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Isolation and characterization of actinobacteria from Yalujiang coastal wetland, North China

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ABSTRACT

Objective: To evaluate various types of samples from the different marine environments as sources of actinomycetes from the Yalujiang coastal wetland, North China, and to screen their antimicrobial properties. Further, the identified actinomycetes were characterized based on morphological, biochemical, and physiological characteristics.

Methods: Eight different production media were used to isolate actinomycets from different stations of marine soil sediments in Yalujiang coastal wetland and the genotypic positions were established by 16S rDNA.

Results: A total of 172 actinomycetal isolates were obtained from 13 samples using five media. The most effective culture media in the isolation of actinobacteria were Gause's Synthetic agar and Starch-casein agar. Among 172 isolates, 46 isolates (26.74%) showed antibacterial activity, 70.93% belonged to the genus *Streptomyces*, others were *Micromonospora* spp. and *Rhodococcus* spp. Out of the 46 isolates, two cultures were further supported by morphological characterization analysis.

Conclusions: This is the first report about actinomycetes isolated from Yalujiang coastal wetland and it seems that the promising isolates from the unusual/unexplored wetland may prove to be an important step in the development of microbial natural product research.

1. Introduction

The marine ecosystem has been widely recognized as a source that nurtures abundant compounds that contain novel composition and organic characteristics. Wetlands are considered to be the most biologically important and productive ecosystems on earth[1]. They provide habitat, food, and spawning grounds for a large number of plants and animals and therefore exhibit great biodiversity[2].

Actinomycetes are the dominant group of soil population together with bacteria and fungi. Some reports described China wetland as a major source of actinomycetes[3,4]. Actinomycetes are one of the major microbial dominant groups and are well known for their saprophytic behaviour as well as for production of diverse bioactive secondary metabolites. They are also recognised for their capacity to survive in extreme habitats[5].

Recently, many scientists are searching new antibiotics from different untouched wetland to find out for their productions of antibiotics. Although hundreds of Chinese rivers discharge into the Northwest Pacific Ocean, little is known about the diversity of actinomycetes in marine sediments, which is an inexhaustible resource that has not been properly exploited for search and discovery of novel actinomycetes. The Yalujiang coastal wetland is one of the largest wetlands near Dandong City, located in North China and covers 108 057 hectares. The temperature is minimum of -28.2 °C, maximum of 33.9 °C and the mean annual temperature is 9.9 °C.

In order to exploit more laudable actinomycetes, it is necessary to better understand the diversity of actinomycetes in a single ecosystem of the Yalujiang coastal wetland, which covers both fresh water as well as marine ecotones and possesses hostile environments enriched with immense biodiverse values. The present study was designed to evaluate various types of samples from the different marine environments as sources of

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actinomycetes from the Yalujiang coastal wetland, North China, and to screen their antimicrobial properties. Further, the identified actinomycetes were characterized based on morphological, biochemical, and physiological characteristics.

2. Materials and methods

2.1. Sample collection

Our research was located in the Yalujiang coastal wetland (120°12'9" E to 123°30'50" E, 39°40'50" N to 40°30' N), North China. Altogether 15 points were sampled, in October 2012 and October 2013, from different zones, areas and marine source of Yalujiang coastal wetland (Table 1). Random sediment samples were collected in triplicates by using sterile bottles and they were stored at 4 ℃ in the laboratory for further study.

Table 1
Characterization of the collection sites from Yalujiang coastal wetland, North China.

No.	Locations	Sample Code	Site denomination	Sampling material
1	Yalu River estuary	Z1	Yalu River estuary (salinity, 0%)	Sediment
2		Z12	Yalu River estuary (water resource)	Sediment
3		Z2	Yalu River estuary (salinity, 1.21%)	Sediment
4		Z4	Yalu River estuary (salinity, 7.63%)	Sediment
5		Z6	Yalu River estuary (salinity, 15.04%)	Sediment
6		Z9	Yalu River estuary (salinity, 21.6%)	Sediment
7		Z17	Yalu River estuary (salinity, 30.4%)	Sediment
8	Trepang habitat	T4	500 m from trepang habitat	Sediment
9		T6	Outside the trepang habitat	Sediment
10		T21	Inside the trepang habitat	Sediment
11	Rhizosphere	T7	Straw rhizosphere	Sediment
12		T8	Seepweed rhizosphere	Sediment
13		T8jl	Between the seepweed and root rhizosphere	Sediment
14		T9	Outside of the root rhizosphere	Sediment
15		T20	Central of the root rhizosphere	Sediment

2.2. Bacterial isolation

The samples were processed using the dilution and heat-shock method as the selective method[6]. After the dilution, 100 μL of the 10^{-3} and 10^{-4} diluted suspension was inoculated by spreading onto eight different production media [glycerol asparagine agar (ISP5), starch casein agar (SC), Gause's synthetic agar (GS), starch-yeast extract-peptone agar (M1, modified), minimal salts agar (M5), glycerol arginine agar (M2), humic acid vitamin (HV), and trehalose dehydrate proline (T-p)]. All the sample aliquots were analyzed in duplicate. The plates were incubated at 28 ℃ for 1 to 3 weeks. All media were prepared with 50% filtered natural seawater. After autoclaving, all of the isolation media were complemented with 50 μg/mL nystatin and 25 μg/mL nalidixic acid in order to minimize contamination with fungi and Gram-negative bacteria.

2.3. Morphological characterization

The isolated strains were initially characterized by morphological criteria according to Bensultana *et al.*[7].

2.4. Antimicrobial testing

The antimicrobial activity was tested by the plate diffusion method[8]. Three strains of pathogenic microbes [*Staphyloccocus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Fusarium oxysporum* (*F. oxysporum*)] were chosen as the test organisms. Isolates were inoculated onto the modified ISP5 medium at 28 ℃ for 7 days and discs (6 mm in diameter) were cut and placed on Luria-Bertani agar medium (for *S. aureus* and *E. coli*) or potato dextrose agar medium (for *F. oxysporum*) which was seeded with appropriate test organism. Plates were incubated at 37 ℃. Inhibition zones were determined after 48 h for *S. aureus* and *E. coli* and after 72 h for *F. oxysporum*.

2.5. Molecular identification

Selected isolates were subjected to 16S rDNA sequence analysis for establishment of their genotypic position. DNA was prepared according to the method described earlier[9]. The 16S rDNA was amplified as described by with eubacterial specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522R (5'-TGCGGCTGGATCACCTCCTT-3')[10]. The PCR cycling conditions included an initial denaturation at 94 ℃ for 3 min, followed by 35 cycles of denaturation at 94 ℃ for 1 min, annealing at 55 ℃ for 1 min and extension at 72 ℃ for 1 min, followed by a final extension for a period of 10 min at 72 ℃. The amplified PCR product was checked on 1% agarose in 1× TAE buffer, and purified with a mixture of 20% polyethylene glycol and 2.5 mol/L NaCl. The sequence obtained was compared with 16S rRNA gene sequences of cultured species available from EzBioCloud using BLAST (http://eztaxon-e.ezbiocloud.net/)[11]. Sequences were aligned using CLUSTAL W software[12]. Phylogenetic analyses were performed by using three treemaking algorithms and phylogenetic trees were constructed using the Neighbour-Joining[13], Maximum-Parsimony[14] and Maximum-Likelihood[15] tree-making algorithms by using the software packages MEGA version 5.0[16]. Kimura's two parameter model was used to calculate evolutionary distance matrices of the Neighbour-Joining method[17]. The topologies of the resultant trees were evaluated by using the bootstrap resampling method with 1 000 replicates[18].

3. Results

3.1. Isolation medium

A total of 15 samples were collected from 3 different locations in Yalujiang coastal wetland, China (Table 1). A total number of 172 different actinomycetes were isolated from different media,

different places and different soil samples on the basis of color of aerial and substrate mycelium, pigmentation and microscopic examination.

The population frequency of actinomycetes isolated per sample in Yalujiang coastal wetland was shown to be different in three locations. The low salinity sector harboured a maximum population frequency (n = 60, 34.88%), while the least was recorded in the high salinity sectors (n = 21, 12.21%). The number of actinomycete colonies was significantly high from trepang habitat of 500 m (n = 19, 11.05%), but low outside the trepang habitat region (n = 13, 7.56%) and inside the trepang habitat (n = 1, 0.58%). The highest number of isolates were recovered from the root rhizosphere (n = 29, 16.86%) as compared with straw rhizosphere (n = 22, 12.79%), followed by seepweed rhizosphere (n = 7, 4.07%).

The most effective culture media in the isolation of actinobacteria were GS (n = 58, 33.72%) and SC (n = 39, 22.67%), followed by M1 (n = 33, 19.19%) and ISP5 (n = 13, 7.56%). Of all the isolation media tested, the T-p medium (n = 2, 1.16%) was the only one found to be unsuitable in isolating actinobacteria from Yalujiang coastal wetland. In terms of diversity, GS also produced isolates belonging to the highest number of genera (eight), followed by SC (seven genera), HV (six genera), M2 (five genera), and the lowest diversity was observed with ISP5 and M1 which recovered only one genus. This variation likely reflects the effect of media composition, which is consistent with previous observations[19,20].

3.2. Molecular profiling

All the isolates were identified up to genus level on the basis of 16S rRNA gene sequences (Table 2). Phylogenetic analysis revealed the maximum similarity of these putatively novel isolates that were affiliated to the genera *Streptomyces, Micromonospora,*

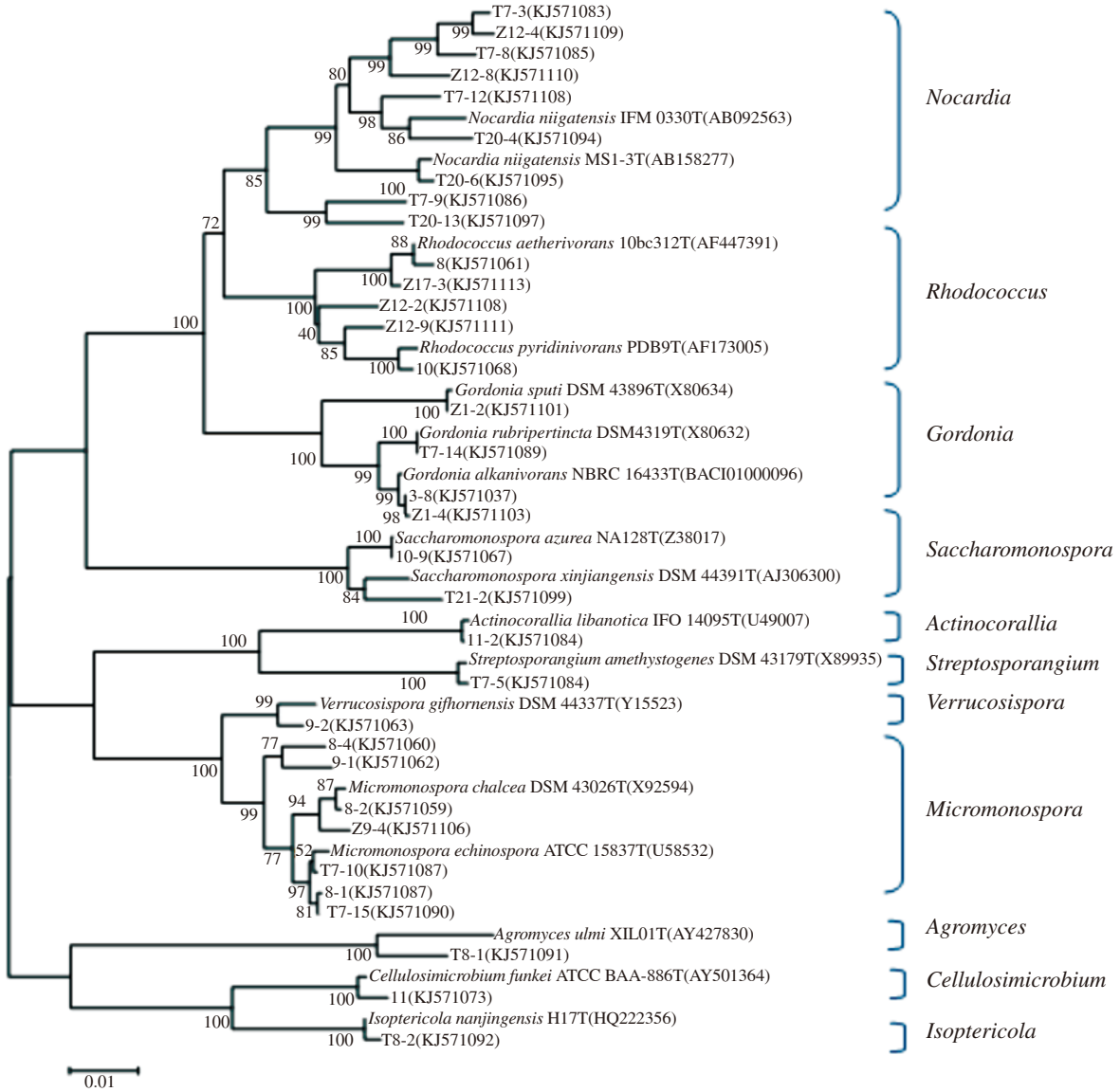

Figure 1. Neighbor-joining tree of the strains and representative species in the Yalujiang costal wetland based on nearly complete 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) of above 50 % are shown at the branch points. The scale bar indicates 0.01 substitutions per nucleotide position.

Rhodococcus, Saccharomonospora, Verrucosispora, Gordonia, Cellulosimicrobium, Brevibacterium, Actinocorallia, Nocardia, Streptosporangium, Agromyces, and *Isoptericola* (Figure 1).

Table 2

Primers used for PCR amplification.

Target taxa	Primer pair	Sequence(5'-3')	Annealing temperature	Approx product size (bp)
Actinomycetaceae	F27	AGAGTTTGATCCTGGCTCAG	60 ℃	1 500
	R1522	TGCGGCTGGATCACCTCCTT		
Amycolatopsis	AMY2	GGTGTGGGCGACATCCACGTTGT	55 ℃	450
	ATOP	GCTGGTACAGAGGGCTGCGATAC		
Micromonospora	M2F	SAGAAGAAGCGCCGGCC	65 ℃	1 000
	A3R	CCAGCCCCACCTTCGAC		
Pseudonocardia	AMP2	GTGGAAAGTTTTTTCGGCTGGGG	60 ℃	640
	AMP3	GCGGCACAGAGACCGTGGAAT		
Streptomyces	Sm6F	GGTGGCGAAGGCGGA	65 ℃	600
	Sm5R	GAACTGAGACCGGCTTTTTGA		
Thermomonospora	T3F	GGGAGAATGGAATTCCC	59 ℃	800
	T8R	CCCCACCTTCGACC		

The occurrence of species in Yalujiang coastal wetland is given in Figure 2. A total of thirteen genera were recovered from Yalujiang coastal wetland. About 70.93% of the isolates were *Streptomyces* spp., 9.88% *Micromonospora* spp. and 5.81% *Rhodococcus* spp. The genus *Streptomyces* was recovered more frequently as compared to other genera, confirming previous reports from various types of soil, and environments[21,22]. Five genera were identified in low-salinity sediments such as *Micromonospora, Rhodococcus, Gordonia, Cellulosimicrobium* and *Nocardia*, while other genera such as *Saccharomonospora* and *Verrucosispora* could not be isolated from low-salinity sediments; 19 actinomycetes belonging to these five genera: *Streptomyces*, *Micromonospora*, *Rhodococcus*, *Verrucosispora* and *Brevibacterium* were recovered from trepang habitat of 500 m, and only one genera was found from trepang habitat which belonged to *Micromonospora*; Furthermore, a noticeably low number of *Agromyces* spp. and *Isoptericola* spp. was only isolated from the sample between the seepweed and root rhizosphere.

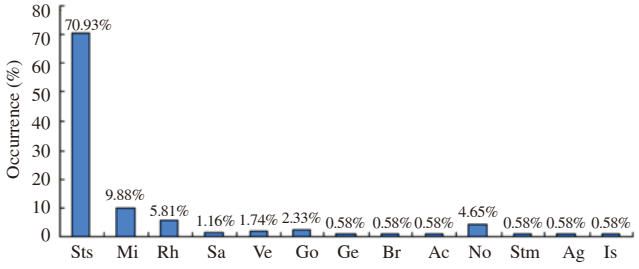

Figure 2. The occurrence of species isolated from Yalujiang costal wetland, North China.

Sts: *Streptomyces*; Mi: *Micromonospora*; Rh: *Rhodococcus*; Sa: *Saccharomonospora*; Ve: *Verrucosispora*; Go: *Gordonia*; Ge: *Gellulosimicrobium*; Br: *Brevibacterium*; Ac: *Actinocorallia*; No: *Nocardia*; Stm: *Streptosporangium*; Ag: *Agromyces*; Is: *Isoptericola*.

3.3. Antibacterial and antifungal activity

All the 172 actinomycetes isolates were evaluated for their antibacterial and antifungal activity. It has been established that solid medium is more adequate to the development of the isolates and production of antibiotics[23,24]. Most of the actinomycetes showed activity against bacteria. Among the isolates, 44 (25.6%) exhibited highest antibacterial activity against *S. aureus* and 2 isolates (1.2%) showed antibacterial activity against *F. oxysporum*. The maximum inhibition zone of 30.5 mm was recorded against *S. aureus*. Out of the 46 isolates, two cultures *i.e.* S601 and S402003 were the two strains selected for further analysis, since they showed significant activity against *F. oxysporum*, which are supported by morphological characterization.

3.4. Morphological characterization of selected isolates

As for the details of cultural characteristics of the selected isolates, S601 and S402003 are given in the Table 3. The scanning electron microphotograph of the strain revealed that the spore surface of S601 was smooth. Long spore chains, borne on the aerial mycelium, were straight to rectiflexibiles and the spore chains were composed of non-motile and coccoid spores with a warty surface (Figure 3). The isolates of S601 grew well on all the media except for HV and T-p and the growth of the isolates was excellent in ISP5. The colonies of developed S601 were observed as smooth with aerial and substrate mycelia of varying colors usually with entire margins and no diffusible pigment was produced in any media. The strain S601 can tolerate a salt concentration up to 6%; the optimum temperature for the isolate ranged between 10 and 28 ℃ and the optimum growth was observed at pH range of 5 to 14. The strain S601 varied in terms of utilization of carbon sources such as fructose, rhamnose, sucrose, and glucose (Table 4).

Cells of S402003 were non-motile, non-spore-forming and rods (Figure 3). The growth of the strain was excellent in ISP5 with undeveloped aerial mycelium and substrate mycelium (Table 3). Colonies were circular and low convex with entire margin. Physiological and biochemical characteristics result indicated that the isolates of S402003 were able to hydrolysis starch and gelatin. The tested actinomycete of S402003 showed resistance capacity to grow in 6% concentration of sodium chloride. The optimum temperature for the growth of the S402003 exceeded up to 37 ℃.

Table 3

Morphological characteristics of actinomycete isolated from Yalujiang coastal wetland, North China.

Isolates	Culture media	Growth	Colony characteristics	Pigments
S601	ISP5	Excellent	White gray	None
	SC	Very good	White gray	None
	GS	Very good	White gray	None
	M1	Excellent	White gray	None
	HV	Good	White gray	None
	T-p	Good	White gray	None
S402003	ISP5	Excellent	Yellow	None
	SC	Good	Yellow	None
	GS	Good	Yellowish	None
	M1	Undeveloped	Transparent	None
	HV	Undeveloped	Transparent	None
	T-p	Good	Yellowish	None

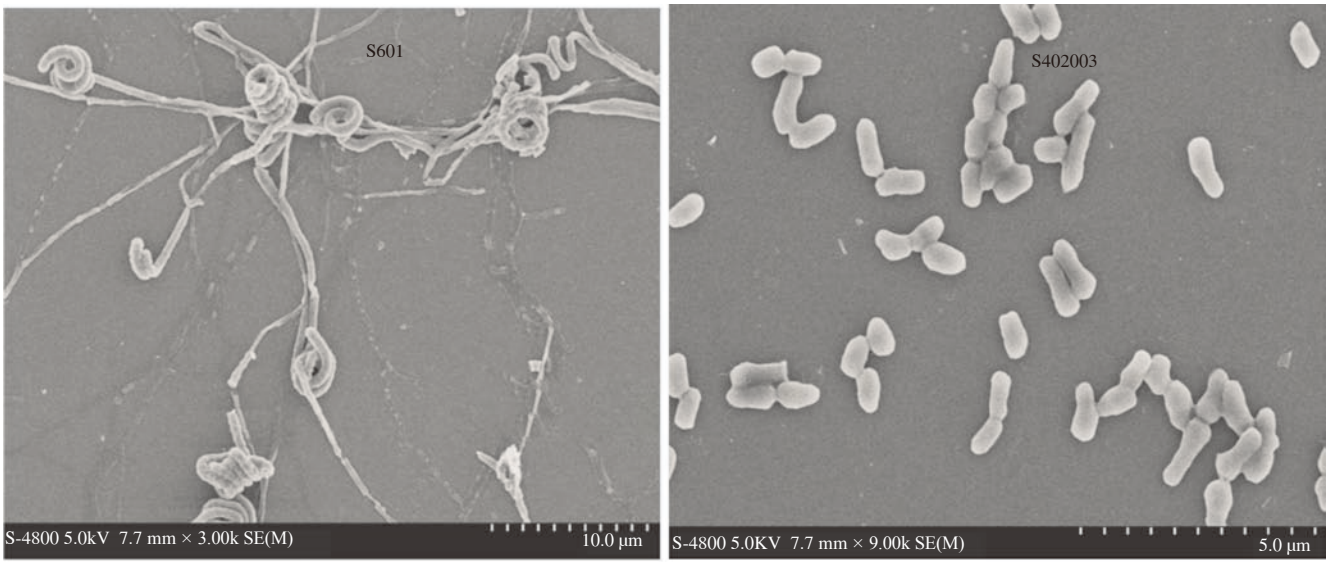

Figure 3. Scanning Electron Micrographs of *Streptomyces*s trains isolated from Yalujiang costal wetland, North China.

Table 4

Biochemical characters of actinomycete isolates isolated from Yalujiang coastal wetland, North China.

Types of test	Characteristics of isolates	
	S601	S402003
Starch hydrolysis	-	-
Gelatin hydrolysis	+	-
Resistance to NaCl	6%	6%
Optimum temperature	10-28 ˚C	4-37 ˚C
Optimum pH	5-14	5-12
Urease activity	+	-
Lipase activity	+	+
N-utilization		
Nitrate reduction	+	+
Indole test	-	-
VP test	-	-
MR test	-	-
H_2S Production	+	-
C-utilization		
Fructose	+	+
Rhamnose	+	-
Sucrose	+	-
Glucose	+	-
Arabinose	-	-
Mannose	-	+
Xylose	-	-
Maltose	-	-

4. Discussion

The present study focused on isolation and identification of active actinomycete from unusual source of Yalujiang coastal wetland. However, still it has not been explored and there is tremendous potential to identify novel organisms with various biological properties. In this line, the present work was undertaken to isolate and screen actinomycete having promising or potent activity against antibacterial and antifungal activity.

Actinomycetes have been evaluated as a source of bioactive compounds based on their distribution in various habits. The present study concluded that the physiological characteristics of actinomycetes varied by available nutrients in the medium and the physical conditions. In this study, a total of 172 actinomycetes isolates were evaluated for their *in vitro* biological activities and the majorities of isolated actinomycetes belonged to *Streptomyces* strains. *Streptomyces* strains could form heat and desiccation-resistant spores and most of them are nonpathogenic to plants and animals, so *Streptomyces* strains isolated from soil have been regarded as potential biocontrol agents for controlling plant diseases[25]. In our study, the isolation rates of antibiotic-producing actinomycetes were approximately 27%. Many studies on the antimicrobial activity of the actinomycetes isolated from marine environment have been done, but the results are different. It was reported that the components of the medium, samples differ in types and the exact reason could influence the antimicrobial activities of actinomycetes[26]. To our knowledge, the isolates, namely, S601 and S402003 were the first identified which was based on colony morphology and microscopic morphology in this study.

The recent discovery of novel primary and secondary metabolites from taxonomically unique population of actinomycetes suggests that these organisms could add a new dimension to microbial natural product research. The results of this study strongly support the idea that actinomycete species isolated from wetland possess a significant capacity to produce compounds having unique antibacterial activity. In conclusion, the results suggest that the Yalujiang coastal wetland is a source of bioactive actinomycetes and hence merits further studies concerning purification, characterization and identification of the active secondary metabolites.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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