SCREENING, CHARACTERIZING, GENE CLONING AND NUCLEOTIDE ANALYZING A NOVEL EXTRACELLULAR LIPASE FROM THERMOTOLERANT *BACILLUS* ISOLATED FROM RANONG HOT SPRING

A THESIS

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TABLE OF CONTENTS

Chapter Page

TABLE OF CONTENTS (continued)

Chapter Page

TABLE OF CONTENTS (continued)

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Many microorganisms such as bacteria, yeast, and fungi are known to secrete lipases during their growth on hydrophobic substrates, which renders the lipid substrates available to the cells. In recent years, there has been great interest in lipase-producing-microorganisms. Lipases act at the interface and catalyse hydrolysis of fats, mono- , di-glycerides and tri-glycerides to free fatty acid and glycerol. The advantages of lipase-catalysed hydrolysis over the conventional high-temperature and high-pressure-steam splitting methods are that the product obtained is higher quality and low energy consumption (1-4). The main disadvantage of lipase hydrolysis processes is the cost of the enzyme.

Lipases (EC 3.1.1.3) catalyse both the hydrolysis and the synthesis of ester form from glycerol and long-chain fatty acids (Figure 1). These reactions usually proceed with high regioselectivity and/or enantioselectivity and make lipases as an important group of biocatalysts in organic chemistry. The reasons for the enormous biotechnological potential of microbial lipases are as follow; (I) their stability in organic solvents, (II) no need cofactors, (III) possess a broad substrate specificity and (IV) exhibition of a high enantioselectivity. A number of lipases have been produced commercially, with the majority of their origin from fungi and bacteria. A recent publication of commercially available triacylglycerol lipases is from 34 different sources including 18 from fungi and 7 from bacteria (5). The steadily growing interest in microbial lipases is reflected by an increasing number of excellent monographs and review articles covering the molecular biology, biochemical properties and, in particular, the biotechnological applications of these enzymes (3, 6-12).

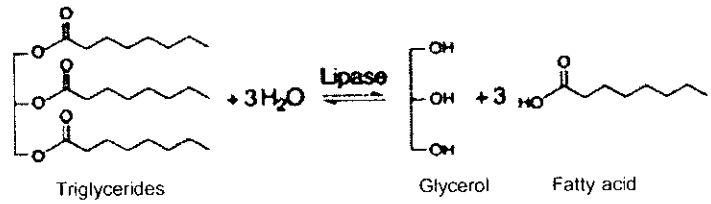

FIGURE 1 THE CATALYTIC ACTION OF LIPASES. A TRIGLYCERIDE CAN BE HYDROLYSED TO FORM GLYCEROL AND FATTY ACIDS, OR THE REVERSE (SYNTHESIS) REACTION CAN COMBINE GLYCEROL AND FATTY ACIDS TO FORM THE TRIGLYCERIDE.

Temperature range for microbial growth

Microorganisms can be placed in one of tree classes based on their temperature ranges for growth.

1. Phychrophiles grow well at 0°C and have an optimum growth temperature of 15°C or lower. The maximum is around 20°C.

2. Mesophiles are microorganisms with optimum growth temperature between 20 and 45°C. Their maximum is about 45°C or lower. Almost all human pathogens is a fairly constant temperature growth at 37°C.

3. Thermophiles are microorganisms that can grow at temperatures of 55°C or higher. Their growth minimum is usually around 45°C and they often have optima between 55 and 65°C. (13).

The genus *Bacillus* (Aerobic spore-forming bacteria)

The genus *Bacillus* is a large and hetrerogeneous collection of aerobic or facultative anaerobic, rod shape endospore-forming bacteria that are widely distributed in the environment. Many kinds of species belong to this genus which have acidophilic, alkalophilic and also exhibit great variation in temperatures of growth. Some thermophiles grow from a minimum temperature of 45°C to maximum temperature of 75°C or higher (14). Most species are harmless saprophytes occurring in soil, fresh water, or sea water. Many form extracellular enzymes that hydrolyze proteins or complex polysaccharides (15).

Bacillus sp. are capable of forming endospores. These are dormant form of the bacterium that allows it to survive sub-optimal environmental conditions. Spores have a tough outer covering made of keratin and are highly resistant to heat and chemicals. Endospores may be located in the middle of the cells (central), at the end (terminal), or between the end and the middle of the cells (subterminal). The endospores themselves may be round or oval.

Applications of lipases as hydrolases

Detergents

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. Enzyme sales in 1995 have been estimated to be US$30 million, with

detergent enzymes making up 30%(9). An estimated 1000 tons of lipases are added to the approximately 13 billion tons of detergents produced each year. In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase™, which originated from the fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced – Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *P. alcaligenes*, both produced by Genencor International. The challenges of which producers of detergent lipases need to meet include: (1) the high variation in the triglyceride content of fat stains, requiring lipases with low substrate specificity; (2) the relatively harsh washing conditions (pH 10–11 and 30–60^{o}C), requiring stable enzymes; and (3) the effects of chemical denaturation and/or proteolytic degradation caused by detergent additives such as the surfactant linear alkyl benzene sulfonate (LAS) and proteases. Solutions to these problems are being sought by a combination of continuous screening for improving lipases and attempting to enhance lipase properties on the basis of protein engineering (16).

Food ingredients

The position, chain length and degree of unsaturation are greatly influence not only the physical properties but also the nutritional and sensory value of a given triglyceride. Cocoa butter contains palmitic and stearic acids and has a melting point of approximately 37°C, leading to its melting in the mouth, which results in a perceived cooling sensation. In 1976, Unilever filed a patent describing a mixed hydrolysis and synthesis reaction to produce a cocoa-butter substitute using an immobilized lipase (17). This technology is now commercialized by Quest-Loders Croklaan. It is based on immobilized *Rhizomucor miehei* lipase which carries out a transesterification reaction replacing palmitic acid with stearic acid to give the desired stearic–oleic–stearic triglyceride. Polyunsaturated fatty acids (PUFAs) play an increasingly important role as biomedical and so-called 'nutraceutical' agents (18). Many of them belong to the essential fatty acids. Its uptake is required for membrane-lipid and prostaglandin synthesis. Microbial lipases are used to enrich PUFAs from animal and plant lipids such as menhaden, tuna, or borage oil. Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals including anticholesterolemics, anti-inflammatories, and thrombolytics (19).

Pulp and paper industry

Another application field of increasing importance is the use of lipases in removing the pitch from pulp produced in the paper industry. 'Pitch' is a term used to describe collectively the hydrophobic components of wood, namely triglycerides and waxes, which cause severe problems in pulp and paper manufacture (20). Nippon Paper Industries in Japan developed a pitchcontrol method that uses a fungal lipase from *C. rugosa* to hydrolyse up to 90% of the triglycerides.

Biomedical applications

Because of their excellent capability for specific regioselective reactions in a variety of organic solvents with broad substrate recognition, lipases have emerged as an important biocatalyst in biomedical applications. Recently, Parmar and his coworker (21) reviewed a variety of substrates accepted by hydrolytic enzymes, including lipases, to produce compounds in high enantiomeric excess, which can be used as chiral building blocks for the synthesis of compounds of pharmaceutical interest. There are other reports on the application of microbial lipases to the hydrolysis of racemic esters, to trans-esterification and to racemization *in situ* to yield optically pure enantiomers for the manufacture of chiral synthons (22). In addition to racemization *in situ*, lipases are also capable of catalysing synthetic reactions, which lead to the production of life-saving drugs. Efficient kinetic resolution processes are in vogue for the preparation of optically active homochiral intermediates for the synthesis of nikkomycin-B, non-steroidal anti-inflammatory drugs (naproxen, ibuprofen, suprofen and ketoprofen), the potential anti-viral agent lamivudine (which can be used against HIV) and for the enantiospecific synthesis of anti-tumour agents, alkaloids, antibiotics and vitamins (23).

Biosensors

Sensing lipids and lipid-binding proteins are a developing technology (24). The quantitative determination of triacylglycerols in the fat and oil industry, in food technology and in clinical diagnosis is of great importance (25). Chemical methods for the analysis are rather costly and time-consuming. A promising new method involves the manipulation of microbial lipases as a biosensor. Biosensors can be a chemical, biochemical, or electronic nature. Biochemical biosensors can have enzymes, antibodies, other proteins, large organs, cells or cell extracts, immobilized or linked to suitable signal producers (transducers). Besides

potentiometric (ion-selective) amperometric arrangements, transducers can involve optic fibres, piezoelectric and heat-toning systems. These can be minimized in suitable ceramic, piezo, or optical elements, which are compatible with microelectronics that permit the direct linking of signal production to the sensors (26).

Cosmetics and perfumes industry

The overwhelming interest of technocrats in screening lipases for use in the cosmetics and perfumes industry has mainly been due to its activity in surfactants and in aroma production, which are the main ingredients in cosmetics and perfumes. Monoacylglycerols and diacylglycerols, prepared by the lipase-catalysed esterification of glycerol, are useful as surfactants in cosmetics (27). Mixed-acid-type polyester and acylglycerol ester fatty acids (26) are the main components in lipase-mediated cosmetics.

Other applications

A large number of additional hydrolytic applications have been described for microbial lipases, including flavour development for dairy products (cheese, butter, margarine, alcoholic beverages, milk chocolate and sweets), achieved by selective hydrolysis of fat triglycerides to release free fatty acids. These can act as either flavours or flavour precursors. In the future, the treatment of waste by lipases looks to become very important including the breakdown of fat solids, the prevention or cleaning of fat films and the cleaning of fat-containing waste effluents.

Applications of lipases as synthetases

Lipases have been employed by organic chemists for a long time to catalyse a wide variety of chemo-, regio- and stereoselective transformations (6-8, 28-30). The majority of the lipases used as catalysts in organic chemistry are of microbial origin. One of the most exciting aspects of this fast-growing field is the possibility of enantioselective lipase catalysis on an industrial scale. There are two basic types of enantioselective organic transformations amenable to lipase catalysis: (1) the reaction of prochiral substrates (6-8, 28-30); and (2) kinetic resolution of racemaces (6-8, 28-31). Traditionally, prochiral or chiral alcohols and carboxylic-acid esters served as the two main classes of substrates but, over the years, the range of compounds has expanded rapidly to include diols, α- and β-hydroxy acids, cyanohydrins,chlorohydrins, diesters, lactones, amines, diamines, amino-alcohols, and α- and β-amino-acid derivatives (6-8,

28-31). Thus it is no exaggeration to note that the most important classes of functionalized organic compounds can, in principle, be prepared enantioselectively by lipase catalysis. Typical catalysts include lipases from the bacteria *P. aeruginosa*, *P. fluorescens* and other *Pseudomonas* species, *Burkholderia cepacia*, *Chromobacterium viscosum*, *B. subtilis*, *Achromobacter* sp., *Alcaligenes* sp., and *Serratia marcescens*, as well as from fungi such as *Candida antarctica* B and *Candida rugosa*. Despite the fact that there are the large number of publications in this area, the number of industrial enantioselective processes based on lipase catalysis is limited (9, 32, 33). Successful examples include the synthesis of chiral amines, catalysed by the lipase from *Burkholderia plantarii* (34) and the *S. marcescens* lipase-based production of (2*R*,3*S*)-3-(4-methoxyphenyl)methyl glycidate, which is used in the synthesis of the calcium antagonist Diltiazem™ (35). In general, however, problems may arise from: (1) insufficient enantioselectivity; (2) limited enzyme activity; (3) difficulties in recycling the lipase; and (4) inherent practical limitations of the kinetic resolution arising from the fact that 50% conversion is the maximum possible. Nevertheless, solutions to these problems are emerging, which means that industrial applications are more likely in the near future. In addition to the optimization of reaction conditions such as the choice of solvent, temperature, acylating agent etc., the most important current developments include: (I) *in vitro* evolution as a method to increase enantioselectivity; (II) novel immobilization techniques, resulting in the enhancement of enzyme activity and stability in organic solvents (6-8, 20, 28-29, 36); (III) recycling techniques enabling multiple reuse of lipases (36); and (IV) methods for dynamic kinetic resolution in which the complete conversion of a racemace into a single enantiopure product is possible (30). Lipase-catalysed dynamic resolution generally requires a second catalyst to induce the racemization of the enantiomer that is not accepted by the enzyme (30). Most often, transition-metal catalysts are used, which must be compatible with the lipase. An example is the production of the *N*-acylated amine as the sole nitrogen-containing product of the reaction of the racemate using the lipase from *C. antarctica* and palladium on charcoal as the two catalysts (37).

Future technologies with lipases

Enzyme crystals

In addition to sol–gel encapsulation of lipases (36, 38, 39), several other novel immobilization methods have recently been reported. One interesting new technology uses lipases in the form of cross-linked enzyme crystals (CLEC). Lipases from *C. rugosa* and *B. cepacia* are crystallized to give microcrystals approximately 50–100 mm in length, which are subsequently crosslinked by the addition of glutaraldehyde and dried, preferably in the presence of a detergent (40). These crystals have been used for the chiral resolution of commercially important organic compounds by ester hydrolysis giving high enantiomeric excesses (ee): *S*-ibuprofen (95% ee), *S*-naproxen (97% ee) and (2)-menthol (95% ee) (41). In addition, CLECs can catalyse esterification and transesterification reactions in low-water organic solvents to achieve chiral resolution of racemic alcohols and acids with ee values in the range 95–99%. CLECs produced from *P. cepacia* and *C. rugosa* lipases are tested for their potential to catalyse the chiral resolution of various acids, alcohols and amines by acylation in organic solvent. They are proved to be more active than the crude enzyme powders by a factor of between 10 and 90, determined by measuring the reaction rates in μmol min^{-1} mg (based on the weight of lipase protein) (42).

Lipid-coated enzymes

Lipase obtained from *P. fragi* has been coated with a lipid monolayer at a ratio of about 150 lipid molecules per enzyme molecule. This lipase catalysed the esterification of racemic (*R,S*)-1-phenylethanol and lauric acid in isooctane with high enantioselectivity and conversion rate (43). Lipid coating seems to render at least some lipases homogeneously soluble and stable in organic solvents (benzene, ethyl acetate, isooctane, isopropyl ether, dimethyl sulfoxide and ethanol). The enzymatic activity of *P. fragi* lipase coated with different glycolipids, zwitterionic, anionic and cationic lipids is measured as the initial rate of ester synthesis. Lipases coated with didodecyl *N*-D-glucono-L-glutamate and didocyl-*N*-D-glucono-D-glutamate show the highest enantioselectivity at enzymatic activities of 50 and 58 μ$Msec^{-1}$(mg protein) towards the preferred (*R*)-enantiomer of 1-phenylethanol (44).

TABLE 1 SOME IMPORTANT APPLICATIONS OF MICROBIAL LIPASES IN BIOTECHNOLOGICAL PROCESSES AND PRODUCTS (26).

Source	Biomedical application	Pesticide	Waste management
Acinetobacter calcoaceticus	-	-	Heating oil/furnace oil
Arthrobacter spp.	-	Pyrethroids	-
Aspergillus spp.	Prostaglandins	-	-
Aspergillus oryzae	-	-	Waste hair
Bacillus subtilis	Cephalosporin	-	-
Candida spp.	Pyrolidinedione derivatives	-	-
Candida cylindracea	Racemic naproxen	-	-
Chromobacterium viscosum	VitaminD, Verlukast	-	-
Rhizopus miehei	Ketoprofen	Geraniol	-
Penicillium urticae	Patuloide A	-	-
Pseudomonas spp.	Pyrolidinedione derivatives, (-) - Indolmycin	Triazole/morpholine	Petroleum-contaminated soil, poisonous gas
Pseudomonas cepacia	Rapamycin-42, Nikkomycin-B	Pyrenophorin, fenpropimorph, racemic vinylglycine, racemic, morpholine, cyanohydrin acetate, pyrethroids, dicyclopentadiene	-
Pseudomonas fluorescens	Hydantoins, Lamivudine(3TC), racemic 2-tetradecyloxirane-carboxylate	Tetraconazole	-
Rhizopus delemer	Aryglyceral derivatives	-	-
Rhizopus oryzae	-	-	Palm oil mill waste
Streptomyces spp.	Penicillins	-	-

CHAPTER 2

LITERATURE REVIEW

Many new bacterial lipolytic enzymes have been studied since the publication of a comprehensive review article in 1994 (45). However, no attempt has been undertaken to organize this information. Some biochemical properties (such as the dependence of activity on Ca^{2+} ions, pH and temperature) of the best studied families of lipases (from the genera *Bacillus*, *Pseudomonas* and *Staphylococcus*) have been summarized previously(11, 12, 46, 47). In 1999 Jarger and his coworkers (48) classified 53 sequences of bacterial lipases and esterases which are compared and classified according to conserved sequence motifs and the biological properties of these enzymes. Relevant information obtained from the 3D structures is also highlighted when available. This work presents an overview of bacterial lipases and esterases currently known and permits the classification of newly isolated lipolytic enzymes, there by giving a hint about their general characteristics as a starting point to their investigation (Table 2). Amino acid sequence similarities are determined with the program MEGALIGN (DNASTAR), with the first member of each family (subfamily) arbitrary set at 100%. In 2002, Jarger and his coworkers (49) were also classified 47 representative bacterial lipases into seven families (families I-VII) and further classified family I into seven subfamilies (subfamilies I.1-I.7, Table 3). Within family I, *Bacillus* lipase belonged to either subfamily I.4 or subfamily I.5 based on sequence homology and protein molecular size, with most thermostable lipases from thermophilic *Bacillus* sp. belong to subfamily I.5. These *Bacillus* lipases are relative large in molecular size and have high temperature optima.

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) catalyze the hydrolysis of water-insoluble esters and triglycerides at the interface between the insoluble substrate and water. Apart from their natural substrates, lipases catalyze the enantio- and regioselective hydrolysis and synthesis of a broad range of natural and non-natural esters.

Due to their extracellular nature, most microbial lipases can be produced in large quantities and are quite stable under non-natural conditions such as high temperatures and nonaqueous organic solvents employed in many applications. Their stability, inexpensive manufacturing, and broad synthetic potential make microbial lipases ideal biocatalysts for

oleochemistry and organic synthesis. At present, the applications of lipases in oleochemistry, in detergents, in the paper and food industry, and in organic synthesis, have recently been reviewed (50-52). The molecular biology and biochemical properties of microbial lipases have also been summarized (12). Many microbial lipase genes have been cloned and characterized over the past few years, including important lipases such as *Candida rugosa* (formerly *Candida cylindracea*), *Candida antarctica*, *Tidestromia lanuginosa* (formerly *Humicola*), *Rhizopus miehei*, *Rhizopus delemar*, *Geotrichum candidum*, *Burkholderia cepacia* (formerly *Pseudomonas*), *Pseudomonas pseudoalcaligenes*, *Pseudomonas mendocina* (originally *Pseudomonas putida*), *Bacillus subtilis*, *Bacillus thermocatenulatus and Burkholderia glumae*.

Classification and three-dimensional structure of lipases

The three-dimensional (3D) structures of the fungal lipase from *Rhizomucor miehei* and the human pancreatic lipase were determined in 1990 (53, 54). Since then, eleven more lipase structures from microbial origin have been solved with the exception of pancreatic lipase (55). These enzymes, which span a molecular weight range of 19 to 60 kDa, exhibit a characteristic folding pattern known as the α/β-hydrolase fold (56). The lipase core is composed of a central β sheet consisting of up to eight different β strands (β1–β8) connected by up to six α helices (A–F). The active site is formed by a catalytic triad consisting of the amino acids serine, aspartic (or glutamic) acid and histidine. The nucleophilic Ser residue is located at the C-terminal end of strand β5 in a highly conserved pentapeptide GXSXG (Table 4), forming a characteristic β–turn–α motif named the 'nucleophilic elbow'. Substrate hydrolysis starts with a nucleophilic attacked by the catalytic-site-Ser oxygen on the carbonyl carbon atom of the ester bond. Thus leads to the formation of a tetrahedral intermediate stabilized by hydrogen bonding to nitrogen atoms of main-chain residues that belong to the socalled 'oxyanion hole' (Table 4). An alcohol is liberated, leaving behind an acyl–lipase complex, which is finally hydrolysed with liberation of the fatty acid and regeneration of the enzyme. Lipolytic reactions occur at the lipid–water interface. This implies that the kinetics cannot be described by Michaelis–Menten equations because these are valid only if the catalytic reaction takes place in one homogenous phase. Lipolytic substrates usually form an equilibrium between monomeric, micellar and

emulsified states, resulting in the need for a suitable model system to study lipase kinetics. The monolayer technique (57) has been used extensively and, more recently, an oil-drop technology has been put forward in which lipase kinetics are monitored by automatic analysis of the profile of an oil drop hanging in water. The decrease in the interfacial tension between the oil and water caused by lipase hydrolysis is measured as a function of time (58). The best-known phenomenon emerging from early kinetic studies of lipolytic reactions are known as 'interfacial activation'. It is the activity of lipases enhanced towards insoluble substrates that form an emulsion. Lipases, in contrast to esterases, are therefore defined as carboxylesterases acting on emulsified substrates. The determination of their 3D structures seems to provide an elegant explanation for interfacial activation. The active site of lipases is found to be covered by a surface loop, which is called the lid (or flap). Upon binding to the interface, this lid moves away and turns the 'closed' form of the enzyme into an 'open' form with the active site which now can accessible to the solvent. At the same time, a large hydrophobic surface is exposed to facilitate binding of the lipase to the interface (59). More recently, it turns out that the presence of a lid-like structure is not necessarily correlated with interfacial activation. Lipases from *P. aeruginosa*, *B. glumae* and *C. antarctica* B, and a coypu pancreatic lipase do not show interfacial activation but nevertheless have an amphiphilic lid covering their active sites. This observation lead the conclusion that the presence of a lid domain and interfacial activation are unsuitable criteria to classify an enzyme as a lipase. Therefore, the current definition of lipase is that it is a carboxylesterase that catalyses the hydrolysis of long-chain acylglycerols (60).

TABLE 2 FAMILIES OF LIPOLYTIC ENZYMES (48).

Family	Subfamily	Enzyme-producing strain	Acession	Similarity (%) Family	Subfamily	Properties
I	1	*Pseudomonas aeruginosa**	D50587	100		True lipase
		Pseudomonas fluorescens	AF031226	95		
		Vibrio cholerae	X16945	57		
		Acinetobacter calcoaceticus	X80800	43		
		Pseudomomas fragi	X14033	40		
		Pseudomonas wisconsinensis	U88907	39		
		Proteus vulgaris	U33845	38		
	2	*Burkholderia glumea**	X70354	35	100	
		*Chromobacterium viscosum**	Q05489	35	100	
		*Burkholderia cepacia**	M58494	33	78	
		Pseudomonas luteola	AF050153	33	77	
	3	*Pseudomonsa fluorescens* SIK	D11455	14	100	
		Serratia marcescens	D13253	15	51	
	4	*Bacillus subtilis*	M74010	16	100	
		Bacillus purqilus	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	
II		*Aeromonas hydrophila*	P10480	100		Secreted
		*Streptomyces scabies**	M57297	36		Secreted esterase
		Pseudomas aeroginosa	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 esterase1
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 immobilus	X67712	34		Extracellular esterase
		Moraxella sp.	X53869	34		Extracellular esterase 3

TABLE 2 (CONTINUED)

Family	Subfamily	Enzyme-producing strain	Accession	Similarity (%) Family	Similarity (%) Subfamily	Properties
VI		*Synechocytis* sp.	D90904	100		Esterace
		Spirulina paltensis	S70419	50		Carboxylesterase
		*Pseudomonas fluorescens**	S79600	24		
		Rickettsia prowazekii	Y11778	20		
		Chlamydia trachomatis	AE001287	16		
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	AAC60471	10		Esterase III

* Lipolytic enzymes with known 3D structure.

TABLE 3 UPDATED CLASSIFICATION OF BACTERIAL LIPOLYTIC ENZYMES CONSTITUTING FAMILY I (50).

Subfamily	Enzyme-producing strain	Acession no.	Similarity (%)	
			Family	Subfamily
1	*Pseudomonas aeruginosa (LipA)**	D50587	100	
	Pseudomonas fluorescens	AF031226	95	
	Vibrio cholerae	X16945	57	
	Acinetobacter calcoaceticus	X80800	43	
	Pseudomomas fragi	X14033	40	
	Pseudomonas wisconsinensis	U88907	39	
	Proteus vulgaris	U33845	38	
2	*Burkholderia glumea**	X70354	35	100
	*Chromobacterium viscosum**	Q05489	35	100
	*Burkholderia cepacia**	M58494	33	78
	Pseudomonas luteola	AF050153	33	77
3	*Pseudomonsa fluorescens* SIK W1	D11455	14	100
	Serratia marcescens	D13253	15	51
4	*Bacillus subtilis* (LipA)*	M74010	16	100
	Bacillus pumilus	A34992	13	80
	Bacillus licheniformis	U35855	13	80
	Bacillus subtilis (LipB)	C69652	17	74
5	*Geobacillus stearothermophilus* L1	U78785	15	100
	Geobacillus thermocatenulatus P1	X95309	15	94
	Geobacillus thermocatenulatus	X95309	14	94
	Geobacillus thermoleorans	X02844	14	92
6	*Staphylococcus hyicus*	M12715	14	100
	Staphylococcus aureus	AF090142	15	45
	Staphylococcus epidermidis	X02844	13	44
	Staphylococcus xylosus	AF208229	15	36
	Staphylococcus warneri	AF208033	14	36
7	*Propionibacterium acnes*	X99255	14	100
	Streptomyces cinnamoneus	U80063	14	50

* Lipolytic enzymes with known 3D structure.

TABLE 4 SEQUENCES FOUND AT THE OXYANION HOLE AND ACTIVE SITE OF SELECTED LIPASES (61).

Lipase origin	Accession no.	Oxyanion region	Active site
Group 1			
Staphylococcus aureus	A24545	PVVFVHG	GHSMGG
Staphylococcus aureus	NP_375790	PIVLVHG	GHSMGG
Staphylococcus epidermis	A47705	PIILVHG	GHSMGG
Staphylococcus epidermis	AAC67547	PVVFVHG	GHSMGG
Staphylococcus hyicus	A24075	PFVFVHG	GHSMGG
Staphylococcus haemolyticus	AAF21294	PVVLVHG	GHSMGG
Archaeoglobus fulgidus	B69470	PVVFVHG	GHSMGT
Mycoplasma pneumoniae	NP_110161	NFIFLHG	GHSMGG
Mycoplasma mycoides	AAA95966	NIIFCHG	GHSMGG
Mycoplasma genitalium	A64238	TVVFAHG	GHSMGG
Ureaplasma urealyticum	NP_077851	SIVFIHG	GHSLGA
Mus musculus	NP_035974	LIFVSHG	GHSMGG
Saccharomyces cerevisiae	NP_010343	PIVFCHG	AHSMGG
Bacillus pumilus	CAA02196	PVVMVHG	AHSMGG
Bacillus subtilis	NP_388152	PVVMVHG	AHSMGG
Bacillus licheniformis	CAB95850	PVVMVHG	AHSMGG
Geobacillus sp. TP10A	AAF63229	PIVLLHG	AHSQGG
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 wisconsinesis'	AAB53647	PIVLVHG	GHSQGS
Proteus vulgaris	AAB01071	PIVLVHG	GHSQGP
Pseudomonas aeruginosa	NP_253500	PIVLSHG	GHSQGG
Bos taurus	JC4017	VVFLQHG	GHSQGT
Homo sapiens	NP_004181	VVFLQHG	GHSQGT
Vibrio cholerae	NP_232620	PIVLVHG	GHSHGG
Pseudomonas aeruginosa	NP_251552	PIVLAHG	GHSHGG
Acinetobacter calcoaceticus	S61927	PIVLSHG	GHSHGS

Table 4 (CONTINUED)

Lipase origin	Accession no.	Oxyanion region	Active site
Group 2			
Yarrowia lipolytica	Q99156	VFVWIHG	GESAGS
Moraxella sp.	A39556	AMLFFHG	GDSAGG
Pseudomonas sp. B11-1	AAG47649	LLVFFHG	GDSAGG
Mycobacterium tuberculosis	NP_215915	VVVYYHG	GDSAGG
Mycobacterium tuberculosis	NP_217486	LLVFYHG	GDSAGG
Mycobacterium tuberculosis	NP_215916	VVLYFHG	GDSAGG
Rattus norvegicus	LIRTH	LVVHIHG	GDSAGG
Clostridium perfringens	BAA81642	ILMWIHG	GDSAGA
Group 3			
Serratia marcescens	AAA81002	IGISFRG	GHSLGG
Pseudomonas brassicacearum	AAF87594	IGVSFRG	GHSLGG
Thermomyces lanuginosus	O59952	IVLSFRG	GHSLGG
Fusarium heterosporum	JX0343	IVVSVRG	GHSLGG
Rhizopus niveus	BAA31548	IYLVFRG	GHSLGG
Rhizomucor miehei	A34959	IYIVFRG	GHSLGG
Aspergillus oryzae	BAA12912	IVVAFRG	GHSLGA
Penicillium camemberti	S32403	VVLAFRG	GHSLGA
Miscellaneous			
Moraxella sp.	S14276	PLLLIHG	GNSMGG
Psychrobacter immobilis	S57275	PLLLIHG	GNSMGG
Saccharomyces cerevisiae	NP_012782	VVYLHHG	GFSQGS
Homo sapiens	A28997	LVMIIHG	GYSLGA
Mycoplasma mycoides	JC4110	NIIYIHG	GKSMGG
Propionibacterium acnes	CAA67627	PVILIPG	GHSQGG
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
Sulfolobus acidocaldarius	AAC67392	PLIMIMG	GWSMGG
Candida rugosa	JN0553	VMLWIFG	GESAGS
Galactomyces geotrichum	PN0493	VMVWIYG	GESAGA

Rhizomucor family – lipase from *Rhizopus oryzae* (ROL)

Over the last decade, more than 30 lipases were isolated from *Rhizopus* strains and many of them characterized (62). Crude enzyme preparation of non-recombinant *Rhizopus* lipases are commercially provided by several suppliers, e.g. Amano N, D and L lipases derived from *R. niveus* (RNL), *R. delemar* (RDL), and *R. javanicus* (RJL). *Rhizopus* lipases, as well as the homologous lipase from *R. miehei* (>55% homology), have been used in a wide range of technical applications. Their high 1,3-regiospecificity make them particularly versatile enzymes in lipid modifications. At present, the lipase genes from *R. oryzae* (ROL) DSM 853 (63), *R. niveus* IFO 9759 (65) and *R. delemar* ATCC34612 (64) have been cloned. All known sequences of lipases from *Rhizopus* species (e.g. *R. delemar* (20, 64), *R. nivenus* (19, 65) and *R. javanicus* (29, 67) have an identical amino acid sequence. In contrast, the lipase from *R. oryzae* contains two different amino acids (His134Asn and Ile234Leu). *R. nivenus* secretes a lipase that possesses the last 28 amino acids of the propeptide at its N-terminus and is converted to the smaller lipase form via limited proteolysis catalysed by a specific extracellular protease (30, 70).

Pseudomonas family -lipase from *Burkholderia cepacia* (PCL)

Lipases from *Pseudomonas* represent probably the largest group of microbial lipases at present, considering the quantity of publications on isolation, purification and cloning of various *Pseudomonas* lipases. Besides their application in laundry detergents, e.g. lipase from *P. pseudoalcaligenes* (52) which are widely used by organic chemists in enantioselective synthesis (8, 68) and is reflected by the various *Pseudomonas* lipase preparations commercially available (e.g. Amano YS, P, AH; Fluka SAM-II). *Pseudomonas* lipases can be divided into three homology groups (assigned as classes I–III), of which class III is only distantly related to the other classes. Lipases of classes I+II, including lipases of *B. cepacia*, *B. glumae* (class II) and *P. aeruginosa* (class I), need a chaperone located downstream of the lipase gene for efficient secretion and folding of active lipase.

Candida family-lipases from *Geotrichum candidum* (GCL)

G. candidum CMICC 335426 secretes two homologous lipase isoforms (84% homology) named A and B with significant different substrate specificity (69-72). Although

both lipases prefer long-chain fatty acids as substrates, lipase A is less specific than lipase B which is highly specific cis-9 unsaturated fatty acids. Therefore lipase B is more particular interest for application in lipid modification. Both lipases are found in the supernatant of *G. candidum* and their close physical and biochemical properties make purification difficult (73, 74). Cloning and separate expression of both isoforms is, therefore, the most reliable way to obtain pure isoforms. Functional production, albeit at very low level, in *S. cerevisiae* has been reported for lipases I (97% homology to lipase B) and II of *G. candidum* ATCC 34614 (75, 76).

Candida rugosa lipase (CRL)

Crude CRL preparations are among the commercial lipases most often employed in the hydrolysis and synthesis of a wide range of esters of commercial interest (8, 52). However, as in the case of the lipases from *G. candidum*, application of crude lipase preparation of *C. rugosa* suffers from the secretion of several isoforms by the yeast. Until now, 7 different lipase genes with an average homology of 66% have been cloned(77, 78). Efforts to purify different lipase forms from commercial enzyme preparation identified the gene product of LIP1 as the major constituent and also demonstrated that the isoforms differ in their catalytic properties (79).

Bacillus family-lipase

Bacillus species are among those microorganisms capable to promote lipid conversion by means of their lipolytic systems. Several lipases from *B. subtilis* (80), *B. pumilus* , *B. licheniformis* (81), *B. megaterium* (82), *B. thermocatenulatus* (83), *B. thermoleovorans* (84), and *B. stearothermophilus* (85) have already been described, cloned or purified. Among them, the small secreted *B. subtilis* lipases LipA (84) and LipB (86) have been shown to be well suited for biotechnological applications (48).

Bacterial lipases and esterases have been grouped into eight families based on conserved sequence motifs and biological properties (49), where the small (19 kDa) *B. subtilis* LipA (80) and LipB (86) were grouped into subfamily I.4 of bacterial true lipases (EC 3·1·1·3), sharing 75% identity with lipases from the moderately mesophilic *B. pumilus* (87)

and *B. licheniformis* (49), and 98% identity to *B. megaterium* LipA (82), all of them belonging to the same subfamily (Table 5).

TABLE 5 BIOCHEMICAL PROPERTIES OF *BACILLUS* LIPASES (88).

Bacillus isolate	M_r[a] (kDa)	pI[a]	Optimum pH	Temperature stability	Reference
Subfamily I.4					
B. licheniformis[b]	19.2	9.46	10-11.5	<25%	(81)
B. subtilis	19.3	9.25	10	<25%	(88)
B. pumilus	19.3	9.43	9.5-10	<25%	(90)
Subfamily I.5					
B. thermocatenulatus	43.2	6.37	8.5	>90%	(88)
B. thermoleovorans	43.1	6.19	7.5	>90%	(89)
B. stearothermophilus	43.2	6.15	9.5	>90%	(91)

[a] The experimental values have been overlooked in favour of theoretical M_r and pI deduced by the compute pI/MW software programme (16, 17, 18, 92-94).

[b] Reflects properties of the recombinant lipase.

[c] Represents residual activity obtained after incubation of the lipase protein at 50°C for 30 min.

Alkaliphilic and thermophilic *Bacillus* have been found to produce lipolytic enzymes. Lipases from *B. subtilis* (95) and *B. pumilus* (87) have been of particular interest as they exhibit optimal activity and stability at extreme alkaline pH values (pH values above 9.5). These enzymes, however, are thermolabile. This is in contrast to lipases from *B. thermocatenulatus* (87), *B. thermoleovorans* (88), and *B. stearothermophilus* (89), which are thermotolerant, and display maximal activity and stability at moderate alkaline pH values (pH 7-9.5). The genes encoding these *Bacillus* lipases and their deduced amino acid revealed that they lack of the characteristic Gly-X-Ser-X-Gly motif centered on the lipase active serine residue.

Staphylococcus family-lipase

Lipases from *B. thermocatenulatus*, *B. stearothermophilus*, *B. thermoleovorans* be classified as subfamily I.5 (88), represents a group of large prokaryotic lipases with molecular weights of 40-45 kDa. Homologous lipase genes (70% homology) have been cloned and functionally expressed from different *Staphylococcus* strains (95,96). Recent investigations of the physicochemical properties of *Staphylococcus* lipases revealed that lipases from *S. aureus* and *S. epidermidis* are closely related: both lipases prefer short-chain triglycerides. The lipase from *S. hyicus*, on the other hand, prefers phospholipids as substrate (95) . A three-dimensional structure, however, has not been solved yet. To clone a thermophilic lipase resulted in the isolation of a 43 kDa lipase named BTL-2 from an expression library generated from genomic DNA from the thermophile *B. thermocatenulatus.* No sequence homology was found with other microbial lipases including those from other *Bacillus* strains, but instead BTL-2 shows a significant homology of 30-35% with the different *Staphylococcus* lipases. A first brief characterization of BTL-2, expressed at low level in *E. coli,* revealed its high activity at temperatures between 50 and 75°C and alkaline conditions. Lipase BTL-2 shares with lipases from *S. eperdimidis* and *S. aureus* the same preference for short chain triglycerides as substrate. However, activation by calcium as reported for the *Staphylococcus* lipases was not observed. Although *Staphylococcus* pro-lipases are cleaved to yield mature proteins with predicted molecular masses similar to that of lipases identified in *B. thermocatenulatus*, *B. stearothermophilus* and *B. thermoleovorans*, the amino acid sequence identity is less than 30% , confirming the lack of close evolutionary relatedness.

The interest in bacterial lipases has increased due to the fact that they are more stable than those from other organisms, especially when exposed to high temperature or other severe condition. Thermotolerant *Bacillus* spp. are among those micro-organisms capable to promote lipid conversion by means of their lipolytic systems and high stability under such condition. The increasing interest for thermotolerant bacterial lipases and new thermotolerant lipase-producing *Bacillus* strains lead us to perform the analysis of the new *Bacillus* spp. lipolytic system. Such study and the cloning of a novel extracellular lipase from the strain are important for industrial application.

Objectives:

1. To isolate thermotolerant lipase production bacteria from hot spring environment in Thailand and identify the strains using HV16S rDNA and biochemical test.
2. To characterize the lipase activity of *Bacillus* sp. RN2.
3. To isolate the gene encoding for lipase from thermotolerant *Bacillus* sp. RN2.

CHAPTER 3

MATERIALS AND METHODS

1. Bacterial strains collection & screening of the lipase producing bacteria

Soil and water samples from hot spring water at Ranong province of Thailand were previously collected and screened for thermotolerant bacteria using dilution technique, the isolated colonies were further tested for lipase acitivity onto tributyrin agar plate. A colony forming clear zone was selected after 72 h incubation at 45 °C.

2. Identification of the isolate *Bacillus* sp. RN2

2.1 Biochemical characteristic test using API 50 CHB kit

The carbohydrate metabolism of *Bacillus* sp. RN2 isolate which showed lipase activity was determined using the API 50 CHB kit (BioMerieux, Lyon, France). The isolate was grown on TSA plate (18-24 h at 45°C). Colony was suspended in 2 ml sterile 0.85% saline solution to correspond with tube No. 2 of the McFarland scale of standard opacities and 0.1 ml of this suspension was diluted in 10 ml of API 50 CHB medium. The strip was inoculated, incubated for 48 h at 45°C and read after both 24 and 48 h. The results were scored according to the manufacturer's instructions and the emerging biochemical profile was identified by means of APILAB software Version 2.1, 1990 (BioMerieux, Lyon, France).

2.2 PCR amplification and sequencing of hypervariable region (HV region) 16S rDNA

For the PCR amplification and sequencing of HV region, two primers were constructed based on the result of multiple alignments of 16S rRNA gene sequences from 69 bacillus type strains using CLUSTAL W version 1.82. A forward primer was 5' TGT AAA ACG ACG GCC AGT GCC TAA TAC ATG CAA GTC GAG CG -3'. A reverse primer consisted of 5' CAG GAA ACA GCT ATG ACC ACT GCT GCC TCC CGT AGG AGT -3'. The PCR product of 320 bp in sizes was expected. The PCR mixture was consisted of 1X PCR buffer (GIBCO BRL®), 1.5 mM $MgCl_2$, 10mM dNTPS, 20μM of each primer and 3 unit of *Taq* DNA polymerase (GIBCO BRL®), sterile distilled water was used to make total volume to 25 μl and the reaction was performed using the Peltier Thermal cycle (MJ Research, PTC-200). The PCR condition contained one cycle of 95°C for 3 min followed by the additional 30 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 1 minute and extension at 72°C for 1 min. The PCR

fragment was excised from the 1.2 % agarose gel at 100 volt for approximately 30 min and purified by using QIAGEN PCR purification kit (QIAGEN). The sequences of PCR product was determined by using Big Dye Terminator Cycle sequencing procedure and analyzed by using ABI PRISM 377 (Perkin Elmer).

The nucleotide sequencing data was detected by the input of the data into BLAST program from http://www.ncbi.nlm.nih.gov/BLAST. The nucleotide sequences of 16S rRNA gene were compared with nucleotide sequences of various organisms that have been previously submitted in GenBank.

3. Culture conditions for lipase production

Cultivation was achieved by aseptically removing the selected colony, activated by sub-culture in 10 ml of inoculum medium containing of nutrient broth 3% (w/v) and $CaCl_2$ 0.01% (w/v) in distilled water and incubated at 45°C for 16 h with agitation. Then, the inoculum size of 1.0% was added to the sterile production medium in 0.5 l Erlenmeyer flask with a working volume of 100 ml medium containing nutrient broth 0.35% (w/v); $CaCl_2$ 0.1% (w/v); gum arabic 1% (w/v); soy bean oil 2.5% (v/v) in distilled water and continued incubated at 45°C for 54 h with shaking (250 rpm).

4. Lipase activity assay

The activity of the extracellular lipase was assayed in the cell-free liquid after separation of biomass by centrifugation at 5,000 rpm for 20 min. Lipolytic activity was determined by measuring the optical density (OD) at 410 nm using *p*-nitrophenyl laurate (*p*NPL) (SIGMA) as substrate (97).

The substrate solution consisted of solution A (0.4 g *p*NPP dissolved in 12 ml isopropanol) and solution B (0.4 g tritonX-100; 0.1 g gum arabic; 90 ml potassium phosphate buffer pH 7). Subsequently solution A was added to solution B to a final composition 1:30 (v/v). The cell free supernatant (1ml) was added to 9 ml of substrate solution and then, the mixture was mixed and incubated at 37°C. After 20 min, the reaction was stopped by boiling and enzyme activity was measured by monitoring the change in absorbance at 410 nm that represented the amount of released *p*-nitrophenol (PNP). One unit of lipase activity is defined as the amounts of enzyme releasing 1 µmol PNP per minute under the assay conditions.

5. Characterization of the new isolate

5.1 pH optimum and stability

The effect of pH on the lipase activity was determined spectrophotometrically at O.D. 410 nm using *p*NPL as substrate as described above. The substrate was prepared in 50 mM buffer volumes of various pH values. To accomplish the pH optimum and stability of the lipase enzyme, various buffers of pH ranging from 7-12 were prepared. The sodium phosphate, glycine / NaOH, carbonate and Na_2HPO_3/NaOH buffers were prepared for pH 7–8, 8.5-9, 9.5-10.5 and 11-12, respectively. The effect of pH on lipase stability was determined by incubating aliquots of crude lipase in buffers of different pH values for 4 h at 30°C. Residual activity was assayed spectrophotometrically.

5.2 Temperature optimum and stability

The optimum temperature for lipase activity was determined over the range of 30-80°C using the spectrophotometric assay. The mixture was equilibrated at the required temperature before the addition of enzyme. The effect of temperature on lipase stability was determined by incubating the aliquots of lipase for 30 min in 50 mM appropiate buffer at various temperatures. The OD of residual activity was measured using the spectrophotometric assay at 410 nm.

5.3 Substrate specificity

Substrate preference of the enzyme was determined at optimum temperature and optimum pH by using the spectrophotometric assay. The *p*NP fatty acyl esters including butyric acid (C_4), caprylic acid (C_8), capric acid (C_{10}), lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}), stearic acid (C_{18}) were used as substrates. The results were reported as the percentage of the substrate that gave maximal activity.

5.4 Effect of various agents on lipase activity

The activity of the purified lipase was determined by incubation with 1 mM of metal chlorides, ethylene diaminetetraacetic acid (EDTA), dithiothreitol (DTT) (Sigma) and β-mercaptoethanol (Phamacia Biotech) at 30°C for 30 min. The remaining activity was determined spectrophotometrically by using the standard *p*NPL assay in the presence of a given agent and was expressed as a percentage of the activity without the agent.

6. DNA extraction

The strain RN2 was cultured in LB medium at 45°C for 12-14 h The cells were collected by centrifugation at 5,000 rpm for 10 min prior to lysis in lysis buffer (10 mM Tris-HCl, 20 mM glucose, lysozyme 100 mg/ml and 2% SDS). Proteinase K (BRL™) of 5 µl was added to the final concentration of 1 mg/ml prior to incubated at 45°C for 2 h. Phenol/chloroform was added to the solution and centrifuged to separate phases. The upper aqueous phase was transfered to new tubes and precipitated with 2 volumes of absolute ethanol and kept at -70°C for 15 min. The solution was centrifuged at 10,000 rpm for 10 min. DNA pellet was dissolved in TE buffer and stored at 4°C. The DNA concentration was determined at 260 nm using UV/VIS spectrophotometer. Molar extinction of DNA was performed as; 1 O.D. 260 nm = DNA concentration of 50 ng/ml.

7. PCR amplification of the *Bacillus* sp. RN2 lipase gene

A pair of degenerate primers was designed based on the nucleotide and amino acid sequence comparison among the conserved regions of the lipase gene of *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. stearothermophilus*, *B. thermoleovorans*, *Bacillus* sp. TP10A.1 and *B. thermostable*. The forward primer was 5′ CAY AAY CCN GTN GTN HTN GT 3′ and a reverse primer consisted of 5′ CCR TTN ARN CCY TCY TTA AT 3′. The set of primers for PCR amplification were synthesized from SIGMA. The PCR product of 519 bp in sizes was expected. All reactions were manipulated in 20 µl volume containing 100-200 ng of genomic DNA sample. The PCR mixture was consisted of 10X PCR buffer (TAKARA), 25 mM $MgCl_2$,2 mM dNTPs,20 µM of each primer and 3 units of *Taq* DNA polymerase (TAKARA), the sterile distilled water was used to make total volume to 20 µl and the reaction was performed using the PC-960G Gradient Thermal Cycler (Australia). The PCR conditions was as follows: 1 initial denaturation step at 94° C for 2 min, 30 cycles at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min, except for the final cycle of which extension proceed for 10 min. The PCR fragment was excised from the 1.2 % agarose gel at 100 volt for approximately 30 min prior to ethidium bromide staining and was observed under ultraviolet light.

Another set of primers was designed for PCR to complete the lipase gene. The forward primer contained 5' ATTCTATTGATTTGCATGCTGTCTG 3' and the reverse primer was 5'CCTTGAAGAAGTTAAGCTCTTCAAG 3'. The expected PCR fragment sized was 641 bp. The

PCR condition contained one cycle of 95 °C for 2 min and followed by the additional 30 cycles of denaturation at 94 °C for 45 sec, annealing at 57°C for 1 min and extension at 72 °C for 1 min The post extension was proceeded for another 10 min at 72 °C. The PCR mixture was prepared according to the protocol described above. The PCR fragment was excised from the 1.2 % agarose gel at 100 volt for approximately 30 min prior to ethidium bromide staining and was observed under ultraviolet light .

8. PCR Product purification

The PCR fragments was eluted from the gel and purified by using MinElute Gel Extraction Kit (QIAGEN). The procedure of purification was performed according to the instruction manual provided by the company. The DNA fragments was excised from the agarose gel with the clean sharp scalp. Gel products was bladed with razor on a fluorescent light box into small chunks and transfered to a microcentrifuge tube. The gel slices was weight in a coloress tube and added 2.5 volumes of 6.6 M sodium iodide to 1 volume and mix gels. The gel slices was incubated at 45°C until the gel is melted completely. The mixture solution was added 1.5 volume of binding buffer and placed into a S.N.A.P.™ purification column. The DNA was bound with the column, centrifuged at 5,000 g for 30 sec (repeat 3 times), discarded flow-though and placed column back in the same collection tube. After that, the DNA pellet was added with 0.6 ml of washing buffer to S.N.A.P.™ column and centrifuged at 5,000 g for 30 sec. The column was washed for another 2 times with washing buffer. The DNA pellet was centrifuged at 10,000 g for 1 min and placed column into a clean microcentrifuge tube. The DNA pellet was eluted with 30 µl of TE buffer and centrifuged the column for 1 min at maximum speed. The product was analyzed using electrophoresis in 1.2% (w/v) agarose gel at 100 volt approximately 30 min prior to ethidium bromide staining and was observed under ultraviolet light.

9. Cloning and transformation of lipase gene

9.1 Ligation of PCR fragment and vector

The purified of PCR fragment was further ligated to the pGEM®-T Easy Vector (Promega). The reaction mixture of 10 µl contained with 1µl of pGEM®-T Easy Vector, PCR fragment (200 ng), 5 µl of 2X Rapid Ligation Buffer, and 1µl of T4 DNA Ligase. The sterile

distilled water was used to make total volume to 10 µl. The reaction was gently mixed and incubated for 1 h at room temperature (25°C). Alternatively, if the maximum number of transformants was required, the reactions could be incubated for 12-14 h at 4°C.

9.2 Transformations

E. coli XL1-Blue was transformed with the ligation mixture by electroporation (BIO-RAD).The mixture containing 1.5 µl of ligation mixture was mixed with 40 µl of the competent cells. A single pulse of 0.7 kV was applied after transferring the mixture to a prechilled 0.1 cm electroporation cuvette. The cells were immediately added to 1 ml LB medium, incubated with shaking for 1 h at 37°C, and spreaded on LB agar plate (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl and 1.5% agar) contained 100 µl/ml ampicillin. The white colonies were selected. DNA sequencing and PCR amplification were used to check the inserted DNA.

10. Analysis of transformants

10.1 Analysis of transformants by PCR

The positive transformants was analyzed by using PCR amplification as described in Materials and Methods section 5.2. While colonies were selected and transferred to the new eppendorf tube contained 10 µl of PCR mixture. The reaction mixture was then heated at 94°C for 10 min to lyse the cell and inactivate nuclease. The PCR condition was performed according to the detail described in Materials and Methods section 5.2. Post-extension was undergone at 72°C for 10 min and stored at 4°C. The PCR product of positive clones were analyzed by loading 10 µl of PCR mixture onto 1.2% (w/v) agarose gel and electrophored at 100 volt for approximately 30 min prior to staining by ethidium bromide and observation under ultraviolet light.

10.2 Sequencing

The DNA sequence was determined using the ABI PRISM® BigDye Primer Cycle Sequencing Ready Reaction Kit (Appiled Biosystems) and analyzed using GeneAmp PCR Systems 9700 (Perkin Elmer) according to the protocol provided by each company. The T7 forward and reverse sequences were used as primers and pGEM®-T Easy vector was used as template. The nucleotide and amino acid sequences data were analyzed using the BLAST program of http://www.ncbi.nlm.nih.gov/BLAST. The nucleotide sequences of interested gene fragments were compared to those of the various organisms that have been previously

submitted in GenBank. The percentage of nucleotide sequence homology in comparison to various organisms was reported. All sequence data were analyzed with Clustal W sequence alignment program for The European Molecular Biology Laboratory (EMBL, U.K.) at http://www.ebi.ac.uk. Multialign program, and signal peptide identification were performed through SignalP V.2·0 software. The physico-chemical parameters of the deduced amino acid sequences were analyzed using ExPASY (http://www.expasy.org) and the pI/MW were determined by using http://us.expasy.org.tools/pi_tool.html.

CHAPTER 4

RESULTS

1. Bacterial strains collection and screening of the lipase producing bacteria

Soil and water samples from hot spring water at Ranong province of Thailand were previously collected and screened for thermotolerant bacteria by using dilution technique, the isolated colony were further tested for lipase acitivity. Among them, the isolate designated RN2 produced largest clear zone after 72 h of incubation at 45 °C on tributyrin agar plate as shown in Figure 2. The strain was an aerobic, Gram-positive, rod-shaped, endospore-former and grew at 45°C (Figure 3).

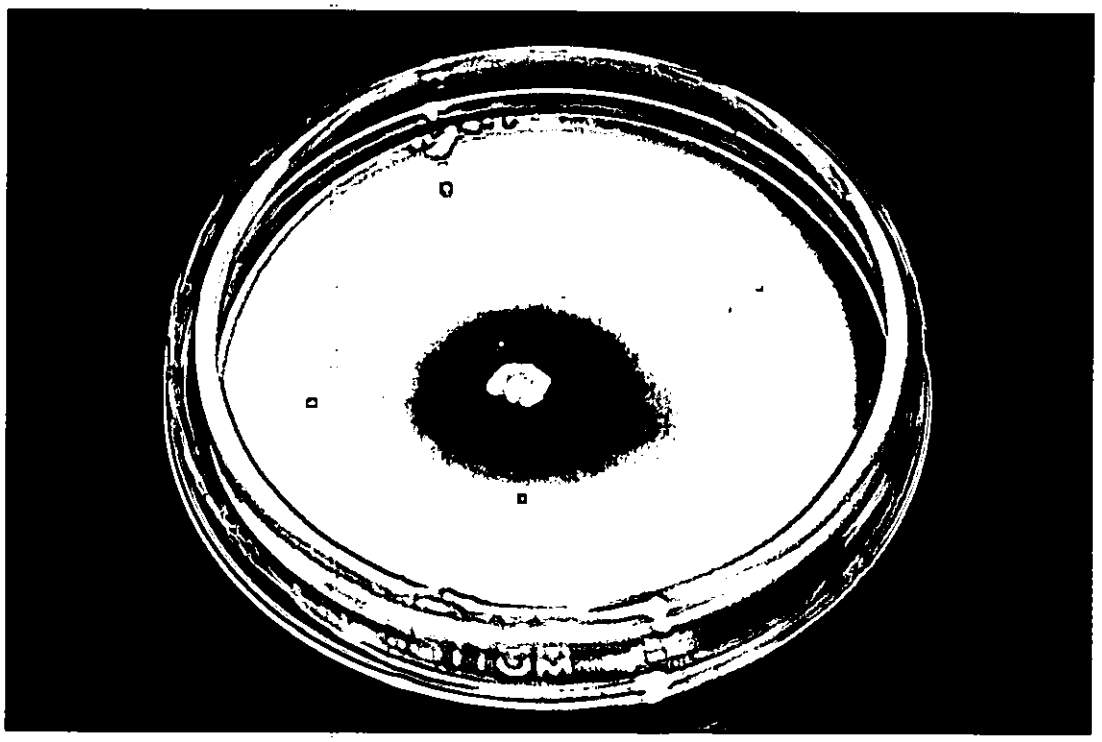

FIGURE 2 CLEAR-ZONE FORMATION OBTAINED FROM THE ISOLATE *BACILLUS* SP. RN2 ON TRIBUTYRIN AGAR PLATE.

FIGURE 3 GRAM STAIN OF *BACILLUS* SP. RN2

2. Identification of the isolate *Bacillus* sp. RN2

2.1 Biochemical characteristic test using API 50 CHB kit

The biochemical characteristic of the isolate *Bacillus* sp. RN2 was determined by using the API 50 strips according to the Materials and Methods 2.1. The results were scored according to the manufacturer's instructions and the emerging biochemical profile was identified by means of API LAB software Version 2.1. The results of carbohydrate metabolism of *Bacillus* sp. RN2 strains were presented in Table 6. The utilization patterns revealed that *Bacillus* sp. RN2 was considered to be *Bacillus licheniformis*.

2.2 PCR amplification and sequencing of hypervariable region (HV region) 16S rDNA

PCR amplification of hypervariable region of *Bacillus* sp. RN2 was performed according to Materials and Methods 2.2 . The PCR product of 320 bp in size was observed from *Bacillus* sp. RN2 (Figure 4). The nucleotide sequences data were analyzed by using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences of HV region of 16S rRNA gene of *Bacillus* sp. RN2 were compared to those of other *Bacillus* sp. that have been previously submitted in GenBank. Sequence alignment was performed by using software Clustal W version 1.82 and revealed that the sequences shared 96% identity with the type strain of *B. licheniformis* accession no. AY336076.1. Phylogenetic analysis using PAUP 4.0 indicated that *Bacillus* sp. RN2 was clustered with *B. licheniformis* (bootstrap value = 98%) based on DNA pasimony method. The reliability of internal branches of the dendrogram generated by cluster analysis was measured using the bootstrap analysis with 1,000 samplings (Figure 5).

TABLE 6 BIOCHEMICAL TEST USING API 50 CHB KIT OF *BACILLUS* SP. RN2

Characteristics	RN2	Characteristics	RN2
Glycerol	+	Esculin	+
Erythritol	-	Salicin	+
D-Arabinose	-	Cellobiose	+
L-Arebinose	+	Melibiose	+
Ribose	+	Sucrose	+
D-Xylose	+	Trehalose	ND
L-Xylose	-	Inulin	-
Adonitol	-	Melezitose	-
ß-methyl-D-Xyloside	-	Raffinose	-
Galactose	ND	Starch	-
Glucose	+	Glycogen	-
Fructose	+	Xylitol	-
Mannose	+	Gentiobiose	-
Sorbose	-	D-Turanose	-
Rhamnose	-	D-Lyxose	-
Dulcitol	-	D-Tagatose	+
Inositol	+	D-Fucose	-
Manitol	+	L-Fucose	ND
Sorbitol	+	D-Arabitol	-
α-Methyl-D-Manoside	-	L-Arabitol	-
α-Methyl-D-Gluoside	ND	Gluconate	+
N-Acetyl-Glucosamine	+	2-keto-Gluconate	-
Amygdalin	+	5-keto-Gluconate	-
Arbutin	+	Lactose	-
Maltose	+	Type of *Bacillus* sp.	*B. licheniformis*

ND = not detected

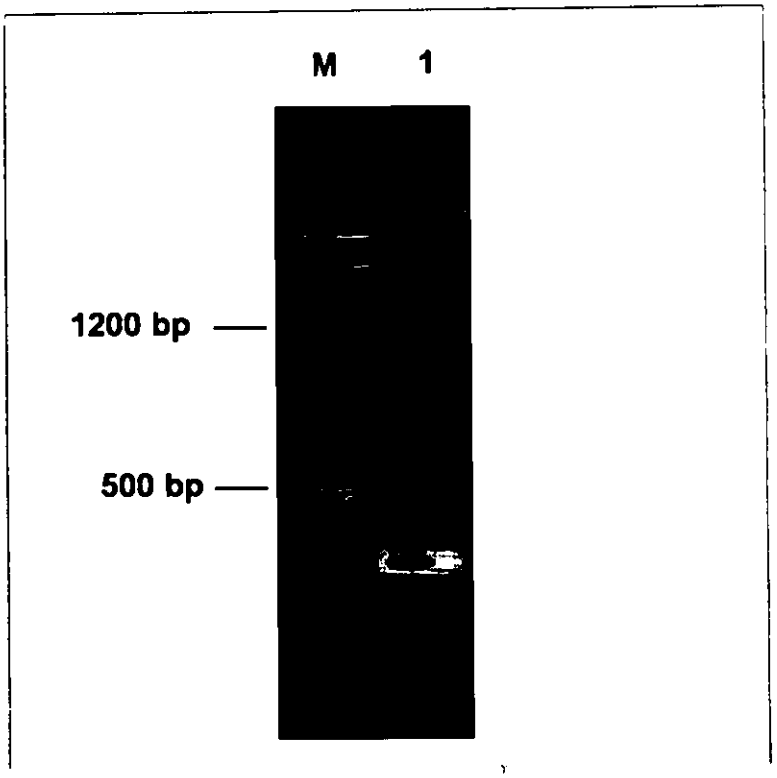

FIGURE 4 THE PCR AMPLIFICATION OF HYPERVARIABLE REGION FROM 16S RDNA OF *BACILLUS* SP. RN2, SHOW 320 BP IN SIZE OF PCR PRODUCT ON 1.2% AGAROSE GEL ELECTROPHORESIS AFTER ETHIDIUM BROMIDE STAINING.

Lane M = 100 bp marker

Lane 1 = PCR product of *Bacillus* sp. RN2

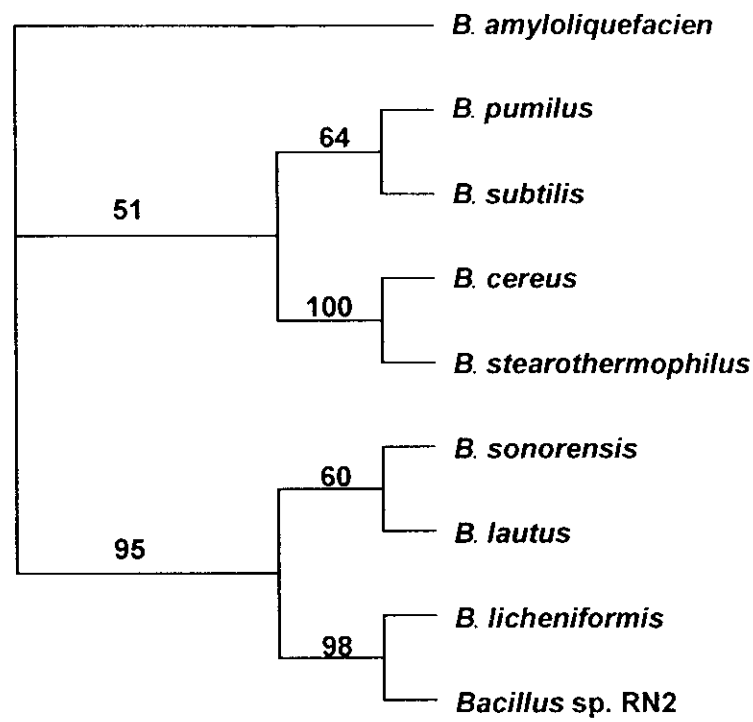

FIGURE 5 DNA PARSIMONY PHYLOGENETIC TREE OF *BACILLUS* SP. BASED ON HV REGION OF 16S RDNA SEQUENCES.

3. Characterization of lipase from *Bacillus* sp. RN2

3.1 pH optimum and stability

The determination of the optimal pH of the lipase activity from *Bacillus* sp. RN2 using p-nitrophenyl laurate (*p*NPL) as substrate in various buffers of the pH range 7-12 was shown that the crude lipase from *Bacillus* sp. RN2 displayed optimal catalytic activity towards *p*NPL in the alkaline region of pH 10-11 (Figure 6). In addition, lipase from RN2 was retained more than 80% of its activity following incubation for 4 h at 30°C in various buffers over a pH ranging from 7-8.5 and lipase activity was decreased less than 70% activity in the pH range of 9-10, with no activity remaining after incubation at pH 10.5 (Figure 7).

3.2 Temperature optimum and stability

The lipase from *Bacillus* sp. RN2 exhibited high activity at 50 to 65°C. The optimum temperature for lipase activity was at 60 °C (Figure 8). The enzyme retained more than 80% activity after incubation for 30 min at 45°C. Above this temperature, the stability of the enzyme was decreased to less than 30% of activity after incubation at 60°C, and no activity remaining after incubation at 70°C (Figure 9).

3.3 Substrate specificity of lipase from *Bacillus* sp. RN2

The substrate specificity of the crude lipase was studied with *p*-nitrophenyl fatty acyl esters of various chain lengths used as substrates (Table 11). The highest hydrolysis rates were obtained with *p*NP-laurate (C_{12}), which exhibited activities of 1.36 U/mg protein. The enzyme also showed the good activity on hydrolysis of *p*-nitrophenyl fatty acyl esters with the carbon chain lengths between C_8-C_{16}.

3.4 Effect of different agents on the activity of lipase from *Bacillus* sp. RN2

The effect of different agents on the activity of crude lipase was determined by using *p*NP-laurate as substrate (Table 12). In the presence of Ca^{2+}, K^+ and Zn^{2+}, the catalytic activity was enhanced. Mg^{2+}, Na^+ and NH_4^+ did not effect the activity of the enzyme. The metal ion as Ag^+ and Ni^{2+} partially inhibited the lipase activity.

The lipase was inhibited to about 70% when incubated and assayed in the presence of 1 mM β-mercaptoethanol while SDS and DTT caused partial inhibition. EDTA had a negligible effect on the activity of the enzyme.

TABLE 7 EFFECT OF pH ON ACTIVITY OF LIPASE FROM *BACILLUS* SP. RN2.

pH	Optimum	
	Enzyme activity (U/mg protein)	Relative activity (%)
7	0.37	18
7.5	0.74	36
8	1.29	63
8.5	1.58	77
9	1.66	82
9.5	1.75	86
10	1.84	90
10.5	2.03	100
11	1.94	95
11.5	1.46	71
12	1.22	60

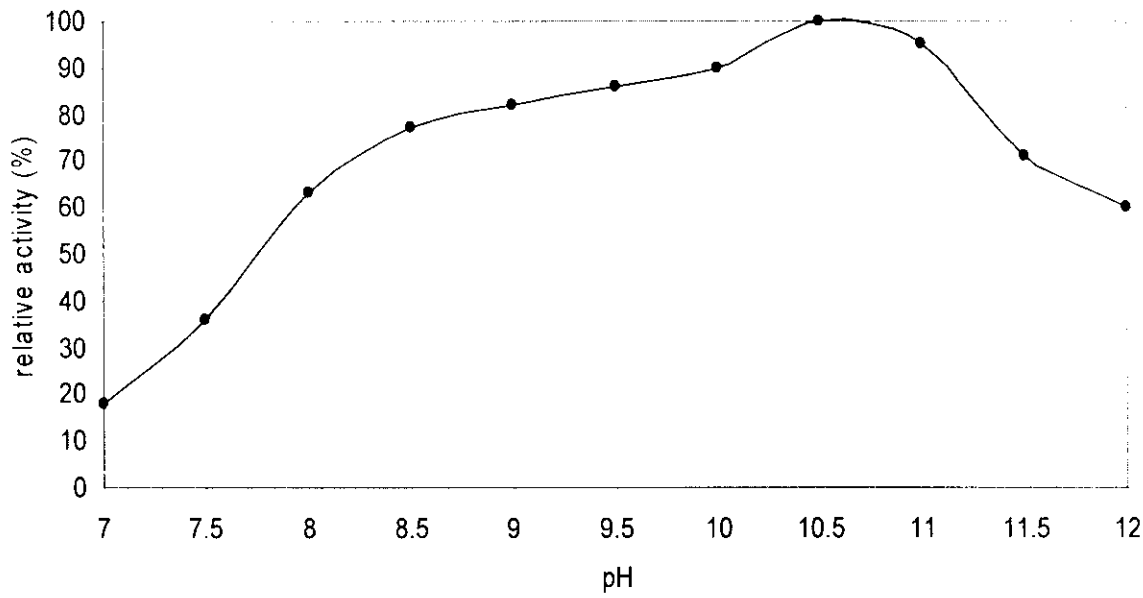

FIGURE 6 EFFECT OF pH ON ACTIVITY OF LIPASE FROM *BACILLUS* SP. RN2. FOR DETERMINATION OF THE OPTIMAL pH, THE FOLLOWING 50 mM BUFFERS WERE USED: SODIUM PHOSPHATE BUFFER (PH 7–8), GLYCINE / NaOH BUFFER (pH 8.5-9), CARBONATE BUFFER (pH 9.5–10.5), AND Na_2HPO_3/NaOH BUFFER (pH 11–12). THE VALUE WAS SHOWN AS PERCENTAGE OF MAXIMUM LIPASE ACTIVITY OBSERVED AT pH 10.5, WHICH WAS TAKEN AS 100%.

TABLE 8 EFFECT OF pH ON STABILITY OF LIPASE FROM *BACILLUS* SP. RN2.

pH	Stability	
	Enzyme activity (U/mg protein)	Residual activity (%)
7	0.36	97
7.5	0.71	95
8	1.19	92
8.5	1.33	84
9	1.11	66
9.5	0.83	47
10	0.14	7
10.5	0	0
11	0	0
11.5	0	0
12	0	0

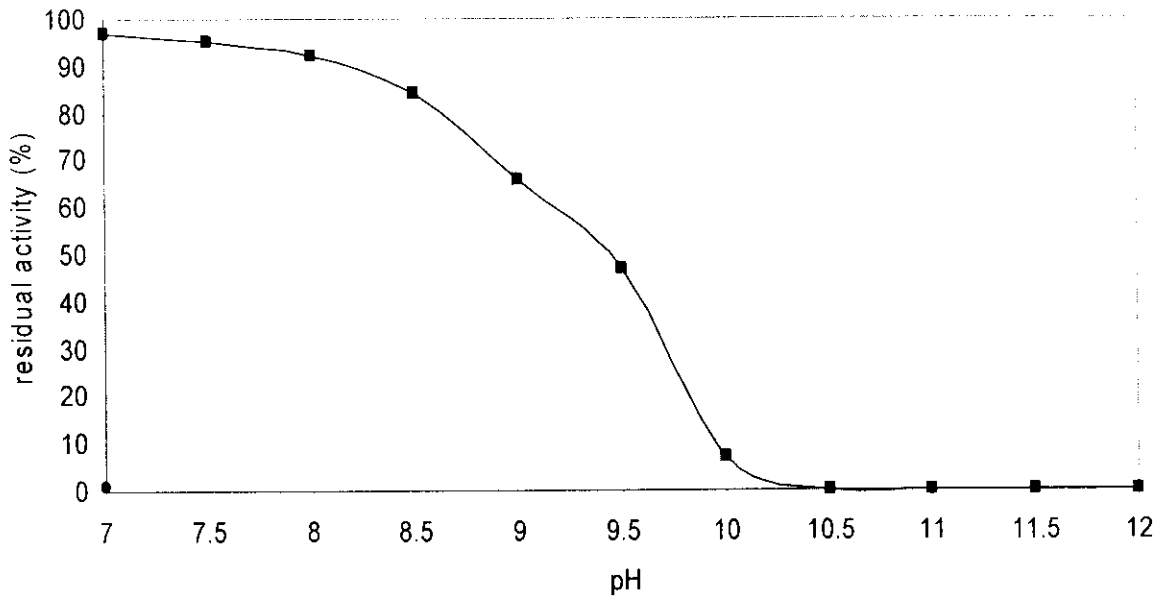

FIGURE 7 EFFECT OF pH ON STABILITY OF LIPASE FROM *BACILLUS* SP. RN2. TO ACCESS THE pH STABILITY, THE ENZYME WAS INCUBATED AT THE INDICATED PH IN VARIOUS BUFFERS AT 30°C FOR 4 H. THE VALUE WAS SHOWN AS PERCENTAGE OF ORIGINAL LIPASE ACTIVITY, WHICH WAS TAKEN AS 100%.

TABLE 9 EFFECT OF TEMPERATURE ON ACTIVITY OF LIPASE FROM *BACILLUS* SP. RN2.

Temperature (°C)	Optimum	
	Enzyme activity (U/mg protein)	Relative activity (%)
30	0.36	33
35	0.40	38
40	0.46	43
45	0.73	68
50	0.79	74
55	0.92	86
60	1.06	100
65	0.97	91
70	0.51	47
75	0.06	5
80	0	0

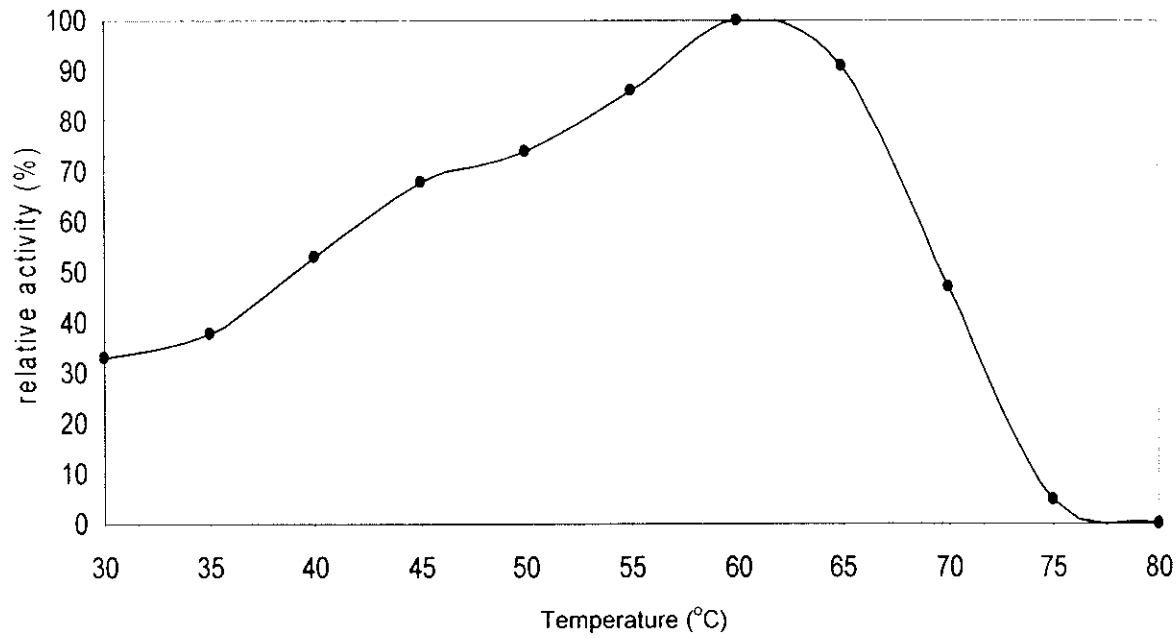

FIGURE 8 EFFECT OF TEMPERATURE ON ACTIVITY OF LIPASE FROM *BACILLUS* SP. RN2. LIPASE ACTIVITY WAS MEASURED AT VARIOUS TEMPERATURES UNDER STANDARD ASSAY CONDITION AT VARIOUS TEMPERATURE. THE VALUE WAS SHOWN AS PERCENTAGE OF MAXIMUM LIPASE ACTIVITY OBSERVED AT 60 ^{o}C, WHICH WAS TAKEN AS 100%.

TABLE 10 EFFECT OF TEMPERATURE ON STABILITY OF LIPASE FROM *BACILLUS* SP. RN2.

Temperature	Stability	
	Enzyme activity (U/mg protein)	Residual activity (%)
30	0.63	100
35	0.62	99
40	0.57	90
45	0.55	87
50	0.39	61
55	0.22	34
60	0.13	20
65	0.06	9
70	0	0
75	0	0
80	0	0

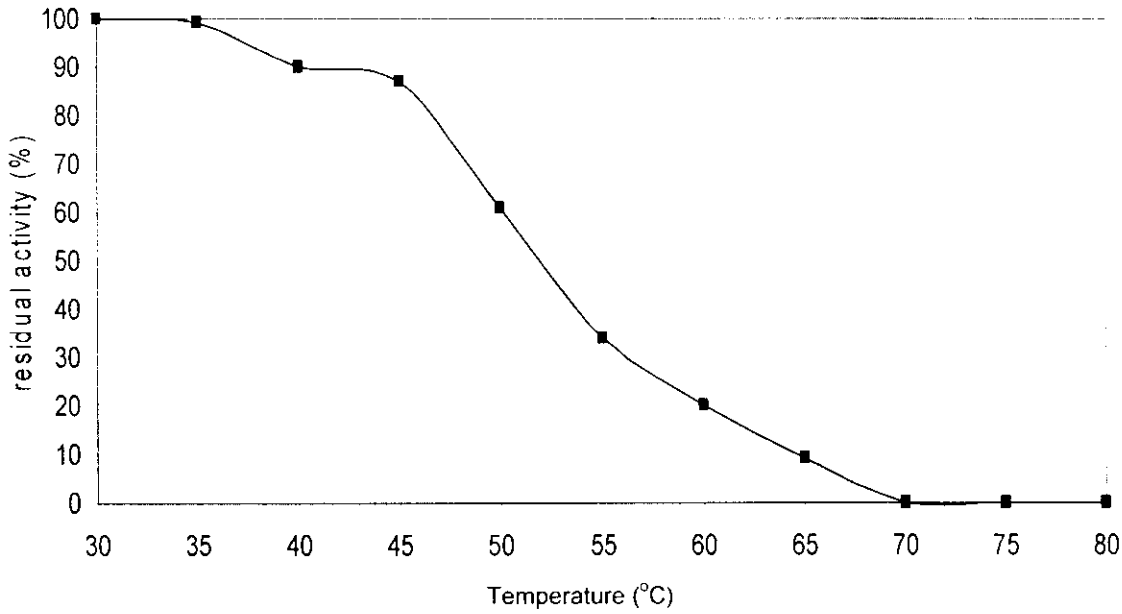

FIGURE 9 EFFECT OF TEMPERATURE ON STABILITY OF LIPASE FROM *BACILLUS* SP. RN2. THE DETERMINATION OF THE THERMAL STABILITY OF LIPASE, THE ENZYME WAS INCUBATED AT 30-80°C IN 50 mM CARBONATE BUFFER (pH 10.5) FOR 30 MIN. THE VALUE WAS SHOWN AS PERCENTAGE OF RESIDUAL LIPASE ACTIVITY OBSERVED AT 30°C, WHICH WAS TAKEN AS 100%.

TABLE 11 LIPASE ACTIVITY FROM *BACILLUS* SP. RN2 ON *p*-NITROPHENYL FATTY ACYL ESTERS OF VARIOUS CHAIN LENGTHS. THE ENZYME ACTIVITIES WERE ASSAYED UNDER STANDARD CONDITION.

Substrates	Enzyme activity (U/mg protein)
*p*NP-butyrate (C4)	0.73
*p*NP-caprylate (C8)	1.46
*p*NP-caprate (C10)	1.24
*p*NP-laurate (C12)	1.82
*p*NP-myristate (C14)	1.07
*p*NP-palmitate (C16)	0.98
*p*NP-stearate (C18)	0.64

TABLE 12 EFFECT OF DIFFERENT AGENTS ON THE ACTIVITY OF CRUDE LIPASE FROM *BACILLUS* SP. RN2.

Agents	Residual activity (%)
none	100
NaCl	95
KCl	106
$CaCl_2$	107
$MgCl_2$	92
NH_4Cl	97
AgCl	80
$NiCl_2$	69
$ZnCl_2$	102
SDS	58
β -mercaptoethanol	74
DTT	68
EDTA	90

4. PCR amplification of thermotolerant *Bacillus* sp. RN2 lipase gene

The DNA was purified and manipulated essentially as described in Materials and Methods 6. Degeneracy primers for PCR (whose nucleotide sequences were based on conserved regions of reported lipases in Genbank) were synthesized and used for the PCR amplification. A PCR product of 519 bp in length was expected.

After 30 cycles of PCR using the degeneracy primes, electrophoresis of the products on a 1.2% (w/v) agarose gel showed several bands including the expected 519 bp fragment. The 519 bp product was isolated by excising the band from the gel and the DNA was eluted from the agarose by MinElute Gel Extraction Kit.

5. Cloning and transformation of the lipase gene

The purified DNA fragment was ligated with pGEM-T Easy vector and transformed into *E.coli* XL1-Blue as previously described in Materials and Methods 9.2. The transformants were analyzed by PCR amplification and DNA sequencing.

6. Analysis of transformants by PCR amplification

After transformation of the *E.coli* XL1-Blue with the recombinant pGEM-T Easy plasmid by electroporation, clones containing the 519 bp insert were selected by PCR amplification. It was found that 12 white positive colonies were obtained and the recombinant plasmids were purified for sequencing. The data of DNA sequencing of the 12 positive colonies revealed that 5 clones containing the nucleotide sequencing corresponded to lipase gene of *B. licheniformis* (Accession number AJ 297356 with identity of 58 out of 69 nucleotides) and *B. subtilis* (Accession number Z99108 with identity 49 out of 58 nucleotides) with the same homology of 84%.

By using specific primers which were designed according to the known nucleotide sequences of partial lipase gene, the product of 641 bp was obtained (Figure 10). The PCR fragment was isolated and cloned into pGEM-T Easy vector. After incubation of the transformants at 37°C for 16 h, 30 white colonies were obtained. Four positive white colonies were selected for further DNA sequencing.

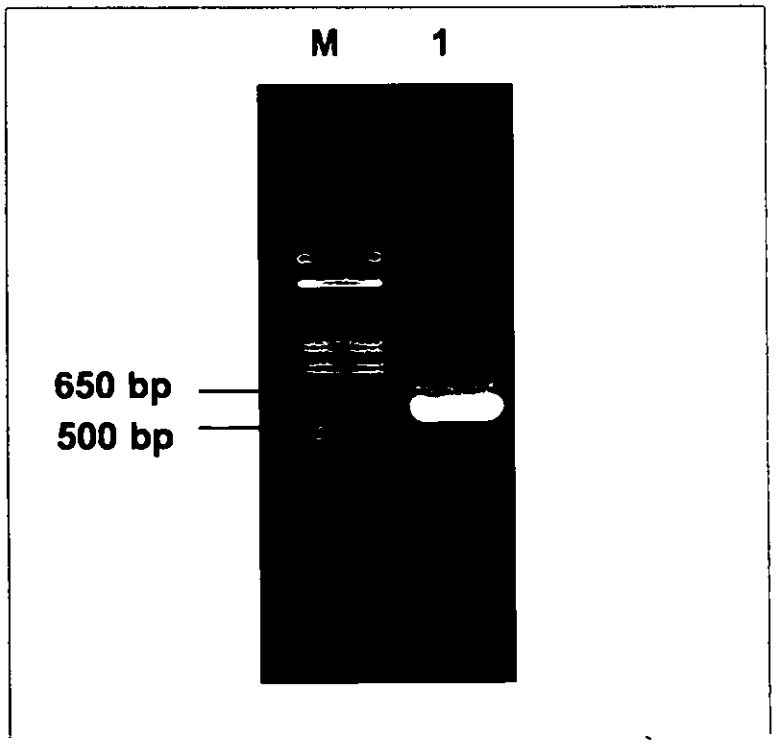

FIGURE 10 ETHIDIUM BROMIDE STAINING OF 1.2% AGAROSE GEL ELECTROPHORESIS OF 641 BP PCR PRODUCT.

7. DNA sequencing of lipase gene

7.1 Nucleotide sequence analysis

The nucleotide sequence of the *Bacillus* sp. RN2 lipase gene composed of an open reading frame of 576 bp that encoding for 192 amino acids with a deduced molecular mass of 20,282 Dalton. As revealed by Signal P program, a 17-residue typical *Bacillus* signal peptide was found at the N-terminal region of the protein, indicating its extracellular location. The cleavage site of the signal peptide was located between Ala 17 and Ala 18 according to the rules for a signal peptide sequence. The mature protein was composed of 174 amino acids with a calculated molecular weight of 18,544 Dalton with the isoelectric point of 9.05. The motif AHSMG, corresponding to the consensus pentapeptide –A-X_1-S-X_2-G- common in all known *Bacillus* lipases was found. Additionally, the cloned lipase contained a His residue at position X_1 like other *Bacillus* lipases, while position X_2 was occupied by a Met, exclusive of subfamily I.4 (81) The catalytic apparatus of lipases, involving the triad serine, glutamate or aspartate, and histidine (12) was placed in the newly isolated lipase at position 77 (S), 129 (D) and 152 (H) (Figure 11 and 12). The identification of restriction enzyme sites presented in the *Bacillus* sp. RN2 lipase gene was shown in Figure 13.

Genetic Code : Universal

```
        10         20         30         40         50         60
ATGCTGTCTGTTGTGTCCGTATTTTCGTTCCGGCCTTCAGCAGCTTCCGCCGCTTCCCAC
 M  L  S  V  V  S  V  F  S  F  R  P  S  A  A  S  A  A  S  H

        70         80         90        100        110        120
AATCCGGTCGTCATGGTCCACGGCATCGGCGGAGCCGATTATAACTTCATCGGCATTAAA
 N  P  V  V  M  V  H  G  I  G  G  A  D  Y  N  F  I  G  I  K

       130        140        150        160        170        180
TCGTATTTACAATCTCAAGGCTGGACAAGCAGTGAGCTTTACGCCATCAACTTTATCGAT
 S  Y  L  Q  S  Q  G  W  T  S  S  E  L  Y  A  I  N  F  I  D

       190        200        210        220        230        240
AAAACGGGAAATAATATAAACAATGCTCCGAGATTATCCGAATACATCAAGCGTGTGCTG
 K  T  G  N  N  I  N  N  A  P  R  L  S  E  Y  I  K  R  V  L

       250        260        270        280        290        300
AATCAAACAGGAGCATCAAAAGTCGATATTGTCGCCCACAGCATGGGCGGGGCCAATACG
 N  Q  T  G  A  S  K  V  D  I  V  A  H  S  M  G  G  A  N  T

       310        320        330        340        350        360
CTCTATTATATTAAAAATCTGGATGGTGCGGATAAAGTCGGACATGTCGTCACCCTTGGG
 L  Y  Y  I  K  N  L  D  G  A  D  K  V  G  H  V  V  T  L  G

       370        380        390        400        410        420
GGCGCTAATCGTCTCGTTACAAACACGGCGCCTCAGAATGACAAAATCTCATACACTTCG
 G  A  N  R  L  V  T  N  T  A  P  Q  N  D  K  I  S  Y  T  S

       430        440        450        460        470        480
ATTTACAGCACAAGCGACTATATCGTCTTAAACAGCCTCTCCAAACTTGATGGTGCAAAC
 I  Y  S  T  S  D  Y  I  V  L  N  S  L  S  K  L  D  G  A  N

       490        500        510        520        530        540
AATGTGCAAATCTCAGGCGTAAGCCATGTCGGTCTTTTGTTCAGCAGCAAAGTAAATGCC
 N  V  Q  I  S  G  V  S  H  V  G  L  L  F  S  S  K  V  N  A

       550        560        570        580
TTGATTAAAGACGGGCTGACCGCCAGCGGGAAATAA
 L  I  K  D  G  L  T  A  S  G  K  *
```

FIGURE 11 NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCES OF THE LIPASE GENE FROM *BACILLUS* SP. RN2. A SEQUENCE THAT EXHIBITED THE CHARACTERISTICS OF A SIGNAL PEPTIDE WAS UNDERLINED. THE CONSERVED PENTAPEPTIDE OF LIPASE GENE WAS INDICATED BY SQUARE. THE COMMON CATALYTIC TRIAD OF LIPASE GENE FAMILY I.4 (SER77, ASP129 AND HIS152) WAS INDICATED IN THE BOLD CAPITAL LETTER.

CLUSTAL W (1.82) multiple sequence alignment

```
Bacillus sp. RN2        ------------------MLSVVSVFSFRPSAASAASHNPVVMVHGIGGADYNFIGIKSYL 43
B. licheniformis        ----MRRHSFLSILLICMLSVVSVFSFRPSAASAASHNPVVMVHGIGGADYNFIGIKSYL 56
B. subtilis             --MKFVKRRIIALVTILMLSVTSLFALQP-SAKAAEHNPVVMVHGIGGASFNFAGIKSYL 57
B. pumilus              MKVILFKKRSLQILVALALVIGSMAFIQPKEVKAAEHNPVVMVHGIGGASYNFASIKSYL 60
                                     * : *:  ::*  ..**.************.:** .*****

Bacillus sp. RN2        QSQGWTSSELYAINFIDKTGNNINNAPRLSEYIKRVLNQTGASKVDIVAHSMGGANTLYY 103
B. licheniformis        QSQGWTSSELYAINFIDKTGNNINNAPRLSEYIKRVLNQTGASKVDIVAHSMGGANTLYY 116
B. subtilis             VSQGWSRDKLYAVDFWDKTGTNYNNGPVLSRFVQKVLDETGAKKVDIVAHSMGGANTLYY 117
B. pumilus              VGQGWDRNQLYAIDFIDKTGNNRNNGPRLSRFVKDVLDKTGAKKVDIVAHSMGGANTLYY 120
                         .***  .:***::* ****.* **.* **.::: **::***.****************

Bacillus sp. RN2        IKNLDGADKVGHVVTLGGANRLVTNTAPQ----NDKISYTSIYSTSDYIVLNSLSKLDGA 159
B. licheniformis        IKNLDGADKVGHVVTLGGANRLVTNTAPQ----NDKISYTSIYSTSDYIVLNSLSKLDGA 172
B. subtilis             IKNLDGGNKVANVVTLGGANRLTTGKALPGTDPNQKILYTSIYSSADMIVMNYLSRLDGA 177
B. pumilus              IKNLDGGDKIENVVTIGGANGLVSSRALPGTDPNQKILYTSVYSSADLIVVNSLSRLIGA 180
                        ******.:*: :***:**** *.:. *      *:** ***:**::* **:* **:* **

Bacillus sp. RN2        NNVQISGVSHVGLLFSSKVNALIKDGLTASGK--- 191
B. licheniformis        NNVQISGVSHVGLLFSSKVNALIKDGLTASGK--- 204
B. subtilis             RNVQIHGVGHIGLLYSSQVNSLIKEGLNGGGQNTN 212
B. pumilus              KNVLIHGVGHIGLLTSSQVKGYIKEGLNGGGQNTN 215
                        .** * **.*:*** **:*:. **:**...*:
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FIGURE 12 ALIGNMENT OF AMINO ACID SEQUENCES OF LIPASES FROM *BACILLUS* SPECIES SUBFAMILY I.4. THE ALIGNMENT WAS PERFORMED WITH CLUSTAL W. THE ASTERISKS (*) INDICATED AMINO ACID IDENTITY IN ALL SHOWN SEQUENCES. THE POINT (.) AND COLON (:) DENOTED AMINO ACID IDENTITY IN AT LEAST TWO ALIGNED SEQUENCES. THE OXYANION HOLE OF LIPASE GENE FAMILY I.4 (PVVMVHG) WAS INDICATED BY BOLD LETTERS. THE PUTATIVE "CONSERVED" PENTAPEPTIDE OF LIPASE GENE (A-X_1-S-X_2-G) WAS INDICATED BY UNDERLINE. THE COMMON CATALYTIC TRIAD OF LIPASE GENE FAMILY I.4 (SER77, ASP129 AND HIS152) WAS INDICATED IN SHADING LETTER. LIPASE GENE SEQUENCE ACCESSION NUMBERS OF *B. LICHENIFORMIS*, *B. SUBTILIS* AND *B. PUMILUS* WERE YP_079680.1, NP_388152.1 AND AAR84668.1, RESPECTIVELY.

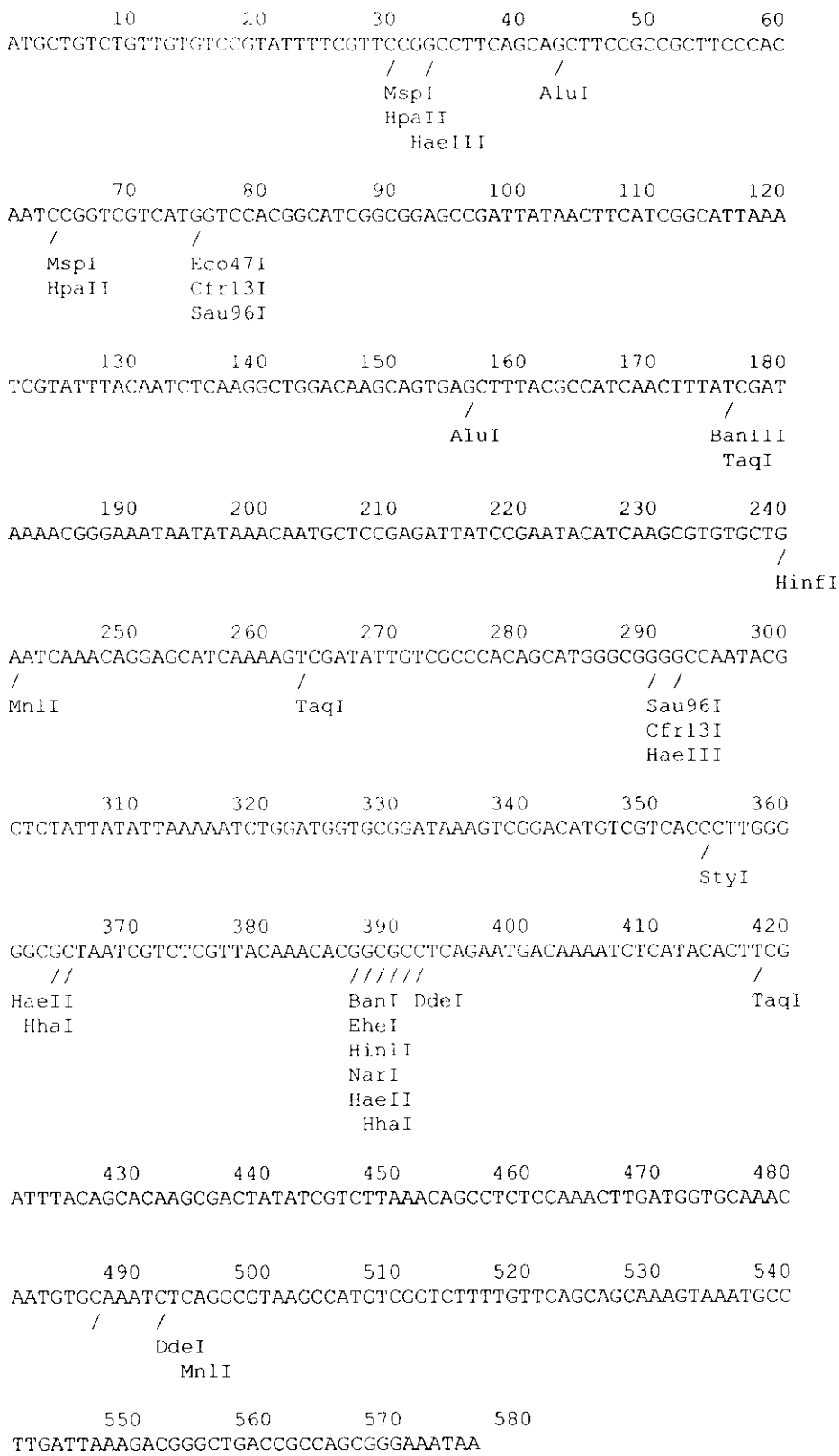

FIGURE 13 RESTRICTION ENZYME SITES USAGE FOR THE 576 BP OF SEQUENCES WITHIN AND ADJACENT TO THE LIPASE GENE OF *BACILLUS* SP. RN2.

Res. Ezm.	Recog. Seq.	Count	Sequence Pos.		
AluI	AGCT	(2):	42	155	
BanI	GGYRCC	(1):	387		
BanIII	ATCGAT	(1):	175		
Cfr13I	GGNCC	(2):	75	290	
DdeI	CTNAG	(2):	392	492	
Eco47I	GGWCC	(1):	75		
EheI	GGCGCC	(1):	387		
HaeII	RGCGCY	(2):	361	387	
HaeIII	GGCC	(2):	32	291	
HhaI	GCGC	(2):	362	388	
HinlI	GRCGYC	(1):	387		
HinfI	GANTC	(1):	240		
HpaII	CCGG	(2):	30	64	
MnlI	CCTG/CAGG	(2):	248	494	
MspI	CCGG	(2):	30	64	
NarI	GGCGCC	(1):	387		
Sau96I	GGNCC	(2):	75	290	
StyI	CCWWGG	(1):	354		
TaqI	TCGA	(3):	176	263	418

Selected Enzymes With No Recognized Positions

AatI	AatII	AccI	AflII	Alw44I	ApaI	AseI	AvaI
BamHI	BanII	BbrPI	BclI	BfrI	BglI	BglII	Bpu1102I
Bsh1236I	BsiWI	BsmI	BssHII	BstEII	BstXI	Cfr10I	Cfr9I
Csp45I	CspI	DpnI	DraI	DraIII	Eam1104I	Eco105I	Eco47III
Eco52I	Eco81I	Eco0109I	EcoRI	EcoRII	EcoRV	EcoT22I	FspI
HincII	HindIII	HpaI	KpnI	MboII	MluI	MroI	MscI
MunI	MvaI	NaeI	NciI	NcoI	NdeI	NheI	NotI
NruI	NspV	PacI	PpuMI	PstI	PvuI	PvuII	RsaI
SacI	SacII	SalI	SanDI	Sau3AI	ScaI	ScrFI	SfiI
SmaI	SpeI	SphI	SrfI	SspI	TspEI	XbaI	XhoI
XhoII	XmnI						

W=A/T, R=A/G, M=A/C, K=T/G, Y=T/C/U, S=G/C, N=A/T/G/C,

H=not G, B=not A, V=not U/T, D=not C

7.2 The consensus sequence and homology in *Bacillus* lipase

The deduced amino acid sequences of the lipase from *Bacillus* sp. RN2 showed the homology of 85% to *B. licheniformis* (Accession number YP_079680.1), 64% to *B. subtilis* (Accession number NP_388152.1) and 63% to *B. pumilus* (Accession number AAR84668.1). The conserved pentapeptide sequences from *Bacillus* lipases were observed among *Bacillus* in subfamily I.4 and subfamily I.5. The lipase possessed the common conserved pentapeptide of Ala-X-Ser-X-Gly (Table 13).

TABLE 13 CONSERVED AMINO ACID SEQUENCES OF *BACILLUS* IN LIPASE SUBFAMILY I.4 AND I.5.

Enzyme	Amino acid sequence[a]	Identity (%)	Accession no.
Bacillus sp. RN2	DIVAHSMGGANT		
Subfamily I-4			
B. licheniformis	DIVAHSMGGANT	85	YP_079680.1
B. subtilis	DIVAHSMGGANT	64	NP_388152.1
B. pumilus	DIVAHSMGGANT	63	AAR84668.1
Consensus sequence[b]	DIVAHSMGGANT		
Subfamily I-5			
B. thermoleovorans	HIIAHSQGGQTA		AF134840
B. thermocatenulatus	HIIAHSQGGQTA		X95309
B. sterothermophilus	HIIAHSQGGQTA		U78785
Consensus sequence[c]	HIIAHSQGGQTA		

[a] The consensus pentapeptide was shown in bold type.

[b] Consensus sequences for *Bacillus* sp. RN2 and subfamily I-4 lipases.

[c] Consensus sequences in subfamily I-5 lipases.

7.3 Codon usage

The codon usage of the *Bacillus* sp. RN2 lipases was shown in Table 14 of which the 192 codons (including the stop codon) were listed. In general, purines were preferred for the first position of the codon (67.2% A+G *cf* 32.8% C+T), the second position was shared more evenly between R and Y while C was found in the third position of codons in a 36.5% of cases giving a pro-pyrimidine (C+T) bias of 63.6% for this position. The codon usage for the *Bacillus* sp. RN2 lipase gene, therefore, tended towards the consensus sequence "RNY" for codons (where N was any nucleotide). Perhaps a more remarkable outcome of codon usage analysis was the clear observation of bias in the use of synonymous codons. Example of bias were noticeable for valine of which, of 4 synonymous codons, usage was striking bias towards GTC (used 10 out of 18 times) while all other valine codons were used relatively sparingly. Other low usage codons exist for isoleucine (ATA), glutamine (CAG), lysine (AAG) and serine (AGT).

TABLE 14 CODON USAGE FOR THE *BACILLUS* SP. RN2 LIPASE GENE.

Phe	TTT	2	Ser	TCT	2	Try	TAT	5	Cys	TGT	0
	TTC	3		TCC	5		TAC	4		TGC	0
Leu	TTA	3		TCA	4	TER	TAA	1	TER	TGA	0
	TTG	2		TCG	3		TAG	0	Trp	TGG	1
	CTT	4	Pro	CCT	2	His	CAT	2	Arg	CGT	2
	CTC	3		CCC	0		CAC	3		CGC	0
	CTA	0		CCA	0	Gln	CAA	4		CGA	0
	CTG	4		CCG	2		CAG	1		CGG	1
Ile	ATT	5	Thr	ACT	1	Asn	AAT	11	Ser	AGT	1
	ATC	8		ACC	2		AAC	6		AGC	9
	ATA	1		ACA	4	Lys	AAA	10	Arg	AGA	1
Met	ATG	3		ACG	3		AAG	1		AGG	0
Val	GTT	2	Ala	GCT	4	Asp	GAT	6	Gly	GGT	3
	GTC	10		GCC	7		GAC	3		GGC	7
	GTA	3		GCA	3	Glu	GAA	1		GGA	4
	GTG	3		GCG	2		GAG	1		GGG	4

7.4 Amino acid composition of lipase gene from *Bacillus* sp. RN2

The data of the nucleotide sequence of the *Bacillus* sp. RN2 lipase gene pemitted the calculation of the RN2 amino acid composition were shown in Table 15. The composition of the RN2 lipase contained high content of Ser and low content of Glu and Trp with the lack of Cys.

TABLE 15 AMINO ACID COMPOSITION OF LIPASE GENE FROM *BACILLUS* SP. RN2.

Length = 191 amino acids (stop codon unexists)

Amino Acid	Number	Mol%
Ala (A)	16	8.4
Cys (C)	0	0.0
Asp (D)	9	4.7
Glu (E)	2	1.1
Phe (F)	5	2.6
Gly (G)	18	9.4
His (H)	5	2.6
Ile (I)	14	7.3
Lys (K)	11	5.8
Leu (L)	16	8.4
Met (M)	3	1.6
Asn (N)	17	8.9
Pro (P)	4	2.1
Gln (Q)	5	2.6
Arg (R)	4	2.1
Ser (S)	24	12.6
Thr (T)	10	5.2
Val (V)	18	9.4
Trp (W)	1	0.5
Tyr (Y)	9	4.7

7.5 Analysis of the hydrophobicity of Bacillus sp. RN2 lipase gene

The hydropathy profile of *Bacillus* sp. RN2 lipase gene was shown as the ratio of hydrophilicity and hydrophobicity along the sequence of lipase (Figure 14). The hydrophilicity of the lipase was slightly rather than its hydrophobicity. Values below the axis denoted hydrophilic regions, which may be exposed on the outside of the analyzed molecule whereas those values above the axis indicated the hydrophobic regions, which tended to be buried inside the lipase structure molecule.

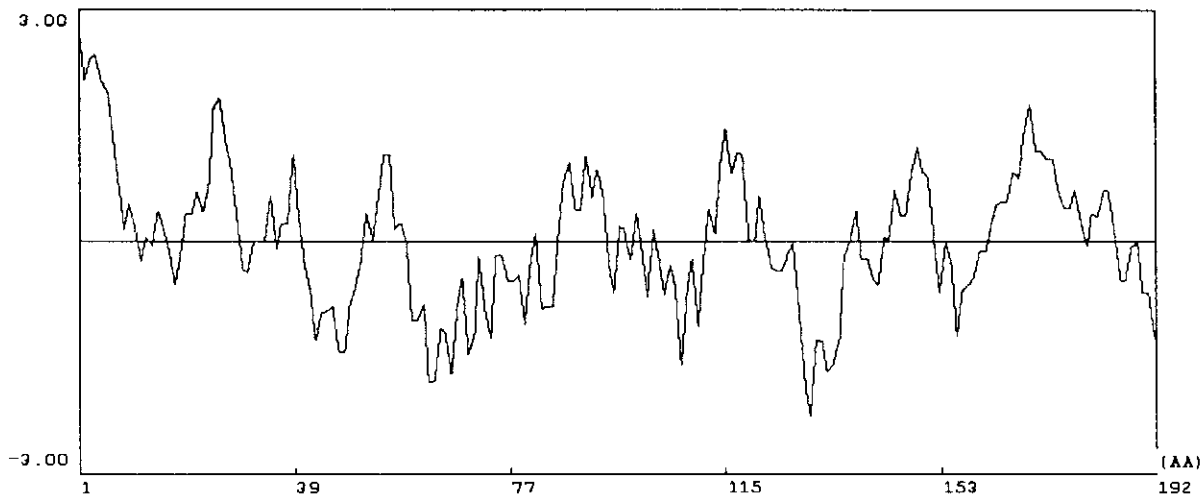

FIGURE 14 THE ANALYSIS OF THE LOCAL HYDROPHOBICITY OF THE LIPASE STRUCTURE WAS BASED ON THE METHOD OF KYTE AND DOOLITTLE (98).

CHAPTER 5
DISCUSSION

Production of an extracellular lipase have been reported previously (81, 82, 85). In this study, the lipase producing bacterial strains were isolated from a hot spring of Ranong province, Thailand. Upon analysis for lipolytic activity on tributyrin agar plate, the isolated *Bacillus* designated as *Bacillus* sp. RN2 generated the highest activity. The phylogenetic analysis of hypervariable region of 16S rDNA showed the similarity of 98% to *B. licheniformis*. The result was corresponded to the biochemical characteristic test using API 50 CHB kit. The strain was able to utilise glycerol, L-arabinose, ribose, D-xylose, glucose, fructose, mannose, inositol, manitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, maltose, esculin, salicin, cellobiose, melibiose, sucrose, D-tagatose and gluconate.

Characterization of lipase from *Bacillus* sp. RN2 were determined. The effect of pH and temperature on the activity of the crude enzyme *Bacillus* sp. RN2 was examined by using *p*-nitrophenyl laurate as a substrate. From this study, the *Bacillus* sp. RN2 lipase has the pH and temperature optimum at 10.5 and 60°C, respectively. The pH was similar to other lipases of subfamily I.4 (Table 5). In contrast, the *Bacillus* sp. RN2 lipase was more stable than those of the same subfamily but not as stable as the lipase in subfamily I.5.

The substrate specificity of the strain RN2 was tested on several *p*-nitrophenyl fatty acyl esters with high activity against *p*-nitrophenyl esters with chain lengths between 8 and 12 carbon atoms. The best substrate for lipase from *Bacillus* sp. RN2 was *p*NP-laurate (C_{12}). Moreover, the enzyme could also hydrolyzed long-chain triglycerides (C_{14}- C_{18}). This was similar to the enzymes from the other strains of *Bacillus* sp., such as *Bacillus* sp. THL027 (99) and *B. thermoleovorans* ID-1 (84). The results indicated that the lipase from *Bacillus* sp. RN2 possessed the wide range activity of substrates which could be beneficial for hydrolysis in environmental and industrial applications.

The effect of several agents (1 mM) on the activity of crude enzyme was assayed using *p*NP-laurate as substrate. It was found that only Ca^{2+}, K^{+} and Zn^{2+} slightly enhanced the activity of the lipase whereas Ag^{+} and Ni^{2+} led to a reduction of enzyme activity. It was also found that the others ions such as Mg^{2+}, Na^{+} and NH_4^{+} did not significantly effect the activity of the enzyme. Moreover, SDS, DTT, EDTA and β-mercaptoethanol caused a partial inhibition of the enzyme.

The data suggested that the native lipase was ion-independent enzyme and appeared to be a monomeric structure which were the common features of lipase in subfamily I.4.

The nucleotide sequences of known *Bacillus* lipases were aligned and stretches of sequence identity was used for the isolation of *Bacillus* sp. RN2 lipase-coding gene by means of PCR (as described in Materials and Methods 7). The sequences of gene encoding for the lipase compared to the sequences of those in prokaryotes deposited in GenBank showed no significant similarities. This indicated that the lipase of RN2 may be the novel enzyme. Nevertheless, to identify the newly isolated enzyme, more experiments such as X-ray crystallization and 3D-structure analysis should be elucidated. The resulting mature lipase showed 192 amino acids (including stop codon) corresponded to a deduced molecular mass of 20,282 Dalton with the pI of 9.05. This lipase was synthesized as pre-proteins with an amino-terminal signal peptide sequence. The signal peptide was essential for proper targeting of the protein, and was removed before the excretion to the extracellular fluid (100). As revealed by SignalP program, a 17-amino acid stretch with the features of the typical *Bacillus* signal peptides (101) was found at the N-terminal region of the protein, indicating its extracellular location.

The results obtained from amino acid sequence homology analysis indicated that the lipase was the member of a reduced cluster of highly conserved bacterial serine-esterases grouped (A-H-S-M-G) and oxyanion hole (PVVMVHG) in subfamily I.4, exclusive from the mesophilic or moderately thermophilic members of the genus *Bacillus* (49). Sequence comparison of lipase RN2 showed that Ser77, Asn129 and His152 were catalytic triad of lipase RN2 protein. This serine was usually embedded in pentapeptide sequence motif A-X_1-S-X_2-G. No cysteine was found in the deduced protein sequence of the lipase. Proteins lacking cysteine or with a low content of cysteine were generally more flexible molecules whose tertiary structure relied on weaker bonds. This data was corresponded to those of many extracellular bacterial proteins that they were likely to contain a low level of cysteine (102). This could suggest that proteins with high flexibility passed more readily through the cell wall. Furthermore, in the case of lipase, the lack of cysteine could allow conformational changes (60) which could be necessary for enzymatic activity when a water-soluble enzyme reacted with a hydrophobic lipid.

The coding nucleotide sequences of the RN2 lipase was 576 bp in length which corresponded to 192 amino acids. The gene was slightly A+T rich with an A+T content of 53.3%. Analysis of codon usage revealed a pronounced bias towards the use of cytosine (C) in the third

(degenerate) position of many synonymous codons (especially in the codons for Ile, Val, Ala, Ser and Gly). Codon choices might usefully be divided by gene sequence location (amino end, carboxyl end, central parts) (103) and by protein structural domains and protein secondary structure determinations (104). In prokaryotes, codon choice was presumably influenced by structure via evolutionary selection for the most accurately translated sequences at structurally important locations (105).

The amino acid composition of the lipase gene from *Bacillus* sp. RN2 revealed that the RN2 lipase protein apparently contained 60 neutral (Ala, Asn, Cys, Gln, Gly, Pro), 51 hydrophobic (Ile, Leu, Met, Val), 15 aromatic (Phe, Trp, Tyr), 11 acidic (Asp, Glu), 20 basic (Arg, His, Lys) and 34 hydroxyl-containing amino acid residues (Ser, Thr). This composition, with the slight excess of basic over acidic residues, would give an isoelectric point (pI) which was expected to be slightly alkaline. The hydrophilicity of the lipase was slightly greater than its hydrophobicity and the nonpolar amino acids were slightly greater than the polar amino acids in the amino acid composition of the lipase gene structure. This indicated that the lipase molecular structure was turned the nonpolar group of amino acid into its core structure and turned the polar group of the amino acid outside the molecule.

In conclusion, the overall properties of *Bacillus* sp. RN2 novel lipase indicated that it was a mesophilic enzyme acting on a broad range of pH that becomes inactivated only by a limited number of lipase inhibitors (81, 86). The high homology among lipases of *B. licheniformis* (81), *B. subtilis* (86), *B. pumilus* (106), and *Bacillus* sp. RN2 indicated that the enzyme was highly ubiqutous and well conserved among mesophilic *Bacillus* species. It probably played an essential role in the transformation of lipids and was independent of the habitat of the corresponding strains. Additionally, the overall data suggested that the enzyme could be evaluated and used in certain manufacturing processes such as antibiotic production (107), biosurfactant and biofilm production, biodegradation of recalcitrant substances (108), or in the conversion of low-cost fats into added value products (109).

REFERENCES

REFERENCES

1.Jaeger KE, Reetz MT. Microbial lipases form versatile tools for biotechnology. Trends Biotechnol 1998;16(9):396-403.

2.Jaeger K, Schneidinger B, Rosenau F, Werner M, Lang D, Dijkstra B, et al. Bacterial lipases for biotechnological applications. J Mol Catalysis B: Enzymatic 1997;3:3-12.

3.Malcata FX, Reyes HR, Garcia HS, Hill CG, Jr., Amundson CH. Kinetics and mechanisms of reactions catalysed by immobilized lipases. Enzyme Microb Technol 1992;14(6):426-46.

4.Dandik L, Arioglu G, Aksoy H. The enzymatic hydrolysis of used frying oil by native lipase. Appl Biochem and Biotechnol 1993;42:119-26.

5.White J, White D. Source Book of Enzymes: CRC Press; 1997.

6.Rubin B, Dennis E. Methods in Enzymology: Lipases, Part A: Biotechnology: Academic Press; 1997.

7.Rubin B, Dennis E. Methods in Enzymology: Lipases, Part B: Enzyme Characterization and Utilization: Academic Press; 1997.

8.Kazlauskas R, Bornscheuer U. Biotechnology and Biotransformations with Lipases: Weinheim; 1998.

9.Godfrey T, West S. Industrial Enzymology. 2nd edn ed: Macmillan Press; 1996.

10.Woolley P, Petersen S. Lipases: Their Structure, Biochemistry and Application: Cambridge University Press; 1994.

11.Gilbert EJ. Pseudomonas lipases: biochemical properties and molecular cloning. Enzyme Microb Technol 1993;15(8):634-45.

12.Jaeger KE, Ransac S, Dijkstra BW, Colson C, van Heuvel M, Misset O. Bacterial lipases. FEMS Microbiol Rev 1994;15(1):29-63.

13.Cano RJ, Colome JS. Temperature range for microbial growth. St.Paul: West Publishing Company; 1986.

14.Gilfillan WN. Thermophilic Anaerobic Sludge Digetion Characteristics Using Mesophilic Inoculum: University of Queensland; 1998.

15.Davis BD, Dulbecco R, Eisen H.N., Ginsberg H.S. The genus *Bacillus*. Singapore: The Benjamin / Cummings Company, Inc; 1990

16.Pelczar j, M.J., Reid RD, Chan ECS. The aerobic spore-forming bacteria. New Delhi: Tata mcGraw-Hill Publishing Company Ltd; 1997.

17.Malcata F. Nato ASI Ser., Ser. E: Engineering of/with Lipases: Kluwer; 1996.

18.Coleman M, Macrae A, inventors. UK Patent No.1 577 933. 1980.

19.Gill I, Valivety R. Polyunsaturated fatty acids, Part 1: Occurrence, biological activities and applications. Trends Biotechnol 1997;15(10):401-9.

20.Gill I, Valivety R. Polyunsaturated fatty acids, Part 2: Biotransformations and biotechnological applications. Trends Biotechnol 1997;15(11):470-8.

21.Farrell R, Hata K, Wall M. Solving pitch problems in pulp and paper processes by the use of enzymes or fungi. Adv Biochem Eng Biotechnol 1997;57:197-212.

22.Parmar VS, Gupta S, Bisht KS, Mukherjee S, Boll PM, Errington W. Synthesis and X-ray structure of 2-(3-methyl-2-butenyl)-3,4,5-trimethoxyphenol: a potent anti-invasive agent against solid tumours. Acta Chem Scand 1996;50(6):558-60.

23.Akita H. [The use of microbial and enzymatic function in organic synthesis. Total synthesis of the aglycone of venturicidins A and B]. Yakugaku Zasshi 1990;110(11):789-806.

24.Jones BC, Silverton JV, Simons C, Megati S, Nishimura H, Maeda Y, et al. Synthesis, absolute configuration, and enantioselectivity of antiretroviral effect of (R)-(-)- and (S)-(+)-cytaliene. Lipase-catalyzed enantioselective acylations of (+/-)-N4-acylcytallenes. J Med Chem 1995;38(8):1397-405.

25.Graffner-Nordberg M, Sjodin K, Tunek A, Hallberg A. Synthesis and enzymatic hydrolysis of esters, constituting simple models of soft drugs. Chem Pharm Bull (Tokyo) 1998;46 (4):591-601.

26.Pouderoyen Gv, Eggert T, Jaeger K-E, Dijkstra BW. The crystal structure of *Bacillus subtilis* lipase: a minimal / hydrolase fold enzyme. J Mol Biol 2001;309:215-26.

27.Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Soccol VT. The realm of microbial lipases in biotechnology. Biotechnol Appl Biochem 1999;29 (Pt 2):119-31.

28.Matsumura F. Mechanism of action of dioxin-type chemicals, pesticides, and other xenobiotics affecting nutritional indexes. Am J Clin Nutr 1995;61(3 Suppl):695S-701S.

29.Zha D, Wilensek S, Hermes M, Jaeger KE, Reetz MT. Complete reversal of enantioselectivity of an enzyme-catalyzed reaction by directed evolution. Chem Commun 2001:2664-5.

30.Drauz K, Waldmann H. Enzyme Catalysis in Organic Synthesis; 1995.

31.Faber K. Biotransformations in Organic Chemistry. 3rd edn ed: Springer; 1997.

32.Boland W, Frobl C, Lorenz M. Synthesis; 1991.

33.Stecher H, Faber K. Synthesis; 1997.

34.Collins A, Sheldrake G, Crosby J, eds. Chirality in Industry: The Commercial Manufacture and Applications of Optically Active Compounds: Wiley; 1992.

35.Collins A, Sheldrake G, Crosby J, eds. Chirality in Industry II: Developments in the Commercial Manufacture and Applications of Optically Active Compounds: Wiley; 1997.

36.Balkenhohl F, Ditrich K, Hauer B, Ladner W. Optisch aktive Amine durch Lipase-katalysierte Methoxyacetylierung. J. Prakt. Chem. Chem. Z. 1997;339:381-4.

37.Shibatani T, Nakamichi K, Matsumae H, inventors; European Patent Application. 1990.

38.Reetz M. Entrapment of biocatalysts in hydrophobic sol–gel materials for use in organic chemistry. Adv. Mater. 1997;9:943-54.

39.Reetz M, Schimossek K. Stereospecific synthesis of chiral alkinylogous amino acids. Chimia 1996;50:668-9.

40.Reetz M, Zonta A, Simpelkamp J. Efficient Heterogeneous Biocatalysts by Entrapment of Lipases in Hydrophobic Sol-Gel Materials. Angew. Chem.,Int. Ed. Engl. 1995;34:301-3.

41.Reetz M, Zonta A, Simpelkamp J. Efficient Immobilization of Lipases by Entrapment in Hydrophobic Sol-Gel Materials. Biotechnol Bioeng 1996;49:527-34.

42.Margolin A. Novel crystalline catalysts. Trends Biotechnol 1996;14:223-30.

43.Lalonde J, Navia M, Margolin A. Cross-linked enzyme crystals (CLECs): efficient and stable biocatalysts for preparative organic chemistry. Methods Enzymol 1997;286:443-64.

44.Khalaf N, al. e. Cross-linked enzyme crystals as highly active catalysts inorganic solvents. J. Am. Chem. Soc. 1996;118:5494-5.

45.Okahata Y, Mori T. Lipid-coated Enzymes as Efficient Catalysts in Organic media. Trends Biotechnol 1997;15:50-4.

46.Okahata Y, Fujimoto Y, Ijiro K. A lipid-coated lipase as an enantioselective ester synthesis catalyst in homogeneous organic solvents. J. Org. Chem. 1995;60:2244-50.

47.Svendsen A, Borch K, Barfoed M, Nielsen TB, Gormsen E, Patkar SA. Biochemical properties of cloned lipases from the *Pseudomonas* family. Biochim Biophys Acta 1995;1259(1):9-17.

48.Gotz F, Verheij HM, Rosenstein R. Staphylococcal lipases: molecular characterisation, secretion, and processing. Chem Phys Lipids 1998;93(1-2):15-25.

49.Arpigny J, Jaeger K-E. Bacterial lipolytic enzymes: classification and properties. Biochem J 1999;343:177-83.

50.Jaeger KE, Eggert T. Lipase for biotechnology. Protein technologies and comercial enzymes 2002;13:390-7.

51.Kazlauskas RJ, Weber HK. Improving hydrolases for organic synthesis. Curr Opin Chem Biol 1998;2(1):121-6.

52.Schmid RD, Verger R. Lipases: interfacial enzymes with attractive applications. Angew Chem Int Ed Engl 1998;37:1603-33.

53.Brady L, Brzozowski AM, Derewenda ZS, Dodson E, Dodson G, Tolley S, et al. A serine protease triad forms the catalytic centre of a triacylglycerol lipase. Nature 1990;343 (6260):767-70.

54.Winkler FK, D'Arcy A, Hunziker W. Structure of human pancreatic lipase. Nature 1990;343 (6260):771-4.

55.Cygler M, Schrag JD. Structure as basis for understanding interfacial properties of lipases. Methods Enzymol 1997;284:3-27.

56.Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, et al. The alpha/beta hydrolase fold. Protein Eng 1992;5(3):197-211.

57.Ransac S, Gargouri Y, Marguet F, Buono G, Beglinger C, Hildebrand P, et al. Covalent inactivation of lipases. Methods Enzymol 1997;286:190-231.

58.Labourdenne S, Cagna A, Delorme B, Esposito G, Verger R, Riviere C. Oil-drop tensiometer: applications for studying the kinetics of lipase action. Methods Enzymol 1997;286:306-26.

59.Brzozowski AM, Derewenda U, Derewenda ZS, Dodson GG, Lawson DM, Turkenburg JP, et al. A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. Nature 1991;351(6326):491-4.

60.Verger R. 'Interfacial activation' of lipases: facts and artifacts. Trends Biotechnol 1997;15:32-38.

61.Bell PJ, Sunna A, Gibbs MD, Curach NC, Nevalainen H, Bergquist PL. Prospecting for novel lipase genes using PCR. Microbiology 2002;148(Pt 8):2283-91.

62.Haas MJ, Joerger R. D. . In Food Biotechnology Microorganisms. Hui, Y. H.; Khachatourians, G. G., Eds., VCH: Weinheim 1995:549-88.

63.Beer HD, J.E.G. McCarthy, U.T. Bornscheuer, Schmid. RD. Biochim. Biophys. Acta. 1998:173-80.

64.Haas M, Allen J, Berka T. Cloning, expression and characterization of a cDNA encoding a lipase from *Rhizopus delemar*. Gene 1991;109:107-13.

65.Kugimiya W, Otani Y, Kohno M, Hashimoto Y. Cloning and sequence analysis of cDNA encoding Rhizopus niveus lipase. Biosci Biotechnol Biochem 1992;56(5):716-9.

66.Uyttenbroeck W, Hendriks D, Vriend G, Baere I, Moens L, Scharpe S. Molecular characterisation of an extracellular acid resistant lipase produced by *Rhizopus javanicus*. Biol. Chem. Hoppe Zeiler 1993;374:245-54.

67.Kohno M, Kugimiya W, Hashimoto Y, Morita Y. Purification, characterization and crystallization of two types of lipase from *Rhizopus niveus*. Biosci Biotechnol Biochem 1994;58:1007-12.

68.Santaniello E, Ferraboschi P, Grisenti P, Manzochi A. The biocatalytic approach to the preparation of enantiomerically pure chiral building blocks. Chem Crit Rev 1992;92:1071-140.

69.Sidebottom C, Charton E, Dunn P, Mycock G, Davies C, Sutton J, et al. *Geotrichum-Candidum* produces several lipases with markedly different substrate specificities. Eur J Biochem 1991;202:485-91.

70.Bertolini M, Laramee L, Thomas D, Cygler M, Schrag J, Vernet T. Polymorphism in the lipase genes of *Geotrichum candidum* strains. Eur J Biochem 1994;219:119-25.

71.Charton E, Macrae A. Specificities of immobilized *Geotrichum candidum* CMICC 335426 lipases A and B in hydrolysis and ester synthesis reactions in organic solvents. Enzyme Microb Technol 1993;15:489-93.

72.Charton E, Macrae A. Substrate specificities of lipases A and B from *Geotrichum candidum* CMICC 335426. Biochim Biophys Acta 1992;1123:59-64.

73.Hedrich H, Spener F, Menge U, Hecht H, Schmid RD. Large-scale purification, enzymic characterization, and crystallization of the lipase from *Geotrichum candidum*. Enzyme Microb Technol 1991;13:840-7.

74.Jacobsen T, Poulsen O. Separation and characterization of 61- and 57-kDa lipases from *Geotrichum candidum* ATCC 66592. Can. J. Microbiol. 1992;38:75-80.

75.Bertolini M, Schrag J, Cygler M, Ziomek E, Thomas DY, Vernet T. Expression and characterization of *Geotrichum candidum* lipase I gene, comparison of specificity profile with lipase II. Eur J Biochem 1995;228:863-9.

76.Vernet T, Ziomek E, Recktenwald A, Schrag JD, de Montigny C, Tessier DC, et al. Cloning and expression of *Geotrichum candidum* lipase II gene in yeast. Probing of the enzyme active site by site-directed mutagenesis. J Biol Chem 1993;268(35):212-9.

77.Lotti M, R. Grandori, F. Fusetti, S. Longhi, S. Brocca, A. Tramontano, et al. Genes Dev 1993;124:45-55.

78.Lotti M, A. Tramontano, S. Longhi, F. Fusetti, S. Brocca, Pizzi E, et al. Protein Eng 1993;7:531-4.

79.Rua ML, T. Diaz-Maurino, V.M. Fernandez, C. Otero, Ballesteros. A. Biochim. Biophys. Acta 1993;1156:181-9.

80.Dartois V, A B, K S, C C. Cloning, nucleotide sequence and expression of a lipase gene from *Bacillus subtilis* 168. Biochim Biophys Acta 1992;1131:253-60.

81.Nthangeni MB, Patterton H, van Tonder A, Vergeer WP, Litthauer D. Over-expression and properties of a purified recombinant *Bacillus licheniformis* lipase: a comparative report on Bacillus lipases. Enzyme Microb Technol 2001;28(7-8):705-12.

82.Ruiz C, Blanco A, Pastor FIJ, Diaz P. Analysis of *Bacillus megaterium* lipolytic system and cloning of LipA, a novel subfamily I.4 bacterial lipase. FEMS Microbiology Letters 2002;217:263-7.

83.Schmidt-Dannert C, Rua ML, Atomi H and Schmid RD. Thermoalkalophilic lipase of *Bacillus thermocatenulatus*.I. Molecular cloning, nucleotide sequence, purification and some properties. Biochim Biophys Acta 1996;1301:105-14.

84.Lee D, Koh Y, Kim K, Kim B, Choi H, Kim D, et al. Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol. Lett 1999;179:393-400.

85. Kim HK, Park SY, Lee JK, Oh TK. Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1. Biosci Biotechnol Biochem 1998;62(1):66-71.

86.Eggert T, Pencreac'h G, Douchet I, Verger R, Jaeger KE. A novel extracellular esterase from *Bacillus subtilis* and its conversion to a monoacylglycerol hydrolase. Eur J Biochem 2000;267(21):6459-69.

87.Kim HK, Choi HJ, Kim MH, Sohn CB, Oh TK. Expression and characterization of Ca^{2+}-independent lipase from *Bacillus pumilus* B26. Biochim Biophys Acta 2002;1583(2):205-12.

88.Lesuisse E, Schanck K, Colson C. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur J Biochem 1993;216(1):155-60.

89.Moller B, Vetter R, Wilke D, Foullois B, inventors; Alkaline *Bacillus* lipases, coding DNA sequences therefore and *Bacilli* which produce the lipase. 1991.

90.Rua ML, Schmidt-Dannert C, Wahl S, Sprauer A, Schmid RD. Thermoalkalophilic lipase of *Bacillus thermocatenulatus* large-scale production, purification and properties: aggregation behaviour and its effect on activity. J Biotechnol 1997;56(2):89-102.

91.Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, et al. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 1993;14(10):1023-31.

92.Bjellqvist B, Basse B, Olsen E, Celis JE. Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. Electrophoresis 1994;15(3-4):529-39.

93.Wilkins M, Gasteiger E, Bairoch A, Sanchez J, Williams K, Appel R, et al. Protein Identification and Analysis Tools in the ExPASy Sever. New Jersy: Humana Press; 1998.

94.Wong SL. Advances in the use of *Bacillus subtilis* for the expression and secretion of heterologous proteins. Curr Opin Biotechnol 1995;6(5):517-22.

95.Simons JW, van Kampen MD, Riel S, Gotz F, Egmond MR, Verheij HM. Cloning, purification and characterisation of the lipase from *Staphylococcus epidermidis*--comparison of the substrate selectivity with those of other microbial lipases. Eur J Biochem 1998;253 (3):675-83.

96.Simons JW, Adams H, Cox RC, Dekker N, Gotz F, Slotboom AJ, et al. The lipase from *Staphylococcus aureus*. Expression in *Escherichia coli*, large-scale purification and

comparison of substrate specificity to *Staphylococcus hyicus* lipase. Eur J Biochem 1996;242(3):760-9.

97.Vorderwulbecke T, Kieslich K, Erdmann H. Comparison of lipases by different assays. Enzyme Microb Technol 1992;14:631-9.

98.Kyte J and Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol 1993;157:105-32.

99.Dharmsthiti S, Luchai S. Production, purification and characterization of thermophilic lipase from *Bacillus* sp. THL027. FEMS Microbiol. Let 1999;179:241-6.

100.Simonen M, Palva I. Protein secretion in *Bacillus* species. Microbial Rev 1993;57:109-37.

101.Tjalsma H, Bolhuis A, Jongbloed JDH, Bron S, Dijl JM. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol Mol Biol Rev 2000;63:515-47.

102.Pollock MR, Richmond MH. Gene structure and extracellular secretion of Neisseria gonorrhoeae Ig A protease. Nature 1962;194:446-9.

103.Chen GFT, Inouye M. Role of the AGA-AGG codons, the rarest codons in global gene expression in *Eschericia coli*. Genes Dev 1994;8:2641-52.

104.Thanaraj TA, Argos P. Protein secondary structural types are differentially coded on messenger RNA. Protein Sci 1996;5:1973-83.

105.Netzer WJ, Hartl FU. Recombination of protein domains facilited by cotranslational folding in eukaryotes. Nature 1997;388:343-9.

106.Moeller B, Vetter R, Wilke D, Foullois B. Alkane lipases from *Bacillus* and the cloning of their genes. Patent number WO9116422. Applicant(s) Kali Chemie A.-G.

107.Zock J, Cantwell C, Swartling J, Hodges R, Pohl T, Sutton K, Rosteck P, McGilvray D, Queener S. The *Bacillus subtilis pnb*A gene encoding *p*-nitrobenzyl esterase: cloning, sequence and high-level expression in a *Escherichia coli*. Gene 1994;151:37-43.

108.Pohlenz HD, Boidol W, Schuttke I, Streber W. Purification and properties of an *Arthrobacter oxydans* P52 carbamate hydrolase specific for the herbicide phenmedipham and nucleotide sequence of the corresponding gene. J Bacteriol 1992;174:6600-7.

109.Schmidt-Dannert C. Recombinant microbial lipases for biotechnological applications. Bioorganic and Medical Chemistry 1999;1301:105-14.

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SCREENING, CHARACTERIZING, GENE CLONING AND NUCLEOTIDE ANALYZING A NOVEL EXTRACELLULAR LIPASE FROM THERMOTOLERANT *BACILLUS* ISOLATED FROM RANONG HOT SPRING

AN ABSTRACT

BY

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Kanokrat Namsrinuan. (2005). *Screening, characterizing, gene cloning and nucleotide analyzing a novel extracellular lipase from thermotolerant Bacillus isolated from ranong hot spring*. Master thesis, M.Sc. (Molecular Biology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Assoc.Prof.Dr. Kosum Chansiri, Asst.Prof. Kajeenart Photivejkul

The aim of this thesis was to perform the isolation, cloning, sequencing, nucleotide analyzing and characterization of lipase from thermotolerant *Bacillus* isolated from Ranong hot spring. Analysed for lipolytic activity on tributyrin agar plate, the isolated *Bacillus* designated as *Bacillus* sp. RN2 gave highest activity. The identification by biochemical characteristic test and hypervariant region of 16S rDNA phylogenetic analysis revealed that it is 98% homology to *Bacillus licheniformis*. The strain was Gram-positive, aerobic, rod-shaped, endospore-former and grew at 45°C. The highest activity of crude lipase was found at 60°C and pH 10.5 and revealed thermostability at 50°C by retaining 61% of acitivity with *p*-nitrophenyl laurate as a substrate. The enzyme exhibited high activity against *p*-nitrophenyl esters with chain lengths between 8 and 12 carbon atoms.

The sequence of the lipase gene from *Bacillus* sp. RN2 was isolated on the chromosomal amplified by polymerase chain reaction (PCR). The complete 576 nucleotide ORF was corresponded to the 192 amino acids with a predicted molecular mass of 20,282 Dalton with isoelectric point of 9.05. As revealed by SignalP program, a 17-amino acid stretch with the features of the typical *Bacillus* signal peptides was found at the N-terminal region of the protein. The motif AHSMG, corresponding to the consensus pentapeptide Ala-X_1-Ser-X_2-Gly common in all known *Bacillus* lipases was found. The catalytic apparatus of lipases, involving the triad serine, glutamate or aspartate, and histidine was placed in the newly isolated lipase at position 77 (S), 129 (D) and 152 (H). No significant similarities with other lipase sequences which deposited in GenBank. It suggested that the lipase of *Bacillus* sp. RN2 is novel. Based on the amino acid sequence homology analysis indicated that it was classified into subfamily I.4 bacterial lipase which have shown to be well suited for biotechnological applications.

การคัดเลือก, การศึกษาคุณสมบัติ, การโคลนยีนและวิเคราะห์นิวคลีโอไทด์ที่ทำการแยกจากบาซิลัสทนร้อนจากน้ำพุร้อน จังหวัดระนอง

บทคัดย่อ

ของ

กนกรัตน์ นามศรีนวล

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา

ตามหลักสูตรปริญญาวิทยาศาสตร์มหาบัณฑิต สาขาอณูชีววิทยา

เมษายน 2548

กนกรัตน์ นามศรีนวล. (2548). *การคัดเลือก, การศึกษาคุณสมบัติ, การโคลนยีนและวิเคราะห์นิวคลีโอไทด์ที่ทำการแยกจากบาซิลัสทนร้อนจากน้ำพุร้อน จังหวัดระนอง*. ปริญญานิพนธ์ วท.ม. (อณูชีววิทยา). กรุงเทพฯ: บัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ. คณะกรรมการควบคุม: รองศาสตราจารย์ ดร. โกสุม จันทร์ศิริ, ผู้ช่วยศาสตราจารย์ ขจีนาฏ โพธิเวชกุล

จุดมุ่งหมายของการศึกษาในครั้งนี้เพื่อทำการคัดเลือก, การศึกษาคุณสมบัติ, การโคลนยีนและวิเคราะห์นิวคลีโอไทด์ที่ทำการแยกจาก *Bacillus* ทนร้อนจากน้ำพุร้อน จังหวัดระนอง เมื่อทำการทดสอบบนอาหารแข็ง tributyrin พบว่า *Bacillus* sp. ที่มีชื่อว่า RN2 ให้ผลดีที่สุด จากการจัดลำดับอนุกรมวิธานระดับโมเลกุลโดยทำการศึกษายีนบริเวณ 16S rDNA มาใช้หาความสัมพันธ์ทางวิวัฒนาการของ *Bacillus* sp. RN2 กับ *Bacillus* สายพันธุ์ต่างๆ พบว่ามีความใกล้เคียงกับยีนดังกล่าวที่พบใน *Bacillus licheniformis* สูงถึง 98% ซึ่งให้ผลสอดคล้องเมื่อทำการทดสอบคุณสมบัติทางชีวเคมี จากลักษณะทางสัณฐานวิทยาพบว่า *Bacillus* sp. RN2 สามารถย้อมติดสีแกรมบวก ต้องการอากาศในการเจริญเติบโต มีลักษณะเป็นรูปแท่ง สามารถสร้าง endospore และเจริญได้ที่อุณหภูมิ 45 องศาเซลเซียส ส่วนสภาวะที่เหมาะสมต่อการทำงานของเอนไซม์ไลเปสจาก *Bacillus* sp. RN2 เมื่อใช้ *p*-nitrophenyl laurate เป็นสับเสตรทคือ อุณหภูมิ 60 องศาเซลเซียส pH 10.5 นอกจากนี้เอนไซม์ยังสามารถใช้อนุพันธ์ของ *p*-nitrophenyl ที่มีความยาวของคาร์บอนอะตอมระหว่าง 8 ถึง 12 เป็นสับเสตรทได้ดี

เมื่อทำการแยกยีนไลเปสโดยนำ *Bacillus* sp. RN2 มาสกัด DNA และเพิ่มปริมาณโดยวิธี PCR แล้วทำการหาลำดับนิวคลีโอไทด์ พบว่ายีนไลเปสประกอบด้วย 576 นิวคลีโอไทด์ ซึ่งจะสามารถถูกแปลออกมาเป็นลำดับของกรดอะมิโนได้ 192 ตัว และสามารถทำนายมวลโมเลกุลได้ 20282 ดาลตัน ค่า pI เท่ากับ 9.05 ต่อมาเมื่อทำการวิเคราะห์ด้วยโปรแกรม SignalP พบว่ายีนไลเปสมีกรดอะมิโนที่เป็นตำแหน่งของ signal peptide อยู่ 17 ตัว อยู่ที่บริเวณปลาย N นอกจากนี้ยังพบว่าตำแหน่งที่เป็นบริเวณอนุรักษ์ของยีนไลเปสที่พบใน *Bacillus* ประกอบด้วยลำดับของ Ala-X_1-Ser-X_2-Gly ซึ่งสอดคล้องกับบริเวณอนุรักษ์ที่พบในยีนไลเปสของ *Bacillus* sp. RN2 โดยมีลำดับของกรดอะมิโนเป็น อะลานีน, ฮีสติดีน, เซอรีน, เมทไธโอนีน และไกลซีน ส่วนกรดอะมิโน เซอรีน ตำแหน่งที่ 77, กลูตาเมท ตำแหน่งที่ 129 และฮีสติดีน ตำแหน่งที่ 152 น่าจะเป็นตำแหน่งที่มีบทบาทสำคัญในการเข้าทำปฏิกริยาระหว่างเอนไซม์กับสับเสตรท อย่างไรก็ตามเมื่อทำการเปรียบเทียบลำดับของกรดอะมิโนระหว่างยีนไลเปสของ *Bacillus* sp. RN2 กับสิ่งมีชีวิตชนิดอื่นๆ พบว่าไม่มีความใกล้เคียงกันอย่างมีนัยสำคัญ แสดงให้เห็นว่ายีนไลเปสจาก *Bacillus* sp. RN2 น่าจะเป็นชนิดใหม่ โดยยังสามารถถูกจัดให้อยู่ใน subfamily ที่ I.4 ซึ่งเป็นเอนไซม์ไลเปสในกลุ่มของ *Bacillus* ที่มีการนำมาประยุกต์ใช้ประโยชน์ทางด้านเทคโนโลยีชีวภาพกันอย่างกว้างขวาง