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Studies on the Growth Temperature Ranges of Bacteria Isolated from Fresh Sardine at Different Primary Incubation Temperatures

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The effect of primary incubation temperature on the growth temperature range was studied with reference to 296 bacterial cultures isolated from sardine using streak plate technique. The primary incubation temperature used during bacteriological sampling caused a selection of strains according to their growth temperature requirements. Incubation at 8°C caused greater recoveries of psychrotrophs while 37°C favoured mesophiles. An incubation temperature of 30°C facilitated the growth of both psychrotrophs and mesophiles.

Temperature is an important environmental factor which affects the growth and development of micro-organisms (Hanus & Morita, 1968). Bacteria have an optimum growth temperature and growth above or below this temperature may cause changes in the physiological and metabolic activities (Ingraham & Bailey, 1959). The psychrophilic bacteria which prefer low temperature have an optimum temperature between 10°C and 20°C, mesophiles 30°C and 40°C and thermophiles 50°C and 65°C. However these groups have no sharply distinguished boundaries between them and are somewhat hazy. Moreover the cardinal points are also affected by other factors such as supply of nutrients, pH of the medium and products of metabolism (Rose, 1968; Upadhyay & Stokes, 1963).

Fresh fish carrying as it does a heterogenous population of all these types of bacteria presents an interesting material for study. Part of the natural flora associated with marine environment and fish is psychrophilic or psychrotrophic. The latter has a very wide growth temperature range while the former has a restricted temperature range from 0°C to 20°C. The fish caught from tropical areas may carry mesophilic terrestrial types as well. The percentage of these different types may vary with season; mesophiles predominate during warmer months while in winter biochemically less active groups are encountered (Karthiayani & Iyer, 1971 a). The incubation temperature used for the primary isolation of bateria from fish samples causes a selection of strains based on biochemical characteristics within the species or from among the various genera (Karthiayani & Iyer, 1971 b). It may also effect a selection of strains based on their growth temperature ranges. Hence this investigation was undertaken to study the growth temperature ranges of micro-organisms associated with fresh marine fish and also to gain an insight into the appropriate incubation temperature needed during bacteriological examination of fresh uniced fish, since such a temperature is likely to facilitate maximum recoveries of bacteria from the sample being examined.

Materials and Methods

Fresh sardines *(Sardinella longiceps)* landed by local country crafts at Cochin were brought to the laboratory in sterile bottles and sampled immediately. The duration between procurement of fish and its examination in the laboratory never exceeded two hours. While sea water agar (SWA) and nutrient agar (NA) were employed for the primary isolation of the cultures, their growth temperature studies were carried out in sea water peptone (SWP) and nutrient broth (NB). Micro-organisms were isolated by spread plating the diluted homogenates of the skin and muscle portion of the fish on SWA and NA. 0.5 ml of 10^3 or 10^4 dilution of the homogenate prepared by homogenizing 10 g of the

fish muscle with 90 ml of sterile sea water was spread on appropriate media using a bent glass rod. Duplicate plates were incubated at 8, 30 and 37°C for 21, 3 and 2 days respectively. After incubation, all the microbial colonies that developed on countable plates were picked and transferred to SWA or NA slants.

296 cultures were studied from July to April on an average of 30 cultures a month. The growth of each pure isolate was studied at a series of temperatures. A uniform and slightly turbid cell suspension in SWP or NB of the isolate was prepared. SWP was used for the cultures isolated from SWA and NB for those isolated from NA. About 0.05 ml of the culture suspension was inoculated into duplicate test tubes containing SWP and NB. The inoculated tubes were incubated at 0, 10, 20, 30, 37, 45 and 56°C. Simultaneously duplicate SWA and NA slants were also inoculated and incubated at the above temperatures. The time required for the appearance of growth was noted in each case and also the degree of growth on slants which was assessed by visual comparison. In addition to growth studies the cultures were also subjected to Gram staining.

Results and Discussion

The percentage of the microbial cultures that grew at different test temperatures is presented in Table 1. It is evident from the data that while all the cultures isolated from SWA at 37°C could grow well at the test temperature of 30°C, only 50% of the cultures isolated using a primary incubation temperature of 30°C, could grow at the test temperature of 37°C. In nutrient agar, however, 66 % of the cultures isolated from 30°C plates could grow at 37°C. Irrespective of the media used, as the primary incubation temperature increased from 8 to 37°C, a reduction was noted in the number of cultures that could grow at the lower temperatures of 0 to 20°C. But at test temperatures of 30, 37 and 45°C, their number increased with increase in the primary incubation temperature. It is apparent that primary incubation at 8°C caused greater recoveries of psychrotrophs while 37°C favoured mesophiles. Thus the 30°C incubation can be considered as most suitable for the growth of both psychrotrophs and mesophiles.

The prevalence of different cultures with respect to their Gram reaction was also noted (Table 2). At all the three primary incubation temperatures and in the two media, Gram negative rods predominated, followed by Gram positive cocci and Gram positive rods. This was in accordance with the previous observations of Karthiayani & Iyer (1967), which revealed a high preponderance of Gram negative rods, mainly *Achromobacter*, *Vibrio* and *Pseudomonas* groups, on the skin and muscle of fresh sardines.

The effect of primary incubation was found to reflect on the secondary incubation temperatures also. Majority of the cultures

Table 1. *Growth of bacteria at test temperatures*

Media	Temperature of primary isolation °C	No. of cultures isolated	Percentage growth						
			0°C	10°C	20°C	30°C	37°C	45°C	56°C
Sea water agar	8	50	100	100	100	90	40	Nil	Nil
	30	60	37	37	70	100	50	20	Nil
	37	42	18	18	28	100	100	57	Nil
Nutrient agar	8	42	100	100	100	74	34	Nil	Nil
	30	60	34	34	60	100	66	26	Nil
	37	42	4	4	21	100	100	48	2
Total		296							

Table 2. *Types of bacteria isolated at the three primary incubation temperatures*

Type	Percentage of different types isolated from NA			SWA		
	8°C	30°C	37°C	8°C	30°C	37°C
Gram positive cocci	7	8	17	10	16	12
Gram positive rods	4	5	7	2	2	7
Gram negative rods	89	87	76	88	82	81

Table 3. *Recovery of psychrophiles, psychrotrophs and mesophiles on SWA and NA at the three primary incubation temperatures*

Type	Percentage recovery SWA			NA		
	8°C	30°C	37°C	8°C	30°C	37°C
Psychrophiles (0–20°C)	10	Nil	Nil	13	Nil	Nil
Psychrotrophs (0–37°C)	90	34	8	87	30	1
Mesophiles (20–45°C)	Nil	66	92	Nil	70	99

showed good growth at the initial isolation temperature, that is, majority of the cultures isolated at 8, 30 and 37°C had their maximum growth at 20, 30 and 37°C respectively. Another observation was that in plates initially incubated at 8°C, it required 12 to 14 days for the first appearance of colonies on the plates. Picked colonies could, however, grow well within 5 days when inoculated into the respective liquid or solid media and incubated at 8°C. With cultures isolated at primary incubation temperatures of 30 or 37°C, no such lag existed and growth, either in liquid media or solid agar took place within 24 h at the respective temperatures.

Thus the primary incubation temperature used in the bacteriological sampling caused a selection of strains according to their growth temperature requirements. Organisms could be placed in psychrophilic (0-20°C), psychrotrophic (0-37°C) and mesophilic (0-45°C) groups (Table 3). Primary incubation at 8°C facilitated the recovery of psychrophiles (10-13%) and psychrotrophs (87-90%). When the primary incubation temperature was raised to 30°C, the percentage of the psychrotrophs decreased to 30-34%, while the mesophiles formed 66-70%. At 37°C, however, psychrotrophs were negligible (1-8%) but the mesophiles constituted 92-99% of the total.

According to the definition of Ingraham & Stokes (1959) and later by Morita (1975) those cultures which grow well at 0°C within a week's time and have a growth range from 0 to 30°C can be considered as psychrotrophs. But some of the psychro-

trophic cultures isolated by us grew well at 37°C. Higoshi *et al.* (1975) have reported the existence of psychrotrophic *Pseudomonas* in milk, whose maximum growth temperature ranged from 0 to 45°C or from 33 to 37°C. The ability of some psychrotrophs to grow at 37°C has been attributed to environmental factors such as available substrate, salinity, temperature and hydrostatic pressure (Morita, 1975). They can also be variants of original mesophiles which adapted to a lower temperature (Larkin & Stokes, 1966).

Between SWA and NA, the growth pattern was found to be almost similar. However it was seen that irrespective of the temperature of primary incubation, the number of cultures with growth temperature range of 0°C-37°C were slightly more in SWA than in NA. (Nirmala Thampuran—unpublished data).

Different incubation temperatures are recommended for the bacteriological examination of food. An incubation temperature of 37°C enhances the growth of mesophilic organism from meat and meat products. Roughley *et al.* (1974) recommend incubation at 30-32°C to include both psychrophiles and mesophiles. An incubation temperature of 20-25°C is used for cold tolerant bacteria from soil, water and foods (Clark, 1967), while a temperature as low as 1°C is suggested for psychrotrophic organisms from refrigerated food (Barnes & Impey, 1968). According to Nottingham *et al.* (1975) incubation of plates at 25 and 30°C gave similar counts, but the 37°C count was usually lower and more variable for aerobic bacteria, due to the inability to include psychrotrophs at this temperature. In raw milk, incubation at 20°C for 4 days is presumed to be superior to incubation at 35°C for 2 days to include both psychrotrophs and mesophiles (Higoshi & Hamada, 1975).

Our study indicates that in marine environment, psychrotrophs occur commonly and in appreciable numbers. Primary incubation temperature of 37°C will exclude some of these types. Lowering the incubation temperature to 30°C can however bring in both psychrotrophs and mesophiles.

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