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Characterization of *Bacillus* sp. PU-T8 Isolated from Tiger Puffer Fish *Takifugu rubripes* as Probiotics

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Key words : *Bacillus* sp., probiotics, antimicrobial activity, viability

Abstract

Bacillus sp., PU-T8 strain, isolated from tiger puffer fish *Takifugu rubripes* showed growth-inhibitory activity against bacterial fish pathogens such as *Edwardsiella tarda* and *Vibrio anguillarum*. Physiological characterization and the phylogenetic analysis based on 16S rDNA sequences indicated that the strain PU-T8 is most closely related to *Bacillus subtilis* strains. The strain was considerably stable in acidic buffers (pH 2.0-4.0) and in buffers with digestive enzymes such as pepsin (pH 2.0) and pancreatin (pH 8.0). Cells suspended in PBS also remainded viable at 10^3 cfu/ml after heat treatment at 100°C for 60 min. The strain PU-T8 grew well in Z-AII broth media with 0.1 M to 2.0 M NaCl. Crude extract obtained with ethyl acetate from the culture supernatant of the strain PU-T8 showed growth-inhibitory activity against fish pathogens on the double-layer agar plates.

The aquaculture industry suffers from many disease problems by fish pathogens. The routine use of various antibiotics as therapeutic agents has resulted in the increase of resistant populations of pathogens.[1,2] Probiotics are generally defined as live microbial food supplements which improve the balance of the host animal's intestinal microflora and have been considered as an alternative means for controlling fish pathogens.[3] In aquaculture systems, probiotics can be administered either as food supplements or as additives to the water.[4] Prerequisites for potential probiotics are as follows: non-pathogenicity to animals, the resistance to digestive enzymes and bile salts, the ability to grow in the gut, the production of antimicrobial substances, and the resistance to heat-treatment for feed preparation.[5, 6]

The genus *Bacillus* constitutes a diverse group of rod-shaped Gram-positive bacteria, and is characterized by the ability to produce endospores.[7] Although the genus *Bacillus* is not common in marine environments, it has been isolated from the guts of marine animals and sediments.[8-10] Because the genus *Bacillus* competes with other bacteria for nutrients and space in the gut,

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and interferes with other bacteria through the production of antimicrobial substances such as antibiotics and enzymes, it has been widely used as potent probiotics.[11, 12)]

In this study, the strain PU-T8, which was isolated from the digestive tract of tiger puffer fish and showed antimicrobial activities against fish pathogens, was characterized and examined as a potential candidate of probiotics.

Materials and Methods

Bacterial strains and growth conditions

A bacterial strain, tentatively named PU-T8, was isolated from the content of a digestive tract of tiger puffer fish *Takifugu rubripes*. Bacteriological characterization was conducted according to the procedure described in "Methods in Microbiology".[13)] *Vibrio harveyi* strain 9M-P5-1 was isolated from a shrimp culture pond in the Philippines and *V. harveyi* strain ATCC 14126 was obtained from the American Type Culture Collection (ATCC). Bacterial strains of *Edwardsiella tarda* from diseased eels, *V. anguillarum* from diseased juvenile yellow tails and *Lactococcus garvieae* from a diseased yellow tail, which are pathogenic to fish, were kindly provided by Dr. Yamamoto of the Laboratory of Fish Pathology, Faculty of Fisheries, Kagoshima University. The strain PU-T8 was generally cultured in Z-AII broth medium containing polypeptone (Nihon Seiyaku, Japan) 5 g/l and yeast extract (Nihon Seiyaku) 1 g/l in artificial seawater (ASW, Herbest's formula composed of NaCl 30 g, KCl 0.7 g, $MgCl_2 \cdot 6H_2O$ 10.8 g, $MgSO_4 \cdot 7H_2O$ 5.4 g and $CaCl_2 \cdot 2H_2O$ 1.0 g per l) at 25°C with shaking at 120 rpm. *V. harveyi, E. tarda, V. anguillarum*, and *L. garvieae* strains were cultured in Z-CII,[14)] nutrient broth (NB), NB with 1 % (w/v) NaCl, and brain heart infusion (BHI) broth at 25°C, respectively. All bacterial strains were maintained on appropriate agar slants.

Antagonistic activity against bacterial fish pathogens

For the determination of antagonistic activity of the strain PU-T8, the double-layer agar plates containing each test strains were used. The fish pathogenic bacteria were cultured in appropriate broth media for 24 h. The basal agar medium (1.5% agar) was overlaid with 3.5 ml of soft agar medium (0.5% agar) containing 0.1 ml of fish pathogen cultures. The strain PU-T8 was inoculated on the surface of the double-layer agar plates and incubated at 25°C for 24 h. After incubation, the inhibition zones around the colonies of the strain PU-T8 were observed.

Preparation of washed bacterial cell suspensions

The strain PU-T8 was cultured in Z-AII broth medium for 24 h with shaking at 120 rpm. Cells were collected by centrifugation (5,000 rpm for 10 min at 4°C), washed and suspended in 0.1 M PBS buffer (pH 7.0). Absorbance of washed cell suspension at 600 nm was monitored with a spectrophotometer U-2010 (Hitachi, Japan) and adjusted to 0.1 ($\fallingdotseq 10^7$ cfu/ml) with 0.1 M PBS buffer (pH 7.0). Total viable counts of the cell suspensions were determined by the plate count method. The inoculated Z-AII agar plates were incubated at 25°C for 72 h.

Viability in acidic pH buffers

Acidic pH buffers were prepared by 0.1 M glycine-HCl buffer (pH 2.0, 2.5 and 3.0) or 0.1 M formic acid buffer (pH 3.5 and 4.0) and sterilized by a membrane filter, DISMIC-13cp (pore size 0.2 μm, Advantec MFS, Japan). Two hundred μl of the washed cell suspension was mixed with 0.3 ml of 0.9% saline solution and 1.0 ml of acidic pH buffer (pH 2.0-4.0). The mixtures were then agitated for 10 sec and incubated at 37°C in a water

bath. After 60, 90, 120 and 180 min incubation, 0.1 ml of the mixture was taken for the determination of total viable counts using the plate count method.

Viability in the solutions simulating gastric and intestinal juices

The solution simulating gastric juice was prepared by dissolving pepsin (Nacalai Tesque, Kyoto, Japan) in 0.1 M glycine-HCl buffer (pH 2.0, 2.5 and 3.0) or 0.1 M formic acid buffer (pH 3.5 and 4.0) to obtain a final concentration of 3 g/l. The solution simulating intestinal juice was prepared by dissolving pancreatin (Sigma-Aldrich, USA) in 0.1 M Tris-HCl buffer (pH 8.0) to obtain a final concentration of 1 g/l with or without 0.45% (w/v) gall powder (Wako Chemicals, Japan). These solutions were sterilized by using a membrane filter (pore size, 0.2 μm).

Viability of the strain PU-T8 in the simulating solutions was determined according to the following method reported by Huang *et al*.[15] An aliquot (0.2 ml) of the washed PU-T8 cell suspension was mixed with 0.3 ml of 0.9% saline solution, and 1.0 ml of the simulating gastric juice (pH 2.0-4.0) or intestinal juice (pH 8.0) solutions with or without 0.45% (w/v) gall powder. The mixtures were then agitated for 10 sec and incubated at 37°C. After 60, 90, 120, 180 and 240 min incubation, aliquots (0.1 ml) were taken for the determination of total viable count.

Viability after heat-treatment

An aliquot (0.4 ml) of the washed PU-T8 cell suspension was mixed with 2.6 ml of 0.1 M PBS buffer (pH 7.0). The mixture was then agitated for 10 sec and heat-treated at 40, 60, 80 or 100°C. An aliquot (0.1 ml) was taken after 10, 20, 30 and 60 min and subjected to the plate count method described above.

Growth in broth media with various NaCl concentrations

For the determination of optimal NaCl concentrations in broth culture, 0.1 ml of the washed PU-T8 cell suspension was inoculated to 10 ml of Z-AII broth media with 0.1, 0.5, 1.0, 2.0 or 3.0 M NaCl and incubated at 25 °C with shaking at 120 rpm. Absorbance of the culture broth at 600 nm was measured after 0, 12, 24, 48 and 72 h.

Antimicrobial activity of crude extract from the culture supernatant

The shaking culture of the strain PU-T8 grown in Z-AII broth at 25°C for 72 h was centrifuged at 10,000 rpm at 4°C for 20 min to remove cells. Antimicrobial substances were extracted from the culture supernatant with ethyl acetate using a separation funnel. The ethyl acetate layer was evaporated to dryness using a rotary vacuum evaporator R-114 (Sibata, Japan). The residual materials were redissolved in distilled water and then the same volume of chloroform was added. After mixing and separation, the chloroform layer was evaporated to dryness using a rotary vacuum evaporator. The residues were dissolved in methanol as crude extract. Twenty μl of the crude extract solution was infiltrated to a sterile paper disk (diameter of 5 mm) and placed on the double layer agar plate, whose upper layer contained 0.1 ml of cell suspensions of fish pathogens. After incubation at 25°C for 24 h, the diameter of the inhibition zone around the paper disk was measured.

PCR amplification and homology search of 16S rDNA

The strain PU-T8 was cultured in Z-AII broth medium at 25°C for 24 h with shaking at 120 rpm. The total DNA was extracted from the cells and purified using the standard phenol-chloroform method. Polymerase chain

reaction (PCR) amplification of the 16S ribosomal RNA gene of the strain PU-T8 was carried out using universal primers, PrSSU.1F (5'-AGAGTTTGATCCTGGCTCA G-3') and PrSSU.1R (5'-AAAGGAGGTGATCCAG CC-3') specific to prokaryotic 16S rRNA genes. Polymerase chain reaction solutions were prepared according to the instruction manual of ExTaq DNA polymerase (Takara Biochem., Japan). The sequences of the 16S rDNA amplification products was determined by using an ABI PRISM Big dye terminator Cycle Sequencing Kit Ver. 3.1 (Applied Biosystems, USA). Homology search for each 16S rDNA sequence was performed using BLAST at the National Center for Biotechnology Information (NCBI) server.

Table 1. Characteristics of the strain PU-T8

Characters	PU-T8*
Gram stain	+
Cell form	Rod
Motility	+
Spore-forming	+
Pigmentation of colony	-
Oxidase test	+
Catalase test	+
Glucose (O/F test)	O
V-P reaction	+
Growth in anaerobic agar	-
Growth at pH	6-10
Growth at NaCl (%)	0-10
Growth at 50°C	+
Growth at 65°C	-
Acid and gas from glucose	-
NO_3 reduction	+
pH in V-P medium < 6.0	-
NH_3 from Arginine	-
Hydrolysis of	
Starch	+
Casein	+
Gelatin	+
Tween 80	-
Olive oil	-
Trybutirin	-
Utilization of citrate	+

*+, Positive; -, Negative; O, oxidative.

Results

Characterization and identification of strain PU-T8

Characterization of the strain PU-T8 was carried out based on morphological and physiological tests according to the standard method of bacteriology. As shown in Table 1, the strain PU-T8 was Gram-positive, spore-forming, motile rod, catalase and oxidase positive, oxidative in Hugh and Leifson's O/F-test, Voges-Proskauer reaction-positive and positive in starch, casein and gelatin hydrolysis, indicating that it belongs to the genus *Bacillus*. In addition, the phylogenetic analysis based on the 16S rDNA sequence exhibited that the strain PU-T8 has 98% similarity with *B. subtilis* strain B-FS01 and *B. subtilis* strain B432 (Table 2).

Table 2. Homology between the 16S rDNA sequence (1402 bp) of the strain PU-T8 and the DNA datebase sequences

The most similar sequences (Accession numbers)	Organisms and strains	Similarity (%)
DQ520955	*Bacillus subtilis* strain B-FS01	98
DQ523502	*Bacillus subtilis* strain B432	98

Table 3. Antagonistic activity of the strain PU-T8 against bacterial fish pathogens

Fish pathogens	Antagonistic activity*
Edwardsiella tarda strain E22	+
Edwardsiella tarda strain SU100	+
Edwardsiella tarda strain SU226	+
Edwardsiella tarda strain E381	+
Vibrio anguillarum strain NUF113	+
Vibrio anguillarum strain NUF114	+
Vibrio anguillarum strain NUF302	+
Vibrio anguillarum strain NUF443	+
Vibrio anguillarum strain NUF482	+
Vibrio anguillarum strain NUF691	+
Vibrio harveyi strain 9M-P5-1	-
Vibrio harveyi ATCC14126	+
Lactococcus garvieae strain NO1	+

*Antagonistic activity was determined by the double-layer agar method as described in Materials and Methods. +, positive; -, negative.

Antimicrobial activity of the strain PU-T8 against bacterial fish pathogens

Antimicrobial activity of the strain PU-T8 against 13 strains of bacterial fish pathogens was determined by using the double-layer agar method. Of 13 strains of fish pathogens, 12 strains were apparently inhibited by the strain PU-T8 as shown in Table 3. Only *V. harveyi* strain 9M-P5-1 was found to be insensitive to the strain PU-T8.

Viability of PU-T8 under different physico-chemical conditions

The effect of acidic pH buffers on the viability of the strain PU-T8 was examined as shown in Fig. 1. When the pH range of the suspension bufferes was from pH 2.0 to pH 4.0, viability of the strain PU-T8 decreased to one-hundredth level within 60 min and kept the same level until 180 min of incubation.

The effects of the gastric juice- or intestinal juice-simulating solutions on the viability of the strain PU-T8 were exhibited in Figs. 2 and 3. The simulating solutions did not affect apparently the viability of the strain PU-T8 during 180 or 240 min of incubation. The viability of the strain PU-T8 was kept constant during 240 min of incubation in the intestinal juice-simulating solution without gall powder. However, in the presence of 0.45% gall powder, the viability of the strain PU-T8 was shown to decrease slightly during 240 min of incubation.

As shown in Fig. 4, the strain PU-T8 was exposed to

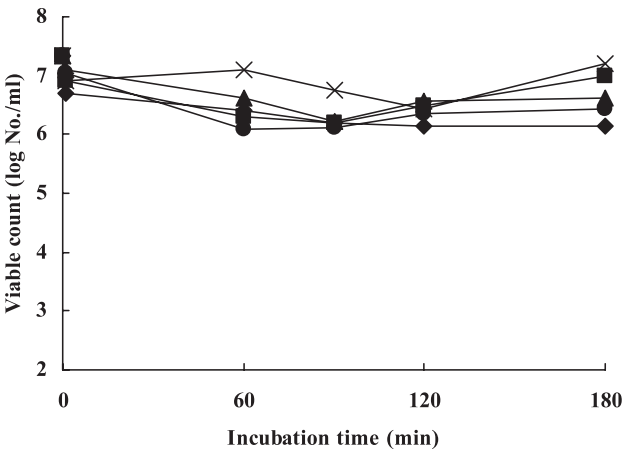

Fig. 2. Effect of the gastric juice-simulating solution with different pH on the viability of the strain PU-T8 during 180 min. The gastric juice-simulating solution was prepared by dissolving pepsin in 0.1 M glycine-HCl buffer (pH 2.0-3.0) or 0.1 M formic acid buffer (pH 3.5 and 4.0) to a final concentration of 3 g/l. ◆, pH 2.0; ■, pH 2.5; ▲, pH 3.0; ●, pH 3.5; ×, pH 4.0.

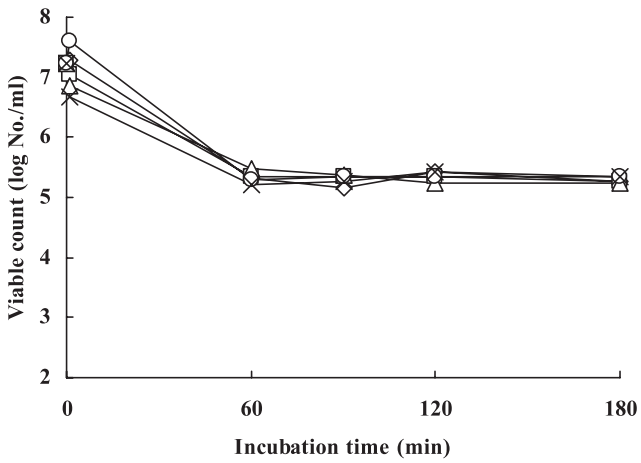

Fig. 1. Effect of acidic pH buffers with different pH on the viability of the strain PU-T8. Acidic pH buffers including 0.1 M glycine-HCl buffer (pH 2.0-3.0) and 0.1 M formic acid buffer (pH 3.5 and 4.0) were used. ◇, pH 2.0; □, pH 2.5; △, pH 3.0; ○, pH 3.5; ×, pH 4.0.

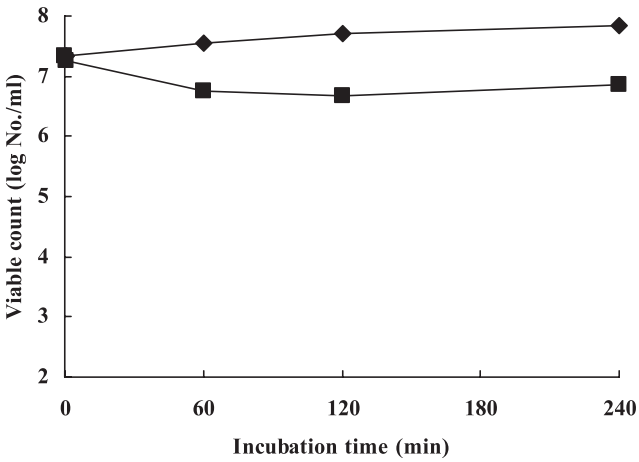

Fig. 3. Effect of the intestinal juice-simulating solution on the viability of the strain PU-T8. The intestinal juice-simulating solution was prepared by dissolving pancreatin in 0.1 M Tris-HCl buffer (pH 8.0) to a final concentration of 1 g/l. ■, with 0.45% gall powder; ◆, without 0.45% gall powder.

heat treatment at 40, 60, 80 or 100°C for 60 min in 0.1M PBS buffer (pH 7.0). The viability of the strain PU-T8 decreased gradually at over 60°C, but did not fall to 10^3 cfu/ml or less, indicating that the cell suspensions contained dormant spores along with vegetative cells.

The strain PU-T8 was demonstrated to grow well in Z-AII broth media with 0.1, 0.5 or 1.0 M NaCl, while it could multiply after a long lag period in the medium containing 2.0 M NaCl and did not multiply in the medium added with over 3.0 M NaCl (Fig. 5).

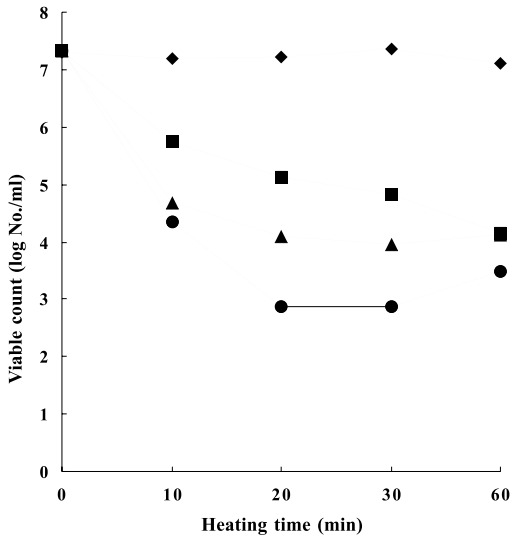

Fig. 4. Effect of heat treatment on the viability of the strain PU-T8. The cell suspensions of the strain PU-T8 were heated at various temperatures for 60 min. ◆, 40°C; ■, 60°C; ▲, 80°C; ●, 100 °C.

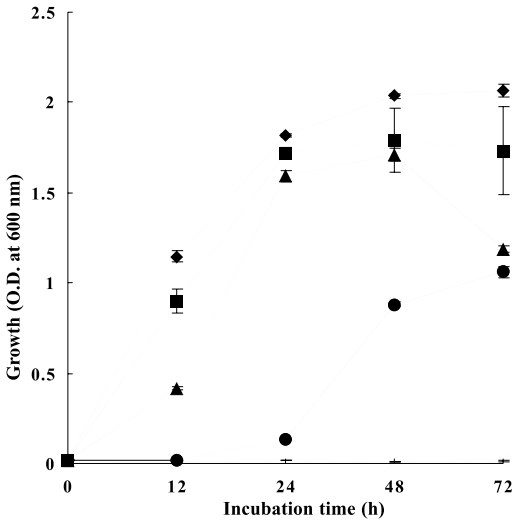

Fig. 5. Effect of NaCl concentrations on the growth of the strain PU-T8. ◆, 0.1 M of NaCl; ■, 0.5 M; ▲, 1.0 M; ●, 2.0 M; ×, 3.0 M. Optical density of the culture was measured at 600 nm after 0, 12, 24, 48 and 72 h of incubation.

Antimicrobial spectrum

Antimicrobial activity of the crude extract from the culture supernatant of the strain PU-T8 is shown in Table 4. Of 13 strains of bacterial fish pathogens, 12 strains were found to be sensitive to the crude extract and especially 4 strains of *E. tarda* were strongly inhibited. Of *Vibrio* strains tested, the strain 9M-P5-1 was insensitive to the crude extract but other *Vibrio* strains were slightly inhibited as well as the *L. garvieae* strain NO1.

Discussion

Bacillus spp. have been widely used as potent probiotics, because they interfere with some other bacteria in the gastrointestinal tract of animals through the production of various antimicrobial substances such as antibiotics and enzymes.[11, 12)]

In this study, antagonistic activity of the strain PU-T8 isolated from a digestive tract of tiger puffer fish was

Table 4. Antimicrobial activity of the crude extract from the culture supernatant of the strain PU-T8 against bacterial fish pathogens

Fish pathogens	Antimicrobial activity*
Edwardsiella tarda strain E22	++
Edwardsiella tarda strain SU100	++
Edwardsiella tarda strain SU226	+++
Edwardsiella tarda strain E381	++
Vibrio anguillarum strain NUF113	+
Vibrio anguillarum strain NUF114	+
Vibrio anguillarum strain NUF302	+
Vibrio anguillarum strain NUF443	+
Vibrio anguillarum strain NUF482	+
Vibrio anguillarum strain NUF691	+
Vibrio harveyi strain 9M-P5-1	-
Vibrio harveyi ATCC14126	+
Lactococcus garvieae strain NO1	+

*+++, diameter of inhibition zone > 15 mm; ++, diameter from 10 to 15 mm; +, diameter < 10 mm; -, no inhibition zone.

demonstrated against fish pathogens including 4 strains of *E. tarda*, 6 strains of *V. anguillarum, V. harveyi* ATCC14126 and *L. garvieae* strain NO1 was detected. The strain PU-T8 also exhibited growth inhibition against *Fusarium solani* causing black gill disease of kuruma shrimp (data not shown).

In aquaculture systems, probiotics can be administered either as food supplements or as additives to the water.[4)] Probiotic bacteria that are delivered through food supplements would be exposed to digestive enzymes such as pepsin and pancreatin but should survive under the conditions in the gut to provide positive effects to the host animals.[4, 5)]

The present study demonstrated that the strain PU-T8 was considerably tolerant to low pH , gastric juice- and intestinal juice-simulating solutions for 3 or 4 h. Huang *et al*.[15)] suggested that pH values of 2.0 and 3.0 could be considered as critical for the selection of potential probiotic bacteria. In the presence of gall powder, the viability of he strain PU-T8 showed slight decrease, while it kept constant in the intestinal juice-simulating solution without gall powder.

The strain PU-T8 tolerated and grew well in Z-AII broth media containing up to 2.0 M NaCl, suggesting that this feature is favorable to the application to marine aquacultures. Abdelkafi *et al*.[16)] reported that *Bacillus* sp. strain YAS1 grew in a range of 0-15% (w/v) NaCl concentrations.

In general, heat-tolerance of probiotics is not considered to be a selection criterion for potential probiotics, but it is necessary when fish feed materials with probiotics are heat-treated at 50~100°C.[6)] In this study, the strain PU-T8 was shown to keep the constant level of viability at 40°C for 60 min and the surviving cells were found in 10^4 and 10^3 cfu/ml levels after treatments at 60 and 80°C for 60 min, respectively. The results indicated that the cell suspensions of the strain PU-T8 contained a small amount of heat-resistant spores as well as vegetative cells. Gatesoupe[17)] suggested that the spores of *Bacillus* spp. are especially easy to introduce in dry feed, and this is an additional advantage of these promising probiotics candidates. Yanbo and Zirong[18)] reported that the addition of probiotics (*Bacillus* sp.) to basal diets improved the growth performance of common carp with feed utilization and digestive enzyme activities.

Antimicrobial substances from the culture supernatant of the strain PU-T8 were extracted with ethyl acetate and chloroform, and its antimicrobial activity against various fish pathogens was examined. The crude extract from the culture supernatant exhibited antimicrobial activity against fish pathogens including *E. tarda* and *V. anguillarum* strains.

Antimicrobial substance produced extracellularly by the strain PU-T8 is found to inhibit the growth of fish pathogens, but the chemical structure and action mechanism have not yet been determined. Further study including the isolation, purification and chemical analysis of antimicrobial substance produced by the strain PU-T8 is expected.

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