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Characterization of the Proteolytic Activity of Bacteria Isolated from a Rotating Biological Contactor

In Jae Park[1], Jerng Chang Yoon[1], Seong Joo Park[1], Eung Ho Kim[2], Yeon-Jae Cho[3], and Kwang-Soo Shin[1,]*

[1]*Department of Microbiology, College of Sciences, Daejeon University, Daejeon 300-716, Korea*
[2]*Major in Civil Engineering, School of Urban & Civil Engineering, College of Engineering, Hong-Ik University, Seoul 121-791, Korea*
[3]*Seil Technology Co., LTD., Seoul 150-800, Korea*

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Four proteolytic bacteria were isolated and identified from a rotating biological contactor based on *Bacillus*. The four isolates, Ni 26, 36, 39 and 49 were identified as *B. vallismortis, B. subtilis, Aeromonas hydrophila* and *B. amyloliquefaciens*, respectively, based on their biochemical properties and 16S rDNA sequence analyses. The optimal proteolytic activity was observed in the temperature and pH ranges of 40-70°C and 8.0-8.5, respectively. The proteolytic activities of all the isolates were partially inhibited by phenylmethylsulfonylfluoride (PMSF), and the isolates Ni 26, Ni 39 and Ni 49 were inhibited by the metalloprotease inhibitor, 1,10-phenanthroline. Zymographic analyses of the culture supernatants revealed the presence of at least two proteases in all isolates.

Key words: *Aeromonas, Bacillus,* proteolytic bacteria, rotating biological contactor

Bacillus is a Gram-positive, endospore-forming bacterium, which is well known for its production of extracellular protease, amylase and lipase. *Bacillus* also plays an important role in the removal of nitrogen, phosphorus and malodor in biological wastewater treatment processes (Ahn *et al.*, 2001). The popular strains that have been used in sewage treatment process are; *B. subtilis, B. licheniformis, B. polymyxa, B. marcerans, B. pasteuri, B. sphaericus* and *B. thuringiensis* (Murakami *et al.*, 1996; Mizuki *et al.*, 2001). Recently, attempts have been made to use *Bacillus* in sewage treatment processes using a rotating contactor. In this process, a *Bacillus* biofilm is immobilized on a rotating polyvinylidene chloride fiber net, designated a rotating activated *Bacillus* contactor (RABC), for sewage treatment.

Proteinaceous organic compounds are one of the most abundant components in sewage, and removal of these compounds is critical for the effective sewage treatment. In the present study, we tried to isolate and identify proteolytic bacteria, and to characterize their proteolytic activities to determine their suitabilities with respect to the rotator contactor process.

Materials and Methods

Isolation of proteolytic bacteria

Biofilm and water samples were serially diluted, plated onto skim milk agar plates, and incubated at 30°C for 48 h. Individual colonies producing clear zones were purified by subculturing, and those isolates producing the largest clear zones were selected and designated; Ni 26, Ni 36, Ni 39 and Ni 49.

Identification of the isolates

The isolates were identified by their morphological and physiological properties, according to Claus and Berkeley (1986) and Michel (1984). The 16S rRNA gene of each isolate was amplified using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGY TACCTTGTTACGACTT-3'), and the purified PCR products were directly sequenced using an ABI PRISM™ model 377 DNA sequencer. The 16S rDNA sequences were aligned using CLUSTAL X software (Thompson *et al.*, 1997), and evolutionary distance matrices were calculated using the DNADIST program within the PHYLIP package (Felsenstein, 1993) using the Kimura two-parameter mo- del. The phylogenetic relationships of the isolates were determined using BLAST Search (GenBank database, www.ncbi.nlm.nih.gov/GenBank/). The phylogenetic trees of the isolates were constructed, based on the calculated distance matrices, by using the neighbor-joining method (Saitou and Nei, 1987).

Preparation of crude protease

Isolates were cultured at 30°C in a medium containing 1.0 % (w/v) skim milk in nutrient broth. After incubation for 24 h, cells were harvested by centrifugation (10,000

* To whom correspondence should be addressed.
(Tel) 82-42-280-2439; (Fax) 82-42-280-2608
(E-mail) shinks@dju.ac.kr

×g, 10 min), and the supernatant was used as crude enzyme.

Proteolytic activity assay
Enzyme activity was assayed using Azocasein as the substrate, according to the method of Sarath *et al.* (1989). The reaction mixture consisted of 0.25 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 2.0% (w/v) of Azocasein and 0.15 ml of enzyme solution. After incubating at 25°C for 15 min, the reaction was stopped by adding 1.2 ml of 10.0% (w/v) TCA, incubating at room temperature for an additional 15 min, and then the precipitate was removed by centrifugation at 8,000×g for 5 min. 1.4 ml of 1.0 M NaOH was added to 1.2 ml of the supernatant, and its absorbance was measured at 440 nm. The protein concentrations were determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Characterization of protease activities
The influence of pH on proteolytic activities was determined using Azocasein with citrate-phosphate buffer (0.1 M), and the influence of temperature was determined by incubating the reaction mixtures for 15 min at temperatures ranging from 30 to 90°C in 50 mM sodium phosphate buffer (pH 7.0). 10 μl of each of the three inhibitor stock solutions was added separately to the reaction mixtures, and the remaining activity was determined. Stock solutions of each protease inhibitor were prepared, namely: serine protease inhibitor-100 mM phenylmethylsulfonyl- fluoride (PMSF) solution in DMSO, metalloprotease inhibitor-100 mM 1,10-phenanthroline solution in DMSO and aspartic acid protease inhibitor-100 μg/ml pepstatin solution in DMSO.

Zymographic analysis
Casein (0.05%) was co-polymerized with polyacrylamide gel and SDS-PAGE was performed according to the method described by Laemmli (1970). Following electrophoresis, the gels were washed successively with 2.5% (v/v) Triton X-100 and then with Triton X-100 containing 20 mM sodium phosphate buffer (pH 7.0) for 10 min each. Finally, the gels were equilibrated for 10 min with the same buffer, and incubated at 37°C for 5 min. The gels were stained with Coomassie brilliant blue, and destained to reveal zones of substrate hydrolysis.

Results and Discussion

Isolation and identification of proteolytic bacteria
In skim milk media, all of the isolates showed zones of substrate hydrolysis. The Ni 26, Ni 36 and Ni 49 isolates were aerobic, endospore-forming, Gram-positive and rod-shaped bacteria, but isolate Ni 39 was an aerobic, Gram-negative and rod-shaped bacterium. The other biochemical

Table 1. Morphological and biochemical properties of proteolytic bacteria

Characteristics	Ni 26	Ni 36	Ni 39	Ni 49
Gram staining	+	+	–	+
Morphology	rod	rod	rod	rod
Spore formation	+	+	–	+
β-galactosidase	+	–	+	+
Arginine dihydrolase	–	+	+	+
Lysine decarboxylase	+	–	–	–
Ornithine decarboxylase	–	–	–	–
Citrate utilization	+	+	–	+
H_2S production	–	–	–	–
Urease	–	–	–	+
Tryptophane deaminase	–	–	–	–
Indole production	–	–	–	–
Acetoin production	–	–	–	–
Gelatinase	+	+	+	+
Utilization of				
Glucose	+	–	+	+
Mannitol	+	–	+	+
Inositol	+	–	–	+
Sorbitol	+	–	+	–
Rhamnose	+	–	–	–
Sucrose	+	–	+	+
Melibiose	–	–	–	–
Amygdalin	+	–	+	+
Arabinose	+	–	+	+

properties of the isolates are shown in Table 1. Isolates Ni 26, Ni 36 and Ni 49 were found to be *Bacillus* sp. based on their biochemical and physiological characteristics, and isolate Ni 39 was similar to *Aeromonas* sp. The 16S rDNA sequences were analyzed to determine which of the *Bacillus* or *Aeromonas* species cited in the GenBank matched each of the isolates with the highest homology. Sequence data were aligned to construct phylogenetic trees using the neighbor-joining method, and are shown in Fig. 1. The phylogenetic position of isolates Ni 26, Ni 36 and Ni 49 were then compared with *Bacillus* species in a dendrogram. Isolate Ni 26 was found to be closest to *B. vallismortis* (99%), isolate Ni 36 to *B. subtilis* C15 (99%) and isolate Ni 49 to *B. amyloliquefaciens* CMB01 (99%) (Fig. 1A). The 16S rDNA sequence of isolate Ni 39 was almost identical to that of *A. hydrophila* ATCC 35654, with 98% similarity (Fig. 1B). The 16S rDNA sequences of isolates Ni 26, Ni 36, Ni 39 and Ni 49 have been deposited in GenBank under accession numbers, AF539667, AF539677, AF539680 and AF539689, respectively.

Characterization of proteases
The optimum temperature for the proteolytic activities of the crude enzyme extracts, prepared from the four isolates, ranged from 40°C for strain Ni 26 to 70°C for strain Ni 36 (Fig. 2). The optimum pH for the proteolytic activities of all the *Bacillus* isolates (Ni 26, Ni 36 and Ni 49) was 8.0 (Fig. 3), which is similar to that of other proteases produced from *Bacillus* sp. and *B. stearothermophilus* F1

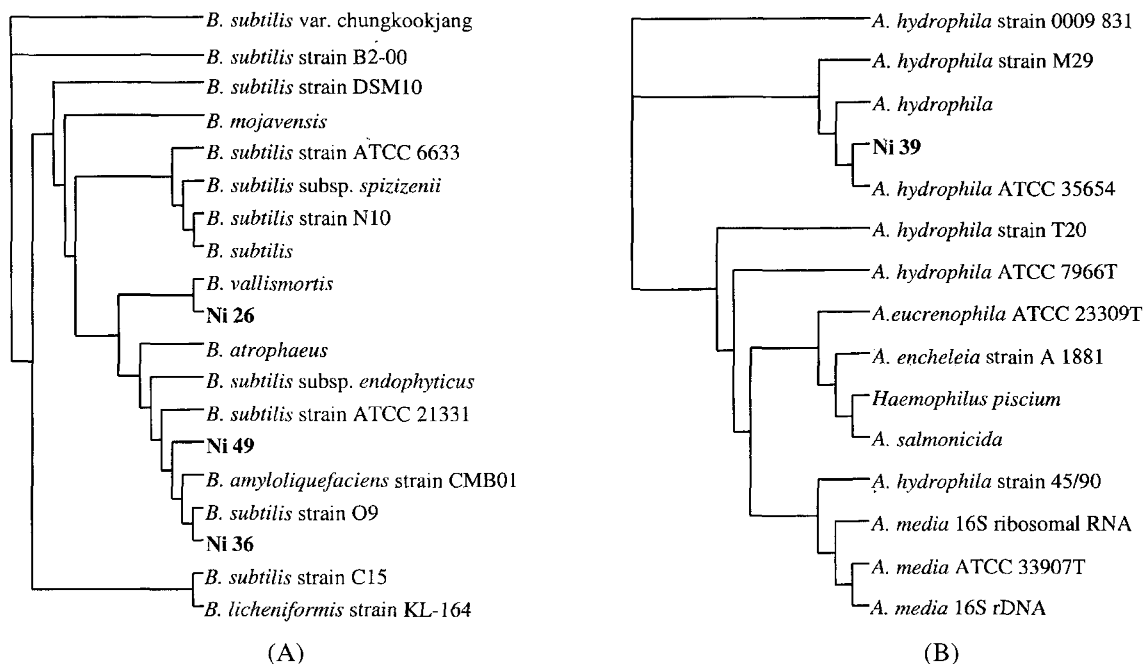

Fig. 1. Neighbor-joining tree showing the phylogenetic affiliation of the proteolytic isolates to a selected number of members of *Bacillus* spp. (A) and *Aeromonas* spp. (B).

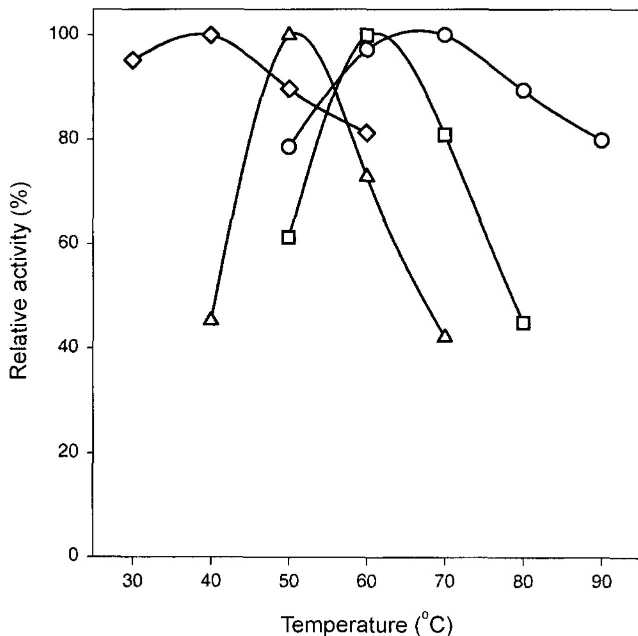

Fig. 2. Effect of temperature on the proteolytic activities of isolates Ni 26 (◇), Ni 36 (○), Ni 39 (□) and Ni 49 (△). Activity was measured in 50 mM sodium phosphate buffer (pH 7.0) at the indicated temperatures; values are means of duplicate experiments.

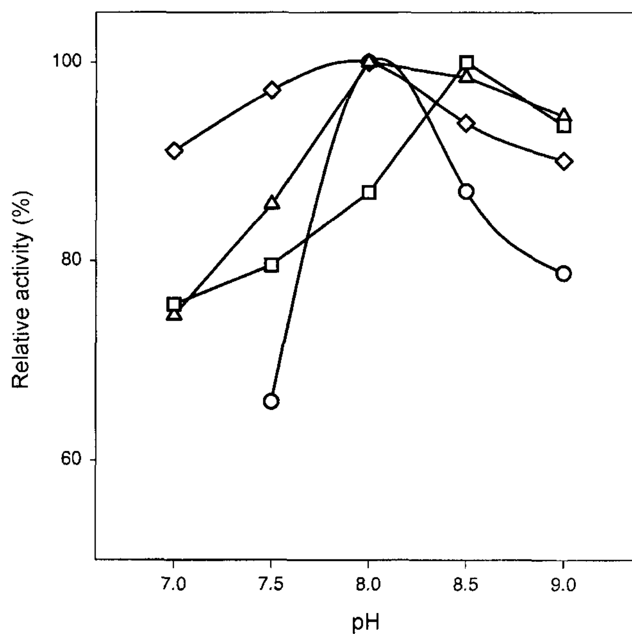

Fig. 3. Influence of pH on the proteolytic activities of isolates Ni 26 (◇), Ni 36 (○), Ni 39 (□) and Ni 49 (△). Activity was measured at 25°C in 0.1 M citrate buffer adjusted to the indicated pH; values are means of duplicate experiments.

(Hamer and Zwiefelhofer, 1986). However, the optimum pH of the Ni 39 isolate was 8.5, which was slightly higher than the pH 7.0 shown by the zinc proteinase of *A. hydrophila* (Loewy *et al.*, 1993). In the presence of PMSF proteolytic activities of all isolates were inhibited (Fig. 4), suggesting they contained serine protease. The proteolytic activities of isolates Ni 26 and Ni 39 were significantly inhibited by 1,10-phenanthroline by about 47 and 50 %, respectively, suggesting the presence of metalloprotease. At least two proteolytic activities were observed in all isolates (Fig. 5), the exact classification of the protease type of each isolate was not possible (Fig. 4).

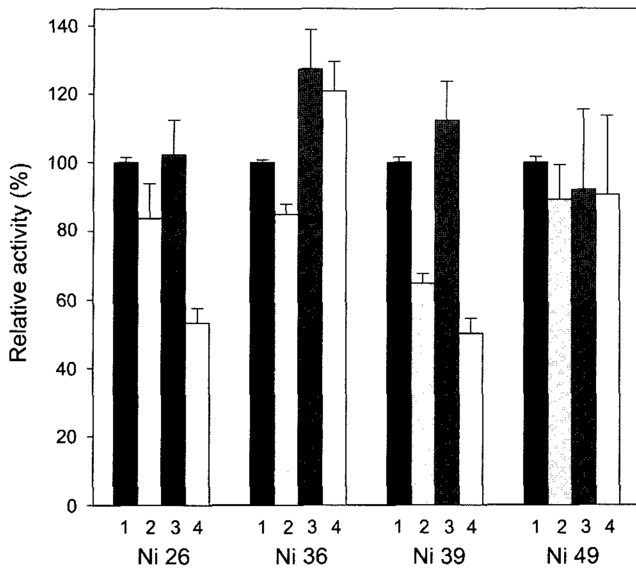

Fig. 4. Effect of protease inhibitors on the proteolytic activities of isolates. 10 μl of inhibitor stock solutions were added individually to enzyme reaction mixtures, and the remaining activity was determined. 1, no inhibitor; 2, PMSF; 3, pepstatin; 4, 1,10-phenanthroline.

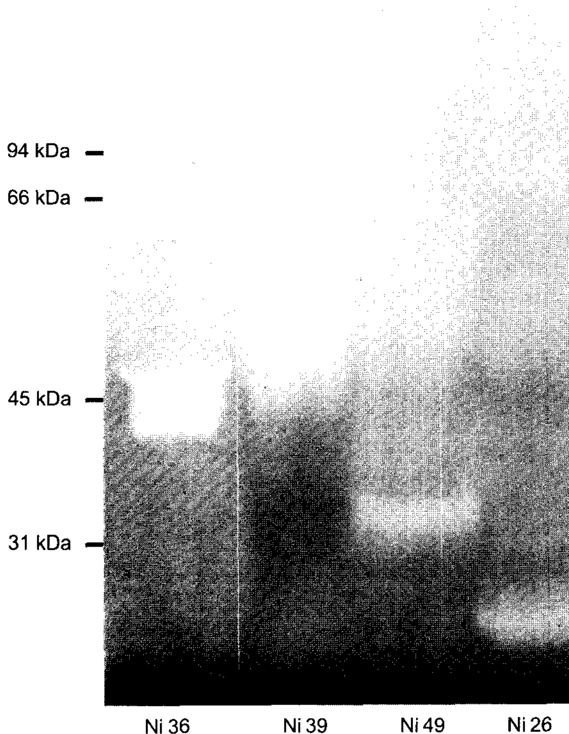

Fig. 5. Analyses conducted to establish the presence of caseinolytic proteases in the cell free supernatants of isolates. Aliquots of culture supernatants were used to analyze for the presence of caseionlytic proteases by zymography.

Zymogram analysis

The extracellular enzyme solutions prepared from the four isolates had different caseinolytic activities, as determined by zymography. On the zymogram obtained for the Ni 39 isolate, the protease activity was smeared from molecular masses of 45 kDa. The largest zone of hydrolysis was observed at about 110 kDa (Fig. 5), indicating the presence of numerous proteases. Many reports have described the numbers and natures of proteases found in the supernatants of *A. hydrophila* cultures. Some have reported that one of two proteases produced is a temperature stable metalloprotease, and that the other is a temperature labile serine protease (Dahle, 1971; Neito and Ellis, 1986; Leung and Stevenson, 1988). Loewy *et al.* (1993) reported another novel zinc-protease in cultures of *A. hydrophila*, which was grown in a buffered tryptone yeast extract broth. Isolate Ni 36 showed two bands with protease activity, with molecular masses of 50 and 40 kDa. The zymograms for isolates Ni 49 and Ni 26 showed the presence of high molecular mass proteases that did not easily migrate in 10% gels. Other distinct proteins with protease activities were found to have molecular masses of approximately 32 and 15 kDa for isolates Ni 49 and Ni 26, respectively (Fig. 5). Similarly to many reported *Bacillus* species (Priest, 1977), isolates Ni 26, Ni 36 and Ni 49 also produced multiple extracellular proteases.

In the present study, we describe the isolation and identification of proteolytic bacteria from RABC, together with some characteristics of their proteolytic enzymes. These bacteria may play an important role in the degradation of proteinaceous organic compounds in wastewaters. Further studies on the purification, and on the molecular properties of each protease are required.

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