BIOHYDROGEN PRODUCTION BY DARK FERMENTATION OF ACID HYDROLYZED SAGO WASTEWATER USING *Enterobacter aerogenes*

BY

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ABSTRACT

As the global fuel hike is inevitable, it is essential to find other options which can substitute fossil fuel. Hydrogen appears as the promising energy alternative which not only meets the demand of energy but also results in the clean environment. However, the current production of hydrogen is releasing much energy and pollution. Therefore, biological approach to produce hydrogen by using microorganism and waste becomes prominent. In the development of biohydrogen research, there is still limited number of records on utilizing sago wastewater as a source of energy. Thus, the main aim of this study is to produce biohydrogen from sago wastewater using *Enterobacter aerogenes (E. aerogenes)*. In this lab scale study, several sequential methods were used in evaluating the optimization process which was included in the research objectives. Firstly, 10 physico-chemical factors (sago wastewater concentration, temperature, pH, inoculum size, malt extract, yeast extract, iron, magnesium, copper, and nitrogen sparging) affecting biohydrogen production was selected in the Plackett-Burman design. Secondly, the factors were optimized using OFAT method followed by FCCCD under RSM. Thirdly, the kinetics parameters of *E. aerogenes* cell growth, substrate uptake, and biohydrogen production were determined. It was found that early screening using Plackett-Burman design, yeast extract (positive effect), temperature (negative effect) and inoculum size (negative effect) had the most profound effect to the biohydrogen production. The three factors were then subjected to OFAT to find the possible optimum range. It was discovered from OFAT that the inoculum size was already at the optimum condition at 5%. Meanwhile, the possible optimum range for yeast extract concentration and temperature were nearly at 3 g/L and 30ºC, respectively, which were then applied as the middle points in the RSM. A total of 11 runs were generated in RSM. The highest hydrogen production was obtained from Run 7 (hydrogen concentration and yield were 629.80 µmol/L and 12.13 mmol H_2/mol glucose, respectively). The statistical analysis of ANOVA revealed that the linear and quadratic term of yeast extract as well as the quadratic term of temperature were indeed significant to the biohydrogen production. After the whole optimization processes, the maximum hydrogen concentration and yield were recorded to be 630.67 µmol/L and 7.42 mmol H_2/mol glucose, respectively, which were obtained under the optimum condition (inoculum size 5%, yeast extract concentration 4.8 g/L, and temperature 31ºC). The kinetic study was then conducted under the optimum condition using 1 L of Schott bottle. It was found that the exponential phase of *E. aerogenes* along with biohydrogen production occurred between the 9^{th} and 30^{th} hour of fermentation period. It was then concluded that biohydrogen produced by *E. aerogenes* is a growth-associated product. Several kinetic parameters that were successfully derived from Monod model were Y_{xs} (0.87 g/g), Y_{ps} (0.003 mol/mol), Y_{px} (0.029 g/g), μ (0.12 h^{-1}), t_d (6 h) and q_p (0.0035 $hour^{-1}$). Moreover, a cumulative hydrogen production curve fitted by the modified Gompertz equation suggested that H_{max}, R_{max}, and λ from this study were 15.10 mL, 2.18 mL/h, and 9.84 h, respectively. Although biohydrogen was successfully produced from sago wastewater, the improvement of the yield for further investigation is still needed due to the limitations of this study, especially on improvement of the strain, pre-treatment method of the waste, effect of the by-products, and scale up process.

ملخص البحث

بما أن ارتفاع الوقود العالمي أمر لا مفر منه فإنّ من الضروري إيجاد خياراتٍ أخرى يمكن أن تحل محل الوقود الأحفوري. يبدو الهيدروجين كبديل للطاقة الواعدة والذي لا يلبي الطلب على الطاقة فحسب ولكن أيضا يؤدي إلى بيئة نظيفة. ومع ذلك، فإن الإنتاج الحالي من الهيدروجين ينتج الكثير من الطاقة والتلوث. لذلك، فإنّ النهج البيولوجي لإنتاج الهيدروجين باستخدام الكائنات الحية الدقيقة والنفايات أصبح بارزاً. لايزال هناك عدد محدود من البحوث في تطوير الهيدروجين الحيوي باستخدام مياه الصرف الصحي sago كمصدر للطاقة. وهكذا، فإن الهدف الرئيسي من هذه الدراسة هو إنتاج الهيدروجين الحيوي من مياه الصرف الصحي sago باستخدام بكتيريا ***Enterobacter aerogenes***. في هذه الدراسة، تم استخدام عدة طرق متتابعة في تقييم عملية التحسين التي تم تضمينها في أهداف البحث. أولاً، تم اختيار عشرة عوامل فيزيائية – كيميائية (تركيز مياه الصرف الصحي ، درجة الحرارة، درجة الحموضة، حجم اللقاح، مستخلص الشعير، مستخلص الخميرة، والحديد، والمغنيسيوم، والنحاس، والنيتروجين) والتي تؤثر على إنتاج الهيدروجين الحيوي في تصميم بلاكيت – بورمان Plackett–Burman . ثانياً، تم تحسين العوامل باستخدام طريقة عامل في وقت واحد OFAT تليها FCCCD تحت RSM. ثالثاً، تمّ دراسة حركية نمو خلايا ***E. aerogenes*** ، امتصاص الركيزة، وإنتاج الهيدروجين الحيوي. وقد وُجد عند الفحص المبكر باستخدام تصميم بلاكيت–بورمان أنّ مستخلص الخميرة (تأثير إيجابي) ودرجة الحرارة (تأثير سلبي) وحجم اللقاح (تأثير سلبي) كان لها الأثر الأكثر عمقاً من حيث إنتاج الهيدروجين الحيوي. ثمّ تمّ إخضاع العوامل الثلاثة إلى OFAT لإيجاد المدى الأمثل المحتمل. ومن ذلك وُجِد أنّ حجم اللقاح كان بالفعل في الحالة المثلى عند 5%. وفي الوقت نفسه، كان المدى الأمثل الممكن لتركيز مستخلص الخميرة ودرجة الحرارة تقريباً عند 3 جرام / لتر وَ 30 درجة مئوية، على التوالي، والتي تم تطبيقها كنقاط مركزية في RSM. تمّ توليد ما مجموعه إحدى عشرة تجربة من تصميم منهجية سطح الاستجابة. وقد تم الحصول على أعلى إنتاج للهيدروجين من التجربة رقم 7 (تركيز الهيدروجين وعائده 629.80 ميكرومول/لتر و 12.13 ملليمول هيدروجين / مول من الجلوكوز، على التوالي). كشف التحليل الإحصائي ANOVA أن المعادلة الخطية والتربيعية لمستخلص الخميرة وكذلك المعادلة التربيعية لدرجة الحرارة كان لهما في الواقع أكبر الأثر لإنتاج الهيدروجين الحيوي. بعد جميع عمليات التحسين ، تم تسجيل تركيز الهيدروجين الأقصى و عائده عند قيمة 630.67 ميكرومول / لتر و 7.42 ملليمول هيدروجين / مول من الجلوكوز، على التوالي، والتي تم الحصول عليها في ظلّ الظروف المثلى (حجم اللقاح 5% ، تركيز مستخلص الخميرة 4.8 جرام/لتر ، ودرجة الحرارة 31 درجة مئوية). ثم أُجريت الدراسة الحركية عند الظروف المثلى باستخدام لتر واحد من زجاجة سكوت. ووُجِد أن المرحلة الأسيّة لبكتيريا ***E. aerogenes*** جنباً إلى جنب مع إنتاج الهيدروجين الحيوي كانت واقعةً بين 9 و 30 ساعة من فترة التخمير. ثم أُستنتج أن الهيدروجين الحيوي الناتج بواسطة ***E. aerogenes*** هو منتج مرتبط بالنمو. وكذلك تم بنجاح اشتقاق عدة معلّمات حركية من نموذج مونود وهي عائد الخلايا/الركيزة 0.87 جرام/جرام، عائد الإنتاج/الركيزة 0.003 مول/مول، عائد الإنتاج/الخلايا 0.029 جرام/جرام، معدل النمو 0.12 لكل ساعة، وقت التضاعف 6 ساعات، ومعدل تكوّن الهيدروجين 0.0035 لكل ساعة. وعلاوة على ذلك، فإن منحنى إنتاج الهيدروجين التراكمي تلاءم مع معادلة غومبرتز المعدّلة مايشير إلى أنّ H_{max}، R_{max} وَ λ من هذه الدراسة كانت 15.10 مللتر، 2.18 مللتر / ساعة، 9.84 ساعة، على التوالي. وعلى الرغم من أنّه تم إنتاج الهيدروجين الحيوي بنجاح من مياه الصرف الصحي ، إلاّ أن تحسين العائد ومزيداً من التحقيق لايزال مطلوباً بسبب محدودية هذه الدراسة، وخاصة من حيث دراسة خصائص مياه الصرف الصحي sago ، وتحسين السلالة البكتيرية، وطريقة معالجة النفايات، وتأثير المنتجات الثانوية، وتوسيع نطاق العملية.

APPROVAL PAGE

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

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DECLARATION

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

Tami Astie Ulhiza

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To you, a knowledge seeker.

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May Allah reward all of your efforts with His blessing and forgiveness and carve you all path of heaven. *In Sha Allah, Aameen.*

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$(NH_4)_2SO_4$	ammonium sulfate
μ	specific growth rate
μ_{max}	maximum specific growth rate
µmol/L	micromole/litre
Ca	calcium
$CaCl_2.2H_2O$	calcium chloride dihydrate
$CaCO_3$	calcium carbonate
Co	cobalt
CO_2	carbon dioxide
Cr	chromium
Cu	copper
$CuCl_2$	copper(II) chloride
$CuSO_4$	copper sulphate
e	exponential constant 2.718
Fe	iron
$FeSO_4$	ferrous sulfate
g/L	gram per litre
$H(t)$	cumulative volume of hydrogen production
H_2	hydrogen
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
H_{max}	hydrogen gas product potential
K	potassium
K_2HPO_4	dipotassium phosphate
KH_2PO_4	monopotassium phosphate
K_s	saturation constant
M	molar
Mg	magnesium
$MgSO_4$	magnesium sulfate
mL	mililitre
Mn	manganese
Mo	molybdenum
m_p	specific rate of product formation due to maintenance
Na	sodium
$Na_2MoO_4.2H_2O$	sodium molybdate dihydrate
Na_2SeO_3so	sodium selenite
$NaNO_3$	sodium nitrate
NaOH	sodium hydroxide
Ni	nickel
$NiCl_2$	nickel(II) chloride
^oC	degree Celcius
P	phosphate
Pb	lead
q_p	specific rate of product formation
q_s	specific rate of substrate uptake

R_m	maximum production rate
rpm	rotation per minute
S	concentration of the limiting substrate
Se	selenium
t	time
v/v	volume per volume
X	biomass concentration
X_0	initial biomass concentration
Y_{px}	yield of product from biomass
Y_{xs}	yield of biomass from substrate
Y_{ps}	yield of product from substrate
Zn	zinc
γ-Fe_2O_3	gamma-iron(III) oxide
λ	lag time
ANOVA	analysis of variance
ATP	adenosine triphosphate
CDW	cell dry weight
CFO	colony forming unit
FCCCD	face centred central composite design
FHL	formate hydrogen lysate
GHG	greenhouse gas
HPLC	high performance liquid chromatography
LB	luria bertani
MYG	malt yeast glucose
NADH	nicotinamide adenine dinucleotide
OFAT	one factor at a time
PFL	pyruvate formate lysate
PFOR	pyruvate ferrodoxin oxidoreductase
PSI	photosystem I
PSII	photosystem II
RSM	response surface method
SCB	sugarcane bagasse
SMR	steam methane reforming
TDS	total dissolve solid
TSS	total suspended solid
VFA	volatile fatty acid
VSS	volatile suspended solid

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Nowadays, the need of energy has become a global issue that is challenging to the humanity due to its high demand, and fossil fuel is still the main source of energy. It was predicted that by 2035 human population will be increased by 1.6 billion (*BP Statistical Review of World Energy*, 2014). Yet, the current oil reservoir would not be sufficient to fulfil this overwhelming population.

The persistent use of fossil fuel and petroleum leads to the environmental problems. Combustion of fossil fuel releases carbon dioxide in the atmosphere that causes greenhouse gas (GHG) effects. GHG emission will increase the surface temperature of the earth which results in global warming and climate change of the earth. Not only carbon dioxide, other pollutants like sulfur oxide, nitrogen oxide, ash, droplets of tars, soot and other organic compounds, are also emitted into the atmosphere as a result of their combustion (Das & Veziroglu, 2001).

Hydrogen has gained a global attention as an energy carrier for an alternative clean fuel in the future by which it is able to produce water as by-product (Momirlan & Veziroglu, 2005). Moreover, as compared to other fuels, hydrogen carries the highest energy per unit mass and lowest CO_2 content (Das, Khanna, & Dasgupta, 2014). Kapdan and Kargi (2006) reported that hydrogen has a 2.75 times greater high energy yield than hydrocarbon fuels. However, the major problem in utilization of hydrogen gas as a fuel is that, it does not available in nature as a single product. Therefore, it needs expensive production methods.

The current hydrogen gas production is not eco-friendly as it is being generated from fossil fuels through thermo-chemical processes, such as hydrocarbon reforming, coal gasification and partial oxidation of heavier hydrocarbons (Show, Lee, & Chang, 2011). Thus, to overcome this, researchers have found other alternatives on how to harvest hydrogen by utilizing the least energy. Production of hydrogen by biological means has attracted attention of researchers to investigate its potential for inexhaustible, low-cost and renewable source of clean energy.

On the other hand, the expansion of industries also contributes to the environmental problems. Industries discharge chemicals and organic waste that needs to be treated in such a way that it will not harm ecosystem when it is released to the environment. The major challenge is how to utilize sludge or waste to become useful and viable product. Accordingly, the main objective of these facts is to reduce the health or environmental side effects to the lowest level as well as to maintain the sustainability of raw material.

From this notion, many researchers nowadays focus on utilization of organic wastes as the substrate. However not all wastes can be used to produce biohydrogen. Cost, availability, carbohydrate content and biodegradability are the major criteria to be considered in choosing the appropriate waste (Kapdan & Kargi, 2006). Some of the wastes that are known as biohydrogen substrate include fruit and vegetable waste (Saidi et al., 2018), waste wheat (Kirli & Karapinar, 2018), waste paper (Eker & Sarp, 2017), waste peach pulp (Argun & Dao, 2017), whey waste (Patel, Vaisnav, Mathur, Gupta, & Tuli, 2016) and dairy wastewater (Gadhe, Sonawane, & Varma, 2015).

Sago palm is widely planted in Malaysia. The plantation has been well established and become one of the sources of national income by export. About 12% of the total Sarawak area is covered by sago palm (Karim, Tie, Manan, & Zaidul, 2008).

To produce 1 ton of starch in industry, 20-60 m^3 of wastewater is discharged. The wastewater composed of carbohydrates, nitrogen and phosphorus at a ratio of 24:0.14:1 (Adeni, Aziz, Bujang, & Hassan, 2010). Based on this composition, it showed that sago wastewater contains a high amount of carbohydrates and it is a potential substrate to produce biohydrogen. Therefore, this research investigated biohydrogen production from sago wastewater as the substrate using microbial organism.

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

The continuous demand of energy supply leads to the depletion of fossil fuel since energy in the world is still focused to this kind of energy. Yet, fossil fuel is a non-renewable energy source. Biohydrogen from waste may become an alternative energy in the future. Moreover, the environmental problem that usually caused by the waste can be addressed simultaneously. Therefore, it is considered as the clean energy.

Initially, the research for biohydrogen production utilized substrate from the laboratory grade of chemicals such as glucose, glycerol, acetate and butyrate which require a high production cost. Even though the yield is high, the use of synthetic chemical is not economical for the continuous production of biohydrogen. On the other hand, waste is considered as a cheap option for the sustainability of raw material (Khanna & Das, 2013). In Malaysia, sago starch is one of the major food industries. Sago waste becomes very potential as a substrate for biohydrogen production because it is available abundantly in Malaysia and it contains soluble carbohydrate. Unfortunately, there is a limited study addressing utilization of sago wastewater as a substrate for biohydrogen production. So far, the scope of investigation is also limited to studying the effect of pH, temperature and inoculum size (Puad, Sulaiman, Azmi, Shamsudin, & Mel, 2015). Other investigation studied the feasibility of biohydrogen

production from sago wastewater by utilizing a mixed microbial consortia (Yunus et al., 2014). The experiment results confirmed the potential use of sago mill effluent with a significant improvement of hydrogen yield when the pH was optimized. Later investigation studied the effect of enzymatic hydrolysis as the pre-treatment method for biohydrogen production from sago mill effluent (Yunus, Jahim, Anuar, Abdullah, & Kofli, 2014). Therefore, by considering above justification that the scope of investigation from previous studies are still having a gap to be fulfilled, this study is aiming to improve biohydrogen production from sago wastewater by optimizing some process parameters that was investigated throughout the experiments in the lab scale.

Up to date, the research of biohydrogen production from sago waste used mixed culture instead of single culture. However, the drawback of mixed culture is that, there may be a possibility that the bacteria will inhibit each other due to by-product which may be toxic for other bacteria. Nevertheless, the researches of biohydrogen production from sago waste are still limited, especially using *E. aerogenes*. Therefore, in this study biohydrogen production from sago wastewater using the single culture of *E. aerogenes* was carried out. However, since it is single culture, the system should be in sterile condition. It was hypothesized that sago wastewater can provide the adequate glucose content required as a substrate for *E. aerogenes* in producing biohydrogen. Optimization of several physico-chemical factors such as sago wastewater concentration, pH, inoculum size, temperature, nitrogen sparge, and the addition some metals to the media was conducted to produce high yield of biohydrogen using sago wastewater as the substrate by *E. aerogenes.*

1.3 RESEARCH OBJECTIVES

The main objective of this study is to evaluate the ability of sago wastewater as the main substrate for biohydrogen production by *E. aerogenes.* Meanwhile the specific objectives are as follow:

1. To select the physico-chemical factors that affect biohydrogen production from sago wastewater by *E. aerogenes.*
2. To optimize the physico-chemical process conditions of biohydrogen production by *E. aerogenes* in serum bottles using Face Centred Central Composite Design (FCCCD).
3. To determine the kinetic parameters of *E. aerogenes* cell growth, substrate uptake, and biohydrogen production.

1.4 RESEARCH SCOPE

Biohydrogen attracts much attentions from the environment and energy sectors due to its viability. The idea of using bacteria as an agent and waste as a substrate is not only to produce biohydrogen, but is also to degrade waste in the environment. Moreover, it also can suppress the total cost of production as compared to hydrogen production by the conventional methods.

This research was intended to utilize sago wastewater for biohydrogen production as well as to perform bioremediation of wastewater. Microbial fermentation technique was employed using single culture of *E. aerogenes* instead of co-culture or mixed culture in dark fermentation. Acid hydrolysis was used as the pre-treatment method to break down starch in sago wastewater into the fermentable sugars. Ten physico-chemical factors (sago wastewater concentration, pH, inoculum size, temperature, yeast extract, malt extract, iron, magnesium and copper concentration, and

nitrogen sparge) were subjected to the screening process using Plackett-Burman design. The 3 most significant factors were optimized for a maximum biohydrogen production using One-Factor-At-a-Time (OFAT) method followed by Face Centred Central Composite Design (FCCCD) under Response Surface Method (RSM). The screening and optimization process of factors were investigated in serum bottles with the aid of statistical software namely Design Expert (V.9.0.6). Kinetic study was carried out in 1 L Schott bottle for *E. aerogenes* in term of cell growth, cumulative biohydrogen production, and substrate consumption. The data output obtained were hydrogen concentration and yield from glucose. The biohydrogen produced was measured using a hydrogen gas analyzer.

1.5 RESEARCH METHODOLOGY

The attainment of the set of objectives should be according to a well-planned methodology (Figure 1.1), which is outlined as follow:

1. Characterization of sago wastewater and pre-treatment process using acid hydrolysis to obtain more fermentable sugars.
2. Early screening of 10 physico-chemical factors affecting biohydrogen production by *E. aerogenes* including sago wastewater concentration, pH, temperature, inoculum size, malt extract, yeast extract and some metals concentration and sparging of nitrogen which were conducted using statistical analysis experimental design; Plackett-Burman design.
3. Reduction of the 10 physico-chemical factors into three most significant variables.
4. Examination of the possible optimum range, which used OFAT approach.
5. Genuine optimization of the significant variables using FCCCD under RSM.

6. Evaluation of the kinetics study which covered several parameters including substrate uptake (Y_{ps}), the yield of biomass formation based on substrate uptake (Y_{xs}), the yield of product formation based on biomass formation (Y_{px}), specific growth of bacteria (μ), doubling time (t_d) and specific rate of product formation (q_p) which used Monod model.
7. Determination of cumulative hydrogen production using Gompertz equation.

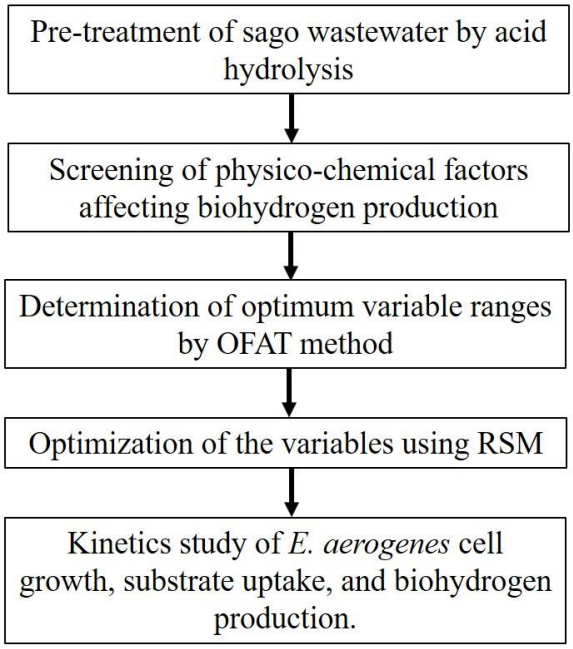

Figure 1.1 Overview of the research methodology

1.6 THESIS ORGANIZATION

This thesis is organized into five chapters; Introduction, Literature review, Methodology, Results and discussion and finally, Conclusion. Chapter 1 describes a brief background of the study, problem statement and its significant, research scope, research methodology and thesis organization. Chapter 2 reviews the available literature related to the subject of study and provide knowledge and information based on the limitation set in the scope of study. Chapter 3 discusses the detailed methodology,