The ability of marine *Bacillus* spp. isolated from fish gastrointestinal tract and culture pond sediment to inhibit growth of aquatic pathogenic bacteria

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Abstract

In this research, antagonistic activity of two *Bacillus* species isolated from digestive tract of marine fish and culture pond of sea cucumber was studied. The inhibitory activity of *Bacillus* spp. isolates against some common pathogenic bacteria of fish was assessed using the agar diffusion method. The strain of *B. subtilis* G024 exhibited antimicrobial activity against *Vibrio anguillarum*, *V. harveyi*, *V. vulnificus*, *Streptococcus* sp. and *Staphylococcus aureus*; the isolate of *B. amyloliquefaciens* N004 inhibited growth in *V. anguillarum*, *V. campbellii*, *V. vulnificus*, *V. parahamolyticus*, *Edwardsiella tarda*, *Streptococcus* sp., *B.* cereus. Scanning electron microscopy (SEM) investigation of indicator bacteria showed that cell morphologies were strongly affected by the cell-free supernatant of the two *Bacillus* spp. isolates. It is determined that the culture filtrates contained inhibitors against growth of some pathogenic bacteria with different degrees of inhibition, although none of the culture filtrates could inhibit the growth of *V. fluvialis*, *V. alginolyticus*, *V. splendidus*. Based upon these characteristics, both of the antagonistic *Bacillus* spp. isolates could be the potential probiotics used in the aquaculture production.

Keywords: *Bacillus*; Antagonistic activity; Scanning electron microscopy; Probiotic; Aquaculture

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Introduction

In the past several decades, aquatic animal breeding in large-scale production facilities was exposed to the higher susceptibility to disease, and consequently the disease outbreak led to serious economic losses (Subasinghe *et al*., 2005). To prevent and treat the infectious aquatic animal-diseases, in China, including a limited number of Veterinary Bureau-approved antibiotics and chemotherapeutics can be used to assist the health management. However, the abuse of antibiotics could lead to immune depression, outbreaks of antibiotic resistant bacteria, environmental hazards and food safety concerns (Resende *et al*., 2012).

The best alternative strategy to the use of antimicrobial substances is to use natural substances that have a broad spectrum of inhibitory activities, including antagonistic activity against the aquatic pathogenic bacteria. In recent years, some reports regarding the antagonistic activity of isolates of *Bacillus* against aquatic pathogens were increasingly common (Kaynar *et al*., 2012; Zokaeifar *et al*., 2012; Xu *et al*., 2013). Xu *et al*. (2013) determined that the lipopetides produced by the isolate of *B. amyloliquefaciens* M1 exihibit antimicrobial properties against multidrug-resistant *Vibrio* spp. and *Shewanella aquimarina* isolated from diseased marine animals. Kaynar *et al*. (2012) illustrated that *Bacillus* species isolated from various fish samples could exhibit antimicrobial activity against *B. subtilis*, *Pseudomonas fluorescens*, *Lactobacillus coryneformis*, *L. plantarum* and *L. xylosus*. Zokaeifar *et al*. (2012) stated that *Bacillus* spp. isolated from fermented pickles could antagonize the *V. harveyi* and *V. parahaemolyticus* (shrimp pathogens). On the basis of these results, these *Bacillus* spp. strains might be considered for future use in aquaculture practice as the very promising substitutes for antibiotics and other fish drugs.

Promisingly, probiotics may provide an alternative way to reduce the use of fish drugs in aquaculture and simultaneously may avoid the development of antibiotic-resistant bacteria. At present, the reports about probiotics belonging to *Bacillus* spp. used as biocontrol agents in aquaculture are increasingly common (Mahdhi *et al*., 2010; Mahdhi *et al*., 2011; Janarthanam *et al*., 2012).

The main purpose of this investigation was to evaluate the antagonistic activity of *Bacillus* spp. strains isolated from marine fish and sediment of sea cucumber culture pond. The strategy was to evaluate the antagonistic activity of *Bacillus* spp. isolates against common aquatic pathogenic bacteria *in vitro*; and to investigate the inhibitory effect of cell-free supernatant of the two *Bacillus*

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spp. isolates on cell morphologies of indicator bacteria by using scanning electron microscopy (SEM).

Materials and methods

Isolation of antagonistic Bacillus spp. strains

Bacterial strains were isolated from the digestive tract of *Paralichthys lethostigma* (collected from the aquarium of the Institute of Oceanology, Chinese Academy of Sciences) and sediment (collected from the culture pond of sea cucumber (36° 1′ N, 120° 18′ E) of Oriental Ocean Group CO., LTD, Yantai, Shandong, China).

Isolation of gut bacterial flora and sediment bacterial strains was performed using the method according to the previous literatures (Kar *et al*., 2008; Kaynar *et al*., 2012) with some modifications. To isolate endogenous bacteria, sample fishes were starved for 24 h to clear the gastrointestinal tract before killing. Before dissection, the ventral surfaces of each fish were thoroughly scrubbed with 70% ethanol. Standard aseptic procedures were used. The gastrointestinal tracts were removed, cut into pieces and homogenized with sterilized 0.9% NaCl solution (1:5; wt/vol). Moreover, the culture pond sediment was diluted 1:1 (wt/vol) in the same sterile saline and resuspended by vigorous vortexing until an evenly distributed suspension was obtained. Subsequently, fish gastrointestinal tract homogenates, sediment suspension were heated at 60℃ for 60 min in a water bath. After that, the treated samples were used after 10 serial 1:10 dilutions. Plating of 0.1 mL aliquots of appropriate 10-fold serial dilutions in sterilized 0.9% NaCl solution (up to 10^{-5}) was done aerobically on 2216E agar plates. Although no quantification was attempted, a measurable number of colonies (30 to 300) were routinely obtained on 10^{-1} to 10^{-3} dilution plates after 24 to 48 h of incubation at 28°C. Colonies representing different morphological appearance (e.g. colony shape, color, elevation) were picked at random and purified by restreaking on 2216E agar. All the single isolated colonies from the streaked plates were retained and preserved in the laboratory and their purity routinely checked during the investigation. Stock cultures were frozen at –80°C with 20% (v/v) glycerol.

The agar diffusion assay was used to test the antimicrobial activity of cell-free supernatant of the selected *Bacillus* spp. isolates according to the previous literatures (Sun *et al*., 2010; Zhao *et al*., 2013) with some modifications. The isolates were inoculated in 10 mL of TSB (tryptic soy broth, containing 1.5% NaCl) liquid medium at 28℃ and 200 rpm until OD_{600} of the culture reached 0.8-1.0 ($\sim 10^8$ CFU/mL). The obtained culture was centrifuged at 12000 rpm at 4℃ for 10

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min. The cell-free supernatant was sterilized by passing through a 0.22-μm Millipore filter (Millipore, Bedford, MA, USA). The ability of the selected *Bacillus* spp. isolates to produce antimicrobial substances was assessed using the oxford cup method, which is briefly described as follows. The 100 μL cultures of indicator bacteria (OD_{600} = 0.8-1.0，～10^8 CFU/mL) were coated on TSA (tryptic soy agar, containing 1.5% NaCl) plate with oxford cup in. Then 200 μL cell-free supernatant was added to the midpoint of the oxford cup, incubated at 28℃ for 24 h, and antimicrobial zone diameter was determined. The spectrum of antimicrobial activity was determined by screening against cultures of *V. anguillarum* M3, *V. campbellii* 1.1596, *V. fluvialis* 1.1608, *V. harveyi* 1.1593, *V. vulnificus* L32, *V. parahamolyticus* 1.1587, *V. alginolyticus* 1.1607, *V. splendidus* 1.1604, *Edwardsiella tarda* LSE40, *Streptococcus sp.* CF, *Staphyloccocus aureus*, *B. cereus*. The antagonistic *Bacillus* spp. strains were retained and preserved at our laboratory and their purity routinely checked during the investigation. Stock cultures were frozen at –80°C with 20% (v/v) glycerol.

16S rRNA gene sequencing and identification of the antagonistic isolates

Based on the results from agar diffusion assay, molecular identification of the active strains (G024, N004) was carried out by 16S rRNA gene (16S rDNA) amplification and sequencing.

DNA extraction

The two antagonistic isolates were each mass cultured in 5 mL TSB (tryptic soytone broth, 1.5% NaCl, wt/vol) liquid medium at 28°C for 24 h on a shaker. The biomass of such selected isolates was harvested by centrifugation (3000 rpm/min for 10 min). Bacterial pellet was washed twice in sterile Tris–EDTA buffer and approximately 150 mg (wet weight) biomass was used for DNA extraction based on cetyltrimethylammonium bromide (CTAB) purification following (Yang *et al*., 2012).

DNA amplification and sequencing

PCR with extracted chromosomal DNA was conducted using 16S universal primer -16S-27F (5′-AGAGTTTGATC（A/C）TGGCTCAG-3′) and 16S-1492R (5′-ACGGCTACCTTGTTACGA-3′) (Sangon, China) in a thermal cycler (Takara, Japan). Polymerase Chain Reaction was performed in 50 μL volumes containing 2.5 mM $MgCl_2$, 2.5 U Taq polymerase (Sangon, China), 100 μM of each dNTP, 0.2 μM of each primer and 2 μL template DNA. The PCR programme used was an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 40 s, 52°C for 45 s and 72°C for 1 min and a final

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extension at 72°C for 10 min. The sequencing reactions were carried out by Sangon Biotech (Shanghai) Co., Ltd., China with 27F, 529F, 518R, 1073R and 1492R primers to compare the chromatograms and get a clear consensus sequence for each strain.

Sequence data were compiled and consensus sequence was obtained by using Geneious 3.8.5 programme and examined for sequence homology with the archived 16S rDNA sequences from GenBank at www.ncbi.nlm.nih.gov/nucleotide, employing the BLAST.

Visualization of bacterial damage by scanning electron microscope (SEM)

The bacteriolytic effect of cell-free supernatant from the antagonistic *Bacillus* spp. cultures on indicator bacteria was observed by SEM using the method developed by Kato *et al*. (1993), Cordovilla *et al*. (1993), Kim *et al*. (2004) and Ringø *et al*. (2008) with some modifications.

The fresh cultures of indicator bacteria containing cells ～10^8 CFU/mL (OD_{600} = 0.8-1.0) were respectively suspended in 0.9% NaCl solution at an optical density (600nm) of 0.8-1.0, and mixed with the same volume of the 0.22 μm culture filtrates from the antagonistic *Bacillus* spp. fresh cultures (～ 10^8 CFU/mL, OD_{600} = 0.8-1.0). As negative control, the sterile TSB medium (tryptic soytone broth, containing 1.5% NaCl) was used. After incubation at 28℃ for 3-6 h, the cells were dropped onto a coverslip previously treated with 0.2% poly-L-lysine and fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) at 4℃ for 3 h. The specimens were dehydrated with 30% ethanol (15min), 50% ethanol (15min), 70% ethanol (15min), 90% ethonal (15min) and three times with 100% ethanol (15min). After drying, the samples were critical-point-dried in CO_2, ionspatter-coated with gold and then observed with a scanning electron microscope (KYKY-2800B, KYKY, China) operating at an accelerating voltage of 25 KV.

Results

In vitro antagonism

A total of two antagonistic *Bacillus* spp. strains were isolated. Of such two strains, G024 was isolated from the sediment and N004 was the gastrointestinal isolate of *Paralichthys lethostigma*. The cell-free supernatants of antagonistic isolates of G024 and N004 showed inhibitory activity against the common aquatic pathogens (mainly *Vibrio*). Antagonistic profile of isolate G024 was broader, compared with which of isolate N004. Both of the antagonistic strains showed inhibitory activities against *Streptococcus* sp. CF, *V. anguillarum* M3, *V. vulnificus* L32. The diameters of the

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inhibitory zones around the colonies of both probiotic strains were about 11-23mm (Table 1).

Identification of antagonistic Bacillus spp. strains

Based on the sequence of the 16S rRNA gene, the isolates of G024 and N004 were respectively identified as *B. subtilis* and *B. amyloliquefaciens*. Names of isolated species and their codes are given in Table 2.

Table 1. The diameter of inhibition zone formed by the antagonistic isolates against aquatic pathogens (mainly the *Vibrio*) *in vitro* (mm).

strain nos	Inhibition zones against aquatic pathogens (mainly *vibrio*) (mm)													
	Edwardsiella tarda LSE40	*Streptococcus* sp.CF	*Staphylococcus aureus*	*Bacillus cereus*	*Vibrio anguillarum* M3	*Vibrio.campbellii* 1.1596	*Vibrio. fluvialis* 1.1608	*Vibrio. harveyi* 1.1593	*Vibrio.vulnificus* L32	*Vibrio. parahamolyticus* 1.1587	*Vibrio. alginolyticus* 1.1607	*Vibrio. splendidus* 1.1604
G024		23±1.0	16.67±0.58		13.33±0.58			9.67±0.58	13.67±0.58			
N004	20±0	21.33±1.15		17±0	13.33±1.15	14.67±0.58			11.67±0.58	15.33±0.58		

Values (mean ± SD) are from three replicates (n = 3).

Table 2: 16S rDNA gene sequence identification of the two selected antagonistic isolates.

strain nos	Species	accession number	similarity(%)
G024	*Bacillus subtilis*	NR027552	99
N004	*Bacillus amyloliquefaciens*	NR041455	99

Scanning electron microscopic observation of cell-free supernatant-induced structural changes in indicator strains

SEM was used to examine the morphological features of the indicator bacteria treated with cell-free supernatant from the antagonistic isolates of G014 and N004 (Figs. 1 and 2). In the control areas, the indicator bacterial cells were healthy and did not show any visible distortion of cell structure (Fig.1 a', b', c', d', e' and Fig. 2 f', g', h', i', j', k', l'). The 0.22 μm culture filtrate of the selected isolate G024 destroyed the structure of indicator bacteria, and large masses of coaggregated bacteria were evident in the scanning microscope preparations (Fig. 1 a, b, c, d, e). Besides that, the lysed cells could be observed in Figures 1a, b, c, d, e. Similarly, the cell-free supernatant from the antagonistic strain of N004 induced morphological changes in indicator

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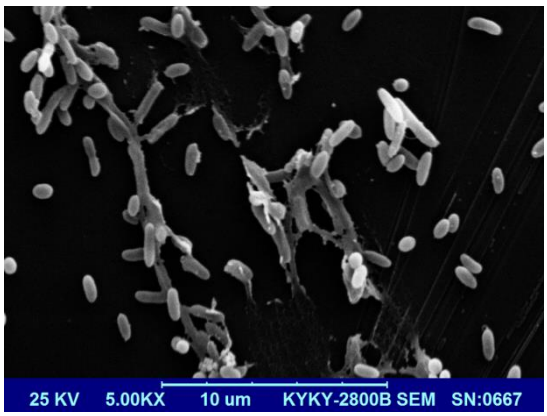
bacteria cells. SEM images of indicator bacteria showed that the cells coaggregated (Fig. 2 f', g', h', i', j', k', l') and some of them appeared to be lysed (Fig.2 f', h', j', k', l'). Moreover, there was typically a mesh-like between the cells found internally in these coaggregates (Fig. 2 f, g, i).

Areas of close cell-to-cell contact between the coaggregated cells are evident on SEM (Fig. 2 f, g, i). The surface of such indicator bacterial cells had what appear to be thin filaments of exopolymers emerging from the outer membrane.



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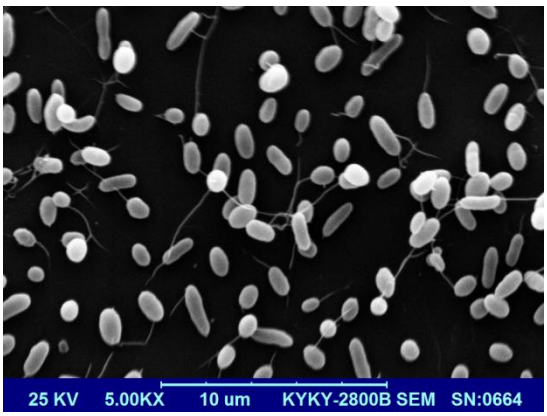

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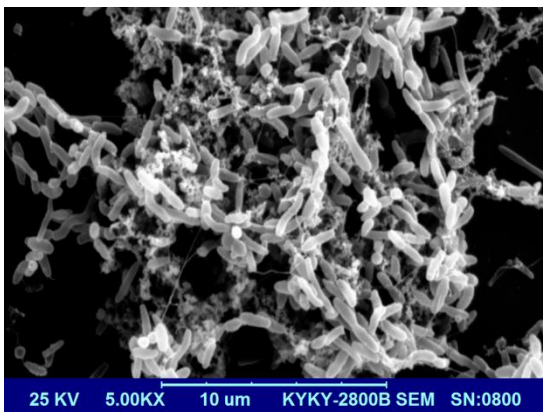

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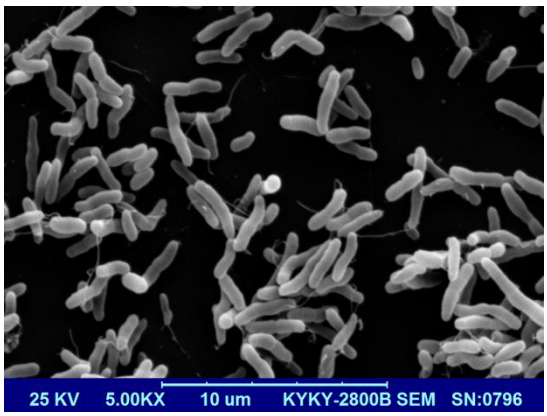

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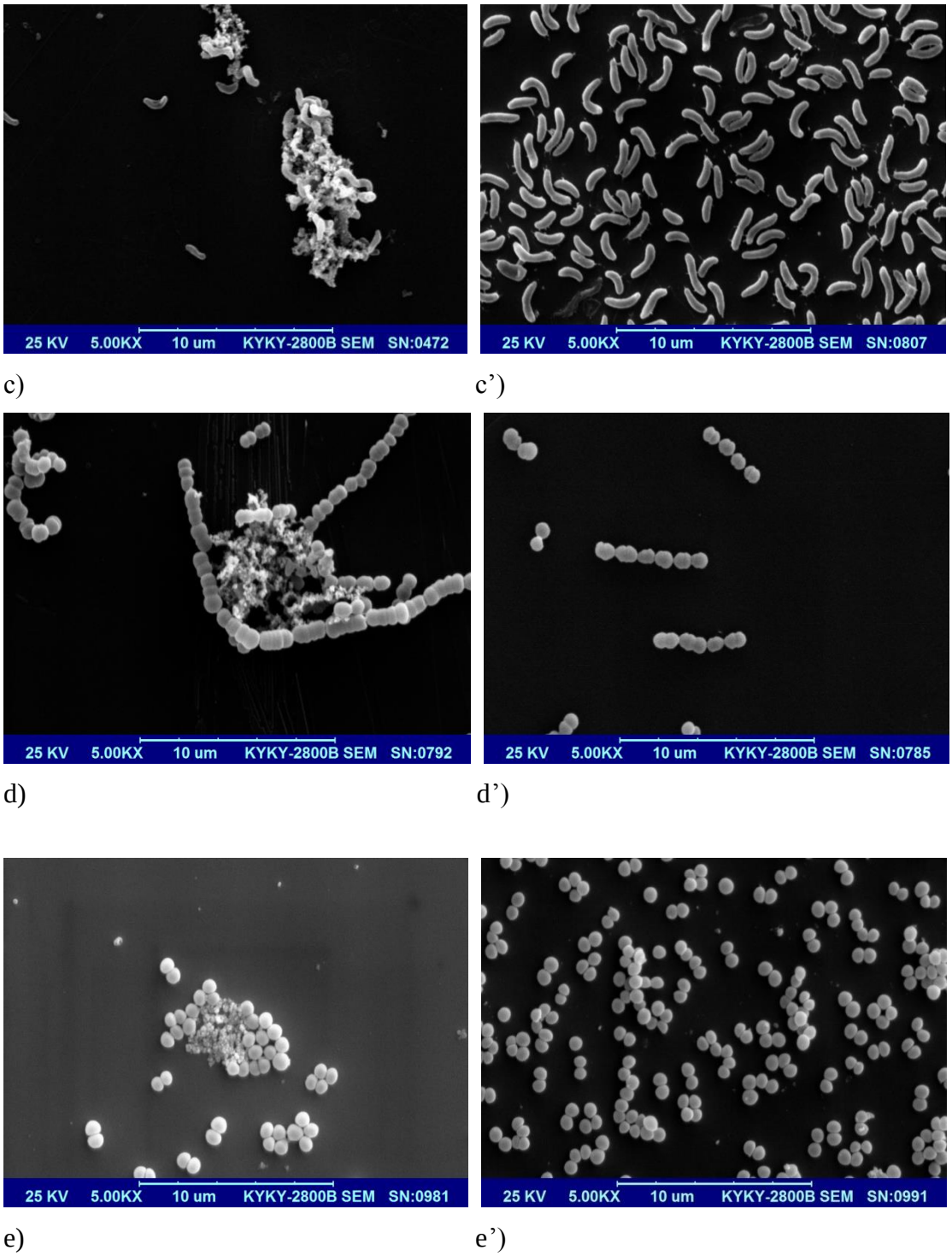

Figure 1: Scanning electron microscopy of indicator bacteria treated with the 0.22 µm culture filtrate of antagonistic isolate G024 for 3-6 h. After incubation, morphological changes were examined. a,b,c,d,e are treatments of indicator bacteria of *Vibrio Harveyi* 1.1593, *V. vulnificus* L32, *V. anguillarum* M3, *Streptococcus* sp. CF, *Staphyloccocus aureus*; a', b', c', d', e' are controls of the corresponding indicator bacteria.

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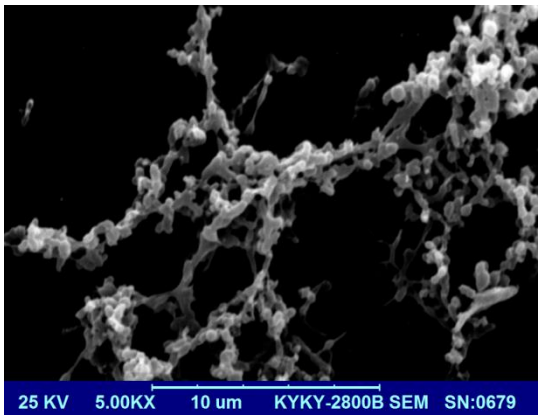
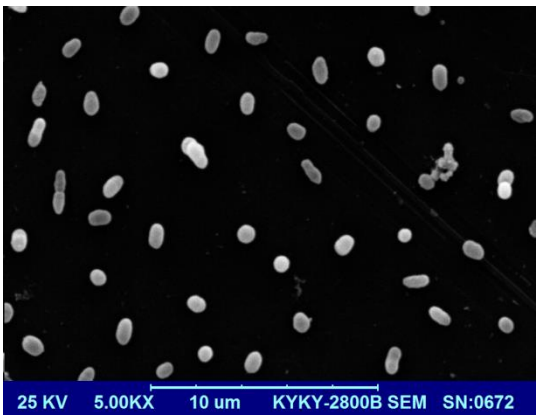


f)



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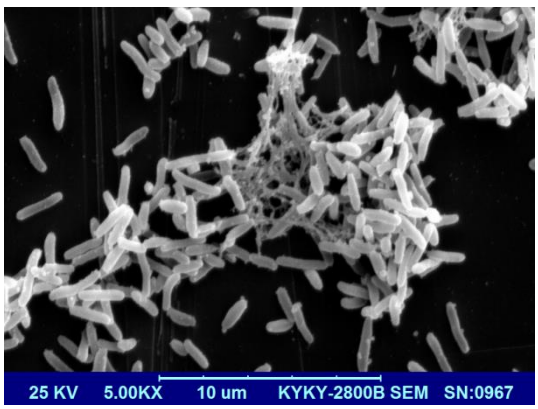

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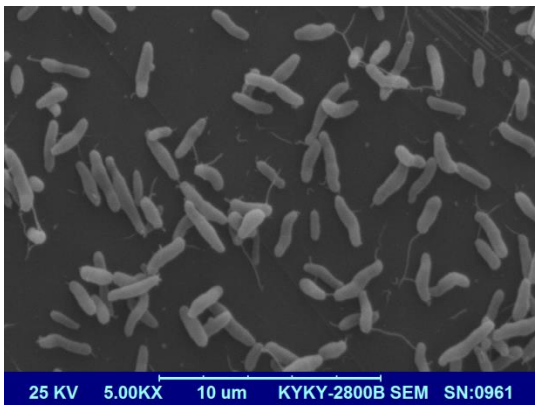

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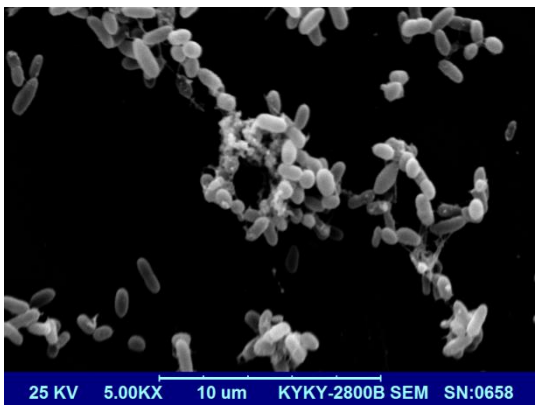

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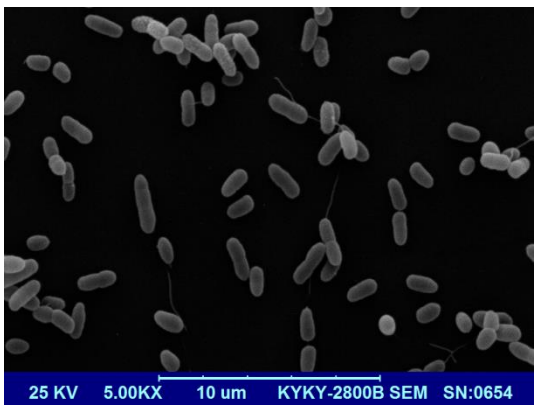

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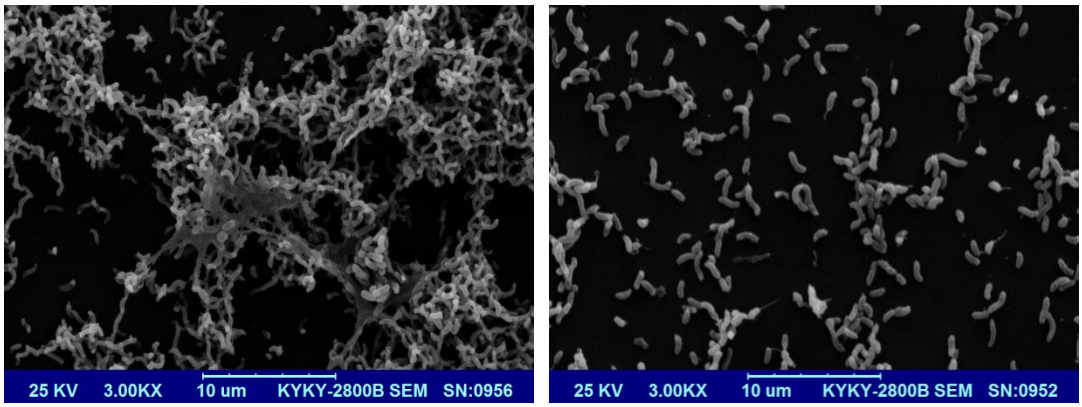

i) i')

Figure 2: Scanning electron microscopy of indicator bacteria treated with the 0.22 µm culture filtrate of antagonistic isolate N004 for 3-6 h. After incubation, morphological changes were examined. f, g, h, i, j, k, l are treatments of indicator bacteria of *Vibrio campbellii* 1.1596, *V. vulnificus* L32, *V. parahamolyticus* 1.1587, *V. anguillarum* M3, *E. tarda* LSE40, *Streptococcus* sp. CF, *B. cereus*; f', g', h', i', j', k', l' are controls of the corresponding indicator bacteria.

Discussion

In the present investigation, we determined that the two *Bacillus* spp. isolates of G024 and N004 were capable of inhibiting growth of various species of aquatic pathogens (mainly *Vibrio*). However, characterization of the exact mechanism of the inhibitory effect was not performed in this study. But some reports had indicated that the inhibitory effects of *Bacillus* spp. might be due to either alteration of pH in the growth medium, competition for essential nutrients, or production of antimicrobial agents (Gullian *et al*., 2004; Chaurasia *et al*., 2005; Yilmaz *et a*l., 2006). In addition, several studies have reported that *Bacillus* spp. produces polypeptide antibiotics, such as bacitracin, gramicidin S, polymyxin, and tyrotricidin, which are efficiently against a wide range of bacterial species (Morikawa *et al*., 1992; Perez *et al*., 1993; Drabløs *et al*., 1999). Importantly, the use of the agar diffusion technique indicated that the antimicrobial compounds produced by the antagonistic isolates were water soluble compounds as they readily diffused through the agar medium. The nature of the antimicrobial activity of the antagonistic isolates is currently under study.

When investigating the action of antagonistic effect on target bacterial cells such as fish pathogens, electron microscope investigations could be a useful tool, and during the last decade, some reports have used transmission

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electron microscopy and/or SEM to evaluate cell structure changes (McDougall *et al*., 1994; Bottazzi *et al*., 1996; Maisnier-Patin *et al*., 1996; Ringø *et al*., 2008; Rattanachuay *et al*., 2010; Xu *et al*., 2013;). The outcomes of SEM examination demonstrated that the normal shapes of indicator bacterial cells were strongly affected by the cell-free supernatant from the antagonistic *Bacillus* spp. isolates. Unlike common antibiotics that penetrate into the target bacteria cells to show their antimicrobial effect (Xu *et al*., 2013), the cell-free supernatant from the antagonistic isolates affected and/or killed target cells by destroying their membrane and/or whole cell, thereby imitating the effects of porins (Xu *et al*., 2013). This mode of action remarkably reduces the chance of development of drug resistance in microbes and hence, offers an effective alternative in the treatment of raging multidrug-resistant infectious diseases in marine animals. The broad-spectrum antimicrobial activities, even against many multidrug-resistant strains make the antagonistic *Bacillus* spp. to be attractive alternatives to conventional antibiotics.

In conclusion, the results from this study indicated that the antagonistic isolates of *B. subtilis* G024 and *B. amyloliquefaciens* N004 could produce soluble extracellular antibacterial substances responsible for inhibiting the growth of some pathogenic bacteria of fish and cause bacterial cell lysis. From the above results, we suggest that both of the two antagonists would seem to be good biocontrol candidates, although questions remain as to how effective these antagonists would be under field conditions. Our work is continuing to characterize the specific composition of the cell-free supernatant of this antagonistic *Bacillus* spp. for use in the aquaculture production.

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