1 Research background

The increasing global warming issue has prompted the development of safe and natural hydrogen production via many approaches, including waste. Production of hydrogen from organic waste as a renewable energy source has gained global attention due to the high – dependency on fossil fuels, which their combustion brings to environmental nuisance such as harmful $CO_x$, $NO_x$, $SO_x$, and $C_xH_x$ gases emission (Dahlgren et al., 2019).

Biohydrogen has become the most popular energy recovery since it produces a clean energy source that emits only water upon combustion with high energy content per unit weight (122 kJ/g) among all other fuels (Silva-Illanes et al., 2017). Biological methods are being investigated to ensure that hydrogen production is safer and more cost-effective than the thermochemical method. Production of biohydrogen through biological pathways is challenging due to the limited conditions that could inhibit the production rate, especially when dealing with the microorganism (Pu et al., 2019).

Among the biological methods of hydrogen production, dark fermentation appears to be one of the environmentally and cost-effectively processes. Dark fermentation is being recognized as an excellent biological process for producing hydrogen due to its ability to operate without light energy or oxygen source (Marques et al., 2019). Dark fermentation is an anaerobic fermentation where the catabolic process of converting organic substrate into hydrogen, carbon dioxide, and other solutes such as acetate and ethanol happens using specific microorganisms. As reported by (Barca et al., 2015), utilizing waste such as carbohydrates found abundantly in biomass or organic wastewater is the most effective substrate for dark fermentative hydrogen production. Carbohydrate-rich waste and wastewater, such as palm oil mill effluent (POME), food manufacturing wastes, cheese whey, sugar factory wastewater, rice winery, and wastewater appear to be suitable feedstocks for the production of hydrogen (Owusu-Agyeman et al., 2021)

Production of hydrogen from organic waste, especially POME, has gained interest over the last few years due to the dual benefits of alternative energy source recovery and controlling environmental pollution. POME poses a great threat to the environment because of its high biochemical and chemical oxygen demands (Zainal et al., 2020). POME is well-known wastewater from the palm oil agro-industry in Malaysia that composes an abundance of cellulosic carbohydrates and available monomeric sugars. The high amount of sugars found in POME made its high potential to be used as a fermentable substrate in dark fermentation. Therefore, this substrate is dedicated as a promising solution for the increasing need for renewable energy (Sekoai et al., 2021).

The practical feasibility of hydrogen production from various synthetic and real wastewaters has been mostly conducted in batch bioreactors. The reactors will be well designed and operated, producing continuous biogas generation under steady-state conditions.

Immobilization technology has been recognized to increase hydrogen production by providing a favourable environment and good support for the microbial cells during the fermentation process. Cells' immobilization also offers high substrate conversion within a shorter time by reducing the lag phase. Entrapment is part of immobilization methods used widely to improve enzyme or microbial cell productivity. The entrapment of hydrogen-producing bacteria using alginate beads as a carrier is not only requires low cost but also reported can improve hydrogen production (Canbay et al., 2018). In addition, chitosan as a second layer of the beads can also enhance the beads' stability due to polymer and interaction with each other by hydrogen and electrostatic bonding (Krunić et al., 2016). Therefore, the efficiency of alginate beads as a carrier will be increased by coating them with chitosan as a second layer. The covalent cross-linking between these two polymers can improve carriers' mechanical strength and stability, thus reducing the potential of cell leakage from the support-carrier.

### 1.2 Problem statement

Malaysia has been reported to produce almost 80 million dry tonnes of solid biomass. The number is expected will be increased to 100 million by 2020 since the industry has gone through an excellent development (Nor et al., 2015). The continuous development and production contribute to the increase of POME discharge, which is identified as the most significant pollutant impact from industry into rivers in Malaysia. Due to the presence of untreated palm oil residue, raw POME consists of a high value of degradable organic matter. The colloidal suspension of POME consists of 95-96% water, 0.6-0.7% oil, and 4-5% total solids which have a high concentration of organic matter, oil, grease, and suspended solids (Zainal et al., 2020).

Biological treatment with the aerobic, anaerobic or facultative process is the most suitable method to degrade or treat POME (Khemkhao et al., 2016). This is because biological treatment requires less energy demand, does not liberate foul odor, can minimize sludge accumulation and can produce biohydrogen by anaerobes under fermentation and digestion processes. The plenty of sugars found in POME also made it high potential to be used as a fermentable substrate in dark fermentation. Therefore, research on POME as a suitable fermentation feedstock to produce biohydrogen has grown rapidly and concurrently can manage the waste via anaerobic fermentation.

Production of biohydrogen via a suspended culture system has gained attention due to its ability of higher hydrogen productivity rate and hydrogen yield (Mahmod et al., 2017). However, according to a previous study on hydrogen production, lower microbial cell density has been the disadvantage (Canbay et al., 2018; Pugazhendhi et al., 2019; Zhu et al., 2018). Washout of the cells is often occurred using free cells (suspended culture system), making the microbial population difficult to be retained in the

bioreactor. Therefore, the biological immobilization approach becomes a special interest to researchers seeking to enhance cell density, especially through the cell attachment method in developing attached-biofilm on the microbial support carrier (Kumar et al., 2016).

Granular activated carbon (GAC) as a support carrier has been thoroughly documented as a support matrix in thermophilic fermentation (Syakina & Jahim, 2016). It has good mechanical strength and inert properties without any additional reaction that might disturb the system. The GAC also has a high surface area and highly porous structure that facilitate sustaining cell viability and colonization density helps to enhance cell density during the fermentation process. The surface area and pores size of the carrier plays an important role in the adsorption mechanism of microbe adhesion and accumulation of the microbe on the GAC surface before further attachment and colonization steps (Idris et al., 2018). Lutpi et al. had reported that the efficiency of hydrogen fermentation is influenced by the amount of biomass retained on the GAC system seems very promising to increase hydrogen production(Lutpi et al., 2016).

Besides, to achieve the highest possible rate of hydrogen production, the excellent fermentation process requires a new idea for immobilization technology to enhance the bacterial population by providing them with a better support carrier and suitable environment for them to grow and colonize and improve the fermentation rates within a more extended period. The innovation of immobilized cells in biohydrogen production is to encounter the problem of cell wash-out phenomenon in a continuous biosystem.

Therefore, the new design of immobilization is an approach to maximizing and maintaining biomass, such that it can work at a higher rate of dilution without biomass washout from the reactor. Entrapment technology has been developed to increase hydrogen production by providing a favorable environment or support for microbial cells during fermentation. The selection of the supporting material is imperative because it affects the overall performance of biohydrogen production.

Polymeric materials are widely used in entrapment methods such as calcium alginate, chitosan, k-carrageenan, poly-acryl amide (PAM) gel, gelatin, and agar (Sekoai et al., 2016). The work by Canbay et al., 2018 reported that alginate is the most extensively studied as a support carrier for immobilization because of its low cost, strong biocompatibility, and resistance to microbial inhibitors (Canbay et al., 2018). Alginate is one of the excellent support materials to make it a practical choice in immobilization. It has been reported that biohydrogen production increased about two times greater when alginate beads were enhanced with activated carbon (Damayanti et al., 2018). Nonetheless, even though alginate beads have been used widely in immobilization, they are reported to still suffer from certain limitations like weak mechanical strength and reduced porosity (Duarte et al., 2013). Therefore, several approaches have been studied to improve the permeability and mechanical stability of alginate matrices, such as incorporating other materials like cellulose, metal, and carbon source.

The attachment of GAC biofilm into alginate (GAC-Alg) immobilized beads alone cannot hold the microbial cells longer due to the cells' detachment and the carrier's low ability to protect the cells from the harsh environment degraded quickly and led to cell leakage. Therefore, the entrapment technique was approached to improve the detachment of the cells. Collagen, carrageen and gelatin are the most common biopolymers found as second layer in the immobilization process (Krunić et al., 2016). However, limitation of significant diffusion and weak enzyme activity might occur during the long–term operation, disturbing the micro-environmental conditions (Basile et al., 2010). A previous study reported that chitosan possesses good mucoadhesion behavior resulting from the cationic properties and free hydroxyl and amino groups, which allow the polymer to interact with each other by hydrogen and electrostatic bonding (Szymańska & Winnicka, 2015). Thus, the additional second layer of chitosan as co-polymerization is needed to improve the stability of the beads.

The work by (Ngah & Fatinathan, 2008) reported the entrapment between chitosan and alginate formed a strong ionic interaction between amino groups of chitosan and carboxyl groups of alginate, thus resulting in an improvement in mechanical properties of the matrix support In other studies, the formation of high cross-linked, porous beads with the better mechanical and chemical stability of support matrix in the buffered medium was produced from the ionotropic gelation of and chitosan, was leading to the low rates of cell leakage even at higher cell loading (Žuža et al., 2011). It was also reported the effectiveness of chitosan coating enables the physical isolation of bacteria from the outer environment and reduces cell detachment during fermentation, besides improving the mechanical strength of alginate beads carrier during storage (Obradović, Krunić, Damnjanović, et al., 2015; Stojkovska et al., 2014). Thus, in this study, the GAC-Alg immobilized beads subsequently need to be entrapped in chitosan (GAC-AlgC), which significantly contributes to preserving carrier strength during fermentation.

### 1.3 Research objectives

This project aims to explore the actions of chitosan–coating alginate beads (GAC-AlgC) on microbial cells behavior on biohydrogen production. Initially, an investigation of microbial behavior by the three different immobilization approaches will be conducted, followed by determining hydrogen productivity. The specific objectives are as follows.

1. To investigate the use of GAC as a microbial support carrier in the formation of GAC-attached biofilm for hydrogen production.
    a. To characterize sugar composition in POME for synthetic medium preparation in immobilization using GAC
    b. To determine the effect of different pH on biofilm development in sequencing batch hydrogen production.
    c. To determine the effect of 10 g/L and 20 g/ L sugar concentration on biofilm development in sequencing batch hydrogen production.

2. To determine the best ratio of GAC-attached biofilm beads using alginate and chitosan as a carrier polymer in producing biohydrogen

a. To characterize the physical properties of different types of alginate and chitosan in the formation of immobilized beads.
b. To examine the microbial performance of GAC-attached biofilm entrapped in alginate (GAC-Alg) in different ratios, immobilize beads for the biohydrogen production
c. To examine the microbial performance of GAC-Alg subsequently entrapped in chitosan (GAC-AlgC) in different ratios immobilize beads for biohydrogen production.

3. To assess the performance of GAC-Alg and GAC-AlgC immobilized beads using palm oil mill effluent (POME) as a fermentation substrate in the production of biohydrogen.
   a. To examine the hydrogen production performance at different ratios of GAC-Alg immobilized beads in POME media fermentation.
   b. To examine the hydrogen production performance at different ratios of GAC-AlgC immobilized beads in POME media fermentation.

## 1.4 Scope and limitations of research

This project was divided into three subdivision objectives with concern to the one preferred product of biohydrogen production via immobilized beads.

In this study, an examination of the GAC-attached biofilm into alginate and chitosan as a carrier polymer via an anaerobic digestion process was investigated. The preliminary study was conducted to find the best microbial environmental conditions that were examined and optimized using GAC as a support carrier. The developing attach biofilm microflora on GAC microbial support was done through immobilization and was conducted in a water bath shaker at 120rpm in 800 ml working volume. The acclimatization was operated in a sequencing batch system at a thermophilic temperature of 60℃ and the initial feedstock was set at pH 6. The next step was the entrapment of GAC attach biofilm into alginate and chitosan. The process was done by entrapment of GAC attached biofilm into the different ratios of alginate and chitosan (GAC-Alg and GAC-AlgC) which is 0.5g,1g, 2g,3g and 4g respectively. The immobilized beads for both alginate and chitosan were conducted in batch fermentation using a synthetic medium at a temperature of 60°C, pH 6.0 and in 200 ml working volume. The effect of GAC attaches biofilm using alginate and chitosan immobilized beads properties on the adhesion and colonization of bacteria through entrapment were characterized in terms of variations in beads sizes and the ratio of concentration polymer. The beads formed were then characterized using scanning electron microscope (SEM) analysis to observe the morphology of cell culture on the carrier surface. The gas samples generated during the fermentation were collected using water displacement method when the biogas amount achieved the stationary phase of microbial growth profile. Biogas production was calculated at standard temperature and pressure (STP) of 273.15 K and 101.325 kPa by converting pressure readings to gas volume in the headspace. The biogas composition is analyzed using gas chromatography (GC). The performance of the beads was further

investigated using complex POME as the fermentation feedstock. This study was conducted to assess the performance of GAC-Alg and GAC-AlgC immobilized beads by using POME as a fermentation medium. It is expected that the immobilization study of these co-polymerization techniques will provide new insight into biohydrogen production.

There are some notable limitations in this research. There is a lack of previous studies on the co-polymerization of GAC with other natural polymers. Therefore, the literature that aims to improve immobilized beads performance using activated carbon towards hydrogen production is referred to. The development of the immobilized beads was performed in variations in the size of beads and cannot get the same size for each ratio of polymer. Generally, the differences in the size of beads were caused by the gravity and surface tension imbalance when the beads dropped from the syringe. The bead's shape formation also was affected by the viscosity of alginate and chitosan concentration and the distance of the dropper to the gel solution. The temperature for the fermentation is under thermophilic conditions (60°C). This is because thermophilic bacteria presented in POME sludge (inoculum) had an optimal growth temperature of 60°C, thus making it more favorable for biohydrogen production. Other than that, the method used to collect data is restricted. The biogas produced during the fermentation exposed to leakage into the environment due to the inadequate design of the fermenter. Hence, the hydrogen composition generated from the fermentation cannot be calculated accurately. Thus, it is essential to ensure the experimental setup is done properly to prevent biogas losses through leakage. However, this condition was minimized by modifying the bioreactor with a tightly covered to prevent the gas flow from the fermenter.

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