BATCH FERMENTATION SYSTEM FOR BIOHYDROGEN PRODUCTION BY
*Klebsiella* sp. ABZ11 ISOLATED FROM ANTARCTICA

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DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.
ACKNOWLEDGEMENT

In preparing this thesis, I was in contact with many people, researchers, academicians, and practitioners. They have contributed towards my understanding and thoughts. In particular, I wish to express my sincere appreciation to my main thesis supervisor, Professor Dr Zaharah Ibrahim, for encouragement, guidance, critics and friendship. I am also very thankful to my co-supervisor Dr Mohd Firdaus Abdul Wahab for their guidance, advices and motivation. Without their continued support and interest, this thesis would not have been the same as presented here.

I am also indebted to Universiti Teknologi Malaysia (UTM) for funding my Ph.D. study. Librarians at UTM, Cardiff University of Wales and the National University of Singapore also deserve special thanks for their assistance in supplying the relevant literatures.

My fellow postgraduate student should also be recognised for their support. My sincere appreciation also extends to all my colleagues and others who have provided assistance at various occasions. Their views and tips are useful indeed. Unfortunately, it is not possible to list all of them in this limited space. I am grateful to all my family member.
ABSTRACT

The study of cold-adapted bacteria for biohydrogen production has attracted much interest in the last few decades due to the lower energy input required during the fermentation process. However, an extended lag phase of growth and slow metabolic rate of the bacteria remain the obstacles for the process to be feasible, particularly for obligate psychrophilic and psychrotolerant bacteria. Bacteria with the oxygen-tolerant ability are also favourable for large-scale fermentation. Thus, there is a need to find oxygen-tolerant bacteria capable of producing biohydrogen at mesophilic temperature. In this study, Antarctic soil and seawater samples were used for bacterial isolation, before being screened for biohydrogen production ability. Twelve bacteria were successfully isolated and six were found capable of producing biohydrogen. The bacterium with the highest biohydrogen production was characterised. The optimum physicochemical parameters, such as temperature, pH and carbohydrate concentration were determined using one-factor-at-a-time (OFAT) approach. Appropriate nitrogen source, temperature tolerance and the effects of dissolved oxygen on the growth and biohydrogen productivity were also investigated. Precise optimal factors for biohydrogen productivity were then examined using the three-level factorial design of Response Surface Methodology (RSM). Identification of bacterium with the highest biohydrogen production showed that it was closely related to Klebsiella pneumoniae with 99% similarity based on the 16S rRNA analysis. The bacterium was therefore designated as Klebsiella sp. ABZ11. It was a Gram-negative bacillus, with no capsule detected and grew at a temperature range of 20–40°C, and exhibited 95% uptake of dissolved oxygen in two hours. Screening using OFAT suggested that the optimum conditions for biohydrogen production were 30°C, an initial pH of 6.5, and with glucose supplemented with concentration of 10 g/L. The bacterium utilised various types of carbon and nitrogen sources for biohydrogen production but preferred glucose as the carbon source and beef extract as the nitrogen source. Further optimisation using RSM revealed that the highest biohydrogen productivity (110.15 mol/L) was obtained at 33.5°C, with an initial pH of 6.75 and glucose concentration of 9.15 g/L. For each gram of glucose supplied, the yield for biohydrogen and cell-biomass was 122 mol/L/g and 0.87 g, respectively. Kinetics showed that the bacterium used more of the glucose for biohydrogen production than for biomass formation in the fermentation process. A scale-up culture using the optimised conditions recorded a biohydrogen production of 137.56 mol/L in 36 h with a cumulative yield of 533.51 mol/L. In conclusion, batch fermentation using Klebsiella sp. ABZ11 under mesophilic temperature was found to have decreased lag phase of growth and increased metabolic rate, thereby influencing faster and higher biohydrogen production.
Kajian bakteria adaptasi sejuk untuk pengeluaran biohidrogen telah menarik minat banyak pihak sejak beberapa dekad yang lalu berikut input tenaga yang rendah diperlukan semasa proses fermentasi. Walau bagaimanapun, fasa lamban pertumbuhan bakteria yang panjang dan kadar metabolisma yang perlahan menjadi halangan utama bagi proses ini dilaksanakan, terutamanya bagi bakteria psikrofilik obligat dan psikrotoleran. Bakteria dengan keupayaan toleransi oksigen juga lebih sesuai untuk fermentasi berskala besar. Oleh itu, terdapat keperluan untuk mencari bakteria toleransi oksigen yang mampu menghasilkan biohidrogen pada suhu mesofilik. Dalam kajian ini, sampel tanah dan air laut Antartika digunakan untuk pengasingan bakteria, sebelum disaring berdasarkan keupayaan penghasilan biohidrogen. Dua belas bakteria telah berjaya diasikan dan enam didapati mampu menghasilkan biohidrogen. Bakteria yang menghasilkan biohidrogen tertinggi dicirikan dan parameter fizikokimia optimum, seperti suhu, pH dan kepekatan karbohidrat ditentukan menggunakan kaedah satu-faktor-pada-satu-masa (OFAT). Sumber nitrogen yang sesuai, toleransi suhu dan kesan oksigen terlarut terhadap pertumbuhan dan produktiviti biohidrogen juga dikaji. Faktor optimum yang tepat untuk produktiviti biohidrogen turut diperiksa dengan menggunakan reka bentuk faktorial tiga-taha Kaedah Gerakbalas Permukaan (RSM). Bakteria yang menghasilkan biohidrogen tertinggi didapati berkait rapat dengan Klebsiella pneumoniae sebanyak 99% persamaan berdasarkan analisis 16S rRNA. Bakteria itu kemudiannya dinamakan sebagai Klebsiella sp. ABZ11. Bakteria ini adalah basilus Gram-negatif, tanpa kapsul dan hidup dalam julat suhu 20-40°C, dengan kadar pengambilan oksigen terlarut sebanyak 95% dalam masa dua jam. Penyaringan OFAT mencadangkan bahawa keadaan optimum untuk penghasilan biohidrogen ialah 30°C, pada pH awal 6.5 dan kepekatan glukosa 10 g/L. Bakteria ini menggunakan pelbagai jenis sumber karbon dan nitrogen untuk penghasilan biohidrogen, tetapi lebih memilih glukosa sebagai sumber karbon dan ekstrak daging sebagai sumber nitrogen. Pengoptimuman lanjut menggunakan RSM menunjukkan bahawa penghasilan biohidrogen tertinggi (110.15 mol/L) diperoleh pada 33.5°C, dengan pH awal 6.75 dan kepekatan glukosa 9.15 g/L. Kajian kinetik menunjukkan bahawa bakteria ini lebih banyak menggunakan glukosa untuk menghasilkan biohidrogen berbanding untuk pembentukan biojisim dalam proses fermentasi. Sebanyak 122 mol/L/g biohidrogen dan 0.87 g biojisim dihasilkan bagi setiap gram glukosa. Pada kesimpulannya penyelidikan skala besar pada keadaan yang optimum menunjukkan penghasilan biohidrogen sebanyak 137.56 mol/L dalam 36 jam dengan hasil kumulatif sebanyak 533.51 mol/L. Fermentasi kelompok menggunakan Klebsiella sp. ABZ11 di bawah suhu mesofilik membuktikan dapat memendekkan fasa lamban pertumbuhan dan meningkatkan kadar metabolisma, sehingga mempengaruhi pengeluaran biohidrogen yang lebih cepat dan tinggi.
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<td>DO</td>
<td>Dissolve oxygen</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>CO</td>
<td>Carbon monoxide</td>
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<tr>
<td>NOX</td>
<td>Nitrogen oxide</td>
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<tr>
<td>CCD</td>
<td>Central Composite Design</td>
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<tr>
<td>SO₂</td>
<td>Sulphur oxide</td>
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<tr>
<td>CH₄</td>
<td>Methane</td>
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<tr>
<td>PEM</td>
<td>Proton Exchange Membrane</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>GC/TCD</td>
<td>Gas Chromatography/ Thermal Conductivity Detector</td>
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<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<td>DCW</td>
<td>Dry Cell Weight</td>
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<tr>
<td>OD₆₀₀</td>
<td>Optical Density</td>
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<tr>
<td>DNA</td>
<td>Dioxiribonucleic acid</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>SPSS</td>
<td>Statistical Analysis</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobyte</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search</td>
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<thead>
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<tr>
<td>$\delta$</td>
<td>Minimal error</td>
</tr>
<tr>
<td>$D, d$</td>
<td>Diameter</td>
</tr>
<tr>
<td>$F$</td>
<td>Force</td>
</tr>
<tr>
<td>$v$</td>
<td>Velocity</td>
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<tr>
<td>$p$</td>
<td>Pressure</td>
</tr>
<tr>
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<td>Moment of Inertia</td>
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<tr>
<td>$r$</td>
<td>Radius</td>
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CHAPTER 1

INTRODUCTION

1.1 Problem Background

Energy is very important owing to its role as critical input in the creation of goods and services and in other human activities. Before the emergence of industrialised societies, the balance energy going into and out of the atmosphere was at equilibrium, as it was mainly recirculated between the naturally occurring plants and animals. This balance, however, was altered as a result of man’s reliance on fossil fuels (coal, petroleum, and natural gas) as the major sources of energy for domestic and industrial uses (see figure 1). This energy imbalance is brought about due to the increasing emission of toxic gases such as $\text{CO}_2$, $\text{NO}_x$, $\text{CO}$ and sulphur into the atmosphere (Bächtold, 2018). This indicates that global energy sources are largely dependent on coal, petroleum and natural gas.

Excessive reliance on these sources exposed the environment to dangers such as ozone depletion and drought. It also emphasizes the need to explore biological sources of renewable energy that are cost-effective and pose a minimum possible danger to the environment. Figure 1.1 presents the various energy sources and their percentage contribution to the global energy supply: Biohydrogen production and associated sources of energy supply constitute an insignificant 1% of the global energy supply. This status quo is not due to lack of potentials in the use of hydrogen as a fuel source, but due to the emergent state of research and development in the field. There is, therefore, the need for upscaling research and development activities in this field of enquiry.
At present, the world satisfies 80% of its energy needs from fossil sources that are associated with problems of greenhouse gas emission, climate change and environmental pollution (Xu et al., 2018). Gasification of coal and natural gas, as well as the burning of petroleum oil, release large quantities of greenhouse gases (e.g., black carbon and ozone) into the atmosphere. These gases are toxic and heat up the atmosphere. Also, the greenhouse gases return as sulphur back to the ecosystem in the form of acid rain, damaging building- roofs and other iron-containing material by making them rust.

Moreover, the gases have long-term adverse effects on human health, including respiratory, cardiovascular and cerebrovascular infections such as asthma, lung, colon and breast cancer, and heart diseases (Patz et al., 2005; Haines et al., 2009). In addition, toxic gases from fossil fuel exacerbate climate change problems directly linked to drought and famine. Currently, 800 million people have been estimated to be malnourished as a result of climate change-induced problems (McMichael, 2017). To mitigate the foregoing environmental, economic, and social problems, scientists are on the relentless search for better and safer sources of energy. One source that has so far shown promising potentials but has not been fully investigated is hydrogen.

Hydrogen is a timely option for fossil fuel owing to its high energy yield per unit mass. It yields 122kJ per gram, which is 2.75 times higher than the conventional fossil fuels (Singh and Wahid, 2015). Moreover, fermentative hydrogen production as
energy source generates water (H\textsubscript{2}O) and a small amount of NOx as by-products. The more fossil fuels are replaced with hydrogen, the greater will be the reduction of greenhouse gas emission (Huang and Tan, 2014). In fact, science has moved from a justification of hydrogen as an energy source to looking into ways hydrogen could be produced in commercial quantities and used on a sustainable basis. It is to contribute to this global scientific drive that this study was carried out. We investigated and reported on hydrogen production through microbial fermentation using bacteria isolated from a naturally low temperature (±5°C) environment.

Hydrogen as biofuel can be produced through microbial fermentation of naturally available waste materials. These materials are diverse, cheaper and eco-friendly compared to the energy from fossil fuel sources. Thus, biological methods of generating hydrogen as biofuel offer a better solution to a wide range of environmental problems associated with the conventional methods that rely on fossil sources. Biohydrogen production is not only a credible alternative to energy-need satisfaction but also a potent environmental conservation strategy for the reduction of wastes, a safer way for the degradation of many toxic organic substrates, and the promotion of a healthier atmosphere.

The success of biohydrogen generation through biological process is much dependent on the efficient microorganism in the system. Different types of bacteria from various environments have been evaluated for biohydrogen production. However, the efficiency of these bacteria in hydrogen production remains a challenge. This is due to their slow metabolic rate which negatively affects their capability to breakdown substrates for hydrogen production. This is evident in a prolonged carbohydrate uptake and hydrogen production under fermentative process demonstrated by this strain of bacteria (Gupta et al, 2016). Thus, studies are still focusing on the search for highly efficient bacteria from different environments for biofuel production.

The polar environment is inhabited by many organisms including bacteria, yeasts, fungi and algae. These microorganisms have undergone physiological adaptation and acquired specific enzymes needed to survive in the harsh Antarctic
environment. This adaptability greatly boosts their biochemical efficiency and activity similar to mesophilic microorganisms (De Maayer et al, 2014). They are often explored for commercial production of drugs, detergents and fertilizer due to possession of enzymes such as proteases, lipases, α-amylases, cellulases and β-galactosidase (Saxena, 2015). However, less attention has been given to the use of cold-adaptive bacteria in the generation of energy as an alternative replacement for fossil fuels. The increasing dangers of climate change and its potentially disturbing values warrants concerted effort at exploiting every available and environmentally friendly alternative energy source. Polar bacteria offer such an alternative

The potential of polar bacteria in hydrogen production is strategic in renewable energy production. Thus, they are viewed as a good biological source of renewable energy. Most importantly, the energy-saving potential and activity under low temperature associated with polar bacteria are characteristics that could enhance renewable energy production and its sustainability. Only a few psychrophilic bacteria from the polar environment have been investigated for biohydrogen production. However, the slow metabolic rate and carbohydrate uptake by psychrophilic bacteria reported remains problematic in fermentative hydrogen production. Specifically, psychrophilic bacteria are known for prolonged production start-up and carbohydrate take-up (Alvarez-Guzmán et al, 2016). Hence, the need for a cold-active bacterium with hydrogen production potential under moderate temperature for improved metabolic activity.

Generally, microorganisms grow very slowly at low temperatures. Low temperatures affect the metabolic rate and substrate degradation capabilities of microorganisms, resulting in low biogas yield in a typical fermentation process. Studies have shown that temperature does not only affect bacterial growth but will also affect the timeframe for growth. For instance, Dobrić and Bååth, (2018) investigated the effect of temperature on lag period and exponential growth of bacteria. They found that the lag phase was around 12 hours at 25°C and 30°C. However, the lag phase increased to almost 200 hours at 0°C. Since growth involved substrate intake to build cell components, this implies that temperature also impacts the rate of substrate breakdown and metabolic rate of enzymes involved in the process.
Similarly, Alvarez-Guzman et al. (2016) observed lag-phase of 20 h, 50 h and 20 h for Antarctic psychrophilic G088 strain with hydrogen production starting after 43 h, 21 h and 34 h for glucose, fructose and sucrose respectively. This prolonged growth lag-phase and hydrogen production start-up are linked to the fermentation at 20°C that affected the metabolic rate and breakdown of the substrates. Moderate temperature would have more influence on biogas productivity than low temperature since every 10°C increase in temperature has been shown to double the microbial growth and their metabolic rate (Robador et al., 2016). In support of this, Deepanraj, et al., (2015) reported high biogas production of 7556 ml with better biodegradation efficiency and reduced lag phase at 50°C compared to 30°C and 40°C in their investigation.

Biogas yield under a temperature above 20°C would be more favourable for biogas production due to the increase in the doubling time. Thus, ambient temperature condition can stimulate rapid production of hydrolytic enzymes by psychrotolerant bacteria for fast substrate degradation in order to generate more energy in the fermentative process (Morgan-Kiss et al., 2018; Saratale et al., 2018). This means that moderate temperature can be used to increase the productive capabilities of cold-active bacteria. Microorganisms with such capabilities are the psychrotolerant strains that proliferate in the polar environment and in seawater where the influx and ingestion of organic matter are greater. However, psychrophiles may not have such ability due to a mean cell turnover of about 1 year and a restricted growth temperature (Wang et al., 2018; Robador et al., 2016).

Psychrotolerant bacteria can thrive at a mesophilic temperature because of their natural physiological characteristics that give them the capability to survive at various temperature conditions and oxygen concentration. Thus, the exploration of hydrogen productivity of psychrotolerant bacteria is important because of their bioactivity at moderate temperature, which has been shown to inactivate psychrophilic strain in fermentative process. It is obvious that they will improve biohydrogen production by greatly enhancing hydrogenase activity compared to psychrophiles due to the influence of the moderate fermentation temperature. Hence, contributing to improvement of low
biohydrogen yield that has been the main constrain for its industrialization process (Xiao et al, 2013).

Psychrotolerant bacteria survive the harsh condition of polar environment and more abundant in that habitat. Oxygen tolerance (Sandle et al, 2013), ability to degrade vast nutrients and grow in vast pH condition, exchange of traits with mesophilic bacteria through plasmid (Dziewit and Bartosik, 2014). These potentials have not been intensively tapped in fermentative hydrogen production. This study is aimed at tapping these potentials of psychrotolerant bacteria to improve biohydrogen production through utilisation for biogas production at ambient condition. It is expected that these potentials will contribute significantly to production of hydrogen through utilisation of this strain of bacteria for production.

1.2 Problem Statement

Reliance on fossil fuels for energy supply has contributed immensely to global warming, environmental pollution and acid rain due to the enormous greenhouse gases often emitted into the atmosphere following the use of fossil fuel. Fermentative biohydrogen production through thermophilic bacteria is unsustainable renewable energy generation process owing to the high energy required.

The activity of psychrophilic microorganisms at low temperature has positive influence on biofuel production owing to the energy-saving and the sustainability properties. However, frequently reported slow metabolism and growth within a narrow temperature range (0-20°C) delay their biosynthetic characteristics.

In view of the foregoing, therefore, using psychrotolerant bacteria in hydrogen production offers better potentials as input in alternative energy production. This potential is feasible due to the improved substrate uptake of psychrotolerant bacteria and their adaptation to a wider temperature range (0-40°C). Since the temperature has been identified as a major factor that influences enzyme activity, this study relies on the temperature adaptability of the of psychrotolerant bacteria in tapping the maximum
biohydrogen producing potentials of these bacteria. Further, the bacteria rely on its adaptability characteristic to bring about improved substrate degradation through rapid metabolic activity, thereby improving their biohydrogen production.

1.3 Research Objectives

1 To isolate and characterise psychrotolerant bacteria isolated from Antarctica for biohydrogen production.

2 To evaluate the physicochemical conditions for biohydrogen production and determine the oxygen uptake capability of bacterium.

3 To optimise biohydrogen production using Central Composite Design of Response Surface Methodology (RSM).

1.4 Scope of the Study

The study covered the isolation of facultative psychrotolerant bacteria from Antarctic seawater. The bacteria obtained were screened for hydrogen production and the potential bacterium identified. Selected biochemical tests and screening were carried out to determine the virulent properties and oxygen take-up capability of the bacterium. Effects of different carbohydrate and nitrogen sources on the biohydrogen productivity of the bacterium were studied. Then optimization for biohydrogen production was finally examined by Central Composite Design (CCD) component of the Response Surface Methodology (RSM) design expert.

1.5 Significance of the Study

Cold-active bacteria have become attractive microorganisms for hydrogen production owing to their potential energy-saving activity at ambient-temperature
REFERENCES


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Kulkarni, H.M., Swamy, C.V. and Jagannadham, M.V. (2014). Molecular characterization and functional analysis of outer membrane vesicles from the


reducing microorganisms in polar, temperate and tropical marine sediments. *The ISME Journal*, 10(4), 796.


