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Screening and molecular classification of low-temperature protease from Antarctic microorganism and its characterization

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Abstract 107 strains producing protease were screened from 260 strains of Antarctic psychrophilic bacteria, among which proteolytic activity of five strains was more than 45 U ml^{-1}. The 16S rRNA gene sequences homology and phylogenetic analysis of five Antarctic psychrophilic bacteria showed that NJ276、NJ5-9、NJ16-70、NJ345 belonged to the described genus *Pseudoalteromonas* and NJ341 belonged to the genus *Colwellia*. The growth and the protease characteristic of four Antarctic psychrophilic bacteria had been studied, and the result showed that the optimal temperature for growth and protease-producing of four strains was about 10 ℃. Their growth and protease-producing were still high during incubating 2-5 days. The maximum proteolytic activity occurred at pH 9 for four Antarctic psychrophilic bacteria. The optimal temperature of protease action of both strains NJ276 and NJ5-9 was about 50 ℃, however, the optimal temperature of protease action of both strains NJ341 and NJ345 was about 40 ℃, and their proteolytic activity under 0 ℃ exhibited nearly 30% of the maximum activity, but their thermal stabilities were weaker. These results indicated that proteases from NJ341 and NJ345 were low-temperature proteases.

Key words 16S rRNA gene, psychrophilic bacteria, protease, screening, Antarctic.

1 Introduction

Low-temperature enzymes are produced by organisms existing in permanently cold habitats. Low-temperature protease had been applied largely in industries, including cleaning detergents, leather processing, food processing and molecular biology (Cavicchioli *et al.* 2002). The continental Antarctica is considered the unique, mostly pristine and extreme environment, thus it would be the new and potential sources of low-temperature enzymes from Antarctic microorganism. Although many proteases from cold- active bacteria have been characterized (Davail *et al.* 1994; Vazquez *et al.* 1995; Marianna *et al.* 2003), few studies dealt with comparisons be-

tween different protease-producing strains.

In order to isolate Antarctic microorganisms able to produce proteases, we performed a screening. 107 strains producing a protease were screened from 260 strains of Antarctic psychrophilic bacteria. We described the molecular classification of four protease-producing Antarctic strains and the characterization of their extracellular protease. These enzymes would be a good candidate not only for industrial applications, but also as additives in baking flour and food processing and preservation.

2 Materials and Methods

2.1 Bacteria and their cultivation

Two hundred sixty strains were isolated from the sea ice in Antarctica (68°30′E, 65°00′S) in 2002. Strains were conserved in the key Laboratory of Marine Bio-active Substances *SOA*. Strains were inoculated in the sea water medium (peptone 0.5% and yeast extract 0.1%, pH 7.5) at 8 ℃ with shaking at 100 rpm. All chemicals employed in the studies were purchased from standard sources and were of reagent grade at least.

2.2 Screening for Antarctic microorganism producing low-temperature protease

Proteolytic activity of the cultures was screened qualitatively in a medium containing 1.25 % of agar and 1 % of casein digest (Difco) pH 7.0-7.5. After a medium was added 10% (w/v) trichloroacetic acid, clear zones around the colonies appearing after 48 h at 20 ℃ were taken as evidence of proteolytic activity and measure the proteolytic zones. The method for the second screening was that clear zones around the colonies appeared after 96 h at 20 ℃

2.3 Assay of protease activity

Protease activity was determined by a modified method of Folin and Ciocalteu (1927). Briefly, 1 ml of the purified protease was added to the reaction mixture, containing 2% (w/v) casein in 1 ml of 50 mM Tris-HCl (pH 8). The mixture was incubated at 40 ℃ for 10 min. The reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid (TCA), followed by centrifugation at 9600 rpm for 15 min. The supernatant were determined by the Folin-phenol reagent. One unit of protease activity was defined as the amount of enzyme that liberated 1 μg tyrosine per min. A blank was run in the same manner, except the enzyme was added after the addition of 10% (w/v) TCA. All experiments were done in duplicate.

2.4 Characterization of protease

The optimal pH for the protease was measured by the hydrolysis of casein at different pH buffer at 4 ℃ for 2 h. The pH was adjusted by the following buffer sys-

tems (50 mM each): NaAC/ HAC (pH 5.0); KH_2PO_4/Na_2HPO_4 (pH 6.0-7.0); Tris-HCl (pH 8.0-9.0) and Na_2HPO_4/NaOH (pH 10.0-12.0). For determination of optimum temperature, 1 ml of the protease were incubated with Tris-HCl (7.5) and assayed as described above except that the enzymatic activities were determined at 0, 10, 20, 30, 35, 40, 45, 50, 55 and 60 ℃ separately. For the determination of thermostability, 1 ml of protease were incubated for 10, 20, 30, 40, 50 min at 30, 40, 50 and 60 ℃, respectively, then directly put into an ice water bath prior to the protease activity assay as described above. The highest protease activity was used as control (100% of relative activity).

2.5 Isolation of genomic DNA and 16S rDNA sequence analysis

DNA of five strains NJ16-70, NJ276, NJ345, NJ341 and NJ5-9 was extracted and precipitated following the CTAB protocol for bacterial genomic DNA preparations. Polymerase chain reaction (PCR) products were amplified using the two PCR primers 16F27 (5'-dAGAGTTTGATCCTGGCTCAG-3') and R1488 (5'-dGGTTACCTTGTTACGACTT-3'). PCR were performed with a thermal cycler by using 20 μl reaction mixtures containing deoxynucleoside triphosphates at concentrations of 0.02 mM, 1.0 μM of primers, 1 μl of five strains' genomic DNA, 2 μl reaction buffer, and 1U Taq DNA polymerase (Takara). The program used for the PCR was as follows: 94 ℃ for 5 min, then denaturation at 94 ℃ for 1 min, annealing at 53 ℃ for 1min, and extension at 72 ℃ for 1 min 30 s, 30 cycles, and extension at 72 ℃ for 10 min.

For sequence analysis, about 1,500-bp PCR products were subcloned into the vector pMD18-T. The nucleotide data for NJ16-70, NJ276, NJ345, NJ341 and NJ5-9 have been deposited in the GenBank nucleotide sequence database under the Accession No. AY781157, AY781155, AY781153, AY781156, AY781154. The evolutionary distances were calculated by the MEGALIGN program of DNASTAR (DNASTAR Inc.). The multiple sequence alignment analysis was performed using CLUSTAL W program. And the phylogenic tree was constructed by the neighbour-joining phylogenic tree (50% majority rule). A boot strap analysis of 1000 replicates was performed.

3 Results

3.1 Screening for Antarctic microorganism producing low-temperature protease

The first screening results of 260 strains of Antarctic microorganism for producing low-temperature protease indicated that there were 107 strains formed clear proteolytic zones, meaning that they secreted the extracellular protease. For the second screening of these 107 strains, there were 44 strains which formed 15-20 mm of proteolytic zones, and 20-25 mm for 30 strains, 25-30 mm for 13 strains. Further the result of assaying the activity of protease indicated protease activity from five strains NJ276, NJ341, NJ5-9, NJ16-70 and NJ345 was higher than 45 U ml^{-1} and that of

three strains was about 50 U ml^{-1} (Table 1). During the second screening, the results showed that the correlation with the proteolytic zones size and protease activity of protease from 107 strains($R^2=0.8256$).

3.2 16S rRNA gene sequence and phylogenetic analysis of five strains

In order to determine the phylogenetic position of five strains NJ276, NJ345, NJ5-9, NJ16-70 and NJ341, 16S rRNA gene sequencing was performed. Comparative sequence analyses of the 16S rRNA gene of four strains NJ276, NJ345, NJ5-9, NJ16-70 and other 16S rRNA available in the database found the highest similarity to the 16S rRNA of strains belonging to the genus *Pseudoalteromonas*, and the highest percentage of the sequence similarity of four strains was found with *Colwellia piezophila* were 98.1%, 97.8%, 97.4% and 96.9%, respectively.

Table 1. Screening for protease from Antarctic microorganism

Strains	Clear Zones	Diameter/mm	Protease activity
	First Screening	Second Screening	(U ml^{-1})
NJ 276	24.00	28.20	58.58
NJ 341	23.00	31.40	58.31
NJ 5-9	22.75	30.00	54.92
NJ 16-70	26.25	27.60	46.96
NJ 345	21.75	28.30	45.17
NJ 272	24.00	26.90	43.33
NJ308	19.50	26.80	40.17
NJ 545	23.50	29.50	37.30
NJ 274	22.00	27.10	29.25
NJ 43	25.00	26.20	23.70
NJ 297	22.00	26.90	23.60

Phylogenetic analyses based on 16S rRNA gene sequence information are shown in Figure 1. Five strains belonged to the γ-proteobacteria subclass which was one of the greatest group *proteobacteria*. Strains NJ276, NJ276, NJ345, NJ5-9 and NJ16-70 should belong to Alteromonadales, Alteromonadaceae, *Pseudoalteromonas*. The strain NJ341 fell into the genus *Colwellia*, which had been identified as a new genus in 1997, and there were only seven identified *Colwellia* genus in the GenBank . What's the most important is that the highest similarity of the strain NJ341 with its most closely related *Colwellia piezophila* was only 94.8%, which strongly suggested that strain NJ341 was a distinct or new species in the genus *Colwellia*.

3.3 Characteristic of producing protease from four psychrophilic bacteria

Four strains NJ16-70, NJ276, NJ341and NJ345 could grow in a temperature range from 0 to 20 ℃, and the optimal temperature for growth was about 10 ℃, but these strains could not grow at 25 ℃. According to the the definitions of psychrophiles and psychrotrophs follow those of Morita (1975), that is both psychrophile

and psychrotrophic have the ability to grow at 0 ℃ and psychrophile have the optimum temperature for growth of 15 ℃ or lower, and the maximum temperature for growth of 20 ℃ or below, whereas psychrotrophic have the optimum temperature for growth of about 20-30 ℃. Thus four strains were psychrophile. The optimum temperature of producing protease from the strains NJ345、NJ276、NJ341 were about 10 ℃, and that for NJ16-70 was about 15 ℃ (Fig. 2). The growth of four strains reached the late logarithmic phase after two days, and came into the stable phase, falling at the fifth day. The curve of the growth and protease from four strains was different, that is, strains NJ341 and NJ345 reached the maximum producing protease on the 3rd day, but strains NJ276 and NJ16-70 reached the maximum during the 4-5th day. Then, the content of the protease from strain NJ341 kept high, and that of other strains falling (Fig. 3).

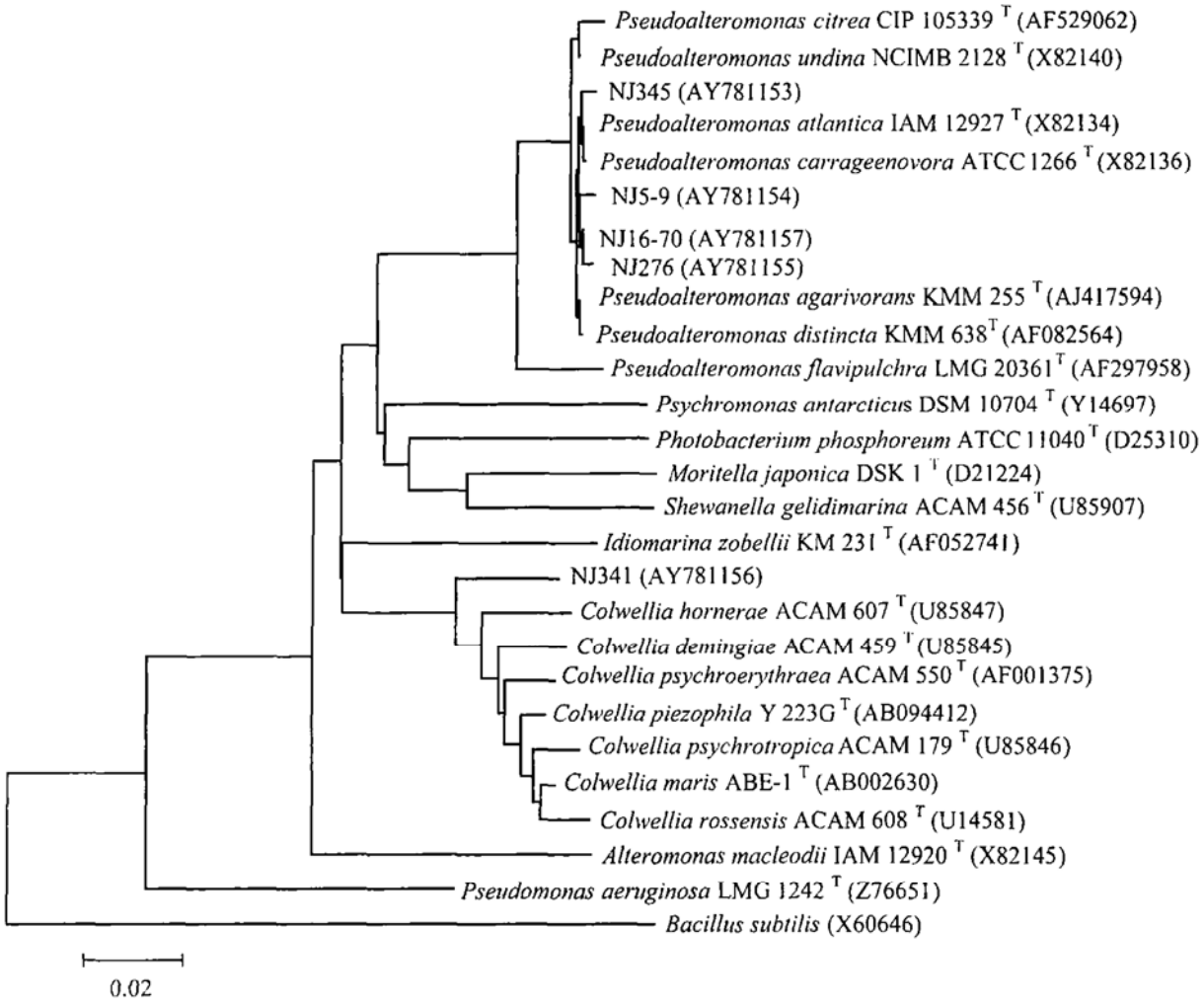

Fig. 1 Phylogenetic tree showing the relationships of five strains of Antarctic psychrophilic bacteria producing protease with the sequences of relating genera constructed by the neighbour-joining method and based on 16S rRNA gene sequences . Scale bars correspond to a 2% different in nucleotide sequence. Bootstrap values are shown at the branch points , submission number of the relating genera is available as supplementary data in IJSEM Online.

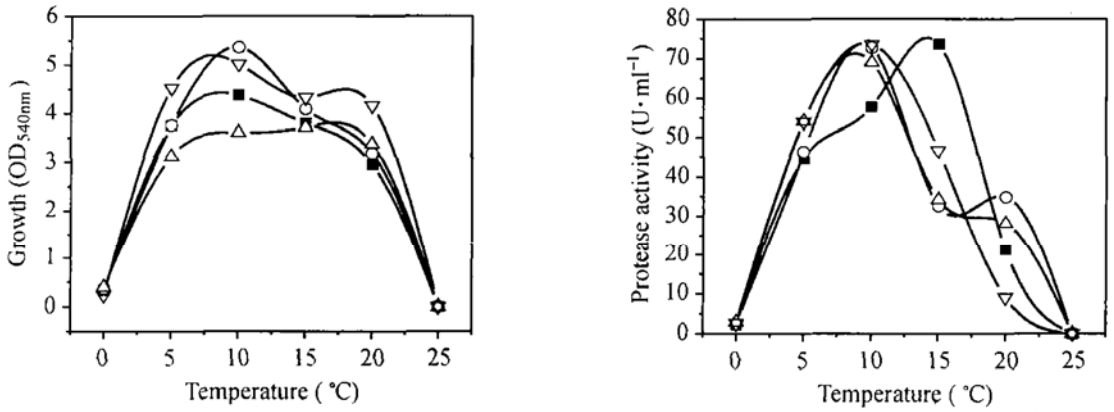

Fig. 2 Effect of temperature on the growth and protease from four Antarctic psychrophilic bacteria.
▽ NJ345; ■ NJ16-70; ○ NJ276; △ NJ341.

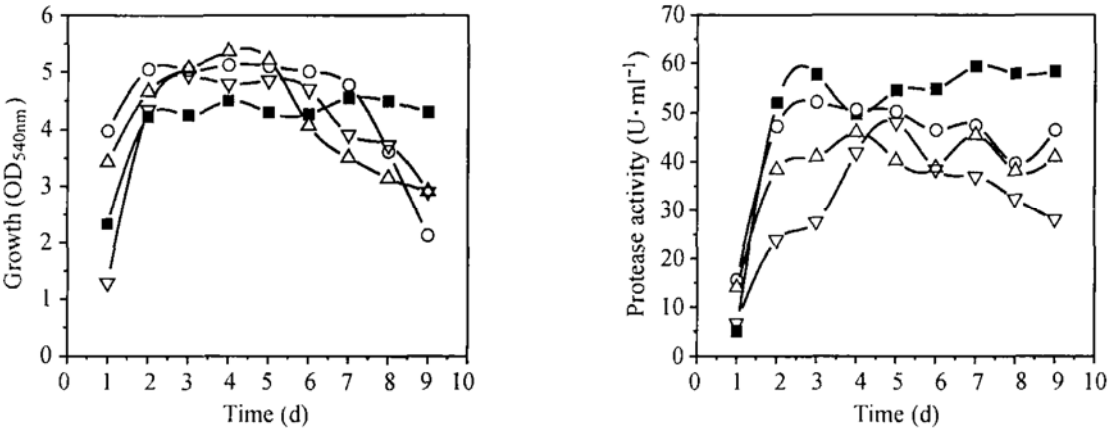

Fig. 3 The curve of the growth and protease from four Antarctic psychrophilic bacteria.
■ NJ341; ○ NJ345; △ NJ276; ▽ NJ16-70.

3.4 Characteristic of protease from four strains

Proteases from four strains NJ16-70, NJ276, NJ341 and NJ345 were all active in pH ranging from 5 to 12. Proteases activity retained more than 50% of their maximal activity at pH 12 and their maximal activity occurring at pH 8.0-9.0 (Fig. 4a). For four strains, the optimal temperature of protease activity was different. The optimal temperature for strains NJ276 and NJ5-9 were about 50 ℃. The optimal temperature of strains NJ341 and NJ345 were about 40 ℃, and their proteolytic activity exhibited nearly 30% of the maximum activity under 0 ℃ (Fig. 4b). These results indicated that proteases from NJ341 and NJ345 were low-temperature proteases. The protease thermostability for four strains was different. With increasing the temperature, strain NJ341and NJ345 protease were sensitive, and strain NJ341 had the t_i of 50 min at 40 ℃ and inactivated at 50 ℃ for 10 min. The protease from strain NJ345 retained 80% of its activity after incubation at 40 ℃ for 50 min. However, for NJ276 and NJ 16-70, the protease were more stable than those of the other two strains, t_i for the strain NJ276 case, was 15 min at 50 ℃ and inactivated at 60 ℃ for 10 min (Fig. 5). Thus, these results indicated that proteases from NJ341 and NJ345 were low-temperature proteases.

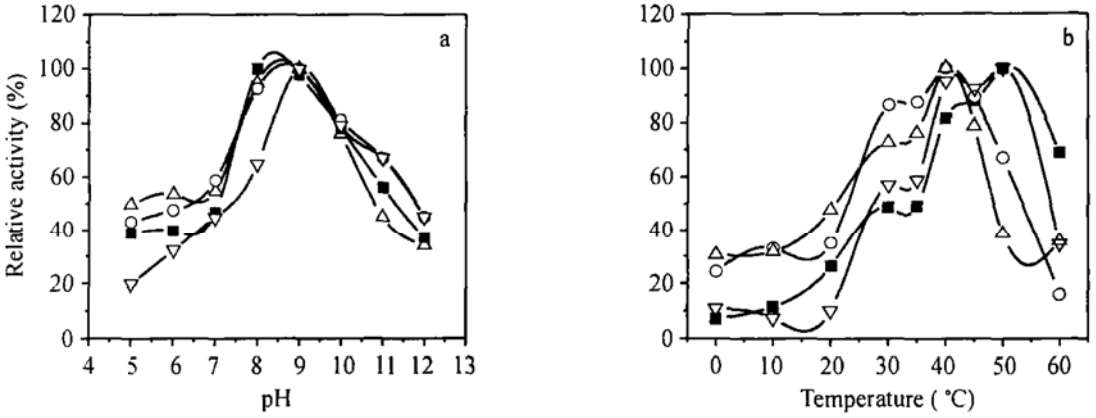

Fig. 4 Effect of pH and temperature on the activity of the protease from four Antarctic psychrophilic bacteria. △ NJ341; ○ NJ345; ■ NJ276; ▽ NJ16-70.

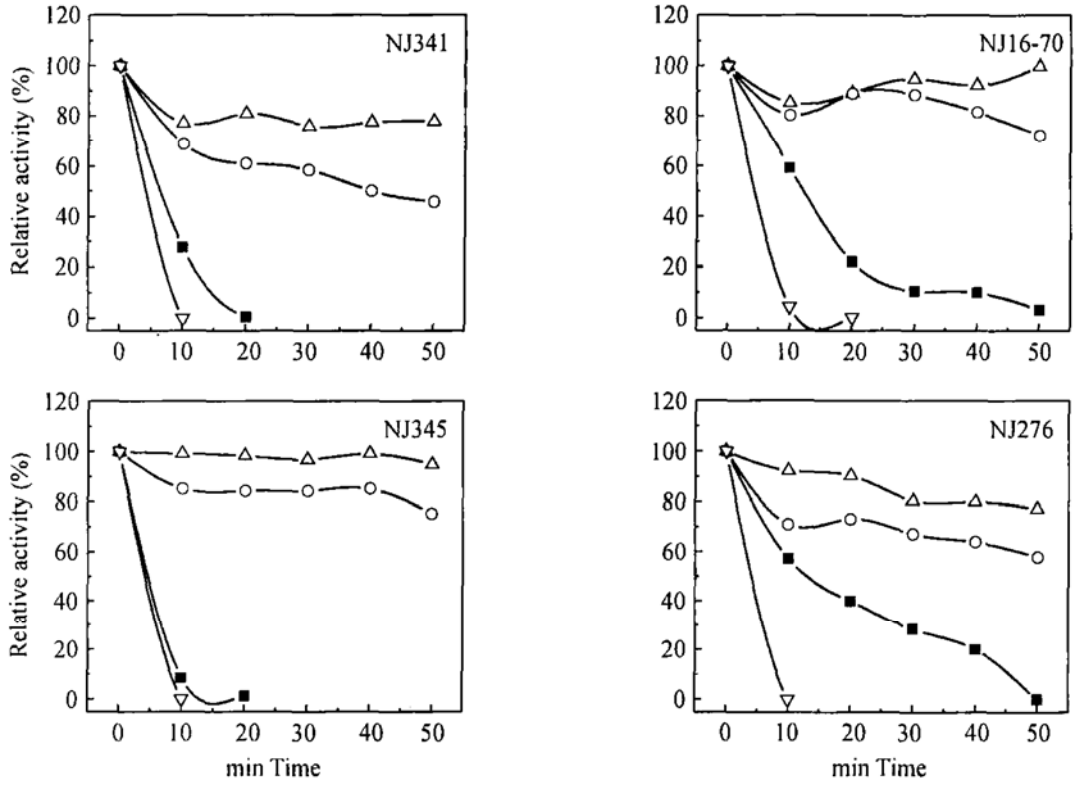

Fig. 5 Effect of the temperature on the stability of the protease from four Antarctic psychrophilic bacteria. △ 30 ℃; ○ 40 ℃; ■ 50 ℃; ▽ 60 ℃.

4 Discussion

4.1 Genus of psychrophiles and psychrotrophs producing low-temperature protease

For the genus of psychrophiles and psychrotrophs producing low-temperature protease, the most genus were *Pseudomonas* and *Bacillus*, a few were reported as such *Flavobacterium*, *Xanthomonas*, *Clostridium*, *Aeromonas*, *Vibrio*, *Altero-*

monas, *Shewanella*, *Sphingomonas*, *Arthrobacter* and *Pseudoalteromonas*. In this study, the strain NJ16-70、NJ276、NJ5-9、NJ345 belonged to the described genus *Pseudoalteromonas*, and NJ341 belonged to the genus *Colwellia* on the basis of 16 S rRNA gene sequence analysis. Previous result indicated that the dominant sea ice bacterial groups represented are from the gamma subdivision of the *Proteobacteria* and the flavobacteria, and that *Colwellia* was described among the psychrophiles , and was found the predominant groups in clean ice include *Shewanella frigidimarina* (52% of clones), *Pseudoalteromonas* (29%) and *Marinobacter* (11%) (Bowman *et al.* 1997). Since the 16S rRNA gene sequence similarity level within genera was 93% or above, which can be a useful additional criterion for genus discrimination (Ivanova *et al.* 2004). This study demonstrated that the the highest similarity of strain NJ341with the most closely related *Colwellia piezophila* was only 94. 8%, which should be described the genus *Colwellia*. But this strain could not be clustered with seven identified species and formed the distinct filiation. These result implied that the strain NJ341 is a distinct or new species in the genus *Colwellia*, but the implication needs further evidence such as the phenotypic characteristics, the whole-cell fatty acid compositions, G+C contents, and DNA-DNA hybridization of strain. To our knowledge, few studies were performed on the low-temperature protease from the the genus *Colwellia*.

4. 2 Screening of low-temperature protease and their application

Although the psychrophiles and psychrotrophs producing the low-temperature protease had been isolated, few studies dealt with comparisons between different the strains producing protease. 260 strains of Antarctic microorganism were screened here for producing low-temperature protease, the result indicated that there were 107 strains secreted the extracellular protease, and for five strains NJ276, NJ341, NJ5-9, NJ16-70, NJ345, the produced protease amount was higher than 45 U ml^{-1}. At the same time, growth characteristic of strains and protease producing were different among the strains. The similar result was found (Margesin *et al.* 1991; Marianna *et al.* 2003; Vazquez *et al.* 2004). The differences between proteases produced by strains of one species were in relation to the respective genetic and physiological adaptation of the strains with the environmental conditions of the sites where the producing strains thrive. In order to adapt to the low temperature, psychrophiles and psychrotrophs living in the Antarctic continent could keep higher enzyme activity in the mechanism and had higher activities at low temperatures than enzymes found for mesophilic microorganisms, which could be related with the long evolution of psychro-adaptation. The optimal temperature for the activity of proteases from strains NJ341 and NJ345 were about 40 ℃, and their protease activity under 0 ℃ exhibited nearly 30% of the maximum activity, which could not produced by the mesophilic microorganisms. Proteases from other strains inhabiting cold environments usually exhibit higher optimal temperatures. Good examples are proteases from Alpine strain of *P. fluorescens* 114, most active at 40-45 ℃ for 30 min (Hamamoto *et al.* 1994) and from *Xanthomonas maltophilia* with maximal activity at 50 ℃ for 15 min (Margesin and Schinner, 1994). Even, protease activity was about 55 ℃ from Arctic and Antarctic mi-

croorganisms (Zeng and Cheng 2002). In this paper, protease activity from strain NJ276 and NJ16-70 reached maximum activity at 50 ℃. So not all enzymes found in psychrophilic organisms were cold-adapted, thermolabile enzymes. This observation suggested that low-temperature enzymes in psychrophilic organisms may catalyze rate-limiting steps in metabolism and play essential roles in survival at a low temperature. Strains NJ16-70, NJ276, NJ341, NJ345 were psychrophilic bacteria and theirs protease were active at pH ranging from 5 to 12 and their maximal activity occurring at pH 8. 0-9. 0. Their properties would make them potentially useful for industrial applications and numerous biotechnological processes, especially for those which require a supply of exogenous energy and are exposed to higher risk of microbial contamination or temperature instability of reactants or products. At the same time, the thermal instability of these proteases could apply to some industries where the proteases quickly inactivate after their action with the protein and protease, such as in case of additives in baking flour and food processing and preservation.

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