Growth kinetics of *Staphylococcus aureus* and *Streptococcus faecalis* under the influence of liquid nitrogen and subsequent storage of treated cooked prawn meat

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Growth kinetics of *Staphylococcus aureus* and *Streptococcus faecalis* in treated cooked prawn meat after liquid nitrogen freezing and subsequent frozen storage indicate that *S. aureus* survived up to 10 months of storage in case of liquid nitrogen frozen sample and more than 12 months in case of conventional frozen sample. In contrast, survival period of *S. faecalis* was limited to 2 months and 4 months in case of liquid nitrogen and conventional frozen sample respectively. Natural coliform bacteria were found to be nil after one month and five months of storage in case of liquid nitrogen and conventional frozen sample. Total bacterial count was much less in case of liquid nitrogen frozen sample during the total period of one year.

THE rate of production of fish in India has increased greatly during the last 20 years. About 3.0 million metric tonnes of fish are caught annually, 10% of which are frozen and exported. The increased production of fish notwithstanding the unbecoming part is the wastage due to lack of proper storage. Nearly 35–40% of the total catch goes waste every year. Fish spoilage[1] is normally caused by microorganisms either directly by their growth or indirectly by secreting enzymes which exert a disintegrating action on the host tissue. There are usually two types of pathogenic and spoilage bacteria naturally occurring in fishes: *Clostridium botulinum* type E, and *Vibrio parahaemolyticus*[2]. The incidence of gastroenteritis due to these organisms is maximum in summer[3,4]. Most of the bacterial gastroenteritis in Japan, which have been reported, are due to *V. parahaemolyticus* infection[3]. However, not all *V. parahaemolyticus* variety are pathogenic and as the infective number for infection is high, food poisoning occurs due to mishandling of the sea food products. Further, most of the gastroenteritis outbreaks in Japan were due to consumption of raw fish[5], and elsewhere due to taking shrimp and crab recontaminated after cooking at temperatures permitting their rapid growth[6]. Other pathogenic organisms which contaminate fish at various stages of processing include *Streptococci*, *Staphylococcus aureus*[7,8] etc. This communication describes the effects of liquid nitrogen freezing and subsequent frozen storage on the survival pattern of total bacterial count and natural coliform bacteria in fresh prawn meat and also *S. aureus* and *S. faecalis* in treated cooked prawn meat.

Grass prawns (*P. monodon*) were purchased from Razban Sea Food Pvt. Ltd., Calcutta. After collection, the samples were taken to the laboratory in an insulated bucket under ice. Then the samples were washed, peeled and again washed. The samples were then divided according to the experimental need. Two pathogenic fish spoilage bacteria, *S. aureus* and *S. faecalis* were used in the experiments. Tryptone-glucose-beef extract agar (TGBA) medium used for total bacterial count (TBC) in fish sample was composed of 0.5% (w/v) peptone, 0.3% (w/v) beef extract, 0.1% (w/v) dextrose and 2.0% (w/v) agar and the pH was adjusted to 7.0. Desoxycholate-agar medium used for natural coliform bacteria in fish sample was composed of 1.0% (w/v) bacto-peptone, 1.0% (w/v) bacto-lactose, 0.1% (w/v) Na-desoxycholate, 0.5% (w/v) NaCl, 0.1% (w/v) Na-citrate, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) ferric citrate, 1.5% (w/v) bacto-agar and the pH was adjusted to 7.3 and then 0.003% (w/v) bacto-neutral red was added. Pink and red colonies were counted within 18–24 h of incubation at 37°C. The medium used for growth of *S. faecalis* consisted of proteose–peptone No. (3) 1% (w/v), yeast extract 1% (w/v), NaCl 0.5% (w/v), sodium glycerophosphate 1% (w/v), maltose 2% (w/v), lactose 1% (w/v), and 2% (w/v) agar in water and NaN_3, Na_2CO_3 and bromocresol purple were used in appropriate quantities[9]. The medium used[9] for growth of *S. aureus* was composed of tryptone (oxoid L_2) 1% (w/v), Lab-Lemco beef extract 0.5% (w/v), yeast extract (oxoid L_{21}) 0.1% (w/v), sodium pyruvate 1% (w/v), glycine 1.2% (w/v), lithium chloride 0.5% (w/v), oxoid agar No. (3) 2% (w/v) in water and pH 6.8 (ref. 9). Nutrient broth medium, composed of peptone 0.5% (w/v), beef extract 0.3% (w/v) and pH 6.8–7.0, was used for the preparation of cell mass of *S. aureus* and *S. faecalis*. The pathogenic bacteria *S. aureus* and *S. faecalis* were each inoculated from agar slant culture to nutrient broth and incubated at 37°C under stationary condition for 24 h. After the incubation period, the culture was harvested by centrifugation under sterile condition and the pellet so obtained was resuspended in sterile isotonic saline. Then the cooked prawn meat was immersed in bacterial suspension for a period of 30 min. The above process was conducted for both *S. aureus* and *S. faecalis*. Subsequently the samples were packed in mylar pouches for liquid nitrogen freezing and in polyethylene packets for conventional freezing. The mylar pouches (containing bacteria infested prawn meat) were sealed by an automatic sealer and immediately after sealing were transferred to liquid nitrogen freezing chamber. The freezing was performed by liquid nitrogen as a two-step process with the help of a semiautomatic electronically controlled freezer, designed and developed in our laboratory. The average

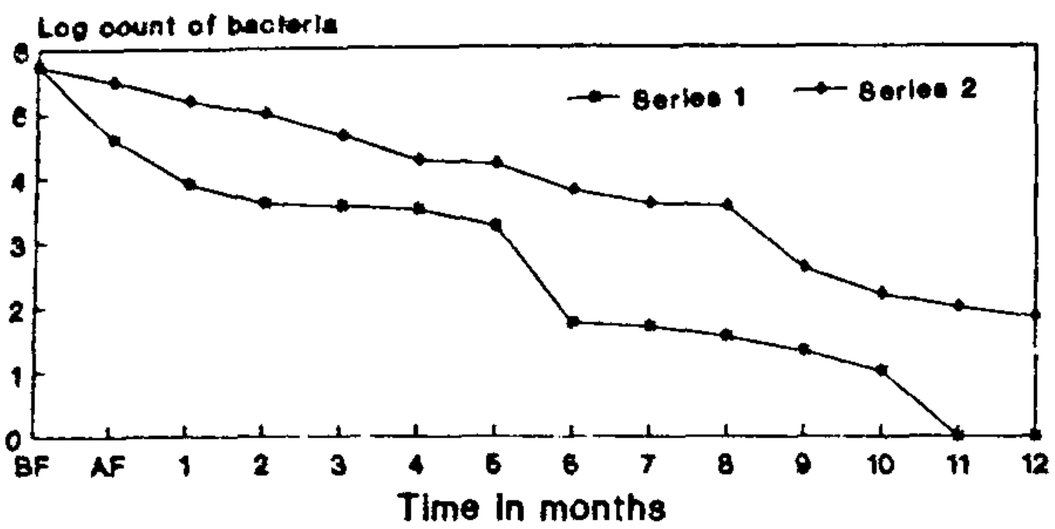

Figure 1. Growth kinetics of *Staphylococcus aureus* in cooked prawn meat under LN_2 and conventional freezing and frozen storage. BF indicates count before freezing, AF indicates count after freezing. Series 1, LN_2 frozen sample; Series 2, conventional frozen sample.

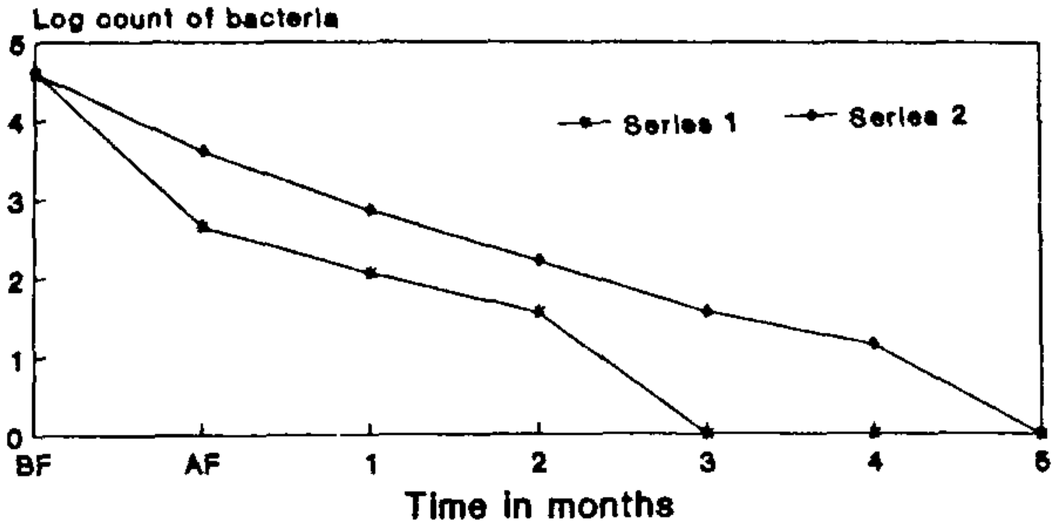

Figure 2. Growth kinetics of *Streptococcus faecalis* in cooked prawn meat under LN_2 and conventional freezing and frozen storage. BF indicates count before freezing, AF indicates count after freezing. Series 1, LN_2 frozen sample; Series 2, conventional frozen sample.

cooling rate was 3°C/min from ambient to –10°C and then 1.5°C/min from –10°C to –40°C. Then liquid nitrogen frozen sample and conventional frozen sample were stored at –18°C for a period of one year. Bacterial counts were made by blending a definite portion of the frozen sample with sterile saline water in the ratio 1 : 10 in a mortar. The different dilutions were made by usual serial dilution technique. Bacterial colonies were developed in nutrient agar medium by pour plate culture method. The plates were incubated at 37°C for 24 to 48 h to get the bacterial colonies. Then the number of colonies were counted by a colony counter.

It appears from the results (data not shown) that total bacterial count (TBC) diminished slowly during the total period of one year in case of conventional frozen sample. But in case of liquid nitrogen frozen sample, the count diminished rapidly compared to conventional method up to 9 months. After 9 months of storage, steep fall in count was observed and the count became almost nil after 1 year of storage in case of liquid nitrogen frozen sample. But more than 50% cells remained viable even after 1 year of storage for conventional frozen sample. It appears from the results (data not shown) that the count of natural coliform bacteria reduced drastically in case of liquid nitrogen frozen sample and became nil after one month of storage. In contrast, fairly good count was observed up to 5 months in case of conventional frozen sample and then the count became nil during the period under consideration. Growth kinetics of *S. aureus* for a period of one year under different conditions of freezing (both conventional and liquid nitrogen) and frozen storage after being applied to prawn meat showed 93% reduction in case of liquid nitrogen frozen sample; whereas only 43.8% reduction was observed in case of conventional frozen sample (Figure 1). It was observed from figure 1 that liquid nitrogen frozen sample showed a little variation up to 5 months of storage, whereas from 6 months onwards the count was drastically reduced and became nil after 10 months of storage. Figure 2 indicates the growth kinetics of *S. faecalis* for a period of one year under different conditions of freezing (both conventional and liquid nitrogen) and frozen storage after being applied to prawn meat. The count was drastically reduced in case of liquid nitrogen frozen sample and the count became nil after two months of storage. But in case of conventional method of freezing, fairly good count was observed up to 4 months of storage after an initial drop of 90% and became nil during the period under consideration. The interesting part of the experiment was that no cryoprotectant had been used during freezing of the sample. This is because of the fact that the cryoprotectants protect cells and tissues during freezing. But in our case, protection of prawn tissues and destruction of pathogenic bacterial cells are equally important. It was observed that at the rates of cooling used in our experiments bacterial cells, particularly the pathogenic bacteria, were destroyed but the prawn tissues got least damaged.

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Nerves innervate the ectopic limbs

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In this communication, we report the innervation of the ectopic hindlimbs formed as a result of homeotic transformation mediated by vitamin A in the Indian jumping frog, *Polypedates maculatus* (Anura: Rhacophoridae). Histology and nerve staining revealed that the ectopic limbs are innervated.

IT is well known that vitamin A causes severe embryonic malformations in several animals ever since their discovery in 1909 (refs 1–3). However, its effect is more pronounced in amphibians causing proximodistal, anteroposterior and dorsoventral duplication of parts of the limbs during limb regeneration in several species of amphibians[4–14]. But the most remarkable of all the effects of vitamin A is the homeotic transformation of tails to limbs in *Uperodon systoma*[15]. Since then several workers have confirmed similar phenomenon in other species of amphibians[16–19]. As many as 8–9 ectopic limbs arise from the tail tissue. The limbs arise either singly or in pairs. Most of the ectopic limbs have the normal hindlimb structure but none of them is functional. The tadpoles dragged the extra limbs and died as a result of overgrowth. As the ectopic limbs were functionless, it was possible that they were devoid of nerves. The present study was therefore undertaken to ascertain whether the ectopic limbs of *P. maculatus* were supplied with nerves.

The egg mass of *P. maculatus* was collected from Utkal University campus in July, 1995 and reared in the laboratory up to the hindlimb bud stage following the standardized procedure of Mohanty-Hejmadi[20]. Prior to amputation in the middle of the tail, the tadpoles were anaesthetized in MS 222 (tricaine methanesulphonate) and exposed to vitamin A 10 IU/ml for 72 h. Following the above treatment they were transferred to aerated, conditioned water and allowed to grow till the emergence of ectopic limbs. Throughout the experiment they were fed with boiled egg and *Amaranthus ad libitum*. Once the ectopic limbs had developed and the forelimbs emerged, the tadpoles were fixed in 10% buffered formalin.

For histological studies of the tail region of tadpoles with ectopic limbs, the tails of those tadpoles who possessed ectopic limbs (Figure 1 *a*) were amputated and fixed in aqueous Bouin's fluid, embedded in paraffin (m.p. 58°C–60°C), sectioned longitudinally at 10 µm thickness and stained with Mallory's triple stain for examination under the light microscope.

Interestingly, histological studies revealed that multiple sections of the nerve cord were found in the extreme distal and ventral regions of the tail (Figure 1 *b*). As the limbs always arise from the ventral side, it is quite likely that the nerve cord which is located on the dorsal side, takes several turns in the distal region of the tail to reach the ventral side, perhaps to supply the ectopic limbs. As a result, multiple sections of the nerve cord were visible on the ventral side in the distal region of the tail.

To be more sure that the ectopic limbs were innervated, Sihler's differential nerve staining technique[21] was used on the tadpoles with ectopic limbs. In both the tadpoles (Figure 1 *c*, *d*) a thin, faint nerve could be discerned in the ectopic limbs. The continuity of the nerves could not be tracked due to the shrinking of the specimens during fixation. On the other hand, the nerves innervating the normal hindlimbs were thick and prominent (Figure 1 *c*) and were therefore easily distinguishable.

The present study therefore confirms that, because of sparse and weak innervation the ectopic limbs are not fully functional, although morphologically they are well-developed hindlimbs. They are richly vascularized too as revealed from histology (unpublished data). Singer[22] transplanted the upper arm segments of the adult *Notophthalmus viridescens*, in the flank region of the same animal and found that a few of the grafts regenerated after some delay. The arms which had regenerated contained an average less than one-third of the normal density of nerve fibres. A similar fraction of the total nerve supply can induce the formation of supernumerary limbs when diverted to a surface wound. Thornton and Tassava[23] also recorded regeneration in orthotypically transplanted arms of *Ambystoma mexicanum* larvae which were kept sparsely innervated by repeated denervation. On the other hand, if the hind brain and trunk nerve cord are excised when they first form a neural tube, a defective embryo develops which survives and develops normally but is incapable of normal movements or feeding. The arms develop quite well with either very few or no detectable nerve fibres and can reach the four digit stage in this species[22]. This might be the

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