

## ANTARCTIC MARINE BACTERIUM *PSEUDOALTEROMONAS* SP. KNOUC808 AS A SOURCE OF COLD-ADAPTED LACTOSE HYDROLYZING ENZYME

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### ABSTRACTS

Psychrophilic bacteria, which grow on lactose as a carbon source, were isolated from Antarctic polar sea water. Among the psychrophilic bacteria isolated, strain KNOUC808 was able to grow on lactose at below 5°C, and showed 0.867 unit of o-nitrophenyl β-D-galactopyranoside (ONPG) hydrolyzing activity at 4°C. The isolate was gram-negative, rod, aerobic, catalase positive and oxidase positive. Optimum growth was done at 20°C, pH 6.8-7.2. The composition of major fatty acids in cell of KNOUC801 was C<sub>12:0</sub> (5.48%), C<sub>12:0 3OH</sub> (9.21%), C<sub>16:0</sub> (41.83%), C<sub>17:0 ω8</sub> (7.24%) and C<sub>18:1 ω7</sub> (7.04%). All these results together suggest that it is affiliated with *Pseudoalteromonas* genus. The 16S rDNA sequence corroborate the phenotypic tests and the novel strain was designated as *Pseudoalteromonas* sp. KNOUC808. The optimum temperature and pH for lactose hydrolyzing enzyme was 20°C and 7.8, respectively. The enzyme was stable at 4°C for 7 days, but its activity decreased to about 50% of initial activity at 37°C in 7 days.

**Key words:** antarctic marine bacterium, *Pseudoalteromonas* sp., cold adapted lactose hydrolyzing enzyme.

### INTRODUCTION

Marine and terrestrial Antarctic ecosystems are a rich source of cold-evolved microorganisms, whose specific molecular mechanisms confer thriving at low temperatures via an adjustment of their metabolism to harsh environmental conditions (13). Microorganisms isolated from these extreme environments have developed adaptive mechanisms, including enzymes that catalyze reactions at temperatures near 0°C. Another attribute of these cold-active enzymes is their thermolability, i.e., they are inactivated at moderate

temperatures close to those at which enzymes from mesophiles are functional (14, 19). Cold active enzymes have a huge biotechnological potential (7, 13, 19) in detergent formulations (e.g., proteinases, lipases, amylases, and cellulases), in dairy industry (e.g., β-galactosidases), as environmental biosensors (e.g., dehydrogenases), for biotransformation (many specific enzymes, e.g., methylases and aminotransferases). One particularly interesting enzyme is the psychrophilic lactose hydrolyzing enzyme. This enzyme is potentially useful: (i) for fast lactose digestion below 20°C, to produce lactose-free milk-derived foods for lactose-intolerant

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individuals (approximately 30% of the world population), (ii) to avoid lactose crystallization in dairy foodstuffs, and (iii) to degrade lactose in the bulk pollution in dairy sewage. A number of cold active  $\beta$ -galactosidases from different sources of *Arthrobacter psychrolactophilus* (23), *Bacillus subtilis* KL88 (16), *Carnobacterium pisciocola* BA (3), *Pseudoalteromonas haloplakis* (8), and *Planococcus* (22) have been studied. However, the cold active lactose hydrolyzing enzyme useful for practical process has not been discovered yet. Mesophilic *Kluyveromyces lactis*  $\beta$ -galactosidase is currently used for lactose hydrolysis at low temperature, and it does not show so good activity at and below 20°C. Replacement of it with a psychrophilic counterpart will shorten the process of lactose cleavage at refrigerated temperature, eliminate any contamination with mesophilic microflora, and avoid nonenzymatic browning products formed at high temperature. Therefore more study is required to find the lactose hydrolyzing enzyme active at low temperature and useful practically.

In the present study, we conducted to screen psychrophilic micro-organisms that are able to hydrolyze lactose at low temperature, and examined the cold active lactose hydrolyzing enzyme produced by the isolated psychrophilic microorganism.

## MATERIALS AND METHODS

### Screening and isolation conditions

In Antarctic polar sea, 15 samples of sea water were collected and enriched in the medium of sea water complex (SWC) media (3.0g yeast extract, 5.0g bacto-peptone, 3.0ml; glycerol, 750ml filtered sea water, 250ml distilled water, pH 7.2-7.3), and Zobell media (25) at 4°C for 30days aerobically by shaking at 200rpm. The enriched samples were spread onto brain heart infusion (BHI) agar containing 1% (w/v) lactose, 1% (w/v) isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), and 0.01% (w/v) 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal; Duchefa Biochemie, Holland), and incubated at

4°C for 15 days. The colonies of thick blue color were picked out, isolated as pure ones, and tested for intracellular activity of lactose hydrolyzing enzyme.

### Assay of lactose hydrolyzing enzyme activity

Microorganisms were cultivated at 4°C aerobically by shaking (200rpm) for 7 days to the late of log phase, and harvested by centrifugation at 8,000 X g for 10min at 4°C, suspended in sodium phosphate buffer (0.01M, pH 6.8), washed 2 times by the same buffer, suspended in the same buffer again, and sonificated at 4°C. Cell debris was eliminated by centrifugation at 12,000g and 4°C for 20min. The cell free extracts were used for assay of lactose hydrolyzing enzyme. Lactose hydrolyzing enzyme activity was determined by measuring the rate of hydrolysis of ONPG (Sigma) as substrate by the procedure of Miller (15). An aliquot of cell free extract (0.5ml) was added to 2.5ml of ONPG (0.04M) dissolved in sodium phosphate buffer (0.01M pH 6.8) and incubated at 4°C for 2hrs. The reaction was stopped by addition of 3ml of 0.5M Na<sub>2</sub>CO<sub>3</sub> and the absorbance at 420nm was measured. One unit of enzyme activity is defined as the activity hydrolyzing 1 $\mu$ mol of ONPG per min by cell free extract from 1ml of culture whose cell concentration was concentrated to 8 of A<sub>600</sub>.

### Morphological and biochemical characterization

The strain with the highest lactose hydrolysis activity was identified by Gram staining, morphological, biochemical, and physiological tests. Cell was grown on BHI agar to determine growth conditions for various temperatures (5~50°C) and in BHI broth for various pH (5.0~8.5). The pH of BHI broth was adjusted with HCl or NaOH. Acid production from carbohydrate and utilization of carbon sources were determined using API tests (BioMerieux), including API 20E (identification system for Enterobacteriaceae and other Gram-negative rods) and API 20NE (identification system for gram-negative non-enterobacterial rods).  $\beta$ -Hemolysis was confirmed by lysis and complete digestion of red blood cell contents surrounding colony on sheep blood agar (KOMED. Co. Ltd) after incubation for 3 days at 15°C.

### Fatty acids determination

The cell biomass for cellular fatty acid composition analysis was collected from BHI agar plates after incubation at 15°C for 3 days. Cells were harvested, and the cellular fatty acid was saponified, methylated and extracted, following the instructions in the manual for Sherlock Microbial Identification System (MIDI, USA). The fatty acids were analyzed by gas chromatography (Hewlett Packard 6890, USA) and identified using the Microbial Identification software package (21).

### 16S rDNA sequence determination and phylogenetic analysis

Isolation of genomic DNA, PCR amplification of the 16S rDNA and sequencing of the purified PCR products were carried out as described by Rainey *et al.*(17). Universal primers of fD1 (5'-gagtttgatcctggctcag-3') and rD1 (5'-agaaggaggtgatccagcc-3') were used for PCR. PCR products were purified by ethanol precipitation and electrophoresis, and sequenced with a model 377 Genetic Analyzer (Perkin-Elmer Co.). The 16S rDNA sequence obtained in this study was aligned against the previously determined sequences of the genus of *Pseudoalteromonas* sequences available from the Ribosomal Database Project (12). The phylogenetic tree for the dataset was inferred using the neighbor-joining method (20). And the tree was constructed by PHYLIP package (4).

### Characterization of lactose hydrolyzing enzyme

Effect of temperature on enzyme activity in cell free extracts was measured at various temperatures from 4°C to 50°C in 0.01M sodium phosphate buffer (pH 6.8). The effect of pH on the enzyme activity was determined by measuring the activity in Na-phosphate (0.01M) for pH 6.0 to 7.0, and in Tris-HCl buffer (0.01M) for pH 7.0 to 9.0 at 4°C. For evaluation of stability at 4°C and 37°C, cell free extracts was incubated for 7 days in Na-phosphate buffer (0.01M, pH 6.8) at 4°C and 37°C. During incubation, residual activity was measured at 4°C for 2hours.

Zymogram of the intracellular fractions from the isolated

strain for X-gal hydrolysis was performed. After native-PAGE, which was performed on 10% (w/v) polyacrylamide gel (11), the gel was stained with 0.25mM X-gal (23) at 4°C for 2 hours. Hydrolysis of X-Gal was confirmed as blue band within the polyacrylamide gel.

## RESULTS

### Distribution of bacteria producing lactose hydrolyzing enzyme in Antarctic sea water and selection of an active producer

15 samples of sea water, collected from Antarctic polar sea water, were tested for bacterial strains producing lactose hydrolyzing enzyme. From 10 samples, 28 colonies of blue color showing hydrolysis of X-Gal at 4°C on BHI medium were picked, and tested for lactose hydrolyzing activity at 4°C for 2h using ONPG as substrate (Table 1). Among 28strains, the strain KNOUC808 showing the highest activity was selected and tested for identification.

### Identification of strain KNOUC808

Strain KNOUC808 was rod, Gram negative staining, aerobic, motile, and positive in oxisase test. The strain did not form spore, grew at 4~30°C, but not at 40°C. Optimal temperature for growth was 20°C. By those above characteristics, the strain KNOUC808 could be presumably identified as *Pseudoalteromonas* genus (6). The range of pH for growth was from 5.0 to 8.5, and it reproduced optimally at pH 6.8~7.2. Strain KNOUC808 showed positivity in tests of catalase and Voges-Proskauer, and in hydrolysis of gelatin, p-nitrophenyl  $\beta$ -D-galactopyranoside (PNPG) and ONPG. The strain was negative in tests of nitrate reduction, urease, indol production, citrate utilization, gas production, H<sub>2</sub>S formation, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and  $\beta$ -hemolysis. Lactose, sorbitol, saccharose, amygdaline, arabinose, D-glucose were assimilated, and D-galactose, mannitol, inositol, rhamnase, sucrose, xylose, melibiose, trehalose, N-acetyl-glucosamine, mannose, maltose

and fructose were not assimilated by the strain KNOUC808. The major cellular fatty acids of strain KNOUC808 were C<sub>16:0</sub> (41.83%), C<sub>12:0</sub> 3-OH (9.21%), C<sub>17:0</sub> ω8 (7.24%), C<sub>18:0</sub> ω7 (7.04%), C<sub>12:0</sub> (5.48%), C<sub>14:0</sub> (3.789%), C<sub>15:1</sub> ω8 (3.54%), and with additional fatty acids comprising less than 1%. To investigate the taxonomic position of the marine bacterium,

16S rDNA sequence analysis was performed. The 16S rDNA of strain KNOUC808 was determined as 1,491bp as shown in Fig. 1. A phylogenetic tree was derived from 16S rDNA sequences, and it showed the phylogenetic position of this bacterium to closely related species in the genus *Pseudoalteromonas* sp. (Fig. 2).

**Table 1.** Distribution of bacteria producing lactose hydrolyzing enzyme in Antarctic polar sea water

Area	Isolated strains	Growth at <sup>1</sup>		Hydrolysis of <sup>2</sup>		
		4	15	X-gal	ONPG(unit)	
KR <sup>3)</sup> 6 Surface sea water	KNOUC801	+	+	+	0.12886	
	KNOUC802	+	+	+	0.10064	
	KNOUC803	+	+	+	0.13514	
	KNOUC804	+	+	+	0.12409	
	KNOUC805	+	+	+	0.09410	
	KNOUC806	+	+	+	0.11475	
	KNOUC807	+	+	+	0.13006	
KR7 Surface sea water				Not found		
KR9 Surface sea water	KNOUC808	+	+	+	0.86706	
KR10 Surface sea water	KNOUC809	+	+	+	0.56484	
SOI <sup>4)</sup> 2 Surface sea water	KNOUC810	+	+	+	0.05449	
Penguin Village	KNOUC811	+	+	+	0.57087	
KARP <sup>5)</sup> MB-86m	KNOUC812	+	+	+	0.17949	
ST6-200m	KNOUC813	+	+	+	0.50872	
KR 150m	KNOUC814	+	+	+	0.22467	
KR4 150m	KNOUC815	+	+	w	n.d	
	KNOUC816	+	+	-	n.d	
	KNOUC817	+	+	+	0.29148	
	KNOUC818	+	+	+	0.38203	
	KNOUC819	+	+	+	0.33251	
	KNOUC820	+	+	+	0.10480	
	KNOUC821	+	+	+	0.34419	
	KR6	KNOUC822	+	+	+	0.25195
		KNOUC823	+	+	+	0.29334
		KNOUC824	+	+	+	0.46992
		KNOUC825	+	+	+	0.36229
		KNOUC826	+	+	+	0.26909
	KNOUC827	+	+	+	0.34528	
KNOUC828	+	+	w	n.d		
KR7 Surface sea water 150m				Not found		
KR9 Surface sea water 150m				Not found		
SOI-2 Surface sea water 100m				Not found		
SOI-2v Surface sea water 150m				Not found		

<sup>1)</sup> +; growth  
<sup>2)</sup> Unit; +; distinct hydrolysis  
 -; no hydrolysis  
 w; weak hydrolysis  
 n.d; not detected  
<sup>3)</sup> KR; Krill collected site  
<sup>4)</sup> SOI; South Orkney Island  
<sup>5)</sup> KARP; Korea Antarctic Research Program

AACGCATTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACCTCAAATGA  
 ATTGACGGGGGCCCCGACAAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG  
 AAGAACCTTACCTACACTTGACATACAGAGAACTTACCAGAGATGGTTTGGTGCCT  
 TCGGAACTCTGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAGATGT  
 TGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCCTTAGTTGCTAGCAGGTAATGC  
 TGAGAACTCTAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTC  
 AAGTCATCATGGCCCTTACGTGTAGGGCTACACACGTGCTACAATGGCGCATACAG  
 AGTGCTGCGAACTCGCGAGAGTAAGCGAATCACTTAAAGTTCGTCGTAGTCCGGA  
 TTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGTATCAGA  
 ATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGA  
 GTGGGTTGCTCCAGAAGTAGATAGTCTAACCCCTCGGGAGGACGTTTACCACGGAG  
 TGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCCTAGGGGAACCTGG

**Figure 1.** 16S rDNA sequence of strain KNOUC808 (1491bp)

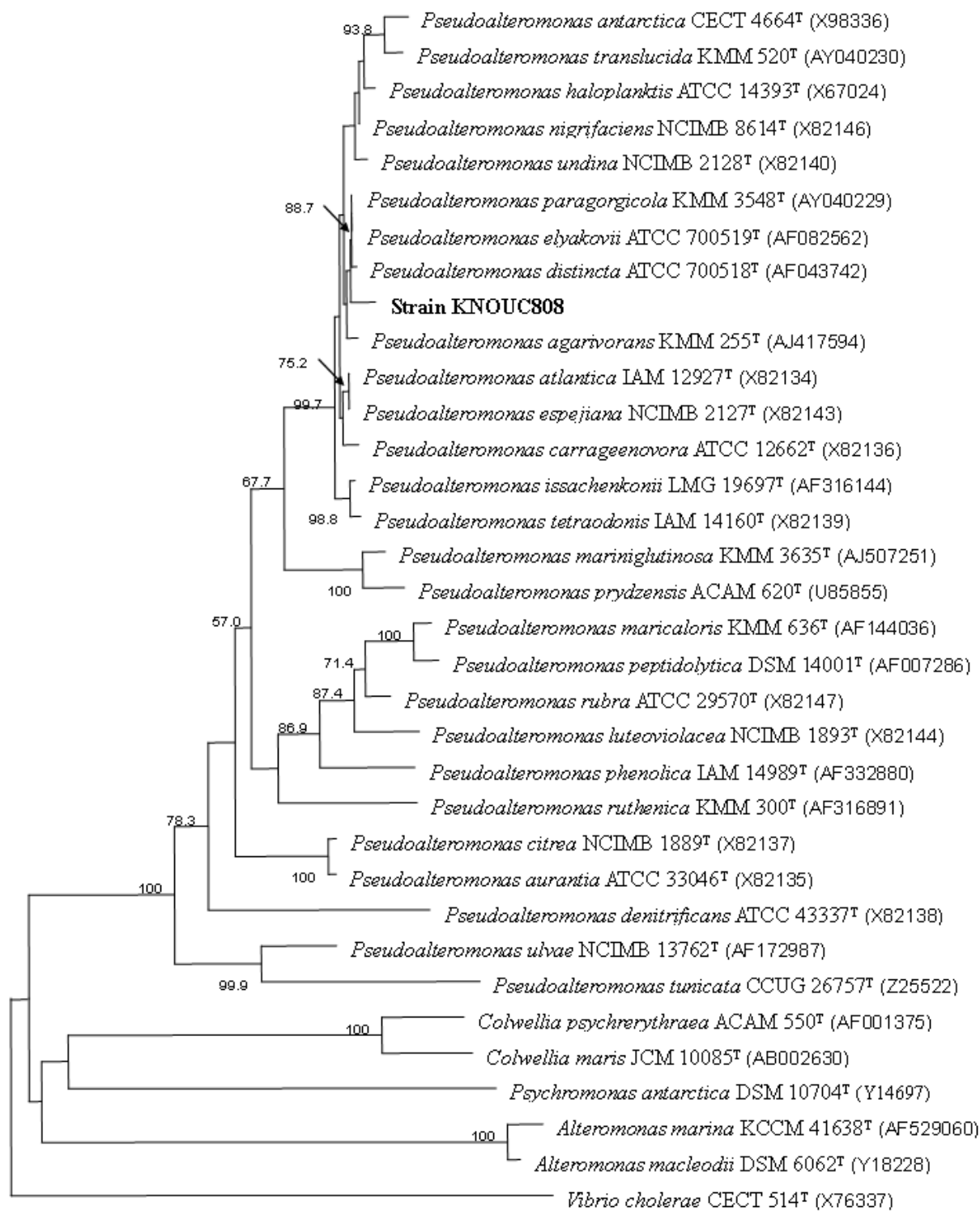
On the basis of morphological and biochemical characteristics, cellular fatty acid composition, by the phylogenetic tree based on 16S rDNA structure, the strain was designated as *Pseudoalteromonas* sp., and named as *Pseudoalteromonas* sp. KNOUC808.

#### Characteristics of lactose hydrolyzing enzyme

Lactose hydrolyzing enzyme from strain KNOUC808 was examined for optimum pH and temperature, and stability at 4°C and 37°C. Optimal temperature of the lactose hydrolyzing enzyme in Na-phosphate buffer (pH6.8) was 20°C, and the enzyme showed high activity between 5°C and 20°C (Fig. 3a).

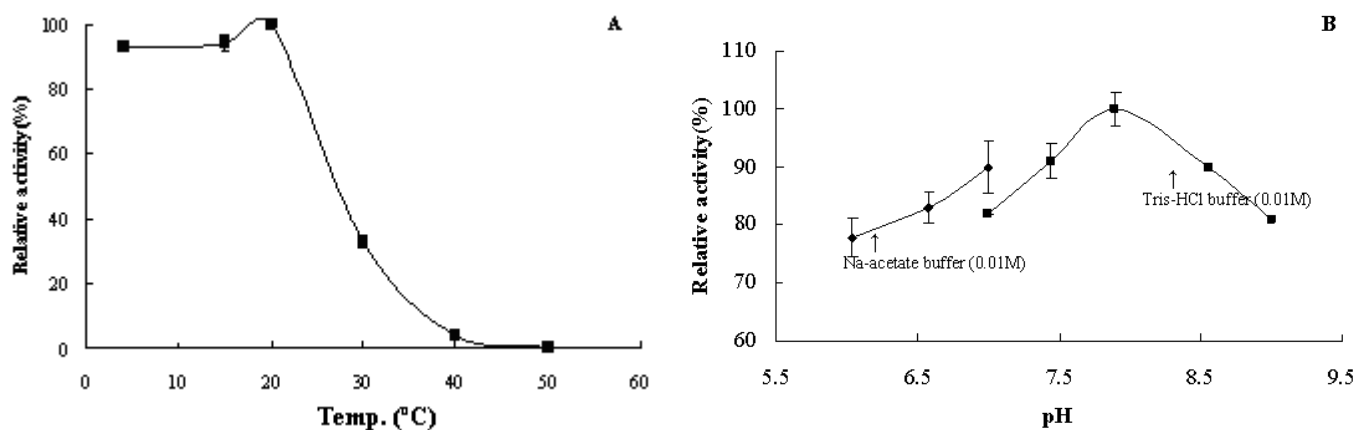
It was active in wide pH of 6 to 9.0, optimally at pH 7.8 (Fig. 3b). Stability of the lactose hydrolyzing enzyme was examined by incubating at 4°C and 37°C for 7 days. It was stable at 4°C for 7 days but decreased about to 50% of initial activity at 37°C in 7 days (Fig. 4).

Cell free extracts from strain KNOUC808 was subjected to non-denaturing polyacrylamide gel electrophoresis and the gel was incubated with X-Gal to detect X-gal hydrolysis activity. There were three blue bands between the sites that standard molecules of 117 kDa and 192 kDa migrated to (Fig. 5).



0.01

**Figure 2.** Phylogenetic tree based on 16S rDNA sequences showing the position of strain KNOUC808, and the representative of some related taxa. Bootstrap values (1000 replications) are shown as percentages at each node only if they are 50% or greater. Scale bar represents 0.01 substitution per nucleotide position.

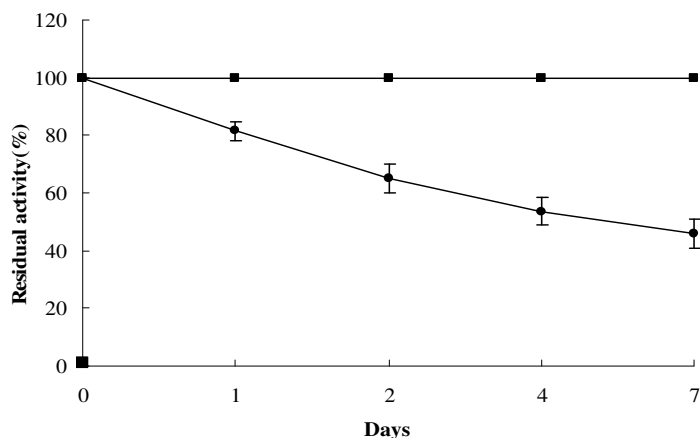


**Figure 3.** Effects of temperature (A) and pH (B) on the activity of lactose hydrolyzed enzyme in cell free extracts of strain KNOUC808

\* Effect of temperature was tested in Na-phosphate buffer (0.01M, pH 6.8).

\* Effect of pH was examined in Na-phosphate (0.01M) for pH 6.0 to 7.0, and in Tris-HCl buffer (0.01M) for pH 7.0 to 9.0 at 4°C.

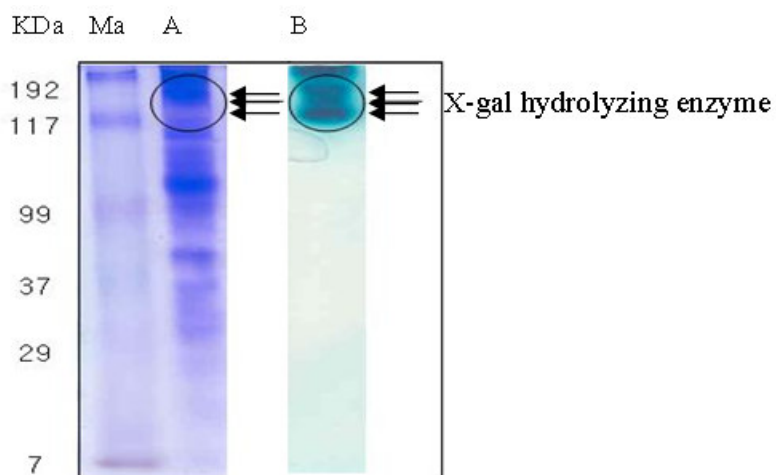
\* Values are means of triplicates ± S.D.



**Figure 4.** Stability of lactose hydrolyzing enzyme in cell free extracts of *Pseudoalteromonas* KNOUC808 at 4°C (■) and 37°C (●)

\* Cell free extracts of KNOUC801 was incubated in Na-phosphate buffer (0.01M, pH 6.8).

\* Values are means of triplicates ± S.D.



**Figure 5.** Native polyacrylamide gel electrophoresis of cell free extracts from *Pseudoalteromonas* sp. KNOUC808. The protein was analyzed on a 10% polyacrylamide gel. Ma: Kaleidoscope Polypeptide Standards Marker. A: The gel was stained with Coomassie Brilliant Blue R-250. B: The gel was soaked in 0.1M X-gal solution (in Z-buffer) at 4°C for 2hr.

## DISCUSSION

Antarctic polar sea water is relatively rich source of cold-active  $\beta$ -galactosidase producing bacteria (24). Cold-active lactose hydrolyzing enzyme with high activity at low temperature is useful for hydrolyzing lactose in refrigerated milk enabling it to be consumed by lactose-intolerant individuals, and for converting lactose in refrigerated whey into glucose and galactose (5).

In the search for cold active lactose hydrolyzing enzyme, we isolated strain KNOUC808 producing cold active lactose hydrolyzing enzyme from Antarctic polar sea water and identified through examining biochemical and physiological properties, fatty acid composition of cell, and 16S rDNA sequence. The biochemical and physiological properties of KNOUC808 were consistent with those of genus *Pseudoalteromonas*, especially in the properties of rod, Gram negative staining, aerobic growth, motile, non spore formation, oxidase positive and hydrolysis of gelatin that are typical ones for this genus (6, 18). Strain KNOUC808 showed positive in hydrolysis of PNPG and ONPG, and utilization of lactose, and negative in  $\beta$ -hemolysis that are the properties required for the microorganism to be used as a source of lactose hydrolyzing enzyme. Although *Pseudoalteromonas* was reported to utilize a wide range of carbohydrates as a carbon source (9), strain KNOUC808 was unable to utilize galactose, sucrose, maltose, and mannitol. The major cellular fatty acids of strain KNOUC801 were C<sub>16:0</sub>, C<sub>12:0</sub> 3-OH, C<sub>17:1</sub>  $\omega$ 8, C<sub>18:1</sub>  $\omega$ 7, C<sub>12:0</sub>, C<sub>14:0</sub>, C<sub>15:1</sub>  $\omega$ 8, especially C<sub>16:0</sub> was the primary fatty acid comprising 41.83% of total fatty acids. Ivanova *et al.* (10) reported that the predominant fatty acids of C<sub>16:0</sub>, C<sub>16:1</sub>  $\omega$ 7, C<sub>17:1</sub>  $\omega$ 7 and C<sub>18:1</sub>  $\omega$ 7 were typical for the genus *Pseudoalteromonas*. The amounts of C<sub>16:0</sub> and C<sub>18:1</sub>  $\omega$ 7 reached up to about 49% of total fatty acids in cell of KNOUC808. A phylogenetic tree was derived from 16S rDNA sequences, and it showed the phylogenetic position of this bacterium is closely related with species in the genus *Pseudoalteromonas* sp. The highest

similarity found by BLAST (NCBI) searching was with *Pseudoalteromonas elyakovii* ATCC 700519<sup>T</sup> (99.6% identity). And it showed high similarity also with *Pseudoalteromonas distincta* ATCC 700518<sup>T</sup> (99.5% identity). The 16S rDNA of this bacterium was also observed to have high homology (over 99%) with those of *P. paragorgicola*, *P. agarivorans*, *P. nigrifaciens*, and *P. haloplankis*. Therefore the bacterium KNOUC808 was identified as *Pseudoalteromonas*, a genus abundant in the Antarctic coastal water (1). It has been reported that several *Pseudoalteromonas* strains produce cold-active  $\beta$ -galactosidase (5, 8, 24).

Lactose hydrolyzing enzyme of *Pseudoalteromonas* sp. KNOUC808 hydrolyzed  $\beta$ -D-galactopyranoside derivatives, such as lactose, ONPG and PNPG, and it showed optimum activity at 20°C, 5°C lower than that of the mesophilic  $\beta$ -galactosidase of *Pseudolateromonas* sp. 22b (24).

The highest yield of  $\beta$ -galactosidase by *Pseudolateromonas* sp 22b (good growth at 4°C, T<sub>opt</sub> of 15°C, T<sub>max</sub> of 30°C), was observed at 6°C, much lower than its optimum growth temperature (24), similar to an Antarctic halotolerant psychrophilic bacterium *P. haloplankis* TAE 79 that produced  $\beta$ -galactosidase maximally at 4°C and grew well between 0°C and 25°C (8). Lactose hydrolyzing enzyme of *Pseudoalteromonas* KNOUC808 showed optimum activity at pH 7.8, which is different from those of *P. haloplankis*  $\beta$ -galactosidase (pH 8.5) (8) and *Pseudoalteromonas* sp.  $\beta$ -galactosidase (pH 9.0) (5).

Thermal stability of Antarctic enzymes is generally weak, since it undergoes fast denaturation above 40°C (24). Lactose hydrolyzing enzyme of *Pseudoalteromonas* sp. KNOUC808 lost about 50% of its activity in 7 days at 37°C, however at 4°C, its stability was excellent by keeping full activity without any stabilizers for 7 days. A decline in the activity of *P. haloplankis* TAE 79b  $\beta$ -galactosidase amounted to 60% in 2 weeks at 4°C (5).

In nondenaturing polyacrylamide gels electrophoresis of cell free extracts from KNOUC808, three blue bands showing X-gal hydrolysis were detected, meaning that



*Pseudoalteromonas* sp. KNOUC808 produces three forms of lactose hydrolyzing enzyme. A strain of *Pseudoalteromonas* produced a  $\beta$ -galactosidase as homotetramer, and Mw of its monomer was 115kDa (2).

From these facts, we conclude that *Pseudoalteromonas* sp. KNOUC808 produces cold-active lactose hydrolyzing enzyme that can have advantageous applications in the foods industry, e.g., the treatment of chilled dairy products while avoiding flavor tainting and the risk of microbial contamination.

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