The Journal of Microbiology, March 2003, p.22-27 Vol. 41, No. 1
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Purification and Characterization of Cold Active Lipase from Psychrotrophic *Aeromonas* sp. LPB 4

Han-Ki Lee, Min-Jung Ahn, Sung-Ho Kwak, Won-Ho Song, and Byeong-Chul Jeong*

Department of Biological Science, Myong Ji University, Yongin, Kyonggi-do, 449-728, Korea

(Received December 27, 2002 / Accepted February 10, 2003)

A lipase from *Aeromonas* sp. LPB 4, a psychrotophile isolated from a sea sediment was purified and characterized. The lipase was purified 53.5 fold to a homogeneous state by acetone precipitation and QAE sephadex column chromatography and its molecular weight was determined to be 50 kDa by SDS-PAGE. The enzyme exhibited maximum activity at 10°C and was stable at temperatures lower than 50°C. This lipase favored substrates containing medium carbon chain of acyl group, while too low and high carbon chain decreased its activity. The lipolytic activity of purified lipase was slightly increased by the addition of 0.1% detergent, but decreased by 1% of detergent. Butanol severely decreased the lipase activity while methanol increased the activity about 15%.

***Key words*:** psychrophile, *Aeromonas* sp. LPB 4, lipase

Lipases (glycerol-ester-hydrolases, E.C.3.1.1.3.) are carboxyl esterases that hydrolyze glycerides present as aqueous emulsions. Certain microorganisms are able to utilize lipids of plant and animal origin as the source of carbon and energy for growth by producing extracellular lipases (Fogarty, 1983). New applications of the microbial lipases such as hydrolysis of fats, transesterfication, stereospecific hydrolysis of racemic esters, organic synthesis, and the use of lipases in detergents have been developed (Mckay *et al.*, 1993; Gerday *et al.*, 1997; Marshall *et al.*, 1997). Large area of the earth's surface is occupied by cold environments, such as the Arctic, Antarctic, and alpine region, and abysses. Even under such inhospitable conditions, many cold-adapted microorganism's, called psychrophiles, can grow at 15°C or lowter, whereas psychrotroph is essentially a mesophilic microorganism that can also grow under cold conditions below 15°C (Morita, 1975; Takeshi *et al.*, 2001). The cold adaptation of these microorganisms is likely to be due to, at least in part, their ability to produce cold-active enzymes which exhibit higher catalytic activities at low temperatures than their mesophilic and thermophilic counterparts do (Margesin *et al.*, 1994). Recently, much attention has been made to the application of psychrophiles, psychrotrophs, and their cold-active enzymes in biotechnology (Choo *et al.*, 1998; Kulakova *et al.*, 1999; Gerday *et al.*, 2000). For example, cold-active hydrolases such as proteinases, amylases, cellulases and lipases from microorganisms have been used as additives in laundry detergent (Margesin *et al.*, 1994). Also, cold-adapted microorganisms and their enzymes have been used as catalysts for organic synthesis of unstable compounds at low temperatures (Langen *et al.*, 1999). Cold-adapted microorganisms could also be developed as agents for bioremediation at low temperatures. Bioremediation (Timmin and Pieper, 1999) is a recently-established technology that utilizes microorganisms for the restoration of contaminated environments. In some cases, bioremediation can be enhanced by adding microorganisms with specific metabolic functions, a procedure that is refered to as bioaugumentation. (Tanaka, S., 1998). In this study, we isolated a psychrotrophic bacterium, *Aeromonas* sp. LPB 4, from Sapkyo sea sediment, Korea, and the lipase was purified from this bacterium and characterized.

* To whom correspondence should be addressed.
(Tel) 82-31-330-6196; (Fax) 82-31-335-8249
(E-mail) bcjeong@korea.com

Materials and Methods

Enrichment and screening of psychrotrophic lipase producing bacteria

For screening of psychrotrophic lipase producing bacteria, enrichment cultivation was done from Sapkyo sea sediment, Korea. Enrichment culture contained 100 ml of LB (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter), 1 g of Sapkyo sea sediment, and 1% (v/v) of tributylin (Sigma, USA) as lipase substrate. After 30 days of enrichment cultivation at 10°C at 150 rpm, enrichment culture broth was diluted with cold distilled water and 100 μl of the 1/000 diluent was spread on tributylin-LB agar (LB agar containing 1% (v/v) of tributylin) and cultivated at 10°C. Psychrotophic bacterial colony appeared after 3 days and lipase producing bacteria was indentified by the tributylin-hydrolyzed clear zone after 5 days (Ana *et al.*, 1997).

Identification of isolated bacteria

For identification of isolated bacterium, 16S rDNA sequencing was done. The degenerated primer based on conserved sequence of 16S rDNA was used to amplify 16S rDNA from isolated bacterium using PCR. Nucleotide sequence of forward primer, called N16sF, was 5'-AGA-GTTTGATCMTGGCTCAG-3' (posed at 27 on 16S rDNA) and sequence of reverse primer, called N16sR, was 5'-GGYTACCTTGTTACGACTT-3' (posed at 1492 on 16S rDNA). 1.4kb PCR fragment was ligated into pGEM-T easy vector (Promega), and generated p16DNA was sequenced by BIONEX (Korea).

Enzyme purification

Aeromonas sp. LPB 4 was cultured by shaking at 200 rpm for 5 days at 10°C using 500 ml baffled flask containing 100 ml of LB, and then transferred to a 15 L jar with a working volume of 10 L of LB. The cells were grown for 8 days at 10°C, and the airflow rate was 1 vvm using air compressor. To purify the lipase, the culture supernatant was collected from culture broth by centrifugation (6000 g, 4°C, 10 min). The supernatant was precipitated by adding cold-acetone up to 70% (v/v) concentration of acetone. After centrifuging at 4°C and 6000 rpm for 30 min, the precipitate was resuspended in 50 ml of 50 mM Tris-Cl (pH 7.0) and dialyzed against 3 changes of 5 L of 50 mM Tris-Cl (pH 7.0) overnight. The protein solution after dialysis was applied onto QAE sephadex column (Pharmacia), pre-equilibrated with 50 mM Tris-Cl buffer, pH 7.0, and eluted with step gradient of 0 N, 0.1 N, 0.2 N, 0.3 N, 0.4 N, and 0.5 N NaCl. The active fractions were collected and then concentrated by ultrafiltration using YM30 (Amicon).

Lipase activity assay

Generally, lipase activity assay was performed using ρ-nitrophenyl substrate as described by Winkler and Stuckmann (Winkler and Stuckmann, 1979) with some modifications. In brief, the reaction mixture consisted of 135 μl of 0.4% (w/v) Triton-X (Sigma), 0.1% (w/v) gum Arabic (Sigma) in 50 mM Tris-Cl buffer, pH 7.0) and 15 μl of substrate (16.6 mM ρ-nitrophenyl substrate in 2-propanol). The mixture was pre-reacted at 10°C for 10 min and 50 μl of enzyme solution was subsequently added. After 30 min of incubation at 10°C, the color change was measured at 405 nm using multi-well plate reader (BIOTEK). One unit (U) of lipase activity was defined as the amount of enzyme that released 1 μmol of ρ-nitrophenol per min. For routine enzyme assay, ρ-nitrophenyl carproate (Sigma) was used as a lipase substrate. Protein concentration was measured by the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Electrophoretic analysis

Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel (Laemmli, 1970). SeeBlue® Plus2 (Invitrogen) was used as protein molecular size marker. When activity staining was performed, the sample was not boiled, and after electrophoresis, SDS was removed by washing the gel once for 20 min with 20% isopropanol and then twice for 10 min with distilled water. For lipase activity detection, the gel was transferred to a 1.3% agar plate containing 1% tributylin, 25 mM Tris-Cl (pH 7.0), and 5 mM $CaCl_2$. After incubation for 6 h at 10°C, lipase was visualized as clearing bands of tributylin hydrolysis (Patricia *et al.*, 1997)

Specific activity of purified lipase on different carbon chains

For determination of the enzyme specificity on carbon chain numbers, the following substrates (Sigma) were used: ρ-nitrophenyl acetate (C2), ρ-nitrophenyl propionate (C3), ρ-nitrophenyl butylate (C4), ρ-nitrophenyl carproate (C6), ρ-nitrophenyl carprate (C10), ρ-nitrophenyl laulate (C12), and ρ-nitrophenyl palmitate (C16).

Effect of temperature, organic solvents, detergents, and various chemicals on activity and stability of lipase

The relative activity of *Aeromonas* sp. LPB 4 lipase at various temperatures (0~100°C) was measured by using the standard procedure described above. To determine thermal stability, the enzyme samples were incubated in 50 mM Tris-Cl buffer (pH 7.0) at the each temperatures (0 ~100°C) for 30 min, cooled on ice, and then the residual activity was measured. Effect of detergents on lipase activity was analyzed by incubation of enzyme for 1 hr at 10°C in 50mM Tris-Cl buffer (pH 7.0) containing 0.1% or 1% (w/v) detergent. The control contained no detergent. Activity was measured at the beginning and at the end of incubation by the photometric assay with ñ-nitrophenyl carproate as a substrate. The detergent concentration in the assay mixture was 0.005 and 0.05%, respectively. The effect of organic solvents, 50% (v/v), on lipase was analyzed in a similar way to the effect of detergents. The mixture was incubated for 1 h at 10°C and the control contained no organic solvent. The solvent concentration in the assay mixture was 0.2% (w/v). The influence of various chemicals on lipase was determined by incubating of the enzyme for 1 h at 10°C in 50 mM Tris-Cl (pH 7.0) containing the reagent to be determined. Residual activity was measured by the photometric assay with ñ-nitrophenyl carproate as a substrate.

Results and Discussion

Isolation and identification of psychrotrophic lipase producing bacteria

To isolate psychrotrophic lipase producing bacteria,

enrichment cultivation was performed with Sapkyo sea sediment as described in Materials and Methods. After enrichment cultivation, thirty candidates which hydrolyzed tributylin during 5 days of incubation on tributylin-LB agar were isolated. They were re-screened on tributylin-LB agar and the bacterium forming the largest clear zone was selected. 16S rDNA sequence of isolated bacterium exhibited the highest similarity to that of a marine bacterium *Aeromonas hydrophila* (95% homology). A marine bacterium *Aeromonas hydrophila* can grow at various temperatures between 4~37°C and produce lipolytic enzyme (Pemberton *et al.*, 1997). And habitat of this bacterium is mainly sea sediment and colony of that is very viscous on agar plate because of lipopolysaccharide of this bacterium (Pemberton *et al.*, 1997). Isolated bacterium in this research showed the same characteristics. So the isolate was named as *Aeromonas* sp. LPB 4, for lipase producing bacterium 4. Aeromonas sp. LPB 4 could grow at temperature 4, 10, 20, and 30°C but not at temperature over 30°C on LB agar unlike *Aeromonas hydrophila* researched. So this bacterium may be a psychrotrophic bacterium. The production of lipase from *Aeromonas hydrophila* (Nieto *et al.*, 1987; Thornton *et al.*, 1988; Anguita *et al.*, 1993; Eggset *et al.*, 1994; Pongtharin, *et al.*, 1997;Yin *et al.*, 1997) was maximum at 30°C. But production of lipase from *Aeromonas* sp. LPB 4 was maximum at 10°C. The reason of different production of extracellular lipase by *Aeromonas* sp. LPB 4 compared to other *Aeromonas hydrophila* (Nieto *et al.*, 1987; Thornton *et al.*, 1988; Anguita *et al.*, 1993; Eggset *et al.*, 1994; Pongtharin *et al.*, 1997;Yin *et al.*, 1997) considered either by temperature-dependent production of it or the extracellular lipase from *Aeromonas* sp. LPB 4 is thermolabile (Naeem *et al.*, 2001).

Purification of the lipase from Aeromonas sp. LPB 4

The extracellular lipase activity was the highest when *Aeromonas* sp. LPB 4 was grown at 10°C for 8 days in LB medium. For purification of lipase from *Aeromonas* sp. LPB 4, we performed 10 L cultivation at 10°C for 8 days. The cell free supernatant was precipitated by cold acetone after centrifugation. After dialysis against 50 mM Tris-Cl, pH 7.0, the protein sample was applied onto QAE sephadex column and fractionated by step gradient. The active fraction was eluted by the 50 mM Tris-Cl, pH 7.0, containing 0.2 N NaCl and concentrated by ultrafilteration. After acetone precipitation and QAE sephadex chromatography, the enzyme was purified 53.5 fold to homogeneity with 7.5% yield (Table. 1). Two major protein bands were displayed after acetone precipitation on SDS-PAGE (Fig. 1) but a single protein band of 50 kDa after QAE sephadex chromatography. The zymographic analysis indicated lipolytic activity was associated with the 50 kDa band in both acetone precipitated proteins and the active fraction concentrate after QAE sephadex chromatography. Lipolytic activity of this protein was also detected on Native PAGE (data not shown). Lipases from *Aeromonas* sp., *A. hydrophila* MCC-2 (Yin *et al.*, 1997), *A. sorbia* LP004 (Pongtharin *et al.*, 1997), *A. hydrophila* (Nieto *et al.*, 1987), *A. hydrophila* (Thornton *et al.*, 1988), *A. salmonicida* (Eggset *et al.*, 1994), and *A. hydrophila* (Anguita *et al.*, 1993) were sized at approximately 80, 97, 15.5, 31, 26, and 72 kDa. But there have been no report on 50 kDa size of cold active lipases researched (Feller *et al.*, 1990; Mayorodomo *et al.*, 2000; Suzuki *et al.*, 2001; Alcuati *et al.*, 2002) except a low temperature lipase, psychrotrophic *Pseudomonas* sp. KB700A (Naeem *et al.*, 2001).

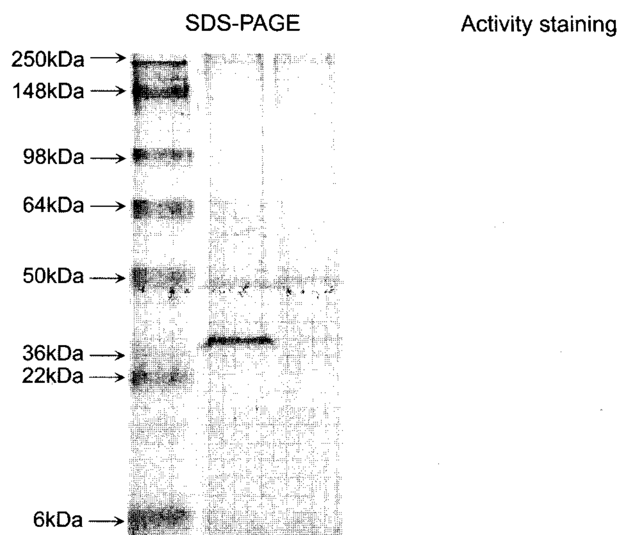

Fig. 1. Electrophoretical analysis of purified lipase from *Aeromonas* sp. LPB 4. Left panal showed SDS-PAGE and right panal showed zymogram with tributylin as a substrate. In zymogram, the reaction was performed at 10°C overnight.

Biochemical characterization of purified lipase

Generally, lipase activity showed different patterns depending on carbon chain length of acyl group. To determine the effect of carbon chain length of group on lipase

Table 1. Purification of lipase from *Aeromonas* sp. LPB 4.

Step	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Yield (%)	Purification fold
Culture broth (15 *l*)	2059	800	1.5	100	1
Acetone precipitation	224	138	14.7	17.3	9.8
QAE sephadex	16	60	80.3	7.5	53.5

[a] means μmole of p-nitrophenol released per min.

activity, various ρ-nitrophenyl substrates with different carbon chain lengths were used; ρ-nitrophenyl acetate (C2), ρ-nitrophenyl propionate (C3), ρ-nitrophenyl butylate (C4), ρ-nitrophenyl carproate (C6), ρ-nitrophenyl carprate (C10), ρ-nitrophenyl laulate (C12), and ρ-nitrophenyl palmitate (C16). The lipase showed high activity when ρ-nitrophenyl propionate (C3 acyl group) and ρ-nitrophenyl carproate (C10 acyl group) were used as substrates (Fig. 2). Thus medium chain acyl group ρ-nitrophenyl esters seemed to be good substrates for the purified lipase, while too short chain length of acyl group of ρ-nitrophenyl esters, such as ρ-nitrophenyl acetate (C2 acyl group), or ρ-nitrophenyl esters consisted of too long chain of acyl group more than C10 were poor substrates. This type of substrate specificity depending on the length acyl group was similar to lipase from *A. hydrophila* MCC-2 (Yin *et al.*, 1997). To determine the temperature effect on purified lipase, enzyme assay was performed at 4, 10, 20, 30, 40, 50, 70, and 100°C using ρ-nitrophenyl carproate (C6) as a substrate. The activity of purified lipase was similar at all temperatures tested (Fig. 3). The purified lipase was stable up to 50°C but stability of it decreased dramatically over 50°C (Fig. 3). Thus it was more active up to 50°C than other lipases from another *Aeromonas* sp. in terms of stability and activity at different temperatures (Yin *et al.*, 1997; Pongtharim *et al.*, 1997). And it was interesting that purified lipase from *Aeromonas* sp. LPB 4 maintained the lipolytic activity at low temperature. The activity of the cold enzyme presents an apparent optimal activity around 35°C and retains about 20% of its activity at 0°C, whereas the activity of the mesophilic lipases is close to zero at temperatures below 20°C and increases at temperatures above 60°C (Gerday *et al.*, 1997). So we suggest that the lipase of *Aeromonas* sp. LPB 4 has the characteristic of cold adapted enzyme. And lipase from *Aeromonas* sp. LPB 4 may be better in application to low temperature and more stable alternative to other lipases. The effects of various detergents on the purified lipase are shown in Table 2. In general, we observed an increase in lipase activity with the addition of detergents. Interestingly,

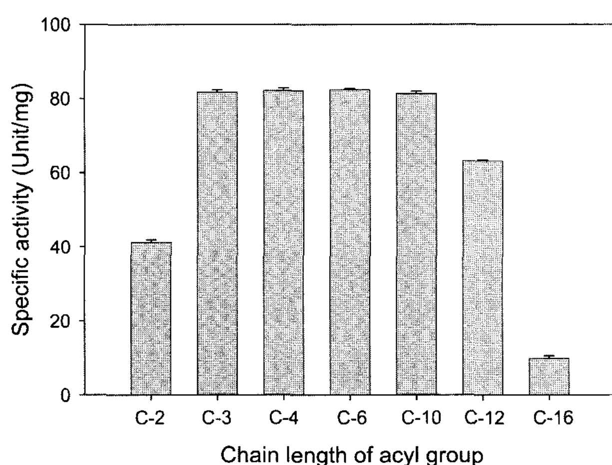

Fig. 2. Substrate specificity on different carbon chains of acyl group. Reaction was performed at 10°C for 30 min. C-2, C-3, C-4, C-6, C-10, C-12, and C-16 mean chain length of acyl group of substrate used . Substrates used were *p*-nitrophenyl acetate (C2), *p*-nitrophenyl propionate (C3), *p*-nitrophenyl butylate (C4), *p*-nitrophenyl carproate (C6), *p*-nitrophenyl carprate (C10), *p*-nitrophenyl laulate (C12), and *p*-nitrophenyl palmitate (C16). The data represent three separate experiments.

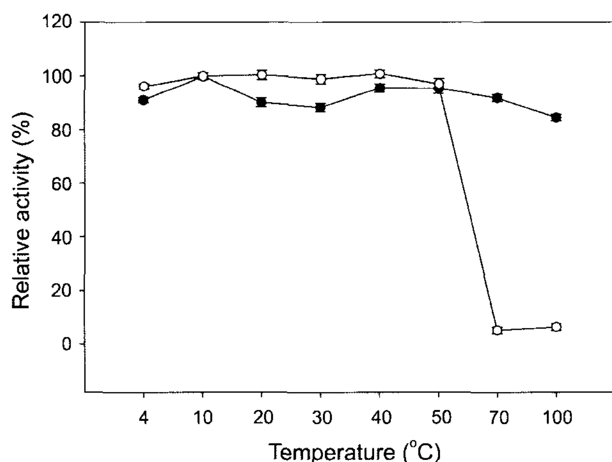

Fig. 3. Effect of temperature on the activity and stability of purified lipase from *Aeromonas* sp. LPB 4. Enzyme activity was assayed at each temperature (●). For thermal stability experiments (○), the enzyme was preincubated at indicated temperature for 30 min and the remaining activity was subsequently determined. The experiment was performed three times.

Table 2. Effect of various detergents on activity of purified lipase from *Aeromonas* sp. LPB 4.

Detergent	Concentration (%, w/v)	Relative activity (%)*
Control	0	100±0.1
Brij30	0.1	119±0.2
	1	102±0.5
Tween 80	0.1	131±0.3
	1	104±0.4
Triton X-100	0.1	121±0.5
	1	105±0.6
SDS	0.1	119±0.7
	1	109±0.5

*This experiment was performed triple.

Table 3. Effect of various solvents on activity of purified lipase from *Aeromonas* sp. LPB 4.

Solvent	Concentration(%, v/v)	Relative activity*
Control	0	100±0.1
Methanol	50	125±0.3
Ethanol	50	86±0.2
Isopropyl alcohol	50	67±0.4
Butanol	50	16±0.3

*This experiment was performed triple.

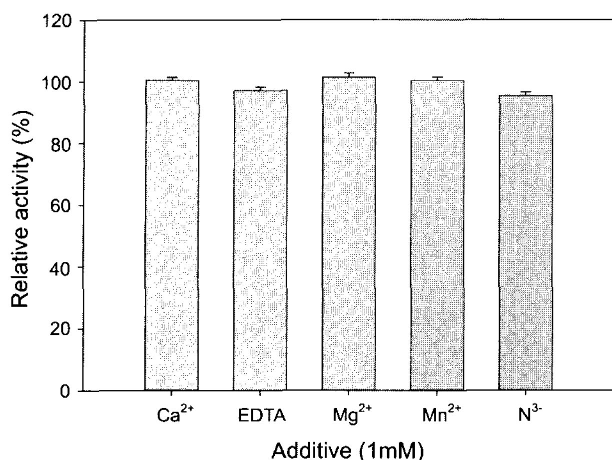

Fig. 4. Effect of various chemicals on activity of purified lipase from *Aeromonas* sp. LPB 4.

there was a 31% increase in activity when 0.1% (w/v) tween 80 was added. However, when detergent concentration increa-sed from 0.1% to 1%, the activity of the purified lipase slightly decreased. The activity of the purified lipase was decreased by the addition of 50% of organic solvents except methanol (Table 3). In the presence of 50% of methanol, the activity increased by 25% but significantly decreased in the presence of 50% of butanol. Several lipases showed enhanced lipolytic activity in the presence of Ca^{2+} and hence called Ca^{2+} dependent metalloenzyme (Matsumae and Shibatani, 1994). No influence on the lipase by 1 mM divalent cations, as Ca^{2+}, Mg^{2+}, and Mn^{2+} was observed after 1 h of incubation at 10°C (Fig. 4). So purified lipase may not be a Ca^{2+}-dependent lipase or a metalloenzyme.

Acknowledgments

This study was supported by grant No. 97-05 02-07-013 from the Korea Science and Engineering Foundation and Regional Research Center in Myongji University.

References

Alquati, C., L. De Gioia, G. Santarossa, L. Alberghina, P. Fantucci, and M. Lotti. 2002. The cold-active lipase of *Pseudomonas fragi*: Heterologous expression, biochemical characterization and molecular modeling. *Eur. J. Biochem.* 269, 3321-3328.

Ana, I., M. Moscovici, P. Cristina, A. Vamaru, O. Popa., and D. Laura. 1997. Screening of yeast and fungal strains for lipolytic potential and determination of some biochemical properties of microbial lipases. *J. Mol. Catal. B-Enzym.*. 3, 147-151.

Anguita, J., L.B.R. Aparicio, and G. Naharro. 1993. Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophyla* human isolate. *Appl. Environ. Microbiol.* 59, 2411-2417.

Birschbach, P. 1994. Origins of lipases and their characteristics. *Bull. Int. Dairy Fed.* 294, 7-10

Bjrkling, F., S.E. Godtfredsen, and O. Kirk. 1991. The future impact of industrial lipases. *Trends Biotechnol.* 9, 360-363.

Bradford, M.M. 1976. A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Choo, D. W., T. Kurihara, T. Suzuki, K. Soda, and N. Esaki. 1998. A cold adapted lipase of an alaskan psychtotroph, *Pseudomonas* sp. strain B11-1: gene cloning and enzyme purification and characterization. *Appl. Environ. Microbiol.* 64, 486-491.

Eggset, G.,R. Bjornsdottir, R.M. Leifson, J.A. Arnesen, and D.H. Coucheron. 1994. Extracellular glycerophospholipid-chorest-erol acyltransferase from *Aeromonas salmonicidia* activation by serine protease. *J. Fish. Dis.* 17, 17-29.

Feller, G., E. Narinx, J.L. Arpigny, M. Aittaleb, E. Baise, S. Genicot, and C. Gerday. 1996. Enzymes from psychrophilic organism. *FEMS Microbiol. Lett.* 18, 189-202.

Feller, G., M. and T., C. Gerday. 1990. Sequence of a lipase gene from the Antarctic psychrotroph *Moraxella* TA 144. *Nucleic Acids Res.* 18, 6431.

Forgarty, W.M. 1983. Microbial Enzymes and biotechnology, p. 187-207. Applied Sciences Pubilishers Ltd. London. New York.

Gerday, C., M. Aittaleb, J.L. Arpigny, E. Baise, J.P. Chessa, G. Garssoux, I. Petrescu, and G. Feller. 1997. Psychtrophilic enzymes: a thermodynamic challenge. *Biochim. Biophys. Acta.* 1342, 119-131

Gerday, C., M. Aittaleb, M. Bentahir, J.P. Chessa, P. Claverie, T. Collins, S. D'Amico, J. Dumont, G. Garsoux, D. Georlette, A. Hoyoux, T. Lonhienne, M. Meuwis, and G. Feller. 2000. Cold-adapted enzymes: from fundamentals to biotechnology. *Trends. Biotechnol.* 18, 103-107.

Kulakova, L., A. Galkin, T. Kurihara, T. Yoshimura, and N. Esaki. 1999. Cold-active serine alkaline protease from the psychrotrophic bacteria Shewanella strain Ac10: gene cloning and ezyme cloning purification and characteriazation. *Appl. Environ. Microbiol.* 65, 611-617.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277, 621-626.

Langen, L.M., E. Vroom, F. Rantwijk, and R. Sheldon. 1999. Enzymatic syhthesis of -lactamase antibiotics using penicillin-G acrylase in frozen media. *FEBS. Lett.* 456, 89-92.

Margesin, R. and F. Shinner. 1994. Properties of cold-adapted microorganisms and their potential role in biotechnology. *J. Biotechnol.* 33, 1-14.

Marshall, C.J. 1997. Cold-adapted enzymes. *Trends Biotechnol.* 15, 359-364.

Matsumae, H. and T. Shibatani. 1994. Purification and characterization of lipase from *Serratia marcescens* Sr41 8000 responsible for asymmetric hydrolysis of 3-phenylglysidic acid ester. *J. Ferment. Bioeng.* 77, 152-158.

Mayordomo, I., F. Randez-Gil, and J.A. Prieto. 2000. Isolation, purification, and characterization of a cold-active lipase from *Aspergillus nidulans*. *J. Agric. Food Chem.* 48, 105-109.

Mckay, A.M. 1993. Microbial carboxylic ester hydrolases (EC 3.1.1.) in food biotechnology. *Lett. Appl. Microbiol.* 16, 1-6.

Morita, R.Y. 1975. Psychrophilic bacteria. *Bacteriol. Rev.* 39, 144-167.

Naeem, R., S. Yuji, E. Satoshi, A. Haruyuki, and I. Tadayuki. 2001. Low-temperature lipase from psychrotrophic *Pseudomonas* sp.

strain KB700A. *Appl. Environ. Microbiol.* 2001, 4064-4069.

Nieto, J.M., J. Tomas, and A. Juarez. 1987. Secretion of an *Aeromonas hydrophyla*aerolysin by mutant strain of *Escherichia coli. FEMS Microbiol. Lett.* 48, 413-417.

Patricia, S., B. Christiane, and G. Friedrich. 1997. Genetic and biochemical characterization of a new extracellular lipase from *Streptomyces cinnamomeus. Appl. Environ. Microbiol.* 63, 3553-3560.

Pemberton, J.M., P.K. Stephen, and S. Radomir. 1997. Secreted enzymes of *Aeromonas. FEMS Microbiol. Lett.* 152, 1-10.

Pongtharin, L. and D. Saovanee. 1997. Purification and characterization of lipase from *Aeromonas sobria* LP004. *J. Biotechnol.* 54, 113-120.

Takeshi, S., N. Toru, K. Tatsou, N. Tokuzo, and E. Nobuyoshi. 2001. Cold-active lipolytic activity of psychtrophic *Acinetobacter* sp. Strain No. 6. *J. Biosci. Bioeng.* 92,144-148.

Tanaka, S. 1998. Pollutions of inland waters and remedial technology. *Foods Food Ingred. J. Jpn.* 176, 27-36.

Thornton, J., S.P. Howard, and J.T. Burkley. 1988. Molecular cloning of a phospholipids-cholesterol acyltransferase from *Aeromonas hydrophyla.* Sequence homologies with lecithin-cholosterol acyltransferase and other lipases. *Biochim. Biophys. Acta.* 959, 153-159.

Timmis, K.N. and D.H. Pieper. 1999. Bacteria designed for bioremediation. *Trends Biotechnol.* 17, 201-204.

Winkler, U.K. and M. Stuckmann. 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens. J. Bacteriol.* 138, 663-670.

Yin, C.C., F.C. Shu, H.S. Jer, L.W. Mei, and C.C. Ming. 1997. Molecular analysis and expression of the extracellular lipase of *Aeromonas hydrophila* MCC-2. *Microbiology.* 143, 803-812.