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PRODUCTION, EXPRESSION AND CHARACTERIZATION OF A HEAT-STABLE ORGANIC SOLVENT TOLERANT LIPASE FROM BACILLUS SP, STRAIN 42

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By

MOHAMED ABDALLAH ELTAWEEL

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

October 2005

DEDICATION

I dedicate this humble effort, the fruit of my thoughts and study,

to the great and helpful wife Hawa Safar,

dear sons Abdallah, Abdalrahman, Abdalraouf

and sweet daughter Halima

who have inspired me to higher ideals of life.

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

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Chairman: Associate Professor Raja Noor Zaliha Raja Abd Rahman, PhD

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Ninety two bacterial strains were isolated from oil palm effluent from Bangi,Selangor; Kluang, Johor; Alor Gajah hot spring (up to 54 °C) Melaka and Slim River hot spring (up to 91°C) Perak. An enrichment culture technique was used to isolate bacteria utilizing olive oil as a substrate. Cultures were incubated at 60°C to select for the thermophilic bacteria. Eight isolates showed lipolytic activity on tributyrin and triolein agar plates. In order to screen for highest lipase producer, six production media were used. Isolate 42 was observed to produce the highest level (0.059 U/ml) after 72h. Its crude lipase retained its full activity when preincubated at 70°C for 30 min. It also showed high stability in several organic solvents (25% v/v). Furthermore, its activity was enhanced in benzene, hexane and hexadecane while, completely inhibited by butanol. Isolate 42 was

identified as *Bacillus* sp. Strain 42 using 16S rDNA. The nucleotide sequence deposited at GenBank under accession number AY 763118.

Further optimization studies were done in order to determine the best lipase production condition. Inoculum size of 3% proved to be the best for lipase production, with an optimum temperature of 50°C when, grown under shaking condition of 150 rpm. A combination of tryptone and yeast extract was the best nitrogen source. Lipase production was stimulated by olive oil.

The lipase gene was amplified by polymerase chain reaction using consensus primers based on multiple aligned sequences of thermophilic genes from other thermophilic *Bacillus* species. Nucleotide sequence comparison shared high homology with the thermostable genes in *Geobacillus* sp., *Bacillus stearothermophilus* and *Bacillus thermoleovorans*. Nucleotide sequence deposited at GenBank under accession number AY 787835. The amplified gene was successfully cloned using a pQE-30 UA expression vector and induced by IPTG at the optimum concentration of 0.75 mM.

The recombinant lipase was facilitated by the fusion of 6-histidine and this allowed a one-step purification of the lipase enzyme using Ni-NTA affinity chromatography. The histidine-tagged lipase was purified 6-fold with a yield of 21.7%. Purified lipase migrated as a single band with a molecular mass of ~43 KDa on SDS-PAGE.

The purified lipase showed high activity at 70°C with its optimum at pH 8.0. The enzyme was stable over a broad range of pH from 6.0 to 10.0. It also showed high stability with half-lives of 315 min at 60°C, 120 min at 65°C, and 45 min at 70°C. Preincubation enzyme activity was stimulated with Na^+, K^+ and Ca^{2+}. While, Zn^{2+}, Mn^{2+} and Fe^{2+} at high concentration (10 mM) were greatly inhibitory. Protease inhibitors Bestatin and pepstatin stimulated the lipase activity while, phenylmethylsulfonyl fluoride (PMSF) completely inhibited the lipase activity. Tween 80 (0.1%) enhanced the lipase activity while higher concentration (1%) dramatically decreased the lipase activity. The activity of preincubated enzyme in heptanol (log *P* 2.4) and octanol (log *P* 2.9) was slightly enhanced while, remains very stable with other organic solvents tested. Solvents such as ethylbenzene (log *P* 3.1) and dodecane (log *P* 6.6) reduced the lipase activity up to 35% and 38%, respectively. The highest specificity was observed towards tricaprylin (C_8), followed by tricaprin (C_{10}). Its hydrolyzed all the natural oils tested, with highest hydrolysis rate on olive oil.

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

PENGHASILAN, PENGEKSPRESIAN DAN PENCIRIAN LIPASE TAHAN PELARUT ORGANIK DAN TERMOSTABIL DARI *BACILLUS* SP. STRAIN 42

Oleh

MOHAMED ABDALLAH ELTAWEEL

Oktober 2005

Pengerusi: Profesor Madya Raja Noor Zaliha Raja Abd Rahman, PhD

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Sebanyak 92 strain bakteria telah dipencilkan daripada sisa kumbahan sawit yang di perolehi dari Bangi, Selangor; dan Kluang Johor, serta kolam air panas di Alor Gajah, Melaka (suhu 54°C) dan di Slim River, Perak (91 °C). Minyak zaitun sebagai substrat telah digunakan untuk memencilkan bakteria melalui kaedah pengkayaan kultur. Pengeraman pada suhu 60°C digunakan untuk menggalakkan pertumbuhan bakteria yang rintang suhu atau termofilik. Sebanyak lapan isolat telah menunjukkan aktiviti lipolitik tertinggi di atas plat agar tributirin and triolein. Di dalam media cecair, penghasilan tertinggi didapati pada strain 42. Bagi meningkatkan penghasilan lipase, sebanyak enam jenis media digunakan. Media M3 didapati menghasillkan lipase tertinggi, iaitu 0.059 U/mL pada 72 jam pengeraman, dengan kadar goncangan 150 rpm, pada suhu 60 °C.

Kajian dilanjutkan bagi menentukan penghasilan terbaik enzim lipase. Inokulum bersaiz 3% terbukti menghasilkan enzim lipase tertinggi dengan suhu optimum pada 50°C. Penghasilan lipase paling tinggi adalah pada kadar goncangan 150 rpm per min. Penghasilan lipase adalah dirangsang oleh penambahan minyak zaitun sebagai substrat. Lipase mentah menunjukkan kestabilan yang tinggi sehingga mencapai suhu 70°C.

Ekstrak enzim mentah diuji terhadap beberapa pelarut organik berkepekatan 25% v/v, selama 30 min untuk menentukan kestabilannya. Peningkatan aktiviti berlaku di dalam pelarut benzena, heksana dan heksadekana, tetapi ia juga stabil di dalam pelarut toluena, xylina, dekanol, isooktana dan tetradekana. Aktiviti enzim menurun sebanyak 34.5% di dalam pelarut propanol dan 63.6% di dalam perlarut propilasetat berbanding kawalan dan direncatkan sepenuhnya oleh butanol. Gen lipase dari *Bacillus* sp. strain 42 telah digandakan melalui tindakbalas rantaian polimerase (PCR) menggunakan primer konsensus berdasarkan padanan jujukan berganda gen termofilik daripada spesis *Bacillus*. Perbandingan jujukan nukleotida menunjukkan gen lipase strain 42 mempunyai homologi yang tinggi dengan gen termostabil dari *Geobacillus* sp, *Bacillus stearothermophilus* dan *Bacillus thermoleovorans*. Gen yang digandakan ini telah berjaya diklon ke dalam vector pQE-30 UA dan telah diekspreskan dengan kehadiran IPTG pada kepekatan optimum 0.75 mM.

Penulenan enzim lipase rekombinan dipermudahkan dengan kehadiran 6-histidina pada vektor, ini membolehkan penulenan satu langkah dengan

menggunakan Ni-NTA kromatografi afiniti. Lipase pembawa histidina telah ditulenkan sebanyak 5.65 kali dengan hasilan 21.7%. Lipase rekombinan tulen bergerak sebagai satu jalur dengan jisim molekular ~43 KDa pada SDS-PAGE. Lipase tulen menunjukkan aktiviti tertinggi pada suhu 70°C dengan pH optimum 8.0. Enzim adalah stabil pada julat pH dari 6.0 ke 10.0. Ia juga menunjukkan kestabilan tertinggi dengah tempoh separuh hayat 315 min pada 60°C, 120 min pada 65°C dan 45 min pada 70°C.

Lipase tulen menghidrolisiskan kesemua minyak semulajadi yang diuji dengan kadar hidrolisis tertinggi terhadak minyak zaitun. Spesifisiti substrat tertinggi adalah terhadap trikaprilin (C_8) diikuti oleh trikaprin (C_{10}). Aktiviti lipase meningkat dengan penambahan ion logam seperti Ca^+ dan Na^+. Tween 80 pada kepekatan 0.1% meningkatkan aktiviti enzim tetapi aktiviti menurun pada 1%. Bestatin dan pepstatin juga meningkatkan sedikit aktiviti enzim tetapi EDTA tidak meningkatkan sebarang kesan. Sebaliknya aktiviti enzim direncatkan oleh fenilmetilsulfonilfluorida iaitu perencat protease serine.

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LIST OF ABBREVIATIONS

POME	Palm Oil Mill Effluent
cm	Centimeter
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic Acid
mM	Millimole
g/L	Gram per Liter
g	Gram
ng	Nanogram
Mg	Milligram
µg	Microgram
pmol	Picomole
L	Liter
M	Molar
mL	Milliliter
µl	Microliter
%	Percentage
µmole	Micromole
rpm	Rotation per Minute
x g	Gravity
UV	Ultraviolet
IPTG	Isopropyl-β-D- Thiogalactopyranoside
X-gal	5-bromo-4chloro-3-indolyl-β-D galactopyranoside
h	Hour
Min	Minute
PCR	Polymerase Chain Reaction
ORF	Open Reading Frame
GTE	Glucose Tris-HCl-EDTA
PCI	Phenol Chloroform Isoamylalcohol
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
APS	Amonium Persulphate
TCA	Trichloroacetic Acid
TSB	Tryptone Soy Broth
LB	Luria-Bertani
BSA	Bovine Serum Albumin
U/mL	Unit per Milliliter
cfu	Colony Form Units
v/v	Volume per Volume
w/v	Weight per Volume

psi	Pound Persquare Inch
bp	Base Pair
Ni-NTA	Nickle-nitrilotriacetate acid
Kbp	Kilo Base Pair
Da	Dalton
KDa	Kilo Dalton
mA	Milliampere
sp	Specie
spp	Species
NCBI	National Center for Biotechnology Information