COMPARATIVE CAPACITIES OF VARIOUS PLATING MEDIA FOR SATISFYING THE CULTURAL REQUIREMENTS OF AEROBIC BACTERIA IN MATERIALS OF MARINE ORIGIN.

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SUMMARY

Eight formulas of plating medium have been studied to determine their comparative abilities in satisfying the cultural requirements of the bacterial population in materials of marine origin. Five series of experiments comprising a total of 56 trials have been performed. The procedure and technique employed are thoroughly discussed.

Media prepared with "aged" sea water as menstruum were found to be definitely superior to those prepared with distilled water, alone or with NaCl.

The effect of different diluents, various plating temperatures of the medium and various incubation temperatures on the total bacterial colony counts were also investigated.

The principal problem which arises when planning any bacteriological investigation requiring quantitative bacterial determination in materials of marine source is the selection of a medium that gives the most nearly accurate picture of their bacterial population. Such a medium should satisfy the cultural requirements of a great number of microorganisms in the material under study.

The large number of nutrient media recommended for this purpose attests to the basic importance of an appropriate medium for enumerating marine bacteria. Comparative studies conducted by the Scripps Institution of Oceanography, on a large number of media which have been recommended by various workers show that medium 2216 (vide infra) is one of the best for supporting the growth of a maximum number of bacteria and for ability to reproduce the plate counts on marine material (ZoBell, 1946).

Fischer (cited by ZoBell, 1942) recommended a medium which differs slightly from 2216. This medium contains 1.0 per cent peptone and 0.5 per cent fish extract in sea water. ZoBell (1941), however, failed to produce any increase in plate counts by fortifying Medium 2216 with various fish extracts. Lloyd (cited by ZoBell, 1942) following experiments with ex-

tracts of various marine organisms including fish, states that they are not essential.

Bacteriologists of the Woods Hole Oceanographic Institution (cited by ZoBell, 1946) adopted the following formula which consistently gave them the best results:

Peptone	g.	1.0
Glucose	do	1.0
K_2HPO_4	do	0.05
Agar	do	15.0
Sea Water	cc.	1000.0

Berkeley, Lipmann, Bavendamn, Gee, and others (cited by ZoBell, 1942) used media containing nitrate. ZoBell (1941), using 0.005 to 0.10 per cent potassium nitrate, was unable to demonstrate any effect, "beneficial or detrimental" on the quantitative or qualitative plate count.

Kiser and Beckwith (1944) in their study of the bacterial flora of mackerel, compared mackerel infusion with 2216 medium for quantitative bacteriological examination and found the latter superior.

To the writer's knowledge no work on this particular phase of bacteriology has been conducted under local conditions. In view of the necessity of a suitable medium for making total and comparative counts of the shellfish-growing water and oysters from the Bureau of Fisheries oyster farm at Binakayan, in connection with investigations being conducted on these materials, the following series of experiments were performed.

MATERIALS

Media.—Eight formulas of plating medium were tried in this study.

Formula 1—2216 Medium

Bacto peptone	g.	5.0
Ferric phosphate	do	0.1
Bacto-agar	do	15.0
Sea Water ("aged")	cc.	1,000.0

pH not adjusted, about 7.6 after sterilization.[1]

Formula 2—Nutrient agar[2] with sea water menstruum

Bacto-beef extract	g.	3.0
Bacto-peptone	do	5.0
Bacto-agar	g.	15.0
Sea Water ("aged")	cc.	1,000.0

pH adjusted to 7.8 before sterilization.

Formula 3—Nutrient agar with 0.5 per cent sodium chloride

Bacto-beef extract	g.	3.0
Bacto-peptone	do	5.0
Bacto-agar	do	15.0
Sodium chloride	do	5.0
Distilled water	cc.	1,000.0

pH adjusted to 7.8 before sterilization.

Formula 4—Nutrient agar with 15 per cent sodium chloride

Bacto-beef extract	g.	3.0
Bacto-peptone	do	5.0
Bacto-agar	do	15.0
Sodium chloride	do	150.0
Distilled water	cc.	1,000.0

pH adjusted to 7.8 before sterilization.

Formula 5—"Alamang" infusion agar.[3]

Alamang infusion	cc.	1,000.0
Bacto-peptone	g.	5.0
Bacto-agar	do	15.0

Adjusted to pH 7.8 before sterilization

Formula 6—Fish infusion agar.[4]

Fish infusion	cc.	1,000.0
Bacto-peptone	g.	5.0
Bacto-agar	do	15.0

Adjusted to pH 7.8 before sterilization.

Formula 7—Eyre's Oyster Agar (1944).[5]

Oyster infusion	cc.	1,000.0
Agar	g.	20.0

pH not adjusted about 7.6.

Formula 8—A modification of the formula recommended by the Woods Hole Oceanographic Institution.[6]

Peptone	g.	1.0

[1] All media were sterilized in the autoclave at 15 lbs. pressure for 20 minutes.

[2] Bacto-nutrient broth (Difco-dehydrated) was used with the other

[3] Preparation of "alamang" infusion. To 500 g. fresh "alamang' (post-larval stages of Penaeidae) 1,000 cc. of "aged" sea water was added. The mixture was blended and infused overnight in the refrigerator, and then boiled and strained through cheesecloth and then through filter paper. The infusion was sterilized at 15 lbs. pressure for 20 minutes.

[4] The procedure for the preparation of fish infusion is the same as given for "alamang" infusion. Dalagang bukid (*Caesio caerulaureus*, Lacépéde) flesh was used, this being one of the most easily available at the time.

[5] The procedure was slightly modified by infusing the oysters in the refrigerator overnight instead of at 37° C. for 30 minutes. "Aged" sea water was used.

[6] The original formula calls for K_2HPO_4.

Glucose	g.	1.0
KH_2PO_4	do	0.05
Agar	do	15.0
Sea Water	cc.	1,000.0

pH not adjusted, about 7.6 after sterization.

Sea water.—Whenever sea water was required, filtered, sterile, "aged" sea water[7] was employed.

Source of specimens plated.—A total of 56 specimens were plated. Fifty of these were obtained from the Bureau of Fisheries oyster farm at Binakayan. These included 34 shellfish-growing water specimens and 16 oyster specimens. The other six included one of "aged" sea water, one of pooled "alamang" (postlarval stages of Penaeidæ), three of sea water obtained at the breakwaters in Manila Bay, and one specimen of pooled intestinal contents of "dalagang bukid" (*Caesio caerulaureus* Lacépéde).

All specimens were obtained and handled aseptically.

METHODS

Five series of experiments comprising a total of 56 trials were conducted. In each series a given group of media was examined for the number of bacterial colonies growing in each.

Preparation of specimens.—Ten-fold dilutions of the specimens were prepared with sterile "aged" sea water. One milliliter or fractions thereof of the two highest ten-fold dilutions was plated separately in each medium being compared in a given trial. Considerations calculated to facilitate an accurate enumeration of the bacterial colonies growing in the various media employed determined the choice of the dilutions plated. For instance, in the first few trials with oysters, 0.25 milliliter of a 1:100 dilution and 0.5 milliliter of a 1:1000 dilution were plated. Due to difficulties encountered in taking the counts in some of the plates, higher dilutions (0.25 milliliter of 1:1000 and 0.5 milliliter of 1:10,000) were tried and in the later trials still higher dilutions (0.25 milliliter of 1:10,000 and 0.5 milliliter of 1:100,000) were plated.

Technique for plating (plating temperature of media).—The technique evolved and employed was designed to reduce the actual temperature of the medium to a minimum without

[7] Fresh sea water filtered through paper and aged in glass bottles in the dark and at room temperature for at least three weeks before being used.

causing it to congeal before it was actually poured into the plate and mixed with the inoculum.

Duplicate tubes of each of the media under experiment were melted and kept in a 50° C. water bath. The petri dishes required (two for each media tested) were readied by pipetting into each the required amount of the appropriate dilution of the inoculum and each properly labeled. The tubes of media were taken from the water bath one at a time and cooled to body temperature by twirling continually in a breaker of cold water. The tube was further twirled a few seconds in air and the medium poured into the corresponding petri-dish. Then the inoculum and the medium were mixed thoroughly. By actual check, the temperature of the medium at the time of pouring ranged between 32° C. to 35° C.

Incubation temperature.—All culture plates were incubated at room temperature ranging from 23.5° C. to 27° C. taken at about 8:00 A. M. and 27° C. to 30° C. taken at about 3:00 P. M. during the period of these experiments.

Incubation period.—On the basis of observations made on preliminary trials, an incubation period of 96 hours for all inoculated plates was allowed before their corresponding colony counts were taken. This short period of incubation was adopted to minimize difficulties in taking accurate counts occasioned by the presence of spreaders which were frequently encountered in a good number of the media tried. Molds which become more troublesome after 96 hours and the rapid growth of some colonies in certain media which after 96 hours when close to one another usually coalesced and contributed their share of difficulty were avoided by this short period of incubation.

Observations made in this connection showed significant increases in the number of colonies up to about a week and that the plate indicating the highest count after 96 hours incubation generally gave the highest count after 7 days incubation.

Method of enumeration.—A direct total count of all the colonies in a plate was made whenever possible. When the colonies were close (over 300 to 400), the Quebec colony counter was used. After computing for the count per cubic centimeter (or per gram) of the undiluted specimen, the counts in the two dilutions in which the specimens were invariably plated, were averaged to obtain the final count per cubic centimeter (or per gram) of the specimen in each of the media tested.

To complement the data obtained in the series of trials performed under the subject of this investigation as indicated in the caption under which this report is being presented, the effects of the following factors on the bacterial colony counts were investigated.

1. *Diluents.*—Distilled water and physiological saline (0.85 per cent NaCl) were tried. "Aged" sea water was used as control diluent.

2. *Plating temperature of medium.*—Plating temperatures of 55° C., 50° C., and 45° C. were tried. The technique described above to attain a temperature of 32° C. to 35° C. in the plating medium at the time of pouring was used with the control plates.

Incubation temperature.—Incubation temperatures of 37° C. and 45° C. were tried. The controls were incubated at room temperature (vide supra).

In all these complementary experiments, the same procedure as discussed previously was employed, except where the particular variation under consideration was concerned. Medium 2216 was used in these trials.

RESULTS AND DISCUSSION

The eight formulas investigated had to be compared in groups of three or four owing to lack of adequate facilities. Five series of trials were run comparing a given group of formulas in each. Formula 1 was included in all the trials to afford a basis of comparison for all the formulas tested. The counts obtained in the individual trials are given in Table 1 and are expressed in per cent. A value of 100 per cent has been assigned to the count in Formula 1 and the proportionate percentage computed for the counts obtained in the other formulas in a given trial.

The results of the first trial illustrate the superiority of media with sea water as menstruum over those with distilled water as menstruum. Formulas 2, 3, and 4 are identical (vide supra) except for the menstruum employed and amount of sodium chloride in each. This observation conforms with those of ZoBell (1941, 1946), ZoBell and Feltham (1934), and others. Besides supporting the growth of very much fewer organisms, spreaders were particularly troublesome in Formula 3, both plates being completely overgrown with them making an accurate count impossible.

TABLE I.—*Bacterial counts in the various media tested.*

Media	Series I		Series II			
	1	2	3	4	5	6
Formula 1	100	100	100	100	100	100
Formula 2	3.22	96.58	64.54	62.5	267.62	69.76
Formula 3	[b]					
Formula 4	0.25					
Formula 5			54.32	84.61	247.12	50.95
Formula 6						
Formula 7						
Formula 8						

Media	Series III							
	7	8	9	10	11	12	13	14
Formula 1	100	100	100	100	100	100	100	100
Formula 2	78.39	22.85	80.00	50.00	26.07	58.73	23.68	81.37
Formula 3								
Formula 4								
Formula 5	59.79	21.42	153.00	50.00	84.95	19.04	24.56	18.06
Formula 6	45.22	22.85	40.00	8.33	37.82	25.39	27.19	46.06
Formula 7								
Formula 8								

Media	15	16	17	18	19	20	21	22
Formula 1	100	100	100	100	100	100	100	100
Formula 2	35.71	24.65	134.50	2.73	33.33	400.00	76.92	12.69
Formula 3								
Formula 4								
Formula 5	75.89	76.13	66.07	15.06	70.00	223.25	215.38	79.36
Formula 6	14.28	5.68	71.42	9.58	11.66	255.34	230.76	95.23
Formula 7								
Formula 8								

Media	23	24	25	26	27	28	29	30
Formula 1	100	100	100	100	100	100	100	100
Formula 2	61.19	8.39	28.57	45.55	24.00	39.58	374.61	21.31
Formula 3								
Formula 4								
Formula 5	27.79	10.58	33.03	11.48	32.00	45.88	51.02	63.93
Formula 6	27.79	3.05	6.25	32.76	22.00	18.75	83.46	36.88
Formula 7								
Formula 8								

Media	Series IV							
	31	32	33	34	35	36	37	38
Formula 1	100	100	100	100	100	100	100	100
Formula 2	140.38	40.34	36.36	11.71	65.56	1.19	13.82	76.82
Formula 3								
Formula 4								
Formula 5	76.92	48.92	14.54	1.17	50.40			
Formula 6	53.84	49.35	52.72	4.31	56.09			
Formula 7						1.40	1.34	31.63
Formula 8								

[a] All counts are expressed in per cent. See text for method of computation.
[b] Count not determined due to spreaders.

given in Table 3. In general, they conform to the pertinent observations made by ZoBell (1941, 1942, 1946), ZoBell and Conn (1940), and ZoBell and Feltham (1935 and 1938).

Distilled water and physiological saline have a definite detrimental effect on the bacterial population when used for the preparation of the tenfold dilutions of the inoculum. Counts 41.89 per cent to 100 per cent lower were obtained when distilled water and physiological saline dilution blanks were used, or an average of 81.81 per cent and 60.97 per cent lower, respectively, than when "aged" sea water was used.

Plating temperatures of 55°C., 50°C., and 45°C. compared with the control plating temperature of 32° C., to 35° C. have definite, though less marked adverse, effects on the counts than distilled water and physiological saline diluents have compared with the corresponding controls. Counts 12.82 per cent to 64.70 per cent lower were obtained when plating temperatures of 55°C., 50°C., and 45°C. were employed, or an average of 30.87 per cent, 36.40 per cent, and 39.04 per cent lower, respectively, than that of the controls. The comparative closeness of the temperatures tried and the difficulty of insuring a uniformity of the factors affecting the rate of cooling of the medium after it is poured, however, has resulted in counts which while consistently lower than those obtained in the corresponding controls, show no definite trend in relation to the plating temperatures. These factors include, among others, the amounts of plating medium actually poured into the plate, the precise moment of contact between the inoculum and the plating medium and the rate and duration of rotation of the petri dish.

TABLE 3.—*Effect of various diluents, varying plating temperatures of medium and varying incubation temperatures on the total bacterial plate counts using 2216 medium.*

Trial	Control[a]	Diluent		Plating temperature			Incubation temperature	
		D.W.*	S.S.*	55° C	50° C	45° C	37° C	45° C
1	7,750[b]	500	1,000	4,250	6,750	4,500	2,750	1,500
2	8,500	0	3,500	6,500	3,000	4,750	5,750	500
3	12,500	2,500	5,500	7,250	6,000	8,250	6,750	3,000
4	85,750	12,250	49,750	74,750	72,000	54,750	79,250	43,250

* D. W. = Distilled water. S. S. = Physiological saline (0.85 per cent NaCl).

[a] The controls were prepared according to technique described under "Methods" using "aged" sea water diluent, a plating temperature of 32°–35°C. and room temperature incubation.

[b] Figures represent total bacterial count per 1 cc of the undiluted material.

Plates incubated at 37° C., and 45° C. show definitely lower counts than the controls which were incubated at room temperature. Counts 7.58 per cent to 80.64 per cent lower were obtained in plates incubated at 37° C. and 45° C., or an average of 37.61 per cent and 75.07 per cent lower, respectively, than those of the controls incubated at room temperature.

CONCLUSIONS

From the results obtained and under the conditions and limitations set forth in this paper, the following conclusions are drawn:

Formula 1, 2216 medium of ZoBell, satisfies the cultural requirements of the greatest number of bacteria in the materials of marine origin examined as evidenced by the counts obtained. This is, therefore, the most suitable among the 8 plating media tried for determining the total aerobic bacterial population in materials of marine origin.

Formula 8, a modification of the media recommended by the Woods Hole Oceanographic Institution in which KH_2PO_4 has been substituted for K_2HPO_4 which the original formula requires gave the second highest count. It is recommended as second choice.

While the counts in Formula 2 gave a lower average than those in Formula 5, the former is preferred to the latter due to the possibility of greater variations in composition and the presence of precipitates and frequency of spreaders in the latter. Formula 2, therefore, is recommended as third choice.

"Aged" sea water for preparing dilutions of the inoculum is definitely superior to either distilled water or physiological saline (0.85 per cent sodium chloride in distilled water) when the objective is for maximum plate counts.

A plating temperature of 32°–35° C. of the medium, attainable by a technique described, results in definitely higher counts than those obtained with plating temperatures of 55° C., 50° C., and 45° C.

Plates incubated at room temperature gave definitely higher counts than duplicate plates incubated at 37° C. or 45° C.

Significant increases in colony counts have been observed up to about 7 days' incubation at room temperature. Molds and spreaders, however, become correspondingly more troublesome with the increase in incubation period. Hence, where a com-

parative periodic check on the total bacterial count of a given material is the objective, the shorter incubation periods are to be preferred.

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