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IMPROVEMENT OF AGROBIOMASS QUALITY AND LOVASTATIN PRODUCTION FOR INHIBITION OF METHANOGENESIS BY Aspergillus terreus

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By

MOHAMMAD FASELEH JAHROMI

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

April 2012

DEDICATION

TO MY FAMILY

MY WIFE PARISA AND MY MOTHER

MY BROTHERS AND MY SISTERS

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
In fulfilment of the requirement for the degree of Doctor of Philosophy

IMPROVEMENT OF AGROBIOMASS QUALITY AND LOVASTATIN PRODUCTION FOR INHIBITION OF METHANOGENESIS BY *Aspergillus terreus*

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April 2012

Chairman: Liang Juan Boo, PhD

Institute: Bioscience

Methane (CH_4) is a greenhouse gas with 23 to 25 times greater warming effect than carbon dioxide on the ecosystem. Agricultural activities contributes 40% of total anthropogenic source of CH_4 with 15 to 20% produced by enteric fermentation from ruminant animals. At the same time, huge quantities of agricultural byproducts are produced annually and because of their high fiber content, these byproducts are of limited use as animal feed. The objective of this thesis was to elucidate the use of *Aspergillus terreus* as a biological agent to mitigate methanogens activity.

The potential of two strains of *A. terreus* (ATCC 20542 and ATCC 74135) to produce lovastatin in solid state fermentation (SSF) using rice straw (RS) and oil palm frond (OPF) as substrates was investigated. Results of this study showed that RS was a better

substrate than OPF for lovastatin production (with maximum production of 157.07 mg/kg DM using *A. terreus* ATCC 74135) and reduction of cellulose and hemicellulose content. In a follow-up study, different factors (moisture, temperature, amount of inoculum, pH and incubation time) known to affect SSF process were optimized for lovastatin production by both strains of *A. Terreus* using RS as substrate. A combination of 25°C incubation temperature, 10% inoculum size, pH 6, 50% initial moisture content and 8 days fermentation time provide the best condition for lovastatin production with the maximum yield of 260.85 mg lovastatin/kg DM.

The ability of *A. terreus* (ATCC 74135) to produce cellulolytic enzymes and to reduce lignocelluloses content of RS in SSF using the optimized condition obtained in the previous experiment was investigated in Chapter 4. Results of the study suggested that 8 days fermentation was suitable for production of the required cellulolytic enzymes. Fungal treatment significantly reduced neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose and hemicelluloses contents of RS by 19.96, 13.8, 16.32 and 32.87%, respectively, and resulted in significant increase in content of reducing sugar in the treated RS.

Lovastatin is an inhibitor of HMG-CoA reductase, a key enzyme responsible for the production of isoprenoid which is a component of the membrane in Archaeal cell, thus lovastatin can be used as an anti-methanogenesis agent. Effect of pure lovastatin and fermented rice straw extract (FRSE) containing lovastatin on pure culture of methanogenic Archaea (*Methanobrevibacter smithii*) was investigated in the third

experiment (Chapter 5). Results of this experiment showed that both treatments significantly reduced the growth (optical density of 0.390 for control, 0.065 for 50 µg/ml lovastatin and 0.031 for 500 µg/ml FRSE), CH_4 production (8.67%, 0.31% and 0% for control, 50 µg/ml lovastatin and 500 µg/ml FRSE treatments, respectively) and methanogenesis activity of *M. smithii*. However, lovastatin in the FRSE which primarily made up of the more active hydroxyl form, recorded stronger suppression on the growth of *M. smithii* and CH_4 production**.** Both treatments (pure lovastatin and FRSE) significantly increased the expression of HMG-CoA reductase gene in *M. smithii* (6.92 and 9 fold increased in 10 µg/ml lovastatin and 100 µg/ml FRSE treatments, respectively)**.** Transmission Electron macrographs showed that lovastatin distorted the morphological structure in *M. smithii* which could be due to the inhibition of isoprenoid production that is involve in the phospholipids formation in the cell membrane of this microorganism.

In the final experiment (Chapter 6), *in-vitro* gas production technique was used to study the effect of fungal treated RS containing lovastatin on mixed culture of rumen microbiota. Fermented rice straw (FRS) significantly reduced total gas (from 55.9 mL for control to 47.0 mL for FRS) and CH_4 productions (from 281.148 µM for control to 47.0 µM for FRS), ratios of CH_4:gas (from 0.113 for control to 0.102 for FRS), gas:VFA (from 0.965 for control to 0.862 for FRS) and CH_4:VFA (from 0.054 for control to 0.044 for FRS). *In-vitro* dry matter digestibility of FRS (49.01%) was significantly higher than that for the untreated RS (45.81%). The population of total methanogenic bacteria and fungi in the FRS treatments was significantly lower than those in the

control but population of *Ruminococcus albus* (cellulolytic bacteria) increased significantly in the FRS treatments.

Results of this thesis showed that lovastatin can be produced from agro-biomass such as RS using *A. terreus* ATCC 74135 in SSF. Besides reducing the fiber content of RS which resulted in higher nutrients digestibility, the FRS also contain higher protein content (due to increased fungal cell-mass) and cellulolytic enzymes which may continue to be active in the rumen of host animals. In addition, lovastatin in the FRS can effectively reduce methanogenesis, primarily through the inhibition of HMG-CoA reductase in methanogenic Archaea. Results of this thesis also showed that efficiency of methanogenesis inhibition by the FRS is higher than pure lovastatin due to the higher quantity of the more active hydroxyl form of lovastatin in the FRS. It can be concluded that fermentation of rice straw using *A. terreus*, is applicable method for enhancing the quality of this agro-biomass as ruminant feed and reduction of ruminal methane production.

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

PENAMBAHBAIKAN KUALITI BIOJISIM DAN PENGELUARAN LOVASTATIN SEBAGAI BAHAN PERENCAT METHANOGENESIS OLEH *Aspergillus terreus*

Oleh

MOHAMMAD FASELEH JAHROMI

April 2012

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Metana (CH_4) ialah sejenis gas rumah hijau yang mempunyai kesan pemanasan 23 hingga 25 kali lebih besar dari karbon dioksida ke atas ekosistem. Aktiviti pertanian menyumbang sebanyak 40% dari jumlah keseluruhan sumber CH_4 yang mana 15 hingga 20% dihasilkan dari proses fermentasi oleh haiwan ruminan. Pada masa yang sama, sejumlah besar hasil sampingan pertanian dihasilkan sepanjang tahun dan disebabkan oleh kandungan fiber yang tinggi, hasil sampingan ini tidak dapat dimanfaatkan sepenuhnya sebagai makanan haiwan. Objektif tesis ini adalah untuk menjelaskan kegunaan *Aspergillus terreus* sebagai agen biologi untuk memecahkan lignoselulose dalam bahan pertanian dan menghasilkan lovastatin yang mempunyai aktiviti anti metanogen.

Potensi dua jenis strain *A. terreus* (ATCC 20542 dan ATCC 74135) menghasilkan lovastatin dalam fermentasi fasa pepejal (SSF) menggunakan jerami padi (RS) dan pelepah kelapa sawit (OPF) sebagai substrat telah dikaji. Hasil dari kajian ini menunjukkan RS merupakan substrat yang lebih baik berbanding OPF bagi penghasilan lovastatin (dengan penghasilan maksima sebanyak 157.07 mg/kg DM menggunakan *A. terreus* ATCC 74135) dan penurunan kandungan sellulosa dan hemiselulosa. Dalam kajian lanjutan, pelbagai faktor (kelembapan, suhu, jumlah inokulum, pH dan masa pengeraman) yang diketahui memberi kesan kepada proses SSF telah dioptimakan bagi penghasilan lovastatin oleh kedua-dua strain *A. terreus* menggunakan RS sebagai substrat. Kombinasi suhu pengeraman 25°C, saiz inokulum 10%, pH 6, kandungan kelembapan 50% dan masa fermentasi 8 hari merupakan keadaan yang terbaik untuk penghasilan lovastatin dengan penghasilan maksimum sebanyak 260.85 mg lovastatin/kg DM.

Keupayaan *A. terreus* (ATCC 74135) menghasilkan enzim sellulolitik dan mengurangkan kandungan lignoselulose RS dalam SSF menggunakan keadaan optima yang diperoleh di dalam eksperimen yang lepas telah dikaji dalam Bab 4. Keputusan kajian mencadangkan yang fermentasi 8 hari adalah sesuai untuk penghasilan enzim sellulotik yang diperlukan. Perawatan menggunakan kulat adalah berkesan untuk menurunkan kandungan neutral detergen fiber (NDF), asid detergen fiber (ADF), sellulosa dan hemisellulosa dalam RS sebanyak 19.96, 13.8, 16.32 dan 32.87% masing-masing, dan menunjukkan peningkatan ketara dalam kandungan gula penurun dalam RS terawat.

Lovastatin dikenali sebagai bahan perencat kepada penurun HMG-CoA, enzim utama berperanan dalam penghasilan isoprenoid yang merupakan komponen dalam sel membran Archaeal, oleh itu lovastatin boleh digunakan sebagai agen anti-methanogenesis. Kesan lovastatin tulen dan ekstrak jerami padi terfermentasi (FRSE) mengandungi lovastatin dalam kultur tulen methanogenic Archaea (*Methanobrevibacter smithii*) telah dikaji dalam eksperimen ketiga (Bab 5). Keputusan kajian ini menunjukkan kedua-dua rawatan adalah berkesan dalam menurunkan pertumbuhan (OD sebanyak 0.390 bagi kawalan,0.065 bagi 50 µg/ml lovastatin dan 0.031 bagi 500 µg/ml FRSE), penghasilan CH_4 (8.67%, 0.31% dan 0% bagi kawalan, 50 µg/ml lovastatin dan 500 µg/ml FRSE rawatan) dan aktiviti methanogenesis *M. smithii*. Walaubagaimanapun, lovastatin di dalam FRSE yang dihasilkan dari bentuk hidrosil yang lebih aktif, menunjukkan penekanan yang lebih kuat kepada pertumbuhan *M. smithii* dan penghasilan CH_4. Kedua-dua rawatan (lovastatin tulen dan FRSE) menunjukkan peningkatan berkesan terhadap gen penurun HMG-CoA dalam *M. smithii* (Sebanyak 6.92 dan 9 kali ganda ditambah dalam rawatan yang menggunakan 10 µg/ml lovastatin dan 100 µg/ml). Makrograf Elektron Transmisi menunjukkan lovastatin mengubah struktur morfologi *M. smithii* yang mana mungkin disebabkan oleh penghasilan penyekat isoprenoid yang terlibat di dalam formasi phospholipids di membrane sel mikroorganisma ini.

Dalam eksperimen terakhir (Bab 6), teknik pengeluaran gas *in-vitro* digunakan untuk mengkaji kesan kulat dalam RS terawat yang mengandungi lovastatin pada campuran

kultur mikrobio rumen. Jerami padi terawat (FRS) berkesan menurunkan penghasilan gas secara keseluruhan (dari 55.9 mL bagi kawalan ke 47.0 mL bagi FRS) dan pengeluaran CH_4 (dari 281.148 µM bagi kawalan ke 47.0 µM bagi FRS), nisbah CH_4:gas (dari 0.113 bagi kawalan ke 0.102 bagi FRS), gas:VFA (dari 0.965 bagi kawalan ke 0.862 bagi FRS) dan CH_4:VFA (dari 0.054 bagi kawalan ke 0.044 bagi FRS). Penghadaman bahan kering *In-vitro* FRS (49.01%) lebih tinggi berbanding dengan RS tidak terawatt (45.81%). Populasi keseluruhan bakteria methanogenic dan kulat dalam rawatan FRS juga lebih rendah berbanding kawalan tetapi populasi *Ruminococcus albus* (bakteria sellulolitik) meningkat di dalam rawatan FRS.

Keputusan di dalam tesis ini menunjukkan lovastatin boleh dihasilkan dari biojisim pertanian seperti RS menggunakan *A. terreus* ATCC 74135 dalam SSF. Selain dari menurunkan kandungan serat dalam RS yang menunjukkan penghadaman nutrien yang lebih tinggi, FRS juga mengandungi kandungan protein yang lebih tinggi (disebabkan oleh peningkatan sel-jisim kulat) dan enzim sellulolitik yang mana mungkin aktif secara berterusan dalam haiwan rumen. Sebagai tambahan, lovastatin dalam FRS sangat efektif dalam menurunkan methanogenesis, pertamanya melalui penyekatan penurun HMG-CoA dalam Archaea metanogenik. Keputusan tesis ini juga menunjukkan keberkesanan perencat methanogenesis oleh FRS lebih tinggi berbanding lovastatin tulen disebabkan FRS mengandungi bentuk hidrosil yang lebih aktif dalam kuantiti yang lebih tinggi. Sebagai kesimpulan pemeraman RS menggunakan *A. terreus*, adalah merupakan kaedah yang boleh diaplikasikan untuk meningkatkan kualiti bahan sampingan pertanian ini sebagai makanan ruminan dan penurunan penghasilan metana dalam rumen.

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I certify that a Thesis Examination Committee has met on **2012** to conduct the final examination of **MOHAMMAD FASELEH JAHROMI** on her Doctor of Philosophy thesis entitled "**IMPROVEMENT OF AGROBIOMASS QUALITY AND LOVASTATIN PRODUCTION FOR INHIBITION OF METHANOGENESIS BY *Aspergillus terreus***" in accordance with the Universities Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. 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 fulfilment of the requirement for the degree of Doctor of Philosophy. 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 other institutions.

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MOHAMMAD FASELEH JAHROMI

Date: 19 April 2012

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LIST OF ABBREVIATION

ADF	Acid Detergent Fibre
ADL	Acid Detergent lignin
bp	Base pair
Cm	Centimeter
CT	Threshold cycle
DM	Dry matter
DNA	Deoxyribonucleic acid
FRS	Fermented rice straw
FRSE	Fermented rice straw extract
GLM	General linear model
Kg	Kilogram
Kg	Kilogram
Kpa	kilo pascal
Kv	Kilovat
L	Litter
mg	Milligram
mg/mL	milligram per milliliter
MgCl	Magnesium Chloride
Mic	Microliter
min	Minute
mL	Milliliter
mM	Millimolar
mm	Millimeter
Mmol	Millimole
NDF	Neutral Detergent Fibre

ng	Nanogram
nm	Nanometer
OPF	Oil palm frond
PCR	Polymerase chain reaction
Ppm	Part per million
RNA	Ribonucleic acid
RS	Rice straw
Rpm	Revolutions per minute
s	Seconds
SD	Standard deviation
UV	Ultraviolet
V	Volt
Vvm	volume per volume per minute

CHAPTER 1

GENERAL INTRODUCTION

Over the last 250 years, the concentration of atmospheric methane (CH_4) increased by approximately 150% (IPCC, 2007), with agricultural activities contributing 40% of the total anthropogenic source, of which 15 to 20% is from enteric fermentation in ruminants (Crutzen *et al.*, 1986). On the other hand, ruminal CH_4 production accounts for between 2 to 15% of dietary energy loss for the host animals (Moss, 1993). Because of the negative effects on environment and the host animal nutrition, mitigation of enteric CH_4 emission in ruminant livestock had been extensively researched, including the use of various mitigating agents such as ionophores (Wildenauer *et al.*, 1984), organic acids (Martin, 1998), fatty acids (Dohme *et al.*, 2001), methyl coenzyme M reductase inhibitors (Lee *et al.*, 2009), vaccine (Williams *et al.*, 2009) and oil (Mohammed *et al.*, 2004). However, these technologies have limited application primarily because they, besides suppressing CH_4 also decrease nutrients digestibility (such as oil and fatty acids), has negative effect on human and animal health (antibiotics) or high cost (methyl coenzyme M reductase inhibitors and vaccine).

Lovastatin ($C_{24}H_{36}O_5$, M.W. 404.55), a secondary product of fungi in their second phase of growth (idiophase) (Lai *et al.*, 2003), is an inhibitor of 3-hydroxy- 3-ethylglutaryl coenzyme A (HMG-CoA) reductase [EC 1.1.1.34], a key enzyme in cholesterol production pathway in human (Alberts, 1988). There is similarity in the biosynthesis

pathways of cholesterol in human and cell membrane in the Archaea. The lipid side of phospholipids in the cell membrane of Archaea is isoprenoid chains (Konrad *et al.*, 2002) while isoprenoid is an intermediate product of cholesterol production pathway (Mevalonate pathway) with HMG-CoA reductase as a key enzyme for production of this component (Smit *et al.*, 2000). Therefore, being an inhibitor of HMG-CoA reductase, lovastatin can suppress isoprenoid formation and thus methanogenesis in the Archaea. Using pure lovastatin, Wolin and Miller (2006) showed significant reduction in growth and activity of methanogenic Archaea without any negative effect on cellulolytic bacteria. Since pure lovastatin is too expensive to be used as feed additive, it thus has limited application for mitigation of CH_4 in ruminants.

Rice straw (RS) is one of the most important agricultural byproduct, with 90% production come from Asia (Karimi *et al.*, 2006). The traditional method for disposing RS after grain harvest is by burning (Summers *et al.*, 2001) resulting in environmental pollution. This agro-biomass is a potential feed for ruminant animals but its high lignocelluloses content is limiting its use. Biological treatment has been suggested to be a usable method for improvement the quality of agricultural biomass as ruminant feed (Alborés *et al.*, 2006).

Aspergillus terreus is a filamentous fungus able to produce cellulolytic enzymes for degradation of lignocelluloses components and has the ability for production of lovastatin in solid state fermentation (SSF) using rice, corn and wheat as substrates (Lai *et al.*, 2003; Gao *et al.*, 2008b; Jaivel *et al.*, 2010). These substrates are food and feed

for human and livestock, thus are too costly to produce lovastatin for mitigation of CH_4 in ruminants under farm conditions.

The primary objectives of this thesis were, firstly to investigate the efficacy of *Aspergillus terreus* for production of lovastatin using agricultural biomass as substrates in SSF, secondly to examine the extent of lignocelluloses breakdown in the agro-biomass and finally to elucidate the effect and mechanism of the produced lovastatin on ruminal CH_4 emission.

Specific Objectives

1. To evaluate the effect of different nitrogen source, substrate and fermentation condition on lovastatin production by *A. terreus* ATCC 20542 and 74135
2. To determine the ability of *A. terreus* to enhance the quality of RS as animal feed by reduction of lignocellulose content and production of cellulolytic enzymes.
3. To study the effect of lovastatin and fermented rice straw containing lovastatin on pure culture of methanogenic archaea.
4. To examine the effectivnes of fermented RS on inhibition of methanogenesis activity and reduction of ruminal methane production.

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