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보 문

Isolation and identification of protease-producing bacteria from the intertidal zone in Jeju Island, Korea

Young-Gun Moon, Subramanian Dharaneedharan, Dong-Hwi Kim, So-Hyun Park, and Moon Soo Heo*

Marine Applied Microbes and Aquatic Organism Disease Control Lab, Department of Aquatic Biomedical Sciences, School of Marine Life Sciences, Jeju National University, Jeju 63243, Republic of Korea

제주 조간대로부터 단백질 가수분해효소를 생산하는 세균의 분리 및 동정

문영건 · 수브라마니안 다라니다란 · 김동휘 · 박소현 · 허문수*

제주대학교 해양생명과학대학 수산의생명과학부

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ABSTRACT: Eleven protease-producing bacteria were isolated from the organisms' external shells and the inorganic materials collected from intertidal zone of Jeju Island, Republic of Korea. The samples were diluted serially, inoculated on Zobell agar plates with 1% skim milk and incubated at 20°C. Clear zone forming colonies were selected as protease-producing bacteria and each strain was identified based on the phylogenetic analysis with their 16S rDNA sequences. Strains JJM125, JJM129, YG47 and YG49 belong to the marine bacterial genus *Pseudoalteromonas*; strain JJM122 belong to the genus *Microbulbifer*; strains YG51, YG52, YG62 and YG63 belong to the genus *Vibrio*; and strain YG65 belong to genus *Bacillus*. Hence, the present study suggests that these protease producing bacteria could be further used to develop new varieties of protease with various biotechnological applications.

Key words: bacteria, intertidal zone, Jeju Island, protease

Proteases [serine protease (EC. 3.4.21), cysteine (thiol) protease (EC.3.4.22), aspartic proteases (EC.3.4.23) and metallo-protease (EC.3.4.24)] constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales (Ellaiah *et al*., 2003; Nascimento and Martins, 2004). Microbial proteases represent one of the third largest groups accounting for approximately 60% of the total enzyme production in the world (Rao *et al*., 1998). According to the recent market research report on world enzymes, global enzyme demand was forecasted to increase annually by 6.4% to $6.9 billion in 2017 (World enzyme market, 2014). Among the proteases, alkaline proteases are one of the most widely studied group of enzymes because of their wide use in many industrial applications such as food, pharmaceutical and leather (Rao *et al*., 1998); and with two-third of its share in detergent industry alone (Kumar and Hiroshi, 1999). Proteases have applications in leather processing, food processing and production of protein hydrolysates (Banik and Parkash, 2004) and also in the production of certain oligopeptides had received great attention as a viable alternative to chemical approach (Lee *et al*., 1993). Their application has attracted worldwide attention to exploit their physiological and biotechnological applications (Rao *et al*., 1998). Previous studies have shown the isolation of proteases from several marine bacteria (Alfredsson *et al*., 1995; Marcello *et al*., 1996; Irwin *et al*., 2001; Lee *et al*., 2002). These bacterial proteases showed algicidal activity or enhanced chitinase activity (Lee *et al*., 2002; Miyamoto *et al*., 2002). There have been a few reports on protease-producing Arctic bacteria (Irwin *et al*., 2001; Lee *et al*., 2004). In this context, present study was done to isolate and identify potent protease-producing bacteria from the intertidal zone and tide pool around in the Jeju Island.

***For correspondence.** E-mail: msheo@jejunu.ac.kr;
Tel.: +82-64-754-3473; Fax: +82-64-756-3493

Materials and Methods

Sample collection

Samples such as sea plants, shells or external cover of organisms and inorganic sea sand (Table 1) were collected from intertidal zone in the Jeju Island (Fig. 1).

Water temperature was measured *in situ*, ranged from 18.0 to 23.0°C. Samples were immediately transported to the laboratory at 4°C for further processing.

Table 1. List of organisms and inorganic components used for in isolation of protease producing bacteria

Species name	Isolate
Sargassum thunbergii	JJM122 JJM123
Aplysia juliana	JJM125 JJM129
Littorina brevicula (Philippi)	YG47
Sea sand	YG49
Anthopleura midori	YG51
Chlorostoma turbinata	YG52
Haliclona permollis	YG62
Nerita (Heminerita) Japonica	YG63
Hypselodoris festiva	YG65

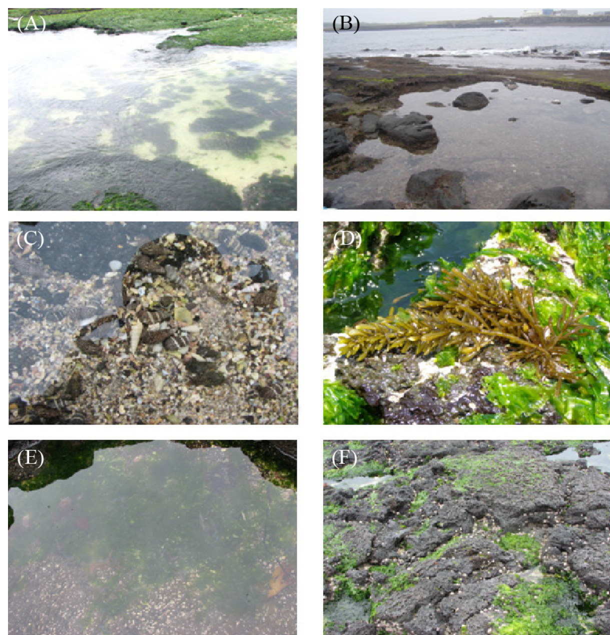

Fig. 1. Sampling site of eleven isolates. (A) A tide pool of sediment; (B) tide pool of rocky; (C) shell which exists in a tide pool; (D, E) seaweed which inhabits the tide pool in the intertidal zone; (F) top shell which inhabits a bedrock intertidal zone.

Isolation and cultivation of protease-producing bacteria

The samples were incubated with 5 ml of sterile distilled water for 30 mins and diluted 50 to 1000-fold in sterilized seawater and spread on Zobell agar plates fortified with 1% skim milk (skim milk 10 g, peptone 5 g, yeast extract 1 g, $FePO_4$ 0.01 g, bacto agar 15 g, distilled water 250 ml, aged sea water 750 ml, pH 7.0). The plates were incubated at 20°C and observed daily for signs of clearing of the agar around the colonies during 3 days. The strains were purified by repeated streaking in the same medium. Following 3 days incubation period at 20°C, colonies forming clear zones were selected as protease-producing bacteria. Stored at -80°C in marine broth 2216 (MB; Difco) supplemented with 20% (v/v) glycerol until use.

Protease assay

The isolated bacterial cultures were centrifuged at 5,000 rpm for 6 min and 0.15 ml of each supernatant was added to a tube containing 0.3 ml of 1% (w/v) casein (dissolved in 20 mM Tris-HCl buffer, pH 7.4) and incubated at 37°C for 30 min. Subsequently, 0.45 ml of a 10% (w/v) tri-chloroacetic acid solution at a final concentration of 5% w/v was added to stop the proteolysis. The mixture was incubated at room temperature for 1 h. After incubation, the reaction mixture was centrifuged at 12,000 × *g* for 5 min and the absorbance of the supernatant was measured at 280 nm. One unit of protease is defined as the amount of enzyme that hydrolyses casein to produce equivalent absorbance to 1 μmol of tyrosine/min with tyrosine as standard (Kembhavi *et al*., 1993).

DNA extraction and PCR amplification

Total bacterial genomic DNA was extracted using AccuPrep genomic DNA extraction kit (Bioneer). The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primer set 27F (5′-AGAGTTTGATCNTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixture consisted of 5 μl of 10× PCR mixture (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl of each primer, 1 μl of template DNA, and 2.5 units of Taq polymerase (TaKaRa) in a final volume of 50 μl. The PCR was performed in a thermal cycler (Biometra) using following cycling conditions,

an initial denaturation at 95°C for 5 min and then 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis, and purified with an AccuPrep PCR purification kit (Bioneer). The purified PCR products were sequenced by Solgent Co. Ltd.

Sequence analysis

The full 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR). The nucleotide sequences were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Sequences of the 16S rDNA were submitted to the Advanced BLAST search program to identify sequences of closely related organisms. The related sequences were preliminarily aligned with CLUSTAL W (Thompson *et al*., 1994), and complete sequence alignments were performed using PHYDIT (Chun, 1995) and manual comparison. Phylogenetic analyses were performed with PHYLIP (Felsenstein, 1993), and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei, 1987). Evolutionary distances for the neighbour-joining method were calculated using the method of Jukes & Cantor (Juke and Cantor 1969). Strains numbers and their corresponding accession numbers are indicated on the phylogenetic trees.

Results and Discussion

Proteases are widespread in nature; particularly microbes serve as a potential source of these enzymes due to their rapid growth in limited space and ease for genetic manipulation to produce new enzymes with altered properties with various applications (Kocher and Mishra, 2009). In the present study, about eleven proteolytic bacterial strains were isolated from the marine organic and inorganic substances collected from the tidal and inter-tidal zones of Jeju Island. The isolated 11 strains were designated as JJM122, JJM123, JJM125, JJM129, YG47, YG49, YG51, YG52, YG62, YG63, and YG65. They formed a clear zone around the colonies on Zobell agar media containing skim milk (Fig. 2) and their concentration of protease produced

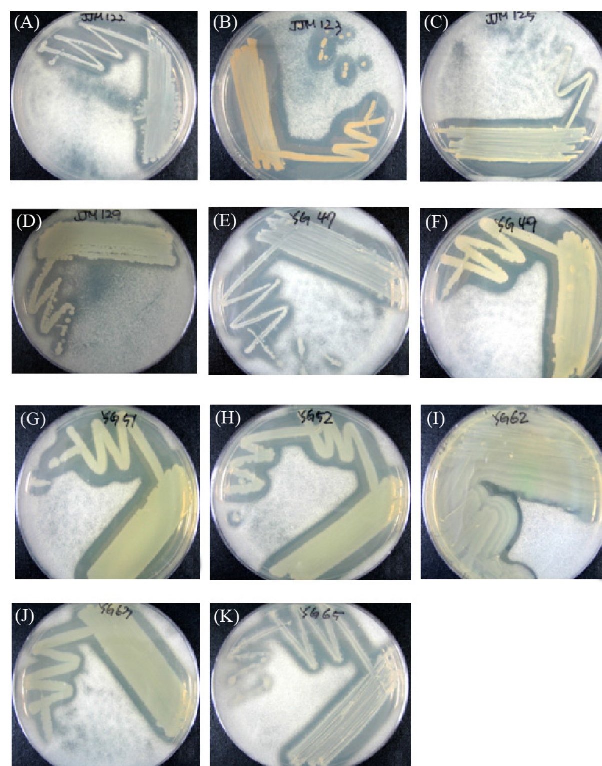

Fig. 2. Marine bacterial isolates showing protease activity. (A) Genus *Microbulbifer*; (B, K) genus *Bacillus*; (C, D, E, F) genus *Pseudoalteromonas*; (G, H, I, J) genus *Vibrio*.

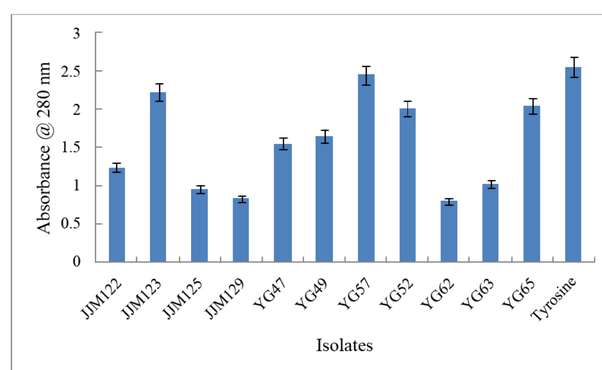

Fig. 3. Marine bacterial isolates showing the different level of protease activity.

by each isolates were shown in Fig. 3. Similar results of clear zone formation by proteolytic action of marine arctic bacteria had been reported by Lee *et al*. (2004).

After incubation at 25°C, eleven morphologically different protease-producing bacteria were selected for further analyses. Except the *Bacillus* isolates (JJM123 and YG65), the other nine strains belonged to the class *Gammaproteobacteria*. Of the nine strains four belongs to the genus *Vibrio* (isolates YG51, YG52,

YG62, and YG63), three to *Pseudoalteromonas* (JJM125, JJM129, and YG47) and one to *Microbulbifer* (JJM122).

Advancement in the PCR technology and its application has revolutionized the field of microbial ecology. It has now been possible to determine the precise phylogenetic position of marine bacterial isolates using 16S rRNA gene sequences. In the present study, a comparative analysis of more than 1,330 bases of the 16S rRNA gene of 11 isolates, chosen from the different genera was performed. The sequence with high similarities and their corresponding percentage of similarity are presented in Table 2 and four phylogenetic trees were constructed (Fig. 4A–D).

Strain JJM122 was isolated from of a specimen of the green algae *Sargassum thunbergii* collected from intertidal zone. The closest match of the sequences of *Microbulbifer* isolate (98% similarity) was to that from a sponge in the Mediterrean Sea, isolate UST040317-107. The highest similarity value (99.75%) with a strain characterized to the species level was to *Microbulbifer mediterraneus* UST040317-107T (accession number DQ096578). Previous report on isolation of cultivable marine proteobacteria such as *Thalassomonas agarivorans*, *Oleiphilus messinensis*, *Brevundimonas vesicularis*, and *Vibrio diazotrophicus* from marine sponge *Hyattella cribriformis* (Prasanna Kumar *et al*., 2013).

Strain JJM125, JJM129, YG47, and YG49 were isolated from of organisms such as *Aplysia Juliana*, *Littorina brevicula*, and inorganic sea sand. The phylogenetic analysis indicated that these strains JJM125, JJM129, YG47, and YG49 belong to the representative marine bacterial genus *Psedoalteromonas*. Sequences were 98–99% similar to those of *Pseudoalteromonas atlantica* AR06T (Gauthier *et al*., 1995), *Pseudoalteromonas nigrifaciens* NCIMB 8614T (Gauthier *et al*., 1995), *Pseudoalteromonas tetraodonis* Do-17T (Ivanova *et al*., 2001), and *Pseudoalteromonas mariniglutinosa* Do-52T (Romanenko *et al*., 2003) respectively. The *Pseudoalteromonas* isolates shared more than 98% similarity with sequences of the type strains of the species described in this genus. *Pseudoalteromonas* sp. possesses broad range of bioactive compounds (Bowman, 2007) and particularly, Vazquez *et al.* (2008) reported extracellular protease activity of Antarctic marine isolates.

Strain YG51, YG52, YG62, and YG63 were isolated from the surface of marine organic and inorganic substances like *Anthopleura midori*, *Chlorostoma turbinate*, *Halichondria japonica*, and *Haliclona permillis*, collected in a tidepool near the seashore. The phylogenetic analysis indicated the strain YG51, YG52, YG62, and YG63 belong to the representative marine bacterial genus *Vibrio*. It showed high 16S rDNA

Table 2. Comparison of phenotypic and molecular features of protease-producing bacteria from tidepools in the Jeju intertidal zone, Korea

Phylogenetic affiliation (phylum, class, family)	Isolate	Growth at		16S rDNA sequence length (bp)	Top matches, GenBank accession number	Nucleotide Identity (%)
		NaCl (%)	Temp. (°C)			
Proteobacteria Gammaproteobacteria Alteromonadaceae	JJM122	1.0 - 12	10 - 37	1419	*Microbulbifer* sp. 7X/A02/240 (AY576773) *Microbulbifer mediterraneus* UST040317-107 (DQ096578) *Microbulbifer cystodytense* (AJ620879)	98
Proteobacteria Gammaproteobacteria Pseudoalteromonadaceae	JJM125	1.0 - 12	4 - 40	1338	*Pseudoalteromonas atlantica* AR06 (AB049728)	99
	JJM129	1.0- 9.0	10 - 37	1411	*Pseudoalteromonas nigrifaciens* NCIMB 8614T (EU195931)	98
	YG47	1.0- 8.0	10 - 35	1407	*Pseudoalteromonas* sp. BCBw003 (DQ492817) *Pseudoalteromonas* tetraodonis Do-17T (AB257325)	98
	YG49	1.0 - 10	5 - 30	1413	*Pseudoalteromonas* sp. MACL07 (EF198247) *Pseudoalteromonas mariniglutinosa* Do-52T (AB257336)	98
Proteobacteria Gammaproteobacteria Vibrionaceae	YG51	1.0 - 11	10 - 37	1428	*Vibrio diabolicus* (X99762)	98
	YG52	1.0- 9.0	7 - 35	1430	*Vibrio splendidus* ATCC 33125T (X74724)	99
	YG62	1.0 - 12	10 - 35	1424	*Vibrio diabolicus* (X99762)	98
	YG63	1.0 - 11	10 - 37	1429	*Vibrio splendidus* ATCC 33125T (X74724)	98
Firmicutes Bacillaceae	JJM123	0.0 - 14	10 - 45	1442	Bacillus aquimaris TF-12T (AF483625)	98
	YG65	0 - 12	5 - 45	1420	*Bacillus subtilis* CM19 (EU660332)	99

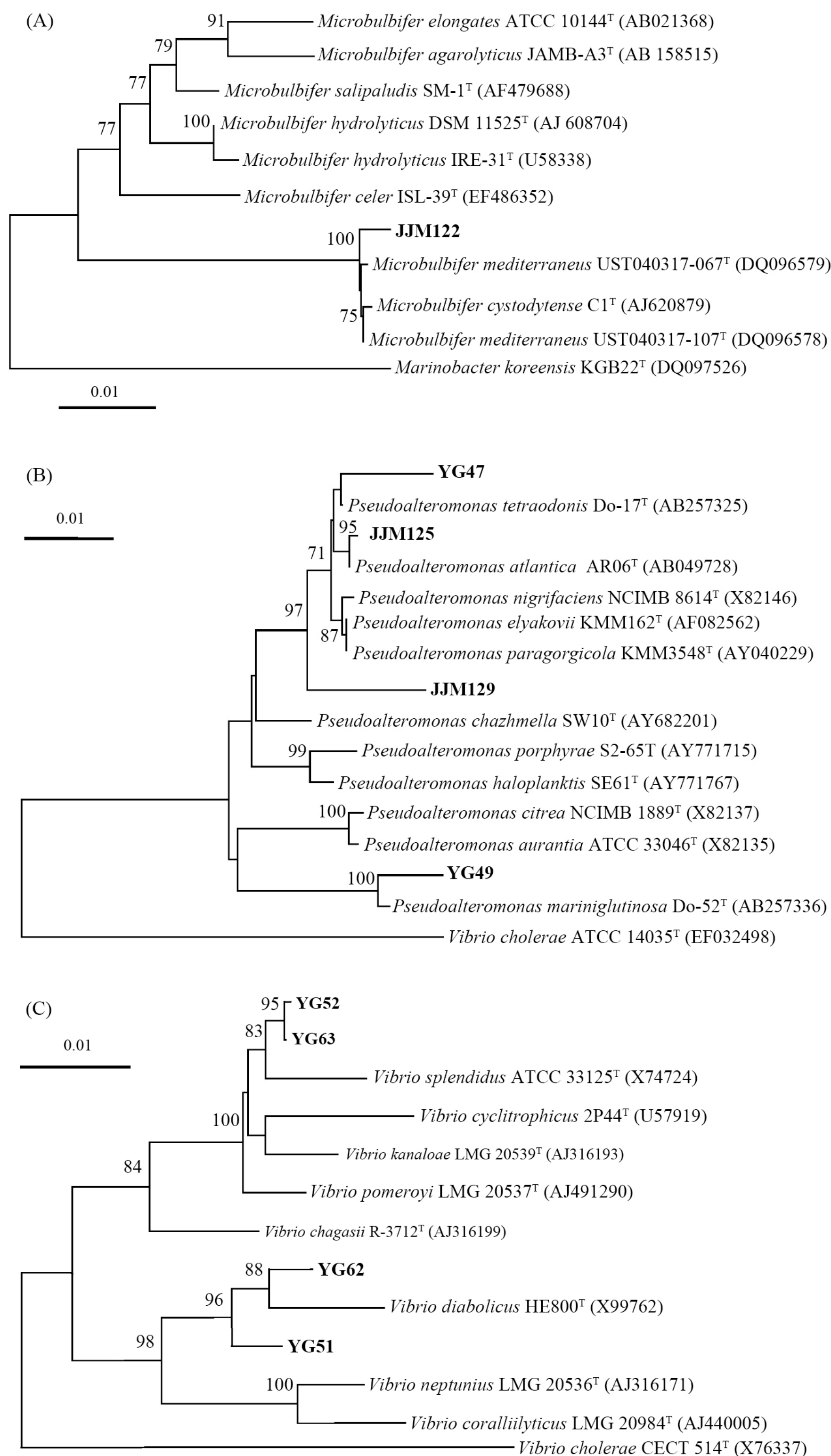

Fig. 4. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of protease-producing bacteria. The tree was constructed from evolutionary distance matrix by using neighbour-joining method. (A) genera of the genus *Micriobulbifer*; (B) the genus *Pseudoalteromonas*; (C) the genus *Vibrio*; (D) the genus *Bacillus*. The 16S rRNA gene sequence of *Marinobacter koreensis* KGB22T was chosen arbitrarily as the outgroup sequence for trees (A) *Vibrio cholerae* ATCC 14035T for (B, C) and *Alkalibacillus haloalkaliphilus* DSM 5271T for (D) Bootstrap percentages (from 1,000 replications) >50% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

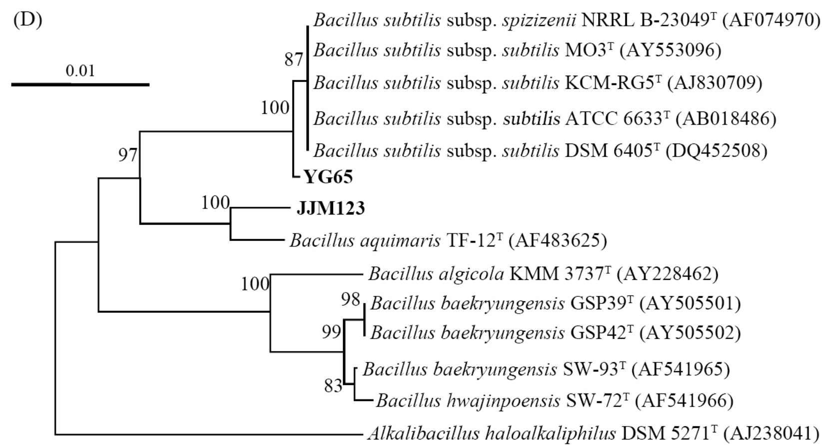

Fig. 4. Continued.

sequence similarity with *Vibrio diabolicus* (YG51 [98.54%], YG62 [99.13%]), *Vibrio splendidus* ([YG52 (99.49%), YG63 (99.56%)]. Similar results have been reported in the isolation and identification of marine *Vibrio* sp. (Marcello *et al*., 1996).

Strain YG65 was isolated from external surface of *Hypselodoris festiva.* The phylogenetic analysis indicated that strain YG65 belongs to the representative *Bacillus*. It showed a high 16S rDNA sequence similarity with *Bacillus subtilis* subsp. *spizizenii* NRRL B-23049T (99.80%) (Nakamura *et al*., 1999), *Bacillus subtilis* subsp. *subtilis* KCM-RG5, *Bacillus subtilis* subsp. *subtilis* DSM 6405, *Bacillus subtilis* subsp. *subtilis* ATCC 6633 (99.70%), and *Bacillus subtilis* subsp. *subtilis* MO3 (99.78%). Strain JJM123 was isolated from the surface of *Sargassum thunbergii* collected from the tidepool near the seashore in the Jeju Island. The colonies of JJM123 were orange in color. Strain JJM123 shared 99.18% 16S rDNA sequence similarity with *Bacillus aquimaris* TF-12T (Yoon *et al*., 2003), of which the type strain was isolated from seawater of a tidal flat of the yellow sea in Korea. Previous studies have reported that many Bacillus species are involved in extracellular protease production (Shumi *et al*., 2004) and also an important source of industrial alkaline proteases commercialized for alkaline protease production (Ferrari *et al*., 1993).

Further studies are needed to elucidate specific activity and optimal conditions for these bacterial proteases. This study suggests that these protease producing bacteria could be potentially used for various biotechnological and pharmaceutical applications.

적 요

단백질가수분해효소를 생산하는 11개의 세균들은 유기생물체의 외부 표면 서식하며, inorganic materials는 제주도 조간대로부터 수집되었다. 시료들은 냉동 상태로 실험실로 옮겨졌으며, 멸균 해수와 1% skim milk가 들어가 있는 Zobell plates에서 배양시켰다. 다음 clear zone이 나타난 11개의 균주들은 단백질분해효소를 생성하는 세균으로서 선택되었으며, 각각의 균주들은 16S rDNA을 기반으로 동정하였다. 분석결과, Psedoalteromonas속 해양 세균 JJM125, JJM129, YG47과 YG49, Microbulbifer속 JJM122, Vibrio속 YG51, YG52, YG62, YG63, Firmicutes문과 Bacillaceae강에 속하는 JJM129, YG65로 나타났다. 따라서, 본 연구에서는 단백질분해가수효소를 생성하는 세균들을 다양한 생명 공학 응용프로그램과 함께 새로운 다양성의 개발 및 이용이 가능할 것이다.

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