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Bacteriological evaluation in *Penaeus monodon* during processing for export

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Abstract

Bacterial contamination in *Penaeus monodon* during processing for export was investigated. Viable counts of bacteria in shrimps under three phases of processing and from two different sources were evaluated. Bacterial strains, pathogenic to man were isolated, characterized and quantified. Attempts were also made to evaluate the seasonal differences in pathogen species composition. Altogether, 4 pathogenic strains *viz., Escherichia coli, Staphylococcus aureus, Vibrio cholerae* and *Salmonella typhi* were isolated and identified. Results also showed high counts in headed raw shrimps than processed and finished product. Comparatively, high bacterial abundance was observed in samples from mechanized boats than those from deep-sea trawlers. Seasonal variations of bacterial abundance were marked and possible cause of contamination and remedies were discussed.

Introduction

The Indian shrimp production witnessed an upsurge during last decade with a total production of 3.89 lakh metric tonnes (CMFRI Records, 1999). This rapid growth made shrimp, a major marine export product. In India, the shrimp export increased 125 metric tonnes in 1987 (Vijayakumar, 1988) to - 1.5 lakh metric tonnes in late nineties. Major part of Indian shrimp has been exported to developed countries, which mainly include Japan and USA. This recognition in shrimp export provoked the growth of several shrimp processing centres along the east coast of India, without proper microbiological laboratory and scientific personnel which ultimately resulted in reduced quality. For instance, Reilly *et al.*, (1986) reported that during 1980-'85 nearly 58.14 metric tonnes of Indian shrimp was rejected by Japan. This quality loss was due to bacterial contamination while handling on board and during different stages of processing.

World wide, studies on the pathogenic bacteria in shrimps under processing were well documented (Green 1949 a, b, c, d; Fieger, 1950; Novak *et al.*, 1956; Cook, 1970; Vanderzant *et al.*, 1970; Lee and Pfeifer, 1976; Sumner *et al.*, 1982). However, in Indian context, reports on bacterial contamination in processed shrimp were few and fragmentary and, were also restricted to west coast (Shaik Mohamud and Magar, 1956; Iyengar *et al.*, 1960; Rao and Gupta, 1978; Iyer *et al.*, 1966, 1979, 1986). Since, with the result of some consignments of shrimp failed to meet the stringent requirements of importing countries, it is essential to assess the quality of shrimp, right from the time of harvest

through the processing stages until the finished product. Assessment of bacterial population will give an idea of various measures that have to be implemented to minimize the bacterial contamination. The present study is therefore intended to :

- Isolate and identify the pathogenic microorganisms contaminating the iced and frozen shrimp during different stages of processing.
- Evaluate total aerobic bacterial load and intensity of different pathogenic strains of the shrimp under process and
- To analyze seasonal fluctuations in bacterial abundance, if any.

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Material and methods

The tiger shrimp, *Penaeus monodon* (Fabricius) harvested from Visakhapatnam coast by deep-sea trawlers and mechanized boats were used in the present study. Shrimps were headed onboard and stored in cold storage in deep-sea trawlers and, in crushed ice in mechanized boats. Samples were collected from the fishing harbour in Visakhapatnam and from a processing plant in Gambheeragedda, some 22 km north of Visakhapatnam for a period of five months, from October 1998 to February '99. Periodic fortnightly sampling were made from the trawlers and mechanized boats upon arrival at the landing site (raw shrimp), processing center after the shrimps were washed in the potable water (processed shrimp) and from the insulated cartons stored in a frozen storage at - 18°C (finished product). In all cases, individuals of each sample were placed in a separate sterile polythen bags and were then transported to the laboratory in an iced chamber within two hours of collection.

In the laboratory, isolation of pathogenic bacteria was made by spread plate method (Cowan and Steel, 1993) using Nutrient Agar (NA), Alkaline Peptone Broth (APB) and Alkaline Peptone Water (APW). Though, bacteria grew readily on most ordinary media, enrichment on selective media was necessary as shrimp samples contained mixed flora. Selective agars used for specific strains include Thiosulphate Citrate Bile salt Sucrose (TCBS) agar for *Vibrios*, Tergitol -7 agar for *Escherichia*, Baird - Parker agar for *Staphylococcus* and Brilliant Green Agar (BGA) and/ or Bismuth Sulfate Agar (BSA) for *Salmonella*. The plates were inoculated and incubated at 37°C for overnight and up to 7 days. Specific colony from mixed culture was sub-cultured and pure culture was used for further biochemical tests.

Isolated bacteria were identified according to "Adansonian Concept" *i.e.* "for classification, equal weight should be given to each character or feature" (Mackie and Mc Cartney, 1989). The biochemial tests include salt tolerance, motility, decarboxylation, oxidase, indole, catalase, coagulase, VP, sensitivity, hydrolysis, acid production etc.

Bacterial enumeration from the tissues were carried out following "surface viable count method" (Mackie and Mc Cartney, 1989). While nutrient agar was used for total aerobic bacterial count, selective enrichment agars were used for specific strain counts.

Results

During the present study, invariably all samples showed the incidence profiles of various pathogenic strains. Altogether four species of pathogenic bacteria namely, *Staphylococcus aureus, Escherichia coli, Vibrio cholerae* and *Salmonella typhi* were isolated.

Staphylococcus aureus : On Baird - Parker agar, the colonies were typical black in color, convex, 1.0-1.5 mm diameter with a narrow white margin surrounded by a zone of clearing, measured 1.5-2.5mm wide (Table 1). The isolated colonies showed positive reaction to gram stain; motile; oxidase negative; catalase positive; acid production from glucose (Table 2), lactose, maltose, mannitol, fructose, sucrose, trehalose, mannose; VP and coagulase positive and reduces nitrate. The colonies showed arginine decarboxylation and sensitive to Novobiocin (Table 3) and hence identified as the strain of *Staphylococcus aureus* (Mackie and Mc Cartney, 1989).

Vibrio cholerae : Gram negative, motile, rod shaped, aerobic bacteria forming round, raised, dome shaped, yellow, large (1.0-2.0 mm) colonies on Thiosulphate Citrate Bile salt Sucrose (TCBS) agar (Table 1). Profused growth seen in nutrient agar (Hl media), Mc Conkey agar and in alkaline peptone water supplemented with 2.5% NaCl. Growth was also observed in the absence of NaCl. As the isolated bacteria were sensitive to O/129 (2, 4 Diamino 6, 7 - tri isopropyl pteridine phosphate) compound (Furniss *et al.*, 1978), it was identified as the bacteria belonging to genus *Vibrio* (Collins *et al.*, 1989). Since the bacteria showed growth in TCBS, lysine and ornithine decarboxylation (Moeller, 1955), oxidase positive (Kovacs, 1956), sensitive to both 10µg and 150µg O/129 com-

Table 1. *Bacterial isolation*

	E. coli	*S. aureus*	*V. chloerae*	*S. typhi*
Nutrient agar	+	+	+	+
Thiosulphate Citrate Bile salt Sucrose agar	-	-	+	d
Alkaline Peptone water	d	d	+	+
Alkaline peptone broth	d	d	+	+
Tergitol - 7 agar	+	-	-	-
Baird-Parker Agar	-	+	-	-
Brilliant green agar	-	-	-	+
Bismuth sulfate agar	-	-	-	+

d = doubtful

Table 2. *First stage table for bacterial identification*

	E. coli	*S. aureus*	*V. chloerae*	*S. typhi*
Grams stain	-	+	-	-
Shape	R	S	R	R
Motility	D	-	+	D
Aerobic growth	+	+	+	+
Anaerobic growth	+	+	+	+
Catalase	+	+	+	+
Oxidase	-	-	+	-
Carbohydrates attack	F	F	F	F
Acid from Glucose	+	+	+	+

S - Spherical
R - Rod shaped
D - Different results with different species
F - Fermentation

pound, sensitive to 10µg Ampicillin, resistant to Polymyxine B 50 (Table 3), growth in 0% NaCl, Indole production, nitrate reduction, no gas production from glucose, hydrolysis of starch and acid production from sucrose, it was identified as *Vibrio cholerae.*

Escherichia coli : Gram negative, rod shaped, aerobic bacteria forming circular non-mucoid, flat, yellowish pink colonies in the Tergitol - 7 agar (Table 1). Hence the isolated colonies were gram negative, aerobic and facultatively anaerobic, catalase positive, oxidase negative, attack sugars fermentatively (Table 2), citrate (simmon's medium) negative, the strains were grouped under genus *Escherichia* (Cowan and Steel, 1993). The isolated strains of *Escherichia* are feebly motile; produces acid from xylose, trehalose, mannitol, maltose, glycerol, arabinose; VP negative; MR positive; no growth in KCN medium, negative to both yellow and red pigments; negative to urease production, gelatin hydrolysis and phenylalanine and, hence identified as the strain of *Escherichia coli.*

Salmonella typhi : Pink in color, translucent to opaque with surrounding colony pink to red was observed on Brilliant green agar. On Bismuth Sulfate agar, the colonies were brownish grey to black in color with a metallic sheen (Table 1). The bacteria were gram negative, motile, aerobic, facultatively anaerobic, catalase positive, oxidase negative, KCN negative, lysine decarboxylation positive and, hence identified as the genus *Salmonella* (Table 2). The isolated colonies showed citrate negative (Simmon's medium), no production of gas from glucose, the typical characters of the species *Salmonella typhi* (Table 3).

The bacterial enumeration analysis showed high abundance of pathogenic strains in both the iced shrimp from

Table 3 . *Second stage table for bacterial identification*

S. aureus		*V. cholerae*		*E. coli*		*S. typhi*	
Aerobic	+	Swarming	-	Motility	d	Motility	+
Anaerobic	+	Arginine	_	Yellow pigment	-	Yellow pigment	-
Oxidase	-	Lysine	+	Red Pigment	-	Red Pigment	-
VP	+	Ornithine	+	Citrate		Citrate	
Coagulase	+	Nitrate	+	Simmons	-	Simmons	-
Acid from		Oxidase	+	Christensons	d	Christensons	+
Lactose	+	Gas from glucose	-	Urease	-	Urease	-
Maltose	+	Indole	+	Gelatin hydr.	-	Gelatin hydr.	-
Mannitol	+	ONPG	+	Growth in KCN	-	Growth in KCN	-
Fructose	+	VP	+	H_2S from TSI	-	H_2S from TSI	d
Sucrose	+	O/129 10µg	S	Gluconate	-	Gluconate	-
Trehalose	+	O/129 150µg	S	Malonate	-	Malonate	-
Xylose	-	Ampicillin 10 µg	S	ONPG	d	ONPG	-
Cellobiose	-	Starch hydrolysis	+	Argninie	d	Arginine	+
Raffinose	-	Starch hydrolysis	+	Arginine	d	Arginine	+
Mannose	+	Urea	-	Ornithine	d	Ornithine	-
Phosphatase	+	Acid from		Lysine	d	Lysine	+
Nitrate	+	Arabinose	-	Gas from glucose	d	Gas from glucose	-
Arginine	+	Arbutin	-	Acid from		Acid from	
Urea	d	Salicin	-	Adonitol	-	Adonitol	-
Protease	+	Sucrose	+	Arabinose	+	Arabinose	-
Novobiocin	S	Xylose	-	Cellobiose	-	Cellobiose	-
		Growth in		Dulcitol	d	Dulcitol	d
		0% NaCl	+	Glycerol	+	Glycerol	-
		Ethanol	-	Inositol	-	Inositol	-
		Propanol	-	Lactose	d	Lactose	-
		D-galacturonate	-	Maltose	+	Maltose	+
		D- glucosamine	+	Mannitol	+	Mannitol	-
				Raffinose	d	Raffinose	-
				Trehalose	+	Trehalose	+
				Xylose	+	Xylose	+
				Sucrose	+	Sucrose	-
				MR	+	MR	+
				VP	-	VP	-

mechanized boats and frozen shrimps from deep-sea trawlers. However, in all the phases of processing, the iced shrimps seemed to harbour high numbers of potential bacteria than frozen shrimp. The differences were marked and uniform (Table 4). In raw shrimp, while the iced individuals showed bacterial counts rang-

ing from 10-66 x 10^3 nos./g (mean, 33.8 x 10^3 nos./g), in the frozen individuals it ranged from 7-59 x 10^3 nos./g (mean, 29.2 x 10^3 nos./g). On the other hand, in processed shrimp, the range was 5-48 x 10^3 nos./g (mean, 23.2 x 10^3 nos./g) in iced and it was 3-36 x 10^3 nos./g (mean, 19.2 x 10^3 nos./g) in frozen shrimp. Like wise, in the finished product, the observed range was between 4 x 10^3 nos./gm to 26 x 10^3 nos./g (mean, 16 x 10^3 nos./g) in iced shrimp and 3-25 x 10^3 nos./gm to 26 x 10^3nos./g (mean, 16 x 10^3nos./g) in iced shrimp and 3-25 x 10^3 nos./g (mean, 12.6 x 10^3 nos./g) in frozen shrimps.

From the foregoing it may be seen that, invariably in all three stages of processing, the iced shrimp harboring remarkably high numbers of potential pathogens. Comparatively, the observations also showed that the counts in iced and frozen shrimp was more in raw shrimp (Fig. 1A) than the processed (Fig. B) and finished product (Fig. C).

Seasonally, the counts were less during December 1998 and more in February 1999. During December '98, while the bacterial counts in iced shrimp ranged between 4-10 x 10^3 nos. /g (mean, 6.33 x 10^3 nos./g), the counts of frozen shrimp was between 3-7 x 10^3 nos./g (mean, 4.33 x 10^3 nos./g). On contrary, during February '99, the counts of iced shrimps were between 26-66 x 10^3 nos./g (mean, 46.67 x 10^3 nos./g) and for frozen shrimp it was 25-59 x 10^3 nos./g (mean, 40 x 10^3nos./g). The observations also revealed a gradual decrease in bacterial counts from October '98 to December '98 and an

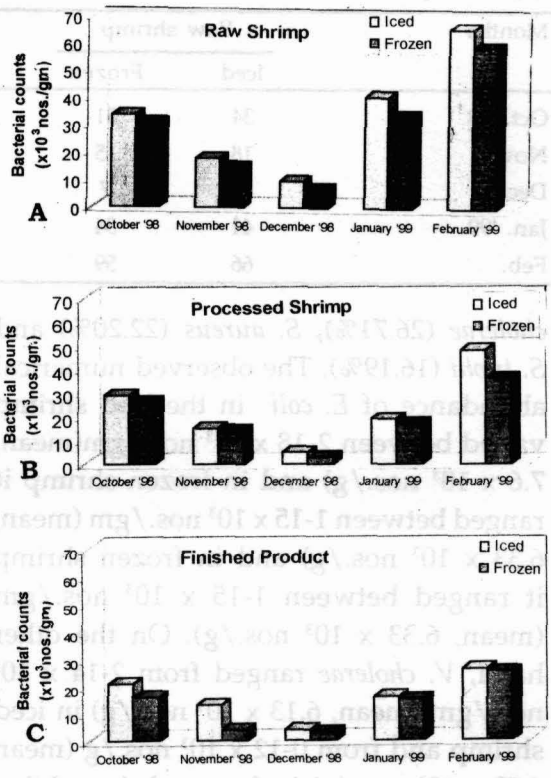

Fig. 1A. *Total viable bacterial counts (monthly mean) in the raw shrimps from both mechanized boats (iced) and deep-sea trawlers (frozen).*

Fig. B. *Total viable bacterial counts (monthly mean) in the processed shrimps from both mechanized boats (iced) and deep-sea trawlers (frozen).*

Fig. C. *Total viable bacterial counts (monthly mean) in the finished product from both mechanized boats (iced) and deep-sea trawlers (frozen).*

increase from there onwards reaching all time high values in February '99. This seasonal variations could be perhaps due to the corresponding atmospheric temperature alterations.

Species wise, *E. coli* (34.89%) dominated all the other species, followed by *V.*

Table 4. *Monthly mean total viable counts (x 10^3nos./g) of pathogenic bacteria in P. monodon at different phases of processing.*

Months	Raw shrimp		Processed shrimp		Finished Product	
	Iced	Frozen	Iced	Frozen	Iced	Frozen
Oct. '98	34	31	29	26	21	16
Nov.	18	15	15	14	13	4
Dec.	10	7	5	3	4	3
Jan. '99	41	34	19	17	16	15
Feb.	66	59	48	36	26	25

cholerae (26.71%), *S. aureus* (22.20%) and *S. typhi* (16.19%). The observed numerical abundance of *E. coli* in the iced shrimp varied between 2-18 x 10^3 nos./gm(mean, 7.6 x 10^3 nos./g) and in frozen shrimp it ranged between 1-15 x 10^3 nos./gm (mean, 6.33 x 10^3 nos./g) and in frozen shrimp it ranged between 1-15 x 10^3 nos./gm (mean, 6.33 x 10^3 nos./g). On the other hand, *V. cholerae* ranged from 2-14 x 10^3 nos./gm (mean, 6.13 x 10^3 nos./g) in iced shrimp and from 0-12 x 10^3 nos./g (mean 4.53 x 10^3 nos./g) in frozen shrimp. Likewise, *S. aureus* ranged from 0-12 x 10^3 nos./g (mean, 4 x 10^3 nos./g) in frozen shrimp. On contrary, significantly low levels of *S. typhi* were recorded in both iced and frozen shrimps. It varied from 0-14 x 10^3 nos./g (mean, 3.73 x 10^3 nos./g) in iced and 0-10 x 10^3 nos./g (mean, 2.73 x 10^3 nos./g) in frozen shrimps. However, the distribution pattern of all species were unique as regards the seasonal abundance and the category of process viz., raw shrimps (47.4%), processed shrimp (31.55%) and finished product (21.03%).

Discussion

During the present study, higher abundance of pathogenic bacteria was observed in raw shrimps. Similar observations were made with a range of potential bacterial load varying between 1.6 x 10^3 - 1.6 x 10^5 nos./g (Green, 1949; Shaik Mohamud and Magar, 1956), 3.6 x 10^3- 2.3 x 10^6 nos./g (Camber *et al.*, 1957, Carol *et al.*, 1968; Cook, 1970; Vanderzaant *et al.*, 1970; Cobb *et al.*, 1973; Nickelson and Vanderzaant 1976), 1.0 x 10^6-1.0-10^7 nos./g (Summer *et al.*, 1982) and 1.0 x 10^5 nos./g (Alvarez, 1983; Iyer *et al.*, 1986). These counts are higher than those obtained in the present study. However, it has been pointed out by various workers, that higher bacterial counts were obtained at 20°C incubation. In the present study, the plates were incubated at 37°C and hence the abundance observed showed the consistent recovery exhibited by the pschyrophilic and mesophilic organisms.

The counts observed during the present study in the processed shrimp were significantly low in both iced and frozen individuals than the raw shrimp. This could be due to the shrimp processing, removal of shell, antennae, legs, veins etc., and the washing, which will effectively reduce the bacterial contamination (Green,

Table 5. *Monthly mean counts (x 10^3 nos./g) of Staphylococcus aureus, Escherichia coli, Virbio cholerae and Salmonella typhi in P. monodon at different phases of processing.*

Species	Months	Raw shrimp		Processed shrimp		Finished Product	
		Iced	Frozen	Iced	Frozen	Iced	Frozen
	Oct. '98	4	2	7	5	4	2
	Nov.	2	2	3	3	2	0
S. aureus	Dec.	2	2	3	3	2	0
	Jan. 99	10	7	4	3	5	3
	Feb.	12	16	11	9	6	7
	Oct. '98	12	10	8	7	6	4
	Nov.	7	6	4	3	4	2
E. coli	Dec.	4	4	2	1	2	1
	Jan. '99	13	12	6	4	6	5
	Feb.	18	15	14	11	8	10
	Oct. '98	8	7	7	6	6	4
	Nov.	4	3	3	2	5	1
V. cholerae	Dec.	3	1	2	1	1	0
	Jan. '99	14	10	5	3	3	4
	Feb.	13	12	11	8	7	6
	Oct. '98	7	6	6	5	3	2
	Nov.	4	3	3	2	1	0
S. typhi	Dec.	0	0	0	0	0	0
	Jan. '99	3	3	2	1	0	1
	Feb.	14	10	9	7	4	1

1949). Feiger (1950) reported that effective washing could reduce the count as much as 75%. Similarly, significant reduction of potential bacteria in the finished product was observed when compared to raw and processed shrimp. This reduction could be attributable to the plate freezing at 18-41°C for 3 hours. As regards the species distribution, invariably the counts of all the four bacterial species were higher in shrimps even after processing. This could be due to the contamination through human handling.

From the foregoing it may be concluded that the considerable delay at jetty before shrimp are transported to processing plant, inadequate ice for extended period of time are the prime factors that affect the quality of shrimps in both the mechanized and deep-sea trawlers. It is necessary that the shrimp should be transported with minimum delay to the processing

plant. Similarly, in processing plant, shrimps were at times washed with tap water instead of chlorinated water. Like wise, hand wash by the curators was also by using the tap water. Hence it is sugested that shrimp washing by chlorinated water and the hand wash with 200 ppm chlorinated water to eradicate the micro-organisms from the fingers will effec-tively reduces the potential bacterial load.

Examination of bacterial data obtained in the present study showed that the total aerobic counts in the tiger shrimp *Penaeus monodon* for export were well within the recommended limit. However, further reduction is the need of the hour as the isolated organisms are extensively pathogenic to man.

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