



UNIVERSITI PUTRA MALAYSIA

***PRODUCTION OF LIPASE AND RECOVERY OF BY-PRODUCTS IN SOLID
STATE FERMENTATION BY *Aspergillus niger* DSMZ 2466 USING PALM
KERNEL CAKE AS SUBSTRATE***

ANUSHA NAIR

FBSB 2014 30



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KERNEL CAKE AS SUBSTRATE**

By

ANUSHA NAIR

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

July 2014

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DEDICATION

.... Many things in life will catch your eye.....

.... but few will catch your heart...

I dedicate this thesis

To my wonderful and ever-loving parents, sister and in-laws who has always been my shoulder of happiness

To my husband, Sugumar Munisamy and children, Yeshvint and Danshikaa who has always been the pillar of my success

To my supervisor, Prof Dr. Arbakariya B. Ariff for his kind patience and the constant generosity in providing knowledge

To my advisor and immediate manager, Dr Hirzun Mohd. Yusof for endless support and motivations

And to my beloved friends; Dr. Lim Chin Ming, Ho Li Sim, Jaime Low Yoke Sum and Kua Shwu Fun for their constant support and encouragement.

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

PRODUCTION OF LIPASE AND RECOVERY OF BY-PRODUCTS IN SOLID STATE FERMENTATION BY *Aspergillus niger* DSMZ 2466 USING PALM KERNEL CAKE AS SUBSTRATE

By

ANUSHA NAIR

July 2014

Chairman: Professor Arbakariya B. Ariff, PhD

Faculty: Biotechnology and Biomolecular Sciences

Bacteria, yeast, and fungi are known potential producers of extracellular lipases. Fungal species are preferably cultivated in solid-state fermentation (SSF), while bacteria and yeast are usually cultivated in submerged fermentation (SLF). Lipase production in SLF systems has been well established. The use of SSF as a robust platform for upstream and downstream processing has yet to be reported. Thus, this research study focuses on the fungal growth morphology, fermentation and downstream processing of lipase as well as the analysis of other valuable products from the SSF system using palm kernel cake (PKC) as substrate.

Lipase production by several fungal strains (*Trichoderma viride* SDTC EDF 002, *Aspergillus niger* SDTC SRW-4, *Aspergillus terreus* DSMZ 5770 and *Aspergillus niger* DSMZ 2466) using SSF and SLF systems in 500 mL flask were investigated. Lipase production in SSF was substantially higher compared to SLF except for *Trichoderma viride* SDTC EDF 002. The highest lipase activities and total protein content were obtained in SSF with *A. niger* SDTC SRW-4 (44.43 U g^{-1} ; 176.36 mg g^{-1}) and *A. niger* DSMZ 2466 (42.05 U g^{-1} ; 176.28 mg g^{-1}). The highest lipase activity (15.34 U mL^{-1}) in SLF was also obtained with *A. niger* DSMZ 2466. Relationship studies showed positive correlation between spore diameter (quadratic, R^2 ; 0.999) and branch diameter (linear, R^2 ; 0.971) with lipase activity for both of these *A. niger* strains.

Further enhancement of lipase production (305.09 U g^{-1} ; 7.3-fold) in SSF from one the best performing fungal strain, *A. niger* DSMZ 2466 was achieved by

supplementing PKC with glucose (0.1%, w/w), NH_4NO_3 (0.05%, w/w) and palm oil (2%, v/w). Improvisation on extraction method showed that cellulase treatment and grinding of the solid fermented material as additional steps was able to increase enzyme recovery by 193% and 185%, respectively. The crude extracellular lipases extracted from SSF was purified using 10 kDa MWCO PLC membrane, QFF anion exchanger and gel filtration (Sephacryl S-200 HR) with a yield of 22% and purification fold of 44. Lipase from *A. niger* DSMZ 2466 has a molecular weight of 45.4 kDa, highly active and stable under moderate alkaline conditions (pH 6.0 to 8.0) and at moderate temperatures (35°C to 45°C). Citric acid (5.020 g kg⁻¹), malic acid (2.370 g kg⁻¹) and oxalic acid (1.435 g kg⁻¹) were present in the buffer extract of the fermented substrate. The total amino acid and reducing sugars in the solid fermented material after lipase extraction process showed increment of 2-fold and 3-fold, respectively. The substantial increment in all essential amino acids (EAAs) content in the fermented SSF substrate suggests its suitability to be used as animal feed ingredient.

The proposed SSF platform for lipase production in this study; in which low-cost agricultural by-product is used as substrate with the ability to utilize the solid fermented substrate as animal feed ingredient, has potential for industrial application. This platform is able to reduce the lipase production cost and also eliminate environmental problems related to solid agriculture wastes disposal. The potential added value of the fermented substrate for application as animal feed is another advantage of the SSF system for enzyme production as compared to SLF.

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

**PENGHASILAN LIPASE DAN PEMULIHAN PRODUK SAMPINGAN DALAM
FERMENTASI FASA PEPEJAL OLEH *Aspergillus niger* DSMZ 2466
MENGUNAKAN HAMPAS ISIRONG KELAPA SAWIT SEBAGAI
SUBSTRAT**

Oleh

ANUSHA NAIR

Julai 2014

Pengerusi: Professor Arbakariya Ariff, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Bakteria, yis dan kulat dikenali sebagai pengeluar berpotensi lipases ekstraselular. Spesies kulat biasanya dikultur dalam fermentasi substrat pepejal (FSP), manakala bakteria dan yis biasanya dikultur dalam keadaan fermentasi substrat cecair (FSC). Pengeluaran enzim lipase dalam sistem FSC telah mantap. Penggunaan FSP sebagai platform yang mantap untuk proses huluan dan hiliran masih belum dilaporkan. Oleh itu, kajian penyelidikan ini memberi tumpuan kepada morfologi pertumbuhan kulat, fermentasi dan pemprosesan hiliran lipase serta analisis produk berharga daripada sistem FSP dengan menggunakan hampas isirong kelapa sawit sebagai substrat.

Pengeluaran lipase oleh beberapa jenis kulat (*Trichoderma viride* SDTC EDF 002, *Aspergillus niger* SDTC SRW-4, *Aspergillus terreus* DSMZ 5770 dan *Aspergillus niger* DSMZ 2466) menggunakan FSP dan FSC dalam kelalang 500 mL telah dikaji. Daripada semua jenis kulat yang telah diuji, pengeluaran lipase dalam FSP adalah jauh lebih tinggi berbanding dengan FSC kecuali *Trichoderma viride* SDTC EDF 002. Aktiviti lipase tertinggi dan kandungan protein jumlah diperolehi dalam FSP adalah strain *A. niger* SDTC SRW-4 (44.43 g U^{-1} ; 176.36 mg g^{-1}) dan *A. niger* DSMZ 2466 (42.05 U g^{-1} ; 176.28 mg g^{-1}). Aktiviti lipase (15.34 U mL^{-1}) yang tertinggi dalam FSC juga diperolehi dengan *A. niger* DSMZ 2466. Kajian menunjukkan hubungan korelasi positif antara diameter spora (kuadratik, $R^2, 0.999$) dan diameter cabang (linear, $R^2, 0.971$) dengan aktiviti lipase bagi kedua-dua jenis *A. niger*.

Pengeluaran lipase (305.09 U g^{-1} ; 7.3 kali ganda) oleh kulat paling berprestasi tinggi, *A. niger* DSMZ 2466 telah ditingkatkan dengan menambah PKC dengan glukosa (0.1 % w/w), NH_4NO_3 (0.05 % w/w) dan minyak sawit (2%, v/w). Pengubahsuaian ke atas kaedah pengekstrakan menunjukkan bahawa rawatan selulase dan pengisaran bahan pepejal selepas fermentasi sebagai langkah proses penambahan dapat meningkatkan pemulihan enzim masing-masing sebanyak 193% dan 185%. Kaedah pengekstrakan lipase yang diekstrak daripada FSP telah dituliskan menggunakan membran 10 kDa PLC, kolum penukar anion dan penapisan gel (Sephacryl S-200 HR) menghasilkan 22% enzim dengan penulenan 44 kali ganda. Enzim lipase dari *A. niger* DSMZ 2466 mempunyai berat molekul sebanyak 45.4 kDa, sangat aktif dan stabil di bawah keadaan alkali yang sederhana (pH 6.0 hingga 8.0) dan pada suhu yang sederhana (35°C hingga 45°C). Penimbal substrat fermentasi juga dikesan mengandungi asid sitrik (5.020 g kg^{-1}), asid malik (2.370 g kg^{-1}) dan asid oksalik (1.435 g kg^{-1}). Jumlah asid amino dan gula terturun dalam bahan pepejal terfermentasi selepas proses pengekstrakan menunjukkan peningkatan masing-masing sebanyak 2 dan 3 kali ganda. Peningkatan yang besar dalam kandungan asid amino perlu dalam substrat FSP selepas fermentasi menunjukkan kesesuaiannya sebagai ramuan makanan haiwan.

Platform FSP yang dicadangkan dalam kajian ini bagi pengeluaran lipase, di mana bahan kos rendah digunakan sebagai substrat dan penghasilan substrat fermentasi pepejal sebagai ramuan makanan haiwan mempunyai potensi dalam bidang perindustrian. Platform ini juga mampu mengurangkan kos pengeluaran lipase dan juga memberi penyelesaian kepada masalah alam sekitar yang berkaitan dengan pelupusan sisa pertanian. Nilai potensi tambahan sistem FSP untuk pengeluaran enzim berbanding FSC adalah penggunaan substrat selepas fermentasi sebagai makanan haiwan.

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I certify that a Thesis Examination Committee has met on 9 July 2014 to conduct the final examination of Anusha Nair a/p Chandran on her thesis entitled "Production of Lipase and Recovery of By-products in Solid State Fermentation by *Aspergillus Niger* DSMZ 2466 using Palm Kernel Cake as Substrate" in accordance with the 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 Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Rosfarizan Mohamad, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Suraini Abd Aziz

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mahiran Basri

Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Jo-Su Chang

Professor
Department of Chemical Engineering
National Cheng Kung University
(External Examiner)

ZULKARNAIN ZAINAL, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 19 March 2015

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:

Arbakariya Ariff, PhD

Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Umi Kalsom Md Shah, PhD

Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Ling Tau Chuan, PhD

Professor
Faculty of Science
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LIST OF ABBREVIATIONS

ATPS:	Aqueous Two-Phase System
AKTA:	High Performance Purification System
CE:	Capillary Electrophoresis
EAA:	Essential Amino Acid
EFB:	Empty Fruit Bunch
ELSD:	Evaporative Light Scattering Detector
GC:	Gas Chromatography
HMM:	Hidden Markov Model
HPLC:	High Performance Liquid Chromatography
IC:	Ionic Chromatography
NEAA:	Non-essential Amino Acid
RQ:	Research Question
SLF:	Submerged Liquid Fermentation
SSF:	Solid State Fermentation
PDA:	Potato Dextrose Agar
PKC:	Palm Kernel Cake
PKM:	Palm Kernel Meal
POME:	Palm Oil Mill Effluent
PPF:	Palm Press Fibre
SEM:	Scanning Electron Microscope
TCA:	Tricarboxylic acid
UV:	Ultraviolet

CHAPTER 1

INTRODUCTION

Enzymes have emerged as one of the most important product from biotechnology industry leading the current demand of green and sustainable technology. 75% of the industrial enzyme market are occupied by hydrolytic enzymes such as proteases, lipases and amylases (Sharma *et al.*, 2001; Li and Zong, 2010). Lipases have numerous industrial applications from food technology to detergent and bioremediation which has urged researchers for the discovery of new processes in producing this enzyme at low cost.

Lipases are found in many species of animals, plants, and microorganisms. Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) are able to catalyze reaction on primary hydroxyl groups of triglyceride to release fatty acids from positions 1 and 3 to give free fatty acid and di- and, or monoglycerides. The benefits of lipases are high substrate specificity under mild conditions and in organic solvents. (Sharma *et al.*, 2001; Kempka *et al.*, 2008).

Microbial lipases can be produced using submerged liquid fermentation (SLF) or solid state fermentation (SSF). Most enzymes or approximately 90% of all industrial biocatalysts produced currently are using SLF system with genetically modified microorganisms (Contesini *et al.*, 2010) to obtain higher productivity which is of higher production cost. On the other hand with SSF system, agro-industrial residues were used as fermentation substrate due to their low cost, easy accessibility and high nutrient compositions (Mateos Diaz *et al.*, 2006; Azeredo *et al.*, 2007). However, SSF has some limitations in selection of microbes that are able to grow in low moisture conditions, and problems in controlling temperature, pH, humidity and air flow in the solid matrix (Contestini *et al.*, 2010). These parameters has to be optimized to obtain an effective and efficient process.

Carbon and nitrogen sources in the growth medium are among important factors to increase lipase production besides cultivation conditions of pH and temperature (Cihangir and Sarikaya, 2004). Carbon source is able to increase growth rate at early phases. Product formation that are growth-associated or partially growth-associated processes will benefit from these supplements because the production of primary metabolites are associated with good microbial growth (Pau and Omar, 2004). Therefore, the medium formulation that is required for optimal microbial growth for specific strains and the products will have to be determined.

The importance of good microbial growth leads to early fungal spore germination and the cell extension to a form hyphae which colonizes the substrate material. This growing hyphae tips able to secrete various enzymes (Wösten *et al.*, 1991) which then degrades large molecules in the substrate into smaller forms to be taken up by the fungi as nutrients. These fungal characteristics such as spore development, hyphae and branching patterns are able to be investigated and may influence product yield (Wösten *et al.*, 1991; Moukha *et al.*, 1993). However, accurate quantification of these complex morphologies is still a challenge in elucidating morphology and product formation relationship in SSF.

Several agricultural wastes that was experimented in SSF for lipase production includes rice bran (Venkata Rao *et al.*, 1993), olive cake and sugar cane bagasse (Cordova *et al.*, 1998), gingelly oil cake (Kamini *et al.*, 1998), babassu oil cake (Gombert *et al.*, 1999), wheat bran (Mahadik *et al.*, 2002), and *Jatropha curcas* seed cake (Mahanta *et al.*, 2008). In our study, the use of agricultural residue as fermentation substrate for SSF was focused on using palm kernel cake (PKC) which is a by-product from the extraction of palm kernels and produced in abundance. In accordance to Malaysian Palm Oil Board report, production of PKC in Malaysia was 2.2 million tons in 2007 and estimated to be 5 million tons worldwide. PKC contains 50% carbohydrate and 15-20% protein (Knudsen, 1997), and categorized as a medium quality animal feed due to the low nutrition content (O'Mara *et al.*, 1999; Ramachandran *et al.*, 2007).

Several reports has been published utilizing PKC as the SSF fermentation substrate such as for production of alpha-amylase (Ramachandran *et al.*, 2004), xylanase (Kheng and Omar, 2005), phytase (Roopesh *et al.*, 2006) and beta-mannanase (Abdeshahian *et al.*, 2010; Ab *et al.*, 2011). No studies has been conducted for production of lipases utilizing PKC in SSF system. Most of the reports focused on investigating the ability of the specific fungus to grow on PKC and optimizing the production of enzymes by nutrient supplementations.

SSF was also successful in the production of other secondary products such as organic acids, pesticides, and aromatic compounds (Virupakshi *et al.*, 2005; Botella *et al.*, 2007; Li *et al.*, 2011). The main reason that the microbial fermentation process is able to produce high yields of organic acids is due to the synthesis of tricarboxylic acid (TCA) pathway (Kapritchkoff *et al.*, 2006). Apart from the microorganism used, formations of these products were enhanced by the usage of agro-industrial residues (Koutinas *et al.*, 2007) that are rich in the nutrient composition and suitable to be used as fermentation substrate. More recently, due to the high abundance of this substrate, significant attention has been drawn to increase the nutritional value of PKC as animal feed using fermentation processes or supplementation of enzymes. This technique is employed to increase the amino acid composition in the solid media (Iluyemi *et al.*, 2006) and digestibility of the solid material.

The high suitability of PKC to be used as fermentation substrate in SSF and lower capital investment in SSF processes compared to SLF (Castilho *et al.*, 2000) suggest the need to investigate and develop an efficient SSF system for lipase production using PKC as the fermentation substrate. The use of a competitive platform such as SSF that uses low cost material as the main fermentation substrate may be able to bring higher returns in enzymes production.

The specific objectives of this study were:

- i. To compare the ability of various fungal strains (*A. niger* DSMZ 2466, *A. niger* SRW-4, *T. viride* EDF 002 and *A. terreus* DSMZ 5770) in producing lipase using SLF and SSF process utilizing palm kernel cake (PKC) as fermentation substrate
- ii. To analyze the relationship between morphological characteristics of the fungi and their ability in producing lipase in SSF
- iii. To formulate media based on palm kernel cake (PKC) as substrate with the supplements of nutrients for improvement of lipase production in SSF by the selected fungal producing strain, *A. niger* DSMZ 2466
- iv. To propose efficient lipase extraction methods from solid fermented material of SSF
- v. To analyze organic acids from liquid extracted from SSF grown with *A. niger* DSMZ 2466 using PKC as substrate
- vi. To identify the suitability of using the solid fermented material of PKC as animal feed by profiling the sugars and amino acids content

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