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

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By

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

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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 H_2/L-medium and volumetric biohydrogen production rate of 172 mL H_2/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 H_2/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 H_2/L-medium and 1034 mL H_2/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 quadratively (P<0.05) with some exception. The CCD results indicated that pH 5.6, 15.7 g/L glucose and 0.39 g/L $FeSO_4$ was the optimum condition for biohydrogen production which gave a yield of biohydrogen at 2.2 mol H_2/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 (Pc) was pH 5.69, temperature 36ºC and 92 g COD/L, with an estimated value of 306 mL H_2/g carbohydrate. The optimum conditions for maximum biohydrogen production rate (R_{max}) was pH 6.52, temperature 41ºC and 60 g COD/L, with an estimated value of 914 ml H_2/ 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 36ºC and 94 g COD/L.

[Fe]-hydrogenase (*hyd*A) gene of *C. butyricum* EB6 was successfully amplified from the genomic DNA. Sequencing results of the *hyd*A gene was identified with open reading frames of 1725 bp which encodes *hyd*A 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 *hyd*A. A *hydA*-expressing recombinant EB6 was successfully obtained with higher biohydrogen production from 4.2 L-H_2/ L-medium to 4.8 L-H_2/ 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 PEMPROSESAN KELAPA SAWIT OLEH *CLOSTIRIDUM 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 membaiki mikroorganisma yang terpencil 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 H_2/L-medium dan kadar penghasilan biohidrogen mencapai 172 mL H_2/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 H_2/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 H_2/L-medium and 1034 mL H_2/L-medium/h. Peratus hidrogen yang didapat di biogas adalah dalma 60% ke 70%

Proses pengoptimasasi penghasilan biohidrogen dalan 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 quadratik (P<0.05) dengan beberapa pengecualian. Keputusan CCD menunjukkan pH 5.6, 15.7 g/L glukosa dan 0.39 g/L $FeSO_4$ adalah yang optimum untuk menghasilkan biohidrogen dengan hasil hidrogen sebanyak 2.2 mol H_2/mol glukosa. Untuk memastikan model ekseperimen 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. butyriucm* 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 36ºC and 92 gCOD/L, dengan nilai anggaran pada 306 mL H_2/g karbohidrat. Keadaan optimum untuk kadar penghasilan hidrogen maksimum (R_{max}) adalah pH 6.52, suhu 41ºC dan 60 gCOD/L dengan nilai anggaran 914 mL H_2/h. Kajian pertindihan telah dilakukan untuk mendapat optimum model keseluruhan. Keadaan optimum untuk model keseluruhan adalah pH 6.05, suhu 36ºC 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 diamplifikasikan daripada DNA genomik. Keputusan jujukan nukleotida gen *hydA* telah dikenalpasti mempunyai ‘open reading frames’ sebanyai 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 diupahsuai untuk *C.*

butyricum EB6 telah digunakan untuk transformasi *hydA*. Satu recombinan sel telah berjaya didapati dengan mempunyai kenaikan penghasilan hidrogen sebanyak 4.2 L-H_2/ L-medium kepada 4.8 L-H_2/ 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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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosphy. The members of the Supervisory Committee were as follows;

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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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CHONG MEI LING

Date:

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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 = ith linear coefficient

β_{ii} = ith quadratic coeffient

β_{ij} = ijth 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 H_2/ g carbohydrate)

R_m = maximum biohydrogen production rate (mL/h)

R_{max} = Biohydrogen production rate per liter medium (mL H_2/ 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_1 = coded values of pH