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Survival of Certain *Vibrio* species at Low Temperature Storage

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Vibrios are indigenous to marine creatures and the marine environment. The survival pattern of two important pathogenic *Vibrio* species, namely *V. parahaemolyticus* and *V. vulnificus* isolated from seafood were studied at -18±2°C and at 6±2°C in three different substrates, *viz*., fish muscle homogenate, tryptic soy broth and 3% sodium chloride solution .The rate of survival was influenced by the suspending menstrum. Maximum survival of *Vibrio parahaemolyticus* was noted in fish muscle substrate at both test temperatures and they were found to be viable till the end of study period of 90 days. For *V. vulnificus* also, the maximum survival was in fish muscle substrate at the two test temperatures, but the viability decreased gradually with storage time, reaching undetectable levels by the end of 60 days at -18±2°C. However, at 6±2°C, they continued to survive till the end of storage period. The study indicates that even though multiplication of pathogenic vibrios are arrested at low temperatures, they can remain viable in fish muscle for long duration and that *V. vulnificus* is more sensitive to low temperature than *V. parahaemolyticus*.

Key words: Low temperature storage, *Vibrio vulnificus*, *Vibrio parahaemolyticus*

Vibrios constitute a considerable portion of the bacterial flora of both finfishes (Cahill, 1990; DePaola *et al.*, 1994) and shellfishes (Surendran & Gopakumar, 1982; Chandrashekharan *et al.*, 1987; Montilla *et al.*, 1994) in the tropics. More than 37 *Vibrio* species have been isolated and characterised so far and among these 12 species have been notified as human pathogens. *Vibrio parahaemolyticus* and *Vibrio vulnificus* are two important members of this group that are considered as inherent pathogens associated with seafood.

Even though *V. parahaemolyticus* is considered as a mesophilic bacteria of aquatic origin, it can withstand lower temperatures better than the other

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common terrestrial mesophiles. Despite the general agreement that *V. parahaemolyticus* exhibits poor resistance to cold, there has been much debate during recent years over its optimal low temperature storage characteristics and there are many reports indicating their survival without growth for reasonably long intervals in muscle substrates at low storage temperatures (Magalthaes *et al.*, 2000; Thampuran & Gopakumar, 1993). The frequent isolation of these two pathogens from iced and frozen fish and shellfish products meant for export from India (Sanjeev *et al.*, 2000) evidently points to the capacity of the pathogens to survive during storage at low temperatures.

Many workers have reported good growth and multiplication of *V. parahaemolyticus* when held in seafood at low temperature (Bradshaw *et al.*, 1974). These results demonstrate that *V. parahaemolyticus* in seafood may grow to enormous numbers when held even for short periods under improper refrigeration conditions. This is further substantiated by the low generation times noted for this organism. Nataragen *et al.* (1979) reported a generation time of 8-13 min at ambient temperature. One of the common practices is to store the fish and shellfish under ice or refrigerated condition, until needed. In many countries like Japan and Korea, shellfish is eaten raw without further cooking or in some countries in semi-cooked state. Hence, the presence of these bacteria in such products may pose as a health hazard.

The aim of the present study was to examine the pattern of survival of *V. parahaemolyticus and V. vulnificus*, in 3 types of substrates, namely fish muscle homogenate (FMH), trypticase soy broth with 3% sodium chloride (TSB) and 3% sodium chloride solution at a typical frozen storage temperature (-18±2°C) and the refrigeration temperature (6±2°C).

Materials and Methods

Growth patterns of *Vibrio parahaemolyticus* and *V. vulnificus*, in the fish muscle homogenate (FMH), trypticase soy broth (Oxoid) supplemented with 3% sodium chloride (TSB) and 3% sodium chloride solution (NaCl), at two temperatures *viz.*, 6±2°C and -18±2°C were studied. Two cultures of each organism, one from the type culture collection of the Central Institute of Fisheries Technology (CIFT), Cochin, India and the other from National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, UK were used for the study.

The fish media for the study was prepared as per Matches *et al.* (1971) with the following modifications. A lot of 150 g of the flesh from the dorsal side of mackerel (*Rastrelliger kanagurta*) was blended with 200 ml aged seawater to make a fine paste. This was made up to 1 litre and boiled for 10 min and dispensed in 10 ml quantities in test tubes and sterilized by autoclaving at 121°C for 15 min.

Inoculum was prepared by washing 18 h old agar slant culture with sterile 3% sodium chloride diluent. Optical density of the inoculum was adjusted to 1.0 at 650 nm. Around 10^8 cells were used as the inoculum per tube as this was the maximum cell density reported to be present in the intestines. An aliquot of 0.1 ml inoculum was added to test tubes containing fish muscle homogenate (FMH), trypticase soy broth (Oxoid) supplemented with 3% sodium chloride and 3% sodium chloride solution. One set of the test tubes was kept in the freezer (Labline Instruments, India) set at a temperature of -18±2°C and another set in BOD incubator (YOMA, India) at 6±2°C. Samples were drawn at an interval of 1 day, 2 days, 1 week, 2 weeks, 1 month, 2 months and 3 months and analysed. Frozen samples were thawed in minimum time under the flow of tap water. Survived cells were recovered by dilution plating on trypticase soy agar supplemented with 3% sodium chloride.

Result and Discussion

Fate of *V. vulnificus,* and *V. parahaemolyticus,* when stored at 6±2°C, for a period of three months is presented in Fig. 1 to 4. Fig. 1 depicts the changes in the survival of *V. vulnificus* in the initial period, namely 1 to 14 days, during which period maximum changes occurred. Fig. 2 gives the results

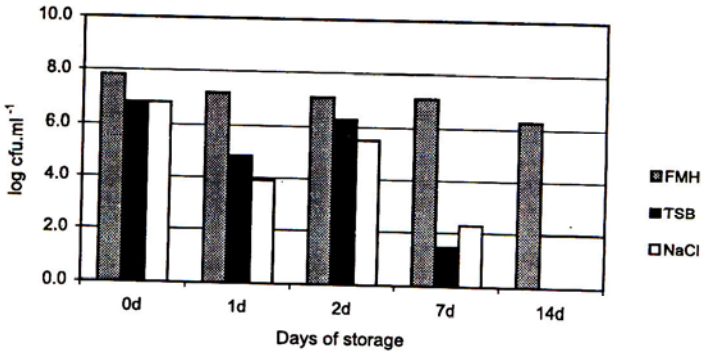

Fig. 1. Survival of *Vibrio vulnificus* during 14 days storage at 6±2°C in different growth media

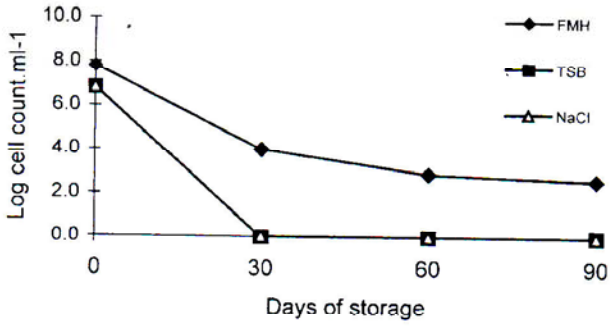

Fig. 2. Survival of *Vibrio vulnificus* during 3 months storage at 6±2°C in different growth media

of the same storage study for 90 days. As seen from the figures, fish muscle afforded maximum protection at 6±2°C. Starting from an initial cell count of 10^8, a 5 log reduction was noticed in the 90 days storage period in fish muscle while in the other menstrum, total elimination of *V. vulnificus* occurred in the first 14 days. Between the two suspending fluids, better survival was noted in TSB. The results for *V. parahaemolyticus* is presented in Fig. 3 and 4. Compared to *V. vulnificus*, survival was better for *V. parahaemolyticus* in the TSB and salt solution, and even after 60 days of storage viable cells could be detected.

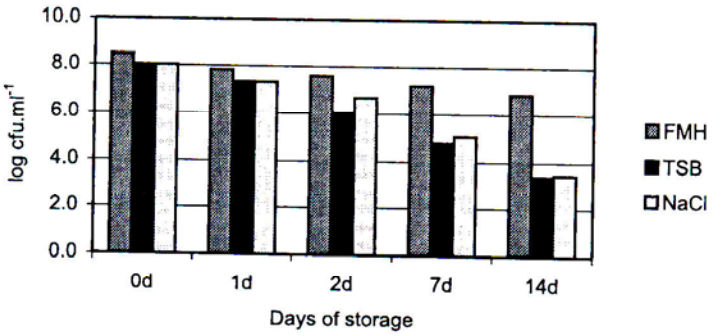

Fig. 3. Survival of *Vibrio parahaemolyticus* during 14 days storage at 6±2°C in different growth media

On storage at -18±2°C, *V. vulnificus* cells were drastically reduced in all the 3 suspending fluids. While complete elimination in fish muscle was evident within 60 days (Fig. 5 & 6), resulting in an 8 log reduction, similar reduction in the other two media was noticed after 14 days. For *V. parahaemolyticus* cells stored at -18±2°C, complete destruction of the cells occurred within 60 days in salt solution and within 90 days in TSB. However,

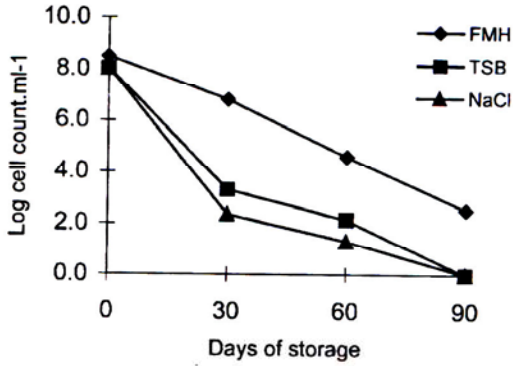

Fig. 4. Survival of *Vibrio parahaemolyticus* during 3 months storage at 6±2°C in different growth media

viable cells remained even after the end of storage period of 90 days in fish muscle, though there was nearly 6 log reduction in the cell number (Fig. 7 & 8).

There is a general agreement that *V. vulnificus* and *V. parahaemolyticus* exhibit poor cold resistance, but there are conflicting opinions over the years regarding the optimum temperature to be selected for storage of flesh foods like seafood. Some workers (Beuchat, 1975; Johnson & Liston, 1973) reported that inactivation occurred more rapidly when *V. parahaemolyticus* was held at chill temperature of 1 to 7°C than when it was frozen at -2 to -30°C. Others described a reverse effect. Matches *et al.* (1971) observed that inactivation of this organism was more at -34°C than at -18°C, which in turn was more than at 0.6°C. Such conflicting views have created an ambiguity regarding the selection of optimum storage temperature during seafood handling.

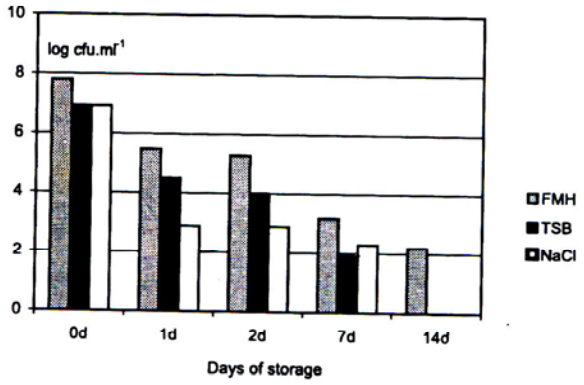

Fig. 5. Survival of *Vibrio vulnificus* during 14 days of storage at -18±2°C in different growth media

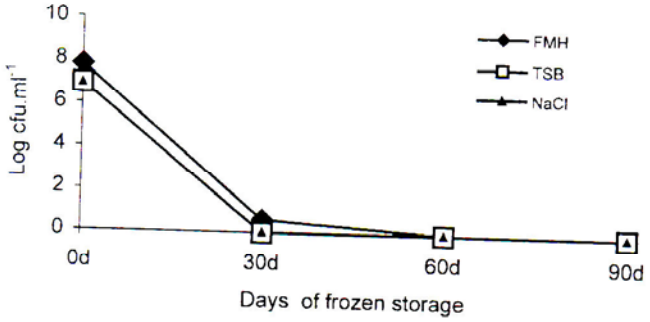

Fig. 6. Survival of *Vibrio vulnificus* during 3 months storage at -18±2°C in different growth media

In the present study, in both 3% salt solution and TSB, *V. vulnificus* could survive only up to 14 days at 6.0±2°C while *V. parahaemolyticus* remained viable for a longer period of up to 60 days. Except for a slightly better survival, especially in the early period of storage, there was not much difference between the two states for both organisms. In a study conducted by Kasper & Tamplin (1993) viable cells of *V. vulnificus* could not be detected in seawater having 10 ppt salinity after 15 days of storage at 5°C. This is further substantiated by the results of the environmental surveys of the coastal seawater and estuaries (Oliver *et al.*, 1982; Tamplin *et al.*, 1982; Kaysner *et al.*, 1989).

Compared to 3% NaCl solution or TSB, fish muscle medium afforded better protection for both *V. parahaemolyticus* and *V. vulnificus*. The survival was better for *V. parahaemolyticus* when compared to *V. vulnificus*. Growth and survival in different seafood products such as shrimp (Boutin *et al.*, 1985; Kasper & Tamplin, 1993), oyster (Oliver, 1981), fish (Covert & Woodburn, 1972) and crab meat (Johnson & Liston, 1973) have been reported in addition

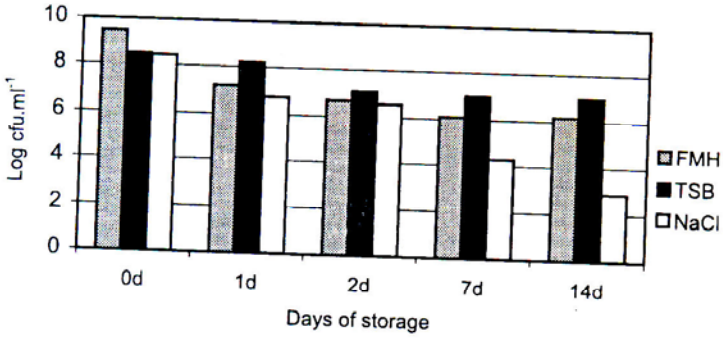

Fig. 7. Survival of *Vibrio parahaemolyticus* during 14 days of storage at -18±2°C in different growth media

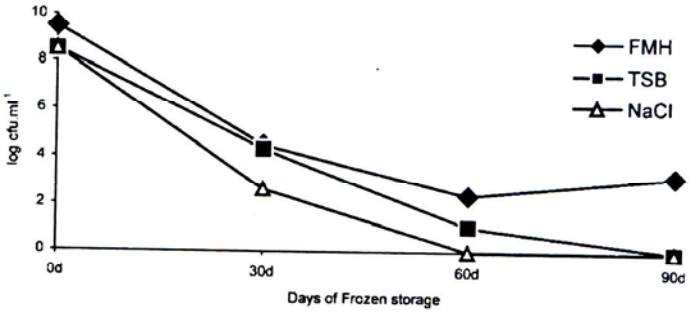

Fig. 8. Survival of *Vibrio parahaemolyticus* during 3 months storage at -18±2°C in different growth media

to others (Oliver *et al.*, 1982; Wolf & Oliver, 1992; Thampuran & Gopakumar, 1993; Venugopal *et al.*, 1999).

In FMH, the plate count declined gradually and $4.0x10^3$ cells survived after 3 months storage of *V. parahaemolyticus* at 6.0±2°C from an initial level of $6.4x10^8$ cfu.g^{-1}. Thus 5 log reduction was noticed during the 3 month storage period. Similar observation of low temperature sensitivity of *V. vulnificus* was reported earlier (Wolf & Oliver, 1992). They opined that the reduction in the viable cell count cannot be ascribed entirely to death, but it can also be due to the formation of viable but not cultivable (VBNC) cells. In this state, bacterium survived for days at low storage temperatures. Protective effect of fish muscle for the *Vibrio* species have been reported previously by Covert & Woodburn (1972) and Thampuran & Gopakumar (1993) and is supported by the present study. In three month's storage in FMH, *V. vulnificus,* was completely eliminated, whereas *V. paraheamolyticus* survived the period indicating better survival capacity for this pathogen. Venugopal *et al.* (1999) reported that *V. parahaemolyticus* was cold sensitive, but survived in cooked prawn, particularly peeled and undeveined prawn up to 17 days at -20°C. Covert & Woodburn (1972) have also reported the detection of viable *V. parahaemolyticus* cells at the end of 30 days of storage at -18±2°C. Muntada-Garriga *et al.* (1995) could detect *V. parahaemolyticus* inoculated into oyster homogenate even after 10 months of storage at -20°C. The longer survival in FMH might be due to the variations among the strains used, difference in menstrua, difference in inoculam size, factors like salt content of the menstrua, etc. Apart from the protective effect of muscle constituents on vibrios, an adverse toxic effect has also been reported (Oliver, 1981). They reported that a heat stable factor is released from oyster which selectively inhibits *V. vulnificus* but not *V. parahaemolyticus* cells.

Greater susceptibility to low temperature of *V. vulnificus* compared to *V. parahaemolyticus* has been emphasised by this study. Storage at 4°C and -20°C in shrimp homogenate, foetal bovine serum and dimethyl sulphoxide caused a decline in cell numbers of *V. parahaemolyticus* and *V. vulnificus,* (Boutin *et al.,* 1985). At 4°C, *V. vulnificus* remained viable up to 32 days while at -20°C viability was noticed at the end of storage period of 40 days. For *V. parahaemolyticus* inactivation occurred at 4°C and -20°C in shrimp homogenate after 32 and 39 days, respectively. They reported a higher inactivation rate for *V. parahaemolyticus* at 4°C compared to -20°C while for *V. vulnificus* death rate at these two temperatures was almost identical.

In frozen shrimp products originating from tropical countries the presence of *V. vulnificus* was reported in 7% in raw frozen samples and none of the cooked frozen samples (Dalsgaard & Hoi, 1997). Sanjeev *et al.* (2000) reported that the iced and frozen fish and shellfish samples collected from processing factories of Kerala and Tamil Nadu were contaminated with pathogenic vibrios. They reported that 32 % samples of iced shellfishes and 5% samples of frozen fish and shellfish meant for export carried *V. parahaemolyticus*. The values for *V. vulnificus* were 7% and 4%, respectively. Since about 5 log reduction in count is possible even in the protected environment, namely fish muscle, the detection of these organisms in such products point to an enormous initial load in raw material or cross contamination and the resulting multiplication.

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