UNIVERSITI PUTRA MALAYSIA

FUNGAL SCREENING AND ISOLATION OF CELLULOLYTIC, MANNAN AND PROTEIN DEGRADING ENZYME PRODUCERS IN PALM KERNEL CAKE SOLID STATE FERMENTATION

MOHD. FAZLI BIN FARIDA ASRAS

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By

MOHD. FAZLI BIN FARIDA ASRAS

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

July 2009

DECLARATION

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

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MOHD. FAZLI BIN FARIDA ASRAS

Date: 2 July 2009

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfilment of the Requirement of Degree of Master Science

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July 2009

Chairman: Rosfarizan Mohamad, PhD

Faculty: Biotechnology and Biomolecular Sciences

Palm Kernel Cake (PKC), an agro-industrial by-product obtained after extraction of oil palm from oil palm seeds is used extensively in the animal feed industry but has limited used in poultry feed due to its high fiber and low protein contents. In this study, PKC was used as a substrate in solid state fermentation (SSF) by locally-isolated strains and their feasibility for cellulase and mannanase enzymes production were investigated. The potential isolates were obtained from various sources such as peat soil, rotten rice chaff, *'Tanah Bakar'*, rotten palm frond and raw PKC. The isolates were screened based on the clearing zone method and on selective agar media containing substrates such as locust bean gum (LBG), carboxymethylcellulose (CMC), potato dextrose, mannan and PKC.

Forty-eight fungal cultures have been screened and isolated based on the selective agars. Only thirty-one isolates were able to grow well after multi-subculturing techniques. The microbial activities of the isolates were accessed through clearing zone by chromogenic substrates such as Azo-carob galactomannan and Azo-CM cellulose. The diameter of clearing zone on the agar plate was observed every 24 h until 120 h.

Cultivation of the strains was carried out at 50% moisture content using shake flask and pre-germinated spores were preferred as the inoculum. The effects of cultivation conditions such as moisture level, inoculum concentration and agitation were investigated with the aim to achieve maximum production of cellulase and mannanase enzymes. Shaking at 120 rpm was found as the best agitation speed in the pre-germination process to be used as the inoculum. The samples were analyzed for neutral detergent fiber, acid detergent fiber, crude fiber and crude protein using Near Infrared Reflectance Spectroscopy analysis.

The best enzymes producer was fungal isolate D1 with specific enzyme exoglucanase activity of 17.9323 U/mg, specific enzyme endoglucanase activity of 41.6008 U/mg and specific enzyme β–glucosidase activity of 79.2626 U/mg using the pre-optimized conditions on the fifth day of fermentation process. About 50.1036 U/mg of specific enzyme mannanase activity was achieved on the fourth day of fermentation process using PKC as the substrate. The fibre degradation increased significantly. Neutral and acid detergent fibers were reduced from 85.16 to 21.72% on the sixth day and 45.18 to 17.18% at eighth day of fermentation process, respectively. The protein content

increased from 13.31 to 31.53% on the eighth day. Lower cellulase and mannanase enzymes activities were obtained in other isolates.

The highest cellulolytic and mannan-degrading enzymes producer was identified using microscopic. Under the microscopic view, isolate D1 was identified as *Aspergillus* sp. The identity of the isolate was further confirmed and belongs to *Aspergillus* sp. after observation under Scanning Electron Microscope (SEM). As a result, isolate D1 was identified as *Aspergillus* sp.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PEMENCILAN DAN PENYARINGAN KULAT PENGHASIL ENZIM BERSIFAT PENGURAI SELULOSA, MANNAN DAN PROTEIN DALAM ISIRUNG KELAPA SAWIT FASA FERMENTASI PEPEJAL

Oleh

MOHD. FAZLI BIN FARIDA ASRAS

Julai 2009

Pengerusi: Rosfarizan Mohamad, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Isirung Kelapa Sawit (IKS) adalah sisa buangan industri pertanian yang diperolehi selepas pengekstrakan minyak daripada buah kelapa sawit telah digunakan secara meluas dalam industri makanan ternakan tetapi penggunaannya adalah terhad dalam makanan ternakan berpunca daripada kandungan fiber yang tinggi dan protein yang rendah. IKS digunakan sebagai substrat dalam fermentasi fasa pepejal oleh pencilan tempatan dan dikaji kebolehannya untuk penghasilan enzim selulase dan mannanase. Pencilan yang berpotensi dipencilkan daripada pelbagai sumber seperti tanah gambut, tanah bakar, sekam padi dan tandan kelapa sawit yang telah reput serta isirung kepala sawit itu sendiri. Pencilan-pencilan itu disaring berdasarkan pada zon penjernihan dan juga pada media agar yang bersifat pemilih mengandungi substrat seperti *locust bean gum, carboxymethylcellulose (CMC), potato dextrose,* mannan dan IKS.

Empat puluh lapan pencilan dipencilkan dan disaring berdasarkan agar bersifat pemilih. Hanya tiga puluh satu pencilan boleh hidup dengan baik selepas teknik *multi-subculturing.* Aktiviti-aktiviti mikrob kultur tersebut dinilai melalui zon penjernihan pada substrat berwarna seperti Azo-carob galactomannan dan Azo-CM selulosa. Diameter zon penjernihan pada piring agar diperhatikan setiap 24 jam sehingga 120 jam.

Pengkulturan pencilan dilakukan pada kelembapan 50% menggunakan kelalang berkocak dan spora pra-percambahan diutamakan sebagai inokulum. Kesan keadaan pengkulturan seperti aras kelembapan, kepekatan inokulum dan pengocakan ditingkatkan dengan sasaran untuk mencapai penghasilan enzim selulase dan mannanase yang maksimum. Kelajuan 120 rpm dipilih sebagai kelajuan pengocak yang terbaik dalam proses pra-percambahan dan digunakan sebagai inokulum. Sampel dianalisa untuk serat neutral detergen, serat asid detergen, serat kasar dan protein kasar menggunakan analisis Spektroskop Pemantulan Cahayamerah Bersebelahan.

Penghasil enzim yang terbaik ialah pencilan D1 dengan aktiviti enzim exoglucanase spesifik 17.9323 U/mg, aktiviti enzim endoglucanase spesifik 41.6008 U/mg dan aktiviti enzim β–glucosidase spesifik 79.2626 U/mg pada hari kelima proses fermentasi menggunakan keadaan yang optimum. Sebanyak 50.1036 U/mg aktiviti enzim mannanase spesifik dicapai pada hari keempat proses fermentasi menggunakan IKS sebagai substrat. Penguraian serat meningkat dengan nyata sekali. Serat neutral dan asid detergen masing-masing menurun daripada 85.16 kepada 21.72% pada hari

keenam dan 45.18 kepada 17.18% pada hari kelapan. Aktiviti enzim selulase dan mannanase lebih rendah dicapai oleh pencilan yang lain.

Kulat penghasil enzim pengurai selulosa dan mannan dikenalpasti menggunakan pemerhatian secara mikroskopik. Di bawah pemerhatian mikroskopik, pencilan D1 dikenalpasti sebagai spesies *Aspergillus.* Pencilan ini dipastikan sebagai spesies *Aspergillus* melalui pemerhatian yang dibuat menggunakan Mikroskop Pengimbas Elektron.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as the fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Rosfarizan Mohamad, PhD
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Professor Arbakariya Ariff, PhD
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
HASANAH MOHD GHAZALI, Ph.D
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 October 2009

I certify that a Thesis Examination Committee has met on 2 July 2009 to conduct the final examination of Mohd. Fazli Bin Farida Asras on his thesis entitled "Fungal Screening and Isolation of Cellulolytic, Mannan and Protein Degrading Enzyme Producers in Palm Kernel Cake Solid State Fermentation" in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

Members if the Examination Committee were as follows:

Shuhaimi Mustafa, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Suraini Abd. Aziz, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mohd. Noor Abd. Wahab, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Kopli Bujang, Ph.D.
Professor
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
(External Examiner)

BUJANG KIM HUAT, Ph.D.
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 17 September 2009

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ABBREVIATIONS

ADF : Acid Detergent Fibre

ADL : Acid Detergent Fibre

AOAC : Association of Official Analytical Chemists

a_w : Water Activity

BLAST : Basic Local Alignment Search Tool

CF : Crude Fibre

CMC : Carboxymethylcellulose

CP : Crude Protein

CF : Crude Fibre

°C : Degree Celcius

DCP : Digestible Crude Protein

DE : Digestible Energy

DM : Dry Matter

DNS : Dinitro Salicylic Acid

DW : Dry Weight

EDTA : Ethylenediaminetetraacetic Acid

EE : Ether Extract

EFB : Empty Fruit Bunch

Fig. : Figure

LBG : Locust Bean Gum

LF : Liquid Fermentation

h : Hour

µm : Micrometer

ml : Mililiter

NAG : *N*-acetyl-glucosamine

NCBI : National Center of Biotechnology Information

NDF : Neutral Detergent Fibre

NFE : Nitrogen Free Extract

NIRS : Near Infrared Reflectance Spectrophotometer

nm : Nanometer

NMR : Nuclear Magnetic Resonance

NTG : Nitrosoguanidine

OUR : Oxygen Uptake Rate

PDA : Potato Dextrose Agar

PDB : Potato Dextrose Broth

PKC : Palm Kernel Cake

PKO : Palm Kernel Oils

POME : Palm Oil Mills Effluent

pNPG : 4-nitrophenyl-β-D-glucopyranoside

pNP : 4-nitrophenol

rpm : Rotation Per Minute

rRNA : Ribosomal RNA

SACGLM : Azo-Carob Galactomannan

SACMC : Azo-Cm Cellulose

SEM : Scanning Electron Microscope

SmF : Submerged Fermentation

SSF : Solid State Fermentation

SSU : Small Subunit

TCA : Trichloroacetic Acid

TDN : Total Digestible Nutrient

THAM :Tris-hydroxymethylaminomethane

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CHAPTER I

INTRODUCTION

The palm oil industry produces a considerable amount of solid waste by-products. These are in the form of fibers, shells and empty bunches discharged from the mills. Currently, shell and fiber are used extensively as fuel for the production of steam in the palm oil mills, combining waste disposal and energy recovery. After combustion, about 5% ash by weight is produced and must be disposed of by other means. Palm kernel cake (PKC), the major agro-industrial by-product of the palm oil industry in Malaysia, Thailand and Indonesia is another good source of energy and protein for ruminants (Setthapukdee *et al*., 1991). In 2006, Malaysia produced 2.20 million tonnes of PKC and exported 2.12 million tonnes (Malaysian Palm Oil Board, 2007).

Palm kernel cake is one of the oilseed by-products widely used in ruminant feeds. PKC being derived from an oil crop is expected to have relatively high oil content. In practice, however, the oil content of PKC depends on the efficiency of oil extraction from the kernel. The nitrogen free extract (NFE) contains variable quantities of sucrose, reducing sugars and starch. PKC is used to supply both energy and protein to animal. However, it is only sparingly used in poultry feeds because of high fiber content and low digestibility. Because of the high fiber content, the metabolizable energy content of PKC is very low. Furthermore, PKC protein has a poor amino acid balance, with lysine being a major limiting amino acid (Onwudike, 1996). Palm kernel cake being derived from an oil crop is expected to have relatively high oil content. In

practice, however, the oil content of PKC depends on the efficiency of oil extraction from the kernel.

The PKC comprises mainly of cell wall. This cell wall consists largely of polysaccharides such as mannan which is responsible for the low digestibility of PKC by poultry. In the total cell wall, mannose is the principle neutral sugar (56.4 %), followed by glucose (11.6 %), xylose (3.7 %) and galactose (1.4 %) (Anon. 2002). Many microorganisms are capable of decomposing celluloses and mannans; however, enzymes from fungi such as *Aspergillus niger* (Ademark *et al*., 1998), *Trichoderma reesei* (Arisan-Atac *et al*., 1993) and *Sclerotium rolfsii* (Gubitz *et al*., 1996) deserve the most attention. The main components of lignocelluloses are cellulose, hemicelluloses and lignin (Sjöströrn, 1981). In nature, lignocellulolytic microbes interact in mixed culture to degrade lignocellulose e.g., wood decay (Bayer and Lamed, 1992). Resembling the natural habitat of filamentous fungi growing on solid lignocellulosic particles; solid substrate fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free water (Murthy *et al*., 1993; Tengerdy, 1996).

Cellulose is known to consist of relatively easily accessible amorphous regions with few lateral interactions between the cellulose chains as well as of crystalline domains that are much harder to hydrolyze. Cellulosic materials can be decomposed into fermentable sugars, which can be converted into other valuable products such as ethanol, single-cell proteins, and hydrogen. Acid treatment and enzymatic hydrolysis are the most common ways to break down cellulose into glucose. Compared with acid