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Screening and Characterization of Psychrotrophic, Lipolytic Bacteria from Deep-Sea Sediments

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Abstract Of 23 psychrotrophic bacteria isolated from the west Pacific deep-sea sediments, 19 were assigned to the γ-*Proteobacteria*, 3 to the β-*Proteobacteria*, and 1 to the Gram-positive bacteria, as determined by their 16S rDNA sequences. Ten psychrotrophs, affiliated to the *Psychrobacter*, *Pseudoalteromonas*, and *Pseudomonas* genera in the γ-*Proteobacteria* group, were screened for lipolytic bacteria. The majority of the lipolytic isolates had growth temperatures between 4–30°C, and all of them were neutrophilic, aerobic, or facultatively anaerobic, and some were able to produce multiple kinds of ectohydrolytic enzymes. The deep-sea strains *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27 were chosen for further lipase production analysis. Both strains had the highest lipase production when grown at 10 to 20°C; their highest lipase production occurred at the late-exponential growth stage; and the majority of the enzymes were excreted to the outside of the cells. Lipases from both strains had the same optimal reaction temperature and pH (20–30°C, pH 7–8) and could retain about 60% of their highest activity at 4°C. Furthermore, SDS-PAGE and an in-gel activity test showed that they had the same high molecular mass of about 85 kDa.

Key words: Deep-sea sediment, psychrotroph, phylogenetic analysis, lipase

The deep-sea is regarded as an extreme environment with commonly high hydrostatic pressure and predominantly low temperatures below 4°C. Deep-sea bacteria have attracted more and more research interests as they are not only essential in some fundamental scientific study areas, but also they provide potential for commercial development [7, 11]. Recent microbial studies of the deep ocean have led to significant new discoveries of unusual microbial diversity, metabolic activity, and natural products of interest to biotechnology and bioremediation [9, 16, 23, 26, 29, 30]. However, partly due to the great difficulties in collecting deep-sea samples, it remains as one of the most unknown areas on the earth.

Lipases (EC3.1.1.3), which are characterized by their activity towards hydrolyzing water-insoluble emulsified long-chain triacylglycerols, are important industrial enzymes [1, 14]. They could be useful in the areas of detergent manufacture, synthesis of drugs, flavor production, and so on [15]. Most commercially available lipases are of bacterial or fungal origin. Lipolytic bacteria are widely distributed in nature, with around 20% of several thousand microbes isolated from soil shown to be lipase-producers, as tested on solid and liquid media for lipase production [14]. Lipases of specific characteristics such as active at low or high pH/temperature, and having different substrate specificity, are of particular value in industry. Interest in searching for novel lipase-producers, or cloning novel lipase genes with distinct characters, is growing [20, 28], although more efforts are needed. This is particularly the case for cold active lipases which have great potential in the fields of wastewater treatment, bioremediation in fat contaminated cold environment, active compounds synthesis in cold conditions, and so on [28].

Studies have revealed that the majority of marine bacteria have extremely high protease and lipase activities, owing to their significant role in organic matter cycling in the ocean [21]. The deep-sea provides a rich resource to explore lipolytic psychrotrophs or psychrophiles. Psychrophiles are defined as an organism having optimal growth temperature of 15°C or lower, whereas a psychrotroph is an essentially mesophilic organism which could also grow under cold conditions below 15°C [18]. In this study, the aim was to investigate the west Pacific deep-sea psychrotrophic,

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lipolytic bacterial community, and also to partly characterize the lipases from representative deep-sea bacteria.

MATERIALS AND METHODS

Collection of Samples

Deep-sea sediment samples were collected by a multi-core sampler during a cruise of HaiYang No. 4 in the west Pacific at the sites of wp02-1 (E125°00.0' N16°56.9', 3,000 m in depth), wp02-2A (E148°44.8' N19°24.1', 5,080 m), wp02-3 (E148°00.0' N13°00.0', 4,500 m), and wp02-4 (E141°43.7' N09°46.8', 2,900 m) during May 2002. The sediment cores were transferred to sterile polypropylene tubes on a clean bench and kept at 4°C for shipping to the laboratory, then stored at -20°C.

Isolation of Psychrotrophic, Lipolytic Deep-Sea Bacteria

Sediments were suspended in artificial sea water containing 0.3% NaCl, 0.07% KCl, 0.53% $MgSO_4 \cdot 7H_2O$, 1.08% $MgCl_2 \cdot 6H_2O$, and 0.1% $CaSO_4 \cdot 7H_2O$. Aliquots were taken out and spread onto marine 2216E agar plates containing 0.5% Tryptone, 0.1% Yeast Extract, 0.01% $FePO_4$, 3.4% NaCl, and 1.5% agar. The plates were incubated at 10°C for up to 14 days. The isolated strains were classified by morphological characters. The distinctive strains recovered from marine 2216E agar plates were tested for their lipolytic activities by inoculating on minimal medium agar plates containing 0.1% Yeast Extract, 0.01% FePO4, and 3.4% NaCl, supplemented with 2.5% (vol/vol) olive oil and 0.001% rhodamine B, or with 1% (vol/vol) Tween 80 or 1% Tween 20. Lipolytic bacteria selected under UV light at 350 nm showed orange-red fluorescent halos on the Rhodamine B-Olive oil agar plates, or formed fuzzy halos around the colonies on Tween 80 or Tween 20 agar plates. Furthermore, other enzymes produced by the strains, including amylase, protease, alginase, chitinase, and β-galactosidase, were also tested according to the methods described previously [3].

DNA Isolation, PCR Amplification, and DNA Sequencing

DNAs were extracted from the strains and used as templates for PCR amplification of the 16S rDNA fragments according to the methods described previously [10]. Primers Eubac27F and Eubac1492R were used to amplify a 1.5 kb fragment of the bacterial 16S rRNA gene.

PCR fragments were recovered from the gel by a Qiagen purification kit, and cloned into a pGEM-T vector following the instructions of the manufacturer (Promega). The 16S rDNA fragments were sequenced by the Sangon Company (Shanghai). The 16S rDNA sequences of the deep-sea strains had already been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databanks under accession numbers: wp14: AJ551095; WP16: AJ551096; wp17: AJ551097; wp18: AJ551098; wp19: AJ551099; wp20: AJ551100; wp21: AJ551101; wp24: AJ551102; wp25: AJ551103; wp26: AJ551104; wp27: AJ551105; wp29: AJ551106; wp30: AJ551107; wp31: AJ551108; wp32: AJ551109; wp33: AJ551110; wp34: AJ551111; wp37: AJ551112; wp38: AJ551113; wp39: AJ551114.

Phylogenetic Tree Construction

The 16S rRNA gene sequences were analyzed in the Ribosomal Database Project (RDP) database, manually aligned to 16S rDNA sequences from the RDP and GenBank using the PHYLIP package (Felsenstein, J., PHYLIP, version 3.5c) and DNAMAN (version 5.1, Lynnon Biosoft). A phylogenetic tree was constructed by the neighbor-joining method using the PHYLIP package and DNAMAN programs [25]. Bootstrapping analysis was used to evaluate the tree topology of the neighbor-joining data, and 1,000 trials were performed.

Enzyme Activity Assay

p-Nitrophenylacetate (PNPA) and *p*-nitrophenylpalmitate (PNPP) were used separately for the esterase and lipase activity assay as described previously [2]. Cell culture supernatant or cell sonicate was used for testing lipolytic activity excreted to the outside of the cell or inside. The assays were performed by supplementing 200 µl of cell culture supernatant or cell sonicate with 1.8 ml of substrate assay solution [2]. The activity was calculated by measuring the absorbance of liberated *p*-nitrophenol at 405 nm. One unit of activity was defined as the amount of enzyme needed to release 1 µmol of *p*-nitrophenol per min. All measurements in this experiment were made in triplicate.

Protein Isolation and Gel Electrophoresis

Enzymes in the culture supernatant were isolated and concentrated by using a centrifugal concentrator, with a molecular mass cutoff of 10 kDa (Pall Filtron). Cytoplasmic proteins of the strains were isolated and concentrated by sonication as described previously [2]. Proteins were separated by SDS-PAGE according to Laemmli [17]. The gel was suspended in 20% (vol/vol) isopropanol and incubated for 30 min, then transferred to distilled water and incubated for 30 min. Lipolytic activity was detected by placing the re-natured gel onto an agarose gel containing 1% Tween 80. The incubation was done at 30°C for 3 h, and examined for transparent bands on the Tween 80 containing agarose gel. After the activity test, the polyacrylamide gel was stained by Coomassie Brilliant Blue R250.

RESULTS AND DISCUSSION

Isolation of Psychrotrophic Lipase-Producers

Based on the morphological differences, such as colony color, edge-shape, etc, 27 morphologically distinct bacterial

Table 1. Physiological characteristics of the lipolytic psychrotrophs from deep-sea sediments.

Strain number	Growth temp range (°C)	Growth pH	Oxygen requirement	Gram reaction[a]	Oxidase[b]	Lipolytic enzyme production test[c]	Other enzymatic activities
wp17	4–35	neutrophile	facultatively anaerobic	-	+	Oliv+, Tw20+	amylase, protease
wp18	4–30	neutrophile	aerobic	-	+	Oliv+	
wp21	4–30	neutrophile	aerobic	-	+	Oliv+, Tw20+	protease
wp24	4–30	neutrophile	aerobic	-	+	Tw80+, Tw20+	gelatinase
wp25	4–30	neutrophile	facultatively anaerobic	-	+	Tw80+, Tw20+	gelatinase
wp27	4–30	neutrophile	aerobic	-	+	Oliv+, Tw80+	β-galactosidase
wp30	4–30	neutrophile	aerobic	-	+	Oliv+, Tw80+	
wp32	4–30	neutrophile	aerobic	-	+	Tw80+, Tw20+	gelatinase
wp33	4–35	neutrophile	aerobic	-	+	Oliv+	amylase, protease
wp37	4–30	neutrophile	aerobic	-	+	Oliv+, Tw80+, Tw20+	

[a]Represents negative.

[b]+ represents positive oxidase.

[c]Lipolytic enzyme production tests were carried out by inoculating the strains on agar plates supplemented with olive oil or Tween 80 or Tween 20 as described in the Materials and Methods. Oliv+, Tw80+, and Tw20+ mean positive signal on plates containing olive oil, Tween 80, or Tween 20, respectively.

strains were isolated from the west Pacific deep-sea sediments by using marine 2216E agar plates. Then, the strains were cultivated at both 4°C and 37°C. Twenty-three strains, which did not grow at 37°C but grew at 4°C, were screened for cold-adaptive bacteria by further lipolytic activity tests. Ten of the 23 strains showed positive lipolytic activity on the olive oil or Tween agar plates (Table 1).

All the ten deep-sea lipolytic bacteria were not psychrophilic, and their optimal growth temperature was higher than 15°C at around 25°C. As all the strains have some growth at 4°C, they could be assigned as psychrotrophs. The physiological characters of the strains were partly characterized. All the 10 strains are Gram-negative, aerobic, or facultatively anaerobic. Some strains could produce other ectohydrolytic enzymes besides lipase or esterase, such as protease, gelatinase, amylase, and β-galatosidase.

Forty-three % (10 of 23) of the psychrotrophic strains isolated from the deep-sea sediments showed lipolytic activity. As far as is known, this percentage of lipase-producers in the bacterial community from the deep-sea sediments is much higher than the number (20%) mentioned in a previous report on the screening of lipase-producers from a soil sample [14]. It is presumed that, possibly, the west Pacific deep-sea sediments used in this study may contain lipids or related materials, and that the deep-sea lipolytic bacteria are involved in the recycling of these organic matters. This study's result supports the previous study, which found that the majority of marine bacteria had high lipase activity [21].

Phylogeny of Psychrotrophic Bacteria from Deep-Sea Sediments

The 16S rRNA gene fragments of the 23 psychrotrophs including the 10 lipase-producers from the west Pacific deep-sea sediments were cloned and sequenced. The phylogenetic tree of these psychrotrophs with related reference strains were constructed as shown in Fig. 1. Nineteen strains were affiliated to the γ-*Proteobacteria* group including the *Halomonas*, *Stenotrophomonas*, *Psychrobacter*, *Pseudomonas*, and *Pseudoalteromonas* genera; 3 fell into the β- *Proteobacteria* group, and 1 into the Gram-positive bacteria group (Fig. 1).

The determined 16S rRNA gene sequences of the 10 lipase-producers named as wp17, wp18, wp21, wp24,

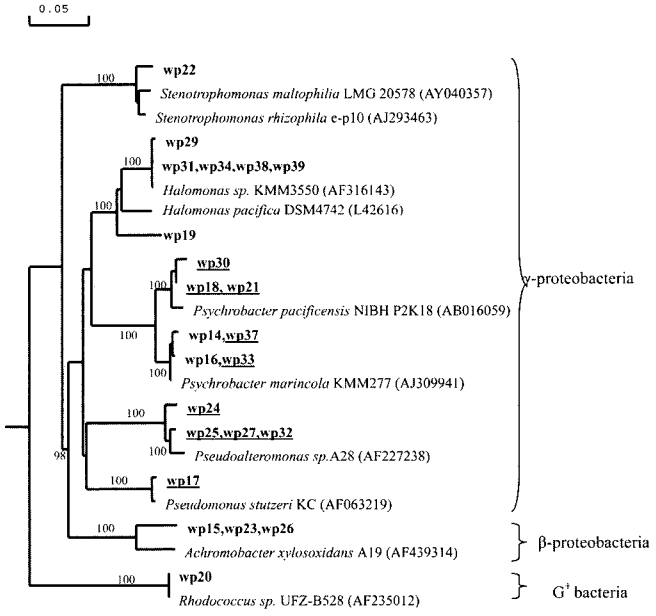

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of the isolates.

The phylogenetic tree was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method using the DNAMAN program, and 1,000 trials of bootstrap analysis were undertaken. All the 23 psychrotrophs isolated from the west Pacific deep-sea sediments named as wp are included in the phylogenetic tree. The 10 lipase-producing strains are underlined; the other 13 strains are indicated by bold letters.

wp25, wp27, wp30, wp32, wp33, and wp37 were compared with the 16S rDNA sequences obtained from the EMBL, GenBank, and DDBJ databases. Five strains were assigned to the *Psychrobacter* genus, 4 to the *Pseudoalteromonas* genus, and 1 to the *Pseudomonas* genus in the γ-*Proteobacteria* group: wp18, wp21, wp30 are most closely related to *Psychrobacter pacificensis* NIBH P2K18 (16S rDNA sequences had 98-99% similarity); wp33, wp37 are most closely related to *Psychrobacter submarines* KMM225 and *Psychrobacter marincola* KMM277 (98-99% similarity); wp24 has the closest relation with *Pseudoalteromonas gracilis* B9 (96% similarity); wp25, wp27, and wp32 have the closest relation with *Pseudoalteromonas ruthenica* kmm290 (96% similarity); and wp17 is most closely related to *Pseudomonas sutzeri* KC (99% similarity).

The deep-sea *Psychrobacter* strains isolated could be divided into two groups according to their 16S rDNA sequences. One group is most related to *P. pacificensis*, and the other most related to *P. submarines*- *P. marincola*. The first described *P. pacificensis* strains were isolated from deep seawater (6,000 m) in the Japan Trench, and they were found to be the dominant psychrophilic bacteria in this deep-sea environment [22]. It was noted that almost the same psychrophilic *Psychrobacter* strains dominated the extremely cold, remote environments such as the Antarctic surface and the Japan Trench's deep seawater, therefore it was supposed that some microorganisms in the Japan Trench probably originated in polar regions such as the Antarctic Ocean [4, 5, 22]. The deep-sea strains wp18, wp21, and wp30, which were isolated from west Pacific deep-sea sediment, are very probably the same *P. pacificensis* species. It is highly likely that they may have the same origin as the Japan Trench isolates. Strains wp14, wp16, wp33, and wp37 are most related to *P. submarines* and *P. marincola*. Type strains of these two species were isolated from seawater and the internal tissues of an ascidian Polysyncraton species [19]. These two species are psychrophilic, Gram-negative, aerobic coccoid, and their 16S rDNA sequences share 99.9% similarity with each other. Wp33 and wp37 are lipolytic bacteria, and they partly share characteristics of the key features of *P. submarines* and *P. marincola*. Wp33 and wp37 differ in that wp33 could produce amylase and protease, in addition to lipase. The possibility that the two groups of *Psychrobacter* strains in the west Pacific deep-sea sediments may have different origins could not be excluded.

Pseudoalteromonas genus has attracted intensive study, as the strains in this genus play important roles in marine environments, and some are producers of biologically active compounds and enzymes [12, 13]. In fact, all the *Pseudoalteromonas* strains isolated from the west Pacific deep-sea sediments could produce lipase, and could also produce either gelatinase or β-galactosidase. *Pseudoalteromonas* strains capable of thiosulfate oxidation [31] and octasaccharide production [24] have been isolated previously from deep-sea sediments and hydrothermal vents. The deep-sea *Pseudoalteromonas* strains wp24, wp25, wp27, and wp32 are probably new *Pseudoalteromonas* species as suggested by their 16S rDNA sequences. No similar strains isolated in the deep-sea have ever been reported.

Lipase Profile of *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27

The strains of *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27 were chosen as representatives for further enzyme analysis, as they showed the highest lipase activity on the Rhodamine B-olive oil agar plates, and they belong separately to the two different genera which encompass nearly all the isolated lipolytic strains in the study. Both of

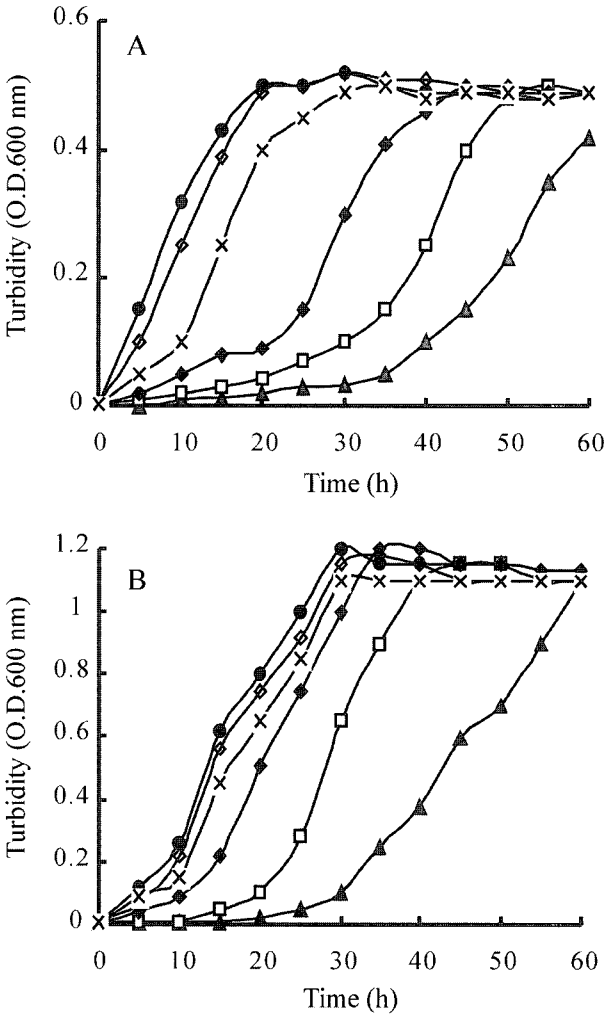

Fig. 2. Growth of *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27 under various temperatures.
Psychrobacter sp. wp37 (A) and *Pseudoalteromonas* sp. wp27 (B) were cultivated in marine 2216E broth at 4°C (▲), 10°C (□), 15°C (◆), 20°C (◇), 25°C (●), and 30°C (×).

the two strains had a growth temperature range between 4 to 30°C with optimal growth temperature around 25°C (Fig. 2). The lipase and esterase activities of the strains were measured after being grown in different media including marine 2216E, the minimal medium (MM) including 1% olive oil or 1% Tween 80 at 4°C, 10°C, 15°C, 20°C, 25°C, and 30°C at 4-h intervals. The results showed that the two strains had a similar lipase production profile: they had the highest lipase and esterase activity at 10 to 20°C grown in marine 2216E media, only had little lipolytic activity at 30°C, and had some lipolytic activity at 4°C. Most of the produced lipolytic enzymes were excreted to the outside of the cells. The addition of Tween 80 or olive oil to the media decreased the production of lipase to different extents (data not shown). This might be caused by the transcriptional inhibition of the lipase gene by the long-chain fatty acids resulting from hydrolysis of the olive oil or Tween 80. Previous studies have revealed that the presence of long-chain fatty acids in the growth media inhibited lipase production [15]. Other studies have revealed that psychrotrophs normally have the highest enzyme production at temperatures lower than the optimal growth temperature [6]. This was also the case for *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27.

Furthermore, the relationship between lipase production and the growth phase of the strains was analyzed. Results revealed that the highest lipase production occurred at the late-exponential growth stage of the strains, as shown in Fig. 3, using *Psychrobacter* sp. wp37 grown at 15°C and 20°C as examples.

Properties of Enzyme

The cytoplasmic proteins of *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27 were isolated and separated by SDS-PAGE (Fig. 4A). After electrophoresis, the gel was re-natured, and the lipase bands were visualized by placing the polyacrylamide gel onto Tween 80 containing agarose gel and incubating at 30°C for 3 h. A single lipase band around 85 kDa was detected in each strain, as shown in Fig. 4B. The molecular masses of reported lipases vary extensively, mostly around 30–40 kDa [15, 27], with the largest lipase ever reported being isolated from *Aeromonas hydrophila* of 80 kDa [8]. The molecular mass of the lipase from the deep-sea strains wp27 and wp37 are unusually large, among the largest lipases found to date.

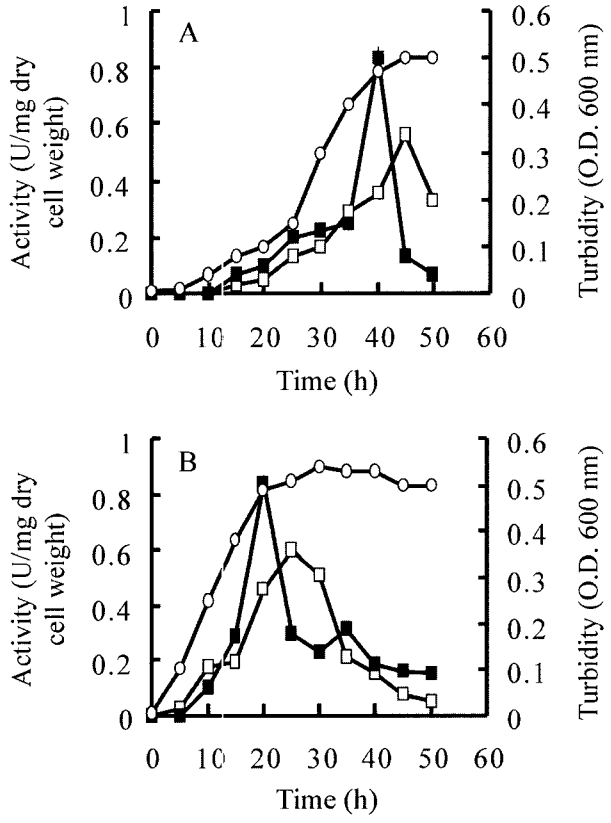

Fig. 3. Lipolytic activities of the culture supernatants of *Psychrobacter* sp. wp37.

Psychrobacter sp. wp37 was cultivated in marine 2216E broth at 15°C (A) and 20°C (B). Growth (O.D.) is indicated by (○); esterase and lipase data (using PNPA and PNPP as the respective substrates) are represented by (■) and (□), respectively.

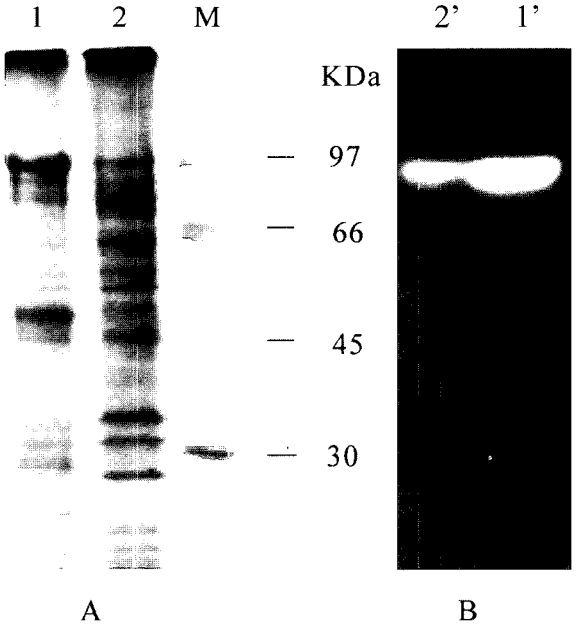

Fig. 4. SDS-PAGE analysis of proteins from *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27 and in-gel lipase activity test.

Cytoplasmic proteins of *Psychrobacter* sp. wp37 and *Pseudoalteromonas* sp. wp27 were extracted, and separated by SDS-PAGE. Proteins were stained with Coomassie blue R250 (A). Lipase activity was revealed by overlaying the re-natured polyacylamide gel onto the gel containing Tween 80 and incubating at 30°C for 3 h (B). Lane 1, 1' cytoplasmic proteins of *Pseudoalteromonas* sp. wp27; Lane 2, 2' cytoplasmic proteins of *Psychrobacter* sp. wp37; Lane M: Protein molecular marker.

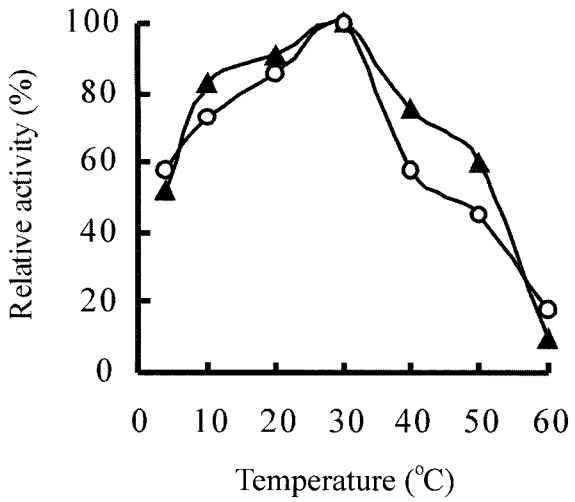

Fig. 5. Effects of temperature on the lipase activities of *Psychrobacter* sp. wp37 and *Pseudoalteromonas*. sp. wp27.
The enzyme activities of *Psychrobacter* sp. wp37 (○) and *Pseudoalteromonas* sp. wp27 (▲) were measured at various temperatures (4°C, 10°C, 20°C, 30°C, 40°C, 50°C, and 60°C) at pH 8.0 using pNPP as substrate. The highest lipase activity was set as 100%.

Concentrated proteins from cell culture supernatant and cell sonicate were applied for an enzyme optimal reaction condition test. With PNPA or PNPP as substrate, the enzymes from wp27 and wp37 showed maximum activity at pH 7-8. Then, assays to gauge the effect of temperature on the enzyme activity were carried out at 4°C, 10°C, 20°C, 30°C, 40°C, 50°C, and 60°C at pH 8. The optimal temperature for each of the two enzymes was determined to be around 20-30°C. Both enzymes were active in the cold, exhibiting lipolytic activity at 4°C, and retaining about 60% of their highest activity at the optimal temperature (Fig. 5).

The two strains wp27 and wp37 belong to different genera, but they were shown to produce a lipase of identical molecular mass by SDS-PAGE and an in-gel activity test. Furthermore, they showed the same optimal reaction pH and temperature, and it is highly possible that the lipases from the two strains are identical. The results suggest that a lipase gene horizontal transfer may have occurred among the bacteria in the west Pacific deep-sea sediments. To confirm this, the researchers of this study will need to purify the enzyme or clone the lipase gene from the two strains and make comparisons in detail.

Acknowledgments

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