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Identification of bacteria isolated from rockworm viscera and application of isolated bacteria to shrimp aquaculture wastewater treatment

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Contribution to Environmental Biology

- Aerobically denitrifying bacteria were isolated from rockworm viscera.
- The isolates strongly degraded organic matters, which are good candidates for shrimp aquaculture wastewater treatment.

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Abstract: Large amounts of waste and wastewater from aquaculture have negatively impacted ecosystems. Among them, shrimp aquaculture wastewater contains large amounts of nitrogen contaminants derived from feed residues in an aerobic environment. This study isolated candidate strains from adult rockworms to treat shrimp aquaculture wastewater (SAW) in an aerobic environment. Among 87 strains isolated, 25 grew well at the same temperature as the shrimp aquaculture with excellent polymer degradation ability (> 0.5 cm clear zone). Six isolates (strains AL1, AL4, AL5, AL6, LA10, and PR15) were finally selected after combining strains with excellent polymer degradation ability without antagonism. 16S rRNA sequencing analysis revealed that strains AL1, AL4, AL5, AL6, LA10, and PR15 were closely related to *Bacillus paramycoides*, *Bacillus pumilus*, *Stenotrophomonas rhizophila*, *Bacillus paranthracis*, *Bacillus paranthracis*, and *Micrococcus luteus*, respectively. When these six isolates were applied to SAW, they reached a maximum cell viability of 2.06×10^5 CFU mL^{-1}. Their chemical oxygen demand (COD_{Cr}) and total nitrogen (TN) removal rates for 12 h were 51.0% and 44.6%, respectively, when the COD_{Cr}/TN ratio was approximately 10.0. Considering these removal rates achieved in this study under batch conditions, these six isolates could be used for aerobic denitrification. Consequently, these six isolates from rockworms are good candidates that can be applied to the field of aquaculture wastewater treatment.

Keywords: rockworm viscera, indigenous bacteria, identification, shrimp aquaculture wastewater, bacterial wastewater treatment

1. INTRODUCTION

Aquaculture is a potential solution for providing relatively cheap proteins worldwide for poor and food-insecure populations (Kobayashi *et al.* 2015). This important role of aquaculture has made it the fastest-growing sector in the past few decades (Ngo *et al.* 2016). As fish consumption steadily increases because of its health benefits, farmed fish production for human consumption has finally surpassed wild-captured

fish (World Bank 2014). Although aquaculture benefits humans, its flourishing causes problems because a large amount of waste and wastewater is generated from the aquaculture industry, negatively exerting environmental impacts on aquatic ecosystems. These impacts include pollution of nearby waters, habitat destruction, disease transfer, and release of farmed exotic species such as escape (Diana 2012). Aquaculture wastewater generally has different composition and concentration than municipal or industrial wastewater. Marine aquaculture wastewater contains excess nitrogen contaminants from fish excrement and feed residue (Schwartz and Boyd 1994). In shrimp aquaculture, 5,345–7,157 m^3 of wastewater is typically discharged per ton of shrimp production (Anh *et al.* 2010), and there is a problem of continual nitrite accumulation and discharge into the environment (Song *et al.* 2011; Kim *et al.* 2019). Given environmental and social aspects, efficient treatment of effluent discharges is essential to develop sustainable aquaculture. So far, microbial treatment for nitrogen contaminants has been known to be the most economical and applicable technology (Vinothkumar *et al.* 2021). For the removal of nitrogen contaminants, the denitrification process under anaerobic/anoxic conditions is known to exhibit excellent removal efficiency (Song *et al.* 2011). However, anaerobic denitrification systems are difficult to apply to aquaculture farms due to aerobic cultivation condition (Song *et al.* 2011). Therefore, the discovery of aerobically denitrifying microorganisms is needed to efficiently treat shrimp aquaculture wastewater (SAW), since oxygen is maintained at a certain acceptable level in the shrimp aquaculture environment for high-density shrimp culture (Song *et al.* 2011).

Polychaetes purify deposits via their feeding behavior, thereby mitigating the environmental impact of pollution. This role of polychaetes was reported in the bioremediation of deposited waste at the bottom of fish ponds (Kinoshita *et al.* 2008). Therefore, polychaetes are ecologically important taxa, particularly in marine benthic communities (Giangrande *et al.* 2005). The organic pollutant degradation ability of polychaetes is derived from their gut microbes, including *Capitella teleta* (Hochstein *et al.* 2019) and *Osedax* (Rouse *et al.* 2004). *Nereis*, a polychaete species, efficiently used unconsumed feed and fecal materials collected from a marine recirculating system (Brown *et al.* 2011). In addition, a *Bacillus* sp. strain EBW4 isolated from a polychaete, could degrade organic hydrocarbons (Shin *et al.* 2013). Moreover, polychaetes are often used as indicators for detecting marine pollution and show excellent potential in accumulating bioavailable heavy metals from benthic habitats (Fuirst *et al.* 2021) and tolerating high levels of organic compounds in soft bottoms (Tomassetti and Porrello 2005).

As a polychaete species, rockworms are filter feeders showing the potential ability to degrade environmental pollutants. Extracellular enzymes produced by bacteria present in the excrement of a rockworm, *Marphysa sanguinea*, exhibited some potential degradation of polycyclic aromatic hydrocarbons (Onozato *et al.* 2012). The food intake of rockworms is inactive in the larval stage, whereas their food intake is active in the juvenile stage. According to a marine polychaete microbiome study (Dale *et al.* 2019; Fuirst *et al.* 2021), the bacterial community is highly dependent on the surrounding sediment ingestion. This gut flora also plays an important role in the immune system by influencing the life cycle of rockworms (McFall-Ngai *et al.* 2013). Besides, marine polychaete gut flora contains a variety of nitrogen cycles microbes than the environment in which they live (Fuirst *et al.* 2021). Therefore, rockworms are beneficial resources for water purification and nutritiously valuable resources.

Considering the potential ability of rockworms to degrade environmental pollutants, microorganisms inhabited in rockworm viscera are good candidates for wastewater treatment. Therefore, symbiotic viscera microorganisms are useful for purifying shrimp aquaculture effluent (Robinson *et al.* 2015). However, further studies are required to understand the role of microorganisms in rockworm viscera. Herein, therefore, native bacteria were isolated from rockworm (*M. sanguinea*) viscera to utilize their potential ability for water purification. Among the isolates, those showing potential ability were first sorted and identified. The identified isolates were then applied to simulated SAW to evaluate their potential ability.

2. MATERIALS AND METHODS

2.1. Isolation of microorganisms and culture conditions

Rockworms (*Marphysa sanguinea*) in the adult growth

stage were collected from the Fisheries Sciences Technology Center (Pukyong National University, Goseung, Korea). The collected rockworms were washed in the order of phosphate buffer saline (PBS) solution (1× pH = 7.4, Thermofisher Scientific, USA) and 70% (*v*/*v*) ethanol. After the skins of the washed rockworms were cut lengthwise, internal parts were dissolved in the PBS solution to collect microorganisms in the viscera. The prepared sample was centrifuged at 15,520 × g for 10 min. To detect microorganisms, even in small amounts, the supernatant was poured onto various solid culture media: nutrient broth (NB) agar (Neogen, USA), skim milk (SM) agar (Bioshop, Canada), laminarin (LA) agar (TCI, Japan), alginate (AL) agar (Sigma-Aldrich, USA), spirit blue (SB) agar (Sigma-Aldrich), and phenol red (PR) tween agar (Sigma-Aldrich). The NB agar medium consisted of 8 g L^{-1} nutrient broth and 15 g L^{-1} agar (Junsei, Japan). The SM agar medium consisted of 10 g L^{-1} skim milk and 15 g L^{-1} agar. The LA agar medium contained 1 g L^{-1} laminarin, 0.1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} NaCl, 0.1 g L^{-1} $CaCl_2$, 2 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} KH_2PO_4, 0.5 mL L^{-1} mineral solution, 0.5 mL L^{-1} vitamin solution, and 15 g L^{-1} agar (pH = 6.8). The AL agar medium consisted of 0.5 g L^{-1} peptone (Bioshop), 0.1 g L^{-1} sodium alginate, and 15 g L^{-1} agar (pH = 6.8). The SB agar medium comprised 32.15 g L^{-1} SBA and 30 mL L^{-1} tributyrin (TCI). The PR tween agar medium contained 8 g L^{-1} nutrient broth, 10 mL L^{-1} tween, 0.018 g L^{-1} phenol red, and 15 g L^{-1} agar (pH = 6.8). All agar plates were incubated at 25°C for 72 h. Colonies formed on each agar medium were separated using morphological differences under microscopic observation and colonial shape. The sorted colonies were finally streaked onto fresh nutrient broth agar media, and then each isolated colony was used for identification.

2.2. Characterization and identification of isolates

Each sorted colony was characterized using Gram staining, stabbing into nutrient broth agar medium, and microscopic observation. In addition, the degradation ability of each colony on polymers was investigated using carboxymethyl cellulose (CMC) agar (Sigma-Aldrich), SM agar, and SB agar media. The carboxymethyl cellulose agar medium consisted of 2 g L^{-1} peptone, 5 g L^{-1} yeast extract, 1 g L^{-1} CMC, and 15 g L^{-1} agar (pH = 6.8). Then, 10 μL of each isolate grown in a late-log growth phase were dropped into 8-mm paper disks (Advantec, Japan) and incubated at 25°C for 24 h. After incubation, the sizes of clear zones formed on carboxymethyl cellulose agar and SM agar media were measured for cellulose and protein degradation ability, respectively, and the formation of blue colonies on the SB agar medium was observed for lipid degradation ability. All the isolates were incubated at 25°C for 24 h after cross-straining on the nutrient broth agar medium. The possibility of any hindrance in cell growth was observed after incubation to check the occurrence of antagonism among the isolates.

16S rRNA sequence analysis was conducted at Cosmogenetech (Seoul, Korea) to identify the isolates. The primers used were universal oligonucleotides corresponding to 27F (5′ AGA GTT TGA TCM TGG CTC AG 3′), 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′), and 518F (5′ CCA GCA GCC GCG GTA ATA CG 3′). The sequences of isolates were matched with the 16S-based ID in EzBioCloud (https://www.ezbiocloud.net/) (Yoon *et al.* 2017), and the identified 16S rRNA sequence of each isolate was used to constitute a phylogenetic tree using MEGA version 7. The sequences were first aligned with Clustal W using the BioEdit program (Version 7.2.0), and then a phylogenetic tree was constituted via neighbor-joining tree analysis with 1,500 bootstrap replicates.

2.3. Simulated shrimp aquaculture wastewater treatment

To examine the potential degradation ability of indigenous microorganisms in rockworm viscera, selected isolates were applied to treat SAW. SAW was treated using a formulated diet for whiteleg shrimp (*Litopenaeus vannamei*). The whiteleg shrimp diet was ground and sieved through the 38-μm mesh, which consisted of 38% crude protein, 5% crude lipid, 2.7% phosphorus, 1.2% calcium, 4.0% crude fiber, and 17% crude ash. The SAW was prepared by dissolving 1 g of whiteleg shrimp diet in 500 mL distilled water. Afterward, the SAW was autoclaved at 121°C for 15 min. Before treating SAW, isolates were preliminarily adapted in SAW at 25°C for 24 h. The adapted isolates (10%, *v*/*v*) were seeded into a 1-L flask containing 500 mL of autoclaved SAW, and the flask was incubated at 25°C and 130 rpm for 36 h. Samples were periodically collected,

Table 1. Characteristics of 13 isolates and their polymer-degrading ability

Isolates	Gram staining	Cell morphology	Motility	Cell dimensions (L × W, in μm)[a]	Degradation ability	
					Protein	Lipid
AL1	+	rod	+	5.41 × 1.30	+	+
AL4	–	rod	+	3.06 × 0.61	+	+
AL5	–	rod	+	3.56 × 0.52	+	+
AL6	+	rod	+	5.42 × 1.37	+	+
LA9	+	rod	–	3.56 × 0.54	+	–
LA10	+	rod, 4–5 chains	+	4.03 × 1.19	+	+
SB13	–	rod, 1–2 chains	+	2.93 × 0.50	+	–
NB14	–	rod, 1–2 chains	+	2.90 × 0.42	+	+
PR15	–	tetrad-forming cocci	+	1.02 × 1.03	+	+
SM16	–	rod, 1–2 chains	+	3.57 × 0.48	+	–
SM19	–	rod	+	3.02 × 0.36	+	–
PR21	+	cocci	+	0.75 × 0.75	+	–
AL22	–	rod	+	2.21 × 0.54	+	+

[a]L and W mean the length and width of cell, respectively.

and reaction parameters were determined.

2.4. Analytical methods

During the treatment of SAW, reaction parameters (pH, optical density (OD_{600})), viable cell number, chemical oxygen demand chromium (COD_{Cr}) concentration, and total nitrogen (TN) concentration) were determined to examine the waste treatment ability of isolates. The pH and OD_{600} of each sample collected from the flask were measured using a pH meter (Mettler Toledo, Switzerland) and a spectrophotometer (Hanson Technology Co., Korea) at 600 nm, respectively. The collected sample was also appropriately diluted with sterile distilled water, spread on the nutrient broth agar medium, and incubated at 25°C for 36 h to determine viable cell numbers. The viable cells formed on the nutrient broth agar medium were counted and represented as colony-forming units (CFUs) per mL. The concentrations of COD_{Cr} and TN in the samples were determined using an HS 2000 Water-quality Analyzer (Humas Co., Ltd, Korea). All analyses were triplicated. Differences in all groups were analyzed by one-way analysis of variance with Tukey's HSD post-hoc test using SPSS 17.0. The p-value < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Isolation of potential microorganisms

Potential microorganisms from adult rockworm viscera were successfully screened. Colonies with different shapes formed on six agar media were first sorted out, and their morphological differences were carefully observed under a microscope. The colonies displaying unique characteristics were serially picked from the six-agar media (NB, SM, LA, AL, SB, and PR tween agar media), and each isolate was named after the originally isolated spot of such agar medium in the given order. Finally, 87 colonies were isolated. Considering the shrimp aquaculture environment (approximately at 25°C), these microbial species were further screened based on their ability to degrade polymers at 25°C (Wyban *et al.* 1995). Only 25 colonies were eligible for these criteria with sufficient growth ($OD_{600} > 0.8$) at 25°C, and potential degradation ability (> 0.5 cm clear zone) toward protein or lipid. Afterward, the antagonism tests were performed between the 25 isolates, and 12 colonies were excluded because they inhibited the growth of other microbial strains. Among the remaining 13 isolates (Table 1), isolates of LA9, SB13, SM16, SM19, and PR21 were finally excluded owing to their inability to degrade lipids.

Table 2. Polymer-degrading ability and viable cell number in each test group after 24-h cultivation at 25°C

Test group	Composition of combined isolates	Degradation ability on		Viable cells (CFU mL^{-1})
		Protein (cm)[a]	Lipid	
1	AL1, AL4, AL5, AL6, LA10	1.6±0.3	+[b]	$5.3\times10^{6}\pm0.02$
2	AL1, AL4, AL5, AL6, LA10, PR15	2.0±0.2	+	$4.2\times10^{6}\pm0.03$
3	AL1, AL4, AL5, AL6, LA10, NB14	1.8±0.1	+	$3.3\times10^{6}\pm0.02$
4	AL1, AL4, AL5, AL6, LA10, AL22	1.7±0.3	+	$2.1\times10^{6}\pm0.04$
5	AL1, AL4, AL5, AL6, LA10, NB14, AL22, PR15	1.4±0.4	+	$1.7\times10^{7}\pm0.09$
6	AL4, AL5, AL6, LA10	1.8±0.2	+	$3.2\times10^{6}\pm0.03$
7	AL4, AL5, AL6, LA10, PR15	1.7±0.2	+	$2.2\times10^{6}\pm0.10$
8	AL4, AL5, AL6, LA10, NB14	2.1±0.3	+	$1.0\times10^{4}\pm0.02$
9	AL4, AL5, AL6, LA10, AL22	1.1±0.3	+	$1.7\times10^{6}\pm0.06$
10	AL4, AL5, AL6, LA10, NB14, PR15,	1.7±0.1	+	$4.2\times10^{5}\pm0.03$
11	AL4, AL5, AL6, LA10, PR15, AL22	1.6±0.3	+	$2.0\times10^{6}\pm0.08$
12	AL4, AL5, AL6, LA10, NB14, AL22	1.8±0.2	+	$2.3\times10^{5}\pm0.01$

[a]Mean diameter of each clear zone.
[b]'+' means degradation ability of lipid.

To select appropriately mixed microorganisms suitable for treating SAW containing complex components, 12 different combinations of isolates were examined based on the degradability of polymers (protein and lipid) generally present in SAW (Iber and Kasan 2021). This experiment was performed because a mixed culture is more advantageous than a single culture in the degradation of complex components in nature (Jankowska *et al.* 2015). In particular, mixed cultures of microorganisms could utilize the substance in nature well since a broad range of enzymes is synthesized by the mixed cultures and such enzymes can degrade various polymers. In this respect, combined isolates cultivated in 1 g L^{-1} SAW at 25°C for 24 h were tested for their polymer-degrading ability. All test groups exhibited degradation abilities on both protein and lipid under the conditions of diversely combined microorganisms (Table 2). Notably, Groups 2 and 8 exhibited higher protein degradation activity than other groups. In addition, the viable cell number of Group 2 was better than that of Group 8. This indicates that the proliferation of mixed culture in Group 2 was the most active owing to its high degradation ability of polymers in SAW. Meanwhile, the mixed culture in Group 5 exhibited the highest number of viable cells but was excluded from this study owing to its low protein degradation ability. Therefore, Group 2, composed of six isolates, was selected for SAW treatment.

3.2. Characterization and identification of isolates

Since Group 2 exhibited the best polymer-degrading activity, the characteristics of each isolate included in Group 2 were examined to distinguish the role of each isolate when applied. The isolates AL1, AL4, AL6, and LA10 were Gram-positive and rod bacteria, whereas the isolate AL5 was Gram-negative and rod bacterium. In particular, the isolate LA10 was a chain-forming bacterium. Unlike these bacteria, isolate PR15 was observed to uniquely form tetrad cocci. All isolates were motile and different in cell dimensions. The colors of colonies formed on the nutrient broth agar medium were ivory (AL1, AL6, and LA10), pale yellow (AL4), and yellow (AL5 and PR15).

The 16S rRNA sequence analysis was conducted for the six selected isolates in Group 2. Homology searches showed that the isolates AL1, AL4, AL5, AL6, LA10, and PR15 were the most closely matched to *Bacillus paramycoides* (99.86%), *Bacillus pumilus* (99.52%), *Stenotrophomonas rhizophila* (100%), *Bacillus paranthracis* (99.66%), *Bacillus paranthracis* (99.52%) and *Micrococcus luteus* (99.58%), respectively (Table 3). Based on partial 16S rRNA sequences of each isolate, a neighbor-joining phylogenetic tree was drawn with other phylogenetically related strains (Fig. 1). *Bacillus* species are known to produce diverse extracellular en-

Table 3. Results of identification for the six selected isolates

Isolate	Length (bp)	GenBank accession No.	Identification	Similarity (%)	Variation ratio
AL1	1462	MAOI01000012	*Bacillus paramycoides*	99.86	2/1462
AL4	1462	ABRX01000007	*Bacillus pumilus*	99.52	7/1462
AL5	1440	CP007597	*Stenotrophomonas rhizophila*	100	0/1440
AL6	1459	MACE01000012	*Bacillus paranthracis*	99.66	5/1459
LA10	1460	MACE01000012	*Bacillus paranthracis*	99.52	7/1460
PR15	1445	CP001628	*Micrococcus luteus*	99.58	6/1445

zymes; thus, microorganisms showing potential proteolytic activity in industrial fields belong to the genera *Bacillus* (Pant *et al.* 2015). *B. paramycoides* had some potential ability to treat hospital wastewater (Rashid *et al.* 2020), degrade organophosphate compounds (Ren *et al.* 2020), and remove toxic selenite from contaminated sites (Borah *et al.* 2021). *B. pumilus* produced potential xylanase (Kapoor *et al.* 2008) to efficiently degrade toxic aromatic compounds (Surendra *et al.* 2017) and improve the health and growth rate of striped catfish when it was used with *B. amyloliquefaciens* as a dietary additive (Thy *et al.* 2017). *B. paranthracis* also degraded a pharmaceutical pollutant, diclofenac (Chopra and Kumar 2020), and polycyclic aromatic hydrocarbons (Wang *et al.* 2020). Moreover, *Stenotrophomonas* sp. produced various extracellular enzymes, such as proteases, lipases, nucleases, chitinases, and elastases (Ryan *et al.* 2009). The genus *Stenotrophomonas*, isolated from surface waters, can denitrify in the presence of O_2 (Lv *et al.* 2017). *M. luteus* had the potential ability to degrade cyclic organic compounds (Zheng *et al.* 2009) and hydrocarbons (Shin-ya and Kajiuchi 2002; Tuleva *et al.* 2009), enabling it to be used in bioremediation (Kanjilal *et al.* 2015). Moreover, five isolates, i. e., bacteria of the genus *Bacillus* and *Strenotrophomonas*, are known for their ability to perform aerobic denitrification (Lv *et al.* 2017; Yang *et al.* 2020). Therefore, the above-reported results imply that the six selected isolates herein were eligible for treating SAW.

3.3. The application of six isolates to SAW treatment

The six selected isolates were applied to simulated SAW to investigate their potential degradation ability on substances mainly from uneaten feed and feces. During the treatment of simulated SAW, the pH increased from 6.21 to 7.45 for the first 12 h. Afterward, it was maintained (Fig. 2A). Simultaneously, the cell density (OD_{600}) and the number of viable cells rapidly increased from 1.37 to 2.34 and 5.90×10^4 to 2.06×10^5 CFU mL^{-1}, respectively, for the first 12 h. Moreover, this tendency was exactly reflected in the reductions of COD_{Cr} and TN as a result of biodegradation by the isolates (Fig. 2B). This indicates that biodegradation occurred actively for the first 12 h with cell growth. During the first 12 h, the removal percentages of COD_{Cr} and TN were 51.0% and 44.6%, respectively. The ratio of COD_{Cr} to TN was maintained at 10.0, except for the treatment time of 8 h because the reduction rate of TN by the mixed culture surpassed that of COD_{Cr} between 3 and 8 h. During this period of biodegradation, cells gradually proliferated, and thus nitrogen source was consumed considerably for cell growth. After 12 h, the decrease in the reduction rates of COD_{Cr} and TN became dull, and those reduction rates were slightly changed between 24 h and 36 h. Simultaneously, the numbers of viable cells did not increase further after 24 h, terminating the determination of all reaction parameters at 36 h.

Herein, biodegradation by the mixed culture was not distinct after 12 h of the saw treatment. The final removal efficiencies of COD_{Cr} and TN by the mixed culture of six isolates were 51.0% and 44.6%, respectively. SAW treatment was performed in a 1-L flask containing 500 mL SAW to ensure treatment under limited O_2. The six isolates reached a maximum cell viability of 2.06×10^5 CFU mL^{-1} in the SAW treatment (Fig. 2A), but according to the number of viable cells in Table 2, the six isolates in Group 2 are in an exponential growth state where they rapidly grow. The C/N (COD/TN) ratio and other factors, such as pH and concen-

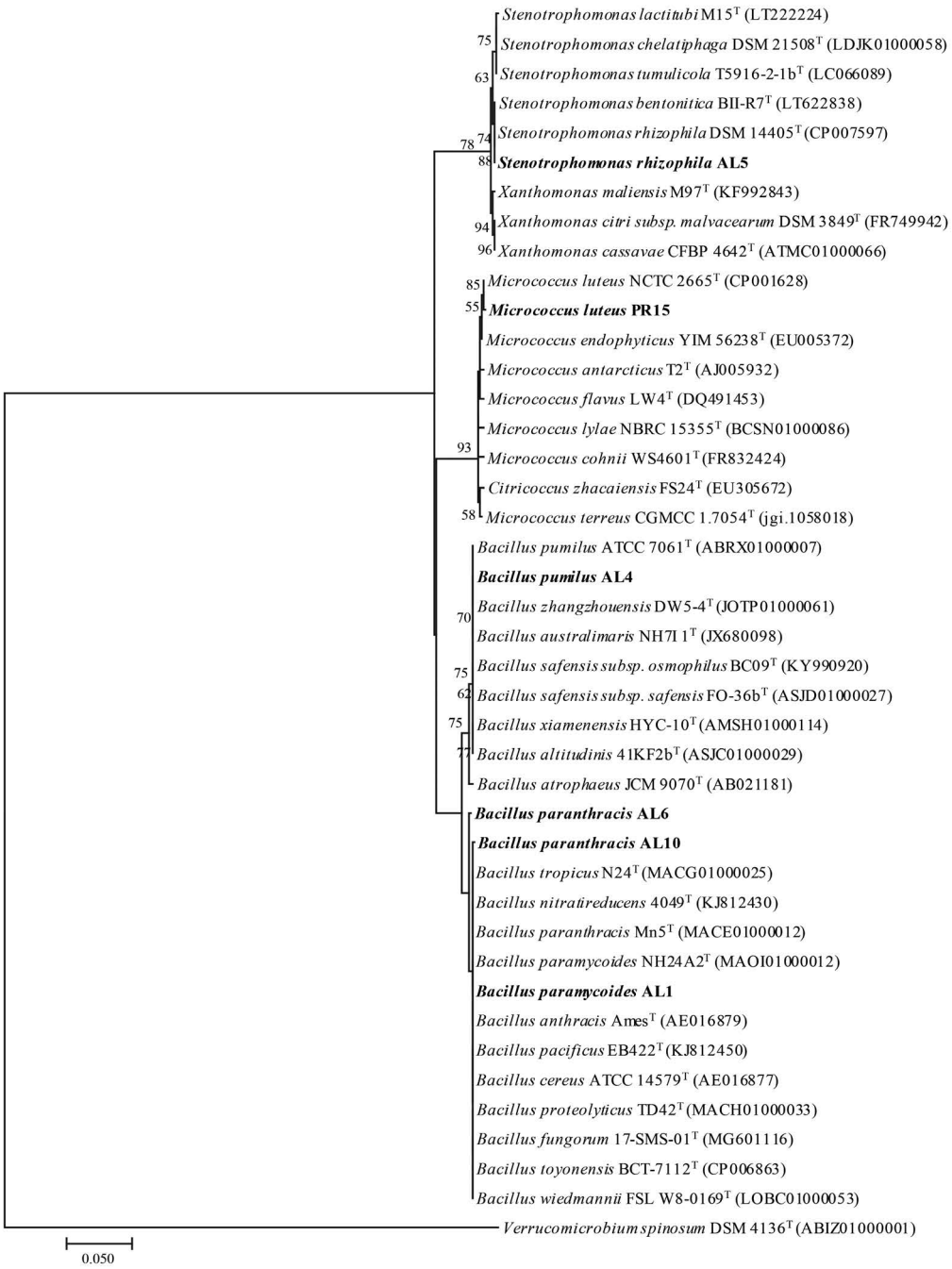

Fig. 1. A neighbor-joining phylogenetic tree drawn with other phylogenetically related strains based on partial 16S rRNA sequences of each isolate in Group 2. Sequences were aligned with Clustal W using the BioEdit program (Version 7.2.0). A phylogenetic tree was constituted via neighbor-joining tree analysis with 1,500 bootstrap replicates by MEGA version 7. NCBI accession numbers are presented in parentheses.

trations of fat and lipid, affected the efficiency of the biological wastewater treatment (Sunny and Mathai 2015). In this experiment, pH was maintained at ~7.45 throughout 36 h, and air was not injected continuously. Therefore, the C/N ratio and batch condition are important parameters affecting the biodegradation process in this study of wastewater treatment (Gao *et al.* 2010). For carbon utilization, microorganisms have

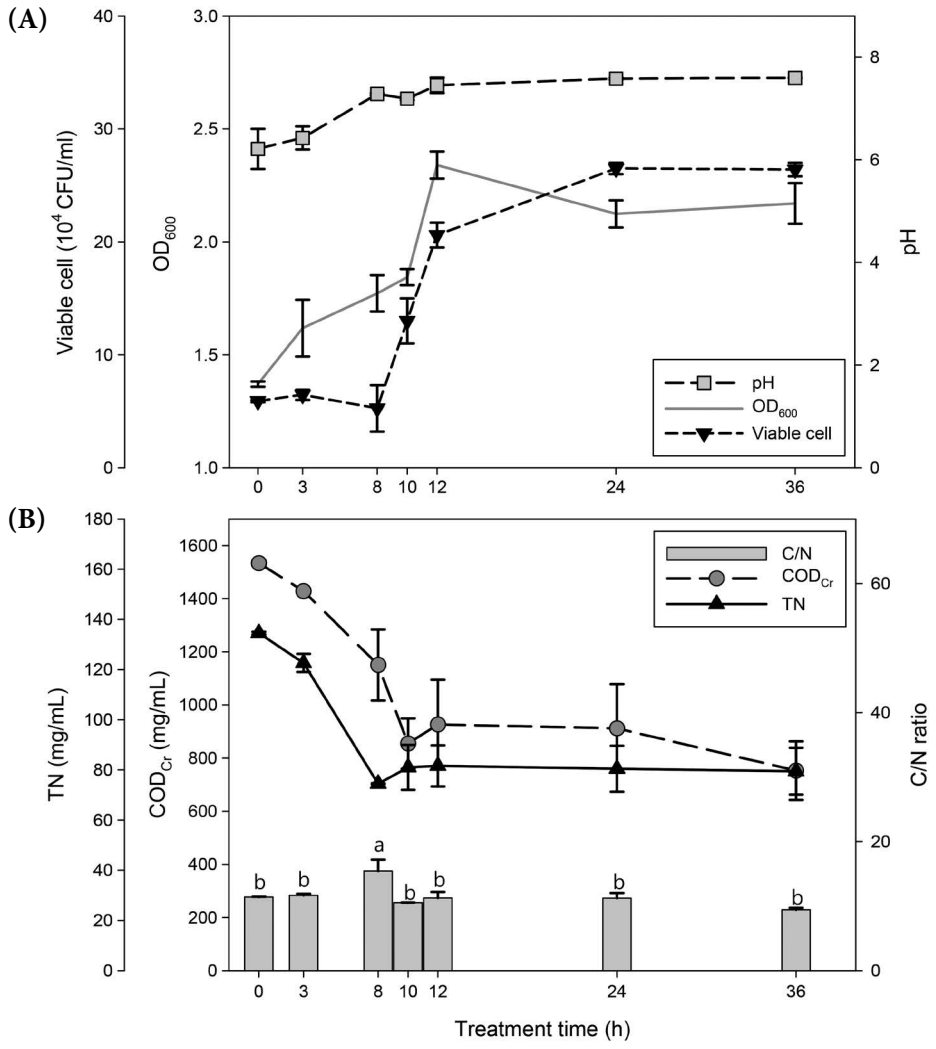

Fig. 2. Variations of (A) viable cell, OD_{600}, and pH; and (B) TN, COD_{Cr}, and C/N ratio as a function of SAW treatment time for the six selected isolates from Group 2. Data are presented as means standard deviation ($n=3$). Different letters on bars indicate a significant difference ($p<0.05$). OD_{600}, optical density; COD_{Cr}, chemical oxygen demand; TN, total nitrogen; C/N, COD/TN; SAW shrimp aquaculture wastewater.

difficulty synthesizing their necessary enzymes under a low N content, whereas microbial growth is inhibited under a high N content, particularly in the form of ammonia (Fontenot *et al.* 2007). In general, the C/N ratios of various wastewaters vary from 20 : 1 to 30 : 1 (Fontenot *et al.* 2007). However, the efficient treatment of wastewater occurs at a C/N ratio of 10 : 1 (Fontenot *et al.* 2007). Since the wastewater of residual feed from shrimp farms contains high nitrogenous concentrations (Roy *et al.* 2010; Iber and Kasan 2021), the C/N ratio is indispensably optimized for efficient treatment. Thus, the removal of nitrates under various C/N ratios (5 : 1, 10 : 1, 20 : 1, and 30 : 1) was studied for SAW, and the most effective nitrate removal was achieved at a C/N ratio of 10 : 1 (Fig. 2B). A C/N ratio of 10 : 1 was reported to produce the best results for maximum nitrogen and carbon removal from SAW in a sequencing batch reactor (Fontenot *et al.* 2007). A COD/TKN (total Kjeldahl nitrogen) ratio of 10 : 1 was also reported to be optimum for high nitrogen removal efficiency in the activated sludge process under very low operating O_2 concentration, with oxidation-reduction potential for aeration control (Chevakidagarn *et al.* 2012). In addition, a C/N ratio of 15 : 1 was reported to display the highest removal of ammonia (98.7%) in fish farm wastewater (Bakar *et al.* 2015). All these results indi-

cate that optimizing the C/N ratio is an important factor for efficiently removing nitrogen-containing residues (Roy *et al.* 2010; Bakar *et al.* 2015; Nguyen *et al.* 2018). Considering the above results, herein, the C/N ratio that remained at 10 : 1 for 36 h is appropriate for efficient nitrate removal. Therefore, a considerable reduction of residual COD_{Cr} and TN would occur with continuous aeration (Avnimelech 2009; Iber and Kasan 2021). This inference can be adequately supported by previous reports. Boopathy *et al.* (2007) performed biological treatment of shrimp farm wastewater under anoxic and aerobic modes, and they obtained a greater reduction of COD under the aerobic mode than in the anoxic mode. Sun *et al.* (2016) reported that reducing the carbon source was strongly related to cell growth.

In the case of nitrogen removal, anaerobic denitrification is considerably efficient, and wastewater is treated using a combination of aerobic and anaerobic (or anoxic modes) conditions (Lv *et al.* 2017). In this study, five among six isolates were found to be bacteria having ability of aerobic denitrification. Especially, the aerobic denitrification of the genus Bacillus strains is closely related to bacterial cell density in connection with COD reduction (Yang *et al.* 2020). The six isolates also showed a TN removal rate of 44.6% under batch conditions. Considering the average TN reduction rate of the aerobic denitrification strains is known to be 6−50% (Lv *et al.* 2017), six isolates are suitable for the treatment of aerobic denitrification. In conclusion, six isolates can remove COD_{Cr} and TN with additional aeration and are particularly valuable for aquaculture wastewater treatment in aerobic environments. Since shrimp farms are an aerobic environment, discovering strains that can simultaneously treat COD_{Cr} and TN under the same conditions is essential. Thus, the six isolates from rockworms are good candidates that can be applied to the field of aquaculture wastewater treatment in the future.

4. CONCLUSION

In shrimp aquaculture, large amounts of nitrogen contaminants are derived from feed residues in an aerobic environment, impacting negatively on ecosystems. To efficiently treat shrimp aquaculture wastewater (SAW), candidate strains were isolated from adult rockworms. Six isolates (strains AL1, AL4, AL5, AL6, LA10, and PR15) were finally selected by combining strains with excellent polymer degradation ability without antagonism. The six strains were identified to be *Bacillus paramycoides*, *Bacillus pumilus*, *Stenotrophomonas rhizophila*, *Bacillus paranthracis*, *Bacillus paranthracis*, and *Micrococcus luteus*, respectively with $>99.52\%$ similarity. The six isolates reached a maximum cell viability of 2.06×10^5 CFU mL^{-1} in 12 h of SAW treatment, with 51.0% of chemical oxygen demand (COD_{Cr}) and 44.6% of total nitrogen (TN) removal rates under the COD_{Cr}/TN ratio approximately at 10.0. These removal rates achieved under batch conditions indicate that the six isolates could be applied to the treatment of aerobic denitrification. Thus, the six isolates are particularly valuable for aquaculture wastewater treatment in aerobic environments, extending their application to the field of aquaculture wastewater treatment.

CRediT authorship contribution statement

JY Cho: Conceptualization, Methodology, Investigation, Visualization, Writing - Original Draft, Writing - Review & Editing. KS Cho: Methodology, Investigation, Data Curation, Writing - Original Draft. CH Kim: Resources, Project administration, Funding acquisition. JK Kim: Conceptualization, Data Curation, Supervision, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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REFERENCES

Anh PT, C Kroeze, SR Bush and APJ Mol. 2010. Water pollution by intensive brackish shrimp farming in south-east Vietnam: Causes and options for control. Agric. Water Manage. 97:872–882. https://doi.org/10.1016/j.agwat.2010.01.018

Avnimelech Y. 2009. Biofloc Technology: A Practical Guide Book. World Aquaculture Society. Louisiana, US.

Bakar NSA, NM Nasir, F Lananan, SHA Hamid, SS Lam and A Jusoh. 2015. Optimization of C/N ratios for nutrient removal in aquaculture system culturing African catfish, (*Clarias gariepi-*

nus) utilizing Bioflocs Technology. Int. Biodeterior. Biodegrad. 102:100–106. https://doi.org/10.1016/j.ibiod.2015.04.001

Boopathy R, C Bonvillain, Q Fontenot and M Kilgen. 2007. Biological treatment of low-salinity shrimp aquaculture wastewater using sequencing batch reactor. Int. Biodeterior. Biodegrad. 59:16–19. https://doi.org/10.1016/j.ibiod.2006.05.003

Borah SN, L Goswami, S Sen, D Sachan, H Sarma, M Montes, JR Peralta-Videa, K Pakshirajan and M Narayan. 2021. Selenite bioreduction and biosynthesis of selenium nanoparticles by *Bacillus paramycoides* SP3 isolated from coal mine overburden leachate. Environ. Pollut. 117519:1–12. https://doi.org/10.1016/j.envpol.2021.117519

Brown N, S Eddy and S Plaud. 2011. Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, *Nereis virens*. Aquaculture 322:177–183. https://doi.org/10.1016/j.aquaculture.2011.09.017

Chevakidagarn P, O Wanaglad and S Chiayvareesajja. 2012. Optimum Carbon/Nitrogen Ratio for upgrading single-stage activated sludge process in the Frozen Seafood Industry. Sci. Technol. Asia 17:11–21.

Chopra S and D Kumar. 2020. Characterization, optimization and kinetic study of diclofenac degradation by novel bacterial strains and their synthetic consortia. Bioremediat. J. 24:150–170. https://doi.org/10.1080/10889868.2020.1793723

Dale H, M Solan, P Lam and M Cunliffe. 2019. Sediment microbial assemblage structure is modified by marine polychaete gut passage. FEMS Microbiol. Ecol. 95:fiz047. https://doi.org/10.1093/femsec/fiz047

Diana JS. 2012. Is lower intensity aquaculture a valuable means of producing food? An evaluation of its effects on near-shore and inland waters. Rev. Aquac. 4:234–245. https://doi.org/10.1111/j.1753-5131.2012.01079.x

Fontenot Q, C Bonvillain, M Kilgen and R Boopathy. 2007. Effects of temperature, salinity, and carbon: nitrogen ratio on sequencing batch reactor treating shrimp aquaculture wastewater. Bioresour. Technol. 98:1700–1703. https://doi.org/10.1016/j.biortech.2006.07.031

Fuirst M, CS Ward, C Schwaner, Z Diana, TF Schultz and D Rittschof. 2021. Compositional and functional microbiome variation between tubes of an intertidal polychaete and surrounding marine sediment. Front. Mar. Sci. 8:656506. https://doi.org/10.3389/fmars.2021.656506

Gao M, F Liang, A Yu, B Li and L Yang. 2010. Evaluation of stability and maturity during forced-aeration composting of chicken manure and sawdust at different C/N ratios. Chemosphere 78:614–619. https://doi.org/10.1016/j.chemosphere.2009.10.056

Giangrande A, M Licciano and L Musco. 2005. Polychaetes as environmental indicators revisited. Mar. Pollut. Bull. 50:1153–1162. https://doi.org/10.1016/j.marpolbul.2005.08.003

Hochstein R, Q Zhang, MJ Sadowsky and VE Forbes. 2019. The deposit feeder *Capitella teleta* has a unique and relatively complex microbiome likely supporting its ability to degrade pollutants. Sci. Total Environ. 670:547–554. https://doi.org/10.1016/j.scitotenv.2019.03.255

Iber BT and NA Kasan. 2021. Recent advances in Shrimp aquaculture wastewater management. Heliyon 7:e08283. https://doi.org/10.1016/j.heliyon.2021.e08283

Jankowska E, J Chwiałkowska, M Stodolny and P Oleskowicz-Popiel. 2015. Effect of pH and retention time on volatile fatty acids production during mixed culture fermentation. Bioresour. Technol. 190:274–280. https://doi.org/10.1016/j.biortech.2015.04.096

Kanjilal T, C Bhattacharjee and S Datta. 2015. Bio-degradation of acetamiprid from wetland wastewater using indigenous *Micrococcus luteus* strain SC 1204: Optimization, evaluation of kinetic parameter and toxicity. J. Water Process. Eng. 6:21–31. https://doi.org/10.1016/j.jwpe.2015.03.002

Kapoor M, LM Nair and RC Kuhad. 2008. Cost-effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of *Prosopis juliflora*. Biochem. Eng. J. 38:88–97. https://doi.org/10.1016/j.bej.2007.06.009

Kim SK, JW Jang, YR Jo, JH Kim and SK Kim. 2019. Influence of denitrified biofloc water on the survival rate and physiological characteristics of Pacific white shrimp juveniles, *Litopenaeus vannamei*. Korean J. Environ. Biol. 37:136–143. https://doi.org/10.11626/KJEB.2019.37.2.136

Kinoshita K, S Tamaki, M Yoshioka, S Srithonguthai, T Kunihiro, D Hama, K Ohwada and H Tsutsumi. 2008. Bioremediation of organically enriched sediment deposited below fish farms with artificially mass-cultured colonies of a deposit-feeding polychaete *Capitella* sp. I. Fish. Sci. 74:77–87. https://doi.org/10.1111/j.1444-2906.2007.01498.x

Kobayashi M, S Msangi, M Batka, S Vannuccini, MM Dey and JL Anderson. 2015. Fish to 2030: the role and opportunity for aquaculture. Aquac. Econ. Manag. 19:282–300. https://doi.org/10.1080/13657305.2015.994240

Lv P, J Luo, X Zhuang, D Zhang, Z Huang and Z Bai. 2017. Diversity of culturable aerobic denitrifying bacteria in the sediment, water and biofilms in Liangshui River of Beijing, China. Sci. Rep. 7:10032. https://doi.org/10.1038/s41598-017-09556-9

McFall-Ngai M, MG Hadfield, TC Bosch, HV Carey, T Domazet-Lošo, AE Douglas, N Dubilier, G Eberl, T Fukami, SF Gilbert, U Hentschel, N King, S Kjelleberg, AH Knoll, N Kremer, SK Mazmanian, JL Metcalf, K Nealson, NE Pierce, JF Rawls, A Reid, EG Ruby, M Rumpho, JG Sanders, D Tautz and JJ Wernegreen. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. U.S.A. 110:3229–3236. https://doi.org/10.1073/pnas.1218525110

Ngo HH, W Guo, T Tram Vo, LD Nghiem and FI Hai. 2016. Aerobic treatment of effluents from the aquaculture industry. pp. 35–77. In: Current Developments in Biotechnology and Bioengineering: Biological Treatment of Industrial Effluents (Lee D, V Jegatheesan, HH Ngo, PC Hallenbeck and A Pandey, eds.). Elsevier. Amsterdam, Netherlands.

Nguyen TNP, SJ Chao, PC Chen and C Huang. 2018. Effects of C/N ratio on nitrate removal and floc morphology of autohydrogenotrophic bacteria in a nitrate-containing wastewater treatment process. J. Environ. Sci. 69:52–60. https://doi.org/10.1016/j.jes.2017.04.002

Onozato M, T Sugawara, A Nishigaki and S Ohshima. 2012. Study on the degradation of polycyclic aromatic hydrocarbons (PAHs) in the excrement of *Marphysa sanguinea*. Polycycl. Aromat. Compd. 32:238–247. https://doi.org/10.1080/10406638.2012.659831

Pant G, A Prakash, JVP Pavani, S Bera, GVNS Deviram, A Kumar, M Panchpuri and RG Prasuna. 2015. Production, optimization and partial purification of protease from *Bacillus subtilis*. J. Taibah Univ. Sci. 9:50–55. https://doi.org/10.1016/j.jtusci.2014.04.010

Rashid A, SA Mirza, C Keating, S Ali and LC Campos. 2022. Indigenous *Bacillus paramycoides* spp. and *Alcaligenes faecalis*: sustainable solution for bioremediation of hospital wastewater. Environ. Technol. 43:1903–1916. https://doi.org/10.1080/09593330.2020.1858180

Ren J, C Wang, C Li, B Fan and D Niu. 2020. Biodegradation of acephate by *Bacillus paramycoides* NDZ and its degradation pathway. World J. Microbiol. Biotechnol. 36:1–11. https://doi.org/10.1007/s11274-020-02931-1

Robinson G, GS Caldwell, CJW Jones, MJ Slater and SM Stead. 2015. Redox stratification drives enhanced growth in a deposit-feeding invertebrate: implications for aquaculture bioremediation. Aquac. Environ. Interact. 8:1–13. https://doi.org/10.3354/aei00158

Rouse GW, SK Goffredi and RC Vrijenhoek. 2004. Osedax: bone-eating marine worms with dwarf males. Science 305:668–671. https://doi.org/10.1126/science.1098650

Roy D, K Hassan and R Boopathy. 2010. Effect of carbon to nitrogen (C : N) ratio on nitrogen removal from shrimp production waste water using sequencing batch reactor. J. Ind. Microbiol. Biotechnol. 37:1105–1110. https://doi.org/10.1007/s10295-010-0869-4

Ryan R, S Monchy, M Cardinale, S Taghavi, L Crossman, M Avison, G Berg, D Lelie and J Dow. 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat. Rev. Microbiol. 7:514–525. https://doi.org/10.1038/nrmicro2163

Schwartz MF and CE Boyd. 1994. Effluent quality during harvest of channel catfish from watershed ponds. Prog. Fish-Cult. 56:25–32. https://doi.org/10.1577/1548-8640(1994)056<0025:EQDHOC>2.3.CO;2

Shin-ya Y and T Kajiuchi. 2002. Enzymatic activity of PEOMA-modified lysozyme obtained from degradation of *Micrococcus luteus* cell and N-acetylated chitosan. Biochem. Eng. J. 11:73–75. https://doi.org/10.1016/S1369-703X(02)00013-X

Shin S, K Yundendorj, SS Lee, DH Lee, KH Kang and HY Kahng. 2013. Characterization and organic hydrocarbons degradation potential of euryhaline marine microorganism, *Bacillus* sp. EBW4 isolated from polychaete (*Perinereis aibuhitensis*). Korean J. Microbiol. 49:38–45. https://doi.org/10.7845/kjm.2013.005

Song ZF, J An, GH Fu and XL Yang. 2011. Isolation and characterization of an aerobic denitrifying *Bacillus* sp. YX-6 from shrimp culture ponds. Aquaculture 319:188–193. https://doi.org/10.1016/j.aquaculture.2011.06.018

Sun Z, Y Lv, Y Liu and R Ren. 2016. Removal of nitrogen by heterotrophic nitrification-aerobic denitrification of a novel metal resistant bacterium *Cupriavidus* sp. S1. Bioresour. Technol. 220:142–150. https://doi.org/10.1016/j.biortech.2016.07.110

Sunny N and L Mathai. 2015. Physicochemical process for fish processing wastewater. pp. 113–122. In: Efficient Management of Wastewater from Manufacturing: New Treatment Technologies (Monsalvo VM, ed.). Apple Academic Press. New York.

Surendra SV, BL Mahalingam and M Velan. 2017. Degradation of monoaromatics by *Bacillus pumilus* MVSV3. Braz. Arch. Biol. Technol. 60:1–18. https://doi.org/10.1590/1678-4324-2017160319

Thy HTT, NN Tri, OM Quy, R Fotedar, K Kannika, S Unajak and N Areechon. 2017. Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish (*Pangasianodon hypophthalmus*). Fish Shellfish Immunol. 60:391–399. https://doi.org/10.1016/j.fsi.2016.11.016

Tomassetti P and S Porrello. 2005. Polychaetes as indicators of marine fish farm organic enrichment. Aquac. Int. 13:109–128. https://doi.org/10.1007/s10499-004-9026-2

Tuleva B, N Christova, R Cohen, D Antonova, T Todorov and I Stoineva. 2009. Isolation and characterization of trehalose tetraester biosurfactants from a soil strain Micrococcus luteus BN56. Process Biochem. 44:135–141. https://doi.org/10.1016/j.procbio.2008.09.016

Vinothkumar R, JY Dar, VS Bharti, A Singh, A Vennila, IA Bhat and PK Pandey. 2021. Heterotrophic nitrifying and aerobic denitrifying bacteria: Characterization and comparison of shrimp pond and effluent discharge channel in aspects of composition and function. Aquaculture 539:736659. https://doi.org/10.1016/j.aquaculture.2021.736659

Wang M, H Zhang and G Li. 2020. Biodegradation of fluoranthene contaminated soil by different combinations of bacteria. pp. 1–4. In: IOP Conference Series: Earth and Environmental Science. IOP Publishing. Bristol, UK. https://doi.org/10.1088/1755-1315/526/1/012014

World Bank. 2014. Fish to 2030: Prospects for Fisheries and Aquaculture (World Bank Report Number 83177-GLB). World Bank. Washington D.C.

Wyban J, WA Walsh and DM Godin. 1995. Temperature effects on growth, feeding rate and feed conversion of the Pacific white shrimp (*Penaeus vannamei*). Aquaculture 138:267–279. https://doi.org/10.1016/0044-8486(95)00032-1

Yang T, Y Xin, L Zhang, Z Gu, Y Li, Z Ding and G Shi. 2020. Characterization on the aerobic denitrification process of *Bacillus* strains. Biomass Bioenergy 140:105677. https://doi.org/10.1016/j.biombioe.2020.105677

Zheng C, B Qu, J Wang, J Zhou and H Lu. 2009. Isolation and characterization of a novel nitrobenzene-degrading bacterium with high salinity tolerance: *Micrococcus luteus*. J. Hazard. Mater. 165:1152–1158. https://doi.org/10.1016/j.jhazmat.2008.10.119

