A Method for the Rapid Cultivation of *Desulfovibrio aestuarii* on Filter Membranes[1]

N. Tsuneishi[2] and A. Goetz

California Institute of Technology, Pasadena, California

Received for publication July 8, 1957

The cultivation of sulfate-reducing bacteria on agar plates is a somewhat involved and time-consuming process. This is partly due to the requirement for fairly strict anaerobic conditions but mainly because of the length of the incubation period which may vary from 6 days to weeks with the resulting delay of the required information.

The work reported herein was undertaken in an attempt to apply the advantageous features of the membrane filter method to the detection of anaerobic organisms. As originally developed, the membrane filter (Goetz and Tsuneishi, 1951; Clark *et al.*, 1951) permits the recovery of small numbers of organisms from large volumes of water and the rapid cultivation of many aerobic bacteria. The ultimate purpose was to develop a method which would not require more complex manipulation than the membrane procedure according to *Standard Methods for the Examination of* Water, Sewage, and Industrial Wastes (A.P.H.A. 1955) and to reduce the incubation period for anaerobic bacteria.

The fact that *Desulfovibrio aestuarii* can be cultured on membranes was first shown by Oppenheimer (1952) who applied the standard technique of providing for an oxygen-free atmosphere to the membrane surface with considerable success.

Methods and Results

The cultures employed in these experiments were two strains of *Desulfovibrio aestuarii* isolated from waste and swamp waters of local oil wells. Both strains are characterized by an incubation requirement of 3 to 5 days for the first evidence of growth and 10 to 14 days for complete development on broth culture.

The basic principle of the method is illustrated schematically in figure 1 which represents a vertical cross-section of a culture dish. (*M*) indicates two halves of a filter membrane on the surface of which the organisms have been concentrated by the filtration of an adequate volume of a water sample. After filtration, the membrane is cut in two and one section is inserted with its surface facing downward (*a*), the other section with its surface facing upward (*b*). If this is done in such a manner that (*M*) is covered on both sides with a suitable anaerobic nutrient agar, growth should be expected as this procedure is, in principle, identical with the customary anaerobic broth culture method. Growth is indicated by the topical formation of (black) FeS in the environment of each colony.

Experience showed that the development of colonies was very slow, the first visible trace may be delayed for many days. Growth occurred consistently earlier if the membrane surface (*a*) carrying the organism faced the bottom of the dish, and much later, if at all, for the reverse position (*b*). Furthermore, it was observed that the nearer the bacteria-bearing surface of the membrane was to the bottom of the dish, the earlier the growth occurred. These findings seemed to indicate the possibility of substantially decreasing the diffusion rate of O_2 (and of other soluble components) across the agar (that is, in a vertical direction) by imbedding into the plate a membrane parallel to the surface of the nutrient which contacts the air. The organisms on the downward side of the membrane (*a*) in figure 1 can thus only be reached by those O_2-molecules which, after diffusing across the agar, pass also the (liquid-filled) extremely fine pores of the membrane—in contradistinction to the upward side (*b* in figure 1) which is directly exposed to the O_2-transfer through the agar. At (*a*) the rate of O_2-influx is apparently small enough for quantitative neutralization by the reducing agents contained in the nutrient. The mode of procedure which resulted in optimal growth development seems to justify this assumption.

An attempt was made to add a rapidly growing aerobic flora (for example, *Serratia marcescens*) to the water sample prior to filtration, in order to introduce

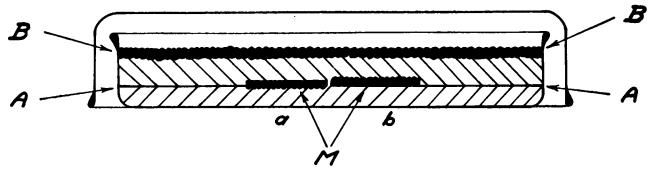

Figure 1. Vertical cross section of culture dish: (*M*) filter membrane imbedded between sterile agar layer (*A*) and aerobe-bearing layer (*B*), culture facing downward (*a*), and upward (*b*).

[1] Presented at the 56th General Meeting of the Society of American Bacteriologists, Houston, Texas, May 1956. Bacteriol. Proc. 1956, Soc. Am. Bacteriologists, p. 54.

[2] Present address: AG Chemical Co., Inc., Pasadena, California.

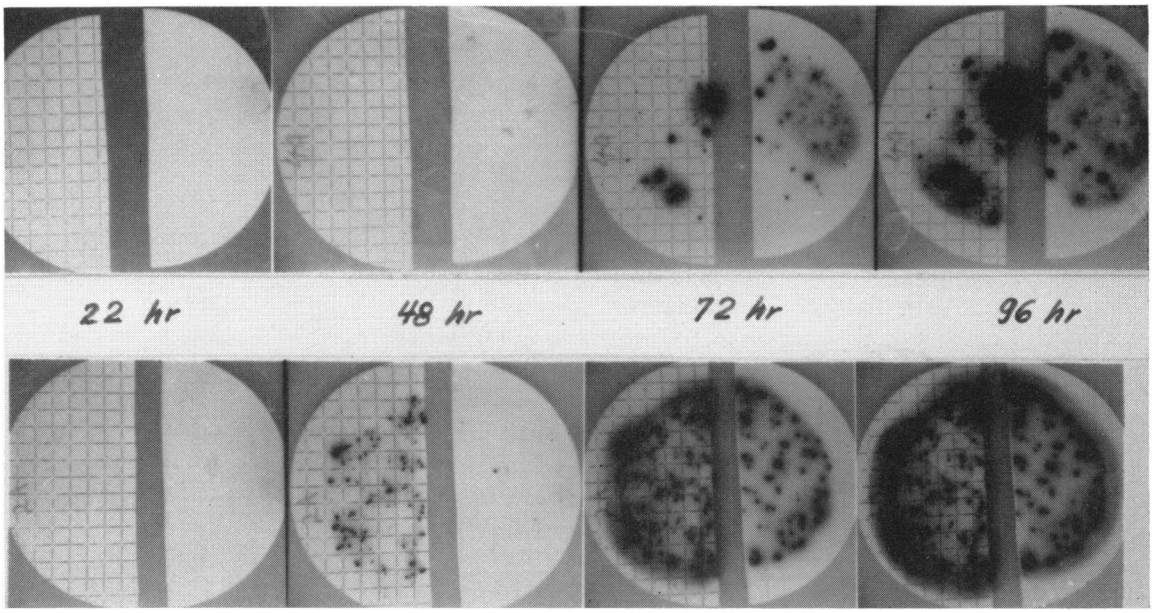

Figure 2. Membrane cultures of *Desulfovibrio aestuarii* at different stages of incubation, *without* (upper row), and *with* (lower row), aerobic infection of top agar layer (*B*, *cf* figure 1). Line pattern on surface bearing the organisms. Photographed through bottom of dish.

an "O_2-scavenger" in the early growth stages on the submerged membrane. This procedure proved unsuccessful evidently due to the inhibitory effect of the metabolic by-products of the aerobes when developing in the immediate vicinity of the anaerobes on the same membrane surface. However, the use of the aerobic flora as an "O_2-scavenger" was successful if the aerobic flora were separated by the membrane from the anaerobes, that is, if the diffusion barrier represented by the pores of the membrane prevented or delayed the inhibitory products of the aerobes from reaching the anaerobes.

The separation was accomplished in the following manner: The sterile nutrient agar was poured into the dish to level (*A*) in a thin layer (about $\frac{1}{8}$ in.), the membrane was then laid upon this layer, face downward, and a thick layer (*B*) (about $\frac{3}{8}$ in.) of the same nutrient heavily inoculated with a 24-hr culture of a fast growing aerobe (such as *S. marcescens*) was poured on top of the first layer. In this manner, a rapid and uniform development of the aerobes occurred on the level (*B*), blocking the access of oxygen into the depth of the agar, and at the same time consuming the dissolved oxygen within the agar at the start of the culture period. With this procedure, the development of the anaerobes (and the formation of FeS) rendered colony counts easily made after 24–36 hr incubation at 30 C.

To facilitate good liquid contact between the membrane surface and the first agar layer (*A*), the agar content should be low (0.75 per cent), while larger in the inoculated layer (*B*) (2 per cent).

The nutrient formula evaluated as optimal for these

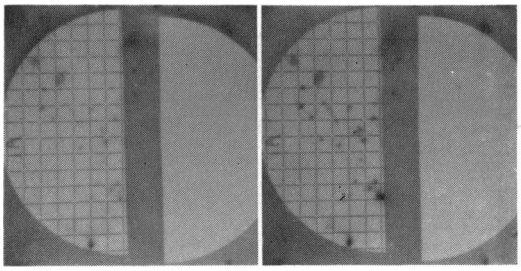

Figure 3. Filtrate of *Desulfovibrio aestuarii* under optimal growth conditions after 24 and 48 hr incubation.

conditions contains in principle the constituents given by Miller (1949), Starkey and Wright (1945), as well as Grossman and Postgate (1953), though with substantial variations in concentration. The nutrient composition is as follows: K_2HPO_4, 0.6 g; $MgSO_4$, 0.3 g; $Fe(NH_4)_2(SO_4)_2$, 0.1 g; sodium lactate, 18.0 g; ascorbic acid, 0.3 g; sodium thioglycollate, 0.3 g; Bacto-yeast extract, 3.0 g; NaCl, 30.0 g; Bacto-peptone, 3.0 g; distilled water, 1000 ml; pH, 7.2.

The $Fe(NH_4)_2(SO_4)_2$ indicator is heat labile and is prepared as a 1 per cent solution in sterile water and added (1 ml per 100 ml nutrient) to the liquid agar just prior to the pouring of the plates.

The over-all concentration of the nutrient is about 3 times higher than that used by previous workers for broth cultures. This concurs with previous findings (Goetz and Tsuneishi, 1951) that the peculiar physical growth conditions of the bacteria on the membrane surface permits a tolerance of substantially higher concentrations of nutrients and minerals with resultant rapid colony development.

The colony count is effected by inspecting the

membrane through the bottom of the dish. Figures 2 and 3 show colonies photographed through the bottom of the plate. Figure 2 shows four development stages on two different membranes, each after 22, 48, 72, and 96 hr, where each membrane had been cut in half after filtration, and placed in the positions (*a*) and (*b*) as shown in figure 1. The surface bearing the organisms (characterized by imprinted line pattern) and facing the bottom of the dish, shows a distinctly higher development of the FeS-growth indicator. The upper row represents a development without aerobes in (*B*); the lower row represents the identical culture in the presence of aerobes uniformly dispersed in the upper layer. In this case, the incubation period was reduced from 72 to less than 48 hr and gave a more uniform growth development.

Figure 3 shows the development of colonies following filtration of *D. aestuarii* tested under optimal conditions after 24 and 48 hr incubation to show that a complete development had taken place after 24 hr as evidenced from identical counts at both times.

Two phenomena, which appear to characterize the development of this organism on membranes under the conditions above described, seem worthy of mention: If a large number of organisms is present on the membrane surface, the initial growth period is accompanied by the development of numerous gas bubbles. These appear on the under side of the membrane and are visible sometimes after 12 to 18 hr. These bubbles disappear in the later growth stages. The appearance of black centers of FeS at each colony occurs after 24 hr (or even less). However, in the case of dense growth, these black zones are replaced by an intense black rim around the membrane. The individual colony centers do not develop apparently due to a condition concurrent with excessive growth activity of the anaerobe which prevents the reduction of the sulfate complex to FeS. The same phenomenon may be responsible for the gradual disappearance of most or all black coloring after extended incubation.

The rapidity of development of the black zone of FeS and the subsequent fading of the color is dependent on the thickness of the upper (scavenger-bearing) layer (*B*). If the upper layer is about the thickness (about $\frac{1}{8}$ in.) of the lower layer (*A*), development occurs as early as 24 hr, but the fading of the color sets in at about 72 hr. If the upper layer is made 3 to 4 times as thick, the color development is delayed for 12 to 16 hr, but, once it has developed, it persists for a week or more.

Experiments were made to investigate whether an increase of incubation temperature from 30 to 37 C would shorten the growth period further (because of the more rapid development of the aerobes). The results were not promising. Although the initial growth was obviously stimulated by the higher incubation temperature, gas bubbles which invariably developed below the membrane interfered with the countability.

ACKNOWLEDGMENTS

The authors wish to acknowledge the advice in the matter of nutrient composition received from Mr. H. F. Keller, Research Process Department of Union Company, Brea, California and from Mr. H. Wiener, Research Laboratory of Shell Oil Company, Los Angeles, California, as well as for supplying cultures of *Desulfovibrio aestuarii*.

The senior author (A. G.) wishes to express his obligation for some stimulating discussions on the subject with Prof. Claude E. ZoBell, Scripps Institution of Oceanography, La Jolla, California.

SUMMARY

An anaerobic culturing method, tested with *Desulfovibrio aestuarii*, is described which does not require the control of the atmosphere contacting the agar plate. It utilizes a dense aerobic flora (*Serratia marcescens*) as O_2-scavenger in the upper layer, and an inverted filter membrane as carrier of the anaerobes in the lower layer of an agar plate. In this manner, the diffusion resistance of the membrane shields the anaerobes against oxygen as well as against the inhibitory effect of the aerobic growth. Discrete colonies having black centers of FeS-formation are obtained after 24 to 36 hr incubation at 30 C. Apparently due to subsequent oxidation these centers can disappear after 72 hr.

REFERENCES

American Public Health Association, American Water Works Association, and Federation of Sewage and Industrial Wastes Association. 1955 *Standard methods for the examination of water, sewage, and industrial wastes*, 10th ed., pp. 395–404. American Public Health Association, Inc., New York, New York.

CLARK, H. F., GELDREICH, E. E., JETER, H. L., AND KABLER, P. W. 1951 The membrane filter in sanitary bacteriology. Public Health Repts. (U.S.), **66**, No. 30, 951–977.

GOETZ, A. AND TSUNEISHI, N. 1951 Application of molecular filter membranes to the bacteriological analysis of water. J. Am. Water Works Assoc., **43**, 943–969.

GROSSMAN, I. P. AND POSTGATE, J. R. 1953 The estimation of sulphate-reducing bacteria (*Desulfovibrio desulphuricans*). Proc. Soc. Appl. Bacteriol., **16**, 1–9.

MILLER, L. P. 1949 Rapid formation of high concentration of hydrogen sulfide by sulfate reducing bacteria. Contrib. Boyce Thompson Inst., **15**, 437–465.

OPPENHEIMER, C. H. 1952 The membrane filter in marine microbiology. J. Bacteriol., **64**, 783–786.

STARKEY, R. L. AND WRIGHT, K. M. 1945 *Anaerobic corrosion of iron in soil*. American Gas Association, New York, New York.