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Chapter 2

Isolation and Cultivation Methods of Actinobacteria

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61457

Abstract

Actinobacteria (actinomycetes) have been received much attention, as these bacteria produce a variety of natural drugs and other bioactive metabolites. The distribution of actinomycetes in various natural habitats, including soil, ocean, extreme environments, plant, lichens and animals, is described. The collection and pretreatment of test samples from different sources, design principle of selective isolation media, selection of inhibitors, selective isolation procedures of special actinomycetes, and cultivation methods are introduced and discussed.

Keywords: Actinobacteria, isolation, cultivation

1. Introduction

Actinomycetes (actinobacteria) have been received much attention, as these bacteria produce a variety of natural drugs and other bioactive metabolites, including antibiotics, enzyme inhibitors, and enzymes. More than 22,000 bioactive secondary metabolites (including antibiotics) from microorganisms have been identified and published in the scientific and patent literature, and about a half of these compounds are produced by actinomycetes. Currently, approximately 160 antibiotics have been used in human therapy and agriculture, and 100–120 of these compounds, including streptomycin, erythromycin, gentamicin, vancomycin, vermectin, etc., are produced by actinomycetes [1, 2]. However, the use of general approaches to develop new drugs from actinomycetes is more and more difficult [3, 4]. Although a large number of microorganisms have been identified, described, screened, and used, more than 90% of all microorganisms remain uncultivable [5–8]. These uncultivable microbes might offer a new hope for the development of new drugs.

To overcome the challenges of drug development from microbes, new concepts based on genomics have been described, i.e., “new habitats, new methods, new species, new gene

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clusters, new products and new uses" [4, 9]. In other words, novel microbes should contain new gene clusters synthesizing novel secondary metabolites. Many laboratories and companies have focused on new actinomycete sources from new habitats, such as oceans [10–15], extreme environments [16, 17], plants [18–20], faeces of animals [21-23] and lichens [24, 25], for the development of new drugs. So Baltz proposed a "renaissance in antibacterial discovery from actinomycetes"[26].

Becoming the uncultured to cultivable actinomycetes and providing new sources for the discovery of new drug leads are the tasks of this chapter.

Dispersion and differential centrifugation (DDC) and high-throughput methods (HTM) [27, 28] can be used for the isolation of actinomycetes. However, the dilution plate method as a key procedure for the isolation of actinomycetes will be described and discussed in this chapter.

2. Distribution of actinomycetes in nature habitats

More than thousands of test samples were collected from western China and Batic Sea, and the diversity of cultural actinomycetes was studied in our laboratories in recent decades. A part of the results is summarized in Table 1. Twenty-nine genera of actinomycetes were isolated and identified in soil samples collected from tropical rain forest in Xishuangbanna (Fig. 1D) and 19 genera from primeval forest in Grand Shangri-La. In contrast, only 13 genera were isolated from secondary growth forest in the Sichuan. The results showed that diversities of actinomycetes in primeval forest soil are more complex than secondary forest, and the diversities in tropical rain forest soil are remarkably complex than frigid forest. It is worth emphasizing extreme environments that have extreme acidity, alkalinity, salt, radioactivity, heat (hot springs), or cold (Polar Regions and snowy mountains); we found many unique microorganisms living in these environments [38]. Members of 21 actinobacteria were isolated from hypersaline soil in Qinghai and Gansu Province. *Haloactinopolyspora*, *Haloglycomyces*, *Jiangella*, *Myceligererans*, *Salinimicrobium*, *Streptomonospora*, *Yania*, and *Zhihengliuella* are novel genera published by our colleagues.

Rock Gypsum-Salt Forest (Gaolin) is located in Yuanjiang, Yunnan, China, only 3 km^2 and a special geological wonder. It is formed by various factors in a long term, and calcium sulfate is a main constituent part. The test samples were collected from there. Actinomycetes were isolated and identified. Twenty-five genera of actinobacteria were identified. It shows that the actinomycete community is very diverse (Fig. 1C).

Test samples were collected from 90 species of medical plant in Yunnan. Thirty-four genera of pure cultural actinobacteria were identified. Like this, 28 genera actinobacteria were identified from only three species of lichens (Fig. 1B).

Fifty-one genera of actinomycetes were identified in feces samples collected from 42 species of animals (Fig. 1A). One new genus, *Enteractinococcus*, was described and published. More than 250 compounds were found from animal fecal actinomycetes. These results unfolded a bright prospect.

Habitats	Diversity of cultured actinomycetes	References
Subtropical every-green forest in Sichuan	*Actinomadura, Actinopolymorpha, Micromonospora, Mycobacterium, Nocardia, Nocardioides, Nonomurae, Promicromonospora, Pseudonocardia, Rhodococcus, Saccharomonospora, Streptomyces, Verrucosispora*	[29]
Primeval frigid forest in Grand Shangri-La	*Actinomadura, Actinopolymorpha, Agromyces, Allokutzneria, Arthrobacter, Dactylosporangium, Georgenia, Kocuria, Lentzea, Mycetocola, Nocardia, Nocardioides, Oerskovia, Promicromonospora, Pseudonocardia, Rhodococcus, Streptomyces, Streptosporangium, Tsukamurella*	[30]
Primeval tropical rain forest in Xishuangbanna	*Actinomadura, Actinoplanes, Actinopolymorpha, Actinomycetospora, Agrococcus, Agromyces, Arthrobacter, Citricoccus, Dactylosporangium, Friedmanniella, Kribbella, Lentzea, Microbacterium, Microlunatus, Micromonospora, Mycobacterium, Nocardia, Nocardioides, Nonomuraea, Oerskovia, Planosporangium, Promicromonospora, Pseudonocardia, Rhodococcus, Saccharopolyspora, Sphaerisporangium, Stackebrandtia, Streptomyces, Streptosporangium*	[31]
Hyper saline soil in Qinghai	*Actinopolyspora, Citricoccus, Corynebacterium, Haloactinopolyspora, Haloglycomyces, Isoptericola, Jiangella, Marinococcus, Microbulbifer, Myceligererans, Nesterenkonia, Nocardiopsis, Prauserella, Rhodococcus, Saccharomonospora, Salinimicrobium, Streptomonospora, Streptomyces, Thermobifida, Yania, Zhihengliuella*	[32]
Rock Gypsum-Salt Forest in Yuanjiang	*Actinoplanes, Actinokineospora, Aerococcus, Amycolatopsis, Arthrobacter, Brevibacterium, Cellulomonas, Glycomyces, Kineococcus, Kocuria, Kribbella, Microbacterium, Micrococcus, Micromonospora, Neisseria, Nocardia, Nocardioides, Nocardiopsis, Prauserella, Promicromonospora, Pseudonocardia, Rhodococcus, Saccharothrix, Streptomyces*	Qinyuan Li et al. unpublished
Baltic Sea in Kiel Bay in Germany	*Actinomadura, Actinoplanes, Amycolatopsis, Arthrobacter, Cellulomonas,Isoptericola, Kocuria, Microbacterium, Micromonospora, Myceligererans, Mycobacterium, Nocardiopsis, Promicromonospora, Rhodococcus, Streptomyces*	[33]
90 species of medicinal plant in Yunnan	*Actinocorallia, Actinomadura, Amycolatopsis, Arthrobacter, Blastococcus, Catellatospora, Dactylosporangium, Dietzia, Delftia, Glycomyces, Gordonia, Herbidospora, Janibacter, Jiangella, Kineococcus, Kineosporia, Lentzea, Microbacterium, Micrococcus, Micromonospora, Mycobacterium, Nocardia, Nocardiopsis, Nonomurae, Oerskovia, Phytomonospora, Plantactinospora, Plantactinospora, Pseudonocardia, Rhodococcus, Saccharopolyspora, Streptomyces, Streptosporangium, Tsukamurella*	[34]
3 species of lichens in Yunnan	*Actinomadura, Actinoplanes, Amnibacterium, Arthrobacter, Candidatus, Cellulomonas, Cellulosimicrobium, Curtobacterium, Corynebacterium, Friedmanniella, Kineococcus, Kocuria, Kribbella, Microbacterium,*	Yi Jiang et al., unpublished

Habitats	Diversity of cultured actinomycetes	References
	Micrococcus, Microlunatus, Micromonospora, Mycobacterium, Nocardia, Oerskovia, Pseudonocardia, Pseudosporangium, Rhodococcus, Saccharopolyspora, Saccharothrix, Streptomyces, Streptosporangium, Williamsia	
42 species of animal feces in Yunnan, China	*Actinocorallia, Actinotalea, Agrococcus, Arthrobacter, Blastococcus, Brachybacterium, Brevibacterium, Cellulomonas, Cellulosimicrobium, Citricoccus, Corynebacterium, Curtobacterium, Dietzia, Enteractinococcus, Gordonia, Gulosibacter, Isoptericola, Janibacter, Jiangella, Kineococcus, Kocuria, Labedella, Leucobacter, Luteococcus, Microbacterium, Micrococcus, Microlunatus, Micromonospora, Mobilicoccus, Mycobacterium, Nocardia, Nocardiopsis, Oerskovia, Patulibacter, Plantibacter, Promicromonospora, Pseudoclavibacter, Pseudonocardia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Salinibacterium, Sanguibacter, Sphaerobacter, Streptomyces, Tessaracoccu, Tsukamurella, Verrucosispora, Williamsia, Yaniella, Zimmermannella*	[22]

Table 1. Diversity of cultured actinomycetes in different habitats cited from the study results in author's laboratories

A. Panda

B. lichen

C. Rock Gypsum-salt Forest in Yuanjiang

D. Primeval tropical rain forest in Xishuangbanna

Figure 1. Sources of a part of test samples.

3. Basic principle for the isolation of actinobacteria

In general, the isolation of actinomycetes has three targets.

First is the study on the community of actinomycetes in a special environment. In this condition, all of actinomycetes as the pure cultures should be isolated and identified. In order to manage to this target, the isolation media used should be propitious to the growth of possible more actinomycetes, and other microbes do not grow. Three to five media with different components should be used. Inhibitors against Gram-negative bacteria and fungi should be added into the media.

Second is the isolation of special actinomycetes, for example, a known species or genus, or some kind of actinomycetes with special physiological characteristics, including the resistance to antibiotics, chemicals, alkaline, acid, salts, and high and low temperatures. The isolation media should meet the requirement of target actinomycetes and inhibit the growth of unwanted microbes at the same time. For example, in order to isolate halophytic and alkalophytic actinomycetes, the salt concentration of isolation media should be 15% to 25%, and the pH level of the media should be adjusted to 10 to 12.

Third is the isolation of unknown actinomycetes. Up to now, countless actinomycetes have been isolated and identified from various habitats in the whole world. Thus, isolating unknown actinomycetes is the most difficult but most important. It requires the restraint of the growth of not only Gram-negative bacteria, some Gram-positive bacteria, and fungi but also most of the common actinomycetes.

In order to isolate as more as unknown actinomycetes, researchers should be familiar with all of the knowledge about the physiology and taxonomies of actinobacteria and other microbes and the role of each isolation factor (including components and concentration of media, pH, inhibitor, cultural temperature, etc.), and they should have rich experience. Isolation procedures should be ceaselessly renewed and improved. The isolation method of actinomycetes is on road and has no end ever.

4. Collection and pretreatment of test samples

4.1. Collection of test samples from different sources

Actinomycetes occur as saprophytes in diverse natural habitats, including soil, lake, ocean, plant, and animal. Soil remains a fruitful source of novel actinobacteria. The numbers and kinds of actinobacteria found in soil and other substrates are greatly influenced by primary ecological factors, such as nutrient, aeration, pH, temperature, salinity, and moisture and organic matter content. Indeed, the success in isolating large numbers of specific actinobacteria can be highly dependent on the choice of environmental samples. It is best to collect the soil samples from pristine area, including primeval forest, saline, alkaline soils, and desert. Soil samples in depth 5–20 cm are collected and put in sterile paper or plastic bag.

Actinomycetes are widely distributed in ocean, and a large number of natural products were found from them. Sediment in deep ocean is collected with sampler, and the samples are put in sterilized glass bottle and conserve at 4°C.

Actinomycetes exist widely in plant. Novel plant endophytic actinomycetes, especially from traditional Chinese drug, are also a promising source of antimicrobial and antitumor agents. Fresh samples of different plant tissues are collected and immediately put in sterilized container. The fresh samples should be used for the isolation of actinomycetes as soon as possible.

Recently, Mohamed et al. analyzed the biosynthetic gene cluster in human microbiome and discovered new bioactive substance, lactocillin, and considered that human microbiome is a huge molecular drug house [35]. There are uncountable species of animal in the whole world; animal feces are a huger actinomycete community, and animal microbiome should be huger molecular drug house. Discovering new drug leads from actinomycetes of animal feces is very important and tempting. In order to isolate actinomycetes, the fresh fecal samples should be put in sterilized container, conserve at 4°C, and used for the isolation of actinomycetes as soon as possible.

4.2. Pretreatment of test samples

Pretreatment is very important for the selective isolation of actinomycetes, which grow slower than other bacteria and fungi. In general, pretreatment regimes select target actinomycetes by inhibiting or eliminating unwanted microorganisms. Several chemical and physical pretreatments have been used for the isolation of actinomycetes. Actinomycete spores are more resistant to desiccation than most bacteria; hence, simply air-drying soil, sediment, lichen, and fecal samples at room temperature will eliminate most unwanted Gram-negative bacteria, which might otherwise overrun isolation plates. Air-dried soil heated or soil suspensions heated treatment can be used for selectively isolating special actinobacterial taxa (Table 2).

Sample suspensions can be treated with ultrasonic waves at 180 W for 40′. It can release the saprophytes fixed by soil granule into the suspension, increased account of actinomycetes, and reduced bacteria in the sample [36] (Fig. 2).

Based on the differential resistant ability of actinobacterial spores to withstand treatment with chemicals, such as benzethonium chloride, chlorhexidine gluconate, phenol, SDS, and various antibiotics, these different chemicals were used to isolate special actinobacterial taxa. Treatment with these agents for 30 min at 30°C can kill Gram negative cells of aerobic, endospore-forming bacilli and pseudomonads, increase frequency of actinomycete, and reduce bacteria (Table 3).

Pretreatment	Target
Air-dried soil heated at 120°C for an hour	*Microbispora* *Streptosporangium* .
Air-dried soil heated at 100°C for 15 min	*Actinomadura* spp.
Water or soil suspensions heated at 45°C or 50°C for 10 min	*Streptomyces* spp.
Water or soil suspensions heated at 60°C for 30 min	*Micromonospora* spp.
Air-dried soil heated at 120°C for an hour	*Dactylosporangium* and *Streptosporangium* spp.
Air-dried soil heated at 28°C for a week	*Herbidospora cretea*
Soil suspension heated at 110°C for an hour	*Microtetraspora glauca*

Table 2. Selective heat pretreatments for the isolation of actinobacteria.

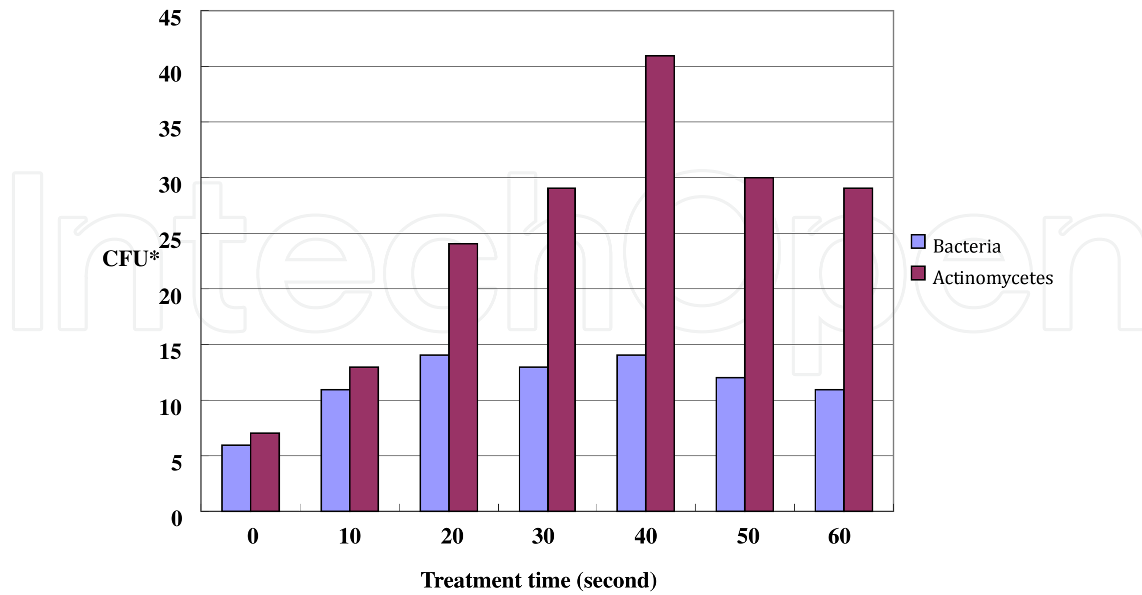

Figure 2. Influence of ultrasonic wave treatment to CFU between actinomycetes and other bacteria. *CFU = colony-forming units.

Pretreatment	CFU (×10^5/g)			
	Actinomycetes		Bacteria	
Control	117	100%	152	100%
YE, 2%	192	164%	169	110%
HA, 2%	183	156%	92	60%
CA, 1%	170	145%	165	109%
VA, 0.2%	159	136%	178	117%
ME, 0.2%	163	139%	136	87%
SDS, 0.05%	152	157%	10	7%
SDS + YE, 6%	183	157%	22	14%
SDS + HA, 1%	173	148%	9	5%
SDS + CA, 1.5%	153	131%	15	9%
SDS + VA, 0.6%	147	126%	19	12%
SDS + ME, 0.2%	158	135%	14	8%

YE = yeast extract; HA = humic acid; CA = acasein hydrolysate; VA = valine; SDS = sodium dodecyl sulfate; ME = mercaptoethanol.

Table 3. Chemical pretreatment for the isolation of actinomycetes.

5. Principle designing medium

The design of selective Isolation media needs to colligate each factor, such as isolation goals, target actinobacterial taxa, medium component, and inhibitors. The component (carbon and nitrogen sources) of selective isolation media can be formulated by using information from taxonomic databases and phenotypic databases. Appropriate inhibitors should be selected based on ability of target actinobacterial spores to withstand with antibiotics and chemicals.

6. Isolation methods of actinobacteria from different habitats

6.1. Isolation of thermophilic actinobacteria

In order to isolate thermophilic actinobacteria, the samples from hot spring or hot environments were air-dried at room temperature for 7 to 10 days, treated at 120°C for 1 h.

Isolation media (for 1000 ml distilled water):

YIM 14 improved Czapek medium: sucrose 20 g, $NaNO_3$ 2 g, K_2HPO_4 1 g, $MgSO_4$ $7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g, vitamin mixtures [37] 3.7 mg, agar 25 g, pH 7.2.

YIM 17 glycerol asparagine medium: L-asparagine 1 g, glycerol 10 g, K_2HPO_4 1 g, vitamin mixtures 3.7 mg, trace salt* 1 ml, agar 20 g, pH 7.2–7.4.

YIM 21 oatmeal medium: oatmeal 20 g (cook or steam 20 g oatmeal in 1000 ml distilled water for 20 min, filter through cheese cloth, and add distilled water to restore volume of filtrate to 1000 ml), vitamin mixture 3.7 mg, trace salts 1 ml, agar 20 g, pH 7.2.

*Trace salts solution: $FeSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, distilled water 100 ml.

Fifty milligrams of potassium dichromate and 1 mg of penicillin are added in the isolation media.

The plate dilution method was used to isolate actinobacteria from the sample suspension. Approximately 0.1–0.2 ml of each sample (10^{-2} and 10^{-3} dilutions) was used to coat the plates and cultivated for 7 days in a moist chamber at 55°C. Single actinomycete colony is picked to inoculate an agar slant containing the same isolation medium.

6.2. Isolation of halophilic and alkalophilic actinobacteria

Media for halophilic actinobacteria (for 1000 ml distilled water):

YIM 6 Starch-casein medium: soluble starch 10 g, casein 0.3 g, KNO_3 2 g, $CaCO_3$ 0.02 g, $FeSO_4$ 10 mg, salt mixtures*, agar 25 g, pH 7.2–7.4.

YIM 17 glycerol asparagine medium (the same as above), salt mixtures*, agar 25 g, pH 7.2–7.4.

YIM 47 soil extracts medium: soil extracts (soil 400 g, 120°C for 1 h, filter through cheese cloth, and add distilled water to restore volume of filtrate to 1000 ml), meat extracts 3 g, peptone 5 g, salt mixtures*, agar 25 g, pH 7.2–7.5.

T3 medium [38]: cellulose 10 g, casein 0.3 g, KNO_3 0.2 g, K_2HPO_4 1 g, $CaCO_3$ 0.02 g, $FeSO_4$ 10 mg, salt mixtures*, agar 25 g, pH 7.5.

Horikoshi medium [39]: glucose 10 g, yeast extracts 5 g, peptone 5 g, K_2HPO_4 1 g, $MgSO_4$ $7H_2O$ 0.2 g, salt mixtures*, agar 25, pH 7.2–7.5.

Salt mixtures (for 1 L): NaCl 100–150 g, KCl 20 g, $MgCl_2$ $6H_2O$ 30 g, $MgSO_4$ $7H_2O$ 5 g, K_2HPO_4 1 g.

Medium preparation: The salt mixtures are dissolved in a half volume of water, other components of medium are dissolved in other half volume of water, and they both have to be sterilized separately. Then the whole medium is spread into the plates after mixing the both while hot.

In order to isolate the alkalophilic actinomycetes, the five media as above can be used. However, it does not need the salt mixtures; pH should be adjusted to 10 to 11 with sterilized NaOH or Na_2CO_3 before spreading plate.

Twenty-five to 40 mg (for 1 L medium) of nalidixic acid should be added into all of media for inhibiting Gram-negative bacteria.

The growth of halophilic actinomycetes is always very slow. Thus, isolation media should be thicker, and the cultivation time of isolation plates should be lengthened to 20 to 35 days in keeping humidity. Single actinomycete colony is picked to inoculate a slant with the same isolation medium.

6.3. Isolation of acidophilic actinobacteria

Study on acidophilic actinomycetes is few worldwide, and only some report on acidophilic streptomycetes exists. The isolation of this actinomycete is difficult because of the fast growth of fungi and other bacteria in the test samples in isolation plate with lower pH. YIM 6, YIM 17, YIM 21, and YIM 47 media can be used for isolating acidophilic actinomycetes. Twenty-five grams of agar for 1 L should be used, and pH should be adjusted to 4.0 to 4.5 with sterilized HCl before spreading plate. All media are supplemented with filter-sterilized mixtures [50 mg cycloheximide + 50 mg nystatin + 20 mg nalidixic acid, or 50 mg sterilized potassium dichromate ($K_2Cr_2O_7$)].

6.4. Isolation of plant endophytic actinobacteria

Isolation media (for 1000 ml distilled water):

Water yeast extract medium [40]: yeast extract 0.25 g, K_2HPO_4 0.5 g, agar 18 g, pH 7.2.

Sodium propionate medium [41]:sodium propionate 1 g, L-asparagine 0.2 g, KH_2PO_4, 0.9 g, K_2HPO_4 0.6 g, $MgSO_4$ $7H_2O$ 0.1 g, $CaCl_2$ $2H_2O$ 0.2 g, agar 15 g, pH 7.2.

YIM 7 HV medium [37]:humic acid 1.0 g, Na_2HPO_4 0.5g, KCl 1.7 g, $MgSO_4$ $7H_2O$ 0.05 g, $FeSO_4$ $7H_2O$ 0.01 g, $CaCl_2$ 1 g, B-vitamins (0.5 mg each of thiamine–HCl, riboflavin, niacin, pyridoxin, Ca-pantothenate, inositol, *p*-aminobenzoic acid, and 0.25 mg of biotin), agar 18 g, pH 7.2.

Supplied in each medium were the following: 50–100 mg cycloheximide, 100 mg nystatin, 25 mg nalidixic acid, and 5 mg penicillin for 1000 ml.

Samples were air-dried for 48 h at room temperature and were then washed with an ultrasonic step (160 W, 15 min) to remove the surface soils and adherent epiphytes completely. After drying, the samples were subjected to a five-step surface sterilization procedure: 4- to 10-min wash in 5% NaOCl, followed by 10-min wash in 2.5% $Na_2S_2O_3$, 5-min wash in 75% ethanol, wash in sterile water, and final rinse in 10% $NaHCO_3$ for 10 min. After being thoroughly dried under sterile conditions, the surface-sterilized tissues were subjected to continuous drying at 100°C for 15 min [34]. Surface-treated tissues are aseptically crumbled into small fragments and homogenized with a glass homogenizer; 0.1 ml of the suspension at three dilutions is spread on the isolation plate. The inoculated plates were incubated at 28°C for 2 to 4 weeks.

6.5. Isolation of actinobacteria in animal feces

Isolation media (for 1000 ml distilled water):

YIM 7 HV medium

YIM 47 soil extracts medium

YIM 171 improved glycerol-asparagine medium: glycerol 10 g, asparagine 1 g, K_2HPO_4 H_2O 1 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCO_3$ 0.3 g, vitamin mixture of HV medium 3.7 mg, and agar 15 g, pH 7.2.

YIM 212 mycose-proline medium: mycose 5 g, proline 1 g, $(NH_4)_2SO_4$ 1 g, NaCl 1 g, $CaCl_2$ 2 g, K_2HPO_4 1 g, $MgSO_4$ $7H_2O$ 1 g, vitamin mixtures, agar 15 g, pH 7.2.

YIM 601 improved starch-casein medium: solution starch 10 g, casein 0.3 g, KNO_3 2 g, $MgSO_4$ $7H_2O$ 0.05 g, NaCl 2 g, K_2HPO_4 2 g, $CaCO_3$ 0.02 g, $FeSO_4$ 10 mg, vitamin mixtures, agar 15 g, pH 7.2~7.4.

Fresh fecal samples were collected. The samples were immediately transferred to sterile glass dishes and dried for 10 days at 28°C. Two grams of each dried sample was pretreated at 80°C for 1 h and subsequently dissolved in 18 ml of sterile water containing 0.1% $Na_4P_2O_5$, followed by shaking at 220 rpm/min for 60 min. The suspension is treated with ultrasound waves for 40 s at 150 W before coating. The suspension was diluted from 10^{-1} to 10^{-7}, and three dilutions, 10^{-5}, 10^{-6}, and 10^{-7}, were used for isolating actinomycetes.

The abundance of Gram-negative bacteria in animal feces presents a major challenge for the isolation of fecal actinobacteria. To eliminate Gram-negative bacteria and fungi and to obtain more unknown actinobacteria, some key points for sampling and isolation should be given attention.

First, based on the results of previous experiments, it is best to collect fresh fecal samples from wild animals living in original habitats. Second, the fresh samples should be dried at 25–28°C for 7 to 10 days. Third, the dried samples should be treated for 60 min at 80°C, and the fecal suspension should be treated with ultrasound waves for 40 s at 150 W before coating [34]. Fourth, potassium bichromate 50 mg and 5 mg penicillin or nystatin 50 mg, nalidixic acid 20 mg, and 5 mg penicillin per 1 L should be added into isolation medium to inhibit the growth of Gram-negative bacteria and fungi. Fifth, the samples should be diluted to 10^{-5}, 10^{-6}, and 10^{-7}, and the optimum dilution concentration for each animal fecal sample should be determined in advance. Sixth, YIM 212, YIM 171, and HV medium are better for the isolation of fecal actinobacteria, and these media should be improved and constantly updated with respect to different samples. Seventh, all experiments should be performed under strict sterile conditions for avoiding spread of pathogen.

6.6. Isolation of actinobacteria associated lichens

Isolation media (for 1000 ml distilled water)

YIM 6 starch-casein medium

YIM 171 improved glycerol-asparagine medium

YIM 709 Fungus polysaccharides medium: Chinese caterpillar fungus polysaccharides 1 g, $(NH_4)_2SO_4$ 2.64 g, NaCl 2 g, KCl 2 g, $MgCl_2$ $6H_2O$ 2 g, K_2HPO_4 1 g, KNO_3 0.2 g, $CaCO_3$ 0.2 g, $FeSO_4$ 10 mg, vitamin mixtures, trace salts 1 ml, agar 15 g, pH 7.5.

YIM 711 Casein Soybean peptone medium: casein 1.5 g, soybean peptone 0.5 g, K_2HPO_4 H_2O 1 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCO_3$ 0.3 g, NaCl 5 g, vitamin mixtures, agar 15 g, pH 7.5.

Inhibitors: all media were supplemented with filter-sterilized mixtures of 50 mg cycloheximide, 50 mg nystatin, and 25 mg nalidixic acid as inhibitors against fungi and Gram-negative bacteria.

The plate dilution method was used to isolate the actinobacteria. Two grams of each dried sample was grinded with a sterile glass homogenizer and dissolved in 18 ml of sterile water containing 0.1% $Na_4P_2O_5$, followed by shaking at 220 rpm/min for 60 min. The suspension was treated with ultrasound waves for 40 s at 150 W before coating. The suspension was diluted from 10^{-1} to 10^{-5}, and 0.1 ml of three dilutions, 10^{-3}, 10^{-4}, and 10^{-5}, was used to coat the plates and cultivated for 10 to 25 days at 28°C. Subsequently, single actinomycete colony was picked up and inoculate to a slant with the same isolation medium.

6.7. Isolation of rare actinobacteria

The actinomycetes except streptomycetes are named rare actinomycetes. In recent years, a large number of novel bioactive substances were discovered from the rare actinomycetes. Thus, isolation methods of rare actinomycetes have been received much attention.

1. Basic media: YIM 7 HV medium, YIM 212 histidine–raffinose medium (histidine 1 g, raffinose 5 g, K_2HP_4 $3H_2O$ 1 g, $MgSO_4$ $7H_2O$ 0.5 g, agar 20 g, pH 7.2), oligotrophic medium

(peptone 1 g, ye$_a$st extracts 0.5 g, K_2HPO_4 H_2O 1 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCO_3$ 0.3 g, NaCl 5 g, vitamin mixtures, agar 15 g, pH 7.5), and minimal medium:glucose 0.5 $_g$, yeast extract 0.5 g, $MgSO_4$ $7H_2O$ 0. 5 g, NaCl 0.5 g, K_2HPO_4 1 g, agar 15 g, pH 7.5–8.0. Special carbon or nitrogen sources (e.g., chitin, lignin, xylan, methanol, propionate, keratin, coconut milk, special amino acids, etc.) can be used to replace the carbon or nitrogen of the four media.

2. Various chemicals or antibiotics (Table 4) can be used for the selective isolation of different rare actinomycetes; for example, leucomycin can be used for isolating selectively the members of *Actinomadura* and *Streptosporangium*; Tunicamycin for *Actinoplanes*, *Dactylosporangiu*, and *Micromonospora*.

Chemicals	Target genera	Chemicals	Target genera
Bruneomycin	*Actinomadura*	Benzoate	*Micromonospora*
Streptomycin	*Actinomadura*	Polymyxin	*Streptomyces*
Gentamicin	*Actinomadura*	Kanamycin	*Microtetraspora*
	Streptosporangium	Nalidixic acid	*Microtetraspora*
Leucomycin	*Streptosporangium*	Nofloxacin	*Microtetraspora*
Fradiomycin	*Actinokineospora*	Penicillin	*Saccharothrix*
Kanamycin	*Actinokineospora*	Neomycin sulfate	*Amycolatopsis*
	Thermomonospora	Lysozyme	*Streptoverticillium*
Nalidixic acid	*Actinokineospora*	Novobiocin	*Glycomyces*
Trimethoprim	*Actinokineospora*	Streptomycin	*Glycomyces*
Tellurite	*Actinoplanes*	Lincomycin	*Micromonospora*
Tunicamycin	*Actinoplanes*	Novobiocin (25°C)	*Micromonospora*
	Dactylosporangium	(50°C)	*Thermomonospora*
	Micromonospora	Oxytetracycline	*Streptoverticillium*
Rifampicin(30°C)	*Actinomadura*	Rubomycin	*Actinomadura*
(50°C)	*Saccharomonospora*	Tetracyclines	*Nocardia*

Table 4. Selective chemicals for the isolation of rare actinomycetes.

3. Combination of Chemical pretreatment and different media can isolate different rare actinomycetes. For example, HV medium with chloramine T treatment can isolate the members of *Herbidospora, Microbispora, Microtetraspora*, and *Streptosporangium* (Table 5)

Chemical treatment	Target genera
Phenol, benzethonium chloride, and chlorhexidine gluconate	*Microbispora, Streptomyces, Streptosporangium*
Quaternary ammonium compounds	*Mycobacterium, Rhodococcus*
Antibiotics	
Nalidixic acid, penicillin G	*Rhodococcus*
Kanamycin, nalidixic acid, trimethoprim	*Actinokineospora*
Gentamicin	*Streptosporangium, Actinomadura, Micromonospora*
Novobiocin	*Actinoplanes, Thermoactinomyces*
Penicillin, nalidixic acid	*Saccharothrix*
Rifampicin, Streptomycin, Kanamycin	*Actinomadura*
Tunicamycin	*Micromonospora*
Isolation media	
Humic acid vitamin agar (HV agar) with Chloramine T treatment	*Herbidospora, Microbispora, Microtetraspora, Streptosporangium*
Hair hydrolysate vitamin agar	*Actinoplanes, , Microbispora, Micromonospora, Streptosporangium*
HV agar containing nalidixic acid with SDS and yeast extract treatment	*Actinomadura, Microbispora, Micromonospora, Microtetraspora, Streptosporangium, Nocardia*

Table 5. Combinatory methods for the isolation of rare actinomycetes.

7. Cultivation of actinobacteria

7.1. Liquid fermentation

The cultivation here is limited to small liquid and solid fermentation for studying the bioactive substances produced by actinomycetes. Fermentation is extremely important procedure for the discovery of new drug leads. Different strains need different fermentation conditions, including components, concentration, and pH of broth, and time, temperature, and aeration of fermentation. In general, the goals of the fermentation are as follows:

1. All of potential bioactive substances in actinomycete strains should be produced in fermentation broth as much as possible.
2. Studying main or target compounds should be produced as much as possible.

3. Background of the fermentation broth should be as less as possible for eliminating the obstruction from broth itself.

The following fermentation broths can be used for studying the bioactive substances of actinomycetes. Each strain should be fermented with 4 to 8 broths for 4 to 7 days in choosing the optimum broth and fermentation times.

7.1.1. Seed broth (for 1000 ml water)

YIM 38 broth: yeast extracts 4 g; glucose 4 g; malt extracts 10 g; thiamine–HCl, riboflavin, niacin, pyridoxin-HCl, inositol, calcium pentothenate, *p*-aminobenzoic acid, each 0.5 mg, and biotin 0.25 mg; pH 7.2.

YIM 306 broth: glucose 10.0 g; glycerol 10.0 g; casamino acids 15.0 g; oatmeal 3.0 g; peptone 10.0 g; yeast extract 5.0 g; $CaCO_3$ 1.0 g; pH 7.0.

Fermentation time of seed broth on shaker is 36–60 h.

7.1.2. Fermentation broth

YIM 61 broth: soybean meal 20 g; peptone 2 g; glucose 20 g; soluble starch 5 g; yeast extracts 2 g; NaCl 4 g; K_2HPO_4 0.5 g; $MgSO_4$ $7H_2O$ 0.5 g; $CaCO_3$ 2 g; pH 7.8.

YIM 301 broth: soluble starch 24.0 g; meat extracts 3.0 g; yeast extracts 5.0 g; peptone 3.0 g; glucose 1.0 g; $CaCO_3$ 4.0 g; pH 7.0.

YIM 302 broth: soybean meal 20 g, mannitol 20 g; pH 7–7.5.

YIM 305 broth: mannitol 30.0 g; glucose 10.0 g; yeast extracts 5.0 g; $(NH_4)C_4H_4O_4$ (ammonium succinate) 1.0 g; K_2HPO_4 1.0 g; $MgSO_4$ $7H_2O$ 0.1 g; pH 7.0.

YIM 307 broth: mannitol 20.0 g; peptone 20.0 g; pH 7.5.

YIM 308 broth: glucose 10.0 g; meat extract 3.0 g; peptone 3.0 g; soluble starch 20.0 g; yeast extract 5.0 g; $CaCO_3$ 3.0 g; pH 7.0.

YIM 310 broth: glucose 5.0 g; peptone 3.0 g; soluble starch 10.0 g; yeast extract 3.0 g; $CaCO_3$ 2.0 g; NH_4 NO_3 3.0 g; pH 7.2.

YIM 312 broth: glucose 10.0 g; glycerol 10.0 g; cornsteep powder 2.5 g; peptone 5.0 g; soluble starch 10.0 g; yeast extract 2.0 g; $CaCO_3$ 3.0 g; NaCl 1.0 g; pH 7.3.

7.2. Solid fermentation

Solid fermentation procedures were also used for cultivation of actinomycete sometimes in research stage. The content of bioactive substances produced by actinomycetes in solid fermentation is more than in liquid fermentation. A handy method is introduced as follows: rice 100 g + YIM 61 broth 100 ml, sterilized for 1 h

Five milliliters of seed broth was inoculated into solid medium, mixed, and incubated for 5 to 7 days at 28°C. Figure 3 is the photography of two strains of streptomycetes with solid

fermentation. The optimum component of solid medium for different actinomycetes is different from each other. It is has to emphasize that no all of actinomycetes can grow in solid fermentation.

Figure 3. Solid fermentation of two streptomycete strains in rice+YIM 61 broth for 7 days at 28°C.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (no. 31270001 and no. 31460005), the Yunnan Provincial Society Development Project (2014BC006), and the National Institutes of Health USA (1P 41GM 086184 -01A 1). We are grateful to M. Goodfellow for sincere help, and Ms Chun-hua Yang for excellent technical assistance.

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