OPTIMIZATION OF BIOHYDROGEN PRODUCTION BY *Enterococcus* sp. NF6 USING STARCH AS SUBSTRATE

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To my family,

Always and no matter what.

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ABSTRACT

The rising gap between the demand for fossil fuel-based energy and insufficient energy supply has led to the vast investigation of potential alternative clean energy, biohydrogen. In this study, starch utilizing bacteria *Enterococcus* sp. NF6 was isolated from cow manure; the effective starch utilization and biohydrogen production of the biocatalyst were investigated. To investigate the optimum conditions for biohydrogen production, batch fermentations were conducted by optimization of physicochemical parameters using one factor at a time (OFAT) method using Modified Reinforced Clostridial Media (MRCM) as fermentation media. Batch fermentations were carried out at several temperatures (30°C, 37°C & 45°C), initial medium pH (5.5, 7.0, 8.0 & 9.0) and initial substrate concentration of (0.5 g/L, 1 g/L & 3g/L). The highest biohydrogen production obtained at pH 5.5, incubation temperature of 37°C, and initial substrate concentration of 1g/L. These conditions, the biohydrogen production rate is 0.23 mL/H, with 100% of substrate utilization, 0.0790 h^{-1} of specific growth rate and maximum biohydrogen production of 1.85 mL. The outcome suggests the potential use of *Enterococcus* sp. NF6 for biohydrogen production in a single culture system.

ABSTRAK

Peningkatan jurang antara permintaan bagi tenaga berasaskan bahan api fosil dan bekalan tenaga yang tidak mencukupi telah membawa kepada penyelidikan akan tenaga alternatif yang bersih dan berpotensi iaitu biohydrogen. Dalam kajian ini, bakteria *Enterococcus* sp. NF6 yang telah diasing daripada baja yang berasaskan najis lembu telah digunakan untuk mengesan penggunaan kanji sebagai substrat untuk pengeluaran hidrogen. Kajian ini dijalankan untuk menyiasat keadaan optimum bagi pengeluaran biohidrogen melalui proses fermentasi selompok telah dijalankan dengan mengoptimumkan parameter fizikal dengan menggunakan kaedah satu faktor pada satu masa (OFAT) dengan menggunakan Modified Reinforced Clostridial Media (MRCM) sebagai media. Fermentasi telah dijalankan pada beberapa suhu (30°C, 37°C & 45°C), pH awal media (5.5, 7.0, 8.0 & 9.0) dan kepekatan substrat awal (0.5 g/L, 1 g/L & 3g/L). Pengeluaran biohydrogen paling tinggi diperolehi ialah pada pH 5.5, suhu fermentasi 37 ° C, dan kepekatan substrat awal 1 g/L. Pada keadaan ini, kadar pengeluaran biohydrogen adalah 0.23 mL / H, dengan 100% daripada penggunaan substrat, 0,0790 h^{-1} kadar pertumbuhan tertentu dan pengeluaran biohydrogen maksimum 1.85 mL. Kesimpulannya, menunjukkan potensi penggunaan *Enterococcus* sp. NF6 untuk pengeluaran biohydrogen dalam sistem fermentasi yang menggunakan kultur tulen.

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LIST OF ABBREVIATIONS AND SYMBOLS

$CH_3COCOOH$	-	Pyruvic acid
$C_6H_{12}O_6$	-	Glucose
CH_3COOH	-	Acetic acid
CH_3CH_2COOH	-	Propanoic acid
$COOHCH_2CH_2OCOOH$	-	Succinic acid
CH_3CH_2OH	-	Ethanol
CH_4	-	Methane
DNS	-	3,5-dinitrosalicyclic acid
Fd	-	Ferredoxin
Fe	-	Ferum (Iron)
g	-	Gram
g/g	-	Gram per gram
H_2	-	Hydrogen
H^+	-	Hydrogen ion
HCl	-	Hydrochloric acid
kJ/g	-	Kilojoule per gram
kPa	-	Kilopascal
L	-	Liter
M	-	Molar
mL	-	Milliliter
mL/g	-	Milliliter per gram
mL/H	-	Milliliter per hour
mL/min	-	Milliliter per minute
mm	-	Millimeter
NaOH	-	Sodium hydroxide
NADH	-	Nicotinamide Adenine Dinucleotide-Hydrogen

NAD^+	-	Nicotinamide Adenine Dinucleotide
nm	-	Nanometer
Ni	-	Nickel
pH	-	Potential of hydrogen
sp	-	Species
µL	-	Microliter
°C	-	Degree celsius
α		Alpha

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

The rising gap between the shortages of energy supply and demand for fossil fuel-based energy has led to the outsized scale of industrial development. This leads to substantial depletion of natural resource and alarming changes in the climate, with increasing of greenhouse gasses. Consequently, this encouraged for the investigation for an alternative renewable energy to suffice the global energy demand (Lee *et al.*, 2011). A potential alternative energy that is currently being discussed and broadly investigates is biohydrogen (H_2). Biohydrogen is a sustainable, clean, environment-friendly and promising alternative to fossil fuel based energy.

H_2 is resourceful energy that could amend the use of liquid base fossil fuel as the biohydrogen based fuel has high energy yield per unit mass 122 kJ/g, which is 2.75 fold higher than hydrocarbon based fuel. Moreover, the combustion of H_2 produces water (H_2O) as a by-product, hence reassuring outcome for the reduction of greenhouse gas emission (Bockris, 2002; Christopher & Dimitrios, 2012). H_2 can be generated in four approaches i.e., i) electrochemical process ii) thermochemical process iii) photochemical process and iv) microbial process (Reungsang *et al.*, 2004).

The first three approaches are at a disadvantage as the processes do not produce energy, do not reduce waste and involve electricity produced from fossil fuel combustion. Alternatively, the microbial process reduces both energy and waste. The microbial process is classified as the following approaches; (i) biophotolysis of water using algae and cyanobacteria, (ii) photodecomposition of organic compounds, (iii) fermentative hydrogen production from organic compounds and (iv) hybrid system using photosynthetic and fermentative bacteria (Reungsang *et al.*, 2004).

For energy efficiency and practicality fermentation using carbohydrate-based substrates depicts promising route for biohydrogen production compared to photosynthetic or chemical routes (Vendruscolo, 2014). This is due to the higher production rate, low operational cost and simple set up of the experiment compared to the rest of microbial approaches (Ghimire *et al.*, 2015). Therefore, the selection of suitable biocatalyst and inoculum is essential for fermentation, either by a pure or mixed consortium of bacterial species. The common research typically used pure culture as biocatalyst and defined substrate as a carbon source (Chandrasekhar *et al.*, 2015). Carbohydrate based substrate for instance like starch is preferred substrate for fermentative hydrogen production.

The foremost H_2 producing biocatalysts for fermentation are heterotrophs that do not require solar energy and able to tolerate oxygen deficiency condition (Chandrasekhar *et al.*, 2015). By understanding the metabolic pathway of the biocatalyst or the fermentative hydrogen producing bacteria, the fermentation conditions reroute the metabolic pathway to increase the substrate utilization.

The operational parameters are the key factor in the hydrogen production through the microbial process. The physiochemical parameters affect mainly the yield and the biohydrogen production rate, through the growth of the microbe, enzymatic and metabolic activity and eventually to the production rate. Parameters that are crucial like pH, temperature and starch concentration for the fermentation process are the important parameter involved (Choi & Ahn, 2014).

pH is reflected as the crucial parameter that affects the hydrogen production by alternating the metabolic pathways by inhibiting the enzymatic activity of the biocatalyst (hydrogen producing bacteria) involved and plays a critical role in fermentative hydrogen production. The minor changes with cause the change in its metabolic pathway and affects the hydrogen yield.

Meanwhile, the changes in temperature have an effect on the hydrogen production rate also in the consumption and degradation of the substrate in the process, biohydrogen yield, and formation of metabolites, Volatile Fatty Acids (VFA) with the growth of the microbe. The optimum temperature directly correlates with the enzyme activity which leads to the higher yield of hydrogen.

Apart from that, the activity and metabolic pathway of the biohydrogen producing bacteria is affected by the substrate and its composition. The metabolic byproduct increases with the concentration of the substrate. The shift in the metabolic pathway forms more reduced compound when the electron flow interrupts with the increase in the byproduct concentrations with the increasing starch concentration confirms the theory.

1.2 Problem Statement/ Significance of Research

The dependence and the outburst of the industrial revolution have led to challenges with the supply of enough fossil energy for continued growth of the economy and related emissions. Moreover, the increasing demand and the extensive use of the fossil fuel-based energy also have led to the emission of greenhouse gasses and climate change.

The depositions of fossil fuels are limited physically or economically, hence making them finite and non-renewable natural resources. United States Energy Information Administration (EIA) has estimated that the natural reserves of fossil fuel will be completely exhausted by 2069 to 2088 (Energy Information Administration, 2017). This has accustomed unconstrained demand of fossil fuel supply that has conveyed to major financial distress by the hike in the fossil fuel price globally.

Moreover, fossil fuel dependence has brought major impacts to the environment including the global warming. National Aeronautics and Space Administration (NASA) has recorded that the level of carbon dioxide in the atmosphere are higher that they have been in the recent years, recording exceeding 400 parts per million (ppm) which results in increasing global temperature and climate change.

Hence, the escalating gap between the supply and demand of fossil fuel had led to the global and national economic impact, increasing competition for fossil fuel reserves and climate change effects has prompted for the exploration for an alternative renewable energy to replace fossil fuel driven energy. Hydrogen, sustainable energy that shows potentially versatile energy currency to replace fossil fuel based energy. Accordingly, focuses on the biological approaches for the production of the hydrogen (biohydrogen).

In this study, a biohydrogen-producing bacteria and starch degrading bacteria *Enterococcu*s sp. strain NF6, isolated from the cow manure used as a biocatalyst for fermentation using starch as a substrate with Modified Reinforced Clostridial Medium (MRCM). The effective starch utilization and biohydrogen production of the biocatalyst are investigated, along with the optimum conditions for biohydrogen production.

The physiochemical parameters pH, temperature and starch concentration was optimized to obtain the higher production of hydrogen along with the analysis on the correlation of enzymatic activity with the hydrogen production. Hence, this study leads to the potential use of the biocatalyst in an upscale study of biohydrogen production or the use in mixed culture of biohydrogen production for higher yield of hydrogen.

1.3 Research Objectives

In this study, *Enterococcus* sp. NF6 is used as the biocatalyst. The main objectives of the study are as follows,

i. To measure the performance of *Enterococcus* sp. NF6 for biohydrogen production on Modified Reinforced Clostridial Medium (MRCM) in batch experiment.
ii. To optimize the production of biohydrogen based on temperature, starch concentration and pH using one-factor-at-time (OFAT).
iii. To study the kinetic of biohydrogen production by *Enterococcus* sp. NF6.

1.4 Scope of the Research

This study focuses on the optimization of the biohydrogen production by *Enterococcus* sp. NF6 through different fermentation conditions using Modified Reinforced *Clostridial* Media (MRCM). The physiochemical parameters pH, temperature and initial starch concentration are optimized using one-factor-at-time (OFAT) method. At each parameter, the bacteria were screened for biohydrogen production, cell biomass, starch utilization, the concentration of reducing sugar and amylase activity. The optimum condition was analyzed with the results obtained. The kinetics of biohydrogen production was investigated by determining the specific growth rate, doubling time, yield of the product over the substrate and cell concentration.

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