UNIVERSITI PUTRA MALAYSIA

MOLECULAR CLONING, SEQUENCING AND EXPRESSION OF AN ORGANIC SOLVENT-TOLERANT LIPASE GENE FROM *BACILLUS SPHAERICUS* 205Y

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MOLECULAR CLONING, SEQUENCING AND EXPRESSION OF AN ORGANIC SOLVENT-TOLERANT LIPASE GENE FROM *BACILLUS SPHAERICUS* 205Y

By

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

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

MOLECULAR CLONING, SEQUENCING AND EXPRESSION OF AN ORGANIC SOLVENT-TOLERANT LIPASE GENE FROM *BACILLUS SPHAERICUS* 205Y

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June 2002

Chairman: **Associate Professor Raja Noor Zaliha Abd. Rahman, Ph.D.**

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Previously, a total of 131 organic solvent tolerant isolates originating from soil samples were successfully isolated via direct plating method using 1% (v/v) of either benzene, toluene, or a mixture of benzene and toluene as their sole carbon sources. Six out of these isolates demonstrated high tolerance of up to 75% (v/v) concentration of BTEX (benzene, toluene, ethyl benzene and xylene). These isolates were screened for lipolytic activity using triolein plates. Isolate 205y was found to produce the highest lipase yield in liquid medium and later identified as *Bacillus sphaericus* via 16S ribosomal DNA gene sequencing. In order to increase lipase yield, four production media were used and medium M3 yielded the highest lipase activity of 0.42 $Uml^{-1}min^{-1}$ at 36h incubation time. Stability studies in various organic solvents of 25% (v/v) for 30 min showed that lipase from *B. sphaericus* 205y was not only stable but also can be activated by hexane and xylene by 3.5 and 2.9 folds respectively. The activity, however, was greatly reduced in dimethyl sulfoxide and completely inactivated by hexadecane and acetonitrile. The gene coding for the extracellular lipase of *Bacillus sphaericus* 205y was isolated via genomic

library and cloned into pUC 19. Genomic DNA extracted from *B. sphaericus* 205y was partially digested with *Sau* 3AI. Optimisation was performed to obtain the highest concentration of DNA fragments in the range of 2 and 9 kb. The DNA fragments were purified and ligated to pUC 19, which was previously digested with *Bam* HI. The ligation products were transformed into *Escherichia coli* TOP 10. Five putative positive recombinant clones were obtained from the library through primary screening with tributyrin agar. These clones were subsequently screened with triolein agar to exclude false positive clones. One clone was isolated and plasmid from the clone was extracted. Restriction enzyme analysis showed that the clone carried a 9 kb fragment. Restriction sites of the 9 kb fragment were mapped and subclonings were performed to obtain the smallest fragment, which was responsible for expression of lipase. Sequence analysis revealed one major open reading frame (ORF) of 1194 bp, which was predicted to encode a polypeptide of 397 amino acid residues. This open reading frame was preceded by the putative Shine-Dalgarno (SD) sequence (AAGGAGG), 10 bases from the start codon and two putative *E.coli* –10 (GATAAT) and –35 (TGCAT) promoters. Signal peptide was deduced based on computer analysis to consist of 32 amino acid residues and a mature protein of 365 amino acids. This was followed by an inverted repeat sequence downstream of a stop codon. Sequence analysis deduced the lipase molecular weight of approximately 44.5 kDa. The native SD and promoter region of the gene were removed and fused to the *Eco* RI digested pUC 19. Expression was dictated by the *lac operon* and resulted in an 8-folds increase of enzyme activity after 3h induction with 1mM IPTG. The expressed enzyme retained 100% and 90% of its activity after 30 min incubation (37°C) in hexane (25%) and p-xylene (25%), respectively.

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

PENGKLONAN, PENGKODAN DAN PENGEKPRESAN GEN LIPASE DARI *BACILLUS SPHAERICUS* 205Y YANG TOLERAN TERHADAP LARUTAN ORGANIK

oleh

CHIN JOHN HUN

Jun 2002

Pengerusi: Profesor Madya Dr. Raja Noor Zaliha Abd. Rahman

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Sejumlah 131 bacteria yang berasal dari tanah telah dipencilkan melalui penggunaan plat yang mengandungi 1% (v/v) benzena, toluena atau campuran benzena dan toluena. Enam bacteria yang berjaya hidup dalam kandungan BTEX (benzena, toluena, etil benzena dan xilena) sehingga 75% (v/v) telah dipencilkan. Bacteria ini kemudiannya diuji kebolehannya untuk menghasilkan lipase dengan menggunakan plat triolin. Bakteria 205y dikesan menghasilkan lipase yang tertinggi dalam media cecair. Bakteria ini dikenalpasti sebagai *Bacillus sphaericus* melalui gen 16S ribosom DNA. Empat media produksi telah diuji untuk meningkatkan penghasilan lipase dan media M3 didapati menghasilkan lipase yang tertinggi iaitu sebanyak 0.42 $Uml^{-1}min^{-1}$ pada 36 jam pengeraman. Ujian kestabilan dijalankan dengan 25% (v/v) pelbagai larutan organik selama 30 min, menunjukan lipase dari *B.sphaericus* 205y bukan sahaja stabil dalam hexana dan xilena tetapi masing-masing terdapat peningkatan aktiviti sebanyak 3.5 dan 2.9. Walau bagaimanapun, aktiviti berkurangan dalam dimetil sulfosida dan aktiviti terbantut dalam heksadekane dan asetonitril. Gen yang mengkodkan lipase *B.sphaericus*

205y telah diasingkan melalui pencernaan separa enzim *Sau* 3AI. Pengoktimaan dijalankan terlebih dahulu untuk menghasilkan fragmen DNA dalam lingkungan 2 sehingga 9 pasangan kilobes. Fragmen DNA yang telah dibersihkan, dicantumkan pada pUC 19 yang telah dicerna dengan *Bam* HI terlebih dahulu. Lima klon diasingkan dari plat tributirin dan diuji dalam plat triolin untuk mengetepikan klon negatif. Hasilnya satu klon berjaya diasingkan dan plasmidnya sepanjang 9 kb telah diekstrakan. Tapak penghad plasmid ini dikenalpasti dan pengasingan fragmen DNA yang minimal untuk menghasilkan lipase dijalankan. Penjujukan mengenalpasti bahawa terdapat satu jujukan Shine-Dalgarno (SD) iaitu (AAGGAGG) 10 bp dari kodon permulaan dan diikuti oleh jujukan promoter –10 (GATAAT) dan –35 (TGCAT). Peptida signal sepanjang 32 asid amino dan protein matang sepanjang 365 asid amino dikesan melalui analisis komputer. Ini diikuti oleh satu fasa pengulangan dan satu kodon pemberhentian Analisis jujukan menjangka berat molekul sebanyak 44.5 kDa. Jujukan asal SD dan promoter telah dibuang dan gen lipase digabungkan dengan promoter *lac* dari pUC 19. Ekspresi dikawal oleh operan *lac* menyebabkan 8 kali ganda peningkatan selepas 3 jam induksi dengan 1mM IPTG. Enzim yang dirembes juga stabil dan mengekalkan 100% dan 90% aktiviti dalam heksana (25% v/v) dan xilena (25% v/v) masing-masing.

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I certify that an Examination Committee met on 3rd June 2002 to conduct the final examination of Chin John Hun, on his Master of Science thesis entitled "Molecular Cloning, Sequencing, and Expression of an Organic Solvent-Tolerant Lipase Gene from *Bacillus sphaericus* 205Y" 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 are as follows:

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DECLARATION

I hereby declare that the thesis is based on my 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 other institutions.

CHIN JOHN HUN

Date:

ABSTRACT..... ii
ABSTRAK..... iv
ACKNOWLEDGEMENTS..... vi
APPROVAL..... vii
DECLARATION..... ix
LIST OF TABLES..... xiii
LIST OF FIGURES..... xiv
ABBREVIATIONS..... xvi

CHAPTER

1 INTRODUCTION..... 1

2 LITERATURE REVIEW..... 4
2.1 Lipase..... 4
2.1.1 Sources and General Properties of Microbial Lipases..... 4
2.1.1.1 Fungal Lipase..... 5
2.1.1.2 Bacterial Lipase..... 7
2.1.2 Classification of Microbial Lipase..... 10
2.1.3 Lipases of Genus *Bacillus*..... 15
2.1.4 Organic Solvent-Tolerant Lipase..... 16
2.2 Application of Lipases..... 19
2.3 Genetic Engineering of Lipase..... 21
2.3.1 Cloning of Lipase Gene..... 21
2.3.1.1 Genomic DNA Library..... 23
2.3.1.2 Selection of Vector..... 25
2.3.1.3 Selection of Host..... 27
2.3.2 Direct Selection Strategy..... 28
2.3.3 Expression of Lipase Gene..... 30

3 MATERIALS AND METHODS..... 32
3.1 Media, Buffers and Solutions..... 32
3.2 Bacterial Strains and Plasmid..... 35
3.3 Screening and Isolation of Organic Solvent-Tolerant Lipase Producer..... 36
3.3.1 Plate Assay for Primary Screening of Lipolytic Activity..... 36
3.3.2 Broth Assay for Screening of Lipolytic Activity..... 36
3.4 Effect of Different Production Media on Lipase Yield..... 37
3.5 Effect of Cultivation Time on Lipase Production..... 37
3.6 Effect of Organic Solvents on Crude Enzyme Activity..... 38

3.7 Bacterial Identification..... 38
3.7.1 Characterisation by Biochemical Test and Bacteria Morphology..... 38
3.7.2 16S rDNA Sequence Identification..... 39
3.7.3 Phylogenetic Tree Analysis..... 40

3.8 Isolation and Cloning of Lipase Gene..... 40
3.8.1 Genomic DNA Extraction..... 40
3.8.2 Quantitation and Quality Assessment of Genomic DNA..... 41
3.8.3 Genomic Library Construction (*Sau* 3AI Partial Digestion).. 41
3.8.4 Purification of DNA..... 43
3.8.5 Plasmid DNA (pUC 19) Extraction..... 43
3.8.6 Restriction Enzyme (RE) Digestion of pUC 19..... 44
3.8.7 Ligation of *Sau* 3AIPartially Digested Genomic DNA with *Bam* HI Digested pUC 19..... 44
3.8.8 Preparation of Competent Cells and Transformation..... 45
3.8.9 Screening of Positive Recombinant Clones..... 46
3.8.10 Subcloning and Restriction Site (RE) Mapping of Putative Fragment Containing Lipase Gene..... 46
47

3.9 Sequencing and Analysis of The Lipase Gene..... 48

3.10 Expression of Lipase Gene..... 48
3.10.1 Degenerate Primer Design..... 48
3.10.2 Amplification of Lipase Gene..... 48
3.10.3 Cloning of Lipase Gene and Transformation into *E.coli* BL21..... 49
3.10.4 Expression and Detection of Lipase Via SDS-PAGE..... 49
3.10.5 Recombinant Enzyme Assay and Organic Solvent-Tolerant Test..... 51

4 RESULTS AND DISCUSSIONS..... 52
4.1 Screening and Isolation of Organic Solvent-Tolerant Lipase Producer..... 52
4.2 Effect of Different Production Media on Lipase Yield..... 56
4.3 Effect of Cultivation Time on Lipase Production..... 60
4.4 Effect of Organic Solvents on Crude Enzyme Activity..... 62

4.5 Bacterial Identification..... 65
4.5.1 Biochemical and Bacterial Morphological Characteristics.... 65
4.5.2 16S rDNA Identification and Phylogenetic Tree Analysis.... 65

4.6 Lipase Gene Isolation, Cloning and Analysis..... 75
4.6.1 Extraction of Genomic DNA..... 75
4.6.2 Lipase Gene Isolation via PCR..... 77

4.6.3 Lipase Gene Isolation via Genomic Library..... 78
4.6.4 Restriction Mapping of Putative Lipase Gene..... 84
4.6.5 Nucleotide Sequencing..... 86

4.7 Gene Analysis..... 87
4.7.1 Nucleotide Sequence Analysis..... 87
4.7.2 Amino Acid Composition..... 93
4.7.3 Hydrophobicity Profile and Signal Peptide Prediction..... 96
4.7.4 DNA Sequence Homology with *B.subtilis* and *B.pumilis* Lipase Genes..... 99
4.7.5 DNA Sequence Homology with Microbial Lipase Genes..... 101

4.8 Expression under *lac* Promoter Regulation..... 106

5 CONCLUSION..... 112

REFERENCES..... 114
APPENDICES..... 123
BIODATA OF THE AUTHOUR..... 124

LIST OF TABLES

Table		Page
1	Properties of microbial lipases.....	9
2	Conserved catalytic sequence among lipolytic enzymes.....	12
3	Classification of microbial lipolytic enzyme.....	14
4	Properties of cloned lipase gene.....	22
5	Number of clones needed for genomic libraries of a variety of organisms.	24
6	Media, buffers and solutions.....	32
7	Bacterial strains and plasmid.....	35
8	The dilutions of *Sau* 3AI in ten individual microcentrifuge tubes.....	42
9	Composition for SDS-PAGE [15% (w/v) gel preparation].....	50
10	Lipase activity of 8 isolates on production medium M5 after 24 h incubation.....	55
11	Effect of different organic solvents on crude lipase activity.....	64
12	Biochemical tests and morphology summary of the isolate 205y.....	66
13	Comparison of the amino acid sequence of the lipase from *B.sphaericus* 205y with others lipase sequences of various organisms.....	92
14	Comparison of amino acid composition of lipases from various organisms.....	95
15	Effect of different organic solvents on crude recombinant lipase activity..	111

LIST OF FIGURES

Figure		Page
1	Production of lipase by isolate 205y on triolein (Victoria Blue) agar.....	54
2	Effect of different production media on lipase yield.....	59
3	The time course of lipase production by isolate 205y.....	61
4	16S rDNA gene (~1500 bp) of isolate 205y gene amplified via PCR.....	69
5	16S rDNA sequence of *Bacillus sphaericus* 205y.....	71
6	Comparison study of 16S rDNA between *Bacillus sphaericus* DSM 96 (AJ 311894) and *Bacillus sphaericus* 205y (AF 435435).....	73
7	Rooted phylogenetic tree showing the relationship of isolate 205y to other *Bacillus* species.....	74
8	Extraction of genomic DNA from *Bacillus sphaericus* 205y.....	76
9	*Sau* 3AI partial digestion of genomic DNAs extracted from *Bacillus sphaericus* 205y.....	79
10	Recombinant plasmid of 205y pLIP containing 9 kb insert.....	81
11	Recombinant clone 205y pLIP producing halo on tributyrin-amp agar after 48 h incubation at 37°C.....	82
12	Recombinant clone 205y pLIP producing blue zone on triolien-amp Victoria Blue agar after 48 h incubation at 37°C.....	82
13	Recombinant clone 205y (pLIP) producing orange fluorescent under UV radiation on triolein-amp (Rhodamine B) agar after 72 h incubation at 37°C.....	83
14	Restriction Endonuclease Map of 205y pLIP	85
15	Subcloning of the DNA coding *Bacillus sphaericus* 205y lipase from pLIP 205y.....	85
16	Nucleotide sequencing strategy of the region containing the putative lipase gene.....	86

17	Nucleotide sequence of the lipase gene from *B.sphaericus* 205y……..	91
18	Hydrophobicity profile of lipase from *B.sphaericus* 205y…….……	97
19	Similarity between lipase from *B.sphaericus* 205y (AF 453713) and *B.subtilis* (M74010) and *B.pumilis* (A34992)……....	100
20	Alignment of amino acid sequences from ten lipases of various organisms……...	104
21	Phylogenetic analysis of lipase gene from *Bacillus sphaericus* 205y……	105
22	Expression of recombinant plasmid under native and *lac* promoter of pUC19 with and without IPTG induction……..	109
23	SDS-PAGE of total cellular proteins stained for protein in 12% (w/v) gel……...	110

LIST OF ABBREVIATIONS

A	adenine
bp	base pair
C	cytosine
°C	degrees centrigrade
DNA	deoxyribonucleic acid
dH_2O	distilled water
EDTA	ethylene diamine tetra acetic acid
G	guanine
g	gram
h	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair
kDa	kilo Dalton
M	molar
mg	miligram
min	minute
ml	mililiter
mM	milimolar
MW	molecular weight
ng	nanogram
ORF	open reading frame

PCR	polymerase chain reaction
pI	isoelectric point
RNA	ribonucleic acid
RBS	ribosome binding site
rpm	revolutions per minute
SD	Shine-Dalgarno
SDS	sodium dodecyl sulphate
TBE	tris borate-EDTA
TEMED N, N, N, N'	tetramethyl-ethylene diamine
T	thymine
U	Unit of activity
uv	ultraviolet
µg	micro gram
µl	micro liter
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase

CHAPTER 1

INTRODUCTION

Lipases (EC 3.1.1.3) are widely distributed in animal, plants and microorganisms (Brockerhoff and Jensen, 1974). These enzymes exhibit a great potential for commercial applications. The most commercially important field of application for hydrolytic lipases is their addition to detergents. A study conducted by Fujii *et.al.* (1986) showed that detergents that contained lipase were 15 to 20% more efficient in removing olive oil from cotton fabrics. Besides that, lipases also are used in flavour production in dairy industries such as butter, cheese and margarine. The enzymes are used to accelerate the maturation of cheese and for production of a typical flavour. During the process, there is formation of free fatty acids which act as flavours or flavours precursors (Gandhi, 1997). Microorganisms producing lipase are also exploited to reduce lipid pollution.

In 1997, Scioli and Vollaro studied the effect of using *Yarrowia lipolytica* to reduce pollution in olive mill wastewater. They have demonstrated the use of *Yarrowia lipolytica* ATCC 20225 lipase in reducing the chemical oxygen demand (COD) level in olive oil wastewater. In Italy alone, approximately 200,000 m^3 of wastewater is produced each year by olive mill industry. Therefore their results have interesting industrial implications for the reduction of pollution at reasonable cost in olive oil producing countries. Malaysia is a palm oil producing country, therefore lots of waste which rich in oil are produced. Lipase can be applied to reduce pollution.

With respect to industrial applications, organic solvents tolerant lipases are more promising, since organic solvent-tolerant proteins have several advantages. Water often participates in undesirable side reactions, such as hydrolysis of acid anhydrides and halogenates. Moreover microbial contamination resulting in the release of proteases would inactivate enzymes. Therefore the use of organic solvents would ease recovery of products from aqueous solutions, reduces undesirable side reactions and reduces contamination. Furthermore substrates of the lipase are often insoluble in aqueous and organic solvents or organic-aqueous two-phase media are favourable for some reactions.

It has been reported that certain wild-type strains can grow in media in the presence of toxic organic solvents such as toluene, xylene and others (Aono *et. al.*, 1992). It was predicted that these solvent-tolerant bacteria secrete organic solvent-tolerant enzymes. Based on this hypothesis, several research have been conducted to search for enzymes that are naturally stable in the presence of organic solvents as they will be very useful industrially. In fact, lipases are among the first enzymes tested and found to be relatively stable in organic solvents (Ogino, *et. al.*, 1994).

Lipases mainly of microbial origin represent the most widely used class of enzymes in biotechnological applications and organic chemistry. The main reason for the use of microorganisms to produce compounds such as microbial lipase that can be otherwise be isolated from plants and animals, or synthesized by chemical reactions, is the ease of increasing production by environmental and genetic manipulation. Microbes are extremely good at producing an amazing array of valuable compounds; however, they

usually produce these compounds in small amount that are needed for their own benefit in natural conditions. Therefore the use of recombinant DNA technology has played a tremendous role in increasing the level of lipase production. For instance, Rua, *et. al.*, (1998) have demonstrated a 100-fold increase in production of lipase via recombinant DNA.

Today, biotechnology is a major participant in global industry, especially in the pharmaceutical, food and chemical industries. Industrial enzymes alone have reached an annual market of US$ 1.6 billion. Some of the important enzymes are protease, lipases, carbohydrases, recombinant chymosin that used in cheese manufacture, and recombinant lipase for use in detergents (Demain, 2000). However, currently lipases only account for about 3% of the total worldwide sale of enzymes. Due to their pivotal roles and more findings about lipases revealed in recent year, it has attracted worldwide attention in attempts to exploit their physiology and biotechnological applications.

The immense potentials of lipase in the industrial applications have promoted this study to be carried out. Thus, the main objectives of this study are:

1) To screen for an organic solvent-tolerant lipase producer
2) To identify the organic solvent-tolerant lipase producer
3) To clone the organic solvent-tolerant lipase gene
4) To sequence and express the organic solvent tolerant lipase gene

CHAPTER 2

LITERATURE REVIEW

2.1 Lipases

Lipases (E.C. 3.1.1.3) belong to the carboxylic ester hydrolase family. Besides lipases, esterases also belong to this family. They both hydrolyse ester bonds. A classification was proposed by Brockerhoff and Jensen in 1974 was based on their specificity for acid moiety of the substrate. Esterases preferentially hydrolyse ester bonds of shorter chain fatty acids whereas lipases display maximal activity breaking ester bonds of insoluble long chain fatty acids or partially soluble (emulsion) fatty acids. Lipases have broader substrate range than the esterases. Verger in 1997 has defined lipases as carboxylesterases that have the ability to hydrolyse long-chain acylglycerols ($\geq C_{10}$) whereas esterases hydrolyse ester substrate with short-chain fatty acids ($\leq C_{10}$).

2.1.1 Sources and General Properties of Microbial Lipase

Lipases are widely distributed in plants, animals and microorganisms and have a broad range of properties with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc (Philips *et. al.*, 1995). Most of the lipases used in biotechnology industries were originated from microorganisms. The versatility of lipases especially those from microbial lipases have made them a good candidate for industrial

exploitation. Various lipases have been isolated from fungal and bacteria. Since microbial extracellular lipases are usually more easily obtained and manipulated than animal or plant lipases, they have received much attention for their potential use in industry and diagnostics.

2.1.1.1 Fungal Lipase

Many lipases from various species of fungal have been isolated. The properties of fungal lipases such as optimum pH, molecular weight, heat stability and substrate specificity differ from lipase to lipase. Among the important and productive strains are *Geotrichum, Rhizopus, Candida,* and *Mucor*. *Geotrichum candidum* lipase has been widely investigated since the early 1960. Since then several papers have been published on strains of *G.candidum* isolated in Germany, United States and Denmark (Charton *et. al.*, 1991). *Geotrichum candidum* secreted several lipase isoenzymes, differing in their selectivity towards esters of long chain fatty acids with *cis*-9 double bond. The genus *Geotrichum* has twenty-three recognized taxa and fourteen species, however little is known about extracellular lipases of species other than *Geotrichum candidum.* Shimada *et. al.*, (1989) identified two genes coding for two different lipases from *G.candidum* ATCC 34614 strain and subsequently in 1990, Sugihara *et al.* isolated and purified the two lipases. The two lipases have molecular weight of 64 and 66 kDa respectively and shared 85% in amino acids homology with pI 4.3. However they differ in optimum pH and heat stability. Both lipases demonstrated significant hydrolysis of saturated C8, C12, C16 and triolein.

Two strains of *Rhizopus rhizopodiformis,* S1 and S2 producing thermostable lipases were characterised by Samad *et. al.* (1990). These lipases were stable at 50°C over 30 min and completely deactivated after 30 min at 90°C. Increment of lipase yield was observed when the organisms was cultivated with different composition of carbon and nitrogen sources. Addition of maltose and lactose (1% w/v) during cultivation was found to enhanced lipase production. Besides that peptone (5% w/v) was found to improved lipase yield as well. Another well characterised species of *Rhizopus* is *Rhizopus oryzae*. This species was found to produce extracellular as well as intracellular lipase (Razak, *et. al.*, 1999). The intracellular lipase of *R.oryzae* was membrane-bounded which has an advantage of natural immobilized enzyme system. This lipase has an optimum pH of 6.0, optimum temperature at 37°C, and hydrolysed a wide range of triglycerides with high preference towards short and medium chain triglycerides (C4-C8).

Meanwhile, Hiol *et. al.* (1999) have isolated a lipolytic *Rhizopus oryza*e strain from palm fruit. The extracellular lipase was purified 1200-fold and has a molecular mass of 32 kDa. The enzyme has an isoelectric point of 7.6 and has an optimum pH of 7.5 for enzyme activity. However the lipase displayed stability in the pH range from 4.5 and 7.5. The optimum temperature for lipase activity was 35°C and retained 65% of its activity at 45°C for 30 min. Triton X100, SDS, and metal ions such as Fe^{3+}, Cu^{2+}, Hg^{2+} and Fe^{2+} inhibited the lipolytic enzyme, in contrast sodium cholate or taurocholate enhanced lipase activity against triolein. The purified lipase had a preference for the hydrolysis of saturated fatty acid chains (C8 –C18) and a 1, 3-position specificity. The lipase showed good stability in organic solvents and especially in long chain-fatty alcohol.

2.1.1.2 Bacterial Lipases

Bacterial lipases have been isolated from a wide range of genera and species, mainly from *Pseudomonas* genera (Bashkatova and Severina, 1978; Ihara *et. al.*, 1991; Kordel *et. al.*, 1991; Gilbert *et.al.*, 1991; Lee *et. al.,* 1993; McKenney and Allison, 1995; Lin *et. al.,* 1996; Yeo *et. al.,* 1998); *Bacillus* genera (Schmidt-Dannert *et. al.,* 1994; Fakhreddine *et. al.,* 1998; Dharmsthiti and Luchai, 1999; Lee *et. al.*,1999) and Staphylococcus genera (Gotz *et. al.*, 1998). The properties of bacterial lipases are also very versatile.

Pseudomonas lipases typically have molecular weight of 30 kDa except *P.fluorescens* type lipases having molecular weights around 55 kDa. Isoletric points for *Pseudomonas* lipases are generally between 4 and 7. Optimum pH of *Pseudomonas* lipases were around 6 and 9, and pH stabilities were reported between 5 and 10. However *P.cepacia* DSM 50181 produced lipase that have pH stabilities from pH 2 and pH 12 (Dunhaupt *et. al.*, 1991). One typical characteristic of *Pseudomonas* lipases was its requirement for an additional gene product, which probably acted as a chaperone in folding of the lipase protein. Ilzumi and Fukase, (1993) have demonstrated that recombinant *E.coli* carrying both lipase and activator gene produced an active lipase. However recombinant *E.coli* carrying only the lipase gene produced same amount of inactive lipase protein. The inactive lipase protein was activated by *in vitro* addition of crude extract containing the activator gene product.