The Influence of Plating Technique and Incubation Temperature on Bacterial Count from Fish and Fishery Products

NIRMALA THAMPURAN, H. KRISHNA IYER and K. MAHADEVA IYER
Central Institute of Fisheries Technology, Cochin-682 029

For bacterial sampling of raw unprocessed fish and frozen fishery products, spread plate method is preferable to pour plate method; incubation of plates at 30°C gives a higher count than incubation at 37°C. Analysis of variance of the data shows that sample variation between different types of fishes is highly significant whereas the variations between triplicate plates is not significant at 5% level.

Increasing attention is paid nowadays to the microbiological quality of foodstuffs and this necessitates a thorough study of the techniques adopted for assessing their microbial content. Total aerobic bacterial count (TABC) or total plate count (TPC) is the most important means of establishing whether a product has been prepared under strict sanitary condition.

Different techniques are in use for determining the TPC of which the spread plate technique and the pour plate technique are the most common and widely accepted. Pour plate technique involves the use of melted agar maintained at 45°C, while spread plate technique employs pre-set plates of agar equilibrated to the desired temperature.

The ISI method for the bacteriological examination of fresh fish as well as frozen fish products stipulates that pour plate technique be used along with an incubation temperature of 37°C (IS: 1977 a, b). The same procedure is also suggested for microbiological analysis of shell fish and its products (AOAC, 1970).

There are several reports on the role of transient warm agar temperature in decreasing the microbial recoveries by pour plate technique (Zobell & Conn, 1940; Stapert *et al.* 1962; Van Soestbergen & Lee, 1969; Klein & Shenyu Wu, 1974). Majority of the marine micro-organisms are found to succumb when exposed to a temperature of 30–40°C for 10 min. Recently several communications have come forth, which claim spread plate method to be superior to pour plate method especially in the case of seafoods (Lee & Harward, 1970; Nottingham *et al.* 1975; Shenyu Wu & Klein, 1976). On the other hand Mossel & Van der Moosdijk (1963) found no difference between pour plate (agar 44–46°C) and spread drop plate (agar at room temperature) of 42 food samples.

A study was undertaken in this laboratory to find out the suitability of these two procedures for use in bacteriological sampling of fish and fishery products and to evaluate their performance.

Materials and Methods

Raw fish samples were procured either from local country crafts or landing centres near Cochin. Samples were analysed within 2h after collection. Raw fish samples analysed were in very fresh condition as judged by their appearance. 39 samples of raw fish and 48 samples of frozen materials were studied during 1976–79. Frozen samples were obtained from a local seafood processing plant and hence of unknown history. The frozen blocks were kept in cold storage till required. Muscle homogenates of the raw or frozen samples were prepared by grinding about 10 g of the material with 90 ml of normal saline in a sterile mortar. Subsequent dilutions were prepared in the same diluent by mixing 1 ml of the sample dilution with 9 ml of the diluent in a vortex mixer. Sea water agar (SWA) for sampling raw fish and tryptone glucose agar (TGA) for frozen material were used. The agar

media for spread plating had been poured 6 h before the experiment and excess moisture evaporated by keeping the plates at 45°C for one hour. Care was taken to prevent syneresis fluid on the agar surface to assure that colony spreading would be minimised. 0.5 ml of the appropriate dilutions were spread evenly on the agar surface with an alcohol flamed bent glass rod. Pour plating was done using 1 ml of the dilution. The molten agar was maintained at 45°C in a thermostatic water bath. All samples were plated in triplicate. One set of plates from each method was kept at room temperature, (29 ± 1°C; RT) and another at 37° C. In addition, a set of spread plates was also kept at 8°C for psychrotrophic count. RT and 37°C counts were taken at the end of 48 h of incubation and low temperature count after three weeks.

Results and Discussion

Average counts from three spread plates and three pour plates were used for statistical analysis. For raw minced fish, the microbial count varied from 8.4 x 10^4/g of the muscle to 1.3 x 10^8/g (Table 1.) The maximum count was noted in spread plates incubated at room temperature. The lowest count was obtained by pour plating and incubating at 37°C. For frozen products the count varied from 3 x 10^3/g of the material to 1.2 x 10^6/g (Table 2). In frozen and raw fish, spread plates incubated at room temperature gave highest count and pour plates at 37°C the lowest. The count at 8°C was used to estimate the prevalence of psychrotrophic bacteria in the sample. Analyses of variance for different fishes are presented in Tables 3 to 10.

From Table 3, it is found that for sardines *(Sardinella longiceps)* there is no significant difference in bacterial count between temperatures. But difference existed between methods, spread plate count being greater than pour plate count. The analysis of variance (Table 4) for mullets *(Mugil cephalus)* showed significant difference in bacterial count between temperatures, RT giving highest count than 37°C. However there was no significant difference with methods. In silver jew fish *(Johnius dussumieri)* and Indian halibut *(Psettodes erumei)* the trend was similar (Tables 5 and 6). The variation in bacterial count between temperatures as well as methods were highly significant. Room temperature and spread plates yielded higher bacterial counts.

Regarding frozen sardines, significant difference in count was observed between the two temperatures as well as the two methods (Table 7). RT and spread plates consistently gave higher counts. Fillets of frozen jew fish *(Pseudosciaena* sp.)..and the mixed fish muscle paste popularly known as 'Kheema' (Tables 8 and 10) showed more or less similar behaviour. But for fillets of cat fish *(Tachysurus* sp.) differences

Table 1. *Total bacterial count for raw fish (total plate count/g x 10^4)*

	(29 ± 1)°C		37°C		8°C
	S.P.	P.P.	S.P.	P.P.	S.P.
Sardine	230*	190	200	203	—
Sardine	6812	3110	1900	1400	48
Sardine	1275	594	4300	3400	3005
Sardine	17	21	20	27	13
Mullet	170	780	85	91	68
Mullet	93	781	8	8	—
Mullet	540	87	96	160	—
Indian halibut	13100	13100	12000	11000	6000
Silver jew fish	742	306	290	280	102

* Average of three values S.P—Spread plate method
P.P—Pour plate method

existed only between the two temperatures, RT giving higher bacterial count than 37°C (Table 9).

In all, sample variation was highly significant, but variation between triplicate plates was not significant at 5% level. A similar finding was also reported by Nottingham *et al.* (1975).

For raw fish as well as frozen samples, room temperature gave higher count than 37°C. The only exception was raw sardines for which the count variation due to temperature difference was not very much marked. Identical counts obtained at RT and 37°C for sardine can be accounted by the fact that the samples had been exposed to ambient temperature for a longer time. Jan Gjerde (1975) had reported that the higher count at 37°C for raw fish can be considered as an indication that the fish has been exposed to higher temperatures, since normally psychrotrophic bacteria predominate in raw fish. It has also been suggested that a comparison of count at 25 and 37°C gives a measure of relative proportion of psychrotrophs and mesophiles (Nottingham *et al.* 1975).

Table 2. *Total bacterial count for frozen fish (total plate count/g x 10^3)*

	(29 ± 1)°C		37°C		8°C
	S.P.	P.P.	S.P.	P.P.	S.P.
	g	g	g	g	g
Sardine	21*	6.7	13	7.6	—
Sardine	180	109	160	108	4.6
Sardine	181	76	74	68	37
Sardine	65	46	56	32	3.2
Sardine	210	150	190	65	—
Sardine	21	9	16	8	—
Jew fish	46	19	36	11	14
Jew fish	57	81	39	48	21
Cat fish	1200	638	530	570	30
Cat fish	188	170	108	77	—
Cat fish	314	206	29	20	8
Kheema	449	317	278	242	11

*Average of three values
S.P— Spread plate method
P.P— Pour plate method

Table 3. *Analysis of variance for raw sardine*

Source	S.S.	df	MS	F
Total	47.9681	71	—	—
Samples	44.1282	5	8.8256	188.18[c]
Temperature	0.0040	1	0.0040	0.085
Methods	0.8828	1	0.8828	18.82[c]
Triplicate	0.0002	2	0.0001	0.002
Error	2.9529	63	0.0469	—

c – Significant at 0.1% level

Table 4. *Analysis of variance for raw mullet*

Source	S.S.	df	MS	F
Total	20.4909	59	—	—
Samples	9.1154	4	2.27885	15.45[c]
Temperature	3.6608	1	3.6608	24.819[c]
Methods	0.1937	1	0.1937	1.313
Triplicate	0.0003	21	0.00015	1
Error	7.5207	51	0.1475	—

c – Significant at 0.1% level

Table 5. *Analysis of variance for raw Indian halibut*

Source	S.S.	df	MS	F
Total	0.0119	11	—	—
Temperature	0.0094	1	0.0094	67.14[c]
Methods	0.0012	1	0.0012	8.57[a]
Error	0.0013	9	0.00014	—

a, c – Significant at 5 and 0.1% respectively

Table 6. *Analysis of variance for raw silver jew fish*

Source	S.S.	df	MS	F
Total	0.3691	11	—	—
Temperature	0.1483	1	0.1483	13.12[b]
Methods	0.1188	1	0.1188	10.51[a]
Error	0.1020	9	0.0113	—

a, b – Significant at 5 and 1 % respectively

Table 7. *Analysis of variance for frozen sardine*

Source	S.S.	df	MS	F
Total	11.8636	71	—	—
Samples	9.9333	5	1.98666	179.46[c]
Temperature	0.2351	1	0.23510	21.24[c]
Methods	1.0061	1	1.00610	90.89[c]
Triplicate	0.0027	2	0.000135	21
Error	0.6864	62	0.01107	—

c – Significant at 0.1% level

Table 8. *Analysis of variance for frozen jew fish*

Source	S.S.	df	MS	F
Total	1.6288	23	—	—
Samples	0.7593	1	0.7593	27.51[c]
Temperature	0.2280	1	0.2280	8.26[a]
Methods	0.1450	1	0.1450	5.25[a]
Triplicate	0.0003	2	0.0002	—
Error	0.4962	18	0.0276	—

a, c – Significant at 5 and 0.1% respectively

Table 9. *Analysis of variance for frozen cat fish*

Source	S.S.	df	MS	F
Total	9.5148	35	—	—
Samples	5.8035	2	2.9028	63.80[c]
Temperature	2.2526	1	2.2526	49.51[c]
Methods	0.1383	1	0.1383	3.04
Triplicate	0.0002	2	0.0001	1
Error	1.3182	29	0.0435	—

c – Significant at 0.1% level

Table 10. *Analysis of variance for frozen kheema*

Source	S.S.	df	MS	F
Total	0.1191	11	—	—
Temperature	0.0793	1	0.0793	113.29[c]
Methods	0.0334	1	0.0334	47.71[c]
Error	0.0064	9	0.0007	—

c – Significant at 0.1% level

Since the primary objective of the aerobic plate count is to evaluate the sanitational aspect of food handling, aerobic mesophilic count at 37°C would be deemed sufficient for this purpose. But such a count will not reflect the actual microbial load on the food where psychrotrophic organisms are also involved as in the case of raw fish or frozen products. The microbial flora of raw fish contained species of which a good part was classified as psychrotrophs. Our earlier studies had indicated that raw fish *(Sardinella longiceps)* contained about 44% of psychrotrophs while mesophiles constituted only 56%. It was also found from their growth temperature studies that 31% of the bacteria isolated from fresh fish could not grow at 37°C (Thampuran & Iyer, 1979). It was not surprising therefore that 30°C incubation is giving higher counts.

In frozen samples, the predominating flora is Gram positive cocci belonging mainly to genus *Micrococcus* (Thampuran & Iyer unpublished data). Selection of cold resistant species due to freezing and frozen storage coupled with the elimination of a large number of strict mesophilic types would have been the reason for getting higher counts at 30°C.

Generally it was found that spread plates yielded more colonies than pour plates for raw and frozen fish. Clark (1967) had suggested that spreading by means of a glass rod would allow the clumped cells to get disintegrated thereby improving the dispersion of cells resulting in higher counts. But analysis of our data shows that the count difference between spread plates and pour plates at 30°C is greater than the count difference at 37°C. Had the effect been due to clumping of the cells alone this difference would not have arisen. It appears therefore that higher counts obtained in spread plates could be due to other factors as well.

One possibility may be the sensitivity of certain marine micro-organisms to the action of hot agar used in pour plating. Zobell & Conn (1940) pointed out the harmful action of agar at 45°C on certain marine bacteria. The extreme heat sensitivity of a psychrotrophic bacterium *Pseudomonas flourescens* was recently shown by Gray *et al.* (1973). Nottingham *et al.* (1975) suggested that the lower count in pour plates could be attributed to the inability of psychrotrophs to survive in the hot agar media used in pour plating.

Postgate (1967) stated that bacteria subjected to a stress becomes hypersensitive to a secondary stress. It has been shown that microbial starvation which can occur in low nutrient environments can also lead to increased susceptibility to a transient secondary warming stress (Klein & Shenyu Wu, 1974). This was shown by decreased recoveries of heterotrophic microorganisms from aquatic environments in pour plate as compared to spread plate method. In frozen foods, stress due to intense cold can cause the microbial flora to be more sensitive to warm agar temperature of 45°C leading to decreased recoveries with pour plate method.

The implication of this study was limited by the number of samples analysed and methods of enumeration adopted. The data nevertheless points out the significance of adopting a procedure by which greater accuracy is attained in the estimation of bacterial count of sea foods.

The authors are grateful to the late Shri. G. K. Kuriyan, former Director, Central Institute of Fisheries Technology for his keen interest and encouragement. They are also thankful to the technicians in the laboratory for specimen collection and laboratory assistance.

References

AOAC (1970) *Official Methods of Analysis,* 11th edn. Association of Official Analytical Chemists, Washington

Clark D. S. (1967) *Can. J. Microbiol.* **13,** 1409

Gray, R. J. H., Witter, L. D. & Ordahl, Z. J. (1973) *Appl. Microbiol.* **26,** 78

IS: 8653 (1977a) *Indian Standard Specification for Sardines,* Fresh. 1st edn. Indian Standards Institution, New Delhi

IS: 8652 (1977b) *Indin Standard Specification for Sardine, Frozen.* 1st edn. Indian Standards Institution, New Delhi

Jan Gjerde (1975) *Fisk Dir Skr.* **1,** 17

Klein, D. A. and Shenyu Wu (1974) *J. Appl. Mlcrobiol* **27.** 429

Lee, J. S. & Lee ann Harward (1970) *J. Milk Fd Technol.* 33, 237

Mossel, D. A. A. & Van der Moosdijk, A. (1963) *J. appl. Bact.* **26,** 10

Nottingham, P. M., Rush brook, A. J. & Jury, K. E. (1975) *J. Fd Technol.* **10,** 273

Postgate, J. R. (1967) *Advanced Microbial Phys.* **1,** 1

Shenyu, Wu & Klein, D. A. (1976) *J. Appl. Environ. Microbiol.* **31,** 216

Stapert, E. M., Sokolski, W. T. & Northam, J. I. (1962) *Can. J. Microbiol.* **8,** 809

Thampuran Nirmala & Iyer, K. M. (1979) *Fish. Technol.* **16,** 15

Van Soestbergen, A. A. & Lee, C. H. (1969) *Appl. Microbiol.* **18,** 1092

Zobell, C. E. & Conn, J. E. (1940) *J. Bact.* **40,** 223