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Isolation of Actinomycetes from Soils in Kanas, Xinjiang Province, China

L.R. Han, Y.W. Zhou, B.J. Yan, J.T. Feng* and X. Zhang*

Biopesticide Technology and Engineering Center, Shaanxi Province/Research & Development Center of Biorational Pesticide, Northwest A&F University, Yangling - 712 100, China.

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In this study, we used efficient, selective methods to systematically isolate actinomycetes from 23 soil samples collected from Kanas National Nature Reserve, Xinjiang, China, and obtained 246 strains. Morphological observation and cell wall amino acids analysis indicated that they belonged to seven genera, in which streptomyces was the dominant genus, accounting for 82.9% of the total. Among the different soil pretreatment methods, 0.05% SDS plus 6% yeast extract is the best, using which, we isolated 62.6% of the obtained actinomycetes only on TPA. Activity screening using six commonly used plant pathogenic fungi and TMV found that 6.1% strains exhibited inhibitory activity greater than 80% to at least one of the six fungi and 5.7% strains could inactivate TMV *in vitro* to certain degrees. The results from this study provide evidence that the actinomycetes in Kanas soils could be promising sources for antimicrobial bioactive agents.

Key words: Actinomycetes; Biological activity; Selective isolation.

Actinomycetes are important microbes and can produce various biologically active substances such as antibiotics, and enzyme preparations used in medicine and agriculture[1]. Among the 12,000 biologically active substances found from microorganisms, nearly 70% are produced by actinomycetes. Many of them have been used in agricultural production[1-4]. However, with the development of agricultural pests and diseases and widespread use of agricultural antibiotics, pathogens' drug resistance has sharply increased and the efficacy of commonly used antibiotics has reduced significantly[5]. Therefore, screening new strains and searching for new, highly active antibiotics are imminent.

Isolating unexploited microbial communities which are good producers of secondary metabolites is one of the important strategies for microbial drug development[6]. Long-term use of traditional microbe isolation technologies in the screening processes of natural products has resulted in repeat isolation of a large number of strains and gradually reduced probability of new compounds discovery, significantly affecting the efficiency of screening new bioactive substances. To isolate more unknown streptomyces and rare actinomycetes, it is necessary to explore new and unique isolation methods. Chinese researchers started to develop new methods to isolate rare actinomycetes since 90s of last century and have developed several highly selective isolation methods for different types of actinomycetes[7,8].

Soil is an important habitat for actinomycetes. Its physical and chemical properties strongly influence and determine the types of soil actinomycetes[9]. Kanas National Nature Reserve is located in the northern Xinjiang, China, which is in the middle of Altai Forest in the hinterland of Eurasia. This area is the only southern Siberia flora

* To whom all correspondence should be addressed.
Tel.: +86-29-87093344; Fax: +86-29-87093344
E-mail: zhxing1952@126.com

and fauna in China. Its special geographical location endows it with unique, rich natural resources and biological species[10] and may also results in special microbial resources, as well as production of biologically active substances with special features. Therefore, we collected 23 soil samples from different regions of Kanas National Nature Reserve and used them to systemically isolate and screen actinomycetes with inhibitory effects on plant pathogenic fungi and plant viruses, hoping to obtain unexploited, secondary metabolites producing microbial communities.

MATERIAL AND METHODS

Soil sample collection

The soil samples were collected from Kanas National Nature Reserve (48°36'~48°42'N, 86°55'~87°03E '). The Reserve has typical continental climate features, which is warm in spring and fall and cold but not frozen in winter. Its average annual precipitation is 1065 mm. In this study, 23 soil samples were collected in August 2009 from underground of 5~20 cm depth. Soils from three collection sites were combined as one sample. Among the 23 samples, 9 were from brown coniferous forest composed of larch, aconitium, fern, wintergreen, etc, at altitude of 1200-1800 m; 14 were from lakeside with vegetation of *Poa pratensis*, *Artemisia annua*, *Cacalia aconitifolia,* etc, at altitude of 1300 m. All samples were placed into sterile plastic bags and stored at 4 °C.

Isolation of actinomycetes

Soil samples were pretreated using the following methods: (a) 1 g soil samples were suspended in solution containing 0.05% SDS and 6% yeast extract and oscillated at 40 °C for 20 min; (b) 1 g soil samples were heated at 100 °C for 15 min and then treated with 1.5% phenol and 0.03% chlorhexidine gluconate for 15 min at 30 °C; (c) 1 g soil samples were treated with 0.03% benzethonium chloride at 30 °C for 30 min. After diluted by 1000-fold, 100 mL soil suspensions were spread on plates of TPA, CMKA and HVA 3 media supplemented with 20 μg/mL nalidixic acid and 50 μg/mL cycloheximide to inhibit the growth of fungi and bacteria, and incubated at 28 °C for 4 weeks[1]. All colonies were selected and purified by further incubation on either Gauze's No. 1 medium or ISP2 medium based on their morphology, color, and time of colony formation. Purified strains were stored in Gauze's No. 1 medium at 4 °C and in 20% glycerol at -70 °C.

One liter TPA medium composes of 5 g trehalose, 1 g proline, 1g $(NH_4)_2SO_4$, 1 g NaCl, 2 g $CaCl_2$, 1 g K_2HPO_4, 1 g $ZnSO_4 \bullet 7H_2O$, 1 ml VB solution, and 20 g agar, pH 7.2 [11]. One liter CMKA medium composes of 0.5 g casein hydrolysate, 1.5 g mannitol, 1 g KNO_3, 2g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.5 g $CaCO_3$, 10 g NaCl, 5 g KCl, and 1 g $MgCl_2$, pH 7.2 [12]. One liter HVA medium composes of 1 g humic acid, 0.02 g $CaCO_3$, 0.5 g Na_2HPO_4, 0.01 g $FeSO_4 \bullet 7H_2O$, 1.7 g KCl, 0.5 g $MgSO_4 \bullet 7H2O$, 1 ml VB solution, and 18 g agar, pH 7.4 [13]. VB solution contains 50 mg/L thiamine, 50 mg/L riboflavin, 50 mg/L nicotinic acid, 50 mg/L calcium pantothenate, 50 mg/L vitamin B_6, 50 mg/L inositol, 50 mg/L p-aminobenzoic acid and 25 mg/L biotin.

Classification of actinomycetes

Actinomycetes were cultured using the method reported previously[14] and observed under microscope for the morphology of their substrate mycelium, aerial hyphae, and sporothrix, as well as the characters of their spore production and formation (single, pair or chain). Their colony characteristics such as colony morphology, growth, spore mass color and soluble pigment were recorded according to the book entitled "Actinomycetes Systematics -Principles, Methods and Practice"[15]. Amino acid components of their cell walls were analyzed using rapid thin layer chromatography as reported previously[16].

Actinomycetes bioactivity assay

Screening of actinomycetes inhibiting plant pathogenic fungi

Plate confrontation method was used to determine all the purified actinomycetes' inhibitory effects on the growth of pathogenic fungi *Botrytis cinerea, Phytophthora capsici*, *Alternaria brassicae*, *Thanatephorus cucumeris*, *Pyricularia oryzae*, and *Rhizoctonia cerealis*[17]. In detail, actinomycetes were inoculated on Gauze's No. 1 medium plate. After 12 d of culture, actinomycetes cakes (Φ = 4 mm) were collected with a punch and placed in the center of potato dextrose agar (PDA) plates. Then the tested pathogenic fungal cakes (Φ= 4 mm) were symmetrically placed at the places 25 mm away from the actinomycetes cake. The PDA plate was then cultured at 25 °C for 7~14 d. The inhibition zone (mm) between the colony edge of

actinomycetes and the pathogenic fungal edges was measured and the inhibition rate was defined as:

$$\text{Inhibition rate (\%)} = \frac{\text{(radius of the control colony -radius of the tested colony)}}{\text{radius of the control colony}} \times 100$$

Each treatment was repeated three times

Screening of actinomycetes inhibiting plant viruses

3 mL actinomycetes spore suspension (10^5 spores/mL) were inoculated into 100 mL sterile millet liquid medium in a 250 mL flask and cultured at 180 rpm at 28 °C for 7 d. The fermented broth were collected by centrifugation at 4000 rpm for 20 min and sterilized by filtration with 0.45 ìm membranes.

The common strain of tobacco mosaic virus (TMV) was provided by the Plant Virus Laboratory, College of Plant Protection of Northwest University of Science and Technology, and stored on cigarettes K326. TMV were purified according to the method reported previously[18] and used in the experiment.

TMV inhibiting activity was measured using necrotic lesions/half leaf method[19]. Nicotiana glutinosa at 5-6 leaf stage with vigorous growth rate and similar growth status were selected as TMV host. The left half leaf was inoculated with the mixture of the fermented broth and virus, and the right half leaf was inoculated with the mixture of the virus and distill water as control. The viral inoculation concentration was 10 μg/mL and the passivation time was 0 min. The leaf surface was rinsed with water immediately after inoculation. Each treatment was conducted in four leaves and repeated three times. Three days after inoculation, the number of necrotic lesions was counted and the inhibition rate was defined as:

$$\text{Inhibition rate(\%)} = \frac{\text{(the number of necrotic lesions in control - the number of necrotic lesions in each treatment)}}{\text{the number of necrotic lesions in control}} \times 100$$

RESULTS

Selective isolation of actinomycetes from soils in Kanas, Xinjiang

Major actinomycetes groups

A total of 246 actinomycetes were isolated from 23 soil samples in Kanas National Nature Reserve. Based on morphological observations, they were classified into 7 genera including streptomyces, streptosporangtum, actinomadura, nocardia, actinoplanes, micromonospora and saccharopolyspora. As shown in Table 1, although soil samples were pretreated using different methods and cultured using a variety of selective media, the dominant species of the isolated actinomycetes are streptomyces. A total of 204 streptomyces were isolated, accounting for 82.9% of the total isolates, followed in turn by 15 micromonospora and 7 actinoplanes, accounting for 6.1% and 2.8% of the total isolated actinomycetes, respectively.

The impacts of different pretreatment of soil samples on actinomycetes isolation

Pretreatment of soil samples before isolation dramatically affects actinomycetes isolation (Table 2). In this study, we used three different pretreatment methods. Using method 1, we obtained a total of 154 actinomycetes in TPA medium alone, accounting for 62.6% of the total isolates. Statistics has shown that currently isolated actinomycetes only account for 10%-20% of the total actinomycetes in nature[20,21]. Therefore, the special soil pretreatment methods and selective isolation conditions are extremely important for unknown actinomycetes isolation. Study by Hayakawa and Nonomura et al has shown that SDS

Table 1. The main groups of actinomycetes isolated from soils in Kanas, Xinjiang, China, and their proportions

Genus	Number of strains	Proportion (%)
Streptomyces	204	82.9
Streptosporangium	3	1.2
Actinomadura	2	0.8
Nocardia	5	2
Actinoplanes	7	2.8
Micromonospora	15	6.1
Saccharopolyspora	3	1.2
Unidentified strain	7	2.8

Table 2. Effects of different media and pretreatments of soil samples on isolation of actinomycetes from soil

Treatment	Culture medium		
	TPA	HVA	CMKA
I	154 (62.6%)	101 (41.1%)	75 (30.5%)
II	73 (29.7%)	38 (15.4%)	18 (7.3%)
III	89 (36.2%)	63 (25.6%)	51 (20.7%)

treatment of soil could enrich streptomyces, micromonospora, and microbisporus while inhibit bacteria[11]. In this study, we pretreated soils with 0.05% SDS and 6% yeast extract under shaking and found that most colonies grown on plates are actinomycetes, only few are bacterial and fungal colonies. Different media could also affect the isolation results. The number and type of actinomycetes isolated on TPA are much more than those on other media, which is consistent with a previous report that using TPA supplemented with trehalose and proline as carbon and nitrogen sources could enhance the isolation rate of rare actinomycetes by 50%[11].

Screening actinomyceteswith antimicrobial activity

Determination of actinomycetes antifungal activity

Plate confrontation method was used to determine the inhibitory effects of the 246 actinomycetes on six different pathogenic fungi. As shown in Table 3, at least 15 actinomycetes have greater than 80% inhibition rate against at least one of the pathogenic fungi, accounting for 6.1% of the isolated actinomycetes. Among them, one actinomycetes had greater than 80% inhibition rate against three pathogenic fungi *P. oryzae*, *T. cucumeris* and *R. cerealis*, showing a wide antifungal spectrum, which suggest that the strain has further application potentials. Morphological classification combined with cell wall amino acid composition analysis indicates that all the 15 strains are streptomyces with L-DAP in their cell wall. Among them, 9 were isolated from the lakeside soil samples. In addition, most strains can inhibit *T. cucumeris*, accounting for 38.2% of the total isolated actinomycetes. Five strains have strong inhibition effect on mycelial growth (>80%), accounting for 2% of the total isolated actinomycetes.

Determination of TMV inactivation in vitro

The TMV inactivation by the fermented broth of the isolated 246 actinomycetes was determined by measuring the necrotic lesions on half leaves of *N. glutinosa*. As shown in Table 2, the fermented broth of 14 isolated actinomycetes could inactivate TMV to different degrees, accounting for 5.7% of the total isolated strains. Among which, 4.9% are streptomyces.

Table 3. Inhibition rate and number of actinomycetes inhibiting mycelium growth of 6 plant pathogenic fungi

Inhibition rate	Number of actinomycetes isolates against tested fungi (%)					
	B. cinerea	*P. capasici*	*A. brassieae*	*P. oryzae*	*T. cucumeris*	*R. cerealis*
++++	6 (2.4)	2 (0.8)	0 (0)	1 (0.4)	5 (2.0)	3 (1.2)
+++	9 (3.7)	8 (3.3)	3 (1.2)	5 (2.0)	19 (7.7)	9 (3.7)
++	23(9.3)	13 (5.3)	16 (6.5)	11 (4.5)	27 (11)	17 (6.9)
+	32 (13.0)	19 (7.7)	29 (11.8)	47 (19.1)	43 (17.5)	33 (13.4)
—	176 (71.5)	204 (82.9)	198 (80.5)	182 (74.0)	152 (61.8)	184 (74.8)

Note: ++++: inhibition rate”80%, +++: 70%~80%, ++: 50%~70%, +: 0~50%, and —: no activity

Table 4. Inhibition rate and number of actinomycetes inactivating TMV on *N. glutinosa in vitro*

Inhibition rate	Number of actinomycetes isolates inactivating TMV (%)		
	Streptomyces	Micromonospora	Nocardiaceae
++++	1 (0.4)	0 (0)	0 (0)
+++	1 (0.4)	1	0 (0)
++	3 (1.2)	0 (0)	0 (0)
+	7 (2.8)	0 (0)	1
-	234 (95.1)		

Note: ++++: inhibition rate”80%, +++: 70%~80%, ++: 50%~70%, +: 0~50%, —: no inactivating activity

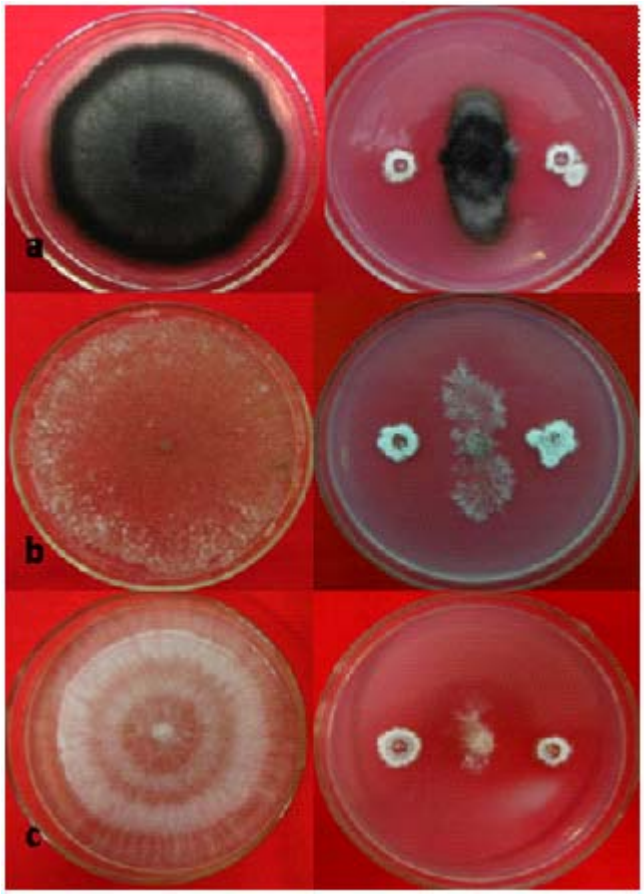

Fig. 1. Inhibitory effects of metabolites from Streptomyces ZX01 grown on potato dextrose agar for 7~14 days on the growth of pathogenic fungi (a) *Pyricularia oryzae*, (b) *Thanatephorus cucumeris,* and (c) *Rhizoctonia cerealis*

Fig. 2. Inactivation of the fermented broth of ZX01 to *Tobacco mosaic virus.*The left half leaf was inoculated with the mixture of the fermented broth and virus, and the right half leaf was inoculated with the mixture of the virus and distill water

DISCUSSIONS

In recent years, scientists at home and aboard have developed selective isolation technologies of actinomycetes from natural ecological environments. Atalan *et al.,* reported that using dispersion and differential centrifugation method to isolate Streptomyces from soil samples could improve isolation efficiency by 3-12-fold than using conventional dilution plate method[22]. Jiang *et al.,* reported several highly selective isolation methods specific for rare actinomycetes[11]. In this study, we systematically isolated actinomycetes from 23 soil samples collected from Kanas National Nature Reserve, hoping to find a relatively simple, but efficient method to isolate more rare actinomycetes species. The results show that the by combining three soil pretreatment methods with three different media, we could isolate a total of 246 actinomycetes of 7 genera including streptomyces, streptosporangium, nocardia, micromonospora, sadura, Sacchararopolyspora and actinoplanaceae. Among them, streptomyces accounts for 82.9%. Khamna *et al.,*[23] used three media and conventional dilution plate coating method to isolate actinomycetes from 16 different soil samples and found that 89% of the isolated actinomycetes are streptomyces, and Li *et al.,,*[24] isolated 95 strains of actinomyces from mangrove forest in Futian, of which 94% were Streptomycetaceae. In the present study, 12.9% of the total isolates are rare actinomycetes including saccharopolyspora and streptosporangium, indicating that using special soil pretreatment and selective medium can improve rare actinomycetes isolation rate from soil.

At present, most of the actinomycetes used in plant diseases biocontrol are streptomyces, thus unknown streptomyces are important objective microbes in actinomycetes isolation. Our results show that vast majority of actinomycetes with anti-microbe activity, especially higher anti-microbe activity, are streptomyces. Of the isolated 246 actinomycetes, only one non-streptomyces could slightly inhibit pathogenic *R. cerealis*. Similarly, among the 5.7% actinomycetes exhibiting anti-TMV activity at certain extent, 4.9% are streptomyces. Tan *et al.,,* isolated 619 actinomycetes from tomato roots, all of which were streptomyces[25]. Ramesh and Mathivanan isolated 208 actinomycetes from the Bay of Bengal, 87.98% of which were streptomyces[26]. In addition, most of actinomycetes with high antifungal activity were streptomyces. Thus, regardless soil environments, relatively special plant environment, or Gulf waters, streptomyces are among the main groups of microorganisms and the main resources of various types of active substances. Forar *et al.,*[27,28] believed that streptomyces is the main source of various types of active substances and will continue to be the main source of new biologically active substances. In this study, we isolated 246 actinomycetes from Kanas National Nature Reserve. These actinomycetes are rich in varieties and have wide spectrum of anti-microbe features. Some of them have the potential for further research. Therefore, developing efficient, selective isolation method is of great significance in finding rare actinomycetes or unknown streptomyces.

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