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A NEW METHOD FOR THE SELECTIVE ISOLATION OF ACTINOMYCETES FROM SOIL

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Summary:

A coal-vitamin medium was developed to isolate actinomycetes from soil, which was superior to other currently used media. It increased the number of actinomycetes and inhibited the growth of other soil bacteria. The pretreatment of soil suspension with peptone (6%) and lauryl sulfate (0.05%) at 50℃ for 10 min, also greatly increased the number of actinomycetes from soil prior to incubation with new medium.

Introduction:

Actinomycetes have contributed largely to economic importance as producers of antibiotics, vitamins and enzymes (Gramajo *et al.*, 1993; Ueda *et al.*, 1992). The vast majority of actinomycetes have originated from soil (Davies and Williams, 1970). The various isolation media have been used for selecting the actinomycetes from mixed microbial populations in soil. The isolation of actinomycetes from soil are usually accomplished by the dilution plating technique using media containing selective nutrients, such as glycerol-asparagine agar, inorganic salts-starch agar, and oatmeal agar (Shirling and Gottlieb, 1966). However, These poorly selective media cannot isolate a large number of actinomycetes and may be overgrown by more abundant or faster growing microorganisms. To overcome these problems, a number of media have been developed for the selective isolation of actinomycetes. Especially, humic acid-vitamin agar (HV agar) is medium which has been the most widely used (Hawakawa *et al.*, 1988; Hawakawa and Nonomura, 1987). In the course of searching for more effective medium than HV agar for the isolation of actinomycetes, It was found that coal as a nutrient was more effective than humic acid. Therefore, a coal-vitamin agar (CV agar) containing coal as the sole sourse of carbon and nitrogen has been developed. On this agar plates, not only *streptomyces* but also many other actinomycetes belonging to *Micromonospora* (Wakisara *et al.*, 1982), *Microbispora* (Nonomura and Ohara, 1960), *Microtetraspora* (Nonomura and Ohara, 1971), *Nocardia* (Orchard and Goodfellow, 1974), and other related genera appeared and developed quite abundantly, whereas the development of microorganisms was restricted. In this paper, we describe a new method for isolating actinomycetes from soil. A selective isolation medium, CV agar containing nalidixic acid have been chosen by trial and error. It includes the pretreatment of soil suspension containing peptone as a spore activating agent and lauryl sulfate as a germicide. It permitted the growth of

larger numbers of soil actinomycetes and limited the growth of soil bacteria more than the other media.

Materials and Methods:

Soil sample:

All soil samples used for the isolation of actinomycetes and were collected from random sites. The soil samples were ground and kept at room temperature until they dried to constant weight.

Isolation and classification of actinomycetes:

One gram of air-dried soil sample was suspended in 10 ml of sterile 5 mM phosphate buffer (pH 7.0) and stirred for 1 min on a super mixer. Serial dilutions of the suspension were prepared by the 10-fold dilution method (Nonomura and Ohara, 1969). The colony morphologies were examined directly under a light microscope. Colonies of actinomycetes and other bacteria which appeared on the plates were counted with the naked eyes. All actinomycetes that appeared on the plates were tentatively classified on the basis of their morphological characteristics (microscopy; ×1000) into the following morphological groups or form-genera; *Micromonospora*, *Microbispora*, *Microtetraspora*, *Nocardia and streptomyces*.

Selective isolation media:

Coal was mined from colliery in Tokye and collected only the raw material of making a briquet. The collected coal was sieved, air-dried at room temperature for two weeks and used for the nutrient of CV agar (1.5 g coal/l). The CV medium also contains vitamins for higher recovery of actinomycetes and 50 mg/l of cycloheximide as a antifungal agent. The composition of vitamins is 0.5 mg/l each of thiamine-HCl, riboflavin, *p*-aminobenzoic acid, niacin, inositol, Ca-pantothenate, pyridoxin-HCl, and 0.25mg/l of biotin. The pH value of CV agar was adjusted to 7.2 with 1M NaOH or 1N HCl before autoclaving. For alkaline pH adjustment of medium, The medium was buffered to 30 mM with 1,3-bis[tris(Hydroxymethyl)-methylamino] propane and then adjusted to 9.0 before autoclaving. Vitamins and cyclohximide were sterilized by membrane filteration and added to the autoclaved media.

Pretreatment of selective isolation for actinomycetes:

To germinate actinomycete spores, the 10 ml of 5 mM-phosphate buffer (pH 7.0) containing one gram of soil sample was heated at 50℃ for 10 min in shaking incubator, before dilutions of the suspension. Peptone (Difco laboratories) and lauryl sulfate (Sigma chemical Co.) were used as spore activating agent and germicide, respectively.

Results:

Soil actinomycetes are so attractive group of bacteria which have great capabilities for production of commerically valuable metabolites. Figure 1 shows on isolation plate of CV agar incubated at 28℃ for 3 weeks. Actinomycete colonies developed well and formed spores or sporangia abundantly on the aerial or substrate mycelium, while the growth of bacterial colonies was restricted. On the CV agar, *streptomyces* genera developed well, and the slower growthers, *Micromonospora*, *Microbispora*, *Microtetraspora*, *Nocardia,* and related genera developed fairly well.

The CV agar plates containing coal as a sole sourse of carbon and nitrogen showed the following advantages when compared with currently used media for the isolation of actinomycetes. Using 3 types of soil samples (Soil no. 350, 357, 358), dilution plates were prepared with CV agar, HV agar, and other currently used media. Table 1 shows the actinomycete counts after 28 ℃ for 3 weeks of incubation. The CV agar produced significantly greater numbers of actinomycetes than did the other media. The selectivity of actinomycetes (% of total population) on CV agar was the highest (67-72%) among the media investigated. The CV agar (pH 9.0) was also much better than the HV agar (pH 9.0) for the number of actinomycetes.

The germination of actinomycete spores by heat treatment may be considered as the causes for an increase in number of actinomycetes on the CV agar plates used for the isolation from soil.

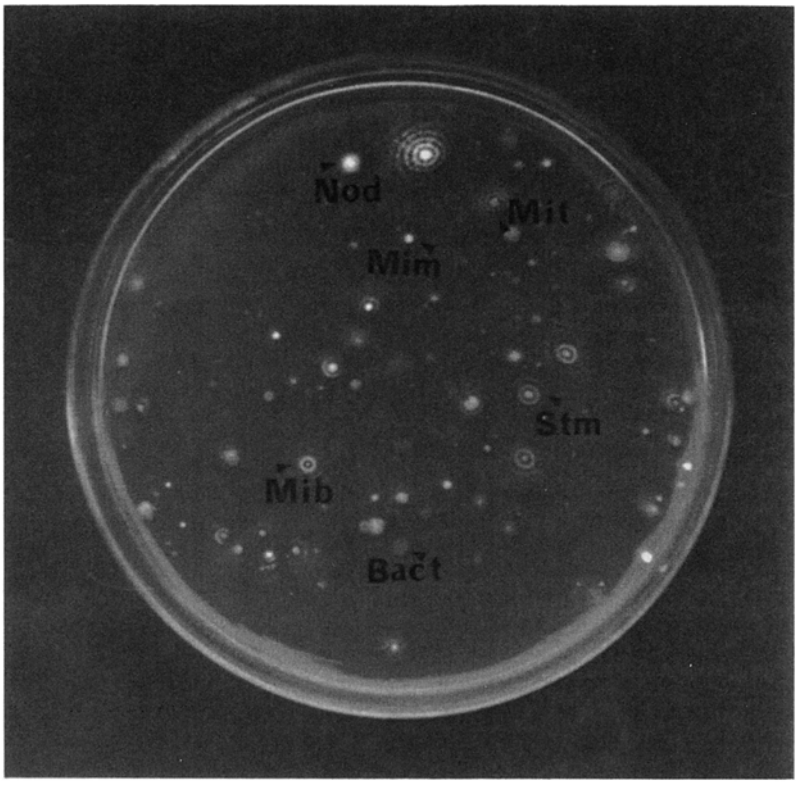

Figure 1. Actinomycete colonies on CV agar isolation plate. Typical colonies of genera are labeled with genus name abbreviation (Soil no. 345). Bact., bacteria; Stm, *streptomyces*; Mim, *Micromonospora*; Mib, *Microbispora*; Mit, *Microtetraspora*; Nod, *Nocardia*.

Table 1. Number of actinomycete colonies appearing on several selective isolation media.

Media examined	No[a]. of actinomycete colonies ($\times 10^4$)/g of soil		
	Soil no. 350	Soil no. 357	Soil no. 358
CV(pH 7.2)	227 67%[b]	241 72%	206 72%
HV(pH 7.2)	208 62%	199 67%	200 70%
CV(pH 9.0)	94 56%	132 64%	75 75%
HV(pH 9.0)	29 41%	28 44%	11 28%
Oatmeal[c]	85 32%	83 54%	35 29%
Inorganic salts-starch[c]	106 52%	115 60%	73 67%
Glycerol-asparagine[c]	72 31%	59 40%	34 27%

[a]After 3 weeks at 30℃. [b]% of total colonies. [c]supplemented with cycloheximide and vitamins. The pH was adjusted to 7.2. The composition of HV medium is humic acid, cycloheximide, and vitamins.

The treatment for proper time increased in number of actinomycetes on the plates, especially at 50℃ for 10 min resulted in the highest recovery of actinomycetes. The pretreatment of soil suspension clearly increased the number of actinomycetes. The pretreatment with peptone (P) gave the largest number of actinomycetes. The number of actinomycetes brought by the pretreatment of P was significantly larger than the number obtained from untreated inocula. On the other hand, elimination of bacteria from actinomycete isolation plates is important. It was found that the pretreatment of soil suspension with lauryl sulfate (LS) significantly decreased the number of bacteria. The action of P and LS were removed easily by dillution with distilled water. Therefore, it was planed to investigate the effects of the combined use of LS (0.05%) as a germicide to soil bacteria with P (6%) as a spore activating agent in the pretreatment of soil sample.

Table 2. Isolation of actinomycetes from various soils by the new method.

Soil sample (type)	Untreated control on CV agar			Treated with P and LS* on CV agar + n.a.**	
	Act. $\times 10^5$	Act. (control)	Bact. %	Act. %	Bact. %
(Vegetable field)					
No. 379	45	100	213	184	20
No. 380	69	100	239	123	44
No. 382	76	100	517	121	23
No. 377	56	100	157	123	117
(Road side)					
No. 389	13	100	946	185	188
No. 392	36	100	517	103	157
(Stream side)					
No. 378	22	100	886	127	82
No. 386	49	100	596	129	67
(Mountain forest)					
No. 384	14	100	521	171	83
No. 390	9	100	1144	189	129
Average		100	574	146	91

*Pretreatment of soil suspension with peptone (P) 6% and lauryl sulfate (LS) 0.05% in 5 mM-Phosphate buffer (pH 7.0) at 50℃ for 10 min,. dilution, inoculation on CV agar plates containing nalidixic acid. Nalidixic acid (n.a.) was sterilized by membrane filteration and added to the autoclaved media.

**No decrease in number of actinomycetes was observed by addition of nalidixic acid 20 mg/l.

The efficiency of the soil pretreatment with P 6% and LS 0.05% at 50℃ for 10 min was confirmed using 10 soil samples collected from vegetable fields, road sides, stream sides and mountain forests (Table 2).

The pretreatment increased actinomycetes by about 46% and decreased bacterial number to about 80%, on the averages. In 4 soil samples (Soil no. 379, 384, 389, 390), the numbers of actinomycetes brought by the pretreatment were significantly larger (71-89%) than the number on control plates. In 7 soil samples (Soil no. 378, 379, 380, 382, 384, 386, 390), bacterial counts were decreased significantly (82-96%) by the pretreatment.

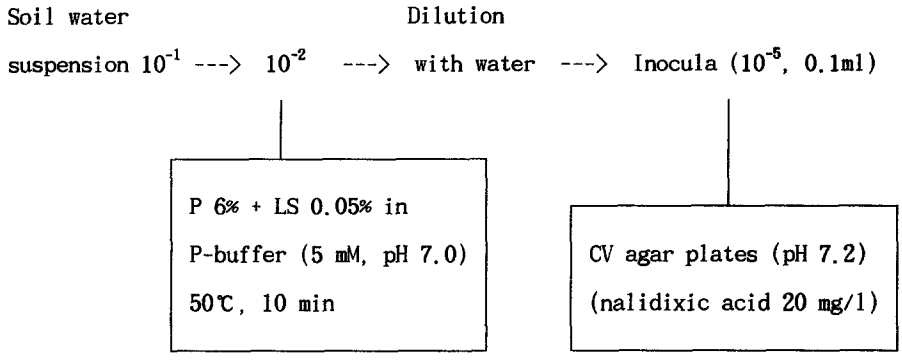

Figure 2. Scheme for isolation of actinomycetes from soil.

Conclusions:

Selective isolation method of soil actinomycetes is important for studying their ecological properties and for finding novel strains producing useful compounds of industrial interest *in vitro*. The three major stages of an isolation procedure are (i) selection of soil sample containing the microbes, (ii) pretreatment of the soil sample, and (iii) growth on laboratory media. Selection of soil sample is obviously determined by the ecological and commercial aims. The pretreatment has been designed to eliminate undesirable bacteria from inocula or to suppress the bacterial growth on isolation media, while preserving the viability of actinomycetes. Heat treatment was quite often used. Examples are the heating of soil up to 100℃ for 1 hour to isolate *Microbispora* and other rare genera (Nonomura and Ohara, 1969), and keeping soil at 40℃ for 2-16 hours to isolate actinomycetes from soil (Williams *et al.*, 1972). Selectivity of isolation medium is influenced primarily by its nutrient composition, pH and addition of selective inhibitors. From the results obtained above, the following procedure (Figure 2) was set up as a new, useful method for the selective isolation of actinomycetes from soil. The dilution after the pretreatment of soil suspension is necessary to remove the action of P and LS in isolation plates, because P promotes bacterial growth, and LS injures germinating spores of actinomycetes. The primary aim of this new method is to construct a probability selective isolation of unknown rare actinomycetes from soil.

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References:

Davies, F. L. and S. T. Williams. (1970) *Soil Biol. and Biochem.* 2, 227-238
Gramajo, H. C., E. Takano, and M. J. Bibb. (1993) *Mol. Microbiol.* 7, 837-845
Hawakawa. M., Ishizawa. K. and Nonomura, H. (1988) *J. Ferment. Technol.* 66, 367-373
Hawakawa. M. and Nonomura, H. (1987) *J. Ferment. Technol.* 65, 609-616
Nonomura, H. and Y. Ohara. (1960) *J. Ferment. Technol.* 38, 401-405
Nonomura, H. and Y. Ohara. (1969) *J. Ferment. Technol.* 47, 463-469
Nonomura, H. and Y. Ohara. (1971) *J. Ferment. Technol.* 49, 1-7
Orchard, V. A. and M. Goodfellow. (1974) *J. Gen. Microbiol.* 85, 160-162
Shirling, E. B. and Gottlieb, D. (1966) *Int. J. Syst. Bacteriol.* 16, 313-340
Ueda, Y., S. Kojima, K. Tsumoto, S. Takeda, K. Miura, and I. Kumagai. (1992) *J. Biochem.* 112, 204-211
Wakisaka, Y., Y. Kawamura, Y. Yasuda, K. Koizumi and Y. Nishimoto. (1982) *J. Antibiotics.* 35, 822-836
Williams, S. T., Shameemullah, M., Watson, E. T. and Moyfield, C. I. (1972) *Soil Biol. and Biochem.* 4, 215-225