Identification, Gene Cloning and Characterization of An Extremely Thermoactive and Thermotolerant Lipase from *Bacillus thermoamylovorans* BHK52

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ชื่อวิทยานิพนธ์ การจัดจำแนก การโคลนยีน และการศึกษาคุณสมบัติของเอนไซม์ไลเปสที่ทนและทำงานได้ดีที่อุณหภูมิสูงจาก *Bacillus thermoamylovorans* BHK52

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บทคัดย่อ

เอนไซม์ไลเปส (triacylglycerol hydrolases; EC 3.1.1.3) เป็นเอนไซม์ที่มีความสำคัญมากที่สุดในกลุ่มของเอนไซม์ย่อยไขมันที่นำมาใช้เป็นตัวเร่งปฏิกิริยาชีวภาพในระดับอุตสาหกรรมเพื่อการประยุกต์ใช้ทางเทคโนโลยีชีวภาพที่หลากหลาย การศึกษาครั้งนี้ได้นำแบคทีเรียทนอุณหภูมิสูงที่คัดแยกจากกองปุ๋ยหมักมาใช้เป็นแหล่งของแบคทีเรียผลิตเอนไซม์ไลเปส โดยคัดเลือกจากการเจริญบนอาหารสังเคราะห์ที่มีองค์ประกอบของน้ำมันปาล์มและ rhodamine B ที่อุณหภูมิ 50°C แบคทีเรียที่ผลิตเอนไซม์ไลเปสจะเรืองแสงสีส้มภายใต้แสงยูวี (254 นาโนเมตร) สามารถคัดเลือกแบคทีเรียที่ผลิตเอนไซม์ไลเปสในระดับสูงได้ทั้งหมด 10 สายพันธุ์ เมื่อจัดจำแนกโดยวิเคราะห์ลำดับนิวคลีโอไทด์ของยีน 16S rRNA พบว่าเป็น *Aneurinibacillus thermoaerophilus* 1 สายพันธุ์, *Bacillus pumilus* 1 สายพันธุ์ และ *Bacillus thermoamylovorans* 8 สายพันธุ์ ในการศึกษาครั้งนี้ได้เลือก *Bacillus thermoamylovorans* BHK52 ซึ่งผลิตเอนไซม์ไลเปสได้สูงและยังไม่พบข้อมูลของเอนไซม์ไลเปสบนฐานข้อมูลมาใช้สำหรับการโคลนยีน การหาลำดับนิวคลีโอไทด์ การศึกษาการแสดงออกของยีน และการศึกษาคุณสมบัติบางประการของเอนไซม์ดังกล่าว โดยทำการออกแบบ degenerate primers จากลำดับนิวคลีโอไทด์ตรงบริเวณอนุรักษ์ของยีนไลเปสในกลุ่ม *Bacillus* spp. เพื่อใช้ในการเพิ่มปริมาณชิ้นส่วนของยีนไลเปสด้วยเทคนิคพีซีอาร์ หลังจากนั้นทำอินเวอร์สพีซีอาร์เพื่อหาลำดับนิวคลีโอไทด์ของยีนไลเปสทั้งหมด จากการวิเคราะห์ลำดับนิวคลีโอไทด์ของยีนไลเปสที่ได้ (*lip52*) พบว่ามีขนาด open reading frame (ORF) ขนาด 1,221 คู่เบส สามารถแปลรหัสเป็นกรดอะมิโน 407 หน่วย เมื่อวิเคราะห์ลำดับของกรดอะมิโนกับโปรตีนในฐานข้อมูลพบว่าเอนไซม์ไลเปส (Lip52) ไม่มีความเหมือนอย่างสมบูรณ์กับโปรตีนที่ปรากฏอยู่บนฐานข้อมูล อย่างไรก็ตาม เอนไซม์ไลเปสดังกล่าวมีความเหมือนในระดับปานกลาง (24–51%) เมื่อเปรียบเทียบกับเอนไซม์ไลเปสที่มีการศึกษามาแล้ว แสดงให้เห็นว่าเอนไซม์ไลเปสที่ได้นี้เป็นเอนไซม์ไลเปสชนิดใหม่ จากการวิเคราะห์ยังพบบริเวณที่มีการเรียงลำดับของกรดอะมิโน (motif) แบบอนุรักษ์ซึ่งพบในกลุ่มของเอนไซม์ไลเปสและเอสเทอเรสคือ GxSxG เมื่อนำยีน *lip52* ไปแสดงออกใน *Escherichia coli* JM109 จากนั้นนำเอนไซม์ที่ได้ไปผ่านขั้นตอนการทำให้บริสุทธิ์แล้วนำไปศึกษาถึงคุณสมบัติบางประการของเอนไซม์พบว่า เมื่อวิเคราะห์ด้วยวิธี SDS-PAGE

เอนไซม์ไลเปสมีน้ำหนักโมเลกุลประมาณ 45 กิโลดาลตัน เอนไซม์มีค่าพีเอชและอุณหภูมิที่เหมาะสมกับการทำงานคือ 8.0 และ 90 องศาเซลเซียส ตามลำดับ เอนไซม์มีความเสถียรสูงในช่วงค่าพีเอช 5.0-10.0 และในช่วงอุณหภูมิ 30–100 องศาเซลเซียส สับสเตรทที่เหมาะสมสำหรับการเกิดกิจกรรมของเอนไซม์ไลเปสในระดับสูงสุดคือ *p*-nitrophenyl caprylate (C_8) จึงพอที่จะสรุปได้ว่าโปรตีน Lip52 คือเอนไซม์ไลเปสไม่ใช่เอนไซม์เอสเทอเรส

Thesis Title	Identification, gene cloning and characterization of an extremely thermoactive and thermotolerant lipase from *Bacillus thermoamylovorans* BHK52
Author	Miss Uschara Thumarat
Major Program	Biotechnology
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ABSTRACT

Lipases (triacylglycerol hydrolases; EC 3.1.1.3) are the most important lipolytic enzymes used as industrial biocatalysts for a variety of biotechnological applications. In this study, the thermotolerant bacteria isolated from composts were used as sources of lipase-producing bacteria. The bacterial strains were screened and scored positive as they showed orange fluorescent colonies under UV light of 254 nm after cultivation on lipase assay medium containing palm oil and rhodamine B at 50°C. A total of 10 bacterial strains exhibiting high levels of lipase activity were selected. Those strains were identified as 1 isolate of *Aneurinibacillus thermoaerophilus*, 1 isolate of *Bacillus pumilus*, and 8 isolates of *Bacillus thermoamylovorans* by 16S rRNA gene sequence analysis. As no report was found on a lipase from *Bacillus thermoamylovorans* so far its target lipase was supposed to be a novel one, *Bacillus thermoamylovorans* BHK52 was selected for further gene cloning, nucleotide sequencing, expression and partial characterization. Degenerate primers were designed according to the sequences of two highly conserved regions of lipases among *Bacillus* spp. and used to amplify a part of lipase gene by PCR. After that, inverse PCR was performed to amplify the whole nucleotide sequence of lipase gene. Sequence analysis of the lipase gene (*lip52*) showed an open reading frame (ORF) of 1,221 bp encoding for a mature lipase of 407 amino acids. Amino acid sequence analysis revealed that the lipase Lip52 did not match perfectly with any known proteins in the databases. However, it showed only moderate sequence identity (24–51%) with the known lipases. This indicates that the lipase Lip52 is a novel enzyme. It contains a typical GxSxG motif found in most esterases and lipases. The *lip52* gene was expressed in *Escherichia coli* JM109, purified and partially characterized. The molecular mass of the lipase Lip52 was determined to be approximately 45 kDa by SDS–PAGE. The enzyme had an optimum pH of 8.0 and showed maximal activity at 90°C. It was highly stable within a pH range of

5.0-10.0 and a temperature range of 30–100°C. The highest activity was observed when *p*-nitrophenyl caprylate (C_8) was used as a synthetic substrate, which can be concluded that the lipase Lip52 is a true lipase but not esterase.

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Uschara Thumarat

CONTENTS

CONTENTS (CONTINUED)

CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

CHAPTER 1

INTRODUCTION

Introduction

Lipases or triacylglycerol hydrolases (EC 3.1.1.3) are lipolytic enzymes that catalyze the hydrolysis of long-chain triglycerides with the formation of diacylglycerides, monoglycerides, glycerol and free fatty acids at the interface between the insoluble substrate and water. However, lipases are also capable of catalyzing the reverse reaction of hydrolysis in the formation of esters from alcohols and fatty acids or via transesterification (Leow *et al.*, 2004). The enzymes are ubiquitous in nature and are produced by various plants, animals and microorganisms (Jaeger and Reetz, 1998). Microbial lipases are widely used for biotechnological applications i.e. detergents, dairy and textile industries, production of surfactants, and oil processing. In recent years, lipases have received considerable attention with regard to the preparation of enantiomerically pure pharmaceuticals, since they have a number of unique characteristics including substrate specificity, regio-specificity, and chiral selectivity (Kim *et al*., 1998). A major requirement for commercial lipases is thermal stability which would allow enzymatic reaction to be performed at higher temperatures and would be helpful to increase conversion rates, substrate solubility, and to reduce the contamination of microorganisms. Recently, lipases that cloned or isolated from extreme thermophiles have led to a special focus due to their higher thermodynamics both at elevated temperatures and in organic solvents, as a consequence of adaptation of the corresponding thermophiles to higher growth temperatures (Gubernator *et al*., 1991). Thermophiles are the good candidates in production of thermostable lipases. However, it is often impractical to use them directly due to the low yield of lipase. In this content, a molecular approach through expression of foreign protein in prokaryotic systems has become a good alternative to economically obtain bulk production of lipase. So far, several lipases have been purified and characterized from thermophilic isolates, mainly from *Bacillus* (Schmidt-Dannert *et al.,* 1994; Luisa *et al.*, 1997; Kim *et al.,* 1994; Lee *et al,* 1999). Researches on lipases are focused particularly on structural characterization, elucidation of mechanism of action, kinetics, sequencing and cloning of lipase genes, and general characterization of performance (Gubernator *et al*., 1991). Lipase is one of lipolytic enzymes which were classified into eight families according to their

conserved sequence motifs and their biological properties (Arpigny and Jaeger, 1999). Most lipolytic enzymes exhibit the catalytic triad formed by serine, histidine, and aspartic acid residues; the active-site serine residue is presented in the -Gly-Xaa-Ser-Xaa-Gly- motif in most families or in the -Gly-Asp-Ser-(Leu)- motif in family II. However, lipolytic enzymes belonging to family VIII have serine residue in the -Ser-Xaa-Xaa-Lys- motif whereas their catalytic center and mechanism are still not clear.

In this study the thermotolerant bacteria isolated from composts were screened. A total of 10 bacterial strains having high level of lipase activity were selected for identification by 16S rRNA gene sequence analysis. Finally, the *Bacillus thermoamylovorans* BHK52 was selected for further gene cloning, nucleotide sequencing, expression, and partial characterization of the purified enzyme.

Review of Literatures

1. Lipases

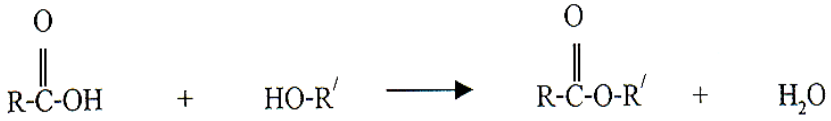
Lipases or triacylglycerol hydrolases (EC 3.1.1.3) are enzymes that catalyze the hydrolysis of long-chain triglycerides with the formation of diacylglycerides, monoglycerides, glycerol and free fatty acids at the interface between the insoluble substrate and water (Leow *et al*., 2004). However, lipases are also capable of catalyzing the reverse reaction of hydrolysis in the formation of esters from alcohols and fatty acid or via transesterification (Fig. 1). Certain microorganisms are the sources of choice for lipase production because they are able to utilize lipids of plant and animal origin as the source of carbon and energy for growth. Practical use of microbial lipases enhance a great interest in biotechnological research in regards to the improvement of both the producing strains and the biochemical properties of the lipolytic enzymes. New applications of the microbial lipases including hydrolysis of fats, transesterification, stereospecific hydrolysis of racemic esters, organic synthesis, and the use of lipases in detergents have been developed in the last years (Ionita *et al*., 1997)

1.1 Source of lipases

Lipases occur widely in animals, plants and microorganisms. Numerous mammalian tissues, organs and fluids, such as pancreas, kidney, adipose tissue, heart, brain, muscle and serum have been also known to contain lipases. Animal pancreatic lipases especially hog pancreatic lipase has been studied most extensively, presumable because of its high concentration and high turnover

1. Hydrolysis

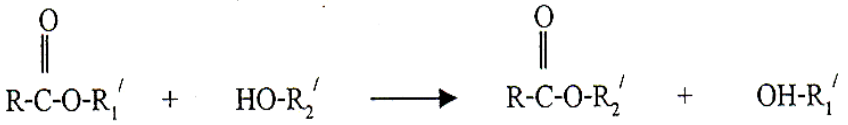
2. Synthesis



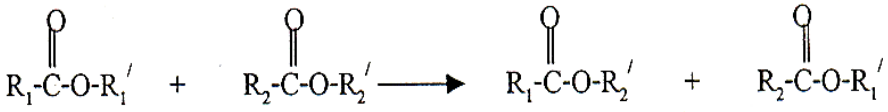
3. Transesterification

3.1 Acidolysis

3.2 Alcoholysis



3.3 Interesterification



3.4 Aminolysis

Figure 1. Industrially important reactions catalyzed by lipases.

Source: Yamane (1987)

number. Moreover, the present of esterolytic enzymes in milk of cow, sow, goat, sheep, and human have been known for a long time (Shahani,1975).

Numerous fruits, vegetables, plant tissues and particularly plant seeds, such as wheat, oat, rye, cotton, soybean, castor bean and *Vernonia anthelmintica*, have been known to exhibit lipase activity. In general, the germ portions of wheat, rye and barley contain a much higher level of lipolytic enzyme activity than endosperm and the scutellum section (Shahani,1975).

During recent years considerable attention has been devoted to intracellular or extracellular lipases produced by microorganisms. Especially, extracellular microbial lipases show high potential for many industrial applications and are appropriated for mass production because of the ease of enzyme collection. A variety of microorganisms capable to produce lipases have been found. These include the genera of *Candida*, *Rhizopus*, *Penicillium*, *Aspergillus*, *Geotrichium*, *Mucor*, *Pseudomonas*, *Achromobacter* and *Staphylococcus* (Godtfredsen, 1993). Examples of lipases from yeast and fungi are listed in Table 1 and lipases from various microorganisms are listed in Table 2. Microbial enzymes are often more useful than the enzymes derived from plants or animals because of the great variety of catalytic activities, high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also often more stable than their corresponding plant and animal enzymes and their production is more convenient and safer. Only about 2% of the world's microorganisms have been tested as enzyme sources. Bacterial strains are generally utilized as they offer higher activities compared to yeasts and tend to have neutral or alkaline pH optima and are often thermostable. Genetic and environmental manipulation have been used to increase the yield of cells (Demain *et al*., 1971) and to increase the enzyme activity of the cells, by making the enzyme of interest either constitutively expressed or inducible expressed or by producing altered enzymes (Betz *et al*., 1974), may be employed easily using microbial cells because of their short generation times, their relatively simple nutritional requirements, and because screening procedures for the desired characteristic are easier.

1.2 External influences on lipase production by microorganisms

1.2.1 Carbon source

Different microorganisms need different medium and carbon source for lipase production. Handelsman and Shoham (1994) reported that lipase activity of *Bacillus* sp. H1 when using Tween 80 as a carbon source was higher than that when using olive oil.

Table 1. Examples of lipase-producing yeast and fugal strains.

Microorganism	Viability (cell no./ml)	R/r	Classification
Absidia repens CPhI-A-3	0.85 x 10^6	< 1.0	weak
Aspergillus niger CPhl-8-N-2	0.70 x 10^7	0	-
Aspergillus niger CPhI-8-N-9	1.80 x 10^8	> 2.0	good
Aspergillus niger CPhl-8-N-2	0.70 x 10^7	0	-
Aspergillus niger CPhI-8-N-9	1.80 x 10^8	> 2.0	good
Aspergillus niger CPhl-8-N-19	0.90 x 10^7	0	-
Aspergillus niger CPhi-8-N-27	1.20 x 10^7	0	-
Aspergillus oqxae CPhI-20	4.70 x 10^7	< 1.0	weak
Aspergillus oryzae CPhI-20-9	3.80 x 10^7	> 2.0	good
Aspergillus oryzae CPhI-20-9-7 1	2.90 x 10^7	1.0-2.0	moderate
Aspergillus oryzae CPhI-20-9-118	2.50 x 10^6	1.0-2.0	moderate
Candida boidinii CPhI-44	4.30 x 10^8	0	-
Candida lipolytica CPhI-SO	0.82 x 10^8	> 2.0	good
Candida rugosa CPhI- 15	3.80 x 10^8	1.0-2.0	moderate
Candida tropicalis CPhl-29	2.20 x 10^8	< 1 .0	weak
Candida utilis CPhI- 10	1.50 x 10^8	< 1.0	weak
Hansenula anomala CPhI-23	2.40 x 10^8	> 2.0	good
Hansenula polymotpha CPhI-3-5	3.70 x 10^8	0	-
Rhizopus nigricans CPhI-Rn-8	2.0 x 10^6	> 2.0	good
Rhizopus stolonifer CPhI-Rs-5	1.2 x 10^6	1.0-2.0	moderate
Saccharomyces cerevisiae CPhI-34	3.90 x 10^8	0	-
Saccharomyces carlsbergensis CPhI-38	2.50 x 10^8	< 1.0	weak

Source: Ionita *et al*. (1997)

Remark: R is the radius of the opacity areas in cm whereas r is the colony radius in cm that related with enzyme activity. Classification of enzyme activity:

R/r >2.0 = Good

1.0 < R/r < 2.0 = Moderate

R/r < 1.0 = Weak

Table 2. Isolation of lipases from various microorganisms.

Microorganism	Reference
Acinetobacter sp.	Snellman *et al*., 2002
Aspergillus niger	Namboodiri and Chattopadhaya, 2000
Aspergillus oryzae	Toida *et al*., 1998
Bacillus sp.	Imamura and Kitaura, 2000; Handelsman and Shoham, 1994 and Sugihara *et al*., 1991
Bacillus coagulans	El-Shafei and Rezkallah, 1997
Bacillus subtilis	Ruiz *et al*., 2005 and Eggert *et al*., 2003
Bacillus thermocatenulatus	Lee *et al*., 1999
Bacillus thermoleovorans	Rua *et al*., 1997
Botrytis cinerea	Commenil *et al*., 1995
Chromobacterium viscosum	Taipa *et al*., 1995
Enterococcus faecalis	Kar *et al*., 1996
Fusarium solani	Knight *et al*., 2000
Geotrichum candidum	Jacobsen and Poulsen, 1995
Lactobacillus plantarum	Lopes *et al*., 1999
Penicillium cyclopium	Chahinian *et al*., 2000 and Ibrik *et al*., 1998
Penicillium simplicissimum	Sztajer *et al*., 1992
Pseudomonas aeruginosa	Chartrain *et al*., 1993; Shabtai and Daya-Mishne 1992
Pseudomonas fluorescens	Kojima *et al*., 1994
Pseudomonas fragi	Nishio *et al*., 1987
Rhizomucor miehei	Herrgard *et al*., 2000
Rhizopus sp.	Macedo *et al*., 2003
Staphylococcus aureus	Gotz *et al*., 1998 and Simons *et al*., 1996
Staphylococcus haemolyticus	Oh *et al*., 1999
Staphylococcus warneri	Talon *et al*., 1995
Staphylococcus xylosus	Mosbah *et al*., 2005
Streptomyces flavogriseus	Mostafa and Ali, 1979
Trichosporon asteroids	Dharmsthiti and Ammaranond, 1997
Trichosporon laibacchii	Liu *et al*., 2004

Source: Hasan *et al*. (2006)

Rhizopus delemar CDBB H313 was found to produce higher level of lipase when dextrin was used as a carbon source than when glucose was used as a carbon source (Espinosa *et al*., 1990).

1.2.2 Nitrogen source

Different microorganisms need different medium and nitrogen source for lipase production. Some bacteria produced lipases under inorganic nitrogen source whereas some bacteria produced lipases under organic nitrogen source. The best nitrogen source for lipase production of *Humicola lanuginose* No.3 was found to be 1.0% (v/v) corn raise water (Omar *et al*., 1987). *Bacillus subtilis* can utilize the purine bases including adenine, hypoxanthine and xanthine as nitrogen sources. High levels of GDEase were found in cells grown with purines and intermediary compounds of the purines catabolic pathway as nitrogen sources (Nygaard *et al*., 2000).

1.2.3 Temperature, pH and aeration

Optimal culture conditions for various lipase-producing bacteria are different from one another. In general, temperature, pH and aeration of the culture were chosen based on requirements for growth of particular bacterial strains. Optimal temperature and pH for growth of *Bacillus* sp. H1 was found to be 60°C and 7.2, respectively (Handelsman and Shoham, 1994), therefore lipase production was done at those conditions. Sometime, aeration also has an effect on lipase production. For example, for *Pseudomonas aeruginosa* EF2, deoxygenization was a cause of decreased lipase activity (Gilbert *et al*., 1991).

1.3 External influence on lipase activity and stability

1.3.1 Temperature

Temperature is an important influence on lipase activity and stability. Different microorganisms have different optimal temperature for growth and lipase production. However, some lipases are unstable at their optimal temperature. For example, the optimal temperature for lipase production of *Humicolar lanuginose* No.3 is 45°C, whereas the optimal temperature for its lipase stability is 60°C (Omar *et al*., 1987).

1.3.2 pH

Normally, thermotolerant lipases have optimum pH in the range of moderate until alkaline pH, and a few lipases are shown to have optimal pH in acidic range. Generally, lipases exhibit stability in the wide range of pH. For example lipase from *Bacillus* sp. 398 has stability in the range of pH 4-11 after incubation for 1 h (Kim *et al*., 1994).

1.3.3 Organic solvent

Lipases are also capable of catalyzing the reverse reaction of hydrolysis in the formation of esters from alcohols and fatty acid or via transesterification under water control system. These reactions must have organic solvents in the production system so studying of their effect on lipase activity were recommended. Different type and concentration of organic solvents have an effect to wide lipase activity. Sugihara *et al*. (1991) reported that lipase activity of *Bacillus* sp. could be increased up to 60% when using 20% (v/v) acetone but when the concentration of acetone was higher than 60% (v/v) the lipase activity was decreased. Isooctane increased the stability of lipase from *Mucor hiemalis f. hiemalis* but decreased the stability of lipases from others (Hiol *et al*., 1999).

1.3.4 Metal ion and chemical

Normally, metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^{+} and Li^{+} can sometimes increase lipase activity. For example, lipase activity of *Bacillus* sp. was increased 7% in the presence of Ca^{2+}, Na^{+}, K^{+} or Co^{2+}, and 32% in the presence of Ba^{2+} (Handelsman and Shoham, 1994). These metal ions are presumably activator for lipases. Ca^{2+} is the majority of microbial lipase activator by changing the conformation of enzyme and increasing adsorption in the surface of water and oil. Metal ions such as Fe^{2+}, Fe^{3+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Sn^{2+}, Co^{2+}, $Ni^{2+}$and Cd^{2+} are inhibitors for some lipases. Thus, some metal ions and chemicals can be activators or inhibitors depending on mechanisms and effects on lipase structure, especially at the active site.

2. Classification of bacterial lipolytic enzymes

Lipolytic enzymes such as esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) catalyze both the hydrolysis and the synthesis of acylglycerides and other fatty acid esters. The true lipases differ from the carboxylesterases in their maximal activity on water-insoluble long-chain esters (Arpigny and Jaeger 1999). Lipolytic enzymes are important biocatalysts for various industrial applications due to their useful features, such as having no requirements for cofactors, remarkable stability in organic solvents, broad substrate specificity, stereoselectivity, and positional selectivity (Jaeger *et al*. 1994; Jaeger and Eggert 2002). Bacteria produce different classes of lipolytic enzyme, including (i) carboxylesterases (EC 3.1.1.1), which hydrolyse small ester-containing molecules at least partly soluble in water, (ii) true lipases (EC 3.1.1.3), which display maximal activity towards water insoluble long-chain triglycerides, and (iii) various types of phospholipase (Arpigny and Jaeger 1999). Many new bacterial lipolytic enzymes have been studied since the publication of a

comprehensive review article in 1994 by Jaeger and his colleagues and the lipolytic enzymes were classified primarily based on structure and amino acid composition as shown in Table 3.

2.1 Family I: True lipases

The bacterial lipase in family I including lipases from *Pseudomonas,* gram-positive bacteria and other organisms. *Pseudomonas* lipases were probably the first one that have been studied and have a preponderant role in industry. The various *Bacillus* lipases have in common an alanine residue that replaces the first glycine in the conserved pentapeptide: Ala-Xaa-Ser-Xaa-Gly. However, the lipases from two mesophilic strains, *B. subtilis* and *B. pumilus*, stand apart from the rest because they are the smallest true lipases known (approx. 20 kDa) and share very little sequence similarity (approx. 15%) with the other *Bacillus* and *Staphylococcus* lipases (Arpigny and Jaeger 1999).

2.2 Family II: The GDSL family

The enzymes grouped in family II do not exhibit the conventional pentapeptide Gly-Xaa-Ser-Xaa-Gly, but rather display a Gly-Asp-Ser-(Leu) [GDS(L)] motif containing serine residue in the active site. In these proteins, this important residue lies much closer to the N-terminus than in other lipolytic enzymes (Arpigny and Jaeger, 1999).

2.3 Family III

This group of enzymes displays the canonical fold of α/β-hydrolases and contains a typical catalytic triad. It also shows approx. 20% amino acid sequence identity with the intracellular and plasma isoforms of the human PAFAH (Arpigny and Jaeger 1999).

2.4 Family IV: The hormone-sensitive lipase (HSL) family

A number of bacterial enzymes in this family display a striking amino acid sequence similar to the mammalian HSL.

2.5 Family V

The lipases in this family are similar to proteins in the HSL family. The enzymes grouped in family V originate from mesophilic bacteria.

2.6 Family VI

The enzymes grouped in this family are among the smallest esterases known. These carboxylesterases hydrolyze small substrates with a broad specificity and display no activity toward long-chain triglycerides (Arpigny and Jaeger, 1999).

Table 3. Families of lipolytic enzymes.

Family	Subfamily	Enzyme-producing strain	Accession no.	Similarity (%)		Properties
				Family	Subfamily	
I	1	*Pseudomonas aeruginosa**	D50587	100		True lipases
		Pseudomonas fluorescens C9	AF031226	95		
		Vibrio cholerae	X16945	57		
		Acinetobacter calcoaceticus	X80800	43		
		Pseudomonas fragi	X14033	40		
		Pseudomonas wisconsinensis	U88907	39		
		Proteus vulgaris	U33845	38		
	2	*Burkholderia glumae**	X70354	35	100	
		*Chromobacterium viscosum**	Q05489	35	100	
		*Burkholderia cepacia**	M58494	33	78	
		Pseudomonas luteola	AF050153	33	77	
	3	*Pseudomonas fluorescens* SIK W1	D11455	14	100	
		Serratia marcescens	D13253	15	51	
	4	*Bacillus subtilis*	M74010	16	100	
		Bacillus pumilus	A34992	13	80	
	5	*Bacillus stearothermophilus*	U78785	15	100	
		Bacillus thermocatenulatus	X95309	14	94	
		Staphylococcus hyicus	X02844	15	29	Phospholipase
		Staphylococcus aureus	M12715	14	28	
		Staphylococcus epidermidis	AF090142	13	26	
	6	*Propionibacterium acnes*	X99255	14	100	
		Streptomyces cinnamoneus	U80063	14	50	

Table 3. Families of lipolytic enzymes (continued).

Family	Subfamily	Enzyme-producing strain	Accession no.	Similarity (%)		Properties
				Family	Subfamily	
II (GDSL)		*Aeromonas hydrophila*	P10480	100		Secreted acyltransferase
		Pseudomonas aeruginosa	AF005091	35		OM-bound esterase
		Salmonella typhimurium	AF047014	28		OM-bound esterase
		Photorhabdus luminescens	X66379	28		Secreted esterase
III		*Streptomyces exfoliatus**	M86351	100		Extracellular lipase
		Streptomyces albus	U03114	82		Extracellular lipase
		Moraxella sp.	X53053	33		Extracellular esterase 1
IV (HSL)		*Alicyclobacillus acidocaldarius*	X62835	100		Esterase
		Pseudomonas sp. B11-1	AF034088	54		Lipase
		Archaeoglobus fulgidus	AE000985	48		Carboxylesterase
		Alcaligenes eutrophus	L36817	40		Putative lipase
		Escherichia coli	AE000153	36		Carboxylesterase
		Moraxella sp.	X53868	25		Extracellular esterase 2
V		*Pseudomonas oleovorans*	M58445	100		PHA-depolymerase
		Haemophilus influenzae	U32704	41		Putative esterase
		Psychrobacter immobilis	X67712	34		Extracellular esterase
		Moraxella sp.	X53869	34		Extracellular esterase 3
		Sulfolobus acidocaldarius	AF071233	32		Esterase
		Acetobacter pasteurianus	AB013096	20		Esterase
VI		*Synechocystis* sp.	D90904	100		Carboxylesterases
		Spirulina platensis	S70419	50		
		*Pseudomonas fluorescens**	S79600	24		
		Rickettsia prowazekii	Y11778	20		
		Chlamydia trachomatis	AE001287	16		

Table 3. Families of lipolytic enzymes (continued).

Family	Subfamily	Enzyme-producing strain	Accession no.	Similarity (%) Family	Similarity (%) Subfamily	Properties
VII		*Arthrobacter oxydans*	Q01470	100		Carbamate hydrolase
		Bacillus subtilis	P37967	48		p-Nitrobenzyl esterase
		Streptomyces coelicolor	CAA22794	45		Putative carboxylesterase
VIII		*Arthrobacter globiformis*	AAA99492	100		Stereoselective esterase
		Streptomyces chrysomallus	CAA78842	43		Cell-bound esterase
		Pseudomonas fluorescens SIK W1	AAC60471	40		Esterase III

Source: Arpigny and Jaeger (1999)

Remark: Amino acid sequence similarities were determined by the program MEGALIGN (DNASTAR), with the first member of each family (subfamily) arbitrary set at 100%.

Abbreviations: OM, outer membrane; PHA, polyhydroxyalkanoate; *, lipolytic enzyme with known 3D structure.

2.7 Family VII

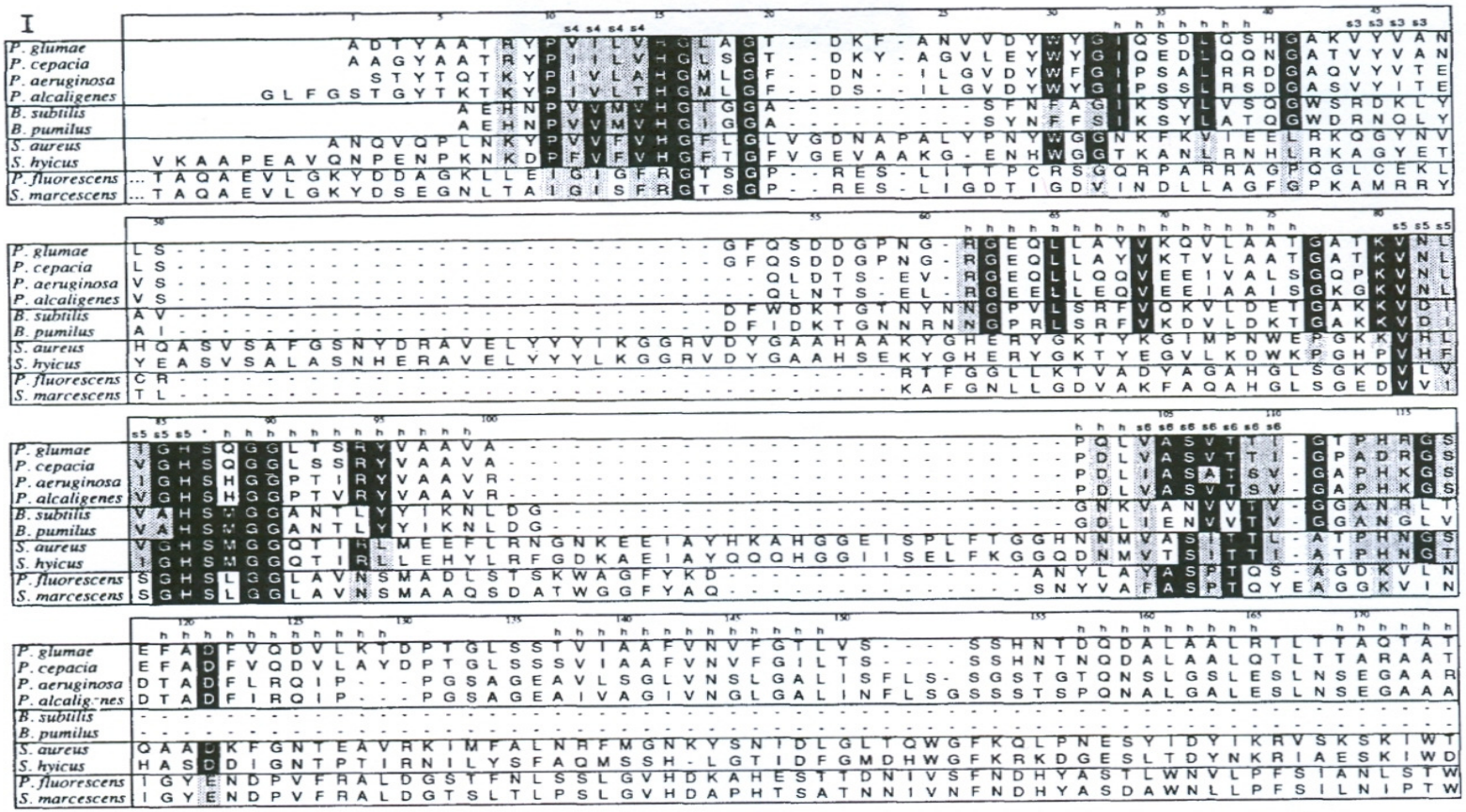
A number of rather large bacterial esterases (55 kDa) in this family share significant amino acid sequence homology with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases (Arpigny and Jaeger, 1999).

2.8 Family VIII

The enzymes forming this family are approximately 380 residues long and show a striking similarity to several class C β-lactamases. This feature suggests that the esterases in family VIII possess an active site more reminiscent of that found in class C β-lactamases, which involves a Ser-Xaa-Xaa-Lys motif conserved in the N-terminal part of enzyme (Arpigny and Jaeger, 1999).

3. Three-dimensional structure of lipases

Bacterial lipases vary considerably in size (20-60 kDa), and although the alignment of their secondary structural elements reveals a certain degree of variation, they presumably all have a similar overall three-dimensional structure. Alignment of several bacterial lipases (Fig. 2) revealed only little sequence homology. Comparison of lipases revealed that


I
P. glumae
P. cepacia
P. aeruginosa
P. alcaligenes
B. subtilis
B. pumilus
S. aureus
S. hyicus
P. fluorescens
S. marcescens

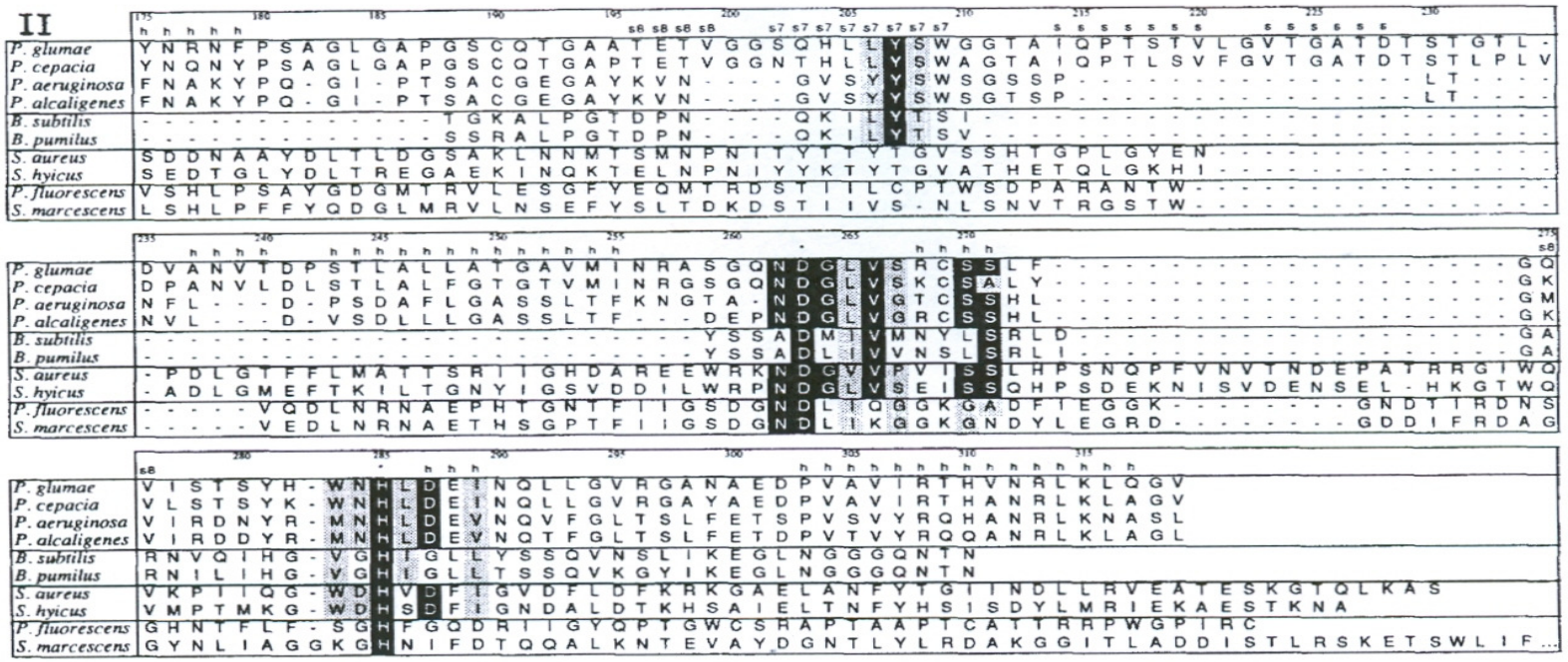


Figure 2. Comparison of amino acid sequences of bacterial lipases. From top to bottom, lipases are from *P. glurnae*, *P. cepaeia*, *P. aerugmosa*, *P. alcaligenes*, *B. subtilis*, *B. pumilus*, *S. aureus*, *S. hyicus* (46-kDa mature lipase), *P. fluorescens* (117 residues at the N-terminal part are not shown) and *S. marcescens* (117 residues at the N-terminal and 151 residues at the C-terminal part are not shown). Black boxes represent amino acid residues highly conserved in at least two of the four groups of lipases; grey boxes represent similar amino acid residues. The numbering of amino acids is given for the *P. glumae* lipase. Secondary structural elements are also indicated above the sequence (h: α-helices, s: β-strands).

Source: Jaeger *et al*. (1994)

sequence homologies are located at the N-terminal part, especially at strand number 4 containing the oxyanion hole (the sequence around the active-site serine residue) with strand number 5 and the following helix (strand number 6) next to the active-site serine residue, and a small region around the active site aspartate residue. These small regions of homology permit researchers to postulate that most lipases will have a similar fold which presumably resembles the so-called α/β hydrolase fold (Ollis *et al*., 1992). Determination of three-dimensional structures of different lipases has confirmed their classification as serine hydrolases. Their active site is composed of three residues: a serine residue hydrogen-bonded to a histidine residue, and a aspartate residue hydrogen-bonded to this same histidine residue. The catalytic centre of the lipases contains Ser-His-Asp residues. The three-dimensional structure of the active site of lipases is showed in Figure 3. Most lipases contain the invariant first and last glycine residues (Gly) in the consensus sequence Gly-X-Ser-X-Gly (where X represents any amino acid). However, this well conserved lipase box differs in lipases from *Bacillus* strains where the first glycine residue (Gly) is replaced by an alanine (Ala) (Dartois *et al*., 1992).

4. Thermostable lipases

Microorganisms adapt to the condition in which they have to live and survive. Thermophiles are reported to contain proteins which are thermostable and resist denaturation and proteolysis at elevated temperature. Specialized proteins known as chaperonins are produced by these organisms to help the refolding of the proteins to their native form and restore their functions after their denaturation at high temperature. The cell membrane of thermophiles is mostly made up of saturated fatty acids. Those fatty acids provide a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures. The archae, which comprises most of the hyperthermophiles, have lipids linked with other on the cell wall. This layer is much more heat resistant than a membrane formed by other fatty acids (Haki and Rakshit, 2003). Examples of sites serving as sources of microorganisms which can provide thermotolerant enzymes are showed in Table 4. The importance of thermostable lipases for different applications has been growing rapidly. Most of the studies so far have been carried out with mesophilic producers. Many lipases from mesophiles are stable at elevated temperatures (Sugihara *et al*., 1991). Proteins from thermophilic organisms have been proved to be more useful for biotechnological applications than similar proteins from mesophiles due to their stability (Imamura and Kitaura, 2000). Thermostable lipases from microbial sources are highly advantageous for biotechnological applications since they can be

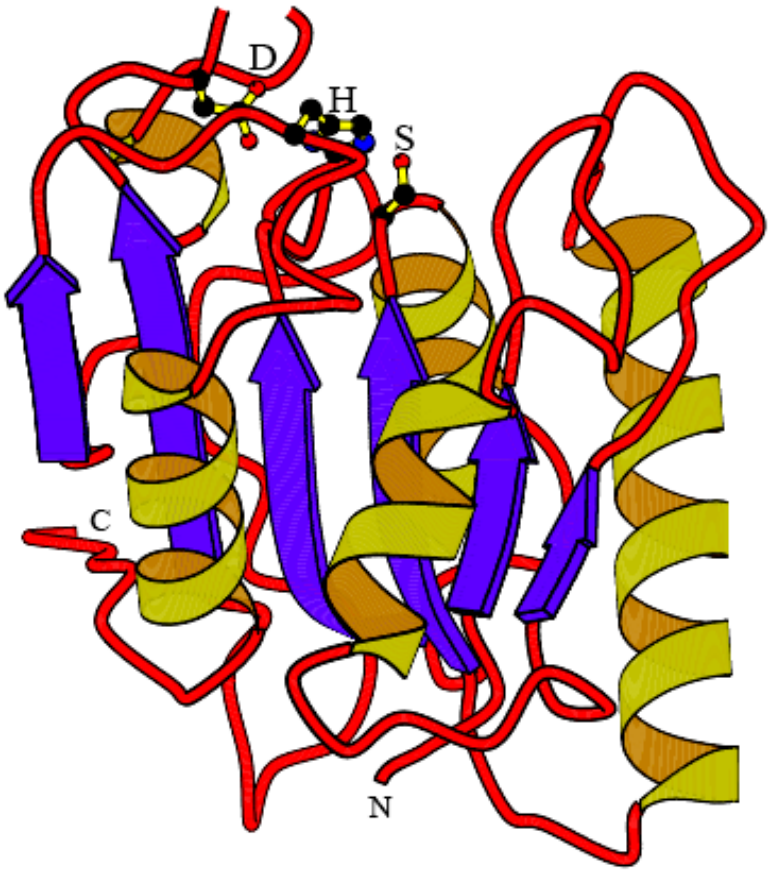


Figure 3. Three-dimensional structure of the active site of the *B. subtilis* lipase. The catalytic triad residues are Ser77 (S), His156 (H) and Asp133 (D). The N and C termini are indicated by N and C alphabets, respectively.

Source: Pouderoyen *et al*. (2001)

Table 4. Example of sites serving as sources of microorganisms which can provide thermotolerant enzymes.

Source	Microorganism	Enzyme
Compost	*Bacillus stearothermophilus* CH-4	β-N acetylhexosaminidase
Compost of fermenting citrus peels	*Bacillus* sp. MH-1	Endochitinase
Compost treated with artichoke juice	*Bacillus* sp.	Inulinase
Decomposed plant samples	*Clostridium absonum* CFR-702	Cellulase free xylanase
Deep sea hydrothermal vent	*Pyrococcus abyssi*	Alkaline phosphatase
Deep sea hydrothermal vent	*Staphilothermus marinus*	α-amylase
Garbage dump	*Bacillus circulans*	Xylanase
Hot spring	*Bacillus thermoleovocans* ID-1	Lipase
Hot spring	*Bacillus* sp. WN.11	α-amylase
Hot spring	*Thermus* sp.	α-amylase
Korean salt fermented anchovy	*Bacillus* sp. KYJ963	β-amylase
Marine solfatare	*Thermococcus litoralis*	Pullulanase
Sediments of hot springs	*Bacillus* sp. 3183	α-amylase-like pullulanase

Source: Haki and Rakshit (2003)

produced at low cost and exhibit improved stability (Handelsman and Shohem, 1994). Thus, in recent years, there has been a great demand for thermostable enzymes in industrial fields, and thermostable lipases from various sources have been purified and extensively characterized. Biocatalyst with high thermostability allows a higher operating temperature, which is clearly advantageous because of a higher reactivity (higher reaction rate, lower diffusional restrictions), higher stability, higher process yield, lower viscosity and fewer contamination problems (Mozhaev, 1993). The advantages of utilizing lipases in bioprocess at elevated temperatures also include the increased solubility of lipids and other hydrophobic substrates in water and increased reactant solubilities. Hence, thermophiles are a valuable source of thermostable enzymes with properties that are often associated with stability in solvents and detergents, giving these enzymes

considerable potential for many biotechnological and industrial applications (Haki and Rakshit, 2003).

Most of the industrial processes in which lipases are employed function at temperatures exceeding 45°C, the enzymes are required to exhibit an optimum temperature of around 50°C (Sharma *et al*., 2002). According to some reports, there are fats exhibiting high melting points which become solids and are able to inhibit enzymatic reactions at a low temperature (Dong-Woo *et al*., 1999). Some enzymatic processes for the physical refining of seed oils have two distinct phases. The first phase usually involves enzymatic reactions at pH 5.0 and temperature around 65°C. It is followed by the second phase which separates lysophosphatide from the oil at approximately 75°C (Klaus, 1998). These reactions are enhanced through the utilization of thermotolerant lipases. Lipases are of widespread occurrence throughout the earth's flora and fauna. Several *Bacillus* sp. were reported to be the main sources of thermotolerant lypolytic enzymes (Kim *et al*., 1994; Schmidt *et al*., 1994; Luisa *et al*., 1997). The optimal pH and temperature for the catalytic activity of some thermostable lipases are given in Table 5.

5. Applications of lipases

Microbial lipases have been widely used for biotechnological applications i.e. detergents, dairy and textile industries, production of surfactants, and oil processing.

5.1 Lipases in fat and oleochemical industry

The physiological role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. In addition, the lipase-catalyzed transesterification in organic solvents is an emerging industrial application such as production of cocoa butter equivalent, human milk fat substitute "Betapol", pharmaceutically important polyunsaturated fatty acids (PUFA), rich/low calorie lipids (Jaeger and Reetz, 1998). Cocoa butter fat is a high-value product because its triacylglycerides (TAGs) are high in stearates, which give a melting point of 37˚C. Thus, cocoa butter fat melts in the mouth to give cooling sensation, smooth 'mouth appeal' effect for chocolate and other products. In contrast, palm oil TAGs are high in palmitate which has a melting point of 23˚C, so it is an oil at room temperature and is a low-value product. Therefore, using lipases to convert palmitate from palm oil to stearates by transesterification to create cocoa butter equivalent is attractive and can add much value to the existing product.

Table 5. Source of microorganisms and properties of thermostable lipases.

Organism	Optimum temperature (°C)	Optimum pH
Bacillus acidocaldariusa (esterase)	70	–
Bacillus sp. RSJ-1	50	8.0–9.0
Bacillus sp. J33a	60	8
Bacillus stearothermophilusa	68	–
Bacillus thermocatenletusa	60–70	8.0–9.0
Bacillus thermoleovorans ID-1	70–75	7.5
Geobacillus sp.	70	9
Pseudomonas sp.	65	9.6
Pseudomonas sp.	90	11
Pyrobaculum calidifontis	90	–
Pyrococcus furiosusa (esterase)	100	–
Pyrococcus horikoshii	97	5.6
Pyrococcus horikoshii	95	7

Source: Haki and Rakshit (2003)

5.2 Production of biodegradable polymers

Lipases have become one of the most important groups of enzymes for its applications in organic synthesis. Lipases can be used as biocatalyst in the production of useful biodegradable compounds. For example, 1-Butyl oleate was produced by direct esterification of butanol and oleic acid to decrease the viscosity of biodiesel for winter use. Trimethylolpropane esters were also similarly synthesized as lubricants (Hasan *et al*., 2006). Lipases can catalyze ester synthesis and transesterification reactions in organic solvent systems has opened up the possibility of enzyme-catalyzed production of biodegradable polyesters. Aromatic polyesters can be synthesized by lipase biocatalysis (Linko *et al*., 1998).

5.3 Lipases in textile industry

Lipases are used in the textile industry to assist in the removal of size lubricants. In the textile industry, polyester has certain key advantages over many other fibers including high strength, soft hand, stretch resistance, stain resistance, machine washability, wrinkle resistance, and

abrasion resistance. Synthetic fibers have been modified enzymatically for the use in the production of yarns, fabrics, textiles, rugs and other consumer items for a long time. Polyesterases (closely related to lipases) are also utilized to improve the ability of a polyester fabric to uptake chemical compounds including cationic compounds, fabric finishing compositions, dyes, antistatic compounds, anti-staining compounds, antimicrobial compounds, antiperspirant compounds and/or deodorant compounds (Hasan *et al*., 2006).

5.4 Lipases in detergent industry

The most commercially important field of the application of hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry, and in household dishwashers. However, the cleaning power of detergents seems to have peaked; all detergents contain similar ingredients and are based on similar detergency mechanisms. To improve detergency, modern types of heavy duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes, such as protease, amylase, cellulase and lipase (Ito *et al*., 1998). Thus, lipase can be used to add value to detergent.

5.5 Lipases in food processing, flavor development and improving quality

Lipases have also been used as food additive to modify flavour by their ability to synthesize esters of short-chained fatty acids and alcohols, which are known as flavour and fragrance compounds because those fatty acids and alcohols are small enough to bind to our taste buds in the mouth (Macedo *et al*., 2003). In addition, lipases are used to remove fat during the processing of the fish meat in the procedure called biolipolysis (Seitz, 1974).

5.6 Lipases in bakery products, confectionery and cheese flavouring

Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. Furthermore, lipases have been used in the improvement of flavour in coffee whiteners to produce the creamy flavour, and buttery texture of toffees and caramel (Godfrey and Rechelt, 1983)

5.7 Lipases in tea processing

The quality of black tea is dependent greatly on the dehydration, mechanical breaking and enzymatic fermentation to which tea shoots are subjected. During manufacture of black tea, enzymatic breakdown of membrane lipids by lipases initiates the formation of volatile products with characteristic flavour properties. This emphasizes the importance of lipase in flavour development. Moreover, lipase produced by *Rhizomucor miehei* enhanced the level of polyunsaturated fatty acid as observed by the reduction in total lipid content (Hasan *et al*., 2006).

5.8 Lipases in cosmetics

Retinoids (Vitamin A and derivatives) are of great commercial potential in cosmetics and pharmaceuticals such as skin care products. Water-soluble retinol derivatives were prepared by catalytic reaction of immobilized lipase (Maugard *et al*., 2002). In addition, lipases have been used in hair waving preparation. Lipases have also been used as a component of topical antiobese creams (Hasan *et al*., 2006).

5.9 Lipase in paper industry

Lipases used for color removal and clarification of paper can increase the pulping rate of pulp, increase whiteness and intensity, decrease chemical usage, prolong equipment life, reduce pollution level of waste water, save energy and time, and reduce composite cost. The addition of lipase from *Pseudomonas* sp. (KWI-56) to a deinking composition for ethylene oxidepropylene oxide adduct stearate was found to improve whiteness of paper and reduced residual ink spots (Hasan *et al*., 2006).

5.10 Lipases as diagnostic tools

Lipases are also important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or their increasing levels can indicate certain infections or diseases. Lipases are also used to generate glycerols, which is subsequently determined by enzyme-linked colorimetric reactions, in the enzymatic determination of serum triglyceride levels (Hasan *et al*., 2006). The level of lipases in blood serum can also be used as a diagnostic tool for detecting conditions such as pancreatic injury (Lott and Lu, 1991).

5.11 Lipases in medical applications

Lipases may be used as digestive aids (Macedo *et al*., 2003). Lipases are the activators of tumor necrosis factor and therefore can be used in the treatment of malignant tumors. In addition, lipases have earlier been used as therapeutics in the treatment of gastrointestinal disturbances, dyspepsias, cutaneous manifestations of digestive allergies, etc. (Hasan *et al*., 2006).

5.12 Resolution of racemic mixtures

Synthesis of specific compounds with high value is currently in great demand. Lipases can be used to resolve racemic mixtures and to synthesize chiral building blocks for cosmetics, pharmaceuticals, agrochemicals, and pesticides. For example, lipase from *C. antarctica* (Novozyme (R) 435) has been used for the kinetic resolution of racemic flurbiprofen by the method of enantioselective esterification with alcohols (Zhang *et al*., 2005). The advantages of using

enzymes in the synthesis of organic compounds relate to their versatility, high reaction rates, regio- and stereospecificities, and the relatively mild reaction conditions involved. Stereospecificity is especially important in the synthesis of bioactive molecules, as only one of the enantiomeric forms usually manifests bioactivity, whereas the other one is often toxic (Iding *et al*., 1998). Therefore, utilization of lipases to effectively synthesize regio- and stereospecific esters and fatty acids should currently be of great interest world wide.

5.13 Lipases as biosensors

The polymer-enzyme system was investigated for use in the sensor, utilizing compounds that were degraded by a lipase. A biosensor based on the enzyme-catalyzed dissolution of biodegradable polymer films has recently been developed. Potential fields of application of such a sensor system are the detection of enzyme concentrations and the construction of disposable enzyme-based immunosensors, which employ the polymer-degrading enzyme as an enzyme label (Sumner *et al*., 2001). Lipases may also be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, and these function as lipid biosensors and may be used in triglycerides and blood cholesterol determinations (Hasan *et al*., 2006).

5.14 Lipases in waste/effluent/sewage treatment

Lipases are utilized in activated sludge and other aerobic waste processes, where thin layers of fats must be continuously removed from the surface of aerated tanks to permit oxygen transport. This skimmed fat-rich liquid is digested with lipases (Hasan *et al*., 2006).

5.15 Lipases in oil biodegradation

Biodegradation of petroleum hydrocarbons in cold environments, including Alpine soils, is a result of indigenous cold-adapted microorganisms able to degrade these contaminants. Margesin *et al*. (1999) have found that monitoring of soil microbial lipase activity is a valuable indicator of diesel oil biodegradation in freshly contaminated, unfertilized and fertilized soils. Fungal species can be used to degrade oil spills in the coastal environment, which may enhance ecorestoration as well as in the enzymatic oil processing in industries (Hasan *et al*., 2006). Thus, crude or purified lipases can be used for treatment of oil waste or oil contamination.

5.16 Lipases in production of biodiesel

The limited resources of fossil fuels, increasing prices of crude oil, and environmental concerns have been the diverse reasons for exploring the use of vegetable oils as alternative fuels (Shah *et al*., 2004). The biodiesel fuel from vegetable oil does not produce sulphur oxide and

minimize the soot particulate three times less than petroleum usage. Because of these environmental advantages, biodiesel fuel can be expected as a substitute for conventional diesel fuel (Iso *et al*., 2001). The usage of lipases in biodiesel production has been explored most extensively recently. Immobilized *P. cepacia* lipase was used for the transesterification of soybean oil with methanol and ethanol (Noureddini *et al*., 2005). Fatty acids esters were produced from two Nigerian lauric oils (palm kernel oil and coconut oil) by transesterification of the oils with different alcohols using PS30 lipase as a catalyst, in the conversion of palm kernel oil to alkyl ester (biodiesel) process (Du *et al*., 2004).

It can be seen that lipases are enzymes that can be utilized in many important industries. Despite its importance, studies on the mechanisms of production of microbial lipases and the role of lipidic substances used as inducers in lipase production are scarce. Lipases represent an extremely versatile group of bacterial extracellular enzymes that are capable of performing a variety of important reactions, thereby presenting a fascinating field for future research (Jaeger *et al*., 1994). For example, the biotechnological applications of bacterial lipases are showed in Table 6.

6. Isolation and screening of lipase-producing microorganism

Recently considerable attention has been devoted to lipases produced by microorganisms. A variety of microorganisms capable to produce lipases have been observed such as *Candida*, *Rhizopus*, *Penicillium*, *Aspergillus*, *Geotrichium*, *Mucor*, *Pseudomonas*, *Achromobacter* and *Staphylococcus* (Godtfredsen, 1993). These microorganisms are culturables because they could be cultured in laboratory and were known conditions for studies. However, we know now that culturability of bacteria in most environments is very poor, most often in the range of 1% or less, so approximately 99% of microorganisms were unculturables which were difficult to grow in laboratory (Schloss and Handelsman, 2003). Thus, identification and isolation of lipase-producing microorganisms were carried out by two methods which cover both of lipases from culturable and unculturable microorganisms.

6.1 Culture-dependent lipases screening

This approach can be done by cultivation of microorganisms which are sources of enzymes under optimal conditions. After that lipases can be identified by activity-based screening to search for lipolytic activity or sequence-based screening to search for gene containing conserved sequence of lipases. The advantages of this approach are (i) lipases-producing bacteria can be

Table 6. Examples of biotechnological applications of bacterial lipases.

Type of reaction	Origin of lipase	Product (Application)
Acidolysis/Alcoholysis of fish oils	*Pseudomonas*	Sugar monoacylesters
Acylation of sugar alcohols	*Chromobacterium riscosum*	(surfactants), Enrichment of
(Trans) esterification to immobilized glycerol	*Chromobacterium riscosum*	PUFAs
Esterification to glycerol	*Chromobacterium riscosum*	Monoacylglycerols
	Pseudornonas fluorescens	(surfactants)
Glycerolysis of fats and oils	*Pseudomonas*	
Hydrolysis of fats and oils	*Pseudomonas*	
lntramolecular esterification	*Pseudomonas*	Macrocyclic lactones
Polytransesterification of diesters	*Chromobacterium*	Oligomers
with diols	*Pseudomonas*	Alkyds
		(polyester intermediates)
	Pseudomonas	Macrocyclic lactones
Resolution of racemic	*Arthrobact,*	Building blocks for
alcohols/esters	*Pseudomonas cepacia*	insecticides and chiral drugs
Transesterification of	*Pseudomonas (cepacia)*	Acrylate esters
monosaccharides		(polyacrylate intermediates)

Source: Jaeger *et al*. (1994)

identified by morphology, taxonomy and DNA analysis, and they can be isolated to be a single strain (pure culture), (ii) amount of genomic DNA can be increased by the growing of bacteria. However, the application of culture-dependent lipases screening is limited due to only 1% of microorganism can be cultured in laboratory.

6.2 Culture-independent lipases screening

This approach can be done by directly extract DNA from samples taken from the environment such as soil or compost (Green and Keller, 2006). Extracted DNA from environment containing both of DNA from culturable and unculturable microorganisms. Extracted DNA will be used for identification of lipases based on sequence-based screening to search for gene containing conserved sequence of lipases by PCR technique or based on activity-based screening to search for lipolytic activity by metagenomic library constructed from recombinant cassette of plasmids, fosmids, cosmids, BACs, etc. (Cowan *et al*., 2005). The advantages of this approach are (i) screening cover both of lipases from culturable and unculturable microorganisms, (ii) enhance the discovery rate of novel one (Schloss and Handelsman, 2003). The disadvantages of this approach are (i) lipase-producing bacteria can not be isolated to be a single strain (pure culture) and identified, (ii) amount of extracted DNA can not be increased by the growing of bacteria.

7. Molecular cloning of lipolytic enzymes

Genetic and protein engineering are modern techniques used for the commercial production of enzymes with improved stability to high temperatures, extreme pH, and presence of oxidizing agents and organic solvents. Cloning and expression of genomic information from a thermophile in a suitable and faster-growing mesophilic host has also provided possibilities of producing the specific thermostable enzyme required for a particular biotransformation process (Haki and Rakshit, 2003). Lipases from many organisms are already studied and cloned (Table 7). Expression of recombinant lipases in *Escherichia coli* provided the enzymes which are reported to demonstrate extreme thermostability. For example, a lipase from *Bacillus thermocatenulatus* was expressed in *E. coli* under the direction of a *Bacillus* promoter upstream of the lipase gene. This enzyme was stable up to pH 11.0 and maximum activity was found at pH 8.0–9.0 and at 60–70°C. This lipase also exhibited high stability in various detergents and organic solvents (Schmidt-Dannert *et al*., 1996). An esterase from *B. licheniformis* was also expressed in an *E. coli* recombinant strain (Alvarez-Macarie *et al*., 1999). The enzyme was stable at pH 7.0–8.5 with optimum activity at pH

Table 7. Properties of bacterial lipases.

Source of lipase	Gene cloned and sequenced	Signal sequence (aa)	Helper protein	Molecular mass (kDa)	Substrate specificity
Aeromonas hydrophila	yes	yes (48)	no	71.8	C6-C8-FA
Bacillus subtilis	yes	yes (31)	no	19.4	1,3 position, C8-FA
Chromobacterium viscosum	yes	n.d.	no	33	broad
Moraxella sp.	yes	no	no	34.7	n.d.
Propionibacterium acnes	no	n.d.	no	41.2	broad
Pseudomonas aeruginosa PAO1/PAC1R	yes	yes (26)	yes	30	broad
Pseudomonas aeruginosa TE3285	yes	yes (26)	yes	30	n.d.
Pseudomonas aeruginosa EF2	no	n.d.	n.d.	29	1,3 position, C18-FA
Pseudomonas cepacia	yes	yes (44)	yes	33	broad
Pseudomonas fragi	yes	no	n.d.	30	broad
Pseudomonas fluorescens B52	yes	no	no	50.2	n.d.
Pseudomonas fluorescens SIKW1	yes	yes (23)	No	48	1,3 position C6-C8-FA
Pseudomonas putida	no	n.d.	n.d.	45	n.d.
Pseudomonas sp. ATCC 21808	no	n.d.	n.d.	35	C8-C10-FA
Pseudomonas sp. KW156	yes	yes (44)	yes	33	n.d.
Staphylococcus aureus	yes	yes (37)	no	76	broad
Staphylococcus epidermidis	yes	yes (31)	no	77	n.d.
Staphylococcus hyicus	yes	yes (38)	no	71.4	broad
Xenorhabdus luminescens	yes	yes (24)	no	68.1	n.d.

Source: Jaeger *et al*. (1994)

Abbreviations: aa, amino acids; C6-, C8-, C10-, C18-FA, fatty acid with a chain length of six, eight, ten, and eighteen carbon atoms, respectively; 1,3 position, the activated position of substrate recognized by lipases.

8.0–8.5. The optimal temperature for enzyme activity was 45°C and the half-life was 1 h at 64°C. Moreover, a new esterase from the thermoacidophile *B. acidocaldarius* was expressed in *E. coli* and its properties for organic synthesis were evaluated by measuring product enantioselectivities for various substrates (Manco *et al*., 1998). Hyperthermophilic enzymes may have potential in specific applications that require high temperature or other special conditions as shown by an esterase from *Pyrococcus furiosus* which has been recombinantly expressed in *E. coli* and displayed optimal activity at 100°C (Ikeda and Clark, 1998).

Microbial lipases can be cloned from lipase-producing microorganisms mostly by (i) activity-based (or function-based) screening to search for lipolytic activity or (ii) sequence-based screening to search for gene containing conserve sequence of lipolytic enzymes. Activity-based screeing can be done using genomic library (a pool or collection of microbial genomic DNA cloned into vector), whereas sequence-based screening can be done by PCR or by affinity pull-down with oligonucleotides conserved for lipase sequence and with a sample of microbial DNA or genomic library.

7.1 Genomic library construction

A DNA library constructed from the lipase-producing microorganisms can be screened with the lipase specific oligonucleotide probe in a sequence-based screening. Alternatively, the probe can be used to facilitate construction of a minilibrary enriched for the target lipase. In addition, a library constructed from a lipase-producing microorganism can be used for activity-based screening to search for recombinant bacteria expressing a functional lipase. The recombinants producing lipases can be identified by direct detection of fat hydrolysis or by fluorescent indicators such as rhodamine B (Philip *et al*., 2002).

A thermophilic lipase of *Bacillus thermoleovorans* ID-1 was cloned and sequenced from a genomic library. A total of 1,000 transformed clones were found to exhibit lipolytic activity on rhodamine B agar plates. The recombinant plasmid of one clone (named pLIP) was chosen for further study and found to contain an insert of 2.7 kb. The lipase gene encodes 416 amino acid residues and contains the conserved pentapeptide Ala-X-Ser-X-Gly, similar to other *Bacillus* lipase genes. The optimal temperature of the lipase is 75°C, which is higher than other known *Bacillus* lipases thus far (Cho *et al*., 2000).

An expression library was generated by partial *Sau*3AI digestion of genomic DNA from the thermophile *Bacillus thermocatenulatus* and subsequent cloning of DNA fragments into pUCI8

using *Escherichia coli* DH5α as a host strain. Screening for lipase activity identified a 4.5 kb insert. The lipase gene was subsequently found to encode a mature lipase of 388 amino acid residues with an Ala residue replacing the first Gly in the conserved pentapeptide Gly-X-Ser-X-Gly found in most lipases. Lipase activity was observed at pH 8.0-9.0 when tributyrin and olive oil were used as substrates and at 60-70°C when *p*-NPP and olive oil were used as substrates (Schmidt-Dannert *et al*., 1996).

7.2 Gene amplification using PCR technique

One strategy for obtaining new lipase genes is to sequence the conserved N-terminal amino acid of lipases and then designs a complementary degenerate oligonucleotide probes based on the conserved portion of the amino acids (Philip *et al*., 2000). Researchers were able to use PCR to amplify small regions of lipase genes directly from chromosomal DNA. This was achieved by using highly degenerate consensus primers to the oxyanion hole (Jaeger *et al*., 1994) and active-site regions of lipase genes to amplify fragments of putative lipases. Genome walking was subsequently done to obtain the full-length gene. The consensus sequences of the oxyanion hole and active site are listed in Table 8.

The PCR and genome walking technique were effectively used to clone microbial lipases. For example, a novel lipase gene (*lipB52*) was cloned directly from the genomic DNA of *Pseudomonas fluorescens* B52. There was an open reading frame (ORF) of 617 amino acids. The optimal temperature of the purified lipase LipB52 was 40 °C at pH 8.0 (Jiang *et al*., 2005). A novel lipase from *Geobacillus* sp. TW1 was cloned and sequenced using designed degenerate primer. A lipase gene encoding 417 amino acids was cloned. The results showed that the recombinant lipase had an activity optimum at pH 7.0–8.0 and at 40 °C. It was active up to 90 °C at pH 7.5, and stable over a wide pH range from 6.0 to 9.0 (Li and Zhang, 2005).

8. Classification of bacteria in a group of thermophiles

Thermophiles are microorganisms that live and grow in extremely hot environments that would kill most other microorganisms. Thermophiles either live in geothermal habitats or in environments that create heat themselves. A pile of compost and garbage landfills are two examples of environments that produce heat on their own. Thermophiles are grouped by the temperature that each microorganisms can live and grow. Three groups of thermophiles have been identified (Fig. 4).

Table 8. Sequences found at the oxyanion hole and active site of selected lipases.

Microorganism	Accession no.	Oxyanion region	Active site
Staphylococcus aureus	A24545	PVVFVHG	GHSMGG
Staphylococcus aureus	NPj375790	PIVLVHG	GHSMGG
Staphylococcus epidermidis	A47705	PIILVHG	GHSMGG
Staphylococcus epidermidis	AAC67547	PVVFVHG	GHSMGG
Staphylococcus hyicus	A24075	PFVFVHG	GHSMGG
Staphylococcus haemolyticus	AAF21294	PVVLVHG	GHSMGG
Archaeoglobus fulgidus	B69470	PVVFVHG	GHSMGT
Mycoplasma pneumoniae	NPj110161	NFIFLHG	GHSMGG
Mycoplasma mycoides	AAA95966	NIIFCHG	GHSMGG
Mycoplasma genitalium	A64238	TVVFAHG	GHSMGG
Ureaplasma urealyticum	NPj077851	SIVFIHG	GHSLGA
Mus musculus	NPj035974	LIFVSHG	GHSMGG
Saccharomyces cerevisiae	NPj010343	PIVFCHG	AHSMGG
Bacillus pumilus	CAA02196	PVVMVHG	AHSMGG
Bacillus licheniformis	CAB95850	PVVMVHG	AHSMGG
Geobacillus thermocatenulatus	CAA64621	PIVLLHG	AHSQGG
Geobacillus stearothermophilus	AAL28099	PIVLLHG	AHSQGG
Burkholderia glumae	A48952	PVILVHG	GHSQGG
Chromobacterium viscosum	AAL28099	PVILVHG	GHSQGG
Burkholderia cepacia	P22088	PIILVHG	GHSQGG
Pseudomonas luteola	AAC05510	PIILVHG	GHSQGG
Pseudomonas fragi	S02005	PILLVHG	GHSQGA
Pseudomonas wisconsinensis	AAB53647	PIVLVHG	GHSQGS
Pseudomonas aeruginosa	NPj253500	PIVLSHG	GHSQGG
Bos taurus	JC4017	VVFLQHG	GHSQGT
Vibrio cholerae	NPj232620	PIVLVHG	GHSHGG
Pseudomonas aeruginosa	NPj251552	PIVLAHG	GHSHGG
Acinetobacter calcoaceticus	S61927	PIVLSHG	GHSHGS

Table 8. Sequences found at the oxyanion hole and active site of selected lipases (continued).

Microorganism	Accession no.	Oxyanion region	Active site
Yarrowia lipolytica	Q99156	VFVWIHG	GESAGS
Moraxella sp.	A39556	AMLFFHG	GDSAGG
Pseudomonas sp. B11-1	AAG47649	LLVFFHG	GDSAGG
Mycobacterium tuberculosis	NPj215915	VVVYYHG	GDSAGG
Mycobacterium tuberculosis	NPj217486	LLVFYHG	GDSAGG
Mycobacterium tuberculosis	NPj215916	VVLYFHG	GDSAGG
Rattus norvegicus	LIRTH	LVVHIHG	GDSAGG
Clostridium perfringens	BAA81642	ILMWIHG	GDSAGA
Serratia marcescens	AAA81003	IGISFRG	GHSLGG
Pseudomonas brassicacearum	AAF87594	IGVSFRG	GHSLGG
Thermomyces lanuginosus	O59952	IVLSFRG	GHSLGG
Rhizopus niveus	BAA31548	IYLVFRG	GHSLGG
Rhizomucor miehei	A34959	IYIVFRG	GHSLGG
Psychrobacter immobilis	S57275	PLLLIHG	GNSMGG
Saccharomyces cerevisiae	NPj012782	VVYLHHG	GFSQGS
Homo sapiens	A28997	LVMIIHG	GYSLGA
Mycoplasma mycoides	JC4110	NIIYIHG	GKSMGG
Streptomyces exfoliatus	3402116	AVVISPG	GHSMGG
Streptomyces albus	AAA53485	AVVVTPG	GHSMGG
Moraxella sp.	P19833	AIAVVPG	GWSMGG
Candida antarctica	S47165	PILLVPG	TWSQGG
Streptomyces cinnamoneus	AAB71210	PVVLVNG	GHSQGG

Source: Philip *et al*. (2002)

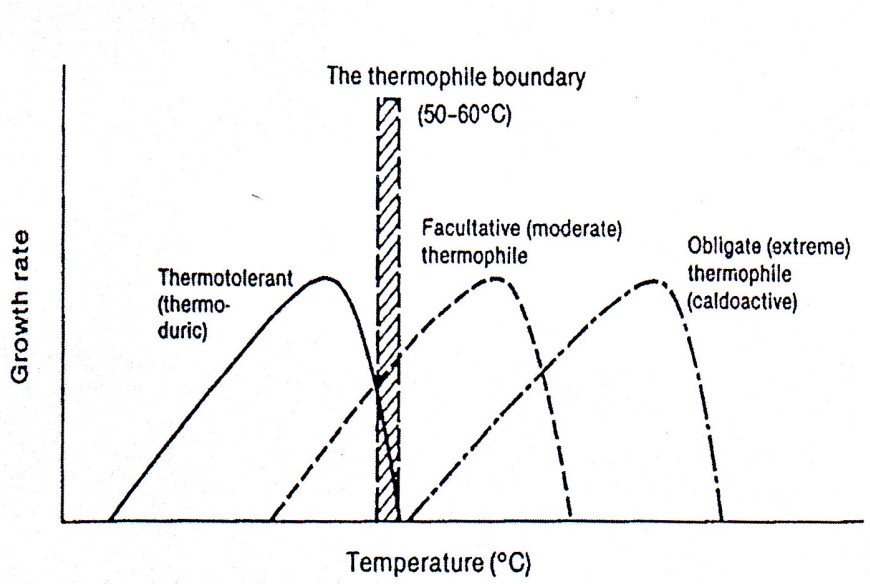


Figure 4. Growth rate of thermophiles at different levels of temperature.

Source: Brock (1986)

8.1 Thermotolerant

Thermotolerant is a group of bacteria which can survive within temperature of 50-60 °C but the optimum temperature for growth is lower than 50-60 °C. Examples of such bacteria include *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Bacillus cereus*, and *Clostridium butyricum* (Saetae, 1998).

8.2 Moderate thermophile

Moderate thermophile is a group of bacteria which can grow at moderate and high temperature but the optimum temperature for growth is higher than 50-60 °C (Saetae, 1998).

8.3 Extreme thermophile

Extreme thermophile is a group of bacteria which can survive only at high temperature and cannot grow at the temperature lower than 50-60 °C. Because of highly saturated, branched and long-chained fatty acids in lipid structure of their cell wall, they can survive at high temperature. These fatty acids are solid only at high temperature, which make extreme thermophile unable to survive at low temperature (Vandermark and Batzing, 1987).

10. Description of *Bacillus thermoamylovorans*

Bacillus thermoamylovorans (*thermoamylovorans* indicating utilization of starch at high temperatures) is Gram positive, straight, and rod-shaped eubacteria which occur singly or in short chains. They are slightly motile by means of peritrichous flagella. Their spores are not detected under a range of conditions and cells are killed by heating at 80°C for 5 min. They are considered facultative anaerobe that produce a range of enzymes including catalase. They also exhibit heterolactic fermentation of hexoses and the end products are lactate, acetate, ethanol, and formate but not H_2. They are chemoorganotrophic since biotrypcase or yeast extract is required for luxuriant growth. Vitamins and nucleic acid derivatives stimulate but are not essential for their growth. The optimum temperature for their growth is approximately 50°C whereas the upper temperature limit for growth is 58°C; thus they can be categorized as moderately thermophilic bacteria. In addition *B. thermoamylovorans* are neutrophilic with the optimal pH for growth is 6.5 to 7.5, whereas the pH range for growth is 5.4 to 8.5 (Combet-Blanc *et al*., 1995).

Objectives

1. To screen the lipase-producing thermotolerant bacteria isolated from composts.
2. To identify the lipase-producing thermotolerant bacteria by 16S rRNA gene sequence analysis.
3. To clone, sequence, express and characterize the lipase gene from the selected strain.
4. To purify and characterize the recombinant lipase cloned from the selected strain.

Scope of research work

Thermotolerant bacteria isolated from composts were used to screen for lipase-producing bacteria. All of the positive strains were identified by 16S rRNA gene sequence analysis and the strain exhibiting the highest lipase activity was selected for further gene cloning. A part of lipase gene was first obtained by PCR technique using degenerate primers designed from conserved regions of known lipase in *Bacillus* spp. After that, inverse PCR (IPCR) was performed

to amplify the complete nucleotide sequence of an open reading frame (ORF) of the lipase gene. Finally, the lipase gene was cloned, expressed in *E. coli*, purified and characterized.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Bacterial strains and plasmids

A thermotolerant lipase producer, strain BHK52, identified as *Bacillus thermoamylovorans* by nucleotide sequence analysis of 16S rRNA, was isolated from compost in Okayama, Japan. The isolation was performed by growing the bacterial strain on lipase assay medium (0.6% (w/v) tryptone, 0.2% (w/v) yeast extract, 0.5% (v/v) palm oil, 0.02% (w/v) $CaCl_2.2H_2O$, 0.02% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) K_2HPO_4 , 0.1% (w/v) KH_2PO_4 and 0.001% (w/v) rhodamine B; pH 7.4) modified from Lee *et al*. (1999). *Bacillus thermoamylovorans* BHK52 was grown in Luria-Bertani (LB) medium 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl; pH 7.0) and used for chromosomal DNA extraction. Plasmids used in this study are listed in Table 9 whereas the multiple cloning site of individual plasmid are shown in Figure 5-7. *Escherichia coli* JM109 [*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(rK^-mk^+), $e14^-$ (*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F' (*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15)] was used as a cloning host. *E. coli* JM109 and *E. coli* transformants were grown in LB medium supplemented with ampicillin (100 μg/ml) when needed.

Table 9. All plasmid vectors used in this study.

Plasmid	Decription of use	Antibiotic resistance	Company
1. pGEM–T Easy	Cloning of PCR products	Amplicillin	Promega, USA
2. pBluescript II SK(+)	Subcloning vector	Amplicillin	Takara, Japan
3. pColdI DNA	Expression vector	Amplicillin	Takara, Japan

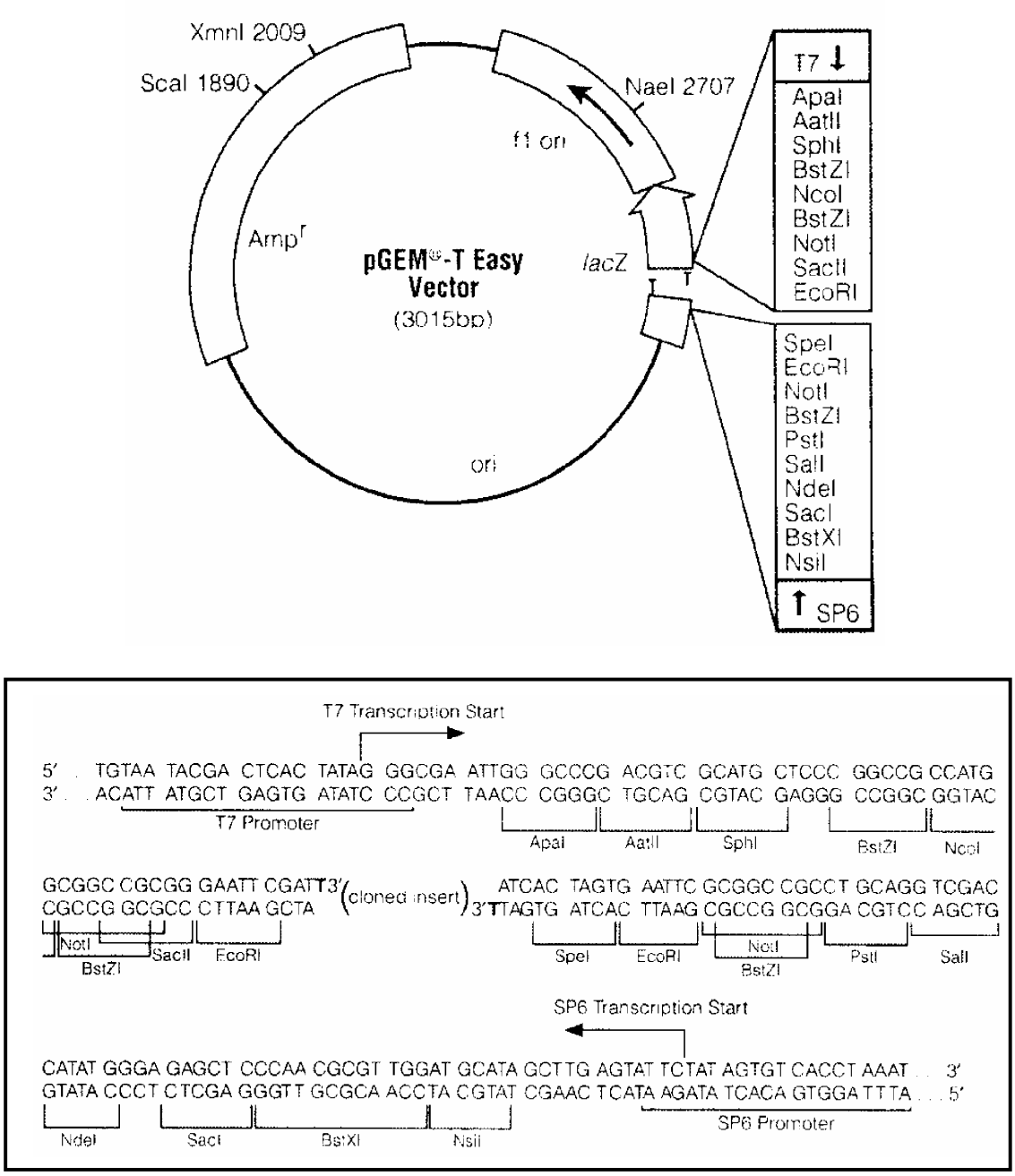
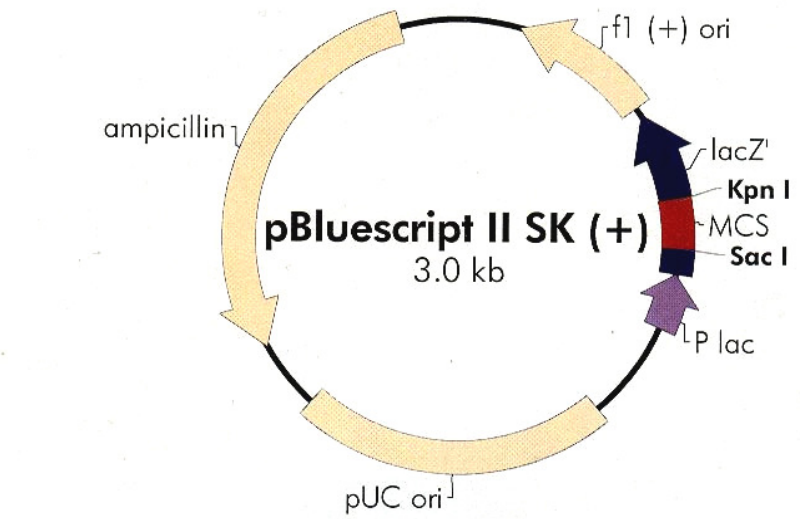


Figure 5. The physical map of pGEM-T Easy cloning vector and its multiple cloning site.

Source: Promega, USA



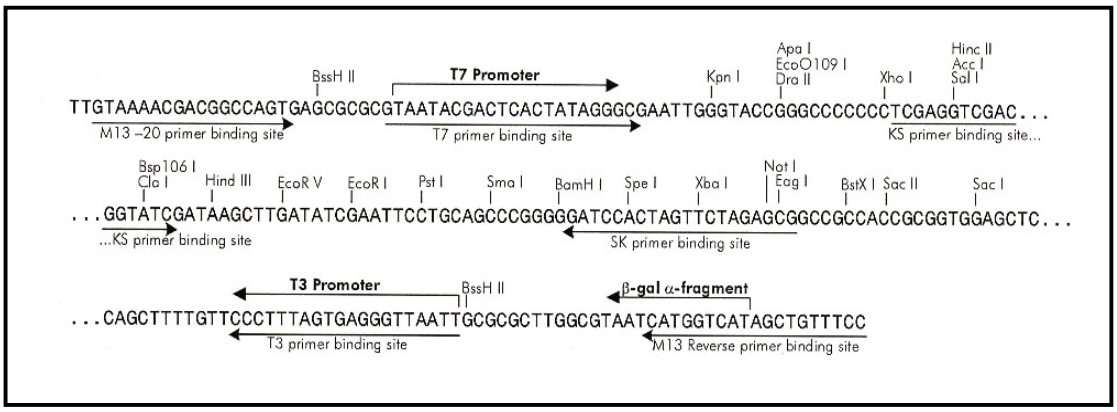
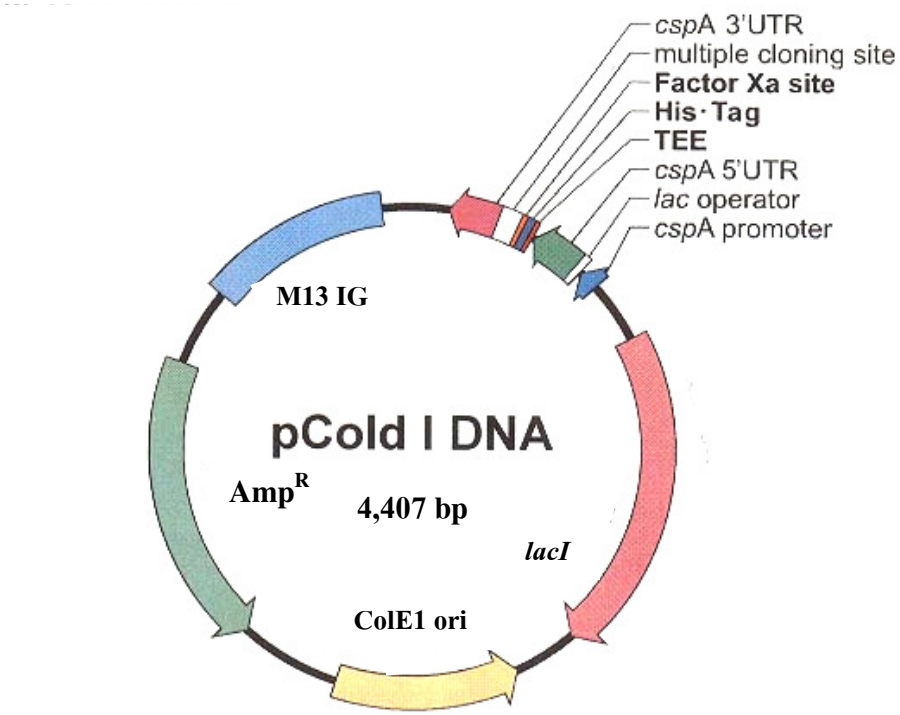


Figure 6. The physical map of pBluescript II SK (+) cloning vector and its multiple cloning site.

Source: Takara, Japan



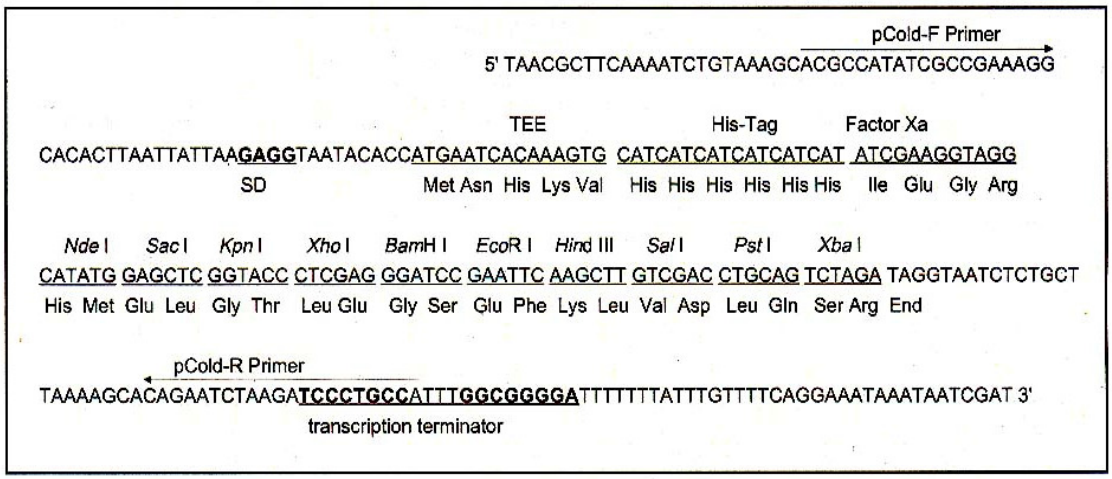


Figure 7. The physical map of pColdI DNA expression vector and its multiple cloning site.

Source: Takara, Japan

2. Instruments

All instruments used were purchased from various suppliers as follows:

Table 10. All instruments used in this study.

Instrument	Company
1. Autoclave (ES-315)	Tomy Digital Biology Co., LTD, Japan
2. Automated DNA sequencer (ABI PRISM 3100)	Applied Biosystems, USA
3. Centrifuge and microcentrifuge (5804R)	Eppendorf, USA
4. Hot air oven	Sanyo Gallenkamp, Japan
5. Incubator (MIR-153)	Sanyo Gallenkamp, Japan
6. Micropipette	Gilson Brand, Eppendorf, USA
7. PCR machine	Bio-Rad, USA
8. pH meter (713)	Metrohm AG, China
9. Power supply (1000/500)	Bio-Rad, USA
10. Shaker	Sanyo Gallenkamp, Japan
12. Vortex mixer (G-560E)	Scientific Industries, USA
13. Water bath (EcoTemp TW20)	Julabo, Japan

3. Chemicals

All chemicals and solvents used were analytical grade and purchased from various suppliers as follows:

Table 11. All chemicals and solvents used in this study.

Chemical	Supplier
1. 30% Acrylamide/bis-acrylamide (29:1) solution	Bio-Rad, USA
2. Chloroform	Nacalai, Japan
3. Comassie Brilliant Blue R250	Bio-Rad, USA
4. Disodium hydrogenphosphate (Na_2HPO_4)	Nacalai, Japan
5. Ethanol (absolute)	Nacalai, Japan

Table 11. All chemicals and solvents used in this study (continued).

Chemical	Supplier
6. Ethylene diamine tetraacetic acid (EDTA)	Nacalai, Japan
7. Gum arabic	Nacalai, Japan
8. Glycine	Nacalai, Japan
9. Hydrochloric acid (HCl)	Nacalai, Japan
10. Magnesium chloride ($MgCl_2$)	Nacalai, Japan
11. *p*-nitrophenyl acetate	Sigma-aldrich, Germany
12. *p*-nitrophenyl caprate	Nacalai, Japan
13. *p*-nitrophenyl caproate	Sigma-aldrich, Germany
14. *p*-nitrophenyl caprylate	Nacalai, Japan
15. *p*-nitrophenyl laurate	Nacalai, Japan
16. *p*-nitrophenyl palmitate	Nacalai, Japan
17. *p*-nitrophenyl stearate	Nacalai, Japan
18. N,N,N,N'-Tetramethylethylenediamine (TEMED)	Nacalai, Japan
19. Potassium chloride (KCl)	Nacalai, Japan
20. Rhodamine B	Nacalai, Japan
21. Sodium chloride (NaCl)	Nacalai, Japan
22. Sodium dodecyl sulphate (SDS)	Nacalai, Japan
23. Sodium hydroxide (NaOH)	Nacalai, Japan
24. Tris (hydroxymethyl) aminomethane	Sigma-aldrich, Germany
25. Triton X-100	Nacalai, Japan
26. Tryptone	Fluka, Switzerland
27. Zinc(II) chloride ($ZnCl_2$)	Nacalai, Japan
28. Ampicillin	Sigma-aldrich, Germany
29. 100 Base pairs DNA ladder	Toyobo, Japan
30. 5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)	Nacalai, Japan
31. *Ex taq* DNA polymerase	Takara, Japan

Table 11. All chemicals and solvents used in this study (continued).

Chemical	Supplier
32. Isopropylthiogalactoside (IPTG)	Nacalai, Japan
33. Proteinase K	Merck, Germany
34. Protein markers	Bio-Rad, USA

4. Primers

The oligonucleotide primers were ordered from Hokkaido System Science Co., Ltd, Japan as follows:

Table 12. All oligonucleotide primers used in this study.

Function	Name	Nucleotide sequence (5' to 3')
16S rRNA primers	Eu8f	AGAGTTTGATCCTGGCTCAG
	Eu1492r	GGCTACCTTGTTACGACTT
Primers for amplification of	LIPF	GTT(A)TTTGGACTT(G)AGC(T)GTT(A)CC
partial sequence of lipase gene	LIPCF	ATTGCA(G)CATTCC(T)CAAGGAGG
	LIPR	TCCAATAATTTCA(C)AGATGAT
	LIPCR	CATG(A)CGT(C)GCCGTTTGTCCTC
	Loxy1	CCA(G)A(G)TT(C)GTT(C)C(A)TGGTT(C)CATGG
	Loxy2	CCAGTTGTTATGGTTCATGG
	Las1	GCCGCCCATA(T)GAATGT(C)GC
Primers for genome walking of	I-Lipase1	CCATGAACCAGGACGACCGG
lipase gene (inverse PCR)	I-Lipase2	GGTCACAGTATGGGAGGACA
	I-Lipase3	TTGCTACATACGTTCTATGA
	I-Lipase4	GGAACACGGTCATGCTCGAT
	I-Lipase5	TCTACTAACATTCTAGATGT
	I-Lipase6	TACCAAATGATGGAATTGTA
Primers for amplification of	N-lipase-XhoI	CTCGAGTTCTTATTTTCTGTTTTTGC
lip52 gene	C-lipase-PstI	CTGCAGTTATTTAGATAACGAATGTA

5. Restriction enzymes

Restriction enzymes were from Toyobo, Inc. (Japan) and T4-DNA ligase was from Takara (Japan). Big Dye Terminator version 1.1 cycle sequencing kit was from Applied Biosystems (USA). Lipase substrates were from Sigma (St. Louis, USA) and Nacalai (Japan) and all chemicals used were of analytical grade.

Analytical methods

1. DNA manipulation

Genomic DNA from *Bacillus thermoamylovorans* BHK52 was prepared by the method of Sambrock *et al* (1989) with some modifications. Plasmid DNA was isolated with a Wizard Plus SV DNA Purification System kit (Promega) according to the manufacturer's instructions. The PCR product was purified with a MagExtractor purification kit (Toyobo, Japan) as decribed by the supplier. Competent cells of *E.coli* JM109 was prepared by a conventional heat-shock method.

2. Degenerate primers designing

The genetic code is degenerate, more than one codon can specify the same amino acid. For example, each of these 3 nucleic acid codons (TCT, TCA, AGC) specifies the same amino acid, serine (Table 13). For amplification of cognate sequences from different sequences, one may increase the chances of getting product by designing degenerate primers: these would in fact be a set of primers which have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences. The degenerate primers are designed as a pool of all the possible combinations of nucleotides that encode for a given amino acid sequence. For degenerate primers designing, the degeneracy of the genetic code for the selected amino acids of the region targeted for amplification must be examined (Table 14). Obviously, selection of amino acids with the least degeneracy is desirable because it provides the greater specificity by checking genetic codes and codon degeneracy. The degeneracy of mixed primer may be reduced by using the codon bias for translation (see codon usage of *Bacillus* spp. in Table 13). The size of the degenerate primers can be as short as 4-6 amino acid codons in length

Table 13. Nucleic acid codon usage of *Bacillu*s spp.

Amino acid	Amino acid code	Nucleic acid codon
Phenylalanine	F	UUU
Leucine	L	UUA, CUG
Isoleucine	I	AUU AUC
Methionine	M	AUG
Valine	V	GUU,GUC
Serine	S	UCU, UCA, AGC
Proline	P	CCA, CCG
Threonine	T	ACA, ACG
Alanine	A	GCA, GCG
Tyrosine	Y	UAU
Histidine	H	CAU
Glutamine	Q	CAA
Asparagine	N	AAU, AAC
Lysine	K	AAA
Aspatic acid	D	GAU
Glutamic acid	E	GAA
Cysteine	C	UGU, UGC
Trptophan	W	UGG
Arginine	R	CGU
Glycine	G	GGA, GGC
Stop	*	UAA, UAG, UGA

Table 14. Degeneracy of nucleic acid codon.

Number of codon	Amino acid code
1	M W
2	F Y H Q N K D E C
3	I
4	V P T A G
6	L S R

and degeneracy at the 3' end of the primer should be avoided, because single-base mismatches may obviate extension. Examples of degenerate primers designing were shown in Figure 8.

3. Lipase assay

Lipase activity was determined using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. The substrate solution was prepared by adding the solution A (30 mg of *p*-NPP in 10 ml of isopropanol) into the solution B (0.1 g of gum arabic and 0.4 ml Triton X-100 in 90 ml of 50 mM Tris–HCl buffer; pH 8.0) with stirring until all was dissolved. The mixture of 9 ml of substrate solution and 1 ml of suitably diluted enzyme solution was incubated at 50°C for 5 min and absorbance was measured for 5 min at λ = 410 nm in a spectrophotometer (Shimadzu UV 2001). One unit of enzyme activity (U) was expressed as μmol of *p*-nitrophenol released per minute under the assay conditions.

4. Protein concentration determination

Protein concentration was measured spectrophotometrically at 595 nm with a Protein assay kit (Bio-Rad, USA) by the method of Bradford (Bradford,1976).

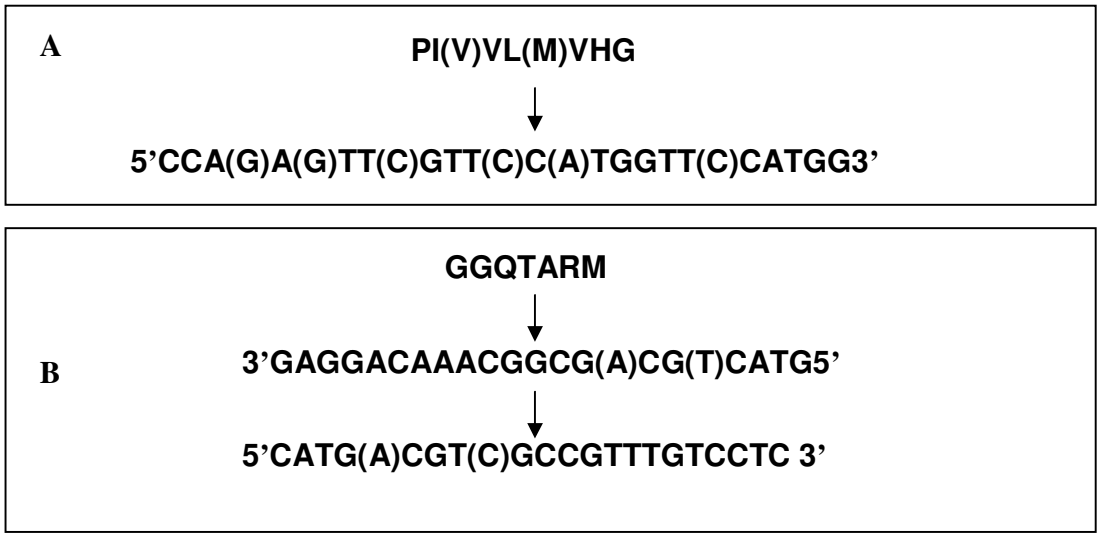


Figure 8. Examples of degenerate primers designing of Loxy1 primer (A) and LipCR primer (B).

5. Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE on a 12% polyacrylamide slap gel was used to determine the purity and apparent molecular weight of the purified lipase by the method of Laemmli (Laemmli,1970). The molecular mass of the lipase was calibrated by using a precision plus protein standards (Bio-Rad, USA) containing the protein molecular mass in the range of 10-250 kDa.

6. Genomic DNA extraction of *Bacillus thermoamylovorans* BHK52

A thermotolerant lipase producer, *B. thermoamylovorans* BHK52, was cultivated overnight in 100 ml of LB medium at 50°C. Cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C. The cell pellet was washed with 50 ml of 0.85% (w/v) NaCl and centrifuged at 6,000 rpm for 10 min at 4°C. The cell pellet was then resuspended with 50 ml of 0.85% (w/v) NaCl and devided into two tubes. Cells were treated with 500 µl of lysozyme solution (300 mg/ml) and shaked at 37°C for 2-3 h. Cells were then disrupted in the presence of 3 ml of 10% (w/v) SDS 1% (w/v) final concentration and 3 ml of proteinase K (10 mg/ml) with shaking at 37°C overnight. After confirming that the solution become transparent, the solution was incubated at 60°C for 20 min before 6 ml of 5 M $NaClO_4$ (1 M final concentration) was added with slightly mixing. Then,

15 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the solution. The solution was mixed gently but sufficiently for approximately 20 min before centrifugation at 5,000 rpm for 20 min. After that, the upper layer was transferred into a new tube and the phenol:chloroform:isoamyl alcohol treatment was repeated for 3 times. All of upper layer after centrifugation was left on ice and chilled absolute ethanol was slowly added. The chromosomal DNA was harvested with autoclaved pasture pipette, washed with chilled 70% (v/v) ethanol, and then dried at room temperature. The chromosomal DNA was dissolved overnight in TE buffer at 4°C before addition of 50 µl of RNase (10 mg/ml) solution and incubation at 37°C for 30 min. The chromosomal DNA solution was then added with 1/10 volume of 3 M CH_3COONa (pH 5.2) and 2.5 volume of cold absolute ethanol, freezed at -80°C for 30 min, and then centrifuged for 5-10 sec at maximum speed. After centrifugation, the supernatant was poured off, air dried, and dissolved in TE buffer. After extraction, the obtained chromosomal DNA was confirmed by agarose gel electrophoresis and the chromosomal DNA was kept at -20°C before used.

7. Plasmid extraction

All plasmids used in this study were extracted by Wizard® *Plus* SV DNA Purification System kit according to the manufacturer's instructions. After extraction, the plasmids were cut with selected restriction enzymes in their multiple cloning sites. Plasmid size was analyzed by agarose gel electrophoresis and the purified plasmid was kept at -20°C before used.

8. Partial digestion of genomic DNA with restriction enzymes

A genomic DNA of *Bacillus thermoamylovorans* BHK52 was partially digested before the inverse PCR (IPCR) was applied. Various restriction enzymes were selected for digestion of genomic DNA including *Bam*HI, *Hind*III, *Eco*RI, *Pst*I, *Not*I, *Sma*I, *Xho*I, *Sua3*AI and *Kpn*I. Each restriction enzyme was added into the reaction consisted of 5 µg of genomic DNA, 1 unit of restiction enzyme, 1X buffer, and milliQ water in the total volume of 100 µl. This reaction was incubated at 37°C overnight. After incubation, the reaction was extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 10,000 rpm for 10 min. The reaction was extracted again with equal volume of chloroform and centrifuged at 10,000 rpm for 10 min. Digested genomic DNA solution was precipitated by addition of 0.1 volume of 3 M CH_3COONa and 2.5 volume of ethanol before incubation -80°C for 30 min and subsequent

centrifugation at 13,000 rpm for 20 min. The DNA pellet was then washed with 30 µl of 70% (v/v) ethanol, centrifuged at 13,000 rpm for 10 min, air dried at room temperature and resuspended with 100 µl of milliQ water. The pattern of partial digestion was confirmed by agarose gel electrophoresis. The digested genomic DNA that showed smear pattern on agarose gel was selected for self-ligation preparation.

9. Plasmid digestion with restriction enzymes

All plasmids used in this study were cut with restriction enzymes in their multiple cloning sites and their sizes were confirmed by agarose gel electrophoresis. The plasmid digestion reaction consisted of 1X buffer, 1 unit of restiction enzyme, 5 µg of plasmid DNA and milliQ water in the total volume of 15 µl. The reaction was incubated at 37°C overnight. The restriction enzyme was then heat inactivation at 80°C for 20 min. The sizes of digested plasmids were then compared with DNA markers (λ/*Eco*T14I) on agarose gel. Plasmid digestion was also performed to get linearized plasmid for further used in the ligation step.

10. Dephosphorylation of DNA

After partial digestion of plasmid DNA with single restriction enzyme, the linearized plasmid can potentially become circular form by self ligation. To solve this problem, the dephosphorylation was done in this study using an alkaline phosphatase. This enzyme caused dephosphorylation at 5' of DNA molecule. A dephosphorylation reation consisted of 1X dephosphorylate buffer, 1 unit of shrimp alkaline phosphatase (Takara, Japan), 5 µg of linearized plasmid DNA (after digestion with single restriction enzyme) and milliQ water in the total volume of 40 µl. The reaction was incubated at 37°C for 10 min. The alkaline phosphatase was heat inactivation at 65°C for 15 min.

11. Ligation of DNA

Ligation was performed to construct the recombinant plasmid from genomic DNA fragment and plasmid vector using enzyme T4 DNA ligase. The ligation reaction was done in small scale under composition of 1X ligation buffer (Takara, Japan), digested DNA fragment, and plasmid vector in 3:1 ratio, 1 unit of T4 DNA ligase (Takara, Japan) (not over than 1/10 of total

reaction) and milliQ water in the total reaction volume of 10 µl. The reaction was incubated at 16°C overnight. The ligation reaction was used for transformation into *E. coli* host cells.

12. Self-ligation of DNA

After the genomic DNA of *B. thermoamylovorans* BHK52 was subjected to partial digestion, the DNA fragments were intramolecularly circularized by self-ligation. The self-ligation reaction was done in a large scale to avoid an intermolecular ligation under the composition of 1X T4 ligation buffer (Takara, Japan), 90 µl of digested DNA, 1 unit of T4 DNA ligase (Takara, Japan) and milliQ water in the total reaction volume of 200 µl. After that, the reaction was incubated at room temperature for 1 h. The reaction was diluted by adding 400 µl of milliQ water and DNA molecules were precipitated by adding 30 µl of 5 M NaCl and 600 µl of isopropanol and subsequent incubating at -80°C for 30 min before centrifugation at 13,000 rpm for 20 min. The DNA molecules were washed with 1 ml of 70% (v/v) ethanol, centrifuged at 13,000 rpm for 10 min, air dried at room temperature and resuspended with 10 µl of milliQ water. This DNA solution can be used as template of inverse PCR (IPCR).

13. *E. coli* JM 109 competent cells preparation

E. coli JM109 was precultured in 5 ml of LB medium at 37°C with shaking (200 rpm) overnight. The culture was then transferred into 500 ml of SOB medium (0.5% (w/v) yeast extract, 2.0% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MnCl_2.4H_2O$, and 10 mM $MgSO_4.7H_2O$), cultivated at 28°C with shaking (200 rpm) until OD_{660} reached to 0.4-0.8. The culture flask was then chilled on ice for 10 min. After centrifugation at 3,000 rpm for 15 min at 4°C, the supernatant was discarded and cell pellet was resuspended with 1/3 volume (relative to culture medium) of transformation buffer (10 mM PIPES, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, adjusted pH 6.6-6.7 with KOH, 55 mM $MnCl_2.4H_2O$). The cell mixture was chilled on ice for 10 min before centrifugation at 3,000 rpm for 15 min at 4°C. The cell pellet was resuspended again in 1/12.5 volume of transformation buffer. Dimethylsulfoxide (DMSO) was added to the final concentration of 7% (v/v). The mixture was then chilled on ice for 10 min and divided into microcentrifuge tube (100-200 µl/tube). The aliquots were freezed in liquid nitrogen and stored at -80°C before being used in transformation step.

14. *E. coli* JM 109 transformation by heat-shock method

A portion of 100 µl of *E. coli* JM109 competent cells were thawed on ice, mixed well with 10 µl of ligation reaction, kept on ice for 1 h, incubated at 42°C for 1 min, and kept on ice for another 5 min. Then, the mixture was added with 600 µl of SOC medium (0.5% (w/v) yeast extract, 2.0% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MnCl_2.4H_2O$, 10 mM $MgSO_4.7H_2O$, and 20 mM glucose) and incubated at 37°C with shaking (200 rpm) for 1 h. The transformants were spreaded on LB-agar plates containing ampicillin (100 µg/ml), IPTG (0.1 mM) and X-gal (40 µg/ml) before incubation at 37°C overnight.

15. Blue/white colony selection

After transformation, the transformants were divided into two groups, blue and white colonies. A white colony is the colony harboring recombinant plasmid whereas a blue colony is the colony harboring plasmid with no inserted DNA. Therefore, only white colonies were selected for next study.

16. Rapid method for white colony selection

After blue/white colony selection, many white colonies were assumed to be the correct recombinant colonies. Those white colonies were subjected to a rapid method to confirm and further select the correct recombinant colonies. The appropriate white colonies were picked up by toothpicks and resuspended in 20 µl of lysis buffer (5 mM EDTA, 10% (w/v) sucrose, 0.25% (w/v) SDS, 60 mM KCl, 0.05% (w/v) bromophenol blue, when in use added 100 mM NaOH), mixed and incubated at 37°C for 5 min before being subjected to centrifugation at 13,000 rpm for 2 min. The supernatant was directly loaded into 0.7% (w/v) agarose gel and electrophoresis was performed at 100 volts for 45 min. The white colony harboring recombinant plasmid showed the larger size of DNA band on agarose gel than the DNA band of plasmid vector, indicating the presence of insert DNA ligated to the plasmid vector.

17. Expression of lipase in pColdI DNA vector

Lipase expression cassette was constructed by ligation of amplified *lip52* from the genomic DNA of *B. thermoamylovorans* BHK52 and linearized pColdI DNA plasmid vector. This expression cassette was transformed into *E. coli* JM109 host cells and the transformants were selected on the selection medium (LB) containing ampicillin (100 µg/ml). For expression, the transformants harboring lipase expression cassette were inoculated into 400 ml of LB-broth medium containing 100 µg/ml of ampicillin and cultivated at 37°C with shaking (200 rpm) until OD_{600} reached to 0.4 - 0.5. To inactivate other proteins, the culture solution was refrigerated at 15°C for 30 min. The induction of lipase expression was performed by adding IPTG at the final concentration of 1.0 mM and continued the cultivation with shaking (200 rpm) at 15°C for 24 h. After cultivation, the cells were collected and the expression of target protein from both soluble and insoluble fractions was investigated with SDS-PAGE and enzyme activity assay.

18. Cell sonication

Cells containing lipase expression cassette were cultivated under the expression conditions as previously described (see expression of lipase in pColdI DNA vector above). Bacterial cells were collected by centrifugation at 6,000 rpm for 15 min, washed cell pellet once with 0.85% (w/v) NaCl and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole) with the ratio of 1 g of cells per 1 ml of lysis buffer before being subjected to sonication with Branson sonifier 250 (3X20 sec on ice with an output set to 2). After centrifugation at 12,000 rpm for 20 min, the supernatant fraction was recovered and used for protein purification and lipase assay.

19. Purification by affinity chromatography with Ni-NTA resin

The Ni-NTA resin purification column was prepared by washing the resin with milliQ water and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole). The resin was then loaded into a column. Before enzyme purification, the column was equilibrated with 5 column volumes of lysis buffer. The cell-free extract after cells sonication was loaded onto the column and the target protein was allowed to bind to the column. The column was then washed with 5-10 column volumes of lysis buffer. To determine the presence of unbounded

target protein in lysis buffer, the flow through fractions were collected for SDS-PAGE, lipase activity assay and protein concentration determination. To wash and release the non-target protein, 5–10 column volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole) were loaded onto column. To determine the target protein in washing buffer, the washed fractions were collected for SDS-PAGE, lipase activity assay and protein concentration determination. The target protein was eluted from the column with 5 column volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole). The 6xHis-tagged target protein was normally eluted in the second and third column volumes. The imidazole ring is part of the structure of histidine (Fig. 9). The imidazole rings in the histidine residues of the 6xHis tag bound to the nickel ions immobilized by the NTA groups on the matrix. The eluted fractions were collected for confirmation of the presence of purified protein and determination of protein molecular mass by SDS-PAGE. This purified lipase was used for lipase activity assay, protein concentration determination and enzyme characterization.

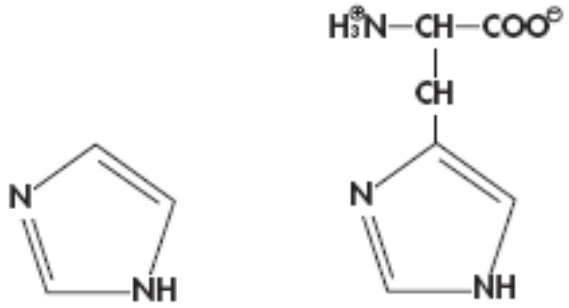
Experimental methods

1. Screening of lipase-producing bacteria

A total of 88 thermotolerant bacteria isolated from composts were cultured and screened for lipase activity on agar plates consisted of: 0.6% (w/v) tryptone, 0.2%(w/v) yeast extract, 0.5% (v/v) palm oil, 0.02% (w/v) $CaCl_2.2H_2O$, 0.02% (w/v) $MgSO_4.7H_2O$, 0.1% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, and 0.001% (w/v) rhodamine B; pH 7.4 (Lee *et al*., 1999). Plates were incubated at 50°C overnight, and lipase production was identified as orange fluorescent colonies under UV light (254 nm) (Kouker and Jaeger, 1987).

2. Identification of bacterial strains

The bacteria were identified by 16S rRNA gene sequence analysis using universal primers 5'AGAGTTTGATCCTGGCTCAG3' (position 8 in the 16S rRNA gene according to *E. coli* numbering) and 5'GGCTACCTTGTTACGACTT3' (position 1,492 in the 16S rRNA gene according to *E. coli* numbering). The PCR mixture consisted of 25 pmol of each primer, 10 ng of chromosomal DNA, 200 µM of dNTPs, 2.5 U of *Taq* polymerase, 1X polymerase buffer, and milliQ water in the total volume of 50 µl. The PCR was carried out for 30 cycles at 94 °C for



Imidazole Histidine

Figure 9. Chemical structures of imidazole and histidine.

Source: Qiagen, USA

30 sec, 55 °C for 1 min and 72 °C for 2 min. The 16S rRNA gene fragment (1,500 bp in length) was sequenced in both direction and BLAST program (www.ncbi.nlm.nih.gov/blast) was used to assess the DNA similarities compared to the existing DNA database.

3. Selection of lipase-producing bacteria

Among numerous numbers of isolated bacteria, 10 strains that showed very high level of lipase activity were selected for further identification, gene cloning, nucleotide sequencing, expression and partial characterization of the purified enzyme based on thermostability test and 16S rRNA gene sequence analysis.

4. PCR amplification, gene cloning, nucleotide sequencing of partial sequence of lipase gene

By comparing the amino acid sequences of more than 10 lipases from *Bacillus* spp. by using ClustalW program, two highly conserved regions namely oxyanion hole and active-site regions of lipase genes flanking a fragment of approximately 300 bp were identified (Jaeger *et al*., 1994). Degenerate primers Loxy1 5'CCA(G)A(G)TT(C)GTT(C)C(A)TGGTT(C)CATGG3' and LipCR 5'CATG(A)CGT(C)GCCGTTTGTCCTC3' were designed according to the sequences of the two highly conserved regions and principles of primer designing (Rose *et al*., 1998). The genomic DNA of *Bacillus thermoamylovorans* BHK52 was used as the template for PCR amplification of the partial sequence of lipase. PCR was performed as follows: 94°C for 5 min, followed by 35 cycles of amplification (95°C for 1 min, 55°C for 1.45 min, and 72°C for 2 min)

and 72°C for 7 min. The PCR product was run on 2% (w/v) agarose gel and the target band with approximately 300 bp in size was purified by MagExtractor Kit (Toyobo, Japan). The purified DNA was cloned into pGem-T easy vector (Fig. 5) and sequenced with M13 primers from both ends by Big Dye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, USA).

5. Cloning of upstream and downstream sequences by inverse PCR

Homology analysis using BLAST program confirmed that the partial sequence obtained was a fragment of a novel lipase gene. To amplify the upstream and downstream sequences flanking the known fragment, inverse PCR (IPCR) was performed by using I-lipase 1-6 as primers based on genome-walking (Table 12). Target DNA was partially cut into smaller fragments of several kilobase pairs by digestion with restriction endonuclease (Toyobo, Japan). Self-ligation was performed under low concentration of DNA, which causes the phosphate backbone to reform and gives a circular DNA ligation product. This can now be used for IPCR which was conducted with primers complementary to the now known internal sequences. IPCR was performed as follows: 94°C for 5 min, followed by 30 cycles of amplification (95°C for 1 min, 50°C for 45 sec, and 72°C for 2 min) and 72°C for 7 min. After that, the purified PCR product was cloned into pGem-T easy vector, and sequenced with M13 primers from both ends by Big Dye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, USA). Homology analysis using BLAST program confirmed that the sequence obtained was a novel lipase gene.

6. Construction of recombinant plasmid containing *lip52* and expression in *E. coli* JM109

Primers N-lipase-XhoI and C-lipase-PstI (Table 12) containing *Xho*I and *Pst*I recognition sites, respectively, were synthesized (based on the analysis of upstream and downstream sequence obtained from genome-walking IPCR) to amplify the complete ORF of *lip52* gene directly from the genomic DNA of *Bacillus thermoamylovorans* BHK52. The PCR product encoding the whole *lip52* gene was digested with *Xho*I and *Pst*I and then ligated into the expression vector pColdI DNA (Takara, Japan). The recombinant plasmid was transformed into *E. coli* JM109 for expression. The transformants were selected on LB-agar plate containing ampicillin (100 μg/ml). The selected transformant was inoculated into 400 ml of LB medium containing 100 μg/ml of ampicillin, and cultivated at 37°C with shaking (200 rpm) until OD_{600} reached to 0.4-0.5.

The cell culture was refrigerated at 15°C and left to stand for 30 min before addition of IPTG at the final concentration of 1.0 mM, and continued the cultivation with shaking (200 rpm) at 15°C for 24 h. Bacterial cells were collected by centrifugation at 6,000 rpm for 15 min at 4°C. The cell pellets were then washed once with 0.85% (w/v) NaCl, resuspended in 50 mM of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole; pH 8.0) and cells were lysed by sonication. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatant was checked for the presence of lipase by SDS–PAGE and lipase activity assay.

7. Enzyme purification

After expression and disruption of the target cells, the supernatant containing lipase was used to purify by Hi-Trap affinity column containing nickel-nitrilo-triacetic acid as described by the manufacturer (Qiagen, USA). The purified protein was subjected to SDS-PAGE and visualized by protein assay kit (Bio-Rad, USA). The purified enzyme was used for further characterization of the lipase Lip52.

8. Enzyme characterization

8.1 Substrate specificity

Substrate specificity for different *p*-NP esters of saturated fatty acids was determined by spectrophotometric assay (Sevgi and Serpil, 2007). The *p*-NP esters with different chain lengths (C2-C18) including *p*-NP–acetate (C2), *p*-NP–butyrate (C4), *p*-NP–caproate (C6), *p*-NP–caprylate (C8), *p*-NP–caprate (C10), *p*-NP–laurate (C12), *p*-NP–palmitate (C16), and *p*-NP–stearate (C18) were used as the synthetic substrates. The highest activity of enzyme assay using these substrates was defined as 100% level of enzyme activity.

8.2 Effect of pHs on lipase activity and stability

To determine the optimum pH, enzymatic activity was carried out at 70°C by spectrophotometric assay. Activity towards *p*-NP–caprylate (C8) was determined at different pH values ranging from 3.0-11.0. The buffers used were 50 mM sodium acetate (pH 3.0-6.0), 50 mM potassium phosphate (pH 6.0-8.0), 50 mM Tris–HCl (pH 7.0-9.0), and 50 mM KOH–glycine (pH 8.0-11.0). The effect of pHs on lipase stability was analyzed by spectrophotometric assay using *p*-NP–caprylate as a substrate after preincubation of enzyme in 0.2 M of different buffers

mentioned above for 6 h at 37°C. After that, the remaining lipase activity was assayed as mentioned in the analytical method.

8.3 Effect of temperatures on lipase activity and stability

To determine the optimum temperature, enzymatic activity was measured at 30, 40, 50, 60, 70, 80, 90, and 100°C by the standard assay at pH 8.0. Thermostability of the lipase was investigated by measuring the remaining lipase activity after incubating the enzyme in 50 mM Tris–HCl buffer; pH 9.0, at various temperatures for 7 h before assaying enzyme activity at 70°C.

8.4 Effect of metal ions on lipase activity

Various metal ions in the forms of $CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, CsCl, LiCl, KCl, NaCl, and $FeSO_4.7H_2O$ were added into the enzyme solution, which was solubilized in 50 mM Tris-HCl; pH 9.0, to get a final concentration of 10 mM. The enzyme solution was preincubated at room temperature for 5 min and then assayed for lipase activity. The lipase activity of the enzyme solution without any metal ion was defined as 100% level of enzyme activity.

9. GenBank Accession Number

Nucleotide sequence of *lip52* gene reported in this article is available in the GenBank databases under the accession number AB381879.

CHAPTER 3

RESULTS AND DISCUSSION

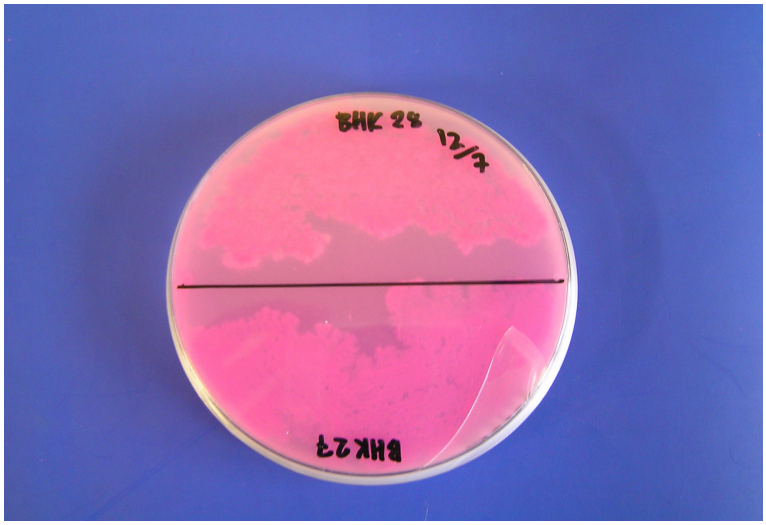
1. Screening, identification and selection of lipase-producing bacteria for gene cloning

A total of 88 thermotolerant bacteria isolated from composts were used as sources of lipase-producing bacteria. These bacterial strains were screened and scored as positives when they showed orange fluorescent colonies under UV light of 254 nm (Kouker and Jaeger, 1987) after cultivation on lipase assay medium containing palm oil and rhodamine B at 50°C (Fig. 10). Finally, 10 positive strains represented the most promising bacteria in terms of enzyme production capability and enzyme advantageous properties were selected (Table 15, 20) (Fig. 26). According to the nucleotide sequence analysis of 16S rRNA, they were identified as 1 isolate of *Aneurinibacillus thermoaerophilus*, 1 isolate of *Bacillus pumilus*, and 8 isolates of *Bacillus thermoamylovorans* (Table 16) (Fig. 27-36). As no report was found on a lipase from *B. thermoamylovorans* and the target lipase was supposed to be a novel one, an isolate named BHK52 showing the highest lipolytic activity was selected for gene cloning, nucleotide sequencing, and partial characterization.

2. Cloning and sequencing of lipase *lip52*

Two highly conserved regions including an oxyanion hole (Jaeger *et al*., 1994) and an active-site region of lipase gene flanking DNA fragment approximately 300 bp were applied to design degenerate primers. Loxy1 and LipCR were designed by principles of primer designing (Rose *et al*., 1998). The genomic DNA of *B. thermoamylovorans* BHK52 was used as DNA template for amplification of partial nucleotide sequence of lipase gene by using degenerate primers. The PCR products were analyzed by agarose gel electrophoresis using 2% (w/v) agarose gel (Fig. 11). An approximately 300 bp DNA fragment was cut and purified by using MagExtractor Kit (Toyobo, Japan). The purified DNA fragment was directly cloned into pGEM-T Easy vector. After that, the recombinant plasmid was cut with suitable restriction enzymes before ligation of the purified DNA fragments with pBluescript II SK (+) vector. Then, the reconstructed plasmid was sequenced with M13 primers from both ends by Big Dye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, USA). After sequencing, a nucleotide sequence of the 338 bp DNA fragment was obtained from genomic DNA of *B. thermoamylovorans* BHK52 (Fig. 12).

A



B

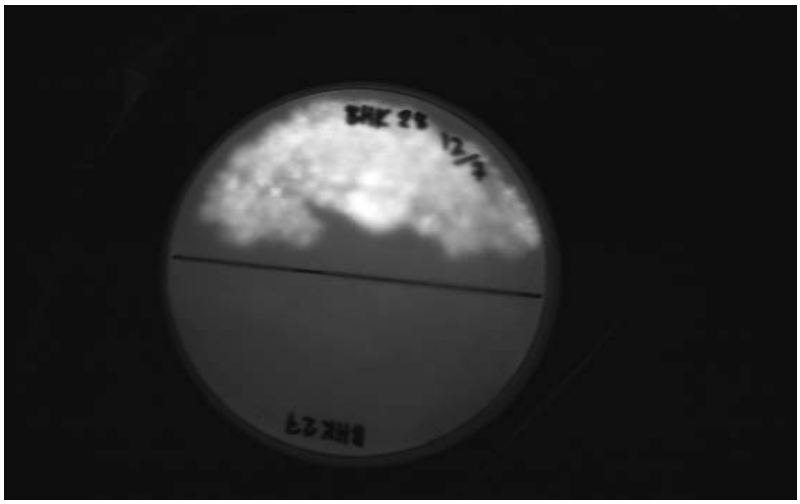


Figure 10. Identification of thermotolerant bacteria exhibiting enzyme activity on lipase assay medium at 50°C. After incubation at 50°C overnight, the lipase-producing strain with pink colonies (A) showed orange fluorescent colonies under UV light (B) whereas non-producing strain formed pink colonies on rhodamine B agar plate (A) but did not showed any orange fluorescent colonies upon UV irradiation (B).

Table 15. Lipase activity from lipase-producing thermotolerant strains growing at different temperatures.

Strain	50 °C		55 °C		65 °C	
	Growth	Activity	Growth	Activity	Growth	Activity
BHK24	+++	+++	+	+++	-	-
BHK26	++++	++++	++++	++	-	-
BHK28	++++	++++	+	+++	-	-
BHK30	++	++	++++	+++	-	-
BHK34	++++	+++	++++	++	-	-
BHK52	++++	++++	+	+++	-	-
BHK60	+	+++	+	++	-	-
BHK65	+	+++	+	++	-	-
BHK66	+	+++	+	++	-	-
BHK67	+	+++	+	+++	-	-

Symbols:			
	-	=	no growth; no activity
	+	=	low growth rate; low activity
	++,+++	=	moderate growth rate; moderate activity
	++++	=	high growth rate; high activity

Table 16. Identification of lipase positive strains by 16S rRNA gene sequence analysis.

Strain	Size (bp)	BLAST result	Identity (%)
BHK24	1,397	*Bacillus thermoamylovorans*	99
BHK26	1,442	*Bacillus thermoamylovorans*	99
BHK28	1,468	*Bacillus pumilus*	100
BHK30	1,437	*Aneurinibacillus thermoaerophilus*	99
BHK34	1,469	*Bacillus thermoamylovorans*	99
BHK52	1,462	*Bacillus thermoamylovorans*	99
BHK60	1,446	*Bacillus thermoamylovorans*	99
BHK65	1,459	*Bacillus thermoamylovorans*	99
BHK66	1,441	*Bacillus thermoamylovorans*	99
BHK67	1,444	*Bacillus thermoamylovorans*	99

Sequence alignment by BLAST analysis revealed that the nucleotide sequence was 69% identical to the partial sequence of lipase gene from *Clostridium botulinum*. This DNA fragment contained a typical GxSxG motif conserved in most esterases and lipases (Arpigny and Jaeger, 1999). In addition, homology analysis with BLAST program confirmed that the nucleotide sequence was a partial sequence of a novel lipase gene since there is only 69% identity to the closest lipase reported in the database.

To obtain the upstream and downstream sequences flanking the partial DNA fragment obtained, inverse PCR (IPCR) was performed after a new set of primers were designed from the known sequence (I-lipase 1-6) for further genome-walking step (Table 12). The target DNA is partially cut into smaller fragments of several kilobase pairs by restriction endonuclease digestion (Toyobo, Japan). The smear pattern of digested DNA on agarose gel were cut and purified for self-ligation (Fig. 13). Intramolecular self-ligation was induced under low concentration causing the phosphate backbone to reform which gave a circular DNA molecule. The ligation product was used as DNA template for IPCR which was conducted with primers complementary to the known internal sequences. The IPCR product was analyzed on 0.7% (w/v) agarose gel whereas the target band was cut and purified by using MagExtractor Kit (Fig. 14, 15). The purified IPCR product was directly cloned into pGEM-T Easy vector and pBluescript II SK (+) vector, respectively.

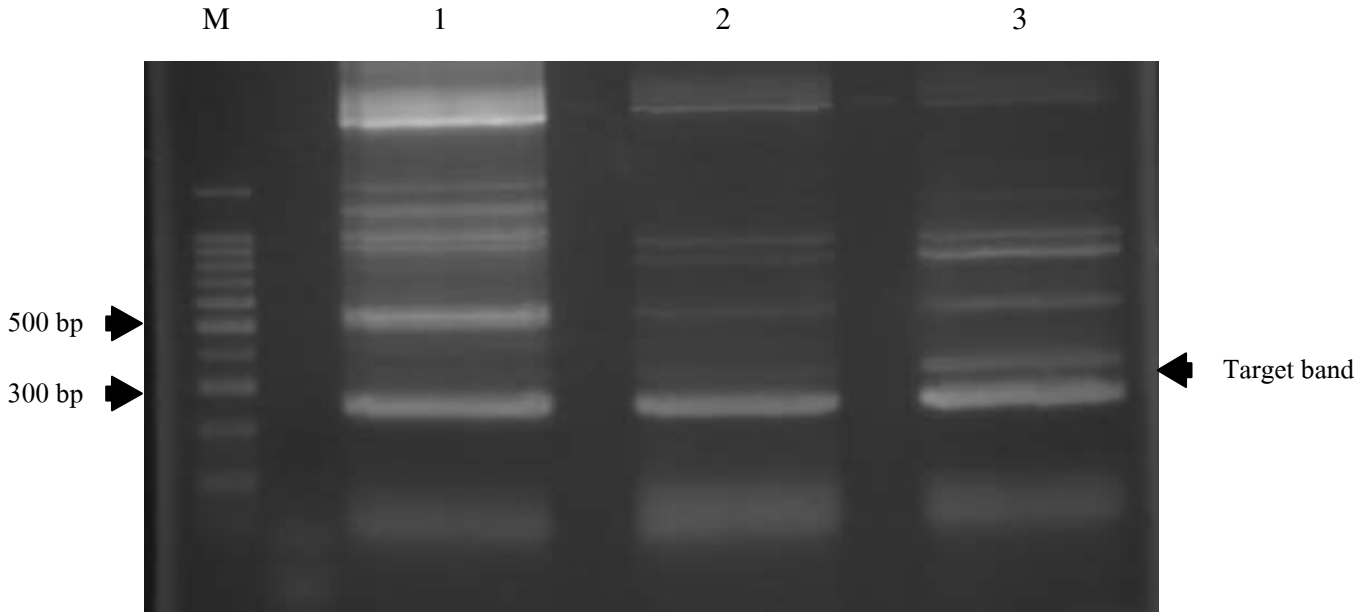


Figure 11. PCR products after gene amplification by using degenerate primers (Loxy1, LipCR) at different annealing temperatures (47-55°C). An approximately 300 bp target band of lipase gene from *B. thermoamylovorans* BHK52 was observed in lane 3. Lanes: M, 100 bp ladder DNA markers; 1, 2, 3; PCR products after gene amplification by using degenerate primers (Loxy1, LipCR) at 47°C, 52.7°C and 55°C, respectively.

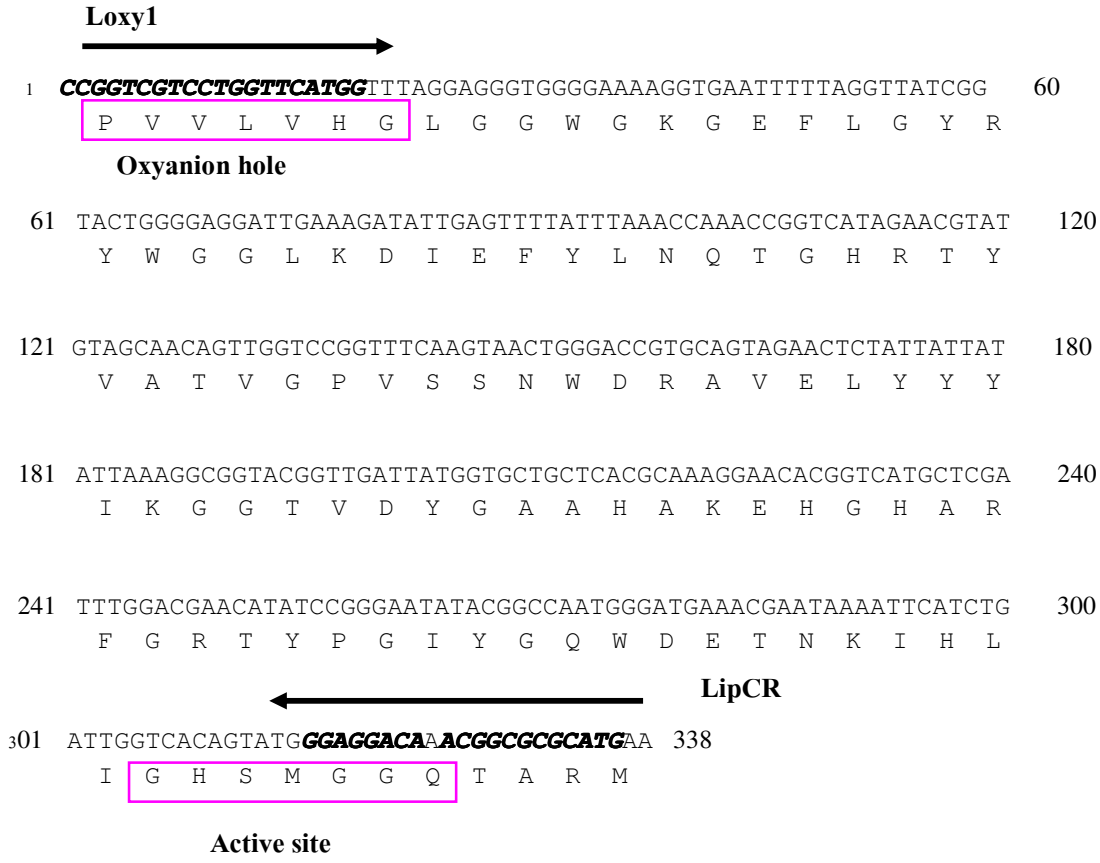


Figure 12. A 338 bp nucleotide sequence of lipase gene from *B. thermoamylovorans* BHK52 after gene amplification by using degenerate primers (Loxy1, LipCR). This nucleotide sequence showed two conserved sequences shown in boxes including oxyanion hole and active site (GxSxG) which were used to design degenerate primers (Loxy1, LipCR).

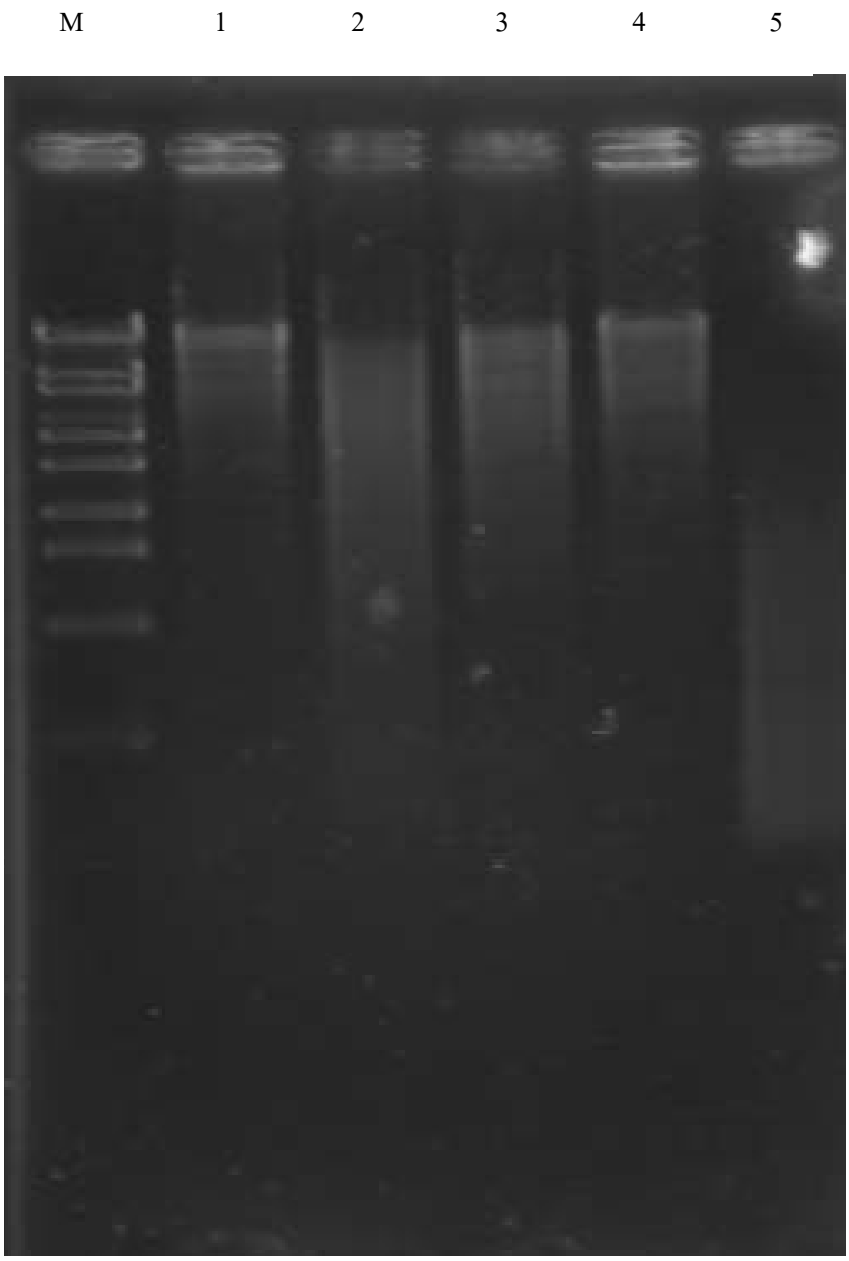


Figure 13. Digestion pattern of genomic DNA from *B. thermoamylovorans* BHK52 after partial digestion with various restriction enzymes and further used as DNA template in inverse PCR (IPCR). Lanes: M, λ/*EcoT*14 markers; 1, 2, 3, 4, 5; digested genomic DNA of *B. thermoamylovorans* BHK52 after partial digestion with *Bam*HI, *Hin*dIII, *Eco*RI, *Pst*I and *Sau*3AI, respectively.

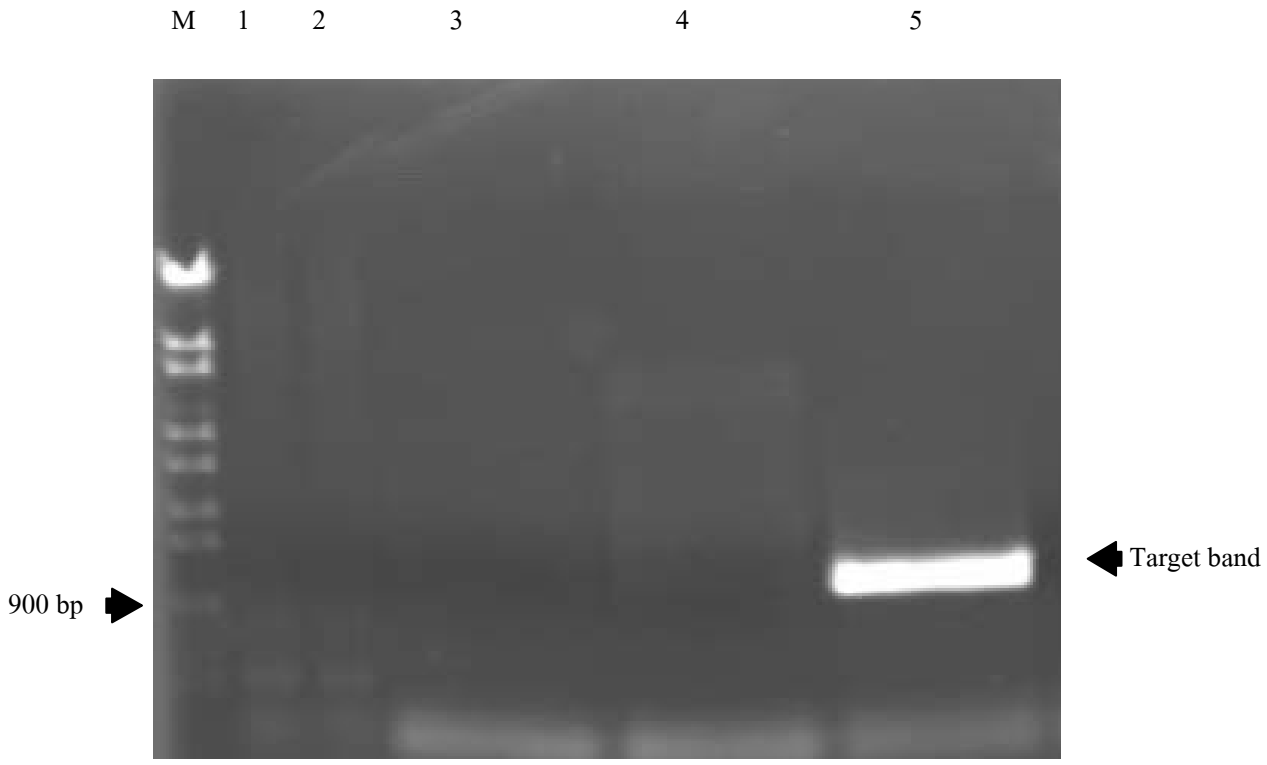


Figure 14. Inverse PCR products after gene amplification by using inverse PCR primers (I-lipase2, I-lipase3) and self-ligated products of digested genomic DNA of *B. thermoamylovorans* BHK52 with various restriction enzymes. An approximately 900 bp target band of lipase gene was observed in lane 5. Lanes: M, λ/*Eco*T14 markers; 1, 2; negative controls (non digested DNA); 3, 4, 5; inverse PCR products of digested DNA with *Bam*HI, *Hin*dIII and *Eco*RI, respectively.

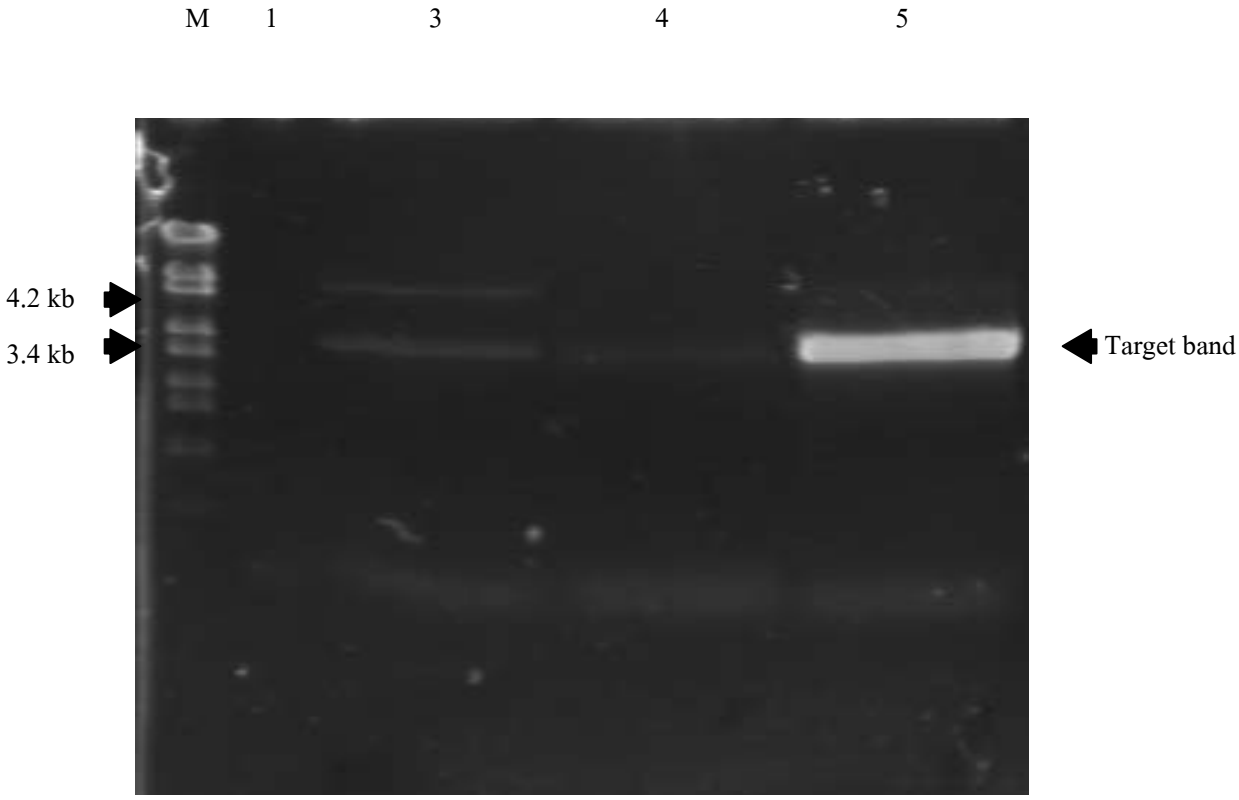
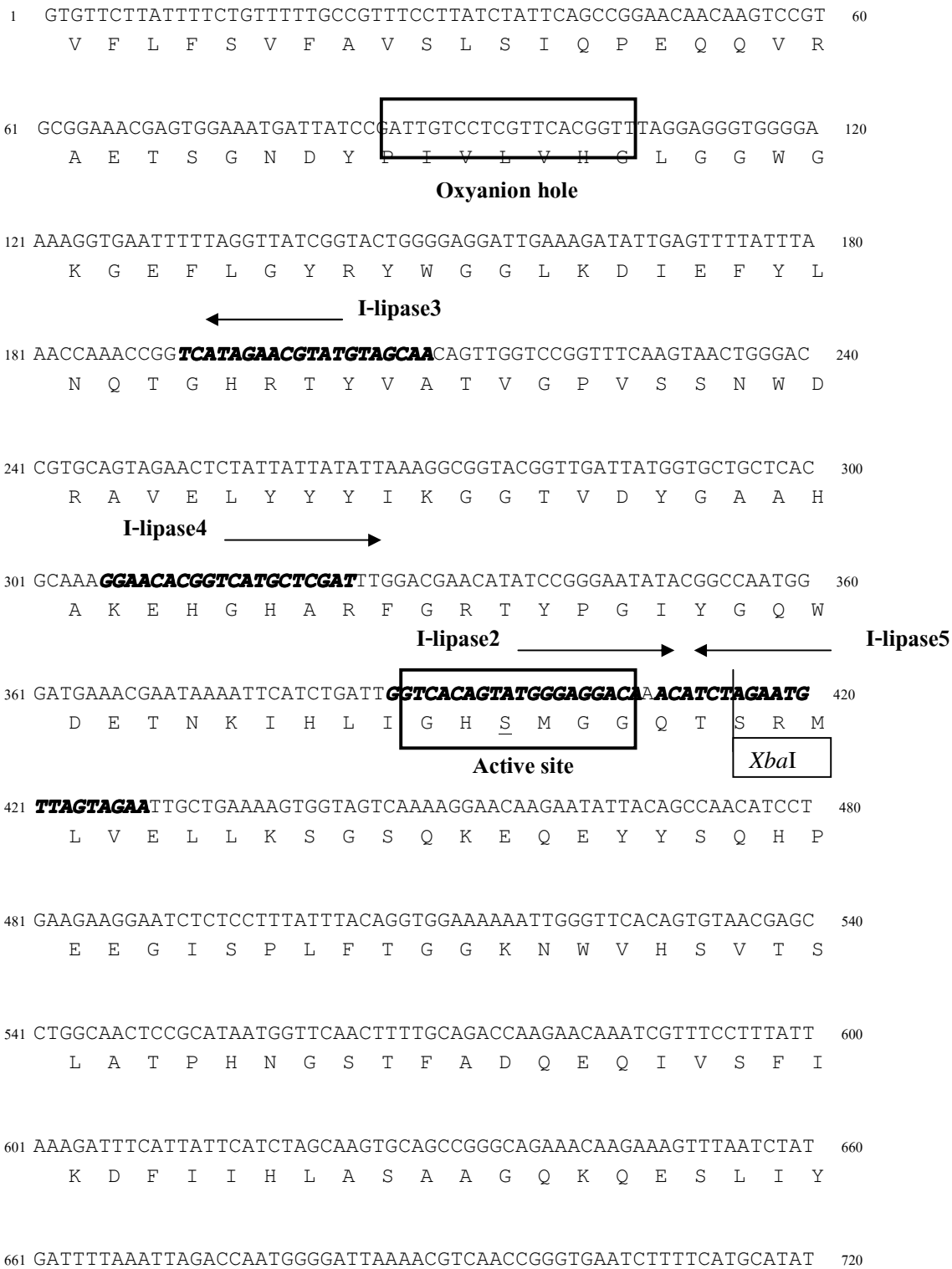


Figure 15. Inverse PCR products after gene amplification by using inverse PCR primers (I-lipase5, I-lipase6) and self-ligated products of digested genomic DNA of *B. thermoamylovorans* BHK52 with various restriction enzymes. An approximately 3,000 bp target band of lipase gene was observed in lane 5. Lanes: M, λ/*Eco*T14 markers; 1, negative controls (non- digested DNA); 3, 4, 5; inverse PCR products of digested DNA with *Sal*I, *Xba*I and *Bcl*I, respectively.

Then, the reconstructed Plasmid was sequenced with M13 primers from both ends by Big Dye Terminator version 1.1 cycle sequencing kit. The primers named N-lipase-XhoI and C-lipase-PstI (Table 12) containing *Xho*I and *Pst*I restriction sites, respectively, were synthesized (based on the analysis of upstream and downstream sequences obtained from genome walking by IPCR) to amplify the complete ORF of *lip52* gene directly from the genomic DNA of *B. thermoamylovorans* BHK52. The PCR product encoding the mature lipase Lip52 was ligated with pGEM-T Easy vector. The recombinant plasmid was named pGem-TLip52 containing an ORF of 1,221 bp (GeneBank Accession No. AB381879), which encoded 407 amino acid residues (Fig. 16). The deduced amino acid sequence was subjected to homology analysis by the BLAST program. The BLAST analysis showed moderate homology to known lipases (Fig. 17). Therefore, this ORF was suggested to be the lipase gene of *B. thermoamylovorans* BHK52 and it was named as *lip52*. The nucleotide sequence analysis of the *lip52* gene confirmed that the DNA fragment was amplified from genomic DNA of *B. thermoamylovorans* BHK52. Restriction site analysis of the *lip52* gene by GENETYX program showed the position of *Xba*I, *Nde*I, *Eco*RI and *Bam*HI which is useful for further cloning work (Fig. 16).

Amino acid composition analysis by GENETYX program revealed that a majority of this ORF contained hydrophobic residues (48.89%) whereas the others are neutral residues (23.59%) and hydrophilic residues (27.27%) (Table 17). A similar result has been reported for the ability of *Bacillus* lipase to aggregate due to its own hydrophobicity (Claudia *et al*., 1994). The amino acid sequence comparisons using the BLASTP program revealed that the lipase Lip52 did not match perfectly with any known proteins in the databases. However, it showed only moderate sequence identity (24–51%) with the known lipases. This indicates that the lipase Lip52 is a novel enzyme. The most similar lipases to the lipase Lip52 were the lipases from bacteria such as *B. thuringiensis* strain Al Hakam (YP895150), *B. anthracis* strain Ames (NP844969), *B. cereus* ATCC (NP978931), *B. weihenstephanensis* KBAB4 (YP001645360), and *Clostridium tetani* E88 (NP781602) with 51, 51, 50, 50, and 50% identities, respectively (Fig 17). Result from phylogenetic analysis based on the amino acid sequences, which was prepared by using ClustalX program version 1.83, revealed that Lip52 is most closely related to lipases from *Bacillus* spp. and *Costidium* spp. (Fig. 18). The lipase Lip52 contains a typical GxSxG motif where the active site serine residue is belong to one of the catalytic triad found in most esterases and lipases (Arpigny and Jaeger, 1999). On the basis of gene


1 GTGTTCTTATTTTCTGTTTTTGCCGTTTCCTTATCTATTCAGCCGGAACAACAAGTCCGT 60
V F L F S V F A V S L S I Q P E Q Q V R
61 GCGGAAACGAGTGGAAATGATTATCCGATTGTCCTCGTTCACGGTTTAGGAGGGTGGGGA 120
A E T S G N D Y P I V L V H G L G G W G
Oxyanion hole
121 AAAGGTGAATTTTTAGGTTATCGGTACTGGGGAGGATTGAAAGATATTGAGTTTTATTTA 180
K G E F L G Y R Y W G G L K D I E F Y L
I-lipase3
181 AACCAAACCGGTCATAGAACGTATGTAGCAACAGTTGGTCCGGTTTCAAGTAACTGGGAC 240
N Q T G H R T Y V A T V G P V S S N W D
241 CGTGCAGTAGAACTCTATTATTATATTAAAGGCGGTACGGTTGATTATGGTGCTGCTCAC 300
R A V E L Y Y Y I K G G T V D Y G A A H
I-lipase4
301 GCAAAGGAACACGGTCATGCTCGATTTGGACGAACATATCCGGGAATATACGGCCAATGG 360
A K E H G H A R F G R T Y P G I Y G Q W
I-lipase2
I-lipase5
361 GATGAAACGAATAAAATTCATCTGATTGGTCACAGTATGGGAGGACAAACATCTAGAATG 420
D E T N K I H L I G H S M G G Q T S R M
Active site
XbaI
421 TTAGTAGAATTGCTGAAAAGTGGTAGTCAAAAGGAACAAGAATATTACAGCCAACATCCT 480
L V E L L K S G S Q K E Q E Y Y S Q H P
481 GAAGAAGGAATCTCTCCTTTATTTACAGGTGGAAAAAATTGGGTTCACAGTGTAACGAGC 540
E E G I S P L F T G G K N W V H S V T S
541 CTGGCAACTCCGCATAATGGTTCAACTTTTGCAGACCAAGAACAAATCGTTTCCTTTATT 600
L A T P H N G S T F A D Q E Q I V S F I
601 AAAGATTTCATTATTCATCTAGCAAGTGCAGCCGGGCAGAAACAAGAAAGTTTAATCTAT 660
K D F I I H L A S A A G Q K Q E S L I Y
661 GATTTTAAATTAGACCAATGGGGATTAAAACGTCAACCGGGTGAATCTTTTCATGCATAT 720

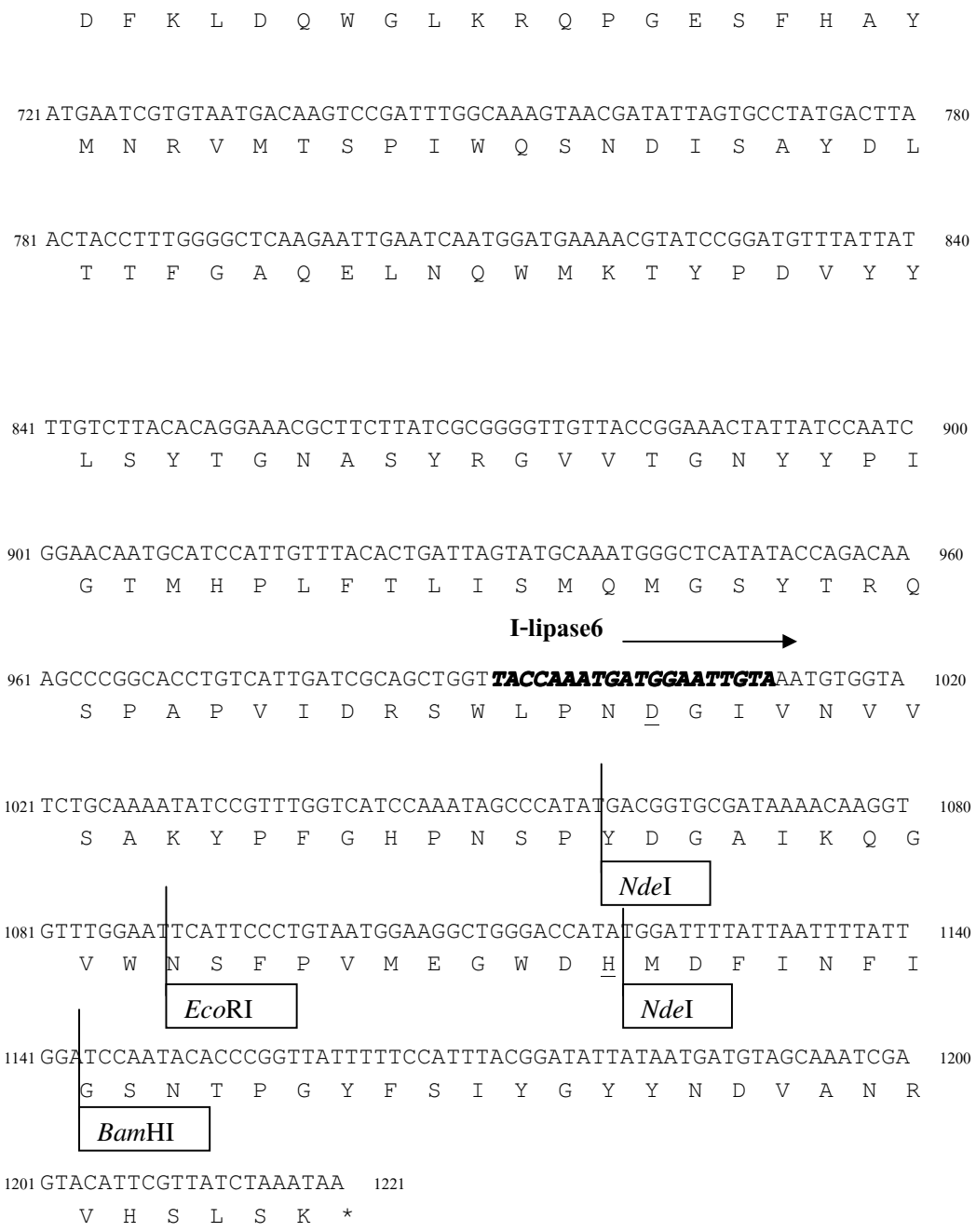
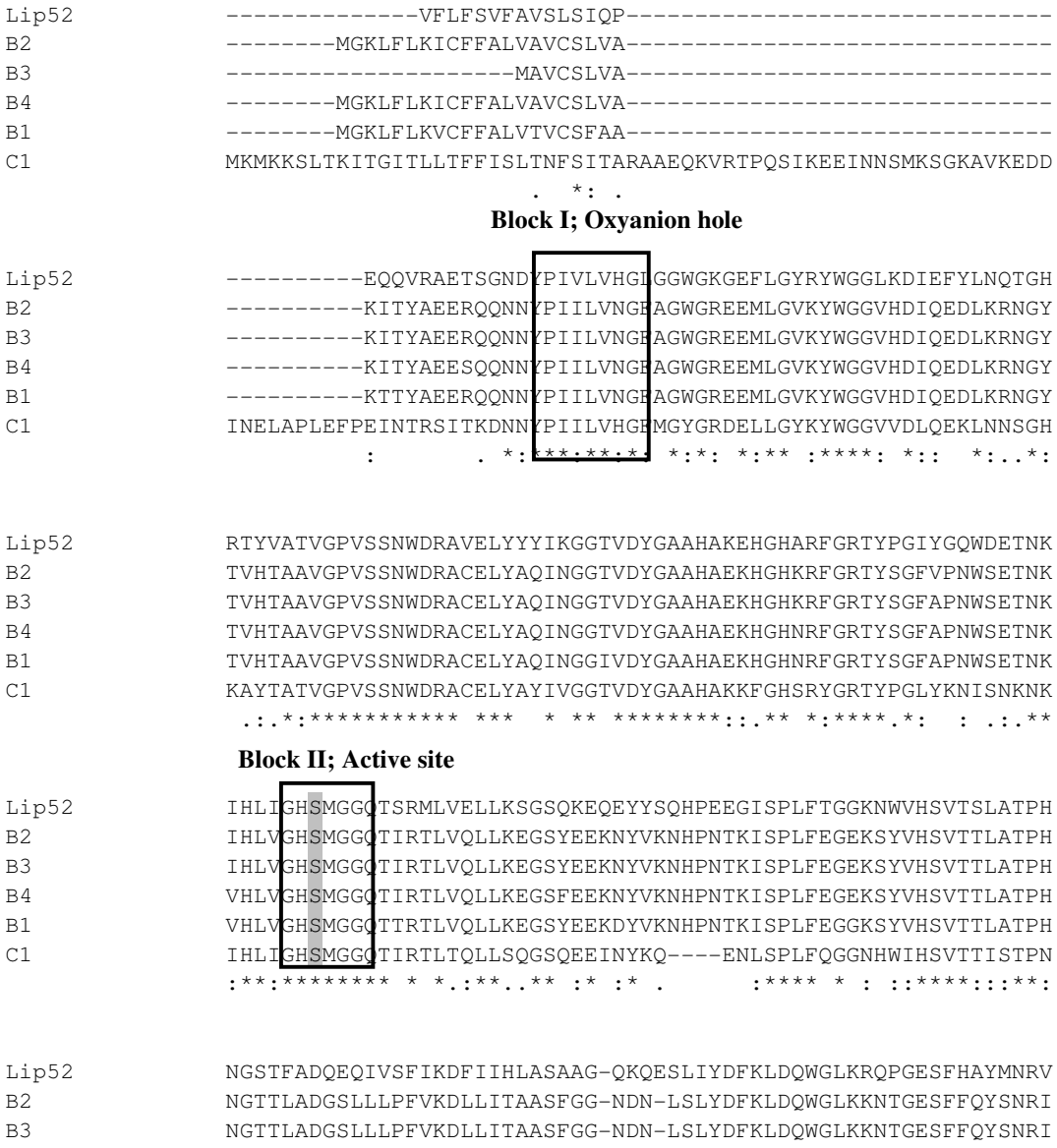


Figure 16. Nucleotide sequence of the *B. thermoamylovorans* BHK52 lipase *lip52* gene. This sequence contains 1,221 bp and encodes for 407 amino acid residues. The predicted amino acid sequence is given below the nucleotide sequence in the standard one-letter code. Two conserved sequences including oxyanion hole and active site are boxed. The predicted catalytic triad residues; serine, aspartic acid, and histidine, are underlined. The position and direction of IPCR primer sequences (I-lipase2-6) are shown by bold

letters and arrows. Restriction sites of endonucleases analyzed by GENETYX program including *Xba*I, *Nde*I, *Eco*RI and *Bam*HI are also boxed. The stop codon is marked by an asterisk (*).



```
B4              NGTTLADGSLLLPFVKDLLITAASFGG-NDN-LSLYDFKLDQWGIKKNVGESFFQYSNRI
B1              NGTTLADGSLLLPFVKDLLITAASLGG-NNN-LSLYDFKLDQWGIKKNTGESFFQYTDRI
C1              DGTTLSDLMPAGELLSSAFGALGTITGNNGIFNSLYDFKLDQWGLKKQEGESQRKYIKRV
                :*:*::*      ::.. :   .:  * :     :*********:*:: ***   * .*:

Lip52           MTSPIWQ-SNDISAYDLTTFGAQELNQWMKTYPDVYYLSYTGNASYRGVVTG-NYYPIGT
B2              LNSSIWKNTKDISQWDLSTDGAKELNNWVKTQPNVYYLSYSGHASQAAPITG-LHLPHIT
B3              LNSSIWKNTKDISQWDLSTDGAKELNNWVKTQLNVYYLSYSGHASQAAPITG-LHLPHIT
B4              LNSSLWKNTKDISQWDLSTDGAKELNNWVKTQPNVYYLSYSGHASQAAPITG-LHLPHIT
B1              LNSSIWENTKDISQWDLSTDGAKELNNWVKTQSDVYYLSYSGHASQAAPITG-LHLPHIT
C1              LDSDIWKRTKDIATYDLSTKGAEELNKWVKAQPDVYYFSWTTQATKESALTGHSIAQIGP
                : * :*: ::**: :**:* **:***:*:*:  :***:*:: :*:  . :**       .

Lip52           MHPLFTLISMQMGSYTRQSP-APVIDRSWLPNDGIVNVVSAKYP---FGHPNSPYDGAIK
B2              MNKVLMGNAFFLGSYARYEENRPLIDTTWWQNDGVVNTSSMIAP---SSNATVNNNESLQ
B3              MNKVLMGNAFFLGSYARYEENRPLIDTTWWQNDGVVNTSSMIAP---SSNATVNNNESLQ
B4              MNKVLMGNAFFLGSYARYEENRPLIDTTWWQNDGVVNTSSMIAP---SSNATVNNNESLQ
B1              MNKVLMGNAFFLGSYARYEENRPLIDTSWWQNDGVVNTNSMIAP---SSNIAANDNESLQ
C1              MNPLLYVPANLMGKYSRNEPNLPIINKEWFPNDGVVNCISQNGPKLGSSDIIEQYDGTAK

                *: ::   :  :*.*:* .   *:*:  *  ***:**  *   *    ..     : : :

Lip52           QGVWNSFPVMEGWDHMDFINFIGSNTPGYFSIYGYYNDVANRVHSLSK
B2              IGKWNHIETKANWDHLDMVGLSVSDTLSFSSIQEFYRAIAEKLSRLPK
B3              IGKWNHIETKANWDHLDMVGLSVSDTLGFSSIQEFYRAIAEKLSRLPK
B4              IGKWNHIETKANWDHLDMVGLSISDTLGFSNIQEFYRTIAEKLSRLPK
B1              VGKWNHIETKANWDHLDMVGLSVSDTLGFSNIQEFYRTIAEKLSRLPK
C1              KGRWNAMPLIINTDHMDITGTFG-------NVKDWYIDYAKILNKLPE
                 * ** :    . **:*: .          .:  :*   *: :  *.:
```

Figure 17. Multiple protein sequence alignment of lipases. Lipase from *B. thermoamylovorans* BHK52 (Lip52) (in this study) was compared to sequences of lipases from *B. thuringiensis* strain Al (B1), *B. anthracis* strain Ames (B2), *B. cereus* ATCC 10987 (B3), *B. weihenstephanensis* KBAB4 (B4) and *Clostridium tetani* E88 (C1), respectively. Amino acids conserved in all organisms are indicated with an asterisk

(*). Two conserved sequences including oxyanion hole and active site are boxed. The predicted catalytic triad residues are marked with grey boxes.

Table 17. Amino acid composition analysis by GENETYX program showing hydrophobic, neutral, hydrophilic and other residues in lipase *lip52* gene.

Hydrophobic residues	Neutral residues	Hydrophilic residues	Other residues
199 (48.89%)	96 (23.59%)	111 (27.27%)	1 (0.25%)
Gly(G) 43 (10.57%)	Ser(S) 35 (8.60%)	Asp(D) 19 (4.67%)	Asx(B) 0 (0.00%)
Ala(A) 21 (5.16%)	Thr(T) 22 (5.41%)	Glu(E) 17 (4.18%)	Glx(Z) 0 (0.00%)
Val(V) 27 (6.63%)	Asn(N) 18 (4.42%)	Lys(K) 16 (3.93%)	Xaa(X) 0 (0.00%)
Leu(L) 25 (6.14%)	Gln(Q) 21 (5.16%)	His(H) 16 (3.93%)	Stop (*) 1 (0.25%)
Ile(I) 23 (5.65%)	Cys(C) 0 (0.00%)	Arg(R) 13 (3.19%)	
Met(M) 10 (2.46%)		Tyr(Y) 30 (7.37%)	
Phe(F) 19 (4.67%)			
Trp(W) 11 (2.70%)			
Pro(P) 20 (4.91%)			

structure analysis, the lipase Lip52 was assigned to be a member of α/β-hydrolases. This family of lipolytic enzyme comprises a wide variety of enzymes whose activities rely mainly on a catalytic triad usually formed by Ser, His, and Asp residues. In the amino acid sequences of α/β-hydrolases,

the three residues follow the order Ser-Asp-His. The serine residue usually appears in the conserved pentapeptide Gly-Xaa-Ser-Xaa-Gly. Their marked preference for water insoluble substrates and their adsorption on the oil water interface before hydrolysis involve substantial conformational changes of the enzyme's architecture during catalysis. These have been particularly well documented for lipases of eukaryotic origin (Arpigny and Jaeger,1999).

3. Expression and purification of lipase Lip52

After nucleotide sequence analysis of the *lip52* gene, pGem-TLip52 was cut with *Xho*I and *Pst*I. The digested DNA fragment was directly cloned into the expression vector pColdI DNA (Takara, Japan) and the reconstructed plasmid was named pColdILip52. In order to determine the biochemical properties of the lipase Lip52 from *B. thermoamylovorans* BHK52, the ORF was expressed as N-terminal 6xHis tagged fusions in *E. coli* JM109. The selected transformant was inoculated into 400 ml of LB-broth medium containing 100 µg/ml of ampicillin,

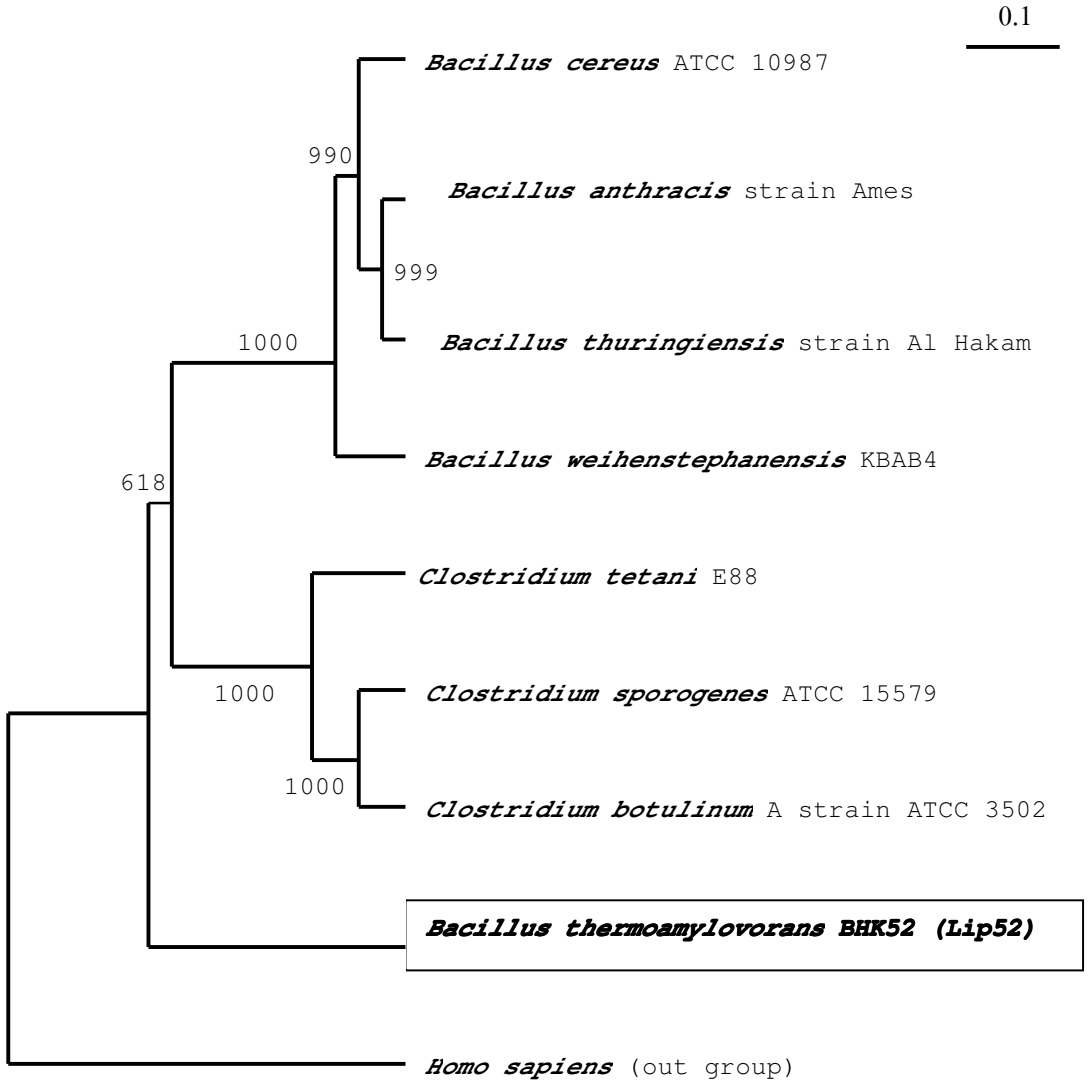


Figure 18. Phylogenetic analysis of the lipase Lip52 based on the amino acid sequences which was prepared by using ClustalX program version 1.83.

and cultivated at 37°C with shaking (200 rpm) until OD_{600} reached to 0.4 - 0.5. The culture solution was refrigerated at 15°C for 30 min before addition of IPTG at the final concentrations of 1.0 mM, and continued cultivation with shaking (200 rpm) at 15°C for 24 h. Bacterial cells were collected

by centrifugation at 6,000 rpm for 15 min at 4°C. The cell pellet was then washed once with 0.85% (w/v) NaCl, resuspended in 50 mM of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole; pH 8.0) and disrupted by sonication. After centrifugation, the supernatant (cell-free extracted) was checked for the presence of the lipase Lip52 by SDS–PAGE. To confirm the expression, the supernatants from uninduced *E. coli* JM109 harboring pColdI DNA, uninduced *E. coli* JM109 harboring pColdILip52, and induced *E. coli* JM109 harboring pColdILip52 were analyzed and compared by SDS–PAGE. A target band approximately 45 kDa (1 amino acid has molecular mass approximately 110 kDa) was observed in the induced recombinant bacteria containing lipase gene. No protein was found at the same positions when the supernatants from uninduced and induced *E. coli* JM109 harboring only plasmid vector were applied (Fig 19). These comfirmed that the lipase *lip52* gene was expressed in the recombinant strain. Furthermore, 1.89 expression fold was increased when the lipase Lip52 was used to determine the enzyme activity compared to the wild-type (*B. thermoamylovorans* BHK52) (Table 18).

To improve expression level of the *lip52* gene, changing expression systems were recommended such as vector, promoter, host cell and induction time. Cho *et al.* (2000) reported that expression of the lipase gene from *B. thermoleovorans* under native promoter is stronger than expression under T7 promoter. Results from various studies revealed that most of the lipase genes expressed in *E. coli* seemed to exist as an insoluble form, as an inclusion body, or as a membrane-bound protein. There are the reports that some lipases are not fully processed by *E. coli* (Claudia *et al*., 1997). Thus, the over expression of lipase was also developed in *Bacillus* for efficient protein secretion and correct maturation (Dartois *et al*., 1994). Successful lipase gene expression of *Geobacillus* sp. strain T1 was obtained by using pBAD, pRSET, pET and pGex vectors as under the control of *araBAD,* T7, T7 *lac* and tac promoters, respectively. The expression level obtained by the pGex vector system was found to be higher than the others (Leow *et al*., 2004). To examine an optimum condition for L1 lipase expression in fed-batch culture, L1 lipase expression was induced at three different growth phases (early, mid- and late exponential growth phases). Maximum production of lipase was observed when the culture was induced at the early growth phase (Oh *et al*., 1999).

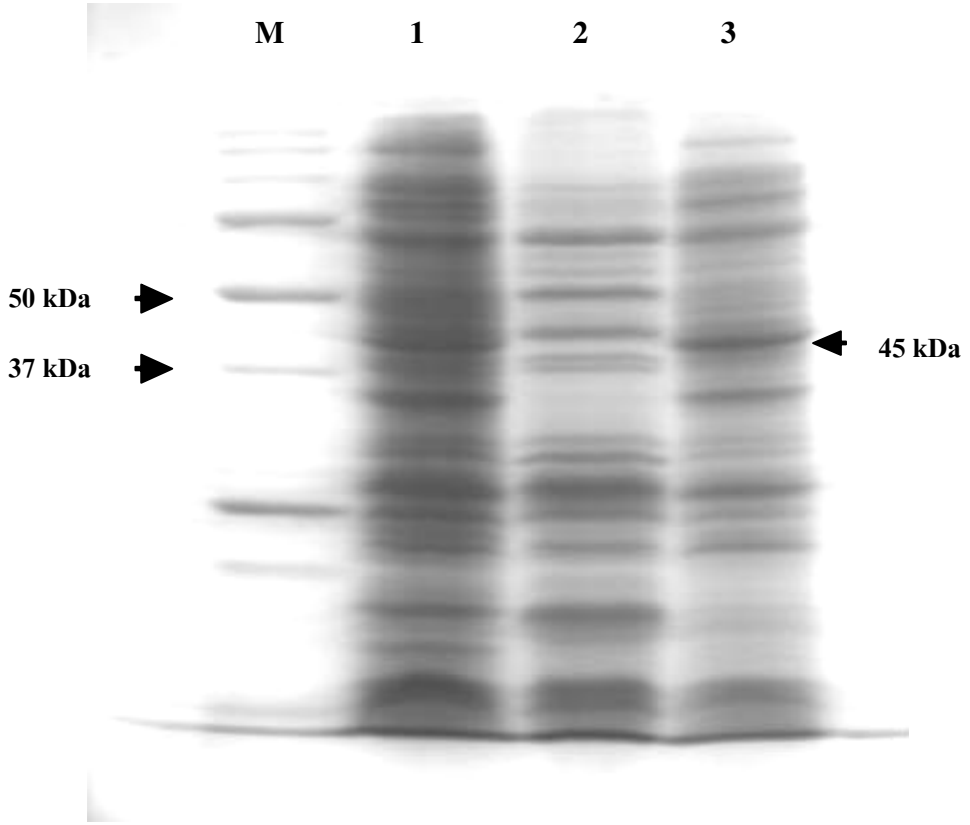


Figure 19. 12% SDS-PAGE of crude cell lysates of *E. coli* JM109 expressed the Lip52 protein. The protein gel was stained with Coomassie Brilliant Blue R-250. Lanes: M, protein standard (Bio-Rad, USA); 1, uninduced *E. coli* JM109 harboring pColdI DNA; 2, uninduced *E. coli* JM109 harboring pColdILip52 and 3, induced (1 mM IPTG) *E. coli* JM109 harboring pColdILip52.

Table 18. Comparison of crude enzyme activity from wild-type (*B. thermoamylovorans* BHK52) and the lipase Lip52 .

Source of enzyme	Activity (mU/ml)	Total activity (mU)	Expression fold
wild-type	0.12	6.023	1
Lip52	0.23	11.41	1.89

For lipase purification and characterization, cell free-extract was purified by Hi-Trap affinity column containing nickel-nitrilo-triacetic acid as described by the manufacturer

(Qiagen, UK). The purified protein was subjected to SDS-PAGE, lipase activity was spectrophotometrically measured using *p*-nitrophenyl palmitate (C16) as substrate and protein concentration was determined by protein assay kit (Bio-Rad, USA). The purified proteins migrated as a single band on SDS-PAGE with the expected molecular mass of approximately 45 kDa (Fig. 20). The purification procedure is summarized in Table 19. The purified enzyme has a purification fold of 5.79 and a protein yield of 38.17%. This purification method is a single step of protein purification with a single affinity column, which is conveniently carried out and is not time-consuming, by tagging the target protein with histidine molecules. The imidazole ring is a part of the structure of histidine (Fig. 9). The imidazole rings in the histidine residues of the 6xHis tag bind to the nickel ions immobilized by the NTA groups on the matrix. The imidazole itself can also bind to the nickel ions and disrupts the binding of dispersed histidine residues in nontagged background proteins. At low imidazole concentrations, nonspecific, low affinity binding of background proteins is prevented, while 6xHis-tagged proteins still bind strongly to the Ni-NTA matrix. Because of the lipase expression plasmid contains 6xHis residues, it is easily to purify the lipase Lip52 by using the Ni-NTA column.

4. Characterization of lipase Lip52

4.1 Substrate specificity of lipase Lip52

Substrate specificity of the lipase Lip52 on *p*-nitrophenyl esters were determined by incubation of purified enzyme in 10 mM of various *p*-nitrophenyl esters with different acyl chain lengths (C2-C18). Hydrolysis of *p*-nitrophenyl esters indicated that the lipase from *B. thermoamylovorans* BHK52 exhibited preferential hydrolysis of *p*-nitrophenyl esters with medium acyl chain lengths (C6-C10) whereas the maximum activity was observed when *p*-NP–caprylate (C8) was used as substrate (Fig. 21). The enzyme also showed hydrolytic activity on esters with short chain fatty acids (C2, C4), but the relative activity was less than 5% (3.34%) when *p*-NP–acetate (C2) was used as substrate. The lipase activity on long chain synthetic substrates (C12-C18) was also observed. Among the substrates with long chain fatty acids tested, *p*-NP–palmitate (C16) provided the highest enzyme activity. The results suggested the ability of lipase Lip52 to hydrolyze palm oil, a sole carbon source in lipase assay medium used in

Table 19. Summary of the purification procedure for the *B. thermoamylovorans* BHK52 lipase Lip52 expressed in *E. coli* JM109.

Purification step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Purification fold	yeild (%)
Cell free extract	27.51	737.42	26.81	1.00	100.00
Ni-NTA	1.81	281.47	155.15	5.79	38.17

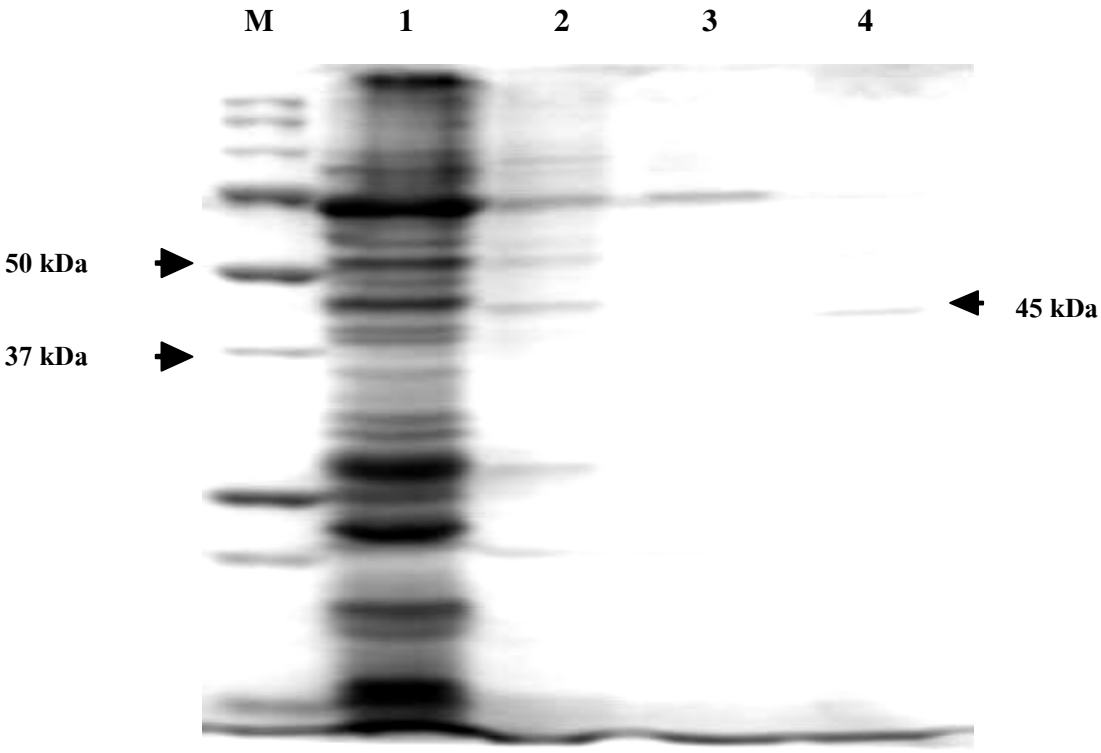


Figure 20. 12% SDS-PAGE of purified lipase Lip52 samples expressed in *E. coli* JM109 with the induction of 1 mM IPTG. The polyacrylamide gel was stained with Coomassie Brilliant Blue R-250. Lanes: M, protein standard (Bio-Rad, USA); 1, cell free extract; 2, flow-through fraction after using lysis buffer; 3, wash fraction and 4, purified lipase Lip52 eluted from the column.

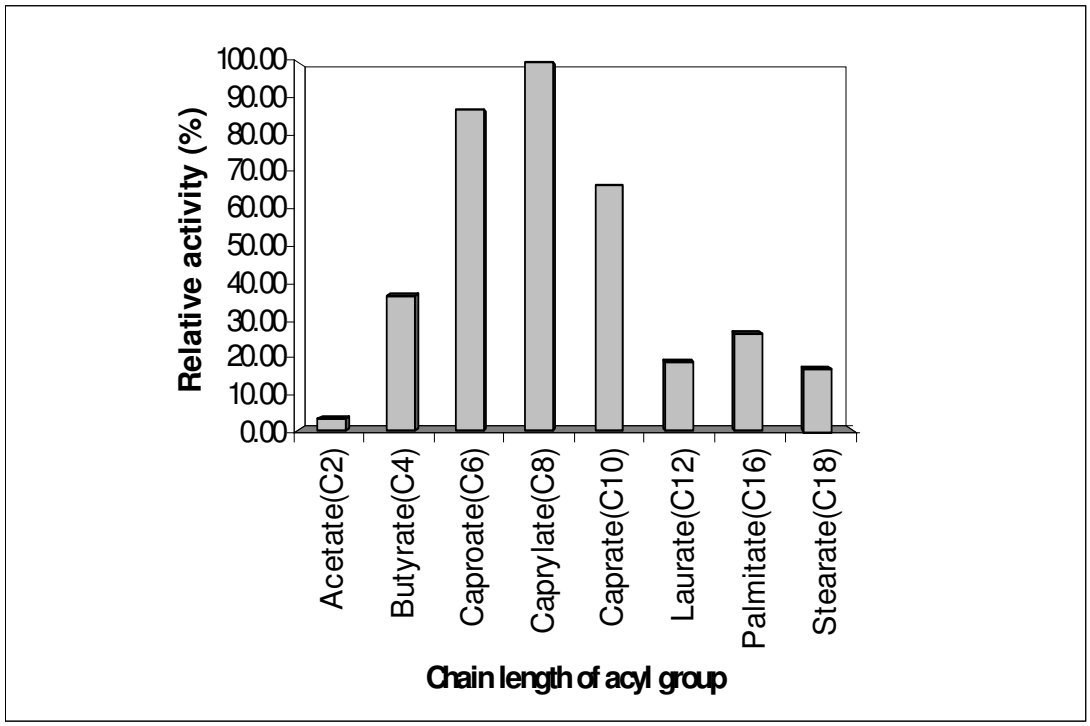


Figure 21. Hydrolysis of *p*-nitrophenyl esters with carbon chain length up to C18 by purified lipase Lip52. Result was exhibited in term of relative activity towards *p*-nitrophenyl caprylate (C8) under stardard assay conditions.

the screening of lipase-producing thermotolerant bacteria. Therefore, it can be concluded that *p*-NP–caprylate (C8) is the best substrate for hydrolytic action of the lipase Lip52. The other

substrates including *p*-NP–butyrate (C4), *p*-NP–caproate (C6), and *p*-NP–caprate (C10) are also good substrates, whereas very short chain ester (*p*-NP–acetate, C2) is a poor substrate. It can be concluded that the lipase from *B. thermoamylovorans* BHK52 (Lip52) prefers medium chain fatty acids more than short and long chain fatty acids as substrate. Similar results have been reported for lipases from *B. thermocatenulatus* (Kim *et al.,* 1994), *B. stearothermophilus* L1 (Kim *et al.,* 1998), *B. stearothermophilus* P1 (Supachok *et al*., 2001), *B. coaglulans* MTCC-6375 (Castro-Ochoa *et al*., 2005), and *B. thermoleovorans* CCR11 (Kam *et al*., 2006).

4.2 Effect of pHs on lipase activity and stability

The effect of pHs on lipase activity at 70°C with *p*-NP–caprylate as substrate was examined at various pH values in the range of 3.0–11.0 (in sodium acetate, potassium phosphate, Tris–HCl, KOH–glycine buffers). The enzyme was active at a broad pH range, with more than 50% of its maximal activity occurring at pH from 6.0–9.0 and the optimal pH was shown to be 8.0 (Fig. 22). The similar results were also reported for lipases from others thermophilic *Bacillus* which were active in the range of pH 7.2–8.5 (Schmidt-Dannert *et al.,* 1994; Luisa *et al.*, 1997; Kim *et al.,* 1994; Lee *et al.,* 1999). To evaluate the pH stability of the enzyme, the purified enzyme was incubated in various 0.2 M pH buffers for 6 h at 37°C. The lipase Lip52 was stable at a broad pH range (5.0-10.0) where the enzyme displayed more than 50% of its maximal activity. However, the enzyme activity was dramatically decreased at pH values below 5.0 (Fig. 23). The similar result was also reported for the lipase from *Geobacillus* sp. (Leow *et al*., 2004). These results indicate potential usage of the lipase Lip52 in a wide range of applications, especially around neutral or mide basic pHs.

4.3 Effect of temperatures on lipase activity and stability

In order to evaluate the effect of temperatures on lipase activity, the purified lipase was allowed to function at various temperatures (30-100°C). The lipase Lip52 exhibited more than 80% of its maximal activity at 80-100°C whereas the highest activity was observed at 90°C (Fig. 24). The thermostability of the lipase Lip52 was examined by incubating the purified enzyme for 7 h at 30-100°C before measuring its residual activity under standard assay conditions. The lipase Lip52

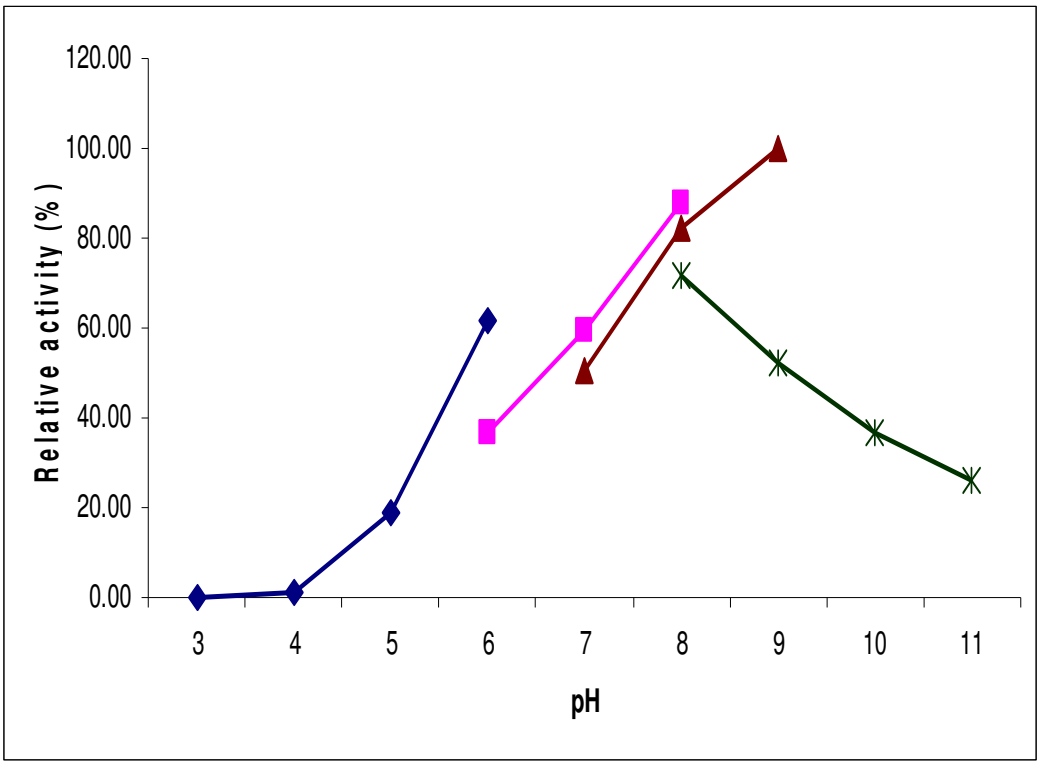


Figure 22. Effect of pHs on lipase activity. The purified lipase was assayed at pHs ranging from 3.0-11.0 under standard assay conditions. The buffers used were 50 mM sodium acetate (pH 3.0-6.0) (♦); 50 mM potassium phosphate (pH 6.0-8.0) (■); 50 mM Tris–HCl (pH 7.0-9.0) (▲); and 50 mM KOH–glycine (pH 8.0-11.0) (✳).

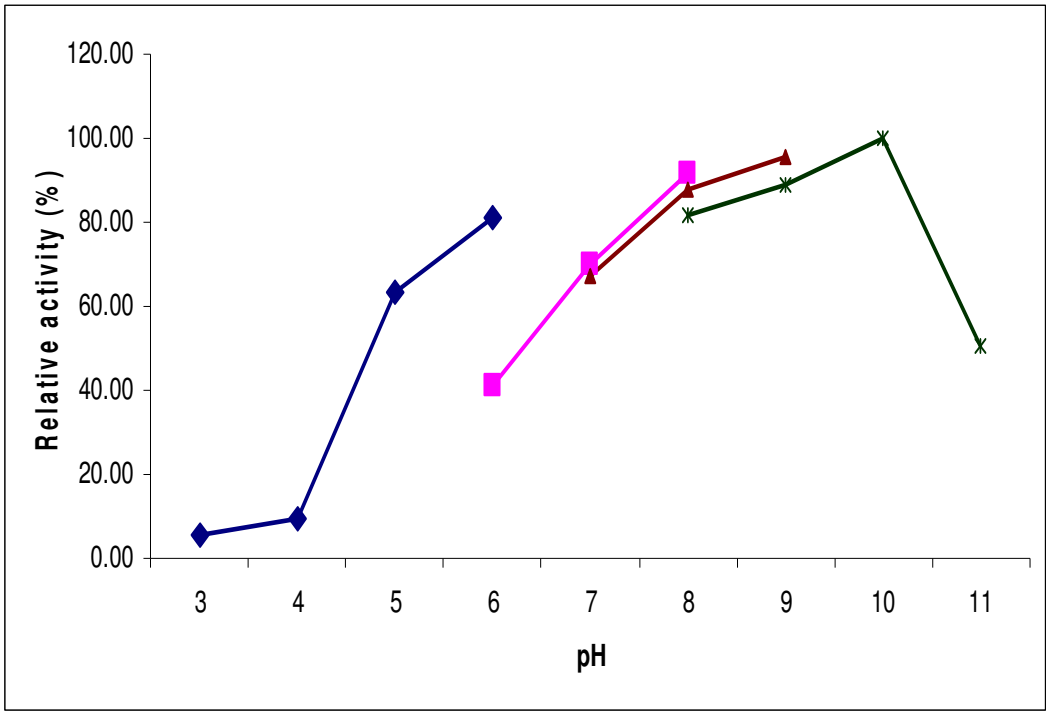


Figure 23. Effect of pHs on lipase stability. The purified lipase was assayed under standard assay conditions. The buffers used were 0.2 M sodium acetate (pH 3.0-6.0) (♦); 0.2 M potassium phosphate (pH 6.0-8.0) (■); 0.2 M Tris–HCl (pH 7.0-9.0) (▲); 0.2 M KOH–glycine (pH 8.0-11.0) (∗).

was stable at a wide range of temperatures from 30°C to 100°C with a residual activity greater than 80% of its initial activity (Fig. 24). The results showed high thermal stability of this enzyme at temperatures up to 100°C which is slightly higher than that of lipases from others *Bacillus* and

much higher than that of lipases from other organisms, which usually display thermostability at 30-60°C (Schmidt-Dannert *et al*., 1996; Kim *et al*., 1998; Cho *et al*., 2000; Supachok *et al*., 2001). At 90°C, the optimal temperature for its activity, the enzyme was stable for more than 7 h where the residual activity was decreased for approximately 20%. However, prolongation of incubation at all temperatures resulted in loss of enzyme activity. The optimal temperature and thermostability of the purified enzyme correspond well to the temperature of the compost where the samples were obtained (60-70°C). Therefore, the lipase Lip52 could be applied within a broad temperature range of 30-100°C and are suitable for biotechnological applications performed at high temperatures.

4.4 Effect of metal ions on lipase activity

The effect of metal ions on the activity of the lipase Lip52 was investigated including determination of metal ions that can activate and inactivate the enzyme. In the presence of all metal ions tested (at 10 mM), no metal ions can activate the activity of the lipase Lip52, indicating no extra requirement for metal ions (Fig. 25). Moreover, the Zn^{2+} became the most effective metal ion which inhibited the activity of the lipase Lip52. Similar results were reported for the lipase from *B. stearothermophilus* L1 that was inactivated by 1 mM $ZnCl_2$ and $FeSO_4$ with 65 and 47% inhibiting activity, respectively (Kim *et al.,* 1998). The lipase from *B. stearothermophilus* P1 was strongly inhibited by 10 mM $ZnCl_2$ and $FeSO_4$ with 98.4 and 99.3% inhibition, respectively (Supachok *et al*., 2001). In addition, the lipase from *B. sphaericus* 205y was also strongly inhibited by 1 mM $ZnSO_4$ and $FeCl_3$ with 90 and 94% inhibition, respectively (Sulong *et al*., 2006). However, the lipase from *B. thermoamylovorans* BHK52 (Lip52) was partially inhibited by 10 mM $ZnCl_2$ with only 34% inactivation whereas the lipase activity was nearly equal to the control (98.6%) when 10 mM $FeSO_4$ was applied.

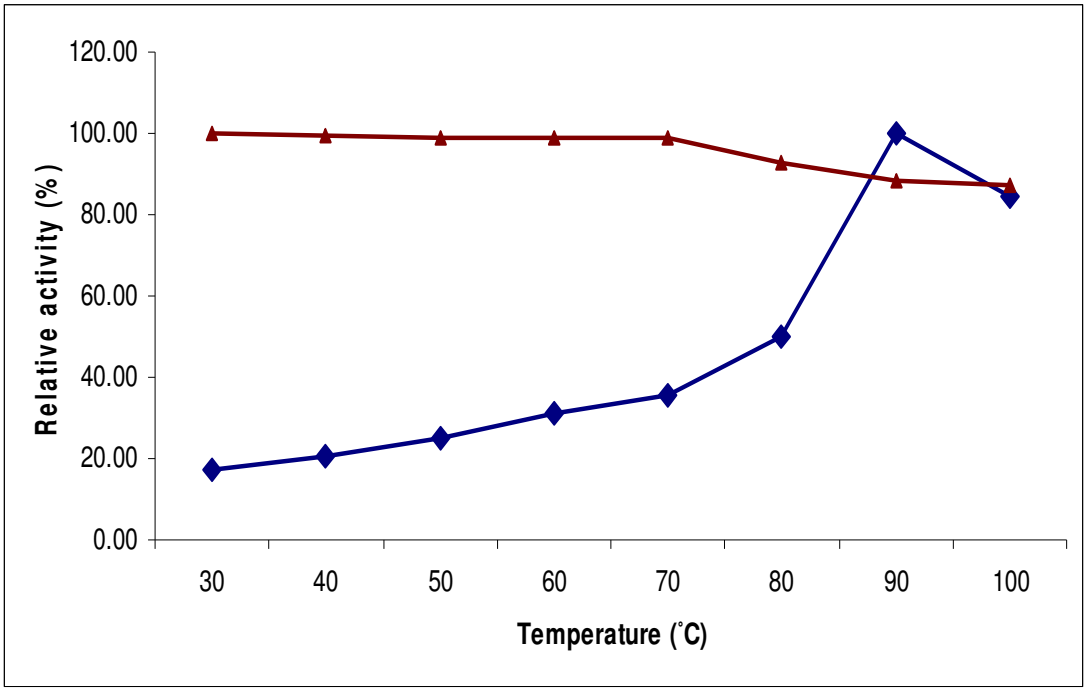


Figure 24. Effect of temperatures on lipase activity (♦) and stability (▲). The optimum temperature for lipase activity was determined by assaying enzymatic reactions at temperatures ranging from 30 to 100 °C under standard assay conditions. For determination of thermal stability, the purified lipases were incubated in 50 mM Tris-HCl (pH 9.0) at temperatures ranging from 30 to 100 °C for 7 h. The residual activity is defined as the ratio of the activity after and before incubation at the specified temperatures.

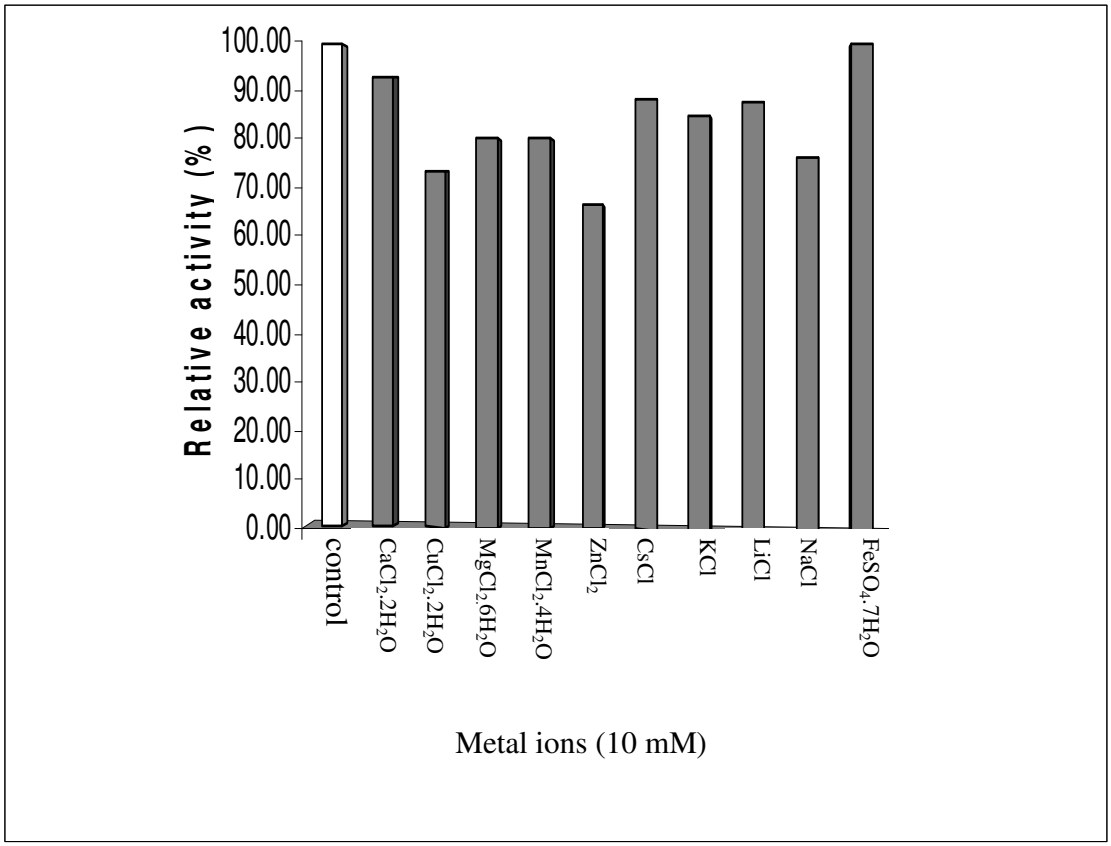


Figure 25. Effect of metal ions on lipase activity. The lipase activity was determined by assaying each enzymatic reaction in 50 mM Tris-HCl (pH 9.0) containing 10 mM of $CaCl_2.2H_2O$, $CuCl_2.2H_2O$, $MgCl_2.6H_2O$, $MnCl_2.4H_2O$, $ZnCl_2$, CsCl, LiCl, KCl, NaCl, or $FeSO_4.7H_2O$ under standard assay conditions. The enzymatic reaction without metal ion was set as control (100%).

CHAPTER 4

CONCLUSION

In this study, a total of 88 thermotolerant bacteria isolated from composts were used as sources of lipase-producing bacteria. The bacterial strains were screened and scored as lipase-producing strains when they showed fluorescent colonies under UV light (254 nm) after cultivation on lipase-assay medium containing palm oil and rhodamine B at 50°C. The most promising strains, in terms of production capability and enzyme advantageous properties, were genetically identified by 16S rRNA gene sequence analysis, and found to belong to 1 isolate of *Aneurinibacillus thermoaerophilus* (BHK30) with 99% identity, 1 isolate of *Bacillus pumilus* (BHK28) with 100% identity, and 8 isolates of *Bacillus thermoamylovorans* (BHK24, BHK26, BHK34, BHK52, BHK60, BHK65, BHK66 and BHK67) with 99% identity. Because of no report was found on a lipase from *Bacillus thermoamylovorans*, therefore the target lipase was supposed to be a novel one. The strain BHK52, which was identified as *Bacillus thermoamylovorans*, was selected for gene cloning, nucleotide sequencing, expression and partial characterization since it showed the highest lipolytic activity. Degenerate primers were designed according to sequences of two highly conserved regions of lipases among *Bacillus* spp. including oxayanion hole and active site region whereas the primers were named Loxy1 and LipCR. The Loxy1 and LipCR primers were used to amplify an approximately 300 bp DNA fragment which belongs to a part of an open reading frame (ORF). For a part of full-length lipase gene, inverse PCR (IPCR) was applied. Inverse PCR primers were designed from known sequences and used as degenerate primers to amplify an ORF of the lipase gene by genome-walking technique. The lipase gene from *Bacillus thermoamylovorans* BHK52 was named *lip52* whereas nucleotide sequence analysis of the lipase *lip52* gene revealed an open reading frame of 1,221 bp encoding a mature lipase of 407 amino acid residues. The amino acid sequence comparisons by using the BLASTP program revealed that the lipase Lip52 did not match perfectly with any known proteins in the databases, while this enzyme showed only moderate sequence identity (24–51%) with the known lipases. This indicates that the lipase Lip52 is a novel enzyme. The most similar lipases to the lipase Lip52 were the lipases from bacteria such as *B. thuringiensis* strain Al Hakam (YP895150), *B. anthracis* strain Ames (NP844969), *B. cereus* ATCC (NP978931), *B. weihenstephanensis* KBAB4 (YP001645360), and *Clostridium tetani* E88 (NP781602) with 51, 51, 50, 50, and 50% identities, respectively. Result

from phylogenetic analysis based on the amino acid sequences, which was prepared by using ClustalX program version 1.83, revealed that the lipase Lip52 is most closely related to lipases from *Bacillus* spp. and *Costidium* spp. The lipase Lip52 contains a typical GxSxG motif found in most esterases and lipases. On the basis of gene structure analysis, the lipase Lip52 was assigned to be a member of α/β-hydrolases. The *lip52* gene was expressed in pColdI DNA system, purified by affinity chromatography with Ni-NTA resin, and partially characterized. The molecular mass of the lipase Lip52 was determined to be approximately 45 kDa by SDS–PAGE. The lipase Lip52 had an optimum pH of 8.0 and showed maximal activity at 90°C. It was highly stable within a pH range of 5.0-10.0 and a temperature range of 30–100°C. The effect of metal ions on the activity of the lipase Lip52 was also investigated, indicating no extra requirement for metal ion because no metal ions tested could activate the enzyme activity after incubation of the lipase Lip52 in 10 mM of each metal ion for 5 min. The highest activity was observed when using *p*-nitrophenyl caprylate (C8) as a synthetic substrate. Further work for improving of the expression level of the *lip52* gene can be done by changing promoter, plasmid vector, host cell, concentration of IPTG, and induction time. The lipase Lip52 is the extremely thermoactive and thermotolerant lipase, therefore it can be applied in environmental and industrial applications. One of the prospecting work in future is the application of the lipase Lip52 for biodeisel production since it worked properly at high temperature and alkaline pH conditions.

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APPENDIX

Table 20. Lipase activity from lipase-producing bacteria growing on lipase assay medium after incubation at 50°C overnight.

Number	Strain	Activity
1	BHK1	++
2	BHK2	-
3	BHK3	-
4	BHK4	++
5	BHK5	-
6	BHK6	++
7	BHK7	++
8	BHK8	++
9	BHK9	++
10	BHK10	+++
11	BHK11	++
12	BHK12	+++
13	BHK14	+
14	BHK15	+
15	BHK16	+
16	BHK17	+
17	BHK18	+
18	BHK19	+
19	BHK20	+
20	BHK21	+
21	BHK22	+++
22	BHK23	+++
23	BHK24	++++
24	BHK25	++
25	BHK26	+++++
26	BHK27	+

Table 20. Lipase activity from lipase-producing bacteria growing on lipase assay medium after incubation at 50°C overnight (continued).

Number	Strain	Activity
27	BHK28	++++
28	BHK29	++
29	BHK30	+++++
30	BHK31	++
31	BHK32	-
32	BHK33	++
33	BHK34	++++
34	BHK35	++
35	BHK36	+
36	BHK37	+
37	BHK38	++
38	BHK39	-
39	BHK40	-
40	BHK41	++
41	BHK42	++
42	BHK43	-
43	BHK44	++
44	BHK45-1	++
45	BHK45-2	++
46	BHK51	++
47	BHK52	+++++
48	BHK53	-
49	BHK54	+
50	BHK55	++
51	BHK56	-
52	BHK57	-

Table 20. Lipase activity from lipase-producing bacteria growing on lipase assay medium after incubation at 50°C overnight (continued).

Number	Strain	Activity
53	BHK58	+++
54	BHK59	-
55	BHK60	+++++
56	BHK61	-
57	BHK62	++
58	BHK63	+
59	BHK64	+
60	BHK65	++++
61	BHK66	++++
62	BHK67	++++
63	BHK68	+
64	BHK69	++
65	BHK70	-
66	BHK100	-
67	BHK101	++
68	BHK102	++
69	BHK103	++
70	BHK105-1	-
71	BHK105-2	-
72	BHK106	-
73	BHK107	+
74	BHK108	+
75	BHK109	-
76	BHK110	++
77	BHK111	-
78	BHK112	+++
79	BHK113	++

Table 20. Lipase activity from lipase-producing bacteria growing on lipase assay medium after incubation at 50°C overnight (continued).

Number	Strain	Activity
80	BHK114	++
81	BHK115	++
82	BHK116	++
83	BHK117	++
84	BHK160	++
85	BHK180-5	-
86	BHK200-1	-
87	BHK200-2	++
88	BHK200-3	++

Symbols:	-	=	no growth; no activity
	+	=	low growth rate; low activity
	++,+++	=	moderate growth rate; moderate activity
	++++	=	high growth rate; high activity

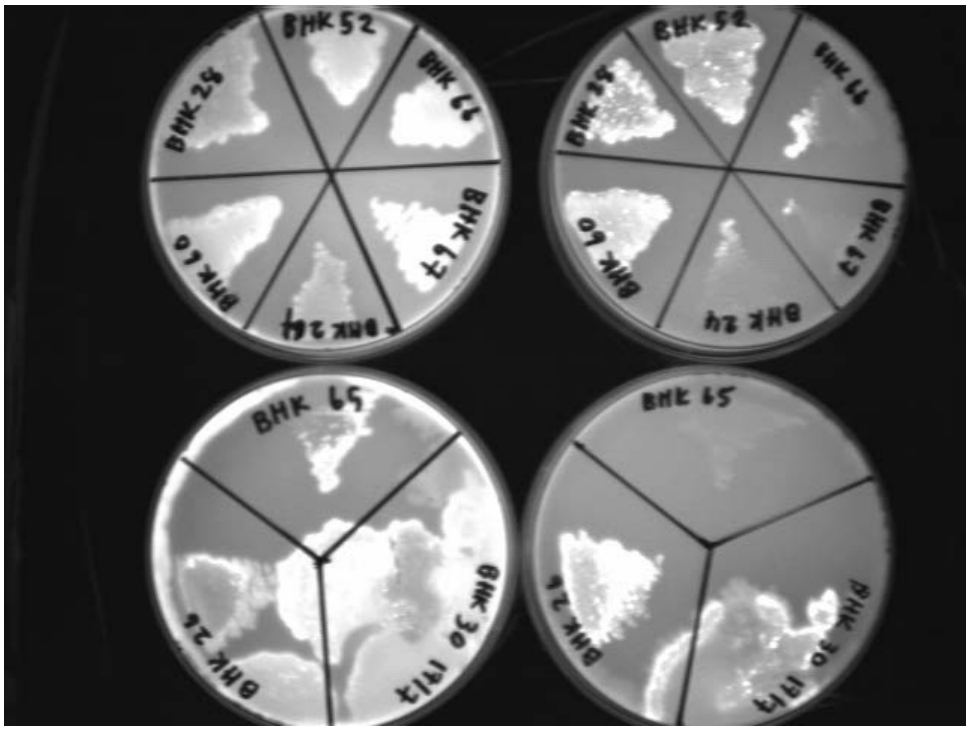

Figure 26. Identification of enzyme activity on lipase assay medium. After incubation at 50°C overnight, lipase-producing thermotolerant strains showed orange fluorescent colonies under UV light (254 nm). Lipase positive strains were selected for further screening by activity assay using spectrophotometer.

CGAACCAATAAGAAGCTTGCTTTTTGTTGGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCTGTAAGACCGGGATAACTCCGGGAAACCGGTGCTAATACCGGATAG
ATTATCTTTCCGCCTGGAGAGATAAGGAAAGATGGCTATTGCCATCACTTACAGATGG
GCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGC
GTGAGTGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCG
GAGGAAATGCCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGC
GCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTC
ATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAA
CTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCT
AACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGA
ACCTTACCAGGTCTTGACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTCGGGGA
CAGGATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGGTTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGA
GCGACTGCCGGCGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCAAAGCGGC
GACGCATGAGCGAATCCCAGAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGC
CTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGT
GAGGTAACCGTA

Figure 27. The 1,397 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK24.

CCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACCAAT
AAGAAGCTTGCTTTTTGTTGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
CCTGTAAGACCGGGATAACTCCGGGAAACCGGTGCTAATACCGGATAGATTATCTTTC
CGCCTGGAGAGATAAGGAAAGATGGCTATTGCCATCACTTACAGATGGGCCCGCGGC
GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAA
GAAGGTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCGGAGGAAATG
CCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGC
GGTCCTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACT
GGGGGACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTGA
GGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAA
GCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTCGGGGACAGGATGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTGGTTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGCGACTGCC
GGCGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG
GGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCGGCGACGCATGAG
CGAATCCCAGAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAG
CCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCG

Figure 28. The 1,442 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK26.

TAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCG
AGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATA
GTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATG
GACCCGCGGCGCATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATGCGTA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCG
AGAGTAACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGG
GCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTC
ATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTA
ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCTTCGGGG
ACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAA
GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCAAGACCG
CAAGGTTTAGCCAATCCCATAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGAC
TGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAGTCGGTG
AGGTAACCTTTATGGAGCCAGCCGCCG

Figure 29. The 1,468 bp nucleotide sequence of 16S rRNA gene from *Bacillus pumilus* BHK28.

GATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACC
GATGGAGTGCTTGCATTCCTGAGGTTAGCGGCGGACGGGTGAGTAACACGTAGGCAAC
CTGCCTGTACGACCGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGGATGCC
GAACCGCATGGTTCGGCATGGAAAGGCCTTTGAGCCGCGTACAGATGGGCCTGCGGCG
CATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAG
AGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAGG
AAGGTCTTCGGATCGTAAAACTCTGTTGTCAGGGAAGAACCGCCGGGATGACCTCCCG
GTCTGACGGTACCTGACGAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTTC
TTAAGTCAGGTGTGAAAGCCCACGGCTCAACCGTGGAGGGCCATCTGAAACTGGGGA
GCTTGAGTGCAGGAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGA
TGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGTAACTGACGCTGAGGCGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAGGTGTTGGGGAGTCCACCTCCTCAGTGCCGCAGCTAACGCAATAAGCACTC
CGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGA
CATCCCGCTGACCCCTCCAGAGATGGAGGTTTCCTTCGGGACAGCGGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGTCCTTTGTTGCCAGCATTCAGTTGGGCACTCTAAGGAGACTGCCGTCGACAA
GACGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCCTGGGCTACAC
ACGTGCTACAATGGACGGTACAACGGGCGTGCCAACCCGCGAGGGTGAGCCAATCCC
TAAAAACCGTTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGCAAACACCCGAAGTCGGTGAGGTAACCTT

Figure 30. The 1,437 bp nucleotide sequence of 16S rRNA gene from *Aneurinibacillus thermoaerophilus* BHK30.

TAGAGTTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTC
GAGCGAACCAATAAGAAGCTTGCTTTTTGTTGGTTAGCGGCGGACGGGTGAGTAACAC
GTGGGTAACCTGCCTGTAAGACCGGGATAACTCCGGGAAACCGGTGCTAATACCGGAT
AGATTATCTTTCCGCCTGGAGAGATAAGGAAAGATGGCTTTTGCCATCACTTACAGAT
GGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGT
AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTAT
CGGAGGAAATGCCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAA
GCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGG
TCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGT
AACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAG
CTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTCGGG
GACAGGATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGGTTCTAGTTGCCAGCATTCAGTTGGGCACTCTA
GAGCGACTGCCGGCGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCG
GCGACGCATGAGCGAATCCCAGAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTC
GCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG
GTGAGGTAACCGCAAGGAGCCAGCCGC

Figure 31. The 1,469 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK34.

CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACCAATAAGA
AGCTTGCTTTTTGTTGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTG
TAAGACCGGGATAACTCCGGGAAACCGGTGCTAATACCGGATAGATTATCTTTCCGCC
TGGAGAGATAAGGAAAGATGGCTATTGCCATCACTTACAGATGGGCCCGCGGCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAG
GTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCGGAGGAAATGCCGG
TACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGCGCGCAGGCGGTC
CTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACTGGGG
GACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCAC
TCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTCGGGGACAGGATGACAGGTG
GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTGGTTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGCGACTGCCGGCGA
CAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTA
CACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCGGCGACGCATGAGCGAAT
CCCAGAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGA
ATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCGCAAG
GAGCCAGCCGCCGAAGGTGGG

Figure 32. The 1,462 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK52.

CCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACCAAT
AAGAAGCTTGCTTTTTGTTGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG
CCTGTAAGACCGGGATAACTCCGGGAAACCGGTGCTAATACCGGATAGATTATCTTTC
CGCCTGGAGAGATAAGGAAAGATGGCTTTTGCCATCACTTACAGATGGGCCCGCGGCG
CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAG
AAGGTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCGGAGGAAATGC
CGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGCGCGCAGGCG
GTCCTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACTG
GGGGACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTGAG
GCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAG
CACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CTTGACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTCGGGGACAGGATGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGGTTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGCGACTGCCGG
CGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
CTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCGGCGACGCATGAGCG
AATCCCAGAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCC
GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCGCA
AG

Figure 33. The 1,446 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK60.

ATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACCA
ATAAGAAGCTTGCTTTTTGTTGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC
TGCCTGTAAGACCGGGATAACTCCGGGAAACCGGTGCTAATACCGGATAGATTATCTT
TCCGCCTGGAGAGATAAGGAAAGATGGCTTTTGCCATCACTTACAGATGGGCCCGCGG
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTG
AGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGA
AGAAGGTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCGGAGGAAAT
GCCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGCGCGCAGG
CGGTCCTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAAC
TGGGGGACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTG
AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTA
AGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTCGGGGACAGGATGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTGGTTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGCGACTGCC
GGCGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG
GGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCGGCGACGCATGAG
CGAATCCCAGAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAG
CCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCG
CAAGGAGCCAGCCGC

Figure 34. The 1,459 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK65.

GTGCCTAATACATGCAAGTCGAGCGAACCAATAAGAAGCTTGCTTTTTGTTGGTTAGC
GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACCGGGATAACTCCGGG
AAACCGGTGCTAATACCGGATAGATTATCTTTCCGCCTGGAGAGATAAGGAAAGATGG
CTATTGCCATCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCTCT
GTTGTTAGGGAAGAACAAGTATCGGAGGAAATGCCGGTACCTTGACGGTACCTGACG
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGATTTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAA
TCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAG
GAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGT
GGCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAG
GGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGG
TCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTCCTGACCGCCC
TGGAGACAGGGTCTTCCCTTCGGGGACAGGATGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGGTTCTAGTTG
CCAGCATTCAGTTGGGCACTCTAGAGCGACTGCCGGCGACAAGTCGGAGGAAGGTGG
GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGA
TGGTACAAAGGGCAGCGAAGCGGCGACGCATGAGCGAATCCCAGAAAACCATTCTCA
GTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACG
AGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCGCAAGGAGCCAGCCGCCGAAGGT
GGG

Figure 35. The 1,441 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK66.

TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACCAATA
AGAAGCTTGCTTTTTGTTGGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC
CTGTAAGACCGGGATAACTCCGGGAAACCGGTGCTAATACCGGATAGATTATCTTTCC
GCCTGGAGAGATAAGGAAAGATGGCTATTGCCATCACTTACAGATGGGCCCGCGGCG
CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAG
AAGGTCTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTATCGGAGGAAATGC
CGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGCGCGCAGGCG
GTCCTTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACTG
GGGGACTTGAGTGCAGAAGAGGAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTA
GAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTGAG
GCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAG
CACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CTTGACATCTCCTGACCGCCCTGGAGACAGGGTCTTCCCTTCGGGGACAGGATGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGGTTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGCGACTGCCGG
CGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
CTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAAGCGGCGACGCATGAGCG
AATCCCAGAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCC
GGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCGCA
AG

Figure 36. The 1,444 bp nucleotide sequence of 16S rRNA gene from *Bacillus thermoamylovorans* BHK67.

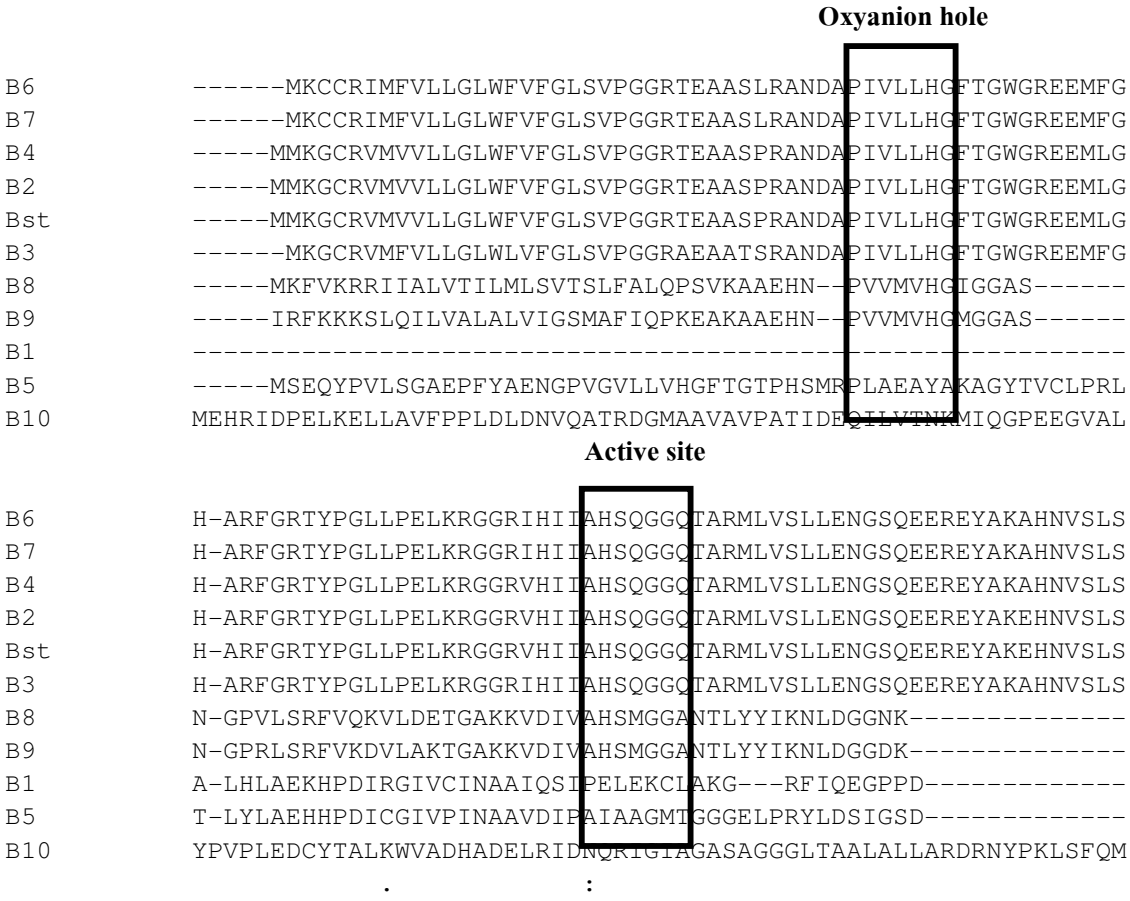

Figure 37. Multiple protein sequence alignment of bacterial lipases. Two conserved regions including oxyanion hole and active site were selected to design degenerate primers. Amino acid sequences were obtained from the following references: B1; *Bacillus coagulans*, B2; *Geobacillus stearothermophilus,* B3; *Geobacillus thermoleovorans*, B4; *Geobacillus thermocatenulatus*, B5; *Bacillus* sp. H-257, B6; *Bacillus* sp. L2, B7; *Bacillus* sp. 42, B8; *Bacillus subtilis*, B9; *Bacillus pumilus,* B10; *Bacillus* sp. B14905, and Bst; *Bacillus stearothermophilus*.

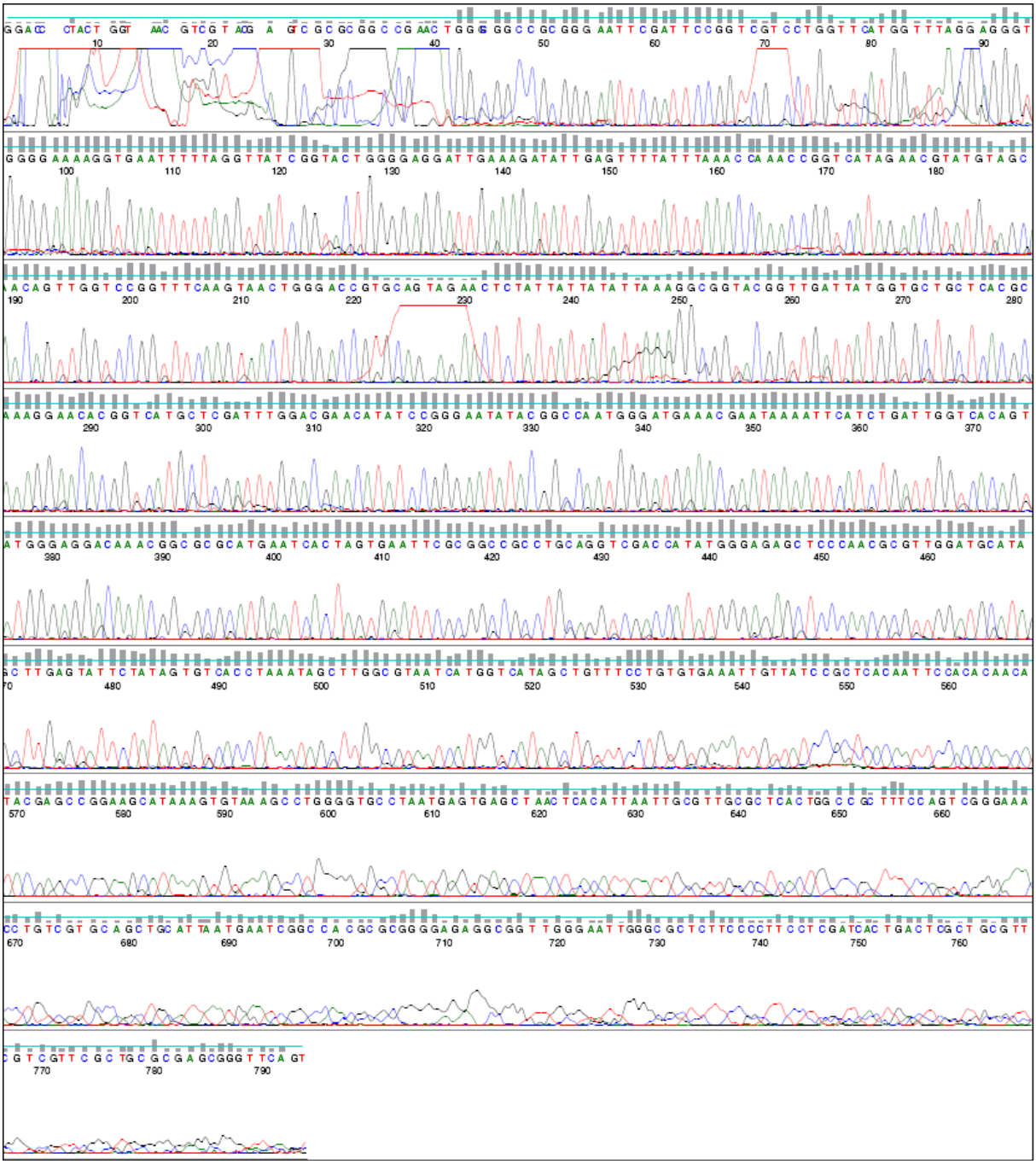

Figure 38. Nucleotide sequence analysis of amplified PCR product when degenerate primers (Loxy1, LipCR) were used in PCR reaction. The amplified DNA fragment was directly cloned into pGEM-T Easy cloning vector and pBluescript II SK (+) vector, respectively. The nucleotide sequence analysis was performed by FinchTV v.1.3.1 program.

```
 Score =  350 bits (897),  Expect = 1e-94
 Identities = 192/413 (46%), Positives = 259/413 (62%), Gaps = 16/413 (3%)
 Frame = +1

Query  1     VFLFSVFAVSLSIQPEQQVRAETSGNDYPIVLVHglggwgkgeflgYRYWGGLK-DIEFY  177
             V L   F   LS+   +   A    ND PIVL+HG  GWG+ E  G++YWGG++ DIE +
Sbjct  10    VLLGLCFVFGLSVPGGRTEAASLRANDAPIVLLHGFTGWGREEMFGFKYWGGVRGDIEQW  69

Query  178   LNQTGHRTYVATVGPVSSNWDRAVELYYYIKGGTVDYGAAHAKEHGHARFGRTYPGIYGQ  357
             LN  G+RTY   VGP+SSNWDRA E Y  + GGTVDYGAAHA +HGHARFGRTYPG+  +
Sbjct  70    LNDNGYRTYTLAVGPLSSNWDRACEAYAQLVGGTVDYGAAHAAKHGHARFGRTYPGLLPE  129

Query  358   WDETNKIHLIGHSMGGQTSRMLVELLKSGSQKEQEYYSQHPEEGISPLFTGGKNWVHSVT  537
                  +IH+I HS GGQT+RMLV LL++GSQ+E+EY   H    +SPLF GG ++V SVT
Sbjct  130   LKRGGRIHIIAHSQGGQTARMLVSLLENGSQEEREYYAKAH-NVSLSPLFEGGHHFVLSVT  188

Query  538   SLATPHNGSTFADQ----EQIVSFIKDFIIHLASAAGQKQESLIYDFKLDQWGLKRQPGE  705
             ++ATPH+G+T  +     ++     K  +   A A+     S +YDFKLDQWGL+RQPGE
Sbjct  189   TIATPHDGTTLVNMVDFTDRFFDLQKAVLEAAAVASNVPYTSQVYDFKLDQWGLRRQPGE  248

Query  706   SFHAYMNRVMTSPIWQSNDISAYDLTTFGAQELNQWMKTYPDVYYLSYTGNASYRGVVTG  885
             SF  Y  R+  SP+W S D + YDL+  GA++LNQW++  P+ YYLS++   +YRG +TG
Sbjct  249   SFDHYFERLKRSPVWTSTDTARYDLSVSGAEKLNQWVQASPNTYYLSFSTERTYRGALTG  308

Query  886   NYYPIGTMHPLFTLISMQ-MGSYTRQSPAPVIDRSWLPNDGIVNVVSAKYPFGHPNS---  1053
             N+YP   M+    ++    +GSY  ++P   ID  WL NDGIVN +S   P    N
Sbjct  309   NHYPELGMNAFSAVVCAPFLGSY--RNPTLGIDSHWLENDGIVNTISMNGPKRGSNDRIV  366

Query  1054  PYDGAIKQGVWNSFPVMEGWDHMDFINFIGSNTPGYFSIYGYYNDVANRVHSL  1212
             PYDG +K+GVWN      G  ++D +  IG +    F I  +Y  +A ++ SL
Sbjct  367   PYDGTLKKGVWNDM----GTYNVDHLEIIGVDPNPSFDIRAFYLRLAEQLASL  415
```

Figure 39. Aligment result of the lipase Lip52 from *Bacillus thermoamylovorans* BHK52 (Query) and the lipase from *Geobacillus stearothermophilus* (AAF40217.1) (Sbjct) showed 62% homology.

VITAE

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Scholarship Awards during Enrolment

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- The Japan Student Services Organization (JASSO) Scholarship

List of Publication and Proceeding

Proceeding

Thumarat, U., Kawai, F., Harnpicharnchai, P. and Upaichit A. 2008. Screening of lipases and cloning a lipase-coding gene from thermotolerant *Bacillus thermoamylovorans* strain BHK52 isolated from compost. 9th National Grad Research Conference. 14-15 March 2008, Chonburi,Thailand.