UNIVERSITI PUTRA MALAYSIA

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE LIPASE FROM AN EXTREMOPHILIC *BACILLUS SUBTILIS* NS 8

AKANBI TAIWO OLUSESAN

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE LIPASE FROM AN EXTREMOPHILIC *BACILLUS SUBTILIS* NS 8

AKANBI TAIWO OLUSESAN

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2010

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE LIPASE FROM AN EXTREMOPHILIC *BACILLUS SUBTILIS* NS 8

By

AKANBI TAIWO OLUSESAN

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

January 2010

Specially for my loving parents

Mr Philip Akintola Akanbi (J.P) and Mrs. Bolanle Grace Akanbi

and my siblings

Dr. Akanbi O.A., Abidemi, Bukky, Blessing and Kenny

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

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE LIPASE FROM AN EXTREMOPHILIC *BACILLUS SUBTILIS* NS 8

By

AKANBI TAIWO OLUSESAN

January 2010

Chairman: **Professor Dr. Nazamid Saari, PhD**

Faculty: **Food Science and Technology**

Lipase is one of the most versatile biocatalysts and has a wide biotechnological application particularly in the production of functional lipids. This work aimed at producing, purifying and characterizing thermostable lipase from an extremophilic *Bacillus subtilis* NS 8 isolated from Setapak hotspring.

Lipase production by an extremophilic *Bacillus* strain which has been previously identified by phenotypic methods and confirmed by the beneficial genotypic techniques of 16S rRNA sequence analysis as *Bacillus subtilis* was carried out. Optimization of the culture conditions which are; nutritional (carbon, nitrogen and mineral sources) and physical (temperature, pH and agitation) conditions was conducted using the conventional shake-flask system. It was observed that the most suitable components of

the basal medium for the lipase production were 2.5% Olive oil (carbon); 1.5% Peptone (nitrogen) and 0.1% $MgSO_4.7H_2O$ (mineral) at an optimum temperature of 50°C, pH 7.5 and 150 rpm agitation, giving an enzyme yield of 4.23 U/ml from the original yield of 2.48 U/ml. Statistical optimization using Response Surface Methodology (RSM) was carried out. An optimum lipase production of 5.67 U/ml was achieved when olive oil concentration of 3%, peptone 2%, $MgSO_4.7H_2O$ 0.2% and agitation rate of 200 rpm were combined. Lipase production was further carried out inside a 2 L bioreactor with a 1.5 L working volume which yielded an enzyme activity of 14.5 U/ml after 15 hours of incubation.

Crude lipase produced was purified by ultrafiltration, DEAE – Toyopearl 650M and Sephadex G-75 column. The enzyme was purified 500-fold with a recovery of 16%. The purified enzyme showed a prominent single band on SDS–PAGE and its molecular weight was determined to be 45 kDa. The optimum pH and temperature for activity of lipase were 7.0 and 60°C. The enzyme was stable in the pH range 7.0 – 9.0 and temperature range 40 – 70°C. It showed high stability with half lives of 273.38 min at 60°C, 51.04 min at 70°C and 41.58 min at 80°C. The D-values at 60, 70 and 80°C were 788.70, 169.59 and 138.15 min respectively. The enzyme's enthalpy, entropy and Gibb's free energy were in the range of 70.07 to 70.40 $KJmol^{-1}$, -83.58 to -77.32 $KJmol^{-1}$ K^{-1} and 95.60 to 98.96 $KJmol^{-1}$ respectively. It was stable in presence of divalent metal ions like Mg^{2+}, Ca^{2+} and markedly inhibited by Zn^{2+}, Cu^{2+} and Fe^{2+}. The enzyme was able to hydrolyze most of the natural oil tested, with the highest hydrolytic activity on soy bean oil. On TLC plate, the enzyme was non-regiospecific as it showed random positional specificity for triolein hydrolysis.

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

PENGHASILAN, PUNULINAN DAN PERINCIAN LIPASE STABIL TERMA DARIPADA *Bacillus subtilis* NS 8 EKSTREMOFILIK

Oleh

AKANBI TAIWO OLUSESAN

Januari 2010

Pengerusi: **Profesor Dr. Nazamid Saari, PhD**

Fakulti: **Sains dan Teknologi Makanan**

Lipase adalah salah satu biokatalis yang versatil dan mempunyai applikasi yang meluas dalam bidang bioteknologi khususnya dalam penghasilan lipid fungsian. Walaupun terdapat banyak pengeluar enzim, hingga ke saat ini, tiada sumber tempatan bagi lipase didapati, walaupun pada hakikatnya pasarannya adalah luas. Kajian ini bertujuan untuk menghasil, menulin dan menciri lipase stabil terma daripada *Bacillus subtilis ekstremofilik* NS 8 yang asingkan daripada kolam air panas Setapak.

Perghasilan lipase oleh Bacillus ekstremofilik yang terlebih dahulu dikenalpasti melalui kaedah fenotipik dan disah menggunakan Teknik Genotipik Manafaat analisis turutan 16S rRNA sebagai *Bacillus subtilis* telah dijalankan. Pengoptimuman keperluan kultur telah dijalan menggunakan kaedah kelalang goncang bagi menentukan kondisi fizikal dan

nutrisi yang terbaik bagi penghasilan lipase. Didapati komponen yang paling sesuai bagi menyokong penghasilan lipase adalah 2.5% minyak zaitun (carbon); 1.5% pepton (nitrogen) dan 0.1% $MgSO_4.7H_2O$ (mineral) pada suhu optimum 50°C, pH 7.5 dan 150 rpm kadar agitasi, meningkatkan penghasilan enzim kepada 4.23 U/ml daripada penghasilan asal iaitu 2.48 U/ml. Pengoptimuman statistik menggunakan Kaedah Ransangan Permukaan (RSM) telah dijalankan. Penghasilan optimum lipase sebanyak 5.67 U/ml telah berjaya diperolehi apabila 3% minyak zaitun, 2% pepton, 0.2% $MgSO_4.7H_2O$ dan 200 rpm kadar agitasi digunakan. Penghasilan lipase seterusnya dijalankan di dalam 2 L biorektor yang mempunyai 1.5 L isipadu kerja yang mana menghasilkan aktiviti enzim sebanyak 14.5 U/ml selepas 15 jam pemeraman.

Lipase kasar yang diperolehi ditulin menggunakan penurasan ultra, DEAE – Toyopearl 650M dan Sephadex G-75. Enzim ditulinkan sebanyak 500 kali ganda degan nilai hasil sebanyak 16%. Enzim yang telah ditulinkan menunjukkan satu jalur utama pada SDS-PAGE dan didapati berat jisimnya adalah 45 kDa. pH dan suhu optimum bagi aktiviti lipase, masing - masing adalah 7.0 dan 60°C. Enzim berada di dalam keadaan stabil pada lingkungan pH 7.0 – 9.0 dan suhu antara 40 – 70°C. Ini menunjukkan kestabilan yang tinggi dengan separuh hayat 273.38 min pada 60°C, 51.04 min pada 70°C dan 41.58 min pada 80°C. Nilai D pada suhu 60, 70 dan 80°C masing – masing adalah 788.70, 169.59 dan 138.15 min. Nilai entalpi, entropi dan tenaga bebas Gibb adalah di antara 70.07 hingga 70.40 $KJmol^{-1}$, -83.58 hingga -77.32 $KJmol^{-1}$ K^{-1} dan 95.60 hingga 98.96 $KJmol^{-1}$. Ianya stabil dengan kehadiran ion-ion divalen seperti Mg^{2+}, Ca^{2+} dan terencat dengan kehadiran Zn^{2+}, Cu^{2+} and Fe^{2+}. Enzim yang diperolehi didapati mampu menghidrolisis

kebanyakan minyak asli yang diuji, dengan aktiviti hidrolysis tertinggi didapati pada minyak kacang soya. Melalui Kaedah kromatografi lapisan nipis, dapati enzim adalah tak-regiospesifik kerana ia menunjukkan spesifikasi kedudukan yang rawak untuk hidrolisis triolein.

ACKNOWLEDGEMENTS

I want to give thanks to the Almighty God for His goodness, mercy and kindness over me, without whom I am frail. He gave me the strength needed to start and complete this project safe and sound.

My deepest thanks go to the chairman of my supervisory committee, Professor Dr. Nazamid Saari, who doubled as a supervisor and as a mentor throughout the entire period of my study. Thanks for the advice, guidance, patience, perseverance, encouragement, love, care and concern. In actual fact, the unquantifiable experience I gained as one of his postgraduate students will forever be remembered and appreciated. I would also want to express my heartfelt gratitude to the members of my supervisory committee, Professor Dr. Yazid AbdulManap and Associate Professor Dr. Fatimah Abu bakar for their accurate guidance, corrections and reproofs throughout the project.

My appreciation goes to Associate Professor Dr. Shuiami for giving me the opportunity to work in his lab. I also want to appreciate all members of staff of Biochemistry and Microbiology laboratory of UPM, for their technical supports throughout my study. Furthermore, I want to appreciate Mrs. Aida for helping me to translate my abstract to Bahasa Melayu, thanks. Big thanks go to all members of Food Biotechnology and Functional Food research group for their understanding even when we needed to step on our toes. Thanks a lot.

Lastly, I want to thank all my family members home and abroad, for their full support throughout my entire programme, thanks and God bless you all.

APPROVAL

I certify that an Examination Committee met on **date of viva** to conduct the final examination of **Akanbi Taiwo Olusesan** on his **Master of Science** thesis entitled "**Production, Purification and Characterization of Thermostable Lipase from an Extremophilic *Bacillus subtilis* NS 8**" 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:

Chairman, PhD
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Examiner 1, PhD
Professor
Faculty of Graduate Studies
Universiti Putra Malaysia
(Internal Examiner)

Examiner 2, PhD
Professor
Faculty of Graduate Studies
Universiti Putra Malaysia
(Internal Examiner)

External Examiner, PhD
Professor
Faculty of Graduate Studies
UniversitI Putra Malaysia
(External Examiner)

_____

BUJANG KIM HUAT, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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

Nazamid Saari, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Mohd Yazid Abdul Manap, PhD
Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Fatimah Abu Bakar, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

_____

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 April 2010

DECLARATION

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

_____

AKANBI TAIWO OLUSESAN

Date: 8 April 2010

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

rpm	revolution per minute
v/v	volume per volume
min	minute
h	hour
g	gram
ml	millileter
mg	milligram
µm	micrometer
nm	nano meter
mM	milliMolar
µmol	micromole
ng	nanogram
µl	microliter
M	Molar
N	Normality
Gly	Glycine
Ser	Serine
kDa	kilo Dalton
U/ml	unit per millileter
U/g	unit per gram
w/v	weight per volume

UV	ultraviolet
DNA	deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
bp	base pair
GP	Gram-positive
RSM	Response Surface Methodology
FCCD	Face Centered Composite Design
k_d	Thermal inactivation rate constant
DG	Di-glyceride
TG	Tri-glyceride

CHAPTER 1

INTRODUCTION

In nature, lipases are ubiquitous and are produced from various sources including animals, plants and microorganisms. However, lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reaction including alcoholysis, aminolysis, hydrolysis, esterification and interesterification (Pandey *et al*., 1999; Rahman *et al.,* 2005). Besides that, microbes can be easily cultivated and the lipases produced are mostly extracellular. It is in the last decade that lipases have gained importance to a certain extent over proteases and amylases, especially in the area of organic synthesis (Rahman *et al.,* 2005).

A large number of lipase producing microorganisms have been found in diverse habitats (Sharma *et al.,* 2002; Eltaweel *et al.,* 2005; Ertu˘grul *et al.,* 2007; Abdel-Fattah and Gaballa, 2008; Horchani *et al.,* 2009). A number of publications have also reported the presence of thermophilic lipase-producing bacteria found in hot springs (Kambourova *et al*., 2003; Castro-Ochoa *et al.,* 2005; Bora and Kalita 2007; Tirawongsaroj *et al.,* 2008). Most of these bacteria belonged to different *Bacillus* sp., grew optimally at temperature of 60 to 65°C and in the pH range from 6 to 9. Thermostable lipases, which have been isolated from thermophilic organisms, play an important role of commercial application because of their overall inherent stability (Demirjian *et al*., 2001). They are also stable and active at temperature which is higher than optimum temperatures for the growth of the microorganisms (Kambourova *et al*., 2003). A considerable number of bacteria strains

isolated for industrial applications have been deposited with the culture collections in connection with patent applications and are designated only by genus name (Khyami-Horani, 1996).

Some commercially available microbial lipases are being produced by Amano, Genencor, Novozymes, Fluka, Biocatalysts and Asahi companies (Sharma *et al.,* 2001). But, Sharma *et al.* (2001) reported that most of the lipases produced for use in food processing by most of these companies are of fungal origin. However, most researchers reported on the production and characterization of bacterial lipases with scarce information on those of extracellular lipases from *bacillus subtilis.*

The conventional method for optimization of medium and fermentation conditions involves varying one factor at a time and keeping the others constant in a shake flask system (En-Shyh *et al.,* 2006). The study of the effect of various components in the basal medium like carbon, nitrogen and mineral sources have been achieved by the use of shake flask (Rodriguez *et al.,* 2006). Statistical optimization using Response Surface Methodology (RSM) has been widely used to augment the conventional shake flask method especially when a large number of variables are to be evaluated. It has helped to determine the optimum concentration of selected media components (Sunitha *et al.,* 1998). However, to the best of our knowledge, no published information is available for the statistical optimization of extracellular lipase from *Bacillus subtilis.*

Submerged fermentation involving continuous bioreactor processes for scaling up of lipase production is also of immense importance. The continuous fermentation processes are characterized by the addition of one or more nutrients to the bioreactor and maintaining the products inside the bioreactor until the end of fermentation (Treichel *et al.,* 2009). Samples are withdrawn at intervals for analysis and the fermentation conditions are digitally checked to ensure conformation to set standards. This is so because microbial lipase fermentations are affected by the medium pH, temperature, medium composition, aeration and agitation and many other factors which must be well monitored (Dalmau et al., 2000; Kambourova *et al.,* 2003; Puthli *et al.,* 2006). Report published by Montesinos *et al.* (2003) which investigated lipase production in the continuous cultures of *Candida rugosa* showed that lipase production increased by 50% when compared to lipase production in batch fermentation cultures. There is therefore no single information on the use of continuous bioreactor for scaling up lipase production in *Bacillus subtilis* whose production medium has been statistically optimized.

Furthermore, purification of lipase allows for better understanding of the kinetic mechanisms of lipase action on hydrolysis, synthesis and group exchange of esters (Chakraborty, *et al.,* 2009). Many bacillus lipases have been purified to homogeneity using variety of methods involving ammonium sulphate precipitation, ion exchange chromatography followed by gel filtration (Kim *et al.,* 2000). The use of ammonium sulphate precipitation has been reported to cause low enzyme yield (Nawani and Kaur, 2000). Purified microbial lipases have also been characterized in terms of their activity