# BIOHYDROGEN GENERATION BY DARK FERMENTATION OF STARCH USING BACTERIA ISOLATED FROM TAPIOCA WASTEWATER

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UNIVERSITI TEKNOLOGI MALAYSIA

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*Specially dedicated to my beloved husband, daughter, mother, father, supervisor and co-supervisor*

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# ABSTRACT

Hydrogen is a desirable alternative energy carrier of the future. Hydrogen can be sustainably produced by microorganisms through biological processes, such as fermentation. Hydrogen produced in this way is termed 'biohydrogen'. The amount of biohydrogen produced varies between genus and species of microorganisms and also depends on the substrate and experimental physicochemical conditions. Starch is a potentially good substrate that can be used for fermentative biohydrogen-producing bacteria. In this study, tapioca starch wastewater from tapioca processing factory was used as substrate. The aim of this study was to isolate bacteria with the ability to utilise tapioca starch wastewater and produce biohydrogen by dark fermentation. Tapioca wastewater and waste sludge were used as isolation source, giving 45 unique isolates. Fifteen isolates were found to be positive starch degraders. The best starch degrader was identified to be *Bacillus* sp. strain LFSF20 with GenBank accession number KY399968. However, this isolate is unable to produce biohydrogen. In addition, *Acinetobacter* sp. AY-SDB4 (accession number KY923069) was found to have both abilities starch degrader and biohydrogen producer. Biohydrogen production was measured using gas chromatography-thermal conductivity detector (GC-TCD). The starter culture medium contained tapioca starch at an initial concentration of 1.0 g/L, initial pH 5.5 with incubation carried out at 30°C. *Acinetobacter* sp. AY-SDB4 was subjected to further optimisations to investigate parameters affecting biohydrogen production, using one-factor-at-a-time (OFAT) method. The effects of incubation temperature (30°C, 35°C and 40°C), initial pH (5.0, 5.5 and 6.0) and initial starch concentration (0.5, 1.0 and 1.5 g/L) were investigated. Several parameters were analysed during the fermentation process, which are biohydrogen production, starch utilisation, reducing sugar content, cell growth, and α-amylase activity. Kinetic analysis of biohydrogen production by *Acinetobacter* sp. AY-SDB4 suggested that the optimum conditions for biohydrogen production to be at initial substrate concentration of 1.0 g/L, initial pH of 5.0 and incubation temperature of 30°C. At these conditions, the highest biohydrogen productivity obtained was $3.183 \times 10^{-3}$ mL/h, highest biohydrogen yield of 34.73 mL/g/L starch, and cumulative biohydrogen production of $19.8 \times 10^{-2}$ mL. These findings suggest that *Acinetobacter* sp. AY-SDB4 has the potential to be used to produce biohydrogen using starch wastewater as substrate.

**ABSTRAK**

Hidrogen adalah pembawa tenaga alternatif yang wajar dipertimbangkan untuk masa hadapan. Hidrogen boleh dihasilkan secara mampan oleh mikroorganisma melalui proses biologi, seperti penapaian. Hidrogen yang dihasilkan dengan cara ini dinamakan 'biohidrogen'. Jumlah biohidrogen yang dihasilkan berbeza-beza antara genus dan spesies mikroorganisma dan juga bergantung kepada keadaan fizikokimia substrat dan eksperimen. Kanji adalah substrat yang berpotensi tinggi untuk digunakan dalam penapaian menggunakan bakteria penghasil biohidrogen. Dalam kajian ini, sisa kanji ubi kayu dari kilang pemprosesan ubi kayu digunakan sebagai substrat. Tujuan kajian ini adalah untuk memencilkan bakteria dengan keupayaan untuk menggunakan air sisa kanji ubi dan menghasilkan biohidrogen melalui penapaian gelap. Air sisa ubi kayu dan lumpur sisa digunakan sebagai sumber pengasingan, memberikan 45 pencilan unik. Lima belas pencilan didapati positif pengurai kanji. *Bacillus* sp. LFSF20 telah dikenal pasti sebagai pengurai kanji yang terbaik, dengan nombor penerimaan GenBank KY399968. Namun, pencilan ini tidak dapat menghasilkan biohidrogen. Di samping itu, *Acinetobacter* sp. AY-SDB4 (nombor penerimaan KY923069) didapati mempunyai kedua-dua keupayaan sebagai pengurai kanji dan pengeluar biohidrogen. Pengeluaran biohidrogen diukur menggunakan kromatografi gas-pengesan konduktiviti haba (GC-TCD). Medium kultur pemula mengandungi kepekatan kanji ubi awal sebanyak 1.0 g/L, pH awal 5.5 dengan pengeraman dilakukan pada suhu 30°C. Pengoptimuman lanjut dijalankan ke atas *Acinetobacter* sp. AY-SDB4 untuk menyelidik parameter yang mempengaruhi pengeluaran biohidrogen, menggunakan kaedah satu-faktor-pada-satu-masa (OFAT). Kesan suhu inkubasi (30°C, 35°C dan 40°C), pH awal medium kultur (5.0, 5.5 dan 6.0) dan kepekatan awal kanji di dalam medium kultur (0.5, 1.0 dan 1.5 g/L) telah diselidiki. Beberapa parameter telah dianalisis semasa proses penapaian, iaitu pengeluaran biohidrogen, penggunaan kanji, pengeluaran gula, pertumbuhan sel, dan aktiviti enzim α-amilase. Analisis kinetik pengeluaran biohidrogen oleh *Acinetobacter* sp. AY-SDB4 yang telah dilakukan, menunjukkan keadaan optimum bagi pengeluaran biohidrogen berada pada kepekatan substrat awal 1.0 g/L, pH awal 5.0 dan suhu inkubasi 30°C. Pada keadaan ini, produktiviti biohydrogen tertinggi yang diperolehi adalah $3.183 \times 10^{-3}$ mL/j, hasil biohidrogen tertinggi 34.73 mL/g/L kanji, dan pengeluaran biohidrogen kumulatif sebanyak $19.8 \times 10^{-2}$ mL. Penemuan ini menunjukkan bahawa *Acinetobacter* sp. AY-SDB4 mempunyai potensi untuk digunakan untuk menghasilkan biohidrogen dengan menggunakan sisa kanji sebagai substrat.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| APHA | - | American public health association |
| ATP | - | Adenosine triophosphate |
| BOD | - | Biochemical oxygen demand |
| COD | - | Chemical oxygen demand |
| $C_6H_{12}O_6$ | - | Glucose |
| $CH_3CH_2CH_2COOH$ | - | Butyrate |
| $CH_3CH_2COOH$ | - | Propionate |
| $CH_3CH_2OH$ | - | Ethanol |
| $CH_3COOH$ | - | Acetate |
| $CO_2$ | - | Carbon dioxide |
| COD | - | Chemical oxygen demand |
| CS | - | Cassava starch |
| $H_2$ | - | Hydrogen |
| $H_2O$ | - | Water |
| HPR | - | Biohydrogen production rate |
| HY | - | Biohydrogen yield |
| $NAD^+$/NADH | - | Nicotinamide adenine dinucleotide |
| $N_2$ | - | Nitrogen |
| OD | - | Optical density |
| $O_2$ | - | Oxygen |
| RPM | - | Round per minute |
| SHPR | - | Specific biohydrogen production rate |
| VSS | - | Volatile suspended solid |

| | | |
|---|---|---|
| $RS_{max}$ | - | Maximum reducing sugar |
| $E_{max}$ | - | Maximum α-amylase activity |
| $X_{max}$ | - | Maximum cell weight |
| $\mu$ | - | Specific growth rate |
| $\mu_{max}$ | - | Maximum specific growth rate |
| $t_d$ | - | Doubling time |
| $Y_{P/S}$ | - | Yield of product on substrate |
| $Y_{X/S}$ | - | Yield of cell on substrate |
| $Y_{P/X}$ | - | Yield of product on cell |

# LIST OF SYMBOLS

| | | |
|---|---|---|
| °C | - | Degree Celsius |
| µL | - | Microliter |
| cm | - | Centimeter |
| ×g | - | Times gravity |
| g | - | Gram |
| g/g | - | gram/gram |
| g/L | - | gram/Liter |
| kg | - | Kilogram |
| kJ/g | - | Kilojoules/gram |
| L | - | Liter |
| M | - | Molar |
| mL | - | milliliter |
| mL/g | - | milliliter/gram |
| mL/h | - | milliliter/hour |
| mL/min | - | milliliter/minute |
| mm | - | milimeter |
| nm | - | Nanometer |
| v/v | - | volume/volume |
| w/v | - | weight/volume |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Research Background

The depletion of fossil fuels and current energy demands increase the need for an alternative source of energy supply. This new source must be clean, cheap and the process obtaining it should not contribute to the increase in carbon emission (low carbon footprint). This is because the production and utilization of conventional fossil fuels are causing various negative impacts on the environment, due to the emission of greenhouse gases into the atmosphere upon combustion. The accumulation of these gases in the atmosphere contributes to global warming and climate change.

Hydrogen is a desirable energy carrier, as an alternative to conventional fossil fuels. This is because when used in combustion process for energy conversion, it does not produce carbon-based emissions. Thus, hydrogen is a clean alternative energy source with high energy content per unit weight of 122 kJ $g^{-1}$ (Carlo *et al.*, 2008). This is reported to be 2.75 times higher than the energy content of conventional fossil fuels (Kapdan and Kargi, 2005). However, before the world could completely rely on hydrogen as fuel source, a feasible and sustainable way of producing it must be investigated. Environmentally friendly and economical are another criteria to be considered (Veziroğlu and Sahin, 2008). Hydrogen currently used for energy generation is from non-renewable sources, like natural gas (48%), heavy oil and nafta (30%), coal (18%), and also via electrolysis (4%) (Logan, 2004).

Hydrogen produced from biological sources is termed 'biohydrogen'. Biological processes have been suggested to be the clean and sustainable way of producing hydrogen. The processes that are commonly used to produce biohydrogen are: i) direct biophotolysis by green algae; ii) indirect biophotolysis by cyanobacteria; iii) photofermentation by anaerobic photosynthetic bacteria; iv) dark fermentation by anaerobic fermentative bacteria; v) water gas shift reaction using photosynthetic bacteria as biocatalyst; and vi) biocatalyst assisted photoelectrochemical hydrogen production (Uttam *et al.*, 2008).

Given the high amount of industrial and municipal wastes generated in Malaysia, fermentation technique is very attractive as the main method for biohydrogen production in the country. This allows for simultaneous waste treatment and energy generation. Bacterial fermentation for biohydrogen production has been widely investigated (Sen and Suttar, 2012; Sagnak *et al.*, 2011). Bacteria are versatile microorganisms, as they can utilize different types of carbon sources, and can work at ambient temperature and under normal pressure (Das and Veziroglu, 2001). Food processing industries in Malaysia release carbohydrate-containing wastewater (e.g. starch) which is potentially a good substrate that could be used for fermentative hydrogen-producing bacteria. The industries reported to release starch-containing wastewater are the sago factories (Phang *et al.*, 2000), distilleries (Krzywonos *et al.*, 2009) and the tapioca-processing industries around the towns of Batu Pahat and Ayer Hitam in Johor, Malaysia.

Many bacterial species reported that are able to produce hydrogen using dark fermentation method, such as *Enterobacter* sp., *Bacillus* sp., *Clostridium* sp., and *Citrobacter* sp., with carbohydrates as the preferred substrates (Levin *et al.*, 2004; Das and Veziroglu, 2001). But the amount of hydrogen produced varies between genus and species, in either pure or mixed cultures. These bacteria have been isolated from various sources, such as leaf extract (Kumar and Das, 2000), hydrothermal vent (Woodward *et al.*, 2000), industrial wastes (Chong *et al.*, 2009), domestic wastewater (Lu *et al.*, 2011) and organic wastewater (Oh *et al.*, 2003). Most of the studies used the starch-processing wastewater from industries as a substrate, but using inoculum from different sources. Inoculum from the same source has the potential to be a better culture system as it has adapted to the substrate environment. Andreani *et al.* (2015) reported a successful study of biohydrogen production using tapioca starch processing wastewater as substrate, with inoculum from the same source. Substrate composition and operating factors affecting biohydrogen production and chemical oxygen demand (COD) removal efficiency was also investigated.

Hence, this study seeks to investigate the potential of locally-isolated bacteria for biohydrogen generation using dark fermentation with tapioca starch-containing wastewater as substrate. Tapioca is usually referred to the starch produced from cassava plant (*Manihot esculenta*). Dark fermentation was chosen as this method has the potential to produce a high amount of biohydrogen using waste materials as substrates.

## 1.2 Significance of Research

This study will be of great interest to other researchers working on alternative methods for hydrogen generation specifically, and cleaner alternative energy sources generally. In addition, biohydrogen research is gaining attention in Malaysia, especially related to using industrial wastes, such as palm oil wastes and effluents. However, starch-containing wastewater as potential substrate for biohydrogen generation is not that well investigated yet, despite being produced by several industries in the country. This is possibly because of the challenge to identify suitable culture systems that are able to simultaneously degrade starch and produce biohydrogen.

This study used newly-isolated native bacteria from the same source as the substrate used in fermentation process, in order to obtain a robust culture able to survive in the actual wastewater. Furthermore, this project utilized tapioca starch wastewater generated by starch-processing industries that is normally untreated before being released. In a case study of one medium scale general food processing industry in Parit Raja, Johor, tapioca and cassava-based waste is among the largest waste type generated (25 kg of tapioca peels per month representing 25% of all food waste type generated) (Kadir *et al.*, 2017). It has been reported that for a small scale production of tapioca starch, from 1 tonne of fresh cassava roots, 12-20 $m^3$ of wastewater is produced, containing residual starch content (FAO, 2001). This residual starch in the wastewater has the potential to be converted into value-added products, like biohydrogen.

## 1.3 Scope of Study

The scope of this research is to isolate and identify bacteria able to produce biohydrogen using tapioca starch wastewater as substrate via dark fermentation process. The strains were characterized using standard microbiological procedures, and their ability to utilize starch as a carbon source and produce biohydrogen was investigated. Strains with good biohydrogen producing ability were subjected to partial 16S rRNA characterization to identify the genus and probable species. Biohydrogen production by the positive strain was then optimized at different pH, temperatures and substrate concentrations using batch culture.

## 1.4 Objectives of Study

1. To isolate and screen starch-utilizing bacteria from tapioca waste sludge using tapioca wastewater as substrate.
2. To screen the starch-utilizing bacteria for biohydrogen production using tapioca wastewater as substrate.

3. To identify the starch-utilizing, biohydrogen-producing bacteria using microbiological characterization and 16S rRNA analysis.
4. To optimize factors affecting biohydrogen production under batch culture using one-factor-at-a-time (OFAT).

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