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Isolation and characterization of aerobic actinomycetes with probiotic properties in Nile tilapia

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ABSTRACT

This study scoped the isolation of aerobic actinomycetes with probiotic properties against bacterial pathogens in Nile tilapia. Eleven rhizosphere soil samples were collected from the agricultural sites in three provinces (Chanthaburi, Nan, and Chachoengsao) of Thailand. A total of 157 actinomycete-like colonies were successfully isolated. The antibacterial testing against four tested bacteria (*Streptococcus agalactiae* 2809, *Aeromonas jandaei* 1929, *Aeromonas veronii* 1930, and *Edwardsiella ictaluri* 2234) was carried out by a modified cross-streaked method. The results showed that 108 strains possessed antibacterial activity against at least one of the bacterial pathogens. Seventeen active isolates were identified in the actinomycetes by the analysis of the partial 16S ribosomal rRNA gene, and the phylogenetic relationships of the isolates and their closely related strains were confirmed by the neighbor-joining method. Isolates LNW002 and YNW004 survived in the liquid cultivation with International Streptomyces Project 2 at pH 2 and the presence of 0.3% bile salt for 2 hours, which mimics the gastric acidity and bile salt in the gastrointestinal tract of Nile tilapia *in vitro*. In conclusion, these strains might be further investigated for their efficacy as probiotics in Nile tilapia. This study is the first to report on anti-*E. ictaluri* activity in streptomycetes.

INTRODUCTION

Nile tilapia or Pla Nin (*Oreochromis niloticus*) is a freshwater fish with great economic importance in Thailand and represents the highest quantity of freshwater aquaculture at two hundred thousand tons and a cost of nearly 12 billion baht since 2012 until 2016 (Ministry of Agriculture and Cooperatives (Thailand), 2016). Moreover, they are also one of the most productive international freshwater food fish at four million tons, with eight billion US dollars in 2016 (Wang and Lu, 2016). Due to the continual increase in global food demand, several countries, including Thailand, have tried to increase aquaculture production. Therefore, intensive farming has been adopted, and subsequently, bacterial or viral infections have been reported in Thailand and other countries (Dong *et al*., 2015; Kannika *et al*., 2017; Win *et al*., 2017) that are caused by several bacterial pathogens, including *Streptococcus agalactiae*, *Vibrio cholera*, *Aeromonas veronii*, and *Edwardsiella ictaluri*. From previous reports, disease outbreaks in the floating cage of cultured Nile tilapia were detected in Mekong river, and the example of observed symptoms caused by *A. veronii* was hemorrhages in the internal organs, e.g., fins and liver (Dong *et al*., 2015).

Nowadays, the conventional treatment for bacterial infection in Nile tilapia is to apply antibiotics. However, antibiotic residue and antibiotic-resistant bacteria in Nile Tilapia were also observed in several countries, like Ghana (Donkor *et al*., 2018) and China (Zhang *et al*., 2018), which lead to rejection due to the

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food safety regulations. Organized animal husbandry practices and the use of antibiotic alternatives, such as vaccination, probiotics, phage therapy, and essential oils, are recommended to reduce the number of antimicrobial residues in aquaculture and consequently in food safety effects (Okocha *et al*., 2018). Probiotics are defined as active microorganisms providing positive health effects to the hosts (de Vrese and Schrezenmeir, 2008). The proposed mechanisms of beneficial properties of probiotics comprise the reduction of pathogenic microorganisms, the promotion of the growth rate of the host, and improving the water quality (Babu *et al*., 2018; Das *et al*., 2010). The application of probiotics in aquaculture was initially evaluated by several research groups to ensure host safety and effectiveness against infectious diseases (Chen *et al*., 2019; Das *et al*., 2008; Tan *et al*., 2016). The criteria concerned with probiotic strains included the ability to tolerate the environment within the gastrointestinal (GI) tract of the host, the ability to adhere to the host epithelial cells, antibacterial activity to the pathogenic bacteria, and also the ability to degrade the complex biomolecules for better nutrient absorption in the host (de Vrese and Schrezenmeir, 2008; Markowiak and Ślizewska, 2018).

Actinomycetes are mostly Gram-positive, filamentous bacteria in the phylum Actinobacteria, with high G+C DNA content in their genomes (Barka *et al*., 2016). They are ubiquitous with a wide range of natural habitats, soil, sediment, mangrove, and marine, and are well known as producers of secondary metabolites, which constitutes about 30% of the total microbial metabolites. Until now, more than 10,000 bioactive metabolites had been isolated from actinomycetes (Bérdy, 2012). Especially, many commercial antibiotics are derived from the genus *Streptomyces*, e.g., chloramphenicol and streptomycin, and from *S. venezuelae* and *S. griseus*, respectively (Kieser *et al*., 2000). Not only are actinomycetes the source for bioactive compounds, but they also include the important industrial enzymes, e.g., lignocellulolytic enzymes (Kumar *et al*., 2016; Saini *et al*., 2015; Shivlata and Satyanarayana, 2015).

Several pieces of research focus on searching for new bioactive compounds or new activity in Thai actinomycetes (Euanorasetr *et al*., 2015; Intra *et al*., 2011; Ser *et al*., 2017). However, no recent research has focused on the actinomycete strains with antibacterial activity against bacterial pathogens in Nile tilapia. Several pieces of research indicate that actinomycetes are promising sources that can be used as probiotics for aquaculture (Das *et al*., 2008; Tan *et al*., 2016). Therefore, in this study, the isolation of actinomycete strains from rhizosphere soil and their antibacterial activity was initially evaluated against four bacterial pathogens (*S. agalactiae* 2809, *A. veronii* 1930, *Aeromonas jandaei* 1929, and *Edwardsiella ictaluri* 2234) in Nile tilapia and the potent strains were also identified by 16S ribosomal ribonucleic acid (rRNA) gene analysis. Moreover, the acid tolerance and bile salt tolerance tests were also investigated to suggest the viability in the GI tract of the fish.

MATERIALS AND METHODS

Tested bacteria

Streptococcus agalactiae 2809, *A. veronii* 1930, and *A. jandaei* 1929 were grown on a nutrient agar (1% beef extract, 1% peptone, 0.5% sodium chloride, and 1.5% agar) for 2 days at room temperature, except *E. ictaluri* 2234 was grown at 30°C for 2 days. Tested bacteria were kindly provided by Associate Professor Triwit Rattanarojpong, from the Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Thailand, and Dr. Saengchan Senapin, from the Fish Health Platform, Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Bangkok, Thailand.

Collection of soil samples and soil analysis

Soils were collected around the plant root at a depth of 10–15 cm from the surface using the procedure from a previous research (Intra *et al*., 2011). There were 11 soil samples from three provinces: Chanthaburi (GPS 12°35′49.5″N 102°01′38.0″E), Chachoengsao (13°42′57.8″N 101°09′29.4″E), and Nan (18°47′53″N 100°32′34″E). The soil pH was evaluated by adding 1 g of soil sample to 5 mL of distilled water, mixed well until homogenous, and kept for 60 minutes. Finally, the pH of supernatants was measured with a pH meter (Hendershot *et al*., 2008). Gravimetric soil water content was determined by oven drying at 110°C for 2 hours and was calculated by the previous procedure (Standards Association of Australia, 1977).

Isolation of actinomycetes and their characteristics

The soil samples were air-dried for 1 week. After that, the samples were divided into two conditions: non-heat and heat. The heat condition was 100°C for 1 hour in the oven. One gram of soil sample was dissolved in 0.85% normal saline solution and serially diluted to 10^{-4}. One hundred µl aliquots was spread on two isolation media: starch casein agar [starch casein agar (SCA); 1% starch, 0.03% casein, 0.2% potassium nitrate, 0.005% magnesium sulfate, 0.2% dipotassium hydrogen phosphate, 0.2% sodium chloride, 0.003% calcium carbonate, 0.001% Iron II sulfate, and 1.8% agar] and water proline agar (1% proline and 1.2% agar), with nalidixic acid and cycloheximide as the final concentrations of 50 and 25 µg/ml, respectively, at room temperature for 4–6 weeks. The total number of actinomycete-like colonies (with leathery, powdery, or butyrous characteristic) was counted as average on two replicating dilution plates. Actinomycete-like colonies were picked and purified in the International Streptomyces Project 2 (ISP2; 0.4% yeast extract, 1% malt extract, 0.4% glucose, and 2% agar) media. The actinomycete morphology was examined on ISP2 agar using a slide culture technique with a bright field light microscope (Kieser *et al*., 2000; Shirling and Gottlieb, 1966).

Antibacterial activity of actinomycetes

The actinomycete strains were grown in 301 production agar (2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, 0.4% calcium carbonate, and 1.5% agar) (Euanorasetr *et al*., 2010) by streaking at the center of the plate for 7 days. The tested bacteria were perpendicular, cross-streaked to the line of actinomycetes, and the plates were incubated at room temperature for 48 h (Balouiri *et al*., 2016). The inhibition zones were observed and recorded in millimeters compared to that of the control plate (without actinomycetes).

16S rRNA gene amplification and phylogenetic tree analysis

The genomic DNA of actinomycetes was extracted by freeze-thawing and boiling methods (Euanorasetr *et al*., 2010), while the fragment of partial 16S rRNA gene was amplified by

polymerase chain reaction (PCR) using the primers 11F and 925R (Tajima *et al*., 2001), with the following conditions: initial denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 3 minutes. The PCR products were confirmed by agarose gel electrophoresis and further purified by AccuPrep PCR Purification Kit (Bioneer, Korea). The purified PCR products were sequenced by the U2Bio Company, Thailand. The partial 16S rRNA sequences (≈900 bp) were analyzed with EzBioCloud (https://www.ezbiocloud.net/) (Yoon *et al*., 2017) and were updated on the National Center for Biotechnology Information (NCBI) database with the accession number MH470491–MH470510. After that, partial 16S rRNA gene sequences from isolates were aligned with multiple available sequences of actinomycete type strains from the NCBI database by using the MUSCLE program (Edgar, 2004), while the 16S sequence of *Bacillus subtilis* DSM 10^T (AJ276351) was used as the out group. The phylogenetic relationship was inferred by the neighbor-joining method (Saitou and Masatoshi, 1987) using the MEGA version 7.0.26 (Kumar *et al*., 2016).

Acidity and bile salt tolerance test

Five milliliters of ISP2 broth with pH ranges from 2 to 7 or 0.3% bile salt was prepared by adding 0.1 M HCl or bile salt (Himedia, India) to the ISP2 broth before sterilization with an autoclave. From the five strains with consistent antibacterial activity (YNW004, SHS004, LNW002, ZNW001, and TNW007), only one loop of each actinomycete strain was inoculated to the adjusted pH or bile salt-containing media. After incubation in the incubator shaker at room temperature with 200 rpm for 2 hours, one hundred microliters of the culture broth was spread on the ISP2 agar and incubated at room temperature for 7 days. The level of the tolerance was evaluated by counting the observed actinomycete colonies: 1–100 colonies represented +, 101–200 colonies represented ++, and more than 200 colonies represented +++, while none of the colony represented −.

RESULTS AND DISCUSSION

Soil analysis

Eleven soil samples were collected from three provinces: five samples from Chanthaburi [Longkong (*Aglaia dookkoo*), lemongrass (*Cymbopogon citratus*), fingerroot (*Boesenbergia rotunda*), salak (*Salacca zalacca*), and durian (*Durio zibethinus*)]; three samples from Chachoengsao [*Crudia chrysantha*, devil tree (*Alstonia scholaris*), *Bruguiear sexangula*]; and three samples from Nan [star apple (*Chrysophyllum caimito*), Ormosia (*Afzelia xylocarpa*) and longan (*Dimocarpus longan*)]. The soil pH was in the range of 3.58–6.71 which was quite acidic, and the percentage of water in the soil was in the range of 2.60–8.26, as summarized in Table 1.

Amount of actinomycete and isolation

The actinomycete-like colonies on isolation plates were observed to have leathery or powdery characteristics according to the general characteristics of the Actinobacteria (Barka *et al*., 2016). The abundance of soil actinomycetes in each isolating condition is shown in Supplementary Figure 1. The comparison of four isolating conditions revealed that non-heat treatment with SCA gave the highest amount of actinomycetes at 6 log colony forming unit (CFU)/g, whereas heat treatment with SCA gave the lowest amount at 3.5 log CFU/g. From Table 1, the average amount of the observed actinomycetes on heat treatment (3.08

Table 1. Detailed information on each soil sample with the amount of actinomycetes isolation.

Rhizosphere	Geographical origin	pH	% Water in soil	Starch casein agar (SCA)				WP (Water proline agar)			
				Non heat		Heat		Non heat		Heat	
				CFU/g soil	No. of isolate	CFU/g soil	No. of isolate	CFU/g soil	No. of isolate	CFU/g soil	No. of isolate
Longkong	12°35′49.5″N 102°01′38.0″E	4.99	3.53	1.31×10^5	2	2.41×10^3	2	2.28×10^5	8	2.45×10^4	7
Lemon grass	12°35′49.5″N 102°01′38.0″E	5.90	5.32	6.55×10^4	1	3.02×10^3	4	1.04×10^6	5	2.02×10^5	7
Fingerroot	12°35′49.5″N 102°01′38.0″E	5.28	8.26	1.83×10^5	4	3.44×10^3	4	3.49×10^5	3	6.00×10^4	5
Salak	12°35′49.5″N 102°01′38.0″E	4.32	4.49	3.75×10^4	0	3.03×10^3	0	1.48×10^5	6	1.98×10^5	3
Durian	12°35′49.5″N 102°01′38.0″E	5.40	4.73	1.74×10^5	1	1.05×10^3	0	4.64×10^5	2	2.25×10^4	4
Star apple	18°47′53″N 100°32′34″E	5.37	4.24	1.52×10^4	3	3.95×10^3	4	4.11×10^5	5	7.01×10^3	0
Ormosia	18°47′53″N 100°32′34″E	6.71	5.12	7.2×10^6	13	2.47×10^3	0	2.88×10^6	4	9.75×10^4	4
Longan	18°47′53″N 100°32′34″E	4.97	3.63	3.20×10^4	3	3.66×10^3	0	1.66×10^5	5	4.35×10^3	3
C. chrysantha	13°42′57.8″N 101°09′29.4″E	4.02	2.60	4.05×10^4	2	3.25×10^3	0	6.98×10^5	7	3.30×10^3	0
Devil tree	13°42′57.8″N 101°09′29.4″E	3.58	3.10	1.55×10^5	1	3.25×10^3	0	3.73×10^4	3	3.25×10^3	1
Bruguiera sexangular	13°42′57.8″N 101°09′29.4″E	6.02	3.14	3.30×10^6	2	3.10×10^3	8	3.47×10^5	11	2.21×10^4	10

× 10^4 CFU/g) was lower than that of non-heat treatment (8.23 × 10^5 CFU/g). In this study, the heat treatment was included in the isolation process in order to increase the chance of discovering rare actinomycetes and exclude other bacteria and fungi. Heat resulted in reducing the amount of fast-growing or filamentous bacteria, including streptomycetes (Hayakawa, 2008). The total isolates were lower than the number of actinomycetes in the soil at 10^4–10^8 CFU/g from a previous report (Bhatti *et al*., 2017), which might be explained by the fact that the soil samples were in acidic condition (Table 1) and most soil actinomycetes are mesophilic (Goodfellow and Williams, 1983). From Table 1, 54 and 103 actinomycete strains were isolated from SCA and WP, respectively. Notably, most heat-tolerant actinomycetes (18 isolates) were isolated from soil under Ormosia, while most heat-sensitive actinomycetes (13 isolates) were also isolated from soil under Ormosia.

All isolates were purified in a single colony ISP2 agar and the characteristics of Actinobacteria were examined under the bright field microscope. One of the isolates, strain LNW002, grew well on ISP2 agar with gray aerial mycelium with sporulation. A brown diffusible pigment was also present in the cultures grown on ISP2 medium. Colonies and sporulating mycelia of strain LNW002 are shown in Figure 1A and B. Observation using light microscopy revealed branching vegetative mycelium and aerial hyphae with 10–30 spores in a chain (Fig. 1B). Morphologically, the characteristics of this strain belonged to the genus *Streptomyces* with the presence of mycelium and a long chain of spores. Streptomycetes are generally characterized as aerobic, Gram-positive, non-acid-fast bacteria that form an extensively branched substrate and aerial mycelia. At maturity, the aerial mycelium forms chains of three or more spores (Goodfellow, 2012).

Antibacterial pathogens in Nile tilapia from actinomycetes

A total of 157 isolated actinomycetes were tested against four bacterial pathogens in Nile tilapia (*S. agalactiae* 2809, *A. veronii* 1930, *A. jandaei* 1929, and *E. ictaluri* 2234). The results showed that 108 strains (69%) showed antibacterial activity against at least one bacterial pathogen. From these active strains, most actinomycetes (98 strains) showed antibacterial

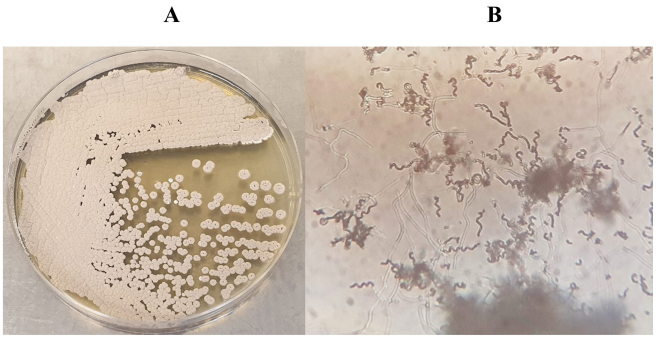
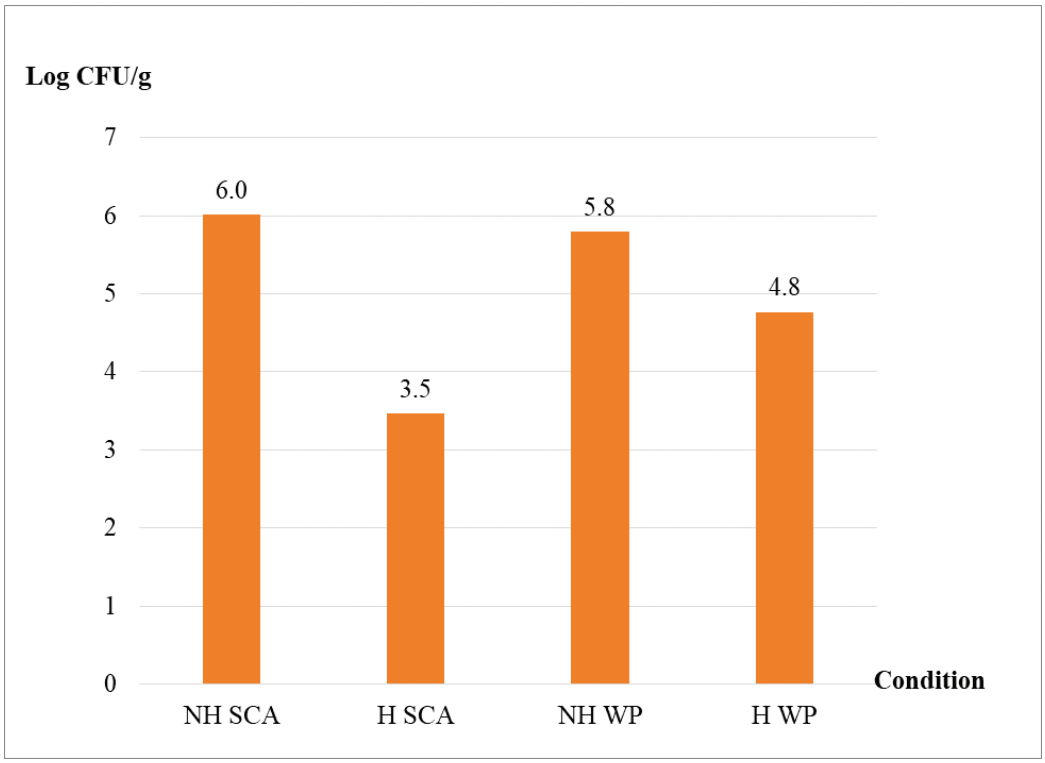
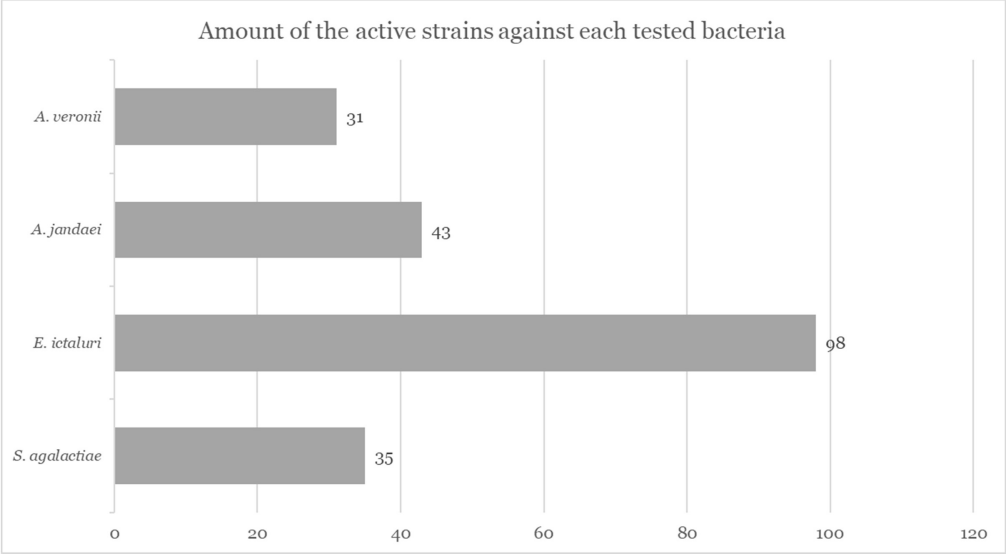


Figure 1. *Streptomyces* spp. LNW002; The colony morphology of LNW002 grown for 8 days at room temperature on yeast extract–malt extract agar (ISP2). Front side (A) and back side (B). The mycelia (×1,000) on ISP2 agar for 8 days at room temperature.



Supplementary Figure 1. Amount of actinomycetes in each condition. NH SCA means non-heat condition with SCA, H SCA means heat condition with SCA, NH WP means non-heat condition with WP, and H WP means heat condition with WP.

activity against *E. ictaluri* 2234, while 31, 43, and 35 strains could inhibit *A. veronii* 1930, A. *jandaei* 1929, and *S. agalactiae* 2809, respectively (Supplementary Fig. 2). Some of the inhibition zone sizes were recorded and are shown in Table 2 and Supplementary Figure 3, respectively. Generally, actinomycetes demonstrate antibacterial activity against Gram-positive bacteria more than Gram-negative bacteria (Euanorasetr *et al*., 2010; Thakur *et al*., 2007). However, in this study, most active isolates showed antibacterial activity against Gram-negative *E. ictaluri*. One possible reason was that *E. ictaluri* was quite sensitive to several classes of antibiotics, such as aminoglycosides and quinolones (Waltman and Shotts, 1986). On the other hand, there was a report of natural *E. ictaluri* with resistance to streptomycin, oxytetracycline, and trimethoprim in Vietnam (Dung *et al*., 2008). To our knowledge, this study was the first to report on anti-*E ictaluri* activity in streptomycetes. Further purification of the bioactive compounds from strains with potent antibacterial activity with all tested bacteria (FNW003, FSH015, WNW026, and KNS006; Table 2) are promising. Moreover, antibiotics in a class of tetracycline (e.g., chlortetracycline, oxytetracycline, and tetracycline), flumequine, and deltamethrin were still allowed to be employed during the early bacterial infection in Nile tilapia in Thailand. Therefore, new bioactive compounds are still essential to treat the bacterial infection in Nile tilapia.



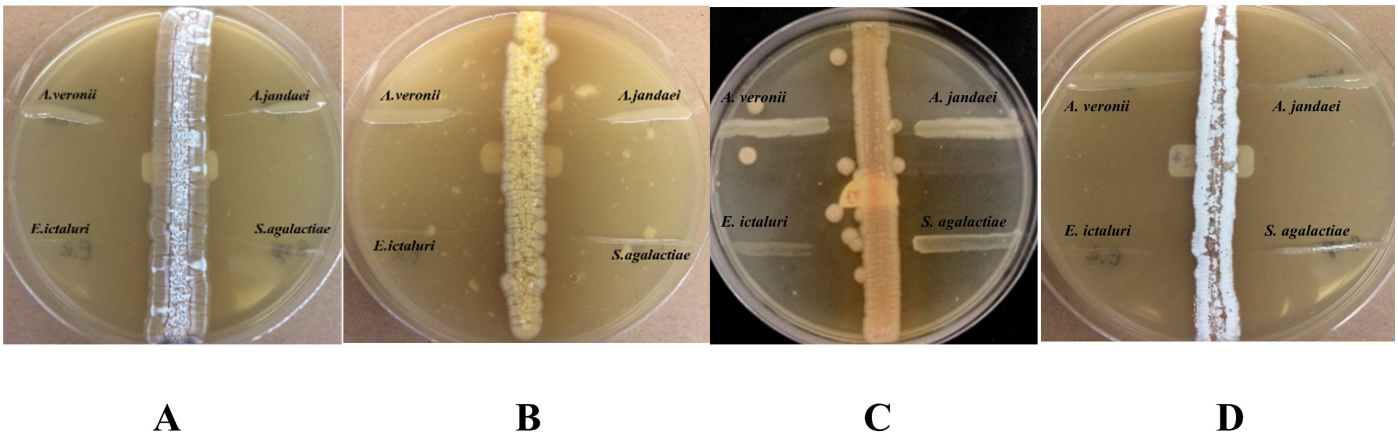
Supplementary Figure 2. Antibacterial activities of isolated actinomycetes against each bacterial pathogen in Nile tilapia.

Table 2. Inhibition zone of twenty actinomycetes isolates and tested bacteria.

Isolates/tested bacteria	Inhibition zone (mm.)			
	***S. agalactiae* 2809**	***A. jandaei* 1929**	***A. veronii* 1930**	***E. ictaluri* 2234**
FNW003	24	29	16	21
LNW002	15	–	–	11
DNW003	–	8	11	7
UNW008	10	7	–	5
WNW026	9	14	13	18
KNW004	5	–	–	15
KNW020	–	–	–	20
KNW005	–	–	–	14
ZNW001	–	13	8	5
YNW004	–	–	–	8
TNW007	–	–	–	8
KHS006	–	14	14	–
FHS015	19	12	30	20
YNS007	3	7	6	9
WHS020	–	–	–	14
KNS006	12	13	19	20
SNS004	4	7	5	15

Molecular identification of actinomycetes with antibacterial activity

Seventeen strains with strong antibacterial activity against one or all tested bacterial pathogens were chosen to be further identified by partial 16S rRNA gene sequence analysis. The expected PCR product size about 900 bp from 11F and 925R primers was observed (data not shown) and was purified and later sequenced. Results from the analysis by the EzBiocloud database revealed that most of the active strains belonged to the genus *Streptomyces* with the similarity percentage above 98%, except for strain KNS006, which was identified as *Nocardia nova* NITE Biological Resource Center (NBRC) 15556^T with 100% similarity (Table 3). Moreover, the phylogenetic tree of 16S rRNA sequences derived from the neighbor-joining analysis also supported the relationships between the isolates with other actinomycetes strains (Fig. 2). Because *Nocardia* sp. KNS006 possessed both antibacterial activity against all tested bacteria (Table 2), and there was no report on the bioactivity from *N. nova* NBRC 15556^T yet, but bioactive compounds purification from this strain is promising. Based on the 16S rRNA gene sequence analysis and the phylogenetic analysis, most of the active isolates were identified in the genus *Streptomyces*. Streptomycetes are abundant in the soil (Goodfellow and Williams, 1983) and several studies indicated the possibility to obtain this group of bacteria by using SCA and WP media supplemented with antibiotics (Euanorasetr *et al.*, 2010; Sripreechasak *et al.*, 2017). This fact is in agreement with a previous report that streptomycetes were the prominent sources of bioactive compounds from Actinobacteria (Bérdy, 2012). Strain SNS004 was interesting as the putative new species because of the lower percentage of 16S rRNA similarity than 99%. Therefore, further study on physiological and genotypic comparison of this strain with *Streptomyces hyaluromycini* NBRC 110483^T and other



Supplementary Figure 3. Examples of inhibiting antibacterial activities of isolated actinomycetes FNW003 (A), WNW026 (B), SNS003 (C), and LNW002 (D) against each bacterial pathogen.

Table 3. Identification of actinomycetes by partial 16S rRNA sequence and Ezbiocloud analysis.

Strain code	Nearest relatives	Accession number	% Similarity
FNW003	*Streptomyces albaduncus* JCM 4715^T	AY999757	100.00
LNW002	*S. fodineus* TW1S1^T	KT820007	99.63
DNW003	*Streptomyces lannensis* TA4-8^T	AB562508	100.00
UNW008	*Streptomyces ramulosus* NRRL B-2714^T	DQ026662	99.24
WNW026	*Streptomyces phaeoluteigriseus* DSM 41896^T	MPOH01000466	99.50
KNW004	*Streptomyces bungoensis* DSM 41781^T	KQ948892	99.63
KNW020	*Streptomyces coelicoflavus* NBRC 15399^T	AB184650	99.63
KNW005	*Streptomyces spiralis* NBRC 14215^T	AB184575	99.87
ZNW001	*S. bungoensis* DSM 41781^T	KQ948892	99.49
YNW004	*S. morookaense* LMG 20074^T	AJ781349	99.74
TNW007	*S. coelicoflavus* NBRC 15399^T	AB184650	99.62
KHS006	*S. bungoensis* DSM 41781^T	KQ948892	99.74
FHS015	*S. albaduncus* JCM 4715^T	AY999757	100.00
YNS007	*Streptomyces chattanoogensis* NRRL ISP-5002^T	LGKG01000206	99.25
WHS020	*Streptomyces omiyaensis* NBRC 13449^T	AB184411	99.87
KNS006	*N. nova* NBRC 15556^T	BDBN01000167	100.00
SNS004	*S. hyaluromycini* NBRC 110483^T	BCFL01000051	98.86

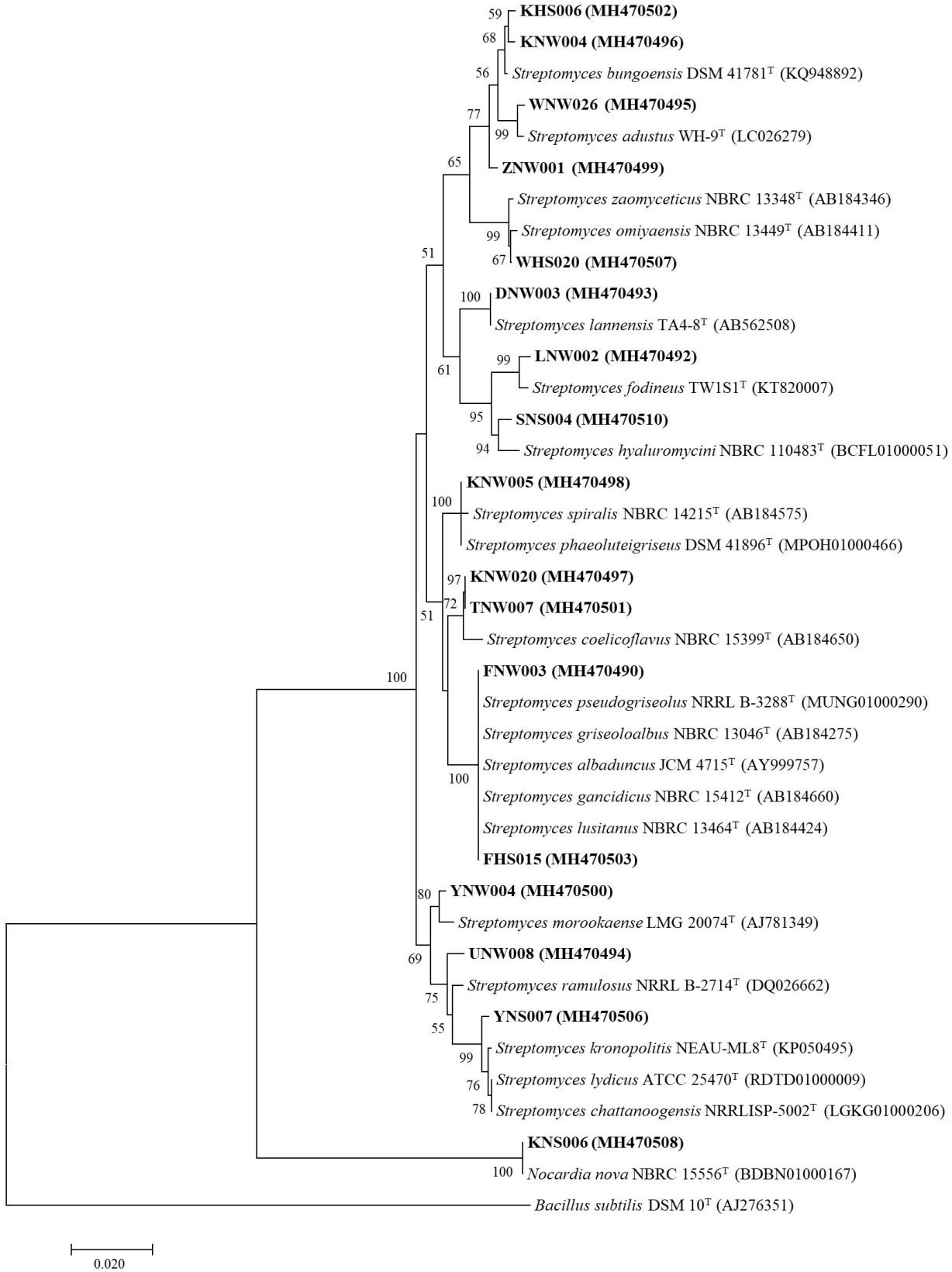


Figure 2. Neighbor-joining tree of the 16S rRNA gene sequences inferred the phylogenetic relationships between the isolated strains with other members of Actinobacteria. Bootstrap values calculated from 1,000 resamples with values over 50% were shown at the respective node. Bar 0.02 nucleotide substitutions per site.

Table 4. Tolerance test against acidic or 0.3% bile salt in ISP2 broth for 2-hours incubation of *Streptomyces* sp.

Strains	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	0.3% bile salt
YNW004	++	++	++	++	+++	+++	+++
SHS004	−	−	+	+	+	+	+
LNW002	++	+++	+++	+++	+++	+++	+++
ZNW001	−	−	+	++	++	++	++
TNW007	−	−	−	++	++	++	++

The level of tolerance was evaluated by counting the observed actinomycete colonies; 1–100 colonies represent +, 2–200 colonies represent ++, and more than 200 colonies represent +++, while none of the colony represent −.

related strains are required to verify the identification of new species. Previous studies indicated the production of bioactive compounds from some predicted strains. For examples, both FNW003 and FSH015, which inhibited all tested bacteria with large inhibition zone (>10 mm) (Table 2), were closely related to *S. albaduncas* which was reported to produce daunomycin with high anti-Gram-positive bacterial activity (Tsukiura *et al*., 1964).

Acid and bile salt tolerance test

After rechecking the antibacterial activity, five streptomyces isolates with consistent and potent activity (YNW004, SHS004, LNW002, ZNW001, and TNW007) were tested for acid and bile salt tolerance test, and the results are summarized in Table 4. LNW002 and YNW004 were tolerated in the liquid cultivation with ISP2 at the variety of acidic pH from pH 2 to 7 at 2 hours. Moreover, it could tolerate the presence of 0.3% bile salt, whereas the others could grow up since pH 4. From the literature, most Actinobacteria are mesophilic and grow in soils with a neutral pH, and they grow in the pH range from 6 to 9 (Barka *et al*., 2016), such that *Streptomyces* sp. MUM212, isolated from mangrove forests, could grow between pH 4 and 7 (Tan *et al*., 2017). In this study, the microorganisms were isolated from the acidic agricultural soil (Table 1), which lead to the excellent tolerance to acidity (Hagedorn, 1976). Isolates LNW002 and YNW004 were identified as *Streptomyces fodineus* TW1S1^T (KT820007) and *Streptomyces morookaense* LMG 20074^T (AJ781349) with similarity at 99.60% and 99.74%, respectively (Table 3). *S. fodineus* TW1S1^T was identified as the Actinobacterium with antifungal activity isolated from a mine area soil in Korea (Kim *et al*., 2019). Its growth occurred at pH 4–9 and in the presence of up to 8% (*w/v*) NaCl.

Probiotics are one of the alternative methods to control the pathogenic bacterial growth in aquaculture (Wang *et al*., 2019). Most of the common probiotic bacteria are lactic acid bacteria, and *Bacillus* (de Vrese and Schrezenmeir, 2008) ,and *Bacillus* sp. has been used in the animals because of their ability to produce antimicrobial compounds with excellent resistance to the gastric juice due to the endospore formation (Papadimitriou *et al*., 2015). In the case of streptomycetes, their practical application as probiotics in aquaculture was scarcely studied despite their potential to produce a variety of bioactive compounds. Not only antibiotics, but streptomycetes also represent the antibacterial activity via siderophore production (Yang *et al*., 2019) and the bacteriocin production (Farris *et al*., 2011). In a previous research, Das *et al*. (2010) conveyed the beneficial effects of streptomycetes-supplemented feed on black tiger shrimp, *Penaeus monodon*, with improved length, wet weight, and survival rate with/without the challenge of *Vibrio harveyi*. Another study by Babu *et al*. (2018) pointed out that granulated streptomycetes could treat the pollutant in *Penaeus monodon* rearing system. Moreover, Bernal *et al*. (2017) suggested that some *Streptomyces* strains alone or in combination with *Bacillus*, exerted probiotic properties through improving growth, modulating the immune response, adjusting host and water microbiota, and increasing disease resistance on white shrimp *Litopenaeus vannamei.* Recently, from microbiome analysis, the Actinobacteria were identified in the gut of Nile tilapia (Hallali *et al*., 2018), which indicated the possibility of using actinomycetes for probiotics. Since *Streptomyces* sp. LNW002 and YNW004 possess vigorous antibacterial activity against the bacterial pathogens in Nile tilapia and tolerate the acidic pH 2 and bile salt up to 0.3%, further study on other probiotic properties, e.g., adherence to the intestinal epithelium, exoenzyme production, as well as the host immunostimulant, is promising (Kaktcham *et al*., 2018; Markowiak and Śliżewska, 2018).

CONCLUSION

Bacterial infection in Nile tilapia causes a significant economic loss in Thailand and other countries. One of the possible preventive methodologies is to apply probiotics in the gut of Nile tilapia to enhance animal nutrition, immune response, and productivity. From this study, *Streptomyces* strains LNW002 and YNW004 proved to be potentially effective against bacterial pathogens in Nile tilapia. They also tolerated the mimic condition in the gut and small intestine of Nile tilapia. The experiments highlight the actinomycetes as alternative microorganisms to be developed as probiotics for an eco-friendly and sustainable aquaculture.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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