

Improvement of an Isolation Medium for Actinomycetes

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Abstract

By comparing differences among the effects of several isolation mediums on soil actinomycetes, we attempted to find a isolation medium that could result in actinomycetes with high diversity. Soil samples were isolated by the dilution plating method, and genomic DNA was extracted using phenol-chloroform method. After amplification by 16S rDNA PCR, PCR products were sequenced and undertaken phylogenetic analysis in order to determine the ownership of isolates. The results showed that the isolated strains belonged to 15 genera; as for the established medium in the present paper, ZSSE was easier to isolate rare actinomycetes with rich diversity, and could replace routine agars such as Gause's No.1, HV and ISP5; CHV was easier to isolate *micromonospora*.

Keywords: Actinomycetes, Separation medium, Species diversity

Actinomycetes were bacterium that could form branches, and such branches were mycelium formed by thallus. They were generally saprophytic, and widely distributed in nature, and only a small number of them coexisted with plants. They distributed in soil, air and water in the form of spores or mycelium, especially in slightly alkaline or neutral soil with low water content and rich organic matters.

Ever since Waksman and Umezwa found the usage diversity of actinomycetes, actinomycetes have been applied as a producing strain for antibiotics, vitamins, enzymes and enzyme inhibitors, and thus have been a microbial population with huge application values.

Acquiring new strains was a necessary condition for modern actinomycetes resource development, and therefore, studies on the separation methods tended to be very critical. There have been great progresses made in selective isolation of rare actinomycetes by researchers at home or abroad in recent years (Jiang, 2006, PP. 181-183; Li, 2002, PP. 105-108; Li, 2003, PP. 114-117; Si, 2004, PP. 61-65). However, a large number of actinomycetes couldn't be separated, and as seen from modern molecular biology studies, there were at least 90% unknown actinomycetes that couldn't be isolated existed in nature (Duan, 2007, PP. 32-33). Accordingly, in the present paper, we investigated the differences among effects of several mediums on isolating soil actinomycetes, and attempted to find a separation medium for isolating actinomycetes with high species diversity.

1. Materials and methods

1.1 Materials

1.1.1 Soil

Soil samples were sampled from different regions in Shanxi Province and all collected 15cm away from the earth's surface (Table 1).

1.1.2 Medium

Preparation of soil extracts: 400g dried soil without miscellaneous stones were added to 960mL tap water,

completely agitated, and sterilized by autoclaving at 121°C. After standing a while, the resultant solution was filtered twice by filter cloth and filter paper subsequently, and sterilized at 121°C for 20 min. The filtrate was used to prepare soil leaching juice agar medium.

Trace salt solution: 0.1g FeSO₄, 0.1g MnCl₂, 0.1g ZnSO₄, and 100mL distilled water.

Soil extracts agar: 5g peptone, 3g beef extract, 1000mL soil extracts, 10g agar, pH7.2.

Glycerol-asparagine agar: 1g asparagine, 10g glycerol, 1g K₂HPO₄, 1mL trace salt solution, 10g agar, 1000mL distilled water, pH 7.2.

Gause's No.1 medium: 20g soluble starch, 1g KNO₃, 0.5g NaCl, 0.5g K₂HPO₄, 0.5g MgSO₄, 0.01g FeSO₄, 10g agar, 1000mL distilled water, pH 7.2;

HV agar: 1g humic acid, 1.7g KCl, 0.5g Na₂HPO₄, 0.5g MgSO₄, 0.02g CaCO₃, 0.01g FeSO₄, 1mL V_B stock solution, 10g agar, 1000mL distilled water, pH 7.2, (V_B stock solution: 50mgV_{B1}, V_{B2}, niacin, V_{B6}, D-calcium pantothenate, inositol, and PABA (para amino acid), 25mg Biotin, and 100mL distilled water);

According to years' laboratory experiences of isolating actinomycetes under different environments, we created CHV (Complex HV Agar) and ZSSE (Zhang'Starch Soil Extract Agar) mediums and first used them in the present paper.

CHV agar: 0.5g humic acid, 10g agar, 1000mL soil leaching juice, 1mL V_B stock solution, pH 7.2;

ZSSE agar: 5g soluble starch, 1g KNO₃, 1000mL soil extracts, 10g agar, pH7.2.

50mg/L nalidixic acid and 100mg/L nystatin were added to each medium as inhibitor, respectively.

1.2 Methods

1.2.1 Soil treatment

Soil samples were dried in ventilated dark room for 7 days.

1.2.2 Separation method

50mg/L nalidixic acid and 100mg/L nystatin were added to six mediums as an inhibitor of bacteria, viz. HV, CHV, ZSSE, Gause's No.1, soil extracts agar and glycerol-asparagine. Samples were diluted in a gradient, and 10⁻⁶ and 10⁻⁷ of stock solution was coated in the plates. The plates were incubated at 28 °C. Actinomycetes colonies with different forms were obtained at different periods, and colonies of all separated plated were counted.

1.2.3 Extraction of genomic DNA

Genomic DNA of actinomycetes was extracted using phenol-chloroform method (Marmur, 1961, PP. 208-218).

1.2.4 PCR amplification of 16S rDNA sequence

Using 16S rDNA sequences universal primers 27f (5'-AGAGTTTGATCMTGGCTC AG-3') and 1525r (5'-AGAAAGGAGGTGWTC ARCC-3') as primers (Lane, 1991, PP. 115-175), 16S rDNA was undertaken PCR amplification, and PCR products were sequenced.

1.2.5 Phylogenetic analysis

Sequencing results were compared using BLAST method, and phylogenetic trees were constructed using neighbor joining module of software MEGA4.1 in order to analyze phylogenetic relationships of strains (Tamura, 2007, PP. 1596-1599; Saitou, 1987, PP. 406-425).

2. Results and Analysis

2.1 Separation effects of mediums

As seen from Figure 1 and Table 2, among six mediums, the number of non-actinomycetes in plates from Gause's No.1 was the least while the size of colony was biggest. The number of non-actinomycetes in plates from HV and CHV was the most. The number of actinomycetes in plates from ZSSE was the most, and CHV followed. Aerial mycelium of actinomycetes in soil extracts agar and glycerol-asparagine grew slowly, and several colonies were tough to distinguish from others.

2.2 Phylogenetic analysis

134 strains with greater morphological difference were selected from the obtained 232 strains to undertake sequence, and results indicated as follows: 68 strains of *Streptomyces*, 23 *Micromonospora*, 11 *Nocardia*, 3 *Actinomadura*, 9 *Promicromonospora*, 2 *Pseudonocardia*, 2 *Rhodococcus*, 4 *Saccharothrix*, 5 *Nonomuraea*, 2

Cellulomonas, 1 *Jiangella*, 1 *Gordonia*, 1 *Cellulosimicrobium*, 1 *Kribbella*, and 1 *Williamsia*. Results were listed in Table 3 in detail.

3. Discussions

Although there were great differences in genus among the obtained rare actinomycetes resulted from different soil samples, genus diversity of the isolated actinomycetes increased with the enrichment of vegetations. Generally, the strains isolated from Gause's No.1 were mainly common *Streptomyces*, other rare actinomycetes were tough to isolate, and other bacteria excluding actinomycetes grew less; the strains isolated from ZSSE, HV, CHV, soil extracts agar and glycerol-asparagine were mainly other bacteria, and such bacteria could be mainly gram-positive. Accordingly, the added inhibitors failed to work effectively; the strains isolated from glycerol-asparagine were mostly *Streptomyces*, the resultant colonies formed aerial mycelium slowly, and thus it was hard to distinguish the rare actinomycetes; genus from HV and had many sorts of actinomycetes, and especially for soil extracts agar, the number of bacteria was so higher that large colony was easy to select other bacteria after long incubation; CHV could enhance the number and diversity of *Micromonospora*, but decreased the diversity of other actinomycetes; ZSSE resulted in a rich species diversity of actinomycetes with the largest sorts and number of rare actinomycetes, and its colony size was uniform. The growth of bacteria couldn't basically hamper the selection of actinomycetes colonies.

Taken together, ZSSE medium established in the present paper possessed the nutritional characteristics of both Gause's No.1 and soil leaching juice, and was easier to isolate rare actinomycetes with rich diversity. It overcame the shortcomings that general mediums couldn't take both specificity and diversity into account, and could basically replace Gause's No.1, HV and glycerol-asparagine to isolate actinomycetes; compared to soil extracts agar, bacteria biomass was less, and it was easier to select actinomycetes. CHV medium was more suitable for the separation of *micromonospora*.

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Table 1. Source of the soil samples

Serial number	Region	Vegetation	Date
S1	Yuncheng	Cropland	2009.6
S2	Jianbei	Secondary forest	2009.6
S3	Linfen	Secondary forest	2009.6
S4	Changsong	Cropland	2009.6
S5	Linfen	Shrub	2009.6
S6	Linfen	Meadow	2009.6
S7	Yuncheng	Cropland	2009.6
S8	Linfen	Secondary forest	2009.6

Table 2. Effects of 6 medium on isolating actinomycetes

Quantity	Gause's No.1	ZSSE	HV	CHV	Glycerol-asparagine	soil extracts agar
cfu (10 ⁶ /mL)	1.78	6.22	4.1	5.56	3	3.66

Table 3. Phylogeny analysis of the isolates

Genus of isolates	Strain number					
	1	2	3	4	5	6
<i>Streptomyces</i>	4	18	7	7	20	12
<i>Micromonospora</i>	4	4	3	6	3	3
<i>Nocardia</i>	0	3	2	2	0	4
<i>Actinomadura</i>	0	0	0	2	0	1
<i>Promicromonospora</i>	1	1	2	3	1	1
<i>Pseudonocardia</i>	0	1	0	1	0	0
<i>Rhodococcus</i>	0	1	1	0	0	0
<i>Saccharothrix</i>	0	3	0	0	1	0
<i>Nonomuraea</i>	1	1	1	0	0	2
<i>Cellulomonas</i>	0	1	0	0	0	1
<i>Jiangella</i>	0	1	0	0	0	0
<i>Gordonia</i>	0	1	0	0	0	0
<i>Cellulosimicrobium</i>	0	0	0	0	0	1
<i>Kribblla</i>	0	0	0	0	1	0
<i>Williamsia</i>	0	0	1	0	0	0
Sum	10	35	17	21	26	25

Note: 1, ZSSE; 2, Gause's No.1; 3, soil extracts agar; 4, glycerol-asparagine; 5, HV; 6, CHV

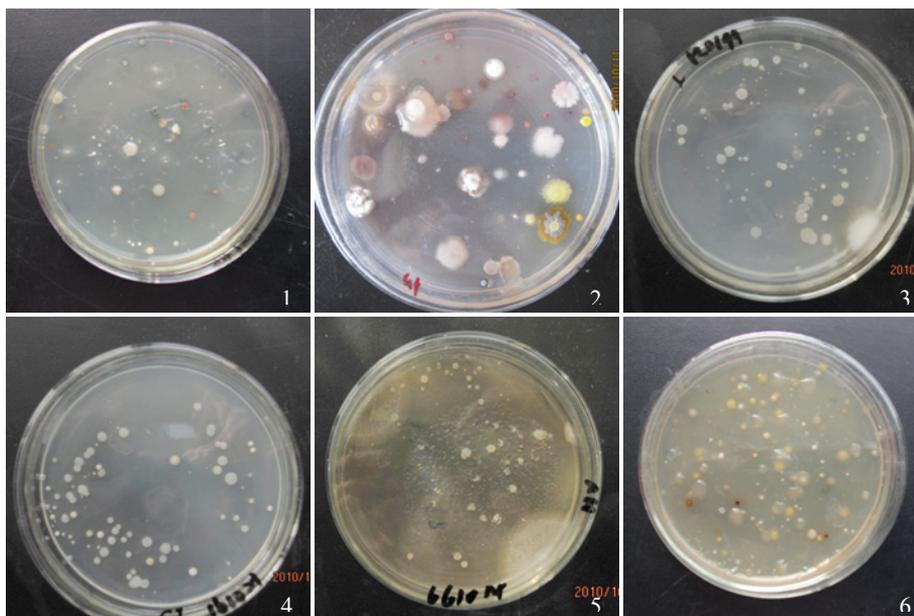


Figure 1. Colonies in 6 isolated mediums

Note: 1, ZSSE; 2, Gause's No.1; 3, soil extracts agar; 4, glycerol-asparagine; 5, HV; 6, CHV