

MICROBIAL ENZYMES

From Earth to Space

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MICROBIAL ENZYMES

From Earth to Space

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ABSTRACT

Biototechnology can provide an unlimited and pure source of enzymes as an alternative to the harsh chemicals traditionally used in industry for accelerating chemical reactions. Enzymes are found in naturally occurring microorganisms, such as bacteria, fungi and yeast, all of which may or may not be genetically modified. Hydrolytic enzymes, such as lipases and proteases are much sought after as the biocatalysts of the future. Proteases have been widely used in industry and there is always scope for new enzymes to be utilized in existing applications as well as new ones. Lipases on the other hand, are projected to have exciting potential in the advancement of the bioprocessing industry in particular oleochemicals. Thermostable enzymes are always sought by the industries while solvent tolerant enzymes are becoming the vogue in view of their ability to function in low aqueous medium, suitable for synthetic reactions. Advances in science and technology have allowed researchers to improve enzymes either through modifications of enzyme producing microorganisms, or via direct changes to the enzymes themselves. By studying the relations between the structure of a protein and how it functions, methods to improve and engineer enzymes can be developed. One of the most widely used methods in studying protein structures is crystallography which can provide an insight into the protein structures and functions from global folds to the atomic details of bonding. The crystals are analyzed by x-ray diffraction to determine their structures, but this procedure is only possible for large and relatively pure proteins. As protein crystals are fragile, it is difficult for some proteins to grow adequately large or to obtain perfect protein crystals in Earth-based laboratories. The influence of gravity on Earth distorts the shape of the crystals resulting in imperfections in the structures. Microgravity can provide an ideal environment for the growth of

crystals. This is due to the fact that in a microgravity environment of space there are no gravity-induced effects such as sedimentation and convection that can disrupt the growth of these fragile protein crystals; thereby increasing the probability of growing larger, more perfect crystals. Crystals grown in microgravity generally have improved morphology, larger volume, better optical properties and higher diffraction limit compared with Earth-grown crystals.

INTRODUCTION

Biotechnology is a major participant in global industry, especially in the pharmaceutical, food and chemical industries. Enzyme reactions are the key to all biotechnological processes. Currently, enzyme applications in various industries have increased tremendously mainly in the US, UK, Japan and many countries in Europe and South America, involving multi-million ringgit of turnover. The global market for industrial enzymes increased from \$2.2 billion in 2006 to an estimated \$2.3 billion by the end of 2007. Worldwide sales of Industrial Enzymes are projected to exceed \$2.9 billion by year 2012, at an estimated compound annual growth rate (CAGR) of 4%. Proteases and lipases are important industrial enzymes. In Malaysia, although there have been numerous reports on the isolation, purification, characterization and application of these useful enzymes, there is no mention of its industrial production. We still remain an importer of these expensive enzymes, which are consumed especially by the detergent, food, textile and related chemical industries. No attempts have been made to produce the enzymes on a commercial scale using local resources. Some of the reasons for this include the high cost for the development of facilities and other equipment and poor support from the local industries in enzyme applications.

A new era of advances in enzyme technology now exists. Genetic engineering is being applied, not only to source valued enzymes in easier-to-grow microorganisms but also to modify and tailor enzyme protein properties to industrial requirements. Researchers are exploring extreme environments in search of enzymes having properties more in tune with industrial needs. They are applying molecular evolution to stretch and alter enzyme specificities. In addition, enzymes are being harnessed to work in partially organic solvents so that they can be used in new applications. The prospects

for this look bright, as new applications under exploration come to fruition and new technologies improve the needed performance characteristics enzymes must have for industrial applications.

More than 20 lipases and proteases are available in our laboratory. These enzymes are locally isolated (except for cold active enzymes) from various sampling sites throughout the country. All of them have been purified and characterized and most are expressed as recombinant enzymes either in *Escherichia coli* or *Pichia pastoris*. A number of them have been successfully crystallized and their crystal structures determined. Six of these enzymes were sent to space. The effect of microgravity on the first two enzyme crystals are now known while the remaining four will spend over 2 months more in space. Based on the above success stories, a number of local and international patents have been generated (Table 1).

Table 1 Patent deposit for lipases and proteases

Patents Granted

Overseas Patents

1. Invention: Lipase from *Geobacillus* sp.strain T1; USA Patent No.: US 7,319,029; Date granted: 15 January 2008; Country: USA
2. Invention: Method for Producing a Recombinant Thermostable *Geobacillus* T1 Lipase; European Patent No. EP-DK 1 624 056; Date granted: 19 November 2008; Country: Europe

Malaysian Patent

1. Invention: Novel *Geobacillus* microorganism; Malaysian Patent No: MY-136932-A; Date granted: 28 November 2008

Patents Filed

Overseas Patents

1. Invention: Production of Protease from *Bacillus stearothermophilus* F1; Application No: 11/062,089; Filing date: 18 February 2005; Country: USA
2. Invention: Novel lipase gene from *Bacillus sphaericus* 205y; Application No: 11/084,508; Filing date: 18 March 2005; Country: USA
3. Invention: Novel *Geobacillus* Microorganism; Application No: 2995-223872; Filing date: 2 August 2005; Country: Japan
4. Invention: Crystallization of Enzyme and Method for Producing Same; Application No: PCT/MY2007/000043; Filing date: 20 June 2007
5. Invention: Crystallization of Enzyme and Method for Producing Same; Application No: 12/151,045; Filing date; 1 May 2008; Country: USA
6. Invention: Crystal Growth under Microgravity Condition; Application No: No.12/415437; Filing date: 31 March 2009; Country: USA
7. Invention: Crystallization of Enzyme and Method for Producing Same (2); Application No: 12/424897; Filing date: 16 April 2009; Country: USA

Malaysian Patents

1. Invention: *Pseudomonas aeruginosa* strain K and production of protease therefrom; Application No: P1 20034117; Filling date: 29 October 2003
 2. Invention: Production of Protease from *Bacillus stearothersophilus* F1; Application No: P1 20040553; Filling date: 20 February 2004
 3. Invention: Novel lipase gene from *Bacillus sphaericus* 205y ; Application No: P1 20040958; Filling date: 18 March 2004
 4. Invention: Novel lipase gene from *Pseudomonas* sp. S5; Application No: P1 20045046; Filling date: 7/12/04
 5. Invention: Crystallization of Enzyme and Method for Producing Same; Application No: P1 20062931; Filling date: 21 June 06
 6. Invention: Production of thermostable L2 lipase from *Bacillus* sp.; Application No: P1 20071008; Filling date: 26 June 2007
 7. Invention: Modified Thermostable Enzyme and Method; Application No: P1 20071387; Filling date: 21 August 2007
 8. Invention: Crystal Growth under Microgravity Condition; Application No: P1 20084994; Filling date: 10 December 2008
 9. Invention: Low temperature enzyme and method thereof; Application No: P1 20090756; Filling date: 25 February 09
 10. Invention: Cold Active Enzyme and Method Thereof; Application No: P1 20092188; Filling date: 28 May 2009
-

THE SEARCH FOR BETTER BIOCATALYSTS

The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions are attractive alternatives to the more tedious and expensive chemical syntheses. Due to their high specificity, efficiency, biodegradability, low level by-products formation and high tendency to react under mild conditions, enzymes are used in a wide range of applications such as in detergent, food

and feed industries, human and animal health, waste treatment, and production of fine chemicals. The largest segment within the industrial enzyme market is the market for technical enzymes whereby nearly two-thirds are made up of detergent additives. The major obstacles that need to be overcome in detergent enzymes include relatively low catalytic activity with regard to the technical substrate, instability and high manufacturing cost.

Biotechnology programmes should begin with the biocatalyst. It is essential that the right enzyme is available and used in a particular process. Although there are numerous enzymes available commercially, the need for new enzymes with novel properties suited to a particular system is tremendous. It is also appropriate for such enzymes to be obtained from specific local environments. Our research programme targeted two types of enzymes from the hydrolases group: the lipases and proteases that play prominent roles in reactions with lipid substrates.

Microbial enzymes such as proteases and lipases have dominated the world market owing to their hydrolytic and synthetic reactions. The proteases are being used in large scale in industries such as the detergent, textile and food sectors (Salleh *et al.*, 2006a). However, their applications in chemical synthesis are still limited. On the other hand, the lipolytic enzymes are not used as much as the other enzymes in industrial applications. The main problems are the rather expensive cost of production, the instability factor and chiefly due to the heterogeneous nature of lipolytic reactions. However, as stated earlier, current technologies may be able to overcome the high production costs and the instability factors. The apparent ability of lipolytic enzymes (for that matter quite a number of other enzymes) to function well in organic solvents has somewhat enhanced their potential usage. Further interest has been kindled by the ability of the enzymes to perform synthetic reactions, thus opening up

a wide spectrum of new applications. It is no coincidence that lately, worldwide interest in the use of enzymes in the oleochemical industry has increased tremendously.

MICROBIAL LIPASES AND PROTEASES

Microbial proteases account for nearly 60% of total enzyme sales, of which detergent enzymes, predominantly alkaline proteases mostly from *Bacillus* sp., make up about half. Alkaline proteases in laundry detergents will facilitate the release of proteinaceous materials in the stains, such as those due to grime, blood and milk. Important commercial detergent proteases available are Subtilisin Carlsberg, Subtilisin BPN', Alcalase, Esparase and Savinase.

Lipases, on the other hand, have developed to be amongst the most important biocatalysts. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity which make them attractive for many industrial applications including detergents, food, flavor industry, ester and amino acid derivatives, baking, fine chemical, bioremediation, hard surface cleaning, leather and paper industry (Rahman *et al.*, 2006d). Their biocatalytic potential in both aqueous and nonaqueous media have shifted industrial inclinations towards utilizing them for a variety of reactions of immense importance, such as in the production of specialty chemicals through organic synthesis. As such, organic solvent-tolerant enzymes are preferred as they have obvious process advantages over other enzymes. In fact, lipases have gained importance, to a certain extent, over other enzymes, especially in the area of organic synthesis. The enantioselective and regioselective nature of lipases were useful for the resolution of chiral drugs, fat modification, biofuels and for the synthesis of personal care products and flavor enhancers. At present, lipases are the enzymes of choice for many disciplines such as biotechnologists,

microbiologists, organic chemists, biophysicists, pharmacists, and biochemists.

It is our objective that new and novel enzymes with high adaptability to local conditions be found. We have conducted an extensive screening programme for thermophilic microbes, mainly of fungi and bacteria, as possible sources of microbial lipases and proteases for industrial application. In addition, our focus is on the isolation of thermophilic and organic solvent-tolerant microorganisms producing thermostable and organic solvent-stable enzymes to be used in synthetic reactions. Samples were obtained from unique soils, palm oil mill effluents, hot springs and waste disposal sites all around the country. Thermal inactivation is the most common mode of enzyme deactivation. Hence, thermostable microbial enzymes, in particular, can provide the solution to the stability problems encountered at elevated temperatures in biotechnological applications, whereas organic tolerant enzymes can be used under non-aqueous solutions. However, screening for novel enzymes through microbial screening is tedious and time consuming while growing and optimizing the production of enzymes by these microorganisms demands a lot of patience. Substantial precise and careful work is required in order to purify and characterise the enzymes. It is not a task enjoyed by everyone, but this part of the work is essential and forms the basis of further development of the enzyme technology programme. Another approach is to use genetic engineering technology.

SCREENING AND ISOLATION OF MICROBIAL LIPASES AND PROTEASES

Over the past few decades, it has become clear that unique microbial communities can be found in the most diverse growth conditions, including extremes of temperature, pressure, salinity

and pH. These microorganisms, called extremophiles, encompass (hyper) thermophiles, psychrophiles, halophiles, alkaliphiles, acidophiles and piezophiles. Added to the list are organic solvent tolerant bacteria which are a novel and unique group of extremophilic microorganisms that thrive in the presence of very high concentrations of organic solvents. Archaea dominates the extremophiles group that has been identified to date. However, many extremophiles have also been recently identified and characterized as belonging to bacteria kingdoms. These microorganisms produce biocatalysts, categorised as extremozymes, that are functional under extreme conditions. Extremozymes have great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications. They can function at high temperatures (thermozyme) and under other extreme conditions such as alkalinity, acidity and in organic solvents. They are therefore, useful enzymes in detergent production, food processing, sugar chemistry and lipid and oil chemistry.

Thermophilic and hyperthermophilic enzymes, also called thermozymes, developed unique structures and structure-function properties of high thermostability and optimal activity at temperatures above 70°C. Some of these enzymes are active at temperatures as high as 100°C and above (Rahman *et al.*, 1997a, 1997b, Jongsareejit *et al.*, 1997, Rahman *et al.*, 1998a). Thermophiles grow optimally between 50 and 80°C producing enzymes with thermostability properties that fall between those of hyperthermophilic and mesophilic enzymes. These enzymes are usually optimally active between 60 and 80°C and typically do not function well below 40°C. Recent research has focused on the identification of extremozymes relevant as industrial biocatalysts resulting in enzymes that are active or stable under extreme conditions being isolated.

Thermostable and organic solvent tolerant lipases have been studied and reported (Salleh *et al.*, 2006). These microbes were isolated from local environments such as soils, palm oil branches, palm oil mill effluent ponds, hot springs (up to 90°C) and garbage dumps (Figure 1). For instance, *Rhizopus rhizopodiformis* was the first among its strains that produces lipase (Samad *et al.*, 1990). Similarly, a strain of *R. oryzae* was the first thermophilic strain that produced lipase (Salleh *et al.*, 1993, Razak *et al.*, 1999). In addition, six thermostable lipase producers, namely *Geobacillus zalihae* strain T1 (AY166603), *Bacillus* sp. strain 42 (AY763118), *Bacillus* sp. strain L2 (AY964643), *Aneurinibacillus thermoaerophilus* subsp. AFNA (EF032876), *Geobacillus* sp strain ARM^T (EF042975) and *Aneurinibacillus thermoaerophilus* strain HZ (DQ89019), were successfully isolated from various sampling sites. *Geobacillus zalihae* strain T1, identified as a new species, was isolated from palm oil mill effluent in Semenyih, Malaysia and it produces an extremely thermostable lipase with an optimum growth temperature of 70°C (Leow *et al.*, 2004). Meanwhile *Bacillus* sp. strain L2 was isolated from a hot spring in Perak, Malaysia (Shariff *et al.*, 2007). The bacterium is Gram-positive producing a thermostable and organic solvent tolerant lipase (Figure 2A). The optimum physical condition for L2 lipase production was determined to be at 70°C after 28 h of cultivation time, at pH 7.0, 150 rpm agitation rate and 1% starting inoculum size. As for nutritional factors, casamino acids, trehalose, Ca²⁺ and Tween 60 were found to be more effective for lipase production. A thermophilic *Bacillus* sp. strain 42 producing thermostable and organic solvent tolerant lipase was isolated from oil palm effluent in Kluang, Johor (Ethawell *et al.*, 2005). The isolate is a spore forming gram-positive bacterium (Figure 2B). Maximum lipase production by *Bacillus* sp. strain 42 was obtained at pH 7.0 when grown under shaking conditions (150 rpm) at 50°C for 72 h.

Aneurinibacillus thermoaerophilus subsp. AFNA and *Geobacillus* sp strain ARM, which are novel isolates producing thermostable and organic solvent stable lipases, were isolated locally from cooking oil-contaminated soil in Selangor, Malaysia. To add to the list, another isolate identified as *Aneurinibacillus thermoaerophilus* strain HZ was isolated from a hot spring in Sungai Kelah, Malaysia, producing a thermostable lipase. Strain HZ is an aerobic, gram-positive, rod shaped and endospore forming bacterium (Figure 2C). Maximum lipase production by *A. thermoaerophilus* strain HZ was obtained at pH 7.5 when grown under shaking conditions (150 rpm) at 60°C for 48h (Rahman *et al.*, 2009a).

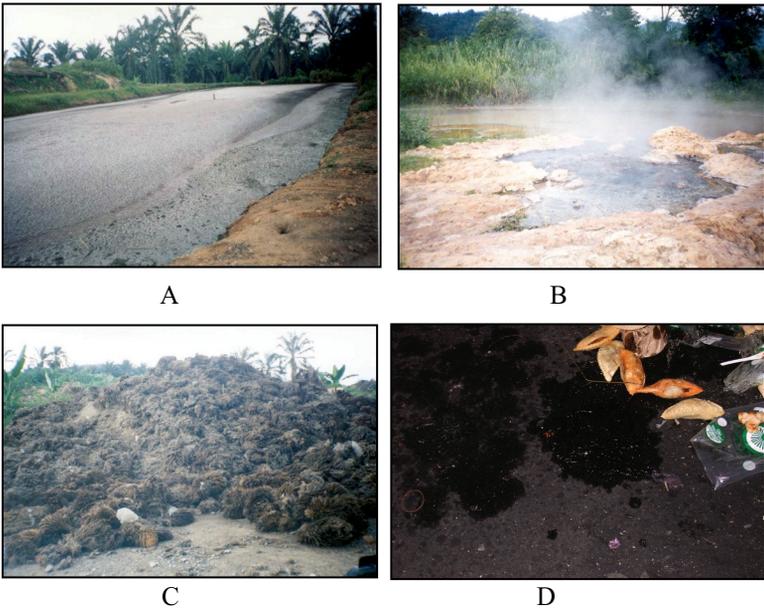


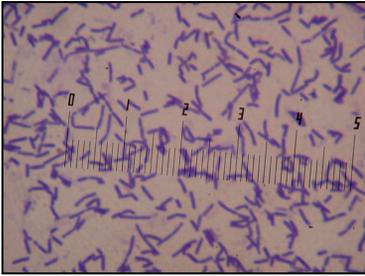
Figure 1 Sampling sites for thermostable lipase producers. **A:** Palm oil mill effluent in Semenyih, Selangor, **B:** Hot spring in Slim River, Perak, **C:** Palm fruit branches near a palm oil factory in Semenyih, Selangor **D:** Cooking oil-contaminated soil at night market in Serdang, Selangor.

Organic solvent-tolerant enzymes are preferred for their obvious process advantages over other enzymes. In organic solvents, the enzyme can catalyse reactions which are not otherwise possible in water. They are more stable with new behaviors such as “molecular memory” (Klibanov 2001). It was reported that enzymatic selectivity (substrate, stereo-, regio- and chemoselectivity) can be markedly affected and inverted (sometimes) by solvents (Klibanov 2001). Several methods have been investigated for stabilizing enzymes in organic solvents. However, little attention has been given to the isolation and study of microorganisms that can produce organic solvent-stable lipases and proteases. Organic solvent-stable enzymes are often found among organic solvent-tolerant bacteria. However, only a few natural enzymes which are organic solvent stable have been reported (Ogino *et al.*, 1994, 1995, 1999, 2000). These include lipases and proteases. The first organic solvent natural lipase reported was LST-03 lipase from an organic solvent tolerant bacterium *Pseudomonas aeruginosa* (Ogino *et al.*, 1994, 2000). The enzyme exhibited longer half-life in the presence of organic solvents, such as *n*-decane, ethyleneglycol, dimethyl sulfoxide, *n*-octane, *n*-heptane, isooctane and cyclohexane, than in their absence.

Different genus of organic solvent tolerant bacteria, known to be BTEX (Benzene, Toluene, Ethyl-Benzene and Xylene) degraders, have been isolated and screened for lipolytic activity. *P. aeruginosa* S5 (AY738722) was locally isolated from soil samples from the Serdang workshop area (Baharum *et al.*, 2003), *Bacillus sphaericus* 205y (AF435435) from a polluted area in Port Dickson (Hun *et al.*, 2003) and *Bacillus* sp. strain 42 from palm oil mill effluents in Johor (Eltaweel *et al.*, 2005). *P. aeruginosa* strain S5 is shown to be a BTEX (Benzene, Toluene, Ethyl-Benzene and Xylene) degrader. Strain S5 generates an organic solvent-tolerant lipase in

the late logarithmic phase of growth. Maximum lipase production was exhibited with peptone being utilized as the sole nitrogen source (Rahman *et al.*, 2006a). The strain produces a lipase that is stable in the presence of organic solvents such as n-hexane, cyclohexane, toluene and 1-octanol. The production of lipase by *P. aeruginosa* strain S5 was optimum at 37°C, pH 7.0 and 6% starting inoculum. Strain S5 is an aerobic, gram-negative rod and motile bacterium (Figure 2D). In another screening exercise, a total of 131 organic solvent tolerant isolates originated from soil samples were successfully isolated via direct plating method using 1% (v/v) of either benzene, toluene or a mixture of benzene and toluene as their sole carbon source. Six isolates demonstrated high tolerance of up to 75% (v/v) concentration of BTEX (n-benzene, toluene, ethyl benzene and *p*-xylene). These isolates were screened for lipolytic activity using triolein plates. Isolate 205y was found to produce the highest lipase yield in liquid medium, later identified as *B. sphaericus* by biochemical test and 16S ribosomal DNA sequence. Morphology study shows that the bacterium is a gram positive rod, occurring singly and in pairs, formed terminal spore and is motile (Figure 2E). The colony appeared to be circular, smooth, convex, entire and opaque on nutrient agar. Isolate 205y is able to grow up to 45°C. It was able to hydrolyse gelatin, casein, triolein and produces gas and acid in glucose and sucrose broth. In addition, it was able to utilize D-xylose and D-mannitol besides having the ability to reduce nitrate to ammonia. No work on *B. sphaericus* producing lipase has been reported thus far. A new strain of *Staphylococcus epidermidis* AT2, isolated from soil sample, is not only tolerant in solvent but also able to secrete an organic solvent-stable lipase. The bacterium demonstrated tolerance in up to 40% (v/v) concentration of Benzene, Toluene, Ethylbenzene and *p*-Xylene (BTEX). *Staphylococcus epidermidis* AT2 which is a Gram positive, cluster

form of coccus shows positive results in catalase and oxidase tests (Figure 2F). The single colony appeared small, white, circular and convex shaped on nutrient agar. All of the bacteria were proven to be organic solvent tolerant lipase producers.



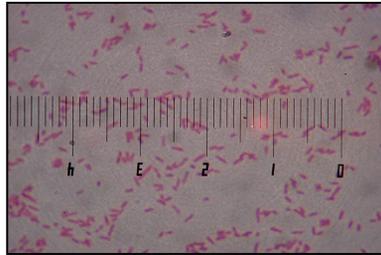
(A)
Bacillus sp strain L2



(B)
Bacillus sp. strain 42



(C)
Aneurinibacillus thermoaerophilus
HZ



(D)
Pseudomonas aeruginosa S5

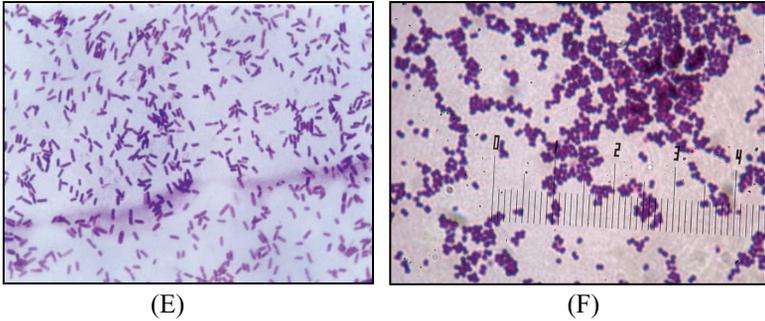


Figure 2 Gram-staining of various lipolytic bacteria isolated locally

Microbial proteases play important roles in biotechnological processes, accounting for approximately 60% of the total enzymes used. They find application in a number of biotechnological processes and are used in many industries. During our screening program for thermophilic protease producers, the *Bacillus stearothermophilus* strain F1 (Figure 3A) was isolated whereby it can not only grow up to 80°C but also produces a remarkable amount of protease activity under alkaline conditions (Razak *et al.*, 1995, Rahman *et al.*, 2003a). The enzyme, which showed remarkably higher thermostability when compared with commercially available enzymes, was isolated from decomposed oil palm branches (Rahman, *et al.*, 1994, Salleh *et al.*, 1997).

In addition to thermostable enzymes, an organic solvent tolerant protease was also isolated from a mesophilic bacterium isolated from contaminated soils of a wood factory in Selangor, Malaysia (Mahamad 2006). The isolate, identified as *B. pumilus* 115b (AY740598) was reported to be Benzene, Toluene, Xylene and Ethylbenzene (BTEX) tolerant and proven to be Polycyclic Aromatic Hydrocarbons (PAHs) degraders. To date, this is the

only report available on organic solvent tolerant protease from *B. pumilus* (Rahman *et al.*, 2007a). The bacterium was gram positive, straight rod, occurred singly or in pairs with terminal spore and motile (Figure 3C). Another interesting isolate is an organic solvent-tolerant bacterium designated as isolate 146 which is capable of producing an organic solvent-stable alkaline protease (Shafee *et al.*, 2005). Strain 146, identified as *Bacillus cereus* is a gram-positive, spore forming, nitrate-positive, rod-shaped organism which has the capability of hydrolyzing gelatine, starch and skim milk (Shafee *et al.*, 2006). An extracellular organic solvent-tolerant protease producer has been successfully isolated out of 11 isolates of benzene-toluene-xylene-ethylbenzene (BTEX) tolerant bacteria. This organic solvent-tolerant microorganism was found to be a polycyclic-aromatic-hydrocarbons (PAHs) degrader and subsequently identified as *Pseudomonas aeruginosa* strain K (Geok *et al.*, 2003). Strain K was an aerobic, gram negative, straight rod, motile bacterium (Figure 3B). It produced water-soluble, yellow-green fluorescent pigments on nutrient agar plate. Physical and nutritional factors affecting the protease production of strain K were investigated and reported (Rahman *et al.*, 2005a, 2005b). Recently, a thermostable organic solvent-tolerant protease producer identified as *Bacillus subtilis* strain Rand (EU233271) was isolated from contaminated soil in Port Dickson (Abushams *et al.*, 2009). Strain Rand was aerobic, rod-shaped, 0.7-0.8 μm in width and 2.5-3.0 μm in length (Figure 3D).

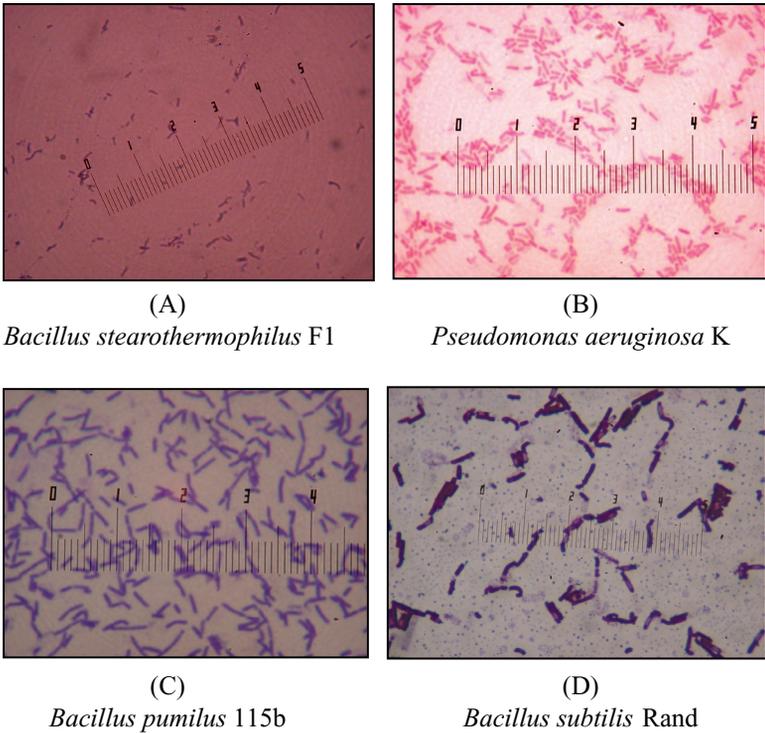


Figure 3 Gram-staining of various proteolytic bacteria isolated locally

Cold-adapted microorganisms, which are expected to produce cold-adapted enzymes, usually grow slowly even under appropriate conditions. Cold-adapted enzymes from psychrophilic microorganisms show high catalytic activity at low temperatures and can be highly expressed in such recombinant strains. Recently, systematic investigation was carried out in order to understand the rules governing their molecular adaptation to low temperatures. Psychrophilic enzymes have high specific activity at low and moderate temperatures and are inactivated easily by a moderate increase in temperature. In fact, many enzymes from psychrophiles

correlate high catalytic activity and low thermal stability at moderate temperatures, which can be partly explained by the increased flexibility of the molecule, compared with mesophilic and thermophilic enzymes. The specific activity of wild type cold enzymes and some of their recombinant forms have been determined for several enzymes produced by Antarctic and Arctic microorganisms including α -amylase, protease, xylanase, lipase, citrate synthase and β -Lactamase.

A lipase and protease producer was isolated from Antarctic sea ice near the Casey station in Antarctica. The organism was able to grow best at 4°C with no growth above 20°C and exhibited both lipolytic and proteolytic activities. The isolate, designated as stain PI12, was later identified as basidiomycete yeast, *Leucosporidium antarcticum* via 18S rDNA, 26S rDNA and ITS1/ITS2 gene sequence (EU621372 and FJ554838). Simple staining was performed to identify the cell morphology, arrangement and size of the psychrophilic isolate PI12. Using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), the surface features and the internal ultrastructure in thin sections of the PI12 cells were studied (Alias 2009). The cells were smooth and oval with measured size of about 3 μ m (Figure 4). Through both simple and negative staining a budding formation of the cells was seen, indicating a yeast strain. Figure 5 represents antarctic yeast Strain PI12 under magnification for the 3-D figure of the budding shape microorganism.

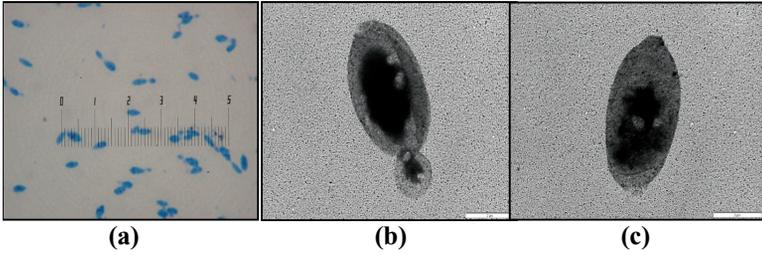


Figure 4 Photograph of Antarctic Microorganism Strain PI12. (a) Simple staining technique; (b) and (c) Negative staining technique with enlargement of 10,000x, bar = 2 μ m.

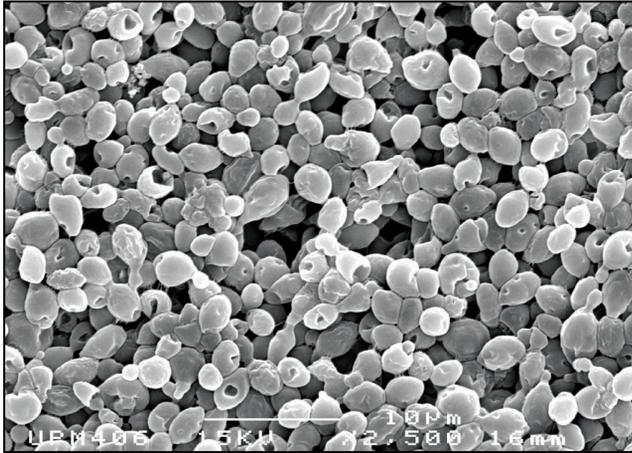


Figure 5 Scanning Electron Microscopy (SEM) Photograph of antarctic yeast Strain PI12.

While proteases and lipases are important in many industrial processes, the production of these enzymes at the level required by industries has remained a challenge. A number of microbial sources exist for the efficient production of these enzymes, however only a few selected strains of fungi and bacteria meet the criteria for commercial production.

NOVEL ISOLATES

During the screening programme to isolate local microorganisms producing lipases and proteases, new isolates were identified. Twenty-nine putative lipase producers were screened and isolated from palm oil mill effluents in Malaysia. Of these, isolate T1^T was chosen for further study as relatively higher lipase activity was detected quantitatively. The growth conditions for strain T1^T was 50-70°C and between pH 7 and 9 with optimum growth temperature and pH of 65°C and pH 6.5, respectively, in nutrient broth. This met the criteria of thermophilic bacteria which usually grow at temperatures beyond 50°C. A study, consisting of morphological and physiological characterization, 16S rRNA analysis, cellular fatty acid analysis, DNA composition, DNA/DNA hybridization, RiboPrint analysis, lipase gene analysis and protein profiling, was performed to authenticate the systematic position of this bacterium. Rahman *et al.*, (2007b) reported on the cellular morphology of isolate T1^T as Gram-positive bacteria, with rod-shaped cells of 0.8–1.0 width and 2.5–6.0 length, spore forming bacterium (Figure 6). The terminal spores are oval/cylindrical with swollen sporangium. The composition of the DNA base of isolate T1 is reported to be around 52.6% mol G + C. The 16S rRNA sequence of strain T1^T is a continuous stretch of 1519 bp (AY166603). The partial sequencing of the 16S rDNA shows 99.5% similarity to *Geobacillus kaustophilus* (DSM 7263^T) and *Geobacillus thermoleovorans* (DSM 5366^T). Construction of phylogenetic trees using the neighbour-joining method in determining the evolutionary relationship among a group of validly described closely related species is indicated in Figure 7. The T1 strain is an obligate aerobic bacterium (incapable of growing in an anaerobic environment) and can tolerate up to 2% NaCl. It gave positive results in the catalase test but not the oxidase test. In addition, it was able to hydrolyze starch but not gelatin

and casein. Acids are produced from L-arabinose and D-lactose but not D-mannitol. Table 2 recaps the comparison of strain T1^T with its closest phylogenetic neighbours in terms of biochemical, morphological and physiological properties.

Strain T1^T can be distinguished from *Geobacillus thermoleovorans* (DSM 5366^T) phenotypically by oxidase test, arabinose, mannitol, inositol, lactose and casein hydrolysis. However, strain T1^T differs from *Geobacillus kaustophilus* (DSM 7263^T) by lysozyme test, arabinose, mannitol, ribose, adonitol, lactose, gelatin and casein tests. Since the sequencing result and physiological data did not allow strain T1^T to be identified with one of the above mentioned species, further analysis needs to be carried out to verify its phylogenetic position. On the basis of 16S rDNA analysis, strain T1^T was shown to belong to the *Bacillus* rRNA group 5, and to be related to *Geobacillus thermoleovorans* (DSM 5366^T) and *Geobacillus kaustophilus* (DSM 7263^T). Chemotaxonomic data of cellular fatty acids supported the affiliation of strain T1^T to the genus *Geobacillus*. The results of physiological and biochemical tests, DNA/DNA hybridization, RiboPrint analysis, the length of lipase gene and protein patterns allowed genotypic and phenotypic differentiation of strain T1^T from its validly published closest phylogenetic neighbors. Strain T1^T therefore represents a novel species, for which the name *Geobacillus zalihae* sp. nov. is proposed, with strain type T1^T (=DSM 18318^T; NBRC 101842^T) (Rahman *et al.*, 2007).

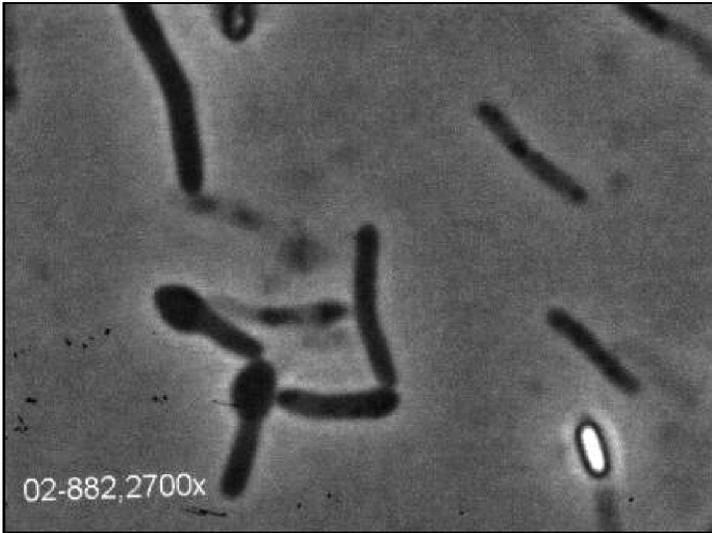


Figure 6 Phase contrast micrograph of strain T1 (magnification x 2700).

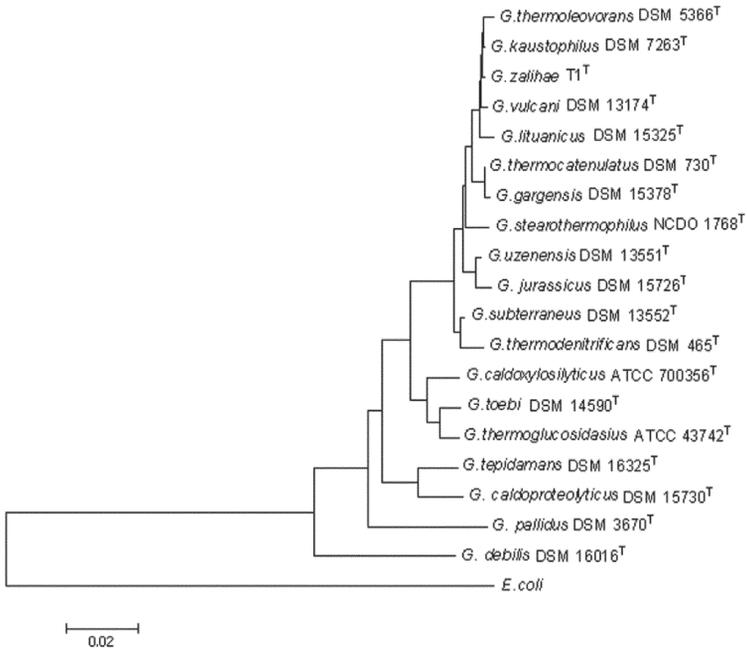


Figure 7 Phylogenetic position of *Geobacillus zalihae* T1^T with other validly described species of the genus *Geobacillus*. The members of genus *Geobacillus* used include *G. thermoleovorans* (DSM 5366^T); *G. kaustophilus* (DSM 7263^T); *G. vulcani* (DSM 13174^T); *G. lituanicus* (DSM 15325^T); *G. thermocatenulatus* (DSM 730^T); *G. gargensis* (DSM 15378^T); *G. stearothermophilus* (NCDO 1768^T); *G. uzenensis* (DSM 13551^T); *G. jurassicus* (DSM 15726^T); *G. subterraneus* (DSM 13552^T); *G. thermodenitrificans* (DSM 465^T); *G. caldxylosilyticus* (ATCC 700356^T); *G. toebi* (DSM 14590^T); *G. thermoglucosidasius* (ATCC 43742^T); *G. tepidamans* (DSM 16325^T); *G. caldoproteolyticus* (DSM 15730^T); *G. pallidus* (DSM 3670^T); and *G. debilis* (DSM 18016^T).

Escherichia coli were used as an out-group. Phylogenetic tree was inferred by using the neighbour-joining methods. The software package MEGA 3.1 was used for analysis

(Source: Rahman *et al.*, 2007b)

Table 2 The differentiating characteristics of the thermophilic strain T1^T and its type strains (Source: Rahman et al., 2007b).

| Characteristics | 1 | 2 | 3 |
|--------------------------|---------|---------|---------|
| Cell width (µm) | 0.8-1.0 | ≥0.9 | 1.5 |
| Cell length (µm) | 2.5-6.0 | ≥3 | 3.5 |
| Spores oval/cylindrical | O/C | O/C | O |
| Spores position | T | T | T |
| Oxidase | - | - | + |
| Growth in: | | | |
| NaCl 2 % | + | + | ND |
| 5 % | - | + | ND |
| Lysozyme broth | - | + | ND |
| Production of acid from: | | | |
| L-arabinose | + | - | - |
| D-xylose | + | + | V |
| D-mannitol | - | + | + |
| M-inositol | + | + | - |
| D-ribose | + | - | + |
| D-cellobiose | w | + | + |
| D-galactose | + | v | + |
| Adonitol | - | + | V |
| D-lactose | + | - | - |
| Hydrolysis of: | | | |
| Gelatin | - | + | - |
| Casein | - | + | + |
| Nitrate reduction | + | + | ND |
| pH range | 5.0-9.0 | 6.0-8.0 | 6.2-7.5 |
| Temperature (°C) | 50-70 | 37-68 | 45-70 |
| DNA G+C content (mol %) | 52.6 | 51-55 | 52-58 |

Taxa 1. *G. zalihae* T1^T; 2. *G. Kaustophilus* (DSM 7263^T); 3. *G. thermoleovorans* (DSM 5366^T). Characteristics are scored as: +, positive; -, negative; w, weak; v, variable within the group; ND, not determined. Data were obtained from the present study (*G. zalihae* strain T1^T), Nazina et al., [16], Sunna et al., [3], Kuisiene et al., [14] and Markossian et al., [54] (*G. thermoleovorans* DSM 5366^T and *G. Kaustophilus* DSM 7263^T). All strains were negative for anaerobic growth, growth at 30°C, VP reaction, indole production and acid from L-rhamnose, sorbitol. All strains were positive for catalase, hydrolysis of starch, use of citrate and acid from D-fructose and D-glucose (Source: Rahman et al., 2007b).

Recently, a novel bacterium producing a thermostable lipase was isolated. This gram-positive, thermophilic lipolytic bacterium, strain ARM, was isolated from cooking oil-contaminated soil from Taman Seri Serdang, Seri Kembangan, Selangor, Malaysia. A modeling study by response surface methodology and artificial neural network on culture parameters optimization for lipase production was carried out (Ebrahimpour *et al.*, 2008). As shown in the three dimensional plots in Figure 8, it was revealed that growth temperature, medium volume, inoculum size and incubation period had significant effects on lipase production of this bacterium.

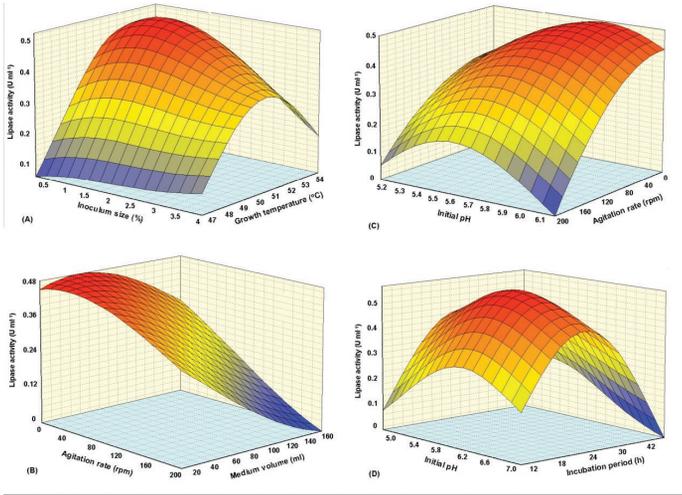


Figure 8 Three dimensional plot showing the effects of: (A) growth temperature, inoculum size; (B) agitation rate, medium volume; (C) initial pH, agitation rate; and (D) initial pH, incubation period, and their mutual effects on lipase production. Other variables are constant: growth temperature (52.3 °C), medium volume (50 ml), inoculum size (1 %), agitation rate (static condition), incubation period (24 h) and initial pH (5.8) (Source: Ebrahimpour *et al.*, 2008).

Colonies of strain ARM observed on nutrient agar plates were 1–2 mm in diameter, opaque, light-cream in colour, circular, convex on plate with entire margins. This strain is an obligate strictly aerobic micro-organism, Gram-positive, spore-forming, motile, occurring singly, in pairs and chains, catalase-positive and oxidase-negative. Cells of strain ARM are 2.5–>6.0 μm long and 0.8–1.0 μm in width with oval/cylindrical endospores at the terminal of the cells within a swollen sporangium [Figure 9 (1)].

Strain ARM grew at 45–70 °C and pH 5.5–9.0, with optimal growth at 55 °C and pH 7.0. The optimum NaCl concentration for growth was almost 0 % (w/v), but strain AFNA was able to grow at NaCl concentrations of up to 2 % (w/v). The DNA G+C content of this strain was 51.5 mol %. The major fatty acids were iso-17:0 (35.58 %), iso-15:0 (26.52 %), and anteiso 17:0 (14.19 %). Phylogenetic analysis based on 16S rRNA gene sequences showed similarities of between 98.8 % and 99.4 % to different species of genus *Geobacillus*. Despite phylogenetic relatedness to different species of the genus *Geobacillus*, the phenotypic signatures did not allow identification of strain ARM with one of the known species in this genus. Based on the identification keys of aerobic spore-forming bacteria, strain ARM^T stands in group IV region D1 that encompasses obligatory aerobic, starch-hydrolysing, nitrate reduction negative, with swollen sporangia, citrate positive, oxidase negative bacilli. On the basis of these findings, strain ARM is considered to represent a novel species of aerobic spore-forming bacteria.

Another gram-negative, thermophilic lipolytic bacterium, strain AFNA, was isolated from the same sampling site. The strain was strictly aerobic, spore-forming rods with oval endospores in the middle of the cells. Colonies of strain AFNA on nutrient agar plates were 5–15 mm in diameter, half-opaque, light-cream in colour, irregular, raised on plate with lobate margins. This strain is an obligate aerobic micro-organism, Gram-negative, spore-forming,

motile, occurred singly, catalase-positive and oxidase-negative. Cells of strain AFNA are 3.0–5.0 μm long and 0.9–1.0 μm in width with oval endospores in the middle of the cells within a swollen sporangium [Figure 9 (2)].

Strain AFNA grew at 45–60 °C and pH 5.5–7.5, with optimal growth at 53 °C and pH 6.9. The optimum NaCl concentration for growth was almost 0 % (w/v), but strain AFNA was able to grow at NaCl concentrations of up to 2 % (w/v). The DNA G+C content of this strain was 45.1 mol %. The major fatty acids were iso-15:0 (60.95 %) and iso-17:0 (30.67 %). Strain ARM^T has a cell wall type A1 γ peptidoglycan with *meso*-diaminopimelic acid-direct. The cell wall sugars of strain ARM^T are glucose and lower amounts of xylose. The polar lipid pattern on TLC revealed the presence of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phospholipid and phosphoglycolipids (PLG1-PLG3). The DNA G+C content of this strain is 51.5 mol %.

Phylogenetic analysis based on 16S rRNA gene sequences showed that strain AFNA was most closely related to *Aneurinibacillus thermoaerophilus* (DSM 10154^T) where their 16S rRNA gene sequences showed 99.8 % similarity. The DNA–DNA hybridization value between strain AFNA and *Aneurinibacillus thermoaerophilus* DSM 10154^T was 81.6 % (75.4 %). Despite similar characteristics and phylogenetic relatedness to *Aneurinibacillus thermoaerophilus* DSM 10154^T, strain AFNA could be clearly distinguished from this strain type, based on Gram staining, NO₂ from NO₃, catalase and oxidase tests, DNA G+C content, growth temperature and pH ranges, acid from L-arabinose, D-xylose and D-fructose, use of citrate, assimilation of fructose, glutamate and mannitol. On the basis of these findings, strain AFNA (=DSM 21497= NCIMB 41584) is considered to represent a novel subspecies of the *Aneurinibacillus thermoaerophilus* DSM 10154^T.

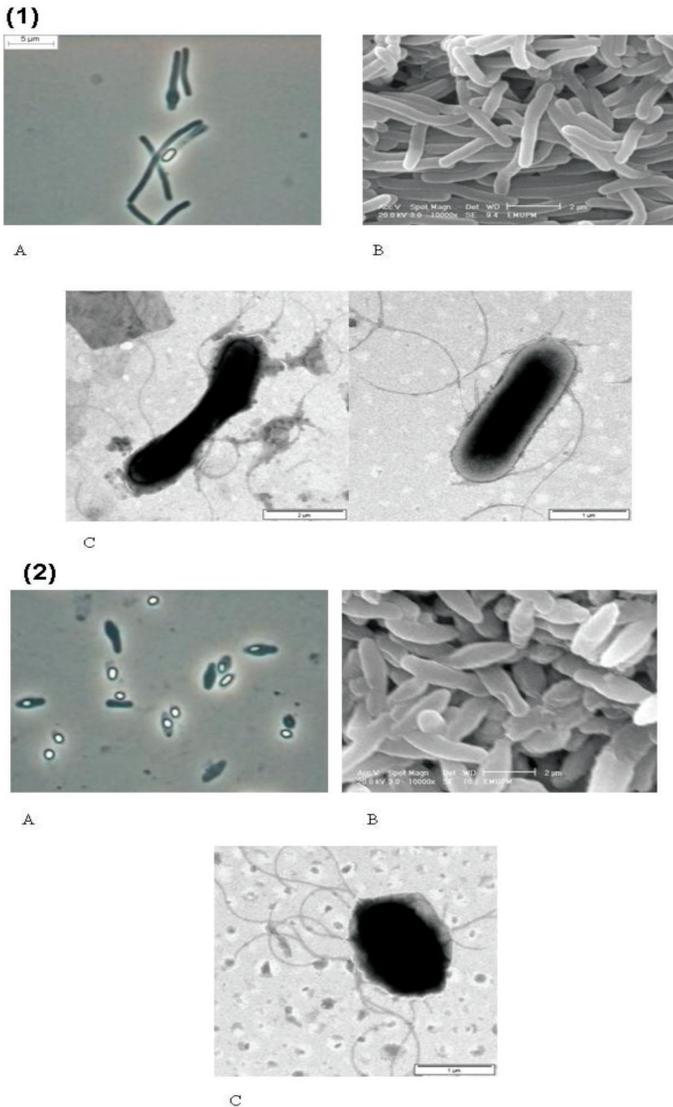


Figure 9 Phase contrast (A) SEM (B) and TEM after **negative staining** (C) images of cells and flagella of strains (1) ARM^T and (2) AFNA^T

CHARACTERIZATION OF MICROBIAL LIPASES AND PROTEASES

Various lipases and proteases were purified and characterized. Thermostable lipases with optimum temperature ranging from 55 to 80°C were locally isolated (Leow *et al.*, 2004, Muked 2005, Eltaweel *et al.*, 2005). With respect to optimum activity temperature, among the 3 enzymes, wild-type L2 exhibited higher temperature with a half-life of 2.5 h at 80°C. Purified T1 mature lipase on the other hand, displayed optimum temperature and pH of 70°C and pH 9, respectively (Leow *et al.*, 2007). It was stable up to 65°C with a half-life of 5 h 15 min at pH 9 (Figure 10). The molecular mass of T1 lipase was determined to be approximately 43 kDa by gel filtration chromatography. It was stable in the presence of 1 mM metal ions Na⁺, Ca²⁺, Mn²⁺, K⁺ and Mg²⁺, but inhibited by Cu²⁺, Fe³⁺ and Zn²⁺. The T_m for T1 lipase was around 72.2°C, as revealed by denatured protein analysis of CD spectra (Figure 11). Various characterizations were carried out on purified Lip 42. The purified fusion lipase was most active at 70°C and pH 8.0, and stable in a broad pH range of 7-10. Results shown in Table 3 summarizes the pH activity and stability, the general affects of metal and surfactants, and its specificities for substrates and natural oils for the enzyme. While T1 and 42 lipases are alkaline lipases, L2 lipase was proved to be a neutral enzyme (Figure 12). Lipase L2 was strongly inhibited by EDTA (100%) at 5mM. The purified lipase was found reactive at a temperature range of 55-80 °C and at pH 6-10. The optimum activity was found to be at 70 °C in pH 9 (Figure 13). The metal ions Ca²⁺ strongly activated the lipase activity by 100% and 200% when treated with the concentrations 1mM and 5 mM, respectively. Presence of K⁺, Na⁺ and Mn²⁺ also increased the lipase activity by more than 100%.

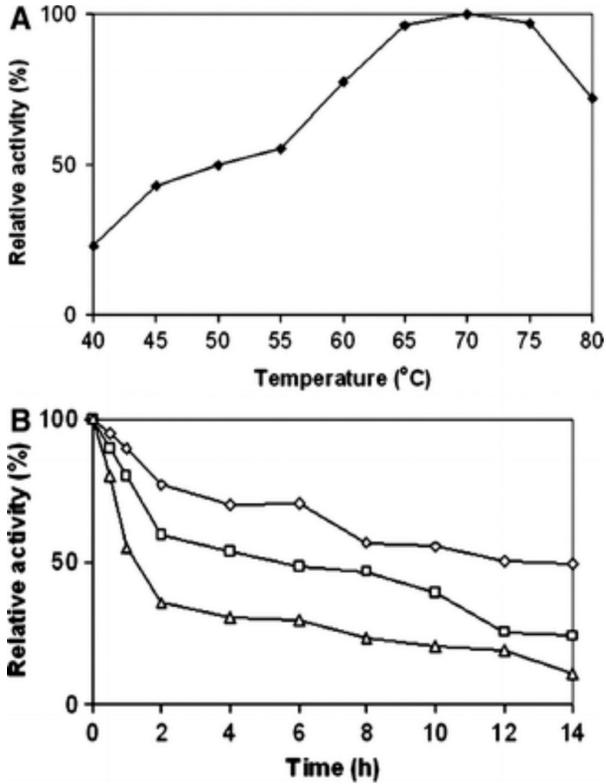


Figure 10 Effect of temperature on T1 lipase activity (a) and stability (b). The purified T1 lipase was assayed at different temperatures (40–80°C). For the stability test, the purified T1 lipase was incubated at different temperatures: 60°C (*open diamond*); 65°C (*open square*); 70°C (*open triangle*) under shaking conditions for different durations. The residual activity was measured colorimetrically with olive oil as substrate (1:1, v/v) under shaking conditions (Source: Leow *et al.*, 2007).

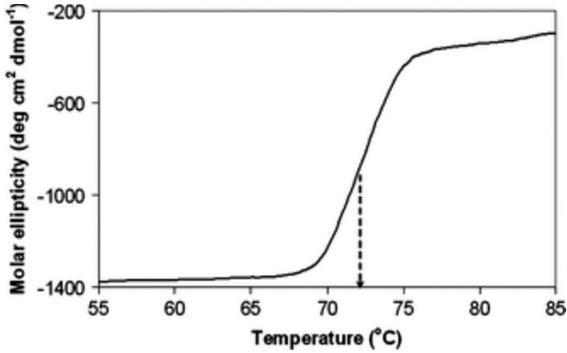


Figure 11 Denatured protein analysis of T1 lipase. The thermal denaturation of T1 lipase was monitored by following the ellipticity at 220 nm from temperatures 55 to 85°C. The arrow indicates the T_m value of T1 lipase (Source: Leow *et al.*, 2007).

Table 3: Characterization of the purified lipase Lip 42. Effects of pH and temperature on stabilities and activities, substrates, metals and surfactants (Source: Hamid *et al.*, 2009a).

| Characteristic | | Purified lipase Lip 42 |
|-----------------------|--------------------|------------------------|
| pH optimum | | 8 |
| pH stability | | 7-9 |
| Temperature optimum | | 70°C |
| Temperature stability | | |
| 60°C | | 315 min |
| 65°C | | 120 min |
| 70°C | | 45 min |
| Substrate specificity | | |
| Tricaprylin C8 | | 100% |
| Tricaprylin C10 | | 92% |
| Olive oil | | 100% |
| Corn oil | | 90% |
| Sunflower oil | | 88% |
| Metal | Concentration 1 mM | Concentration 5 mM |

| | | |
|------------|---------------|------------------|
| K | 115% | 128% |
| Zn | 26% | 0% |
| Mg | 105% | 113% |
| Ca | 113% | 118% |
| Fe | 44% | 36% |
| Surfactant | Concentration | Concentration 1% |
| Tween 80 | 0.1% | 15 to 25% |
| Tween 60 | 100% | |

The '%' represents activities relative to maximal activity being measured. For effects of metal on enzyme, the '%' records the residual activity to that of control.

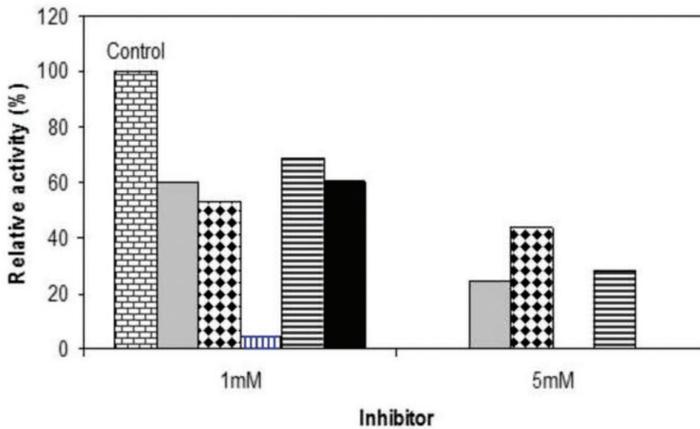


Figure 12 Effect of inhibitor on purified recombinant L2 lipase activity. The L2 lipase was incubated with 1 mM and 5 mM of inhibitor (except Pepstain A) at 65°C for 30 min prior to lipase assay in standard conditions. The inhibitors tested are: DTT (■); PMSF (▣); EDTA (▤); 2-mercaptoethanol (▥); Pepstain-A (■)

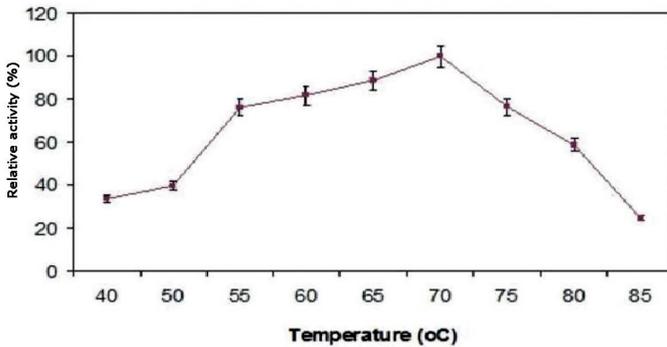


Figure 13 Temperature profile of purified recombinant L2 lipase. The L2 lipase was assayed with olive oil as substrate in temperatures ranging from 40°C to 80°C at pH 9 for 30 min

Protease from *B. stearrowthermophilus* strain F1 was purified, characterized and identified as a head stable alkaline protease (Rahman *et al.*, 1994). With an optimum temperature of 85°C, the F1 protease exhibited a higher optimum temperature than two commercial proteases, i.e. thermolysin and subtilisin (Salleh *et al.*, 1997). However, this property was not retained when the F1 protease was expressed in *E. coli* system. Recombinant F1 protease exhibits optimal temperature protease activity at around 80°C (Zhibiao *et al.*, 2003). The purified enzyme showed a pH optimum of 9.0, temperature optimum of 80 °C, and was stable at 70 °C for 24 h in the pH range from 8.0 to 10.0. The enzyme exhibited a high degree of thermostability with a half-life of 4 h at 85 °C 25 min at 90 °C (Figure 14), and was inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The recombinant enzyme might be improperly folded and/or assembled at 37°C while the native enzyme was produced at a higher growth temperature (65°C). In the case of thermostable proteins, some recombinant proteins

produced by *E. coli* exhibit less thermostability (Rahman *et al.*, 1997a)

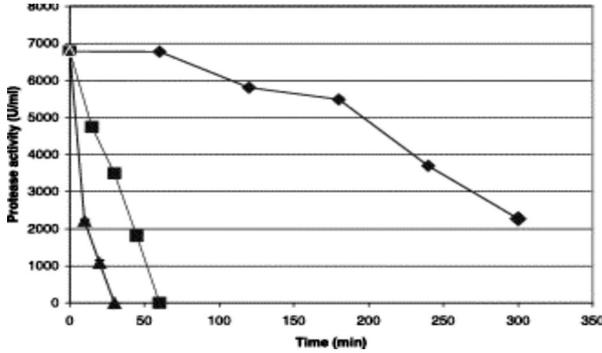


Figure 14 Effect of different temperatures on enzyme stability. *Note:* The enzyme in Tris-HCl-2 mM Ca²⁺ (pH 9.0) was pre-incubated various times at 85 °C (-♦-), 90 °C (-■-), and 95 °C (-▲-). Error bars represent standard deviations of triplicate determinations (Source: Zahibino *et al.*, 2003)

Enzymes in organic solvents have been largely studied and employed in the areas of product synthesis, food production and even biochemical analysis. The use of organic solvents is especially advantageous to transform substrates that are unstable or poorly soluble in water. However, in general, enzymes are not stable in the presence of organic solvents and are apt to denature, which has led to the development of several methods to stabilize them. Nevertheless, enzymes that remain stable in the presence of organic solvents, without the need for special stabilization measures, could be very useful in industry (Ogino *et al.*, 2000). Consequently, many researchers have investigated the stability of enzymes in organic solvents. To obtain lipases and proteases that are active and stable in the presence of organic solvents, we isolated solvent-tolerant microorganisms from various sampling sites. Purified lipases

from *Pseudomonas* sp. S5, *B. sphaericus* 205y and *Bacillus* sp. strain 42 exhibited great stability and high activity in most of the organic solvents tested (Rahman *et al.*, 2005c, Ethawel *et al.*, 2005, Ropaning *et al.*, 2006, Hamid *et al.*, 2009a). The purified organic solvent tolerant lipase from *Pseudomonas* sp. S5 showed optimum temperature and pH at 45°C and 9.0, respectively (Rahman *et al.*, 2005c). The enzyme exhibited highest stability in the presence of various organic solvents such as *n*-dodecane, 1-pentanol, and toluene (Table 4). Therefore, the S5 lipase is naturally stable in organic solvents and holds potential for use in organic synthesis and related applications. The enzyme activity was not affected by 1 mM EDTA, indicating that the lipase from *Pseudomonas* sp. S5 was not a metalloenzyme. The purified OST-205y, on the other hand was very stable with enhanced stability in the presence of methanol and DMSO (Ropaning *et al.*, 2006). This unique enzyme is stable in these hydrophilic solvents, which normally inactivates most enzymes. In addition, a thermophilic *Bacillus* sp. strain 42 producing an organic solvent tolerant lipase was isolated from oil palm mill effluent. The lipase produced was a thermostable enzyme with optimum activity at 70°C. Based on solvent stability the purified Lip 42 showed different residual activity profiles depending on solvents and temperatures (Hamid *et al.*, 2009a). Lip 42 was found to be stable in polar organic solvents such as DMSO, DMF, acetone, methanol, heptanol and octanol, which could make it a potential biocatalyst for use in industrial biodiesel production (Figure 15).

Table 4 Stability of purified S5 lipase in the presence of various organic solvents (Source: Rahman *et al.*, 2005c)

| Organic solvent | Log <i>P</i> | Relative activity | |
|-----------------------|--------------|-------------------|----------------|
| | | 30 min incubation | 2 h incubation |
| None | — | 1.00 | 1.00 |
| 1-Pentanol | 1.3 | 5.40 | 36.70 |
| Benzene | 2.0 | 30.60 | 0.00 |
| Chloroform | 2.0 | 23.30 | 5.33 |
| Toluene | 2.5 | 1.66 | 0.00 |
| 1-Octanol | 2.9 | 0.29 | 44.50 |
| Cyclohexane | 3.2 | 31.80 | 23.20 |
| <i>n</i> -Hexane | 3.6 | 18.40 | 0.00 |
| 1-Decanol | 4.0 | 3.48 | 0.00 |
| <i>n</i> -Decane | 5.6 | 4.51 | 0.19 |
| <i>n</i> -Dodecane | 6.6 | 1.17 | 4.95 |
| <i>n</i> -Tetradecane | 7.6 | 2.84 | 0.68 |
| <i>n</i> -Hexadecane | 8.8 | 1.32 | 0.15 |

Note. Data are means of triplicate determinations. The enzyme and organic solvent were mixed in a 3:1 ratio, and the mixture was incubated at 37 °C with shaking at 150 rpm for 2 h and assayed for the lipase activity. Activities of S5 lipase in the presence of various organic solvents are shown as values relative to those in the absence of an organic solvent.

Microbial Enzymes: From Earth to Space

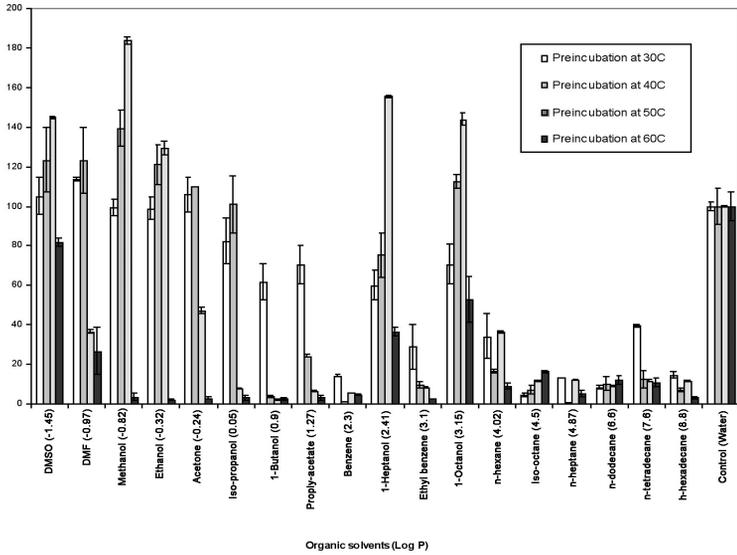


Figure 15 Solvent stabilities of lipase 42 in various organic solvents with range of log P values (in parenthesis). The purified enzymes were pre-incubated for 30 min at 25% v/v solvent with shaking rates at 150 strokes per min, and assayed for residual activity (Source: Hamid *et al.*, 2009a).

Very few organic solvent stable proteases have been characterized. We have isolated an organic solvent tolerant bacterium, *Pseudomonas aeruginosa* strain K, producing an organic solvent tolerant protease (Geok *et al.*, 2003). The purified K protease showed stability and activation in the presence of organic solvents with log $P_{a/w}$ values equal or more than 4. Stability studies using various organic solvents showed that this enzyme, was not only stable but also activated by 1-decanol, isooctane, decane, dodecane and hexadecane and can therefore, be used for reaction in media containing organic solvents (Rahman *et al.*, 2006b). The enzyme showed stability and activation in the presence of organic solvents with log $P_{a/w}$ values

equal or more than 4.0 (Figure 16). After 14 days of incubation, the purified protease was activated 1.11, 1.82, 1.50, 1.75 and 1.80 times in 1-decanol, isooctane, decane, dodecane and hexadecane, respectively (Table 5). Reports on organic solvent tolerant proteases from genus *Bacillus* are rare. Recently, we reported on an organic solvent tolerant alkaline protease from *Bacillus cereus* strain 146 (Shafee *et al.*, 2006). Activity of the protease was drastically increased in the presence of 1-decanol, isooctane, n-dodecane and n-tetradecane, but reduced in the presence of ethyl acetate, benzene, toluene, 1-heptanol, ethylbenzene and hexane.

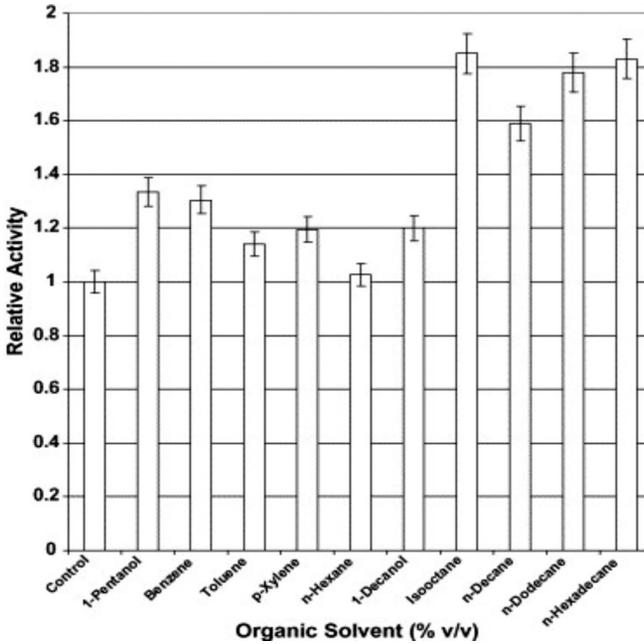


Figure 16 Organic solvent stability of the purified protease. One millilitre of organic solvent was added to 3.0 mL of the purified protease and incubated at 37 °C, 150 rpm for 30 min. The protease activity measured without the presence of organic solvent was taken as 1.0 (control) (Source: Rahman *et al.*, 2006b).

Table 5 Organic solvent stability of the purified protease after 14 days treatment (Source: Rahman *et al.*, 2006b).

| <i>Solvents</i> | $\log P_{o/w}$ | Stability |
|---------------------|----------------|------------------|
| <i>None</i> | – | 1.00 |
| <i>1-Pentanol</i> | 1.30 | 0.67 |
| <i>Benzene</i> | 2.00 | 0.65 |
| <i>Toluene</i> | 2.50 | 0.60 |
| <i>p-Xylene</i> | 3.10 | 0.52 |
| <i>n-Hexane</i> | 3.50 | 0.64 |
| <i>1-Decanol</i> | 4.00 | 1.11 |
| <i>Isooctane</i> | 4.50 | 1.82 |
| <i>n-Decane</i> | 5.60 | 1.50 |
| <i>n-Dodecane</i> | 6.60 | 1.75 |
| <i>n-Hexadecane</i> | 8.80 | 1.80 |

One mL of organic solvent was added to 3 mL of the purified protease and incubated at 37 °C, 150 rpm for 14 days. The remaining proteolytic activity relative to the non-solvent containing control (0%, v/v) was shown as the stability.

A psychrophilic microorganism (cold loving) designated as PI 12 was isolated from the Antarctic sea ice near Casey station, Antarctica. Isolate PI 12 was identified as *Leucosporodium antarcticum*, a psychrophilic yeast producing extracellular lipase and protease. LipPI12 lipase was successfully cloned and sequenced. Its sequence and the predicted structure revealed a bifunctional ability. No such report has been seen so far in terms of enzymes isolated from psychrophiles. Characterization of the bifunctional ability of the LipPI12 as lipase and protease, in terms of optimum temperature, pH, metal ion substrate specificity, organic solvents stability, surfactants and inhibitors, was carried out (Ali 2009). The bifunctional activities of LipPI12 exhibited different enzymatic properties for its lipase and protease. For example, the

enzyme exhibited an optimum temperature of 20°C for its lipase activity and a higher temperature of 40°C for protease (Figure 17). Further, as shown in Figure 18, the lipase was found to have a good stability in the presence of hexadecane, benzene, dodecane and heptane, with highest stability in dimethylsulfonyl (resulting about 80% increase of activity). Surprisingly, for its protease activity, most of the LipPI12 activities were enhanced in the presence of hydrophilic solvents ($-1.22 < \log P_{o/w} < 0$), even though the enzymes were generally inactivated by the solvents at this range of $\log P_{o/w}$. In contrast to lipase, the protease exhibited tolerance towards organic solvents of high hydrophilicity and hydrophobicity, such as the dimethylsulphonyl and hexadecane, respectively. Nevertheless, some LipPI12 activities were observed to be destabilized by water-immiscible organic solvents with $\log P_{o/w} > 4$, although the enzymes are little affected by these solvents. In fact, the protease also showed significant resilience to benzene, dodecane and heptane, with activity still more than 100% as compared to the reaction in water. This led to the understanding that the protease is more tolerant than the lipase. However, different profiles were shown by these two enzymes of LipPI12, although they existed as a single unit which might somehow elucidate a new adaptation in terms of their molecular architecture. LipPI12 lipase and protease are remarkable enzymes which have highlighted a way of surviving the cold and thus, offer potential applications in the future.

Microbial Enzymes: From Earth to Space

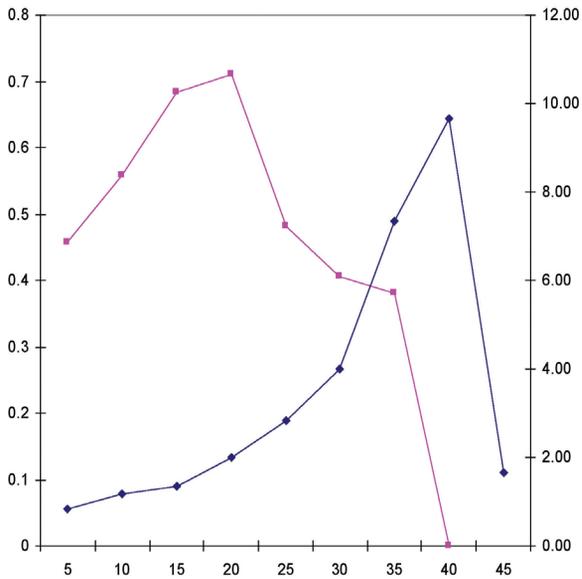


Figure 17 Optimum temperature profile of LipPI12 lipase and protease. Lipase and protease assays were done at 20°C using olive oil and azocasein as substrates respectively. Lipase activity (■); Protease activity (◆).

The temperature stability of lipases and proteases has been regarded as the most important characteristic for use in industry. However, low temperature stability is favorable for some purposes. For example, heat-labile enzymes can be easily inactivated by treatment for short periods at relatively low temperatures after being used for processing of food and other materials. Considering these variable useful properties (stability at elevated temperatures, and in the presence of organic solvents), all the above enzymes may find potential applications such as in the food industry, oil processing, production of surfactants, oil processing, detergents, peptide and oligopeptide synthesis, pesticides, environmental management and

leather industries. We have isolated a number of interesting lipases and proteases, as mentioned above, from microorganisms isolated all over Malaysia. These enzymes possess novel characteristics which offer potential use as industrial catalysts with high thermostability and organic solvent stability. Considering the fact that enzyme consumption in Malaysia will increase tremendously in the near future, production of enzymes within the country will definitely be a cost saving approach for local industries.

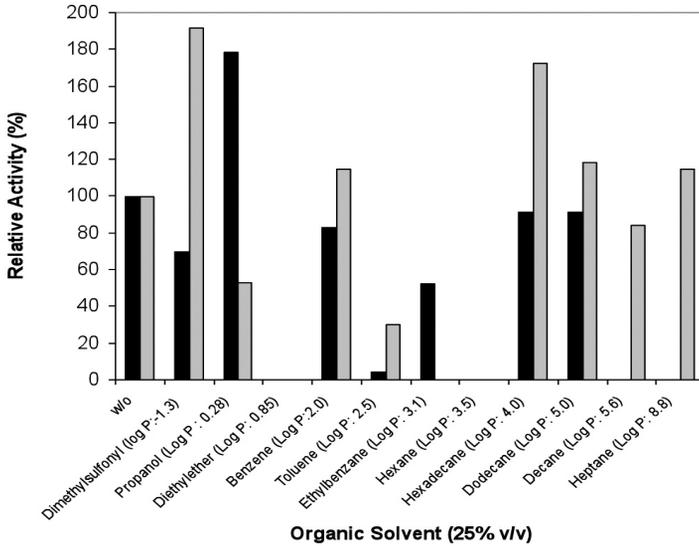


Figure 18 Effect of various organic solvents on LipPI12 activity. Protease activity (■); Lipase activity (■)

Genetic Engineering of Lipases and Protease

Gene cloning is a rapidly progressing technology that has been instrumental in improving our understanding of the structure-

function relationship of genetic systems. It provides an excellent method for the manipulation and control of genes. More than 50% of industrially important enzymes are now produced from genetically engineered microorganisms. Several reports have been published in the past decade on the isolation and manipulation of microbial genes with the aim of (i) enzyme overproduction by the gene dosage effect; (ii) studying the primary structure of the protein and its role in the pathogenicity of the secreting microorganism; and (iii) protein engineering to locate the active-site residues and/or to alter the enzyme properties to suit commercial applications.

Lipase and protease genes from bacteria, fungi, and viruses have been cloned and sequenced. These genes were isolated either via polymerase chain reaction (PCR) or genomic library cloning. In order to utilize PCR technology to isolate the gene, degenerate primers have been designed to amplify the region encoding the enzymes. Several papers regarding cloning of protease and lipases genes have been published. For instance, thermostable lipase from *Geobacillus* sp. strain T1 has been cloned successfully (Leow *et al.*, 2004). The gene encoding T1 lipase was cloned in TOPO TA pBAD vector using PCR technology. Sequence analysis revealed an open reading frame of 1,251 bp in length which codes for a polypeptide of 416 amino acid residues. The polypeptide was composed of a signal peptide (28 amino acids) and a mature protein of 388 amino acids. Instead of Gly, Ala was substituted as the first residue of the conserved pentapeptide Gly-X-Ser-X-Gly (Figure 19). Secretory expression of T1 lipase was achieved through co-expression of BRP. A final lipase activity of 28.459 U/ml was detected in culture medium when the culture of Origami B was induced with 50 μ M IPTG, at $A_{600\text{ nm}}$ of 1.25, in YT medium at 30 °C for 32 h (Rahman *et al.*, 2005d). In addition, we have isolated and cloned other thermostable lipases, namely L2 and 42 lipases isolated from local

Bacillus spp. These lipase genes were cloned in pQE-30UA/M15 (pREP4) for strain 42 lipase (Ethawel 2006), strain L2 in pUC19 (Shariff 2006) and strain S5 in pRSET (Baharum *et al.*, 2005). In another study, *P. pastoris* appears to be a good expression system for L2 lipase production, as compared with those obtained with L2 lipase expressed in *E. coli* (Sabri *et al.*, 2009). The lipase production in *P. pastoris* was best obtained from pP α G2 clone with 125 U/ml after six days of induction time (Figure 20). Strain 42 lipase was also successfully cloned and overexpressed into expression vector pET51b, using heterologous *E. coli* host strain BL21 (DE3) pLysS that employs the T7 expression mechanism which exhibited a higher expression level than previously expressed in the pQE30-Top 10 host-vector system (Hamid *et al.*, 2009a). Similar to the T1 lipase, 42 and L2 lipases possess a polypeptide of 416 amino acid residues and a Gly usually replaces the first Ala in the conserved pentapeptide. A recent addition was an organic solvent lipase from *Staphylococcus epidemidis* AT2. The AT2 gene was cloned in a prokaryotic system. Sequence analysis revealed an open reading frame of 1,933 bp in length which codes for a polypeptide of 643 amino acid residues. The peptide comprised a signal peptide (37 amino acids) and a mature protein of 390 amino acids (Yunus 2009).

Besides lipases, we have successfully cloned an extremely thermostable protease. F1 protease belongs to the subtilisin-like clan of serine protease produced by *Bacillus stearothermophilus* strain F1 isolated from decomposed oil palm branches (Rahman *et al.*, 1994). We subsequently cloned the F1 protease gene and unraveled its nucleotide sequence which comprised 1,206 base pairs encoding a polypeptide of 401 amino acid residues (Zhibiao *et al.*, 2003). From comprehensive qualitative screening on skim milk agar (SMA), we isolated two organic tolerant protease genes from

Pseudomonas aeruginosa strain K and *Bacillus pumilus* strain 115b (Figure 21). These genes were successfully cloned and expressed. Nucleotide sequence analysis of the strain K protease gene revealed an open reading frame of 1440 bp. It was identified as an alkaline protease with molecular weight of 50.4 kDa (Rahman *et al.*, 2009b). The gene encoding the organic solvent tolerant protease from *Bacillus pumilus* strain 115b was cloned and its nucleotide sequence determined. Sequence analysis revealed an open reading frame (ORF) of 1,149 bp that encoded a polypeptide of 383 amino acid residues (Figure 22). The polypeptide composed of 29 residues of signal peptide, a propeptide of 79 residues and a mature protein of 275 amino acids with a calculated molecular mass of 27,846 Da. This is the only report available to date on organic solvent tolerant protease from *B. pumilus* (Rahman *et al.*, 2007a).

| | | | | | |
|------------------------------------|-----|---------|---------|-------|-----|
| <i>Geobacillus</i> sp. strain T1 | 135 | I H I I | A H S Q | G G Q | 145 |
| <i>Bacillus</i> sp. strain L2 | 135 | I H I I | A H S Q | G G Q | 145 |
| <i>Bacillus</i> sp. strain 42 | 135 | I H I I | A H S Q | G G Q | 145 |
| <i>B. thermoleovorans</i> ID-1 | 135 | I H I I | A H S Q | G G Q | 145 |
| <i>B. stearothermophilus</i> L1 | 136 | V H I I | A H S Q | G G Q | 146 |
| <i>B. thermocatenulatus</i> | 136 | V H I I | A H S Q | G G Q | 146 |
| <i>Psychrobacter immobilis</i> B10 | 136 | I H V G | G N S M | G G A | 146 |
| <i>Pseudomonas fluorescens</i> B52 | 201 | V V V S | G H S L | G G L | 211 |
| <i>Staphylococcus haemolyticus</i> | 432 | I H L I | G H S M | G G Q | 442 |

Figure 19 Conserved region of several bacterial lipases. Open box indicates conserved pentapeptide of bacterial lipases. Amino acid sequences were obtained from the following sources: *Geobacillus* sp. T1 (AY260764), *Bacillus* sp. L2 (AY855077), *Bacillus* sp. strain 42 (AY787835), *Bacillus thermoleovorans* ID-1 (AF134840), *Bacillus stearothermophilus* L1 (U78785), *Bacillus thermocatenulatus* (X95309), *Psychrobacter immobilis* B10 (X67712), *Pseudomonas fluorescens* B52 (M863560) and *Staphylococcus haemolyticus* (AF096928) (Source: Leow *et al.*, 2004)

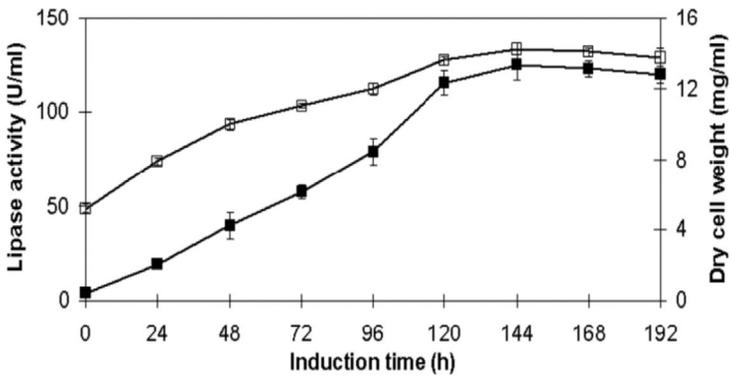


Figure 20 Lipase production and cell growth of recombinant *Pichia pastoris* in shake flask. Cells and supernatant were harvested every 24 h and checked for dry cell weight (□) and lipase activity (■). Error bars are standard deviation of three determinations (Source: Sabri *et al.*, 2009)

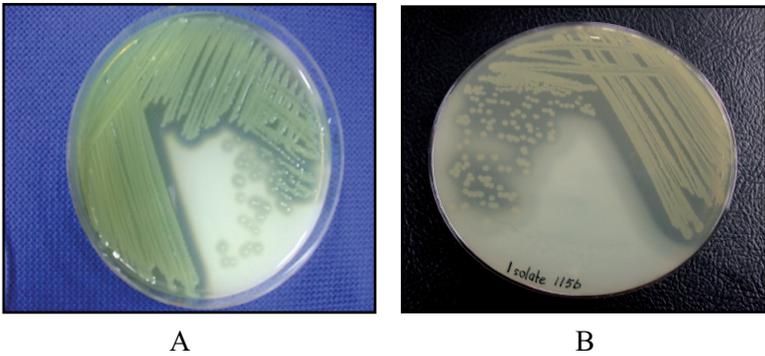


Figure 21 Single colony and pure culture of *P. aeruginosa* strain K (A) and *Bacillus pumilus* strain 115b forming zones of lysis around the colonies on SMA.

Lipases are however very diversified. Identity of amino acids in each subfamily range from being highly to very little conserved. They were classified into the same family due to biochemical properties such as the dependence on the lipase chaperone to secrete active lipase. Unfortunately, a number of these genes were not successfully cloned using PCR technology due to the very low sequence similarity to published and reported lipase genes. Hence, a better approach to isolate a lipase gene would be the construction of a genomic library. No report is available, so far, on lipase produced by any strain of *B. Sphaericus* and thus, an attempt to clone lipase genes from this bacterium by PCR was not successful. Alternatively, the novel *B. sphaericus* 205y lipase gene was isolated via genomic DNA library strategy by direct selection method (Figure 23). Phylogenetic analysis was performed based on an alignment of thirteen microbial lipase sequences obtained from the NCBI database. The analysis suggested that the *B. sphaericus* lipase gene is a novel gene, as it is distinct from other lipase genes in Families 1.4 and 1.5 reported so far (Rahman *et al.*, 2003b).

Similar to the 205y lipase, the S5 lipase was also successfully cloned and expressed by shotgun cloning approach. In this case, the failure to express the S5 lipase cloned by PCR might be because the *Pseudomonas* lipase gene needs a helper or activator gene in the *E. coli* systems. The expression of subfamily I.1 and I.2 *Pseudomonas* lipases are, in most cases, hampered by the fact that a lipase chaperon is necessary for correctly folding to an enzymatically active form. The gene encoding for the intracellular organic solvent tolerant lipase of *P. aeruginosa* strain S5 was isolated via genomic DNA library and cloned into pRSET. The cloned sequence included two open reading frames (ORF) consisting of 1575 bp for the first ORF (ORF1) and 582 bp for the second ORF (ORF2). The ORF2, known as the chaperon, plays an important role in expression systems of

the S5 gene (Baharum 2006). This may be the only study so far to investigate the enzyme activity of solvent stable lipase and its chaperon in the presence of organic solvents.

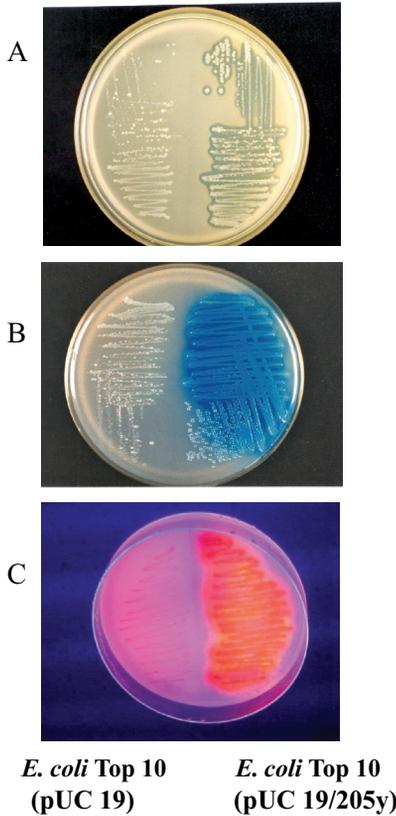


Figure 23 Recombinant clone 205y (pLIP) producing halo on tributyrin-amp agar after 48 h incubation at 37°C (A), producing blue zone on triolein-amp Victoria blue (B) and producing orange fluorescent under UV radiation on triolein-amp Rhodamine B agar (C) on the right. *E. coli* TOP 10 transformed with pUC19 (left) as negative control.

Leucosporidium antarcticum strain PI12 was shown to be a lipase and protease producer. Lipase and protease genes from this yeast were cloned and sequenced. The lipase gene of isolate PI 12 was isolated via shotgun cloning. Gene analysis showed an open reading frame of 783 bp and it was subsequently found to encode a lipase (Ali 2009). The lipase was assayed at 4°C with activity at 0.1 U/ml. The gene was also successfully expressed extracellularly by co-transformation of the pJL3 plasmid which encodes the Bacteriocin Release Protein (BRP). The lipase gene did not show high similarity to other lipases as anticipated. Interestingly, the gene shared high homology to protease. In order to visualize the enzyme, LipPI12 was modeled using the template of the psychrophilic protease from *Pseudomonas* sp. TACII18. The putative 3D structure of the enzyme showed the typical properties of the psychrophilic enzyme, which is an increasing number of loops and a non compact structure to cater to the lipase structural flexibility (Figure 24).

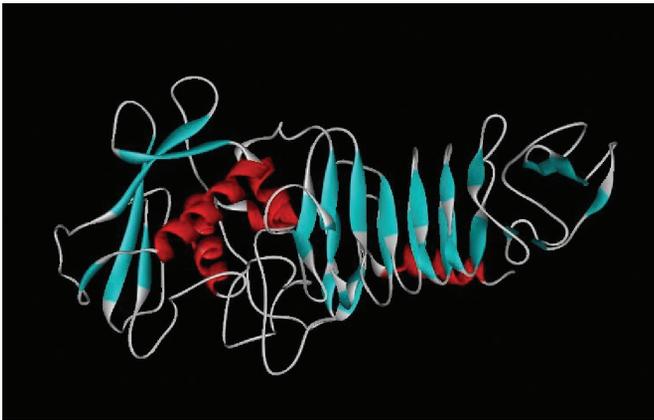


Figure 24 Template model of the cold adapted protease from psychrophilic *Pseudomonas* TAC II 18

Cold-adapted protease holds great potential for fundamental research and development for industrial applications. To date, no published report on recombinant protease from *L. antarcticum* has been described. The gene encoding mature PI12 protease was cloned into *Pichia pastoris* expression vector, pPIC9, which was placed under the control of methanol inducible alcohol oxidase (*AOX*) promoter (Alias 2009). Recombinant PI12 protease was secreted into the culture medium driven by the *Saccharomyces cerevisiae* α -factor signal sequence. The protease production was best obtained from *P. pastoris* GS115 host (GpPro2) with 20.3 U/mL activities after 3 days of induction time at 15°C. The expressed protein was detected by SDS-PAGE and activity staining analysis with molecular weight of 99.3 kDa (Figure 25).

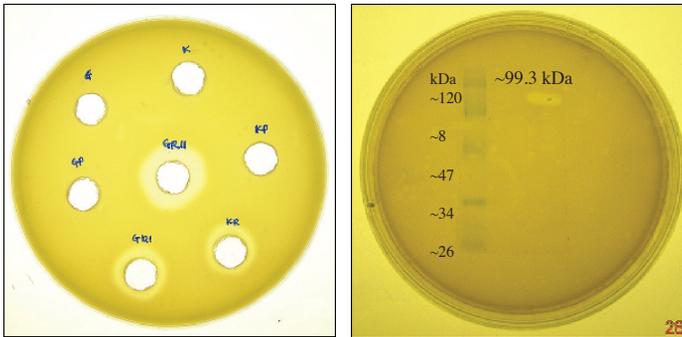


Figure 25 Activity Staining. (a) Plate activity staining of different recombinant *P.pastoris* clones. G: GS115; GP: GS115/pPIC9; GRI: GS115/pPIC9/PI12prot1 (GpPro1); GRII: GS115/pPIC9/PI12prot2 (GpPro2); K: KM71; KP: KM71/pPIC9; KR: KM71/pPIC9/PI12prot (KpPro). (b) Gel activity staining of recombinant PI12 protease in *P. pastoris* GpPro2 clone. Lane 1: Prestained molecular weight marker (Fermentas); Lane 2: GS115/pPIC9 empty (Control); Lane 3: GS115/pPIC9/PI12prot1 (GpPro2)

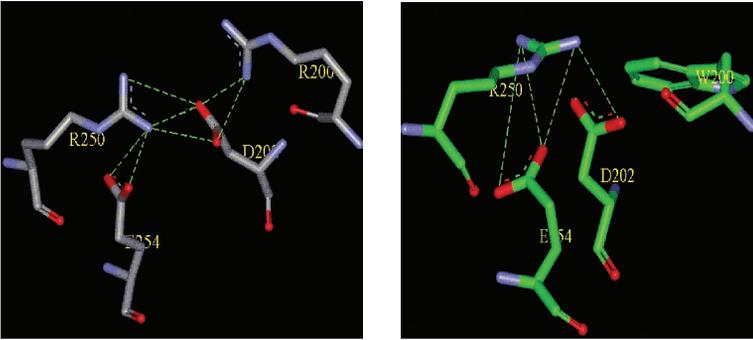
PROTEIN ENGINEERING OF LIPASES AND PROTEASES

The vision of tailor-made proteins is becoming a reality with the advances in protein engineering since proteins with improved properties have enormous commercial potential. Often enzymes do not have the desired properties for industrial applications. One option is to search for better enzymes from nature. The other option is to engineer commercially available enzymes into useful industrial catalysts. Two different methods are presently available for enzyme engineering: a random method called directed evolution and a protein engineering method called rational design. Protein engineering can be used to define protein structure and function and elucidate the catalytic mechanisms of the enzyme of interest. Additionally, it can also be used to alter protein functions in a manner known as rational protein design. Protein design involves the specific alteration of a protein by changing some of the amino acid sequences by site-directed mutagenesis or chemical method. The outcome of this manipulation would be an improvement in protein function. Enzymes modified with new and improved properties would greatly improve their usability as catalysts, for instance, in organic chemistry.

To be effective, protein design requires detailed 3-D structure of a protein and some pre-conceived notion as to the kind of changes to make to produce the desired improvements in function. The 3-D structures of many lipases and proteases of mammalian, yeast and microbial origin have been determined since 1990. Engineering of lipases and proteases is possible by employing recombinant DNA technologies. Recent advances in recombinant DNA technology and the ability to selectively exchange amino acids by site-directed mutagenesis (SDM) have been responsible for the rapid progress in protein engineering. Identification of the gene and knowledge of the three-dimensional structure of the protein in question are the

two main prerequisites for protein engineering. For instance, lipases engineered for specific functions by altering its enantioselectivity, substrate specificity or general performance can be done by either rational design or directed evolution.

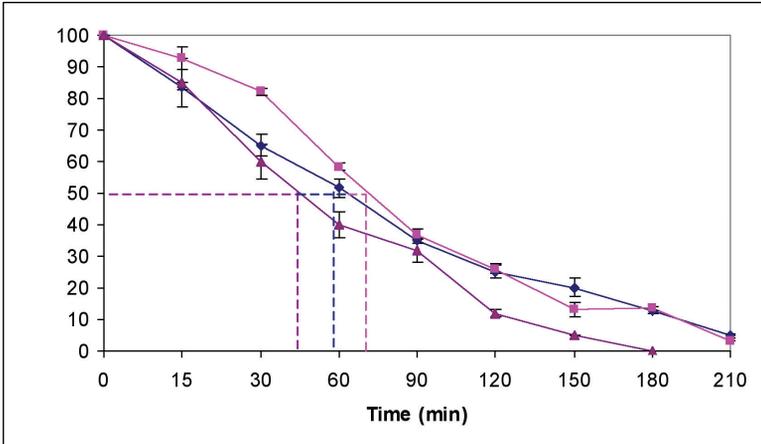
Ion pairs and ion-pair networks have been reported to play a crucial role in stabilizing the thermostable structure of thermostable enzymes (Rahman *et al.*, 1998c). Utilizing rational design, we successfully enhanced the thermostability of F1 protease from *Bacillus stearothermophilus* F1 by introducing additional ion-pairs, thus creating huge ion-pair networks of 4 charged amino acid residues (Ibrahim 2007). Mutations were designed towards additional potential ion pairs as well as towards the disruption of the existing ion pairs. The W200R mutant was designed, adding in more ion pairs on the F1 protease structure while the D58S mutant was designed to disrupt the existing ion pairs on the F1 protease (Figure 26). Figure 27 shows the thermostability of the recombinant wild type F1 protease, W200R mutant and D58S mutant in the presence of 2 mM Ca^{2+} for different durations. The half lives of recombinant W200R mutants were 1.25 to 1.7 times longer and considerably more stable than the recombinant wild type F1 protease. The recombinant D58S mutant was less stable than the recombinant wild type F1 protease. The result obtained indicates that by introducing additional ion pairs (W200R) or by eliminating the existing ion pairs (D58S) the thermostability of the F1 protease was changed.



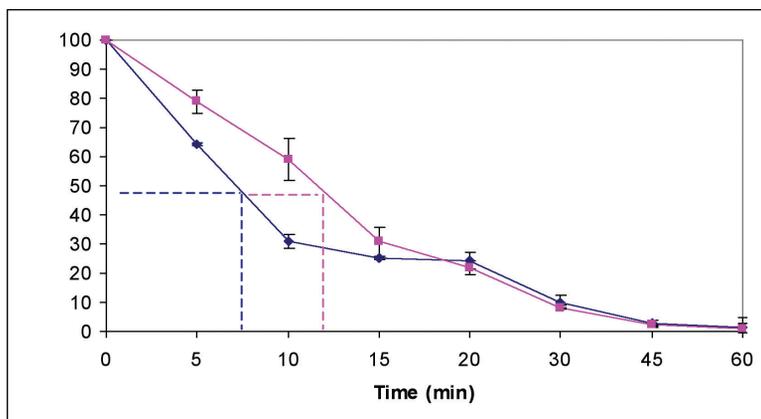
A- W200R with 7 ion-pairs

B- Wild-type F1 with 4 ion-pairs

Figure 26 The addition of additional three new potential ion-pairs in mutated F1 protease W200R structure by replacing Try (W) with Arg (R) at position 200 (A) compared to wild-type F1 (B).



(A)



(B)

Figure 27 Effect of different temperatures on the enzymes' stability. The enzymes (♦) wild type F1 protease, (■) W200R mutant and (▲) D58S mutant in Tris-HCl-2mM Ca²⁺ (pH 9.0) were pre-incubated various times at 85°C (A) and 90°C (B).

The interaction of ligands with biomacromolecular targets can be investigated through docking procedure. To examine the structural basis of the substrate specificity of this enzyme, we have carried out computational docking study using AutoDock 3.0.5. Various sizes of substrates were docked to F1 protease to compare the binding ability of each substrate (Rahman *et al.*, 2006c). The resulting clusters of the substrates were analysed by inspecting the energetic results and the orientation of each cluster to determine the arrangement of the productive binding. Docking of substrates to this enzyme showed that all substrates tested docked near the catalytic residues. The amino acids of the binding site that participated in the hydrophobic and hydrogen-bond interactions were also determined. Bigger-sized substrates such as SucAAPFpNA and SucAAPLpNA were found to show more favourable E_{docked} values compared to the smaller-

sized substrates (Figure 28). Higher activity was also detected for larger substrates while the protease was less active against smaller substrates. It can be stated that substrates with higher activity will generally show more favourable E_{docked} values. Furthermore, it was found that the arrangement of the substrate at the binding site also plays a role in determining the specificity of the enzyme, where productive binding orientation was crucial in obtaining high activity. However, the amount of interactions formed for the enzyme-substrate complex did not play a significant role in substrate specificity. Hence it can be concluded that docking was a useful method in assisting specificity studies for F1 protease. Alternatively, one can resort to more elaborate approaches such as molecular dynamics, which can provide accurate and comprehensive energetic measurements on receptor-ligand molecular interactions.

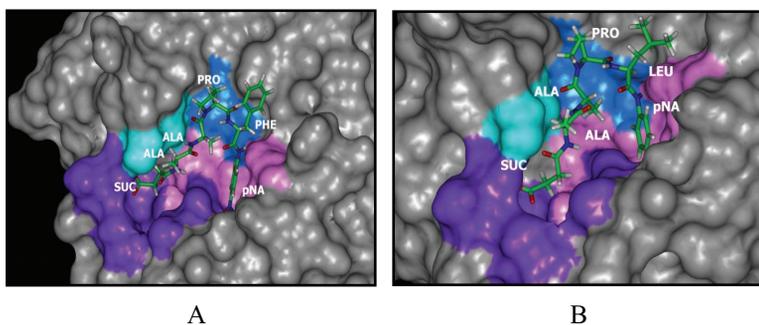


Figure 28 Orientation of the lowest E_{docked} of SucAAPFpNA (A) and SucAAPLpNA (B) docked to the active site of F1 protease (Source: Rahman *et al.*, 2006c)

Lip 42 lipase, isolated from *Bacillus* sp. strain 42 was previously shown to be stable in polar organic solvents such as dimethyl sulfoxide (DMSO) (Hamid *et al.*, 2009a). Stabilities in different solvent compositions were studied based on 40°C pre-incubation in

solvent, and the purified lipase was shown to retain at least 100% residual activity in up to 45% v/v DMSO. Based on these solvent stability profiles, molecular dynamic simulations were subsequently carried out in the presence of water, 60% v/v DMSO + 40% v/v water and 100% v/v DMSO, by using a structure predicted from a highly homologous (97%) lipase (PDB:1JI3) (Hamid *et al.*, 2009b). Molecular dynamic (MD) simulations have been used in order to gain insights into the structure and behavior of enzymes in organic solvents; and in general, there were overall structural conservations with an increase in electrostatic interactions, such as salt bridges and hydrogen bonds, and also a reduced flexibility of the protein compared to corresponding water simulation. Results showed that the flexibility changes in the helix-loop-helix motif covering catalytic triad were found to be associated with a hydrophobic cluster region (Figure 29). The presence of 60% v/v DMSO resulted in the disorganization of the cluster, accompanied by non-native H-bonds formations. The cluster was retained in 100% v/v DMSO which resembled that of water simulation (Figure 30). Mutant form of Lip 42, V171S contained residue substitution in the cluster and within the helix-loop-helix motif. At 50°C pre-incubation, the mutant lost as much of the high temperature enhancements as observed in low DMSO compositions. This indicated the potential role of hydrophobic residues in helix-loop-helix motif and the cluster in interfacial activation

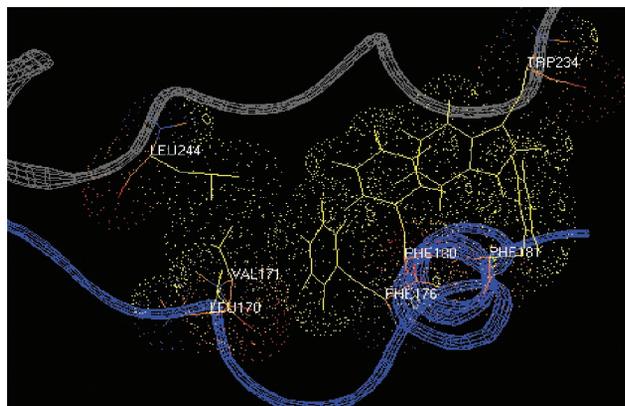


Figure 29 Schematic representation of simulated Lip 42 structure, highlighting the changes in two loops on helix-loop-helix lid upon simulation in the presence of water and 60% DMSO. The Grey coloured ribbon represents the loop of residue 160 to 200 and the red ribbon represents loop of residue 230 to 270. At the top panel, background sticks represents the rest region of protein molecules, with blue colour for N-terminal and red for C-terminal (Source: Hamid *et al.*, 2009b).

The alteration of protein surface characteristics by chemical modification is a good strategy to improve biocatalyst performance. It is a useful way to change their physical or biochemical properties. Attachment of hydrophobic groups using aldehydes to the enzyme surface has been considered to increase enzyme solubility in organic solvents. Methods such as reductive alkylation of amino group, amino group derivatization with PEG and detection and localization of modification site have been employed in enzyme engineering. *Candida rugosa* lipase has been chemically modified to enhance its activity in organic solvents. Salleh *et al.*, (1990) attached a variety of hydrophobic groups to enzymes with different chain lengths via reductive alkylation of aldehydes.

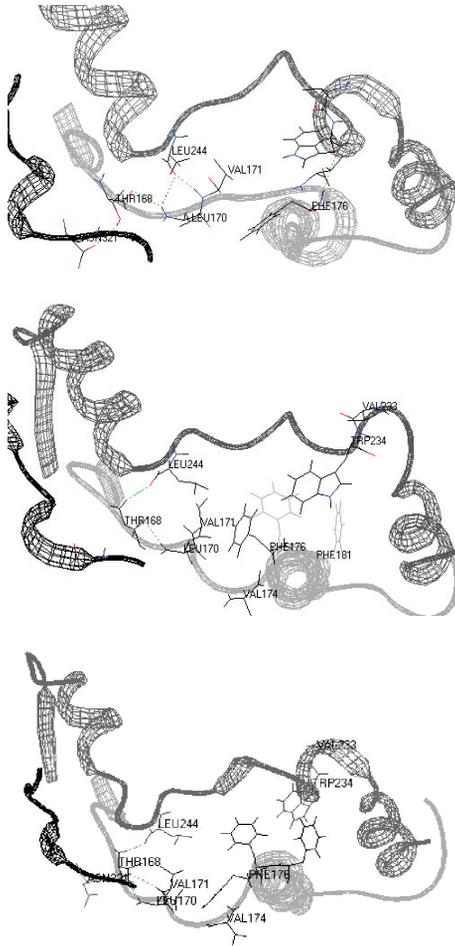


Figure 30 Schematic representation of helix-loop-helix motif on the putative lid of lipase 42 when simulated in water (a); 60% DMSO (b); and 100% DMSO (c). The presence of 60% resulted in re-arrangement of the H-bonds that ultimately resulted in the collapse of the hydrophobic cluster region that formed stably in water simulation. The electron cloud cluster is formed by interaction of mainly the indole ring from Trp side-chain and phenolic rings from Phe side-chains. H-bond is presented in green broken line. (Source: Hamid *et al.*, 2009b).

Prior to this, we have modified *C.rugosa* lipase via reductive alkylation to increase its hydrophobicity to work better in organic solvents (Rahman *et al.*, 2004). The result indicated that at the molecular level alkylation could increase the flexibility of modified lysine and, at the same time, could decrease the possibility of the enzyme forming intramolecular interactions. The existing knowledge about the structure-function relationship of lipases and proteases, coupled with gene-shuffling techniques, promises a fair chance of success, in the near future, in evolving these enzymes that were not made in nature and that would meet the requirements of their multitude applications.

UNDERSTANDING ENZYME STRUCTURE

It is an important assignment in the new era of life sciences to elucidate the structure, function and structural organization of biological machinery. With recent successes in genome-sequencing projects, the focus of structural biology has now progressed from DNA to RNA and proteins, resulting in the new multi-disciplinary sub-field of proteomics. The principal aim of proteomics is to elucidate the three-dimensional structures of all gene products in a genome. Solving the structure of a protein and subsequently comparing it against those of known proteins in the protein structure databases can in turn reveal its functional, biochemical and evolutionary properties that were previously not evident at the sequence level.

Computational modeling utilizes predictive and comparative methods to simulate protein structures. By probing these models, it may be possible to design specific modifications that may or may not affect their structural conformations, but may alter and create novel properties. Computational techniques offer much flexibility and are advantageous in terms of time and resource

savings. However, once determined, each computer model has to be validated. High-throughput technologies employed for resolving such macro-molecular structures range from initial cloning, expression, purification and biophysical characterization, to structural determination using NMR spectroscopy or X-ray crystallography. X-ray crystallography is an experimental technique that exploits the fact that X-rays are diffracted by crystals. It is not an imaging technique. X-rays have the proper wavelength (in the Ångström range, $\sim 10^{-8}$ cm) to be scattered by the electron cloud of an atom of comparable size. Based on the diffraction pattern obtained from X-ray scattering off the periodic assembly of molecules or atoms in the crystal, the electron density can be reconstructed. Crystallography can reliably provide the answer to many structure related questions, from global folds to atomic details of bonding.

We subsequently cloned the F1 protease gene and unraveled its nucleotide sequence which comprised 1,206 base pairs encoding a polypeptide of 401 amino acid residues (Zhibiao *et al.*, 2003). With knowledge of the nucleotide and amino acid sequences, bioinformatic techniques can enable prediction of a three-dimensional structure for the F1 protease by comparative modeling based on sequence similarities to proteins of known structures. The three-dimensional structure of the F1 protease (Figure 31) was predicted by homology modelling. The F1 protease sequence was modelled onto the crystal structure of thermitase, Protein Data Bank (PDB) code 1THM that has 61% sequence identity with F1 protease. The final model comprises nine β strands and six α helices arranged in single domain. The Ramachandran plot for F1 structure showed that about 87.6% of the residues lie in the core or most favoured regions and all the non-glycine and non-proline residues lie within the allowed region of conformational space. The catalytic

triad (Ser226, His72, Asp39) and the subsites of the F1 protease were predicted based on the crystal structure of Ak.1 protease which has 96% similarity with the F1 protease gene. Figure 32 shows the subsites and the catalytic triad of the F1 protease but the S3 site is not shown as it is not a distinct site for this enzyme. The model was also evaluated using the Verify 3D and Errat programmes. Although Structure prediction has progressed and developed to a high level of accuracy, at the moment, biophysical technique still remains the conclusive method.

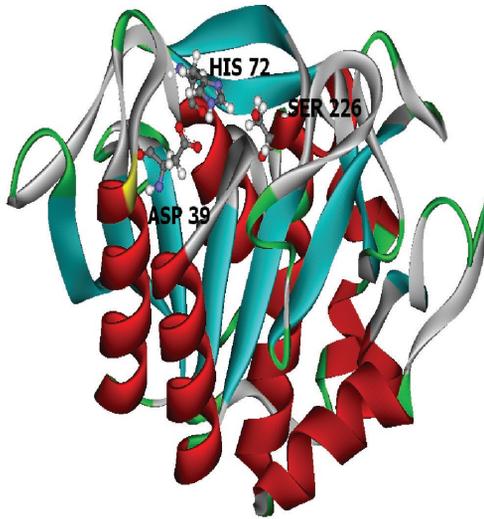


Figure 31 Three-dimensional structure of the predicted F1 protease. The residues rendered as sticks represent the catalytic triads

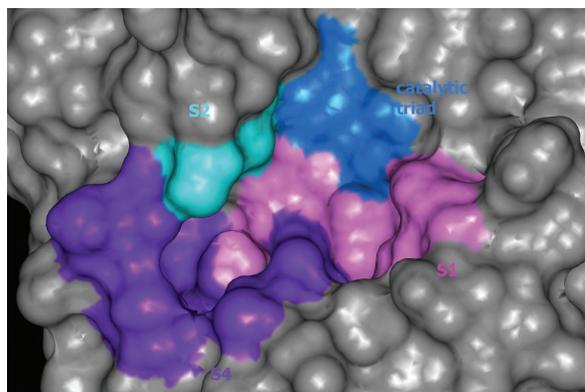


Figure 32 Active site of the F1 protease. The enzyme is represented as atomic surfaces. Pink coloured surface is the S1 subsite, cyan surface is S2 and S4 is the purple coloured surface while the blue surface represents the catalytic triad (Source: Rahman *et al.*, 2006c).

Our work on lipase produced by a thermophilic bacterium isolated from palm oil mill effluent (POME) represents another approach to structural study. One of the most widely used methods to study protein structures is crystallography, which can provide an insight into the protein structures and functions from global folds to the atomic details of bonding. Protein crystallization is necessary for structure elucidation by X-ray diffraction whereby the crystals are analyzed by X-ray diffraction to determine their structures. We have crystallized the wild-type and mutant F16L T1 lipases in the presence of alkali metal cations and determined their crystal structures at 1.5 and 1.8 Å resolutions, respectively (Matsumura *et al.*, 2008). These lipases were crystallized through hanging and sitting drop vapor-diffusion methods. Parameters such as temperature, precipitant and protein concentration were optimized. A protein concentration of 2.5 to 4.5 mg/ml at 20°C was proven to be good for crystallization of these lipases within 24

h with 1 M NaCl as precipitant (Leow *et al.*, 2007). Temperature is being recognized as a non invasive control parameter for protein crystallization, as compared to other parameters such as protein concentration, precipitants, buffer and pH. Temperature can be used as an alternative route in protein crystallization to precisely control nucleation and post-nucleation crystal growth without manipulation of the crystallization solution toward high-quality crystals. As a thermostable enzyme, crystallization of T1 lipase was still possible up to 60 °C (Figure 33).

To further understand the structure, mutant F16L was crystallized. X-ray diffraction data of wild-type T1 and mutant F16L lipases were collected at SPring-8 BL41XU and in-house X-ray beam, respectively. The results of the structural analysis of wild-type and mutant F16L T1 lipase are shown in Table 6. Both wild-type and mutant enzymes have crystallized with the same crystal lattice, molecular packing and cell parameters. The structures of both wild-type and mutant enzymes are very similar (rmsd of 0.22 for 774 C α atoms). The final models of wild-type and F16L mutant enzymes both include 776 amino acid residues (for two molecules), two Zn²⁺, two Ca²⁺ and two Cl⁻ ions (for two molecules). Although the wild-type T1 lipase contains 1148 water molecules in addition to two alkali metal cations (probably Na⁺, *vide infra*), the mutant F16L contains a smaller number of water molecules (686 H₂O) and no metal ions. It was unexpected that Cl⁻ ions were observed in the structures [Fig. 34 (B)], since these ions have never been reported to be part of the structures of lipases. The larger electron density on the spots compared with water prove that this assignment of Cl⁻ is correct, and this was also supported by the relatively long distances between the coordinating side chains and the Cl⁻: distances between Cl⁻ and NH1 or NH2 of Arg227, N atom (main chain) of Arg214, or NE of Gln216 are 3.1, 3.6, 3.3, and 3.6 Å, respectively.

The asymmetric unit of the crystal contains two copies of the T1 lipase. The overall structure is globular in shape, with a central β -sheet consisting of seven strands surrounded by 13 α -helices and 10 3_{10} -helices and loops, which results in an overall topology of a typical α/β hydrolase canonical fold [Fig. 34 (A)]. Lipases are generally known to adopt a closed or open conformation. The structure of the current T1 lipase showed a closed conformation and the active site was buried inside the molecule. The localization and coordination spheres of Zn^{2+} and Ca^{2+} were very similar to those observed in the crystal structure of lipase from *Bacillus stearothermophilus* L1.

During crystallographic refinement of wild-type T1 lipase, the metal-free model phased ($2F_{obs} - F_{calc}$) map indicated the existence of an additional atom near the aromatic ring of Phe16 [Fig. 35 (A)]. The electron density looks spherical, and an aromatic ring of Phe16 faces toward the peak of the electron density. The 1.5 Å resolution map revealed that a specific atom tightly interacts with the aromatic π -system of Phe16. Since the crystallization buffer contains a high concentration of alkali metal cations (600 mM of Na^+ and 100 mM of K^+ , respectively) but with no divalent cations, this atom is likely to be either alkali metal cation or water molecules. The resolved structures revealed that a unique Na^+ - π interaction with Phe16 was only observed in the wild type T1 lipase. Therefore, in addition to the electrostatic and induction interaction between cation and ion-pair electrons of nitrogen and oxygen, the cation- π interaction is vital for the coordination of metal ions in the T1 lipase.

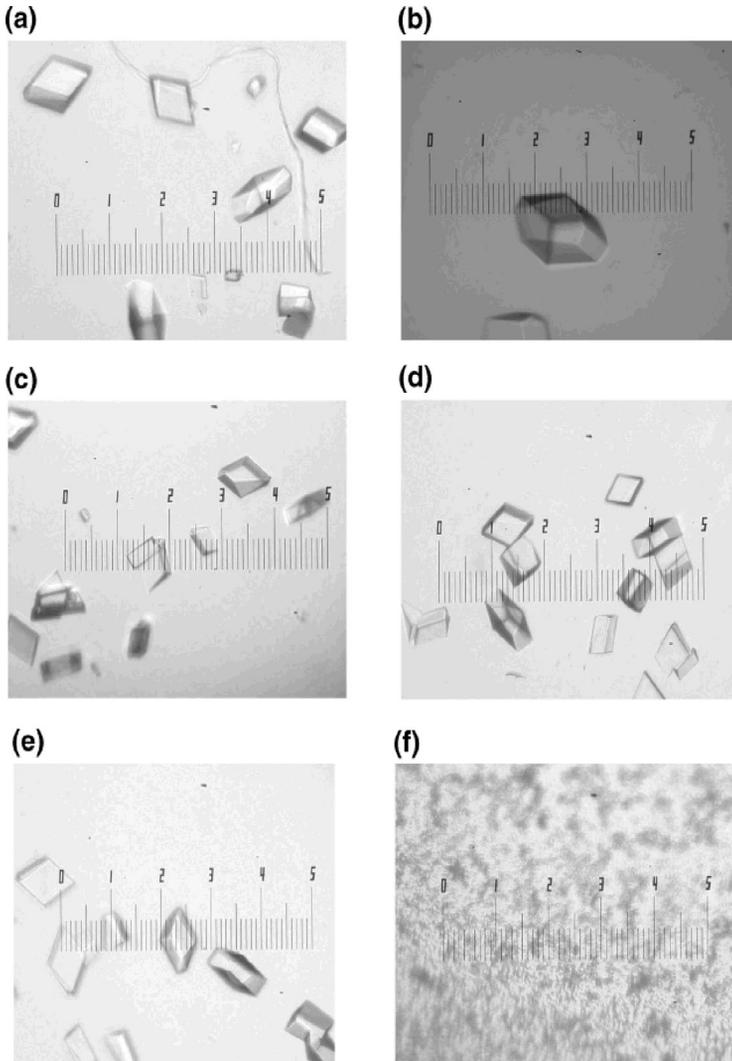


Figure 33 T1 lipase crystallization at various temperatures: (a) 16 °C, (b) 20 °C, (c) 40 °C, (d) 50 °C, (e) 60 °C, (f) 70 °C. The drop volume consists of 4 μ L of T1 lipase and 2 μ L of mother liquor. Scale used was 1:0.1 mm (Source: Leow *et al.*, 2007)

Table 6 Summary of the Crystallographic Data
(Source: Matsumura *et al.*, 2008)

| Data collection statistics | Wild-type | Mutant F16L |
|---|------------------------------------|------------------------------------|
| Unit cell (Å) | $a = 117.73, b = 81.27, c = 99.91$ | $a = 117.78, b = 81.11, c = 99.48$ |
| Space group | C2 | C2 |
| Resolution range (Å) | 40.3-1.5 (1.55-1.5) | 32.3-1.8 (1.90-1.8) |
| R_{merge} (%) ^{a,b} | 5.7 (32.1) | 6.0 (37.6) |
| Completeness (%) | 94.7 (85.1) | 96.5 (95.7) |
| Unique reflections | 140,773 (12,658) | 83,050 (8,178) |
| Redundancy | 3.9 (3.3) | 2.3(2.2) |
| $I/\sigma(I)$ | 12.4 (2.3) | 14.3 (2.9) |
| Refinement statistics | | |
| Resolution range (Å) | 40.3-1.5 (1.55-1.5) | 32.3-1.8 (1.91-1.8) |
| No. of reflections | 136,451 | 136,451 |
| $R_{\text{cryst}}/R_{\text{free}}$ (%) ^{c,d} | 17.4/18.8 | 19.6/21.2 |
| RMSD bond length (Å) | 0.008 | 0.006 |
| RMSD bond angle (°) | 1.3 | 1.2 |
| Protein atoms | 6,106 | 6,100 |
| Heterogen atoms | 8 | 6 |
| Water molecules | 1,148 | 686 |
| Ramachandran plot (%) ^e | | |
| Favored | 90.2 | 90.9 |
| Allowed | 9.2 | 8.5 |

Microbial Enzymes: From Earth to Space

^a Values in parentheses are for the highest resolution shell.

^b $R_{\text{merge}} = \frac{\sum |I - \langle I \rangle|}{\sum I}$, where I is the intensity of observation I and $\langle I \rangle$ is the mean intensity of the reflection.

^c $R_{\text{cryst}} = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}$ where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

^d R_{free} was calculated using a randomly selected 5% of the data set that was omitted through all stages of refinement.

^e Ramachandran plot was performed for all residues other than Gly and Pro.

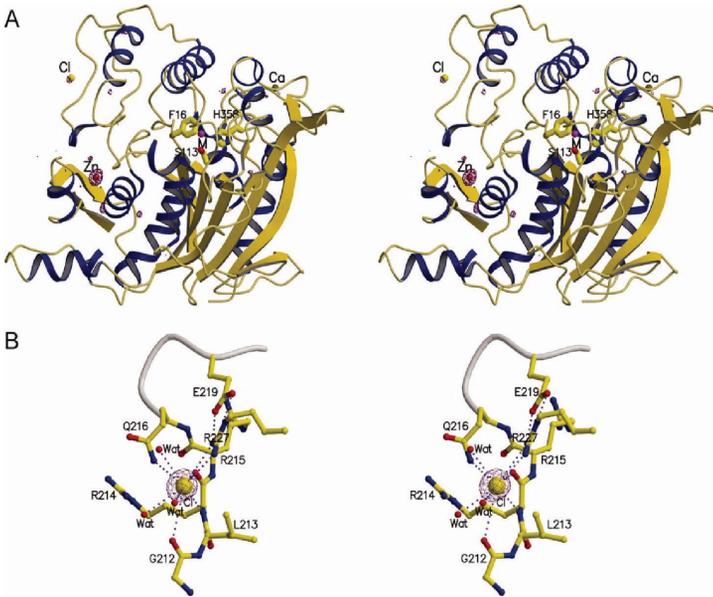


Figure 34 (A) Stereo diagram of the wild-type T1 lipase structure. Sodium, chloride, calcium and zinc atoms are represented as cyan, yellow, grey and brown balls, respectively. Side chains of alkali metal cation binding residues (Phe16, Ser133 and His358) are shown as stick models. The anomalous electron density coming from the anomalous signal of the zinc and chloride atoms is in cyan (4.0σ). (B) Close-up view of the Cl⁻ binding site. The Cl⁻ and Cl⁻ binding residues are shown as stick models with labels. The water molecule is shown as a red sphere labeled “Wat”. The σ_A weighted $F_o - F_c$ electron density map (4σ) was calculated after omission of the relevant moiety from the model at a resolution of 1.5 Å (Source: Matsumura *et al.*, 2008).

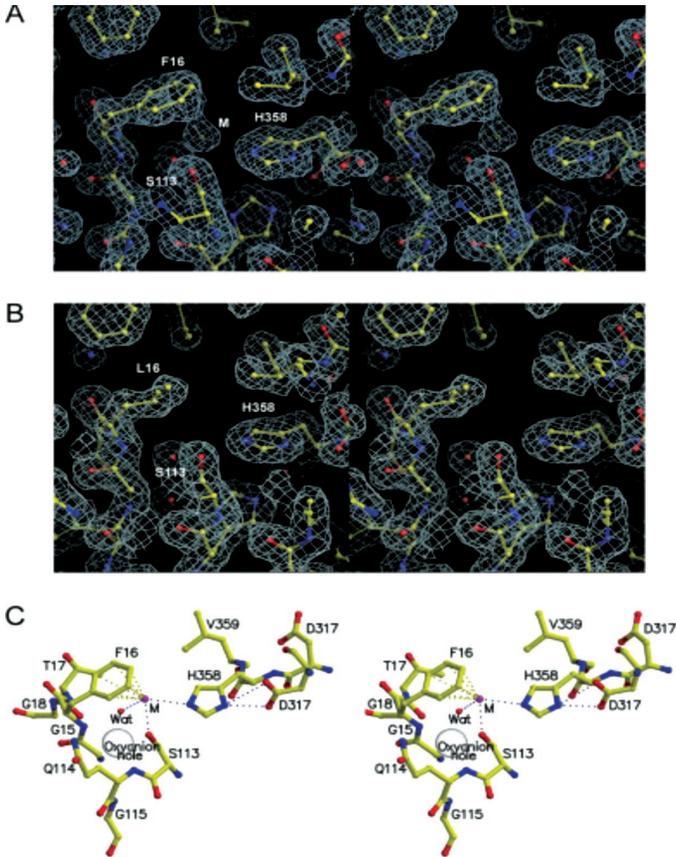


Figure 35 Close-up views of electron density map of wild-type T1-lipase (A) and mutant enzyme F16L (B). The alkali metal cation and the side chains are shown in stick models with labels. The σ_A weighted $2F_o - 2F_c$ electron density map (1σ) was calculated after omission of the relevant moiety from the model at a resolution of 1.5 \AA (C) the alkali metal cation binding site. The cation and cation binding residues are shown in stick models with labels. The water molecule is shown as a red sphere labeled “Wat”. The oxyanion hole is shown as a transparent sphere. Hydrogen bonds and cation-p interactions are represented by blue and yellow dashed lines, respectively

(Source: Matsumura *et al.*, 2008).

In order to acquire a specific catalyst efficiently, there has to be comprehensive knowledge and understanding of the structural and functional relationships of enzymes. The knowledge of accurate molecular structures is a prerequisite for successful molecular design of molecules with desirable features. Based on structural studies, it would be possible to engineer specific mutants that can produce proteins with particular characteristics. A combination of computational, biophysical and molecular biology techniques will enhance these studies and enable development of designer enzymes for industrial uses.

WHY MICROGRAVITY?

Microgravity is a term used by scientists to mean “very little gravity.” The effects of gravity aboard an orbiting spacecraft like the space shuttle are reduced significantly compared to what one experiences on the ground. The unique environment of space, with its near-absence of the effects of gravity, can be used to perform scientific research that cannot be done anywhere else. There are many types of experiments that can be done in these microgravity conditions - experiments that would be affected by convection currents or sedimentation if carried out on Earth. These include important research areas such as fluid flow, protein crystallisation, plasma physics and cell behaviour.

Perfect crystals are difficult to achieve on Earth. Ambient gravity and liquid turbulence disrupt crystal structure formation in that terrestrial samples mix, as a result of gravity-driven convective flow and turbulence caused by other movements and vibrations within the laboratory. Hence, a microgravity environment provides a better alternative for crystal formation, largely due to the absence of turbulence and mixing within the liquid or gaseous samples during crystal formation. A space station located at low Earth

orbits can provide a microgravity environment that is convection- and sedimentation-free, ideal for the study and application of fluid-based systems. After many trials, it became clear that for several proteins, crystallization in microgravity environment can result in the formation of much bigger and better quality crystals. The generation of perfect crystals can sometimes be the limiting factor in determining a protein's detailed structure. By eliminating variables such as gravity, proteins are able to crystallize slowly and more precisely.

The application of microgravity environment is the subject of several ongoing investigations which aim to increase the size and internal order of protein crystals. Successful applications of the microgravity environment for the growth of high quality protein crystals are well documented. Crystals grown in microgravity generally have improved morphology, larger volume, better optical properties, higher diffraction limit and lower mosaicity when compared to Earth-grown crystals (Vergara *et al.*, 2005). Earlier, Ng *et al.*, 2002 reported that the resulting three dimensional structure model of an aminoacyl-tRNA synthetase at 2.0 Å resolution is more accurate than that produced in parallel using the data originating from earth-grown crystals. The above results indicate that the major differences between the structures, including the better defined amino-acid site chains and the higher order of bound water molecules, are emphasized. According to reports, the microgravity environment improved crystal growth over the best-case Earth-grown crystals in the following ways: larger crystals in 45.4% of the cases, new crystal structures in 18% of the cases, at least 10% increase in X-Ray crystallography brightness in 58% of the cases, X-Ray Crystallography resolution improvement of ~0.3 Å in 42.4%, of 0.3 to 0.5 Angstroms in 9.9% of the cases and of 0.5 to 1.0 Å in 9.9% of the cases. For the resolution improvement, an increase of

1 Angstrom could result in the successful determination of the three-dimensional structure and atomic positions in the macromolecule. This is extremely important, since the inability to produce high quality protein crystals has been the limiting step in a number of important macromolecule structural elucidating problems. Thus, protein crystals generated in microgravity environment may significantly accelerate the progress of biotechnological research. The improved X-ray diffraction data from these space-grown crystals has allowed researchers to refine structures in much more detail than ever before, and has allowed the structure of some proteins to be determined for the first time.

MALAYSIAN NATIONAL ANGKASAWAN PROGRAM: PROTEIN CRYSTALLIZATION IN SPACE

As mentioned earlier, wild-type lipase and its F16L mutant were successfully crystallized and their structure determined. They were crystallized through hanging and sitting drop vapor-diffusion methods in our laboratory. Crystallization at 16 °C using formulation 21 of crystal screen II at 2.5 mg/mL yielded bigger and more defined crystals (Leow *et al.*, 2007). The primary objective of this project was to produce high quality protein crystals under microgravity and to understand the dynamics of growing crystals under this condition. For this purpose, our project has undertaken the golden opportunity to study the effects of microgravity on the crystallization process of protein crystals samples by sending several protein samples off to space via the Malaysian National Space Programme.

The Soyuz FG rocket carrying the Soyuz TMA-11 spacecraft, lifted off from the launching pad at Site 1 at Baikonur Cosmodrome in Kazakhstan on October 10, 2007, at 17:22:14 Moscow summer time. The crew included our first Angkasawan, Dr. Sheikh Muszaphar

Shukor Al Masrie, After a nine-minute powered flight, Soyuz TMA-11 reached orbit and after a further two-days of autonomous flight, Soyuz TMA-11 successfully docked at the station on October 12, 2007, at 18:50:07 Moscow time. The high-density protein crystal growth apparatus (HDPCG), six cells of two chambers containing thermostable F16L and T1 Lipases and precipitating agent were used in this experiment. At the ISS (International Space Station) the activation process of HDPCG was initiated on day three of the mission and the deactivation process was carried out at 4.5 h before undocking on day 11. Simultaneous activation and deactivation processes were conducted on Earth using ground control units. Dr. Sheikh Muszaphar Shukor Al Masrie, returned to Earth on October 21, 2007, aboard Soyuz TMA-10. The protein crystals developed in space and those produced on earth were subsequently analysed using X-ray diffraction.

Crystallization under microgravity for both lipases was a success. All chambers developed crystals after 8 days in space. Microgravity apparently improved the size and interface of the crystals significantly. As shown in Figure 36, the crystals were well developed under microgravity, bigger in size compared to the controls performed on Earth. Data were collected using Synchrotron BL 41XU, at SPring-8, Japan, on these space crystals as well as their earth counterparts. Microgravity effect on crystallization of T1 lipase was substantially supported by the finer atomic details at 1.35 Å compared to the 1.8 Å obtained on earth (Figure 37). In conclusion, the bigger size and better interface of the crystals were evidence of the effect of the use of microgravity environment for crystallization of these lipases whereby it ultimately improved the atomic details of the crystal structure.

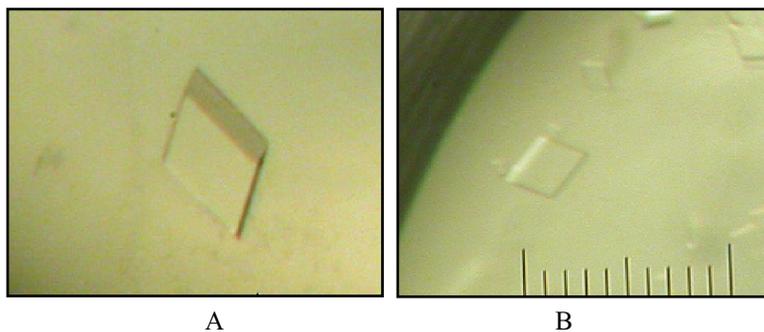


Figure 36 Crystal of T1 lipase grown under microgravity (A) and grown on earth (B)

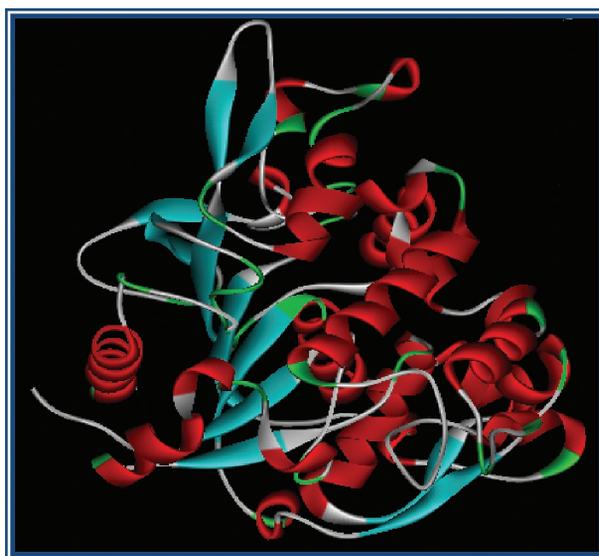


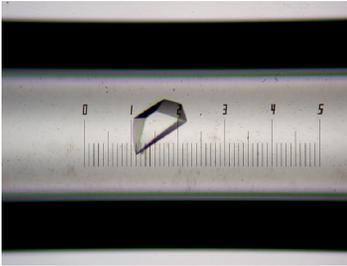
Figure 37 T1 lipase crystal structure grown in microgravity solved at 1.35 Å.

CURRENT MICROGRAVITY EXPERIMENT: SPACE CRYSTALLIZATION OF INDUSTRIAL ENZYMES ONBOARD THE JAPANESE EXPERIMENTAL MODULE (JEM)

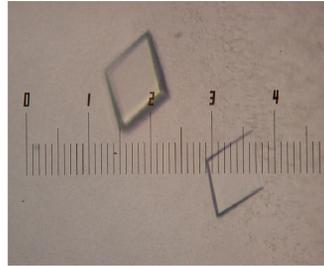
The Japanese Experimental Module (JEM) called “Kibo”, developed by JAXA (Japan Aerospace Exploration Agency), was recently assembled at the ISS. Kibo is now fully established as a permanent on-orbit laboratory that provides both pressurized and exposed experiment environments. The assembly was confirmed as completed when the Exposed Facility was attached to the Kibo Pressurized Module by the STS-127 (2J/A) mission, and all functions were verified to be satisfactory at 10:23 a.m. on July 19, 2009 local time. Kibo operations are jointly monitored and controlled from the Space Station Operations Facility (SSOF) at the Tsukuba Space Center (TKSC), in Japan, and the Space Station Mission Control Center (MCC) at the NASA Johnson Space Center (JSC), in Houston, TX, where the overall operations of the ISS are controlled. The equipment available at JEM for protein crystallization is the Protein Crystallization Research Facility (PCRF) which is a JAXA subrack facility, located in the Ryutai (fluid) Rack, designed specifically for protein crystal growth under microgravity. Collaboration between JAXA and ANGKASA created the opportunity for Malaysian Scientists to send a total of four proteins in each of the six (6) flights being planned from 2009 to 2012. The first mission for protein crystallization onboard Kibo was launched on July 24th 2009 on unmanned spacecraft Progress 34P at 10:56 (GMT). Due to the delay of the Space Shuttle Endeavour, the Progress Cargo Ship successfully docked to the ISS on July 29 at 11:12 (GMT) 5 days after its launch. In this first expedition, a total of 47 proteins were sent to space whereby 32 proteins are from Japan, 10 from Russia and 5 from Malaysia.

The hardware for the crystallization experiments in space, called JCB-HD, utilizes the counter diffusion technique which can eliminate the marangoni convection effect experienced in the vapor diffusion method (the method utilized in our first National Angkasawan Program). Four new proteins (Lip 42, Lip L2, V171S, and D311E) that were successfully crystallized in our lab were chosen for the first mission (Figure 38). In addition, the T1 lipase from the first Angkasawan program was also part of the first mission to study the effects of different crystallization methods on crystal growth. All are lipases with properties exhibiting thermostable and organic solvent stabilities. Crystallization of these proteins is being carried onboard Kibo at $20^{\circ}\text{C} \pm 2$ for 78 days before undocking and return back to earth on October 11. We look forward to the return of our protein crystals from space and their structures will be determined thereafter.

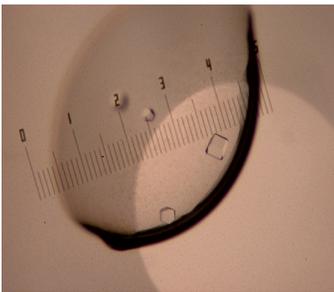
As protein crystals are very fragile in nature, it is usually difficult for the crystals to grow adequately large in size or to obtain perfect protein crystals in normal earth-based laboratories. In view of the aforementioned problems, experiments in space for growing protein crystals under microgravity environment has attracted the attention of various researchers, since such a microgravity environment provides the perfect condition for protein crystallization. Results from microgravity experiments indicate that proteins grown in microgravity may be larger, display more uniform morphologies, and yield diffraction data to significantly higher resolutions than the best of these crystals grown on Earth. As in our first mission, we are expecting that our space grown crystals will be of better quality which in turn will lead to obtaining higher resolution of structures which is necessary for establishing the molecular mechanisms of biological reactions in the design of proteins, particularly lipases, with modified activities and functions for specific target industrial applications.



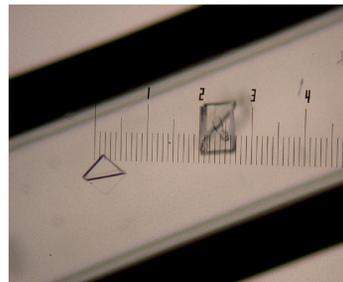
L2



42



V171S



D3111E

Figure 38 Earth grown crystals of four lipases at 20°C. The same proteins were sent to space onboard Kibo, to be crystallized at the same temperature

CONCLUSION

The Industrial enzyme market is growing steadily. The reason for this lies in improved production efficiency that produces cheaper enzymes, for new fields of applications. Using enzymes as crystalline catalysts, with its ability to recycle cofactors, and engineering these enzymes to function in various solvents with multiple activities are important technological developments, which will steadily create new applications. Enzymes are complex biological compounds which serve a variety of functions in living organisms. The information on the three dimensional structure

of enzymes is very useful for our understanding of how they function. Detailed knowledge of the structures of proteins is essential for applications in biotechnology. Critical understanding of how the structure is formed and the parameters affecting the conformation is fundamental and can lead to further innovations such as designing novel drugs for medical and industrial purposes. Tailoring enzymes for specific applications will be a future trend with the continuously improving tools and better understanding of structure-function relationships and increased search for enzymes from exotic environments. Extremophilic organisms have colonized environments that were believed to be hospitable for survival and their true diversity, has not as yet been fully employed. The unique enzymes isolated from these organisms are able to perform reactions that are appropriate for industrial conditions including reactions that are not possible with normal enzymes. Hence screening of these novel microbes which produce new enzymes should be continuously pursued.

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BIOGRAPHY

Born in Arau, Perlis, **Raja Noor Zaliha** had her early education in Kangar and proceeded to obtain her Bachelor of Science with Honors degree in Microbiology from University of Science, Malaysia after obtaining a Diploma in Microbiology, followed immediately by her Master of Science degree from UPM. Subsequently she was awarded the JSPS (Japan Society for the Promotion of Science) fellowship to pursue a doctorate degree in molecular biology at Kyoto University, Japan. This fellowship is awarded to outstanding candidates from each of the 10 Asian countries. She was conferred the doctorate degree of Doctor of Engineering from the university. Her project entitled “*Studies on enzymes for ammonium assimilation in hyperthermophilic archaeon Pyrococcus sp. Strain KOD1*” was under the supervision of the well respected Professor Tadayuki Imanaka.

Her first appointment as Assistant Research Officer enabled her to acquire much skills and knowledge in advanced laboratory techniques, which was fully utilised to maximize her pursuit of tertiary education. She was subsequently appointed as a Tutor upon obtaining her MS degree and as Lecturer upon confirmation of her Doctorate of Engineering in 1998. With this strong academic and technical background, she was able to contribute effectively and was promoted to Associate Professor in 2001 and Professor in 2007. She is the current Deputy Dean (Research & Graduate Studies) at the Faculty of Biotechnology and Biomolecular Sciences. The appointment enables her to play a much bigger role at the university, contributing her ideas and knowledge positively in meetings and discussions, and exhibiting her organisational and effective leadership skills. Prior to that, she was Head of Department of Microbiology at the same faculty. She has received Excellent Service Awards (2), Certificates of Excellent Services (9), and many

other national and international awards. Further, she has won 78 medals at exhibitions held locally and internationally, including the conferment of Knight Degree of the International Order of Merit of Inventors in 2009 and First Prize in the Individual Category at the National Intellectual Property Awards 2008. In 2009, she was awarded the Johan Mangku Negara (JMN) in conjunction with the Official Birthday Celebrations of the Yang Dipertuan Agong on 6 June and earlier an A.M.P from the King of Perlis on commemoration of his 60th birthday in 2003.

Raja Noor Zaliha works very closely with all her post graduate students. The key to the success of her research laboratory is her high commitment, high reliability and easy approachability. She works hard and expects all her students to work just as hard, if not harder. That is demonstrated in her laboratory remaining active for long hours, up to late at night and quite often for seven days in a week. She has developed a positive working culture that has been passed on to all her students. From a total of 65 post graduate students (22 PhD and 43 MS), 36 of her students have graduated. She is the main supervisor for 32 students and co-supervisor for 33 students. A total of 12 of her post graduate students are foreign nationals from Libya, Thailand, Indonesia, China, Iran, Iraq, Sudan, Saudi Arabia and Yemen. In addition to her own students, she serves as external examiner and co-supervisor for postgraduate students from other universities.

Her expertise in two key areas, namely *Science and Applied Science* and *Molecular Biology and Biotechnology* are being utilized by LAN (National Accreditation Board), now know as MQA (Malaysian Qualification Agency), as an assessor for more than 20 programs ranging from Diploma to Doctor of Philosophy for private universities and collages. She is also involved in the setting up of the National Biotechnology Criteria and Standards for public and

private universities, and has contributed to the preparation of the curriculum for science and biotechnology courses, besides being regularly consulted by other researchers and private companies on protein crystallisation, biotechnology criteria and standards, microbiology laboratory techniques, microbiology laboratory safety and microbial remediation. She is currently a Council Member for the Asian Crystallographic Association (AsCA), representing Malaysia since 2004, and Executive Member for the Malaysian Microbiology Society from 2003-2004. She is also a member of several associations in Japan including the JSPS-NRC/DOST/LIPI/VCC Large Scale Cooperative Research Program in the Field of Biotechnology, Society for Fermentation and Bioengineering and Japan Society for the Promotion of Science, enabling her to forge collaborations with prominent scientists from that country. She is also actively involved in the promotion of biotechnology in Malaysia, where she was the resource person and committee member for 7 workshops and symposiums conducted recently whereby she has participated in 50 of these events.

Raja Noor Zaliha is known for being a very committed researcher who excels in her work and is well known locally and internationally in her field. Her initial involvement in research was on cocoa and immobilized enzymes. Under the Intensified Research in Priority Area (IRPA) program, she became involved in the enzyme technology program on Biotechnology of Fats and Oils. One of her major contributions was the isolation of the thermophilic bacterium designated as *Bacillus stearothermophilus* Fl. This bacterium produces a highly thermostable alkaline protease and was the only reported thermostable alkaline protease produced by *B. stearothermophilus* at that time. Study on the use of the enzyme in detergent formulation is already complete. Following that success, her research was geared towards the acquisition of

novel microbial enzymes, with particular interest in the isolation of microbes producing thermostable and organic tolerant proteases and lipases. The focus of her work is currently shifting towards the determination of enzyme structures and the mechanism of reactions of these enzymes. Such knowledge is valuable to future studies, particularly on the engineering of the enzymes to enhance their infinities for specific organic synthesis, followed by the utilization of the enhanced enzymes as catalysts in the production of various industrial products.

Research on microbes producing novel enzymes has been very productive for her, boasting an extensive output of research products in a relatively short period of time. To date, she has published over 100 publications in refereed journals, most of which are renowned internationally. Numerous other papers have been presented at conferences and seminars held locally and overseas. Further to that, she recently contributed to a book chapter on '*Trends in Biotechnology Research*', published by Nova Science Publishers, Inc. New York. The same publisher invited her to write a book on her area of specialisation entitled '*New Lipases and Proteases*', which has been in international circulation since 2006. Her work on Archaea also proved to be very productive, resulting in many papers published in renowned international journals: *Applied Environmental and Microbiology*, *Molecular and General Genetics* and *Biochemistry and Biophysics* and *Research Communication*. Her papers are regularly cited in refereed international journals up to today. Her research findings also attracted a leading biotechnology company in Japan, where her Japanese counterpart, filed for a Japanese Patent for this work. Her expertise in archaea and thermostable and organic solvent tolerant industrial enzymes is well recognized internationally whereby she is on the Editorial Board and is a Reviewer for *Archaea*, *Extremophiles*, *FEMS Microbiology*

Letters, Biotechnology Progress, Enzyme and Microbial Technology, Journal of Hazardous Materials, Critical Reviews In Biotechnology, BMC Biotechnology, Process Biochemistry, Biochemical Engineering Journal and many others.

The most important tool in structural biology is crystallography, which is a new research area in Malaysia. She was the first to have successfully crystallized protein and elucidated the detailed structure of the protein. In the recent Nasional Angkasawan Program, she was one of Principle Investigators for the three scientific projects chosen for the mission. Her project entitled 'Protein Crystallization in Space' was a success. Further to that she is currently collaborating with the Japan Aerospace Exploration Agency (JAXA) on space experiments for the 6 missions planned from 2009 to 2012. Five of her proteins were sent to space recently and are expected to return to earth in October 2009.

Her research group is also actively involved in protecting their research work. She has recently secured funding from MOSTI, amounting to RM 490,000.00, for patent applications, and has successfully filed for 21 patents in Malaysia and 13 patents overseas (Singapore, Indonesia, Europe, Japan and USA). In 2008, one of her patents was granted in USA, another in Europe and one in Singapore, and additionally, two patents were granted in Malaysia. Further she has 4 trademarks applied, 40 sequences submitted for accession number, 25 microorganisms isolated and 6 microorganisms deposited besides having one of the novel bacteria named after her. Scaled up studies on the production of the enzymes for industrial application is one of her current priority research areas, with the aim of providing better alternatives to the existing imported enzymes.

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Finally, my deepest appreciation and gratitude goes to my family, especially my mother, Tengku Zaharah, my husband Zainal and my children Dura, Yana, Jaja, Ammar and Asyraf, for being the source of my inspiration, for their prayers, love, encouragement and understanding. I dedicate this piece of work to all of them.

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19. Prof. Dr. Tengku Azmi Tengku Ibrahim
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