



UNIVERSITI PUTRA MALAYSIA

**BIOHYDROGEN PRODUCTION FROM PALM OIL MILL EFFLUENT BY
LOCALLY ISOLATED *CLOSTRIDIUM BUTYRICUM* EB6**

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By

CHONG MEI LING

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
fulfilment of the requirements for the Degree of Doctor of Philosophy**

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Name of Supervisor : Prof Dr Mohd Ali Hassan

Faculty : Faculty of Biotechnology and Biomolecular Sciences

Hydrogen is a renewable, clean source of energy which has a great potential to be an alternative fuel. Abundant biomass from various industries could be a source for biohydrogen production where combination of waste treatment and energy production would be an advantage. Potential biomass that could be the substrates for biohydrogen generation include food and starch-based wastes, cellulosic materials, dairy wastes, palm oil mill effluent and glycerol. The objectives of this study were to isolate biohydrogen producing bacteria, to maximize the biohydrogen production in a synthetic medium and palm oil mill effluent (POME) and to improve the strain by overexpressing the hydrogenase gene in the host cell.

A biohydrogen producer was successfully isolated from anaerobic POME sludge. The strain, designated as *Clostridium butyricum* EB6, efficiently produced biohydrogen



during active cell growth. Controlled study was done on synthetic medium with 10 g/L glucose resulted in biohydrogen production at 948ml H₂/L-medium and volumetric biohydrogen production rate of 172 mL H₂/L-medium/h at initial pH 5.5. The supplementation of yeast extract at 4 g/L was found to have a significant effect with the highest biohydrogen production of 992 mL H₂/L-medium. The effect of pH on biohydrogen production from POME was investigated, with the optimum biohydrogen production ability at pH 5.5. The maximum biohydrogen production and maximum volumetric biohydrogen production rate were at 3195 mL H₂/L-medium and 1034 mL H₂/L-medium/h, respectively. The biohydrogen content in the biogas produced was in the range of 60 - 70%.

Optimization of biohydrogen production using synthetic medium was done on pH, glucose and iron concentration according to response surface methods (RSM) analysis. By central composite design (CCD) results, pH, glucose concentration and iron concentration were shown to significantly influence the biohydrogen gas production individually, interactively and quadratically ($P < 0.05$) with some exception. The CCD results indicated that pH 5.6, 15.7 g/L glucose and 0.39 g/L FeSO₄ was the optimum condition for biohydrogen production which gave a yield of biohydrogen at 2.2 mol H₂/mol glucose. For the confirmation experiment model, *t*-test result showed that experimental data curve had a high confidence at 95% with $t = 2.225$. Based on the results of this study, optimization of the culture condition for *C. butyricum* EB6 significantly increased the biohydrogen production.

Clostridium butyricum EB6 successfully produced hydrogen gas from POME. Central composite design and response surface methodology were applied to determine the optimum conditions for biohydrogen production (P_c) and maximum biohydrogen production rate (R_{max}) from POME. Experimental results showed that the pH, temperature and chemical oxygen demand (COD) of POME affected both the biohydrogen production and production rate individually and interactively. The optimum conditions for biohydrogen production (P_c) was pH 5.69, temperature 36°C and 92 g COD/L, with an estimated value of 306 mL H₂/g carbohydrate. The optimum conditions for maximum biohydrogen production rate (R_{max}) was pH 6.52, temperature 41°C and 60 g COD/L, with an estimated value of 914 ml H₂/ h. An overlay study was carried out to get an overall model optimization. The optimized conditions for the overall model was pH 6.05, temperature 36°C and 94 g COD/L.

[Fe]-hydrogenase (*hydA*) gene of *C. butyricum* EB6 was successfully amplified from the genomic DNA. Sequencing results of the *hydA* gene was identified with open reading frames of 1725 bp which encodes *hydA* of 574 amino acids with approximate size of 64 kDaltons. The *hydA* of *C. butyricum* was found 80.5% similar to *hydA* of *C. acetobutylicum* P262 and closely similar to *Clostridia* hydrogenase. A modified method of electroporation on *C. butyricum* EB6 was established for transformation of *hydA*. A *hydA*-expressing recombinant EB6 was successfully obtained with higher biohydrogen production from 4.2 L-H₂/ L-medium to 4.8 L-H₂/ L-medium compared to the wild type.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**PENGHASILAN BIOHIDROGEN DARIPADA SISA AIR PEMROSESAN
KELAPA SAWIT OLEH *CLOSTRIDIUM BUTYRICUM* EB6**

Oleh

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Hidrogen adalah sumber tenaga bersih, boleh diperbaharui dan mempunyai potensi yang besar sebagai sumber tenaga alternatif. Sumber biomass yang banyak dari pelbagai industri boleh dijadikan sebagai sumber penghasilan biohidrogen di mana kombinasi antara rawatan sisa dan penghasilan tenaga menjadi kelebihan untuk proses ini. Biomass yang mempunyai potensi menjadi substrat kepada penghasilan biohidrogen termasuk sisa makanan dan asas kanji, bahan cellulose, sisa tenusu, sisa buangan kilang kelapa sawit (POME) dan sisa gliserol. Objektif untuk kajian ini adalah untuk memencilkan mikroorganisma yang boleh menghasilkan hidrogen, mengoptimumkan penghasilan hidrogen daripada medium sintetik and sisa buangan kilang kelapa sawit (POME) and memperbaiki mikroorganisma yang terencil dengan memperbanyak gene hydrogenase dalam bacteria.

Satu penghasil biohidrogen telah berjaya dipencilkan daripada sisa rawatan POME. Bacteria ini, dikenalpasti sebagai *Clostridium butyricum* EB6, menghasilkan hydrogen secara efisien semasa pertumbuhan sel. Kajian kawalan telah dijalankan dengan menggunakan medium sintetik dan penghasilan hydrogen mencapai 948 mL H₂/L-medium dan kadar penghasilan biohidrogen mencapai 172 mL H₂/L-medium/h pada permulaan pH 6.0 dan 10 g/L glucose. Penambahan yis ekstrak didapatkan memberi kesan bermakna di mana penghasilan hidrogen tertinggi adalah 992mL H₂/L-medium semasa 4 g/L yis ekstrak ditambah. Kesan pH kepada penghasilan biohidrogen daripada sisa air kilang kelapa sawit (POME) juga dikaji. Keputusan eksperimen menunjukkan bahawa optimum kebolehan penghasilan biohidrogen adalah pada pH 5.5. Maksimum penghasilan hidrogen dan maksimum kadar penghasilan hidrogen adalah 3195 mL H₂/L-medium and 1034 mL H₂/L-medium/h. Peratus hidrogen yang didapat di biogas adalah dalma 60% ke 70%

Proses pengoptimasasi penghasilan biohidrogen dalam medium sintetik telah dilakukan ke atas pH, kepekatan glukosa dan zat besi melalui kaedah permukaan tindakbalas (RSM). Keputusan dari rekaan komposit pusat (CCD) menunjukkan bahawa pH, kepekatan glukosa dan zat besi mempengaruhi penghasilan biohidrogen secara individu, interaktif and kuadratik ($P < 0.05$) dengan beberapa pengecualian. Keputusan CCD menunjukkan pH 5.6, 15.7 g/L glukosa dan 0.39 g/L FeSO₄ adalah yang optimum untuk menghasilkan biohidrogen dengan hasil hidrogen sebanyak 2.2 mol H₂/mol glukosa. Untuk memastikan model eksperimen adalah betul, keputusan 't-test' menunjukkan model mempunyai keyakinan yang tinggi sebanyak 95% dengan $t = 2.225$. Berdasarkan

keputusan daripada kajian ini, keadaan kultur yang optimum bagi *C. butyricum* EB6 mempunyai peningkatan yang penting dalam penghasilan biohidrogen.

C. butyricum EB6 berjaya menghasilkan biohidrogen daripada sisa air kilang kelapa sawit (POME). Rekaan komposit pusat (CCD) dan kaedah permukaan tindak balas (RSM) diaplikasikan untuk mengenalpasti keadaan yang optimum untuk penghasilan hidrogen (P_c) dan kadar penghasilan hidrogen maksimum (R_{max}) daripada POME. Keputusan eksperimen menunjukkan pH, suhu dan keperluan kimia oksigen (COD) dari POME mempengaruhi penghasilan hidrogen dan kadar penghasilan secara individu and interaktif. Keadaan optimum untuk penghasilan hidrogen (P_c) adalah pH 5.69 dan suhu 36°C and 92 gCOD/L, dengan nilai anggaran pada 306 mL H₂/g karbohidrat. Keadaan optimum untuk kadar penghasilan hidrogen maksimum (R_{max}) adalah pH 6.52, suhu 41°C dan 60 gCOD/L dengan nilai anggaran 914 mL H₂/h. Kajian pertindihan telah dilakukan untuk mendapat optimum model keseluruhan. Keadaan optimum untuk model keseluruhan adalah pH 6.05, suhu 36°C dan 94 gCOD/L. kandungan hidrogen dalam biogas yang terhasil adalah dalam lingkungan 60-70%.

Gen [Fe]-hydrogenase (*hydA*) daripada *C. butyricum* EB6 telah berjaya diampifikasikan daripada DNA genomik. Keputusan jujukan nukleotida gen *hydA* telah dikenalpasti mempunyai 'open reading frames' sebanyak 1725bp yang mengkodkan 574 amino asid dengan anggaran saiz sebesar 64 kDaltons. *hydA* daripada *C. butyricum* EB6 dikenalpasti 80.5% serupa dengan *hydA* daripada *C. acetobutylicum* P262 dan serupa dengan Clostridia hydrogenase yang lain. Kaedah elektroporasi yang diupahsui untuk *C.*

butyricum EB6 telah digunakan untuk transformasi *hydA*. Satu recombinan sel telah berjaya didapati dengan mempunyai kenaikan penghasilan hidrogen sebanyak 4.2 L-H₂/L-medium kepada 4.8 L-H₂/L-medium berbanding *C. butyricum* EB6 induk.



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I certify that a Thesis Examination Committee has met on 14 July 2009 to conduct the final examination of Chong Mei Ling on her thesis entitled “Biohydrogen Production from Palm Oil Mill Effluent by Locally Isolated *Clostridium butyricum* EB6” in accordance with the Universiti Putra Malaysia [P.U(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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LIST OF ABBREVIATIONS

H ₂	-	Hydrogen
O ₂	-	Oxygen
CO ₂	-	Carbon dioxide
MPOB	-	Malaysia Palm Oil Board
POME	-	Palm oil mill effluent
SREP	-	Small Renewable Energy Power Programme
COD	-	Chemical oxygen demand
D	-	Dilution factor
VSS	-	Volatile suspended solid
CHO	-	Carbohydrate
RS	-	Reducing sugar
HRT	-	Hydraulic retention time
CPO	-	Crude palm oil
FFB	-	Fresh fruit bunch
BOD	-	Biological oxygen demand
SS	-	Suspended solid
rRNA	-	Ribosomal ribonucleic acid
ATP	-	Adenosine triphosphate
DCW	-	Dry cell weight
EtOH	-	Ethanol
DGGE	-	Denaturing gradient gel electrophoresis
CO	-	Carbon monoxide
NADH	-	Nicotinamide adenine dinucleotide
RCM	-	Reinforced clostridia medium
SEM	-	Scanning electron microscope
EDTA	-	Ethylenediaminetetraacetic acid
NaOAc	-	Sodium acetate
SDS	-	Sodium dodecyl sulfate



PCR	- Polymerase chain reaction
TS	- Total solid
GC	- Gas chromatography
DNA	- Deoxyribonucleic acid
NCBI	- National Center for Biotechnology Information
$C_6H_{12}O_6$	Glucose
CH_3COOH	Acetic acid
C_3H_7COOH	Butyric acid
RSM	Response surface methodology
CCD	Central composite design
ANOVA	Analysis of variance



LIST OF NOMENCLATURE

β_i = *i*th linear coefficient

β_{ii} = *i*th quadratic coefficient

β_{ij} = *ij*th interaction coefficient

β_o = offset term

$C_{H,i}$ = fraction of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current (%/100)

$C_{H,i-1}$ = fraction of hydrogen gas in the headspace of the bottle measured using gas chromatography in the previous intervals (%/100)

$e = 2.718281828$

H = cumulative hydrogen production (mL)

P = hydrogen production potential (mL)

P_c = biohydrogen production (mL H₂/ g carbohydrate)

R_m = maximum biohydrogen production rate (mL/h)

R_{max} = Biohydrogen production rate per liter medium (mL H₂/ h/ L)

$V_{G,i}$ = total biogas volumes in the current time intervals (mL)

$V_{G,i-1}$ = total biogas volumes in the previous time intervals (mL)

V_H the total volume of headspace in the reactor (mL)

$V_{H,i}$ = cumulative hydrogen gas volumes at the current (*i*) time intervals (mL)

$V_{H,i-1}$ = cumulative hydrogen gas volumes at the previous (*i-1*) time intervals (mL)

x_l = coded values of pH

