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The Introduction of Polyacrylamide Gel into the Solid Culture of *Streptomyces spp.*

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It is proposed that polyacrylamide gel, instead of agar, could be used for the solid cultures of microorganisms including *Streptomyces* strains. Polymerization and gellation of 5% acrylamide solution were done by autoclaving for 5 min at 121℃ and not hindered by the addition of nutrient-rich media. In particular, pH buffer solution suitable for corresponding microorganisms must be used in the preparation of culture media. Comparing with agar, it was discussed that polyacrylamide gel had many advantages such as gellation within the wide range of strong acid or alkaline pH, cultivation of agar-degradable microorganisms, high purity, utilization tests of Carbon and Nitrogen sources, requirement tests of growth factors and minerals, sterilization at high temperature, diffusion assays of products depending on the pore size of gel, and stability and standardization of microbial cultures.

KEY WORDS □ Polyacrylamide gel, Agar, *Streptomyces*, Growth factors

Since 1882, agar originated from algae has been widely used for the isolation and clultivation of microorganisms(8). There are, however, some problems in impurity(11), biodegradation(13), the adjustment of pH to acid condition(5), and the inhibition of anareobes(3,9). In particular, some aquatic bacteria such as *Vibrio*, *Pseudomonas*, *Cytophaga* and soil bacteria, *Streptomyces* have been known to be able to digest agar for their growth(2,13). Because of such a limited usage, carageenan and silica gel are sometimes substituted for agar(5). These gelling agents have not been generally used, because carageenan of red algae is likely to be impure and preparation of silica gel is a little complicate.

In order to solve such disadvantages of agar in cultures of *Streptomyces* strains, that whether or not polyacrylamide gel(PAG) can be applied to solid cultures as a gelling agent is studied. Polyacrylamide gel has long been used for the seperation of the biomolecules by electrophoresis in the fields of biochemistry and molecular biology. Acrylamide and ammonium persulfate are known to be toxic to animals, men, and microorganisms(10), but once polymerization is done, the toxicity is eliminated so that the consequent polyacrylamide gels become safe(1). Therefore, polyacrylamide gel might have many merits as follows. About the biodegradation of polyacrylamide gel no scientific paper has not reported yet. Acrylamide is polymerized and gellated from low temperature to high temperature within the wide range of pH, strong acid or alkaline, and has higher purity than agar(1). Also, pore size and strength of gel can be adjusted easily.

In this paper, the utilization of carbon and nitrogen sources and the requirements of growth factors by *Streptomyces* were studied using polyacrylamide gel. And it was suggested that agar could be replaced by polyacrylamide gel in solid culture of microorganisms including *Streptomyces*.

MATERIALS AND METHODS

Bactrial cultures

Streptomyces avermitilis, *S. albus* ATCC 11385, *S. coelicolor* ATCC 10147 and *Streptomyces* sp. IH-29 were cultured and maintained on ISP-4 and Bennett's agar slants(14), *Escherichia coli* K-12 and ATCC 11775 on EMB agar slants(4), and *Lactobacillus plantarum* KFCC 11322, *L. brevis* KFCC 35464, *L. lactis*, *Streptococcus faecalis* KFCC 11729, *Leuconostoc mesenteroides* KFCC 35471 on MRS-BPB agar slants(6) by general procedures. For the harvest of spores of *Streptomyces* strains, glass beads and distilled water were placed on agar slants, and then agitated and suspended in

Table 1. *Growth of Streptomyces spp. on the various agar plates which were prepared to dissolve only agar in distilled water*

Species of *Streptomyces*	Agar				
	Difco	Merck	Sigma	Calbiochem	Agarose
S. avermitilis	+	+	+	-	+
S. coelicolor	++	++	++	++	++
S. albus	+	+	+	+	+
S. IH-29	+	+	+	+	

+; growth

distilled water in 2.5 by 15 cm screw-capped test tube.

Preparation of agar gels

It was examined that whether or not there is any nutrient(s) enabling *Streptomyces* strains to grow in agar. Agar used was the products of Difco, Merck, Sigma and Calbiochem, and pure agarose one of Sigma.

Fifteen grams of agar or agarose were added to one litre of distilled water and boiled on a heater, and thereafter 20 m*l* of the agar or agarose solutions were poured to 2 by 18 cm test tubes, followed by plugging with cotton stopper. Finally, the test tubes were autoclaved at 121℃ for 15 min. While in hot state, solutions in test tubes were put into sterile and disposable plastic Petri-dishes (1.5 by 8 cm, GCM, Seoul) and solidified at room temperature.

All Petri-dishes were kept in 45℃ incubator for 24 hr to allow water of syneresis to evaporate enough. One tenth m*l* of spore suspension was loaded and spread by a L-form glass rod, and incubated in 25℃ incubator.

Preparation of polyacrylamide gel medium

Tests were made to know how acrylamide and ammonium persulfate contained in polyacrylamide gel media influence the growth of bacteria used. Acrylamide(Sigma) 47.5 g and N,N'-methylene-bis-acrylamide(Sigma) 2.5 g were added to 1 litre of 0.2 M phosphate buffer or distilled water in 1 litre beaker in ice and rendered soluble thoroughly. N,N,N',N'-tetramethylethylenediamine (TEMED) 0.25 m*l* and ammonium persulfate (Sigma) 1.5 g were next added to the above solution and dissolved(1), immediately followed by addition of constituents of media as described in bacterial cultures. Twenty m*l* of the mixture of acrylamide and medium was pipetted by the pipette pump(Bel-Art products, USA) into a glass Petri-dish and for polymerization autoclaved at 121℃ for 5 min, keeping Petri-dish horizontally. All procedures should be finished within about one hour. Evaporation of excess water in Petri-dish and inoculation were performed as the same procedures in the preparation of agar gels. Gram-negative bacteria were incubated at 37℃ and Gram-positive bacteria at 25℃.

In order to examine how *Streptomyces avermitilis* and *Streptomyces* sp. IH-29 are effected in phosphate buffer, its concentrations were adjusted to 0.01, 0.05, 0.1, 0.15 and 0.2 M, which were used as a solvent for polymerization of 5% acrylamide supplemented with Bennett's medium.

Utilization tests of amino acids

It was tested that *Streptomyces avermitilis* can use 20 amino acids as carbon or nitrogen sources on PAG plus ISP-4 medium. The concentration gradients were varied from 0 to 100 μg/m*l* of medium at intervals of 20 μg/m*l*. In C and N source tests of amino acids, soluble starch and ammonium sulfate were excluded from respective test medium. Glucose was used in place of soluble starch in N source tests.

RESULTS AND DISCUSSION

Preparation of polyacrylamide gels for solid cultures

Attempts were made to find conditions that microorganisms are able to grow on PAG with culture media. When 20 m*l* of acrylamide solution in glass Petri-dish was polymerized at room temperature under ambient illumination, the gel formed undulate surface, even though acrylamide solution was degassed by a vaccum pump. It is likely that it was partly because gels were exposed to open air for a long time and oxygen redissolved to gels. When acrylamide solutions were autoclaved at 121℃ for 15 min, the gels formed were distorted and torn irregularly. Through many trials, we found that after autoclaved at 12℃ for 5 min, even surface of gels could be formed to be good enough for solid cultures. This gellation condition permitted also sterilization. Before inoculation, PAG gels have to be rendered dry sufficiently because excess water was evaporated more than in case of agar gel. Otherwise, colonies, were not grown separately.

Growth on agar gel

The growth of *Streptomyces* strains was checked on agar gels prepared with 4 kinds of agar and agarose which were manufactured by different companies, i.e., Sigma, Merck, Calbiochem, and Difco.

Table 2. *Growth responses of different bacterial strains on acrylamide gel prepared with distilled water and 0.2 M phosphate buffer, and added with ingredients of various culture media*

Species	Acrylamide gel with distilled water				Acrylamide gel with 0.2 M phosphate buffer			
	A	B	C	D	A	B	C	D
Escherichia coli	ND	ND	−	ND	ND	ND	+	ND
Lactobacillus brevis	ND	ND	ND	−	ND	ND	ND	+
Lactobacillus plantarum	ND	ND	ND	−	ND	ND	ND	+
Lactobacillus lactis	ND	ND	ND	−	ND	ND	ND	+
Streptococcus faecalis	ND	ND	ND	−	ND	ND	ND	+
Leuconostoc mesenteroides	ND	ND	ND	−	ND	ND	ND	+
Streptomyces avermitilis	−	−	ND	ND	+	+	ND	ND
Streptomyces IH-29	−	−	ND	ND	+	+	ND	ND

ND; not determined, +; growth, −; no-growth A; ISP-4, B; Bennett, C; EMB, D; MRS-BPB

Streptomyces coelicolor showed the normal growth of substrate and aerial mycelia and the formation of spores, but other *Streptomyces* did mycelial growth and were observed to form scanty spores on the colonies(Table 1). These facts indicate that small amounts of various carbon and nitrogen sources, and minerals available for the growth exist in agar itself, although nutrients are mostly eliminated in the purification of agar. Actually agar contains different forms and concentrations of minerals such as Ca^{++}, Mg^{++} and other ions, depending on different origin of algae and purification processes(11), and *Streptomyces* strains are prototrophic and many of them are oligocarbophilic(14).

Thus, strictly saying, agar gel could not be used for the performance of the tests comparing relationship between the growth and nutrients required in the cultivation of *Streptomyces* strains. Because of these disadvantages, not only the utilization of carbon and nitrogen sources and minerals but the detection of metabolic or genetic auxotrophs requiring growth factors could not be determined exactly(7,12). Furthermore, the assays of growth and differentiations would be more difficult in the strains which cometabolism are achieved in the presence of different carbon sources.

Growth on polyacrylamide gel

The growth of *E. coli*, 5 species of lactic acid bacteria and 2 species of *Streptomyces* was compared on the PAG prepared by distilled water and 0.2 M phosphate buffer, which were supplemented with EMB, MRS-BPB, ISP-4, and Bennett's media(Table 2).

All tested bacteria did not grow on PAG with distilled water, whereas they grew on PAG with phosphate buffer and *Streptomyces* showed good growth and sporulation. Thereby, inhibition factors or substances were removed when acrylamide is polymerized in phosphate buffer solution. The reason is that the solution of ammonium persulfate(APS) as a catalyst of polymerization is acid and decomposed to evolve oxygen and form ammonium bisulfate and free oxygen radicals at high temperature(10) and thereby the solution is neutralized by phosphate buffer. In this study, actually the pH of APS solution made of distilled water was about 5.5, if acrylamide, bis-acrylamide, and TEMED were added, the pH of mixture became about 6.7. After autoclaved, the pH of APS solution was decreased to about 2.3.

Table 3. *Effects of phosphate buffer concentrations on the growth of Streptomyces avermitilis and Streptomyces sp. IH-29 on acrylamide gel added with Bennett's medium.*

Species	Phosphate buffer concentrations					
	D.W.	0.01	0.05	0.1	0.15	0.2
S. avermitilis	NG	NG	6.48	6.62	6.64	6.6
S. IH-29	NG	NG	7.10	7.0	7.0	6.8

Arabic numbers represent log values of colonies/ml
NG ; no-growth

The concentration of phosphate buffer was 0.05-0.2 M, which were able to neutralize strong acidity from the decomposition of APS by autoclaving. At the above-mentioned concentrations good growth was shown in *S. avermitilis* and *Streptomyces* sp. IH-29(Table 3). The growth and sporulation of the latter two strains on PAG plus bennett's medium was similar to that on agar, considering many experiences acquired from cultivation on agar plates.

Thus, the gelling property of PAG was not changed by the supplement of nutrient-rich media or sterilization. The inhibition of growth by strong acidity could be removed by the use of proper buffer solution corresponding to each microorganism. Therefore, polyacrylamide gel

Table 4. *Colony numbers of Streptomyces avermitilis utilized amino acids as carbon and nitrogen sources*

Amino acids	Concentrations (μg/m*l*) C source					N Source				
	20	40	60	80	100	20	40	60	80	100
control	–	–	–	–	–	–	–	–	–	–
Asp	47	46	45	57	52	28	32	40	34	29
Arg	–	–	–	–	–	24	27	30	30	20
Glu	–	–	–	–	–	20	22	27	24	24
His	32	30	32	26	22					
Ile	–	–	–	–	–	32	32	39	34	30
Lys	34	42	36	40	37	–	–	–	–	–
Thr	27	25	37	40	34	–	–	–	–	–
Trp	–	–	–	–	–	27	31	40	33	32
Ala, Asn, Cys, Gln,	–	–	–	–	–	–	–	–	–	–
Leu,Met,Gly,Phe.	–	–	–	–	–	–	–	–	–	–
Pro,Ser,Tyr,Val,	–	–	–	–	–	–	–	–	–	–

Arabic numbers represent average colony numbers per 3 PAG media plate. As control test, polyacrylamide plus ISP-4 medium without carbon or nitrogen source was used.
–; no growth

media are stable, because it is chemically inert and homogeneous(1), and available for culture standardization due to its purity.

Utilization of amino acids as C and N sources

As mentioned above, we knew that PAG supplemented with media should be used because *Streptomyces* strains grow to utilize carbon, nitrogen, and minerals in agar gel itself, so that which carbon and nitrogen added into agar media are used can not be decided. The utilization of 20 amino acids was examined on PAG plus ISP-4 as C and N sources.

Streptomyces avermitilis did not grow on PAG plus ISP-4 without carbon and nitrogen as a control experiment. This means that acrylamide, bis-acrylamide, TEMED, and APS which contains amides, amines, and ammonium ions and carbon were not used as energy source. Unavailability of nitrogen in amides and amines indicated that acrylamides and TEMED were not degraded by *Streptomyces*. The reason why ammonium ions of ammonium bisulfate transformed from APS were not uptaken is due to the evaporation of ammonia gas which is produced from ammonium bisulfate by means of high temperature and pressure (autoclaving)(10), and sulfuric acid formed under the same conditions was neutralized in phosphate buffer solution.

In the utilization of amino acids, *S. avermitilis* used aspartic acid, lysine, and threonine as carbon sources, and arginine, histidine, tryptophan, glutamic acid, aspartic acid, and isoleucine as nitrogen sources(Table 4). From the results PAG could be applied to the determination of not only carbon and nitrogen utilization but the detection of metabolic and genetic auxotrophs, observing the differenciation process of *Streptomyces* strains on PAG.

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REFEREMCES

1. **Andrew, A.T.,** 1986. Electrophoresis-Theory and Biochemical and Clinical Application. p. 5-25. 2 nd ed., Oxford Univ., Press OX2 6DP.
2. **Buttner, M., A.M. Smith and M.J. Bibb,** 1988. At least three different RNA polymerase holoenzyme direct transcription of the agarase gene(dagA) of *Streptomyces coelicolor* A3(2). *Cell,* **52**, 599-607.
3. **Cox, C.S., and R.J. Heckly,** 1973. Effects of Oxygen upon freeze-dried and freez-thawed bacteria: viability and free radical sutdies. *Can. J. Microbiol.,* **19**, 189-194.
4. **Difco manual(10th ed.),** 1984. EMB agar, p. 324, ISP-4 agar, p. 467, Difco Laboratories, Detroit Michigan 48232 USA.
5. **Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A.Wood, N.R.Krieg and G.B. Phillips,** 1981. Manual of methods for general Bacteriology. p. 143-145. American Society for Microbiology, Washington DC 20006.
6. **Han, H.U. and H.K. Park,** 1991. Differential Counts of Lactic Acid Bacterial Genera On Bromphenol blue medium. *Bull. Inst. Basic Sci.,* Inha Univ., **12**, 89-94.
7. **Han, H.U., M. Yang, J.M. Kim, and M.S. Kweon,** 1992. Requirements of Cysteine and Methionine of *Streptomyces avermitilis* by Treatment of Triton

X-100. *Bull. Inst. Basic Sci.*, Inha Univ., **13**, (in press).

8. **Hitchens, A.P. and C. Leikind,** 1939. The introduction of Agar-agar into bacteriology. *J. Bacteriol.*, **37**, 485-492.
9. **Hoffman, P.S., H.A. George, N.R. Krieg, and R.M. Smibert,** 1979. Studies of the microaerophilic nature of *Campylobacter fetus* subsp. jejuni. II. Role of exogenous superoxide anions and hydrogen peroxide. *Can. J. Microbiol.*, **25**, 8-16.
10. **Martha W.(ed),** 1983. Acrylamide, p. 19, Ammonium peroxydisulfate, p. 79. In The Merck Index, Merck Co.,Inc. Rahway, New Jersey, USA.
11. **Okami, Y., S. Arima and M. Suzuki,** 1963. Influence of agar on the morphology and pigmentation of Streptomycetes. *Appl. Microbiol.*, **11**, 493-497.
12. **Prosser, B. LA. T., and N.J. Palleroni.,** 1978. Nutritional characterization of some selected Actinomycetes. *Int'l Syst. Bacteriol.*, **28**, 516-522.
13. **Stainer, R.Y.,** 1941. Studies on marine agar-digesting bacteria. *J. Bacteriol.*, **42**, 527-558.
14. **Starr, M.P**(ed.), 1981. Benett's agar, p. 1359, The family Streptomyceaceae,p. *In* The Prokaryotics, Springer-Verlag, Berlin, Heidelverg, New York.

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초 록: *Streptomyces* spp.의 고체배양시 Polyacrylamide gel의 이용

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Polyacrylamide gel을 Streptomyces 및 미생물의 고체배양시 한천대신에 사용할 수 있음을 제시 하였다. Polyacrylamide gel은 51% acrylamide농도로 121℃에서 5분간 중합 및 고화 시켰다. 영양분이 많은 배지를 첨가하여도 중합반응에는 지장이 없었다. 특히, 배지를 제조할때 시험균주에 적합한 pH 완충액을 반드시 사용하여야 했다. 한천과 비교해볼때 Polyacrylamide gel의 장점으로 전 pH 영역에서 고화가 가능하고, 한천 분해균을 배양할 수 있고, 고순도를 유지하며, 고체배지에서 탄소 및 질소원의 이용을 시험할 수 있고, 생장인자 및 무기염의 요구성 실험에도 사용할 수 있고, 고온에서 멸균이 가능하고, 공극에 따른 생성물의 확산을 검정할 수 있고 그리고 생장조건의 표준화 및 안정성에 응용할 수 있음을 토론 하였다.