UNIVERSITI PUTRA MALAYSIA

ISOLATION, IDENTIFICATION AND IN-VITRO FERMENTATION ACTIVITY OF CELLULOLYTIC BACTERIA FROM THE GUT OF TERMITES

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By

MOHAMMAD RAMIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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I wish to dedicate this thesis to my beloved family; my father, my mother, Zohreh, Mahmood, Masoud and my wife Narges who always understand and give me loving support.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

ISOLATION, IDENTIFICATION AND FERMENTATION ACTIVITY OF CELLULOLYTIC BACTERIA FROM THE GUT OF TERMITES

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OCTOBER 2008

Chairman: **Professor Abdul Razak Alimon, PhD**

Faculty: **Agriculture**

Termites are known for their ability to digest high lignocellulolytic compounds, such as wood and fiber materials. Ruminants with the aid of their microorganisms are able to digest fiber materials, however the percentage of digestion is not so high. Therefore, the main objectives of this study were to isolate and identify cellulolytic bacteria from the termites gut and to determine the ability of these bacteria to improve the digestibility of fibrous feed materials by the rumen microflora using the *in-vitro* gas production technique. In this study, cellulolytic bacteria isolated from the gut of termites were used to mix with the rumen microflora on fiber material digestion. Termites were obtained from decayed plant materials and nests from different locations in the vicinity of Universiti Putra Malaysia (UPM). They were identified as the lower termite *Coptotermes curvignathus* (*Holmgren*) and the higher termite *Macrotermes gilvus* (Hagen). Cellulolytic bacteria from the gut of the lower termite; *Coptotermes curvignathus* (*Holmgren*) was isolated. The

isolates were cultured aerobically in a medium containing carboxymethyl-cellulose (CMC) at temperature of 30°C. The five isolates obtained were identified based on the Biolog reader chemical test, Bergy's Manual and 16S rRNA sequence homology. The species were identified as: *Bacillus cereus* (isolate 1), *Acinetobacter baumanni* (isolate 5), *Enterobacter aerogenes* (isolate 2), *Enterobacter cloacae* (isolate 3) and *Chryseobacterium kwangyangense* (isolate 4). The Gene Bank NCBI/EMBL accession numbers for the bacterial isolates are EU294508, EU332791, EU305608, EU305609, and EU169201 respectively. *Acinetobacter baumanni* isolate 5 is an aerobic bacterium, while the other four species are facultative anaerobes. The first *in-vitro* experiment by the gas production technique was conducted to examine the digestion and volatile fatty acid production by the five bacterial species grown in the rice straw medium. There were significant differences ($P<0.05$) in dry matter loss (DM) of rice straw and acetic acid concentration among the five bacterial species. *Acinetobacter baumanni* isolate 5 showed the highest fermentation activity (7.76 mM). The second *in-vitro* experiment also by gas production technique, which was conducted to determine the effect of adding rumen fluid microflora on rice straw digestion. The bacterial cultures were standardized to an OD of 0.5 (10^8 CFU/ml) before adding to the rumen fluid microflora. Rumen fluid was obtained from a fistulated cattle maintained on a grass diet. The facultative bacteria tested were *C. kwangyangense* isolate 4, *E. cloacae* isolate 3 and *E. aerogenes* isolate 2. Digestion of rice straw by rumen fluid microflora was determined with or without adding individual cultures of termites gut bacterial species. The parameters measured were pH, gas (volume), DM loss, acetic, propionic and butyric acid concentrations. The

rumen fluid treated with *E. aerogenes* isolate 2 showed the highest pH (6.76) when compared to the other treatments. The addition of *C. kwangyangense* isolate 4 showed the highest activity ($P<0.05$) for rice straw DM loss (50%), acetic (17.49 mM), propionic (7.02 mM) and butyric acid (1.67mM) concentration when compared to the other treatments. The lowest fermentation activity was obtained in untreated rumen fluid microflora. A similar experiment was conducted with oil palm fronds as the growth substrate. There was a significant effect ($P<0.05$) of adding the three bacterial species (*E. cloacae*, *E. aerogenes* and *C. kwangyangense*) to the rumen fluid microflora for the DM loss of oil palm fronds. However, there was no significant difference among the bacterial isolates. On the other hand, the production of volatile fatty acids (VFA) was significantly ($P<0.05$) higher in the treatment with *C. kwangyangense* isolate 4 when compared to the other bacterial species. All the three bacterial species significantly ($P<0.05$) increased DM loss and VFAs production when added to rumen fluid microflora grown in rice straw or oil palm fronds.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Science

ISOLASI, IDENTIFIKASI DAN AKTIVITI FERMENTASI IN-VITRO BAKTERIA SELULOLITIK DARIPADA SALURAN MAKANAN ANAI-ANAI

Oleh

MUHAMMAD RAMIN

OKTOBER 2008

Pengerusi: **Professor Abdul Razak Alimon, PhD**

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Anai-anai telah diambil daripada bahan pokok yang mereput dan sarang-sarang di beberapa lokasi tertentu di Universiti Putra Malaysia (UPM). Ia telah dikenalpasti sebagai anai-anai peringkat rendah *Coptotermes curvignathus* (Holmgren) dan anai-anai peringkat tinggi *Macrotermes gilvus* (Hagen). Bakteria selulolitic daripada perut anai-anai peringkat rendah; *Coptotermes curvignathus* (Holmgren) telah diasingkan. Pencilan tersebut dikultur di dalam medium selulosa karbomektil (CMC) dan dibiarkan membiak secara aerobik pada suhu 30°C. Lima isolasi yang diambil di kenalpasti berdasarkan kepada bakteria selulolitic yang diambil daripada anai-anai peringkat rendah; telah diasingkan dan dikenalpasti berdasarkan kepada ujian bacaan kimia Biolog, Bergy's Manual dan jujukan homologi 16S rRNA. Pengenalpastian menggunakan prosedur-prosedur ini telah menunjukkan bakteria yang di isolasi adalah isolasi baru. Isolasi baru tersebut telah dinamakan seperti berikut: *Bacillus*

cereus isolasi 1, *Acinetobacter baumanni* isolasi 5, *Enterobacter aerogenes* isolasi 2, *Enterobacter cloacae* isolasi 3 dan *Chryseobacterium kwangyangense* isolasi 4. Nombor akses bank gen NCBI/EMBL untuk isolasi bakteria tersebut adalah EU294508, EU332791, EU305608, EU305609 dan EU169201. *Acinetobacter baumanni* isolasi 5 ialah bakteria aerobik, manakala empat spesis lain bersifat anaerobik fakultatif. Eksperimen pertama *in-vitro* dengan menggunakan teknik penghasilan gas telah dijalankan untuk mengkaji pencernaan dan penghasilan asid lemak meruap oleh lima spesis bakteria yang tumbuh di dalam substrat jerami padi. Terdapat perbezaan bererti ($P<0.05$) dalam kehilangan jerami padi dan kepekatan asid asetik di kalangan lima spesis bakteria. *Acinetobacter baumanni* isolasi 5 menunjukkan aktiviti fermentasi yang paling tinggi. Eksperimen *in-vitro* kedua juga menggunakan teknik penghasilan gas dan dijalankan untuk menentukan kesan penambahan bakteria perut anai-anai terhadap pencernaan jerami padi oleh mikroflora cecair rumen. Kultur bakteria telah dipiawaikan pada OD=0.5 (10^8 CFU /ml) sebelum ditambah kepada mikroflora cecair rumen. Cecair rumen mikroflora diperoleh daripada lembu berfistula yang kekal terhadap diet rumput. Bakteria fakultatif yang diuji adalah *C. kwangyangense* isolasi 4, *E. cloacae* isolasi 3 dan *E. aerogenes* isolasi 2. Pencernaan jerami padi oleh mikroflora cecair rumen telah ditentukan dengan atau tanpa menambah kultur individu, isolasi bakteria daripada perut anai-anai. Parameter yang diukur adalah pH, gas (isipadu), kehilangan BK, kepekatan asid asetik and propionik dan butirik. Cecair rumen dirawat dengan *E. aerogenes* menunjukkan pH terendah (pH 6.76) apabila dibandingkan dengna rawatan lain. Penambahan *C. kwangyangense* isolasi 4 menunjukkan aktiviti yang paling tinggi ($P<0.05$) untuk kehilangan BK jerami padi (50%), kepekatan asid asetik (17.49 mM), asid propionik (7.02 mM) dan asid butirik (1.67 mM) apabila

dibandingkan dengan rawatan lain. Aktiviti fermentasi terendah telah diperolehi dalam mikroflora cecair rumen yang tidak terawat. Eksperimen yg sama dijalankan menggunakan daun pelepah kelapa sawit sebagai substrat pertumbuhan. Terdapat paledzae bererti ($P<0.05$) apabila penambahan tiga isolasi bakteria kepada mikroflora cecair rumen untuk kehilangan BK pelepah kelapa sawit dilakukan. Walau bagaimanapun, tiada perbezaan bererti di kalangan bakteria isolasi. Penghasilan asid lemak meruwap (ALM) adalah bererti ($P<0.05$) dan lebih tinggi dalam rawatan menggunakan *C. kwangyangense* isolasi 4 apabila dibandingkan dengan isolasi bakteria lain. Ketiga-tiga spesis bakteria baru meningkatkan kehilangan BK dan penghasilan asid lemak meruwap dengan bererti apabila ditambah ke dalam mikroflora cecair rumen jerami padi atau pelepah kelapa sawit.

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I certify that an Examination Committee has met on 17 October 2008 to conduct the final examination of Mohammad Ramin on his Master of Science thesis entitled "Isolation, Identification and In-Vitro Fermentation Activity of Cellulolytic Bacteria from the Gut of Termites" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee were as follows:

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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 Master of Science. The members of the Supervisory Committee are as follows:

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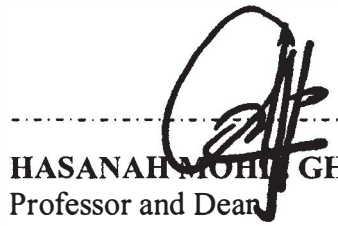

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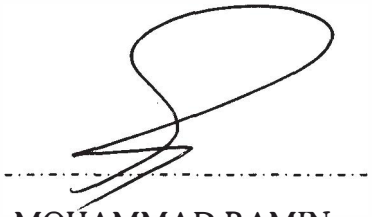
DECLARATION

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.



MOHAMMAD RAMIN

Date: 29/12/08

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LIST OF ABBREVIATIONS

IVDMD	*In-vitro* Dry Matter Digestibility
DM	Dry Matter
SEM	Standard Error of Mean
PCR	Polymerase Chain Reaction
GC	Gas Chromatography
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope
CMC	Carboxymethyl-cellulose
PTA	Phosphate Tungsten Acid
NCBI	National Center for Biotechnology Information
EMBL	European Molecular Biology Laboratory
DNA	Deoxyribonucleic acid
NA	Nutrient Agar
NB	Nutrient Broth
OD	Optical Density
CFB	*Cytophaga-Flavobacterium-Bacteroides*
VFA	Volatile Fatty Acid
CFU	Colony Forming Unit
NDS	Neutral-Detergent Solution
EDTA	Ethylenediamine Tetraacetic Acid
FID	Flame Ionization Detector
SCFA	Short Chain Fatty Acids
SAS	Statistical Analysis System
OPF	Oil Palm Fronds
ANOVA	Analysis of Variance
DFM	Direct Fed Microbial
PKC	Palm Kernal Cake
TSBA	Tryptone Soya Broth Agar

CHAPTER 1

INTRODUCTION

Termites are distributed throughout the world, but they are more abundant in tropical regions especially in countries like Malaysia (Ohkuma *et al*., 2001). There are two types of termites, namely lower or higher termites depending on their morphology and physiological characteristics (Yow, 1992). Both types of termites are important insects due to their ability to digest high fibrous materials which contain cellulose, hemicellulose and lignin (Harazona *et al.*, 2003).

The digestive tract of termites comprises of several compartments, such as fore gut, mid gut and hindgut, which the hind gut contains the intestinal microbiota and are initially considered as "fermentation chambers" (Brune and Friedrich, 2000). The "fermentation chamber" of the termite gut is analogous to the rumen of ruminants like sheep and cattle where the termite gut is considered as the smallest fermentation chamber when compared to the rumen of ruminants (Brune, 1998). The conditions of the rumen as well as the fermentation chamber of the lower termite provided by the host allows the prolific growth of microorganisms which include bacteria, protozoa and fungi (Wenzel *et al.*, 2002) where their main function is to digest fibrous feed materials (Brune, 2007).

Digestion occurs in two stages. Firstly the hydrolysis of cell wall compounds like cellulose and hemicellulose and secondly the fermentation of the products to short chain fatty acids such as acetic, propionic, and butyric acid, which then are absorbed

by the host as a main energy source (Breznak and Brune, 1994; Brune, 1998; Konig, 2006).

The *in-vitro* gas production technique has been well recognized as a tool for estimating fermentation activity of microorganisms and feed digestion under various conditions (Fievez *et al.*, 2005). Various feed materials and feed additives can be easily formulated and tested by using this technique.

Extensive studies have been conducted on rumen microflora and their fermentation activities (Hobson, 1988). It was reported that one of the main constraint in ruminant feeding is the poor digestibility of lignocellulose materials (Hobson, 1988) and certain feed additives such as *Saccharomyces cerevisiae* (Callaway and Martin, 1997; Lynch and Martin, 2002), *Aspergillus oryzae* fermentation extract (Beharka and Nagaraja, 1993) have been utilized to improve fermentation in the rumen. In this case the improvement is on the fermentation process rather on the break down of lignocellulotic materials. Hence, continuous efforts are needed to develop new strategies in improving complex polysaccharide digestion in the rumen. As such, termite gut cellulolytic bacteria which are well known for their high fermentation activity on lignocellulotic materials may have the potential as an alternative feed additive.

To date, there is a lack of information on the cellulolytic bacterial species present in the gut of local termites. Also, there is no study on the possibility of using termite's gut bacteria to improve fibrous feed digestion by using rumen fluid microflora.

Objectives

Therefore, the main objectives of this study were to isolate cellulolytic bacteria from the termite's gut and to determine the ability of these bacteria to improve the digestibility of fibrous feed materials by the rumen microflora using the *in-vitro* gas production technique.

The specific objectives were to:

- collect termites found in the vicinity of UPM and to identify their species
- isolate and identify cellulolytic bacteria from the termites gut
- determine the effects of adding termites bacteria to rumen fluid microflora on fibrous feed digestion *in-vitro*

CHAPTER 2

LITERATURE REVIEW

2. 1 Termites

Termites are insects commonly called white ants, from the order Isoptera which originates from the Greek word, where *'isos'* means equal and '*pteron*' means wing which refers to the two pairs of identical wings in the adult (Thorne and Carpenter, 1992). They are small to medium with white to dark brown body (Varma *et al.,* 1994) as the soldier of the lower family group (*Coptotermes curvignathus*) is given in figure 2.1, which shows the yellow body color and a small length of the head capsule. Their similarity with ants is their shape and behavior. However, morphologically and phylogenetically they are very different from ants. Termites are more closely related to cockroaches rather than ants. Cockroaches and termites are examples of insects used for studying the role of symbionts for cellulose digestion by the microorganisms which live in their gut (Slaytor, 1992). Approximately 1900 living and fossil species of termites have been identified (Lee and Wood, 1971) and the fossil records indicated that termites originated 220 million years ago. Majority of termites have been found within tropical regions like Malaysia (Ohkuma *et al.,* 2001). Termites play a significant role in decomposing lignocelluloses by degrading wood and dead trees and also in soil fertilization (Varma *et al.,* 1994).

Figure 2.1. Soldier termite (Lower family)
With permission from Mr. Cheong Yew Long – Bar: 1mm

2. 1. 1 Termite Classification

Termites can be classified into six families and fifteen subfamilies as shown in Table 2. 1 (Lee and Wood, 1971). Some families are divided to sub families.

Table 2.1. Classification of termites

Family	subfamily
Mastotermitidae	–
Kalotermitidae	–
Hodotermitidae	*Termopsinae, Stolotermitinae* *Porotermitinae, Cretatermitinae* (fossil) *Hodotermitinae*
Rhinotermitidae	*Psammotermitinae, Heterotermitinae* *Stylotermitinae, Coptotermitinae* *Termitogetoninae* *Rhinotermitinae*
Serritermitidae	–
Termitidae	*Amitermitinae, Termitinae* *Macrotermitinae* (fungus growing termite) *Nasutitermitinae*

(Source: Lee and Wood, 1971)

The first five families are known as lower termites and the sixth family (Termitidae), which includes approximately 75% of the known species and is the evolutionary