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Original Article

Secondary metabolites of marine-derived *Bacillus spizizenii* against the enteric redmouth disease in common carp, *Cyprinus carpio*

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Abstract: Looking for effective alternatives, such as secondary microbial metabolites, is needed to restrict the use of antibiotics in farmed fish and their detrimental effects on public health and the environment. Thirty-three water and sediments samples were collected from coastal areas in the Basrah Governorate, southern Iraq, to assess their biological activity against bacterial pathogens isolated from *Cyprinus carpio*, with enteric red mouth disease. 20 spore-forming bacteria were isolated and identified by VITEK BCL cards and amplifying the *gyrA* gene. Furthermore, the secondary metabolites produced by the strains were extracted and analyzed by GC-MS. Four pathogenic bacteria were isolated from common carp infected with the enteric red mouth disease. The antibacterial activity of the extracts of the isolated marine strains was examined on bacteria causing enteric red mouth disease and *Y. ruckeri* and *P. aeruginosa*. Based on the results, the marine isolates were identified as *B. spizizenii* and GC-MS analysis revealed that these strains' extract contained amino acids and their derivatives and esters and hydrocarbons. Also, biochemical identifications showed that the bacteria isolated from fish belonged to the species of *Yersinia ruckeri, Aeromonas hydrophila, Streptococcus agalactiae,* and *Pseudomonas aeruginosa.* According to the antibacterial activity assay, the extracts of *B. spizizenii* strains were considerably active against bacteria involved in enteric red mouth disease, especially *Y. ruckeri.* These findings indicate marine *B. spizizenii* can be replaced with antibiotics in the aquaculture industry to combat infections.

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Introduction

Aquaculture is a fast-growing industry aimed to meet the ever-increasing global population protein demand. In this regard, fish and fish products are considered excellent sources of essential proteins and micronutrients necessary for human health and growth. However, the escalation of aquatic animal disease has been a setback for the aquaculture industry. *Aeromonas*, *Vibrio*, *Streptococcus*, *Yersinia*, *Acinetobacter*, *Lactococcus*, *Pseudomonas*, and *Clostridium* are frequently the most pathogens in aquaculture (Yi et al., 2018). The enteric red mouth disease or yersiniosis is an infectious disease caused by *Y. ruckeri*, a Gram-negative rod-shaped enterobacterium. This is often responsible for causing irreparable economic losses in the fish farming industry. Symptoms such as bleeding at the base of the fins, inside and around the mouth, bilateral exophthalmia with or without haemorrhage, and per-ocular and peri-oral haemorrhages clinically describe the disorder (Ummey et al., 2021).

A few approaches to managing fish diseases, such as applying chemicals and antibiotics, have been introduced, and some of them are valuable for successful farming and high production. Some of these compounds are highly specific to certain illnesses, yet others are non-specific. Hence, over-use of these agents can contaminate water bodies (Li et al., 2020). Besides, the misuse of antibiotics has

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increased the number of antibiotic-resistant bacteria (Khorrami et al., 2020; Jafari-Nasab et al., 2021). The presence of drug residues in seafood is one of the other main issues that motivate scientists to look for safe and efficient therapeutic alternatives (Pal, 2015).

Scientists and aquaculturists have recently introduced successful biological control methods, such as vaccines, bacteriophage therapy, and probiotics (Banerjee and Ray, 2017; Zangeneh et al., 2020). Nowadays, the utilization of marine bacteria metabolites is offered a promising method for the sustainable supply of aquaculture (Sihag and Sharma, 2012). Bacterial strains, such as the *Bacillus* spp., have produced secondary metabolites with various chemical structures, which can be considered to develop novel drugs and lead compounds (Petersen et al., 2012; la Cruz-López et al., 2022; Duc et al., 2022).

Based on the last update (January 2019), the *Bacillus* genus consists of 377 species of Gram-positive, rod-shaped bacteria. Their ability to form endospores, the versatility of physiological properties and produce various antimicrobial compounds facilitate their omnipresent distribution in soil, aquatic habitats, food, and mammals' gut (Caulier et al., 2019). The antibacterial compounds secreted by these bacterial strains are mainly secondary metabolites, such as polyketides, terpenes, and siderophores, and ribosomally and non-ribosomally synthesized peptides (Harwood et al., 2018). *Bacillus* spp. secondary metabolites have been demonstrated to be more effective in controlling many plant diseases, such as white onion rot, than conventional chemicals. With the development of modern biotechnology, increasing numbers of novel antimicrobial agents are isolated from marine *Bacillus* spp. (Liu et al., 2019).

While research on the applying *Bacillus* species against pathogens is proceeding, the effects of the secondary metabolites mainly produced by *Bacillus* strains have been less explored yet. Therefore, this study was aimed to isolate and identify *B. subtilis* strains from water and sediments samples collected from different locations in Basrah Governorate, southern Iraq. The secondary metabolites produced by this strain were analysed using GC-mass and gel filtration chromatography, followed by assessing their biological activity against bacterial pathogens isolated from common carp, *Cyprinus carpio*, with enteric red mouth disease.

Materials and Methods

Sampling to isolate *Bacillus* spp.: Thirty-three water (20 ml per sample) and sediment (20 g per sample) samples were collected from different locations from January to May 2018 from the main aquatic area (Table 1). They were transferred to the laboratory and heated to 80°C via a water bath to eliminate non-spore-forming bacteria. After that, in sterile conditions, 1 g of each sediment sample or 1 ml of water sample was added to a test tube containing 9 ml distilled water, and their serial tenfold dilutions were carried out. Next, the dilutions were filtrated using a Millipore filter paper 0.45 µm. The filter was then placed on Petri dishes containing Lauria-Bertani medium (LB agar, HiMedia) and incubated at 35°C for 18 hours. Single colonies were taken, and after Gram-staining, the pure gram-positive and spore-forming isolates obtained in this stage were stored as glycerol stocks at -20 and -80°C until further experiments.

Isolation and identification of *B. subtilis* strains: The biochemical diagnosis was done using the kit VITEK2 BCL card (bioMérieux, France) according to the manufacturer's instruction, and genetic analyses were performed by amplifying *gyrA* gen as a differential marker of *Bacillus* spp. using the primers of *gyrA*-F (5'-CAGTCAGGAAATGCGT ACGTCCTT-3') and *gyrA*-R (5'-CAAGGTAATGC TCCAGGCATTGCT-3') (Kunst et al., 1997).

According to the manufacturer's instructions, the polymerase chain reaction (PCR) was performed using GoTaq®G2 Green Master Mix (Promega, USA). Briefly, the primers and components were mixed in 25 µl amplification reaction tubes containing 1 µl primer pair mix, 2 µl DNA sample, 12.5 µl Green Master Mix, and 9.5 µl PCR grade

water. The amplification program consisted of a pre-denaturation phase (94°C, 2 min), followed by 40 cycles; denaturation (94°C, 30s), annealing (51 °C, 45s), extension (72°C, 60s) and final extension (72°C, 7 min). Finally, the agarose gel electrophoresis (1%) for PCR reactions was used to ensure the results. PCR products were sent to Macrogen/Seoul-Korea for sequencing of the DNA. The Basic Local Alignment Search Tool program was used to assess the level of convergence between local isolates and *Bacillus* global breeds in the National Biotechnology Center for Information (NCBI) database. The sequences of the isolates were registered in NCBI and their accession numbers are presented. Finally, the neighbor-joining method was performed with MEGA-X to draw the phylogenetic tree.

Isolation and identification of fish bacterial pathogens

The sampling of infected fish: Five female infected common carps were obtained from a fish farm in Basra Governorate. The fish was 750-1000 g in weight and 32-39 cm in total length with an unhealthy appearance, including several external wounds, redness, bleeding, scales loss, erosion of fins, and red mouth spots, which are symptoms of enteric red mouth disease. Cotton swabs were used for sampling from dorsal fin, caudal peduncle, belly near pelvic fins, and the head and jaws. Finally, the samples were cultured on Nutrient Agar (two replicates were made for each site). In addition, three farm water samples were taken and cultured on Nutrient Agar and incubated at 35-37°C. At the next step, the bacterial colonies were cultivated on the following differential media; *Pseudomonas* agar F, selective *Streptococcus* agar, Ampicillin Dextrin agar base, and mFC agar. The presumptive colonies were picked up and subjected to identification using the Vitek II system (Biomerieux, USA).

Secondary metabolites production and purification: The method of Dusane et al. (2017) was adopted to produce bacterial secondary metabolites, followed by extraction according to Amin et al. (2015). Ion exchange chromatography and gel filtration chromatography using the S-25 Sephadex column were performed to purify these compounds (Anju et al., 2015). The gel electrophoresis was carried out as described by Laemmli (1970).

GC-Mass analysis: The ethyl acetate extract of the metabolites was subjected to gas chromatography-mass spectrometry (GC–MS, Agilent 78908/5977A) to identify substances of the sample components. The GC-MS gas chromatography instrument was fitted with a Hp-5MS capillary column (5% phenyl and 95% dimethylpolysiloxane) (30 m x 250 µm x 0.25 µm) and the mass detector turbomass gold working in EI mode. The carrier gas was helium with a 1 ml/min flow rate. The injector was operated at 290°C, and the oven temperature was set as follows; 70°C for 2 min, then was gradually increased within 16 min to 250°C. The component identification was based on comparing their mass spectra and the NIST 2014 Library.

The antibacterial activity tests: The agar well diffusion method (Khorrami et al., 2018) was used to evaluate the antibacterial activity of the metabolites of the four most productive isolates against the pathogens isolated from infected fish, including *Y. ruckeri*, *P. aeruginosa, A. hydrophila*, and *S. agalactiae,* as well as two standard bacterial strains, *Y. ruckeri* ATCC 29473 and *P. aeruginosa* ATCC 27853. Noteworthy, both faecal coliforms and *S. faecalis* were neglected because they are not classical fish pathogens (Al-Imarah 2008).

This test was undertaken via spreading 100 µl of target bacteria suspension (0.5 McFarland) by L-shape spreader on LB agar and incubating at 35°C for 15 min, followed by making wells on the medium using cork poorer. The holes were then filled with 50 µl of the marine bacterial products and incubated for 18 h at 35°C. Finally, the inhibition zone diameters were measured by a ruler and recorded.

Statistical Analysis: The SPSS version 25.0.0.1 was applied to analyse the data at the $P<0.01$. The data were subjected to a revised least significant difference (RLSD) analysis to compare means (SPSS, 2018).

Table 1. Sites and numbers of the studied samples.

No.	Site	Samples No.	Sediments	Water
1	Fish ponds in marine science center	4	2	2
2	Port of Khor Al Zubair	9	5	4
3	Port of Umm Qasr	10	5	5
4	Port of Abu Flus	2	1	1
5	Abu al-Khasib	4	2	2
6	Port of Faw	2	1	1
7	Seeba	2	1	1

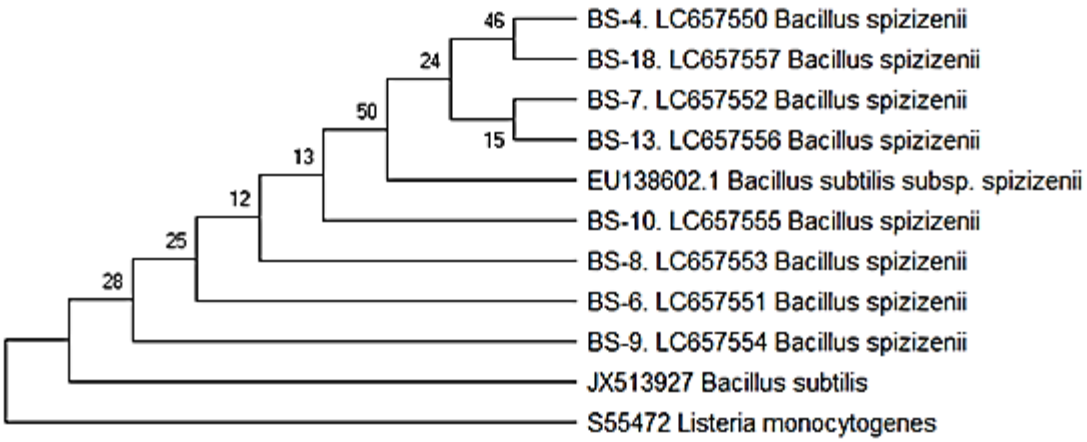

Figure 1. The phylogenetic tree of *Bacillus spizizenii* isolates based on *gyrA* gene sequence. BS-4, BS-6, BS-7, BS-8, BS-9, BS-10, BS-13 and BS-18 were isolated in this study.

Results

Isolation and identification of *Bacillus subtilis* strains: 20 gram-positive *Bacilli* bacterial isolates were isolated from 33 water and sediment samples. Based on the results, most of *Bacillus* spp. isolates belonged to the marine environment. Biochemical identification done using the VITEK II system showed that most isolates belonged to the genus *Bacillus,* including *B. subtilis, B. atrophaeus* and *B. amylolequifaciens*. However, some isolates showed just 75% similarity and could not be identified. To an accurate identification, genetic identification was performed, which revealed that all isolates were *B. spizizenii*, of which twelve isolates had been obtained from sediments and eight from water. Seven isolates were sequenced and registered on NCBI with the accession numbers LC657550-7 (BS-4: LC657550, BS-6: LC657551, BS-7: LC657552, BS-8: LC657553, BS-9: LC657554, BS-10: LC657555, BS-13: LC657556, and BS-13: LC657556). Figure 1 shows the phylogenetic tree of the strains, upon which there is 97-99% identity between them.

Production of secondary metabolites: Based on the results, secondary metabolites were produced after 48 h of incubation. In the early to mid-stationary phase (12-72 h), the microorganisms produce secondary metabolites when cell density increases and their activity decreases after 78 h of incubation (Boottanun et al., 2017).

Secondary metabolites purification: The SDS-PAGE gel electrophoresis showed a sharp band for BS-7, BS-10, BS-13, and BS-18 isolates, indicating the efficiency of the method used to extract and purify secondary metabolites (Fig. 2). Also, Figures 3a and 3b represent the results of ion-exchange chromatography and gel filtration chromatography of secondary metabolites produced by BS7. The appeared single peaks indicate the purity of the compounds. Given that BS-7, BS-10, BS-13, and

Table 2. Comparative chemical composition of the secondary metabolites of the BS-7, BS-10, BS-13 and BS-18. These isolates showed the best performance.

Isolates	GC-Mass characters		
	RT	Name	Formula
BS-7	6.998	Diethyl sulfon	C4H10O2S
	14.716	Phosphorus pentafluoride	F5P
	17.703	3-Ethoxy-4-methoxyphenol	C9H12O3
	18.167	l-Alanine, N-(2-thienylcarbonyl)-, hexyl ester	C14H21NO3S
	18.482	Aniline, N-(3',3'-diphenylspiro[fluorene-9,2'-oxetan]-4'-ylidene)-	C33H23NO
	18.865	l-Norvaline, npropargyloxycarbonyl-,nonyl ester	C18H31NO4
	19.859	DL-Alanine, N-methyl-N-(byt-3-yn-1-yloxycarbonyl)-, tetradecyl ester	C23H41NO4
	20.025	L-Proline, N-valeryl-, decyl ester	C20H37NO3
BS-10	21.711	3,4-Methylpropylsuccinimide	$C_8H_{13}NO_2$
	21.72	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$
BS-13	14.451	Carbamic acid,methyl-, 3-methylphenyl ester	$C_9H_{11}NO_2$
	20.624	Undecanoic acid, 2-methyl-, methyl ester	$C_{13}H_{26}O_2$
BS-18	20.627	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$
	21.716	Phthalic acid,di(6-methylhept-2-yl) ester	$C_{24}H_{38}O_4$

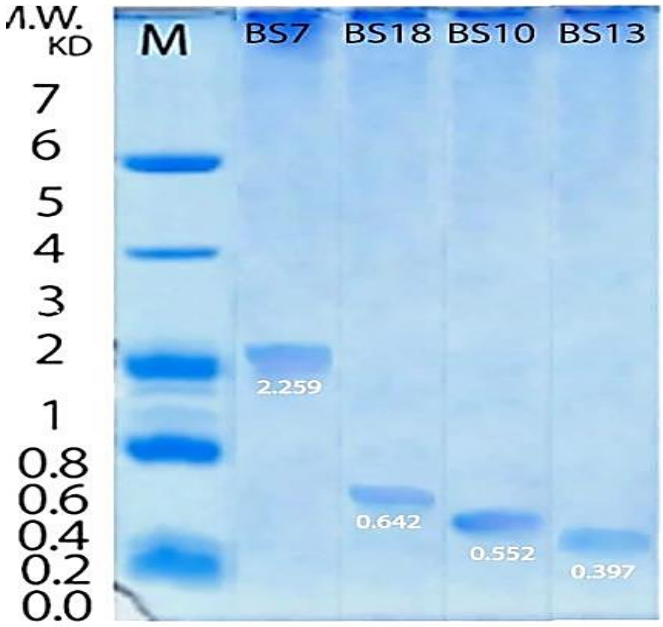

Figure 2. SDS gel electrophoresis of the secondary metabolites of the four *Bacillus spizizenii* isolates. The Aprotinin peptide with the molecular weight (MW) of 6.512 KD was applied as a marker. BS7 MW = 2.259 KD; BS18 MW = 0.642 KD; BS10 MW = 0.552 KD; BS13 MW = 0.397 KD.

BS-18, among all isolates, produced the most metabolites, and their products were selected for further analysis.

GC-Mass analysis: The gas chromatography results of compounds produced by the studied microbial isolates showed that their secretions contain many biocompounds, including amino acids and their derivatives, esters, hydrocarbons, and other compounds (Table 2).

Isolation and identification of fish pathogens: The morphological and biochemical identification of bacteria isolated from infected fish and water by VITEK II system cards are presented in Table 3. Based on the results, *Y. ruckeri* and *P. aeruginosa* were the most prevalent ones.

The antibacterial activity test: The antibacterial activity of the compounds produced by the *B. subtilis* isolated was assessed against the pathogens isolated from infected fish and two standard strains. As shown in Table 4, all bacterial extracts had antibacterial activity against the pathogenic bacteria. The highest antibacterial activity was observed against *Y. ruckeri*, while *P. aeruginosa* showed the lowest susceptibility to these compounds. Among the bacterial compounds, the secondary metabolites of BS-7 showed a more considerable effect than others. Also, the standard strains were slightly more sensitive to these compounds than their wild type (the pathogens isolated from fish).

Discussion

Many studies have suggested that competition between microorganisms for space and nutrients in marine environments is a decisive factor in

Table 3. Source and bacterial pathogens isolated from infected fish and water.

Samples		Isolated pathogens		
Infected fish	Dorsal fin	*S. agalactiae*	*P. aeruginosa*	Fecal coliform
	Caudal peduncle	*Y. ruckeri*	*A. hydrophila*	*P. aeruginosa*
	Belly near pelvic fins	*P. aeruginosa*	*A. hydrophila*	*S. agalactiae*
	Near the head	*A. hydrophila*	*P. aeruginosa*	*Y. ruckeri*
	Jaws	*Y. ruckeri*	*S. agalactiae*	*P. aeruginosa*
Water	Sample No.1	*P. aeruginosa*	*S. sciuri*	Fecal coliform
	Sample No.2	*Y. ruckeri*	*A. hydrophila*	Fecal coliform
	Sample No.3	*P. aeruginosa*	*A. hydrophila*	Fecal coliform

Table 4. The antibacterial activity of *Bacillus spizizenii* against bacterial fish pathogens.

Metabolites of	Pathogenic bacteria					
	S. agalactiae	*Y. ruckeri*	*P. aeruginosa*	*A. hydrophila*	*Y. ruckeri* ATCC 29473	*P. aeruginosa* ATCC 27853
BS-7	18±0.80	21±0.78	12±0.89	17±0.57	22±0.38	13±0.25
BS-10	13±0.57	16±0.89	8±0.99	12±0.80	18±0.40	8±0.37
BS-13	9±0.89	13±0.99	10±0.57	15±0.80	15±0.22	11±0.67
BS-18	11±0.78	15±0.78	10±0.78	16±0.99	14±0.73	12±0.86

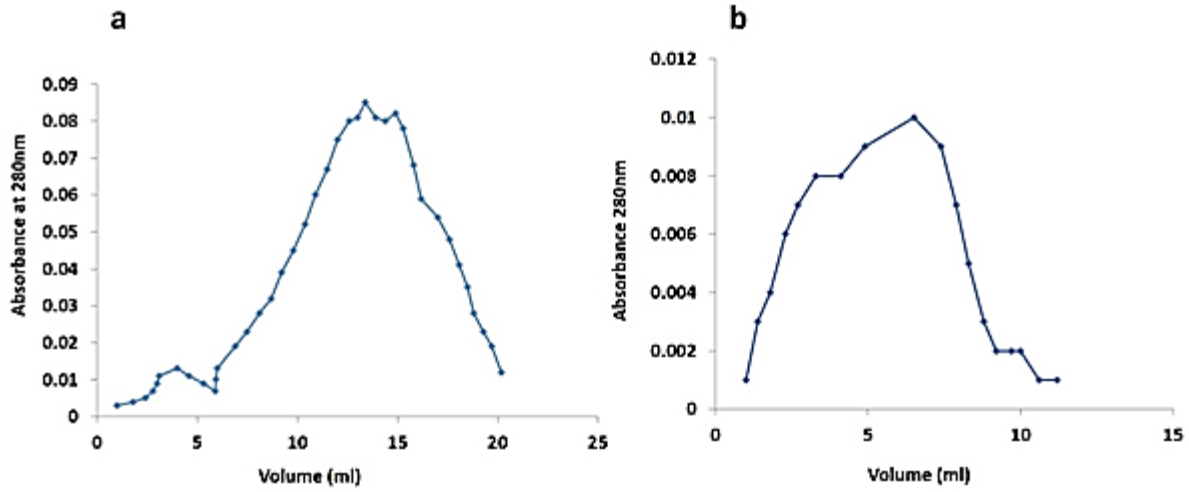

Figure 3. Ion exchange chromatography (a) and Gel filtration chromatography (b) of secondary metabolites produced by the BS7 strain.

producing substances that increase their ability to exclude competitive organisms. These substances have high industrial and medical value and can be used to solve many environmental issues that cause significant economic losses (Matobole et al., 2017; Liu et al., 2019). Based on our results, *B. spizizenii* isolates belonged to the marine environment. This may be because this environment is a harsh, low-nutrient, and high-salinity environment where only microorganisms like the genus *Bacillus* can survive. Furthermore, the production of compounds that inhibit the growth of other competitive microorganisms gives members of this genus the survival and reproduction advantage (Hassan et al., 2015; Zhang et al., 2019). The percentage of identification in this study using the VITEK II system was lower than those reported by Halket et al. (2010). The identification percentage of this method was reported as 93%, and this difference may be due to the thick mucus substance which surrounded some isolates. This substance made some difficulties in diagnosing the isolates; however, this percentage was higher than the report of Mussa and Baqer (2017) (56.25%).

The secondary metabolites' findings agree with Harwood et al. (2018) who showed that industrial

micro-organisms like *B. subtilis* could develop an array of metabolites to improve their survival. The production of compounds can be induced by several factors such as stress, starvation, or environmental factors and also cell-to-cell communication or quorum sensing, which uses small peptides as inducers (Kleerebezem and Quadri, 2001). Our results are in accordance with the report of Kleerebezem and Quadri (2001) that in their report, *B. subtilis* produces several essential metabolites.

Based on our results, the lowest antibacterial activity was observed against *P. aeruginosa.* Poole (2005) reported that the *P. aeruginosa* resistance to antimicrobial agents may be attributed to the impermeable outer membrane and protein channels in the cytoplasmic membrane that release antimicrobial substances outside the cell to protect them. Abbas et al. (2010) confirmed that bio-components driven from *B. subtilis* defended their host against *Y. ruckeri.*

As the results showed, *B. spizizenii* secondary metabolites had activity against *A. hydrophila.* It has been reported that *Bacillus* species either prevent the proliferation of *Aeromonas* species or increase the immunity of the host to resist the virulent *Aeromonas* species (Kuebutornye et al., 2020). Santos et al. (2018) indicated that natural antimicrobial compounds produced by *B. subtilis* are effective against *A. hydrophila, A. salmonicida, A. veronii,* and *A. bivalvium. Aeromonas hydrophila* is the leading cause of hemorrhagic bacterial septicemia in freshwater fishes (Al-Imarah, 2008). The compounds extracted from *B. spizizenii* also showed antibacterial activity against *S. agalactiae.* It has been demonstrated that *Bacillus* strains driven from marine sediments produce metabolites like their terrestrial counterparts. These compounds are usually lipopeptides, including surfactants, iturins, and fengycins, which exhibit a variety of biological activities (de Oliveira et al., 2020). Al-Zereini et al. (2014) also indicated that marine bacteria isolated from sediments are an interesting source of secondary metabolites with antimicrobial and antioxidant properties. In conclusion, *Bacillus spizizenii* from marine sediments and water is a rich source of novel natural products and excellent antibiotics that have advantages in using against bacterial strains to control pathogenic bacteria in the aquaculture industry.

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