CHEMICAL CONTROL OF PSYCHROPHILIC BACTERIAL SPOILAGE OF FISH

I. ISOLATION AND IDENTIFICATION OF PSYCHROPHILIC BACTERIA FROM MARINE FISH

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Making use of the streak plate technique and low temperature incubation, 28 cultures belonging to six genera namely, *Achromobacter*, *Flavobacterium*, *Pseudomonas*, *Micrococcus*, *Vibrio* and *Alcaligenes* were isolated from different varieties of marine fish. The growth studies indicated that all of them were able to grow between −5 and +5°C within a week's time and none of them showed growth at 37°C. The optimum temperature of growth for all these cultures was in the range 25–28°C. Among these only one, *i.e.*, a *Vibrio* sp., was found to be an obligate psychrophile.

INTRODUCTION

Many attempts have been made in the past to isolate and identify the psychrophilic bacteria in fish. The recent reviews on this aspect by Ingraham and Stokes (1959), Witter (1961) and Morita (1975) give a full account of the various attempts made in the past and of the psychrophilic groups identified. The attempts made so far to check the growth of psychrophilic bacteria in ice stored fish by the use of various antibiotics and chemical preservatives have not given satisfactory results for commercial application though chlorotetracycline has been found to be of some use commercially. While most of the earlier studies were directed towards treating the fish with preservative added to water or ice, only recently a few attempts have been made to study the effect of some of these preservatives on isolated cultures (Velankar, 1958; Heather and Vander Zant, 1958; Surendran and Mahadeva Iyer, 1971; Tunstal and Gowland, 1974). Even in these cases the studies were not quite comprehensive in the sense that either only a few isolates from fish have been included for the study or preservatives have been tried at arbitrary concentrations. As seen from the current literature, it appears that this problem could be solved not by any one preser-

vative but by a combination of two or three preservatives as pointed out by Partmann (1952). Therefore, the objective of this investigation was to try these preservatives on the various groups of psychrophilic bacteria that normally exist in ice stored fish and to see which of these groups are inhibited by the particular preservative and then choose that preservative or a combination of preservatives which can effectively check the growth of all these groups. In the present study, attempts have been made to isolate and identify the various groups of psychrophilic bacteria that normally exist in fishes of Mangalore coast..

Materials And Methods

The fish used in the study were collected from the sea at a depth of 20-25 fathoms using the college's boat. The hauled fish were iced immediately, brought to the laboratory and kept in the ante-room of cold storage (0 to + 2°C) prior to sampling. The samples were drawn over a period of 15 days at intervals of 2 days. The types of marine fish used were *Rastrelliger kanagurta*, *Sardinella* spp., *Johnius* sp., *Nemipterus japonicus*, *Lactarius lactarius* and *Tachysurus* sp.

Both sea water agar and nutrient agar were employed for isolation of bacterial cultures from fish samples. The composition of the two media are shown in Table I. While both the media were used for isolation purposes in order to isolate as many types as possible, sea water agar was used for maintenance of isolated cultures.

Isolation of cultures

Streak plate technique was adopted for the isolation of organisms. The streaking was done by means of a glass hockey stick. Whole fish samples were homogenised in sterilized and cooled homogenising cups from which 10 g. material was transferred to 90 ml. of precooled 0.85% saline. The serial dilutions were then prepared from this for inoculating the medium. The diluents and the petriplates containing the medium were precooled (0-5°C) before they were inoculated. The streaked plates were incubated at -3 to -5°C, 0°C (±2) and 3 to 5°C. The morphologically different colonies were picked and transferred to sea water agar slants and pure cultures obtained. The pure cultures were stored in a refrigerator at 5-7°C and were subcultured periodically.

Growth studies

All the 28 pure cultures were studied for their optimum temperature requirements for growth. The incubation temperatures tried were 0°C (±2), 3 to 5°C, 15-20°C, 25-28°C, 30-32°C and 37°C, The studies were carried out on slants using both sea water agar and nutrient agar media and the time of appearance of growth over a period of time were noted for each.

Identification of cultures

Attempts were made to identify the cultures upto their generic level and wherever possible upto their species level. The procedures followed were those described in the Manual of Microbiological Methods (Pelczer *et al.*, 1957) using Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957) as the guideline for identification of cultures. However, for identification and confirmation of *Pseudomonas* cultures Kovac's oxidase test

TABLE I
COMPOSITION OF MEDIA

Components of the medium	Sea water agar medium	Nutrient agar medium
Bacto-peptone (Difco)	5 g.	5 g.
Beef extract (Difco)	3 g.	3 g.
Glucose (BDH)	1 g.	1 g.
Agar (Japan make)	15 g.	15 g.
Sodium chloride (BDH)	—	30 g.
Distilled water	—	1000 ml.
Aged sea water	1000 ml.	—
pH	7	7

as described by Shewan, Hobbs and Hodgkiss (1960) was also made use of.

RESULTS AND DISCUSSION

Making use of the streak plate technique and low temperature incubation 158 cultures were picked out based on their colony morphology. They were further subjected to primary and secondary screening to eliminate the duplicates yielding about 70 isolates. Out of these, 28 isolates which showed distinctly different morphological and cultural characteristics were finally chosen for purification and identification.

The enrichment culture technique recommended by Morita (1975) was tried for isolation of psychrophiles by incorporating a small quantity of fish extract into the sea water agar medium. However, it was observed that this medium was not in any way superior to sea water agar or nutrient agar media, since the number and types of colonies that grew up on these media were almost the same and hence for all further isolations only sea water agar and nutrient agar media were used.

Though the low temperature incubation technique was followed right through their isolation, purification and maintenance, these 28 cultures were again subjected to growth studies to determine their optimum growth temperatures and also to confirm their psychrophilic nature. For this purpose they were incubated at 0°C (±2), 3 - 5°C, 15 - 20°C, 25-28°C, 30-32°C and 37°C. It was observed that all these cultures took 5-7 days to show visible growth at 0°C (±2), 3-4 days at 3 to 5°C, 2 days at 15-20°C, 24 hrs. at 25-28°C and 30-32°C and showed no growth at 37°C. While the growth of all these cultures was found to be abundant at 25-28°C within 24 hours, their growth at 30-32°C was moderate and quite a few of the cultures showed very slight growth at this range. While the growth of these isolates was good on sea water agar and nutrient agar with 3% sodium chloride, the growth in nutrient agar without sodium chloride was,

TABLE II
IDENTIFICATION OF CULTURES

Isolate No.	Name of the organisms
I.	*Flavobacterium halmephilum*
2.	*Vibrio sputigenus*
3.	*Achromobacter* sp.
4.	*Vibrio costicolus*
5.	*Achromobacter* sp.
6.	*Micrococcus conglomeratus*
7.	*Achromobacter superficialis*
8.	*Achromobacter liquefacienes*
9.	*Micrococcus varians*
10.	*Pseudomonas striata*
11.	*Pseudomonas fragi*
12.	*Vibrio* sp.
13.	*Achromobacter guttatus*
14.	*Micrococcus agilis*
15.	*Achromobacter* sp.
16.	*Achromobacter* sp.
17.	*Achromobacter delmarvac*
18.	*Flavobacterivm diffusum*
19.	*Achromobacter* sp.
20.	*Alcaligenes bucheri*
21.	*Achromobacter aquamarinus*
22.	*Achromobacter* sp.
23.	*Alcaligenes faecalis*
24.	*Pseudomonas* sp.
25.	*Acoromobacter delicatulus*
26.	*Flavobacterium* sp.
27.	*Flavobacterium* sp.
28.	*Achromobacter* sp.

very poor even at optimum growth temperature.

It was concluded from this study that the optimum temperature for all these cultures lies between 25-28°C, except the isolate number 12 (*Vibrio* sp.) which could not grow at temperatures above 20°C but grew well between 0 to 5°C.

The 28 cultures thus screened were subjected to various morphological, cultural and physiological tests after ensuring their purity. These cultures were identified as given in Table II.

Of the 28 cultures, 13 were identified as species of *Achromobacter*, 4 as *Flavobacterium*, 3 each as *Micrococcus*, *Pseudomonas* and *Vibrio* and 2 as *Alcaligenes*.

Efforts were made to identify these 28 cultures upto their species level making use of various tests. However, some of the cultures could not be identified upto their species level by the above tests alone as they were not agreeing with any of the type cultures described under their respective genera. It was concluded that either they were new species or further work was needed for proper identification of these cultures.

Though it has been possible to obtain psychrophilic bacteria belonging to the genera *Achromobacter*, *Flavobacterium*, *Pseudomonas*, *Alcaligenes*, *Micrococcus* and *Vibrio*, the psychrophiles belonging to other genera like *Bacillus*, *Serratia*, *Aeromonas*, *Corynebacterium*, *Photobacterium*, *Cytophaga*, *Moraxella*, *Mycoplana*. Enterobacters *etc.* were not encountered in the present study. However, the most commonly occurring psychrophilic genera on fish have been isolated in this study.

The facts that all these cultures grow well at 0°C within a week's time, grow best between 25-28°C, show only a scanty growth above 30°C and no growth at 37°C, go to show that they are definitely psychrophiles as per the definitions of Ingraham and Stokes (1957) and Stokes (1963). Among these only the *Vibrio* sp.

could be called an obligate psychrophile since it could not grow at temperatures above 20°C but could grow well between 0 - 5°C.

While there was no difficulty in identifying these cultures upto their generic level, differentiation of *Pseudomonas* from *Achromobacter* was slightly difficult since both were motile. Kovack's oxidase and fermentative test could however differentiate these two according to the determinative scheme suggested by Shewan *et al.*, (1970).

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REFERENCES

Breed, R. S., E. G. D. Murray and N. R. Smith. 1957. *Bergey's Manual of Determinative Bacteriology*., 7th Edn., Bailliere, Tindall and Cox, Baltimore.

Heather, C. D. and C. Vanderzant. 1958. *Fd. Technol.*, **12** : 263.

Ingraham, J. L. 1958. *J. Bacteriol.*, **76** : 75.

Ingraham, J. L. and J. L. Stokes. 1959. *Bacteriol Rev.*, **23** : 99.

Morita, R. Y. 1975. *Bacteriol Rev.*, **39** : 144.

Partmann, W. 1952. *Z. Levensm. – Untersuch. U. Forsch.*, **94** : 246.

Pelczar, M. J. Jr. 1957. *Manual of Microbiological Methods;* McGraw Hill Book Co., New York.

Shewan, J. M., G. Hobbs and W. Hodgkiss. 1960. *J. Appl. Bacteriol.*, **23** : 379.

Stokes, J. L. 1963. *Recent Progress in Microbiology*; Ed. N. E. Gibbons : 187. University of Toronto Press.

Surendran, P. K. and K. Mahadeva Iyer. 1971. *Fish. Technol.*, **8**, 2 : 128.

Tunstall, A. M. and G. Gowland. 1974. *J. Appl. Bacteriol.*, **37** : 455.

Velankar, N. K. 1958. *Proc. Indian Acad. Sci.*, 47B : 87.

Witter, L. D. 1961. *J. Dairy Sci.*, 44 : 983.