A Simple Medium for Selective Isolation and Enumeration of Soil Actinomycetes*

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Wang-ching Ho**・Wen-hsiung Ko** : 土壌中のアクチノミセスの
選択分離・定量用培地について*

Abstract

Most media developed for enumerating soil actinomycetes do not prevent the growth of bacteria. When the pH of water agar was adjusted to 10.5, more than 90 % of bacterial colonies from natural soil were suppressed, whereas the number of actinomycete colonies remained constant. All six species of bacteria tested were completely or nearly completely inhibited in alkaline water agar, whereas, colony development of all six actinomycetes was not affected. When water agar was used the numbers of actinomycetes from soil amended with glucose or alfalfa meal were very difficult to count because of the presence of numerous bacteria. In alkaline water agar more than 99.9 % of bacteria were suppressed, and the numbers of actinomycetes were easily determined.

(Received August 4, 1980)

Introduction

Population densities of actinomycetes, bacteria and fungi in soil are usually estimated by the dilution-plate method¹⁾. Selective media have been developed for enumerating soil bacteria⁵⁾ and fungi^{7,11)}. However, media previously used for estimating actinomycetes do not prevent the development of bacterial colonies^{7,9)}. In certain nutrient amended soil in which the bacterial population is much higher than that of actinomycetes, it is difficult and often impossible to detect actinomycete colonies using these media. The purpose of this investigation was to search for a medium selective for soil actinomycetes. A brief account of this work has been reported⁶⁾.

Materials and Methods

The method of Lingappa and Lockwood⁹⁾ was used to prepare chitin agar (0.2 % colloidal chitin, 2 % Bacto agar). The amounts of 1N NaOH required for bringing 100 ml of water agar (2 % Bacto agar) and chitin agar to certain pH values were determined in preliminary tests. Subsequently the pH was adjusted by adding known amounts of 1N NaOH to media after autoclaving.

* This study was supported in part by a grant from the East-West Center to the senior author. Journal Series Paper No. 2498 of the Hawaii Institute of Tropical Agriculture and Human Resources.

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Soil suspensions were prepared by grinding 10 g of soil (silty clay loam, pH 6.3) with 90 ml of sterile distilled water in an Omni-Mixer at 4,500 rpm for one min. Soil suspensions were further diluted to 10^{-5} before being plated on agar media. Plates were incubated at 24 C and colony numbers were determined after 14-day incubation. Six plates were used for each treatment and experiments were done at least two times.

The following organisms were grown on potato dextrose agar supplemented with 0.1 % yeast extract at 24 C: *Agrobacterium radiobacter* (Beijerinck & Van Delden) Conn, *A. tumefaciens* (Smith & Townsend) Conn, *Corynebacterium insidiosum* (McCulloch) Jensen, *C. michiganense* (Smith) Jensen, *Erwinia carotovora* (Jones) Holland, *E. chrysanthemi* Burkholder *et al.*, *Pseudomonas fluorescens* Migula, *P. solanacearum* (Smith) Smith, *Rhizobium leguminosarum* Frank, *Xanthomonas campestris* (Pammel) Dowson, *Actinoplanes philippinensis* Couch, *Nocardia erythropolis* (Gray & Thornton) Waksman & Henrici, *Streptomyces antibioticus* (Waksman & Woodruff) Waksman & Henrici, *S. aureofaciens* Duggar, *S. griseus* (Krinsky) Waksman & Henrici, *S. scabies* (Thaxter) Waksman & Henrici. *Rhizobium leguminosarum* was obtained from B.B. Bohlool. The rest of the bacteria and actinomycetes were supplied by A.M. Alvarez and J.L. Lockwood, respectively. For comparing the growth of actinomycetes and bacteria in water agar at pH 10.5, actinomycete and bacterial suspensions were made from 14-day-old and 3-day-old cultures, respectively, and diluted to 10^{-6} before use.

Results and Discussion

When soil suspensions were plated on water agar adjusted to various pH values, the numbers of actinomycetes per plate were constant from pH 6 to pH 10.5 (Fig. 1). Beyond pH 10.5 the numbers of actinomycetes decreased with increasing pH. However, starting from pH 6 the numbers of bacteria decreased with increasing pH. About 90 % of the bacterial colonies were inhibited at pH 10.5, and the development of bacteria was completely inhibited at pH 11.

Fig. 1. Number of actinomycete and bacterial colonies from diluted soil suspensions plated on water agar adjusted to various pH values with 1N NaOH after autoclaving.

Table 1. Numbers of actinomycete and bacterial colonies from diluted soil suspension plated on water agar and chitin agar with and without pH adjustment

Microorganism	No. of colonies/plate ^{a)}			
	Water agar		Chitin agar	
	pH 6.2	pH 10.5 ^{b)}	pH 6.2	pH 10.5 ^{b)}
Actinomycetes	22	22	19	20
Bacteria	67	6	56	19
Fungi	0.7	1	0.5	0.7

a) Average of six plates. Data are from one of two experiments with similar results.

b) The pH of agar media was adjusted with 1N NaOH after autoclaving.

Table 2. Numbers of actinomycete and bacterial colonies from diluted suspensions of identified organisms plated on water agar and alkaline water agar (pH 10.5)

Microorganism	No. of colonies/plate ^{a)}	
	Water agar	Alkaline water agar
Bacteria		
<i>Agrobacterium radiobacter</i>	>1000	11
<i>Erwinia chrysanthemi</i>	>1000	3
<i>Pseudomonas fluorescens</i>	256	0
<i>P. solanacearum</i>	>1000	25
<i>Rhizobium leguminosarum</i>	332	0
<i>Xanthomonas campestris</i>	>1000	0
Actinomycetes		
<i>Actinoplanes philippinensis</i>	152	149
<i>Nocardia erythropolis</i>	36	34
<i>Streptomyces antibioticus</i>	48	44
<i>S. aureofaciens</i>	68	70
<i>S. griseus</i>	67	73
<i>S. scabies</i>	49	55

a) Average of six plates. Data are from one of two experiments with similar results.

Since both water agar and chitin agar have been used for determining population of actinomycetes in soil ^{2,9)}, their pH was adjusted to 10.5 to determine its effect on recovery of actinomycetes. Results showed that the numbers of actinomycete colonies developed on water agar and chitin agar were about the same and that pH adjustment did not affect recovery of actinomycetes (Table 1). However, the numbers of bacteria/plate decreased about 91 % in alkaline water agar, but only about 66 % in alkaline chitin agar. Due to their relatively low population density in soil ²⁾ fungi appeared infrequently on these plates. Water agar at pH 10.5 was, therefore, selected for further studies.

Effect of high pH on colony development in water agar was also studied using six identified species each of bacteria and actinomycetes. All six species of bacteria tested were completely or nearly completely inhibited in alkaline water agar, whereas colony development of all actinomycetes tested was not affected (Table 2). This further supports the previous results that alkaline water agar is selectively inhibitory to most bacteria but not actinomycetes.

The selectivity of alkaline water agar was further evaluated using soils with high bacterial population resulting from glucose or alfalfa meal amendment, or addition of 10 identified bacteria. When water agar was used, the numbers of actinomycetes from amended soils were very difficult to count because of the presence of numerous bacteria (Table 3). In alkaline water agar more than 99.9 % of bacteria were suppressed, and the numbers of actinomycetes were easily determined.

More than 99.9 % of bacteria from pure cultures and nutrient amended soils were suppressed in alkaline water agar (Table 2, 3) whereas only about 91–95 % of bacteria from natural soils were inhibited (Table 1, 3). This probably is due to the relative

Table 3. Numbers of actinomycete and bacterial colonies from diluted suspensions of amended soils plated on water agar and alkaline water agar (pH 10.5)

Treatment	No. of colonies/plate ^{a)}			
	Water agar		Alkaline water agar	
	Actinomycetes	Bacteria	Actinomycetes	Bacteria
Soil amended with 1% glucose ^{b)}	78	1000	82	3
Soil amended with 1% alfalfa meal ^{b)}	20	1000	20	2
Soil amended with 10 species of bacteria ^{c)}	15	1000	14	3
Soil without amendment	16	38	16	2

a) Average of six plates. Data are from one of two experiments with similar results.

b) Platings were made 7 days after amendment.

c) Platings were made right after addition of the following bacteria: *Agrobacterium radiobacter*, *A. tumefaciens*, *Corynebacterium insidiosum*, *C. michiganense*, *Erwinia carotovora*, *E. chrysanthemi*, *Pseudomonas fluorescens*, *P. solanacearum*, *Rhizobium leguminosarum* and *Xanthomonas campestris*.

sensitivity of young cells to high pH in comparison with old cells. When all 10 identified bacteria were incubated for 40 days before plating, the inhibition in alkaline water agar was decreased from more than 99.9 % to only 41 %.

The pH values shown in this report were those before solidification of agar. These values decreased to a certain extent after the agar solidified. For example, when the pH of molten water agar was adjusted to 10.5, the value decreased to 8.7 after solidification. Binding of ions by agar has been reported⁸⁾. Probably more OH⁻ ions were bound by agar in the solid state than in molten state.

Fungi were not inhibited in alkaline water agar. However, due to their relatively low population density in soil²⁾, the numbers of fungal colonies/plate were very low and negligible at the dilution suitable for counting actinomycetes. In the presence of excessive large numbers of fungi in soil, antifungal antibiotics^{3,10,12)} or sodium propionate⁴⁾ may be added to alkaline water agar. These compounds have been shown to be effective in suppressing fungi on agar media without affecting the development of actinomycete colonies. When bacterial suspensions were spotted on alkaline water agar, growth of bacteria was not inhibited. Therefore, for isolating actinomycetes from diseased tissues, they should not be placed directly on alkaline water agar. Diseased tissues should be ground with sterile distilled water, and the suspension should be mixed with molten alkaline agar before pouring plates.

Our results showed that the selectivity of water agar used for enumerating soil actinomycetes was greatly increased when the medium was adjusted to pH 10.5. The alkaline water agar is simple and effective.

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和 文 摘 要

土壌中のアクチノミセスの選択分離・定量用培地について

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これまで土壌中のアクチノミセスの定量に用いられてきた培地の多くは、土壌細菌の生育を抑制しない。アルカリ素寒天培地 (pH 10.5) を用いると土壌細菌の90%以上の集落形成が抑制されるが、アクチノミセスの集落形成は影響をうけない。植物病原細菌を含む6種の細菌のうちには、完全にその生育が抑制されたものと、著しく抑制されたものがあったが、6種のアクチノミセスはいずれも集落形成が抑制されなかった。グルコースあるいはアルファルファ粉末添加土壌のアクチノミセスの定量にあたって、素寒天培地を用いると細菌集落数が著しく多くなり、その定量は困難となるが、アルカリ素寒天培地を用いると、99.9%以上の細菌の生育が抑制され、その定量が容易となる。