Distribution and Ecology of *Vibrio vulnificus* and Other Marine Vibrios in the Coastal Waters, Fishes and Shellfishes in Arabian Sea Off Cochin

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in

MICROBIOLOGY

(Under the Faculty of Marine Sciences)

by

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October 2001

CERTIFICATE

This is to certify that the thesis entitled "**Distribution and Ecology of *Vibrio vulnificus* and other marine vibrios in the coastal waters, fishes and shellfishes in Arabian Sea off Cochin**" is an authentic record of research work carried out by **Smt. Sudha K.** under my supervision and guidance in the Microbiology, Fermentation and Biotechnology Division, Central Institute of Fisheries Technology, Cochin in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part thereof has been submitted for any other degree.

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DECLARATION

I, Sudha K., do hereby declare that the thesis entitled "**Distribution and Ecology of *Vibrio vulnificus* and other marine vibrios in the coastal waters, fishes and shellfishes in Arabian Sea off Cochin**" is a genuine record of research work done by me under the supervision of **Dr. Nirmala Thampuran**, Principal Scientist, Central Institute of Fisheries Technology and has not been previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title of any university or institution.

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Introduction

1. INTRODUCTION

The ever increasing population and the resulting food scarcity made food science an important subject in this era. Decline in the food production and high demand have made it essential the proper management and utilisation of the produce. Seafood forms major source of protein world over. Its high demand attributes to the nutritional quality in terms of its digestibility and amino acid composition. Like the case of other food commodities, sea food also faces deficit in its production (Chitranshi, 2001). The global requirement of fish and shrimp is around 260 million tonnes as against current annual production of 100 million tonnes (Rao, 2000). While considering Indian scenario, in order to meet the demand, we have to produce at least over 5.0 million tonnes per annum in the years to come (Gopakumar, 2001). Proper resource management is the need of the hour to over come this shortage in the production. The main impediments in the proper utilisation of the seafood is its high perishability and health risk due to contaminated pathogens.

Microbial contamination is a major problem as evidenced from the report that on a world wide basis, 3000 people die due to consumption of contaminated food and 40,000 die due to starvation per day (Manja, 1998). Microbiological hazards can only be eliminated by the implementation of international quality assurance based safety systems like *Codex elimentarius* and HACCP systems. For implementing such systems, sound foundation in the ecology, physiology and etiology of food borne microorganisms, and the epidemiology of food borne disease is essential.

Fish and crustaceans are generally safe as food for human consumption. However, the reports on the seafood contamination and food safety hazards due to microorganism are increasing. Microbiological hazards include mainly bacterial pathogens associated food borne diseases. These are

broadly classified into two major groups-those that are naturally present in the environment which are indigenous to the food at the time of harvesting and those that get entry into fish during various stages of handling or those introduced into the environment from external sources such as sewage pollution, land run off during heavy rain etc. One major group of bacteria that belong to former category is the genus *Vibrio.*

Vibrios are autochthonous to the aquatic environment and well known for their halophilic nature. However, a few exceptions are the *Vibrio cholerae* and *V. mimicus*, which are not salt dependent and pathogens. *Vibrio cholerae* has been well known historically because of its very virulent nature and the intensity of the disease out break. And voluminous data has been gathered on various aspects of this organism. However the other vibrios are comparatively less studied and interest in this group of microorganisms is of recent origin.

At present, about 45 species were assigned to the genus *Vibrio* (Table 1). Table 2 shows the different pathogenic *Vibrio* species isolated from human clinical specimens. Disease manifestations in most cases range from simple diarrhoea, allergic reactions to acute gastroenteritis and scepticemia.

Aquatic environment is the established reservoir of vibrios. Depending on the salinity and other physicochemical parameters there exists a selection among genus *Vibrio*. Tropical climate predisposes the flourishing of the *Vibrio.* and thus tropical seas and the aquatic animals there in carry an array of *Vibrio* species. Their inter and intra-relationship forms an interesting topic of study.

Among pathogenic vibrios, *V. cholerae* and its serotypes have gained attention through out the world. Besides, *V. parahaemolyticus* and *V. vulnificus* have also emerged as the food borne pathogens in recent period and are widely studied. Information on the occurrence and properties of the less important

Table 1 List of various species of the genus *Vibrio*.

Sl. No.	Species	Reference	Sl. No.	Species	Reference
1	*V. cholerae*	Baumann *et al.*, (1984)	23	*V. damsela*	Love *et al.*, 1981
2	*V. metschnikovii*	,,	24	*V. diazotrophicus*	Guerinot *et al.*, 1982
3	*V. harveyi*	,,	25	*V. hollisae*	Hickman-Brenner *et al.*, 1982
4	*V. campbellii*	,,	26	*V. mimicus*	Davies *et al.*, 1981
5	*V. parahaemolyticus*	,,	27	*V. ordalli*	Schewe *et al.*, 1981
6	*V. alginolyticus*	,,	28	*V. orientalis*	Yang *et al.*, 1983
7	*V. natriegenes*	,,	29	*V. tubiashii*	Hada *et al.*, 1984
8	*V. vulnificus*	,,	30	*V. penaecida*	Ishimaru *et al.*, 1995
9	*V. neries*	,,	31	*V. carchariae*	Grimes *et al.*, 1984
10	*V. fluvialis*	,,	32	*V. mytili*	Pujalte *et al.*, 1993
11	*V. splendidus*	,,	33	*V. navarrensis*	Urdaci *et al.*, 1991
12	*V. pelagius*	,,	34	*V. salmonicida*	Edigius *et al.*, 1996
13	*V. nigripulchritudo*	,,	35	*V. ichthyoenteri*	Ishimaru *et al.*, 1996
14	*V. anguillarum*	,,	36	*V. cincinnatiensis*	Brayton *et al.*, 1986
15	*V. fischeri*	,,	37	*V. furnissii*	-
16	*V. logei*	,,	38	*V. mediterranei*	Pujalte *et al.*, 1983
17	*V. proteolyticus*	,,	39	*V. vulnificus biovar.II*	Tison *et al.*, 1982
18	*V. gasogenes*	,,	40	*V. pectenicida*	Lambert *et al.*, 1999
19	*V. marinus*	,,	41	*V. aerogenes*	Shieh *et al.*, 2000
20	*V. costicola*	,,	42	*V. iliopsicarium*	Onarheim *et al.*, 1994
21	Marine luminous isolates	Chumakova *et al.*, (1973)	43	*V. viscosus*	Lunder *et al.*, 2000
22	*V. aestuarianus*	Tison and Seider (1983)	44	*V. wodanis*	,,
			45	*V. tapestis*	Borrego *et al.*, 1996

Table 2. *Vibrio* species associated with the human clinical specimens.

Species	Occurrence in human clinical specimens*	
	Intestinal	Extra intestinal
V. alginolyticus	-	+ +
V. carchariae	-	+
V. cholerae		
01	+ + + +	+
Non-01	+ +	+ +
V. cincinnatiensis	-	+
V. damsela	-	+
V. fluvialis	+ +	-
V. furnissii	+ +	-
V. hollisae	+ +	-
V. metschnikovii	+	+
V. mimicus	+ +	+
V. parahaemolyticus	+ + + +	+
V. vulnificus	+	+ + +

*The symbols +, + +, + + + and + + + + give relative frequency of each organism in the specimens; -, not found.
Taken from Dalsgaard (1998)

species needs special focus as they may also arise to prominence in future years.

The vibrios have also gained attention of the researchers and industrialists as a result of the its role as a fish pathogen. Vibriosis is a an important disease manifestation appearing on cultured fish and massive mortality could ensue. In addition to the above problem, there is also no concrete evidence regarding the spoilage potential of the *Vibrio* species as they constitute bulk of the tropical microbial flora of aquatic environment.

In the export trade where India ranks seventh position as the leading exporter of seafood, the menace caused by vibrios species, particularly, *V. cholerae* is tremendous. *V. parahaemolyticus*, another pathogen for which inspection is imposed in some countries like Japan is totally indigenous to the marine environment and seafood. Hence it is argued that while imposing preventive regulations and specifications, the standards are to be formulated after conducting in depth studies revealing the status of the particular seafood in terms of the expected pathogens and indigenous flora contained in them as well as in their habitat. Thus adoption of this type of regulatory measures on *Vibrio* species needs a comprehensive study on the ecology and distribution of vibrios in marine animals and the inhabiting environment.

Hence basic research on the ecology and distribution of the vibrios in the aquatic environment is a thrust area in fisheries development. As a primary solution to this problem, the ecology and features of marine *Vibrio* are attempted in this thesis. It is expected that the data will be valued for improving the processing strategies and enlarging the vision on the concept for high quality, safe seafood in the domestic as well as international trade.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Ecology and distribution of *Vibrio* species in marine environments

The genus *Vibrio* comprises species that are characterised by wide variations in their nutritional versatality, physiological traits and biochemical features. This suggests that different species may vary greatly in their potential to inhabit in environments of differing nature. Vibrios constitute a significant component of the autochthonous bacteria of various marine environments like water, sediment *etc.* and also form the major flora of aquatic animals inhabiting them. Vibrios are also associated with plankton notably zooplankton. Twelve species of the genus *Vibrio* produce various virulence factors and are human pathogens (Dalsgarrd, 1998). A few species are important pathogens in aquacultured animals (Lightner, 1988; Austin and Austin, 1993; Otta *et al.,* 1998). Their role in the environment included biodegradation, nutrient regeneration and bio-geochemical cycling (Okpokwasili and Olisa, 1991; Uchida and Nakayama, 1993; Colwell, 1994). Some of the species *viz. V. diazotrophicus* is capable of nitrogen fixing (Tibbles and Rawlings, 1994). A few species of *Vibrio* are luminescent and form symbiosis with higher invertebrates. They include psychrophilic as well as barophilic strains (Lee and Ohawada, 1995). This divergent physiological capabilities made them to occupy various ecological niche of human interest. An authentic survey on the ecological distribution of *Vibrio* species was made by Colwell (1984).

2.1.1. Distribution of *Vibrio* species in seawater

Studies of *Vibrio* as a flora of seawater is mainly reported from temperate regions (Hlady, 1997; DePaola *et al.,* 1994; O'Neill *et al.,* 1992; Kaysner *et al.,* 1987). Though scarce, reports from tropical region was also not

uncommon. Table 3 shows the geographic distribution of vibrios in various sea waters.

Tropical ocean and its inhabitants were reported to be good reservoirs of *Vibrio* species. Quantitatively, in Cochin coastal water, vibrios constituted 5% of the total heterotrophic bacteria (Alavandi, 1989). Distribution of *Vibrio* species is influenced by the changes in the physico-chemical and ecological parameters. Quantitatively coastal water harbours more *Vibrio* than the open sea (Jung and Shin, 1996). A similar diminishing trend in the density of *Vibrio* towards open sea was reported from Indian coast also (Sreeja and Ravindran, 1999). Horizontal variation in the occurrence was also noted in East China Sea by Shin and Jung (1996) and it contained $0.2x10^1$ to $9.0x10^3$ ml^{-1} while open sea contained $0.8x10^1$ to $3.0x10^1$ ml^{-1}. Qualitative variations due to ecological parameters is evident from the reports of predominance of different *Vibrio* species from non-related countries (Cheng *et al.,* 1995; Miyazaki and Ezura, 1995; Monticelli and Crisafi, 1995; Caruso *et al.*, 1996).

2.1.1.1. Influence of temperature on the vibrios in water

Temperature is the cardinal factor determining the prevalence of *Vibrio*. Caruso *et al.,* (1998) and Barbierri *et al.* (1999) reported a positive correlation between the occurrence of *Vibrio* and temperature. Furuta *et al.* (1994) reported a similar correlation between the size of the heterotrophic bacterial population and temperature in the surface water of deep portion of the Uragami bay, Japan. However, the influence of temperature on the *Vibrio* population is significant only in temperate region were the degree of seasonal fluctuation in the temperature was high (Wright *et al.*, 1996). Oliver *et al.* (1982) reported that the *Vibrio* population in the coastal waters of southern United States reached the peak value in summer.

Table 3. Distribution of vibrios in marine and estuarine waters of different geological realms.

Place of isolation	Reference
Australia	Ghosh and Bowen, 1980
Bahrain	Mahsneh and Al-Sayed, 1997
China- Rushan Bay	Chang *et al.*, 1987
Denmark	Hϕl *et al.*, 1998
Dutch Coast	Veenstra *et al.*, 1994
France	Rollet *et al.*, 1991
England	O'Neill *et al.*, 1990
Hong kong	Yam *et al.*, 2000
India - Bombay Coast	Bhathena and Docter, 1995
India-Gujarat Coast	Mogal, 1997
India-Laccadives	Chandrika, 1996
India-Mangalore Coast	Karunasagar *et al.*, 1990
India-South west coast	Thampuran *et al.*, 1996
Indonesia	Sunarya *et al.*, 1997
Israel	Ghinsberg *et al.*, 1999
Italy-Adriatic Coast	Barbieri *et al.*, 1999
Japan	Urakawa *et al.*, 1999
Korea-Kwangan beach	Kim *et al.*, 1990
Nigeria	Okpokwasili and Akajobi, 1996
Spain	Amaro *et al.*, 1992
Sri Lanka	Fonseka, 1990
US-Great Bay	O'Neill *et al.*, 1992
US-Gulf Coast	Depaola *et al.*, 1994
US-Gulf Coast	Kelly, 1982
US-South east	Oliver *et al.*, 1982
US-West Coast	Kaysner *et al* ., 1987

Seasonal and geographical variations depend on water temperature and the influence of temperature on bacterial count in water and sediment have been reported and reviewed for *V. cholerae* (Roberts *et al.*, 1982; West and Lee, 1982; Nair *et al.*, 1988; Perez-Rosas and Hagen, 1989), *V. parahaemolyticus* (Ayres and Barrow, 1978; Kaneko and Colwell, 1978, Watkins and Cambelli, 1985; Kelly and DanStroh, 1988a), *V. fluvialis* (Barbay *et al.*, 1984), and *V. vulnificus* (Oliver, 1989; O'Neill *et al.*, 1992; Kasper and Tamplin, 1993; Motes *et al.*, 1998).

2.1.1.2. Influence of pH on the vibrios on water

Most of the studies on the pH tolerance of *Vibrio* were *in vitro* in growth medium and low acid containing food systems (Ama *et al.*, 1994, Oliver and Kasper, 1997; Koo *et al.*, 2000). However *Vibrio* population was earlier reported to be negatively correlated to water pH (Oliver, 1982). A pH range of 6-9 was reported for optimum growth of *Vibrio* strains by DeLapena *et al.* (1993).

2.1.1.3. Influence of salinity on the vibrios in water

Vibrio species have halophilic characteristics and occur most frequently in water with salinity ranging from 0.5 to 3%, thus significantly limiting their presence to estuarine and inshore coastal waters (West and Lee, 1982; Seidler and Evans, 1984; Boekmuhl *et al.*, 1986; Tison *et al.*, 1986; Kelly and DanStroh, 1988b; Koh *et al.*, 1994a). But ecological studies conducted by Singleton *et al.* (1982a; 1982b) and Miller *et al.* (1984) revealed that vibrios could survive in fresh water also and the interaction of high water temperature and elevated organic nutrient concentration might be the factors help to tide over the deleterious effect of low salinity. In a similar study of the coastal waters of USA , O'Neill *et al.* (1992) reported that salinity was significantly related to the prevalence of *V. vulnificus*.

2.1.1.4. Influence of other factors on the vibrios in water

Vibrio population was positively correlated to the suspended particulate matter of the water column (Monticelli and Crisafi, 1995). In addition to this *Vibrio* concentration was affected by sampling variables like day, depth and tidal cycles (Koh *et al.*, 1994b). Stratification of the water column could be another source of variation. Shen *et al.* (1996) studied the vertical distribution of *Vibrio* and reported highest quantity in surface water (0m) followed by 100m and lowest in 25m depth. The consistent higher concentration of *Vibrio* obtained from bottom samples were considered to be due to re-suspension of *Vibrio* from the sediment (Koh *et al.*, 1994b). The occurrence of vibrios in water column was also influenced by the their association with plankton and other higher animals (West and Lee, 1982) on which they gain temporary shelter.

Many other factors are also contribute to the occurrence of vibrios. This included the availability of nutrients and products of metabolism and biological factors zooplankton . *V. vulnificus* could survive in sterilised sea water up to 14 days at 4^0C, it could not survive in unsterilised sea water. This observation indicate the influence of unidentified biological factors in their survival in the environment (Kasper and Tamplin, 1993; McCarthy, 1996). Kim and Kwon (1997) reported that at 4^0C, in bottom deposit solution of brackish water, survival time of *V. vulnificus* was longer and rate of decline slower than that of brackish water.

2.1.2. Distribution of vibrios in sediment

Williams and LaRock (1985) reported that the *Vibrio* density in the sediment was nearly three orders of magnitude higher than those in the overlying water. Interestingly, total microbial load of sediment was also ten times higher than that of the water (Pagnocca *et al.*, 1991). It was reported that *Vibrio*

constituted 35% of the total flora of the sediment and water of Madras coast of India (Prabhu *et al.,* 1991). Luminous *Vibrio* has been reported as a major component of sediment of Vellar estuary, India (Ramesh *et al.,* 1999). Association of *V. cholerae* (Hood and Ness, 1984; West and Lee, 1982) and *V. parahaemolyticus* (El-Sahn *et al.,* 1982) with sediment was found to overcome the unfavourable environmental conditions. Association of *V. parahaemolytics* (Kaysner *et al.*, 1990) and *V. vulnificus* (Wright *et al.,* 1996) with sediment and plankton are also established.

2.1.3. Distribution of vibrios in plankton

Bacterial counts of sea water are affected by plankton blooms, especially, zooplanktons (Colwell, 1994); Dilille and Razoul, 1994). Both positive and negative influences are reported. Oppenheimer (1963) found that the bacterial numbers were high in *Prorocentrum* red tides in California. Bacteria can live on the exocrine from dinoflagellate and also can provide growth factors such as vitamin B_{12}. On the other side, a negative correlation was observed between *Vibrio* population and phytoplankton biomass (Jimenez and Carmona, 1995).

Vibrio appear to maintain high numbers and prolong their existence by association with plankton. In particular the chitin component in plankton appears to enhance significantly this phenomenon of prolonged survival (Huq *et al.*, 1986; Karunasagar *et al.*, 1987). Studies have established an association between chitinous zooplankton and *V. cholerae* (Huq *et al.,* 1984) and also with V. *parahaemolyticus* (Kaneko and Colwell, 1978; Sarkar *et al.,* 1985; Watkin and Cambelli, 1985; Venketeswaran *et al.*, 1989a) and *V. vulnificus* (Oliver *et al.*, 1983). Zooplankton dynamics was the major attributing factor for the seasonal

variations in the occurrence of *V. parahaemolyticus* and allied organisms in estuarine water (Abraham, 1981; Nair, 1981).

2.1.4. Distribution of vibrios in finfishes

The presence of *Vibrio* is reported from fishes of almost all countries and is thus ubiquitous in its distribution. Literature on the distribution of vibrios on fish from various parts of the world is presented in Table 4 which reveals their ecological diversity and preponderance in different area. A close scrutiny of the literature surveyed showed that most of the reports were from the coasts of United States and from other temperate countries. It was also noted that most of them are autecological studies confined mainly on *V. cholerae, V. parahaemolyticus or V. vulnificus*. For example, distribution of *V. vulnificus* was studied extensively from United States. The bacterium was reported from West coast (Kaysner *et al.*, 1987), Gulf coast (Kelly, 1982; Levine and Griffin, 1993; DePaola *et al.*, 1994), South-eastern United States (Oliver, 1982), North-eastern United States (Tilton and Ryan, 1987) and Florida estuary (Tamplin *et al.*, 1982).

Majority of the reports from tropics are mainly pertained to the occurrence of vibrios in shrimp, as it form the major export item (Matte *et al.*, 1994b; Dalsgaard, 1998). Matte *et al., (*1994a) reported that in Brazil, 88% of the oyster samples analysed showed presence of pathogenic *Vibrio* . Qualitatively, the highest incidence was observed for *V. alginolyticus*, followed by *V. parahaemolyticus, V. cholerae-non 01, V. fluvialis, V. furnissii, V. mimicus* and *V. vulnificus*. Other reports from the tropics on pathogenic vibrios like *V. vulnificus* (Dalsgaard and Hϕi,1997), *V. parahaemolyticus* (Nair *et al.*, 1980; Honda and Iida, 1993), *V. fluvialis and V. furnissii* (Huq *et al.*, 1980; Magalhaes *et al.*, 1990) and *V. cholerae non* 01 (Kaysner *et al.*, 1987; Dalsgaard *et al.*, 1995) are also documented.

Table 4. Distribution of *Vibrio* species in various body parts of fishes seen in different geological realms

Fish species	Location	body part	Occurrence of *Vibrio*	Reference
Marine Fishes	India	Skin and Muscle,	12 - 27%	Thampuran *et al.*, 1996
		Intestine	26 - 33%	
Puffer (*Fugu rubripus rubripus*)	Japan	Intestine	$\log_{10}$ 2-6	Sujita *et al.*, 1988
Puffer (*Fugu vermicularis*)	Japan	Intestine	70%	Noguchi *et al.*, 1987
Red Sea Bream (*Pagrus major*)	Japan	Intestine	Ca 45%	Muroga *et al.*, 1987
Black Sea Bream (*Acanthopragus schlegeli*)				
African Snake Head (*Channa obscura*)	Nigeria	Intestine	--	Kori and Evbakhare, 1993
Plaice (*Pleuronectes platesta* L.)	UK	skin	--	Gilmour *et al.*, 1976
Turbot (*Scophthalmus maximus*)	UK	Skin	--	Austin, 1983
Turbot (*Scophthalmus maximus*)	UK	Gill	24%	Mudarris and Austin, 1988
Blue fish(*Pomatomus saltatrix*)	USA	Intestine	28.4%	Newman *et al.*, 1972
Estuarine fishes	USA	Skin	3.4%	Colwell, 1962
		Gill	9.5%	
		Intestine	13.9%	
finfishes and shellfishes	USA	Muscle, haemolymph, skin, gut, and gill	--	Oliver *et al.*, 1982
finfishes and shellfishes	USA	Intestine	$\log_{10}$ MPN 1.5 - 8.8	DePaola *et al.*, 1994
Stripped bass(*Morone saxatilis*)	USA	Intestine (estuarine fish)	10%	MacFarlane *et al.*, 1986
		Intestine (marine fish)	27%	

Vibrio has been reported as one of the major flora of Indian coastal waters and its inhabitants (Karthiyani and Iyer, 1971a; Chandrika and Nair, 1994; Thampuran *et al.*, 1996). Occurrence of vibrios in aquaculture farms were also reported (Nayyarahamed *et al.,* 1995; Bhasker *et al.,* 1998; Sanjeev, 1999; Surendran *et al.,* 2000). The occurrence of vibrios was reported from Indian south east coast (Karunasagar *et al.*, 1990; Sreeja and Ravindran, 1999), Bombay coast (Bhathena and Docter, 1995), Laccadives (Chandrika, 1996), Gujarat coast (Mogal, 1997) and south west Cochin coast (Pradeep and Lakshmanaperumalsamy, 1984; Prasad and Rao, 1994; Thampuran and Surendran, 1998; Sanjeev *et al.,* 2000). Majority of these studies were confined to a particular species say, *V. parahaemolyticus* or *V. cholerae* or to specific groups. Information regarding the overall picture of the divergent nature of *Vibrio* population is yet to be unveiled, even though scattered reports are available.

Fish from marine, estuarine and coastal waters are expected to be colonised by vibrios as they are autochthonous to the environment. Association of vibrios with finfishes were reviewed by Cahill (1990). Generally, the range of bacterial genera isolated is related to the aquatic habitat of the fish and varies with factors such as salinity and bacterial load of the water. Reported data of vibrios in different fish species are tabulated in Table 4. Earlier studies were largely centred on *V. parahaemolyticus*, since this organism was commonly associated with gastroenteritis (Joseph *et al.*, 1983; Sarkar *et al.*, 1985; Twedt, 1989). Recently *V. vulnificus* has also gained attention due to its high virulence and mortality rate(Oliver, 1989; Thampuran *et al.*, 1999). Possibility of other *Vibrio* species in sea food is yet to be studied with respect to its public health significance, as many of these species are proved equally virulent (Huq *et al.,* 1980; Karunasagar *et al.*, 1990). Skin, gill and intestine are the established niches for the colonisation of the vibrios (Cahill, 1990).

2.1.4.1. Distribution of vibrios in skin of finfishes

The major component of the skin flora were similar to those in the ambient water, indicating that it is a reflection of their environment (Horsley, 1973). Method of handling and pre-capture environment determine the composition of surface flora (Horsley, 1973). Cahill (1990) reviewed the bacterial flora of fish skin and muscle and stated that various genera on the fish skin are similar although the proportion of different types varied with geographical location. Colwell (1962) reported 3.4% of skin flora were *Vibrio*. Hajji *et al.* (1991) reported a very low *Vibrio* density in skin when compared to gill and intestine. They opined that direct plating could not bring out the true picture of the number of bacteria on the scales and suggested direct epifluorescence microscopic techniques for estimation of bacterial density. Sar and Rosenberg (1987) attributed characters like the ability to attach strongly to skin, high surface phobicity, ability to release drag reducing polymers and mechanisms to overcome the antimicrobial agents in fish skin and mucous, for the association of the bacteria with skin surface

2.1.4.2. Distribution of vibrios in gill of finfishes

Mudarris and Austin, (1988) stated that the gill flora was quite distinct from the surrounding water as well as that of fish skin. On the other hand, Austin and Austin (1987) reported that gill microflora resembled that of the surrounding water. Occurrence of *Vibrio* at a level of 9.5% of total flora in Puget farm fishes (Colwell, 1962) and 24% in *Scophthalmus maximus* (Mudarris and Austin, 1988) were reported. A limited number of studies have been carried out on the density of vibrios on gills, still these result indicate that considerable variation may exist on this aspect.

2.1.4.3. Distribution of vibrios in intestine of finfishes

Microbiology of the intestine of fish has received the attention of many researchers. *Vibrio* forms the predominant component in the intestinal flora of marine fishes (Colwell, 1962; Newman *et al.*, 1972). Higher densities of the order of 10^8 g^{-1} of intestine of fish have been reported (Karunasagar *et al.,* 1987; Miceli *et al.,* 1993; Depaola *et al.*, 1994; Thampuran and Surendran, 1998). Reports of percentage composition of *Vibrio* in the gut flora of different fishes are also presented in Table 3. Their symbiosis in the intestine is not only having a role in digestion process (Rajkumar and Ayyakannu, 1995), but also as a probiotic biological barrier against pathogens (Westerdahl *et al.,* 1994).

Harris *et al.*, (1991) stated that gut flora was distinct from habitat flora. MacFarlane *et al.* (1986) claimed that bacteria present in the aquatic environment influences the composition of the gut flora. Sugita *et al.,* (1983; 1988a) claimed that permanent intestinal flora consisted of bacteria which were present in the surroundings, but only those able to persists and grow in the environment provided by the intestinal tract will flourish.

Intestinal flora is affected by various factors like temperature (Kori and Evbakhare, 1993), microflora of the water (Olafsen, 1994; Sugita *et al.*, 1988b), feeding habit (Bergh *et al*, 1994; Grisez *et al.*, 1997) *etc.* Grisez *et al.*, (1997) showed that the feed determines the colonisation vibrios in the intestine and opined that the fluctuation in the composition of the dominant microflora appeared to reflect the bacterial composition of the ingested live feed. *Vibrio* in ingested live feed also serve as important source of pathogens causing intestinal infections (Muroga, 1995)

Degree of development of digestive tract is another factor determining the indigenous flora. Fish with well developed and long digestive tract contained a resident flora composed of vibrios whereas fish with under-developed digestive tract contained a transient flora which closely reflected that of the diet (Sera and Ishida, 1972). In red sea bream having developed digestive system, the composition of the flora of stomach and intestine changed with time after feeding (Sera and Ishida, 1972). Their studies also confirmed the indigenous nature of vibrios in the intestine by demonstrating its resistance to 2% bile and 5.5 pH.

2.1.5. Distribution of vibrios in shellfishes

Bivalve molluscan shellfish may become rapidly contaminated which filter feeding on plankton material colonised by pathogenic vibrios and so often subsequently incriminated as vectors in food poisoning incidence (DePaola, 1981; Kelly and Dinuzzo, 1985; Morris and Black, 1985; West and Colwell, 1984). Association of pathogenic vibrios with the flesh of filter feeding bivalve molluscs after harvesting can prolong their survival. Hence storage of shellfish at ambient temperature can then lead to rapid proliferation of pathogenic vibrios (Eyles *et al.*, 1985; Karunasagar *et al.*, 1987). Processing of oysters (shucking and washing) coupled with cold storage reduces the *Vibrio* load considerably as compared to shellstock oysters (Hood and Ness, 1984). They further stated that the type of shellfish influence the load of vibrios, clams being less likely source of intestinal illness than oysters.

Quantitatively, freshly landed samples contained more vibrios than market samples. Market samples contained *V. parahaemolyticus* (10.7%), *V. vulnificus* (7.7%) and *V. fluvialis* (5.4%), where as the corresponding value for freshly landed samples were 37.5, 25.0 and 11.1% respectively (Barbay *et al.*,

1984). In India, shrimp samples (41%) and clam samples (42%) harboured heavy load of vibrios (Bhasker *et al.*, 1998). Qualitatively, *V. parahaemolyticus* (27%), *V. alginolyticus* (29%), *V. cholerae* (2%) and *V. vulnificus* (11.8%) were isolated from shrimp. High preponderance of *V. alginolyticus* (Bhasker and Setty, 1994), *V. parahaemolyticus* (Pradeep and Lakshmanaperumalsamy, 1984; Dela Cruz *et al.*, 1990) and *V. vulnificus* (Bhasker and Setty, 1994) in shrimp were reported.

Higher *Vibrio* count were also reported in bivalves (Montilla *et al.*, 1994; Aiyanperumal *et al.*, 1995). Dempsey *et al.* (1989) also proved the role of vibrios in the digestive process in intestine of estuarine and marine crustaceans. Singh *et al.* (1991) opined vibrios inhabiting in the gut of *Penaeus indicus* play dual role, both beneficial and harmful in the life of the animal by enhancing the digestive process and by infecting the host during adverse environmental conditions respectively. These studies indicated that the wide fluctuation occur on the *Vibrio* count of the marine environment as well as main inhabitants was influenced by various factors of which season has a significant role to play.

Distribution of vibrios in shellfish often exhibits seasonality coinciding with the phenomenon in the environment in which the animal live (Rivera *et al.*, 1989; O'Neill *et al.*, 1990; Tamplin *et al.*, 1990; Motes *et al.*, 1998).

2.1.6. Distribution of vibrios as effected by season

The seasonal occurrence, distribution, abundance and the diversity of the bacterial species are greatly influenced by the prevailing environmental parameters (Sochard *et al.*, 1979; Ramathirtham *et al.*, 1987; Alavandi, 1989). In coastal water off Cochin, Alavandi (1989) has observed low bacterial population during monsoon which was suggested to be due to the discharge of flood waters from Vembanad lake. The reason for the major peak in bacterial count observed

during January-February was related to the factors such as optimum temperature, evaporation of surface water and low variation in salinity.

Bacterial flora of the fish is effected by season (Karthiayani and Iyer, 1971a). Pradeep and Lakshmanaperumalsamy (1984) noted strong correlation the season and the count of *V. parahaemolyticus* associated with water, plankton and sediment. Total *Vibrio* and *V. parahamolyticus* peak were found to coincide with the zooplankton blooms in pre monsoon (April-May) and post monsoon (August and November) seasons. Total viable count also followed the same pattern as reported by Chandrika and Nair (1994). They observed maximum count of heterotrophic bacteria ($512x10^6 g^{-1}$) in sediment and ($496x10^6 ml^{-1}$) in sea water was observed in the month of August and minimum during February in sediment ($84x10^6 g^{-1}$) and in sea water ($46x10^6 ml^{-1}$).

Incidence of *V. vulnificus* is reported to be higher during warmer months of the year (Blake *et al.*, 1980; Cook, 1997; Hϕi *et al.*, 1998; Motes *et al.*, 1998). Seasonal effect in the occurrence of *V. vulnificus* was studied by O'Neill (1992). The peak values were observed for oyster and water in early July (temperature,>20^0C). Thereafter the levels peaked in August and slowly decreased , disappearing from oysters in early October (temperature,<15^0C) and from water by the middle of October. Same trend was observed from other temperate countries also (Kaysner *et al.*, 1987; Rivera *et al.*, 1989; Tamplin *et al.*, 1990). Similar higher incidence in warmer months was also reported for *V. parahaemolyticus* (Kaneko and Colwell, 1978; Mahsneh and Al-Sayed, 1997).

Species wise distribution of vibrios was also affected by season (Thampuran *et al.*, 1996). Maximun species diversity was observed in monsoon period, (July-September). In fish skin and muscle, total *Vibrio vis- a-vis* total viable count noted highest in May- June where as it was almost steady from

June to August for intestinal sample. Month-wise difference was also studied by Arias *et al.* (1999) in Spain who noted that when temperature was below 15^{0}C, (March-May), *V. splendidus* dominated in sea water and in transition period when temperature was maintained between 15 to 20^{0}C, *V. splendidus* population began to decline and in June-October, (temperature, >20^{0}C) *V. harveyi* became the predominant species.

2.1.7. Distribution of vibrios in relation to indicator bacteria

Very few studies have been carried out regarding the correlation between vibrios and other indicator bacteria. Watkin (1996) explained the utility and significance of using an array of microbial indicators for the monitoring of pollution by human intervention. This array included total and faecal coliforms, *Escherichia coli,* enterococci, *Clostridium perfringens* and specific bacteriophages. Positive as well as negative correlation of vibrios to these indicators have been reported. Okpokwasihi and Akajobi (1996) found vibrios correlated with indictors and recommended vibrios were also included along with *Aeromonas hydrophila* as indicator bacteria in tropical water quality assays.

Numerous investigators have proved that human contamination indicators do not necessarily reflect the presence of vibrios in marine and estuarine environment (Hood *et al.*, 1981; Bhasker *et al.*, 1998). Rodrick *et al.* (1984) indicated that little relationship existed between the presence of *V. parahaemolyticus, V. cholerae, V. alginolyticus* and standard coliform values for sea water and oyster meat. Negative correlation was noted between the presence of *V. cholerae* and feacal coliforms (Colwell *et al.*, 1977), *V. parahaemolyticus* and faecal coliforms (Pradeep, 1986; Shiharis *et al.*, 1987), *V. mimicus* and total and feacal coliforms (Boekmuhl *et al.*, 1986) and *V. vulnificus* and feacal coliforms (Oliver *et al.*, 1983; O'Niell *et al.*, 1992). Venketeswaran *et al.* (1989b) reported in Japan that *V. parahaemolyticus* count was significantly

related to total and feacal coliforms in plankton. Karunasagar *et al.* (1990) reported poor correlation between the incidence of human pathogenic vibrios and feacal coliforms.

A close scrutiny of the literature showed that most of the studies are centred in temperate regions, where *Vibrio* population is scanty. But in tropical areas where vibrios constituted bulk of the flora of marine environment, a different picture could emerge.

2.2. Characterisation of vibrios

The fate of a microorganism in the environment is ultimately determined by the so called 'microclimate' which include physical and chemical conditions, nutritional status, biological factors like the interactions between and among the species *etc.* The physico-chemical parameters included temperature, light, salinity, turbidity and pH. Physiological and biochemical characteristics *vis-a-vis* the availability of the substrate that can be metabolised by the microbe determine the diversity of the flora of the environment. Another predisposing factor is the ability to survive competitively in an environment.

2.2.1. Taxonomic status of genus *Vibrio*

Vibrio belong to family Vibrionaceae along with the genera *Listionella* and *Photobacterium* (Farmer and Hickmann-Brenner, 1992). Bergey's manual of Systematic bacteriology (Baumann *et al.*, 1984) described 20 different *Vibrio* spp. However, other taxonomic studies differentiated more than 30 species and phenetic groups of *Vibrio* (Bryant *et al.*, 1986a; Austin and Lee, 1992; Farmer and Hickmann-Brenner, 1992; Alsina and Blanch, 1994a; 1994b). Bhat (1998) listed 39 species in genus *Vibrio.* A modified list of the members of Vibrionaceae is presented in Table 1 which included a few new species currently accepted by

International Committee for Taxonomy of Vibrionacae. This lack of parallelism in the reports along with the fast emergence of the reports of new species (Lambert *et al.*, 1999), make the taxonomy of *Vibrio* more cumbersome. The taxonomic position of some species *viz. V. marinus* (Urakawa *et al.*, 1998) and *V. carchariae* (Pederson *et al.*, 1998) was still uncertain. Further, the identification is made more complicated by problems like assigning isolates of apparently same kind into different groups by different authors. (For example, phenon 10 of West *et al.* (1986) have been grouped as *V. anguillarum* like by Bryant *et al.* (1986a). The discrepancies in the results of certain tests for the same species by different authors also made the taxonomic characterisation more complicated (Baumann *et al.*, 1984; Austin and Lee, 1992; Alsina and Blanch, 1994a; 1994b). While scrutinising the literature it is seen that the thrust of the research in this field is concentrated on a few pathogenic *Vibrio* species from temperate regions. Comprehensive biochemical characterisation of marine environmental vibrios is lacking from the tropical area, except a few scattered studies (Bhat, 1998). A complete biochemical characterisation and a classification scheme for the *Vibrio* species from tropical environment is urgently needed.

2.2.2. Biochemical characteristics of the *Vibrio*

Vibrios exhibit wide diversity in physiological and biochemical characteristics. As per Bergey's Manual of Determinative Bacteriology (Baumann *et al.*, 1984) isolates that are Gram negative, oxidase positive (*V. metschnikovii* and *V. gazogenes* are exceptions), capable of growing on TCBS medium and are facultative anaerobes which ferment glucose without gas (+/+ for oxidation/ fermentation test) are presumptively considered as the member of genus *Vibrio.* Attempts to characterise *Vibrio* through various tests including utilisation of many sugars, sugar acids, sugar alcohol, monocarboxylic acids and monocyclic aromatic compounds as sole carbon source, production of many extracellular

hydrolases and other metabolites, growth at various salt concentrations, growth at various temperature, sensitivity to various antimicrobial agents, swarming, bio-luminescence *etc.* were carried out (West and Colwell, 1984; Baumann *et al.*, 1984; Bryant, *et al.*, 1986a; 1986b; Alsina and Blanch, 1994a; 1994b; Bhat, 1998). Variations in the biochemical traits of individual species observed in these studies to the limitation of biochemical identification schemes and also the need for searching a more reliable identification systems based on stable properties. For example, Bhat (1998) proved the unreliability of test using vibriostatic agent, 0/129, a major trait segregating *Vibrio* from *Aeromonas*. However, Subcommittee on Taxonomy of Vibrionaceace, International Committee of Systematic Bacteriology, has accepted the use of 0/129 irrespective of the resistance pattern exhibited, as per the minutes of the meeting on 1998 by Nair and Holmes (1999). Hϕi *et al.* (1998) disapproved the classification of *V. vulnificus* as two biotypes depending on the indole production. Such disparity in Indole production by the environmental strains of *V. vulnificus* was also reported (Dalsgaard *et al.*, 1999). Biochemical heterogeneity among the strains is thus a major problem in identification. This warrants the amendment of existing identification systems with preference to changes in geographical difference. Heterogeneity thus produces constant changes in taxonomy.

2.2.3. Physico-chemical parameters influencing growth of *Vibrio*

Incidence of vibrios in the environment is influenced by various factors like temperature, pH, salinity, abundance of zooplankton, availability of nutrients and other unidentified factors (West and Colwell, 1984; DeLapena *et al.*, 1993). Hence the Incidence of *Vibrio* in the sea food is highly depended on the prevailing physico-chemical condition of the ambient environment. Even though the work on similar lines is in plenty from the temperate regions, such data on the tropical isolates are very limited.

2.2.3.1. Temperature

Temperature is one the most important factor determining the growth and survival of vibrios in environment as well as sea food. Temperature dependence in the environment is evident from the seasonal dominance exhibited by some of the *Vibrio* species. In *in vitro* conditions, tolerance to temperature varies with *Vibrio* species. Most of the species grow at 20^{0}C and some at 30^{0}C; few grow at 4^{0}C and 45^{0}C; none at 50^{0}C (Baumann *et al.*, 1984).

Temperature tolerance of *V. parahaemolyticus* and *V. vulnificus* were extensively studied as both the species were relevant in disease outbreaks through seafood. *V. parahaemolyticus* cells were more sensitive to 0.6^{0}C than lower temperature like -18^{0}C and -34^{0}C (Matches *et al.*, 1971). Beuchat (1975) demonstrated its survival through freezing process and been able to isolate some strains from frozen fishery products. Kaneko and Colwell (1978) reported that 10^{0}C was minimum temperature for growth of *V. parahaemolyticus* in a natural environment. Psychrophilic strains (those isolated from frozen food) of *V. parahaemolyticus, V. mimicus* and *V. fluvialis* survived well at 10^{0}C, 4^{0}C, and -30^{0}C (Wong *et al.*, 1994). *Vibrio parahaemolyticus* introduced into cooked prawn as post process contamination is unlikely to multiply, but survives at 5, 10, and -20^{0}C up to 17 days (Venugopal *et al.*, 1999).

Vibrio vulnificus shows poor survival below 8.5^{0}C. Growth was noted in sterilised sea water in the 6th day of incubation at 13 to 22^{0}C and temperature outside this range reduced the time of survival up to 90% level after 6 days of incubation (Kasper and Tamplin, 1993). Parker *et al.* (1994) also reported a considerable reduction in the number of cells from 10^5 to 10^1 cfu g^{-1} of *V. vulnificus* in oysters stored at -20^{0}C. For *V. vulnificus* low temperature induces the formation of viable but non-culturable state (VBNC) (Oliver *et al.*, 1991; Wolf

and Oliver, 1992; Oliver, 1993; Oliver *et al.*, 1995). In VBNC state, the bacteria survive for at least for 50 days at low temperature (Boisca *et al.*, 1996). Therefore the hazard due to this organism exists even though the contaminated products are stored at low temperature.

Vibrios were equally sensitive to heat. Heat processing is thus a recommendable alternative for the elimination of the pathogens. Vanderzant and Nickelson (1972) recommended one minute heating at 100^{0}C for complete elimination of *V. parahaemolyticus* cells and 15 minutes at 60^{0}C or 80^{0}C. Thermal decimal reduction time is about 4 min. for *V. parahaemolyticus* at pH 7 (Beuchat, 1973). Increasing significance of vibrios in public health and spoilage, especially in warmer countries necessitates an in-depth study in terms of tolerance to extreme temperature.

2.2.3.2. pH

Most of the species of *Vibrio* can tolerate moderately alkaline condition and can grow at pH 9. A few, notably *V. cholerae* and *V. metschnikovii* could grow at pH 10 (Baumann *et al.*, 1984). In a study by Vanderzant and Nickelson (1972) threshold limit of pH 6 for *V. parahaemolyticus* was established and Beuchat (1973) described the same as pH 5 in liquid medium. An optimum pH of 7.5 for *V. vulnificus* was described by Koh *et al.* (1994b).

2.2.3.3. Salinity

Salinity is the other important factor determining the prevalence of vibrios. Growth of all vibrios is stimulated by Na^+. The minimal concentration required for optimal growth ranges from 5-15 mM for *V. cholerae* and *V. metschnikovii* and 600 to 700 mM for *V. costicola* (Baumann *et al.*, 1984). Salinity optima for *V. parahaemolyticus* (Blake *et al.*, 1979) and *V. vulnificus* (Kelly, 1982) were reported as 1.0 to 2.0%. At salinities 30, 35 and 38 ppt,

number of *V. vulnificus* decreased 58, 88 and 83% respectively (Kasper and Tamplin, 1993). Motes *et al.* (1998) have found high levels of *V. vulnificus* in moderate salinity levels above 28 ppt. Salinity was a determining factor in the long term survival of vibrios in sea water (McCarthy, 1996).

2.2.3.4. Low oxygen levels

Use of modified atmosphere storage of fish and products for the extension of shelf life and normal spoilage counts was gaining importance now a days. The combination of low temperature and various gases, especially CO_2 replacing air in the storage atmosphere has proven effective in reducing spoilage bacteria of fish and other foods (Mokhele *et al.*, 1983; Molin *et al.*, 1983; Isenschmid *et al.*, 1995). Inhibition of growth and metabolism of microorganism by CO_2 was reviewed by Dixon and Kell (1989). Kimura and Murakami (1993) observed that *V. parahaemolyticus* cells showed a reduction in the viable cells on CO_2 storage at 5^0C in jack mackerel fillets. Studies on the survival pattern of *Vibrio* species in reduced oxygen levels are noted attempted so far.

2.2.3.5. Other parameters

Many other factors are also critical for the occurrence of vibrios. This included the availability of nutrients, products of metabolism and many other biological factors like presence of zooplankton and sediment etc. *V. vulnificus* could survive in sterilised sea water up to 14 days at 4^0C, but it could not survive in unsterilised sea water. This observation indicated the influence of biological factors in their survival in the environment (Kasper and Tamplin, 1993; McCarthy, 1996). Kim and Kwon (1997) reported that at 4^0C, in bottom deposit solution of brackish water, survival time of *V. vulnificus* was longer and rate of decline slower than that of brackish water.

The pattern of growth at various physico-chemical factors, *in vitro,* was also affected by the menstruum used for the study (Covert and Woodburn, 1972; Beuchat, 1973; Kim and Kwon, 1997). Moreover the survival of cells at low temperature depends up on the inoculated strain, inoculum size and method adopted for enumeration. Direct plating method was reliable and the results varied in relation to plating media used (Goatcher *et al.*, 1974).

2.2.4. Factors influencing the prevalence of *Vibrio* in the Intestine

Intestine of fishes represented very conducive micro environment for the colonisation of *Vibrio* (Cahill, 1990). Vibrios are capable of overcoming the low pH and bile concentration in the digestive tracts and predominate the flora of intestine. Sera and Ishida (1972) stated that most of the *Vibrio* isolated from digestive tract of fish could grow well in presence of 2.0% bile, at pH 5.5. Thus bile resistance may be a contributing factor for their survival in the intestine.

In addition to bile and pH tolerance, many factors may also help to colonise vibrios in the intestine. Their efficiency to thrive competitively in the micro environment of the intestine is the important reason for the preponderance. So far no such studies were reported to evaluate the competitive nature of vibrios explaining their interactive growth. Such experiments possibly spell out the mechanisms favouring the flourished growth of vibrios in the intestinal micro-environment.

However, a few researchers reported the interactive nature of the vibrios in the natural environment. Intercommunication and stimulation of physiological and biochemical activities in bacterial population was not uncommon (Kelly *et al.*, 1995). Cross species control of bacterial metabolism was first described in *V. harveyi* where regulation of bio-luminescence by biological factors were reported (Bassler *et al.*, 1997). Spragg *et al.* (1998) found

that the production of anti-microbial compounds can be induced by the presence of terrestrial bacteria.

2.3. Pathogenicity of *Vibrio* isolates

The genus *Vibrio* causes diseases to human and animals. At present, 12 *Vibrio* species are reported to be associated with human infections (Dalsgaard, 1998). This includes *Vibrio cholera*-01, the most dreaded pathogen ever known to man kind even though this organism (Anon., 1995).

The first of the non-cholerae *Vibrio* to be widely recognised as human pathogen was *V. parahaemolyticus* (Joseph *et al.*, 1983). It is the major etiological agent of sea-food borne gastroenteritis. Another important organism is *V. vulnificus*, which is described as 'the monster of the deep' by Morris (1989) and is concerned with three types of diseases in human: primary septicaemia, gastroenteritis and wound infections (Nakafusa *et al.*, 2001; Klontz *et al.*, 1988). This bacterium is significant for its high virulence potential and mortality rate (Hlady and Klontz, 1996; Hlady, 1997). Several other *Vibrio* species have been involved in diarrhoeal diseases, namely, *V. mimicus, V. fluvialis I, V. fluvialis II, V. furnissii, V. metschnikovii* (Elliot *et al.*, 1995). *Vibrio damsela and V. alginolyticus* were often associated with wound infections (Morris and Black, 1985). Pathogenic potentials of some of these *Vibrio* species had been reviewed in depth by Blake *et al.*, (1980), Joseph *et al.*, (1983) and West and Colwell (1984).

Vibrios are also associated with high mortalities in aquacultured and wild animals. Literature surveyed on the *Vibrio* infections in different aquatic animals are summarised in Table 5. Lightner (1983) reported cent per cent mortality in culture systems due to vibriosis. Animal infections by vibrios were reviewed by Lightner (1988), Austin and Austin (1993), Hjethness and Robert (1993) and Otta *et al.* (1998). Mathew (1996) highlighted the significance of

vibriosis in shrimp culture systems and pointed out as a major threat to the shrimp culture industry in Kerala.

From the scrutiny of literature available on the toxigenic characterisation of species like *V. vulnificus* (Oliver, 1989; Moreno and Landgraf, 1998) *V. parahaemolyticus* (Twedt, 1989; Nishibuchi, 1999), *V. harveyi* (Liu *et al.*, 1996) and *V. alginolyticus* (Balebona *et al.*, 1998), it was evident that an array of characters have been implicated as contributing factor for the pathogenicity. Scoglio *et al.*, (2001) stated that possible virulence factors for vibrios included extracellular enzymes, haemolysins, cytotoxins and adherence ability. Information on the contribution of each of these factors to the pathogenicity of various *Vibrio* species has been discussed here.

2.3.1. Production of extra-cellular cytolysins - cytotoxins.

Sakazaki *et al.* (1968) reported that the Kanagawa phenomenon, the ability of *V. parahaemolyticus* to cause haemolysis in Wagatsuma agar, was associated with gastroenteritis illness. The properties of thermostable direct haemolysin (tdh) responsible for the phenomenon are well elucidated (Joseph et al., 1983; Nishibuchi and Kaper, 1985; Nishibuchi, 1999). Haemolysin production by clinical as well as environmental strains from Indian coastal water were also studied (Karunasagar, 1981; Malathi *et al.*, 1988; Karunasagar *et al.*, 1989). Haemolysins were related to virulence in many other *Vibrio* species also.

Kreger and Lockwood (1981) were the first to describe the production of toxin having haemolytic activity against mammalian erythrocyte in *V. vulnificus*. Gray and Kreger (1985) purified and characterised this toxin. They proved that environmental as well as clinical isolates of *V. vulnificus* showed haemolytic activity in Wagatsuma agar containing 3% sodium chloride. This was also substantiated by malathi *et al.* (1988) that haemolysin was a virulence factor

for *V. vulnificus*. Fan *et al.* (2001) stated that cytolysins produced by *V. vulnificus* confer them the pathogenic potential against mouse. This variable reports indicate that the clear picture of the actual mechanism of pathogenicity of important pathogens like *V. vulnificus* is deciphered out so far.

Enterotoxigenic factors have been characterised and purified for *V. mimicus* (Spira and Fedorka-Cray, 1984) and *V. hollisae* (Kothari and Richardson, 1987). Haemolysins and other toxins were proved to have role in the pathogenicity of *V. damsela* in fish (Sakata and Kawasu, 1992) and *V. harveyi* against tiger prawn, *Penaeus monodon* (Liu *et al.*, 1996). Wong *et al.*, (1993) reported haemolysins and cytotoxins from *V. parahaemolyticus*, *V. fluvialis* I, *V. fluvialis* II and *V. mimicus*.

2.3.2. Production of hydrolytic enzymes.

Role of hydrolytic enzymes in pathogenicity is well established. Oliver (1989) suggested that possible virulence factors, other than haemolysin and cytotoxin, included enzymes like protease, deoxyribonuclease (DNase), lipase, phospholipase, mucinase, chondroitin sulfatase, hyaluronidase, fibrinolysin and alkyl phosphatase. Information regarding the involvement of different enzymes in pathogenicity of the tropical *Vibrio* species is lacking, however, studies on chosen *Vibrio* species from temperate regions *viz. V. harveyi*, (Liu *et al.*, 1996), *V. alginolyticus* (Balebona *et al.*, 1998) *V. vulnificus* biotype I & II (Amaro *et al.*, 1992; Moreno and Landgraf, 1998) were reported.

2.3.3. Virulence determination in animal models.

A variety of animal models have been described for studying the pathogenesis of *Vibrio* infections. Routinely, bioassay for pathogenicity was recommended only for *V. vulnificus*. Poole and Oliver (1978) first studied the

Table 5 Different *Vibrio* species involved in disease of aquatic animals

Disease/Symptom	Animal	*Vibrio* species involved	Reference
Brown ring disease	*Ruditapes philippinarum*	*V. tapestis*	Borrego *et al.*, 1996
Brown ring disease	*Ruditapes philippinarum*	*V. pelagius*, *V. splendidus*	Paillard *et al.*, 1994
Juvenile oyster disease	*Crassostrea verginica*	*Vibrio* sp.	Lee *et al.*, 1996a
		V. anguillarum	Lee *et al.*, 1996b
Sluggish/Aneroxia	*Penaeus monodon*	*V. parahaemolyticus*, *V. damsela*	Ponnuraj *et al.*, 1995
Hitra disease	*Salmo salmon*	*V. salmonicida*	O'Halloran and Henry, 1993
Red appendage disease	*Penaeus* spp.	*V. parahaemolyticus*, *V. alginolyticus*	Zhao *et al.*, 1995
Red appendage disease	*P. chinensis*	*V. harveyi*, *V. natreigens*	Wang *et al.*, 1993
Cold water vibriosis	*Salmo salar*	*V. salmonicida*	Ellias, 1989 Griffiths, 1994
Septicaemia, Abdominal swelling	*Sparus aurata*	*V. alginolyticus*	Balebona *et al.*, 1998
Red spot disease, (ulcers, haemorrahage)	*Chanos chanos*	*V. anguillarum*	Huang, 1987 Tuang *et al.*, 1987
Intestinal necrosis	*Paralichthys olivaces*	*Vibrio* sp.	Masumura *et al.*, 1989
Melanised tissue	*P. indicus*	*V. harveyi*	Abraham and Manley, 1995
Winter ulcer	*Salmo salmon*	*Vibrio* sp.	Lunder *et al.*, 1995
Sea gull syndrome	*P. vennamei*	*V. parahaemolyticus*, *V. vulnificus*	Mohney *et al.*, 1994
Shell disease	*Homarus americanus*	*Vibrio* sp.	Prince *et al.*, 1993
opaque necrosis	*P. japonicus*	*V.damsela*,	Chiang *et al.*, 1992
	P. monodon	*V.alginolyticus*, *V. vulnificus*	
necrosis	*P. monodon*	*V. harveyi*	Jiranvanichpaisal *et al.*, 1994
Haemorrhagic septiceamia	fresh water fishes	*V. fluvialis*	Xu *et al.*, 1993
Ulcers	*Anguilla anguilla*	*V. vulnificus biotype 2*	Amaro *et al.*, 1992
Gill disease	*Scophthalmus maximus*	*V. alginolyticus*	Austin *et al.*, 1993
Systemic disease	*Procambarus clakii*	*V, mimicus*, *V. cholerae*	Thune *et al.*, 1991
Brawn spot shell disease	*P. chinensis*	*V.pelagius*, *V. alginolyticus*	Yang *et al.*, 1992
Septicaemia	*Carassius auratus*	*V. cholerae non-01*	Reddacliff *et al.*, 1993

pathogenicity of *V. vulnificus* in animal models and reported lethal infections in 17 of 18 animals challenged, with some animals dying within 5h. post injection. They also described the importance of rat and rabbit legated ideal loop technique in the study of enteropathogenicity. US-FDA Analytical Manual suggested iron overloaded mice models to differentiate virulent and non-virulent strains as iron is the limiting factor for the pathogenicity of *V. vulnificus* (Elliot *et al.*, 1995).

Lethal dose- 50 (LD_{50}) values were determined mainly using mice and also by other susceptible animals. Many researches have reviewed LD_{50} value for *V. vulnificus* (Oliver, 1989; Moreno and Landgraf, 1998) *V. parahaemolyticus* (Joseph *et al.*, 1983), *V. harveyi* (Liu *et al.*, 1996) and *V. alginolytius* (Balebona *et al.*, 1998). Cytolysin was proved to be the major virulence factor responsible for mice mortality (Fan *et al.*, 2001).

2.3.4. Other virulence factors

Bacterial adherence to mucosal epithelial surface is considered as an important determinant in the pathogenesis of infectious disease of many bacterial species in humans (Beachey, 1981). The most of the pathogenic strains of *V. vulnificus* and *V. anguillarum* possess this ability (Balebona *et al.*, 1995). Several studies have sought evidence of the adhesive capacity of virulent strains of *V. parahaemolyticus* (Hackery *et al.*, 1980) and *V. vulnificus* (Reyes *et al.*, 1985; Amaro *et al.*, 1992). *Vibrio fluvialis, V. parahaemolyticus and V. alginolyticus* were proved to exhibit adhesive ability with aggregative pattern in cell lines (Scoglio *et al.*, (2001)..

Iron might have an important role in *V. vulnificus* pathogenicity towards man. This is evident from the fact that *V. vulnificus* infections is often reported in patients with diseases involving iron metabolism (Oliver, 1989). Wright *et al.* (1981) observed a strong correlation between serum iron levels and

the inoculum size of *V. vulnificus* required to cause mortality in mice. Furthermore, the author also stated that the iron was a limiting factor for the growth and survival of *V. vulnifius* in normal human serum of healthy individuals. Siderophore mediated iron acquisition mechanism was described in *V. vulnificus* biotrype II (Boisca *et al.*, 1990).

The correlation between colony morphology and virulance was reported for *V. vulnificus* (Simpson *et al.*, 1987; Boisca *et al.*, 1993). It was reported that the presence of capsules in *V. vulnificus* biotype-I was related to virulance (Yoshida *et al.*, 1985).

The impact of viable but non-culturable (VBNC) state of *V. cholerae*-01 on the epidemolgy of cholera was established by demonstrating the presence of dormant cells of the bacterium in endemic area in between epidemic periods (Brayton *et al.*, 1987; Xu *et al.*, 1983; Rozak and Colwell, 1987). Studies on experimental animals have indicated that VBNC toxigenic *V. cholerae-01* retained virulence and induce fluid accumulation in ligated ilial loop preparations (Colwell *et al.*, 1985). Similar persistent occurrence for *V. vulnificus* was reported (Oliver *et al.*, 1991). According to the Boisca *et al.* (1996) the dormant cells of *V. vulnificus* biotype II is not virulent for eels and mice, and only culturable cells maintained virulence.

Many of the surveillance studies on marine vibrios point towards the risk of these organisms as a pathogen. The epidemiological studies by Desenclus *et al.* (1991), Bean and Goulding (1997) and (Shaparío *et al.*, 1998) revealed that the information on the role of vibrios as the causative agent of food borne diseases was vague and confusing, particularly from tropics. The status of *V. vulnificus* and related vibrios as a sea food pathogen is seldom discussed in tropical countries where the warm climate can predispose the infection.

Information on the toxigenic characterisation of many of these pathogens common in the tropical water is also lacking. From India, the studies were mainly confined to a few selected species of *Vibrio* and that too as animal pathogens. (Pradeep, 1986; Mathew, 1996).

2.4. Spoilage potential of *Vibrio* isolates

The emphasis of bacteriological studies of fishery science are mainly given to hygiene and sanitation apart from spoilage. However a few reports signifies the *post mortem* spoilage of fish (Surendran, 1980; Hobbs and Hodgkiss, 1982; Surendran and Gopakumar, 1985; Chandrasekharan *et al.*, 1985; Thampuran, 1987). Quality deterioration and loss due to improper preservation of freshly caught fishes is a major concern in tropics. In retail marketing, the low value fishes like Oil sardine and Mackeral, which form the staple animal protein source of our nation, met with a loss 10-15% before it reaches consumer (Surendran *et al.*, 1989).

2.4.1. Role of bacteria in spoilage

Quality deterioration of seafood is mainly by the activity of bacteria (Sikes and Maxcy, 1979; Surendran and Gopakumar, 1985; Kim *et al.*, 1999) and also by autolysis (Dingle *et al.*, 1977). The initial research efforts to elucidate the role of bacteria in spoilage was reviewed by Shewan (1961). The bacterial flora associated with the fresh fish and selection occurring during the preservation practices ultimately determine terminal the spoilage flora. During the storage of tropical fish in ice, there was a selection of bacterial types and by the time of incipient spoilage only one or two species of bacteria constituted the entire flora (Surendran *et al.*, 1989).

In low temperature preservation, psychrophilic or psychrotrophic organisms were the important agents of spoilage (Surendran and Gopakumar, 1981; 1982; Poulter *et al.*, 1985). The genus *Pseudomonas* was the major spoilage bacteria as they dominate the spoilage flora of temperate marine fish (Shewan, 1977) and, to some extend on tropical marine fish (Surendran and Gopakumar, 1985; Chandrasekharan *et al.*, 1985; Shetty *et al.*, 1992). The other genera flourished during spoilage were *Moraxella, Acinetobacter, Flavobacterium* (Shetty *et al.*, 1992). However little attention has been given to understand the role of other bacterial genera like *Vibrio*, in the spoilage of tropical fish in particular.

2.4.2. Role of *Vibrio* in spoilage

Vibrios constituted a considerable portion of the bacterial flora of both fin fishes (Surendran *et al.*, 1989; Cahill, 1990; DePaola *et al.*, 1994; Grisez *et al.*, 1997) and shellfishes (Surendran and Gopakumar, 1982; Chandrashekharan *et al.*, 1987; Montilla *et al.*, 1994). All the members of the native flora might not be involved in spoilage (Markarios-Laham and Lee, 1993). Extent of spoilage is depended on many factors like composition and activity of natural flora, temperature, pH drop during rigor mortis, type of the fish and post harvest handling practices .

In ambient temperature vibrios constituted the major spoilage bacterium for fin fish (Surendran and Gopakumar, 1985) and that of shellfishes (Chandrasekharan *et al.*, 1985). A few reports revealed their spoilage role. At 8±1^{0}C, 25-50% and at 1±1^{0}C less than 25% of the *Vibrio* strains showed spoilage capacity (Surendran and Gopakumar, 1985). Chandrasekharan *et al.* (1985) observed abundance of vibrios in raw and unprocessed prawn, *Penaeus indicus* stored at -18^{0}C. In their study *Vibrio* dominated among the tested genera in some

instances at 4°C and -18°C. Shetty *et al.* (1992) demonstrated that *Vibrio* species isolated from fish preserved at low temperature, were biochemically very active. However, *Vibrio* is highly cold sensitive (Thampuran and Gopakumar, 1993) and does not pose any health problem. This type of limited reports warns the role of vibrios as a potent group involved in the spoilage of seafood at room temperature as well as under the conditions of temperature abuse which are not uncommon in the marketing, storage and transportation in inland trade. The psychrophilic nature of the *Vibrio* species (Markarios-Laham and Traxler, 1991; Wong *et al.*, 1994) and their ability to survive in low temperatures necessitates the need of a detailed study on the spoilage potential of various *Vibrio* species.

2.4.2.1. Survival of *Vibrio* species in low storage temperatures

Vibrios were reported to be sensitive to low temperature. At frozen temperatures, -39±2°C and -20±2°C, the sensitivity of the bacterial genera was in the order of *Vibrio*> *Bacillus*> *Psuedomonas*> *Moraxella*> *Acinetobacter*> *Flavobacterium*> *Micrococcus* (Thampuran and Gopakumar, 1993). However, *Vibrio* species remained viable in fish muscle medium for months at -39±2°C. But, they did not survive for more than three months in other suspending fluids like normal saline and sea water. Cell elimination in this case occurred mostly in the first 24 h., thus freezing menstruum and freezing period remained critical for the survival of bacteria.

Vibrio vulnificus survive poorly below 8.5°C (Kasper and Tamplin, 1993) and the minimum temperature for *V. parahaemolyticus* in natural environments 10°C (Kaneko and Colwell, 1978). A rapid and dramatic decrease in *V. vulnificus* cells was observed during low temperature storage (0.5 to 22°C), when the oysters were shucked prior to storage (Murphy and Oliver, 1995; Cook, 1997). The rapid decrease was explained due to viable but non-culturable

(VBNC) state. Weichart and Kjelleberg (1996) indicated that in spite of the apparent absence of recovery under a wide range of laboratory conditions, VBNC cells underwent changes at low temperature. This potentiality allow them to persists for extended periods. Moreover in prolonged incubation, these cells exhibited sonication resistance, resistance against exposure to ethanol and mechanical stress.

Many mechanisms were put forth to explain this resistance. Role of 'cryoprotective' proteins were explained for *V. cholarae* (Faming *et al.*, 1993) and *V. vulnificus* (Paludan-Mueller *et al.*, 1996). This persistent occurrence is significant as it affects the safety of the food and possible reversion to viable state in cases of temperature abuse common to the retail marketing. This persistent survival again rendered the low temperature preservation less preferable to methods like irradiation, use of preservatives *etc.* Recently, consumers advocates for minimally processed foods that retains the appearance, flavour, texture and nutritional qualities of raw or fresh food.

2.4.2.2. Elimination of *Vibrio* in elevated temperature

Cook and Ruple (1991) described that mild heat treatment and later storage in low temperature were more acceptable from a perceptive and safety point of view. Decimal reduction time for *V. vulnificus* at 47^{0}C was 78 seconds (SD ± 30 sec.) and D_{50} value of 39.8 seconds (SD ± 12.2 sec.) Cook and Ruple (1992). They also stated that heating oysters for 10 min. in water at 50^{0}C was adequate to reduce *V. vulnificus*. Literature survey revealed that most of the heat sensitivity studies were confined only to the elimination of limited pathogenic strains of *V. parahaemolyticus* and *V. vulnificus*. Other vibrios like *V. harveyi* and *V. alginolyticus* wre abundant in the sea foods of this area (Sanjeev *et al.*, 2000) have received very little attention by researchers.

2.4.2.3. Production of hydrolytic enzyme by *Vibrio* and their role in spoilage

Chandrasekharan (1985) has explained the role of various hydrolytic enzymes in the spoilage of prawn, *Penaeus indicus*. Proteolysis, lipolysis, ureolysis and amylolysis were reported to be the major events during spoilage. *Vibrio* spp. exhibited high casienase , gelatinase and chitinase activity among the spoilage flora (Chen, *et al.*, 1991). Deterioration of the quality of fresh fishery products during storage is essentially attributed to the build up of bacterial protease (Nair and Lahiri, 1968; Herbert *et al.*, 1971; Venugopal and Lewis, 1985). About 85% of the vibrios were reported to be spoilers as assessed by the production of protease, lipase and amylase reduction of TMAO to TMA, off odour production in flesh broth and holozone formation in flesh agar (Chandrasekharan *et al.*, 1987). Shetty *et al.* (1992) have assessed the spoilage potential of *Vibrio* strains and found that they are very active as per the traits like gelatine hydrolysis, ammonia production, indole production, H_2S production DNase production, phosphatase activity, hydrolysis of sugars, decarboxylation of aminoacids and TMAO reduction.

In the course of fish spoilage, bacteria first utilises low molecular weight components like amino acids and other non-protein components. The proteolysis thus become an event of advanced spoilage (Jay, 1978). Philip and Lakshmanaperumalsamy (1992) compared the protein degradation and growth of *Vibrio* species in fish, prawn and clam flesh media and casein medium. In all these media protein degradation commenced on third day and progressed rapidly during following days.

Extra cellular protease from the marine fish bacteria, which could degrade actomyosin at 5^0C was isolated and characterised. Psychrophilic *Vibrio*

species which could hydrolyse protein at 4°C and -20°C was reported by Markarios-Laham and Lee (1993). Protease degrading Mackerel actomyosin at 0-2°C was identified (Venugopal and Lewis, 1985).

Among the population of *Vibrio* in the environment of Cochin, 82.12% was reported to be amylolytic (Saramma *et al.*, 1994). *Vibrio parahaemolyticus* and *V. vulnificus* were potent producers of amylase. The optimum culture condition for these organisms were pH 7.0 and 30°C. Amylase production was found to begin at the early logarithmic phase and continued till they entered stationary phase.

Biogenic amines are also formed by the action of bacterial decarboxylase on precursor aminoacid mainly in proteinaceous food such as meat or fish (Maga, 1978; Leisner *et al.*, 1994). Presence of histamine in this kind of food can be used as an index of hygienic quality (Dacher and Simrad, 1985). Consumption of food with high amount of histmine has been involved in a food borne disease called 'scomberotoxin' or histamine food poisoning (Taylor, 1986). Toxicological effect is usually slight in healthy individuals, however, some circumstances such as presence of mono amine oxidase inhibitors or alcohol or the presence of other cadaverine or putrcine can potentiate the toxicity (Shakila, *et al.*, 1999).

Hydrolysis of sulphur containing aminoacids result in the production of H_2S , which contribute to the off odour of spoiled fish (Thampuran and Iyer, 1990). Cent percent of the tested *Vibrio* species isolated from chilled fish produced H_2S (Shetty *et al.*, 1992).

Polyphosphates are good preservatives (Chandrasekharan *et al.*, 1998) and also prevent autolysis of the food (Warrier *et al.*, 1985). Bacterial phosphatases can mineralise the non-available phosphates, this in turn reduces

preservative efficiency (Salim and Purushothaman, 1998). They also reported a high phosphatase activity in vibrios. Most of the *Vibrio* species isolated from the marine environments were capable of phosphatase activity (Venketeswaran and Natarajan, 1983; Eapen *et al.*, 1993). *Vibrio alginolyticus* was the dominant phosphatase producing bacteria in the gills and intestine of fishes (Nayak and Panda, 1998).

Materials & Methods

3. Materials and Methods

3.1. Materials

3.1.1. Media

Media used in the study were categorised as dehydrated and those compounded in the laboratory using analytical reagent grade ingredients. Ingredients for the compounded media such as peptone, tryptone, yeast extract, beef extract, *etc.* were from Oxoid (England) and Difco Laboratories (USA). Agar agar powder was procured from Hi-media Chemicals (India).

Other inorganic chemicals used were obtained from Sigma (USA), Merck (Germany), BDH (England) and Sisco Laboratories (India) and were of analytical reagent grade. Dyes and indicators used were from BDH (England) and Hi-media Chemicals (India).

3.1.1.1. Dehydrated media

Dehydrated media used were of the brand Oxoid (England) and Difco Laboratories (USA). For studies on marine bacteria, the media were supplemented with 3% (w/w) sodium chloride, unless specified separately. Three percentage sodium chloride was opted after comparing the growth in different salt levels and also to simulate the salinity of the sea off Cochin. Dehydrated media used in the study were listed below.

Baird Parker medium (BP)

Brain heart infusion broth (BHI)

Brain heart infusion medium (BHA)

Christensen's Urease Agar (CUA)

Eijekman coliform medium (EC)

Eosine methylene blue medium (EMB)

McConkey broth (MB)

Muller-Hinton medium (MHA)

Nutrient agar (NA)

Simmon's citrate medium (SCA)

Thiosulphate citrate bile salt medium (TCBS)

Trypticase soy agar (TSA)

Trypticase soy broth (TSB)

Violet red bile glucose medium (VRBGA)

3.1.1.2. Compounded media & its composition

Amino acid decarboxylase medium

Yeast extract, 3.0g; glucose, 1.0g; bromocresol purple, 0.016g; distilled water, 1000ml. pH 6.9. Hydrochlorides of respective amino acids were added at the rate of 5g l^{-1}. Dispensed in to 3ml aliquots and sterilised at 115^{0}C for 20 min. Amino acids used were lysine, ornithine, arginine and histidine.

Basal medium for carbon utilisation study.

Solution A : Ammonium chloride, 10g; ammonium nitrate, 2g; sodium sulphate, 4g; dipotassium hydrogen phosphate, 6g; potassium dihydrogen phosphate, 2g; sodium chloride, 20g.

Solution B: Hydrated magnesium sulphate, 0.2g; magnesium chloride, 0.8g; purified agar, 30 g.

Carbon sources used in the study were amygdalin, α-ketoglutarate, D- glucose amine, D-glucose, L-arabinose, lactose and melibiose

Sterilised each solution separately by autoclaving at 115^{0}C for 20 min., cooled to 50^{0}C and mixed thoroughly before addition of filter sterilised aqueous solution of carbon substrate to a final concentration of 0.1%(v/v). Filter sterilisation was done by cellulose nitrate filters of 2μm using sterile syringe.

Blood agar

To sterile and cooled basal nutrient agar (1000ml), added 50 ml human red blood cell suspension. Cell suspension was prepared by centrifuging 50 ml blood at 3000 rpm. for 45 min. and suspended the residue in 50ml physiological saline.

Brilliant green lactose broth

Peptone, 10g; lactose, 10g; bile salt No.3, 20g; brilliant green, 0.0133g; distilled water, 1000ml. pH adjusted to 7.4. Dispensed to 4ml quantities with Durahm's tube and sterilised at 115^{0}C for 20 min.

Cystein- peptone broth

Peptone, 10g; cysteine hydrochloride, 0.1g; sodium sulphate, 0.5g; sea water (aged and filtered), 1000ml. pH 7.2. Sterilisation at 1.05 kg cm $^{-2}$ for 15 min.

Egg yolk medium

Basal nutrient agar, 1000ml; Egg yolk emulsion, 10% (V/V)

Egg yolk emulsion was prepared by mixing egg yolk with sterile distilled water in 1:1(w/v) proportion.

Esculin broth

Tryptone, 10g; sodium chloride, 10g; potassium chloride, 1,0g; magnesium chloride, 4g; esculin, 1g; ferric citrate, 0.05g; distilled water, 1000ml. pH adjusted to 7.0. Sterilisation at 115^{0}C for 20 min.

Fish muscle homogenate

Fish muscle homogenate was prepared as per Matches *et al.* (1971). A lot of 150g of the Indian mackerel *(Rastrelliger kanagurta)* muscle was weighed out and blended with 200ml aged sea water. More

sea water was added whenever necessary. This was made up to 1000ml. with sea water and transferred to a 2000ml. beaker. Boiled for 10 min. and 10 ml quantities were dispensed in test tubes and sterilised at 121^{0}C for 15 min.

Gelatin medium

Peptone, 4g; yeast extract, 1g; gelatin 15g; sodium chloride, 10g; agar agar, 15g; distilled water, 1000ml. pH 7.2. Sterilisation at 115^{0}C for 20 min.

Glucose salt tryptone broth

Beef extract, 3g; Peptone, 10g; sodium chloride, 30g; dextrose, 5g; methylviolet, 0.002g; teepol, 4ml; distilled water, 1l. pH adjusted to 7.4. Sterilisation at 121°C for 15 min.

Hugh-Leifsons medium

Peptone, 10g; sodium chloride, 5g; dipotassium phosphate, 4g; dextrose, 10g; agar, 3g; distilled water, 1000ml. Dissolved, adjusted pH to 7.1 and added 1ml of 0.1% solution of phenol red indicator, Dispensed in 8ml quantities in narrow tubes of 15x12cm and sterilised at 115^{0}C for 20 min.

Kenner- Fecal medium (KF Medium)

Peptone, 10g; yeast extract, 10g; sodium chloride, 5.0g; sodium glycerophosphate, 10g; maltose, 20g; lactose, 1g; sodium azide, 0.4g; bromocresol purple, 0.016g; agar agar, 15g; distilled water, 1000ml.

pH adjusted to 7.2 and autoclaved as 100ml quantities at 0.7 kg cm^{-2} gauge pressure for 20 min. Before pouring, cooled to 45^{0}C and added 1 ml of 1% (w/v) triphenyl tetrazolium chloride solution.

Media for testing luminescence

Nutrient agar, 25g; sodium chloride, 17.5g; magnesium chloride, 4g; potassium chloride, 1g; agar agar, 12g, distilled water, 1000ml. Sterilisation at 121^{0}C for 15 min.

Milk agar

Skimmed milk powder, 100g. dissolved in 500ml distilled water; agar agar, 20g, dissolved in 500 ml distilled water. Sterilised separately at 115^{0}C for 20 min. and cooled to 50^{0}C and mixed well before plating.

Nitrate broth

Peptone, 10.0g; potassium nitrate, 1.0g; sodium chloride, 5.0g; distilled water, 1000ml; pH 7.1±0.1. Sterilisation at 120^{0}C for 15 min.

Phosphatase medium

Nutrient agar, 1000ml; phenolphthalein diphosphate, 0.1g. Phenolphthalein diphosphate was added to cooled sterile nutrient agar before plating.

Starch agar

Basal nutrient agar, 1000ml; soluble starch, 2% (w/v). Sterilisation at 121^{0}C for 15 min.

Sugar fermentation broth

Peptone, 10.0g; yeast extract, 3.0g; bromocresol purple, 0.016g; distilled water, 1000ml; sodium chloride, 0.5g. pH, 7.2. Sterilisation at 115^{0}C for 20 min. Arabinose, arbutin, inositol, mannitol, salicin, sorbitol, sucrose and cellobiose were the sugars tested. Sugar were added at the rate of 1%. In the case of disaccharides and oligosachharides, filter sterilised solution was added to the previously sterilised broth. To detect gas production, the above described method with inverted Durham's tube was used.

Tryptone broth

Tryptone, 10g, dissolved in 1000ml. distilled water and sterilised at 121^{0}C for 15min. pH adjusted to 7.1.

Tween-80 medium

Peptone, 10g; sodium chloride, 10g; calcium chloride, 0.1g; agar agar, 20g; distilled water, 1000ml; Tween-80 (polyoxy-ethelylene sorbitan mono-oleate), 10 ml., pH 7.4. Sterilised by 115^{0}C for 20 min.

Voges- Proskauer medium

Peptone, 5.0g; D-glucose, 5.0g; dipotassium hydrogen phosphate, 5.0g; distilled water, 1000ml; pH, 6.9±0.2. Sterilisation at 115^{0}C for 20 min.

3.1.2. Samples for analysis

Water, sediment, plankton, fin fishes and shell fishes were sampled in the study. Details regarding the samples are given below.

3.1.2.1. Water

Water samples were collected in sterile wide mouthed bottles from a depth of 10 to 15 M. Samples were collected during the cruises of the departmental vessel, Matsyakumary, from different sites of the open sea off Cochin (lat. 9^{0}25' and 10^{0}10'; long. 76^{0}13' and 76^{0}30'). A total of 27 samples were collected from different sampling stations as given in Table 6 and brought to laboratory under ice.

3.1.2.2. Sediment

Sediment samples were collected using Peterson's grab. Samples were collected from the each of the first 12 stations mentioned

in Table 6. From the central portion of the collected sediment samples, about 100g portion was transferred aseptically into a fresh polythene bag and transported to the laboratory in ice box for analysis.

3.1.2.3. Plankton

A total of 18 samples of zooplankton were collected using a Bongo net of 200μm sieve size. Net was operated horizontally at a constant speed of 20 min. The operation of the net was started from the first 18 sampling stations listed in Table 6. A portion of the plankton samples were transferred aseptically into bottles containing sterile sea water for bacteriological analysis. Other portion of the sample was preserved in 10% neutralised formalin for the identification of major component species.

3.1.2.4. Fin fishes

Part wise samples *viz.* skin and muscle, gill and intestine and composite samples comprising whole body parts were analysed in the study. Fishes which were analysed partwise are categorised as fresh samples (Table 7.A) and market samples (Table 7.B). Fresh samples included those collected onboard the departmental research vessel (Matsykumai, Central Institute of fisheries Technology, Cochin) and those collected freshly from fishing boats at the time of landing. Market samples were obtained from markets in and around Cochin. Samples represented the eighteen different locally available edible fish species. The fishes which were sampled composite for the study of *Vibrio*-indicator relationship were described in Table 7.C. Prey fishes for the present

study were collected from the Chinese dip net at the near shore region of Fort Cochin. Five different species were analysed (Table 7.D).

For the seasonal study on the variations of flora, *Sardinella longiceps* and *Arius dussumeirri* were selected as they were available all through the year. Samples were collected in the first week of every month from October, 1998 to September, 1999, from Fort Cochin fish landing centre.

3.1.2.5. Shell fishes

Muscle and intestine of six commonly available prawn species were analysed separately as in Table 8. Composite samples of clam (shucked), mussel and lobster were also included in the study. These samples were obtained from the Fort Cochin fish landing centre.

3.1.3. Bacterial strains

Reference bacterial strains included in the study comprised those received from type culture collection of ATCC (American type culture collection, Maryland, USA) or NCIMB (National Collection of Industrial and Marine Bacteria, Torry Research station, UK) and also NCAFB (National collection of aquatic and fish bacteria, Central Institute of Fisheries Technology, India). Type cultures included in the study were described in Table 9. Selected *Vibrio* species isolated in the course of study were used in the study. From a total of 2000 isolations, 799 representative cultures belonging to 26 different *Vibrio* spp. were selected for biochemical characterisation studies. For hydrolytic enzyme detection

Table 6. Locations of the sampling stations for the collection of water, sediment and plankton samples.

Station No.	Location
1	9°58.7'N 76°10.8'E
2	10°0.1'N 76°0.9'E
3	9°58.7'N 76°0.09'E
4	10°0.01'N 76°0.09'E
5	9°58.1'N 76°11.2'E
6	10°0.5'N 76°0.6'E
7	9°58.1'N 76°0.3'E
8	9°58.5'N 76°10.7'E
9	10°0.2'N 76°0.7'E
10	N off Cochin 340°E-W
11	2.5 km N off I station 340°E-W
12	2.5 km S off II station 340°E-W
13	2.5 km S off III station 200°E-W
14	6 km S off IV station 190°E-W
15	6 km S off V station 190°E-W
16	180° 13 NM off Barmouth
17	180° 2.5 NM off Station I
18	350° 2.5 NM off Station II
19	045° 3.5 NM off Station III
20	045° 3.5 NM off Station IV
21	070° 1.7 NM off Station V
22	180° 12 NM off Barmouth
23	180° 3 NM off Barmouth
24	180° 2 NM off Barmouth
25	180° 4 NM off Barmouth
26	180° 2.5 NM off Barmouth
27	180° 5 NM off Barmouth

Table 7.A. Fish samples collected from various fish markets for the bacteriological observation of different body parts.

Sl.No.	Common name	Scientific name	Habitat	No. of samples
1	Sea cat fish	*Arius dussumieri*	Demersal.	3
2	Russels scad	*Decapterus russellii*	Pelagic	3
3	Little tuna	*Euthynnus affinis*	Pelagic	3
4	mojarras	*Gerres filamentoses*	Demersal.	3
5	Whip tail sting ray	*Himantura bleekeri*	Demersal.	3
6	Giant perch	*Lates calcarifer*	Demersal.	2
7	Red snapper	*Lutjanus malabaricus*	Demersal.	3
8	Grey mullet	*Mugil cephalus*	Pelagic	3
9	Thread fin bream	*Nemipterus japonicus*	Demersal.	3
10	Big eye scad	*Selar crumenophthalmus*	Pelagic	2
11	Mustached anchovy	*Thryssa mystax*	Pelagic	3
12	Sardine	*Sardinella* sp.	Pelagic	2

Table 7.B. Fish samples collected onboard and sampled freshly from landing centers for the bacteriological observation of different body parts.

Sl.No.	Common name	Scientific name	Habitat	No. samples
1	Croaker	*Johnius dussumieri*	Demersal.	2
2	Indian mackeral	*Rastrelliger kanagurta*	Pelagic	3
3	Oil sardine	*Sardinella longiceps*	Pelagic	3
4	Seer fish	*Scomberomerus commerson*	Pelagic	3
5	Thread fin bream	*Nemipterus japonicus*	Demersal.	3
6	Alligator gar	*Strongylura strongylura*	Pelagic	3

studies, 160 cultures belonging to17 different *Vibrio* species were included.

3.1.4. Experimental animals

For mouse lethality studies, Albino Wester mice maintained in the animal house of Central Institute of Fisheries Technology, Cochin, were used.

3.2. Methods

3.2.1. Methods for ecological study of *Vibrio*

3.2.1.1. Analysis of samples

3.2.1.1.1. water

Sea water samples were analysed for physico-chemical and bacteriological parameters.

Physico-chemical parameters

Temperature, pH, salinity and dissolved oxygen of water samples were noted. Temperature was determined *in situ* with a mercury thermometer. The sample for the estimation of dissolved oxygen was fixed and brought to the laboratory for the analysis following Winkler's method (APHA, 1976). pH and salinity were determined in the laboratory by digital pH meter (Digison Electronics, Chennai) and Mohr's argentometric titration method (Strickland and Parsons, 1972) respectively.

Table 7C Fish samples collected from various fish markets for evaluating the relationship of vibrios to indicator bacteria (composite sampling).

Sl.No.	Fish species		No. of samples
	Common name	Scientific name	
1	Whip tail sting tay	*Himantura bleekeri*	3
2	Tuna	*Thunnus thunnus*	4
3	Pig face breams	*Lethrinus spp.*	4
4	Tilapia	*Tilapia mossabicus*	3
5	Pearl spot	*Etroplus suratensis*	3
6	Indian mackeral	*Rastrelliger kanagurta*	4
7	Oil sardine	*Sardinella longiceps*	3
8	mojarras	*Gerres filementosus*	3
9	Reef cod	*Epinephelus sp.*	2
10	Red snapper	*Lutjanus sp.*	2
11	Barra cuda	*Sphyrena obtusa*	3
12	Giant perch	*Lates calcarifer*	3
13	Mustached Anchovy	*Thryssa mystax*	2
14	Russels scad	*Decapterus russellii*	2
15	Common sprat	*Dussumeiria acuta.*	3
16	Horse mackeral	*Melaspis cordyla*	3
17	Grey mullet	*Mugil cephalus*	4

Table 7D Prey fishes collected from Chinese dip net for the analysis of diet-intestinal flora relationship.

Sl.No.	Common name	Scientific name	No. samples
1	Commersons anchovy	*Anchoviella commersonii*	3
2	Anchovies	*Stolephorous sp.*	4
3	Silver bellies	*Leognathus sp.*	3
4	Glassy perchlet	*Ambasis commersonit*	3
5	Cat fish	*Arius dussumeiri*	2

Table 8 Shellfishes sampled for the bacteriological observations during the present study.

Sl.No	Species		
	Local name	Scientific name	No. of samples
1	Kiddi shrimp*	*Parapenaeopsis stylifera*	6
2	Indian white prawn*	*Penaeus indicus*	4
3	Brown prawn*	*Metapenaeus monoceros*	3
4	Tiger prawn*	*Penaeus monodon*	4
5	Brown shrimp*	*Metapenaeus dobsonii*	4
6	King prawn*	*Metapenaeus affinis*	4
7	White clam	*Meretrix casta*	4
8	Green mussel	*Perna viridis*	5
9	Black clam	*Villorita cyprinoides*	4
10	Brown mussel	*Perna indica*	4
11	Lobster	*Panulirus homarus.*	2

*Samples used in part wise study

Table 9. List of reference bacterial strains used in the study.

Species*	Reference number
V. vulnificus	NCIMB 2046
V. alginolyticus	NCIMB 1903
V. fluvialis	NCIMB 2249
V. parahaemolyticus	ATCC 17802
V. harveyi	ATCC 14126
V. mimicus	ATCC 33653
V. furnissii	ATCC 35016
V. parahaemolyticus	**vp42, *vp31, *vp159, vp155, vp149
V. alginolyticus	*va139, *va49, *va147, va134, va302
V. vulnificus	*vv242, *vv70, *vv296, vv69, vv50
V. harveyi	*vh264, *vh318, *vh77, vh113, vh105
V. mimicus	*vm63, *vm38, *pw15, vm99, vm144

** NCAFB (National collection of aquatic and fish bacteria, Central Institute of Fisheries Technology, India).

* Strains used for the in vitro studies on physicochemical parameters, frozen storage studies, heat elimination studies etc.

Bacteriological analysis

Quantitative estimation total *Vibrio*

Water samples were analysed for total halophilic bacterial count (THC) and total *Vibrio* count (TVC). TSA supplemented with 3% sodium chloride (termed, Trypticase soy salt agar, TSSA) was used for determining THC based on preliminary studies comparing the growth in other media like SWA and NA. Both the media were found inferior as the former favoured spreading and the latter exhibited reduced recovery potential. Three percent sodium chloride in distilled water was the diluent of choice. Samples were blended in stomacher 400 (Seward Medical, U.K.) for one minute. Pour plating method was adopted with serially diluted sample. Plates were incubated for 48h. at room temperature (28 ± 2^0C).

Total *Vibrio* count was determined by direct surface plating on TCBS medium. In the present study, direct plate count was preferred to MPN method (though it is renowned as the sensitive method by Koh *et al.*, 1994b), because, even if in the present study, counts obtained by MPN method with GSTB and direct TCBS plating were statistically correlated, ($r = 0.72$, $p<0.05$), the magnitude was two to three logarithmic units lower in MPN method. Moreover this study is intended for the recovery of total *Vibrio* and estimation of individual species becomes difficult by MPN. Thus neglecting the intervention of *Aeromonas* and *Plesiomonas*, which accounted for about 0.15% of the total colonies forming in TCBS plates, direct plate count was opted in the present study.

An aliquot of 0.5ml was spread on surface dried TCBS plate with a sterile bend rod. TCBS medium was slightly modified to get a better result by reducing the sucrose concentration from 20g l^{-1} to 10g l^{-1} .

Plates with colonies 30 to 300 were counted and multiplied with dilution factor to obtain the colony forming units (cfu) per ml of the sample.

Qualitative estimation of *Vibrio* species

Form each plates about 20 to 30 representative colonies were picked randomly and identified into species as per the following section 3.2.1.3. Percentage prevalence of individual species among the isolates is estimated.

3.2.1.1.2. Sediment

Ten gram portions of the sediment were transferred aseptically into fresh Stomacher bags. Added 90 ml diluent and homogenised for one minute. Serial dilution were made and THC and TVC and percentage of individual species were determined as per the section 3.2.1.1.1

3.2.1.1.3. Plankton

Plankton samples were identified microscopically into major groups following the method of Pillai, (1986) (Table 10). Fresh samples were filtered through a previously weighed and sterile nylon net of mesh size 200μm and weighed in aseptic conditions. The whole content was

transferred to stomacher bag and made up to 100 ml with the diluent. Analysis was proceeded in the same manner as in 3.2.1.1.1

3.2.1.1.4. Fin fishes.

Prior to sampling, fishes were identified up to species level using the identification sheets of Fischer and Bianchi (1984). Skin surface with muscle, gill and intestine of 4-6 randomly selected fish were taken separately and pooled for analysis. Care was taken, while sampling the gills and intestines, to reduce surface contamination by swabing the body surface with 70% ethanol. Ten gram portions of each part were analysed for THC and TVC as described in section 3.2.1.1.1.

Prey fishes were analysed as finely cut whole samples for THC and TVC and percentage of individual species as per the section 3.2.1.1.1.

3.2.1.1.4.1. Study on the seasonal variation

An year round study on the seasonal variations of microflora was also conducted by analysing the skin with muscle, gill and intestine of oil sardine (*Sardinella longiceps)* and cat fish (*Arius dussumeir-i*) for THC and TVC following the procedure in section 3.2.1.1.1.

3.2.1.1.4.2. Analysis for indicator bacteria

Composite samples included equal portions of skin, muscle, gills and intestine. Bacteriological parameters like THC, TVC, total aerobic bacterial count (TAC), and indicator organisms like total coliforms, faecal streptococci, *Escherichia coli* and *Staphylococcus aureus* were determined to establish the relationship of vibrios to indicator organisms. THC and TVC were determined as described above (3.2.1.1.1.) and TAC was determined by pour plating on TSA. Total coliforms, faecal streptococci and *S. aureus* were enumerated in VRBGA, KF and BP media respectively as per the procedures out lined in US-FDA bacteriological analytical manual (1995). All the plates were incubated at 37^0C in incubator. Faecal coliforms and *E. coli* were counted by MPN method with MB (US-FDA manual, 1995). For confirmed total coliforms, a loopful of the culture from positive MPN tubes were inoculated to BGLB and incubated at $44.5\pm.5^0$C in serological water bath for 24h. Results noted as positives if there are growth and gas production and it is compared with 3 tube MPN table to obtain the confirmed total coliform count. Similarly, EC broth was used to determine MPN feacal coliforms and coliform bacteria which produced gas in EC broth and indole in tryptone broth at $44.5\pm.5^0$C are considered as the count of *E. coli.* Presence of *E.coli* was further confirmed by streaking on to EMB medium.

The counts obtained were attempted to correlated statistically among themselves as well as with *Vibrio* count.

Table 10. Composition of the plankton samples used in the present study.

Genera	Percentage occurrence in the samples						Total
	I*	II	III	IV	V	VI	
Coscinodiscus	74.36	87.93	74.73	19.83	44.83	21.21	55.74
Pyrophagus	3.85	--	--	--	--	--	0.22
Leptocylindrium	--	2.16	--	3.31	--	13.13	1.90
Nitzchia longisima	--	0.86	4.62	4.96	3.45	--	3.15
Siphonosphore	--	--	1.09	--	--	--	0.29
Cyclops	--	--	--	--	2.87	21.21	2.27
Calanus	16.67	4.31	10.87	8.26	37.36	33.33	18.00
Noctiluca	5.13	--	8.42	63.64	11.49	11.11	17.56
Cladocera	--	4.74	--	--	--	--	9.80
Medusa	--	--	0.27	--	--	--	0.07

* sampling station number as in Table 6

3.2.1.1.5. Shell fishes.

Intestine and muscle and also composite samples of prawn were analysed for THC and TVC. Approximately one to five grams of intestine of prawn samples, according to the availability, were aseptically removed and transferred to stomacher bags and proceeded as per section 3.2.1.1.1.

Composite samples comprised whole body parts sampled randomly and samples were analysed as described in Section 3.2.1.1.1.

3.2.1.2. Isolation of cultures

From each TCBS plate a representative population of vibrios were selected randomly ranging from 20-30 colonies. Selected cultures were purified in TCBS medium and stocked for identification in TSSA slants.

Isolation of cultures were made only from first twelve samples of sea water, sediment and plankton as in Table 6.

3.2.1.3. Identification of cultures

The scheme mainly followed for identification was that proposed by Alsina and Blanch (1994a; 1994b).The cultures that were Gram-negative, positive oxidase test and were facultatively anaerobic (H&L, positive) were considered as presumptive *Vibrio* isolates. A total of 2000 presumptive isolates were further classified into species level as per the scheme of Alsina and Blanch (1994a, 1994b). Out of this 2000, 799

representative cultures were selected and the identification tests described Table 10 were done as per the methodology described lately in section 3.2.2.1. A type strain under each species was also included where ever possible. The identifying characters were cross checked with Bergey's Manual of Determinative Bacteriology (Baumann *et al.*, 1984). Randomly selected strains were further confirmed for their identity by API strips (*Biomeuriux*, France).

3.2.1.4. Maintenance of cultures

Representative strains from different species after comparing with type strains were maintained in the laboratory for further studies. Long term preservation was achieved by stab culturing on TSSA containing agar (0.5%) and salt (1.0%) concentrations. Drying was prevented by a layer of sterile mineral oil (liquid paraffin) over culture. Tubes were kept in ambient temperature with subculturing once in three months.

3.2.2. Methods for detecting biochemical traits and growth.

3.2.2.1. Biochemical studies

A total of 799 representative strains were selected for the characterisation studies. Selection of the tests for biochemical characterisation was done on the basis of the scheme of Alsina and Blanch (1994a; 1994b). In addition to this, production of some important enzymes were also demonstrated. All the tests were performed by standard procedures following the references listed in Table 11.

3.2.2.1.1. Inoculum preparation

Unless otherwise stated, the inoculum for the various tests were prepared as outlined here. Eighteen hour old cultures in TSSA slants were washed into 3%(W/V) sodium chloride diluent. The turbidity of the inoculum was adjusted to 0.2-0.3 absorbency at 560 nm and this contained 10^7-10^8 cells ml^{-1}. For salt tolerance studies, inoculum was prepared in sterile tap water and a loopful was inoculated immediately after preparation. For carbon utilisation studies and hydrolytic enzyme production studies, spot inoculation with a loopful of the cell suspension was adopted.

3.2.2.1.2. Gram staining

For staining, smear preparation was done from 18h old culture in the TSSA slant. The method followed was that of Huckers modifications of Gram's method (Anon, 1957).

3.2.2.1.3. Oxidase test

Oxidase test (Lee *et al.*, 1979) was performed using 24h. old growth from TSSA. Filter paper strips was moistened with oxidase reagent (*N-N-N-N*-tetra methyl *p*-phenylenediamine) and a part of the growth was placed on the moistened filter paper using platinum loop. Those cultures which have turned dark purple or blue with in 1 minute were taken as positives.

3.2.2.1.4. Hugh-Leifson test

The oxidative or fermentative attack of glucose was tested in Hugh- Leifson's medium. Medium was stabbed with 18h old culture as described by Hugh and Leifson (1953). A yellow colour through out, indicated a fermentative reaction typical of *Vibrio*.

3.2.2.1.5. Decarboxylation of amino acids

Decarboxylation of L-arginine, L-lysine and L-ornithine was detected by the method of Mϕller (1955) using decarboxylating medium described in section 3.1.1.3.. Medium was inoculated heavily, over layered with paraffin oil and incubated for 10 days. An alkaline reaction indicated amine formation.

For demonstrating histidine decarboxylase activity of the *Vibrio* spp., Niven's plating method (Niven *et al.*, 1981) was compared with Mϕller's method and Mϕller's method with L-histidine hydrochloride as substrate was selected because colonies in the other medium was faint purple in colour which confused with negative results.

3.2.2.1.6. Salt tolerance studies

Growth in 0, 3, 6, 8 and 10% (W/V) were studied according to West and Colwell (1984). To about 5ml of the tryptone broth, one loopful of the culture was introduced and incubated for 24 h. Positive tubes have visible growth as turbidity in the medium.

Table 11 List of various biochemical tests used for the characterisation of *Vibrio* species

Tests	Reference
Gram staining	Anon, (1957)
Oxidase Test	Elliot *et al.* (1995)
Hugh&Leifson test	Hugh and Leifson (1953)
Decarboxylation of amino acid.	
Lysine dehydrolase	Mϕller (1955)
Ornithine decarboxylase	Mϕller (1955)
Arginine dihydrolase	Thornley (1960)
Salt Tolerance	
Growth at 0,3,6,8 and10% sodium chloride	West and Colwell (1984)
Temperature tolerance	
Growth at 4,20.30 & 40^{0}C	Elliot *et al.* (1995)
Esculine hydrolysis	Anon, (1957)
Amygdalin hydrolysis	
Citrate reaction	Anon, (1957)
Gelatinase production	Anon, (1957)
Gas from Glucose	West and Colwell (1984)
Indole Production	Cowan and Steel (1965)
Luminescence	West and Colwell (1984)
Nitrate reduction	Crosby (1967)
ONPG reaction	Elliot *et al.* (1995)
Swarming	West and Colwell (1984)
Urease Production	Elliot *et al.* (1995)
Voges Proskauer reaction	Lee *et al.* (1979)
Growth on single carbon source	Lee *et al.* (1979)
α- ketoglutarate, xanthine, L-arabinose, lactose D-glucoseamine, melibiose & D- glucose	
Fermentation of carbohydrate	Elliot *et al.* (1995)
arabinose, arbutin inositol, mannitol, cellobiose salicin,sorbitol and sucrose	
Disc diffusion tests	West and Colwell (1984)
ampicillin resistance 10μg	
0/129 resistance 10 &150μg	
Hydrolytic enzyme production	
caseinase	Hoshino *et al.* (1997)
lipase (Tween 80 Medium)	Seirra (1957)
amylase	Karthiayani and Iyer (1964)
lecithinase	West and Colwell (1984)
deoxyribonuclease (DNase)	West and Colwell (1984)
haemolysin production	West and Colwell (1984)
phosphatase	Barber and Kuper (1951)
histidine decarboxylase	Mϕller (1955)
H_2S production	Anon (1957)

3.2. 2.1.7. Temperature tolerance studies

Growth at 4, 20, 30 and 40^{0}C were studied in TSSB. BOD incubator (YOMA, India) set at 4 and 20^{0}C provided low temperatures and the higher temperatures, 30 and 40^{0}C were achieved in serological water baths. Visible turbidity after 24h in respective temperatures were counted as positive.

3.2.2.1.8. Esculin hydrolysis

Esculin hydrolysis was determined as Anon (1957) Blackening of the medium after 48h incubation was considered positive.

3.2.2.1.9. Citrate utilisation

Citrate utilisation was demonstrated in SCA supplemented with 0.75% sodium chloride and incubation for 14 days after comparing different salt levels and incubation periods. At higher salt levels the test failed to gave correct results as utilisation of citrate by the test organisms was often slow and high salt levels enhances the drying of the medium on prolonged incubation. Blue colouration of the media due to alkaline reaction was indicative of positive reaction.

3.2.2.1.10. Gelatinase production

Gelatinase production was demonstrated by liquefaction method (Anon, 1957). Liquefaction was noted by keeping the culture tube in refrigerator where low temperature solidified the non-hydrolysed gelatin.

3.2.2.1.11. Gas from glucose

Cultures were inoculated to sugar fermentation broth containing 1% (W/V) glucose and inverted Durham's tube. Gas production after 24h growth in ambient temperature was taken as positive

3.2.2.1.12. Indole production

Indole production was detected in tryptone broth cultures with Kovac' reagent (Cowan and Steel, 1965).

3.2.2.1.13. Luminescence

Luminescence medium was streak plated with cultures to obtain discrete colonies. The plates were examined at 18^{th} h of incubation. Examination was done in a completely closed dark box after vision has become dark adapted for five minutes (West and Colwell, 1984).

3.2.2.1.14. Nitrate reduction

Cells were grown in nitrate broth. Formation of nitrite was detected by nitrate A and nitrate B reagents described by Crosby (1967).

Nitrate A reagent : Sulphanilic acid, 0.5g; glacial acetic acid, 30ml; and deionised water, 120ml.

Nitrate B reagent : 1-Naphthylamine-7-suphonic acid, 0.2g dissolved in 120ml deionised water, kept for overnight at 37^{0}C and made up to 150ml with glacial acetic acid.

The presence of nitrite was indicated by development of a red colour within 30 seconds. Cultures were tested after 48h incubation . Negative tubes were tested for zinc dust reduction procedure outlined by Cowan and Steel (1965) for organisms which reduce nitrite.

3.2.2.1.15. ONPG reaction

Fermentation of lactose is often slow or week in some species of *Vibrio*. So hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside has been used to detect β-galactosidase activity. To 1 ml cell suspension, ONPG disc (Oxoid, England) was added and incubated overnight at ambient temperature. Yellowing of the suspension represent production of β-galactosidase.

3.2.2.1.16. Swarming

Swarming was noted after 48h. growth from the spot inoculation of the culture in TSSA. Cultures which grew more than a diameter of 0.5 cm was considered as spreading cultures.

3.2.2.1.17 Urease production

Urease production was detected in CUA. Slants cultures of organisms which became pink in colour before 24h incubation was considered positive (Elliot *et al.*, 1995)

3.2.2.1.18. Voges proskauer test

Forty eight hour old culture was tested with VP reagent for the detection of acetoin production from glucose following the method of Lee *et al.* (1979). Reagent constituted solution A (5% α-Naphthol in alcohol) and solution (40% KOH).

3.2.2.1.19. Carbon utilisation study

Carbon utilisation study were conducted as per Lee *et al.* (1979). Xanthine, α-ketoglutarate, D-glucoseamine, D-glucose, L-arabinose, lactose and melibiose were the sugars tested. Plates were spot inoculated and observation of the visible growth was considered as positive.

3.2.2.1.20. Fermentation study of sugars

Carbohydrate fermentation was tested as per Elliot *et al.* (1995). Fermentation of arabinose, arbutin, inositol, mannitol, salicin, sorbitol and sucrose were determined. Sugar fermentation broth was over layered with paraffin oil after inoculation. Reddening of the medium due to acid reaction was indicative of positive test.

3.2.2.1.21. Disc diffusion test

Resistance to vibriostatic agent, 0/129, (2,4-diamino-6,7-diisopropyl pteridine) and ampicillin (50μg) were tested by disc diffusion method in MHA. Discs of 10 and 150μg 0/129 (Sigma, USA) 50μg ampicillin (Himedia, India) were placed on uniformly seeded plates and zone of inhibition was noted. Zone larger than 1mm was taken as positive.

3.2.2.1.22. Hydrolytic enzyme production

Enzymes detected included casienase, lipase, amylase, lecithinase, deoxyribonuclease and phosphatase. Activity of the enzyme were studied by spot inoculation on the respective plate assay media described in section 3.1.1.3. The inoculum contained 18h. old cells grown in BHI broth supplemented 1% sodium chloride. Inoculation site of the spreading cultures were overlaid by a drop of agar cooled to about 48^0C or exposed to alcohol vapours to prevent the swarming of the bacteria over the zone of clearance.

3.2.2.1.22.1. Caseinase

Caseinase activity was determined by following the method of Hoshino *et al.*, (1997). Skimmed milk agar was used. Clearing zones surrounding the growth after four days incubation indicated proteolysis.

3.2.2.1.22.2. Lipase

Lipase production was determined using tween-80 medium. A cloudy zone of precipitation after 7 days were counted as positives.

3.2.2.1.22.3. Lecithinase

Lecithinase was determined in egg-yolk medium according to West and Colwell (1984). The plates were incubated for 7 days at respective temperatures. Zone of precipitation surrounding the colony showed the production of lecithinase. Cultures which also produce clearing zones peripheral to the cloudy area indicated lipase production.

3.2.2.1.22.4. Amylase

For amylase production of various *Vibrio* species were analysed by spot inoculation on starch agar plates. Clearance zones were detected using iodine pellets as described by Karthiyani and Iyer (1964) after an incubation period of 4 days.

3.2.2.1.22.5. Deoxyribonuclease

Method described by Lee *et al.* (1979) and Lachica *et al.* (1971) were followed for detecting the production of deoxyribonuclease. Since both these methods did not yield reliable results, a new simplified method was adopted. Test cultures were grown in BHI broth containing 1% sodium chloride for 24h. at different test temperatures. Culture broth was then centrifuged (5000 rpm. for 15 minutes at 4^0C) and 20 λl of the supernatant was added to the well cut on 0.5% agar containing 0.01g of purified deoxyribonucleic acid (Sigma, USA). After the incubation for 4h., plates were flooded with 1N hydrochloric acid and those with visible clearance zones were taken as positives.

3.2.2.1.22.6. Phosphatase

Phosphatase production was tested following Barber and Kuper (1951). Cultures in the phosphatase agar were exposed to ammonia vapours. Formation of a deep pink colour on the growth and the surrounding area indicated positive test.

3.2.2.1.23. Production of haemolysin

Production of haemolysin was detected by allowing the cultures to grow in plates of blood agar containing 1% sodium chloride. Overnight incubation was done and the plates were observed for the clear zone of haemolysis.

3.2.2.1.24. H_2S production

Hydrogen sulphide production was detected by keeping a filter paper strip impregnated with saturated lead acetate just above the cystien-tryptone broth inoculated with the test organism and incubated at 37^{0}C. Blackening of the paper strip indicated positive result.

3.2.2.2. Study on masking of green colour of *Vibrio vulnificus* by sucrose fermenting bacteria on TCBS agar

Masking of green colour of *V. vulnificus and V. parahaemolyticus* colonies on the TCBS medium by yellow coloured colonies of *V. alginolyticus*, were determined in the study. This is a phenomenon of getting yellow colonies instead of characteristic green colonies for *V. vulnificus* on TCBS agar in presence of other sucrose fermenting bacteria. The strains selected were va139 and va49 for *V. alginolyticus* and vp42 and vp31 for *V. parahaemolyticus* and NCIMB 2046 and vv242 for *V. vulnificus*. Overnight broth cultures were suitably diluted with 3% sodium chloride diluent and plated on to duplicate plate of TCBS agar. Dilution of individual strains as well as the mixture of two species were plated. Yellow and green colonies developed on TCBS agar

were counted and colour of the colony was confirmed again by streaking on a fresh plate of TCBS medium.

3.2.2.3 Evaluation of the proposed key for the identification of *V. vulnificus*

The authenticity of the key was evaluated by test trails with type culture of *V. vulnificus* (NCIMB 2046) as well as with vv70 and vv69, isolated in the course of the study. Sterile muscle portions of fish (*Rastrelleger kanagurta*), prawn (*Paeneus indicus*) and dry fish (*Sardinella longisceps*) were artificially inoculated with low levels of *V. vulnificus* (10^2 g^{-1}) was subjected to the study. *Vibrio vulnificus* was recovered from the respective samples by using the proposed key as well as with that of Alsina and Blanch (1994a; 1994b) and Oliver *et al.*, (1989). Recovery of *V. vulnificus* was also attempted from six samples of uninoculated fresh fish (*Rastrelleger kanagurta*) by these three identification keys.

3.2.2.4. *In vitro* Growth studies

In this section, the evaluation of optimal conditions for the growth like different temperatures, salinity, pH, low oxygen levels were attempted. *Vibrio vulnificus, V. parahaemolyticus, V. alginolyticus, V. mimicus* and *V. harveyi* were the species selected for the study. Three strains of each of these species were experimented. First four species are pathogens to human and the last one for aquaculture animals. Their high preponderance in the area also form a reason for the selection. Identity of the strains experimented was given in Table 9.

3.2.2.4.1. Temperature tolerance

Temperatures selected were 4, 15, 28±2 (room temperature, RT), 37 and 42°C. For incubation at 4 and 15°C, BOD incubator (YOMA, India) and for 37 and 42°C, serological water baths (Inlab Instruments, India) at respective temperatures were used. Growth medium used was TSSB and a loopful of the inoculum was added to 10 ml quantities of TSSB (starting optical density was zero) and incubated at the respective temperatures. Tubes were drawn after 18, 24, 48, 72, and 144h. of incubation and growth was measured as optical density by spectrophotometer (Spectronic 20+, Milton Roy, Rocheston, New York) at 650 nm.

3.2.2.4.2. Salinity tolerance

Salt level tested were 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 15.0 and 20.0% in tryptone broth. pH was kept constant at 7.0 throughout the experiment. Experiment was carried out at room temperature (28±2°C) for 4 days. The growth was assessed turbidimetrically by measuring optical density as in 3.2.2.4.1.

3.2.2.4.3. pH tolerance

The same cultures and medium were used for this experiment also. Tested pH ranges were 3.0, 4.0, 5.0, 6.0, 6.5, 7.0,7.5, 8.0, and 9.0. Optical density of the broth was noted at 6^{th}, 24^{th} and 48^{th} hours of incubation. pH adjusted with dilute hydrochloric acid and sodium hydroxide using pH meter (Elico, India). Growth was monitored turbidimetrically as in 3.2.2.4.1.

3.2.2.4.4. Low oxygen level (reduced redox levels)

Cultures were allowed to grow in TSSB in reduced oxygen levels in a CO_2 incubator (Heraeus, Hanau, Fr. Germany). Tested CO_2 levels were 5 and 10% and growth was monitored spectrophotometrically using spectrophotometer (Spectronic 20+, Milton Roy, New York) as in 3.2.2.4.1.

3.2.2.5. Bile tolerance

Bacterial strains used in the study were the same listed in Table 9. TSSB with 0.1, 0.2 and 0.3% bile concentrations at three different pH levels *viz.*, 0.6, 7.0 and 8.5 was used. Control tubes with 0% bile level were kept in each experiment. This range of bile concentration was selected because no growth was observed out of this range for the tested *Vibrio* species. The selection of the pH was made to simulate the pH at different parts of the digestive tract of fish and considering the range of pH tolerable for the growth of *Vibrio* in presence of bile salt. Bile

salt no.3 (Oxoid, England) was the substrate used. Growth was measured as optical density after 6, 24 and 48h as as in 3.2.2.4.1.

3.2.2.6. Studies to determine competitive growth of *Vibrio* isolates in mixed population.

Ability of *V. vulnificus* to grow competitively was evaluated by comparing the growth of bacterial species individually and in mixed culture with representative members of the same genus, family and members of related family.

3.2.2.6.1. *V. vulnificus Vs V. alginolyticus*

The inoculum preparation was as described in 3.2.2.1.1. Known number of *V. vulnificus* and *V. alginolyticus* were inoculated individually purely and in mixed culture to TSSB. Survival was estimated as cfu ml^{-1} obtained in TSSA at 6^{th} and 24^{th} h of inoculation. The growth of two species in mixed culture was differentiated utilising the colony colour difference, as colonies of *V. alginolyticus* are yellow in colour and that of *V. vulnificus* is green in TCBS medium.

3.2.2.6.2. *V. vulnificus Vs V. parahaemolyticus*

Procedure similar to that described in 3.2.2.4.1. was adopted for the experiment. Since *V. parahaemolyticus* and *V. vulnificus* produced green coloured colonies, a new medium, Chromocult coliformen agar (Merck, Germany), which was originally made for enterobacterial identification, was tried for the differentiation. In the pale purplish back ground of the medium, *V. vulnificus* produced reddish violet colonies and

V. parahaemolyticus produced translucent colourless colonies. pH of the medium was raised to 7.5 for a better colour contrast.

3.2.2.6.3. *V. vulnificus Vs V. harveyi*

Test procedure was similar as in 3.2.2.4.1. In mixed population, species were distinguished by taking advantage of the difference in salt tolerance of *V. vulnificus* and *V. harveyi*. TSA with 3% sodium chloride was used to enumerate *V. vulnificus* and the same medium with 7% sodium chloride was used for *V. harveyi*

3.2.2.6.4. *V. vulnificus Vs Aeromonas hydrophila*

Test procedure was similar as in 3.2.2.4.1. Differentiation among the mixed cultures was achieved by using TSSA with and with out sodium chloride.

3.2.2.6.5. *V. vulnificus Vs Escherichia* coli

Test procedure was similar as in 3.2.2.4.1. TCBS and T-7 medium were employed for the enumeration of *V. vulnificus* and *E. coli* respectively. On T-7 plates *E. coli* colonies were lime yellow, occasionally with rust brown centre and an yellow zone around.

3.2.3. Methods to test pathogenic potential of *Vibrio.*

Pathogenic characterisation was done by detecting hydrolytic enzymes involved in virulence and also by animal inoculation studies

3.2.3.1. Hydrolytic enzymes involved in virulence

Enzymes studied were protease, lipase, lecithinase and deoxyribonuclease. Activity of these enzymes at five different temperatures *viz.* 6±1, 10, 28±2 (room temperature), 37 and 42^{0}C were studied. 6±1^{0}C was achieved in refrigerator and temperature was monitored using a thermocoupler. 10^{0}C was achieved in BOD incubator (YOMA, India) and 37 and 42^{0}C in serological water baths (Inlab instruments, India) at respective temperatures. Procedure was explained in section 3.2.2.1.23.. Haemolysin production at the above temperatures was detected as described in section 3.2.2.1.24..

3.2.3.2. Animal inoculation studies

Lethality to mice was examined as described by Moreno and Landgraf (1998) with slight modifications. Bacterial cultures were grown in BHI broth containing 1% sodium chloride at the room temperatures for 24h. An aliquot of 0.5 ml (approximately 10^8 cell ml^{-1}) was injected intraperitoneally into each of five mice of 17-20g weight. Control mouse received uninoculated BHI broth. Animals were observed for 48h. and the culture which can kill three or more of the mice was considered as the positive for virulance. Cell free filtrate of the pathogenic culture broth filtered through 0.2λm membrane filter was inoculated to mice as described above. Again, another experiment was conducted by injecting heat killed suspensions (kept in boiling water bath for 10 minutes for killing the culture) of the test cultures to the mice.

Influence of iron in enhancing the virulence of *V. vulnificus* was noted as explained by Elliot *et al.* (1995). Lethality of three strains of *V. vulnificus* were determined by injecting cell suspensions diluted to 10^3 to 10^6 from 18h. old culture broth. Diluent used for the serial dilution was BHI broth with 1% sodium chloride. A set of three mice was used for each dilution and the death of two out of three animals was considered positive. The same experiment was conducted using iron loaded mice. For this experiment, test animals were injected with 250 λg of iron dextran per gram body weight by intra muscular route 2h prior to the experiment.

3.2.4. Methods to determine spoilage potential of *Vibrio*

3.2.4.1. Hydrolytic enzyme studies

The spoilage potential of the isolates were determined by the production and activity of enzymes at wide range of temperatures. The test temperatures were 6±1, 10, 28±2 (room temperature), 37 and 42^{0}C. Ability of *Vibrio* species to produce protease, amylase, lipasae, Lecithinase (phospholipase), gelatinase, urease, phosphatase, histamine, H_2S and indole were demonstrated. Details regarding the medium and methods are outlined in section 3.2.2.1..

3.2.4.2. Low Temperature Storage Study

Growth patterns of *Vibrio* strains belonging to five species as mentioned in Table 9, in the fish muscle homogenate (FMH), TSSB and 3% sodium chloride solution, at two temperatures *viz.* -18±2 and -6±1^{0}C

were studied. Most frequently occurring *Vibrio* species were chosen for the study *viz. V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. mimicus,* and *V. harveyi.* Their pathogenic nature and high prevalence were also a criterion for the selection.

Inoculum was prepared by washing 18h old agar slant culture in sterile 3% sodium chloride diluent. Optical density of the inoculum was adjusted to 1.0 at 650nm. Number of cells in the inoculum was determined by dilution plate method. An aliquot of 0.1ml was added to test tubes containing 10ml pre cooled medium. One set of the test tubes was kept in the freezer (Labline Instruments, India) set at a temperature of -18±2^{0}C and another set in BOD incubator (YOMA, India) at 6±1^{0}C.

Organoleptic assessment was done by observing the media for off odour, colour change and change in the texture and clump size. 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 TSSA. Diluent used was 1% sodium chloride solution instead of 3% sodium chloride to reduce the high salinity stress.

3.2.4.3. Determination of bacterial destruction at elevated temperatures

Bacterial strains used were listed in Table 9. Inoculum was prepared in 3% sodium chloride diluent from 18h old culture and it contained about 10^5-10^6 cfu ml^{-1}. Elimination of cells in high temperature was detected inoculating 1ml quantities of the inoculum to 9 ml aliquots of

TSSB previously adjusted to test temperatures in a serological water bath (Inlab Instruments, India). Tested temperatures were 45, 50 and 55^{0}C. Tubes were drawn and total survivers were enumerated after 2.5, 5.0, 7.5 and 10.0 minutes of the experiment. Recovery of the cells were obtained in TSSA.

3.2.5. Statistical analysis

Simple linear correlation (Yamane, 1967) was carried out using computer to find out significant relation between the parameters.

Results & Discussion

4. Results and discussion

4.1. Ecology and distribution of *Vibrio* species in marine environment

4.1.1. Distribution of *Vibrio* species in sea water

Physico-chemical parameters recorded from the sea water samples collected off Cochin coast are presented in the Table 12A. Temperature, pH, salinity and dissolved oxygen were in the range of 27-30^{0}C, 7.4-8.6, 19.50- 36.74 ppt and 2.8-8.8 mg l^{-1} respectively. The values recorded in the present study were in accordance with previous data (Balakrishnan and Shynamma, 1976; Pradeep, 1986). Sreeja and Ravindran (1999) also reported similar results from Mangalore coast. *Vibrio* population is not significantly ($P<0.05$) related to the tested physical parameters like temperature, salinity, pH and dissolved oxygen (Table 13). A negative correlation between bacterial population and temperature (Velankar, 1955; Chandrika and Nair, 1994) and dissolved oxygen (Chandrika and Nair, 1994) was reported previously from this area. But reports from temperate regions showed that occurrence of *Vibrio* were positively correlated to the temperature (Wright *et al.*, 1996; Caruso *et al.*, 1998; Barbierri *et al.*, 1999). Such striking variations in the distribution can be related to the wide seasonal fluctuation in the water temperature in temperate zones, where temperatures often fall below the tolerance limits of the bacterium. O'Neill *et al.* (1992) have reported a temperature variation from $^-1$ to 29^{0}C in US coastal waters and he claimed that the seasonal variations in the occurrence of *V. vulnificus* was due to this

Table 12A Physico-chemical parameters recorded for water samples collected from various stations off Cochin coast.

Station No.	Depth (m).	Temperature (^{0}C)	p^H	Salinity (ppt)	Dissolved Oxygen (ppm)
1*	10	28.0	7.4	30.40	8.4
2	10	27.0	7.7	35.00	6.8
3	10	27.0	7.5	32.10	8.8
4	11	26.0	7.9	36.74	8.7
5	11	27.0	7.6	31.01	6.2
6	10	26.5	8.1	34.60	6.4
7	10	27.0	8.1	34.10	7.8
8	10	27.5	7.9	34.50	8.2
9	10	27.5	7.6	33.10	8.2
10	14	28.0	8.0	35.20	7.5
11	14	28.0	8.1	35.20	5.4
12	14	29.0	8.0	33.00	7.6
13	14	28.5	7.8	32.10	6.3
14	10	29.0	8.0	34.80	5.4
15	6	29.0	8.1	34.50	3.2
16	14	28.0	7.7	25.20	4.5
17	15	28.0	7.6	35.25	2.8
18	12	28.0	7.7	35.20	3.5
19	10	29.0	7.8	35.20	6.8
20	11	29.5	7.6	35.20	3.8
21	14	30.0	8.2	35.25	8.4
22	10	29.0	8.2	23.90	7.5
23	10	29.0	8.0	23.10	7.5
24	10	29.0	7.9	20.40	6.3
25	10	29.0	7.8	19.50	5.5
26	10	29.0	8.1	22.40	6.3
27	10	29.0	7.7	24.95	4.8
Mean ± SD	11.1 ± 0.4	28.2 ± 0.2	7.9 ± .04	31.2 ± 1.0	6.4 ± 0.3

*Details of the sampling stations given in Table 6

ND- Not Determined

Table 12 B Bacteriological parameters recorded for water samples collected from various stations off Cochin coast.

Station No.	Total Bacterial Count x $10^4 g^{-1}$	Total *Vibrio* Count x $10^2 g^{-1}$	Percentage of vibrios to total bacteria
1*	9.40	48.00	5.10
2	64.00	82.00	12.80
3	0.70	13.00	18.57
4	0.96	6.00	6.30
5	1.80	6.00	3.33
6	3.40	13.00	3.82
7	0.81	9.00	11.11
8	1.60	8.20	5.13
9	1.30	8.00	6.15
10	1.50	9.80	6.53
11	12.00	56.00	4.66
12	2.40	12.00	5.00
13	4.00	19.60	4.85
14	3.10	14.00	4.52
15	2.40	12.80	5.33
16	4.50	14.00	3.10
17	10.20	60.00	5.88
18	24.00	120.00	5.00
19	13.50	100.00	7.40
20	ND	ND	ND
21	8.90	60.00	6.74
22	3.90	44.00	11.28
23	2.25	32.00	14.22
24	1.60	12.00	7.50
25	9.10	41.00	4.50
26	14.90	100.00	6.71
27	8.80	33.00	3.75
Mean ± SD	8.1 ± 2.5	35.9 ± 6.6	6.9 ± 0.7

*Details of the sampling stations given in Table 6

ND- Not Determined

Table 13 Relationship between *Vibrio* count (TVC) to total halophilic bacterial count (THC) and different physico-chemical parameters.

	Depth	Temp.	pH	Salinity	DO	THB	TVC
Depth	1.00						
Temp.	0.04	1.00					
pH	-0.03	0.25	1.00				
Salinity	0.21	-0.37	-0.04	1.00			
DO	-0.06	-0.29	0.12	0.03	1.00		
THB	-0.02	-0.07	-0.19	0.14	-0.18	1.00	
TVC	0.08	0.30	-0.06	0.01	-0.30	*0.65	1.00

Temp.- Temperature, DO- Dissolved Oxygen

* Significant($P<0.05$)

temperature fluctuation. But in tropical climate with the temperature always within the tolerance limit for the survival, the influence of temperature may not be significant. In the present study, temperature recorded from the sea water was in the range of 27 to 30^0C. This range falls within the optimal growth temperature recorded for most of the *Vibrio* (Baumann *et al.*, 1984).

4.1.1.1. Quantitative distribution of *Vibrio* in sea water

Total halophilic bacterial count (THC) varied from 7.0×10^3 to 6.4×10^5 cfu ml^{-1} and total *Vibrio* count (TVC) from 6.0×10^2 to 1.2×10^4 cfu ml^{-1} (Table 12B). *Vibrio* population showed a significant relationship ($P<0.05$) to total bacterial flora (Table 13). Density of *Vibrio* in the coastal water of Korea was reported to be 0.2×10^1 to 9.0×10^3 ml^{-1} (Jung and Shin, 1996). Sreeja and Ravindran (1999) reported be 0.2×10^1 to 9.0×10^3 ml^{-1} in coastal water and 0.8×10^1 to 3.0×10^1 ml^{-1} in open water off Mangalore coast of India. *Vibrio* concentration is very much affected by sampling variables like day, depth and tidal cycles (Koh *et al.*, 1994b). Stratification of the water column may also be a likely source of variation.

The percentage of *Vibrio* to the total flora isolated from the sea water varied from 3.33 to 18.57 with a mean value of 6.9%. In 1989, Alavandi has reported 5% value from the same area. However, the percentage of vibrios is on a lower side when compared to the earlier report that 22% of the bacterial flora of Cochin backwaters was constituted by *Vibrio* (Chandrika and Nair, 1994). *Vibrio* as a dominant flora with incidence 35% preponderance in sea water and sediment collected from

Madras coastal waters was also established (Prabhu *et al.*, 1991). High particulate matter and nutrient status due to terrestrial wash off may be the reason of this high load of *Vibrio* in the backwaters. In similar studies conducted at Chesapeake Bay, USA, the percentage of *Vibrio* to total heterotrophic bacterial flora was 56 (Lovelace *et al.*, 1968) and the same value was 37% in Kamogawa Bay, Japan (Simidu and Aiso, 1962). These variations can be attributed to the seasonal and environmental variability as well as to the variations in the procedures adopted.

Wide range in the values of quantitative occurrence of *Vibrio* in water can be due to the variations in the physico-chemical parameters prevailing in the sampling station and time of collection. Similar fluctuation in the *Vibrio* population in various stations of Cochin backwaters was reported earlier (Pradeep and Lakshmanaperumalsamy, 1984). Barbierri *et al.* (1999) also pointed out the inconsistency in the occurrence of *Vibrio* and its strong dependence on physico-chemical parameters. Recurrently, quantitative occurrence was also affected by factors like plankton blooms (Oppenheimer, 1963) and resuspension of sediment in water currents (Williams and LaRock, 1985; Koh *et al.*, 1994a), as plankton and sediment are rich reservoirs of *Vibrio*.

4.1.1.2. Qualitative distribution of *Vibrio* in sea water

Qualitative distribution of individual *Vibrio* species in 12 station was presented in Table 14. *Vibrio alginolyticus, V. parahaemolyticus, V. campbellii* and *V. orientalis* were found to be the predominant species. A single isolate of *V. vulnificus* was identified from the sample collected

Table 14 Percentage of different *Vibrio* species isolated from water sample collected from different locations off Cochin

Sl. No.	*Vibrio* Species	Seawater Samples*												Total (%)**
		1	2	3	4	5	6	7	8	9	10	11	12	
1	*V. alginolyticus*	33.3	13.3	25.0	27.3	46.2	7.1	33.3	--	14.3	21.1	--	--	19.6
2	*V. campbellii*	33.3	20.0	8.3	18.2	--	21.4	27.8	33.3	--	31.6	16.7	5.6	17.3
3	*V. carchariae*	--	--	--	--	7.7	--	--	--	--	--	--	--	1.1
4	*V. cincinnatiensis*	--	--	--	--	--	14.3	--	--	14.3	--	11.1	--	2.8
5	*V. harveyi*	--	13.3	--	--	--	--	--	25.0	--	42.1	--	16.7	8.9
6	*V. logei*	11.1	--	--	--	3.8	--	11.1	16.7	14.3	5.3	--	5.6	5.0
7	*V. meditarrenei*	--	--	8.3	--	--	21.4	--	8.3	--	--	44.4	--	7.3
8	*V. mimicus*	--	--	8.3	--	7.7	--	5.6	8.3	--	--	5.6	5.6	3.9
9	*V. orientalis*	22.2	40.0	--	36.4	15.4	--	16.7	8.3	28.6	--	--	27.8	15.1
10	*V. parahaemolyticus*	--	13.3	50.0	18.2	--	21.4	5.6	--	--	--	22.2	27.8	12.8
11	*V. pelagius II*	--	--	--	--	15.4	14.3	--	--	28.6	--	--	11.1	5.6
12	*V. vulnificus*	--	--	--	--	3.8	--	--	--	--	--	--	--	0.6
Total Number of Isolates		9	15	12	11	26	14	18	12	7	19	18	18	179

* Location of sampling stations given in Table 6..

'--' Not Detected

** Percentage of individual species in total isolates

during March. Clinically important species *viz.*, *V. mimicus* (7.0%), *V. carchariae* (2.0%) and *V. cincinnatiensis* (5.0%) were also isolated from the water samples.

Members of the genus *Vibrio* that were isolated from each samples varied and thus the species distribution did not seem related to parameters like location and depth. Similar variations in the distribution of vibrios were established earlier by Caruso *et al.* (1998). Watkin and Cambelli (1985) found that the density of *V. parahaemolyticus* was highest near the surface of water. Lokabharathi *et al.* (1986) in their study in Laccadives sea, confirmed the fluctuating trend in the distribution of *Vibrio* population in the water column and also the low density of V. *parahaemolyticus* in offshore waters.

A close scrutiny of the literature showed that even if *Vibrio* is ubiquitous in distribution, the species diversity differed with geographical areas. Predominance of *V. parahaemolyticus* from seawater off Laccadives (Lokabharathi *et al.*, 1986) and Cochin coast (Sanjeev and Stephan, 1993) were reported. *Vibrio alginolyticus* was found to be the predominant species in Taiwan sea (Cheng *et al.*, 1995). Seventy-two percentage of the *Vibrio* population of Tanabe Bay, Japan, was found to be constituted by *V. alginolyticus, V. campbellii* and *V. harveyi* (Miyazaki and Ezura, 1995). In straits of Messina, Italy, *V. mediterranei, V. spendidus* II and *V. pelagius* II were the predominant species representing 25, 19 and 13% respectively of total *Vibrio* population (Caruso *et al.*, 1996). Preponderance of *V. anguillarum* (73% of the total *Vibrio*) was reported from a study at the Strait of Magellan, South America (Monticelli

and Crisafi, 1995). From waters of China, 21 *Vibrio* species isolated with *V. campbellii* as the predominant species (Shin and Jung, 1996). From this data, it is evident that the tropical waters keep a trend in distribution of *Vibrio* with *V. alginolyticus, V. campbellii* and *V. parahaemolyticus* as the predominant species. In the present study also the predominant species noted in the waters included the aforesaid species closely followed by *V. orientalis.*

Six *Vibrio* species *viz., V. alginolyticus, V. campbellii, V. logei, V. mimicus, V. orientalis* and *V. parahaemolyticus* were isolated from more than 50% of the samples. Among these species *V. alginolyticus, V. mimicus* and *V. parahaemolyticus* are human pathogens. The high prevalence of V. *parahaemolyticus* (23%) is to be considered seriously as water is an important etiological agent for contamination of seafood. However, *V. cholerae*, the significant pathogen is not isolated from water. *Vibrio vulnificus* was present only in one out of 176 isolates identified. The low prevalence of *V. vulnificus* in Cochin area was reported earlier (Thampuran *et al.*, 1996).

Physico-chemical parameters primarily determine the characteristic distribution of *Vibrio* species. In closed systems like exhibition aquaria tanks, the structure and composition of *Vibrio* population was determined by abiotic factors, mainly temperatures (Blanch *et al.*, 2001). O'Neill *et al.* (1992) suggested that there was a strong correlation between the occurrence of *Vibrio* species and temperature and salinity. For example, in US waters the occurrence of *V. vulnificus* in water and oyster was detected in the estuary in early July and

the organism continued to exist only up to September (O'Neill *et al.*, 1992). Temperature in that area varied from -1 to 29 ^{0}C and salinity from 0 to 28 ppt.. In Chesapeake Bay, USA, 8% of the flora in warmer months (temperature, >8^{0}C) was *V. vulnificus*, whereas, in cooler months it was not detected (Wright *et al.*, 1996). Many biological factors were also hypothesised to be influential. In the present study, only 0.6% of the *Vibrio* flora constituted *V. vulnificus*. Competitive elimination and starvation can be attributed to their low prevalence in water when compared to sediment and plankton where the corresponding values are 10.4 and 13.6% respectively).

4.1.2. Distribution of *Vibrio* species in sediment

4.1.2.1. Quantitative distribution of *Vibrio* in sediment

Sediment samples collected from twelve stations off Cochin area were analysed for THC and TVC and the average values were tabulated in Table 15. In sediment sample, as in sea water, *Vibrio* load is significantly related to total halophilic bacteria ($r=0.71$; $P<0.05$). Percentage of vibrios to total bacteria varied from 4.2 to 25.5% with a mean value of 11.59. THC was also high ranging from 4.1 x 10^7 to 8.1 x 10^8 cfu g^{-1}. This corroborates with the earlier report of 22% of total flora from sediment of Cochin area (Chandrika and Nair, 1994). Similarly, *Vibrio* comprising 35% of total flora was reported from water and sediment of Madras coast (Prabhu *et al.*, 1991). In the present study mean percentage of *Vibrio* in sediment was observed high when compared to water (6.9%). Generally sediment provide better micro environment than water and thus rich flora can flourish. It was reported earlier that the flora

Table 15 Occurrence of vibrios in the sediment samples collected from various stations off Cochin coast.

Station. No.	Total halophilic bacteria (x $10^7 g^{-1}$)	Total *Vibrio* (x $10^6 g^{-1}$)	Percentage of *Vibrio* to total halophilic bacteria
1*	18.00	46.00	25.50
2	12.00	9.20	7.67
3	46.00	31.00	6.47
4	52.00	72.00	7.74
5	62.00	64.00	10.32
6	10.00	18.30	18.30
7	81.00	73.00	9.01
8	9.20	8.20	8.91
9	32.00	37.00	11.60
10	43.00	22.00	5.12
11	4.10	0.98	23.90
12	5.20	2.40	4.62

*First 12 stations mentioned in Table 6

of sediment was 3 times (Williams and LaRock, 1985) and 10 times (Pagnocca *et al.*, 1991) higher than the water. This high value might be due to the comparatively higher nutritional status, availability of substrate for attachment or the positive interactive effect of organisms present in the sediment. Preferential chitinoclastic activity of *Vibrio* gave selective and advantage over other bacteria to flourish in the chitin rich sediments (Ivanova *et al.*, 1993; Montgomery and Kirchman, 1994). It was suggested that association of *Vibrio* with sediment was to overcome the unfavourable environmental conditions (El-Sahn *et al.*, 1982; West and Lee, 1982; Hood and Ness, 1984).

The heavy load of *Vibrio* in the sediment is of great significance as it directly influences the *Vibrio* load of bottom dwelling edible fishes. This is evident from the higher diversity of *Vibrio* population in demersal fishes as well as in detritus feeder (see, Table 20). This finding has economical importance also because of the bottom dwelling nature of shrimps and other demersal fishes can carry a higher load of vibrios.

4.1.2.2. Qualitative distribution of *Vibrio* in sediment

The qualitative occurrence of various *Vibrio* species in the sediment samples are given in Table 16. Species diversity was found to be higher in sediment sample than water samples. Comparative higher diversity implies that sediment form the suitable niche for the growth of *Vibrio.* Most prevalent *Vibrio* species in the sediment sample was *V. parahaemolyticus* (18.1%), followed by *V. campbellii* (14.6%), *V. orientalis*

Table 16. Percentage of different *Vibrio* species isolated from sediment sample collected from various locations off Cochin

Sl. No.	*Vibrio* Species	Sediment Samples												
		1*	2	3	4	5	6	7	8	9	10	11	12	Total(%)**
1	*V. alginolyticus*	18.2	8.3	--	10.0	21.1	15.0	--	12.5	--	--	22.2	10.0	11.1
2	*V. campbellii*	18.2	12.5	25.0	10.0	21.1	15.0	25.0	--	--	16.7	22.2	--	14.6
3	*V. cholarae*	--	4.2	--	--	5.3	--	--	--	--	--	--	--	1.4
4	*V. fluvialis*	--	--	12.5	10.0	--	--	--	12.5	--	--	--	--	2.1
5	*V. furnnissi*	--	--	--	--	--	10.0	--	12.5	--	--	--	--	2.1
6	*V. harveyi*	18.2	8.3	12.5	10.0	--	--	12.5	25.0	20.0	--	--	20.0	8.3
7	*V. marinus*	--	--	--	10.0	--	--	--	--	--	--	--	--	0.7
8	*V. meditarrenei*	--	--	--	--	--	--	--	25.0	20.0	--	11.1	---	2.8
9	*V. mimicus*	9.1	4.2	12.5	10.0	10.5	--	12.5	--	20.0	8.3	--	10.0	6.9
10	*V. orientalis*	9.1	16.7	--	20.0	10.5	20.0	12.5	--	--	25.0	11.1	20.0	13.9
11	*V. parahaemolyticus*	9.1	20.8	12.5	20.0	15.8	20.0	25.0	--	20.0	16.7	22.2	30.0	18.1
12	*V. pelagius I*	--	4.2	--	--	--	--	12.5	--	--	--	--	--	1.4
13	*V. pelagius II*	--	12.5	12.5	--	5.3	5.0	--	--	--	16.7	11.1	--	6.3
14	*V .vulnificus*	18.2	8.3	12.5	--	10.5	15.0	--	12.5	20.0	16.7	--	10.0	10.4
Total Number of Isolates		11	24	8	10	19	20	8	8	5	12	9	10	144

* First 12 stations in Table 6
'—' not detected
** Percentage of individual species in total isolates

(13.9%), *V. alginolyticus* (11.1%) and *V. vulnificus* (10.4%). *Vibrio* has been reported as a major component of sediment of Vellar estuary, India (Ramesh *et al.*, 1999). Association of *V. parahaemolytics* (Kaysner *et al.*, 1990) and *V. vulnificus* (Wright *et al.*, 1996) with sediment were also established.

Vibrio carchariae, V. logei and *V. cincinnatiensis* which were present in sea water are absent in sediment collected from the same area. At the same time, *V. cholerae, V. fluvialis, V. furnissii, V. marinus* and *V. pelagius* I were observed only in sediment samples. While considering the pattern of species distribution, it is revealed that both the microcosms are different and only those species, which can preferentially thrive, will establish irrespective of its origin.

Higher percentage of pathogenic vibrios like *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* in the sediment samples are to be considered in the case of demersal trawling methods of fish capture, as that method can increase in the pathogen load in skin and gill.

4.1.3. Distribution of *Vibrio* species in plankton

4.1.3.1. Quantitative distribution of *Vibrio* in plankton

Zooplankton harboured heavy load of *Vibrio* with a highest concentration of 3.9×10^8 cfu g^{-1}. Heterotrophic bacterial count of the plankton were also found to be high, with value ranging from 2.8×10^7 to 1.1×10^9 cfu g^{-1} (Table 17). *Vibrio* constituted 7.92 to 40.0% (mean value is 24.18) of the total bacteria attached to zooplanktons. Colwell (1994) also

Table 17. Occurrence of vibrios in plankton samples collected from various locations off Cochin coast.

Station* No.	Total Halophilic bacteria x 10^8 g^{-1}	Total *Vibrio* x 10^7 g^{-1}	Percentage of *Vibrio* to Total Halophilic bacteria
1	2.50	4.70	18.70
2	1.40	1.80	12.86
3	5.70	18.40	32.34
4	1.50	3.90	25.83
5	5.00	5.40	10.80
6	3.60	9.40	26.14
7	2.00	8.00	40.00
8	5.50	18.10	32.91
9	11.00	9.20	8.36
10	4.80	11.00	22.92
11	2.40	1.90	7.92
12	7.30	18.50	25.34
13	3.30	9.30	28.18
14	10.00	39.00	39.00
15	2.10	4.80	22.86
16	6.70	6.40	9.55
17	3.60	7.80	21.67
18	2.40	7.10	29.58

* First 18 sampling stations as in Table 6.

established association of *Vibrio* with plankton. Zooplankton blooms might provide favourable microcosm, supplementing substrate for attachment, growth factors and other nourishment as their exocrines. Chitin degrading ability of vibrios were exstensively studied by Ivanova *et al.*, (1993). Vibrios were found to attach on the zooplankton due to its chitinoclastic nature (Montgomery and Kirchman, 1994). Vibrios could tide over the unfavourable conditions in attached form on chitin which is cryoprotective in nature (Shimodori *et al.*, 1989). The role of zooplankton in the seasonal distribution of *V. parahaemolyticus* is highlighted by the work of Venketeswaran *et .al.* (1989b).

4.1.3.2. Qualitative distribution of *Vibrio* in plankton

Plankton contained 10 different *Vibrio* species including human pathogens *V. alginolyticus, V. cincinnatiensis, V. hollisae, V. parahaemolyticus* and *V. mediterranei* (Table 18). *Vibrio parahaemolyticus* was isolated from 10 samples out of 12 tested and percentage to the total flora varied from 5.3 to 40.0, with a mean value of 15%. Similar result was reported earlier from same area (Venketeswaran *et al.*, 1989a). Association of *V. parahaemolyticus* with zooplankton was much discussed earlier and it is portrayed as a major reason for the seasonality exhibited by the bacterium in the environmental samples (Abraham, 1981; Nair, 1981; Watkin and Cambellii, 1985; Pradeep, 1986). Their reports also highlighted that the plankton dynamics in the fish capturing areas are to studied extensively as they are proved reservoirs of pathogenic vibrios.

Vibrio vulnificus comprised 13.6% of the total vibrios in the sediment samples. Association of *V. vulnificus* with plankton samples

Table 18. Percentage of different *Vibrio* species isolated from plankton sample collected from various stations off Cochin coast.

Sl. No.	*Vibrio* Species	Plankton Samples*												
		1	2	3	4	5	6	7	8	9	10	11	12	TOTAL (%)**
1	*V. alginolyticus*	--	--	18.2	--	40.0	17.6	--	26.3	20.0	11.1	--	28.6	12.9
2	*V. campbellii*	--	25.0	--	31.8	--	--	20.0	21.1	10.0	33.3	--	14.3	13.6
3	*V. cincinnatiensis*	--	--	18.2	--	--	--	--	--	--	--	8.3	--	2.0
4	*V. harveyi*	25.0	--	--	--	--	17.6	--	10.5	10.0	--	8.3	14.3	7.5
5	*V. hollisae*	--	25.0	--	--	40.0	--	--	--	--	--	--	--	4.1
6	*V. marinus*	--	--	--	--	10.0	--	20.0	10.5	--	--	8.3	--	4.1
7	*V. mediterranei*	--	12.5	27.3	18.2	--	17.6	20.0	10.5	--	22.2	25.0	--	13.6
8	*V. parahaemolyticus*	25.0	--	18.2	--	10.0	11.8	40.0	5.3	20.0	11.1	33.3	28.6	15.0
9	*V. splendidus II*	--	25.0	--	31.8	--	17.6	--	15.8	20.0	22.2	--	14.3	13.6
10	*V. vulnificus*	50.0	12.5	18.2	18.2	--	17.6	--	--	20.0	--	16.7	--	13.6
Total number of isolates		12	8	11	22	10	17	10	19	10	9	12	7	147

*First 12 sampling stations in the Table 6.
'—' Not detected
** Percentage of individual species in total isolates

(100%) and sediment (45%) was also reported in earlier studies (Wright *et al.*, 1996). Oliver *et al.*,(1983) also established a high preponderance *of V. vulnificus* in plankton samples. Presence of these species in plankton is important as the planktivores fishes may have the risk of contamination. Eight out of the ten planktivores fishes tested contained *V. vulnificus* and six contained *V. parahaemolyticus* in their intestine.

Despite a higher mean value for the percentage of *Vibrio* in plankton samples compared to water and sediment, the species diversity was minimum in plankton. Ten species were isolated from plankton, whereas the respective values in sediment and water were 14 and 12. Chitinous nature of the plankton and selective chitin degrading ability of *Vibrio* can be attributed as a possible reason for their presence in plankton samples. In plankton, *V. parahaemolyticus* was the predominant species followed by *V. campbellii, V. vulnificus, V. mediterranei* and *V. splendidus* II.

4.1.4. Distribution of *Vibrio* species in fin fishes

4.1.4.1. Distribution of *Vibrio* in skin and muscle of finfishes

In skin and muscle samples, the count of total bacteria was in the range of 1.0×10^5 to 6.5×10^7 cfu g^{-1} and that of *Vibrio* was 1.0×10^4 to 2.1×10^7 cfu g^{-1} (Table 19 and Table 20). *Vibrio* counts of fin fishes were found correlated to THC at $P<0.05$ significance level (Table 21). The count of *Vibrio* as well as total halophilic bacteria in the sea water is two to three logarithmic units lower than that in skin and muscle. This higher density substantiated the autocthonous nature of *Vibrio* in the skin. In fresh

Table 19. Total halophilic bacterial count in the skin and muscle, gills and intestine of commercially important fishes of Cochin area.

Sl.No	Fish Species	Total halophilic barcteria x $10^7 g^{-1}$		
		Skin and Muscle	Gills	Intestine
1	*Arius dussumieri*	0.43	0.61	4.10
2	*Decapterus russellii*	0.02	32.00	0.10
3	*Euthynnus affinis*	0.98	8.70	5.80
4	*Gerres filamentoses*	4.20	7.90	6.91
5	*Himantura bleekeri*	2.20	7.50	66.00
6	*Lates calcarifer*	1.20	9.30	57.00
7	*Lutjanus malabaricus*	0.72	8.20	4.50
8	*Mugil cephalus*	4.60	1.00	33.00
9	*Nemipterus japonicus*	1.10	15.00	4.80
10	*Selar crumenophthalmus*	0.13	47.00	6.00
11	*Thryssa mystax*	0.65	16.00	6.00
12	*Sardinella* sp.	4.30	4.30	4.08
13	*Johnius dussumieri*	0.04	0.63	1.50
14	*Rastrelliger kanagurta*	0.70	66.00	28.00
15	*Sardinella longiceps*	4.20	2.80	34.00
16	*Scomberomerus commerson*	0.01	5.30	11.00
17	*Strongylura strongylura*	6.50	44.00	7.00

Table 20. *Vibrio* count and percentage of vibrios to total halophilic bacterial count in the skin and muscle, gills and intestine of commercially important fishes of Cochin area

Sl. No.	Fish Species	Skin and Muscle		Gills		Intestine	
		Vibrio x $10^5 g^{-1}$	Percentage of *Vibrio* to total halophilic bacteria	*Vibrio* x 10^5 g^{-1}	Percentage of *Vibrio* to total halophilic bacteria	*Vibrio* x 10^5 g^{-1}	Percentage of *Vibrio* to total halophilic bacteria
1	*Arius dussumieri*	5.30	12.32	6.00	9.84	230.00	56.09
2	*Decapterus russellii*	< 0.10	ND*	800.00	25.00	2.30	23.00
3	*Euthynnus affinis*	19.00	19.39	530.00	60.92	400.00	68.96
4	*Gerres filamentoses*	55.00	13.10	ND	ND	110.00	15.91
5	*Himantura bleekeri*	51.00	23.18	130.00	17.33	490.00	7.42
6	*Lates calcarifer*	30.00	25.00	230.00	24.73	1350.00	23.68
7	*Lutjanus malabaricus*	< 0.10	ND	120.00	14.63	120.00	26.67
8	*Mugil cephalus*	66.00	14.35	ND	ND	780.00	23.63
9	*Nemipterus japonicus*	12.00	10.91	280.00	18.67	65.00	13.54
10	*Selar crumenophthalmus*	< 0.10	ND	1000.00	21.28	100.00	16.67
11	*Thryssa mystax*	20.00	30.77	390.00	24.36	158.00	26.33
12	*Sardinella* sp.	53.00	12.33	110.00	25.58	230.00	56.37
13	*Johnius dussumieri*	0.10	25.00	< 0.10	ND	120.00	80.00
14	*Rastrelliger kanagurta*	< 1.00	ND	650.00	9.85	1200.00	42.86
15	*Sardinella longiceps*	140.00	33.33	120.00	42.86	630.00	18.53
16	*Scomberomerus commerson*	0.40	40.00	< 0.10	ND	180.00	16.36
17	*Strongylura strongylura*	210.00	32.31	1700.00	38.64	140.00	20.00

*ND- Not determined

Table 21. Correlation between total *Vibrio* count (TVC) and total halophilic bacterial count(THC) in different body parts of fish.

	THB SM	TVC SM	THB GILL	TVC GILL	THB INT	TVC INT
THB SM	1.00					
TVC SM	*0.89	1.00				
THB GILL	-0.07	0.05	1.00			
TVC GILL	0.22	0.43	*0.79	1.00		
THB INT	0.17	0.19	-0.09	-0.20	1.00	
TVC INT	0.08	0.05	0.14	-0.12	*0.75	1.00

THB SM- Total halophilic bacterial count in skin and muscle.
TVC SM- Total *Vibrio* count in skin and muscle
THB GILL- Total halophilic bacterial count in gill
TVC GILL- Total *Vibrio* count in gill
THB INT- Total halophilic bacterial count in intestine
TVC INT - Total *Vibrio* count in intestine
* significant ($P<0.05$)

samples, percentage of *Vibrio* ranged from 12.33 to 40.0% of the total flora whereas in market samples it varied from 12.32 to 30.77%. Comparatively lower value in the market samples may be due to icing preservation (Surendran, 1980) or due to the habit of continuous moistening of the surface with fresh water during the retail marketing to prevent drying which leaches out the surface flora. The average mean value of the percentage of *Vibrio* to total flora is 22.46. Colwell *et al.* (1962) reported only 3.4% *Vibrio* in the skin. Wide fluctuation among the values observed in the study may be due to the characteristics of the fish such as texture of the skin surface, presence of mucilage, *etc.* and habitat of the fish. Sar and Rosenberg (1987) reported that association of bacteria with skin was determined by many characters like the ability to attach strongly to skin, high surface phobicity, ability to release drag reducing polymers and mechanism to overcome the anti microbial agents in fish mucous.

Average value of percentage of *Vibrio* to the total flora is less on the skin and muscle compared to gills and intestine. Similar results were reported earlier (Hajji *et al.*, 1991). Surface flora is primarily related to wound infections, as cuts and scratches are common while handling the fish. In this context, presence of *V. alginolyticus* and *V. mimicus* is to be taken cautiously as both of them causes wound infections.

Qualitatively, 14 different *Vibrio* species were isolated from skin and muscle samples with a high preponderance of *V. alginolyticus, V. campbellii, V. orientalis* and *V. parahaemolyticus* (Table 22). The flora of the fish surface is usually a reflection of the flora of the environment or

Table 22 Percentage of *Vibrio* species in skin and muscle of commercially important fishes collected from Cochin area

Vibrio Species	*Fishes																	Total (%) **
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1 .*V. alginolyticus*	11.8	--	--	--	20.0	14.3	--	--	--	11.1	--	21.4	--	54.5	13.3	87.5	--	16.1
2. *V campbellii*	5.9	--	33.3	--	13.3	14.3	--	75.0	--	33.3	--	21.4	66.7	45.5	--	--	--	16.1
3. *V. cincinnatiensis*	--	--	--	--	6.7	--	--	--	--	--	--	--	--	--	13.3	--	--	1.9
4. *V. costicola*	--	--	66.6	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1.3
5. *V marinus*	--	--	--	--	--	28.6	--	--	--	--	--	--	--	--	--	12.5	23.1	3.9
6. *V mediterranei*	--	66.7	--	100	--	--	--	--	--	--	22.2	7.1	--	--	--	--	--	5.8
7. *V. metschnikovii*	17.6	--	--	--	13.3	--	--	--	--	--	--	--	--	--	--	--	--	3.2
8. *V. orientalis*		33.3	--	--	26.7	--	60.0	--	--	--	--	--	22.2	--	26.7	--	53.8	15.5
9. *V parahaemolyticus*	47.1	--	--	--	--	--	--	--	100	22.2	--	--	--	--	26.7	--	23.1	13 5
10. *V. pelagius* I		--	--	--	--	--	--	--	--	--	11.1	28.6	--	--	--	--	--	3.2
11. *V. pelagius* II	11.8	--	--	--	6.7	--	--	25.0	--	--	66.7	--	--	--	--	--	--	6.5
12. *V. splendidus* II		--	--	--	--	28.6	--	--	--	22.2	--	14.3	--	--	--	--	--	3.9
13. *V. vulnificus*	5.9	--	--	--	13.3	14.3	40.0	--	--	11.1	--	--	11.1	--	20.0	--	--	8.4
14. *V. vulnificus* B_2		--	--	--	--	--	--	--	--	--	--	7.1	--	--	--	--	--	0.6
Total isolates	17	3	3	4	15	7	10	4	4	9	9	14	9	11	15	8	13	155

* Name and details of the fish species given in Table 7A and 7B.
'—' Not detected
** Percentage of individual species in total isolates

those acquired as contaminant in mishandling (Horsley, 1973). The species present in the sample were common in sea water, confirming its origin from habitat. However *V. carchariae*, *V. harveyi* and *V. mimicus* were absent in skin and muscle and present in water. Reciprocally, *V. vulnificus* B_2, *V. pelagius* I, *V. splendidus* II, *V. metschnikovii*, *V. costicola* and *V. marinus* were present only in skin and muscle. The qualitative difference of *Vibrio* species in both the microcosm confirms the selective establishment and colonisation of the species on the skin. However their proportion in the skin and muscle sample was very low and thus the chance of missing them during analysis of water is to be taken into account.

4.1.4.2. Distribution of *Vibrio* in gill of finfishes

The results of the bacterial analysis of gill samples were presented in Table 19 and Table 20. In gills, the halophilic bacterial population was in the range of 6.1×10^6 to 6.6×10^8 cfu g^{-1}. Percentage of vibrios to the total population fluctuated widely among the species from 9.84 to 42.86%. However, the variation was significantly correlated with the variation in THC ($r=0.78$; $P<0.05$; Table 21). Occurrence of *Vibrio* at a level of 9.5% for Puget farm fish (Colwell, 1962) and 24% for *Scophthalmus maximus* (Mudarris and Austin, 1988) were reported. Both these values falls within the range of vibrios reported in the present study. The wide fluctuation in the data might be due to the anatomical difference of the gill of different fishes and the difference in the flora of the water they sieve. Sampling limitations should also be accounted because bacteria could colonise only in very restricted area of the gill (0.1 to 1% of the total

area) as revealed by scanning electron microscopic studies (Mudarris and Austin, 1988).

A total of 11 *Vibrio* species of were isolated from the gill with *V. alginolyticus* (19.9%), *V. vulnificus* (14.9%) and *V. parahaemolyticus* (13.5%) as the major constituents (Table 23). All the species associated with water except, *V. spendidus* II were isolated from gills. This corroborates with the report that gill flora was a reflection of habitat flora (Austin and Austin, 1987). However their proportion is highly varied in both the environments. Only a single strain of *V. vulnificus* was isolated from the sea water (0.6%), whereas gill contained 14.9% *V. vulnificus* in its total flora. It was likely suggested that even though the origin of the flora was from the habitat, only those strains which can competitively survive could colonise and form the resident flora (Sugita *et al.*, 1983; 1988a) and this hypothesis may explain the variation noted here.

Pathogenic species isolated included *V. alginolyticus, V. cincinnatiensis, V. parahaemolyticus* and *V. vulnificus.* A single strain of *V. vulnificus B2*, an eel pathogen, was also isolated from the gills. However the environmental and human isolations of this bacterium were reported earlier (Hφi *et al.*, 1998).

The heavy *Vibrio* load in the gill is important with regard to the spoilage. Gill is hypothesised as the major source from where the bacteria spread in to the muscle, post mortem. *Vibrio alginolyticus, V. campbellii, V. cincinnatiensis, V. metschnikovii, V. orientalis, V. parahaemolyticus, V. pelagius* II, *V. splendidus* II and *V. vulnificus* were demonstrated as

Table 23. Percentage of *Vibrio* species in gills of commercially important fishes collected from Cochin area

	Fishes*																	
Vibrio Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	To
1. *V. alginolyticus*	14.3	25.0	20.0	11.1	50.0	11.1	7.1	25.0	20.0	40.0	20.0	28.6	--	22.2	10.0	28.6	11.1	
2. *V. campbellii*	--	37.5	--	11.1	--	33.3	--	12.5	20.0	10.0	--	14.3	--	11.1	--	21.4	--	
3. *V. logei*	42.9	--	20.0	--	16.7	--	21.4	--	--	10.0	--	--	16.7	--	--	--	--	
4. *V. marinus*	--	--	--	--	--	--	--	--	--	--	20.0	--	--	22.2	--	--	33.3	
5. *V. mediterranei*	--	--	--	--	--	--	--	12.5	--	--	--	--	--	--	--	--	22.2	
6. *V. mimicus*	--	--	--	--	16.7	11.1	7.1	12.5	--	--	60.0	--	33.3	--	--	--	--	
7. *V. orientalis*	--	12.5	--	33.3	--	22.2	14.3	--	--	--	--	--	--	11.1	20.0	7.1	--	
8. *V parahaemolyticus*	--	--	40.0	11.2	--	22.2	28.6	25.0	--	--	--	42.9	--	22.2	--	21.4	--	
9. *V. pelagius* II	28.3	12.5	--	22.2	--	--	--	--	40.0	--	--	--	--	--	30.0	--	--	
10. *V. splendidus* II	--	--	--	--	--	--	14.3	--	20.0	10.0	--	--	--	--	20.0	14.3	--	
12. *V. vulnificus*	14.3	12.5	20.0	11.1	16.7	--	7.1	12.5	--	30.0	--	14.3	50.0	11.1	20.0	7.1	33.3	
Total isolates	7	8	5	9	6	9	14	8	5	10	5	7	6	9	10	14	9	

* Name and detailes of the fish species given in Table 7A and 7B.
'—' Not detected
** Percentage of individual species in total isolates

hydrolytic enzyme producers for major biomolecules like protein. lipid, starch, lecithin *etc.* in section 4.3. of this chapter (see Table 40 to 45.). Thus from the gill, which is a reservoir, *Vibrio* could easily spread to muscle portion to degrade the quality and reduce the shelf life of fish. Contamination with pathogens will have public health significance. Beheading which is suggested as precautionary to prevent the spoilage by the bacteria can also reduce the load of vibrios . But this is practically impossible for small fishes, which form the major protein supplement for rural people. However in such cases proper handling and preservation practices could only eliminate the problem.

4.1.4.3. Distribution of *Vibrio* in intestine of fin fishes

As far as edible fish is concerned, heavy load of bacteria particularly, *Vibrio* in their intestine is of concern as they can invade and contaminate the muscle tissues in post mortal stages. THC was in the range of 1.0 x 10^6 to 6.6 x 10^8 cfu g^{-1}, whereas, TVC varied from of 2.3 x 10^5 to 1.35 x 10^8 cfu g^{-1} (Table 19 and Table 20). Statistically, a significant relationship was noted between THC and TVC (Table 21). Total halophilic bacteria ranging from 1.8 x10^5 to 2.3 x10^8 cfu g^{-1}. Similar observation was reported by Thampuran and Surendran (1998) from the same area. Density in the order of 10^6 cfu g^{-1} of intestine or muscle was also reported earlier for fish (Karunasagar *et al.*, 1987; Miceli *et al.*, 1993; DePaola *et al.*, 1994). Their data cannot be compared with the present data, as the methodology adopted in each case is different. In the present study percentage of *Vibrio* fluctuates widely within the species reaching up to 80%, with a mean value of 30.44%. Instance where Intestinal flora was

exclusively consisted of *Vibrio* was reported (Aiso *et al.*, 1968). This high population of *Vibrio* in the intestine form a reservoir from which *Vibrio* could easily spread to muscle portion, post mortem.

The symbiosis of *Vibrio* in the intestine is having dual role-one in digestion (Dempsey and Kitting, 1987; Rajkumar and Ayyakannu, 1995) and other in the probiotic biological barrier against pathogens (Westerdahl *et al.*, 1994). Singh *et al.* (1991) confirmed that *Vibrio* inhabiting in the gut of *Paenaeus indicus* were both beneficial and harmful to the life of the animal by enhancing the digestive process and at the same time infecting the host during adverse environmental conditions. Its role as an opportunistic pathogen is important in aquaculture practices, as it is being done in closed systems highly susceptible for sudden change in physico-chemical parameters, which can negatively affect the health of the organism. In such stress conditions vibrios can easily raise its head and cause mass mortality. Mass mortalities by opportunistic action of *V. anguillarum* in Sea bass (Salati and Kusuda, 1996) was reported.

Another aspect of the symbiosis of vibrios in the intestine is hypothesised by Natarajan *et al.* (1979) that the gut of fishes might serve as a 'provisional' environment to tide over unfavourable conditions like hypersalinity (tropical systems) and low temperatures (temperate systems). This is especially true for *V. parahaemolyticus* and *V. vulnificus* as both the species showed distinct seasonality in their prevalence.

A total of 16 *Vibrio* species were isolated from the intestine (Table 24). *Vibrio alginolyticus* (15.5%) was the most prominent

Table 24 Percentage of *Vibrio* species in the intestine of commercially important fishes collected from Cochin area

Vibrio Species	Fishes*																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total**
1. *V. alginolyticus*	26.7	--	11.1	12.5	--	10.0	--	16.7	12.5	37.5	55.6	--	27.3	7.1	5.9	7.7	33.3	15.5
2. *V. campbellii*	6.7	33.3	11.1	--	42.9	--	--	--	12.5	--	--	33.3	27.3	--	5.9	7.7	--	8.8
3. *V. damsela*	--	--	--	--	--	--	8.3	--	--	12.5	--	--	--	--	--	--	--	1.1
4. *V. fluvialis*	--	--	--	37.5	--	--	--	33.3	--	--	--	--	--	--	23.5	--	--	6.1
5. *V. furnissii*	13.3	--	11.1	--	--	30.0	--	--	12.5	--	--	11.1	--	--	11.8	--	--	5.5
6. *V. harveyi*	--	--	22.2	37.5	14.3	--	8.3	25.0	--	--	--	--	--	14.3	17.6	15.4	--	9.4
7. *V. logei*	--	--	11.1	--	--	--	25.6	--	--	--	--	--	--	7.1	--	--	8.3	3.3
8. *V. marinus*	--	--	--	--	--	--	8.3	--	--	--	11.1	--	--	7.1	--	--	16.7	2.8
9. *V. mediterranei*	--	--	--	--	--	--	--	16.7	--	--	--	--	--	--	--	--	--	1.1
10. *V. mimicus*	6.7	--	--	12.5	--	--	--	--	37.5	--	22.2	11.1	--	--	--	--	--	4.4
11. *V. natriegenes*	--	--	--	--	--	--	--	--	--	25.0	--	--	--	--	--	--	--	1.1
12. *V. orientalis*	6.7	33.3	--	--	--	--	--	--	12.5	--	--	222	--	42.9	23.5	23.1	16.7	11.6
13. *V. parahaemolyticus*	--	--	22.2	--	28.6	30.0	8.3	--	--	25.0	--	11.1	9.1	21.4	5.9	7.7	25.0	11.0
14. *V. pelagius* II	--	--	--	--	--	10.0	--	8.3	--	--	--	--	36.4	--	--	--	--	3.3
15. *V. vulnificus*	26.7	33.3	11.1	--	14.3	20.0	41.7	--	12.5	--	11.1	11.1	--	--	5.9	38.5	--	13.8
16. *V. proteolyticus*	13.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1.1
Total isolates	16	6	9	8	7	10	12	12	8	8	9	9	11	14	17	13	12	181

* Name and detailes of the fish species given in Table 7A and 7B.

'—' Not detected

** Percentage of individual species in total isolates

component, followed in the descending order *V. vulnificus* (13.8%), *V. orientalis* (11.6%), *V. parahaemolyticus* (11.0%). Pathogenic species like *V. damsela, V. fluvialis, V. furnissii, V. harveyi* and *V. mimicus* were also present in considerable quantities. Species diversity was maximum in the intestine when compared to other parts of the fish. All the species isolated from the water, except *V. carchariae* and *V. cincinnatiensis* were noted in the intestinal contents. Thampuran *et al.* (1996) also reported high preponderance of *V. campbellii* and *V. parahaemolyticus* in the intestine along with *V. vulnificus.* Thampuran and Surendran (1998) reported a level of incidence of *V. vulnificus,* 16.6% in intestine of onboard samples of fishes and 17.8% in market samples. *Vibrio vulnificus* as a major constituent of the flora of intestine has been reported from temperate regions also (DePaola *et al.*, 1994).

It is observed that there is no definite pattern of distribution or occurrence of *Vibrio* in intestine of market fish or fresh fishes. Fresh fish contain comparatively higher percentage of vibrios than the market samples. This indicates that problem of post harvest multiplication of the bacterium was only marginal. However it should be noted that certain market samples like *Arius dussumeirri* and *Euthunnus affinis* contained high load in its intestine, indicating post harvest multiplication.

The intestine was the most conducive environment for the proliferation of the vibrios as evidenced by the high load and highest species diversity. The factors favouring its predominance with respect to its source of collection, habitat and diet and the specific physiological

characters of the intestinal isolates favouring such a behaviour were tried to analyse in this context.

Feeding habit highly influenced the flora of the intestine (Cahill, 1990; DePaola, *et al.*, 1994; Thampuran and Surendran, 1998). Fishes of the demersal group analysed in the present study, are carnivorous which eat small fishes crab and other invertebrates. Exception is *Mugil cephalus*, which is the only detritus feeder in the group. There was no noticeable trend in the quantitative distribution of *Vibrio* species in the intestine of carnivores and planktivores group of pelagic fishes.

Detritus feeder, *Mugil cephalus*, contained 35% vibrios as its intestinal flora. Detritus feeders and other bottom dwelling demersal fishes ingest sediment particles along with its food. The high percentage of *Vibrio* in the sediment is thus a plausible reason to explain the high preponderance of vibrios in their intestine. The species of *Vibrio* noted in both the samples were also comparable (see, Table 16). High percentage prevalence of vibrios, especially *V. parahaemolyticus* in the intestinal content of detritus feeders was established earlier (Natarajan *et al.*, 1979).

Carnivores fishes feed on small fishes, crustaceans and other aquatic animals. Thus present study included the analysis of small prey fishes like *Anchoviella, Stolepheroros, Leognathus* and *Ambasis*, which form the feed of fishes. The prey fishes contained a maximum of 2.1×10^6 *Vibrio* cells per gram (see, Table 28). The similarity in the species distribution in prey fishes and intestinal flora of carnivorous fish (see Table

29) depicts that the prey fishes as a possible portal of entry of *Vibrio* into the intestine.

Plankton eaters also harboured high percentage of *Vibrio* (18.53 to 56.37%), which corresponds to the preponderance of *Vibrio* in the plankton. Zooplankton analysed in the present study harboured a *Vibrio* load up to $3.9x10^8$ cfu g^{-1} (see, Table 17). All the predominant species present in the plankton samples were isolated from the intestinal contents (Table 18), confirming plankton as a portal of entry for vibrios into the fish intestine. Moreover plankton eaters have well elongated digestive system. The degree of development of digestive system is a determining factor for the resident flora and fishes with well developed digestive tract contained *Vibrio* as the major constituent (Sera and Ishida, 1972).

Resistance to bile and low pH is another factor favouring the colonisation of vibrios in the intestine (Aiso *et al.*, 1968; Sera and Ishida, 1972; Sera *et al.*, 1972). In the present study, all the dominant *Vibrio* species in the intestine except *V. alginolyticus*, were found to tolerate 0.3% bile (see Figure 8). However growth as well as bile tolerance was not observed at pH below 6. Contradictory to this observation, Sera and Ishida (1972) reported growth of *Vibrio* at 2% ox bile and at pH 5.5. Heterogeneity among the strains could be attributed for the disparity in the observation. Since high levels of these organisms were present in the intestine, possibly other tolerance mechanisms might also be acting in *in vivo* conditions.

Studies on the survival pattern and tolerance properties depicting the fate of the *Vibrio* species entering the intestinal tract of edible fish are scarce. Such studies could bring out the probability of the occurrence of pathogenic *Vibrio* species in the seafoods. Muroga (1995) in his review of literature on the bacterial diseases stated that the high mortalities in aquacultured animals had its source from live food contaminated with the opportunistic pathogens. Intestinal infections by indigenous and opportunistic *Vibrio* species in larval stages of marine fish are the major cause of loss in aquaculture.

Seven out of 12 described pathogens in the genus *Vibrio* were isolated from the intestine *viz., V. vulnificus, V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. mimicus* and *V. parahaemolyticus.* Higher counts of *V. vulnificus* and *V. parahaemolyticus* were of public health concern. Gills were also established as favourable niche for a few of these pathogens. Thus the practice of degutting and beheading coupled with proper preservation immediately after harvest is recommended to reduce risk of vibrios in large fishes.

4.1.5. Distribution of *Vibrio* in shellfishes

Shrimp muscle harboured 3.2 x 10^5 to 4.3 x 10^7 cfu g^{-1} halophilic bacteria, of which 1.2 to 23.4% (mean value, 6.32%) were constituted by vibrios (Table 25). The corresponding values in the intestine were 8.0 x 10^8 to 7.2 x 10^9 cfu g^{-1} and the *Vibrio* percentage was 16 to 54.16% (mean value, 33.06%). Muscle of shellfishes harboured lower densities of *Vibrio*, except for *Parapenaeopsis stylifera* where it went

up to $1.0x10^6$ cfu g^{-1}, when compared to intestine. Degree of freshness and post harvest handling of the sample can alter the *Vibrio* percentage.

Singh *et al.* (1991) noticed high percentage of *Vibrio* in the intestine of *Penaeus indicus* and opined that vibrios play dual role, both beneficial and harmful in the life of the animal by enhancing the digestive process and at the same time infecting the host during adverse environmental conditions. Role of *Vibrio* in digestion was also suggested by Dempsey *et al.* (1989). Heavy load in the intestine of aquacultured prawn, especially in the larval stages poses threats of mass mortality by opportunistic pathogenic behaviour of *Vibrio* species. It should be noted at this context that infection in different aquacultured and wild organism by the *Vibrio* species (see Table 5) isolated from the intestine is very common. Thus surveillance studies revealing their prevalence in the environment, feed, body parts *etc.* are very essential to take prophylactic measures to cope up with the sudden onset of disease out breaks in aquaculture systems.

Composite sampling revealed that various shellfishes contained $4.6x10^5$ to $1.5x10^8$ *Vibrio* cells per gram (Table 26). Samples representing all body parts of the animal had the *Vibrio* percentage ranging from 7.67 to 82.14%. High counts in *Penaeus indicus*, *Perna viridis* (shucked), and *Villorita cyprinoides* were alarming as it implicated multiplication after harvest. Post-harvest multiplication of *Vibrio* species in general (Chandrasekharan *et al.*, 1987) and *V. vulnificus*, in particular (Oliver, 1981; Matte *et al.*, 1994b) in oysters has been reported. Bivalves were well known *Vibrio* reservoirs, as they are biological concentrators of

Table 25. Occurrence of vibrios in the muscle and intestine of commercially important shrimps collected from the Cochin area

Shrimp	Muscle			Intestine		
	halophilic barcteria x $10^6 g^{-1}$	*Vibrio* x $10^5 g^{-1}$	Percentage of *Vibrio* to total halophilic bacteria	halophilic bacteria x $10^8 g^{-1}$	*Vibrio* x $10^7 g^{-1}$	Percentage of *Vibrio* to total halophilic bacteria
Parapenaeopsis stylifera	43.00	10.00	2.33	13.00	48.00	36.92
Penaeus indicus	4.80	0.88	1.83	10.80	51.00	47.22
Metapenaeus monoceros	4.24	0.51	1.20	72.00	120.00	16.67
Penaeus monodon	1.20	0.61	5.08	8.00	18.00	22.5
Metapenaeus dobsonii	0.32	0.75	23.44	18.00	97.00	54.16
Metapenaeus affinis	0.79	0.32	4.05	34.00	71.00	20.88

Table 26 Occurrence of vibrios in the commercially important shell fishes collected from the Cochin area (Composite sampling)

Shell Fishes	Total halophilic bacteria x $10^6 g^{-1}$	*Vibrio* x $10^5 g^{-1}$	Percentage of *Vibrio* to total halophilic bacteria
Parapenaeopsis stylifera	111.80	110.00	9.84
Penaeus indicus	5.60	46.00	82.14
Meretrix casta	121.00	330.00	27.27
Metapenaeus dobsonii	8.28	25.00	30.19
Perna viridis	20.20	120.00	59.40
Metapenaeus monoceros	8.28	9.30	11.20
Metapenaeus affinis	6.00	4.60	7.67
Villorita cyprinoides	200.00	1500.00	75.00
Perna indica	172.00	230.00	13.37
Panulirus homarus.	56.00	52.00	9.28

pathogens and thus incriminated in the various disease outbreaks (Koenig *et al.*, 1991; Hlady and Klontz, 1996; Hlady, 1997). Filter feeding mechanism of the crustaceans helps the accumulation and colonisation of vibrios in their body (Oliver, 1981; Oliver *et al.*, 1983).

Generally, species diversity of *Vibrio* in the intestine of shellfishes resembled that of the finfishes, except the presence of *V. cincinnatiensis* in the former (Table 27). Most commonly occurred species was *V. alginolyticus* (20.5%) followed by *V. parahaemolyticus* (17.2%) and *V. vulnificus* (15.6%). Berry *et al.* (1994) reported the occurrence of *V. parahaemolyticus* (36.7%), *V. alginolyticus* (26.7%) and *V. vulnificus* (16.7%) among the total flora in the shrimp samples from China, Equador and Mexico.

The number of *V. vulnificus was* very high in the intestine when compared to the muscle portion. Thampuran and Surendran (1998) reported that no marine prawn collected from markets of Kerala contain *V. vulnificus* in either its muscle or in intestine. In the present study, 4 out of 65 strains and 19 out of 122 strains from the muscle and intestine respectively, were identified as *V. vulnificus*. *Vibrio vulnificus* was reported to be present in frozen shrimps exported from tropical areas (Dalsgaard and Hϕi, 1997). Micro flora of the intestine and gill of the Japanese spiny lobster, *Panulirus japonicus,* contained *Vibrio* as the major constituent (Ueda *et al.*, 1995). This observation is at par with the present observation that *Panulirus homanus* contained an average of 9.2% vibrios in the total flora. In the present study, predominant *Vibrio* species constituting the gut

Table 27. Percentage of *Vibrio* species in body parts of commercially important shrimps collected from Cochin area.

Sl. No.	*Vibrio* species	Muscle							Intestine						
		1*	2	3	4	5	6	TOTAL	1	2	3	4	5	6	Total **
1	*V alginolyticus*	33.3	35.7	--	11.1	8.3	--	16.9	60.0	23.5	23.8	9.1	8.7	20.7	20.5
2	*V campbellii*	8.3	--	20.0	22.2	--	37.5	12.3	20.0	11.8	4.8	4.5	8.7	3.4	7.4
3	*V. cincinnatiensis*	--	14.3	--	--	16.7	--	6.2	--	5.9	--	4.5	--	6.9	3.3
4	*V. damsela*	--	--	20.0	--	--	--	3.1	10.0	--	9.5	--	--	--	2.5
5	*V. fluvialis*	--	--	--	--	--	--	--	--	11.8	4.7	--	17.4	--	5.7
6	*V. furnissii*	8.3	--	--	11.1	--	--	3.1	--	--	--	--	--	3.4	0.8
7	*V harveyi*	16.7	--	--	22.2	--	--	6.2	--	--	--	--	--	--	--
8	*V. logei*	--	14.3	10.0	--	--	--	4.6	--	--	23.8	--	--	17.2	8.2
9	*V mediterranei*	--	7.1	--	--	16.7	--	4.6	--	5.9	--	--	4.3	7.9	3.3
10	*V. metschnikovii*	8.3	--	--	11.1	--	--	3.1	--	--	--	--	--	--	--
11	*V. mimicus*	--	--	30.0	--	16.7	12.5	9.2	--	--	14.3	--	--	7.9	4.1
12	*V. orientalis*	8.3	7.1	--	--	--	--	3.1	--	--	--	18.2	13.0	--	5.7
13	*V parahaemolyticus*	16.7	21.4	--	11.1	16.7	37.5	16.9	--	17.6	4.8	36.4	17.4	17.2	17.2
14	*V. splendidus II*	--	--	20.0	--	8.3	--	4.6	--	--	9.5	--	13.0	--	4.1
15	*V. vulnificus*	--	--	--	11.1	16.7	12.5	6.2	10.0	23.5	--	27.3	17.4	13.8	15.6
16	*V. proteolyticus*	--	--	--	--	--	--	--	--	--	4.8	--	--	3.4	1.6
	Total isolates	12	14	10	9	12	8	65	10	17	21	22	23	29	122

*1. *Parapenaeopsis stylifera* 3. *Metapenaeus monoceros* 5. *Metapenaeus dobsonii*
2. *Penaeus indicus* 4. *Penaeus monodon* 6. *Metapenaeus affinis*

** Percentage of individual species in total isolates

flora was comparable to the same in the seawater. But Harris *et al.* (1991) reported that the gut flora was found to be distinct from the habitat flora in the detritivore prawn, *Upogebia africana* and *Callianassa kaussi.*

The occurrence of pathogenic species of *Vibrio* in the shellfish is to be viewed with caution as shrimps contribute the major share of seafood export from India. Earlier reports showed the common species isolated from frozen prawn samples of this area were *V. alginolyticus* and *V. harveyi* (Sanjeev, *et al.*, 2000).

4.1.6. Distribution of *Vibrio* in prey fishes

Higher density of *Vibrio* in the intestine compared to other body parts suggests that it represents a microcosm conducive for the flourishing of the bacterium. Various factors may influence the colonisation, the important among them being the ability of *Vibrio* species to overcome the resistance mechanisms and the food habit (Muroga, 1995; Grisez *et al.*, 1997). Hence small fishes which form the prey were the main object of the study to find out whether any relationship existed between intestinal flora and the food.

Prey fishes collected from near shore area contained 5.1 x 10^5 to 3.6 x 10^9 cfu g^{-1} when determined from composite samples comprising equal quantities of skin, muscle, gill and digestive tract (Table 28). *Vibrio* percentage varied widely from 0.3 to 73.8%. These fishes were collected from chinese dip net operated in the near shore region, where salinity and other physico-chemical parameters were highly susceptible to changes. Highly variable physico-chemical parameters due to terrestrial wash off

Table 28 Occurrence of vibrios in small pray fishes collected from near shore waters off Cochin coast.

Fishes	Halophilic bacteria x $10^6 g^{-1}$	*Vibrio* x $10^5 g^{-1}$	Percentage of *Vibrio* to total halophilic bacteria
Anchoviella commersonii.	0.51	1.10	21.57
Leognathus sp.	1100.00	2100.00	19.10
Ambasis commersonii	0.84	6.20	73.81
Arius dussumeri-i	3600.00	7.00	1.94
Stolephorous sp.	220.00	7.20	0.33

and physiological difference of the fishes might be the reason for the wide fluctuation in the count.

The prey fishes harboured 17 different species of *Vibrio* (Table 29). Most the species isolated from other sources were also present in the prey fishes. Additionally, it contained terrestrial species like *V. cholerae* (3%) and *V. mimicus* (7.5%). Higher counts of non-halophilic *V. mimicus* and presence of *V. cholerae* indicates low salinity prevailed in the water where samples were collected from Chineese dip net operating in the shore regions of Fort Cochin. Terrestrial intervention may be considerably high in this area. The vicinity to shore may also lead to the problem of sewage pollution, which can account for the presence of *V. cholerae.*

Owing to the transient nature, the gut flora of the carnivorous fishes bears some comparison with that of the prey fishes. Qualitatively 17 species were isolated from prey fishes, which included almost all the pathogenic species of *Vibrio*. Pathogenic species present in the intestine of carnivores fishes were *V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. mimicus, V. parahaemolyticus* and *V. vulnificus.* All these species were present in prey fishes and additionally *V. cholerae* and *V. cincinnatiensis.* Grisez *et al.* (1997) suggested that the feed determine the colonisation of *Vibrio*. The assumption that original source of *Vibrio* in gut of carnivores fishes was from the prey fishes is also substantiated by the wide species diversity harboured in both groups.

Table 29. Percentage of *Vibrio* species in small pray fishes collected from the near shore waters of Cochin area.

		Fishes*					
Sl. No.	*Vibrio* species	1	2	3	4	5	Total
1	*V alginolyticus*	7.1	12.5	13.6	16.7	18.29	11.9
2	*V campbellii*	17.1	15.6	12.5	26.7	23.17	16.3
3	*v. cholerae*	1.4	7.8	1.1	--		2.8
4	*V. cincinnatiensis*	1.4	3.1	1.1	--	3.66	1.6
5	*V. fluvialis*	--	3.1	3.4	6.7	1.22	2.8
6	*V. furnissii*	1.4	--	--	3.3	1.22	0.8
7	*V harveyi*	2.3	10.9	1.1	--	6.10	4.0
8	*V. logei*	2.3	--	2.3	--		1.6
9	*V. mediterranei*	--	--	6.8	3.3		2.8
10	*V. mimicus*	10.0	9.4		20.0	3.66	7.5
11	*V. orientalis*	18.6	4.7	17.0	--	6.10	12.3
12	*V. parahaemolyticus*	15.7	7.8	18.2	10.0	19.51	13.9
13	*V. pelagius I*	1.4	--	2.3	--		1.2
14	*V. pelagius II*	4.3	--	4.5	6.7	2.44	3.6
15	*V. splendidus I*	8.9	--	--	--	1.22	0.8
16	*V. splendidus II*	5.7	14.1	3.4	--	6.1	6.3
17	*V. vulnificus*	7.1	10.9	12.5	6.7	7.32	9.9
Total number of isolates		70	64	88	30	82	334

1 *Anchoviella commersonii.*
2. *Leognathus* sp.
3. *Ambasis commersonii*
4. *Arius dussumieri.*
5. *Stolephorous* sp

4.1.7. Distribution of *Vibrio* in fishes as affected by season.

Sardinella longiceps, a pelagic fish, and *Arius dussumieri*, a demersal fish, were selected for studying the seasonality in the occurrence of *Vibrio* species in the fish. The pattern of distribution of total bacteria and vibrios in different body parts of both the fishes in four seasons of the year were comparable (Figure 1; Figure 2). Total halophilic bacterial count and total vibrios were found to be correlated in all the tested parts at significance level $P<0.05$. Two peaks were observed in the quantitative distribution of vibrios, which correspond to summer (March-May) and post monsoon (September-November) periods. The peaks were more prominent in skin and muscle than in gill and in intestine density of the vibrios showed not much variation.

Seasonal variations in the occurrence of vibrios in different marine environments like water, plankton and sediment were reported (Pradeep and Lakshmanaperumalsamy, 1984). They observed that peaks in occurrence of total vibrios and *V. parahaemlyticus* associated with water, plankton and sediment were found to coincide with the zooplankton blooms in pre monsoon (April-May) and post monsoon (August-November) periods. Similarly, seasonality in the occurrence of *V. vulnificus* in water and oysters of temperate region was studied (O'Neill *et al.*, 1992). They could detect the organism, only in July and remained present through September (in water) and this occasional distribution was correlated to extreme changes which often crosses optimum levels in the temperature and salinity of the area.

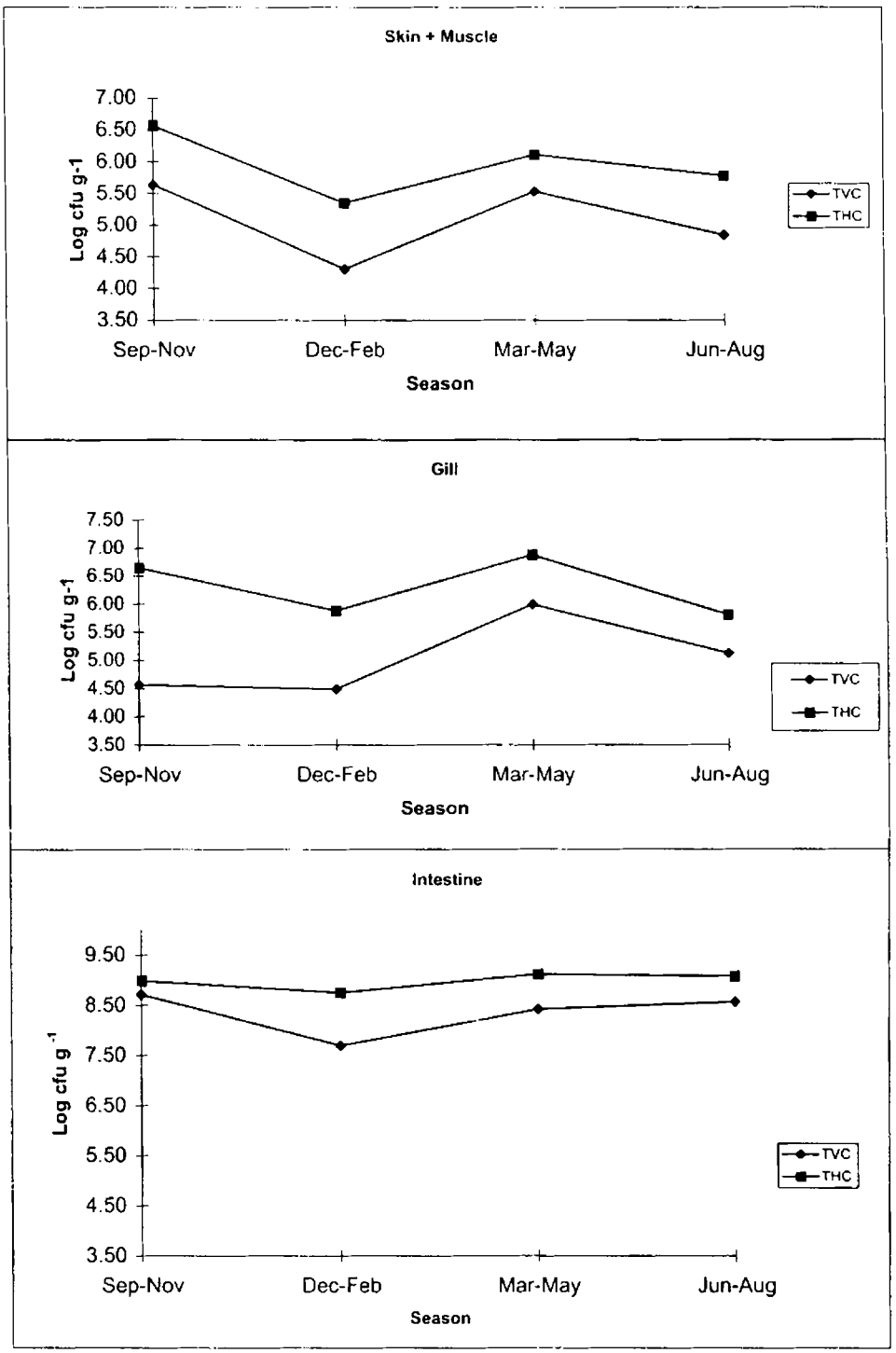


Figure 1. Seasonal variation in the occurrence of vibrios in different body parts of the pelagic fish, *Sardinella longiceps*

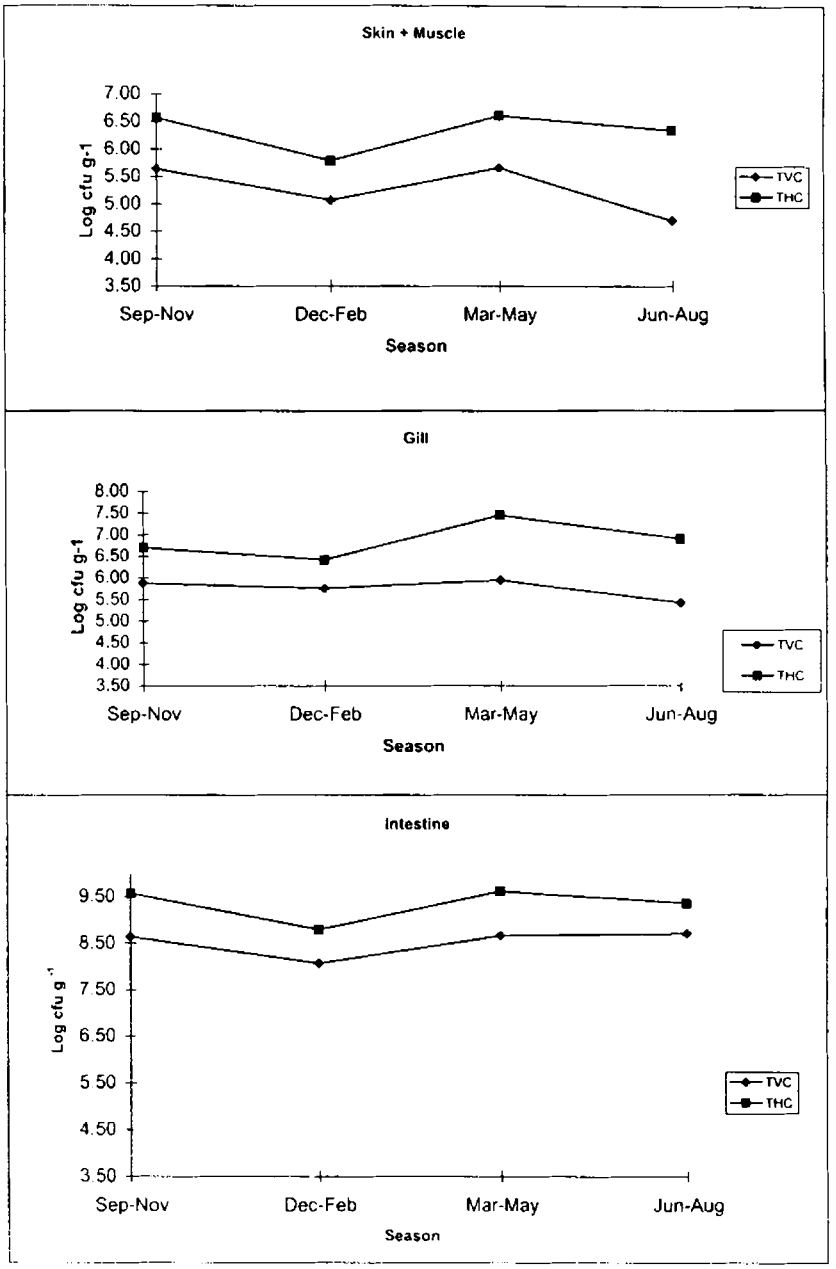


Figure 2. Seasonal variation in the occurrence of vibrios in different body parts of the demersal fish, *Arius dussumieri*

Seasonal variations in the occurrence of vibrios in the body of fishes were not reported earlier. Trend in the distribution of *Vibrio* in skin and muscle of the present study coincide with those reported for water from the same area (Pradeep, 1986). Such a comparable trend was noted for gills of the pelagic fish also. The two peak seasons correspond to plankton blooms, which are proven reservoirs of vibrios and thus gills and skin might reflect its seasonality. For the intestine of both the fishes, the counts were more or less steady through out the period. This infers that intestinal flora is not directly influenced by the seasonal changes of the surroundings.

The greater species diversity was observed in skin and muscle as well as in gills of *S. longiceps* in summer (March-May) period (Table 30). Whereas, in intestine maximum diversity was in December-February period and the least at June-August period. In demersal fish, maximum diversity was noted in post monsoon period (September-November) in skin and muscle, in March-May period in gills and in December- February period in intestine (Table 31). *Vibrio vulnificus* was isolated consistently in December to May from skin and gills. In June to August, monsoon flushes may carry load of terrestrial bacteria and the poor competency of *V. vulnificus* among a mixed community may be one of the reason for their low prevalence(see, section 4.2.2.6). In the intestine *V. vulnificus* was present through out the year except, September, October and November. *Vibrio parahaemolyticus* was isolated through out the year from the tested body parts but not in December- February period in gills. No regular trend was observed in the distribution in different seasons.

Table 30. Prevalance of various *Vibrio* species in the body parts of the pelagic fish, *Sardinella longiceps* during different seasons

		Skin and Muscle				Gills				Intestine				
	Vibrio species.	*1	2	3	4	1	2	3	4	1	2	3	4	Total**
1	*V. alginolyticus*	--	41.2	15.0	--	--	9.4	29.0	25.0	13.0	10.3	--	--	13.3
2	*V. campbellii*	--	29.4	15.0	20.0	26.7	18.8	12.9	--	--	3.4	--	14.3	12.5
3	*V. cholarae*	--	--	--	--	--	--	--	--	--	--	15.4	--	0.8
4	*V. cincinntiensis*	23.1	--	--	--	--	--	9.7	--	--	3.4	--	--	2.8
5	*V. damsela*	--	--	--	--	--	--	3.2	--	--	--	--	--	0.4
6	*V. fluvialis*	--	--	--	--	--	--	--	--	--	3.4	--	--	0.4
7	*V. furnssii*	--	--	--	--	--	--	3.2	--	--	--	--	--	0.4
8	*V. harveyi*	--	17.6	--	--	--	18.8	--	--	--	6.9	--	--	4.4
9	*V. logei*	--	--	25.0	--	--	--	--	--	13.0	--	23.1	--	6.5
10	*V. marinus*	--	--	7.5	--	--	--	--	--	--	--	--	--	1.2
11	*V. mediterranei*	--	--	--	20.0	--	--	9.7	--	--	27.6	--	--	6.0
12	*V. mimicus*	--	--	--	--	--	--	--	--	--	3.4	--	42.9	1.6
13	*V. orientalis*	--	--	--	--	60.0	--	--	--	39.1	--	--	--	7.3
14	*V parahaemolyticus*	46.2	--	22.5	20.0	13.3	6.3	19.4	25.0	26.1	--	38.5	28.6	17.7
15	*V. pelagius II*	--	--	--	--	--	9.4	--	--	8.7	13.8	--	--	3.6
16	*V. splendidus II*	30.8	--	5.0	40.0	--	28.1	--	50.0	--	6.9	7.7	--	12.1
17	*V. vulnificus*	--	11.8	10.0	--	--	9.4	12.9	--	--	20.9	15.4	14.3	8.9
	Total isolates	13	17	40	20	15	32	31	8	23	29	13	7	248

* 1- Sep-Nov, 2- Dec-Feb, 3- Mar-May, 4-Jun-Aug.

** Percentage of individual species in total isolates

Table 31 Prevalence of various *Vibrio* species in the body parts of the demersal fish, *Arius dussumieri* during various seasons

		Skin and Muscle				Gills				Intestine				
	Vibrio species.	*1	2	3	4	1	2	3	4	1	2	3	4	Total**
1	*V. alginolyticus*	22.2	8.8	7.7	44.4	11.8	--	27.3	--	26.3	4.5	12.5	20.0	14.2
2	*V. campbellii*	--	8.8	--	--	--	25.0	27.3	27.3	--	--	12.5	5.0	8.4
3	*V. cincinnatiensis*	3.7	--	7.7	--	11.8	--	9.1	--	--	4.5	25.0	--	4.2
4	*V. cholarae*	--	--	3.8	--	--	--	4.5	--	--	9.1	--	--	1.7
5	*V. costicola*	3.7	--	--	--	--	--	--	--	--	--	--	--	0.4
6	*V. furnissii*	14.8	--	--	--	--	--	--	--	--	--	--	10.0	2.5
7	*V. harveyi*	3.7	--	7.7	--	--	--	9.1	--	21.1	--	--	--	3.8
8	*V. logei*	--	17.6	3.8	--	11.8	16.7	--	--	--	9.1	--	10.0	7.1
9	*V. mediterranei*	--	--	--	--	23.5	--	--	--	--	9.1	25.0	--	3.3
10	*V. metschnikovii*	--	--	--	33.3	--	--	--	27.3	--	--	--	--	2.5
11	*V. mimicus*	--	5.9	--	--	--	--	4.5	--	--	4.5	--	20.0	3.3
12	*V. natreigens*	--	2.9	--	--	--	--	--	--	--	--	--	--	0.4
13	*V. orientalis*	--	--	23.1	--	23.5	12.5	--	--	--	18.2	12.5	--	7.5
14	*V parahaemolyticus*	22.2	20.6	23.1	11.1	--	12.5	--	18.2	31.6	--	--	15.0	14.0
15	*V. pelagius II*	3.7	11.8	7.7	--	11.8	12.5	--	27.3	--	--	--	--	6.3
16	*V. proteolyticus*	--	--	--	--	--	--	--	--	--	--	--	20.0	1.7
17	*V. splendidus I*	3.7	--	--	--	--	4.2	--	--	--	--	--	--	0.8
18	*V. splendidus II*	14.8	--	--	--	--	--	9.1	--	--	18.2	--	--	4.2
19	*V. vulnificus*	7.4	23.6	15.4	11.1	5.9	16.7	9.1	--	21.1	22.7	12.5	--	13.4
	Total isolates	27	34	26	9	17	24	22	11	19	22	8	20	239

* 1- Sep-Nov, 2- Dec-Feb, 3- Mar-May, 4-Jun-Aug.

** Percentage of individual species in total isolates

Species wise distribution of vibrios were also affected by season (Thampuran *et al.*, 1996). Difference in the extent of the effect of the seasonal parameters on various body parts might be the reason for this irregularity in the distribution. Species diversity was minimum in demersal fish samples except in intestine, where the diversity was not seem to be effected by the season. Maximum number of species was noted from December to May in both pelagic and demersal fish.

4.1.8. Distribution of *Vibrio* in fishes as affected by habitat.

Percentage of *Vibrio* to the total halophilic bacteria in the body parts of both pelagic and demersal fishes showed higher values in December-February and June-August periods respectively. Thus habitat wise variation was not profound in the quantitative distribution of vibrios in fishes.

In *S. longiceps*, THC was recorded maximum in July (1.0×10^7 cfu g^{-1}) in skin and muscle, in November (1.34×10^7 cfu g^{-1}) in gills and in May (1.9×10^7 cfu g^{-1}) in intestine. Respective values in *A. dussumeirri* were 1.3×10^6 cfu g^{-1} in September for skin and muscle, 3.1×10^9 cfu g^{-1} in May for gills and 9.3×10^9 cfu g^{-1} in March for intestine. *Vibrio* population of the body parts followed a no traceable trend. Thus it is evident that the habitat of the fish is not influential for the occurrence of vibrios.

Species diversity was greater with 19 species in demersal fish when compared to 17 species in pelagic fish. *Vibrio parahaemolyticus* was the most abundant species in the pelagic fish followed by *V. alginolyticus, V. campbellii* and *V. vulnificus* (see Table 39). Correspondingly, *V.*

parahaemolyticus and V. alginolyticus followed by *V. vulnificus* were the prominent species in demersal fish (see Table 31). *Vibrio marinus, V. fluvialis, V. metschnikovii* and *V. damsela* were present only in pelagic fish, whereas, *V. costicola, V. natriegens, V. spendidus* II and *V. proteolyticus* were found only in demersal fishes. Thus it is assumed that no regular trend was observed in the species wise distribution of *Vibrio* in fishes inhabiting in different layers of water.

With respect to individual body parts the prevalence of various species did not showed a significant habitat wise variation, except for intestine. Intestinal microenvironment is least affected by changing seasonal parameters. But the prevalence percentage of *V. alginolyticus, V. parahaemolyticus,* and *V. orientalis* in the intestine of demersal fishes was higher than their pelagic counterparts. However, pathogenic species like *V. furnissii, V. mimicus* and *V. vulnificus* were more frequently isolated from the pelagic species. Among these species, *V. furnissii* was isolated neither from surface water nor from plankton and *V. vulnificus* was present only in very low concentration in water. This confirms their resident nature in the intestine. The resident nature can further be emphasised by observations of their ability to assimilate complex compounds like protein, lipid, starch, lecithin and DNA (See Table 40 to 45).

4.1.9. *Vibrio* in relation to indicator bacteria

Table 32 give the details of the prevalence of indicator organisms in relation to *Vibrio* in fishes collected from markets in and around Cochin. Higher counts of *E. coli* was noted in *Etroplus suratensis,*

Table 32. Prevalence of indicator organisms and vibrios in fishes collected from the various markets of Cochin area.

Fish species*	Total halophilic bacteria x $10^7 g^{-1}$	Total *Vibrio* x $10^6 g^{-1}$	Total aerobic bacteria x $10^6 g^{-1}$	*Escherichia coli* x $10^2 g^{-1}$	Total coliforms x $10^3 g^{-1}$	Feacal Streptococci x $10^3 g^{-1}$	*Staphylococcus aureus* x $10^2 g^{-1}$
Himantura bleekeri	89.20	5.48	6.36	0.10	0.10	3.00	0.01
Thunnus thunnus	16.70	1.00	ND**	0.12	ND	ND	1.80
Lethrinus sp.	0.32	3.00	ND	0.35	ND	ND	0.12
Tilapia mossabicus	2.16	0.40	5.30	38.00	.01	.010	0.33
Etroplus suratensis	0.23	0.20	0.96	480.00	18.00	0.90	0.01
Rastrelliger kanagurta	1.70	0.40	8.00	0.10	22.00	52.00	0.01
Sardinella longiceps	14.00	60.00	57.00	1.80	24.00	25.70	4.00
Gerres filementosus	2.20	4.60	12.30	0.10	29.00	15.50	0.01
Epinephelus sp.	8.20	5.20	61.00	28.00	1200.00	9.60	60.00
Lutjanus sp.	7.40	12.60	37.00	0.10	52.00	4.90	0.71
Sphyrena obtusa	13.4	102.00	52.00	96.00	1400.00	98.80	20.00
Lates calcarifer	1.27	41.00	2.36	ND	35.40	2.00	120.00
Thryssa mystax	0.60	0.46	7.00	6.00	7.50	3.00	10.00
Decapterus russelli	0.30	2.40	2.07	1.70	5.90	16.10	2.60
Dussumeiria acuta.	0.31	2.40	7.00	0.18	18.00	0.50	6.00
Melaspis cordyla	0.51	2.40	2.35	0.34	4.70	1.20	0.40
Mugil cephalus	4.10	0.025	0.31	3.10	ND	9.00	5.10

* Composite samples comprising skin, muscle, gill and intestine.
**ND -- Not Determined

Epinephelus sp., *Sphyraena obtusa* and *Tilapia mossambicus*. Statistically, there observed no significant relationship between THC and TVC in the composite samples (Table 33). Similarly no significant relationship was observed at significance level, $P<0.05$, between TVC and *E. coli* count, TVC and total coliform count and TVC and *Staphylococcus aureus* count. On the other hand, TVC showed a positive correlation with the faecal streptococci count.

Very little studies have been conducted in India or abroad regarding the correlation between vibrios and other indicator bacteria. Positive as well as negative correlation of vibrios to these indicators have been reported previously. Okpokwasili and Akajobi (1996) found vibrios correlated with indictors and recommended that vibrios should also be included along with *Aeromonas hydrophila* as indicator bacteria in tropical water quality assays. *Escherichia coli* count ranged from 1.0×10^1 to 4.8×10^4 cells g^{-1} in fishes. Total coliforms count reached up to 1.4×10^6 from a lowest value of 1.0×10^1 cell g^{-1}. Corresponding value of faecal streptococci and *Staphylococcus aureus* were 1.0×10^1 to 9.8×10^4 and 1.0 to 1.2×10^4 cells g^{-1} respectively. Good manufacturing practices specification limits for the total plate count is in the range 10^5 to 10^6 cells g^{-1} and for total coliform, *E. coli* and *S. aureus* count is $\leq 10^2$ cells g^{-1} of sea foods (ICMSF, 1978). Wide fluctuation noted in the counts of human contamination indicators reflected the degree of freshness of these samples.

Table 33 Relationship between different indicator bacteria and vibrios in fishes collected from various markets of Cochin area

	THC	TVC	TAC	*E.coli*	coliforms	Fec strep	*Staph aureus*
THC	1.00						
TVC	0.06	1.00					
TAC	0.10	0.51	1.00				
E.coli	-0.11	0.02	-0.11	1.00			
coliforms	0.01	0.58	0.61	0.08	1.00		
Fec strep	0.00	0.72*	0.47	0.00	0.62	1.00	
Staph aureus	-0.11	0.31	0.10	-0.09	0.30	-0.05	1.00

THC - Total halophilic bacterial count
TVC - Total *Vibrio* count
TAC - Total aerobic bacterial count
* Significant at $P < 0.05$ level.

A comparison of the counts of vibrios in the fishes freshly caught from the sea (Table 20) to that of fishes from the market (Table 32) shows that there is no significant rise in the count in post harvest period. This could be attributed to the poor competitive growth of *Vibrio* in a mixed population existing in fish tissue and needs further study.

The finding that vibrios were detected even when faecal bacteria were absent or present in very low number imply a poor correlation between the level of faecal bacteria and presence of *Vibrio* species (Hood *et al.*, 1981; Bhaskar *et al.*, 1998). Poor correlation between *V. parahaemolyticus* and faecal bacteria was also noted (Pradeep, 1986). Oliver *et al.* (1983) documented lack of relation between incidence of *V. vulnificus* and faecal contamination of water. Boekmuhl *et al.* (1986) reported that incidence of *V. mimicus* was not related to the number of coilforms or faecal coliforms. This negative correlation confirms the indigenous nature of *Vibrio* in fishes. Thus using feacal coliform count a index or giving a standard of indicator to vibrios are not satisfactory to monitor vibrios in the fishes.

Eventhough health hazard due to ingestion of *Vibrio* is so far not a problem as the sea foods being cooked properly but cross contamination of processed food is a matter of concern. High *Vibrio* count (up to 1.08×10^8 cfu g^{-1}) in fishes should be considered seriously as *V. parahaemolyticus* and *V. vulnificus* were predominant in them. In addition to this, *V. alginolyticus and V. vulnificus* are capable of initiating wound infections and cellulitis in the cuts and wounds probably produced during the handling of shell fishes and fishes. A thorough surveillance of their

occurrence and survival as well as proper education of seafood handlers could eliminate the problem.

International commission for microbiological specification for food (ICMSF, 1978) has suggested a safety limit for *V. parahaemolyticus* in processed sea foods . It has been early reported that shrimp processors around Mangalore were able to meet ICMSF specification of 10^2 *V. parahaemolyticus* cell g^{-1} of the sample (Karunasagar *et al.*, 1990). In the present study, in certain cases, *V. parahaemolyticus* counts exceeded this limit. As this bacterium is indigenous to marine environment and comprises a major portion of the vibrios of the tropical environments, the specification limit for sea foods from such area is to be reconsidered taking into account their high preponderance.

4.2. Characterisation of *Vibrio* isolates

4.2.1. Biochemical characterisation of *Vibrio* species

A total of 2000 isolated strains of vibrios from various sources that grew on TCBS were selected and subjected to preliminary identification up to genus level following the scheme of Surendran and Gopakumar (1981). A few strains which showed negative oxidases but having 0/129 sensitivity were also chosen if other characteristics exacted with that of vibrios. These strains were further identified up to species level based on the scheme of Alsina and Blanch (1994a; 1994b). Out of these strains, 799 representative cultures belonging to 26 different *Vibrio* species were selected for detailed investigation based on 53 different

identification tests. List of identification tests performed are listed in Table 11.

The results of the identification tests were tabulated in Table 34. Five categories are defined for representing the results of each test. + when the test is positive with a percentage higher than 90, **(+)** when the 75 to 89% showed positive reaction, **v** when the percentage of positive reactions are between 26 and 74%, **(-)** when 11 to 25% of the isolates showed positive reactions and - when less than 10% of the isolates showed positive reactions. The data was compared with the similar data for the same species derived by Alsina and Blanch (1994a) and Bryant *et al.* (1986a; 1986b). The mode of presentation of the data of Bryant *et al.* (1986a) was slightly modified to ease the comparison. The results were also compared with that given in the Bergey's manual by Baumann *et al.* (1984). The incentive behind this analysis is to assess the difference in biochemical traits of the tropical strains from India and that of the strains from other regions and thus to evaluate the utility of the identification schemes based on these traits.

Generally the result of most of the traits of present study were in agreement with the data compared. The identification scheme proposed by Alsina and Blanch (1994a; 1994b) relied mainly on three tests *viz.* the decarboxylation of arginine, lysine and ornithine for the primary grouping of *Vibrio* isolates. The result of these basic decarboxylation tests were at par with the data compared for all the tested species, except for *V. damsela, V. fluvialis* and *V. orientalis*, though the percentage of strains with the trait among the species varied. In the case of aforesaid species,

Table 34. Comparison of various identification tests used in the present study for the characterisation of *Vibrio* species with earlier studies

Species	No. of cultures	Present study								Bryant *et al.* (1986a)								Alsina and Blanch (1994a)							
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
V. alginolyticus	88	-	+	v	-	+	+	+	v	-	+	(+)	-	nt	nt	nt	nt	-	+	v	-	+	+	+	+
V. campbellii	86	-	v	-	-	+	+	v	(-)	-	(-)	-	-	nt	nt	nt	nt	-	nt	-	-	+	nt	v	-
V. carchariae	2	-	+	+	-	+	+	+	-	nt	nt	nt	nt	nt	nt	nt	nt	-	+	+	-	+	+	+	-
V. choleare	12	-	+	+	+	+	-	-	-	-	+	+	+	nt	nt	nt	nt	-	+	+	+	+	-	-	-
V. cincinnatiensis	10	-	+	-	-	+	+	+	+	nt	nt	nt	nt	nt	nt	nt	nt	-	(+)	-	-	+	+	+	v
V. costicola	2	+	-	-	-	+	+	+	-	+	-	-	-	nt	nt	nt	nt	(+)	-	-	-	+	+	+	(+)
V. damsela	6	+	+	-	-	+	-	-	-	+	-	-	-	nt	nt	nt	nt	-	v	-	-	+	+	v	-
V. fluvialis	12	-	+	+	-	+	+	-	-	+	-	-	v	nt	nt	nt	nt	+	-	-	v	+	+	(+)	(+)
V. furnissii	18	+	-	-	+	+	+	+	+	+	-	-	v	nt	nt	nt	nt	+	-	-	nt	+	+	+	+
V. harveyi	44	-	+	+	-	+	+	v	v	-	+	+	-	nt	nt	nt	nt	-	+	+	-	+	(+)	(+)	(-)
V. hollisae	12	-	-	-	-	+	+	+	(-)	nt	nt	nt	nt	nt	nt	nt	nt	-	-	-	-	+	+	+	(-)
V. logei	46	-	+	+	-	+	v	-	-	nt	nt	nt	nt	nt	nt	nt	nt	-	-	(+)	-	+	-	-	-
V. marinus	10	(-)	+	-	-	+	(+)	-	-	nt	nt	nt	nt	nt	nt	nt	nt	-	+	-	-	+	-	-	-
V. mediterranei	54	(-)	v	-	-	+	(+)	v	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	v	-	-	+	+	v	-
V. metschnikovii	6	+	+	-	+	+	-	-	-	(+)	-	v	+	nt	nt	nt	nt	nt	v	-	nt	+	(+)	(+)	(-)
V. mimicus	28	v	+	+	v	+	(+)	(-)	-	-	+	+	+	nt	nt	nt	nt	nt	+	+	+	+	nt	-	-
V. natriegens	2	-	-	-	-	+	+	-	-	-	-	-	-	nt	nt	nt	nt	-	-	-	-	+	nt	+	-
V. orientalis	84	-	-	-	-	+	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	-	-	+	+	+	-
V. parahaemolyticus	72	-	+	+	-	+	+	+	v	-	+	+	-	nt	nt	nt	nt	-	+	+	-	+	+	+	-
V. pelagius I	8	-	-	-	-	+	v	v	-	-	-	-	-	nt	nt	nt	nt	-	-	-	-	+	+	+	-
V. pelagius II	44	-	-	-	-	+	(+)	v	-	nt	nt	nt	nt	nt	nt	nt	nt	-	-	-	-	+	+	-	-
V. proteolyticus	4	+	+	-	-	+	+	-	-	+	+	-	-	nt	nt	nt	nt	+	+	-	-	+	+	+	+
V. splendidus I	4	(+)	-	-	-	+	+	v	-	+	-	-	-	nt	nt	nt	nt	(+)	-	-	-	nt	nt	v	-
V. splendidus II	48	(+)	-	-	-	+	v	(-)	-	+	-	-	-	nt	nt	nt	nt	v	-	-	-	nt	nt	v	-
V. vulnificus	102	-	+	+	-	+	v	-	-	-	+	+	-	nt	nt	nt	nt	-	+	+	-	+	nt	-	-
V. vulnificus B2	1	-	+	-	-	+	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt	-	+	-	-	+	+	-	-

+ - positive for >=90%; (+) -positive for 75-89%; - - negative for <=10%; (-) - negative for 11-25% and v - variable for 26 to 74%

nt- Not Tested

1-Arginine dihydrolase, 2-Lysine decarboxylase, 3-Ornithine decarboxylase, 4-Growth in 0% sodium chloride, 5-Growth in 3% sodium chloride. 6-Growth in 6% sodium chloride 7-Growth in 8% sodium chloride 8-Growth in 10% sodium chloride

Table 34. Continued.....

Species	No. of cultures	Present study												Bryant *et al.* (1986a)											Alsina and Blanch (1994a)											
		9	10	11	12	13	14	15	16	17	18	19	20	9	10	11	12	13	14	15	16	17	18	19	20	9	10	11	12	13	14	15	16	17	18	19	20
V. alginolyticus	88	-	+	+	v	v	-	(+)	+	-	+	-	+	(-)	nt	nt	nt	(-)	-	nt	+	-	+	-	+	-	+	+	+	(-)	-	+	+	-	+	-	+
V. campbellii	86	-	(+)	+	v	v	(-)	v	+	+	+	-	+	-	nt	nt	nt	v	-	nt	+	-	+	(+)	-	-	+	+	-	v	-	V	+	-	(+)	-	(+)
V. carchariae	2	-	+	+	-	-	+	-	+	-	+	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+	+	+	nt	nt	-	+	-	+	-	+
V. choleare	12	-	+	+	+	-	-	+	+	-	+	-	+	-	nt	nt	nt	-	-	nt	+	-	+	-	+	-	+	+	+	-	-	v	+	-	+	-	+
V. cincinnatiensis	10	-	+	+	+	-	-	+	+	-	-	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	nt	+	nt	nt	-	+	-	-	-	-	+
V. costicola	2	-	+	+	-	-	-	-	-	+	+	-	+	+	nt	nt	nt	v	-	nt	+	-	-	-	v	nt	+	+	-	v	-	-	-	-	-	-	v
V. damsela	6	-	+	+	-	-	-	-	-	-	-	-	+	-	nt	nt	nt	v	-	nt	+	+	-	-	+	-	nt	nt	nt	-	-	-	-	nt	-	-	+
V. fluvialis	12	-	+	+	-	-	-	+	+	-	+	-	+	-	nt	nt	nt	v	(-)	nt	+	-	v	-	+	-	+	+	+	v	(+)	+	+	-	(+)	-	+
V. furnissii	18	+	+	+	-	-	-	+	-	+	-	-	-	-	nt	nt	nt	-	-	nt	+	-	-	-	+	-	+	+	+	-	-	+	(+)	+	(-)	-	+
V. harveyi	44	(-)	+	+	(+)	v	-	-	(+)	-	+	v	+	-	nt	nt	nt	v	-	nt	+	-	+	v	-	-	+	+	v	(-)	-	v	+	-	+	v	+
V. hollisae	12	-	+	+	-	-	-	-	-	-	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	+	-	nt	-	-	-	+	nt	+
V. logei	46	(-)	v	+	(-)	(-)	-	-	+	-	-	v	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	+	-	-	nt	nt	-	-	-	-	(+)	+
V. marinus	10	v	(+)	+	v	(-)	-	(-)	(-)	-	-	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	+	-	-	nt	nt	-	+	-	-	nt	+
V. mediterranei	54	-	v	+	v	(-)	-	(-)	-	-	+	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+	+	-	+	nt	-	-	-	+	nt	+
V. metschnikovii	6	-	+	+	-	-	-	-	-	-	+	-	+	+	nt	nt	nt	v	-	nt	+	-	(-)	-	-	nt	+	+	+	v	-	v	+	-	(-)	-	-
V. mimicus	28	(-)	(+)	+	(+)	v	-	v	+	(-)	(+)	(-)	+	-	nt	nt	nt	-	-	nt	+	-	+	-	+	-	nt	+	v	-	-	+	+	-	+	-	+
V. natriegens	2	-	+	+	-	-	-	-	+	-	-	-	+	-	nt	nt	nt	+	v	nt	(-)	-	-	-	+	-	+	+	+	+	v	+	(+)	-	-	-	+
V. orientalis	84	-	+	+	-	-	-	-	+	-	-	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	+	+	-	nt	nt	+	+	-	+	+	+
V. parahaemolyticu	72	-	+	+	v	(-)	-	+	+	-	+	-	+	-	nt	nt	nt	-	-	nt	+	-	+	-	+	-	+	+	+	-	-	+	+	-	+	-	+
V. pelagius I	8	v	v	+	-	-	-	(+)	+	-	-	-	(+)	-	nt	nt	nt	v	-	nt	+	-	-	-	+	v	+	+	-	nt	nt	+	-	-	-	-	+
V. pelagius II	44	(-)	+	+	v	-	-	v	+	-	+	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	v	+	+	-	nt	nt	+	+	-	+	-	+
V. proteolyticus	4	-	+	+	+	-	-	-	+	-	+	-	+	-	nt	nt	nt	-	-	nt	v	-	+	-	+	-	+	+	+	-	-	+	(+)	-	-	nt	-
V. splendidus I	4	-	+	+	-	v	-	-	+	-	+	-	+	-	nt	nt	nt	v	-	nt	+	-	+	-	+	nt	+	+	-	v	-	nt	+	-	+	-	+
V. splendidus II	48	-	(-)	+	(-)	-	-	(+)	(+)	(-)	(+)	-	+	-	nt	nt	nt	v	-	nt	+	-	-	-	+	-	+	+	-	v	-	nt	+	-		-	+
V. vulnificus	102	-	+	+	v	-	-	+	+	-	+	-	+	-	nt	nt	nt	-	-	nt	+	-	+	-	+	-	+	+	+	(-)	-	+	+	-	+	-	+
V. vulnificus B2	1	-	+	+	+	-	-	+	+	+	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+	+	nt	nt	+	+	+	nt	-	-	+

+ - positive for >=90%; (+) -positive for 75-89%; - - negative for <=10%; (-) - negative for 11-25% and v - variable for 26 to 74%

nt- Not Tested.

9- Growth at 4° C, 10- Growth at 20° C, 11- Growth at 30° C, 12- Growth at 40° C, 13- aesculin hydrolysis, 14- amygdalin hydrolysis, 15- citrate utilisation, gelatinase production.

16- gelatinase production, 17- gas from glucose, 18- indole production, 19- lumniscence, 20- nitrate reaction.

Table 34. Continued.....

Species	No. of cultures	Present Study												Bryant *et al.* (1986a)												Alsina and Blanch (1994a)											
		21	22	23	24	25	26	27	28	29	30	31	32	21	22	23	24	25	26	27	28	29	30	31	32	21	22	23	24	25	26	27	28	29	30	31	32
V. alginolyticus	88	v	+	(+)	-	+	-	v	(+)	+	-	(-)	v	-	+	(+)	(-)	(+)	-	+	v	+	-	-	-	-	+	(+)	(-)	+	-	+	v	+	-	-	-
V. campbellii	86	v	+	-	-	+	+	v	v	+	(-)	(-)	v	+	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+	-	-	+	-	+	-	-	-
V. carchariae	2	-	+	-	-	-	-	-	-	+	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+	+	+	-	+	+	nt	nt	+	nt	+
V. choleare	12	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-	+	-	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+	-	-	-
V. cincinnatiensis	10	-	+	+	-	-	-	-	-	+	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	+	nt	-		-	+	nt	+	+	-	nt
V. costicola	2	-	+	-	-	+	-	-	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	(+)	-	-	-	nt	-	-	-
V. damsela	6	-	+	+	-	-	-	-	-	+	-	-	-	-	+	-	+	+	-	-	-	+	+	-	-	-	(+)	-	+	+	-	-	+	+	-	-	-
V. fluvialis	12	-	+	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	(+)	+	-	-	-	-	+	+	+	+	+	-
V. furnissii	18	+	-	-	+	+	-	+	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-
V. harveyi	44	v	+	(-)	-	-	-	v	(-)	v	v	-	v	v	+	(-)	v	-	-	v	-	+	-	-	+	v	+	(-)	v	-	-	(+)	-	+	(-)	-	-
V. hollisae	12	+	-	-	-	-	-	+	+	+	-	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	-	+	nt	-	-	nt	nt	nt	nt	nt	nt	nt
V. logei	46	v	+	-	-	-	-	-	v	(+)	-	-	(+)	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	-	nt	-	nt	-	nt	+	-	-	-
V. marinus	10	v	+	-	-	(-)	-	v	(+)	+	-	-	(-)	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	nt	nt	-	nt	+	nt	+	-	-	-
V. mediterranei	54	v	(+)	-	-	-	(-)	(-)	(-)	v	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	nt	-	-	nt	nt	nt	nt	-	+	(+)
V. metschnikovii	6	+	+	-	-	-	-	-	-	v	v	-	-	(+)	-	-	-	(+)	-	-	v	+	-	v	nt	(+)	-	-	-	(+)	-	-	v	+	-	v	-
V. mimicus	28	(+)	(+)	-	-	(-)	-	v	(-)	v	-	-	v	+	+	-	-	(-)	-	+	+	+	-	-	-	+	+	-	-	(-)	-	+	+	+	-	-	-
V. natriegens	2	-	+	-	-	-	-	+	-	+	+	-	-	v	+	-	v	v	-	+	+	+	+	-	v	v	+	-	v	-	-	+	+	+	+	-	v
V. orientalis	84	-	+	-	-	-	-	+	-	+	+	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	-	nt	-	nt	-	nt	+	-	-	nt
V. parahaemolyticus	72	v	+	v	(-)	-	-	v	(+)	+	v	(-)	v	+	-	+	v	-	-	+	+	+	(+)	-	-	-	+	nt	v	-	-	+	+	+	(+)	-	-
V. pelagius I	8	(+)	+	v	-	-	-	-	-	+	(-)	(+)	(-)	v	+	-	v	-	-	v	v	+	-	v	v		+	-	nt	-	nt	-	nt	+	-	v	-
V. pelagius II	44	v	+	-	-	-	-	(-)	(-)	(+)	-	v	v	-	+	+	v	-	-	+	nt	+	nt	nt	nt	nt	+	-	nt	-	nt	(-)	nt	+	-	v	-
V. proteolyticus	4	+	+	v	-	-	-	-	-	+	-	-	-	nt	nt	nt	nt	nt	nt	nt	+	nt	-	-	-	-	+	+	v	(+)	-	+	+	+	-	-	-
V. splendidus I	4	(+)	+	-	-	-	-	+	-	+	-	-	-	+	+	-	-	-	-	+	-	+	-	-	-	(+)	+	-	-	-	-	+	-	+	-	-	-
V. splendidus II	48	(+)	(+)	-	(-)	-	-	(-)	v	(+)	(-)	-	(-)	v	+	-	-	-	-	v	-	+	-	-	-	v	+	-	-	-	-	nt	-	+	-	-	-
V. vulnificus	102	v	+	(-)	-	-	-	v	v	+	-	v	v	+	+	+	-	-	-	(+)	+	+	-	v	-	+	+	-	-	-	-	(+)	+	+	-	v	-
V. vulnificus B2	1	-	+	+	-	-	-	-	+	+	-	-	-	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+	nt	-	-	nt	nt	nt	nt	nt	nt	nt

+ - positive for >=90%, (+) -positive for 75-89%; - - negative for <=10%, (-) - negative for 11-25% and v - variable for 26 to 74%

nt- Not Tested

21- Ortho nitro phenol galactopyranoside production, 22- oxidase production, 23- swarming, 24- urease production, 25- voges proskaure reaction, 26- xanthine decomposition

27- ketoglutarate utilisation(carbon source), 28- D-glucose amine (carbon source), 29- D-glucose (carbon source),

30- L- arabinose (carbon source), 31-Lactose (carbon source), 32-Melibiose (carbon source). 33- production of acid from arabinose

Table 34. Continued.....

Species	No. of cultures	Present study											
		33	34	35	36	37	38	39	40	41	42	43	44
V. alginolyticus	88	-	v	-	v	v	v	+	v	v	(+)	+	-
V. campbellii	86	(-)	(-)	(-)	v	(-)	(-)	v	+	v	v	+	v
V. carchariae	2	-	-	-	+	-	-	+	-	+	+	+	-
V. choleare	12	+	-	-	v	-	-	+	-	+	+	+	-
V. cincinnatiensis	10	-	-	-	-	-	-	+	v	-	-	+	-
V. costicola	2	-	-	-	-	-	-	+	v	+	+	+	+
V. damsela	6	+	+	-	-	-	-	-	-	+	+	+	+
V. fluvialis	12	-	-	-	+	-	-	+	v	-	-	+	-
V. furnissii	18	-	+	-	-	-	-	+	-	+	+	+	-
V. harveyi	44	-	(+)	-	(+)	(-)	v	v	v	v	+	+	v
V. hollisae	12	+	-	+	+	v	+	-	-	v	+	+	+
V. logei	46	-	(+)	-	v	-	-	v	+	(-)	v	+	v
V. marinus	10	(-)	(-)	-	-	-	-	v	-	(-)	(-)	+	(+)
V. mediterranei	54	-	(-)	-	v	v	-	v	-	v	v	+	(-)
V. metschnikovii	6	-	v	-	+	-	-	+	(-)	-	+	+	+
V. mimicus	28	v	(+)	(-)	v	(-)	(-)	(-)	(-)	v	(-)	+	v
V. natriegens	2	+	-	-	+	+	-	+	+	-	-	+	-
V. orientalis	84	+	-	-	+	+	-	+	v	-	-	+	-
V. parahaemolyticus	72	v	v	(-)	v	(-)	(-)	v	v	v	+	+	v
V. pelagius I	8	-	(-)	-	(+)	-	-	(+)	-	v	v	+	-
V. pelagius II	44	-	-	-	(-)	-	-	v	-	(-)	(-)	+	(-)
V. proteolyticus	4	+	-	+	+	-	+	-	v	-	+	+	v
V. splendidus I	4	-	-	-	+	-	-	(+)	+	-	+	+	v
V. splendidus II	48	(-)	-	-	v	(-)	v	v	v	v	(+)	+	(-)
V. vulnificus	102	-	v	-	v	v	v	v	+	(-)	v	+	v
V. vulnificus B2	1	-	-	-	-	-	-	-	+	-	+	+	+

Species	No. of cultures	Bryant *et al.* (1986a)											
		33	34	35	36	37	38	39	40	41	42	43	44
V. alginolyticus	88	-	-	-	nt	v	-	(+)	+	nt	nt	(-)	nt
V. campbellii	86	-	-	-	nt	(+)	-	-	v	nt	nt	-	nt
V. carchariae	2	nt	nt	nt	nt	nt	nt	nt	-	nt	nt	nt	nt
V. choleare	12	-	-	-	nt	-	-	+	v	nt	nt	-	nt
V. cincinnatiensis	10	nt	nt	nt	nt	nt	nt	nt	v	nt	nt	nt	nt
V. costicola	2	-	-	-	nt	-	-	+	-	nt	nt	-	nt
V. damsela	6	-	-	-	nt	-	-	-	v	nt	nt	-	nt
V. fluvialis	12	+	+	-	nt	(+)	(-)	+	-	nt	nt	-	nt
V. furnissii	18	+	v	-	nt	-	-	+	+	nt	nt	-	nt
V. harveyi	44	-	v	-	nt	(+)	-	v	-	nt	nt	(-)	nt
V. hollisae	12	nt	nt	nt	nt	nt	nt	nt	+	nt	nt	nt	nt
V. logei	46	nt	nt	nt	nt	nt	nt	nt	-	nt	nt	nt	nt
V. marinus	10	nt	nt	nt	nt	nt	nt	nt	-	nt	nt	nt	nt
V. mediterranei	54	nt	nt	nt	nt	nt	nt	nt	(-)	nt	nt	nt	nt
V. metschnikovii	6	-	-	-	nt	v	v	+	(-)	nt	nt	-	-
V. mimicus	28	-	-	-	nt	-	-	-	+	nt	nt	-	nt
V. natriegens	2	+	+	v	nt	+	(-)	+	v	nt	nt	-	nt
V. orientalis	84	nt	nt	nt	nt	nt	nt	nt	v	nt	nt	nt	nt
V. parahaemolyticus	72	(+)	-	-	nt	-	-	-	-	nt	nt	-	nt
V. pelagius I	8	-	-	-	nt	-	-	v	-	nt	nt	-	nt
V. pelagius II	44	nt	nt	nt	nt	nt	nt	nt	v	nt	nt	nt	nt
V. proteolyticus	4	-	-	-	nt	-	v	-	+	nt	nt	v	nt
V. splendidus I	4	-	-	-	nt	-	-	v	v	nt	nt	-	nt
V. splendidus II	48	-	-	-	nt	-	-	-	+	nt	nt	-	nt
V. vulnificus	102	-	-	-	nt	+	-	(-)	v	nt	nt	-	nt
V. vulnificus B2	1	nt	nt	nt	nt	nt	nt	nt	+	nt	nt	nt	nt

Species	No. of cultures	Alsina and Blanch (1994a)											
		33	34	35	36	37	38	39	40	41	42	43	44
V. alginolyticus	88	(+)	-	-	+	-	(+)	+	v	+	v	-	nt
V. campbellii	86	-	-	-	v	nt	-	-	+	+	v	-	nt
V. carchariae	2	-	-	-	+	nt	-	+	v	nt	+	-	nt
V. choleare	12	-	-	-	+	-	-	+	-	(-)	(-)	-	nt
V. cincinnatiensis	10	+	+	+	+	+	-	+	v	-	+	-	nt
V. costicola	2	-	-	-	(+)	-	-	(+)	v	-	+	-	nt
V. damsela	6	-	-	-	-	-	-	(-)	-	-	-	-	nt
V. fluvialis	12	+	(+)	-	+	(+)	(-)	+	v	+	+	-	nt
V. furnissii	18	+	-	-	+	-	-	+	-	+	+	-	nt
V. harveyi	44	(-)	v	-	+	nt	-	v	+	+	v	(-)	nt
V. hollisae	12	+	nt	-	-	-	-	-	-	-	nt	nt	nt
V. logei	46	-	nt	-	(+)	-	-	(-)	+	nt	nt	nt	nt
V. marinus	10	-	nt	-	-	-	-	-	-	nt	nt	nt	nt
V. mediterranei	54	-	nt	-	+	+	+	+	-	nt	nt	-	nt
V. metschnikovii	6	-	v	(-)	(+)	(-)	v	+	(-)	v	-	-	nt
V. mimicus	28	-	v	-	(+)	-	-	-	(-)	-	(-)	-	nt
V. natriegens	2	+	+	(-)	+	+	(-)	+	+	-	+	-	nt
V. orientalis	84	-	nt	-	+	-	-	+	v	nt	nt	nt	nt
V. parahaemolyticus	72	(+)	-	-	(+)	-	-	-	v	+	+	-	nt
V. pelagius I	8	-	nt	-	+	-	-	+	-	nt	nt	nt	nt
V. pelagius II	44	-	nt	-	+	-	-	v	-	nt	nt	nt	nt
V. proteolyticus	4	-	-	-		-	(+)	-	v	-	+	v	nt
V. splendidus I	4	-	-	-	+	-	-	(+)	+	-	-	-	nt
V. splendidus II	48	-	-	-	+	-	-	-	v	+	v	-	nt
V. vulnificus	102	-	v	-	(+)	+	-	(-)	+	-	(-)	-	nt
V. vulnificus B2	1	-	nt	-	-	-	-	-	v	nt	-	nt	nt

+ - positive for >=90%; (+) -positive for 75-89%; - - negative for <=10%; (-) - negative for 11-25% and v - variable for 26 to 74%

nt- Not Tested

33- production of acid from arabinose, 34- production of acid from arbutin, 35- production of acid from inositol, 36- production of acid from mannitol, 37- production of acid from salicin, 38- production of acid from sorbitol. 39- production of acid from arabinose, 40- production of acid from cellobiose. 41- ampicilline resistance, 42- 0/129 resistance(10 g), 43- 0/129 resistance(150 g), 44- green colour producton in TCBS

Table 34. Continued.....

Species	No. of cultures	Present study									Bryant *et al*. (1986a)								
		45	46	47	48	49	50	51	52	53	45	46	47	48	49	50	51	52	53
V. alginolyticus	88	+	+	+	+	+	-	+	+	(+)	+	+	+	+	+	-	nt	nt	nt
V. campbellii	86	v	+	+	+	v	(-)	+	-	+	+	+	+	+	+	v	nt	nt	nt
V. carchariae	2	+	+	+	+	+	-	+	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. choleare	12	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-	nt	nt	nt
V. cincinnatiensis	10	+	(+)	+	+	-	v	v	+	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. costicola	2	-	+	-	+	-	v	-	+	-	-	+	-	+	-	v	nt	nt	nt
V. damsela	6	-	v	v	+	+	v	+	-	-	-	+	+	+	+	v	nt	nt	nt
V. fluvialis	12	+	v	+	+	+	v	(+)	+	+	+	+	+	+	+	+	nt	nt	nt
V. furnissii	18	-	-	-	-	(-)	-	v	v	+	+	+	(+)	+	+	v	nt	nt	nt
V. harveyi	44	(+)	v	+	+	v	v	+	v	+	v	+	+	+	+	-	nt	nt	nt
V. hollisae	12	+	+	+	+	+	v	+	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. logei	46	(+)	+	+	+	(-)	v	+	+	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. marinus	10	-	+	+	+	-	(-)	v	+	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. mediterranei	54	(+)	v	+	v	(-)	+	v	v	v	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. metschnikovii	6	(+)	(+)	v	+	v	v	(+)	+	+	+	(+)	+	+	+	(+)	nt	nt	nt
V. mimicus	28	(+)	+	v	+	+	+	+	v	v	+	+	(-)	(+)	+	+	nt	nt	nt
V. natriegens	2	v	+	+	+	v	-	-	-	+	v	+	+	+	v	-	nt	nt	nt
V. orientalis	84	v	+	+	+	v	-	+	+	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. parahaemolyticus	72	(+)	+	+	+	+	(-)	+	+	+	+	+	+	+	+	-	nt	nt	nt
V. pelagius I	8	+	+	+	+	+	v	+	-	+	+	+	+	+	+	v	nt	nt	nt
V. pelagius II	44	(+)	v	+	+	v	v	+	-	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
V. proteolyticus	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nt	nt	nt
V. splendidus I	4	v	+	+	(+)	+	v	(+)	(+)	+	v	+	+	(+)	+	v	nt	nt	nt
V. splendidus II	48	v	+	+	+	v	v	+	+	+	+	+	+	v	+	v	nt	nt	nt
V. vulnificus	102	v	(+)	+	+	v	v	+	(+)	+	+	+	+	+	+	(+)	nt	nt	nt
V. vulnificus B2	1	+	+	+	+	-	+	+	+	+	nt	nt	nt	nt	nt	nt	nt	nt	nt

+ - positive for >=90%; (+) -positive for 75-89%; - - negative for <=10%; (-) - negative for 11-25% and v - variable for 26 to 74%
nt- Not Tested
45- Protease production, 46- lipase production, 47-Amylase production, 48- lecithinase production,
49- deoxyribonuclease production, 50-haemolysin production. 51- phosphatase production,
52- histidine decarboxylase production, 53- Hydrogen sulphide production
No data available in Alsina and Blanch (1994a)

all other tests were identical to the type strains, except amino acid decarboxylation tests. The identity of these species was also confirmed by comparing the characters described by Bergey's manual (Baumann *et al.*, 1984) and by api 20E strips. This type of disagreement in the result of key identification tests makes the isolation process more cumbersome.

The present data reflects the diversity among the environmental strains of different species of *Vibrio*. Percentage of variable results (v) noted within the species is higher in present data, especially for a few tests like, growth at 8% sodium chloride and at 40^{0}C, ONPG reaction, carbon utilisation tests with α-ketoglutarate and melibiose, acid production from mannitol and sucrose, ampicillin resistance, colony colour in TCBS medium and production of caseinase, haemolysin and nuclease.

Maximum variability in biochemical traits among the strains was exhibited by *V. alginolyticus* (12/52), *V. campbellii* (14/52), *V. harveyi* (13/52), *V. mediterranei* (11/52), *V. parahaemolyticus* (13/52) and *V. vulnificus* (13/52). As the number of tested strains increases, variable results for the tests within the species also increases. This reveals the heterogeneity existing in the species. Heterogeneity among the species can be attributed to the difference in source of isolation, mutation due to the pollution and direct sunlight and long term maintenance in the laboratory by repeated subculturing. Even, a particular test may not give the same result when tested in different laboratories (Bryant *et al.*, 1986a). While interpreting the result it should to be noted that, for a few species like *V. carchariae, V. costicola, V. natriegens V. vulnificus* B2 *etc.* only a

few isolates were isolated in the study and thus the number of tested isolates were meagre to consolidate an authentic result.

Heterogeneity as a main problem in biochemical classification is explained by Hϕi *et al.* (1998). They observed 82 of 97 isolates of *V. vulnificus* B2 were indole positive and questioned the reliability of the sub-division of *V. vulnificus* into two biotype based on the indole reaction. This throws light to the inferiority of the biochemical testing methods for the identification due to the existing heterogeneity and also to the necessity of adopting more sensitive and specific techniques like serotyping, ribotyping and plasmid and other genetic profiling techniques. In isolation process depending on particular traits, the heterogeneity will pose problems. Zuppardo *et al.* (2001) reported that clinical isolates of *V. vulnificus* were more or less homogeneous and the maximum diversity among the strains existed in environmental isolates. However, in countries like India, routine identification methods still relays biochemical traits. Thus the comprehensive information on the distribution and prevalence of *Vibrio* and its characterisation will be helpful to amend the existing identification schemes with respect to characteristics and specificity of the Indian strains.

The tested strains of *V. alginolyticus*, *V. campbellii*, *V. harveyi*, *V. mediterranei*, *V. parahaemolyticus* and *V. vulnificus*, which constituted the bulk of the *Vibrio* flora of tropical waters, exhibited maximum variability in the traits. This variability in the identification traits necessitates a detailed study as the routine identification procedures relay on it. This is especially important for the traits which judge primarily the isolation of

Vibrio, for example, acid production in TCBS medium which determine the primary selection of important *Vibrio* species.

The strains of *V. marinus*, *V. damsela*, *V. parahaemolyticus* and *V. splendidus* II showed maximum variation in acid production from sucrose (Table 35). Acid production from sucrose was the reason for the colour production in TCBS medium. This is a major criterion for the primary isolation of *Vibrio* species as per the standard method like APHA (1976) and US-FDA (1995). Again, in the present study the colour production in the TCBS medium is not found correlated to the acid production from sucrose (Table 35). Though only 30.80% of *V. vulnificus* strains produced acid from sucrose, 79.20% were green in colour TCBS medium. Similarly, 59.44% of the strains of *V. parahaemolyticus* are sucrose acid positive and only 35.54% were yellow in TCBS medium. This discrepancy may be due to the variation in the rate of sucrose fermentation by different strains and some strains could produce only meagre acidity and there by little change in the medium (The original pH of the medium is 8.5.). The green coloured colonies on prolonged incubation was observed to fade away to yellow, especially for *V. parahaemolyticus* cultures. This delayed production of the acid might be due to the late utilisation of lactose present in the medium along with sucrose.

Additionally, *V. alginolyticus*, *V. cholerae* and *V. orientalis* will elaborate the acid to give a yellow halo around the colony. Interestingly, some of the sucrose negative colonies growing in this halo will found to be yellow in colour. This was particularly noted in the mixed culture of *V.*

Table 35. Comparison of percentage of *Vibrio* strains showing positive reaction for acid production from sucrose and colour production in TCBS medium.

Vibrio species	Alsina and Blanch (1994a)	Bryant *et al.* (1986)	Present study	
			Sucrose acid	yellow colour in TCBS
V. campbellii	≤10	46.0	34.9	39.5
V. damsela	11—25	*ND	0.0	0.0
V. logei	11—25	ND	26.1	47.8
V. marinus	≤10	ND	60.0	20.0
V. mediterranei	≥90	ND	74.7	77.8
V. mimicus	≤10	25.0	22.2	33.3
V. parahaemolyticus	≤10	24.0	59.4	35.5
V. splendidus II	≤10	1.0	41.7	83.3
V. vulnificus	11—25	20.0	30.8	20.8

*ND-- Not Determined

alginolyticus and *V. vulnificus*. This colour may mistakes the selection of colony for the isolation and identification. This observation formed the impetus for the study conducted to evaluate masking behaviour noted in TCBS medium.

4.2.1.1. Evaluation of the colour masking behaviour of some *Vibrio* isolates in TCBS medium

The selective medium, TCBS containing 2% sucrose have been considered as the medium of choice for identification of *Vibrio* by the standard microbiological analytical manuals (APHA, 1976; US-FDA, 1995). This medium was originally developed and standardised for the isolation of sucrose positive *V. cholerae* which form typical yellow colonies. Subsequently, the medium came to be used for other vibrios as well. The draw back in the medium as observed here, is that, in presence of large population of sucrose positive colonies, sucrose negative colonies get masked. Hence they escape notice. This might be the reason assumed for the variability in the sucrose reaction observed in the study (see Table 35). Production of yellow or green colonies on TCBS medium by known number of sucrose positive and sucrose negative *Vibrio* species are summarised in Table 36 and Table 37. It is seen from the data that when the levels of sucrose positive and negative are lesser in number and almost equally distributed in the plate, then the recovery of the green colonies is facilitated. On the other hand, in presence of large number of sucrose positive bacterium in the plate, the smaller fraction of the sucrose negatives appeared yellow and get undetected. The acidity elaborated by the sucrose positives may be responsible for this behaviour. This explains

Table 36. The colony characteristics of *Vibrio alginolyticus* and *Vibrio vulnificus* individually and in mixed population (number per plate)

Trial No.	Individual colonies per plate		Mixed colonies per plate	
	V. alginolyticus yellow	*V. vulnificus* green	*V. alginolyticus* yellow	*V. vulnificus* green
1	186	180	269	58
2	135	362	272	169
3	90	548	81	437
4	45	724	538	33
5	9	36	5	34
6	180	36	645	55
7	650	Nil	245	55

Table 37. The colony characteristics of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* individually and in mixed population (number per plate)

Trial No.	Individual colonies per plate		Mixed colonies per plate	
	V. alginolyticus yellow	*V. parahaemolyticus* green	*V. alginolyticus* yellow	*V. parahaemolyticus* green
1	120	115	110	112
2	445	11	449	Nil
3	255	39	242	2
4	89	11	89	3
5	22	226	20	200
6	5	34	5	36

why some yellow colonies on TCBS agar showed a negative sucrose fermentation in broth cultures.

The significance of this finding shows the limitation of the use of TCBS as the primary selective medium for sucrose negative vibrios such as *V. parahaemolyticus* and *V. vulnificus.* As explained in the above section, the isolation was made more confusing in presence of *V. alginolyticus*, which form a major constituent of the *Vibrio* flora of the tropical areas. Hence in the present study, the problem of selection of strains for identification was surmounted by considering all the colonies developed on the TCBS medium irrespective of the colour. It is envisaged that for deriving a rational conclusion on the efficacy of the medium, in depth studies with more trials at different concentrations of sucrose are needed which is beyond the scope of the present work, but points to a future line of research.

4.2.1.2. Selection of identification keys and proposal of an identification scheme for *Vibrio vulnificus*

Tests for the identification should provide high discrimination among the species, for that, they must have a high probability of a definite positive or negative results of a particular test (Rypka *et al.*, 1967). Similarly a particular trait should be less variable within the species. In the present study variable result (26 to 74% positive) for traits were very frequent (see Table 34). Maximum variability was seen for the traits like growth at 8% salt and 40^{0}C, ONPG test, utilisation of α-ketoglutarate and melibiose as sole carbon source, acid production from mannitol and

sucrose, resistance to 10μg ampicillin, colony colour and production of caseinase, haemolysin and nuclease. Since 24 to 76% of the strains showed variable results for these traits, they are not reliable to consider as key test for identification.

The least variability within the species were observed for the growth at 0% and 3% sodium chloride level, arginine dihydrolase, ornithine decarboxylase, amygdalin, gelatinase, gas production, indole production, nitrate reaction, oxidase test, urease production, Voges-Proskauer reaction, xanthine decomposition and acid production from inositol. These tests were ideal for the preparation of identification scheme.

A set of key tests was suggested as per the above results, which will be useful to design an identification scheme for *Vibrio* from tropical environment (Table 38). First set of the test like arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, growth in 3% sodium chloride, oxidase test and resistance to 150 μg 0/129 (vibriostatic agent), were the essential tests for the tentative isolation of *Vibrio* species. These tests have minimum variable results and high discrimination among the species. Growth at 10% and 6% salt, citrate utilisation, gelatinase production, indole production, Voges-Proskauer test, utilization of L-arabinose and lactose as sole carbon source, acid production form arabinose and salicin were included in the second set. These tests showed comparatively less variation. The third set is with minimum discrimination among the species and it could thus be used in the final stages of classification. For example, amygdalin hydrolysis could be useful

Table 38 Key identification tests recommended for the isolation of tropical environmental *Vibrio* species based on the present study.

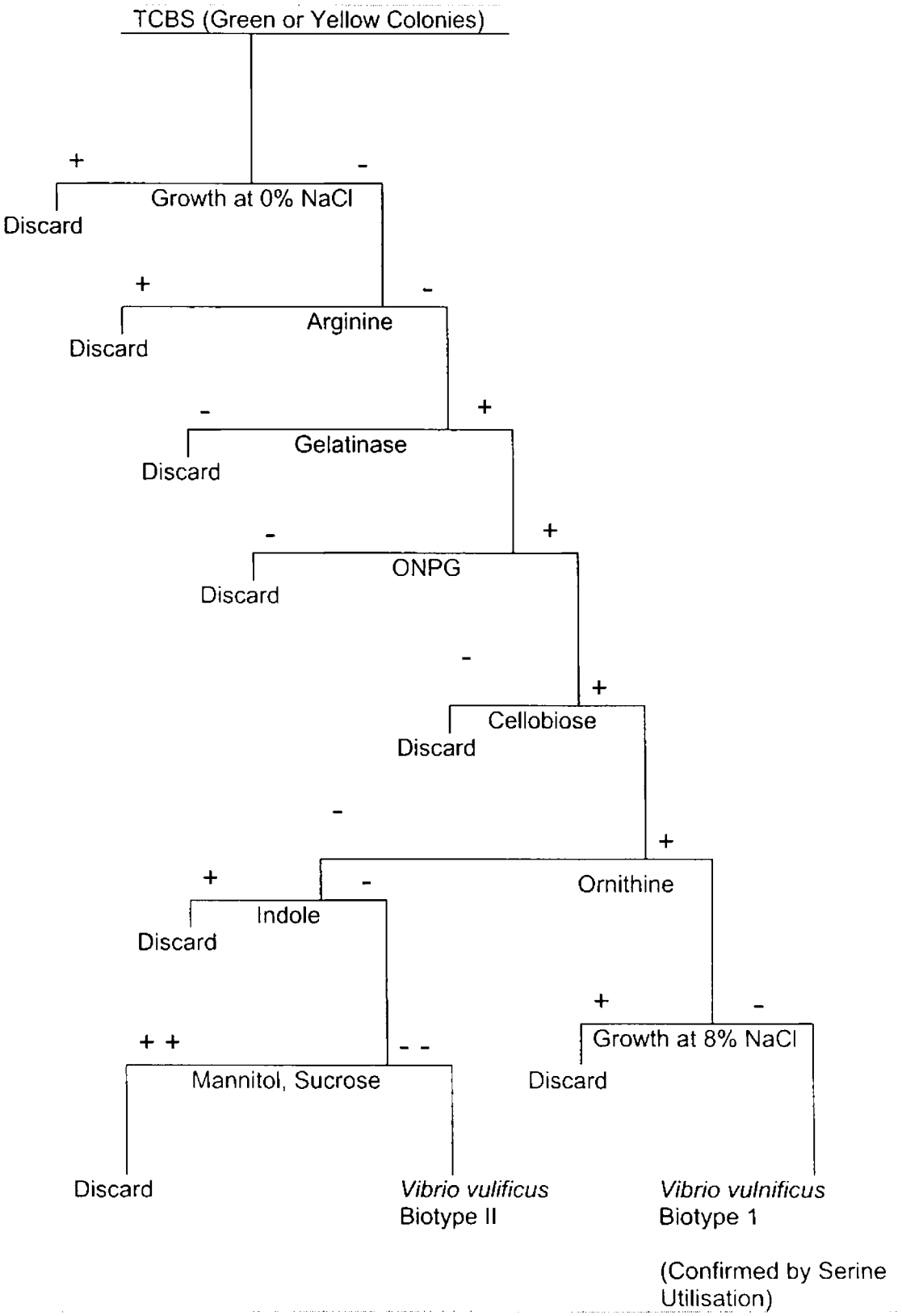
I. For presumptive isolation	
	Decarboxylation of Arginine, Lysine & Ornithine
	Growth in 3% sodiun chloride
	Oxidase test
	Resistance to 0/129 (150 μg)
II. For Confirmation	
	Growth at 10% & 6% sodium chloride
	Citrate utilisation
	Gelatinase production
	Indole production
	Voges Proskauer reaction
	Utilisation of L-arabinose & lactose as sole carbon source
	Acid production from arabinose & salicin
III. For further Confirmation	
	Amygdalin
	Gas production from glucose
	Urease production
	Xanthine decomposition
	Acid production from inositol
	Growth at $0^{o}C$
	Luminescence
	Nitrate reduction

in discriminating *V. charchariae* from other species as *V. carchariae* is the only species in the genus giving positive result for the test. Selection of identification of traits were done as per the criteria proposed by Rypka *et al.* (1967). They stressed that during the selection of identification test, the selected traits must be in agreement with that of the other authors. Here all the recommended tests in the identification key were proposed by either or one of the data matrices compared in the previous section.

Preparation of scheme considering all variable characters for the identification tropical *Vibrio* species is not attempted in this context as the enormity of the study area is beyond the scope of this chore. However, the data matrix of different biochemical traits derived in the study and the key tests recommended here may hope to form the basis for a future research plan since a reliable identification strategy for the tropical vibrios is the need of the time.

Identification of *V. vulnificus* is important in the present context, as it constantly associated with the food borne illness resulting from shellfish (Hlady, 1997). Here, after considering the biochemical properties exhibited by 102 isolates from various marine environments, a sensitive and reliable identification scheme enabling the easy isolation of *V. vulnificus* from the rest of the tropical vibrios is proposed (Figure 3). Such an identification key is much essential as the general schemes of identification proved to be not sensitive for *V. vulnificus* as the isolates may confuse with *V. campbellii* and *V. harveyi* as both shared similar cultural and physiological characters with the former.

Figure 3. Proposed scheme for the isolation of *Vibrio vulnificus* from seafood and marine samples



The characters considered for the preparation of the scheme were colour in TCBS medium, growth at 0% sodium chloride, arginine dihydrolase, gelatinase, ONPG reaction, cellobiose reaction, growth at 8% sodium chloride, ornithine decarboxylase, indole reaction and production of acid from mannitol. These tests showed least variability for the trait among the strains. All of the tested strains were negative for growth at 0% sodium chloride, arginine dihydrolase and growth at 8% sodium chloride (see Annexure 1). Rest of the characters like gelatinase (98.08%), ONPG reaction (96.15%), cellobiose reaction (98.10%), ornithine decarboxylase (98.08%), indole reaction (98.08%) and production of acid from mannitol (96.15%) were positive. For *V. vulnificus* biotype II, selected traits like indole and acid from mannitol were negative. Salient features of the traits used in the key were interpreted as follows:

1. Colour in TCBS colony

Generally green coloured colonies were selected for the identification of *V. vulnificus* (APHA, 1976; US-FDA, 1995). Here 20.77% of the isolates produced yellow coloured colonies. Moreover as discussed earlier, in the vicinity of a large population of yellow colonies, the green colour is masked. Thus all the colonies are considered irrespective of the colour.

2. Growth at 0% sodium chloride

Isolates that tolerate 0% sodium chloride are *V. cholerae*, *Psuedomonas shigelloides*, *Aeromonas* spp. (Bryant *et al.*, 1986a; Baumann *et al.*, 1984), *V. fluvialis*, *V. furnissii*, *V. metschnikovii* and *V. mimicus*. Hence in this step these species/genera are eliminated.

3. Arginine dihydrolase production

A positive reaction is shown by *Photobacterium*, *Aeromonas*, *Psuedomonas*, *V. anguillarum*, (Bryant *et al.*, 1986a) *V. damsela*, *V. proteolyticus*, *V. fluvialis* and *V. furnissii*, whereas *V. vulnificus* is negative for the traits.

4. Gelatinase

This reaction eliminates *V. fischeri* (Bryant *et al.*, 1986a) *V. pelagius* I, *V. logei*, , *V. damsela* and *V. proteolyticus* which are negative for the trait. *Vibrio logei*, which often perplexed the isolation of *V. vulnificus* and was also frequent in tropical waters is eliminated in this step.

5. ONPG reaction

Dominant *Vibrio* species like *V. alginolyticus*, *V. parahaemolyticus* and *V. campbellii* were eliminated by this test. *V. carchariae* is also negative for the test.

6. Cellobiose reaction

Species like *V. marinus*, *V, metschnikovii*, *V. pelagius* II could be discarded after this test as they gave a negative test.

7. Ornithine decarboxylase

Ornithine decarboxylase negative cultures are *V. damsela*, *V. cincinnatiensis*, *V. costicola*, *V. logei*, *V. marinus*, *V. pelagous* II and *V. vulnificus* biotype II are eliminated.

8. Indole and acid production from mannitol

Indole negative isolates that are mannitol negative are *V. vulnificus* biotype II.

Table 39. Evaluation of the key proposed under the present study with the existing keys

Vibrio vulnificus strains used	Sample	No. of Trials	*Vibrio vulnificus* detected by the method of		
			Alsina and Blanch, 1994a&b	Oliver *et al.*, 1989	Present study
NCIMB 2046	Fish	6	6	4	6
vv70	Prawn	6	5	3	6
vv69	Dry fish	6	6	5	6

The identity of the *V. vulnificus* strains are further confirmed by checking their efficiency in serine utilisation, as strains are positive for the trait (Oliver *et al.*, 1982).

The authenticity of the key was evaluated by test trails with artificial inoculation of known quantity of type strains of *V. vulnificus* (NCIMB 2046, vv70 and vv69) as well as the isolation from natural samples. The strains were identified ~~with~~ simultaneously with the key proposed (Figure 3) in the study and the scheme of Alsina and Blanch (1994a; 1994b) and Oliver *et al.* (1989). The results of the study are presented in Table 39. The results indicate that the detection and identification of the *V. vulnificus* is improved by using the proposed key. The improved specificity and sensitivity of the key was also substantiated by the high recovery rate from proposed key. Other advantage of the scheme is that it allow discrimination of *V. vulnificus* from *V. harveyi* and *V. campbellii* (especially from the former) as both are quite identical with *V. vulnificus* and are very frequently observed in the fish samples from the tropical area (Thampuran and Surendran, 1998; Sanjeev *et al.*, 2000).

4.2.2. *In vitro* growth studies of selected *Vibrio* species

Survival of selected *Vibrio* species *viz. V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus* and *V. harveyi* were studied. Parameters tested were different temperatures, salinity, pH, reduced oxygen level and different bile concentrations.

4.2.2.1. Tolerance to temperature by selected *Vibrio* species.

Growth pattern of representative strains of *V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. harveyi* and *V. mimicus* at 4°C, 15°C, 28±2°C (RT), 37°C and 42°C were presented in Figure 4. There was only negligible difference exhibited in the pattern of growth among the three strains tested irrespective of the variations due to difference in inoculation size. Heterogeneity among the strains of *Vibrio* species is widely reported, especially for metabolic properties (Hϕi *et al.*, 1998). Present study also revealed heterogeneity among the strains for individual traits (see Table 34). Eventhough heterogeneity existed among the strains for individual metabolic traits, the effective growth seemed to be not affected.

For all the test cultures, growth was observed at 15°C, RT and 37°C. *V. alginolyticus* and *V. parahemolyticus* showed growth at 42°C and none of the cultures showed growth at 4°C. At room temperature and 37°C all the test cultures showed characteristic growth curve without a lag phase and slight difference in the curve corresponding to the difference in growth rate was observed. Growth was maximum at 37°C and RT for all the strains tested. For *V. mimicus, V. parahaemolyticus* and *V. alginolyticus,* the stationary phase attained at the first 18h of incubation, whereas, the same for *V. harveyi* and *V. vulnificus* were delayed. At 15°C, little growth was noted in the first 24h for *V. harveyi* and *V. vulnificus.*

At 4°C, none of the *Vibrio* strains showed growth. This observation corroborates with Baumann *et al.* (1984). While, Bryant *et al.*

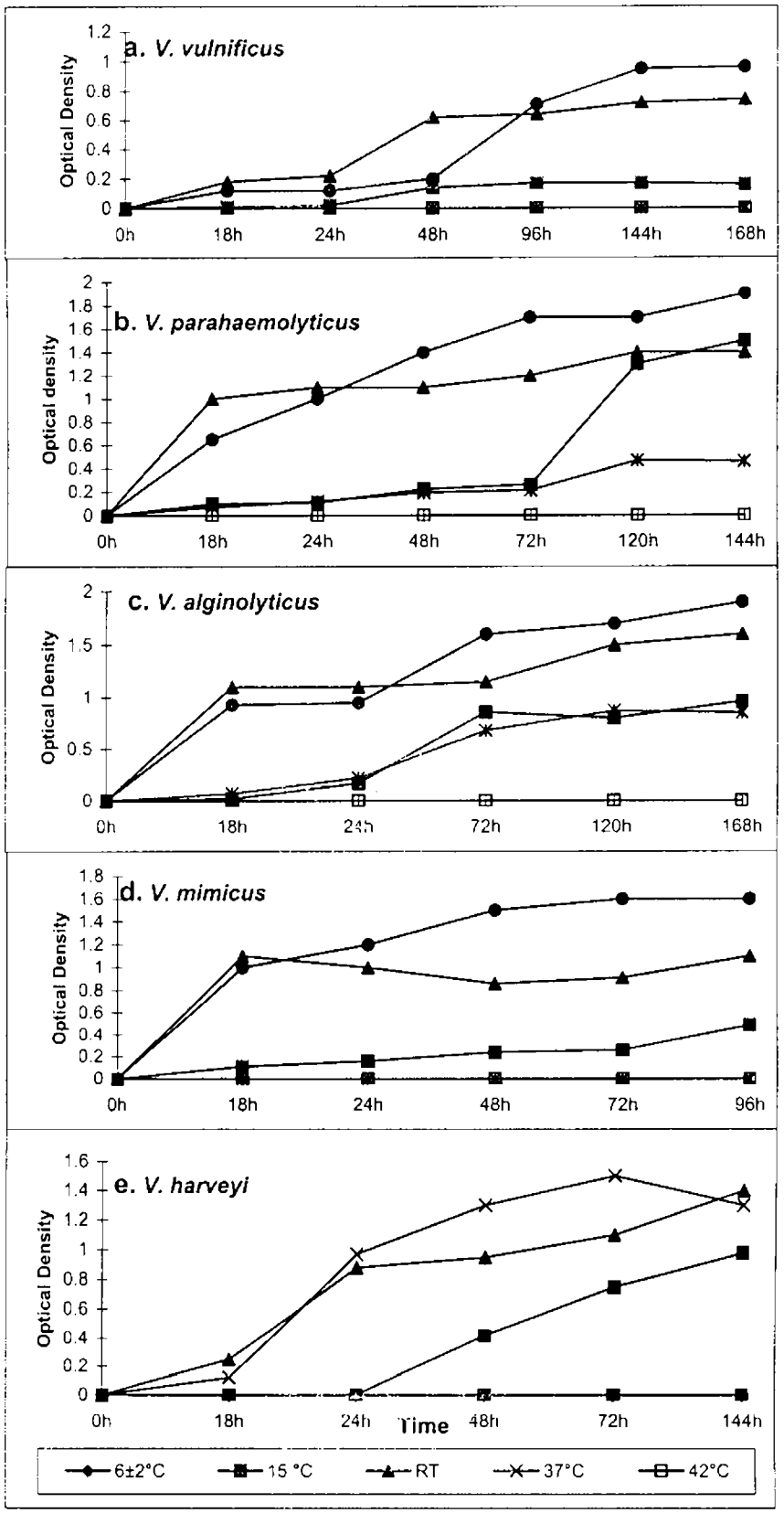


Figure:4 Growth pattern of various *Vibrio* species at differen incubation temperature in Trypticase soy broth

(1986a) reported 11.0% of *V. alginolyticus* showed growth at 4^0C. Strain difference could be attributed to this observation. Survival of *V. vulnificus* (Kim and Kwon, 1997) and *V. parahaemolyticus* (Venugopal *et al.*, 1999) and *V. mimicus* (Wong *et al.*, 1994) were studied extensively at refrigerated temperature. Goatcher *et al.* (1974) stated that inoculum size is highly influential for the elimination of vibrios. Eventhough there was no visible growth in cultures at 4^0C all the test strains except *V. vulnificus* retained viability at the end of seventh day. Existence of viable cells were reported after long term refrigeration in the case of *V. parahaemolyticus* (Beuchat, 1975; Venugopal *et al.*, 1999), *V. mimicus* (Wong, 1994) and *V. vulnificus* (Boisca *et al.*, 1996; Kim and Kwon, 1997). In the present study *V. vulnificus* cells were not detected in the culture (initial count 1.7×10^4 cfu ml^{-1}) on the seventh day of incubation. Similar lose of viability was reported for *V. vulnificus* by Oliver *et al.* (1991), Wolf and Oliver (1992) and Oliver (1993). They explained it may be due to the formation of viable but non culturable (VBNC) state.

Boisca *et al.* (1996) reported that in VBNC state *V. vulnificus* could survive at least 50 days at low temperature. Thus the hazard exists even when the products are stored at low temperature. Low temperature survival is a cause of concern as it can revert back into actively growing stage, at higher temperatures, which is often occur due to mishandling. Fortunately, virulance factors like haemolysin, protease *etc.* will not produce significantly at refrigeration temperature (see Table 47) Moreover the chance of cooked or processed food being cross contaminated from

frozen or refrigerated food through food handlers or their utensils should not be ruled out.

Growth rate for all the five species were high at the room temperature (28±2^{0}C) and 37^{0}C. Maximum growth was observed at 37^{0}C confirming its human pathogenic nature. Optimum temperature for growth of these species was 37^{0}C (Sudha *et al.*, 1998). The enhanced growth rate at ambient temperature is significant as many of the *Vibrio* species are very potent spoilers in this temperature (Chandrasekharan *et al.*, 1985; Surendran and Gopakumar, 1985). Bacteria that are susceptible to the adverse conditions can be easily eliminated at the exponential phase of growth, where multiplication rate is maximum. From the Figure 4, it is evident that the exponential phase falls in the initial hours of growth. In food systems, like fish meat, where growth of the bacteria will be rapid, it can be predicted that the complete elimination of susceptible cells might be possible by resorting to efficient preservation practices during the exponential phase. This suggestion is supported with the observation of Goatcher *et al.* (1974) and Thampuran and Gopakumar (1993) that the maximum destruction *Vibrio* from fish muscle homogenate occurs at first 24h of incubation at low temperature. By adopting techniques like chilling, freezing, blanching and addition of preservatives *etc.* soon after the harvesting can eliminate the risk by the vibrios.

Only *V. alginolyticus* and *V. parahaemolyticus* could grow at 42^{0}C. Growth of this species at 40°C was reported earlier (Alsina and Blanch, 1994a; Baumann *et al.*, 1984). Since higher temperatures were

detrimental for *V. mimicus*, *V. vulnificus* and *V. harveyi*, application of elevated temperature assured an optional preservation techniques. This also ensures minimum alteration from the raw state which is a quality highly appreciable in the modern food trends. This observation form the impetus for the thermal death point study, where all the tested strains were eliminated by heating up to 55^0C for a few minutes (see Figure 16).

4.2.2.2. Tolerance to salinity by selected *Vibrio* species

Growth patterns of *V. vulnificus, V. parahaemolyticus V. alginolyticus V. mimicus* and *V. harveyi* in differing salt concentrations ranging from 0.5 to 20% are presented in Figure 5. The lowest sodium chloride level in which growth observed was 0.5% and the exception was *V. mimicus*, which could grow even at 0.0% sodium chloride level though at a lower rate. This agrees with the earlier report of Baumann *et al.* (1984), Bryant *et al.* (1986a), and Alsina and Blanch (1994a). *Vibrio alginolyticus* and *V. parahaemolyticus* could tolerate 10% sodium chloride whereas the corresponding limit for *V. mimicus* and *V. vulnificus* was 6% (no growth at 8% level).

Optimum salt level for growth of *V. vulnificus* was found to be 1.0 to 2.0% sodium chloride (Kelly, 1982). In the present study maximum optical density was noted at 4% level with a lag phase of 24h for *V. vulnificus*. Fairly good growth observed in 2.0, 2.5 and 3% also. For *V. vulnificus*, the optical density values were not seem to be correspondingly increasing with the increase in the turbidity. This discrepancy was especially noticeable in lower range from 0.5 to 2.5% salt level. This may

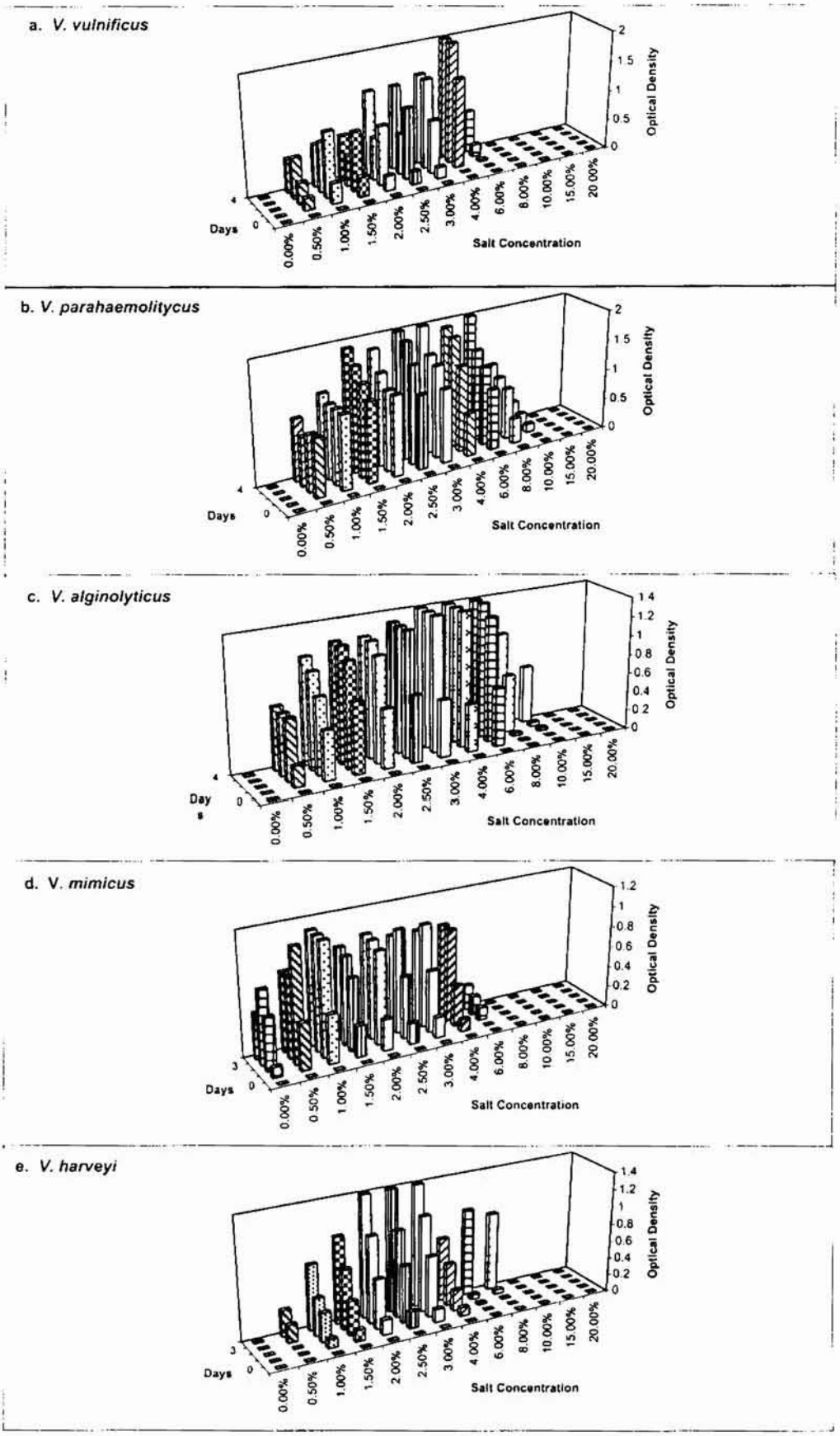


Fig. 5. Growth pattern of *Vibrio* species in trypticase Soy Broth with different sodium chloride levels

be due to some intermediary metabolite which interfere with the optical density reading. Three percentage sodium chloride was considered to be optimum for *V. vulnificus and V. alginolyticus*, 2.5% for *V. parahaemolyticus* and *V. harveyi* and 1% for *V. mimicus*. Since 15% sodium chloride was lethal to all tested *Vibrio* species, salting above 15% was found adequate for elimination of vibrios and thus practice of salting can be considered as a safe method of preservation of sea foods from the risk of vibrios.

4.2.2.3. Tolerance to pH by selected *Vibrio* species

Growth pattern of *V. vulnificus, V. parahaemolyticus V. alginolyticus V. mimicus* and *V. harveyi* in trypticase soy salt broth adjusted to pH ranging from 3 to 9 are shown in Figure 6. In all cases, visible growth was noted only in pH 6 and above. At higher pH all the tested strains fairly good growth and pH 7.5 was found to be optimum for the growth. *V. parahaemolyticus* was the most tolerant species followed by *V. alginolyticus.*

The pattern of growth in different physico- chemical parameters will help to follow the fate of vibrios in the sea foods. While manipulating these *in vitro* patterns of growth to chalk out better preservation technique it should also be consider that in natural conditions the growth and sensitivity will be influenced by many other chemical and biological factors (Kasper and Tamplin, 1993; Kim and Kwon, 1997).

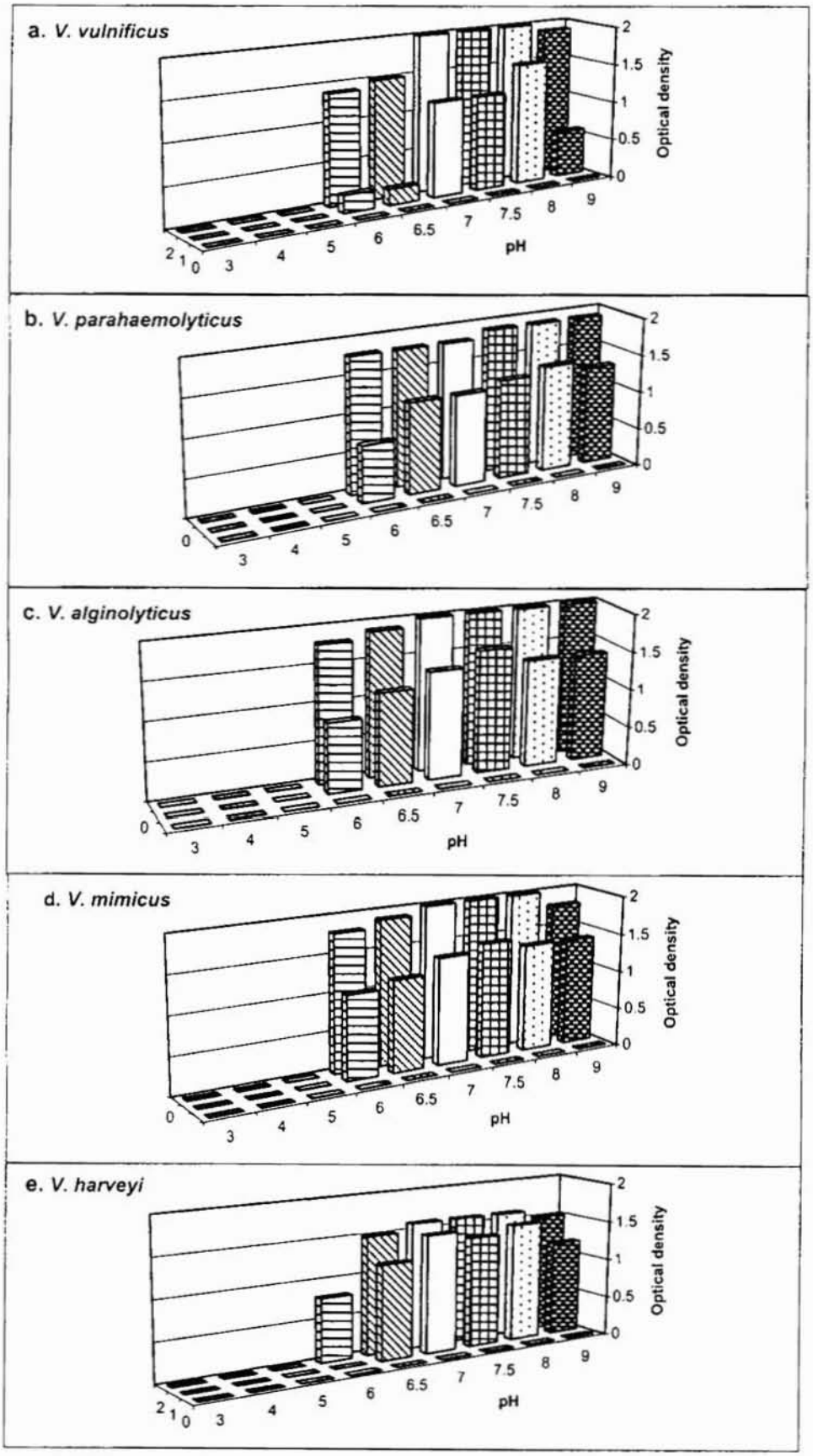


Fig. 6. Growth pattern of various *Vibrio* species in Trypticase soy broth at different pH levels

4.2.2.4. Growth of selected *Vibrio* species as affected by low oxygen levels

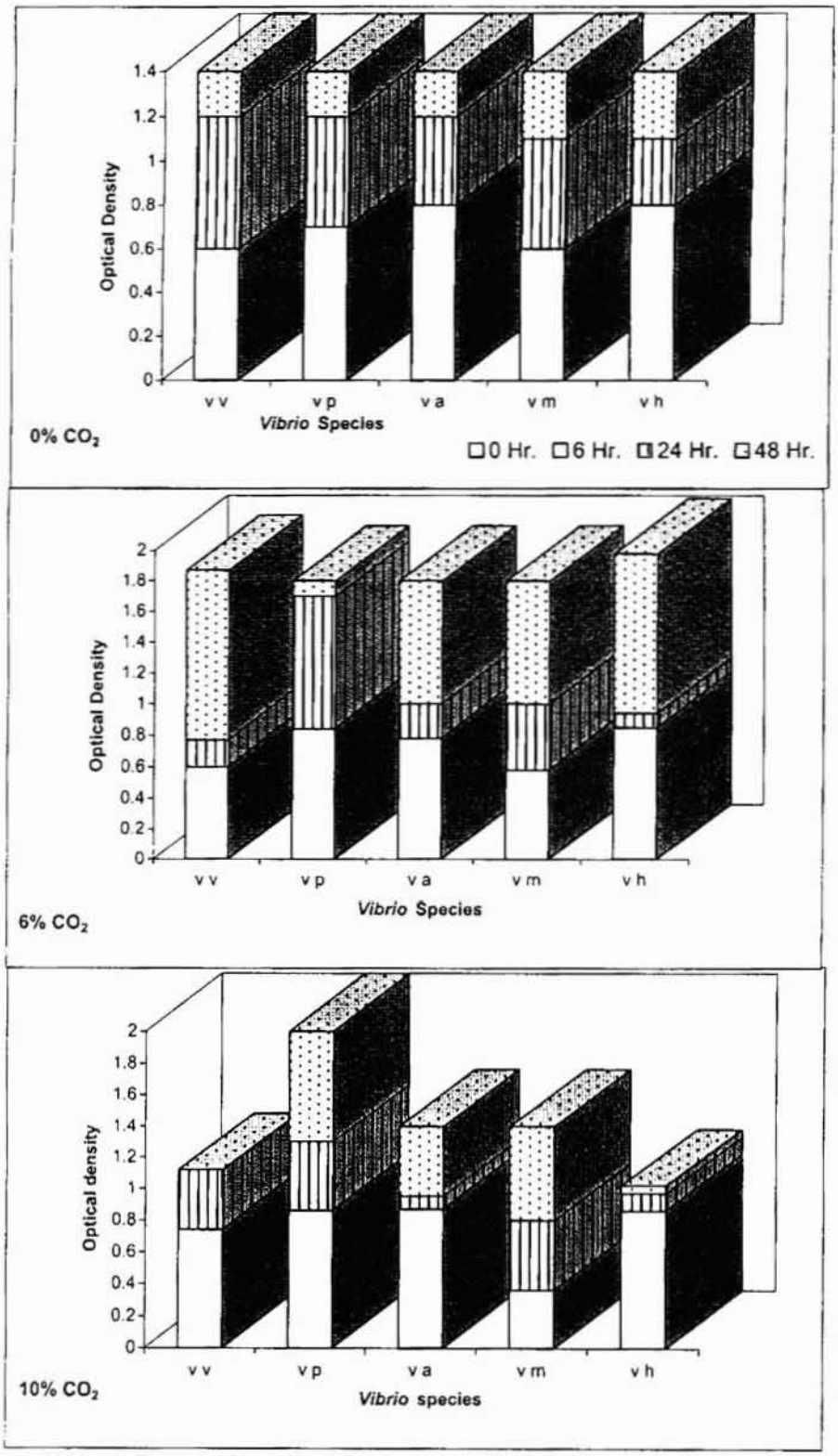
Low oxygen level of the incubating atmosphere was found not affecting the growth of tested *Vibrio* species. Carbon dioxide added at the level of 5 and 10% did not have any deleterious effect on the growth (Figure 7). *Vibrio parahaemolyticus* was found to have an enhanced growth when compared to other species in 10% carbon dioxide levels. No lag phase observed in any of the experimental systems, with considerable growth in first 6h of incubation.

Vibrio species are facultative anaerobes (Baumann *et al.*, 1984) and growth of *Vibrio* in the reduced oxygen levels might be considered as a favourable factor for the colonisation of the bacterium in the intestine.

Use of modified atmosphere storage of fish and products for the extension of shelf life was gaining importance now a days. The combination of low temperature and various gases, especially CO_2 replacing air in the storage atmosphere has proven effective in reducing spoilage bacteria of fish and other foods (Mokhele *et al.*, 1983; Molin *et al.*, 1983; Isenschmid *et al.*, 1995).

4.2.2.5. Tolerance to bile by selected *Vibrio* species

Combined effect of different bile concentrations and pH on the growth of five selected *Vibrio* species were assessed. Growth was noted turbidimetrically for two days, as the control tube without bile showed maximum growth within this time. No growth observed at 0.4% bile level.



v v- *Vibrio vulnificus* ; v p- *V.parahaemolyticus* ; v a - *V.alginolyticus;*
v m- *V. mimicus* ; v h- *V. harveyii*

Fig. 7 Growth pattern of *Vibrio* species in Tripticase soy broth in CO_2 modified atmosphere

For *V. vulnificus*, growth started only and proceeded in slow rate at the extremes of tested pH ranges (Figure 8). In the experimental conditions, bile salt did not considerably affect the growth of *V. parahaemolyticus*, even though growth rate was reduced in higher bile concentrations (Figure 9). At pH 6 and 7, *V. alginolyticus* was sensitive to bile, but in high pH it could tolerate higher levels of bile (Figure 10). *Vibrio mimicus* showed considerable tolerance especially at high pH (Figure 11). Bile concentration of 0.3% and above where found inhibitory at pH 6.0 for *V. harveyi* (Figure 12).

Vibrio parahaemolyticus was the least affected species, followed in the descending order, *V. mimicus, V. harveyi, V. vulnificus* and *V. alginolyticus*. Combined effect of high bile level and low pH was more detrimental and thus high pH has a protective effect in various bile concentrations.

Intestines of marine fishes form a very conducive microcosm for vibrios (Cahill, 1990). Characters helping the colonisation included pH tolerance, bile resistance and the ability to establish competitively. Sera and Ishida (1972) confirmed the indigenous nature of *Vibrio* by stating that *Vibrio* species isolated from the gut of marine fish were resistant to 2% bile and pH 5.5. In the present study, strains belonging to five pathogenic species of *Vibrio* were found inhibitory to 0.4% bile salt. The value could not be compared *in toto* as the constituent of the bile was different in both the studies. As the pH decrease the bile tolerance also decreases, irrespective of the concentration . As per the result, *Vibrio* might be sensitive to the low pH of the stomach of fish . However, presence of food

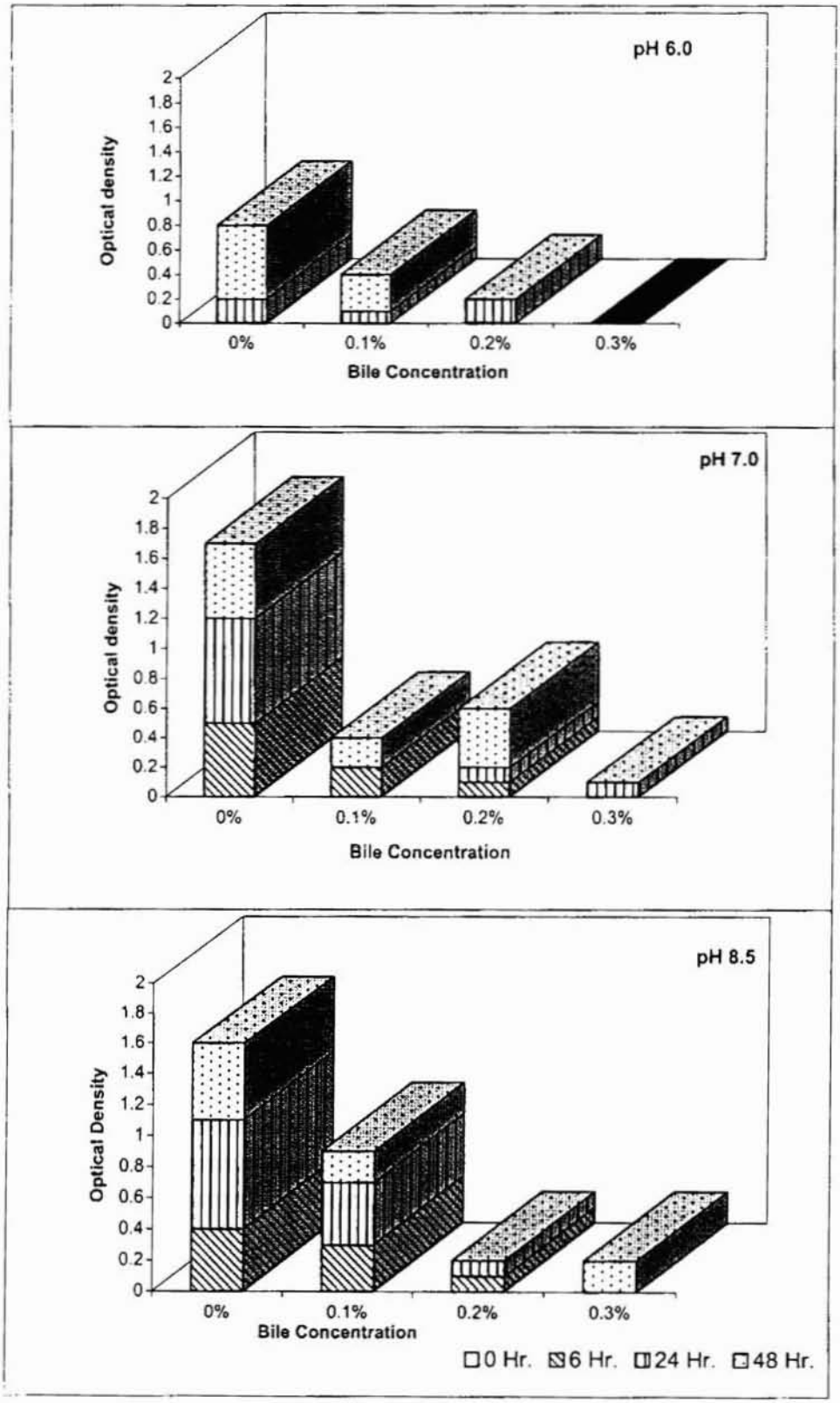


Fig. 8 Growth pattern of *Vibrio vulnificus* in different bile concentrations and pH

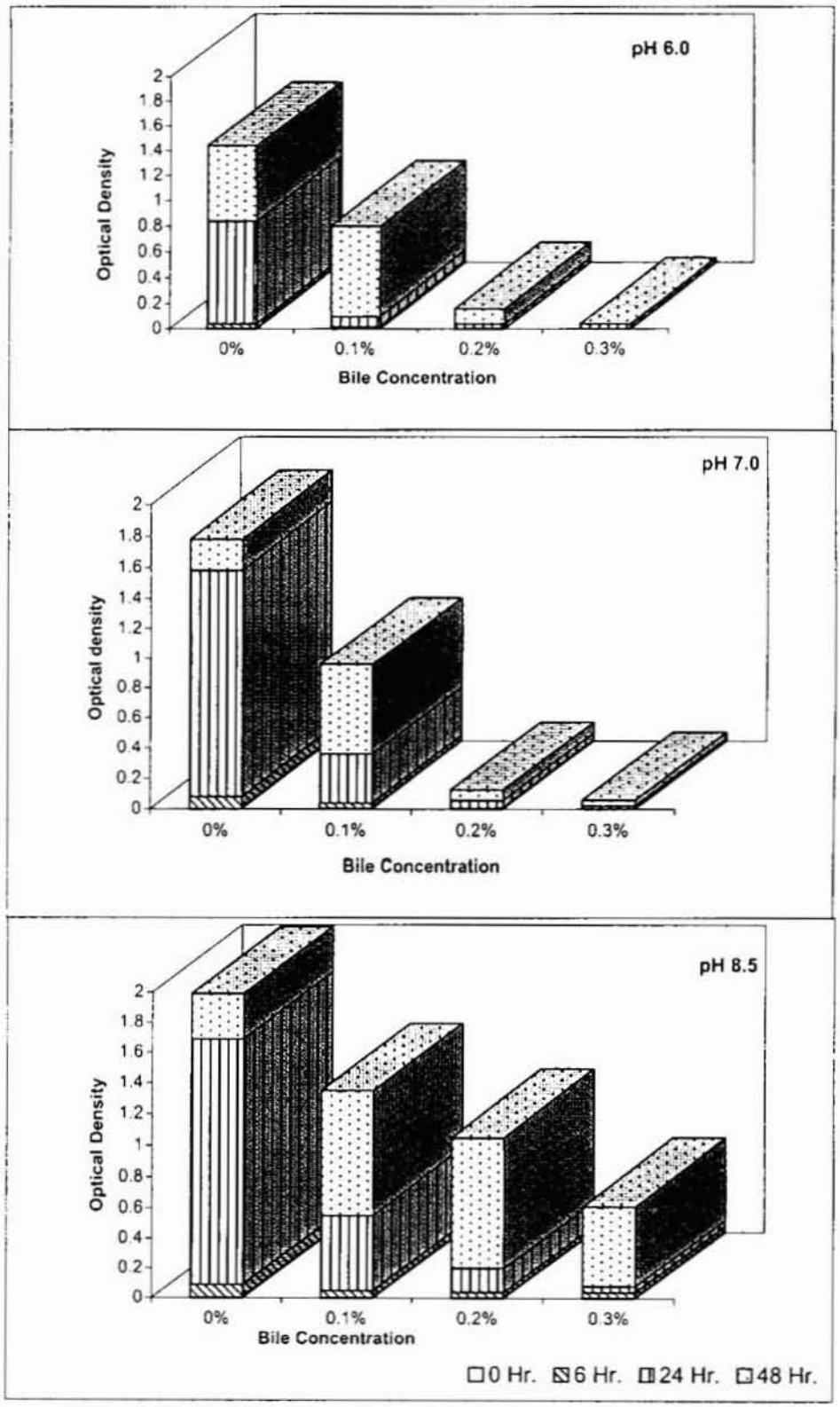


Fig. 9 Growth pattern of *Vibrio parahaemolyticus* in different bile concentrations and pH

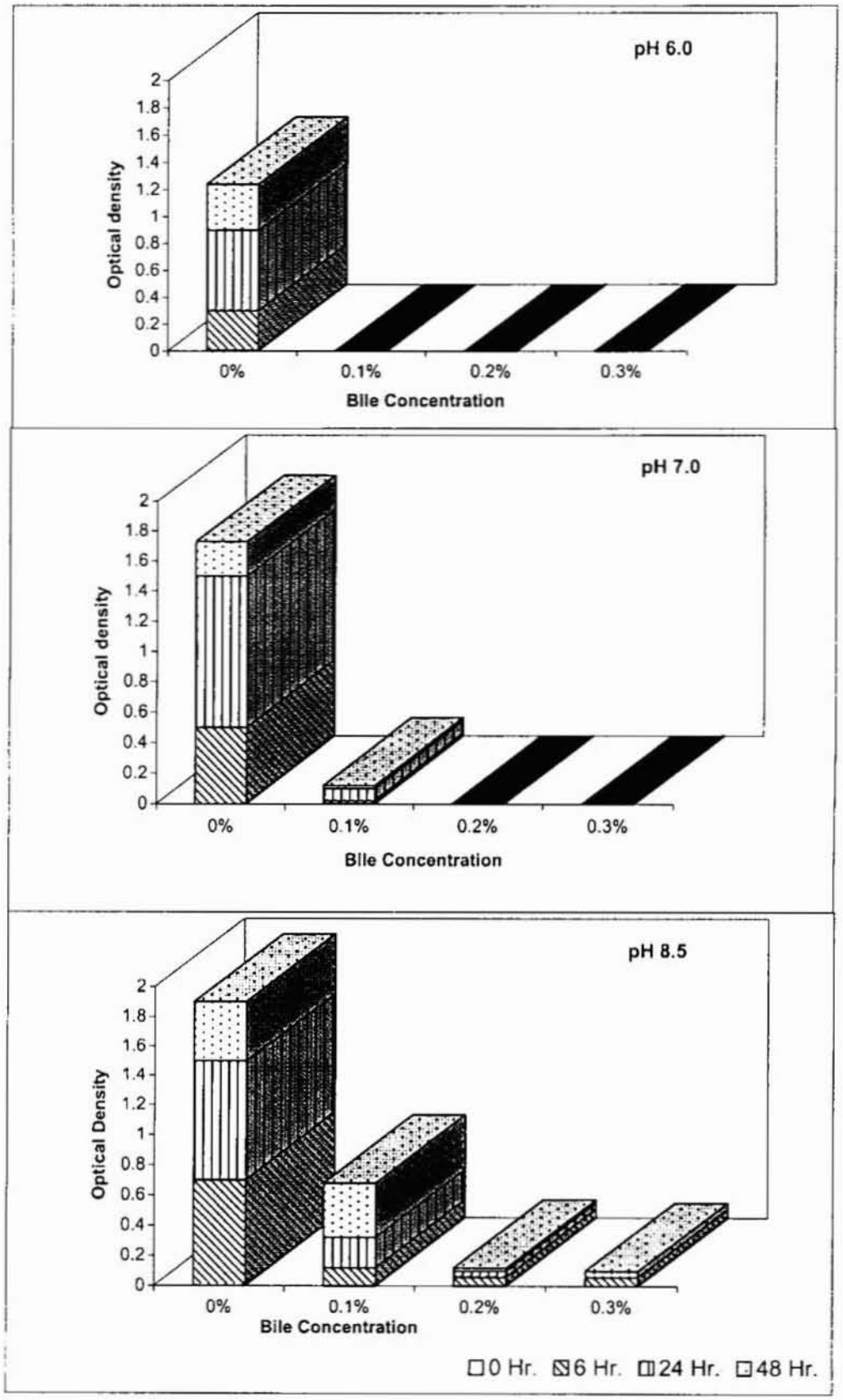


Fig. 10 Growth pattern of *Vibrio alginolyticus* in different bile concentrations and pH

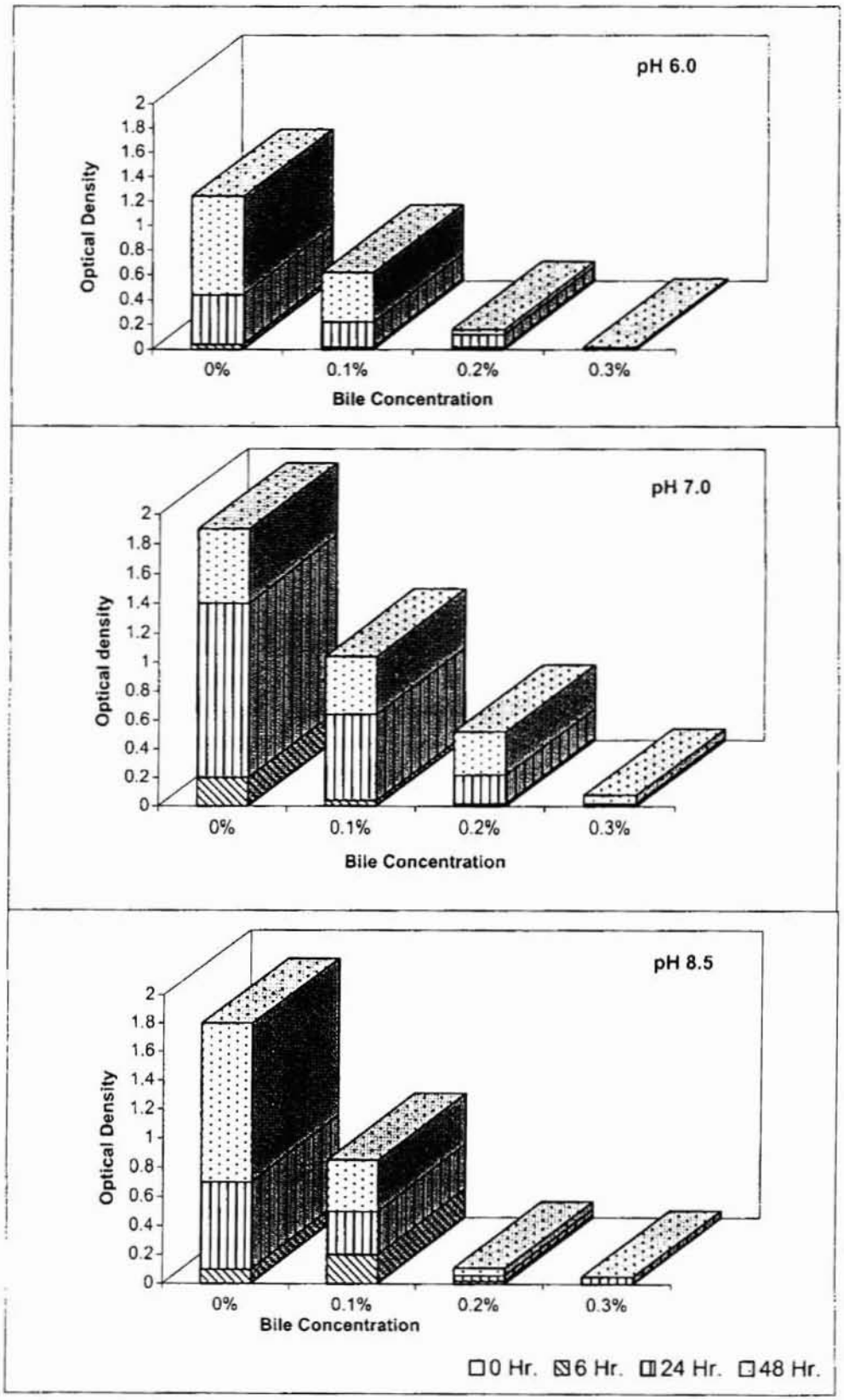


Figure 11. Growth pattern of *Vibrio mimicus* in different bile concentrations and pH

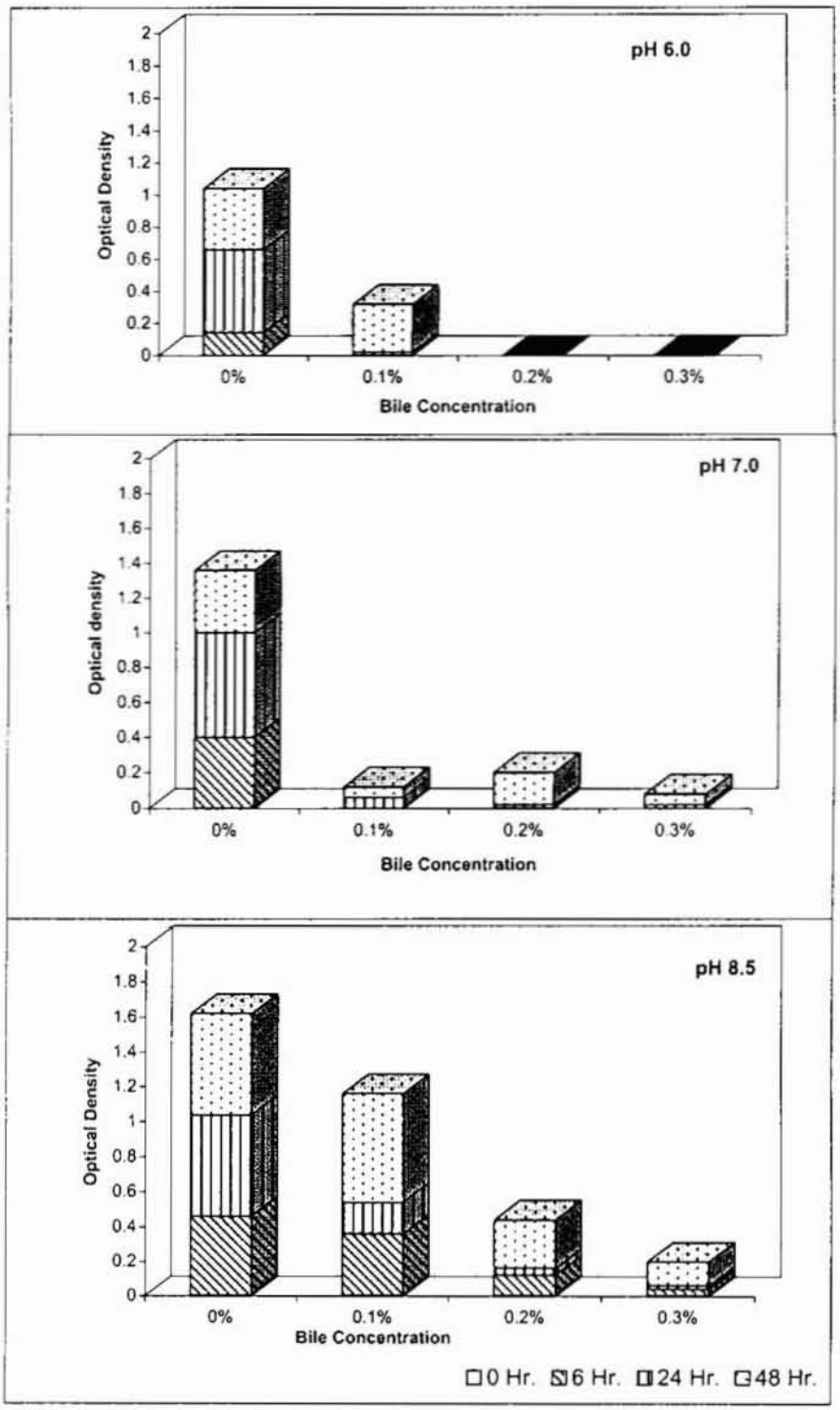


Fig. 12 Growth pattern of *Vibrio harveyi* in different bile concentrations and pH

in the stomach can alter the pH and help the bacterium to overcome the effect of bile. Indirectly, the acidity occurring as a result of the starvation in fish stomach can make bile concentration more lethal to the existence of vibrios in the intestine.

Frequent isolation of *V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveyi* from the intestine can be explained in terms of their ability to withstand bile and low pH of stomach. However, *V. alginolyticus* which was also a frequent isolate were comparatively sensitive to bile at low pH. Since the oral introduction of *Vibrio* species as probiotic (Westerdahl *et al.,* 1994) and vaccines against vibriosis (Newman, 1993; Devi *et al.*, 1996) is gaining importance in aquaculture, the study on the bile tolerance is much relevant.

4.2.2.6. Competitive growth of selected *Vibrio* species in mixed cultures

An important factor favouring the colonisation of *Vibrio* species in the intestine is their ability to establish growth competitively with other bacteria. This aspect was studied with respect to a pathogenic *Vibrio* species of emerging importance namely, *V. vulnificus* by noting the growth performance of this organism in mixed culture with three other members of the same genus-(1) *V. parahaemolyticus*, an organism very similar to *V. vulnificus* in many respects, (2) *V. alginolyticus*, the most dominant among the vibrios and (3) *V. harveyi*, the luminiscent *Vibrio* causing disease in aquatic animals and also with *Aeromonas hydrophila*, a non-vibrio but member of vibrionaceae and *Escherichia coli*, enterobacteriaceae member

quite comparable physiologically to vibrios. Survival were noted as plate counts and shown in Figure 13.

No considerable difference was observed in growth pattern of *V. vulnificus* when grown together with *V. alginolyticus* and *V. harveyi.* In the case of mixed growth with *V. parahaemolyticus, V. vulnificus* showed a three logarathmic unit reduction in their viable cells when compared to the corresponding count in pure culture after 24h of incubation. Thus *V. parahaemolyticus* cells produce a sort of negative impact on the survival of *V. vulnificus.*

One logarithmic reduction in the cell count of *V. vulnificus* were noticed in mixed culture with *E. coli,* in comparison with pure cultures. In mixed culture with *A. hydrophila,* cell count of *V. vulnificus* was found drastically decreased from 1.3×10^5 ml^{-1} to 3.1×10^1 ml^{-1} within 24h growth.

The associative growth of *Vibrio* species has received little attention the world over and reported work in this aspect is practically absent. Hence the present study is highly relevant. The study clearly shows that the growth pattern of *V. vulnificus* in individual and in mixed population is different. The finding that the number of *V. vulnificus* is comparatively low in seawater can be explained by the inability of this pathogen to grow competitively with others and to ascertain its presence.

4.3. Pathogenic potential of *Vibrio* species

The genus *Vibrio* constituted some of the most virulent pathogens known to man. *Vibrio cholerae* is responsible for many fatal

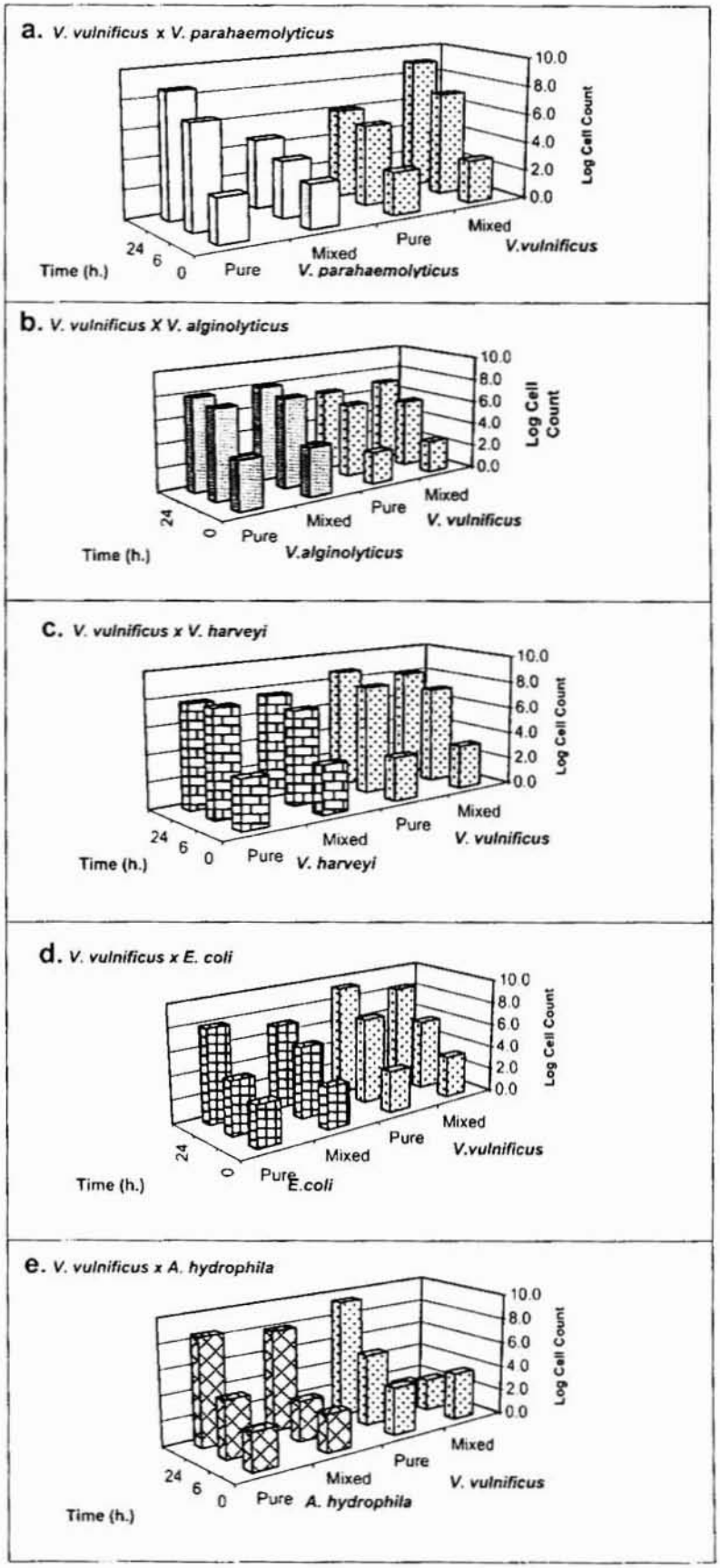


Figure 13. Competitive growth of *Vibrio vulnificus* in mixed culture with *V. parahaemolyticus, V. alginolyticus, V. harveyi, Escherichia coli* and *Aeromonas hydrophila* and their comparison with pure culture

epidemics of food poisoning and its virulence mechanisms were studied in detail. But the virulence mechanisms of halophilic vibrios were yet to be deciphered out. In this context, the pathogenic nature of the common *Vibrio* species in the tropical area studied with reference to their capacity to elaborate one or more of the enzymes that can degrade biomolecules and also by bioassay in mice.

4.3.1. Production of hydrolytic enzyme by selected *Vibrio* species.

Hydrolytic enzymes have been considered as virulence determinants because they allow bacteria to survive, proliferate or invade host tissues. Role of hydrolytic enzymes like protease, lipase, lecithinase, DNase in the virulence of pathogenic vibrios have been established (Liu *et al.*, 1996; Moreno and Landgraf, 1998).

A total of 160 isolates of *Vibrio* comprising 17 prevalent species were tested for the production of various hydrolytic enzymes at temperatures ranging from 6 ± 2^0C to 42^0C. The number of strains of different *Vibrio* species showing production of the various hydrolytic enzymes are presented in Table 40 to Table 45. *Vibrio* strains included in the study were isolated from the marine environments as well as from fishes collected from Cochin area. A few type strains used in the study were listed in the Table 9.

4.3.1.1. Production of protease by *Vibrio* species

Protease production by 17 *Vibrio* species at the different temperatures (6 ± 1^0C, 10^0C, room temperature (28 ± 2^0C), 37^0C, and 42^0C) are tabulated (Table 40). Zone sizes were measured and cultures were clustered as strong, medium and weak producers of protease depending

on the zone radius of 20mm or above, 10mm or above and below 10mm respectively. Maximum number of strains produced protease when the plates were incubated at 37^{0}C (70.6%) and at the room temperature (67.5%). Of the 17 species, 15 were capable of producing protease. At 37^{0}C, *V. alginolyticus, V. carchariae, V. cincinnatiensis, V. fluvialis, V. logei, V. metschnikovii* and *V. mimicus* showed cent percent enzyme production among their strains. All these species, except *V. logei*, are grouped as human pathogens (Dalsgaard, 1998). Other pathogens like *V. hollisae* and *V. parahaemolyticus* produced the enzymes at 80% level at this temperature, whereas *V. damsela* and *V. furnissii* were observed negative for the enzyme production at all the test temperatures.

Proteases are considered most important among the enzymes causing direct tissue damage and enhanced invasiveness (Norqvist *et al.*, 1990). Protease enzyme production in relation to pathogenicity has been reported for *V. alginolyticus* (Balebona *et al.*, 1998*) V. harveyi* (Liu *et al.*, 1996; Liu and Lee, 1999), *V. vulnificus* () and *V. anguillarum* (Farrell and Crosa, 1991). Cystiene protease was discriminated as the major virulence factor of *V. harveyi* against tiger prawn, *Penaeus monodon* (Liu and Lee, 1999*)*.

Even though, the role of protease in the pathogenicity of some important *Vibrio* species were established, controversial reports were also not uncommon. Recently Fan *et al.* (2001) invalidate the role of metalloprotease and cytolysin as major virulence factors by stating that mutant strains of *V. vulnificus*, which lack the genes for both the traits were equally cytotoxic and virulent as wild strains in mice. Fan *et al.*

(2001) also stated that the cytolysin, but not the protease, seemed to be important for causing damage in the elementary tract of the mice.

None of the cultures produced the enzyme at the tested low temperatures like 6±1^{0}C and 15^{0}C and only *V. alginolyticus* and *V. fluvialis* could elaborate the enzyme at 42^{0}C. Hence this study shows that protease enzyme production is common among the *Vibrio* species, but their activity is very much restricted to the mesophilic temperatures.

In the present study proteolytic activity was demonstrated with casein as substrate. This is because, for marine bacteria studied by Sizemore and Stevenson (1970) and Venugopal and Lewis (1985), the proteases could degrade both casein and fish protein equally well. Hence it can be concluded that these *Vibrio* species can cause fish tissue degradation at ambient temperatures.

4.3.1.2. Production of lipase by *Vibrio* species.

Compared to protease enzyme, the *Vibrio* species showed better ability to produce the lipase enzyme even in lower range of temperatures. Two substrates *viz.* tween-80 and egg yolk were tested and the result were presented in Table 41 and Table 42 respectively. At 6±1^{0}C, none of the strains produced lipase in both media, but at 10^{0}C, 12% of the *Vibrio* species produced lipase in Tween-80 medium while their activity was completely absent in egg yolk. At the highest test temperature of this study also, this trend continued. While 37% of the vibrios produced lipase at 42^{0}C in Tween-80, only 7% of them in egg yolk produced lipase. This indicate that the nature of the substrate has a major influence on lipase production at a given temperature. All the species

Table 40. Protease enzyme production at different temperatures by various *Vibrio* species isolated during the present study

Vibrio species	No of strains tested	Number of strains producing protease enzyme at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	0	10	10	2
V. campbellii	10	0	0	4	6	0
V. carchariae	5	0	0	5	5	0
V. cincinnatiensis	10	0	0	10	10	0
V. damsela	10	0	0	0	0	0
V. fluvialis	10	0	0	10	10	4
V. furnissii	10	0	0	0	0	0
V. harveyi	10	0	0	8	7	0
V. hollisae	5	0	0	5	3	0
V. logei	10	0	0	8	10	0
V. metschnikovii	10	0	0	10	8	0
V. mimicus	10	0	0	8	10	0
V. orientalis	10	0	0	6	6	0
V. parahaemolyticus	10	0	0	8	8	0
V. pelagius II	10	0	0	8	10	0
V. splendius II	10	0	0	4	4	0
V. vulnificus	10	0	0	4	6	0
Total strains	160	0	0	108	113	6

Table 41. Lipase enzyme production at different temperatures by various *Vibrio* species isolated during the present study using Tween-80 medium

Vibrio species	No of strains tested	Number of strains producing lipase at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	0	10	10	4
V. campbellii	10	0	0	10	10	6
V. carchariae	5	0	0	5	5	3
V. cincinnatiensis	10	0	5	8	7	4
V. damsela	10	0	2	4	4	4
V. fluvialis	10	0	5	5	5	5
V. furnissii	10	0	0	0	0	0
V. harveyi	10	0	0	4	7	4
V. hollisae	5	0	0	5	5	5
V. logei	10	0	0	10	10	0
V. metschnikovii	10	0	5	8	8	4
V. mimicus	10	0	0	10	6	4
V. orientalis	10	0	0	10	10	4
V. parahaemolyticus	10	0	0	10	10	6
V. pelagius II	10	0	0	7	6	0
V. splendius II	10	0	2	10	10	4
V. vulnificus	10	0	0	8	8	2
Total strains	160	0	19	124	121	59

except *V. furnissii* produced lipase at the ambient and 37^{0}C and seven species showed cent percent activity at these temperatures.

Lipase production by tested *Vibrio* strains was higher in Tween-80 medium (77.5%) when compared to egg yolk medium (50%). Moreno and Landgraf (1998) reported 100% lipase production for *V. vulnificus* in Tween-80 medium and 96.9% in egg yolk medium. It was also noted that the temperature did not have an influential effect on lipase production as 19% of *Vibrio* showed lipase activity at 10^{0}C and 37% at 42^{0}C. Similar results have been reported by Rodrigues *et al.* (1992). Little reference literature is available to establish the direct involvement of lipase in pathogenesis. However, due to its hydrolytic activity it is included as a determinant in some pathogenicity studies.

4.3.1.3. Production of amylase by *Vibrio* species

Table 43 shows the influence of different temperatures on amylase production by *Vibrio* species. All of the tested species except, *V. furnissii* could hydrolyse starch. Out of the tested 17 species, 11 showed cent percent enzyme production among its strains. Refrigeration temperature (6$\pm$1^{0}C), was inhibitory to amylase production, while 42^{0}C was found favourable with 58.13% *Vibrio* species showing positive reaction. Room temperature and 37^{0}C were the most conducive temperatures for the production of the enzyme as 83.75% strains were positive.

In the present study 83.75% of the vibrios produced amylase in the starch agar. Amylase secretion was detected in 58.13% and 5% at 42^{0}C and 10^{0}C respectively. At refrigeration temperature (6$\pm$1^{0}C) vibrios

Table 42. Lipase enzyme production at different temperatures by various *Vibrio* species isolated during the present study using egg yolk agar

Vibrio species	No of strains tested	Number of strains producing lipase enzyme at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	0	8	10	0
V. campbellii	10	0	0	6	10	0
V. carchariae	5	0	0	2	5	0
V. cincinnatiensis	10	0	0	6	8	0
V. damsela	10	0	0	0	0	0
V. fluvialis	10	0	0	5	5	0
V. furnissii	10	0	0	0	0	0
V. harveyi	10	0	0	4	6	4
V. hollisae	5	0	0	5	5	5
V. logei	10	0	0	6	8	0
V. metschnikovii	10	0	0	8	8	0
V. mimicus	10	0	0	6	8	0
V. orientalis	10	0	0	6	6	0
V. parahaemolyticus	10	0	0	6	8	0
V. pelagius II	10	0	0	5	5	2
V. splendius II	10	0	0	8	7	0
V. vulnificus	10	0	0	4	6	0
Total strains	160	0	0	85	105	11

Table 43. Amylase enzyme production at different temperatures by various *Vibrio* species isolated during the present study

Vibrio species .	No of strains tested	Number of strains producing amylase enzyme at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	2	10	10	6
V. campbellii	10	0	2	10	10	6
V. carchariae	5	0	0	5	5	3
V. cincinnatiensis	10	0	0	10	10	10
V. damsela	10	0	0	5	7	5
V. fluvialis	10	0	0	10	10	10
V. furnissii	10	0	0	0	0	0
V. harveyi	10	0	0	10	10	10
V. hollisae	5	0	0	5	5	2
V. logei	10	0	0	10	10	6
V. metschnikovii	10	0	0	5	5	5
V. mimicus	10	0	0	4	4	2
V. orientalis	10	0	2	10	10	6
V. parahaemolyticus	10	0	0	10	10	8
V. pelagius II	10	0	0	10	10	0
V. splendius II	10	0	2	10	10	8
V. vulnificus	10	0	0	10	8	6
Total strains	160	0	8	134	134	93

were found not able to produce amylase. However amylolytic activity by psychrophilic *Vibrio* isolates from deep sea were reported (Hamamota and Horikoshi, 1991; Markarios-Laham and Traxler, 1991). Among the population of *Vibrio* in the estuarine environment of Cochin, 82.12% was previously reported to be amylolytic (Saramma *et al.*, 1994). They found *V. alginolyticus* and *V. parahaemolyticus* as the potent producers of the enzyme and the optimum temperature for the production was 30^{0}C. This observation corroborates with the present result as all the tested strains of both of the species produced the enzyme at 28$\pm$2 and 37^{0}C. Additionally, eight more *Vibrio* species exhibited cent percent enzyme production in the present study.

4.3.1.4. Production of lecithinase by *Vibrio* species

All the species, except *V. furnissii,* studied were positive for production of lecithinase in egg yolk agar at the room temperature and 37^{0}C (Table 44). Thirty percentage of the total strains produced lecithinase at 42^{0}C whereas, only *V. orientalis* showed activity at 6$\pm$1 and 10^{0}C. Enzyme production was exhibited by 100% strains of the all the species positive for the test, except for *V. damsela* where 80% was positive. The result shows that the incubation temperature, is influential for the lecithinase production by the *Vibrio* species.

Cent percent lecithinase production in *V. vulnificus* (Rodrigues *et al.*, 1992; Moreno and Landgraf, 1998) and *V. harveyi* (Liu *et al.*, 1996) with the suggestion that this character enhances virulence of the species was reported. This is in accordance with the result of the present study. Since almost all of the tested strains except *V. furnissii*, produced

lecithinase, the hydrolytic activity on phospholipid compounds might be a factor for the virulence by *Vibrio* species.

4.3.1.5. Production of deoxiribonuclease by *Vibrio* species

Nuclease activity was meagre in most of the *Vibrio* species (Table 45). Though the extend of production of the nuclease within species was low, all the tested species were found positive except *V. cincinnatiensis*. Out of the 160 cultures tested, 96 and 90 elaborated the enzyme at room temperature and 37^{0}C respectively. The extreme temperatures were inhibitory for DNase production.

Fifty six percentage of vibrios tested were destructive to DNA at the room temperature. Among the *Vibrio* species, *V. alginolyticus, V damsela, V. fluvialis, V. mimicus* and *V. parahaemolyticus* were cent percent positive for the hydrolysis. Among *V. vulnificus* isolates tested 40% and 60% of the strains with positive DNase production at 28$\pm$2 and 37^{0}C respectively. Bryant *et al. (*1986a), Rodrigues *et al. (*1992) and, Moreno and Landgraf (1998) reported DNase production by *V. vulnificus* and they confirmed it as a virulence determinant for the bacterium.

4.3.1.6. Production of heamolysin by *Vibrio* species

Data on the haemolysin production is presented in Table 46. *Vibrio carchariae, V. furnissii*, and *V. orientalis* showed negative results. All the strains of *V. mimicus* were found to be positive for haemolysin production at ambient temperature and 37^{0}C. Extremes of temperature were not favourable for production of haemolysin, except for *V. fluvialis* whose 50% strains were positive at 42^{0}C. Twenty percent strains of *V. parahaemolyticus* were positive at room temperature, where as 40%

Table 44. Lecithinase enzyme production at different temperatures by various *Vibrio* species isolated during the present study

Vibrio species	No of strains tested	Number of strains producing lecithinase enzyme at the temperatures				
		6 ± 1°C	10°C	28±1°C	37°C	42°C
V. alginolyticus	10	0	0	10	10	8
V. campbellii	10	0	0	10	10	4
V. carchariae	5	0	0	5	5	0
V. cincinnatiensis	10	0	0	10	10	5
V. damsela	10	0	0	8	10	0
V. fluvialis	10	0	0	10	10	5
V. furnissii	10	0	0	0	0	0
V. harveyi	10	0	0	10	10	3
V. hollisae	5	0	0	5	5	2
V. logei	10	0	0	10	10	0
V. metschnikovii	10	0	0	10	5	5
V. mimicus	10	0	0	10	10	0
V. orientalis	10	2	2	10	10	0
V. parahaemolyticus	10	0	0	10	10	8
V. pelagius II	10	0	0	10	10	3
V. splendius II	10	0	0	10	10	5
V. vulnificus	10	0	0	10	10	0
Total strains	160	2	2	148	145	48

Table 45. Deoxyribonuclease enzyme production at different temperatures by various *Vibrio* species isolated during the present study

Vibrio species	No of strains tested	Number of strains producing deoxyribonuclease enzyme at the temperatures				
		6±1°C	10°C	28±1°C	37°C	42°C
V. alginolyticus	10	0	0	10	10	0
V. campbellii	10	0	0	4	4	0
V. carchariae	5	0	0	4	3	0
V. cincinnatiensis	10	0	0	0	0	0
V. damsela	10	0	0	10	10	0
V. fluvialis	10	0	0	10	10	0
V. furnissii	10	0	0	2	2	0
V. harveyi	10	0	0	7	7	0
V. hollisae	5	0	0	4	5	0
V. logei	10	0	0	2	2	0
V. metschnikovii	10	0	0	7	5	0
V. mimicus	10	0	0	10	10	0
V. orientalis	10	0	0	4	4	0
V. parahaemolyticus	10	0	0	10	10	2
V. pelagius II	10	0	0	4	0	0
V. splendius II	10	0	0	4	2	0
V. vulnificus	10	0	0	4	6	0
Total strains	160	0	0	96	90	2

Table 46. Haemolysin production at different temperatures by various *Vibrio* species isolated during the present study

Vibrio species	No of strains tested	Number of strains producing haemolysin at the temperatures				
		6±1°C	10°C	28±1°C	37°C	42°C
V. alginolyticus	10	0	0	0	4	0
V. campbellii	10	0	0	2	2	0
V. carchariae	5	0	0	0	0	0
V. cincinnatiensis	10	0	0	5	5	0
V. damsela	10	0	0	4	4	0
V. fluvialis	10	0	0	5	5	5
V. furnissii	10	0	0	0	0	0
V. harveyi	10	0	0	4	4	0
V. hollisae	5	0	0	0	0	0
V. logei	10	0	0	6	6	0
V. metschnikovii	10	0	0	5	5	0
V. mimicus	10	0	0	10	10	0
V. orientalis	10	0	0	0	0	0
V. parahaemolyticus	10	0	0	2	4	0
V. pelagius II	10	0	0	4	4	0
V. splendius II	10	0	0	4	4	0
V. vulnificus	10	0	0	6	6	0
Total strains	160	0	0	57	63	5

Table 47 Percentage of positive strains (n=160) of different *Vibrio* species capable of producing hydrolytic enzymes at different temperatures

Enzymes.	Percentage of positive strains with various enzyme production at the temperatures				
	6±1°C	10°C	28±2°C	37°C	42°C
Protease.	0.00	0.00	67.50	70.63	3.75
Lipase (Tween-80 medium)	0.00	11.88	77.50	75.63	36.88
Lipase (Egg Yolk medium)	0.00	0.00	50.00	61.76	66.47
Amylase	0.00	5.00	83.75	83.75	58.13
Lecithinase	1.25	1.25	92.50	90.63	30.00
Deoxyribonuclease	0.00	0.00	56.47	52.94	1.18
Haemolysin	0.00	0.00	36.88	39.38	3.13

showed the same property at 37^{0}C. This is indicative of the comparative low virulence of environmental strains of the bacterium. Haemolysin is a widely discussed virulence factor in vibrios, particularly in *V. parahaemolyticus* (Nishiguchi, 1999; Venugopal *et al.,* 1999). Out of 160 cultures of 17 *Vibrio* species tested at room temperature and 37^{0}C, 36.8% and 39.3% showed haemolyctic activity (see Table 47). Low temperature prevented the haemolysis. *Vibrio mimicus* was the only species that recorded cent percent haemolysin production. Similar observation was reported previously by Wong *et al. (*1993). *Vibrio harveyi* showed haemolysis at 40 % level and the similar observation was made by Liu *et al. (*1996). Haemolysins of *V. parahaemolyticus* are reviewed recently by Venugopal *et al. (*1999). Among the strains of *V. parahaemolyticus*, 20 and 40% were positive for haemolysis at the room temperature and 37^{0}C respectively. This observation was supported by Wong *et al. (*1993).

Morris *et al.* (1987) summarised that though cytotoxin-haemolysin might contribute to virulence, it did not appear to be a marker for pathogenicity as it was produced by both virulent and avirulent strains. Malathi *et al. (*1989) supported the findings stating that the haemolysin as supposed earlier was not the sole factor in virulence. In the present study , 36.88% of vibrios could lyse the human blood. However, both haemolytic and non haemolytic strains were isolated from sea food and former had greater lethality in mice (Malathi *et al.*, 1988). Karunasagar *et al. (*1989) pointed out that lysed blood could enhance virulence.

Table 47 presents the percentage of total *Vibrio* species showing positive hydrolytic enzyme production at different temperatures.

Maximum hydrolytic activity is at ambient temperature and at 37^{0}C. Least activity was at refrigeration temperature and lecithinase is the only exception with production by 1.25% of the tested isolates. Thus in refrigerated conditions the risk by vibrios were only marginal as most of the virulence characters were negative at this temperature.

4.3.2. Animal inoculation studies

The ability of two strains each of the four human pathogens viz. *V. alginolyticus*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* and one fish pathogen, *V. harveyi*, to cause mortality in mice was studied (Table 48). Only live cells of the tested strains were found lethal in mice. Mortality was not observed when the mice were challenged with heat killed and cell free filtrate of the culture of the same bacteria. Wong *et al.* (1993) suggested that the extra cellular toxins were a virulance factor for the lethality of mice. The results of this study ruled out the suggestion as the cell free filtrate was incapable of killing the mice. Wong and his team (1993) also suggested the possibility of the presence of cell mediated lethal factor for the virulence. Thus it is hypothesised that the live cells might signal the production and activation of a potential lethal factor within the body of the host which can cause the sudden mortality. Gerhard *et al.* (2001) found that a fatal infection of *V. vulnificus* in a patient with mutation in hemochromatosis gene, that resulted in iron overload which was a predisposing factor for infection.

The mortality was tried to correlate with the infection dose. The inoculum size and the period within which the death occurred is given in Table 49. *Vibrio vulnificus and V. paraheamolyticus* were the most virulent

Table 48. Percentage and number of mice lethality on intraperitonial injection of *Vibrio* species.

Vibrio species	Culture No.	Mortality		
		Live Cells[1]	Heat Killed suspension	Cell Free Filtrate
V.alginolyticus	139	5/5[2] (100)[3]	0/5 (0)	0/5 (0)
	134	3/5 (60)	0/5 (0)	0/5 (0)
V.harveyi	264	5/5 (100)	0/5 (0)	0/5 (0)
	318	4/5 (80)	0/5 (0)	0/5 (0)
V.mimicus	63	5/5 (100)	0/5 (0)	0/5 (0)
	pw15	5/5 (100)	0/5 (0)	0/5 (0)
V.parahaemolyticus	149	5/5 (100)	0/5 (0)	0/5 (0)
	155	5/5 (100)	0/5 (0)	0/5 (0)
V.vulnificus	2046	5/5 (100)	0/5 (0)	0/5 (0)
	296	5/5 (100)	0/5 (0)	0/5 (0)

1. Inoculum contained 10^7 to 10^8 cells/ml
2. Number of animals dead against number of animals challenged
3. Percentage of animals dead.

species followed in the descending order *V. harveyi, V. mimicus* and *V. alginolyticus*. Of the tested strains, *V. vulnificus* (NCIMB 2046) and *V. parahaemolyticus* (vv155) were the most virulent and were lethal within 6h. after inoculation of $9.0x10^7$cfu intraperitoneally. However, inoculum size of other strains used to inject mice was not found related to virulence potential as expressed as the time taken to kill the mice. This inferred that pathogenic potential varies with the strains. This was also evident from the reports that isolates of *V. vulnificus* from mussels were avirulant to mice, (Matte *et al.*, 1994b), but two out of eight isolates from oysters were virulent (Matte *et al.*, 1994a) Kaysner *et al.* (1987), reported 60% mouse lethality among the strains of *V. vulnificus*. Another report showed (Oliver *et al.*, 1983) all the 22 environmental strains of *V. vulnificus* were lethal to mice Thus lethality of the strains may be a variable factor. After one hour of injection the animals become sluggish with symptoms like tail dropping and watery and drooping eyes. No clinical symptoms were observed at the site of inoculation except a slight inflammation in some cases.

Oliver *et al.* (1983) examined the virulence of 12 *Vibrio* species in the mice model and only *V. vulnificus, V. cholarae* and *V. parahaemolyticus* were found to be lethal. In the present study, in addition to this, *V. alginolyticus, V. mimicus* and *V. harveyi* were also pathogenic to mice. Tested strains of *V. vulnificus* showed lethality within 24h.

The inoculum size required to kill two strains of *V. vulnificus* in healthy mice and iron over loaded mice were showed in Table 50. Iron loaded mice were more susceptible than the normal one for the virulence

Table 49 . Effect of inoculum size of different *Vibrio* species on the mortality of mice

Vibrio species	Culture No.	Number of cells used as inoculum	Total no. of mice tested	Mortality		
				within 6 h.	within 24 h.	within 48 h.
V.alginolyticus	139	7.0×10^7	5	+[1] (1)[2]	+ (2)	+ (2)
	134	7.4×10^7	5	+ (2)	+ (3)	+ (1)
V.harveyi	264	1.5×10^8	5	+ (4)	+ (1)	NIL
	318	8.0×10^7	5	+ (2)	+ (3)	+ (1)
V.mimicus	63	3.0×10^7	5	+ (1)	+ (3)	+ (1)
	pw15	9.0×10^7	5	+ (3)	+ (1)	+ (1)
V.parahaemolyticus	149	3.0×10^8	5	+ (4)	+ (1)	NIL
	155	9.0×10^7	5	+ (5)	NIL	NIL
V.vulnificus	2046	9.0×10^7	5	+ (5)	NIL	NIL
	296	6.0×10^7	5	+ (3)	+ (2)	NIL

1. + indicates death
2. Number of animals killed

Table 50. Effect of iron on lethality of *Vibrio vulnificus* against mice.

Culture No.	Number of cells in the highest dilution which killed three of the five tested mice.	
	Healthy mice	Iron over loaded mice
NCIMB 2046	4.6×10^5	<500
vv 296	3.2×10^6	9.0×10^5
vv 69	4.1×10^6	7.2×10^4.

of *V. vulnificus*. The infection dose is drastically reduced in the strain NCIMB 2046, from $4.6x10^6$ to <500 cells in iron loaded mice. The strain NCIMB 2046 was found highly virulent and killed the mice within 6h after inoculation. For the other strain, vv 296, infection dose in healthy mice was $3.2x10^6$, while in iron loaded mice it was 9.0×10^5 cells ml^{-1}. The strain vv 69 the infection dose reduced from 4.1×10^6 to 7.2×10^4 cells when iron was injected to the body of mice. Thus it is inferred that the infection dose is strain dependant. Bowdre *et al.* (1981) reported a LD_{50} value of 10^6 colony forming units for *V. vulnificus*. US- Food and Drug Administration (1995) recommended that the death of iron loaded mice (250 λg iron dextran per gram body weight) caused by 1000 or fewer *V. vulnificus* cells indicated virulence. Avirulent strains have not been reported to kill iron loaded mice at concentrations less than 10^6 cells per mouse. Thus it is concluded that increased iron levels in the body resulted in *V. vulnificus* infection and persons with impaired iron metebolism are at risk for *V. vulnificus* infection.

When enzyme profile alone is taken into consideration, most of the *Vibrio* possess virulence potential as observed by the study. However, the bioassay in animal models are the most dependable and efficient tool to detect virulence of strains (Oliver, 1981). The scope of the present is limited to the bioassay of only selected species. But the study indicate that the role of vibrios as sea food pathogen is not to be neglected. This points the need to reconsider the present status of the genera *Vibrio* as a public health hazard. The American public health association considers *V. parahaemolyticus*, *V. vulnificus* and enterotoxigenic *V. cholerae* among

the vibrios as risk for public health (Kaysner *et al.*, 1987). *Vibrio fluvialis* and *V. mimicus* were reported as potent pathogens in oysters and suggested to consider in the assessment of risks in sea food (Matte *et al.*, 1994b). To reduce the risk of contamination, a continuous surveillance by monitoring of sea foods as well as their environment for the presence of the pathogens is suggested. This type of monitoring can also help to tackle 'vibriosis', a serious problem in tropical aquaculture.

4.4. Spoilage potential of *Vibrio* species

4.4.1. Effect of low temperature on the survival selected *Vibrio* species

Vibrio species in food is of concern, as these organisms are pathogens as well as spoilers. Since the genus is indigenous to marine creatures and its environments, their presence in sea food is inevitable. Hence the fate of five species of pathogenic and dominant *Vibrio* isolated from the sea food was evaluated for their capacity to survive in low storage temperatures.

Vibrio are known to survive for long period in low storage temperature, even though they are incapable of growth (Boisca *et al.*, 1996; Surendran and Gopakumar, 1985; Thampuran and Gopakumar, 1993; Weichart and Kjelleberg, 1996). Thus the pattern of destruction of *Vibrio* species, at -18±2^{0}C, a typical storage temperature and 6±2^{0}C, the refrigeration temperature, in fish muscle homogenate (FMH), trypticase soy broth with 3% sodium chloride (TSSB) and 3% sodium chloride solution were traced out inn this study. Around 10^8 cells were used as the

inoculum because the intestine and gills of the fishes harboured *Vibrio* in this range.

Rate of elimination of cells of *V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. harveyi* and *V. mimicus* at -18±2^{0}C, for a period of three months, were depicted in Figure 14. *Vibrio vulnificus* was the most sensitive, followed by the decreasing order *V. harveyi, V. mimicus, V. parahaemolyticus and V. alginolyticus.* None of the cultures found to multiply in these temperatures.

The TSSB and 3% sodium chloride diluent were more detrimental for *V. vulnificus* as no viable cells could be detected after 14 days. In FMH, though the plate count declined gradually, 4.0 x 10^3 cells survived after 3 months storage from an initial level of 6.4 x 10^8 cfu g^{-1}. Similar observation of low temperature sensitivity of *V. vulnificus* was reported earlier (Oliver *et al.*, 1991; Wolf and Oliver, 1992; Oliver, 1993). They opined that the reduction in the viable cell count may be due to the formation of VBNC cells. In this state, bacterium survived up to 50 days at low storage temperatures. However, FMH have a protective effect for the cells as reported by Covert and Woodburn (1972) and Thampuran and Gopakumar (1993). The protection afforded by the fish muscle substrate may be due to the fish protein molecules which behave like colloid and interfere with rate of crystallisation water in the fish.

In three month's storage in FMH, *V. vulnificus, V. harveyi* and *V. mimicus* were completely eliminated, whereas *V. alginolyticus* and *V. paraheamolyticus* survived the period. Venugopal *et al.* (1999) however,

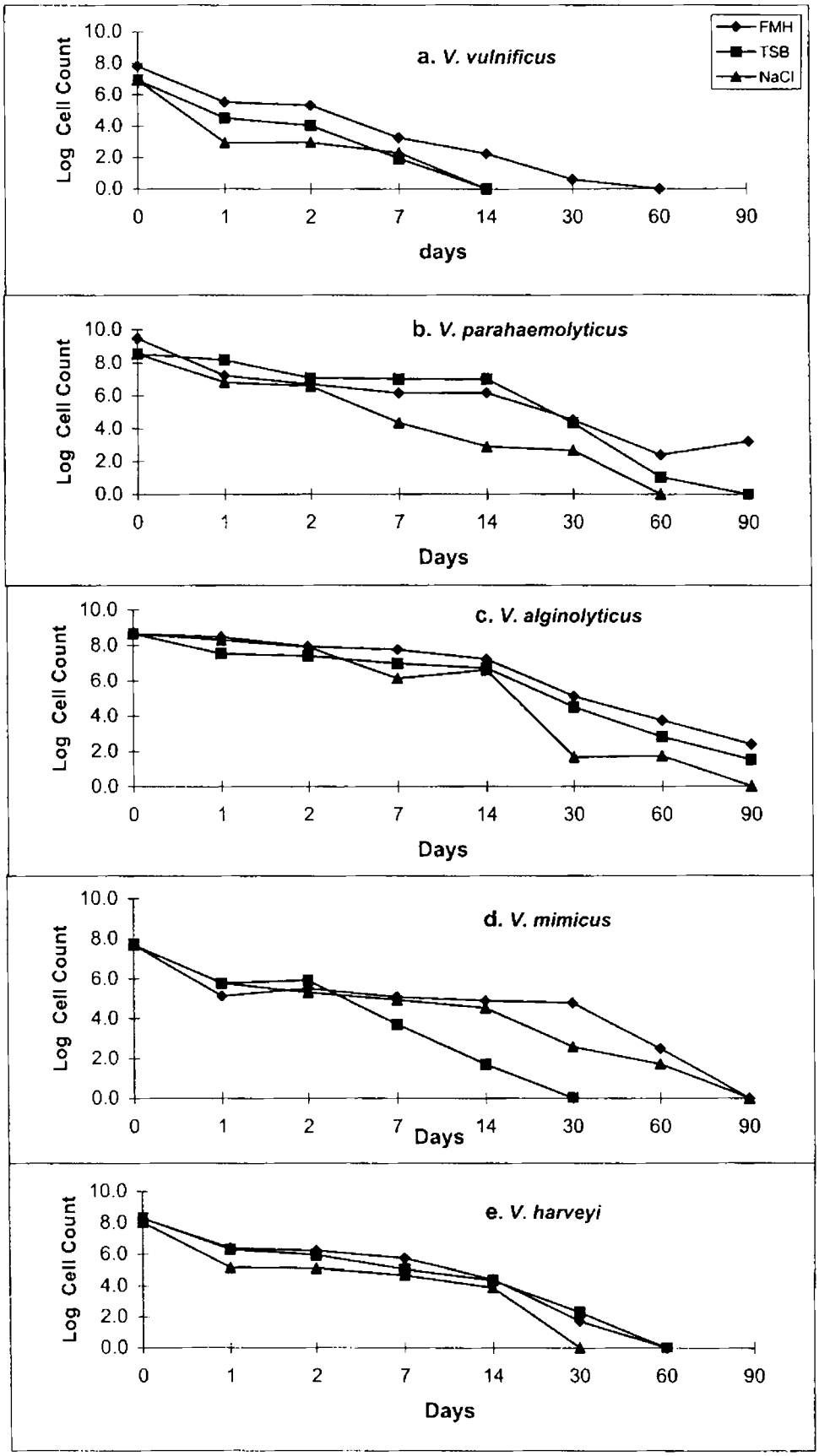


Figure 14. Survival of *Vibrio* species at -18±2°C in different growth media (FMH- Fish Muscle Homogenate; TSB- Trypticase soy broth NaCl- 3% sodium chloride)

reported that *V. parahaemolyticus* was cold sensitive, but survived in cooked prawn, particularly peeled and undeveined prawn up to 17 days at -20^{0}C. The longer survival in FMH in the present study might be due to the variations among the strains used, difference in menstrua, difference in inoculam size, *etc.* Moreover, Covert and Woodburn (1972) had explained that *V. parahaemolyticus* could survive for longer period in presence of higher salt levels than in medium without salt. In the present study, Mackerel muscle homogenate was prepared with aged sea water. Survival in the medium depends on the physiological age of the cell and also on the nutritional history of the inoculating cells (Oliver *et al.*, 1991).

V. parahaemolyticus cells were completely killed within 60 days and 90 days in 3% sodium chloride and TSSB respectively. Covert and Woodburn (1972) have also reported the detection of viable *V. parahaemolyticus* cells still at the end of 30 days of storage at -18±2^{0}C.

Strain variations among *V. parahaemolyticus* and the difference in the freezing menstrua were attributed to the variation in the survival by Karunasagar *et al.* (1986). They reported that Kanagawa positive and Kanagawa negative strains appeared to show a ten fold increase in the population up to fourth day in chilled sea water and after which the number started declining. However, sensitivity of *V. parahaemolyticus* to refrigeration temperature was substantiated by the report of Matches *et al.* (1971).

Growth pattern of *V. parahaemolyticus* and *V. vulnificus* has been studied extensively, however, such studies on *V. alginolyticus, V.*

harveyi and *V. mimicus* is very limited. *V. harveyi* was found to be completely eliminated by 60th day in -18±2°C. *V. harveyi* was noticeably tolerant to 6±2°C. Sanjeev *et al.* (2000) reported that the iced and frozen fish products samples collected from processing factories of Kerala and Tamil Nadu, had *V. harveyi* as the dominant species with a prevalence of 12.3%.

V. alginolyticus, *V. parahaemolyticus* and *V. vulnificus* was also isolated from commercial frozen sea food samples with a prevalence of 15.18, 9.42 and 5.24% respectively (Sanjeev *et al.*, 2000). However the long term survival of the species in sea food can not be compared to that in the *in vitro* experimental conditions. In the former case survival might be favoured by many inter related factors. Still, occurrence of these pathogenic species in fish products meant for export was a cause of concern.

Survival of five *Vibrio* species in different growth media at 6±2°C was presented in Figure 15. Surendran and Gopakumar (1985) have reported that at 8±1°C, 20-50% of the *Vibrio* strains showed spoilage capability. Chandrasekharan *et al.* (1987) reported that in their spoilage study on prawn, at few instance at 4 and -18°C *Vibrio* species dominated in the flora. Thampuran and Gopakumar (1993) stated that *Vibrio* were very sensitive to low storage temperatures. The result of the present study also agrees with it.

Organoleptic assessment of FMH during the three month period showed no considerable changes. By the end of third month the sweet

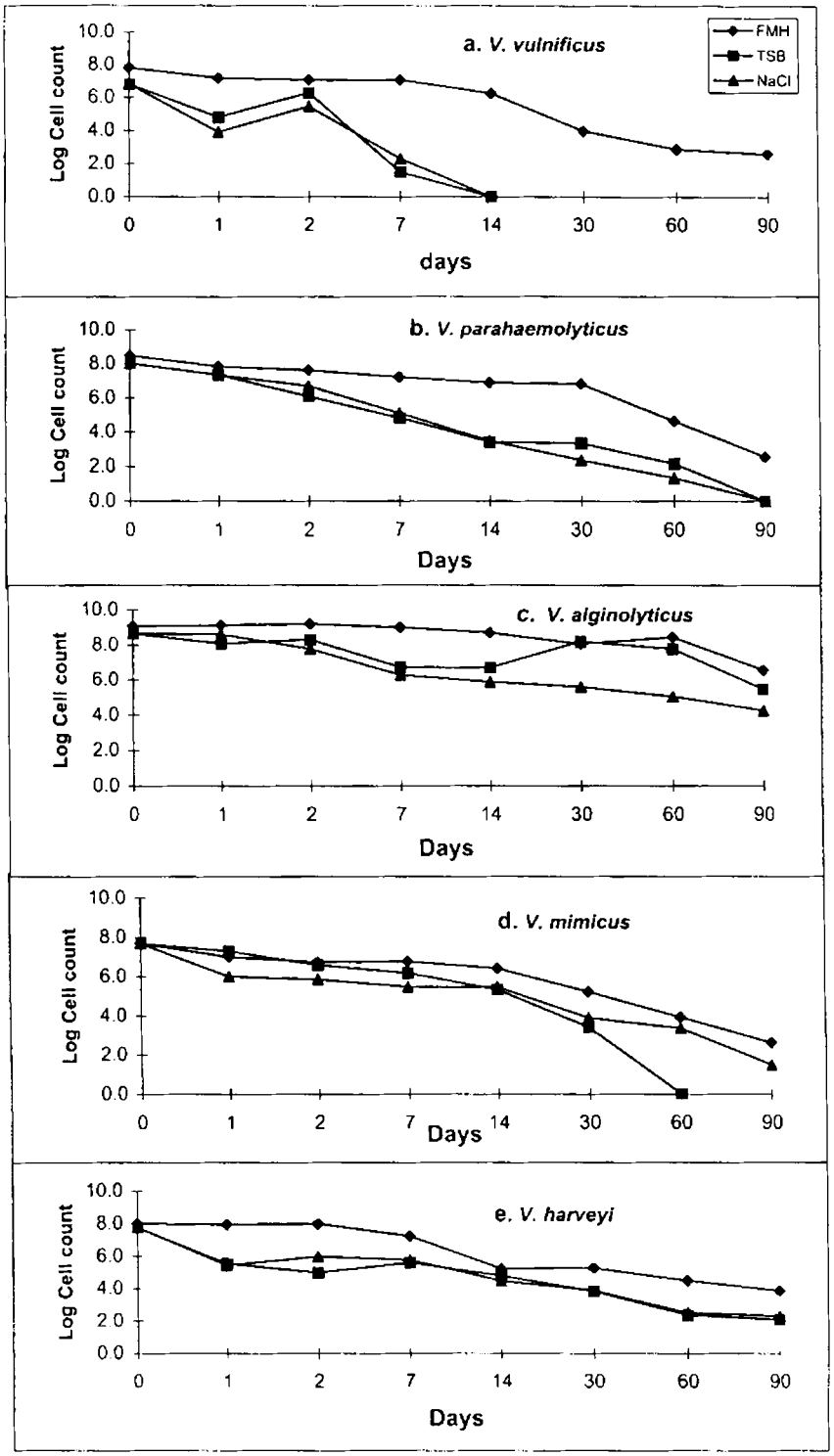


Figure 15 Survival of *Vibrio* species at 6±2°C in different growth media (FMH- Fish Muscle Homogenate; TSB- Trypticase soy broth with NaCl- 3% sodium chloride)

original odour was replaced with a very slight fishy odour. Slight paling of the flesh colour and clumping also observed. These changes can be attributed to the mechanical damage to the muscle due to ice crystal formation rather than microbial action.

4.4.2. Sensitivity of *Vibrio* species to elevated temperatures.

Figure 16 showed the rate of elimination of cells of five *Vibrio* species at 45 and 50^{0}C in TSSB, respectively. At 50^{0}C, within in 7.5 min. all the species tested except *V. mimicus* were killed. At 55^{0}C, *V. mimicus* alone found to survive and cells were completely eliminated within 2.5 minutes. *V. harveyi* was the most susceptible species. *V. parahaemolyticus and V. vulnificus* also showed a drastic reduction in cells within the tested range of temperature.

Studies on the sensitivity of *Vibrio* species to elevated temperature is an area where no work has been reported elsewhere. The present study showed that heating at 55^{0}C for 2.5 min. could completely eliminated the tested *Vibrio* species to zero levels. But the study has the limitation that all the prevailing vibrios have not been attempted. Even then, the study clearly points out that the heating at 55^{0}C for 5 min. can bring down the level of vibrios to a safer limits.

4.4.3. Production of Hydrolytic enzymes by *Vibrio* species.

Number of strains of 17 *Vibrio* species producing L-histidine decarboxylase, H_2S production, urease, phosphatase, indole production

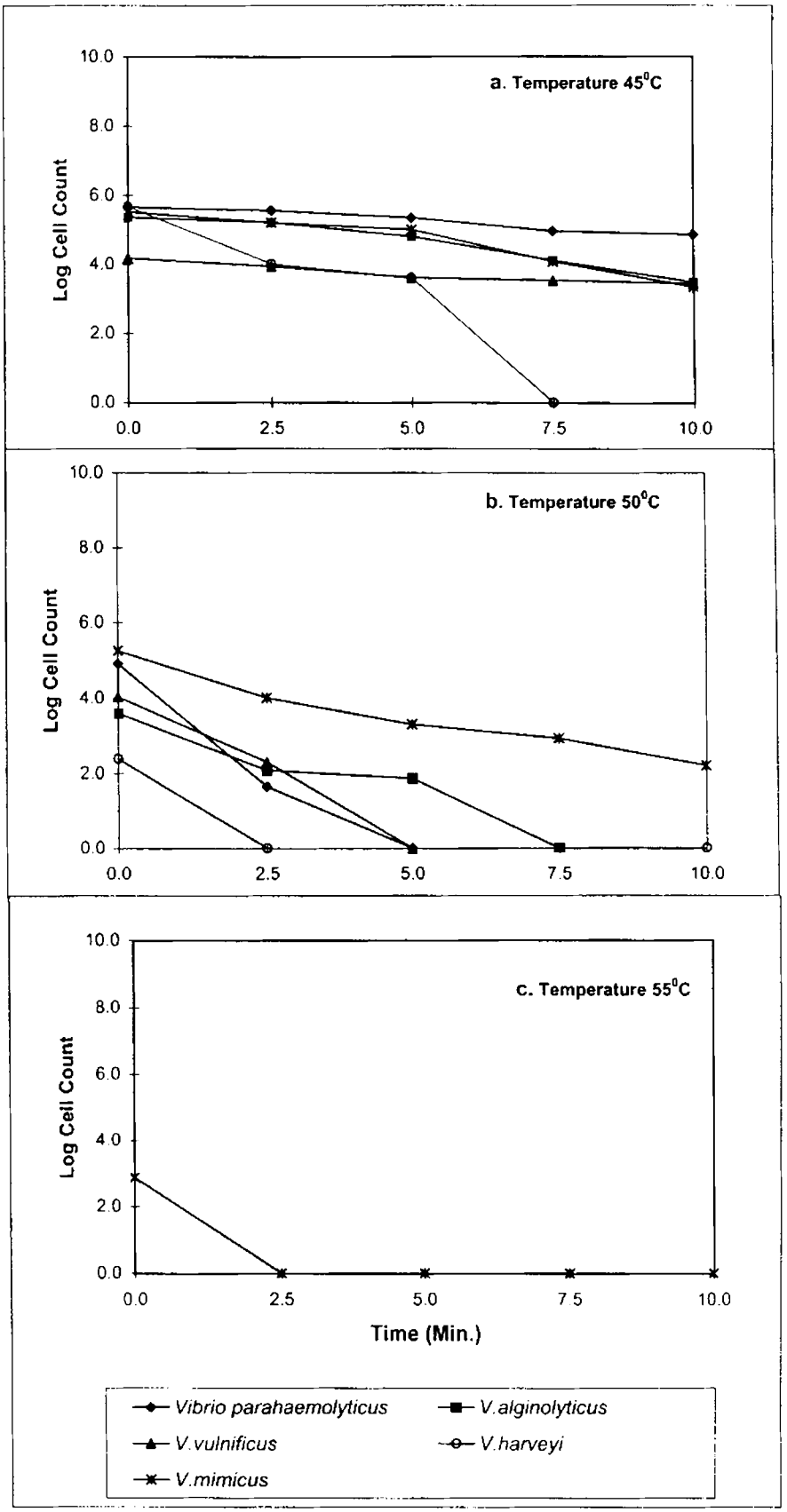


Figure 16. Survial of *Vibrio* species exposed to 45, 50 and 55°C in Trypticase soy broth

and gelatinase production at five different temperatures are given in the Table 51, 52, 53, 54, 55 and 56 respectively.

Production of casienase, lipase, amylase, phopholipase and deoxy ribonuclease (Table 40 to 45) at five different temperature were also studied. At 6 ± 1^0C, none of the species showed the hydrolytic activities, except for lecithinase production by *V. orientalis*. A small percentage of cultures showed the activity at 10^0C, for histidine decarboxylase (3.13%), H_2S production (28.75%), gelatinase (12.5%), indole (9.38%), lipase (11.88%), amylase (5.0), and lecithinase (1.25%). However only reduced activity was exhibited by *Vibrio* species at lowered temperatures. None of the strains produced urease at 10^0C. The ambient temperature and 37^0C were the most conducive temperatures for the enzymatic activities. Thus *Vibrio* have a major role in the spoilage of unprocessed, raw sea foods.

The spoilage potential *Vibrio* was extensively studied by Chandrasekharan (1985), Chandrasekharan *et al.* (1987) and Surendran (1980). Chandrasekharan (1985) has explained the role of various hydrolytic enzymes in the spoilage of prawn, *Penaeus indicus*. Proteolysis, lipolysis, ureolysis and amylolysis were reported to be the major events during spoilage. *Vibrio* species exhibited high caseinase, gelatinase and chitinase activity among the spoilage flora (Chen *et al.*, 1991). Deterioration of the quality of fresh fishery products during storage is essentially attributed to the build up of bacterial proteolysis (Nair and Lahiri, 1968; Herbert *et al.*, 1971; Venugopal and Lewis, 1985). About

Table 51. L-histidine decarboxylase enzyme production at different temperatures by various *Vibrio* species isolated during the present study.

Vibrio species	No of tested strains	Number of strains with L-histidine decarboxylase production at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	1	10	10	1
V. campbellii	10	0	0	0	0	0
V. carchariae	5	0	0	0	0	0
V. cincinnatiensis	10	0	2	9	10	0
V. damsela	10	0	0	0	0	0
V. fluvialis	10	0	1	10	10	0
V. furnissii	10	0	0	6	6	0
V. harveyi	10	0	0	5	6	0
V. hollisae	5	0	0	0	0	0
V. logei	10	0	1	9	10	0
V. metschnikovii	10	0	0	10	10	1
V. mimicus	10	0	0	4	4	2
V. orientalis	10	0	0	10	10	0
V. parahaemolyticus	10	0	0	10	10	1
V. pelagius II	10	0	0	0	0	0
V. splendius II	10	0	0	10	10	0
V. vulnificus	10	0	0	8	10	0
Total strains	160	0	5	101	106	5

Table 52. Hydrogen sulphide production at different temperatures by various *Vibrio* species isolated during the present study.

Vibrio species	No of tested strains	Number of strains with hydrogen sulphide production at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	6	8	10	4
V. campbellii	10	0	4	10	10	0
V. carchariae	5	0	2	4	4	0
V. cincinnatiensis	10	0	5	10	10	0
V. damsela	10	0	0	0	0	0
V. fluvialis	10	0	8	10	10	0
V. furnissii	10	0	0	10	10	0
V. harveyi	10	0	0	10	10	0
V. hollisae	5	0	2	5	5	0
V. logei	10	0	4	10	10	0
V. metschnikovii	10	0	5	10	10	2
V. mimicus	10	0	0	6	10	0
V. orientalis	10	0	4	10	10	0
V. parahaemolyticus	10	0	2	10	10	4
V. pelagius II	10	0	0	10	10	0
V. splendius II	10	0	0	10	10	0
V. vulnificus	10	0	4	10	10	0
Total strains	160	0	46	143	149	10

Table 53. Urease enzyme production at different temperatures by various *Vibrio* species isolated during the present study

Vibrio species	No of tested strains	Number of strains with urease enzyme production at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	0	3	4	0
V. campbellii	10	0	0	0	0	0
V. carchariae	5	0	0	10	10	0
V. cincinnatiensis	10	0	0	0	0	0
V. damsela	10	0	0	4	4	0
V. fluvialis	10	0	0	0	0	0
V. furnissii	10	0	0	0	0	0
V. harveyi	10	0	0	3	3	0
V. hollisae	5	0	0	0	0	0
V. logei	10	0	0	6	6	1
V. metschnikovii	10	0	0	0	0	0
V. mimicus	10	0	0	6	7	1
V. orientalis	10	0	0	8	8	0
V. parahaemolyticus	10	0	0	3	5	0
V. pelagius II	10	0	0	0	0	0
V. splendius II	10	0	0	0	0	0
V. vulnificus	10	0	0	0	0	0
Total strains	160	0	0	43	47	2

Table 54. Phosphatase enzyme production at different temperatures by various *Vibrio* species isolated during the present study

Vibrio species	No of tested strains	Number of strains with phophatase enzyme production at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	0	10	10	2
V. campbellii	10	0	0	10	10	0
V. carchariae	5	0	0	5	5	0
V. cincinnatiensis	10	0	0	5	5	2
V. damsela	10	0	0	10	7	0
V. fluvialis	10	0	0	8	8	0
V. furnissii	10	0	0	6	6	0
V. harveyi	10	0	0	10	10	0
V. hollisae	5	0	0	5	5	0
V. logei	10	0	0	10	10	0
V. metschnikovii	10	0	0	8	8	0
V. mimicus	10	0	0	10	8	0
V. orientalis	10	0	0	10	10	0
V. parahaemolyticus	10	0	0	10	10	0
V. pelagius II	10	0	0	10	10	0
V. splendius II	10	0	1	10	10	0
V. vulnificus	10	0	0	10	8	0
Total strains	160	0	1	147	140	4

Table 55. Indole production at different temperatures by various *Vibrio* species isolated during the present study.

Vibrio species	No of tested strains	Number of strains with indole production at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	2	10	10	2
V. campbellii	10	0	1	7	10	0
V. carchariae	5	0	1	5	7	0
V. cincinnatiensis	10	0	0	2	5	1
V. damsela	10	0	0	2	2	0
V. fluvialis	10	0	1	10	10	0
V. furnissii	10	0	0	2	3	0
V. harveyi	10	0	2	10	10	0
V. hollisae	5	0	1	10	10	0
V. logei	10	0	0	0	0	0
V. metschnikovii	10	0	3	6	6	1
V. mimicus	10	0	2	8	8	1
V. orientalis	10	0	0	10	10	0
V. parahaemolyticus	10	0	1	10	10	0
V. pelagius II	10	0	0	10	10	0
V. splendius II	10	0	1	8	8	0
V. vulnificus	10	0	0	10	10	0
Total strains	160	0	15	120	129	5

Table 56. Gelatinase enzyme production at different temperatures by various *Vibrio* species isolated during the present study.

Vibrio species	No of tested strains	Number of strains with gelatinase enzyme production at the temperatures				
		6 ± 1°C	10°C	28 ± 1°C	37°C	42°C
V. alginolyticus	10	0	1	10	10	2
V. campbellii	10	0	1	10	10	1
V. carchariae	5	0	0	8	8	0
V. cincinnatiensis	10	0	0	1	1	0
V. damsela	10	0	4	0	0	0
V. fluvialis	10	0	6	10	10	2
V. furnissii	10	0	3	10	10	1
V. harveyi	10	0	1	10	10	1
V. hollisae	5	0	0	0	0	0
V. logei	10	0	0	0	0	0
V. metschnikovii	10	0	1	10	10	0
V. mimicus	10	0	2	10	10	0
V. orientalis	10	0	0	10	10	2
V. parahaemolyticus	10	0	1	10	10	1
V. pelagius II	10	0	0	8	8	0
V. splendius II	10	0	0	10	10	0
V. vulnificus	10	0	0	10	9	0
Total strains	160	0	20	127	126	10

85% of the *Vibrio* species were reported to be spoilers as assessed by the production of protease, lipase and amylase reduction of TMAO to TMA, off odour production in flesh broth and holozone formation in flesh agar (Chandrasekharan *et al.*, 1987). The spoilage potential of *Vibrio* strains were assessed by Shetty *et al.* (1992) and found them very active as per the traits like gelatine hydrolysis, ammonia production, indole production, H_2S production DNase production, phosphatase activity, hydrolysis of sugars, decarboxylation of aminoacids and TMAO reduction

Ability of *Vibrio* species to hydrolyse protein was determined by the detection of caseinase at low temperature (Table 40). While all the species, except *V. hollisae* and *V. furnissii* showed the activity at RT and 37^{0}C, none exhibited hydrolysis at 6±1 and 10^{0}C. Bacterial proteolysis is established as a major event during spoilage (Venugopal and Lewis, 1985; Shetty *et al.*, 1992). Philip and Lakshmanaperumalsamy (1992) compared the protein degradation and growth of *Vibrio* species in fish, prawn and clam flesh media and casein medium. In all the media protein degradation commenced on third day and progressed rapidly during following days. In the course of fish spoilage, bacteria first utilises low molecular weight components like amino acids and other non-protein components. The proteolysis thus become the major event in advanced spoilage (Jay, 1978)

Extra cellular protease from the marine fish, which could degrade actomyosin at 5^{0}C was isolated and characterised. Psychrophilic *Vibrio* species which could hydrolyse protein at 4^{0}C and -20^{0}C was reported by Markarios-Laham and Lee (1993). Protease

degrading Mackerel actomyosin at 0-2^{0}C was identified (Venugopal and Lewis, 1985).

Extracellular protease which could degrade actomyosin at 5^{0}C (Mrkarios-Laham and Traxler, 1991) and at 0 - 2^{0}C (Venugopal and Lewis, 1985) were reported. Proteolytic enzymes are significant in spoilage as they can alter several properties of fresh food such as muscle solubility, flavour, colour and functional properties. The loss of initial texture of the fish muscle could thus be attributed to the breakdown of actomyosin by the protease (Chen *et al.*, 1991). Chen *et al.* (1991) stated the *Vibrio* was among the predominant genera with a high caseinase, geletinase and chitinase activity, and a major spoiler of grass prawn (*Penaeus monodon*).

Among the population of *Vibrio* species in the environment of Cochin, 82.12% was reported to be amylolytic (Saramma *et al.*, 1994). *V. parahaemolyticus* and *V. vulnificus* were potent producers of amylase. The optimum culture condition for these organisms were pH 7.0 and 30^{0}C. Amylase production was found to begin at the early logarithmic phase and continued till they entered stationary phase.

Biogenic amines are formed by the action of bacterial decarboxylase on precursor aminoacid mainly in proteinaceous food such as meat or fish (Maga, 1978; Liesner *et al.*, 1994). Presence of histamine in this kind of food can be used as an index of hygienic quality (Dacher and Simrad, 1985). Consumption of food with high amount of histmine has been involved in a food borne disease called 'scomberotoxin' or histamine food poisoning (Taylor, 1986). Toxicological effect is usually slight in healthy individuals, however, some circumstances such as

presence of mono amine oxidase inhibitors or alcohol or the presence of other cadaverine or putrcine can potentiate the toxicity (Shakila, *et al.*, 1999). Histamine production by vibrios were demonstrated (Dacher and Simrad, 1985).

Hydrolysis of sulphur containing aminoacids result in the production of H_2S , which contribute to the off odour of spoiled fish. Hydrogen sulphide production as an index of spoilage by marine bacteria was proved by Thampuran and Iyer (1990). Cent percent of the tested *Vibrio* species isolated from chilled fish produced H_2S (Shetty *et al.*, 1992).

Polyphosphates are good preservatives (Chandrasekharan *et al.*, 1998) and also prevent autolysis of the food (Warrier *et al.*, 1985), whereas, bacterial phosphatases can mineralise the non-available phosphates, which in turn reduces preservative efficiency (Salim and Purushothaman, 1998). They also reported a high phosphatase activity of *Vibrio* species in degradation of phosphate in tissues. Most of the *Vibrio* species isolated from the marine environments were capable of phosphatase activity (Venketeswaran and Natarajan, 1983; Eapen *et al.*, 1993). *Vibrio alginolyticus* was the dominant phosphatase producing bacteria in the gills and intestine of fishes (Nayak and Panda, 1998).

Thus the fore going results conclusively show that the role of *Vibrio* in the quality deterioration in refrigerated condition is negligible. This is due partially to the poor survival of cells at low storage temperature and partially due to the inability to elaborate hydrolytic enzymes at these temperatures. However, their role could not be ruled out as certain

psychrophilic *Vibrio* species has been reported to elaborate enzyme which can degrade fish muscle protein at low temperatures (Markarios-Laham and Traxler, 1991). Also the study emphasis the role of *Vibrio* as a potent spoiler of sea food material when kept at ambient temperature.

Summary

5. Summary

Vibrio species are natural inhabitants with ubiquitous distribution in marine environments and its inhabitants. The genus include species with wide diversity in physiological characters and include some of the most important pathogens. Eleven marine species of *Vibrio* are pathogenic to human, of which *V. parahaemolyticus* and *V. vulnificus* were the major cause of gastroenteritis. Vibrios were also associated with high mortality in animals, especially in larval stages, and thus cause heavy loss in aquaculture industry. Future strategy for prevention of food-borne diseases and vibriosis should be founded in scientifically based evaluations of the whole food production chain 'from farm to table', which warrants a comprehensive information on ecology and physiology of the pathogen as well as the epidemiological details.

The present study gives a comprehensive information on the ecology of the genus *Vibrio* in the tropical marine environment, along the south-west coast of India. The study has been taken up with the following objectives:

1. Survey the distribution of *Vibrio* in fishes and their environment.
2. Bring out the etiology of *Vibrio* in fishes.
3. Characterise the tropical *Vibrio* isolates.
4. Assess the pathogenicity of the *Vibrio* isolates.
5. Establish the spoilage potential of *Vibrio* isolates

The thesis consists of five sections *viz.* Introduction, Review of Literature, Results and Discussion, Summary and References. In section 1,

Introduction, the significance of the study in the present context, its practical implications and extent of work done are highlighted. An up-to-date review of literature pertaining to various aspects of taxonomy, distribution, ecology, physiology, pathogenicity and spoilage of *Vibrio* and other related species were attempted and included in section 2, Review of Literature. Observations of the present study and its interpretations is consolidated in the section 3, Results and Discussion followed by Summary as section 4, in which the important results and future scope of the study are summarized. The fifth section is References, where the bibliography of previous works conducted and the references quoted in the text are given.

Ecological study included the bacteriological analysis of finfish and shellfish and its immediate surroundings like water, plankton and sediment. Quantitative distribution and prevalence percentage of *Vibrio* species was assessed in skin, gill and intestine of finfishes. The bacteriological quality of diet of fish like small pray fishes and plankton, bile tolerance, pH tolerance and the competency of major bacterial species to survive in mixed cultures were determined to explain the high preponderance of *Vibrio* in the intestine.

The distribution of *Vibrio* in fish was tried to correlate to habitat and seasons. Seasonal variation in the flora of representative fishes from the pelagic and the demersal habitat were analysed. Incidence of vibrios was tried to correlate to conventional microbial indicators of human contamination.

A total of 799 strains isolated from different environmental samples were characterised biochemically by studying 53 important traits.

On the basis of observations of the present study, a set of identification tests were proposed which is expected to form the basis for the preparation of a scheme for the identification of vibrios from tropical marine environments. A sensitive and reliable scheme for the easy identification of *V. vulnificus,* which is emerged as a troublesome organism with increasing public health significance, from seafood and other marine environments was formulated. Growth pattern in various eco-physical parameters like temperature, salinity and pH were noted *in vitro* and the optimum conditions for the flourishing of *Vibrio* were determined. Pathogenic potential of selected *Vibrio* species were assessed by noting the enzyme activities predisposing pathogenicity and also by mice inoculation studies. With the view of ascertain the spoilage capability of selected *Vibrio* species, their hydrolytic enzyme production at various temperatures and survival in frozen storage temperatures were studied. In this regard, the elimination of cells of pathogenic vibrios at mild heat treatments were also studied.

Comprehensive reports on the prevalence of vibrios in this area, which contribute to a major share of seafood to the export industry, are lacking and whatever information was available is autecological pertaining to single species or to a particular physiological group. An extensive study is thus relevant as all the tested species except, one or two, showed same virulence determinants and possibility of them emerging out as pathogens also can not be ruled out. It is wished that the study will form the basis for modifying processing strategies which could provide a high quality and safe seafood to compete in international markets.

The important findings in the study were summarised as follows:

1. *Vibrio* population in the seawater in area under the present study is not directly correlated to physico-chemical parameters like temperature, salinity, pH and dissolved oxygen. Total halophilic bacterial count varied from 7.0×10^3 to 6.4×10^5 cfu ml^{-1} and total *Vibrio* count from 6.0×10^2 to 1.2×10^4 cfu ml^{-1} in water samples. The percentage of *Vibrio* to the total flora in water ranged from 3.33 to 18.57 with a mean value of 6.9%. A total of 12 *Vibrio* species were isolated form seawater and *V. alginolyticus* was the most predominant followed by *V. campbellii, V. orientalis* and *V. parahaemolyticus.*

2. Total halophilic bacteria in the sediment samples ranged from 4.1×10^7 to 8.1×10^8 cfu g^{-1}, whereas, *Vibrio* count ranged from 9.8×10^5 to 7.3×10^7 cfu g^{-1}. The percentage of vibrios to total flora in the sediment varied between 4.2 and 25.5% with a mean value of 11.59%. Fourteen different *Vibrio* species were isolated from sediment of which *V. parahaemolyticus* form the most predominant one, following *V. campbellii, V. orientalis, V. alginolyticus* and *V. vulnificus.*

3. Zooplankton samples contained 2.8×10^7 to 1.1×10^9 cfu g^{-1} and *Vibrio* constituted 7.92 to 40.0% of it with a mean value of 24.18. A total of ten *Vibrio* species were detected in zooplankton samples. *Vibrio parahaemolyticus, V. mediterranei. V. splendidus II, V. vulnificus, V. campbellii* and *V. alginolyticus* were the predominant species.

4. Intestine contained the maximum load of vibrios as well as total halophilic bacteria, followed by gill and skin and muscle samples of

finfishes. Total halophilic bacterial count can be statistically correlated to the *Vibrio* count in the respective part.

5. Total bacterial count in skin and muscle samples of commercially important finfishes falls in a range of 1.0×10^5 to 6.5×10^7 cfu g^{-1}. *Vibrio* count varied from 1.0×10^4 to 2.1×10^7 cfu g^{-1}. The average percentage of vibrios to total bacteria was 22.46. Fresh samples harboured more vibrios in their skin and muscle than the market samples inferring that the post harvest multiplication of vibrios is rather negligible.

6. A total of 14 *Vibrio* species were isolated from skin and muscle samples of which *V. alginolyticus*, *V. campbellii*, *V. orientalis* and *V. parahaemolyticus* were the predominant species.

7. Gill contained a bacterial load of the range 6.1×10^6 to 6.6×10^8 cfu g^{-1} of which *Vibrio* constituted 6.0×10^5 to 1.7×10^8 cfu g^{-1}. Percentage of *Vibrio* population to total bacteria in the gill ranged from 9.48 to 42.86%, with a mean value of 25.67%.

8. Eleven species of *Vibrio* were isolated from gill samples of fishes. *Vibrio alginolyticus*, *V. vulnificus* and *V. parahaemolyticus* were the major constituents of the flora.

9. Intestine is the most suitable microcosm for the flourishing of vibrios as evidenced by the high load of 2.3×10^5 to 1.35×10^8 cfu g^{-1}. Total bacterial count ranged from 1.0×10^6 to 6.6×10^8 cfu g^{-1}. Vibrios constituted 7.42 to 80.00% of the total bacterial flora, with a mean value of 30.44%.

10. Sixteen *Vibrio* species were isolated from the intestine. *V. alginolyticus* constituted the most prominent component, followed in the descending order by *V. vulnificus, V. orientalis* and *V. parahaemolyticus*.

11. There was no perceptible trend observed in the distribution of *Vibrio* in the intestine with respect to the feeding habit of the fish. However, planktivores and detritus feeder contained higher quantity of *Vibrio*, corresponding the higher count in plankton and sediment. In carnivores fish, the intestinal flora could be related to the flora of the pray fishes.

12. A few of the dominant species of the intestine were found to tolerate 0.3% bile. Bile tolerance is found inversely proportional to the pH of the test medium and the combined effect of bile and pH is more detrimental.

13. Shrimp muscle harboured 3.2×10^5 to 4.3×10^7 cfu g^{-1} of which 1.2 to 23.4% were vibrios. The corresponding value in the intestine of shellfish was 8.0×10^8 to 7.2×10^9 cfu g^{-1} and the *Vibrio* percentage was 16 to 54.16. Fourteen different *Vibrio* species were isolated from both muscle and intestinal samples. *Vibrio alginolyticus, V. parahaemolyticus* and *V. campbellii* were the dominant species in the muscle whereas, *V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* constituted the bulk of the flora of intestine.

14. No traceable pattern was observed in the quantitative and qualitative distribution of *Vibrio* in different body parts of demersal and pelagic fishes. Species diversity was greater (19 species) in the demersal fish than in its pelagic counterpart (17 species).

15. In the seasonal study, two peaks were obtained at the summer months (March-May) and post monsoon months (September-November) for skin and muscle and intestine of fishes irrespective of the habitat in which it live. Intestine flora was not found affected by season. *Vibrio* count and total halophilic bacteria count were found significantly correlated ($p<0.05$) in all the body parts in most of the seasons.

16. In composite samples of the market fishes, total coliform count ranged from 1.0×10^2 to 1.4×10^6 cfu g^{-1}, *Escherichia coli* from 1.0×10^1 to 4.8×10^4 cfu g^{-1}, faecal streptococci from 1.0×10^1 to 1.4×10^6 cfu g^{-1} and *Staphylococcus aureus* count from 1.0 to 1.2×10^4 cfu g^{-1}. Total *Vibrio* count was not statistically related to any of the indicator bacteria except, feacal streptococci, which indicate the resident and indigenous nature of *Vibrio* species in marine animals.

17. A total of 799 cultures belonging to 26 different *Vibrio* species isolated from various marine environments were characterised by considering 52 important identification tests. The results were compared with the similar data. Certain loop holes in the existing identification systems were pointed out, e.g., colour variations of *Vibrio* colonies in TCBS medium, which confuses the isolation procedure was brought out.

18. A set of key identification tests was suggested which could serve as the basis for the preparation of identification scheme for the isolation of tropical vibrios from seafood and marine environments.

19. A scheme was proposed for easy and precise identification of *V. vulnificus* from the marine environments, as this bacterium is often

found confused with phenotypically similar species like *V. harveyi* and *V. campbellii.*

20. Temperature tolerance limits of *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus, V. mimicus* and *V. harveyi* were determined. 37^{0}C was found to be the optimum for all the tested species. At refrigeration temperature none of the species showed growth and at 42^{0}C, *V. alginolyticus* and *V. parahaemolyticus* showed growth.

21. Salinity tolerance limits of *V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveyi* were determined. The lowest sodium chloride level in which growth observed was 0.5%, except for *V. mimicus*. No growth was observed in any species at 15% salt concentration. Optimum salt level brought out in the present study for *V. vulnificus* and *V. alginolyticus* was 3%, 2.5% for *V. parahaemolyticus* and *V. harveyi* and 1% for *V. mimicus*.

22. Acid tolerance of *V. alginolyticus*, *V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveyi* were determined and none showed growth below pH 6.0. pH 7.5 was found to be the optimum for all the tested strains. *Vibrio parahaemolyticus* was the most tolerant to high pH followed by *V. alginolyticus*, *V. vulnificus, V. harveyi* and *V. mimicus*.

23. Both 6 and 10% CO_2 in the incubating atmosphere were found to have no deleterious effect on the growth of *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus, V. mimicus* and *V. harveyi.*

24. Strains of *V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveyi* could tolerate bile in the range below 0.4%. pH is an influencing factor for bile tolerance and as the pH decreases the sensitivity to bile increases.

25. *Vibrio vulnificus* was found to survive poorly with a competitive population comprising of *A. hydrophila, E. coli* and *V. parahaemolyticus*, whereas, *V. alginolyticus* and *V. harveyi* were found to have no deleterious effect on the growth of *V. vulnificus.*

26. Hydrolytic enzymes which predispose virulence like protease, lipase, amylase, lecithinase, DNase and haemolysin were produced by almost all the tested 17 species of vibrios. Maximum activity was at ambient temperature and at 37^0C followed by 42 and 10^0C. At refrigeration temperature, only lecithinase was produced in a low percentage.

27. In mice inoculation studies, *V. vulnificus* and *V. parahaemolyticus,* were the most virulent species with regard to their infection dose and time taken for the death, followed by *V. harveyi, V. mimicus and V. alginolyticus*. Virulance potential of the species to mice varied with strains. The highest dilution of *V. vulnificus* which killed the healthy mice is 4.6×10^5 cfu for the strain NCIMB 2046 and it was 3.2×10^6 for strain vv296. Iron injection prior to *V. vulnificus* inoculation drastically enhanced the virulence.

28. At the storage temperature, -18 ± 2^0C, the strains of *V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveyi* showed gradual elimination. Survival was influenced by the freezing menstrua

used. In fish muscle homogenate, *V. parahaemolyticus* and *V. alginolyticus* survived even after 90 days of incubation while all other strains were completely eliminated by this period. Most tolerant *Vibrio* species at this temperature was *V. alginolyticus*, followed in the descending order *V. parahaemolyticus*, *V. mimicus, V. harveyi* and *V. vulnificus*. *Vibrio vulnificus,* at -18±2^{0}C, was completely eliminated within 14 days in trypticase soy broth and sodium chloride diluent. In fish muscle homogenate it extended up to 60 days.

29. In refrigeration temperature, all the tested strains of *V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveyi* survived even after 90 days of incubation though considerable reduction in the cell count was noticed. *Vibrio vulnificus* followed the same trend in the elimination of culturable cells as in -18±2^{0}C.

30. All the tested strains of *V. alginolyticus*, *V. parahaemolyticus, V. vulnificus, V. mimicus* and *V. harveyi* were completely eliminated at 55^{0}C, with in 2.5 minutes. *V. mimicus* was most tolerant to elevated temperature, followed by in the descending order, *V. alginolyticus, V. vulnificus, V. parahaemolyticus* and *V. harveyi*.

31. Indices of spoilage potential like protease, lipase, amylase, lecithinase, DNase, gelatinase, urease and phoshatase were produced by almost all tested strains of 17 different *Vibrio* species at ambient temperature and at 37^{0}C. Refrigeration temperature was inhibitory for the elaboration of these enzymes.

32. Other indices of spoilage potential like H_2S, histamine and indole were also produced profusely at ambient temperatures which ascertain its role in spoilage at this temperatures. *Vibrio alginolyticus, V. campbellii, V. parahaemolyticus , V. orientalis, V. harveyi, V. logei, V. vulnificus* and *V. mimicus* were among the potent enzyme producers and possible spoilers.

Future Scope of the Study

Vibrios are consistently associated with seafood borne disease out breaks. Recently, much attention has been gained by *V. vulnificus* as it is associated with septicaemia in raw oyster eaters or debilitated persons. Quick and easy identification of pathogenic vibrios is the need of the hour to cope with the stringent quality control specifications of the world trade. A list of useful tests for the identification of tropical vibrios and a reliable scheme for the identification of *V. vulnificus* has been brought out in the present study. Considering the pathogenic potential studies and ever increasing reports of the emerging pathogens, this study could be extended to chalk out a scheme which encompass all the important vibrios in the tropical environment.

Our processing factories rely on identification tests based mainly on physiological properties, which is highly variable among the strains. This heterogeneity among the strains will make the isolation more cumbersome. In addition to this, most primary isolation methods were out dated and needed a renovation, based on detailed research. For example, still now the accepted selective medium for the isolation of vibrios was TCBS, which have

its own drawbacks as revealed in the study. Green colonies, which were happened to grow in the vicinity of yellow colonies, get colour masked and thus reducing the recovery potential of some important *Vibrio* species. Detailed experimental trails are needed in this aspect to modify the medium with desired characters. Due to these drawbacks in the existing identification systems and high variability among the isolates, it is highly essential to use more reliable method of isolation. It is hoped that in near future the processing factories of our country will shift over to more sensitive and quick identification strategies like genetic profiling to compete in the international market.

Among the 26 *Vibrio* species isolated from various tropical environmental samples, only five important pathogenic species were studied in detail with respect to its optimum growth parameters, pathogenic potential, survival pattern in frozen storage temperature, *etc.* These studies should be extended include the complete characterisation of all the species especially, established pathogens and that information will be useful to plan out processing programs for a safe and quality seafood.

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ANNEXURE 1

Percentage of various Vibrio species showing positive reacions in different Biochemical tests

Sl. No.		No. of cultures tested	PERCENTAGE OF CULTURES SHOWING POSITIVE REACTIONS											
			ARGININE DECARBO-XYLASE	LYSINE DECARBO-XYLASE	ORNITHINE DECARBO-XYLASE	GROWTH IN 0% NaCl	GROWTH IN 3% NaCl	GROWTH IN 6% NaCl	GROWTH IN 8% NaCl	GROWTH IN 10% NaCl	GROWTH AT 4 C	GROWTH AT 20 C	GROWTH AT 30 C	GROWTH AT 40 C
1	*V alginolyticus*	88	0.00	97.73	40.91	0.00	100.00	97.73	90.91	45.45	4.55	95.45	97.73	70.45
2	*V campellii*	86	0.00	25.58	2.33	2.33	100.00	95.35	53.49	20.93	4.65	76.74	100.00	27.91
3	*V carchariae*	2	0.00	100.00	100.00	0.00	100.00	100.00	100.00	0.00	0.00	100.00	100.00	0.00
4	*V cholerae*	2	0.00	100.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00	100.00	100.00	100.00
5	*V cincinnatiensis*	10	0.00	100.00	0.00	0.00	100.00	100.00	100.00	100.00	0.00	100.00	100.00	100.00
6	*V costicola*	2	100.00	0.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00	100.00	100.00	0.00
7	*V damsela*	6	100.00	100.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	100.00	100.00	0.00
8	*V fluvialis*	12	0.00	100.00	100.00	0.00	100.00	100.00	100.00	0.00	0.00	100.00	100.00	0.00
9	*V furnissii*	18	100.00	0.00	0.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00
10	*V harveyi*	44	0.00	100.00	100.00	0.00	100.00	100.00	63.64	27.27	18.18	90.91	100.00	81.82
11	*V. hollisae*	12	0.00	0.00	0.00	0.00	100.00	100.00	100.00	14.00	0.00	100.00	100.00	0.00
12	*V logei*	46	0.00	100.00	100.00	0.00	100.00	65.22	4.32	0.00	13.04	69.57	100.00	13.04
13	*V marinus*	10	20.00	100.00	0.00	0.00	100.00	20.00	0.00	0.00	40.00	80.00	100.00	60.00
14	*V mediterrenei*	54	11.11	37.04	0.00	0.00	100.00	77.78	37.04	0.00	0.00	70.37	100.00	29.63
15	*V metchnikovii*	6	100.00	100.00	0.00	100.00	100.00	0.00	0.00	0.00	0.00	100.00	100.00	0.00
16	*V mimicus*	28	66.67	100.00	1000.00	66.67	100.00	88.89	22.22	0.00	11.11	88.89	100.00	77.78
17	*V natreigenes*	2	0.00	0.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00	100.00	100.00	0.00
18	*V orientalis*	84	0.00	0.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00	100.00	100.00	0.00
19	*V parahaemolyticu*	72	0.00	100.00	97.22	0.00	100.00	100.00	100.00	36.11	5.56	91.67	100.00	50.00
20	*V pelagius I*	8	0.00	0.00	0.00	0.00	100.00	50.00	50.00	0.00	25.00	50.00	100.00	0.00
21	*V pelagius II*	44	4.55	0.00	0.00	0.00	100.00	86.36	27.27	4.55	13.64	95.00	100.00	31.85
22	*V. proteolyticus*	4	100.00	100.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00	100.00	100.00	100.00
23	*V splendidus I*	4	75.00	0.00	0.00	0.00	100.00	100.00	50.00	0.00	0.00	100.00	100.00	0.00
24	*V splendidus II*	48	87.50	0.00	0.00	0.00	100.00	70.83	20.83	0.00	4.17	12.50	100.00	25.00
25	*V vulnificus*	102	0.00	100.00	98.08	0.00	98.08	69.23	1.92	0.00	7.69	94.23	100.00	40.38
26	*V vulnificus B2*	1	0.00	100.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00	100.00	100.00	100.00

ANNEXURE 1 (Continued...)

Sl. No.		No. of cultures tested	PERCENTAGE OF CULTURES SHOWING POSITIVE REACTIONS											
			ESCULIN HYDROLY-SIS	AMYGDA-LIN HYDROLY-SIS	CITRATE REACTION	GELATINA-SE	GAS FROM GLUCOSE	INDOLE PRODUCT-ION	LUMINISC-ENSE	NITRATE REACTION	ORTHO-NITRO-PHENOL-GALACTOPI NOSIDASE	OXIDASE	SWARMING	UREASE PRODUCT-ION
1	*V alginolyticus*	88	27.27	2.27	81.82	97.73	0.00	95.45	0.00	97.73	72.73	100.00	75.00	2.27
2	*V campellii*	86	32.56	11.63	51.16	97.67	90.70	97.67	6.98	95.35	25.58	95.35	6.98	4.65
3	*V carchariae*	2	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	0.00
4	*V cholerae*	2	0.00	0.00	100.00	100.00	0.00	100.00	0.00	100.00	100.00	100.00	0.00	0.00
5	*V cincinnatiensis*	10	0.00	0.00	100.00	100.00	0.00	0.00	0.00	100.00	0.00	100.00	100.00	0.00
6	*V costicola*	2	0.00	0.00	0.00	0.00	100.00	100.00	0.00	100.00	0.00	100.00	0.00	0.00
7	*V damsela*	6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00	100.00	100.00	0.00
8	*V fluvialis*	12	0.00	0.00	100.00	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00	0.00
9	*V furnissii*	18	0.00	0.00	100.00	0.00	100.00	0.00	0.00	0.00	100.00	0.00	0.00	100.00
10	*V harveyi*	44	0.00	0.00	9.09	81.82	0.00	90.91	27.27	100.00	45.45	100.00	18.18	9.09
11	*V. hollisae*	12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
12	*V logei*	46	13.04	4.35	4.35	91.30	0.00	4.35	26.09	100.00	52.17	95.65	0.00	0.00
13	*V marinus*	10	20.00	0.00	20.00	20.00	0.00	0.00	0.00	100.00	40.00	100.00	0.00	0.00
14	*V mediterrenei*	54	22.22	0.00	18.52	7.41	0.00	92.59	0.00	100.00	33.33	88.89	0.00	0.00
15	*V metchnikovii*	6	0.00	0.00	0.00	0.00	0.00	100.00	0.00	100.00	100.00	100.00	0.00	0.00
16	*V mimicus*	28	33.33	0.00	44.44	100.00	11.11	88.89	22.22	100.00	77.78	88.89	0.00	0.00
17	*V natreigenes*	2	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00	0.00	100.00	0.00	0.00
18	*V orientalis*	84	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00	0.00	100.00	0.00	0.00
19	*V parahaemolyticu*	72	16.67	8.33	97.22	100.00	2.78	97.22	2.78	97.22	38.89	100.00	55.56	11.11
20	*V pelagius I*	8	0.00	0.00	75.00	100.00	0.00	0.00	0.00	75.00	75.00	100.00	50.00	0.00
21	*V pelagius II*	44	4.55	0.00	36.36	90.91	0.00	100.00	4.55	100.00	59.00	95.45	0.00	0.00
22	*V. proteolyticus*	4	0.00	0.00	0.00	100.00	0.00	100.00	0.00	100.00	100.00	100.00	50.00	0.00
23	*V splendidus I*	4	50.00	0.00	0.00	100.00	0.00	100.00	0.00	100.00	75.00	100.00	0.00	0.00
24	*V splendidus II*	48	8.33	0.00	83.33	79.17	12.50	83.33	8.33	100.00	75.00	87.50	8.33	20.83
25	*V vulnificus*	102	3.85	3.85	100.00	98.08	1.92	98.08	3.85	100.00	96.15	96.15	1.92	0.00
26	*V vulnificus B2*	1	0.00	0.00	100.00	100.00	100.00	0.00	0.00	0.00	100.00	100.00	0.00	0.00

ANNEXURE 1 (Continued...)

		PERCENTAGE OF CULTURES SHOWING POSITIVE REACTIONS												
Sl. No.		No. of cultures tested	VOGES PROSKAU-ER	XANTHIN DECOMPO-SITION	KETOGL-UTARATE (Carbon Source)	D-GLUCOS-AMINE (Carbon Source)	D-GLUCOSE (Carbon Source)	L-ARABINOSE (Carbon Source)	LACTOSE- (Carbon Source)	MELIBIOSE- (Carbon Source)	ACID FROM ARABIN-OSE	ACID FROM ARBUTIN	ACID FROM INOSITOL	ACID FROM MANNITOL
1	*V alginolyticus*	88	95.45	9.09	61.36	75.00	95.45	9.09	18.18	34.09	9.09	29.55	4.55	68.18
2	*V campellii*	86	90.70	95.35	53.49	48.84	95.35	23.26	16.28	32.56	20.93	16.28	13.95	25.58
3	*V carchariae*	2	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00
4	*V cholerae*	2	0.00	0.00	0.00	0.00	100.00	100.00	0.00	0.00	100.00	0.00	0.00	50.00
5	*V cincinnatiensis*	10	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	*V costicola*	2	100.00	0.00	0.00	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	*V damsela*	6	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00	100.00	0.00	0.00
8	*V fluvialis*	12	0.00	0.00	100.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00	0.00	100.00
9	*V furnissii*	18	100.00	0.00	100.00	100.00	100.00	100.00	0.00	0.00	0.00	100.00	0.00	0.00
10	*V harveyi*	44	0.00	0.00	36.36	18.18	36.36	27.27	9.09	45.45	0.00	81.82	0.00	81.82
11	*V. hollisae*	12	0.00	0.00	100.00	100.00	100.00	0.00	0.00	100.00	100.00	0.00	100.00	100.00
12	*V logei*	46	0.00	0.00	8.70	34.78	86.96	4.35	0.00	73.91	0.00	78.26	8.70	26.09
13	*V marinus*	10	20.00	0.00	40.00	80.00	100.00	0.00	0.00	20.00	20.00	20.00	0.00	0.00
14	*V mediterrenei*	54	0.00	11.11	22.22	14.81	51.85	3.70	3.70	3.70	3.70	14.81	3.70	66.67
15	*V metchnikovii*	6	0.00	0.00	0.00	0.00	50.00	50.00	0.00	0.00	0.00	50.00	0.00	100.00
16	*V mimicus*	28	11.11	0.00	44.44	22.22	66.67	0.00	0.00	33.33	33.33	77.78	11.11	66.67
17	*V natreigenes*	2	0.00	0.00	100.00	0.00	100.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00
18	*V orientalis*	84	0.00	0.00	100.00	0.00	100.00	100.00	0.00	0.00	100.00	0.00	0.00	100.00
19	*V parahaemolytici*	72	2.78	8.33	50.00	86.11	91.67	47.22	22.22	27.78	30.56	27.78	25.00	69.44
20	*V pelagius I*	8	0.00	0.00	0.00	0.00	100.00	25.00	75.00	25.00	0.00	25.00	0.00	75.00
21	*V pelagius II*	44	9.09	4.55	18.18	22.73	81.82	4.55	45.45	45.45	9.09	9.09	0.00	22.73
22	*V. proteolyticus*	4	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	100.00	0.00	100.00	100.00
23	*V splendidus I*	4	0.00	0.00	100.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00
24	*V splendidus II*	48	0.00	4.17	25.00	29.17	87.50	16.67	4.17	12.50	20.83	8.33	4.17	33.33
25	*V vulnificus*	102	3.85	5.77	51.92	55.77	96.15	3.85	34.62	50.00	3.85	63.46	3.85	96.15
26	*V vulnificus B2*	1	0.00	0.00	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

ANNEXURE 1 (Continued...)

PERCENTAGE OF CULTURES SHOWING POSITIVE REACTIONS

Sl. No.		No. of cultures tested	ACID FROM SALICIN	ACID FROM SORBITOL	ACID FROM SUCROSE	ACID FROM CELLOBI-OSE	AMPICILLIN RESISTA-NCE	O/129 RESISTAN-CE (10 Ug)	O/129 RESISTAN-CE (150 Ug)	COLOUR (GREEN)	CASEINASE	LIPASE	AMYLASE	LECIETHINASE	DNase	HAEMOLY-SIN	PHOSPHAT-ASE	HISTIDINE DECARBOXYL ASE	HYDROGEN SULPHIDE
1	*V alginolyticus*	88	38.64	29.55	90.91	54.55	54.55	84.09	100.00	6.82	100.00	100.00	100.00	100.00	100.00	0.00	100.00	100.00	84.09
2	*V campellii*	86	18.60	13.95	34.88	100.00	55.81	25.58	100.00	60.47	55.81	100.00	100.00	100.00	55.81	16.35	95.35	0.00	100.00
3	*V carchariae*	2	0.00	0.00	100.00	0.00	100.00	100.00	100.00	0.00	100.00	100.00	100.00	100.00	100.00	0.00	100.00	0.00	100.00
4	*V cholerae*	2	0.00	0.00	100.00	0.00	100.00	100.00	100.00	0.00	100.00	100.00	100.00	100.00	0.00	0.00	100.00	0.00	100.00
5	*V cincinnatiensis*	10	0.00	0.00	100.00	60.00	0.00	0.00	100.00	0.00	100.00	80.00	100.00	100.00	0.00	30.00	70.00	100.00	100.00
6	*V costicola*	2	0.00	0.00	0.00	50.00	100.00	100.00	100.00	100.00	0.00	100.00	0.00	100.00	100.00	50.00	0.00	100.00	0.00
7	*V damsela*	6	0.00	0.00	0.00	0.00	100.00	100.00	100.00	100.00	0.00	50.00	50.00	100.00	100.00	60.00	90.00	0.00	0.00
8	*V fluvialis*	12	0.00	0.00	0.00	50.00	0.00	0.00	100.00	100.00	100.00	54.00	100.00	0.00	24.00	48.00	60.00	100.00	100.00
9	*V furnissii*	18	0.00	0.00	0.00	0.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00	100.00	50.00	0.00	100.00	50.00	100.00
10	*V harveyi*	44	18.18	27.27	45.45	63.64	63.64	100.00	100.00	72.73	82.45	50.00	100.00	100.00	100.00	38.55	100.00	50.00	100.00
11	*V. hollisae*	12	60.00	100.00	0.00	0.00	60.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	24.00	60.00	100.00	0.00	100.00
12	*V logei*	46	0.00	4.35	26.09	100.00	17.39	30.43	100.00	51.20	86.26	100.00	100.00	100.00	0.00	23.74	52.17	100.00	100.00
13	*V marinus*	10	0.00	0.00	60.00	0.00	20.00	20.00	100.00	80.00	0.00	100.00	100.00	50.00	50.00	20.00	40.00	100.00	100.00
14	*V mediterranei*	54	40.74	3.70	74.70	0.00	29.63	48.15	100.00	22.22	75.00	50.00	100.00	100.00	100.00	100.00	88.89	50.00	88.89
15	*V metchnikovii*	6	0.00	0.00	0.00	24.00	0.00	100.00	100.00	100.00	76.66	76.66	66.67	100.00	50.00	66.66	100.00	100.00	100.00
16	*V mimicus*	28	11.11	11.11	22.22	22.22	33.33	22.22	100.00	66.66	82.00	100.00	50.00	100.00	50.00	66.67	0.00	50.00	66.67
17	*V natreigenes*	2	100.00	0.00	100.00	100.00	0.00	0.00	100.00	0.00	50.00	100.00	100.00	100.00	100.00	0.00	100.00	0.00	100.00
18	*V orientalis*	84	100.00	0.00	100.00	82.45	0.00	0.00	100.00	0.00	64.20	100.00	100.00	100.00	100.00	0.00	100.00	100.00	100.00
19	*V parahaemolyticu*	72	25.00	25.00	59.40	69.44	55.56	91.67	100.00	84.46	91.67	100.00	100.00	100.00	50.00	22.22	100.00	100.00	100.00
20	*V pelagius I*	8	0.00	0.00	75.00	0.00	50.00	50.00	100.00	0.00	100.00	100.00	100.00	100.00	100.00	75.00	100.00	0.00	100.00
21	*V pelagius II*	44	9.09	0.00	68.18	0.00	13.64	13.64	100.00	22.73	45.60	50.00	100.00	100.00	100.00	68.18	100.00	0.00	90.90
22	*V. proteolyticus*	4	0.00	100.00	0.00	75.00	0.00	100.00	100.00	50.00	75.00	100.00	100.00	100.00	50.00	50.00	75.00	100.00	100.00
23	*V splendidus I*	4	0.00	0.00	75.00	100.00	0.00	100.00	25.00	50.00	50.00	100.00	100.00	75.00	50.00	50.00	100.00	75.00	100.00
24	*V splendidus II*	48	25.00	29.17	41.67	41.67	82.50	79.17	100.00	16.67	50.00	100.00	100.00	100.00	50.00	41.67	96.66	100.00	92.00
25	*V vulnificus*	102	51.92	28.85	30.77	98.00	23.08	51.92	100.00	79.20	69.23	69.23	100.00	100.00	50.00	30.77	98.02	80.24	100.00
26	*V vulnificus B2*	1	0.00	0.00	0.00	100.00	0.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	100.00	100.00	100.00	100.00

List of Publications

1. Sudha K., Nirmala Thampuran and Surendran, P.K. (1998) Effect of temperature on growth and biochemical properties of selected species of pathogenic *Vibrio* from fishes caught off Cochin. In; *Advances and Priorities in Fisheries Technology.* Society of Fisheries Technologists India, Cochin. pp 380-382

2. Nirmala Thampuran, Sudha,K. and Surendran, P. K. (1998) The present status and significance of *Vibrio vulnificus,* an emerging seafood associated pathogen- a review. Paper presented in National Symposium on *"Sustainable development of Fisheries towards* 2020 *AD-Opportunities and Challenges* ". Cochin University of Science and Technology

3. Sudha,K., Nirmala Thampuran and Surendran, P. K.(2001) Diversity and density of *Vibrio* populations on tropical fish from different habitats. *Fishery Technology* (Communicated).