UNIVERSITI PUTRA MALAYSIA

MOLECULAR PHYLOGENY AND CHARACTERISTICS OF METHANOGENS FROM A PALM OIL MILL ANAEROBIC TANK

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Philosophy of Doctrine

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To my family that I owe them each single moment of my life

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

MOLECULAR PHYLOGENY AND CHARACTERISTICS OF METHANOGENS FROM A PALM OIL MILL ANAEROBIC TANK

By

MEISAM TABATABAEI

February 2009

Chairman : Professor Mohd. Ali Hassan, PhD

Faculty : Biotechnology and Biomolecular Science

This study was set up to investigate the phylogeny of and characterize the methanogenic population in anaerobic tank for treating palm oil mill effluent. In this study, environmental DNA was extracted and purified from wastewater sludge by using a simplified and less time consuming procedure (Malaysian Patent Pending Number: PI20082842 filed on 30/07/2008) and the results obtained were compared to that of other three existing protocols i.e. Ogram *et al.*, Tsai and Olson, and Jacobsen and Rasmussen methods which are normally used for environmental samples. The DNA isolated from the palm oil mill anaerobic tank in FELDA Serting Hilir, was used for determining the molecular phylogeny of methanogenic archaea by using culture-independent analysis of the 16S rRNA genes amplified directly from sludge. Restriction fragment length polymorphism (RFLP) analysis, denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization (*FISH*) were also used in combination which made the present study, the first wide-scale study carried out in

Malaysia. 1260-bp 16S rRNA PCR products were cloned and sequenced. Phylogenetic analysis showed the microbes were closely affiliated with known cultured methanogenic Archaea, *Methanosaeta concilii.* Based on RFLP (*Hae*III) analysis, just a few clones (clone SamaliEB; Genebank Accession Number: EU580025) seemed to be new species or at least new strains of *Methanosaeta*. This was also confirmed by DGGE analysis which showed the presence of *M. concilii* and *Methanosarcina.* sp. *FISH* was carried out using specifically designed 16s rRNA probes to target methanogens and bacteria. The results were in line with DGGE analysis and revealed the presence of two types of methanogens including *M. concilii* and *Methanosarcina* sp. in the anaerobic tank. Quantitative *FISH* showed that *M. concilii* had a population of 1.4 x 10^8/ml of wastewater sludge, while *Methanosarcina* sp. was 2 x 10^5/ml of wastewater sludge. This could be the reason of failing to get it cloned as for each 1000 clones of *Methanosaeta,* there was just one clone of *Methanosarcina* and therefore, the probability of picking up a clone affiliated to *Methanosarcina* was approximately 0.1 %. *FISH* helped to elucidate the association of methanogens and bacteria together. The findings of this study helped to understand the microbial population of the anaerobic tank for treating POME in Malaysia. The results indicate that filamentous acetate-utilizing methanogens detected in the POME anaerobic tank belong to the genus *Methanosaeta* based on the cell-morphology, and the phenotypic and phylogenetic characteristics described above. The data obtained also suggest that *Methanosaeta* is the most abundant methanogen in POME anaerobic digestion and that it plays an important role in methane production from acetate and its optimum growth conditions should be considered when an attempt is made to treat

POME anaerobically. In future, these findings will provide the chance to optimize the anaerobic tank conditions to increase the methane production and “carbon oxygen demand” (COD) removal.

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

FILOGENI MOLEKULAR DAN PENCIRIAN METHANOGEN DARIPADA TANGKI ANAEROBIK DI KILANG MINYAK KELAPA SAWIT

Oleh

MEISAM TABATABAEI

Februari 2009

Pengerusi : Profesor Mohd Ali Hassan, PhD

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Kajian ini dijalankan untuk menyiasat filogenetik dan ciri populasi methanogenik di dalam tangki anerobik bagi merawat hasil buangan kilang kelapa sawit. Dalam kajian ini, DNA telah dipencilkan daripada sisa kumbahan dengan menggunakan kaedah yang ringkas dan menjimatkan masa (Malaysian Patent Pending Number: PI20082842 difailkan pada 30/07/2008), dan keputusan yang diperolehi telah dibandingkan dengan tiga kaedah yang sedia ada cth. kaedah Ogram *et al.*, Tsai and Olson, and Jacobsen and Rasmussen, di mana kaedah ini biasanya digunakan untuk sampel alam sekitar. Kumpulan Archaea (methanogen) dalam tangki anaerobik di perindustrian kelapa sawit Malaysia telah dikaji dengan menggunakan analisis 16s rRNA gene secara terus dari enapcemar dengan gabungan antara elektroforesis gel gradien nyahasli (DGGE), florescent *in situ* hybridization (*FISH*), mikroskop cahaya dan scanning elektron microscopy (SEM). Hasil 1260-bp 16 rRNA PCR telah diklonkan dan disusun.

Analisis felogenetik menunjukkan mikrob hampir menyerupai dengan kultur yang dikenali sebagai methanogenic Archae, *Methanosaeta concilii.* Keseluruhan susunan klon berdasarkan RFLP (*Hae*III), hanya beberapa klon (SamaliEB) adalah spesis baru dari *Methanosaeta* atau sekurang-kurangnye adalah strain yang baru, dan ianya telah dikenalpasti oleh analisis DGGE. DGGE menunjukkan kehadiran *M. concilii* dan *Methanosarcina.* sp. *FISH* telah dijalankan dengan menggunakan rekaan probe 16s rRNA yang spesifik untuk mengenalpasti methanogens dan bakteria dari keputusan adalah menyokong analisis DGGE and menyokong kehadiran dua jenis methanogen termasuk *M. concilii* dan *Methanosarcina* sp. dalam tangki anaerobik. Analisis kuantitatif *FISH* menunjukkan *M. concilii* mempunyai populasi sebanyak 1.4×10^8 manakala *Methanosarcina* sp. pula hanya 2.0×10^5. Ini mungkin menyebabkan kegagalan untuk menghasilkan klon kerana bagi setiap 1000 klon *Methanosaeta,* hanya ada satu klon *Methanosarcina*. Oleh itu kebarangkalian mendapat klon *Methanosarcina* hanya 0.1 %. Kaedah *FISH* telah membantu untuk lebih memahami hubungan antara methanogen dan bakteria. Kajian ini membantu untuk memahami dimensi mikrob di dalam tangki anaerobik untuk merawat POME di Malaysia buat pertama kalinya. Keputusan kajian ini menunjukkan filamentus bagi penggunaan asetat methanogens ditemui di dalam tangki anaerobik POME adalah dari genus *Methanosaeta* berdasarkan kepada morfologi sel, ciri fenotopik dan filogenetik yang telah diterangkan sebelum ini. Data yang diperolehi menunjukkan *Methanosaeta* adalah methanogen yang paling banyak didapati dalam penguraian anaerobik POME dan ia memainkan peranan penting dalam penghasilan gas metana dari asetat di mana keadaan optimum untuk pertumbuhan perlu dipertimbangkan apabila merawat POME

secara anaerobik. Pada masa hadapan, kajian ini dapat meningkatkan lagi peluang untuk mengoptimumkan keadaaan tangki anaerobik untuk meningkatkan lagi penghasilan gas metana dan mengurangkan permintaan oksigen kimia (COD).

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I certify that an Examination Committee met on ----------2009 to conduct the final examination of Meisam Tabatabaei on his Philosophy of Doctrine thesis entitled "molecular phylogeny and characterization of methanogens in palm oil mill anaerobic tank" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as Follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

MEISAM TABATABAEI

Date: February 2009

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LIST OF ABBREVIATIONS

A_{260}	absorption under 260 nm
A_{280}	absorption under 280 nm
bp	base pair
BLAST	basic logical alignment search tool
BOD	biological oxygen demand
BSA	bovine serum albumin
CCD	charge-coupled device
CDM	clean development mechanism
CDT	closed digester tank
COD	carbon oxygen demand
CPO	crude palm oil
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EFB	empty fruit bunches
FISH	fluorescence *in situ* hybridization
FFB	fresh fruit bunches
M. concili	*Methanosaeta* (*Methanothrix*) *concilii*
M. thermophila	*Methanosaeta thermophila*
MW	molecular weight
NCBI	National Center for Biotechnology Information, USA
PCR	polymerase chain reaction
POME	palm oil mill effluent

RDP	Ribosomal Database Project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
spp.	species
SSU	small subunit
tRNA	transfer ribonucleic acid
UV	ultra violet ray
UASB	up-flow anaerobic sludge blanket

Units

°C	degrees centigrade
× g	unit for measuring centrifugation force
g	gram
µl	microlitre
h	hour
kJ	kilojoule
l	liter
kg	kilogram
l	liter
mg	milligram
min	Minute
mm	millimeter

mL	milliliter
M	mollarity
Nm	nanometer
Pa	pascal
pM/µl	pico mol per microliter
s	second
$	United state dollar
V	voltage
w/	weight by volume

Common abbreviations

e. g.	for example
et al.	and others
i.e.	that is

Statistical terms

ANOVA	analysis of variance
DNMRT	duncan's New Multiple Range Test
P	probability
RCBD	randomized complete block design
SD	standard deviation
SE	standard error

Chemical elements and compounds

$AlCl_3 \cdot 6H_2O$	aluminium chloride hydrate
CO_2	carbon dioxide
$CaCl_2 \cdot 2H_2O$	calcium chloride hydrate
$CoCl_2 \cdot 6H_2O$	cobalt (II) chloride
CsCl	cesium chloride
$CuCl_2 \cdot 2H_2O$	copper(II) chloride hydrate
dH_2O	distilled water
ddH_2O	double distilled water
Fe	Iron
$FeSO_4 \cdot 7H_2O$	ferrous sulfate
H3BO3	boric acid
KH_2PO_4	potassium di-hydrogen phosphate
$MgCl_2.6H_2O$	magnesium chloride hydrate
$MnCl_2 \cdot 4H_2O$	manganese chloride hydrate
N_2	nitrogen
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
$Na_2MoO_4 \cdot 2H_2O$	sodium molybdate
$Na_2S \cdot 9H2O$	sodium sulfide hydrate

Na_2SeO3	sodium selenite
$Na_2WO_4 \cdot 2H_2O$	sodium wolframate hydrate
NH_4Cl	ammonium chloride
$NiSO_4 \cdot 6H_2O$	nickel sulfate
PBS	phosphate buffered saline
SDS	sodium dodecylsulfate solution
TE buffer	tris EDTA buffer
TRIS	tris (hydroxymethyl) aminomethane
$ZnCl_2$	zinc chloride