An Improved Medium for the Isolation of Bacteria from Marine Fish

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SUMMARY

A medium for the isolation of heterotrophic aerobic bacteria from marine fish has been developed. The medium contains inorganic ions with a total concentration equal to about half that of sea water. The ionic ratio of sodium and potassium in the medium is nearly 1:1, and the concentration of the organic nutrients is higher than in media that have been generally used for the isolation of bacteria from sea water.

INTRODUCTION

One of the determining factors in the investigation of the bacterial flora of marine fish is the composition of the media used for the isolation of the bacteria. After the study of ZoBell (1941) on the cultural requirements of bacteria from sea water, many investigators have used the medium developed by him, often with various modifications, for the isolation of bacteria from marine fish.

A study on the salt dependency of the bacteria on marine fish (Simidu & Hasuo, 1968) suggested that these bacteria had different salt requirements from those of bacteria in sea water, and that basic research would be needed on the culture media for bacteria from fish. The present report is concerned with an attempt to obtain the culture medium which will give maximum viable counts on marine fish.

METHOD

Plate counts were carried out on plaice, 'Ishigarei' (*Kareius bicoloratus*) and on a flatfish, 'Hirame' (*Paralichthys olivaceus*) purchased at Funabashi Fish Market, Chiba Prefecture. In most cases, the fish were still alive at the beginning of the experiments.

A piece of skin from the mid dorsal part of the fish, 6.0 cm. in area and with or without underlying muscle was excised from the fish and homogenized with 40–100 ml. salt solution. Two different salt solutions were used for the dilutions, i.e. 0.85% sodium chloride solution and a solution containing 0.85% sodium chloride and 1.0% potassium chloride. From serial dilutions, 0.5 ml. were placed on to triplicate plates using a mechanical pipette devised by Yanagita & Maruyama (1953). Twenty ml. of agar medium which had been held at $49^{\circ} \pm 0.2^{\circ}$ were poured into the plate and mixed with the diluent. Up to six different media were compared in one experiment, and, in each dilution step, the time required for the whole procedure from placing

the diluent on to the first of 18 plates to the end of mixing the agar in the last plate was under 9 min. The colonies developed on the plates were counted after incubation of 3–5 days at 24–25°.

RESULTS

Concentration and composition of salts in the medium

Investigation on the salt dependency of the fish microflora (Simidu & Hasuo, 1968) showed that the highest viable counts were generally obtained at the concentration of salts corresponding to 25–75 % dilution of sea water.

The fact that the ratio of potassium ion to sodium ion is about 2.7 in fish muscle (Thurston, 1958) and about 0.7 in bacteria (*E. coli*) (Guillemin & Larson, 1922) seemed to leave some doubt about the supposition that the ratio of these ions in sea water, i.e. 0.2, would be the most suitable for the growth of the bacteria which had grown or survived on marine fish.

Table 1. *Composition of salt solution used in the experiment shown in Table 2*

	Solution					
	Artificial sea-water	A	B	C	D	E
g/1000 ml.						
NaCl	23.5	5.0	13.4	14.6	19.5	24.3
KCl	6.6	29.8	19.4	18.6	12.4	6.2
Na ₂ SO ₄ . 10H ₂ O	8.9	—	9.7	—	—	—
K ₂ SO ₄	—	5.2	—	—	—	—
MgCl ₂ . 6H ₂ O	10.2	10.2	10.2	—	—	—
MgSO ₄ . 7H ₂ O	—	—	—	6.2	6.2	6.2
CaCl ₂ . 2H ₂ O	1.5	1.5	1.5	1.5	1.5	1.5
Ionic ratio of K:Na	0.2	5.0	1.0	1.0	0.5	0.2

Viable counts were compared among media which have different ratios as to the ionic concentration of sodium and potassium. The composition of the salts in these media are given in Table 1. Two different basal media were used with the salt solutions. The composition of the first one used in the experiment with flatfish was: 1.0 % Polypepton (Daigo Eiyo Kagaku Co., Osaka), 0.5 % Meat Extract (Kyokuto Seiyaku Kogyo Co., Tokyo) and 1.2 % agar. The second one used in the experiment with plaice contained 1.0 % Polypepton, 0.3 % Lab-Lemco (Oxoid), 0.3 % Yeast Extract (Difco) and 1.2 % agar. The pH value of the media was adjusted to 7.0 in both experiments.

The results of the plate counts given in Table 2 showed that the highest counts were obtained in the media in which the ionic ratio of the sodium and potassium was 1:1.

Effect of other nutrients and pH value

The 'Meat Extract' which is widely used in Japan is composed mainly of a concentrate of a hot-water extract of fish (bonito), and is known to contain some substances inhibitory for the growth of bacteria.

The effect of Meat Extract (Kyokuto) on viable counts was compared with Lab-Lemco (Oxoid) and with Lab-Lemco + Yeast Extract (Difco). The constituents

Table 2. *Effect of salt composition of media on viable count of fish*

A. Bacterial counts from a flatfish sample

Dilution of solution (%)	Solution		
	Artificial sea water	A	B
	Counts $\times 10^{-2}/g.$		
100	204	168	227
	186	138	221
	197	151	229
	Average 196	152	226
50	366	231	344
	298	243	305
	304	205	373
	Average 323	226	341
Analysis of variance			
	Degrees of freedom	Sum of squares	Mean square
Dilution	1	49,928	49,928
Solution	2	28,515	14,257.5*
Interaction	2	2,317	1,158.5
Individuals	12	6,570	547.5
Total	17	87,330	—

* $14,257.5/547.5 = 26.0$, significant at 1 % level.

B. Bacterial counts from a plaice sample

Dilution of solution (%)	Solution		
	C	D	E
	Counts $\times 10^{-2}/g.$		
75	50	36	42
	54	51	46
	58	46	41
	Average 54	44.3	43
25	48	46	40
	53	35	39
	53	51	43
	Average 51.3	44	40.7
Analysis of variance			
	Degrees of freedom	Sum of squares	Mean square
Dilution	1	14.2	14.2
Solution	2	390.1	195.1*
Interaction	2	4.8	2.4
Individuals	12	322.0	26.8
Total	17	731.1	—

* $195.1/26.8 = 7.28$, significant at 1 % level.

of the media shown in Table 3 were dissolved into $\frac{1}{4}$ strength solution C (Table 1). The pH was adjusted to 7.0.

The results (Table 3) showed that the combination of 0.3% Lab-Lemco and 0.3% Yeast Extract gave the highest counts.

The growth-supporting effect of two different peptones, i.e. Trypticase (BBL) and Polypepton (Daigo) was compared at two different concentrations. The composition of the basal medium was 0.3% Lab-Lemco, 0.3% Yeast Extract and 1.2% agar.

Table 3. *Effect of extracts in media on viable counts of fish*

A. Composition of organic nutrients in media				
No. of medium	Meat Extract (Kyokuto) (%)	Lab-Lemco (Oxoid) (%)	Yeast Extract (Difco) (%)	Polypepton (Daigo) (%)
1	0.5	—	—	1.0
2	—	0.5	—	1.0
3	—	0.3	0.3	1.0

B. Bacterial counts with a plaice sample			
No. of medium	Counts $\times 10^{-3}/g.$, average of 3 plates	Standard deviation	
1	9.7 a	2.6	
2	15.0 b	4.6	
3	23.3 c	3.3	

Differences between a and c , and b and c were significant at 5% level.

Table 4. *Effect of different concentration of peptones on viable counts of fish*

Bacterial counts from a plaice sample			
Concentration of peptone (%)	Peptone		Counts $\times 10^{-3}/g.$
	Polypepton (Daigo)	Trypticase (BBL)	
1.0		48	56
		53	49
		53	52
	Average	51.3	52.3
0.5		41	48
		41	43
		54	42
	Average	45.3	44.3
Analysis of variance			
	Degrees of freedom	Sum of squares	Mean square
Concentration	1	147.0	147.0*
Peptone	1	0	0
Interaction	1	3.0	3.0
Individuals	8	174.7	21.8
Total	11	324.7	—

* $147.0/21.8 = 6.74$, significant at 5% level.

The constituents were dissolved in diluted C solution and peptones were added to the medium at concentrations of 0.5 and 1.0%. The pH value of the media was adjusted to 7.0.

The results of the plate counts are shown in Table 4. Whereas there was virtually no difference of the viable counts between two peptones, the advantage of the higher concentration was observed in both peptones.

ZoBell (1941) showed with bacteria in sea water that the highest plate counts were obtained in alkaline media and in the presence of minute amount of ferric ion.

The effect of both pH value and added ferric ion was examined with a basal medium composed of 1.0% Polypepton, 0.3% Lab-Lemco, 0.3% Yeast Extract, and 1.2% agar dissolved in a half dilution of C solution.

The results, counted after 5 days incubation at 25°, are given in Table 5. The results showed that although higher counts were obtained at higher pH ranges, the effect of pH was not as remarkable as in ZoBell's experiments with sea water. No appreciable effect was observed with the addition of ferric ion to the medium.

Table 5. *Effect of pH value and ferric ion*

Bacterial counts with a plaice sample			
pH	FeSO ₄ .7H ₂ O (%)	Count × 10 ⁻² /g. average of 3 plates	Standard deviation
6.84	—	133	5.7
7.21	—	155	3.1
7.50	—	169	26.0
7.97	—	165	13.7
7.23	0.01	163	11.9

DISCUSSION

Wood (1953), in his study on the marine environments of Eastern Australia, stated that there was no significant difference in the growth of the fish flora on freshwater and sea-water agar. Liston (1956), on the other hand, reported that higher viable counts were generally obtained from fish with sea-water medium than horse-heart infusion agar using tap water. In his experiments, however, as he noticed on the evaluation of the results on gut flora, the advantage of the higher salt content in the sea-water medium on the one hand seemed to compete with the advantage of higher nutrition in the horse heart agar on the other hand, and this made it difficult to evaluate the effect of the salts themselves.

The heterogeneity of the fish microflora growing on a salt range (Simidu & Hasuo, 1968) suggested that it would hardly be possible to obtain all the heterotrophic aerobes by using a single medium with any particular salt concentration. However, it would be not practical to use many media of different salt concentrations for the isolation and identification of fish bacteria, for it might make difficulties in the interpretation of the results.

Based on the present study, the medium (Table 6), called 'medium B' was developed for the study of the bacterial flora of marine fish. This medium can also be used for the examination of the bacteriological conditions of fish instead of for the usual plate count agar or nutrient agar with or without added 3% salt.

Table 6. *Composition of 'Medium B'*

Polypepton (Daigo)	10.0 g.	MgSO ₄ .7H ₂ O	3.0 g.
Lab-Lemco (Oxoid)	3.0 g.	CaCl ₂ .2H ₂ O	1.5 g.
Yeast Extract (Difco)	3.0 g.	Agar	12.0 g.
NaCl	8.0 g.	Tap water	1000 ml.
KCl	10.0 g.	pH 7.5	

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