Freezing Temperature and Freezing Menstruum on the Survival of Selected Marine Bacteria

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The survival pattern of dominant bacterial genera encountered in fishery environs viz. *Pseudomonas, Moraxella, Acinetobacter, Vibrio, Flavobacterium, Micrococcus and Bacillus* were investigated at frozen storage temperature of -39±2°C and -20±2°C in various suspending fluids over a period of one year. The survival was maximum in fish muscle substrate for *Vibrio, Moraxella, Acinetobacter, Micrococcus, and Bacillus* species. For genera *Pseudomonas and Flavobacterium*, there was no significant difference in survival among the various freezing menstruum. Maximum reduction in cell numbers occurred during freezing period or in the period immediately after freezing. Based on the studies, the sensitivity of the bacterial genera to frozen storage was in the order of *Vibrio* > *Bacillus*, > *Pseudomonas* > *Moraxella* > *Acinetobacter* > *Flavobacterium* > *Micrococcus*.

Microbial safety and stability have been the prime consideration in food processing operations. One of the recent approaches to this problem is the study of microbial growth response to factors that control bacterial growth in a particular food system (Roberts & Jarvis, 1983).

The bacterial count of the frozen product reflects the bacteriological quality of the raw material or its contamination during processing; but the reduction in bacterial count resulting from the frozen state is highly variable making any prediction of quality unreliable (Anon, 1980). The factors responsible for the death of bacteria at low temperature have been widely reviewed (Calcott, 1978; Ingram & Mackey, 1986). The cryosurvival of bacteria significant in health or sanitational programmes have been studied to some extent (Raj & Liston, 1961; Digirolamo *et al.*, 1971; Johnson & Liston,1973). But information is very limited on the freezing survival of other bacterial types many of which are active spoilers (Arpai, 1962).

This paper summarizes the salient observations of the effect of freezing temperatures and freezing menstrua on the survival of selected marine bacteria.

Materials and Methods

The study was initiated with bacterial strains isolated from fish and shellfish in course of the studies. For reference, type cultures received from National Collection of Marine Bacteria (NCMB) were used wherever possible.

Ten ml aliquots of sea water peptone broth (Peptone 1%, Potassium nitrate 0.02% in sea water) inoculated from the stock culture served as the inoculum. Bacterial cells which had been activated by repeated subculturing in the same broth, were centrifuged to harvest the cells, washed 3 times with distilled water and finally suspended in distilled water to a cell density of 0.2-0.3 at 600 nm. One ml of the suspension was used for inoculation.

To study the effect of freezing menstruum, one ml of the suspension was inoculated into tubes of fish muscle medium (FM) (Matches *et al.*, 1971) along with distilled water (DW), sea water (SW, salinity: 30‰), saline (NS, 0.85% w/v sodium chloride in distilled water) phosphate buffer (PB, pH 7.0) and peptone water (PW, 1% w/v aqueous solution of peptone). The inoculated tubes were frozen at -39±2°C in deep freezer and stored at - 20±2°C for

upto one year. To study the effect of freezing temperatures on bacterial survival, duplicate sets of fish muscle medium were inoculated as described above and tubes kept at -20±2°C and -39±2°C for one month

Duplicate tubes from the first lot were removed at the end of each month, whereas the tubes from the second set were removed at the end of 1, 10 and 30 days. Tubes were defrosted at a rate of 6–8°C/min by keeping in running water.

The bacterial count of the initial cells exposed to freezing as well as the frozen cells were determined by spreading 0.1 ml of the saline dilutions on sea water agar plates (peptone 1%, ferric phosphate 0.05% and agar agar 1.5% in sea water) and incubating for 48 h at 29±1°C (RT) and enumerating the colonies.

Results and Discussion

The effect of freezing menstruum on the survival of various bacterial genera are presented in Figs.1-7. Analysis of variance of the components, freezing menstrua and frozen storage period is given in Table 1. The results showed significant differences in the viability of bacterial strains belonging to the genera *Vibrio, Moraxella, Acinetobacter, Bacillus and Micrococcus* and significant differences between various freezing menstrua. In all these cases, fish muscle medium caused maximum survival of bacterial cells. For *Pseudomonas and Flavobacterium*, there was no significant difference in the survival among the freezing menstrua. However the freezing menstrua showing the lowest survival differed among the bacterial genera. *Micrococcus* species showed lowest survival in peptone water. *Bacillus* species indicated lowest survival in sea water and *Vibrio, Moraxella* and *Acinetobacter* strains exhibited lowest survival in distilled water.

Among the bacterial genera studied, significant difference was observed for the frozen storage period at -20±2°C & -39±2°C (Table 3). The mean reduction in the viable count after different time intervals upto one year indicated that significant reduction in count occurred within three months. It was also observed that the viability of bacterial strain was dependent on freezing menstruum. While *Vibrio* species remained viable in fish muscle medium for five months at -39±2°C, they did not survive for more than three months in other suspending fluids (Fig.2).

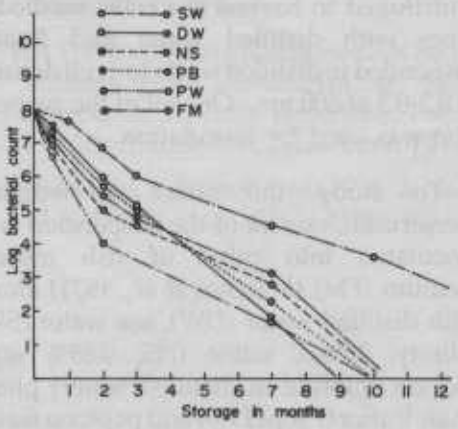

Fig. 1. Survival of a Pseudomonas strain in various suspending fluids during freezing and storage at -20±2°C

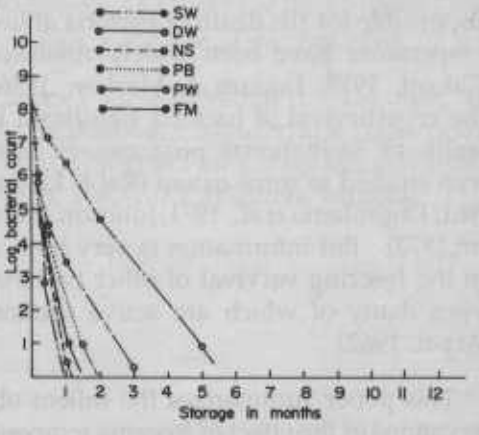

Fig. 2. Survival of *Vibrio* strain in various suspending fluids during freezing and storage at -20±2°C

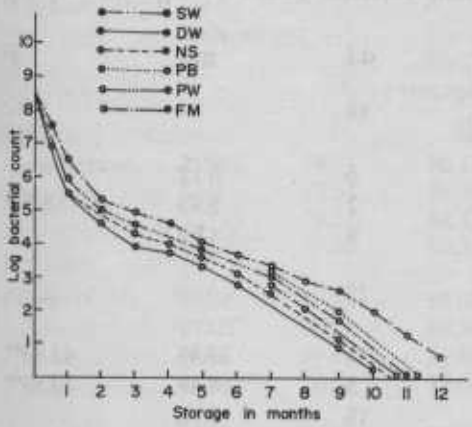

Fig. 3. Survival of a *Moraxella* strain in various suspending fluids during freezing and storage at -20±2°C

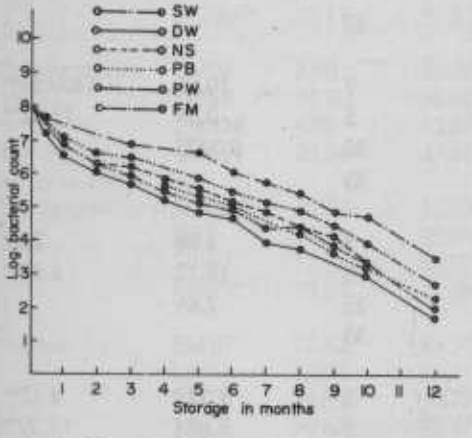

Fig. 4. Survival of an *Acientobacter* strain in various suspending fluids during freezing and storage at -20±2°C

The Figures clearly point to the protective effect afforded by fish muscle and its superiority over other test substrates. According to Raj & Liston (1961), the protection afforded by the fish muscle substrate may be due to the fish protein molecules which behave as a true colloid and interfere with the rate of crystallisation of water in the fish.

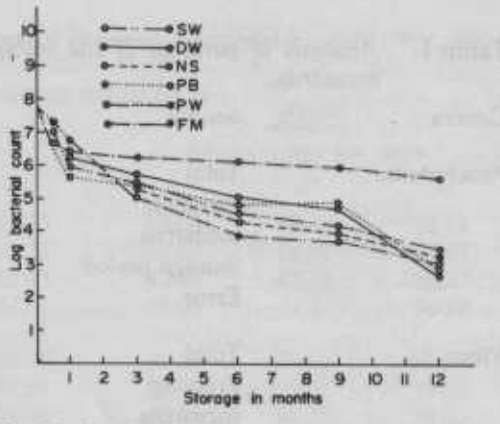

Fig. 5. Survival of *Flavobacterium* strain in various suspending fluids during freezing and storage at -20±2°C

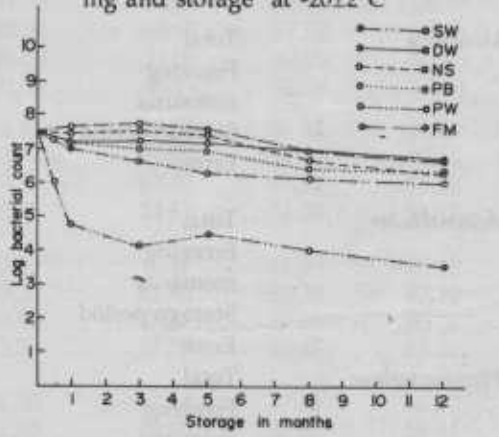

Fig. 6. Survival of a *Micrococcus* strain in various suspending fluids during freezing and storage at -20±2°C

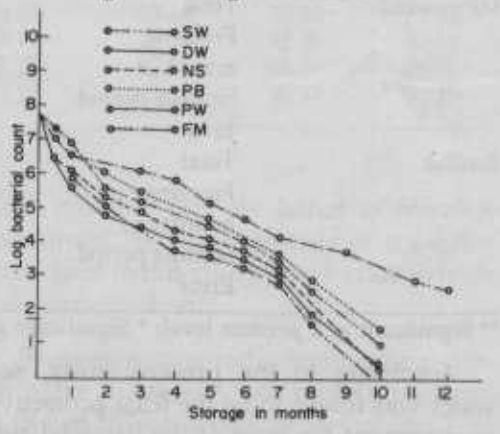

Fig. 7. Survival of a *Bacillus* strain in various suspending fluids during freezing and storage at -20±2°C

Table 1. *Analysis of variance of the viability of various bacterial genera in different freezing menstrua.*

Genera	Source	s.s	d.f	m.s	F
Pseudomonas	Total	22.53	16	-	-
	Freezing menstrua	0.60	5	0.12	1
	Storage period	11.86	2	5.93	5.29*
	Error	10.07	9	1.12	-
Vibrio	Total	154.61	23	-	-
	Freezing menstrua	100.45	3	33.48	42.83**
	Storage period	42.66	5	8.49	10.89**
	Error	11.70	15	0.78	-
Moraxella	Total	101.88	65	-	-
	Freezing menstrua	86.70	10	8.67	95.27**
	Storage period	10.63	5	2.13	23.41**
	Error	4.55	50	0.09	-
Acinetobacter	Total	79.14	47	-	-
	Freezing menstrua	75.97	7	10.85	1409.46**
	Storage period	2.90	5	0.58	75.32**
	Error	0.27	85	0.0077	-
Flavobacterium	Total	125.60	35	-	-
	Freezing menstrua	24.92	5	4.98	2.02
	Storage period	81.11	5	16.22	6.59**
	Error	19.57	25	2.46	-
Micrococcus	Total	41.86	35	-	-
	Freezing menstrua	6.11	5	1.222	4.17**
	Storage period	28.42	5	5.684	19.39**
	Error	7.33	25	0.2932	-
Bacillus	Total			68.93	47
	Freezing menstrua	7.05	5	1.41	21.69**
	Storage period	59.60	7	8.45	130.00**
	Error	2.28	35	0.065	

** Significant at 1 percent level; * Significant at 5 percent level

According to the present study, sea water was found to be the least protective environment for most of the bacterial cultures. Reports on the survival of bacteria in sea water during freezing are scanty. Distilled water was found to be the most toxic to *Moraxella* and *Acinetobacter* sp. While Postgate & Hunter (1961) reported distilled water to be toxic for *Aerobacter aerogenes*, Clement (1961) found the same

Table 2. *Effect of freezing temperatures on the survival of various bacterial genera*

Genera	Culture No.	Percentage reduction at -39±2°C After storage for days			Percentage reduction at -20±2°C After storage for days		
		1	10	30	1	10	30
Pseudomonas	2PW5	78.31	90.12	92.53	65.06	88.07	92.64
	SN4	79.64	88.98	93.73	69.67	88.95	93.01
	3LW5	79.51	84.47	88.43	73.88	82.13	88.53
	MB398*	67.30	83.34	97.51	69.05	94.45	96.59
Vibrio	PW4	98.23	99.07	100.00	98.12	99.75	100.00
	PD2T	97.02	99.96	99.99	96.18	99.87	99.98
	PD6	97.05	99.86	99.96	95.61	97.31	99.95
	MB407*	76.02	97.66	99.97	73.47	97.52	99.74
Acinetobacter	LW2	94.29	97.25	98.75	90.00	96.75	98.29
	MS8	68.89	97.69	98.34	69.07	97.57	98.25
	2PW2	42.29	72.40	81.89	31.09	62.93	90.65
	PD8	32.71	40.84	61.59	40.19	42.06	55.14
Moraxella	LW6	38.87	88.25	92.12	35.00	86.37	91.00
	2PW4	72.40	90.43	93.77	59.05	90.10	93.66
	PW9	63.64	89.50	94.75	57.37	87.64	91.80
	MB308*	37.11	88.22	96.56	24.67	74.00	91.15
Flavobacterium	1MS7	8.00	48.00	59.20	4.00	41.20	42.40
	1MS8	47.20	66.80	79.20	42.80	53.20	63.20
	2MS18	4.00	62.60	90.80	8.00	59.20	90.38
	4MS11	23.64	37.62	53.54	17.28	44.46	53.91
Micrococcus	2LWP2	23.79	31.03	66.20	17.14	47.24	66.90
	CP2	14.58	20.83	37.50	12.50	20.83	35.42
	3PW1	11.70	35.00	55.32	11.18	33.30	35.42
	MB13*	30.69	41.48	71.04	27.59	40.34	70.86
Bacillus	SM10	70.83	89.34	94.67	33.64	70.00	84.34
	LW15	60.00	98.47	99.15	94.00	97.00	98.60
	PW21	94.84	99.77	99.22	92.84	98.70	99.07
	7NG3	90.62	92.47	95.57	88.93	90.82	94.23

* Type cultures from NCMB

environment harmless to the same bacteria. Toxicity of saline was noticed by Nakamura & Dawson (1962) for *Pseudomonas fluorescence* and Calcott & MacLeod (1974) for *Escherichia coli*. According to the present study, saline was less toxic to *Pseudomonas* than distilled water, contarary to the finding of Calcott & MacLeod (1974). According to the same authors salts other than sodium chloride such as lithium chloride, potassium chloride etc,, are lethal as freezing menstrua. The severe toxicity of sea water to bacteria in this study may be due to high salt concentration.

The percentage reduction of the representative isolates in fish muscle medium at frozen storage temperatures of -39±2° C and -20±2° C after exposure for one, ten and thirty days are represented in Table 2.

Table 3. *Analysis of variance of viability of various bacterial genera on storage at different freezing temperatures in fish muscle medium*

Genera	Source	s.s	d.f	m.s	F
Pseudomonas	Total	1647.16	23	-	-
	Bacterial strains	118.56	3	39.52	2.39
	Freezing temperature	39.37	1	39.37	2.38
	Storage period	1207.85	2	603.92	36.49**
	Error	261.38	17	16.55	-
Vibrio	Total	1663.19	23	-	-
	Bacterial strains	339.24	3	113.08	4.72*
	Freezing temperature	1.74	1	1.74	< 1
	Storage period	914.89	2	457.54	19.09**
	Error	407.32	17	23.96	-
Acinetobacter	Total	7458.46	23	-	-
	Bacterial strains	5206.62	3	1402.21	39.11**
	Freezing temperature	4.51	1	4.51	< 1
	Storage period	1637.90	2	818.95	22.84**
	Error	609.43	17	35.85	-
Moraxella	Total	5145.73	23	-	-
	Bacterial strains	346.12	3	115.32	4.99**
	Freezing temperature	95.12	1	95.12	4.12
	Storage period	4311.86	2	2155.93	93.33**
	Error	392.63	17	23.10	-
Flavobacterium	Total	7174.94	23	-	-
	Bacterial strain	634.80	3	211.60	2.65
	Freezing temperature	5.42	1	5.42	< 1
	Storage period	5175.81	2	2587.90	32.37**
	Error	1358.91	17	79.94	-
Micrococcus	Total	2773.43	23	-	-
	Bacterial strain	690.44	3	230.15	7.64**
	Freezing temperature	1.79	1	1.79	1
	Storage period	1568.96	2	784.48	26.04**
	Error	512.24	17	30.13	-
Bacillus	Total	3519.00	23	-	-
	Bacterial strain	1483.50	3	494.50	8.29**
	Freezing temperature	42.69	1	42.69	1
	Storage period	979.07	2	489.54	8.21**
	Error	1013.74	17	59.63	-

** Significant at 1 percent level; * significant at 5 percent level

The periods were selected after observation that maximum changes in the survival of bacteria resulted in the early period (Figs. 1-7). Statistical analysis of the data were undertaken and are presented in Table 3.

The reduction in the bacterial count during frozen storage were quite significant for all the bacterial genera (Table 3). The mean reduction in the viable count at -39±2°C in FM for *Pseudomonas, Vibrio, Moraxella, Acinetobacter, Flavobacterium, Micrococcus* and *Bacillus* after 24 h were respectively 58.90, 75.37, 44.10, 51.03, 20.74, 25.38 and 64.33 percentage. After 10 days the corresponding values were 71.71, 86.00,68.91,64.33, 45.96, 36.32 and 75.61 and after 30 days the values were 75.19, 90.00, 75.12, 70.89, 55.57, 36.49 and 79.35 percentage. This showed that for bacterial species belonging to *Pseudomonas, Vibrio, Moraxella, Acinetobacter* and *Bacillus* maximum reduction in viable count or cell death occurred in the first 24 h. For genera *Flavobacterium and Micrococcus*, maximum cell destruction occurred in the storage period upto 10 days. The order of sensitivity of various bacterial genera to freezing temprature could be represented as *Vibrio* > *Bacillus* > *Pseudomonas* > *Moraxella* > *Acinetobacter* > *Flavobacterium* > *Micrococcus*.

Regarding the two test temperatures, -39±2°C and -20±2°C, there was no significant difference in viability between the two temperatures. In both cases, species variations were quite significant (Table 3).

Tanner & Williamson (1928) stated that during storge of bacteria at low temperature the rate of death of bacteria followed the curve of monomolecular reaction, death being proportional to the number of viable cells. Hence the plot of logarithm of the number of survivors against period of storage should result in a straight line. But the survivor curve obtained in this study was non-linear as in previous observations (Major *et al.*, 1955).

The Gram negatives are generally considered very susceptible to freezing temperatures and Gram positives as resistant (Calcott, 1978, Ingram & Mackey, 1986). While supporting their statement, it is to be added that exceptions are possible as in the case of *Bacillus* spp. and *Flavobacterium* spp. Eventhough spores of *Clostridium perfringens* were resistant to freezer damage, the vegetative cells of the same bacterium showed high sensitivity at -17.2°C (Canada *et al.*,1964). According to Gilliland & Speck (1974), the difference in susceptibility of Gram positive and Gram negative bacteria to freezing is due to the difference in the amount and type of fatty acids and poly glucose residues present in the bacterial cell.

There are conflicting reports about the effect of freezing temperature on bacterial mortality. While Digirolamo *et al.*,(1971) reported -20°C to be less harmful than -10°C, Covert & Woodburn (1972) noted greater cell mortality at -29°C than -5°C. Asakawa (1967) noted greater decline of *Vibrio parahaemolyticus* at -10°C than -20°C in laboratory media, while in raw tuna meat, survival was same at both temperature.

The reduction in bacterial numbers as affected by the period of storage has been noted previously. Digirolamo *et al.* (1971) reported the loss of viability of *Salmonella* sp. and *E.coli* in two stages, a rapid initial stage followed by a gradual decline.

The data accounts well for the persistant appearance of bacterial genera comprising *Micrococcus, Moraxella, Acinetobacter* and *Flavobacterium* in frozen fishery products (Harrison & Lee, 1969; Kawabata *et al.* 1975; Thampuran & Gopakumar, 1991). The relatively lower susceptibility of *Moraxella* and *Acinetobacter* (formerly designated as *Achromobacter* spp.) which are reported as spoilers (Hobbs, 1983) points to the greater possibility of spoilage of frozen product during temperature abuse or defrosting. At the same time, wide variations noted in the survival of species even within a genus

make predictions regarding shelflife impracticable.

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