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Original Research Articles

Isolation, identification, and evaluation of the characteristics of *Streptomyces* sp. as the probiotic strains applied in aquaculture

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The present study was conducted to select *Streptomyces* strains that exhibited characteristics of probiotics for application in aquaculture. *Streptomyces* were isolated from soil and sediment samples collected from various habitats such as riverside, shrimp ponds, pangasius ponds, mangrove forests, or estuaries along the coast of Vietnam. Two strains of *Streptomyces* (AG12.2 and ND10.1) were selected due to (1) their potent antimicrobial activity against common pathogens that cause serious diseases in shrimp and pangasius such as *E. ictaluri*, *A. hydophila*, *V. harveyi*, *V. parahaemolyticus*, and (2) their superior ability to produce several extracellular enzymes that strongly hydrolyze organic compounds such as starch, CMC, and skimmed milk, compared to other strains. AG12.2 and ND10.1 can tolerate salinity from 0 ppt to 5 ppt and pH from 3 to 8; however, their growth is most vigorous at salt concentrations and pH between 0 ppt to 2 ppt, and 7.2 to 8, respectively. *In vivo* evaluation on *L. vannamei* in this study shows that shrimp that were fed with diets containing AG12.2 and ND10.1 had WG (%) increasing by 1.59 times and 1.61 times respectively, DGW (g/day) increasing by 1.64 times, and FCR decreasing by 1.30 times and 1.35 times respectively. The shrimp treated with AG12.2 and ND10.1 were then challenged with *V. parahaemolyticus*, resulting in a survival rate increase of 3.9 times and 3.71 times, respectively. Phylogenetic analysis revealed that strains AG12.2 and ND10.1 belong to *Streptomyces kunmingensis* and *Streptomyces angustmyceticus*, respectively. The findings of the present study led to the conclusion that *Streptomyces kunmingensis* AG12.2 and *Streptomyces angustmyceticus* ND10.1 are excellent candidates for producing beneficial probiotics for aquaculture.

INTRODUCTION

Global consumption of aquatic foods increased by 3.0 percent annually from 1961 to 2019, with the annual per capita consumption reaching a peak of 20.5 kg in 2019. To meet this demand, global aquaculture production has increased rapidly, from 21.8 million tons in the 90s to 214 million tons in 2020.[1]

At the national level, Vietnam is the world's third largest exporter of aquatic products[1]; pangasius, tiger shrimp, and whiteleg shrimp are the main farming species.[2] Exports of these products bring in large amounts of foreign currencies, making an important contribution to the country's economic development, while aquaculture serves as the main livelihood for people in the Mekong Delta region and coastal localities.[3,4] In recent years, Vietnam, like other major global seafood producers, has encountered numerous challenges in expanding aquaculture production. These challenges include environmental degradation such as mangrove depletion, declining water quality, and disease outbreaks in farming.[4-6] The increasing scale of intensive farming leads to more frequent and difficult-to-control disease outbreaks.[7] For pangasius, disease outbreaks in farms are mainly caused by environmental degradation, improper care, high stocking density, and low-quality fingerlings.[8]

Diseases have been reported to cause severe damage to aquaculture.[9] Antibiotics and other veterinary drugs are commonly used in aquaculture to prevent and treat diseases.[10] However, improper antibiotic usage can result in antibiotic-resistant bacteria or residues in processed products.[11,12] Several alternative strategies, including vaccines, probiotics, herbal plants, and immunostimulants, have

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Table 1. Location, salinity, pH of the samples for isolating of *Streptomyces*.

Collection site	Geographic coordinates*	Samples	Salinity (ppt)	pH	No. of samples
My Thanh, Long Xuyen, An Giang	10°20'04N''105°28'49''E	Soil, Riverside	4.5	7.1	03
Tra Su mangrove forest, Tinh Bien, An Giang	10°35'06''N105°03'28''E	Sediments	5.4	7.4	05
Vinh Xuong, Tan Chau, An Giang	10°53'55''N105°10'46''E	Fish pond sediments	3.5	7.3	02
Long Hoa, Phu Tan, An Giang	10°45'26''N105°16'51''E	Fish pond sediments	3.5	7.5	08
Trieu Van, Trieu Phong, Quang Tri	16°53'50''N107°11'54''E	Shrimp pond sediments	22	7.9	06
Con Lo, Tien Hai, Thai Binh	20°29'29''N106°35'56''E	Estuarine and coastal sediments	19	7.5	08
Nam Trung, Tien Hai, Thai Binh	20°15'39''N106°36'03''E	Estuarine and coastal sediments	19.9	7.8	05
Giao Thien, Giao Thuy, Nam Dinh	20°13'52''106°34'53''E	Shrimp pond sediments	18.5	7.9	06
Giao Lac, Giao Thuy, Nam Dinh	20°13'50''N106°31'49''E	Shrimp pond sediments	18.1	8.1	05

*Geographic coordinates were based on Google Maps

been proposed to prevent disease outbreaks and limit the use of veterinary drugs in aquaculture.[13] As one of the best alternatives, probiotics have been strongly recommended because they contain secondary metabolites modulating biological functions, such as growth promotion, appetite stimulation, immune stimulation, disease resistance, antimicrobial activities, and extracellular enzymes that degrade organic compounds.[14] Most of the probiotics proposed as biological control agents in aquaculture belong to the lactic-acid bacteria, the genus *Bacillus*, or the genera *Pseudomonas* and *Burkholderia*.[14,15] *Streptomyces* have also been proven to be potential probiotics in aquaculture because they can produce antibiotics, antifungals, extracellular enzymes, siderophores, and anti-quorum sensing.[16]

This study was conducted to identify the indigenous *Streptomyces* strains with probiotic properties for potential use in aquaculture. These strains were tested on whiteleg shrimp to assess their ability to stimulate growth, improve feed efficiency, and provide protection against pathogens. The findings from this study will serve as a foundation for generating novel biological products to promote sustainable aquaculture practices.

MATERIALS AND METHODS

SAMPLES FOR ISOLATING *STREPTOMYCES*

The soil and sediment samples were taken at a depth of 10-15 cm from the surface and stored in 50 mL falcon tubes at 4°C for 72 h before being used to isolate *Streptomyces*. The sample collection sites are shown in **Table 1**.

EXPERIMENTAL SHRIMP

Whiteleg shrimp (*L. vannamei*) (61–70 days old, 16 g/each) were purchased from Nghe An, Vietnam. They were randomly tested for *V. parahaemolyticus,* and *V. harveyi* by polymerase chain reaction.

BACTERIAL STRAINS

The bacterial strains used to evaluate the antagonistic activities of *Streptomyces* in this study were provided by the Institute of Biotechnology and Food Technology, Hanoi Open University. Of these strains, *E. ictaluri* E.HOU/AG21, *A. hydophila* A.HOU/DT14 were isolated from infected pangasius, and *V. harveyi* VH/BT12, *V. parahaemolyticus* V.HOU/BT01 were isolated from infected shrimp.

ISOLATING AND SCREENING *STREPTOMYCES*

Soil or sediment samples were air-dried at room temperature for 7 days to remove moisture and reduce the number of bacteria (except for *Streptomyces* species). Then, they were crushed and sieved through a sterilization screen to remove large debris. After that, the samples were mixed with $CaCO_3$ (10:1 w/w) in the sterile petri plates and incubated for 2 days at 28°C. Following, 20 g of sample-$CaCO_3$ mixtures were suspended in 180 mL of sterile distilled water in the flasks on a rotary shaker at 200 rpm for 30 min. The pretreated samples were diluted up to 10^{-6}. An aliquot of 0.1 mL of the diluted samples was then overlaid on starch casein agar (SCA-HiMedia) (contained 50 µg/ml of nystatin and 5 µg/ml of ampicillin) and incubated at 28°C for 7 days. The colonies of *Streptomyces* observed on SCA showed a tough or powdery texture and a dry or folded appearance, adherence to the agar surface, and branching filaments with or without aerial mycelia. These colonies were categorized into several colors, namely gray, white, red, yellow, green, brown, pink, orange, and violet (determined through comparison with the color charts of Inter-Society Color Council National Bureau of Standards- ISCCNBS)[17];

regarding morphological and biochemical characteristics, *Streptomyces* is gram-positive, filamentous, non-motile, and catalase-positive.[18]

Colonies of *Streptomyces* were purified using ISP2 agar (HiMedia). Purified *Streptomyces* were stored in ISP2 slants at 4°C or in 20% glycerol at -20°C.

ANTIMICROBIAL ACTIVITY

The agar-diffusion method was used for antagonism detection. *Streptomyces* isolated were cultured on SCA for 7 days at 28°C. Four strains *E. ictaluri* E.HOU/AG21, *A. hydophila* A.HOU/DT14, *V. harveyi* VH/BT12, and *V. parahaemolyticus* V.HOU/BT01 were spread separately on tryptone soya agar (TSA-HiMedia). Subsequently, 6 mm plugs were excised from the *Streptomyces* plates, and placed on the TSA plates inoculated with those bacteria. The antimicrobial activity was monitored by measuring the diameter of the inhibition zone (halos; mm) around the agar plugs after 24 h incubation at 28°C.

ENZYMATIC ACTIVITY

The agar-diffusion method was used to evaluate the ability of isolated strains to hydrolyze macromolecular polymers, such as proteins, starch, and cellulose.

Amylolytic activity: 6mm plugs were circled from the *Streptomyces* plates and placed on ISP2 agar (HiMedia) supplemented with 2% starch. The plates were incubated at 28°C for 3 days. After that, the surface of the agar was flooded with an iodine solution and then allowed to stand for a few minutes. The appearance of clear zones around the colony characterized amylolytic activity. The halo zone indicates the presence of amylase.

Proteolytic activity: *Streptomyces* isolated were screened for their proteolytic activity by using ISP2 agar containing skimmed milk 1%. The plates having the plugs of *Streptomyces* were incubated for 3 days at 28°C. After that, the plates were flooded with an $HgCl_2$ solution ($HgCl_2$ 15 g, HCl 20 mL, and distilled H_2O 100 mL). Protein hydrolysis appeared as a clear or white opaque zone around the colony.

Cellulolytic activity: ISP2 agar plates supplemented with 1% carboxy methyl cellulose (CMC) were used to measure the cellulolytic activity of *Streptomyces*. The plugs of *Streptomyces* were incubated on the above plates for 3 days at 28°C. After incubation, a lugol solution was dripped onto each of the plates. The halo zone around the colony indicates the presence of cellulase.

SALT AND PH TOLERANCE

The salt and pH tolerance of *Streptomyces* isolated was determined by growing in tryptone soya broth (TSB- HiMedia) at pH of 3, 4, 7.2, and 8.0 and salt concentrations of 0 ppt, 0.5 ppt, 2 ppt, 3 ppt and 5 ppt. The isolates were incubated at 28°C for 7 days. The presence or absence of growth was recorded after 7 days by UV-vis spectrophotometer at OD_{600}.

EXPERIMENTAL DESIGN FOR EVALUATION OF GROWTH PERFORMANCE AND FEED CONVERSION RATIO

The experiments were performed in 100L glass tanks disinfected with 30 mg/L chlorine. Before the trials, *L. vannamei* was acclimated for 7 days under controlled temperature conditions (28 ± 0.5°C), salinity 15 ppt, and dissolved oxygen ⩾ 4 mg/L; 30% of the water was changed daily.

The experiments lasted for 3 months (90 days), and experimental conditions were maintained as described above. The shrimp were fed with pellets (Cargill, HH724), which were supplemented with 10^8 CFU/g of isolated *Streptomyces*. In the control tank, the shrimp only consumed commercial pellets (without *Streptomyces)*. The shrimp were fed 4 times daily to their needs. At the end of the experiments, the growth performance of the shrimp, such as weight gain (WG, %), daily weight gain (DWG, g/day), and feed conversion ratio (FCR) were calculated as follows:

> WG (%)= 100×(final body weight (g)- initial body weight (g))/initial body weight (g);
> DWG (g/day) = (final body weight (g)- initial body weight (g))/experimental days;
> FCR= feed intake (g)/ total weight (g)

PROTECTION ACTIVITY OF *STREPTOMYCES* FOR THE CHALLENGED SHRIMP

The experimental shrimp were fed with diets containing *Streptomyces* as described above. After 7 days of being fed with *Streptomyces*, the shrimp were challenged by being immersed in a suspension containing 10^6 CFU/mL of *V. parahaemolyticus* V.HOU/BT01. The shrimp in the *Streptomyces* control tank were fed daily on *Streptomyces* without challenge, while in the challenge control tank, the shrimp were challenged and fed with the diet without *Streptomyces*. All the tanks were continuously cultured for 15 days under the above conditions. The number of surviving shrimp was counted daily, with the survival rate being calculated using the following formula: survival rate (SR, %) = 100 x Wt/Wi, where Wi is the initial number of the experimental shrimp, Wt is the number of the shrimp that survived at the time of observation.

SEQUENCING FOR IDENTIFICATION OF *STREPTOMYCES*

The genomic DNA of the isolated *Streptomyces* was extracted using a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The eubacterial universal primers 27F: 5'-AGAGTTTGATCATGGCTCAG-3' and 1492R: 5'-TACGGTTACCTTGTTACGACTT-3' were used to amplify a product of approximately 1.5 kb of the 16S rRNA gene. The amplification reactions were performed in a final volume of 50 µL, which contained a DNA template, 2× PCR Master Mix solution (iNtRON, Korea), primers, and sterile distilled water. The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 60 s, 57°C for 60 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. The amplified PCR products were checked on a 1% agarose gel, purified, and sequenced at the 1st BASE

sequencing company, Seri Kembangan, Selangor, Malaysia. The sequences were subjected to BLAST (https://blast.ncbi.nlm.nih.gov/Blast) analysis for homology analysis. The relative sequences from the BLAST results were aligned and used to construct phylogenetic trees using CLC Workbench (version 22.0, Qiagen, Hilden, Germany).

STATISTICAL ANALYSIS

All experiments were carried out in triplicate. Data were analyzed using SPSS 22.0 and one-way ANOVA. The result values were expressed as mean ± standard error, and differences were considered significant at the 0.05 probability level.

RESULTS

ISOLATION AND SCREENING OF *STREPTOMYCES*

From 48 soil and sediment samples collected from different regions of Vietnam, a total of 58 strains were identified as *Streptomyces* because they have typical patterns, such as (1) smooth appearance colonies that later developed aerial mycelia which either appeared floccose, granular, powdery, velvet, or leathery; (2) varied colors of the substrate and aerial mycelia, including white, yellow-white, gray, brown, orange (**Figure 1**); (3) positive catalase and positive gram; and (4) filaments with branching mycelium. Eleven of those isolated *Streptomyces* strains were found to be resistant to four tested bacterial strains (pathogens in pangasius and shrimp), namely *E. ictaluri* E.HOU/AG21, A .HOU/DT14, *V. harveyi* VH/BT12, and *V. parahaemolyticus* V.HOU/BT01; among them, the five strains AG8.9, AG12.2, ND10.1, TB1.6, and ND5.3 were determined to be superiorly resistant to all four pathogenic bacterial strains with inhibition zones ranging from 18.70 mm to 28.93 mm (**Table 2**, **Figure 2**). Regarding extracellular enzyme activity, the 3 strains AG12.2, ND10.1, and TBL1 were found to have outstanding ability in the hydrolysis of starch, CMC, and skim milk; the size of hydrolysis zone was in the range of 19.60 mm to 32.13 mm (**Table 3**, **Figure 3**). Based on the evaluation of antibacterial activity and extracellular enzyme activity, *Streptomyces* AG12.2 and ND10.1 were initially identified as potential strains for application in aquaculture.

SALT TOLERANCE AND PH TOLERANCE

Streptomyces AG12.2 and ND10.1 grew best at a salinity of 0 ppt to 2 ppt and pH of 7.2 to 8. **Figure 4** shows that ND10.1 was more adaptive to salinity than AG12.2, and at the same time, it appears to be more acid-tolerant than AG12.2. Comparing the growth efficiency of these two strains at optimal salinity (0 ppt -2 ppt) and higher salinity shows that the biomass density of AG12.2 decreased by 2.43 times to 2.92 times corresponding to salinity of 3 ppt and 4.22 times to 5.09 times corresponding to salinity of 5 ppt; meanwhile, for ND10.1, biomass density decreased by 2.07 times to 2.34 times and 2.67 times to 3.02 times respectively salinity of 3 ppt and 5 ppt. Regarding pH tolerance, the biomass density of AG12.2 and ND10.1 decreased sharply under growth conditions at pH 3 and pH 4. Compare the growth efficiency of these two strains under the condition of optimal pH (7.2 - 8) and pH 3 and pH 4 shows that the biomass density of AG12.2 decreased by 2.82 times to 3.13 times at pH 3, decreased by 1.38 times to 1.53 times at pH 4; meanwhile, the biomass density of ND10.1 decreased by 2.42 times to 2.67 times and 1.40 times to 1.54 times at pH 3 and pH 4 respectively.

GROWTH PERFORMANCE AND FEED CONVERSION RATIO

Compared to the control group, the two strains AG12.2 and ND10.1 significantly improved growth performance in whiteleg shrimp, with the level of growth stimulation efficiency in shrimp of these two strains being equivalent ($p > 0.05$) (**Table 4**). Compared to the control group, weight gain and daily weight gain of shrimp fed with AG12.2 increased by 1.59 times and 1.64 times, respectively; meanwhile, in shrimp fed with ND10.1, these parameters increased by 1.61 times and 1.64 times, respectively.

The inclusion of *Streptomyces* in shrimp diets significantly reduced the feed conversion ratio compared to the control group ($p < 0.05$). Specifically, in groups of the shrimp fed with AG12.2 and ND10.1, FCR decreased by 1.3 times and 1.35 times, respectively (**Table 4**).

SURVIVAL RATE OF CHALLENGED SHRIMP

Both strains AG12.2 and ND10.1 showed the ability to protect whiteleg shrimp challenged with *V. parahaemolyticus*, and AG12.2 protected the shrimp more effectively than ND10.1. After 15 days of observation, compared to the control group, the survival rate of challenged shrimp fed with AG12.2 and ND10.1 increased by 3.9 times and 3.71 times, respectively (**Figure 5**).

IDENTIFICATION OF THE SELECTED *STREPTOMYCES* TRAINS

The amplified 16S rRNA gene sequences of *Streptomyces* AG12.2, ND10.1 were aligned with other sequences in Genbank using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast). The nucleotide sequence data submitted with the Genbank accession numbers are shown in **Table 5**. A phylogenetic tree (**Figure 6**) was constructed according to the bootstrap test using the neighbor-joining algorithm with CLC Workbench 22.0 (version 22.0, Qiagen, Hilden, Germany). It shows the phylogenetic relationships of the two strains (XK8, the code for AG12.2 and XK 22, the code for ND10.1) with some other *Streptomyces* sp. strains in Genbank. Based on the nucleotide sequence data, colony characteristics, shape of spore chains, and biochemical characteristics of these two strains, the results show that XK8 (AG12.2) strain, collected at An Giang province, belongs to *Streptomyces kunmingensis* and XK22 (ND10.1) strain, collected at Nam Dinh province, belongs to *Streptomyces angustmyceticus*.

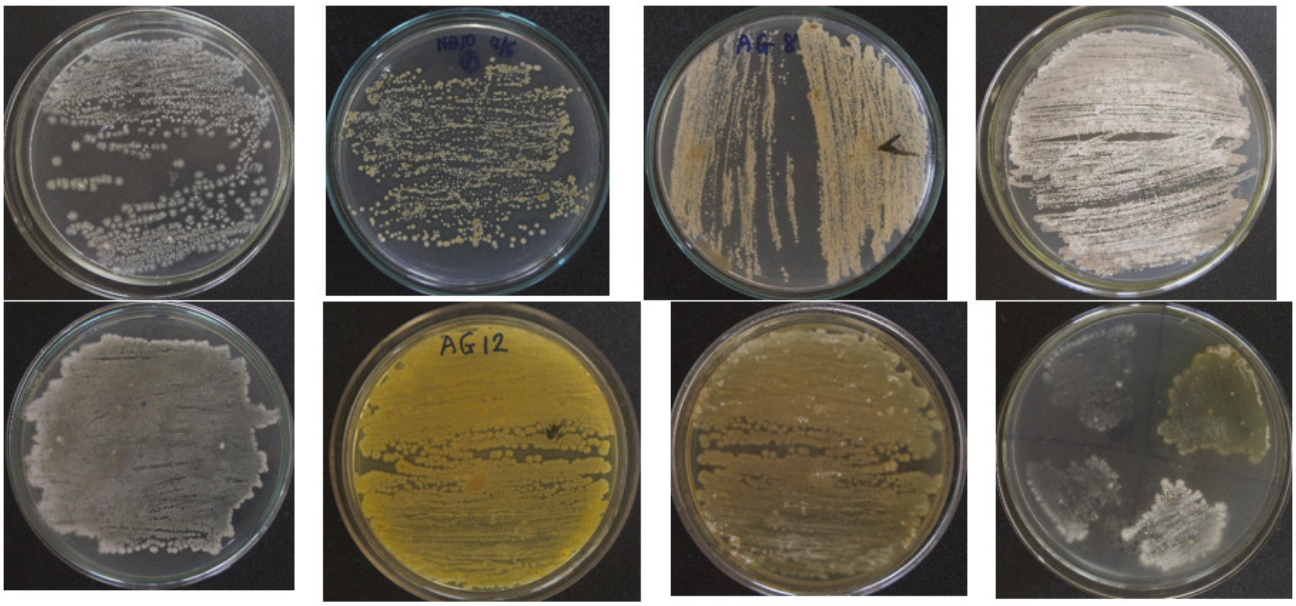

Figure 1. The colonies of isolated *Streptomyces*.

Table 2. Antimicrobial activity of *Streptomyces* strains.

No.	Strains	Inhibition halos (mm)			
		E.HOU/AG21	A.HOU/DT14	HOU/BT01	VH/BT12
1	AG8.9	19.27 ± 0.50	21.77 ± 0.43	27.83 ± 0.27	26.53 ± 0.23
2	AG09	10.43 ± 0.56	14.00 ± 0.72	10.37 ± 0.49	12.47 ± 0.49
3	AG12.2	21.93 ± 0.18	18.70 ± 0.21	28.93 ± 0.35	27.23 ± 0.20
4	TBL7	8.37 ± 0.33	9.07 ± 0.23	8.60 ± 0.23	11.33 ± 0.35
5	TBL1	6.87 ± 0.09	14.00 ± 0.12	16.70 ± 0.26	18.10 ± 0.12
6	ND1.3	11.13 ± 0.27	8.93 ± 0.12	10.60 ± 0.23	11.90 ± 0.15
7	ND10.1	20.80 ± 0.35	23.13 ± 0.20	28.87 ± 0.19	27.23 ± 0.15
8	QT02	15.87 ± 0.35	8.70 ± 0.15	9.03 ± 0.26	11.87 ± 0.24
9	TB 1.6	25.93 ± 0.19	28.53 ± 0.26	28.90 ± 0.21	26.77 ± 0.15
10	TBL4	11.77 ± 0.24	7.67 ± 0.33	11.07 ± 0.43	12.00 ± 0.17
11	ND5.3	21.03 ± 0.33	19.83 ± 0.35	27.30 ± 0.47	22.97 ± 0.44

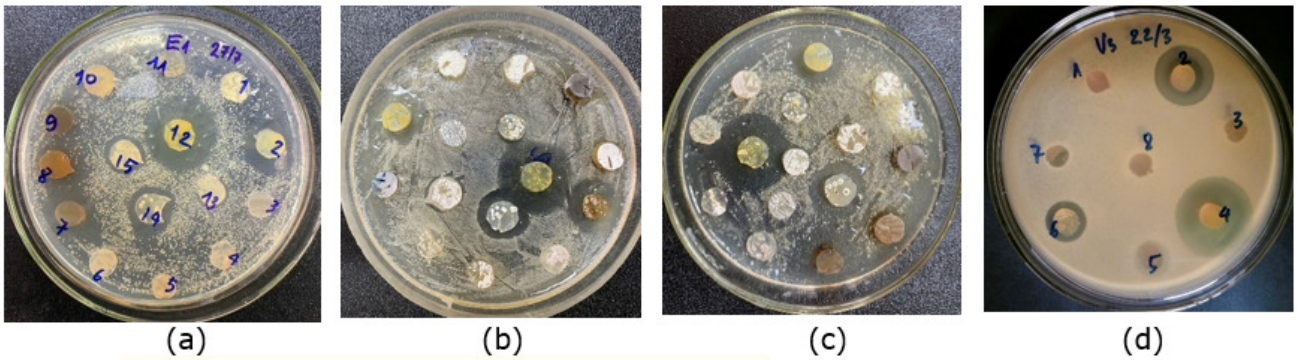

Figure 2. Antimicrobial activity of *Streptomyces* against (a) *E. ictaluri* E.HOU/AG21, (b) *A. hydophila* A.HOU/DT14, (c) *V. harveyi* VH/BT12, (d) *V. parahaemolyticus* V.HOU/BT01.

Table 3. Extracellular enzymatic activity of *Streptomyces* strains.

No.	Strains	Hydrolytic halos (mm)		
		Amylase	Cellulase	Protease
1	AG8.9	6.53 ± 0.24	1.93 ± 0.22	3.60 ± 0.35
2	AG09	4.83 ± 0.32	3.07 ± 0.32	3.73 ± 0.29
3	AG12.2	30.30 ± 0.67	22.50 ±1.13	28.10 ± 0.15
4	TBL7	5.87 ± 0.23	3.40 ± 0.47	6.73 ± 0.24
5	TBL1	26.27 ± 0.24	21.43 ± 0.58	24.73 ± 0.24
6	ND1.3	12.10 ± 0.26	8.93 ± 0.32	11.37 ± 0.52
7	ND10.1	32.13 ± 0.82	19.60 ± 0.55	29.33 ± 0.47
8	QT02	11.47 ± 0.41	10.07 ± 0.35	10.37 ± 0.55
9	TB 1.6	13.37 ± 0.43	13.73 ± 0.19	10.47 ± 0.41
10	TBL4	7.07 ± 0.12	4.27 ±0.43	11.43 ± 0.45
11	ND5.3	11.63 ± 0.43	9.40 ± 0.44	14.23 ± 0.74

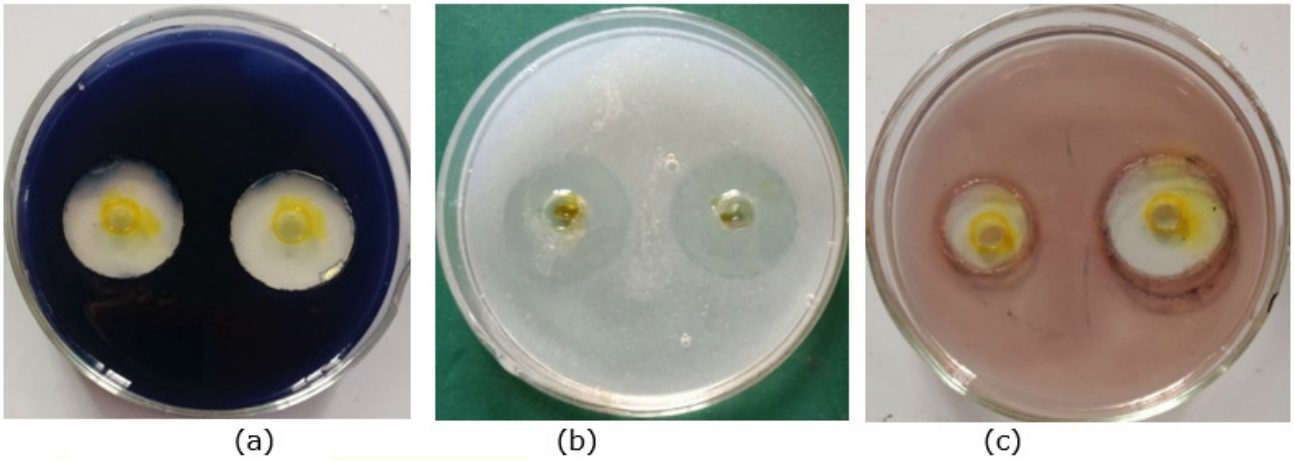

Figure 3. Enzyme activity of *Streptomyces* AG12.2, ND10.1; (a) amylase, (b) protease, (c) cellulase.

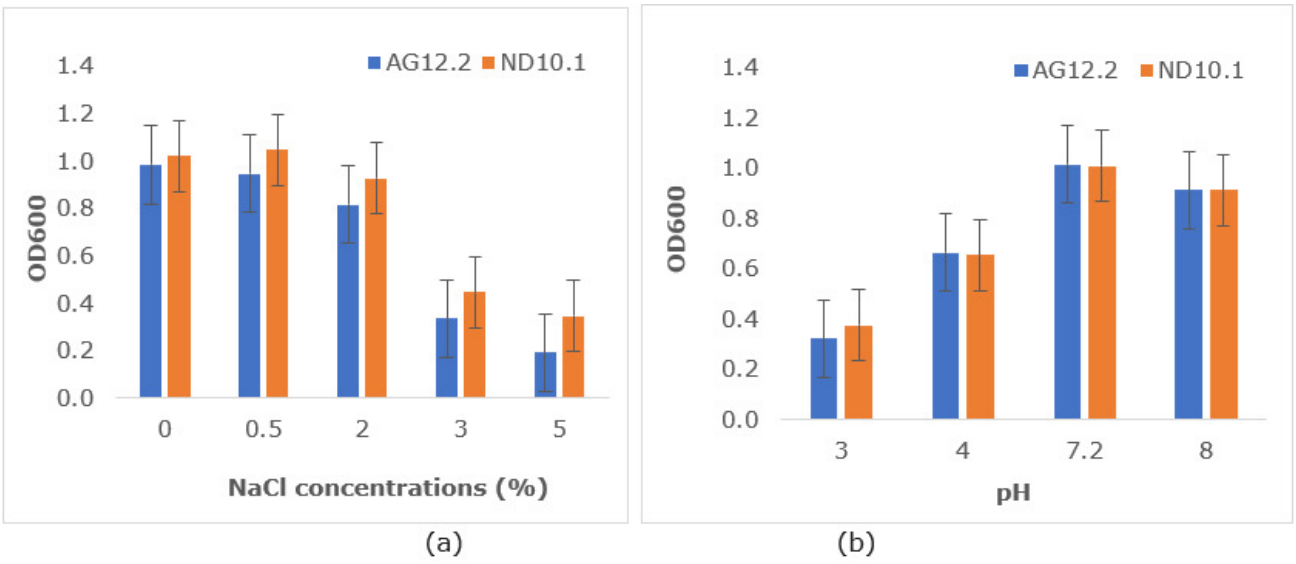

Figure 4. Optical density of *Streptomyces* biomass: (a) salt tolerance, (b) pH tolerance.

Table 4. Effect of *Streptomyces* AG12.2 and ND10.1 in shrimp growth performance.

Trial	WG (%)	DGW (g/day)	FCR
AG12.2	$100.63^{a} \pm 3.34$	$0.18^{a} \pm 0.003$	$1.14^{a} \pm 0.02$
ND10.1	$102.07^{a} \pm 3.67$	$0.18^{a} \pm 0.012$	$1.10^{a} \pm 0.13$
Control	$63.35^{b} \pm 4.35$	$0.11^{b} \pm 0.009$	$1.48^{b} \pm 0.15$

Data with different superscripts in the same column indicate significant differences ($p < 0.05$).

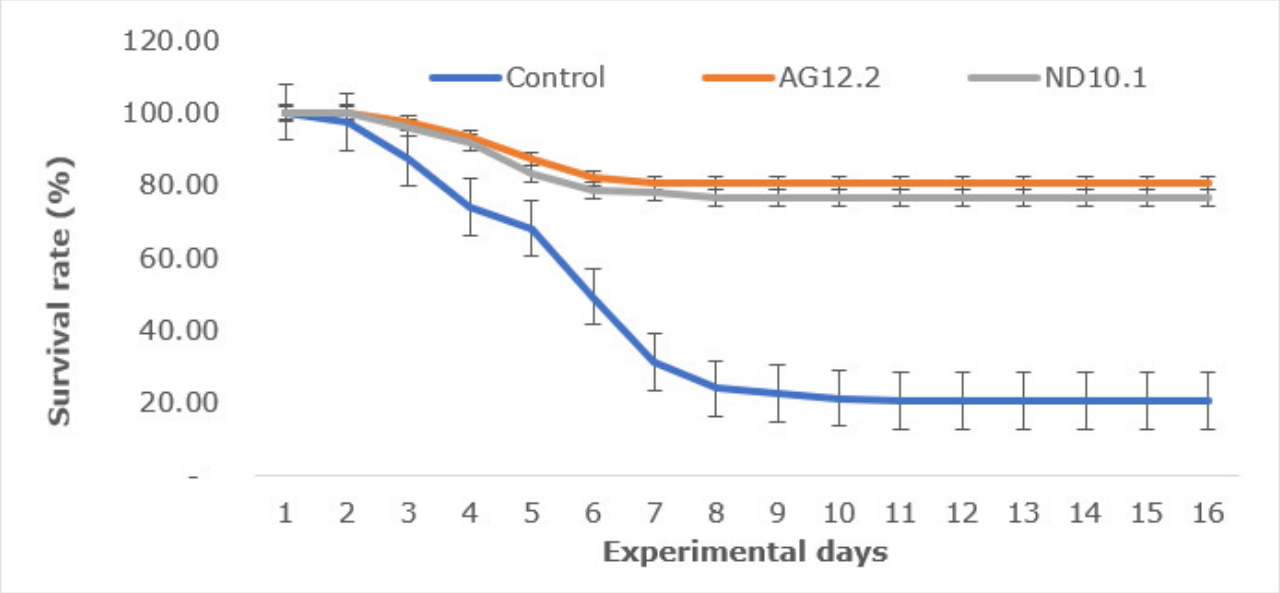

Figure 5. Survival rate of shrimp challenged with *V. paraharmolyticus* fed a diet containing *Streptomyces* AG12.2, ND10.1.

Table 5. Identification of the selected *Streptomyces* trains.

Strain code	Species	Similarity rate	Genbank accession No.
ND10.1 (XK22)	*Streptomyces angustmyceticus*	100% *Streptomyces angustmyceticus* KL6B (OK335988) 100% *Streptomyces angustmyceticus* S2 (F712390) 99,93% *Streptomyces angustmyceticus* K93G4 (T124462)	OR122322
AG12.2 (XK8)	*Streptomyces kunmingensis*	99,21% *Streptomyces kunmingensis* DSD1764 (MW217139) 99,13% *Streptomyces kunmingensis* DSD250 (MW217098) 99,06% *Streptomyces kunmingensis* MJ35 (MH656779)	OR122316

DISCUSSION

ISOLATION AND SCREENING OF *STREPTOMYCES*

In this study, *Streptomyces* were isolated from all samples collected in several different ecological regions of Vietnam. The characteristics of salinity (0 ppt - 22 ppt), and pH (7.1 - 8.1) of the sample collection areas showed that *Streptomyces* are widely distributed in wetlands in Vietnam. The soil along riversides and sediments in shrimp ponds, fish ponds, and coastal estuaries are environments rich in organic matter, nitrogen, and total phosphorus, which are optimal for the growth of *Streptomyces*.[19-21] The results of this study reveal that 18.97% of isolated strains were resistant to pathogenic bacteria in pangasius and shrimp, such as *E. ictaluri*, *A. hydrophila*, *V. parahaemolyticus*, and *V. harveyi*. Those strains can also hydrolyze starch, cellulose, and protein. Previous studies have also isolated and described the antibacterial, antifungal, and enzymatic activities of *Streptomyces* isolated from soil in different ecological regions in Vietnam[20-22]; however, this is the first time that *Streptomyces* have been isolated with a broad antibacterial spectrum against various pathogens that caused severe disease in shrimp and pangasius such as hemorrhagic disease and bacillary necrosis of pangasius, and luminous vibriosis, and

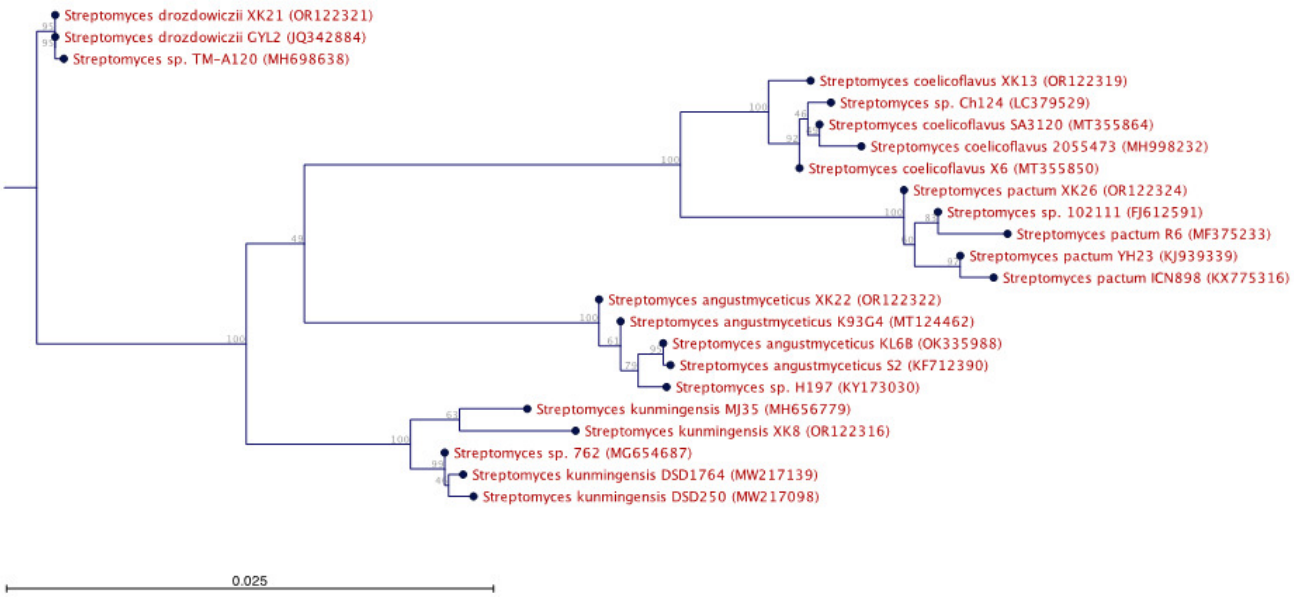

Figure 6. Phylogenetic relationships based on 16S rRNA gene sequences between two selected *Streptomyces* strains and closely related species.

Evolutionary analysis was performed based on the neighbor-joining method using the CLC Workbench software (version 22.0, Qiagen, Hilden, Germany). The bar represents 0.025 nucleotide substitutions per alignment position, and the numbers above the branches are the bootstrap values. The GenBank sequence accession numbers are in brackets after the strain names.

acute hepato - pancreatic necrosis disease of shrimp; furthermore, they also produce a broad spectrum of extracellular enzymes that can hydrolyze several macromolecular polymers.

SALT TOLERANCE AND PH TOLERANCE

As mentioned above, this study aims to find out *Streptomyces* strains with proper probiotic activity to develop biological products for application in aquaculture. Vietnam is a country that cultivates a diverse range of aquatic species that are farmed in inland, marine, and brackish waters[23]; therefore, the probiotic strains selected should be able to adapt to a multitude of environmental conditions, especially salinity and pH. In addition, developing probiotics for the feeding of livestock also needs to be considered in terms of pH tolerance in the digestive tract. Previous studies have reported that invertebrates lack gastric acid in the stomach, while the pH in the digestive tract of freshwater fish, which is normally in the range of 6.5 to 8.0, depends on the environmental temperature.[24,25] The results of this study show that the two selected *Streptomyces* strains (AG12.2 and ND10.1) are adaptive to the salinity in the range of 0 ppt to 5 ppt and pH in the range of 3.0 to 8.0, in which they grow best at the salinity from 0 ppt to 2 ppt, and pH in the range of 7.2 to 8.0. Thus, these two strains are suitable for developing probiotics that are added to feed aquatic animals raised in Vietnam's fresh, brackish, and coastal water.

GROWTH PERFORMANCE, FEED CONVERSION RATIO, AND SURVIVAL RATE OF CHALLENGED SHRIMP

The present study on whiteleg shrimp shows that AG12.2 and ND10.1 could promote growth performance in shrimp, and at the same time, they also significantly improve feed efficiency. In the control group, WG, DWG, and FCR were 63.35%, 0.11 g/day, and 1.48, respectively, while the growth parameters of shrimp fed with AG12.2 and ND10.1 were as follows: WG was as high as 100.63% and 102.07%, DWG was as high as 0.18 g/day, FCR was as low as 1.14 and 1.10, respectively. These *Streptomyces* strains effectively protected whiteleg shrimp against *V. parahaemolyticus*; in the challenged control group (fed with diets without *Streptomyces*), the survival rate of shrimp was only 20.67%, while in groups fed with AG12.2 and ND10.1, the figures were 80.67% and 76.67%, respectively. Previous studies on the effects of *Streptomyces* on the growth in whiteleg shrimp have reported that DWG of shrimp fed with *Streptomyces parvulus* reached 0.12 g/day,[26] and WG of shrimp fed with *Streptomyces* DH3.4 increased by 1.08 times.[27] Reported by García-Bernal et al.[28] showed that *L. vannamei* infected with *V. parahaemolyticus* was protected by *Streptomyces* RL8 and N7, the survival rate of shrimp fed with RL8 and N7 was 84.44 ± 5.7% and 57.77 ± 6.8%, respectively; meanwhile, the survival rate of challenged shrimp fed with the diet without *Streptomyces* was 11.11 ± 9.0%.

IDENTIFICATION OF THE SELECTED *STREPTOMYCES* STRAINS

Through the application of 16s RNA gene sequencing, AG12.2 and ND10.1 were identified to belong to the species *Streptomyces kunmingensis* and *Streptomyces angustmyceticus,* respectively. These species have been shown to possess many beneficial characteristics, such as the ability to produce angustmycin with anti-mycobacterial and cytokinin activities, antifungal compounds, biosurfactant compounds, and antioxidant compounds.[29,30] This finding

shows that *Streptomyces* AG12.2 and ND10.1 are potential probiotics that cannot only be supplemented to the feeding regimen of aquatic animals to improve the growth performance, prevent diseases, and increase feed efficiency but also can be used to enhance the quality of aquaculture environment.

CONCLUSION

The typical characteristics of the two strains *Streptomyces* AG12.2 and ND10.1, which include (1) broad-spectrum antibacterial activity against many pathogenic bacteria, (2) ability to decompose macromolecular compounds, (3) salt and pH tolerance, (4) protective effect of infected whiteleg shrimp, and (5) improvement of growth performance as well as feed conversion ratio, show that these strains are excellent candidates for producing probiotics having various beneficial properties for application in aquaculture.

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COMPETING OF INTEREST – COPE

The authors of the research declare that there is no conflict of interest regarding financial support or personal relationships.

ETHICAL CONDUCT APPROVAL – IACUC

The study was approved by the Animal Ethics Committee of Ha Noi Open University (protocol code 101/TB-HĐTVĐĐĐV, dated March 18, 2021).

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All data are available upon reasonable request.

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