Cey. J. Sci. (Bio.Sci.) Vol. 31,2003,61-67

RECOVERING ABILITY OF FREEZE-STRESSED *SALMONELLA TYPHIMURIUM* AND *STAPHYLOCOCCUS AUREUS* CELLS IN FROZEN SHRIMP

W. Thushani[1], K.W.S. Ariyawansa [2] and P.C. Arampath [1*]
[1] Department. of Food Science and Technology, Faculty of Agriculture, University of Peradeniya, Peradeniya
[2] Institute of Post-harvest Technology, National Aquatic Resources Research and Development Agency, Crow Island, Colombo 15.

ABSTRACT

Viability of *Salmonella typhimurium* and *Staphylococcus aureus* in frozen shrimp sample was investigated. Shrimp slurry was sterilized and divided into two parts. The slurry samples were artificially inoculated with the above organisms separately, frozen and stored at -24^0C. *S. typhimurium* count was reduced by 3 log cycles after 8 weeks of storage at temperature, -24^0C. Increment of one log cycle of *S. aureus* was observed after frozen storage at -24^0C for 24 hours, and more than two log cycle reduction at the end of 8 weeks storage. The result revealed that *S. aureus* was more resistant than *S. typhimurium* under frozen temperature (-24^0C).

The construction of quality control charts for *S. aureus* and aerobic plate count by using reference samples helped to validate and monitor the test procedure. A chart was drawn by analyzing 14 reference samples. The mean values (MV), upper and lower warning limits (MV $\pm$ 2*SD) and upper and lower control limits (MV $\pm$ 3*SD) were transferred into the control charts. These control charts are ideal and useful to decide the quality of the results obtained from the analysis at the laboratory.

INTRODUCTION

Salmonella is known all over the world as an agent of food-borne disease and the animal foods are still the major source of human Salmonellosis (Bachhil and Jaiswal, 1988; Rao, 1983). Staphylococcal food poisoning is a common food borne disease that occurs in most countries of the world, especially in India due to its warm and humid climate (Bergdoll, 1989). Freezing has been used as a means of preserving the biological materials. Presently research on freezing is basically focused on survival ability of cells, lethality and multiplication of microorganisms in perishables and preserving the microbial cells themselves in the case of medically and industrially importance cultures (Davis and Obafemi, 1985).

The microflora on the skin and gill surface of cold-water marine species consist largely of the gram-negative rods. The microflora on warm-water fish is mainly gram-positive type (Liston, 1980; Shewan, 1977). Anaerobes are usually absent from slim and gill but always present in the gut (Shewan, 1962). Gram-negative organisms are generally more sensitive to freezing than gram-positive organisms, while spores and food poisoning toxins are unaffected by freezing (Shewan, 1961). Microbial cells at the exponential phase are more sensitive to freeze-thaw stress than that of at the stationary phase (Ray and Speck, 1973).

**Corresponding author* e-mail: pca.mail@pdn.ac.lk

Frozen seafood industry is one of the profitable industries today. The microbiological test on colony counts, *Staphylococcal* counts, tests on *Salmonella*, *Vibrios* are the regular tests on product quality. The microbiological quality of seafood in the export market is a critical factor. The validated test methods in laboratory analysis are very important in this regard. Quality tests of seafood usually include enumeration and identification of microorganisms that cause food poisoning. The information on survival of *Salmonella typhimurium* and *Staphylococcus aureus* under freeze-thaw condition is important in quality control of fish/shrimp industry. The control charts are ideal to maintain the accuracy and quality of the test results. The objective of the study was to investigate the effect of freeze-thaw stress on survival of *S. typhimurium* and *S. aureus* to construct control charts for the methods of analyzing *S. aureus* and aerobic plate counts using the pure culture.

MATERIALS AND METHODS

De-headed, peeled and chopped shrimps were blended in a stomacher. The shrimp slurry was sterilized (15 psi, 15 minutes) and equal portions were inoculated separately with pure cultures of *S. typhimurium* (ATCC 14028) and *S. aureus*. Colony counts were taken just after the inoculation and also at weekly intervals. The organisms were confirmed as *S. typhimurium* and *S. aureus* by the biochemical tests.

Identification of *S. typhimurium* was done as follows: One loopful of pure culture was streaked on a Nutrient Agar (NA) plate and the plates were incubated at 37°C for 24 hrs. Secondly Xylose Lysine Desoxycholate agar (XLD) was inoculated with culture obtained from the first step and incubated at 37°C for 24 hours. Thirdly, on Triple Sugar Iron Agar (TSIA) and Lysine Iron Agar (LIA) slants were streaked and stabbed butt followed by incubation at 37°C for 24 hours. Finally, Urease test was used to confirm *S. typhimurium*.

For identification of *S. aureus* a loopful of pure culture was streaked on NA plates and the plates were incubated at 37°C for 24 hours. The bacterium was taken from NA, streaked on Baird Parker agar (BP) medium and incubated at 37°C for 72 hours to observe the characters of the colonies. Coagulase test and catalase test were performed to confirm the identity of *S. aureus*.

Freeze-thaw experiments were conducted with inocula of *S. typhimurium* and *S. aureus*. The samples were prepared by inoculating 3 ml of Brain Heart Infusion (BHI) with the working culture in Nutrient agar and incubating at 37^0C for 24 hours.

To enumerate *Salmonella*, appropriate dilutions were made using Minimum Recovery Diluent (MRD) and 1ml of inoculated sample. 0.1 ml from each dilution was placed on a Xylose Lysine Deoxycholate (XLD) plate separately using a sterile pipette and spread evenly over the surface with the help of a spreader, followed by incubation at 37^0C for 24 hours. Duplication sampling was carried out for each dilution. Finally number of colonies was counted.

For enumeration of *S. aureus*, appropriate dilutions were made using MRD with a sterile pipette and spread evenly over the surface of the Baird Parker plate using a spreader. Duplicate plating was carried out for each dilution. The samples were incubated at 37^0C for 72 hours and the number of colonies was counted.

Construction of control charts and validation of test methods (aerobic plate count and enumeration of *S. aureus*) were conducted with a mixed culture, which consisted of *Micrococcus sp, Klebsialla oxytoca, Escherichia coli, Staphylococcus aureus, Clostridium perfringens* and *Salmonella dublin*. The reference samples (14 samples) were analyzed and the control charts were drawn for each analysis (log no, bac./ml vs. date). The mean value (MV) and standard

deviation (SD) of the logarithmic results were calculated. Finally mean value (MV), upper & lower warning limits (MV $\pm$ 2*SD) and upper & lower control limits (MV $\pm$ 3*SD) were determined and transferred into the control charts.

RESULTS AND DISCUSSION

The biochemical tests revealed the presence of *S. typhimurium*. Pink colonies with or without black centers were observed in XLD plates, therefore *Salmonella* was present in the tested samples. Many cultures of *Salmonella* may produce large glossy black centers or appear as completely black colonies. Atypically, a few *Salmonella* species produced yellow colonies with or without black centers. Pink colonies were observed on Brilliant Green Agar (BGA). Red butt (alkaline) and yellow butt (acid) appeared on TSI agar due to the fermentation action of *Salmonella* on dextrose. Blackening of agar was observed due to the production of hydrogen sulphide (H_2S). Purple butt (alkaline), appeared on Lysine decarboxylase (LI) agar revealed the presence of *Salmonella* in the sample. The presence of *S. typhimurium* in the test sample was confirmed based on purple-red colour formation by Urease test.

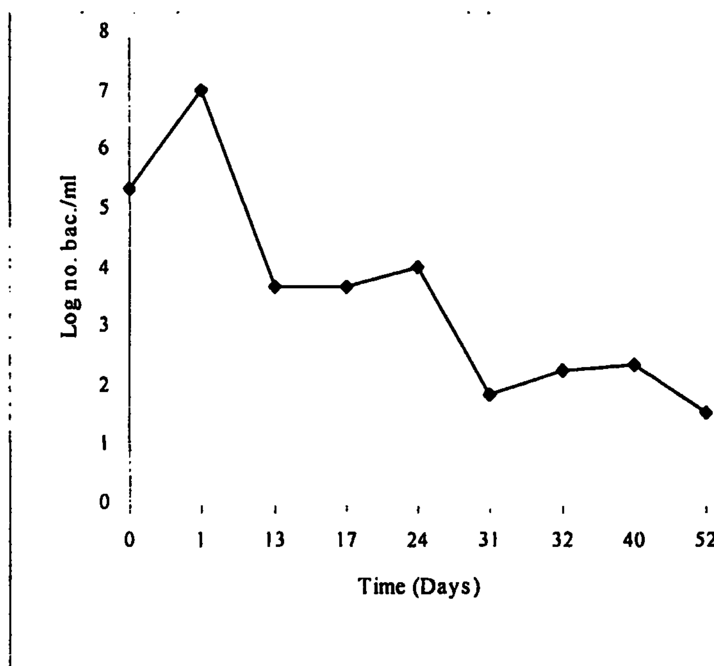

Figure 1. The survival of *S. typhimurium* in frozen shrimp storage (-24^{0}C)

Characteristic colonies of *S. aureus* appeared on Baired Parker agar plates 72 hours after incubation at 37°C. Circular, smooth, convex and moist colonies of 2-3 mm in diameter, with light coloured margin surrounded by opaque and frequently with outer clear zone were observed on the colonies. Presence of *S. aureus* in the sample was confirmed by catalase test and coalgulase test. Production of gas bubbles due to the reaction with hydrogen peroxide was observed (catalase test). A cloudy appearance was observed after 1 hour sitting for coalgulase test. Therefore the presence of *S. aureus* was confirmed. The freeze-thaw experiment revealed that the number of colony forming units of *S. typhimurium* was increased by more than one log cycle within 24 hours at –24^{0}C, due to the multiplication of bacterial cells before it reached the storage temperature (Fig 1). However, a significant reduction (more than 3 log cycles) was shown in *Salmonella* counts during the storage period of 8 weeks at -24^{0}C (P=0.05). The unfavorable temperature may have caused this reduction. 0.02 % of the initial organisms

inoculated remained viable after 52 days. Bacterial cells were subjected to freeze stress resulting death, metabolically or structurally injured or both. Therefore to recover the injured cells an enrichment step is a necessity. Buffered peptone was used as enrichment broth in this study.

The freeze-thaw experiment on *S. aureus* revealed a growth increment of microorganism after 24 hours at -24^0C storage followed by reduction in bacterial count by two log cycles during the storage period of 8 weeks. It was noted that, 21.7% of the *S. aureus* cells remained viable after 52 days storage (Fig. 2). BPA medium was the enrichment broth that was able to recover the injured *S. aureus* cells. The result revealed that *S. aureus* was more resistant than *S. typhimurium* under frozen temperature (-24^0C).

Using the parameters given in Table 1, the control charts were constructed (Fig. 3 & 4). The test methods on enumeration of *S. aureus* and aerobic plate count were validated. The results of the reference samples of the study were fitted within the limits, specified on the control charts of *S. aureus* and aerobic plate count. Therefore the error associated with the test methods was normal. The data fitted beyond the control and warning limits (upper & lower) was probably due to the personal errors. The data fluctuation within the limits was due to flaws inherent with the method of analysis. Therefore the methods of analysis need to be changed or improved.

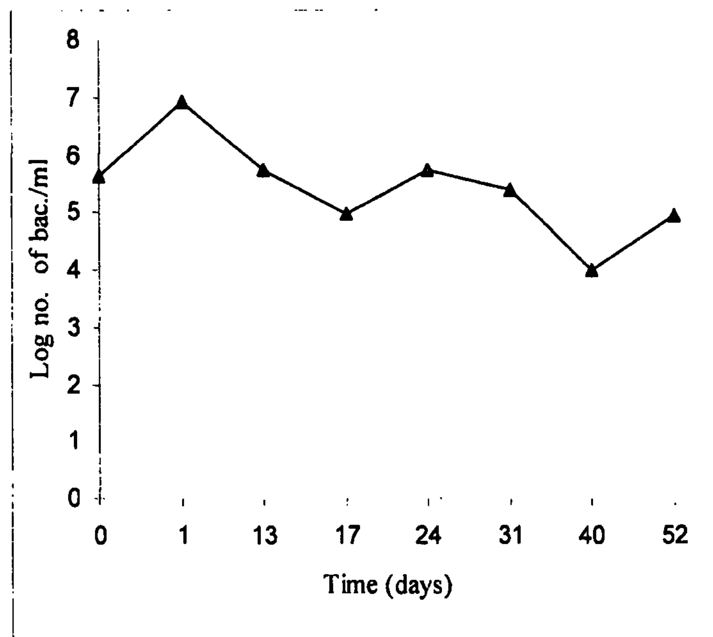

Figure 2. The survival of *S. aureus* in frozen shrimp storage (-24^0C)

Table 1
Parameters included in the control chart

Parameters	*S. aureus* *(Log no. bac/ml)*	Aerobic plate count *(Log no. bac/ml)*
Upper Control Limit (UCL)	5.45	7.1
Upper Control Limit (LCL)	4.5	6.5
Mean value (MV)	4.95	6.8
Lower Warning Limit (LWL)	4.65	6.6
Lower Control Limit (LCL)	4.5	6.5

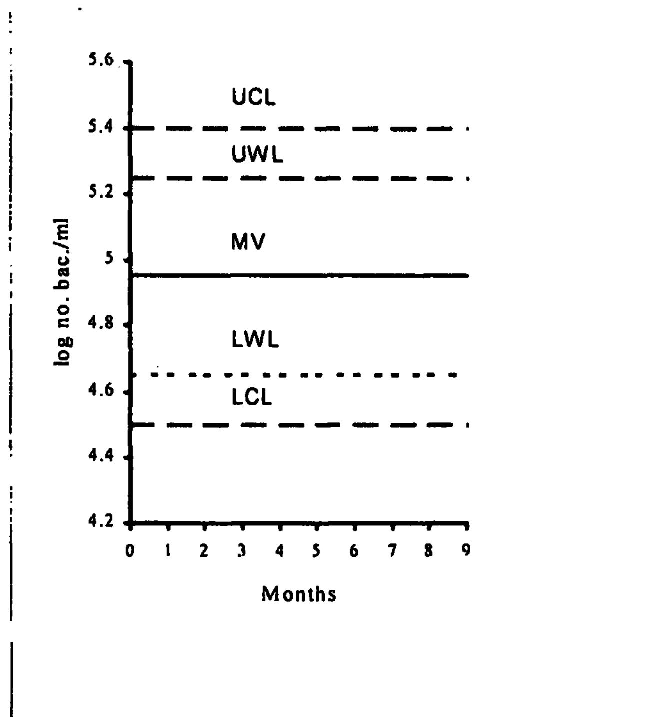

Figure 3. Control Chart: *Staphylococcus aureus*

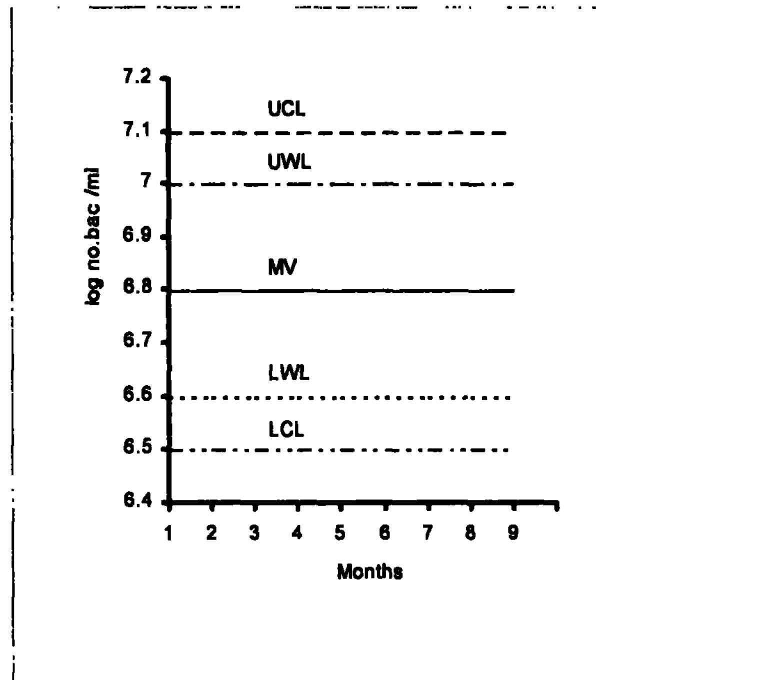

Figure 4. Control Chart: Aerobic Plate Count

CONCLUSION

Both *S. typhimurium* and *S. aureus* survived during storage at -24^0C over a period of 8 weeks. There was a possibility of food poisoning due to contaminated shrimp prior to the storage at -24^0C. *S. aureus* was more resistant than *S. typhimurium* at storage temperature, $-24\ ^0$C. The control chart was very important tool to determine the quality of the test results, to validate & monitor the test methods and to apply the corrective actions.

ACKNOWLEDGEMENTS

Authors thank Mrs. Kumudu Hettiarachchi and Mr. Rohana Wijesinghe for their cooperation.

REFERENCES

Bachhil,V. N, and Jaiswal, T.N (1988). Occurrence of *Salmonella* in meals. *Journal of Food Science and Technology* **25**:310-312

Bergdoll, M.S. (1989). Staphilococcus aureus. In: Doyle MP (ed) Food borne Bacterial Pathogen, Marcel Deccer, Inc., New York. Pp 463-523.

Davis, R. and Obafemi, A (1985). Response of Microorganisms to freeze–thaw stress, microbiology of frozen foods, (Ed) R.Robinson), Pp 83-107.

Liston, J. (1980). Advances in fish science & technology (Ed, J.J.Connel), Fishing News ltd, London. Pp 206 –209.

Rao, N. D. (1983) *Salmonella* in meat. *Journal of Food Science and Technology*. **20**: 156-160.

Ray, B. and Speck, M.L (1973), Freeze injury in bacteria, *Critical Reviews in Clinical Laboratory Sciences* 4, 161 – 213.

Shewan, J.M. (1961) The microbiology of sea water fish. Fish as Food (Ed, G. Borgstrom), Academic Press, London, Pp 167 – 193.

Shewan, J.M. (1962) Recent advances in food sciences (Ed, P. Sutcliffe and J. Disney), Tropical Products Institute, London, Pp 51-66.

Shewan, J.M. (1977) Proceeding conference on handling, processing and marketing tropical fish (Ed. P. Sutcliffe and J. Disney), Tropical Products Institute, London, Pp 121-131.