

Low-Temperature Active Lipase of Deep-Sea Psychrophilic Bacteria and Effect of Hydrostatic Pressure on Enzyme Activity

Nobuhisa TAKATA*¹ Tetsuo HAMAMOTO*¹
Koki HORIKOSHI*¹

Four psychrophilic bacteria, that produced lipases, were isolated from deep-sea sediment samples. The isolates, identified as *Vibrio* spp., and produced low optimum working temperatures lipases. Culture supernatant fluids exhibited optimal temperatures of around 30°C for hydrolyzation of *p*-nitrophenyl laurate (*p*-NP laurate), while these temperature optima decreased by 5–10°C in a reaction mixture containing 50% (v/v) *n*-hexane. Complete inactivation of enzyme activities occurred after a 10 min incubation at 40°C. Culture fluids from all four psychrophiles kept *p*-NP laurate-hydrolyzing activities at temperatures as low as –10°C in the presence of *n*-hexane. In addition, the effect of hydrostatic pressure regarding the reaction system was also examined. All enzymes showed the highest apparent activities at 0.1 MPa (= 1 atm).

Key words : psychrophilic bacteria, lipase

Introduction

Recent advances in the field of marine technology have made it possible to study the deep sea and its floor with more preciseness (Myers and Anderson 1992¹⁾).

One characteristic of the deep sea is its low temperature. The temperature of deep-sea water is constant at about 3°C (Austin (1988)²⁾, Baross and Morita 1978³⁾, Jannasch and Taylor (1984)⁴⁾), an ideal habitat for the isolation of bacteria living at low temperatures. Such bacteria should provide unique examples for investigating low-temperature adaptation^{3,4)}, as well as a new source material of biotechnological importance (Gounot (1986)⁵⁾, Gounot (1991)⁶⁾). Research on bacteria capable of growing at low temperatures (0°C or lower) began when the first bacteria of this kind were isolated by Foster in 1887 from preserved fish (Foster 1887⁷⁾). Later, Morita defined psychrophiles as those bacteria which have an optimal temperature for growth of lower than 15°C, a

maximal temperature for growth of lower than 20°C, and minimal temperature for growth at 0°C or lower (Morita (1975)⁸⁾). Those bacteria that could grow at temperatures as low as 5°C or lower but with higher optimal growth temperatures, he defined as psychrotrophs⁸⁾. A number of properties of enzymes from psychrotrophic and psychrophilic bacteria have been reported (Alichanidis and Andrews (1977)⁹⁾, Feller et al. (1989)¹⁰⁾, Hamamoto and Horikoshi (1991)¹¹⁾, Margesin and Schinner (1991)¹²⁾, Mitchel et al. (1985)¹³⁾, Nakajima et al. (1974)¹⁴⁾, Ochiai et al. (1979)¹⁵⁾). There may be possible applications for enzymes in the processing of food and the biotransformation of chemicals at low temperatures because of their low optimal temperatures^{5,6)}.

Since the deep sea is a high hydrostatic pressure environment, it is of interest to study the influence of hydrostatic pressure on physiological and biochemical reaction of deep-sea microorganisms. For example, the presence of a gene regulated by hydrostatic

* 1 Deep-sea Environment Exploration Program

pressure was reported (Bartlett et al. (1989)¹⁶⁾). Furthermore, a gene which is expressed more efficiently under high hydrostatic pressure was found (Kato et al. (1994)¹⁷⁾). Further research in this field might open a way for the fermentation and production of valuable substances under a high-pressure condition.

This report describes the properties of low-temperature active lipase produced in the culture fluids of several psychrophilic bacteria isolated from deep-sea sediment samples and the effect of hydrostatic pressure on the enzyme activities.

Isolation and characterization of psychrophiles using deep-sea sediment samples

Deep-sea sediment samples have been collected during a series of dives at various depths in Suruga Bay off Shizuoka, about 150km west of Tokyo. The isolation was carried out as previously described¹¹⁾. Bacterial colonies that appeared on the plates were removed and inoculated into 10ml of a modified LB liquid medium (1% Difco Tryptone, 0.5% Difco Yeast Extract, 3% NaCl w/v) and cultivated at 10°C for 2 days with shaking. Among the range of bacteria isolated from deep-sea sediment samples, four strains produced *p*-NP laurate-hydrolyzing enzymes in the culture media. These bacteria, designated No. 5405 (from a depth of 2625m), 5501 (from a depth of

2485m), 5502 (from a depth of 2485m), 5710 (from a depth of 2485m), grew rapidly at 4°C, although they showed poor growth at 20°C. Their taxonomic properties were determined as previously described¹¹⁾. Biochemical properties were examined using the ID test EB-20 (Nissui Pharmaceuticals). The organisms were gram-negative facultative anaerobes, and were curved motile rods in shape. On the basis of sensitivity to the vibrio-stat reagent, O-129 (10μg/disc, Oxoid), and a requirement for 0.5 M NaCl, but not seawater, for growth, we tentatively assign these bacteria to the genus *Vibrio* (Felter et al. (1970)¹⁸⁾, Krieg (1984)¹⁹⁾). The specific growth rates of the isolates at different temperatures by measuring the changes in optical density at 660nm are shown in Fig. 1. The maximum and optimum temperatures were around 20°C, 10–13°C, 10–13°C for three strains (No. 5405, 5501 and 5502), respectively, while one isolate, No. 5710 had its maximum temperature at around 15°C and the optimum temperature was observed at 4–8°C. Therefore, each of these bacteria fit Morita's definition of a psychrophile⁸⁾ for the growth capability at lower temperatures and sensitivity to grow at a temperature above 20°C.

Characterization of lipase activity

Lipases were produced in culture supernatant broths at 10°C in an LB medium containing 3% NaCl. Culture supernatants 40 hr after inoculation were obtained by centrifugation at 10,000g for 15min. Enzyme activity in aqueous reaction mixtures was measured according to the method of Heymann and Mentlein (Heymann and Mentlein (1981)²⁰⁾) by mixing 20μl of the supernatant sample with 960μl of 50mM Na-phosphate buffer, pH 7.0 containing 2μl of 500mM *p*-NP laurate (SIGMA chemicals) acetonitrile solution. The reaction started by adding samples to the pre-warmed reaction mixture and the increase in hydrolyzed *p*-nitrophenol was measured by the increase in absorbance at 405nm in a temperature regulated cell holder using a Hitachi 100 spectrophotometer. Temperature stability of the four activities were determined by incubating supernatant samples at various temperatures for 10min (Table 1).

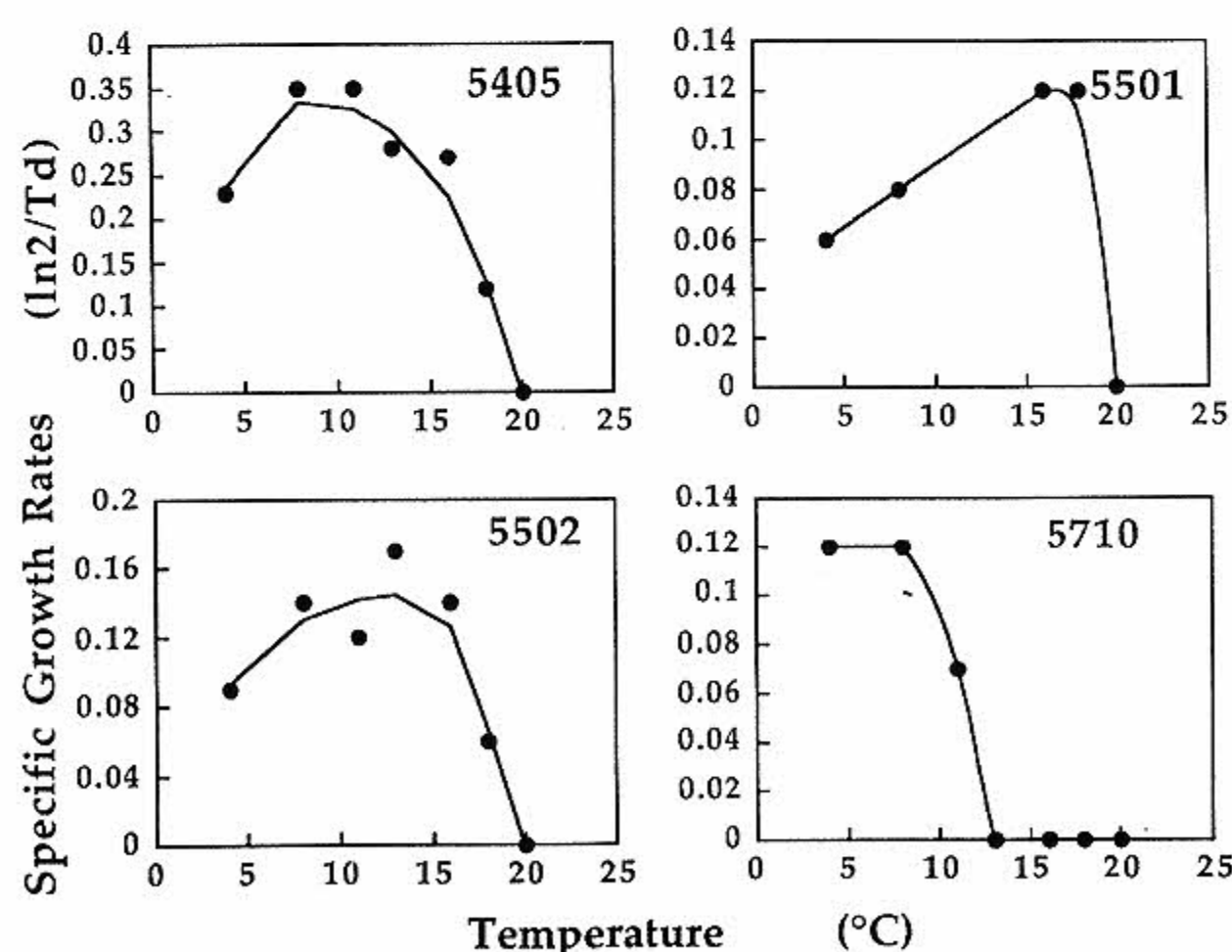


Fig. 1 Effects of temperature on the growth of deep-sea psychrophilic bacteria. Growth is expressed as specific growth rate, $\ln 2/\text{generation time}$.

Table 1 Temperature stability of the activities in the culture supernatants.

Stain No.	Residual activity (%)			
	0°C	30°C	40°C	50°C
5405	100	100	5	0
5501	100	76	3	0
5502	100	86	2	0
5710	100	40	2	0

Residual activities were calculated taking the activity after incubation at 0°C for 10 minutes as 100%.

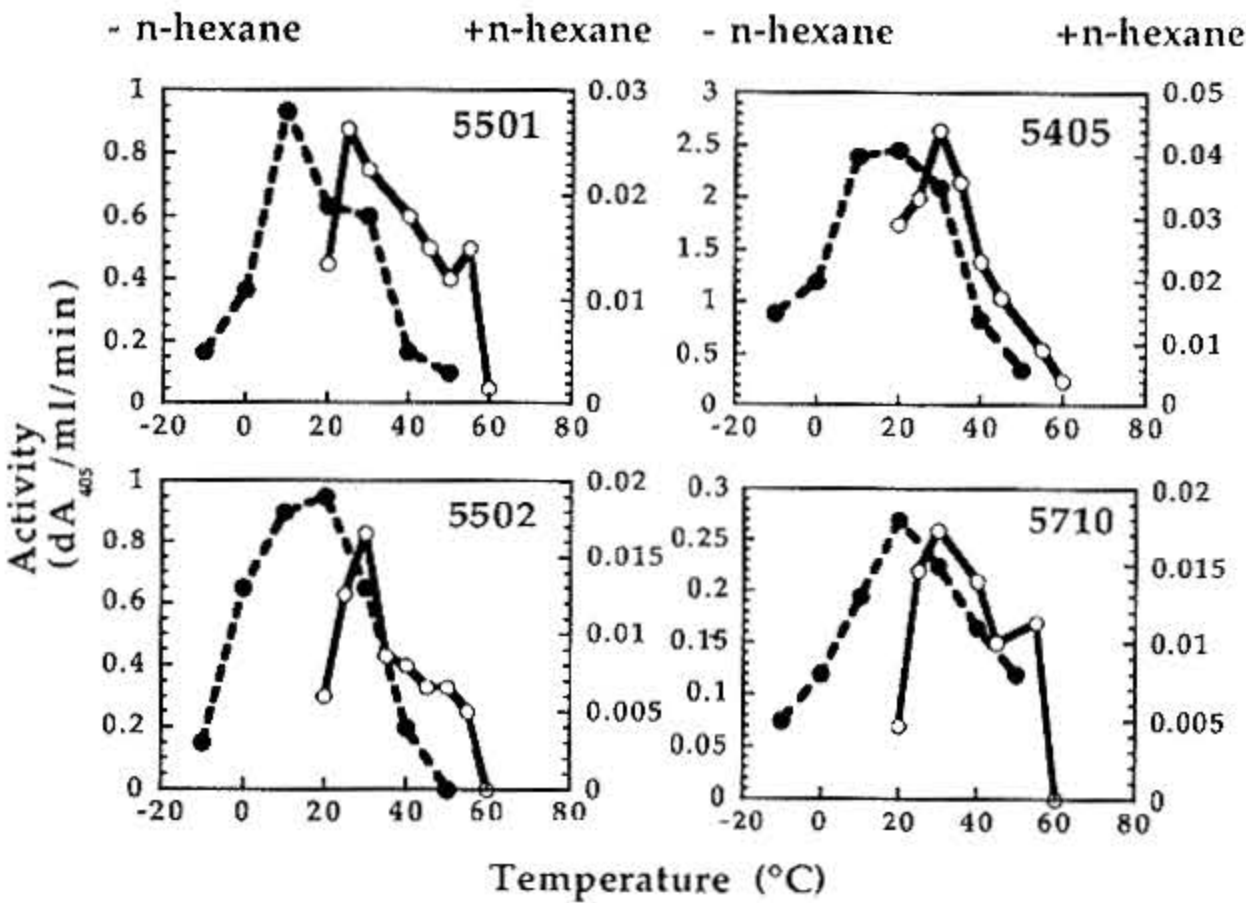


Fig. 2 Effects of temperature on enzyme activities of culture supernatants. Activity was measured by the increase of absorbance at 405 nm either directly (aqueous condition) or after 30 min of incubation for reaction mixture containing *n* hexane. Solid lines indicate the reactions without *n*-hexane, and broken lines indicate the reactions with *n*-hexane. Activity was expressed as the increase in absorbance at 405 nm by 1 ml of sample in 1 min.

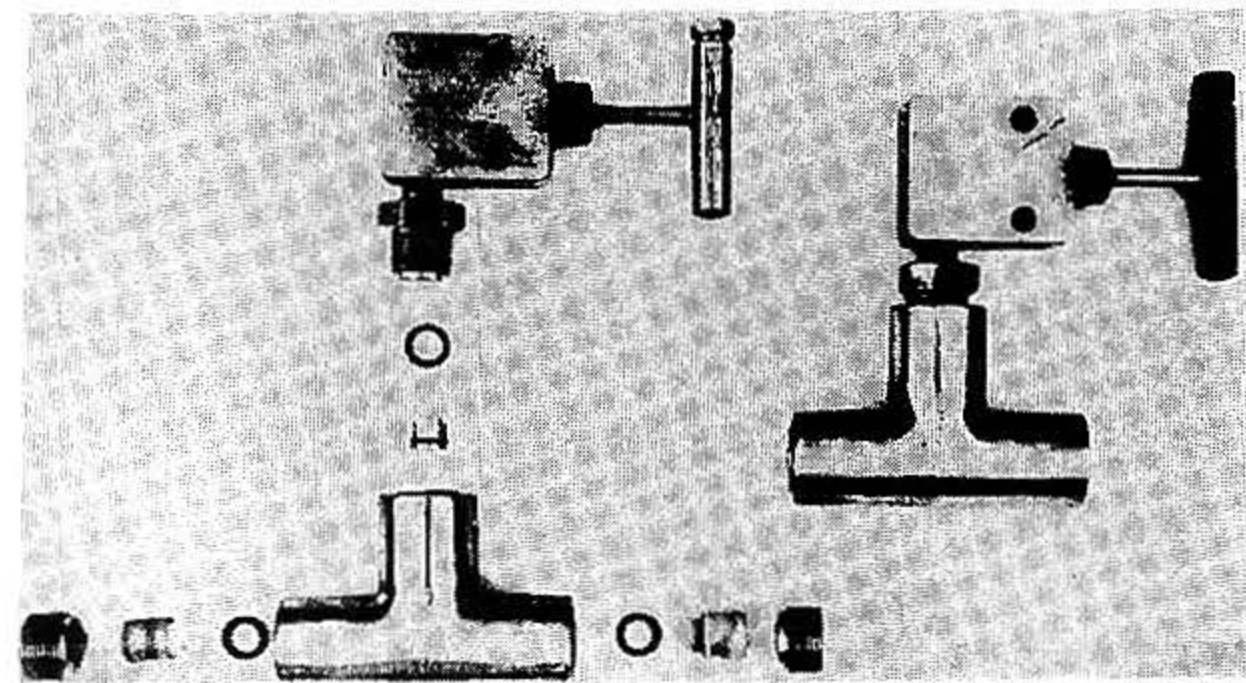


Fig. 3 Photograph of Optical High Pressure Cylinder.

The incubated samples were put into ice, and the activities were measured at 30°C in the aqueous reaction system described above. Activities in the cul-

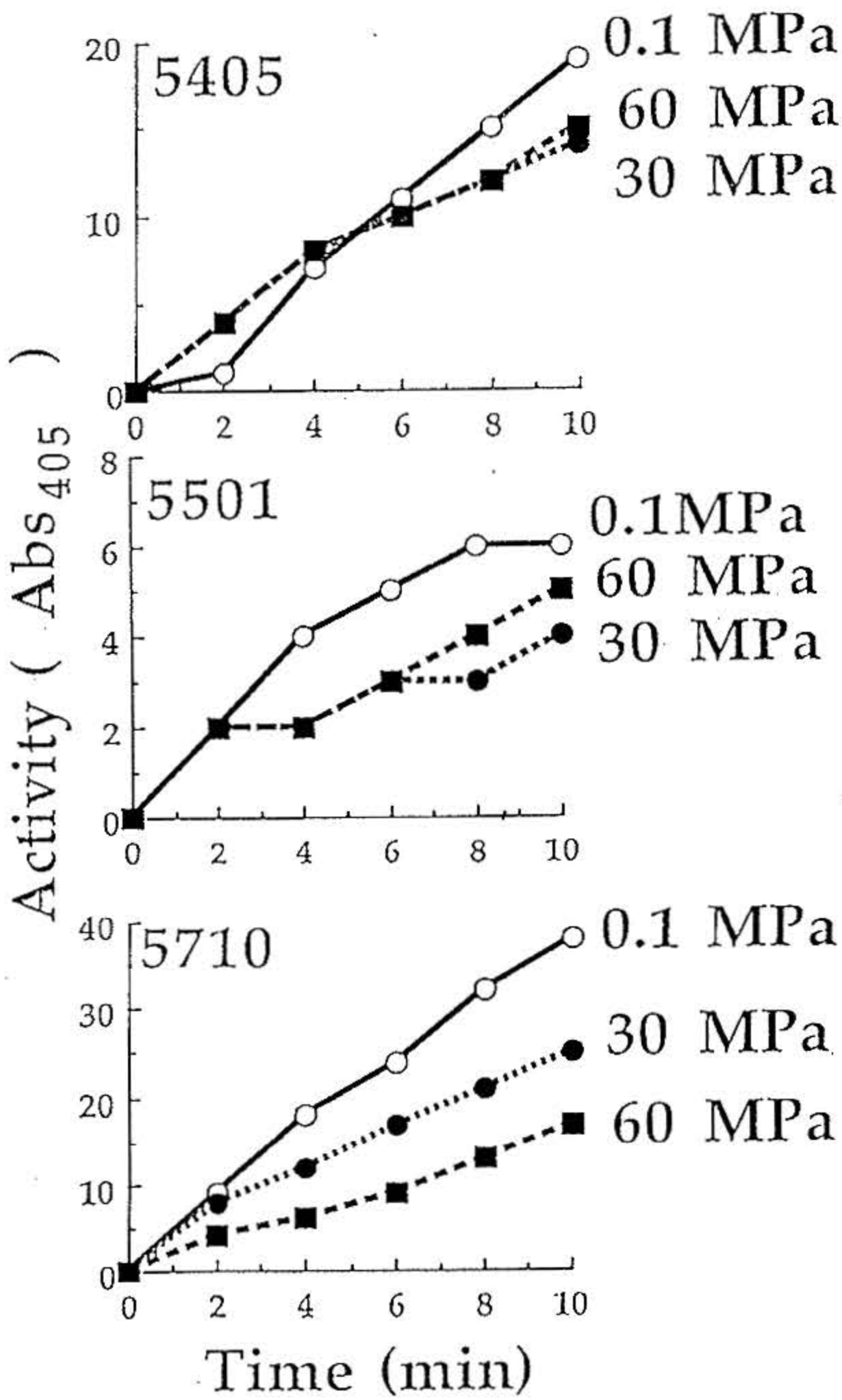


Fig. 4 Effects of hydrostatic pressure on enzyme activities. Activity was measured by the increase of absorbance at 405nm directly. Solid lines indicate the reaction under 0.1 MPa. Fine and rough broken lines indicate the reaction under 30 MPa and 60 MPa. Activity was expressed by the absorbance at 405 nm as a function of reaction time.

ture supernatant of all four psychrophiles were completely lost after being incubated.

Reactions containing *n*-hexane were performed in a reaction mixture containing 300μl of the supernatant sample, 450μl of 50mM Na-phosphate buffer, pH 7.0 and 750μl of 10mM *p*-NP laurate dissolved in *n*-hexane, and vigorously shaken (200 times/min) for 30 min or 1 hr at different temperatures. After the incubation, the reaction mixture were centrifuged at 5,000g for 1 min at 4°C. The lower aqueous phase was diluted to twice the volume with pre-warmed distilled water at 30°C and the absorbance was meas-

ured immediately at 405nm. Linearity of the reaction was confirmed by measuring the increase in absorbance over 15 min or 30 min. Although it was not possible to measure the activities with *p*-NP laurate in an aqueous media at temperatures lower than 10°C because of precipitation of the substrate at such low temperatures, activity in the presence of *n*-hexane could be observed at temperatures as low as -10°C. The effect of temperature regarding activities in an aqueous and *n*-hexane-containing reaction mixture were compared (Fig. 2). At -10°C, enzyme reactions occurred at rates of 10-20% of those at the optimal temperatures in *n*-hexane containing reaction mixtures. The optimal temperatures for enzyme activities for all four strains decreased by 5 to 10°C when *n*-hexane was present in the reaction mixture. About 1-6% of the activities obtained in the aqueous reaction mixture were observed in the presence of *n*-hexane. All the measurements were carried out in duplicate and compared with the reaction mixtures before incubation and reaction mixture containing heat-inactivated (80°C, 1 hr) samples.

The significant retention of enzymatic activities at low temperatures is compared with other lipases isolated from psychrotrophic microorganisms¹⁰⁾. Although the low-temperature characteristics of enzymatic activities reported here observed only with crude culture supernatants, characterization of purified enzymes could provide useful information for a biochemical study of psychrophily. It may also be possible to apply these enzymes in various chemical reactions at low temperatures.

Effect of hydrostatic pressure on enzyme activity

To observe the effect of hydrostatic pressure, an optical high pressure cylinder (Fig. 3), constructed by R. Morita, was utilized (Morita (1957)²¹⁾). Using this equipment, it was possible to measure an enzyme reaction optically without releasing high hydrostatic pressure (up to 100 MPa). It is made of stainless steel with white sapphire windows. A neutral piston prevents the hydraulic fluid and the reaction mixture from mixing. This neutral piston was fitted with a 8/32 in. self-seal screw which had one

side cut away to allow for the escape of gas and excess liquid in the optical high pressure cylinder. After filling the cylinder with the reaction mixture, the excess gas or liquid was replaced by the neutral piston and the self-seal screw was tightened. The valve was attached and the optical high pressure cylinder attached to the pressurizing apparatus and pressurized to the desired pressure. The total liquid volume of the optical high pressure cylinder was 7.5 ml. Enzyme activity was measured at 30°C.

Reaction system used was the same as the aqueous system used in the temperature-activity experiment but the total volume of reaction mixture was increased to 7.5ml.

After pouring the reaction mixture into the optical high pressure cylinder, it was immediately pressurized to the desired pressure, and the increase in absorbance at 405nm was measured. The increase in absorbance at 405nm is shown in Fig. 4.

All enzymes showed their highest activity at 0.1 MPa. A comparison of these enzyme activities with lipases from bacteria, which have no pressure-tolerance, would be a way to estimate the pressure-activation of the enzymes.

Acknowledgements

The authors are very grateful to the staff of the Japan Marine Science And Technology Center for operating the vessels and obtaining the samples, and to R.A. Herbert and M.G. Cockcroft for providing a method for lipase assay. They are also thankful to W.D. Grant for critically reading this manuscript.

References

- 1) Myers, S.F., and A. Anderson. Microbes from 20,000 feet under the sea. *Science* **255**: 28-29. (1992)
- 2) Austin, B. Marine microbiology. Cambridge University Press. (1988)
- 3) Baross, J.A., and R.Y. Morita. Microbial life at low temperatures: ecological aspects. p.9-72. In D.J. Kushner (ed.), *Microbial life in extreme environments*. Academic Press, London. (1978)
- 4) Jannasch, H.W., and C.D. Taylor. Deep sea mi-

- crobiology. Ann. Rev. Microbiol. **38** : 487-514. (1984)
- 5) Gounot, A-M. Psychrophilic and psychrotrophic microorganisms. Experimentia **42** : 1192-1197. (1986)
- 6) Gounot, A-M. Bacterial life at low temperature : Physiological aspects and biotechnological implications. J. Appl. Bacteriol. **71** : 386-397. (1991)
- 7) Forster, J. Ueber einhne Eigenschaften leuchtender Bakterien. Centr. Bakteriolog. Parasitenk. **2** : 337-340. (1887)
- 8) Morita, R.Y. Psychrophilic bacteria. Bacteriol. Rev. **39** : 144-167. (1975)
- 9) Alichanidis, E., and A.T. Andrews. Some properties of the extracellular protease produced by the psychrotrophic bacterium *Pseudomonas fluorescens* strain AR-11. Biochim. Biophys. Acta **485** : 424-433. (1977)
- 10) Feller, G., M. Thiry, J-L Arpigny, M. Mergeay, and C. Gerday. Lipases from psychrotrophic antarctic bacteria. FEMS Microbiol. Let. **66** : 239-244. (1989)
- 11) Hamamoto T., and K. Horikoshi. Characterization of an amylase from a psychrotrophic *Vibrio* isolated from a deep-sea mud sample. FEMS Microbiol. Let. **84**. 79-84 (1991)
- 12) Margesin, R., and F. Schinner. Characterization of a metalloprotease from psychrophilic *Xanthomonas maltophilia*. FEMS Microbiol. Let. **79** : 257-262. (1991)
- 13) Mitchel, P., H.C. Yen, and P.F. Mathemier. Properties of lactate dehydrogenase in a psychrophilic marine bacterium. Appl. Environ. Microbiol. **49** : 1332-1334. (1985)
- 14) Nakajima, M., K. Mizusawa, and F. Yoshida. Purification and properties of an extracellular proteinase of psychrophilic *Escherichia freundii*. Eur. J. Biochem. **44** : 87-96. (1974)
- 15) Ochiai, T., N. Fukunaga, and S. Sasaki. Purification and some properties of two NADP⁺-specific isocitrate dehydrogenase from and obligately psychrophilic marine bacterium, *Vibrio* sp., strain ABE-1. J. Biochem. **86** : 377-384. (1979)
- 16) Douglas Bartlett, Miriam Wright, A. Aristides Yayanos, and Michael Silverman. Isolation of a gene regulated by hydrostatic pressure in a deep-sea bacterium. Nature **342** : 572-574. (1989)
- 17) Kato, C., S. Hata, S. Suzuki, M. Smorawinska, and K. Horikoshi. The isolation and properties of deep-sea bacteria at high hydrostatic pressure. pp. 152-163 In S. Kunugi, S. Shimada, A. Suzuki, and R. Hayashi (ed.), High Pressure Bioscience. Sanei Syuppan, Tokyo. (1994)
- 18) Felter, R.A., S.F. Kennedy, R.R. Colwell, and G. B. Chapman. Intracytoplasmic membrane structures in *Vibrio marinus*. J. Bacteriol. **102** : 552-560. 1970)
- 19) Krieg, N.R. Aerobic/microaerobic, motile, herical/vibroid, Gram-negative bacteria. p. 71-124 In N.R. Krieg and J.G. Holt (ed.), Bergey's manual of systematic bacteriology, The Williams & Wilkins Co. Baltimore. (1984)
- 20) Heymann, E. and R. Mentlein. Carboxylesterases-Amidases. In Methods in Enzymology. **77** : 333-344. (1981)
- 21) R.Y. Morita. Effect of hydrostatic pressure on succinic formic and malic dehydrogenase in *Escherichia coli*. J. Bacteriol. **74** : 251-255. (1957)

(Received : 28 July 1994)

深海から得られた好冷性細菌の生産する低温で 高活性を示す脂質分解酵素と静水圧の 酵素活性に与える影響

高田 信久*² 浜本 哲郎*² 堀越 弘毅*²

深海底において採取された底泥サンプルより、脂質分解酵素を生産する好冷性細菌4株が分離された。これらの菌株はいずれも分類学的にはビブリオ属と同定され、これらにより生産される脂質分解酵素は反応至適温度を低温側に持つものであった。酵素活性は水系および有機溶媒（ノルマルヘキサン）存在系の2条件で測定され、液体培養上清中の酵素のパラニトロフェニルラウレートに対する加水分解反応の至適温度は、水系では約30℃であったが、50%のノルマルヘキサン存在下では約5-10℃へ減少し、-10℃もの低温においてすら活性を示した。これらの酵素はいずれも40℃、10分間の加熱処理で完全に失活した。さらに、反応系の静水圧の酵素活性に与える影響を調べたところ、いずれも大気圧条件下においてもっとも高い見かけの活性を示した。

キーワード：好冷性細菌，脂質分解酵素

* 2 深海環境プログラム