MICROBIOLOGY RESEARCH ADVANCES

MOLECULAR AND STRUCTURAL BIOLOGY OF NEW LIPASES AND PROTEASES

MICROBIOLOGY RESEARCH ADVANCES

Additional books in this series can be found on Nova's website under the Series tab.

Additional e-books in this series can be found on Nova's website under the e-book tab.

BIOCHEMISTRY RESEARCH TRENDS

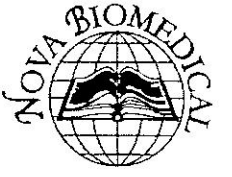
Additional books in this series can be found on Nova's website under the Series tab.

Additional e-books in this series can be found on Nova's website under the e-book tab.

MICROBIOLOGY RESEARCH ADVANCES

MOLECULAR AND STRUCTURAL BIOLOGY OF NEW LIPASES AND PROTEASES

RAJA NOOR ZALIHA RAJA ABD RAHMAN
ABU BAKAR SALLEH
AND
MAHIRAN BASRI
EDITORS



New York

Library of Congress Cataloging-in-Publication Data

ISBN: 978-1-62618-645-3

Library of Congress Control Number: 2013938116

Published by Nova Science Publishers, Inc. † New York

Contents

Preface

Research must progress. In an effort to be on the frontier of enzyme research, we have move on. Since the publication of our book" New Lipases and Proteases" by Salleh, et al., in 2006, our research activities have accelerated and expanded to new frontiers. Although our main focus is still on lipases and proteases, the search for new catalysts has developed into a more detailed approach. Molecular biology has become an essential tool. With the advent of bioinformatics databases and softwares, the studies of protein structure and functions have become critical in catalyst design and catalysis development. And with the introduction of biophysical techniques of protein crystallization, it is now possible to simulate and validate protein structures and functions in a more efficient and economical ways. And it is hoped that we would eventually have in our hands the essentials guidelines to design and produce specific catalysts for specific reactions or applications.

The chapters on thermostable lipases and proteases, as well as, organic solvent tolerant enzymes are still retained but with added recent development. Chapter 1 covers the molecular expression of new thermostable lipases while chapter 2 focuses on organic solvent tolerant lipases. Gene encoding for thermostable or organic solvent tolerant lipases were cloned and expressed in corresponding *Escherichia coli* and *Pichia pastoris* under the regulation of various strong promoters. Single or two step purification strategies were developed for high yield purification.

Chapter 3 follows the development of protease F1. The gene was characterized, and the conditions for optimal expression from the transformant were studied. Chapter 4 discusses the solvent tolerant proteases discovered from *Pseudomonas aeruginosa* strain K and *Bacillus pumilus* 115b. The proteases secreted by these microorganisms were proven stable in most of the organic solvents tested.

Interestingly we have in Chapter 5 a write up on cold-active lipase and protease, which indicates the importance of such enzymes in the understanding of protein structure and functions. Cold-active enzymes offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermostability and unusual specificities.

Efforts to design enzyme function started with chemical modification. Chapter 6 outlines the approaches in modifying or derivitising selected amino acids to affect changes to the properties of the protein. These effects can be monitored or determined by various techniques including molecular dynamics (MD), circular dichroism (CD), and intrinsic and extrinsic fluorescence studies.

Chapter 7 underpins our effort towards understanding and then applying the information in designing a specific property on an enzyme. Changing a specific amino acid may result in modified thermostability, altered substrate specificity, changing response towards chemicals and altered enantioselectivity. We are very excited that we are able to explore biophysical techniques in looking at protein structure. We follow up with chapter 8, to emphasise our endeavor in understanding the various parameters for optimal protein crystallization, critical in the ultimate validation protein structure. We have successfully crystallized a number of lipases and their mutants. T1 lipase could even be crystallised at temperature as high as 60°C. By using these high quality crystals, the 3-D crystal structures of lipases and their mutants were successfully elucidated by X-ray crystallography.

Last but not least in chapter 9, we look at yeast as an enzyme production host. *P. pastoris* was used to express thermostable lipase using *AOX*- and *GAP*-derived promoter system. Our attempt in isolating potentially new local yeasts to be developed as an expression host also resulted in discovery of potential new yeast species. Studies were initiated with molasses in developing potential carbon source for enzyme production by yeasts.

We must acknowledge the generous sponsorship of MOHE and MOSTI in enabling us to undertake the variety of research projects addressing different topics in this book. We are grateful to our employer UPM for its continuous support. We appreciate the contributions of our collaborators nationally and internationally, and may all of us benefit and prosper from our associations. However, it must be emphasized that the bulk of the experimental work was done by our students. Their thirst for knowledge and their commitment and endurance are expressed in this book. We thank them all for making our efforts seem so worthwhile and meaningful.

Raja Noor Zaliha Raja Abd. Rahman
Abu Bakar Salleh
Mahiran Basri
Enzyme and Microbial Technology Research Centre
Universiti Putra Malaysia
Serdang, Malaysia

In: Molecular and Structural Biology of New Lipases ... ISBN: 978-1-62618-645-3
Editors: R.N.Z.R.A.Rahman, A.B.Salleh, M.Basri

Chapter 5

Molecular Studies of Cold Active Lipase and Protease

Mohd Shukuri Mohamad Ali,
Raja Noor Zaliha Raja Abd. Rahman[*],
Norsyuhada Alias, Abu Bakar Salleh and Mahiran Basri
Enzyme and Microbial Technology Research Centre,
Universiti Putra, Malaysia

Abstract

Psychrophilic organisms produce enzymes adapted to function at low temperature. These enzymes are characterized by a catalytic efficiency at low and moderate temperatures but are rather thermolabile. Thermophilic enzymes usually feature in discussion of industrial uses because their heat-stability makes them ideal biocatalyst for many reactions. However, cold-active/heat-labile enzymes also have great potential in industry. Cold-active enzymes might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermostability and unusual specificities. These properties are of interest in diverse fields such as detergents, fabric and food industry, bioremediation and biocatalysts under low water conditions. Furthermore, fundamental issues concerning the molecular basis of cold activity and the interplay between flexibility and catalytic efficiency are of important in the study of structure-function relationships in protein. By means of X-ray crystallography, these properties are beginning to be understood, and the rules governing their adaptation to cold appear to be relatively diverse. To date, extensive studies on psychrophilic enzymes were conducted ranging from purification and characterization, molecular cloning and expression, homology modeling and structural studies and x-ray crystallography studies. Lipases and protease which represents the most important groups of extracellular hydrolytic enzymes were examined due to the attractive properties of these enzymes that constitute a tremendous potential for fundamental research and biotechnological applications.

[*] Corresponding author email address: rnzaliha@biotech.upm.edu.my.

Introduction

Enzymes, which are proteins capable of catalyzing all the biochemical reactions occurring within an organism that render them compatible with life, are an essential target for the adaptation of an organism to a cold environment. Recently a systematic investigation has been carried out in order to understand the rules governing their molecular adaptation to low temperature. Psychrophilic enzymes have a high specific activity at low and moderate temperature 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. This adaptation of the enzymes to low temperatures has been the subject of several studies (Choo et al., 1998; Gerday, 2000; Rashid et al., 2001). The specific activity of wild type cold enzymes and some of their recombinant forms have been determined for several enzyme produced by Antarctic and Arctic microorganisms including α-amylase, protease, xylanase, lipase, citrate synthase and β-Lactamase.Typically the specific activity of these cold enzymes is higher than that of their mesophilic counterparts at temperatures of approximately 0-30°C (Gerday, et al., 2000).

Proteases and lipases are the most widely used biocatalysts in fine chemical applications, largely because the advantages of these catalysts for the production of pure compounds. Lipase (glycerol ester hydrolases E.C. 3.1.1.3) catalyses the hydrolysis of triglycerides at water/oil interfaces (Pernas, et al., 2000) and the synthesis of esters formed from glycerol and long chain fatty acid (Jaeger and Reetz, 1998) (Figure 1). Lipases are produced by different microorganisms including yeast, fungi and bacteria. Lipases have been widely used for biotechnological and industrial applications such as in food industry, oil processing, production of surfactants, oil processing, detergents, pesticides, environmental management and leather industry.

A variety of microbial lipases with different enzymological properties and substrate specificities have been found (Jaeger, et al., 1994). The temperature stability of lipases has been regarded as the most important characteristic for use in industry. However, low 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 (Margesin and Schinner, 1994). One can therefore prevent the materials from damage during heat inactivation of the enzymes. Novel microbial lipases have been isolated, including extremozymes from both thermophilic and psychrophilic species (Demirjian, et al., 2001). All these enzymes were overexpressed in the heterologous host *Escherichia coli*, however, the overexpression efficiency in terms of yield of enzymatically active protein was not always been reported.

Taxonomic identification resulted in the description of unknown lipase-producing species, including filamentous fungi, yeast and bacteria. These lipases were extensively characterized with respect to their hydrolytic or biological properties and synthetic capacities and also with respect to their enantioselectivities towards artificial substrates, such as carboxylic acids, alcohols and amines including the kinetic resolution of racemic (*R,S*)-ibuprofen and (–)-menthol as high-value substrates (Cardenas et al., 2001).

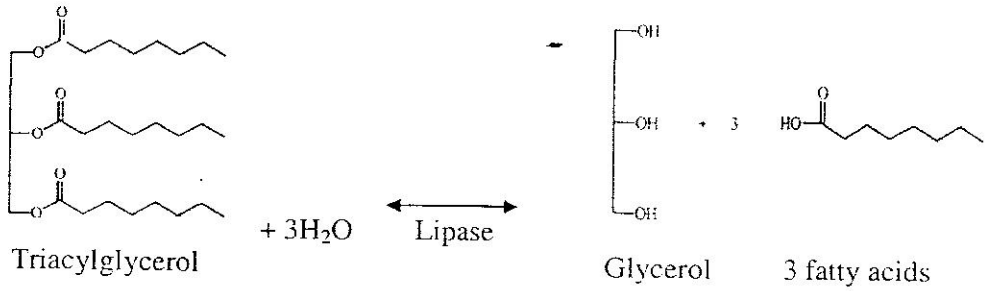


Figure 1. Enzymatic reaction of a lipase catalyzing hydrolysis or synthesis of a triacylglycerol substrate.

Proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. As an essential group of enzymes, proteases are common among animal tissues, plant and microorganisms (Rao et al., 1998). Cold-adapted protease was discovered as a useful catalyst in industrial "peeling" of leather and domestic processes such as low temperature clothes washing and enzyme-added detergents to hydrolyze macromolecular stains (Feller and Gerday, 2003). Undoubtedly, psychrophilic enzymes hold great potential and their characterization will enhance our basic knowledge of microbial physiology and enzyme structures and help develop industrial applications. Future studies could give basic insight into protein structure and could yield industrially useful enzymes.

Numerous psychrophilic bacteria are known to excrete proteases (Vasquez et al., 2004; Zeng et al., 2003). Cold-active proteases from psychrophilic bacteria living in polar region (Vazquez et al., 1995), ice field (Margesin and Schinner, 1994; Margesin et al., 1991) and marine animal intestine (Hoshino et al., 1997) have been studied extensively. Cold-active enzyme represents the lower natural limit of protein stability and is a useful tool for studies in the field of protein folding (Zeng et al., 2003). Turkiewicz et al. (2003) has published the first report of extracellular serine proteinase (LAP2) from Antarctic yeast, *Leucosporidium antarcticum*. The proteinase LAP2 is specific towards synthetic substrates of chymotrypsin and subtilisin and classified as a newly found subtilase of the clan SB (family S8 of subtilisin and subtilases).

Many extremophilic proteases have been studied because of their utility in the laundry detergent industry. Recent interest in this industry is the challenge of finding stable proteases that function in cold water. Psychrophilic subtilisin from the Antarctic *Bacillus* TA39 was found to exhibit a low temperature optimum as expected (Demirjian et al., 2001). Currently, subtilisins from *Bacillus* sp. supplied most of the proteases, mainly alkaline proteases in detergent industry.

A few alkaline proteases from thermophilic and mesophilic *Bacillus* sp. have been commercially formulated such as Proleather, Purafect, Maxacal, Alcalase, Maxapem and Optimase (Gupta et al., 2002; Kumar and Takagi, 1999). To date, Japan Advanced Institute of Science has two patents for cold-active proteases CP-58 and CP-70 while Kao Corporation has filed a number of patents for cold-active proteases for use in detergents (Wang et al., 2007).

Cold Adapted/Active Lipase

Cold-adapted microorganisms, which are expected to produce cold-adapted enzymes, have been isolated. These microorganisms usually grow slowly even under appropriate conditions. However, recent advances in genetic engineering have enabled efficient production of heterologous enzyme genes in an appropriate host strain such as *E. coli*. Thus, cold-adapted enzymes from psychrophilic microorganisms showing high catalytic activity at low temperatures can be highly expressed in such recombinant strains.

In the early 90's, the genes of cold-adapted lipases from psychrotrophic bacteria *Moraxella* TA144 (Gerday, et al., 2000) and *Psychrobacterim mobilis* B10 (Arpigny, et al., 1993) isolated in Antarctica were cloned and sequenced. The enzymes showed high activities at temperatures as low as 3°C. None of these recombinant enzymes, however, have been purified to homogeneity due to strong interaction of the enzymes with lipopolysaccharides secreted by the bacterial cells (Arpigny, et al., 1993).

Nevertheless, the investigations however, continued on semi-purified preparations of the lipase from *Psychrobacterim mobilis* B10, the nucleotide sequence of which is also available (Arpigny et al., 1993). Some properties of the cold lipase were compared to those of the lipase from the mesophilic bacterium *Pseudomonas aeruginosa*.The molecular activities of enzymes from cold-adapted organisms are characteristically much higher at temperatures of 0–10 °C than those of its mesophilic and thermophilic counterparts (Gerday, et al., 2000; Feller et al., 1998). It has been proposed that such high catalytic activity at low temperatures (i.e., cold activity) should arise from molecular flexibility of an active site of these enzymes (Kulakova, et al., 2003). Namely, enzymes generally undergo small conformational changes during catalysis with an energy barrier between such conformational transitions.

At low temperatures, only insufficient energy could be supplied to an enzyme to overcome this barrier, and the kinetic motion of the enzyme would hence be restricted. The hypothesis proposes that cold-active enzymes possess structural flexibility of the active site to obtain a lower energy barrier for the conformational changes. Consistently, it has been observed that crystallographic temperature factors of active-site environments of cold-active enzymes were indeed higher than those of their mesophilic counterparts (Kim, et al., 1999) Further evidence connecting molecular flexibility and cold activity has also been obtained through biochemical and biophysical approaches (Georlette, et al., 2003; D'Amico, et al., 2003).

The Nature of Cold Adapted Enzymes

The well-known low stability of cold-adapted enzymes and the postulated involvement of molecular plasticity or flexibility in cold activity (Svingor, et al., 2001; Fields, et al., 2001) have stimulated the analysis of the structural factors that are responsible for stability in the available X-ray structures. It seems that all the structural factors that stabilize a protein molecule can be attenuated in both strength and number in psychrophilic enzymes (Russel, 2000). The number of proline and arginine residues (which restrict backbone rotations and can form multiple hydrogen bonds and salt bridges, respectively) is reduced, whereas clusters of glycine residues (which essentially have no side chain) provide localized chain mobility.

All weak interactions (ion pairs, aromatic interactions, hydrogen bonds and helix dipoles) are less abundant, and non-polar core clusters have a weaker hydrophobicity, making the protein interior less compact.

Frequently, stabilizing cofactors bind weakly, and loose or relaxed protein extremities seem to favor unzipping. The protein surface is generally characterized by the disappearance of several solvent exposed ion pairs, the exposure of a higher proportion of non-polar groups to the surrounding medium (an entropy-driven destabilizing factor) and an excess of negative charges that favor interactions with the solvent. These factors are thought to improve the resilience or the breathing of the external shell. In multimeric enzymes, the cohesion between monomers is also reduced by decreasing the number and strength of interactions that are involved in association. However, each protein family adopts its own strategy to decrease stability by using one or a combination of these structural alterations (Gianese, et al., 2001).

One astonishing aspect of the active site in cold-active enzymes is the fact that the catalytic centre can be identical to that of mesophilic homologues, at least in the rigid picture that is provided by X-ray crystallography. This has been shown by the perfect conservation of ~30 side chains that are involved in the binding of a transition state analogue in both psychrophilic and mesophilic α-amylases (glycosidases that hydrolyse starch) (Russel et al., 1998). Two other types of molecular adaptation have been shown by X-ray structures. Frequently, the catalytic cavity seems to be larger and more accessible to ligands in psychrophilic enzymes than in mesophilic enzymes (Russel et al., 1998; Aghajari, et al., 2003). This is achieved by the deletion of residues in loops bordering the active site, by the distinct conformation of these loops or by the replacement of bulky side chains with smaller groups at the entrance to the active site. This improved accessibility is thought not only to be responsible for the accommodation of the substrate at low energy cost, but also to facilitate the release and exit of the reaction products. In some enzymes, the electrostatic potential around the active-site region is also improved, so as to attract the oppositely charged ligand and channel the substrate towards the catalytic cavity (Bransdal, et al., 2001).

Recombinant Psychophilic Enzymes

Due to the overwhelming biotechnological application of microbial lipases, the need to use recombinant DNA technology has already begun. Many microbial lipase genes have been cloned over the past few years, including important commercial lipases like *Candida rugosa* (formerly *C. cylindracea*), *Rhizomucor miehei, Rhizopus delemar, Geotrichum candidum, Burkholderia cepacia* (formerly *Pseudomonas). Pseudomonas pseudoalcaligenes, Pseudomonas mendo-cina (originally P. putida),* and *Chromobacterium glumae.* All of them have found their way to major industrial application (Dannert, 1999).

Throughout the last decades, psychrophilic enzymes especially lipases, have broadens the view of lower temperature applications. United States alone has made a profitable income about quarter billion dollars out of their thermostable enzymes, the cold adapted ones will give more via the aid of recombinant DNA technology in the future.A major achievement in the study of psychrophilic enzymes was the availability of recombinant enzymes that have been produced in sufficient amount to determine X-ray structures and for their analysis by sophisticated biophysical methods. This has also opened the way for mutagenesis studies.

The first cold-active lipase *Moraxella* TA144 was cloned in *E.coli* by Feller et al., in 1991 and selected by the activity of the gene product expressed by the recombinant colonies. Later, the high level of sequence identity with mesophilic homologues demonstrated by psychrophilic enzymes prompted the isolation by the polymerase chain reaction of other genes that cannot be detected in expression libraries.

For the purpose of expression of the recombinant cold adapted lipases, various *E.coli* strains were used. These includes *E. coli* BL21 (DE3) (Suzuki, et al., 2003; Rashid, et al., 2001), *E. coli* TG 1 (Kulakova, et al., 2004) and *E. coli* SG13009 (Alquati, et al., 2002) whereas expression in *Bacillus* species or yeast was rarely addressed. The first concern of workers in the field was to establish if an enzyme expressed near 0°C by the wild-type strain maintains its properties when expressed and folded at higher temperatures that are lethal for the parent microorganisms. Indeed, when expressed at a sufficiently low temperature (15–18°C), a recombinant psychrophilic enzyme is undistinguishable from its wild-type parent molecule with regards to kinetic parameters, folding properties (Feller, et al., 1998) and three-dimensional (3D) structure (Aghajari, et al., 1998). By contrast, at 37°C, recombinant psychrophilic enzymes are frequently not expressed or are quickly inactivated. In addition, when expressed at this temperature, the properties of the recombinant psychrophilic enzyme seem to be altered as a result of misfolding or the occurrence of an inactivated population (Feller, et al., 1998).

Molecular Approaches of Cold-adapted Proteases

Currently, extensive molecular approaches on cold-adapted proteases from various sources of psychrophiles have been reported. The gene encoding serine alkaline protease (SapSh) of the psychrotrophic bacterium *Shewanella* strain Ac 10 was cloned in *Escherichia coli*. The amino acid sequence deduced from the 2,442 bp nucleotide sequence revealed that the protein was 814 amino acids long and had an estimated molecular weight of 85,113 Da (Kulakova et al., 1999). The gene encoding a subtilisin-like serine proteinase in psychrotrophic *Vibrio* sp. PA44 has been successfully cloned, sequenced and expressed in *Escherichia coli* with 1593 bp nucleotide sequence encoded a precursor protein of 530 amino acid residues with a calculated molecular mass of 55.7 kDa (Arnórsdottir et al., 2002). According to Chessa et al. (2000), gene encoding psychrophilic alkaline metalloprotease (PAP) produced by a *Pseudomonas* bacterium isolated from Antarctica has been cloned and sequenced and the derived amino acid sequence shows 66% identity with the mesophilic alkaline metalloprotease from *Pseudomonas aeruginosa* IFO 3455. They concluded that structural features responsible for cold-activity could not be deduced simply from the amino acid sequence due to subtle changes that were sufficient to alter the folded state.

Hence, these results are still not sufficient to highlight features for analysis in future studies. Discovering these fundamental rules will require pushing our knowledge beyond analyzing the amino acid sequence into characterizing enzyme structure and eventually towards examining enzyme reaction dynamics. However, the combination of the more rapid analysis of crystal structures and the additional insight gained by comparisons that include cold-active proteins should improve the understanding on protein structure and functions.

Our Work

Cold Active Lipases

Introduction

We have screened several microbial isolates taken from Antarctic sea ice near Casey station, Antarctica. Some of the isolates were able to grow at 4°C and were screened for cold active lipolytic activity. Among these isolates, strain PI12 also known as *Leucosporodium antarcticum* exhibited the highest lipolytic and proteolytic activity and was then used for further study. This chapter discusses some of the work which involved microbial identification, lipase gene isolation, expression and purification of recombinant enzyme and also biochemical and biophysical characterization of the recombinant cold active lipase and protease

Screening for Cold Active Lipase

Extracellular lipase activity was determined using both qualitative and quantitative methods. Screening plates, namely tributyrin, Rhodamine B and triolein were used to carry out the experiment. Clear zones around the culture formed on tributyrin plates indicated extracellular lipase activity (Figure 2). Free fatty acids, liberated by the lipase, react on the colouring dyes in both Rhodamine B and triolein plates containing Victoria blue, forming an orange fluorescent (Figure 3) and an intense blue colour for the respective plates (Figure 4). The formation of the blue zones, on the triolein plates, is a result of the pH reduction of the medium due to the increasing fatty acid composition (Samad et al., 1989). Meanwhile, the exhibition of orange fluorescence is resulted from the formation of Rhodamine B-free fatty acid complex (Kouker and Jaeger, 1987). The bacterium was incubated at 4°C for 7 days. Lipase activity was at 0.051 U/ml when assayed at 4°C.

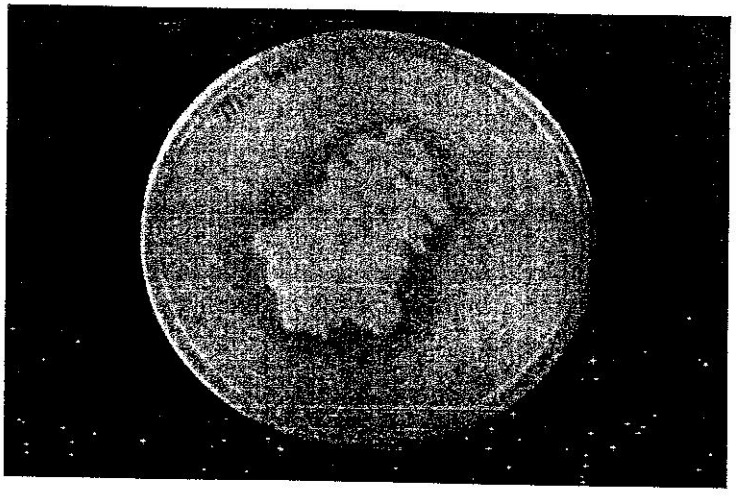


Figure 2. Clear zone formed on tributyrin agar plates. Isolate PI12 was grown on top of nutrient agar containing tributyrin for 7 days. Clearing zone indicates hydrolysis of tributyrin (C4).

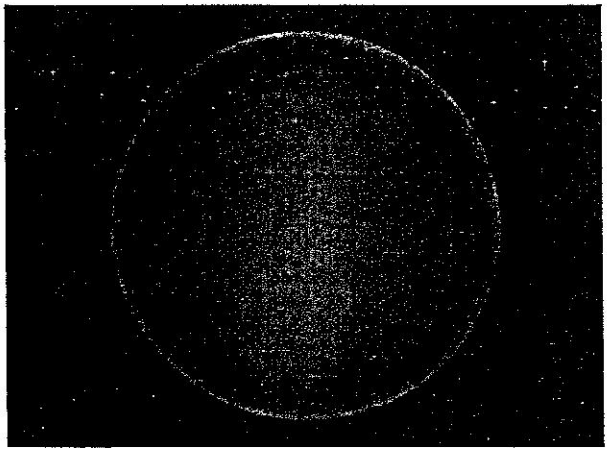


Figure 3. Orange fluorescent formed around the colonies on rhodamine B agar. The free fatty acid interaction with rhodamine B dye was viewed under UV light which exhibited the orange fluorescence indicating the presence of lipase.

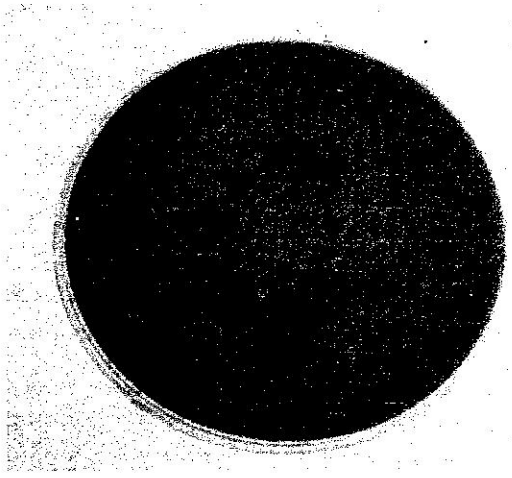


Figure 4. Intense blue colour formed around the colonies on Victoria Blue Plate. Increased acidity as a result of increased fatty acid favoured the formation of a more intensed blue colouration around the colony. This screening method serves as an alternative to the rhodamine B agar plates.

Recombinant Cold Active Lipase

Gene isolation was performed using genomic library approach. Indeed, it has yielded recombinant colonies habouring DNA fragment in various sizes through shotgun cloning method. The word "shotgun" implies a rapid and randomly generated cloned DNA fragment resulting from partial digestion via *Sau*3A1.

Among thousands of transformants from the shotgun cloning, four of them produced clearing zone.The positive recombinants harbouring the putative lipase gene, was assumed to have cold active lipolytic activity when the recombinant clone was initially grown at 37°C for 24 hours and was then transferred at 4°C. The clearing zone only appeared after 3 days of incubation at 4°C.

The lipid hydrolysis appearance in cold temperature was due to the nature of the enzyme itself. At elevated temperature, the activity of lipase was inhibited because of its

thermolability. This has become a consensus to many other investigations circumventing thermal denaturations (Feller et al., 1991; Arpigny et al., 1993). The blue colouration which was formed at low temperature on the LB triolein plates was an indication that *E. coli* had harboured a lipase gene.The transformant was subjected to lipase assay using olive oil as substrate and found to have a lipase activity of 0.106 U/ml. at a temperature as low as 4°C. The enzyme was expressed at 3.5 fold higher than its the wild type, isolate PI 12. The lipase was believed to be cold adapted and probably possessed properties which enabled this enzyme to be cold tolerant.

Hydrolases such as lipases from *Psychrobacterim mobilis* (Arpigny et al., 1993), *Moraxella* TA144 (Feller et al., 1993) and *Pseudomonas* sp B11-I (Choo et al., 1998) exhibited a cold lipolytic activity. All of these lipase genes were isolated via genomic library similar to the approach carried out in this research. Nevertheless, instead of plate screening, the isolation of other cold active/adapted lipase gene via PCR technique had also been carried out as reported by Alquati and co-workers (2002).

Recombinant plasmid denoted as pLipPI12 which was obtained from the genomic library was used to sequence the insert. A DNA sequence with the size of 1509 bp was obtained. The gene was analyzed for any possible open reading frames (ORFs) using the software called the ORF finder from the NCBI. One putative ORF was predicted with the size of 783 bp (denoted as LipPI12 gene). The BLAST result revealed that LipPI12 showed 20% homology with a lipase gene from *Pseudomonas* sp. UB140. This is in agreement with observation of other researchers where other lipase genes from Gram negative bacteria, including *Pseudomonas* sp., were notable in having quite a small ORF ranging from 800-1000 bp. Such functional lipase genes with small ORFs were reported. A functional cold adapted lipase from the *Pseudomonas* sp. B11-1 was shown to possess an ORF of 924 bp, as reported by Choo*et al.* (1998). Other lipase genes such as that from *Pseudomonas fragi* (Alquati et al., 2002) contained an even shorter ORF of 875 bp. Although the genes coding for cold active lipases are small, what matters most was their catalytic function, which could be achieved through proper protein folding.

The most interesting and intriguing part of LipPI12 was its high amino acid sequence similarity when the protein sequence alignment was compared to other types of proteases, including the psychrophilic protease derived from *Pseudomonas* sp. TACII 18 and an organic solvent tolerant protease of *Pseudomonas aeruginosa* Strain K. This suggests that expressing enzymes, with two or more types of activities, is one possible way of adaptation in isolate PI 12. Only one of the enzymes remains as the primary protein, while the rest act as a substitute depending on the availability of substrate. No report has been published with regard to psychrophilic enzymes.

The expression of the lipase gene was carried out to assess its catalytic functions. Nonetheless, the expression of cold adapted proteins was a major task due to its thermolability and structural stability. In addition, expression in mesophilic hosts such as *E. coli* would cost improper folding and result in the formation of aggregates or inclusion bodies. The formation of inactive protein was something inevitable due to the limited availability of expression vector and its suitable host which could express protein efficiently at low temperature. Therefore, to cater for structure and function of the lipase structure, the expression was carried out at various temperatures of 25, 20 and 15°C. *E. coli* Top10 carrying pTrcHis2/LipPI12 was initially grown at 37°C prior to induction. The cell culture was then induced with different concentrations of IPTG ranging from 0 to 1 mM and transferred to a

lower temperature for further incubation. Primarily, the expression was performed at 25°C with 1mM of IPTG and was induced for 24 hours. Meanwhile, the lipase assay was carried out at 4°C to screen for the presence of cold active lipase activity. The lipase activity was detected at 0.1 U/ml.

Figure 5 shows that the crude lipase indicates some effects of deterioration when exposed to higher temperatures. This was due to the thermolability of the protein. As the temperature increased, the recombinant LipPI12 could have undergone some structural changes which led to altered folding and consequently increasing the inactive population. This was a typical characteristic of a cold active enzyme. No activity was detected at 40°C. At high temperature, enzymes from a psychrophilic organism tended to lose their catalytic function as a result of the loss in its stability and flexibility (Gerday et al., 2000). Optimum temperature was observed at 20°C. The lipase exhibited an optimal activity of 0.15 U/ml.

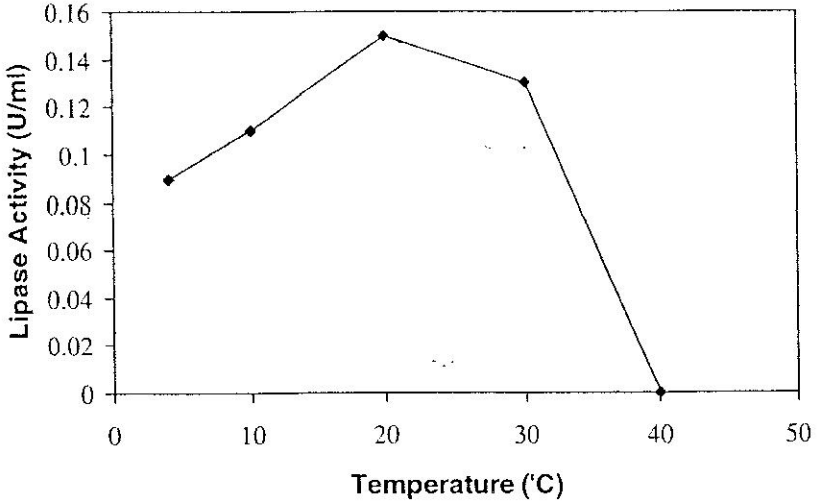


Figure 5. Effect of different temperature on crude lipase activity. The crude recombinant cold active lipase was incubated at various temperatures from 5-40°C and was assayed using olive oil as substrate.

Characterization of Bifunctional LipPI2 Enzyme

The cold adapted LipPI12 was purified to homogeneity with the size of ~30kDa. Purified intracellularly expressed LipPI12 was 75% recovered with a purification factor of 42. Extracellularly expressed LipPI12 also has the same size with recovery and fold of 82% and 8.12 respectively. Characterization of the bifunctional ability of the LipPI12 in terms of optimum temperature showed the lipase had the optimum activity at 20°C and protease was activated at a higher temperature of 40°C. As for pH optimum, the recombinant LipPI12 showed maximal activity at pH 7 and 8 for lipase and protease, respectively. As shown in Table 1, Ca^{2+} enhances the activity for both enzymes. The activity of protease, on the other hand was also activated by Zn^{2+} and Mg^{2+}. Substrate specificity profile for LipPI12 showed that the lipase preferred medium chain length of fatty acids (C12). Highest activity was exhibited in coconut oil (mainly composed of C12 fatty acids). Nonetheless, activity towards hydrolysis of pNP esters showed LipPI12 was more favourable to longer carbon chain on pNPpalmitate. Stability of psychrophilic enzyme has been regarded as important

characteristic. The halflife of LipPI12 lipase at 20°C was 30 min while protease showed a halflife of 15 min at 40°C. Both lipase and protease had shown some tolerances to organic solvents whereby lipase was activated with solvents of high *log P* and interestingly the protease reacted vise versa. LipPI12 protease was activated in the presence of Tween 80 and SLS with 30 and 70% enhancement respectively. LipPI12 lipase also retained its activity when tested with Tween 40, 60 and Triton X-100 compared to the total inhibition effect of Tween 20. Incubation with SDS decreased the catalytic activity of LipPI12 to 27% of its remaining activity. Inhibitor studies revealed that LipPI12 lipase was partially inhibited with EDTA and PMSF whereby the LipPI12 protease was inhibited by pepstatin and partially inhibited by EDTA and PMSF. These put to the understanding that LipPI12 utilizes serine as its nucleophile while aspartate is used by its protease. Table 1 summarizes the biochemical properties of LipPI12 enzyme.

Table 1. Biochemical properties of LipPI12 enzyme

Characteristic	LipPI12 lipase	LipPI12 protease
Molecular weight	27kDa (monomer)	
Temperature profile	20°C	37°C
pH profile	7	8
Metal ion	Ca^{2+}	Ca^{2+}, Zn^{2+}, Mg^{2+}
Inhibitors	PMSF, EDTA	PMSF, Pepstatin, EDTA
Organic solvent	Propanol, Hexadecane,	Dimethylsulphonyl
	Decane, Benzene	Hexadecane, Decane, Dodecane
Substrate	Medium chain fatty acid	
Surfactant	Tween 80, Sodium Lauryl Sulphate	Tween 20
Circular dichriosm analysis (Tm)	38°C	38°C
Fluorescence spectrum analysis	Non compact structure and less hydrophobic core	

Cold Active Protease

Qualitative and Quantitative Determination of Protease Activity

Prior to observation for the ability of this Antarctic yeast for protease production, the cells were streaked on a skim milk agar plate at 4°C for 10 days. Principally, skim milk was used to prepare skim milk agar for detecting proteolytic microorganisms in foods (Downes and Ito, 2001), including dairy products. It can also be used to prepare litmus milk, a differential test medium for determining lactose fermentation and for detecting proteolytic enzymes that hydrolyze casein (milk protein) and cause coagulation (clot formation) (MacFaddin, 1985).

The skim milk agar plate comprised peptone and yeast extract which provided essential nutrients and skim milk powder as a source of casein. Dextrose was supplied as the carbon energy source while agar was the solidifying agent. Casein, the protein that gave milk its white and opaque appearance was successfully hydrolyzed by protease where it was converted into soluble nitrogenous compounds (pieces of peptides and amino acids) with the formation of clear zones around the colonies signifying the production of protease (Figure 6).

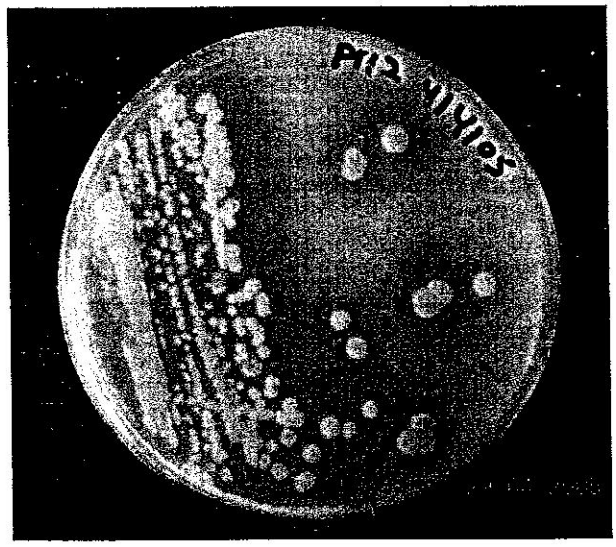


Figure 6. Casein Hydrolysis on Skim Milk Agar Plate. The Antarctic microorganism strain PI12 was grown and screened qualitatively for the protease production on skim milk agar. The plate was incubated at 4°C for 10 days. Protease was excreted out of the cells into the surrounding media, catalyzing the breakdown of casein and formed clearing zones around the growth area.

The production of extracellular protease from wild-type *L.antarcticum* strain PI12 in liquid media was tested. The isolate was cultivated in trypticase soy broth at 4°C without shaking and tested for protease activity after 10 days of incubation. Protease activity was at 1.0 U/mL when assayed at 4°C where azocasein served as the substrate using the modified method of Brock et al. (1982). This is the second extracellular protease reported being synthesized by this obligate psychrophilic yeast, *L. antarcticum*. The first cold-adapted extracellular subtilase from this yeast have been purified and characterized previously by Pazgier et al. (2003) and Turkiewicz et al. (2003). Interestingly, the extracellular serine proteinase (LAP2) from this Antarctic yeast, *L. antarcticum*strain 171 was specific towards synthetic substrates of chymotrypsin and subtilisin and classified as a newly found subtilase of the clan SB (family S8 of subtilisin and subtilases) (Turkiewicz et al., 2003). This subtilase of *L. antarcticum* strain 171 showed specific activity of 40.43 U/mg with *N*-SucAAPF*p*NA as a substrate. Unfortunately, comparative characteristics of cold-adapted PI12 protease with the cold-active protease cited above cannot be done as PI12 protease was only a crude extract. Until now, no published report on recombinant protease from this *L.antarcticum* has been described.

Recombinant Cold Active Protease

Seven recombinant clones harboring putative partial protease gene *L.antarcticum* strain PI12 was successfully achieved from shotgun cloning. Recombinant plasmid carrying 1321 bp partial serine protease gene was chosen for further study. Subsequently, genomic DNA walking was performed and successfully identified a total length of 2687 bp putative partial protease gene. Prediction by Augustus Web server revealed the presence of putative exon/intron boundaries in the sequence. Later, reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to prove the prediction. Overall, the coding sequences were interrupted by 18 introns. Full-length cDNA of PI12 protease gene was amplified by

rapid amplification of cDNA ends (RACE) strategy with an open reading frame (ORF) of 2892 bp that coded for 963 amino acids. Homology search by BLAST web server has discovered the PI12 protease sequence was sharing a significant homology to the subtilisin protease family from fungus and no similarity with other psychrophilic protease. This is the first protease sequence from *L. antarcticum* being deposited into the GenBank Database with accession no. CAQ76821.

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. Only two recombinant clones from GS115 strain (GpPro1 and GpPro2) and one from KM71 strain (KpPro1) were successfully achieved using colony PCR method. Later, all positive recombinant clones were grown in small scale cultures and assayed for the cold-adapted protease activity in the culture supernatants. The clones were first grown in BMGY medium with glycerol as the sole carbon source. For induction of *AOX1* promoter and protein production, the growth medium was transferred to the same medium except with 0.5% methanol in place of glycerol as the carbon source (Clare et al., 1991).

Assay of Recombinant Protease Activity

The supernatant of the recombinant *P. pastoris* expression cultures after 72 h of incubation time was analyzed by the modified method of Brock et al. (1982) to determine the recombinant protease activity. All tested clones secreted protease into the supernatant but in different amounts. This might be due to the variability of each cell to secrete and express the recombinant product. The clone with the highest yield is GpPro2 with 20.3 U/mL activity followed by KpPro1 and GpPro1 with 9.1 U/mL and 5.2 U/mL activities, respectively. Therefore, the recombinant PI12 protease was 20-fold higher than that produced by its wild-type host (1.0 U/mL). It is apparent that the expression system of *Pichia pastoris* is the ideal system for this protein.

The preliminary assay has successfully produced high protease expression at 15°C. Further optimization of the recombinant PI12 protease production needs to be conducted in order to examine for the optimum condition of this protein. To date, cold-adapted proteases showed optimum activity at a range of 15°C-60°C with various protein yields (Wang et al., 2007; Vasquez et al., 2005; Morita et al., 1998; Zeng et al., 2003). This difference was possibly related to the respective genetic and physiological adaptation of the strains. Substrate selection might also affect the specificity and activities of protease since the assay of all protease above were conducted using different substrate. Comparative characteristics of cold-adapted PI12 protease with the cold-active protease cited above cannot be done as PI12 protease was only a crude extract.

Detection of Recombinant Protein by SDS-PAGE

Generally, molecular masses of cold-adapted serine proteases were approximately 20-40 kDa, such as from *L. antarcticum*, 34.4 kDa (Turkiewicz et al., 2003); *Aspergillus ustus* 32 kDa (Damare et al., 2006); *Pseudomonas* sp. NJ276 (Wang et al., 2007) and *Pseudomonas* strain. DY-A, 25 kDa (Zeng et al., 2003). However, some cold-adapted protease also acquired large molecular mass such as from *Flavobacterium balustinum*, 70 kDa (Morita et al., 1998); *Flavobacterium psychrophilum*, 62 kDa (Secades et al., 2003) and *Stenotrophomonas maltophilia*, 65 kDa (Vasquez et al., 2005). Interestingly, cold-adapted serine protease from *L. antarcticum* strain PI12 in this study possessed the

other cold-adapted proteases with predicted molecular mass of 99.3 kDa. Figure 7 illustrates the picture of SDS-PAGE where a band at around 99.3 kDa was visibly seen from the recombinant supernatant. GpPro2 colony which contributed the highest protease activity demonstrated the most intense expression band (Lane 5) which proved that the PI12 protease was successfully expressed as fusion protein.

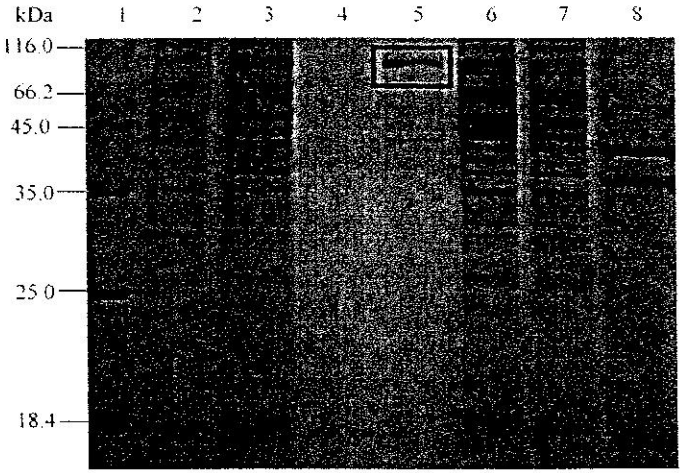


Figure 7. Coomassie-stained SDS-PAGE from Different Recombinant *P.pastoris* Clones. One mL supernatants collected from third-day culture were concentrated 10-fold using trichloroacetic acid, and 10 µL resultant products were applied to SDS–PAGE analysis. Lane 1: Molecular weight marker (Fermentas); Lane 2: GS115 (Control); Lane 3: GS115/pPIC9 empty (Control); Lane 4: GS115/pPIC9/PI12prot1 (GpPro1); Lane 5: GS115/pPIC9/PI12prot2 (GpPro2); Lane 6: KM71 (Control); Lane 7: KM71/pPIC9 empty (Control); Lane 8: KM71/pPIC9/PI12prot (KpPro1). Box indicates the secreted recombinant PI12 protease.

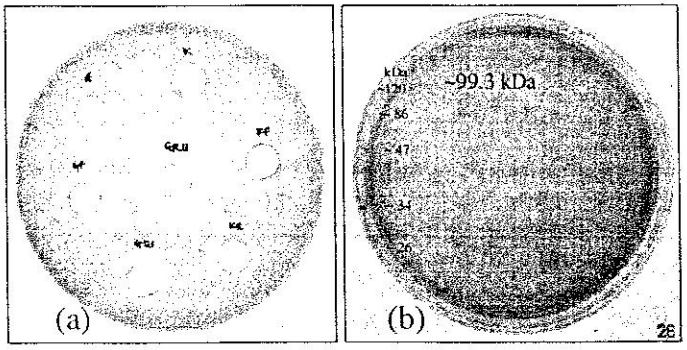


Figure 8. Activity Staining. (a) Plate activity staining of different recombinant *P.pastoris* clones. G: GS115; GP: GS115/pPIC9; GRI: GS115/pPIC9/PI12prot1 (GpPro1);GRII: GS115/pPIC9/PI12prot2 (GpPro2); K: KM71; KP: KM71/pPIC9; KR: KM71/pPIC9/PI12prot (KpPro). (b) Gel activity staining of recombinant 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).

Activity Staining and Cup Plate Assay

Two activity staining assays which were plate staining and gel staining were executed to examine if the recombinant PI12 protease expressed in *P. pastoris* was functionally active. In

the plate staining, the recombinant clones of *P. pastoris* GS115 were first grown and later induced by methanol by putting the methanol to the lid of the inverted skim milk plate. As expected, the supernatant obtained showed strong proteolytic activity when introduced into the plate (Figure 8 (a)). When these colonies were picked and regrown on milk plates, they formed halos regardless of the presence or the absence of methanol. In gel staining, proteolytic activitywas also detected around 99.3 kDa (Figure 8(b)). This is an evidence of protease secretion into the surrounding media whereby it catalyzes the breakdown of casein, thus, forming clearing zones around the activity ring and band. Apparently, the cold-adapted PI12 protease was functionally expressed in *P.pastoris* (GpPro2 clone) and it was proven through activity staining that the size of mature PI12 protease was 99.3 kDa.

Conclusion

Cold ecosystem, including Antarctica remains a place full of challenge and mysteries. Life in the cold is a barrier to overcome but it does not stop the place from being inhabited especially by the microbial community. Questions on microbial adaption along with its enzymes have still to be answered. However, the major prospect from the psychrophilic microorganism posed a huge opportunity to do research for better understanding on cold adaptation. Isolate PI12 or known as *Leucosporodium antarcticum* is an exquisite example of microorganism which has really shown its true potentials. This includes the findings of a unique bifunctional cold active enzyme (LipPI12). In addition, huge serine protease isolated is a new breakthrough since it has a very few identites with other proteases and no homology similarity with other psychrophilic proteases. Sequence analysis through bioinformatics studies revealed novel discoveries about this protein and it was particularly interesting enzyme since very few information had been unveiled from cold-adapted yeast until now. The production of the recombinant LipPI12 and the serine protease of *L. antarcticum* in *E.coli* and *P.pastoris* respectively are notably significant as cold-adapted enzymes are found to have important values from the industrial point of view. Isolation of bifunctional LipPI12 enzyme and the serine protease have led to better understanding of enzyme psychrophilicity. It is imperative that continuous research on these industrially important enzymes be pursued as benefits have been reaped from the molecular studies of cold active lipase and protease.

References

Aghajari, N., Feller G., Gerday, C. &Haser, R. (1998). Structures of the psychrophilic *Alteromonas haloplanctis a*-amylase give insights into cold adaptation at a molecular level. *Structure,* 6,1503–1516.

Aghajari, N.(2003). Crystal structures of a psychrophilic metalloprotease reveal new insights into catalysis by cold adapted proteases. *Proteins,* 50, 636–647.

Alquati, C., De Gioia, L., Santarossa, G., Alberghina, L., Fantucci, P. &Lotti, M. (2002). Thecold active lipase of *Pseudomonas fragi*: heterologous expression, biochemical characterizatiu on and molecular modeling. *European Journal of Biochemistry*, 269, 3321-3328.

Arnórsdóttir, J., Smáradóttir, R.B., Magnússon, O.T., Thorbjarnardóttir, S.H., Eggertsson, G. & Kristjánsson, M. (2002). Characterization of a cloned subtilisin-like serine proteinase from a psychrotrophic *Vibrio* species. *Federation of European Journal of Biochemistry*, 269, 5536-5546.

Arpigny, J. L., Feller, G. & Gerday, C. (1993). Cloning, sequence and structural features of a lipase from the Antarctic facultative psychrophile *Psychrobacter immobilis* B10. Biochim Biophys Acta 1171, 331–333.

Bendtsen, J.D., Nielsen, H., von Heijne, G. and Brunak, S. 2004. Improved prediction of signal peptides-SignalP 3.0. *Journal of Molecular Biology*, 340, 783-795.

Brandsdal, B. O., Smalås, A. O. & Åqvist, J. (2001). Electrostatic effects play a central role in cold adaptation of trypsin, *FEBS Letter*, 499, 171–175.

Brock, F.M., Forsberg, C.W. & Buchanan-Smith, J.G. (1982). Proteolytic activity of rumen microorganism and effect of proteinase inhibitors. *Applied and Environmental Microbiology*,44, 561-569.

Cardenas, F., de Castro M. S.,Sanchez-Montero, J.M., Sinisterra, J.V., Valmaseda, M., Elson, S.W. & Alvarez, E.(2001). Novel microbial lipases: catalytic activity in reactions in organic media. *Enzyme and Microbial Technology*, 28, 145-154.

Chessa, J.P., Petrescu, I., Bentahir, M., Beeumen, J.V. /7 Gerday, C. (2000). Purification,physico-chemical characterization and sequence of a heat labile alkaline metalloprotease isolated from a psychrophilic *Pseudomonas* species. *Biochimica et Biophysica Acta*, 1479, 265 274.

Choo,D.-W.,T., Kurihara, T., Suzuki, K. Soda & Esaki, N. (1998). A cold adapted lipase of an Alaskan psychrotroph, *Pseudomonas* sp. Strain B11-1: gene cloning and enzyme purification and characterization. *Applied and Environmental. Microbiology*, 64, 486-491.

Clare, J.J, Rayment, F.B., Ballantine, S.P., Sreekrishna, K. &Romanos M.A. (1991). High-level expression of tetanus toxin fragment C in *Pichiapastoris* strains containing multiple tandem integrations of the gene. *Biotechnology*, 9, 455-460.

D'Amico, S., Marx, J.C., Gerday, C., & Feller, G.(2003). Activity– stability relationship in extremophilic enzymes, *Journal of Biological Chemistry*, 278.

Demirjian, D.C., Meris-Varas, F. & Cassidy, C.S. (2001). Enzyme from extremophile.*Current Opinion Chemical Biology*, 5, 144-157.

Downes, P. F. and Ito, K.(2001). Compendium of methods for the microbiological examination of foods. 4th ed. Washington, D.C: American Public Health Association.

Efstratiadis, A., Posakony, J.W, Maniatis, T., Lawn R.M., Catherine O'Connell, C., Spritz, R.A, Deriel, J.K, Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C. & Proudfoot, N.J. (1980). The structure and evolution of the human β-globin gene family. *Cell*, 21, 653-668.

Feller, G. &Gerday, C. (2003). Psychrophilic Enzymes: Hot Topics in Cold Adaptations. *Nature* 1: 200-208.

Feller, G., Thiry, M.,Arpigny, J. L. &Gerday, C. (1991). Cloning and expression in *Escherichia coli* of three lipase-encoding genes from the psychrotrophic Antarctic strain *Moraxella* TA144. *Gene*, 102, 111–115.

Feller, G., Le Bussy, O. &Gerday, C. (1998). Expression of psychrophilic genes in mesophilic hosts: assessment of the folding state of a recombinant alpha-amylase. *Applied and Environmental. Microbiology*, 64, 1163–1165.

Fields, P. A. (2001). Protein function at thermal extremes: balancing stability and flexibility. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 129, 417–431.

Georlette, D., Damien, B., Blaise, V., Depiereux, E., Uversky, V.N., Gerday, C. & Feller, G. (2003). Structural and functional adaptations to extreme temperatures in psychrophilic, mesophilic and thermophilic DNA ligases, *Journal of Biological Chemistry*, 278.37015–37023.

Gerday, C, Aittaleb, M, Bentahir, M, Chessa J-P, Claverie, P, Collins T, D'AmicoS., Dumont, J., Garsoux, G.& Georlette, D.(2000). Cold-adapted enzymes: from fundamentals to biotechnology .*Trends in Biotechnology*, 18, 103-107.

Gianese, G., Argos, P. &Pascarella, S. (2001). Structural adaptation of enzymes to low temperatures. *Protein Engineering*, 14, 141–148.

Gupta, R., Beg, Q.K. & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, 59, 15-32.

Hoshino, T., Ishizaki, K., Sakamoto, T., Kumeta, H., Yumoto, I., Matsuyama, H. &Ohyiga,S. (1997). Isolation of a *Pseudomonas* species from fish intestine that produces a protease active at low temperature. *Letters in Applied Microbiology*, 25, 70-72.

Jaeger, K.E. &Reetz,M.T. (1998). Microbial lipases form versatile tools for Biotechnology. *Trends in Biotechnology*, 16, 396-403.

Kim, S.Y., Hwang, K.Y.,Kim, S.H., Sung, H.C., Han, Y.S. & Cho, Y.(1999). Structural basis for cold adaptation. Sequence, biochemical properties, and crystal structure o fmalate dehydrogenase a psychrophile *Aquaspirillu marcticum*, *Journal of Biological Chemistry*, 274, 11761– 11767.

Kouker, G. & Jaeger, K.E. (1987). Specific and sensitive assay for bacterial lipases. *Applied and Environmental. Microbiology*, 53, 211-213.

Kulakova, L. Galkin, A., Kurihara, T., Yoshimura, T. &Esaki, N. (1998). Cold-active serine alkaline protease from the psychrotrophic bacterium *Shewanella* Strain Ac10: Gene cloning and purification and characterization. *Applied and Environmental. Microbiology*, 65, 611-617.

Kumar, C. G. & Takagi, H. (1999). Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnology Advances*, 17, 561-594.

Margesin, R. &Schinner, F.(1994). Properties of cold-adapted microorganisms and their potential role in biotechnology. *Journal of Biotechnology*,33, 1-14.

MacFaddin. (1985). *Media for isolation-cultivation-identification maintenance of medical bacteria*, vol. 1. Baltimore: Williams & Wilkins.

Morita, Y., Sakaguchi, H., Murakami, Y., Yokoyama, K. & Tamiya, E. (1998). Properties ofa cold-active protease from psychrotrophic *Flavobacterium psychrophilum* P104. *Applied Microbiology and Biotechnology*, 50, 669-675.

Nevins, J.R.(1983). The pathway of eukaryotic mRNA formation. *Annual Review of Biochemistry*, 52, 441-466.

Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering*, 10, 1-6.

Pernas, M.A., C. Lopez, L. Pastrana & Rua, M.L. (2000). Purification and characterization of lip2 and lip3 isoenzymes from *Candida rugosa* pilot plant plan scale fed batch fermentation. *Journal of Biotechnology*,84, 163-174.

Rapoport, T.A. (1992). Transport of proteins across the endoplasmic reticulum membrane. *Science*, 258, 931-936.

Rashid, N., Shimada, Y., Ezaki, S., Atomi, H.&Imanaka, T.(2001). Low temperature lipase from psychrotrohic *Pseudomonas*sp. strain KB 700A. *Applied and Environmental Microbiology*, 4064-4069.

Rao, M.B., Tanksale, A.M., Ghatge, M.S. &Deshpande, V.V. (1998). Molecular andBiotechnological Aspects of Microbial Proteases. *Microbiology and Molecular BiologyReview*, 62, 597-635.

Russell, R. J., Gerike, U., Danson, M. J., Hough, D. W. & Taylor, G. L.(1998). Structural adaptations of the cold-active citrate synthase from an Antarctic bacterium. *Structure*, 6, 351-361.

Samad, M.Y.A., Razak, C.N.A., Salleh, A.B., Yunus, W.M.Z.W., Ampon, K., &Basri, M. (1989). A plate assay for primary screening of lipase activity. *Journal of Microbiological Methods*, 9, 51-56.

Secades, P., Alvarez, B. & Guijarro, J.A. (2003). Purification and properties of a new psychrophilic metalloprotease (Fpp2) in the fish pathogen *Flavobacterium psychrophilum*. *FEMS Microbiology Letters*, 226, 273-279.

Suzuki, T.,Nakayama, T., Choo, D.W.,Hirano, Y., Kurihara, T., Nishino, T. & Esaki, N.(2003). Cloning, heterologous expression, renaturation and characterization of a cold adapted esterase with unique primary structure from a psychrotroph *Pseudomonas* sp. strain B11-1.*Protein Expression and Purification*, 30, 171-178.

Svingor, A., Kardos, J., Hajdu, I., Nemeth, A. & Závodszky, P. (2001). A better enzyme to cope with cold. Comparative flexibility studies on psychrotrophic, mesophilic, and thermophilic IPMDHs. *Journal of. Biological Chemistry*, 276, 28121–28125.

Turkiewicz, M., Pazgier, M., Kalinowska H. &Bielecki, S. (2003). A cold-adapted extracellular serine proteinase of the yeast *Leucosporidium antarcticum*. *Extremophiles*, 7, 435-442.

Vazquez, S.C., Coria, S.H. &MacCormack, W.P. (2004). Extracellular proteases from eight psychrotolerant Antarctic strains. *Microbiological Research*, 159, 157-166.

Vasquez, S.C, Ruberto, L. &MacCormark, W.P. (2005). Properties of extracellular proteases from three psychrotolerant *Stenotrophomonas maltophilia* isolated from Antarctic soil. *Polar Biology*, 28, 319-325.

Von Heijne, G. (1990). The signal peptide. *Journal of Membrane Biology*, 115, 195-201.

Wang, Q.F., Hao, Y.H., Xu, Z., Miao, J.L. & Li, G.Y. (2007). Purification and properties ofan extracellular cold-active protease from the psychrophilic bacterium *Pseudoalteromonas* sp. NJ276. *Biochemical Engineering Journal*, 38, 362-368.

Zeng, R., Zhang, R., Zhao, J. & Lin, N. (2003). Cold-active serine alkaline protease from the psychrophilic bacterium *Pseudomonas strain* DY-A: Enzyme purification and characterization. *Extremophiles*, 7, 335-337.