OPTIMIZATION OF MEDIA AND PROCESS PARAMETERS FOR BIOCONVERSION OF CRUDE GLYCEROL TO HYDROGEN BY LOCALLY ISOLATED *Klebsiella pneumoniae*

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ABSTRACT

Bioconversion of crude glycerol to biohydrogen is promising because the cost for capital investment and operation is cheaper, and it can help in reducing waste thus making it a clean process. However, not many microbes can metabolise glycerol efficiently under anaerobic or oxygen-limited conditions. This makes the production and yield of hydrogen produced to be low. Therefore, this study sought to isolate new indigenous bacteria that can consume crude glycerol (85%) and convert it into biohydrogen without the need for pretreatment or acclimatization. Dark fermentation approach was employed since this approach offers much advantages in terms of substrates and microbe producers used. The aim of study was achieved through isolation and screening for potential hydrogen producers followed by identification using 16S rRNA technique. Next, optimisation of medium composition and optimisation of operating parameters to obtain the optimum production and yield of hydrogen and ethanol including glycerol uptake. The study was finalised by a kinetic study for growth and substrate utilisation by kinetic model and potential hydrogen production by modified Gompertz model. The best hydrogen producer had been successfully isolated, screened and identified as *Klebsiella pneumoniae* strain HS11286. The 2-level fractional factorial design (2^{8-3}) for medium composition optimisation and response surface methodology study employing Box-Behnken design for operating parameters were employed to evaluate the interactive effects of several respective factors. The fermentation was conducted in 2 L reactor with a working volume of 1.8 L. Medium optimisation study gave the composition of 30 g/L glycerol, 3.5 g/L K_2HPO_4, 3.98g/L KH_2PO_4, 2.69 g/L $(NH_4)_2SO_4$, 0.03 g/L $CaCl_2.2H_20$, 0.054 g/L $FESO_4.7H_2O$, 3.0 g/L yeast extract, and 0.54 g/L $MgSO_4.7H_2O$ as the optimised medium composition. This optimised composition yielded 588.68±0.04 mmol H_2/mol $glycerol_{consumed}$, 9345±63.64 mL of hydrogen, 97±1.41% glycerol uptake and 0.024±0.001 mmol ethanol/mol $glycerol_{consumed}$. Meanwhile, the optimum operating condition was found best at pH 6.0, temperature 32.5°C and 25% headspace with 82% desirability. This yielded 601.07±10.69 mmol H_2/mol $glycerol_{consumed}$ of hydrogen yield, 9935±176 mL of hydrogen, 97±1.4% of glycerol uptake and 0.045±0.002 mmol ethanol/mol $glycerol_{consumed}$. Finally, kinetic parameters for specific growth rate (μ) was at 0.106 h^{-1}, glycerol consumption rate (Q_{gly}) at 1.572 g/L/h, and yield coefficient $Y_{p/x}$, $Y_{p/s}$ and $Y_{x/s}$ at 30758.51 mL/g cell, 479.26 mL/g substrate and 0.016 g cell/g substrate, respectively. Meanwhile, the modified Gompertz model gave a prediction of 10155 mL of hydrogen at 620 mL/h. In conclusion, *Klebsiella pneumonia* strain HS11286 has the potential to produce almost 10 litre of hydrogen in a short period (less than 48 h) without the need to pretreat the glycerol or to acclimatize the bacteria in crude glycerol.

ABSTRAK

Biopenukaran gliserol mentah kepada biohidrogen sangat berpotensi kerana kos untuk pelaburan modal dan operasi adalah lebih murah dan ia mampu mengurangkan sisa lalu menjadikannya satu proses bersih. Walau bagaimanapun, tidak banyak mikrob yang boleh menggunakan gliserol dengan cekap di bawah keadaan anaerobik atau oksigen terhad. Keadaan ini menyebabkan pengeluaran dan hasil hidrogen yang dikeluarkan rendah. Oleh itu, kajian ini bertujuan untuk mengasingkan bakteria asal yang mampu menggunakan gliserol mentah (85%) dan menukarkannya menjadi biohidrogen tanpa keperluan terhadap prarawatan atau penyesuaian. Pendekatan fermentasi gelap telah digunakan memandangkan ia menawarkan banyak kelebihan dari segi penggunaan substrat dan mikrob pengeluar. Matlamat kajian dicapai melalui pemencilan dan penyaringan mikrob pengeluar biohidrogen berpotensi diikuti dengan pengenalpastian bakteria menggunakan teknik 16S rRNA. Seterusnya, pengoptimuman komposisi media dan pengoptimuman parameter pengoperasian untuk mendapatkan pengeluaran dan hasil hidrogen serta etanol termasuk pengambilan gliserol yang optimum. Kajian ini dimuktamadkan dengan kajian kinetik untuk pertumbuhan dan penggunaan substrat melalui model kinetik dan potensi pengeluaran hidrogen menggunakan model Gompertz diubahsuai. Mikrob pengeluar hidrogen terbaik berjaya dipencilkan, disaring dan dikenal pasti sebagai *Klebsiella pneumoniae* strain HS11286. Reka bentuk faktoran 2 peringkat (2^{8-3}) untuk pengoptimuman komposisi media dan kajian kaedah sambutan permukaan menggunakan reka bentuk Box-Behnken bagi parameter pengoperasian digunakan untuk menilai kesan-kesan interaktif beberapa faktor berkaitan. Fermentasi telah dijalankan di dalam reaktor 2 L dengan isipadu kerja sebanyak 1.8 L. Kajian pengoptimuman media memberikan komposisi 30 g/L gliserol, 3.5 g/L K_2HPO_4, 3.98 g/L KH_2PO_4, 2.69 g/L $(NH_4)_2SO_4$, 0.03 g/L $CaCl_2.2H_20$, 0.054 g/L $FESO_4.7H_2O$, 3.0 g/L ekstrak yis, dan 0.54 g/L $MgSO_4.7H_2O$ sebagai komposisi medium yang optimum. Komposisi optimum ini menghasilkan 588.68±0.04 mmol H_2/mol $gliserol_{diguna}$, 9345±63.64 mL hidrogen, 97±1.41% pengambilan gliserol, dan 0.024±0.001 mmol ethanol/mol $gliserol_{diguna}$. Sementara itu, parameter pengoperasian optimum didapati terbaik pada pH 6.0, suhu 32.5 C dan 25% ruang kosong dengan 82% keberertian. Keadaan ini memberikan hasil 601.07±10.69 mmol H_2/mol $gliserol_{diguna}$ bagi hasil hidrogen, 9935±176 mL hidrogen, 97±1.4% pengambilan gliserol dan 0.045±0.002 mmol ethanol/mol $gliserol_{diguna}$. Akhir sekali, parameter kinetik masing-masing bagi kadar pertumbuhan spesifik (μ) adalah pada 0.061 h^{-1}, kadar penggunaan gliserol (Q_{gly}) pada 1.572 g/L/h, dan pekali hasil $Y_{p/x}$, $Y_{p/s}$ dan $Y_{x/s}$ pada 30758 mL/g sel, 491 mL/g substrat dan 0.016 g sel/g substrat. Manakala model Gompertz diubahsuai memberikan ramalan sebanyak 10,115 mL hidrogen pada 620 mL/h. Kesimpulannya, *Klebsiella pneumonia* strain HS11286 berpotensi menghasilkan hampir 10 liter hidrogen dalam masa yang singkat (kurang 48 j) tanpa perlu prarawat gliserol atau penyesuaian bakteria dalam gliserol mentah.

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LIST OF ABBREVIATIONS

CO_2	-	Carbon dioxide
% w/v	-	Percent weight per volume
3D	-	Three dimensional
3-FI	-	Three-factor interaction
Adj R^2	-	Adjusted regression coefficient
ADP	-	Adenosine diphosphate
ANOVA	-	Analysis of variance
AnSBBR	-	Anaerobic sequencing batch biofilm reactor
ATCC	-	American Type Culture Collection
$atm \cdot L \cdot mol^{-1} \cdot K^{-1}$	-	Litre atmospheres per mole Kelvin
ATP	-	Adenosine triphosphate
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
C	-	Carbon
Ca^{2+}	-	Calcium ion
cal/g	-	Calorie per gram
CG	-	Crude glycerol
cm	-	Centimetre
cm^2/s	-	Centimeter square per second
C–N	-	Carbon–Nitrogen
COD	-	Chemical oxygen demand
COD/L	-	Chemical oxygen demand per litre
cP	-	Centipoise
CSTR	-	Continuous stirred tank reactor
CV	-	Coefficient of variation
DF	-	Dark fermentation
DFE	-	Dark fermentation effluent
DHA	-	Dihydroxyacetone
DHAP	-	Dihydroxyacetone phosphate
DNA	-	Deoxyribonucleic acid

e^-	-	Electron
EFISC	-	European Feed and Food Ingredient Safety Certification
exp	-	Exponential
Fd_{ox}	-	Ferredoxin (oxidised form)
Fd_{rd}	-	Ferredoxin (reduced form)
Fe^{2+}	-	Ferum ion
FHL	-	Formate:hydrogen lyase
g	-	Gram
g/L	-	Gram per litre
g/mol	-	Gram per mol
GC–TCD	-	Gas chromatography–thermal conductivity detector
h	-	Hour
H	-	Hydrogen
H_2	-	Hydrogen gas
HPB	-	Hydrogen-producing bacteria
HPLC	-	High performance liquid chromatography
HydA	-	Hydrogenase
ID	-	Identification
IEA	-	International Energy Agency
kb	-	Kilobase
KH_2PO_4	-	Potassium dihydrogen phosphate
kJ/g	-	Kilojoule per gram
L	-	Litre
LOF	-	Lack of fit
max	-	Maximum
MEC	-	Microbial electrolysis cell
MFC	-	Microbial fuel cell
mg $DCWL^{-1}$	-	Milligram dry cell weight per litre
mg/L	-	Milligram per litre
Mg^2	-	Magnesium ion
$MgSO_4$	-	Magnesium sulfate
min	-	Minute
MJ/kg	-	Megajoule per kilogram

mL	-	Millilitre
mL H_2/g COD	-	Millilitre hydrogen per gram chemical oxygen demand
mL H_2/L-OP sap	-	Millilitre hydrogen per litre oil palm sap
mL/L	-	Millilitre per litre
mL/min	-	Millilitre per minute
mm	-	Millimetre
mM	-	Millimole
mmol H_2/g COD	-	Millimole hydrogen per gram chemical oxygen demand
mmol H_2/L	-	Millimole hydrogen per litre
mmol photon $m^{-2}s^{-1}$	-	Millimole photon per meter square per second
mmol/L/h	-	Millimole per litre per hour
mol H_2/kg COD	-	Mole hydrogen per kilogram chemical oxygen demand
MONG	-	matter organic non-glycerol
N	-	Normal
n/a	-	Not available
N_2	-	Nitrogen gas
N_2ase	-	Nitrogenase
NaCl	-	Sodium chloride
NAD^+	-	Nicotinamide adenine dinucleotide (oxidised form)
NADH	-	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	-	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	-	Sodium bicarbonate
NaOH	-	Sodium hydroxide
NCBI	-	National Center for Biotechnology Information
ND	-	Not detected
NFOR	-	NAD(P)H:ferredoxin oxidoreductase
NL/h	-	Normal litre per hour
nm	-	Nanometre
NO_x	-	Nitrogen oxide
O	-	Oxygen
O_2	-	Oxygen gas

OAA	-	Oxaloacetate
OD	-	Optical density
P	-	Product
PCR	-	Polymerase chain reaction
PEP	-	Phosphoenolpyruvate
PF	-	Photofermentation
PFL	-	Pyruvate:formate lyase
PFOR	-	Pyruvate:ferredoxin oxidoreductase
PG	-	Pure glycerol
PNS	-	Purple non-sulfur
ppc	-	Phosphoenolpyruvate kinase
psi	-	Pound-force per square inch
RID	-	Refractive Index Detector
rpm	-	Rate per minute
rRNA	-	Ribosomal ribonucleic acid
RSM	-	Response surface methodology
S	-	Substrate
SD	-	Standard deviation
sp.	-	Species
t	-	Time
Taxid	-	Taxonomy identification
TCA	-	Tricarboxylic acid
UASB	-	Upflow anaerobic sludge blanket
UFCB	-	Upflow column bioreactor
US	-	United States
v/v	-	Volume per volume
VFA	-	Volatile fatty acid
W	-	Watt
wt %	-	Weight percent
YE	-	Yeast extract

LIST OF SYMBOLS

$\$$	-	Dollar
$\%$	-	Per cent
$(NH_4)_2SO_4$	-	Ammonium sulfate
A_0	-	Regression coefficients for the intercept
A_i	-	Linear coefficients
A_{ij}	-	Interaction coefficients
C_{g_f}	-	Final concentration of the glycerol
C_{g_i}	-	Initial concentration of glycerol
C_2H_5OH	-	Ethanol
$C_3H_6O_3$	-	Dihydroxyacetone
$C_3H_7O_6P$	-	Dihydroxyacetone phosphate
$C_3H_8O_3$	-	Glycerol
X_0	-	Cell mass at time *0*
X_f	-	Cell mass at time *f*
X_i	-	Independent variables in coded units
X_j	-	Independent variables in coded units
$>$	-	More than
×	-	Times sign
°C	-	Degree Celcius
°C/min	-	Degree Celcius per minute
μ	-	Specific growth rate
µL	-	Microlitre
µm	-	Micrometre
$CaCl_2{\cdot}2H_2O$	-	Calcium chloride dihydrate
DOE	-	Design of experiment
EtOH	-	Ethanol
$FeSO_4{\cdot}7H_2O$	-	Ferrous sulfate heptahydrate
IV	-	Four
k	-	Factor

K	-	Potassium
K^+	-	Potassium ion
$K_2HPO_4 \cdot 3H_2O$	-	Dipotassium hydrogen phosphate trihydrate
KCl	-	Potassium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
KOH	-	Potassium hydroxide
$MgCl_2.6H_2O$	-	Magnesium chloride hexahydrate
$MgSO_4 \cdot 7H_2O$	-	Magnesium sulfate heptahydrate
NH_4Cl	-	Ammonium chloride
P	-	Hydrogen potential
p	-	Probability
PO_4^{2+}	-	Phosphate ion
Q_{gly}	-	Glycerol consumption rate
R^2	-	Correlation coefficients
R_m	-	Hydrogen production rate
X	-	Cell mass or biomass
X	-	Representation of factors in optimisation study
Y	-	Yield coefficient
$Y_{p/s}$	-	Maximum hydrogen production per unit of substrate
$Y_{p/x}$	-	Maximum hydrogen production per unit of cell
$Y_{x/s}$	-	Total yield coefficient (cell per substrate)
Δ	-	Delta
λ	-	Lag phase
HCO_2^-	-	Formate
P	-	Pressure
R	-	Ideal gas constant
T	-	Temperature
V	-	Volume of hydrogen
Y	-	Response or independent variable
n	-	Mole of gas
ε	-	Error term

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The world's fuel consumption has risen intensely due to urbanisation and globalisation. Transportation and industrial sectors are among the key sectors in which fuel usage is high. The fuel used is normally petroleum/fossil based, and this has in turn led to several adverse effects, such as depletion of fossil fuel from the extensive exploitation of the present oil and gas reservoirs (Chuah *et al.*, 2017), and anthropogenic CO_2 emission (Lee *et al.*, 2017). Furthermore, fossil fuel has several downsides for humans such as greenhouse gas emission to the atmosphere, climate change, and air pollution; and the limitation of fossil fuel may cause a great increase in oil prices (Omi, 2009).

In 2003, the European Union enforced Directive 2003/30/EC to blend renewable fuel (biofuels) with fossil fuels to control the vast usage of petroleum-based fuel. The enforcement had drastically increased the production of biodiesel from 200,000 tonnes in 2003 to more than 2 million tonnes in 2012 (Ciriminna *et al.*, 2014). Globally, the United States (US) and Europe are the two main producers of biodiesel (United Nations Conference on Trade and Development, 2009). Germany alone had produced approximately 775 million gallons of biodiesel in 2006 (Carriquiry, 2007). Accordingly, the International Energy Agency (IEA) predicted that by the year 2050, biofuels will completely replace the use of petroleum-based fuels as transportation fuel (Hashim *et al.*, 2017). Deloitte (2015) predicted that the production of biodiesel will increase up to 1900 barrels in 2020. This massive generation of biodiesel has led to a huge increase of crude glycerol (CG) (Chookaew, Prasertsan, and Ren, 2014).

In general, biodiesel is produced via transesterification (Figure 1.1) of triglyceride with alcohol in the presence of a catalyst. This process generates 10%–20% of glycerol as its by-product, which is regarded as the main waste produced (Nda-Umar *et al.*, 2019). As a waste, CG contains impurities (Boga *et al.*, 2016), which are mostly salts (e.g. carboxylate salts), catalyst, residual methanol, and water (Tu, Lu, and Knothe, 2017).

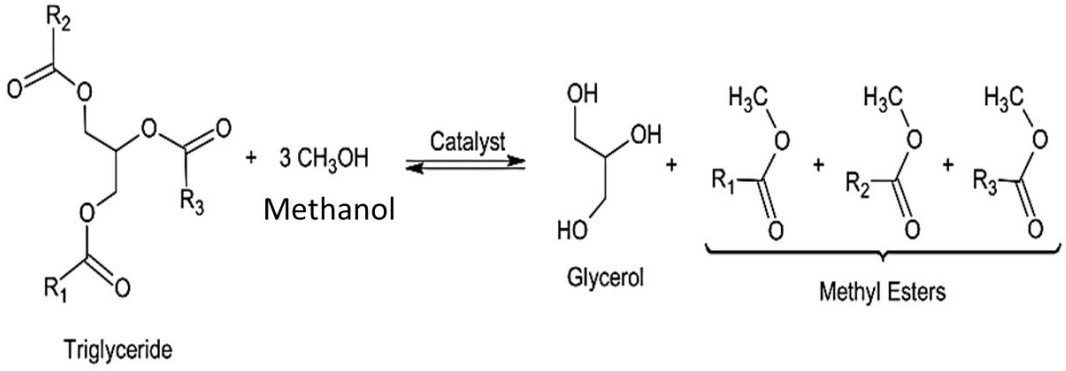

Figure 1.1 Transesterification process producing glycerol as the main by-product via base catalytic reaction with methanol (Ciriminna *et al.*, 2014)

Impurities in CG makes it lack commercial value and harmful to the environment. Therefore, CG can neither be used directly in industry nor disposed to the environment without purification or proper treatment. Eventually, CG is either valorised or regarded as industrial waste. This leads to a drop in glycerol price from $1.15 to $0.66 per kilogram for refined glycerol and from $0.44 to $0.11 per kilogram for CG (Li, Lesnik, and Liu, 2013). Presently, the common practice to destroy CG is by incineration, but Gholami, Abdullah, and Lee (2014) claimed that this method leads to the production of primary greenhouse gases like nitrogen oxide (NO_x) and carbon dioxide (CO_2). Thus, the need for sustainable management of the waste along with the availability of CG has generated interest on glycerol-based cultures.

Amid the green technologies adopted or that have taken interest among researchers and some industry players is to convert CG into hydrogen via biological approach. Hydrogen is said to play an important role in the world's future as a good alternative renewable fuel in replacing petroleum-based fuel. Hydrogen offers a clean energy since it produces only water during its combustion. Until recently, production

of hydrogen from glycerol is mostly from chemical conversion technologies such as steam reforming (Tamošiunas *et al.*, 2016), partial oxidation (Wang, 2010), dry reforming (Zakaria *et al.*, 2015), autothermal reforming (Wang *et al.*, 2016), aqueous reforming (Subramanian *et al.*, 2016), pyrolysis (Ng *et al.*, 2017), and photocatalysis reforming (Lucchetti *et al.*, 2017). Despite the stability of some processes, the mentioned approaches require high temperatures, and higher reaction temperature promotes the formation of encapsulated carbon which negatively reflects on catalyst stability (Chiodo *et al.*, 2010).

Cheaper, simpler compound as a good carbon source for microbial growth, greater degree of reduction to enable higher yield of fuels and reduced chemicals, as well as high energy contents make CG the best substrate in biological conversion, especially hydrogen. Previously, hydrogen was produced using short-chain organic acids (acetate, lactate, malate, acetate, and butyrate) (Barbosa *et al.*, 2001; Asada *et al.*, 2008), glucose (Fang, Zhu, and Zhang, 2006; Chaudary, 2010; Jame *et al.*, 2011), starch (Yokoi *et al.*, 2002; Afsar *et al.*, 2011), lignocellulosic materials (Chong *et al.*, 2009a; Liu, 2008; Noparat, Prasertsan, and O-thong, 2011), and algae (Lam and Lee, 2013) as substrate—which is expensive. Then slowly, the use of those substrates was replaced with cheaper and more abundant substrates such as waste (i.e., lignocellulosic waste, livestock manure, and industrial waste like glycerol). Despite the ability to be converted into hydrogen, lignocellulosic materials require higher temperature to give a higher hydrogen yield (Ghimire *et al.*, 2015). This again causes CG to be a viable and promising substrate for hydrogen production.

Fermentation is one of the biological ways to produce hydrogen from CG. Hydrogen, 1,3-propanediol, carotenoids, citric acid, succinic acid, polyhydroxyalkanoates, polyunsaturated fatty acids, and rhamnolipids are the main products of CG fermentation (Abad and Turon, 2012). Production of hydrogen is more favourable because glycerol has a higher content of hydrogen (8 numbers) and thus can give high energy content of hydrogen (up to 142.9 kJ/g) (Sarma *et al.*, 2012).

The production of hydrogen from CG by using microorganisms as the producer has been reported by many researchers. Ito *et al.* (2005) evaluated the production of hydrogen and ethanol using pure and crude glycerol by *Enterobacter aerogenes* HU-101 strain. The strain cannot tolerate the impurities in the crude glycerol, thus giving low hydrogen yield compared to pure glycerol. Ngo, Kim, and Sim (2011) used hyperthermophilic eubacterium *Thermotoga neapolitana* DSM 4359 to produce hydrogen from CG. Due to impurities in the CG, CG was pretreated first by heating at 45 °C to remove methanol or ethanol, and solids from CG were removed by centrifugation. The hydrogen yield from *Thermotoga neapolitana* DSM 4359 at 1.97 ± 0.09 mol H_2/mol glycerol was observed after 56 h of cultivation.

Chookaew, O-Thong, and Prasertsan (2012) also reported the use of pure culture to produce hydrogen. The reported hydrogen yield was at 0.25 mol H_2/mol glycerol by a newly isolated thermotolerant *Klebsiella pneumoniae* TR17. The fermentation experiment was conducted at 45 °C. Since the bacteria used was isolated from glycerol contaminated soils, the enrichment was conducted three times prior to isolation. However, Mangayil, Karp, and Santala (2012) used mixed cultures to convert CG to hydrogen. Prior to experiment, the cultures were enriched to acclimatise the microbes. The glycerol yield was obtained at 1.1 ± 0.1 mol H_2/mol with *Clostridium* as the dominant species in the mixed culture used. Besides, Marone *et al.* (2015) co-fermented CG with cheese whey to produce hydrogen by using a mixed culture. Dounavis, Ntaikou, and Lyberatos (2015) then investigated the production of hydrogen from CG by using continuous anaerobic upflow column bioreactor (UFCB) packed with cylindrical ceramic beads as support matrix for bacterial cells. The study was run for nine months continuously using a mixed culture as the producer.

The latest bioconversion of CG to hydrogen was reported by Sarma *et al.* (2019) using an engineered strain of *Clostridium pasteurianum*. The *hydA* gene was overexpressed encodes for hydrogenase and combined *dhaD1* and *dhaK* genes, which encode for glycerol dehydrogenase and dihydroxyacetone kinase, respectively. The engineered *hydA*-overexpressed strain produced 1.1 mol H_2/mol glycerol, and 0.93 mol H_2/mol glycerol was produced by the *dhaD1K*-overexpressed strain. Although the yields are high comparable to the wild type, the strains must be engineered first.

Until now, either dark fermentation or photo fermentation or combination of the two systems, most literature reported were conducted in batch mode. The choice to use either pure or mixed cultures is dependent on the use of substrates. Reports so far show that the use of mixed culture is more common compared to pure culture. However, the presence of different types of microorganisms in a mixed culture makes it difficult to be optimised.

Pure culture, on the other hand, is much easier to understand, especially when describing the pathway. *Enterobacter* sp., *Clostridium* sp., *Bacillus* sp., and *Klebsiella* sp. are commonly used bacteria in dark- or anaerobic fermentation of glycerol. A non-hydrogen-producer, *Escherichia coli*, is genetically modified to transform it into a hydrogen producer (Gonzalez *et al.*, 2008; Maru et al, 2016; Karen Trchounian & Trchounian, 2014). However, the yield reported is still very low unless it is co-cultured with other microbes (Maru *et al.*, 2016). In terms of fermentation condition, the reported temperature ranged from 37 (mesophilic) to 80 °C (thermophilic) with a pH range of 5.5 to 8.0. The highest yield reported was 2.84 mol H_2/mol glycerol in batch by a hyperthermophilic bacteria. Although the yield is high, the process needed a higher temperature which may be unsuitable for industrial applications.

1.2 Problem Statement

Crude glycerol (CG) is classified as Scheduled Waste S181 of the Environmental Regulations in Malaysia (Ardi, Aroua, and Hashim, 2015; Ooi *et al.*, 2001); therefore, it cannot be disposed freely. However, the development of processes that can utilise crude glycerol directly to produce value-added chemicals or energy carriers, e.g. hydrogen, would be very advantageous.

Many reports have been published to prove the best biological approaches exploiting CG as a cheap feedstock. Ghosh, Tourigny, and Hallenbeck (2012), Ghosh, Sobro, and Hallenbeck (2012), Pott, Howe, and Dennis (2013), and Ghosh *et al.* (2017) used photosynthetic bacteria to produce hydrogen from CG. However, using this photo-approach (biophotolysis or photofermentation) has drawbacks because of its

complexity in light management and limitation in scale up (Chookaew, Prasertsan, *et al.*, 2014). Therefore, dark fermentation has been claimed by many to have many advantages over other approaches. It is simple and can be carried out using many types of microorganisms, especially enterobacterial species, which are easy to grow and maintain (Abdeshahian *et al.*, 2014; Argun & Dao, 2017; Fuess, Zaiat, & do Nascimento, 2019; Rajhi *et al.*, 2016).

Until recently, most dark fermentation productions of hydrogen from CG use a mixed culture (Faber and Ferreira-Leitão, 2016; Gallardo *et al.*, 2014; Mangayil *et al.*, 2015; Marone *et al.*, 2015; Pachapur *et al.*, 2015; Rodrigues *et al.*, 2019), engineered pure culture (Sanchez-Torres *et al.*, 2013), or a combination of engineered pure culture and another single or mixed culture (Maru *et al.*, 2016; Veeramalini *et al.*, 2019). The use of mixed culture is claimed to be easier but may limit the hydrogen production or yield, besides the need to first adapt the culture before the real experiment can be started. Meanwhile, engineered culture (single or mixed) may offer higher hydrogen yield, but its modification steps are tedious. Moreover, genetic engineering is not always fruitful, as undesirable effects are often encountered in the engineered organisms (Valle, Cantero, and Bolívar, 2019). Therefore, using indigenous pure culture is preferable, especially in understanding the mechanism of the conversion process. In addition, a pure culture is easy to manage.

So, having an isolate that can metabolise CG without the need for pretreatment of CG or extra time to acclimatise the bacteria and produce hydrogen large volume of hydrogen at higher yield would be a great success. In this study, CG was used as a sole carbon source for the locally isolated bacteria via dark fermentation. The best isolate was then identified and further investigated for its capability to produce hydrogen under optimised medium and condition. The kinetics of its growth and substrate consumption, together with hydrogen yield and production were also determined.

1.3 Research Objectives

The aim of this work is to produce hydrogen from CG using microorganisms isolated from biodiesel wastewater treatment plant. The following are the objectives of this study:

1. To identify a potential hydrogen-producing microorganism isolated from biodiesel wastewater treatment plant.
2. To optimise the fermentation media composition of hydrogen production using crude glycerol and other nutrients by the best pure culture of hydrogen-producing microorganism isolated from biodiesel wastewater treatment plant.
3. To optimise the operating parameters of hydrogen production from crude glycerol.
4. To evaluate the kinetic coefficients of hydrogen production from crude glycerol under optimized conditions.

1.4 Research Scopes

This study involves four stages, in which the scopes for each stage are as follows:

1. Isolation, screening, and identification of potential hydrogen producer.

   In this stage, the isolation was performed using anaerobic pour plate method (serial dilution for enriched and direct plating) to choose the potential producer(s). Prior to isolation, sampling of crude glycerol and wastewater was conducted. Crude glycerol was collected from Carotino Sdn. Bhd. and biodiesel wastewater were obtained from Vance Sdn. Bhd. Both plants are located in Pasir Gudang, Johor. Once the best potential microbe had been isolated, screened and chosen, identification of the microbe was conducted using 16S rRNA method.

2. Optimisation of the media composition and determination of the effect of each factor on hydrogen production by the best hydrogen producing microorganisms isolated from biodiesel wastewater treatment plant.

   The optimisation of fermentation media was performed using two-level fractional factorial design (2^{8-3}) designed by Design–Expert version 6.0.4 software. Eight factors were involved: glycerol (30–60 g), dipotassium phosphate (3.5–5 g), potassium phosphate (2–4 g), ammonium sulphate (2–5 g), calcium chloride (30–55 g), ferrous sulphate (0.5–1 g), yeast extract (1.5–3 g), and magnesium sulphate (0.3–0.6 g). All factors were analysed to determine their effect on hydrogen production, hydrogen yield, and metabolite production. The collected gas was analysed using gas chromatography–thermal conductivity detector (GC–TCD) and liquid samples were analysed using high performance liquid chromatography (HPLC). Results from HPLC gave information on substrate utilisation and metabolites produced. Growth pattern was determined through optical density (OD) and cell dry weight. The performance of each run was monitored based on the hydrogen produced and bacterial growth.

3. Optimisation of operating parameters of hydrogen production from crude glycerol.

   This stage was conducted to determine the best condition for the optimal production and yield of hydrogen and metabolite production. This study contained a few other parameters but only three parameters were chosen. Significant operating parameters involved were pH (6–8), temperature (30–40 °C), headspace (25%–70%). Design of experimental work was done using Box–Behnken design by Design–Expert version 6.0.4 software. Gas analysis was conducted using GC–TCD and liquid samples were analysed using HPLC, and growth pattern was obtained using OD and cell dry weight. Substrate utilisation and metabolites production were analysed by using HPLC. The performance of each run was monitored based on the hydrogen produced and bacterial growth.

4. Evaluation of hydrogen kinetics coefficients via dark fermentation from crude glycerol under optimised conditions.

   A comprehensive kinetic analysis elucidates the effect of operational parameters on substrate utilization, biomass growth, and product formation rate. The growth and formation of hydrogen and metabolites were monitored periodically over fermentation time until gas production ceases. This gave specific growth rate (μ) and other yield coefficients, namely growth yield coefficient ($Y_{x/s}$) and product yield coefficient per substrate ($Y_{p/s}$) and per cell mass ($Y_{p/x}$). Since the experiments were conducted in anaerobic condition, the modified Gompertz model was used to was used to obtain the H_2 production potential (P), H_2 production rate (R_m), and lag phase (λ).

1.5 Research Significance

This study utilises indigenous bacteria which originate from the biodiesel wastewater itself. Thus, the potential microorganism that was chosen can greatly reduce the start-up time and give a high volume of hydrogen in a short time. This is because the microbe no longer needs to be acclimatised even though the substrate used, which is crude glycerol, has impurities. Being able to utilise the substrate directly also means that there is no need for substrate pretreatment, which has been reported by many in the literature. Thus, by knowing the effect of fermentation medium composition and optimising the operating conditions, the production of hydrogen and the yield of hydrogen can be enhanced. Furthermore, kinetics study on the bacterial growth, substrate consumption and production of products can help in describing the process better.

The main products—hydrogen and ethanol (the most produced metabolite)—are biofuel. Hydrogen is a clean energy carrier. It can be converted to electricity with efficiencies higher than 80% and energy density of 142 MJ/kg. Research communities regard hydrogen as a promising alternative renewable fuel that can replace the use of petroleum-based fuel in the future, because hydrogen only produces water as product of combustion.

Ethanol, like hydrogen, is one of the renewable fuels that have gained interest in industries and research communities. Apart from its use as a fuel, it is also used in biodiesel production. So, it can be reused in biodiesel production and reduce ethanol consumption in the process. Another metabolite, 1,3-propanediol, is also well-known for its advantages in many applications such as in the medical sector and for production of polymers, cosmetics, foods, adhesives, lubricants, laminates, solvents, and antifreeze.

1.6 Thesis Outline

This thesis consists of five chapters. Chapter 1 focuses on introduction of the study and its background. It also emphasises the problem and why the method in this study is chosen, including its objectives, scopes, and significance.

Chapter 2 presents the literature related to this study, from the glycerol to biodiesel to hydrogen production and related works. The microorganisms or producers used are also elaborated in detail. This chapter is concluded by presenting the factors that might influence the yield and production of hydrogen.

The methodology used is elaborated in detail in Chapter 3. The steps from inoculum preparation to experimental runs are presented and explained in this chapter. The analyses involved are also presented, including the designs used for media composition optimisation, operating condition optimisation, and kinetic study.

Chapter 4 presents the results and discussion. All results on the optimisation of fermentation media composition and RSM analysis on optimisation of operating conditions are discussed in detail in this chapter. Effects of the factors involved in both media optimisation and operating condition optimisation on hydrogen production, yield, and metabolite production are also discussed. Finally, the kinetics correlated with growth and substrate utilisation, as well as the cumulative hydrogen and metabolite production are presented and discussed.

Finally, Chapter 5 concludes the thesis based on the findings and gives recommendations for future study.

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