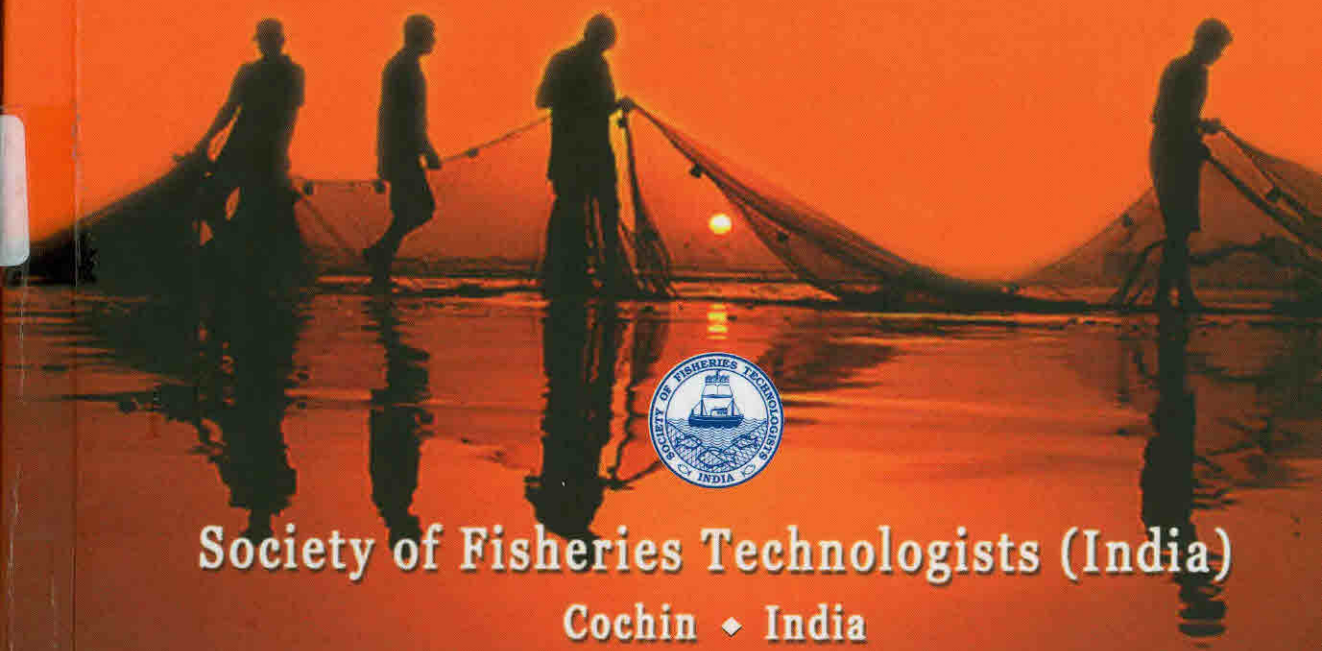
Coastal Fishery Resources of India

Conservation and Sustainable Utilisation



Society of Fisheries Technologists (India)
Cochin ◆ India

Coastal Fishery Resources of India: Conservation and Sustainable Utilisation

Proceedings of the National Seminar on Conservation and Sustainability of Coastal Living Resources of India, 1-3 December 2009, Cochin

Organised by

Society of Fisheries Technologists (India), Cochin
and
Centre for Ocean and Environmental Studies, New Delhi

In association with

Ministry of Earth Sciences (New Delhi)
Central Marine Fisheries Research Institute (Cochin)
National Institute of Oceanography (Goa) and
Central Institute of Fisheries Technology (Cochin)





ISBN: 978-81-901038-7-9

Published by

Society of Fisheries Technologists (India)
P.O. Matsyapuri, CIFT Junction, Cochin - 682 029, India

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Citation:

Rao, G.S. (2010) Current status and prospects of fishery resources of the Indian continental shelf, In: Coastal Fishery Resources of India: Conservation and Sustainable Utilisation (Meenakumari, B., Boopendranath, M.R., Edwin, L., Sankar, T.V., Gopal, N. and Ninan, G., Eds.), p. 1-13, Society of Fisheries Technologists (India), Cochin

Cover design: Vineethkumar, P., CIFT, Cochin

Printed at PAICO, Cochin - 682 035, India

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2010

Bacterial Microflora Associated with Cephalopods from Southwest Coast of India

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Introduction

Cephalopods are believed to be a major and largely untapped source of marine protein. While groundfish landings have remained fairly stable or declined over the past three decades, the cephalopod world catch has increased substantially (Caddy and Rodhouse, 1998), reaching 3.78 million t in 2004 (FAO, 2006). Squid is by far the major cephalopod species (67%) produced worldwide, but octopus (9.5%) and cuttlefish (16%) are becoming increasingly important (FAO, 2003). Of the species that are explored world-wide roughly 41% belong to the genera *Loligo*, *Sepia* or *Octopus*, which are predominantly found on the continental shelf (Guerra, 1996). The consumption of cephalopods has recently increased in countries that were not traditionally cephalopod consumers, mainly as chilled and frozen ready meals (Barbosa and Vaz-Pires, 2004). Squid consumption is limited in large parts of the world, especially in North America and northern Europe. On the other hand, considerable amounts of squid are consumed in east and south-east Asia. General handling, processing, preservation and product properties of cephalopods, mainly based on squid which is the major cephalopod species produced, were published by Kreuzer (1984). The microbiology of fresh and spoiling fish has been extensively studied, as reviewed by Liston (1980) and Gram and Huss (1996), but qualitative microbiological data on cephalopods are scarce as autolytic changes are believed to be the main reason for deteriorating sensory quality (LeBlanc and Gill, 1984).

The microflora on temperate water fish is dominated by psychrotrophic Gram-negative rod-shaped bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella* and *Flavobacterium.* Members of the *Vibrionaceae (Vibrio* and *Photobacterium)* and the *Aeromonadaceae*

(Aeromonas spp.) are also common aquatic bacteria and typical of the fish flora (Huss, 1995). The composition of the natural bacterial flora of a newly caught fish is dependent on its origin and season (Wilson *et al.*, 2008). Only a few studies have covered sensory, chemical and microbiological changes in cephalopods (Licciardello *et al.,* 1985; Paarup *et al.*, 2002; Lapa-Guimarães *et al.,* 2002; 2005 Vaz-Pires and Barbosa, 2004; Vaz-Pires and Seixas, 2006), and microbiological studies have mainly focused on total aerobic counts. Hence, the present investigation was carried out to study the microbiological quality attributes of fresh octopus (*Octopus vulgaris*), squid (*Uroteuthis duvauceli*), and common cuttlefish (*Sepia officinalis*).

Materials and Methods

Six samples each of squid (*Uroteuthis duvauceli*), cuttlefish (*Sepia officinalis*) and Octopus (*Octopus vulgaris*) used in this study were collected from landing centre and retail outlets in and around Cochin. Samples were placed inside plastic bags, surrounded by crushed ice in clean and insulated containers and transported to the laboratory (1 h maximum) and analysed immediately upon arrival at the laboratory. Three to five samples from each lot were subjected to bacteriological analyses. At the laboratory, squid and Octopus in the whole, uneviscerated form were then held at ambient temperature for 6 h and the microbiological load and composition of the microflora was studied. Squid samples were placed in insulated thermocole boxes with crushed ice and kept in a cold room maintained at 0-4°C. The squid:ice ratio was approximately 2:1 and crushed ice was added at regular intervals to maintain the ratio after draining out the melted ice. A total of 18 whole raw squid were used. Samples were analysed after 1, 5 and 8 days of storage. On the day of analysis, four randomly chosen squid were removed from the lot held in ice and total aerobic counts and the composition of the microflora was determined.

From each mantle,10 g samples (flesh and skin) were homogenised with sterile physiological saline (Nacl,0.85% w/v) solution (90 ml) in a stomacher (400 lab blender, Seward, UK) for 60 s at room temperature. Also, appropriate decimal dilutions were prepared in sterile physiological saline solution and plated on agar. Microbial levels of the mantle muscles were determined in duplicate by plating samples (0.1 ml for spread plating and 1 ml for pour plating) of serial dilutions of mantle muscle homogenates on the following nonselective, indicative or selective media : spread-plated Tryptone soy agar (TSA CM 131, Oxoid, Basingstoke, UK) incubated at

30°C for 3 days was used for total aerobic mesophilic (TPC) plate counts, *Enterobacteriaceae* were enumerated by pour plating in Violet Red Bile Glucose Agar (VRBG, CM 485, Oxoid) at 30°C for 48 h. The large colonies with purple haloes were counted (FDA 1998). Enterococci numbers were estimated by pour plating in KF Agar (Difco). Five typical *Enterococcus* colonies were identified by checking growth at 45±1°C and growth in Dextrose Azide Broth containing 6.5% sodium chloride incubated at 35±2°C and confirmed by biochemical tests as described by APHA (1998). *Staphylococcus aureus* counts were estimated on Baird Parker Agar (BP, Oxoid) at 37°C for 2 days and typical colonies were confirmed by coagulase test (FDA, 1998).

Composition of the microflora of fresh cephalopod samples was determined by isolating and identifying a total of 80 bacterial cultures. Bacterial flora associated with squid stored at ambient temperature and in ice were also determined by isolating and characterizing 76 bacterial cultures. Colonies were picked from TSA sampled from the fresh cephalopod samples. All colonies from a sector of the plate or all colonies from a whole plate were isolated, purified and stored on TSA slants. The strains were tested for gram reaction, catalase and oxidase reactions, motility, oxidative/fermentative metabolism and presence of spores. They were then grouped according to the taxonomic schemes of Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984; Sneath *et al.*, 1986), further tested for the most relevant characteristics of each group and identified using the schemes proposed by several authors for identification (Dainty *et al.*, 1979). Further more, the strains were tested for their ability to reduce trimethyl amine oxide (TMAO) to trimethyl amine (TMA) and to produce H_2S. The ability to reduce TMAO and to produce H_2S are regarded as prominent characteristics of fish spoilage bacteria (Gram et al.1987).

Results and Discussion

Microflora associated with fresh cephalopods

The microbial loads of squid (*Uroteuthis duvauceli*), cuttlefish (*Sepia officinalis*) and octopus (*Octopus vulgaris.*) tissues are presented in Table 1. TPC analysis revealed an initial aerobic mesophilic count of ca. $\leq 10^6$ cfu.g^{-1} for squid, cuttlefish and octopus. The mean mesophilic counts of 6.5 $\log_{10}$ cfu.g^{-1} for whole cephalopods in the present study is closely related to the upper limit of acceptability of raw / frozen fish as defined by ICMSF (1998). The counts of Enterobacteriaceae on VRBG agar were

around 4.0 $\log_{10}$ cfug^{-1} in all the three cephalopod samples tested. The microflora was dominated by *Enterobacter cloacae, Morganella morganii, Proteus mirabilis* and *Proteus vulgaris.* Faecal streptococcal counts were significantly higher in all the three cephalopod samples analyzed and were around 10^4 cfu.g^{-1}. *Staphylococcus aureus* could not be detected in any of the samples analyzed.

Table 1: Mean microbial counts of cephalopods landed at Cochin

Microbial parameters	Squid	Cuttlefish	Octopus
Aerobic mesophilic bacteria, $\log_{10}$ cfu.g^{-1}	6.55 ± 0.511	5.81± 0.123	6.62± 0.092
Enterobacteriaceae, $\log_{10}$ cfu.g^{-1}	4.16± 0.044	4.11 ± 0.067	4.18± 0.039
Faecal Streptococci, $\log_{10}$ cfu.g^{-1}	4.25± 0.048	4.33± 0.011	4.27± 0.029

A total of 84 bacterial isolates were characterized from squid, cuttlefish and octopus. A heterogeneous group of Gram-negative bacteria was isolated from fresh squid, cuttlefish and octopus (Table 2). The dominant microflora (55-58%) included motile, Gram-negative, non-fermentative rods with positive oxidase and catalase reactions comprising of Genera *Pseudomonas, Shewanella, Acinetobacter, Flavobacterium* and *Moraxella.* Gram-negative, fermentative rods with positive and negative oxidase reactions constituted 39% of the microflora in squid and 25% of the flora in cuttle fish and Octopus. They belonged to genera *Vibrio, Aeromonas, Enterobacter, Morganella* and *Proteus*. Gram positive flora belonged to *Bacillus* and *Micrococcus.* A microflora consisting of *Pseudomonas, Acinetobacter, Moraxella* and *Vibrio* has been found on newly-caught fish in several Indian studies (Surendran *et al.,* 1989). The microflora on temperate water fish is dominated by psychrotrophic Gram-negative rodshaped bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella* and *Flavobacterium.* Members of the *Vibrionaceae (Vibrio* and *Photobacterium)* and the *Aeromonadaceae (Aeromonas* spp.) are also common aquatic bacteria and typical of the fish flora. several studies have shown that the microflora on tropical fish species is very similar to the flora on temperate species (Huss 1995). The results of the study agree well with that of Surendran *et al.* (1989) and Huss (1995).

Table 2: Percentage compositon of bacterial microflora associated with cephalopods landed at Cochin

Bacterial genera	Squid	Cuttlefish	Octopus
Vibrio	27	20	17
Pseudomonas	11	10	17
Shewanella	16	10	8
Enterobacteriaceae*	6	5	8
Flavobacterium	11	15	21
Acinetobacter	11	10	4
Moraxella	6	10	8
Aeromonas	6		
Micrococcus	6	10	4
Bacillus		10	13

*Enterobacteriaceae identified as *Enterobacter cloacae, Morganella morganii, Proteus mirabilis, Proteus vulgaris.*

Changes in microflora of squid and Octopus during storage at ambient temperature for 6 h

The microbiological changes in squid *(Uroteuthis duvauceli)* and Octopus (*Octopus vulgaris*) during storage at ambient temperature (30±1°C) were studied. At initial time, the total aerobic mesophilic counts were around 6.5 $\log_{10}$ cfu.g^{-1} (Fig 1). Faecal streptococci levels were around 4.0 $\log_{10}$ cfu.g^{-1} respectively in both the samples. After 6 h of storage at ambient temperature, a two log increase was noticed in aerobic mesophilic and faecal streptococcal populations in Octopus. In squid, a similar increase was observed only in faecal streptococcal populations.

At ambient temperature storage for 6 h, the microflora was dominated by mesophilic Gram-negative, fermentative rods (64-67%) belonging to Genera *Vibrio* (42-46%), Enterobacteriaceae (17-18%) and *Aeromonas* (8%). Gram-negative, non-fermentative rods with positive and negative oxidase reactions constituted only 25% of the microflora in squid and 18% of the flora in Octopus. Huss(1995) reported that at ambient temperature (25°C), the microflora at the point of spoilage is dominated by mesophilic *Vibrionaceae* and, particularly if the fish are caught in polluted waters, *Enterobacteriaceae.* The results of the present study confirms the earlier findings (Gram *et al.* 1987; Dalgaard *et al.* 1993; Huss, 1995). TMAO reduction was observed in the genera of bacteria *Vibrio, Aeromonas* and

S. putrefaciens. Huss (1995) reported that the ambient spoilage by *Vibrionaceae* is dominated by H_2S and TMA. Gram *et al.* (1987) and Dalgaard *et al.* (1993) showed that a large proportion of the flora on ambient-stored fish consisted of Gram-negative mesophilic fermentative rods *(Vibrionaceae, Enterobacteriaceae)* and *Aeromonas hydrophila* was identified as specific spoilage organism (SSO). The results indicate that any delay in icing cause increase in mesophilic bacteria such as *Vibrio, Enterobacteriaceae* and faecal streptococci which is of great concern from safety point of view.

Bacterial microflora associated with ice stored squid

The variation on microbial flora of squid stored in ice is shown in Fig. 2. The mesophilic bacterial counts decreased initially and this reduction in bacterial load noticed probably was caused by the inability of some of the mesophilic bacterial species to survive and / or grow at low temperatures. The counts increased and reached 7.6 $\log_{10}$ cfu.g^{-1} on day 8. This increase can be attributed to the growth of psychrotrophic bacteria and also to the adaptation of bacterial species to chill temperatures. The shelf life of ice stored squid based on aerobic mesophilic counts is <8 days in the present study as reported by Vas–Pires and Barbosa (2004) and Paarup *et al.* (2002) who found that the shelf life of iced-stored squid is slightly shorter than that of most finfish and the product is rejected in 8-12 days.

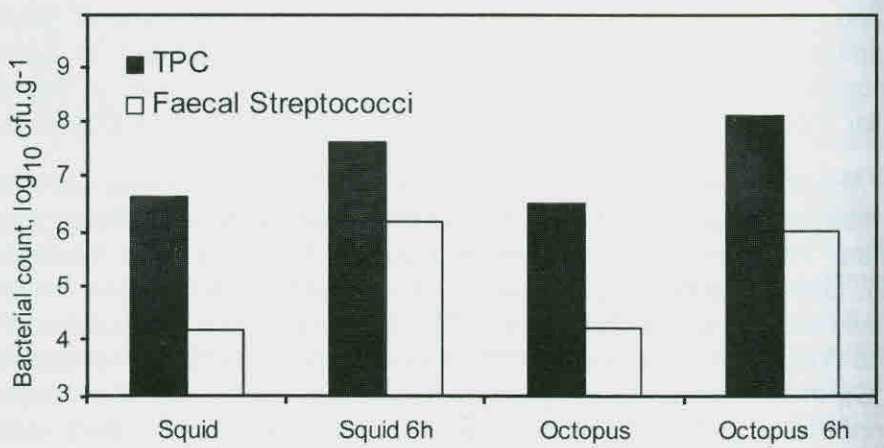

Fig. 1: Changes in the bacterial population of squid and octopus during storage at ambient temperature (30±1°C) for 6h

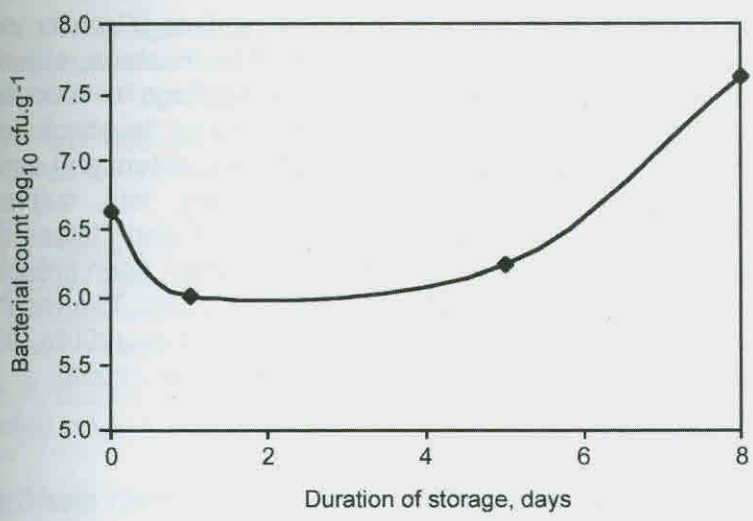

Fig. 2: Changes in aerobic bacterial population of squid stored in ice

At the end of storage, the microflora was dominated by *Shewanella* (40%) followed by *Pseudomonas* (30%) and *Flavobacterium* (15%). *Moraxella and Arthrobacter* were found in lower frequencies. The species *Shewanella putrefaciens* plays a prominent role as a spoilage organism of fish and other food products (Gram and Dalgaard, 2002). The psychrotrophic nature and the ability of the *Shewanella putrefaciens* to reduce TMAO to trimethylamine (TMA) explains its importance in spoilage of fish stored at low temperatures where the "fishy" off-odor of spoiling fish is caused by the production of TMA. *Shewanella putrefaciens* concentrations never reached levels of 10^8–10^9 that is required to produce off-odours (Jørgensen and Huss, 1989) in the present study and this is consistent with the lack of foul, H_2S-like off-odours and off-flavours in spoiling squid. Morohoshi *et al.* (2005) identified *Shewanella* strains in the intestinal microbial flora of ayu fish (*Plecoglossus altivelis*) that exhibited AHL-degrading activity and interrupted the AHL-mediated production of protease by the *Aeromonas* sp. These bacteria may use this AHL-degrading mechanism as a competitive advantage over bacterial competitors, and it may help them to dominate their ecological niche and cause spoilage. Paarup *et al. (2002) reported dominance of Pseudoalteromonas in the spoilage microflora of iced squid. Pseudomonas* spp., together with *S. putrefaciens*, are the spoilers of marine tropical fish stored in ice (Gillespie and MacRae, 1975; Gram *et al*., 1990; Gram and Dalgarrd, 2002; Lalitha *et al.*, 2005; Ravisankar *et al.* 2008). Gram and Huss (2000) reported that Gram-negative, fermentative bacteria (such as *Vibrionaceae*) spoil unpreserved

fish, whereas psychrotolerant Gram-negative bacteria (*Pseudomonas* spp. and *Shewanella* spp) grow on chilled fish. Flavobacteria, together with *Pseudomonas*, have been shown to cause spoilage in food and food products (Forsythe, 2000). Metabolites produced by flavobacteria include alcohols, sulphur compounds, ketones, aldehydes, esters and amines and the resultant odours can be described as fishy, foul, sulphuric and ammonia-like (Nychas and Drosinos, 1999; Gram and Dalgaard, 2002). Most of the food spoiling flavobacteria have, however, been grouped in the new genus *Chryseobacterium* (Bernardet *et al.*, 2002). The results of the present study agree well with that of Gram and Huss (2000), (Forsythe, 2000), Gram and Dalgaard (2002) and Lalitha et al. (2005).

Conclusion

The present study revealed that microflora of fresh squid, cuttlefish and octopus is heterogeneous and dominated by *Pseudomonas, Shewanella, Acinetobacter, Flavobacterium* and *Moraxella.* At ambient temperature storage (30°C), different species of Vibrionaceae and Enterobacteriaceae dominated. However, *Pseudomonas* spp., together with *Shewanella putrefaciens were* the main spoilers of iced squid. AHL-degrading activity in *Shewanella* strains has to be investigated and such information is needed to understand how these bacteria use this AHL-degrading mechanism as a competitive advantage over bacterial competitors and cause spoilage. Bacterial spoilage is influenced by quorum-sensing-regulated phenotypes. *Pseudomonads* produce *N*-acyl homoserine lactones (AHLs). Lipolytic, proteolytic and chitinolytic activities of bacteria potentially involved in spoilage of seafoods are regulated by quorum sensing, suggesting a potential role of such cell-to-cell communication in food spoilage. Understanding these processes may be useful in the development of novel food preservation additives that specifically block the quorum-sensing systems. Hence, elucidation of the role of N-acyl homoserine lactones (AHLs) in squid spoilage and strategies targeting quorum sensing may offer a means to control the growth of undesirable bacteria in foods and control spoilage.

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