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MOLECULAR CHARACTERIZATION OF PSYCHROTOLERANT HISTAMINE PRODUCING BACTERIA FROM INDIAN MACKEREL (*RASTRELLIGER KANAGURTA)*

UGC-MAJOR RESEARCH PROJECT

FINAL REPORT

(01.02.2011 TO 31.01.2014)

Submitted to

UNIVERSITY GRANTS COMISSION
BHADURSHAH ZAFAR MARG
NEW DELHI-110 002

Submitted by

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FINAL REPORT OF THE WORK DONE ON THE MAJOR RESEARCH PROJECT

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2.	Name and Address of the Institution	Department of Oceanography and Coastal Area Studies, School of Marine Science, Alagappa University, Thondi Campus, Thondi-623 409, Ramanathapuram District, Tamilnadu.
3.	UGC Approval No. and Date	F. No. 39-595/2010 (SR); date: 10.01.2011
4.	Date of Implementation	01.02.2011
5.	Tenure of the Project	01.02.2011 to 31.01.2014
6.	Total Grant Allocated	Rs. 7,22,738/-
7.	Total Grant Received	Rs. 7,00,87/-
8.	Final Expenditure	Rs. 7,00,87/-
9.	Title of the Project	Molecular characterization of Psychrotolerant histamine producing bacteria from Indian mackerel (*Rastrelliger kanagurta)*
10.	**Whether objectives were achieved** Yes, the following objectives were achieved ✓ To isolate the total heterotrophic bacteria (THB) from the gut, gill and muscles with skin of Indian mackerel fish ✓ To isolate the histidine decarboxylating bacteria in Moeller's agar medium. ✓ To identify the histamine producing bacteria by chemotaxonomic method. ✓ Molecular characterization of histamine producing bacteria (16 S r RNA method). ✓ To estimate the histamine production by bacteria. ✓ To estimate the histamine content in the muscles tissues of Indian mackerel at frozen condition as well as in fresh fishes.	
11.	**Achievements from the Project** The research findings have revealed the relationship of psychrotolerant and mesophilic histamine producing bacteria and their ability to produce histamine even in very low storage temperature. Probably for the first time mackerel infusion broth was used to determine the histamine producing ability of the isolated bacterial strains. Histamine content from the fish was estimated by simple and rapid detection method using TLC.	

12.	**Summary of the findings** Total heterotrophic bacteria (THB) density and total Histidine decarboxylating bacteria (HDB) density were enumerated from gill, gut and muscle tissues with skin of *Rastrelliger kanagurta*. A total of 159 THB and 70 HDB were isolated. In which 13 selected psychrotolerant and mesophilic bacterial strains were analysed chemotaxonomically. Eight psychrotolerant and mesophilic histamine producing bacterial strains were identified as *Photobacterium* sp. (KP689587), *Klebsiella oxytoca* (KP751412), *Klebsiella* sp. (KP689585), *Proteus mirabilis* (KP751413), *Klebsiella* sp. (KP689584), *Enterobacter* sp. (KP689583), *Proteus* sp. (KP689586) and *Klebsiella* sp. (KP689582) by 16S rRNA method. Two more clusters were observed in phylogenetic tree one which has sequences with more residues and another one has sequences with fewer residues. *Klebsiella* sp. (KP689582) strain was found in both the groups and shows close relationship with each other. In addition that *Enterobacter* sp. (KP689583) strain clustered with the *Klebsiella* sp. (KP689585). Hence it is assumed that *Enterobacter* sp. (KP689583) is closely related with *Klebsiella* sp. (KP689585). The secondary structure of histamine producing psychrotolerant and mesophilic bacteria were predicted using RNA fold-Vienna RNA web service. The free energy and centroid energy were calculated. Furthermore, total loop and stem were calculated. Eight psychrotolerant and mesophilic bacteria were used to analyse the histamine producing ability. The maximum histamine 5.56±0.44 mg/100 ml was obtained from *Photobacterium* sp. followed by 4.04±0.10 mg/ml from *Erwinia* sp., 3.92±0.12 mg/100 ml from *Flavobacterium* sp., 3.5±0.26 mg/ml from *Proteus mirabilis* and 2.53±0.12 mg/100 ml from *Klebsiella* sp. The change in the histamine content in Indian mackerel fish stored at -20, 0, 10, 20, and 30°C were studied. The eviscerated *R. kanagurta* fish stored at 10°C for 1 day possess histamine content 7.6±0.37mg/100g and uneviscreated *R. kanagurta* fish contained 10.1±0.37mg/100g of histamine. *R. kanagurta* fish stored at ambient temperature for 10 h contained the histamine 19.3±0.86 mg/100g in eviscerated muscles. The uneviscreated *R. kanagurta* fish had 22.33±1.44mg/100g of histamine during 30°C storage at 10 h. At 0 and -20° C very low quantity of histamine were formed. Histamine is formed even the fishes are stored at very low temperature by Psychrotolerant histamine producing bacteria.	
13.	**Contribution to the Society** This study has provided better understanding about best practices in fish handling, processing, storage and export of Indian mackerel, *R. kanagurta* at ideal temperatures. Storage of Indian mackerel fish below -20°C for two days will be safe for consumption and export. Indian mackerel fish can be stored at 0°C for 6 h duration with ice. Eviscerated Indian mackerel fishes can be stored for a week at -20°C. Psychrotolerant histamine producing bacteria are able to produce histamine even at very low storage temperatures. Moreover, the study brought more information regarding mesophilic and psychrotolerant fish spoilage bacteria.	
14.	Whether any Ph.D Enrolled /Produced out of the Project	Nil
15.	No. of Publications out of the Project	Three

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DETAILED FINAL REPORT

INTRODUCTION

Indian mackerel (*Rastrelliger kanagurta*) is an important fishery resources in India, especially along the southwest coast of India as well as an important feed item for the higher valued food fishes such as seer fishes and tuna varieties (Vivekanandan *et al*., 2009). It is found at the depth of 0-150 m, often near the thermocline. Adults live in coastal bays, harbour and deep lagoon, usually in turbid plankton rich waters. India and Sri Lanka catch the Indian mackerel fish as part of their small scale coastal gill nets and beach seine fisheries (BOB-LME, 2012). Unlike other meat fishes, Indian mackerels are captured at far away locations, the time between catch and landing is much longer than the landing and selling. Mackerel contains high amount of free amino acids especially histidine in its body. The free amino acids present in fish muscle may be liberated through proteolysis action of bacteria. In addition to the availability of the precursor amino acid histidine, histamine is accumulated in foods requires the presence of microorganisms with amino acid decarboxylase and favourable condition for its growth and amino acid decarboxylase activity (Zarei *et al*., 2011; Bjornsdottir *et al*., 2011; EFSA, 2011).

Histamine food poisoning is a chemical intoxication caused by consumption of food containing elevated amount of histamine (Ababouch *et al*,. 1991: An *et al*., 1998). Fresh fish meat contains negligible amounts of histamine. But improper handling and storage of fish products can increase histamine formation by the presence of bacteria possessing the enzyme histidine decarboxylase (Rawles *et al*., 1996).

Histamine is one of the important mediators of bronchoconstriction and inflammation in human (Goyal, 2003). In the living organisms histamine is synthesized from the naturally occurring α-amino acid, histidine or enzymatic decarboxylation (Cooper et al., 2005). Histamine alone may not cause toxicity at a low level, but the presence of other biogenic amines such as putrescine and cadaverine at concentration five times higher than histamine enhance the toxicity of histamine fish (food) poisoning (Stratton et al.,,

1997; Hernandez-Jover et al., 1997; Emborg and Dalgaard; 2006). Histamine fish (food) poisoning (Scombroid poisoning) is a worldwide problem (Russell and Maretic, 1986). That occurs after consumption of food containing high amount of biogenic amines particularly histamine at concentration higher than 500 ppm (Gonzaga et al., 2009).

The symptoms are include peppery or metallic taste, oral numbness, headache, dizziness, palpitations, rapid and weak pulse (low blood pressure), difficulty in swallowing, and thirst, hives, rash, flushing and facial swelling. Symptoms involving the central nervous system (CNS) such as anxiety are frequently observed. Less specific symptoms such as nausea, vomiting abdominal cramps and diarrhea are also experienced (Kim, 1979; Taylor et al., 1986).

Histamine poisoning is caused by naturally occurring bacteria present in the fish muscle during decarboxylation of the amino acids found in seafoods. Moreover, the gill and intestine of the fishes are exposed to the entry of a variety of bacteria along with diverse food particles and more number of bacteria retained in the intestine. The apparent residence of these bacteria can produce more histamine upon the death. The Enterobacteriaceae such as *Morgonella morganii, Citrobacter freundii, Enterobacter cloacae, Klebsiella pneumoniae, Raoultella plantikola, Providencia rettgri, Proteus mirabilis* and *P. vulgaris* are mainly involved in histidine decarboxylase activity (Kim *et al*., 2003; Tsai *et al*., 2005). In addition, *Clostridium* spp., *Vibrio* spp., *Acinetobacter* sp., *Pseudomonas* spp., and *Photobacterium* spp. also reported as histamine formers (Chen *et al*., 2010). Histamine formation in fish meat during storage is mainly affected by fish species, pre processing and storage condition. Since most high histamine producing bacteria such as *Morganella morganii*, *Citrobacter braakii*, *Hafnia alvei* and *Raoultella planticola* are mesophiles, storage time and temperature had significance on histamine production of these bacteria (Jiang *et al*., 2013). Storage at a temperature lower than 4^{o} C and reducing the length of handling period in an open environment could minimize histamine formation in fishery products (Economou *et al*.,

2007). In general, freshness of fishes is highly affected by the length of storage conditions (Park *et al*., 2010) by psychrotolerant bacteria.

Indian mackerel fish are generally marketed in fresh form and stored in ice, it is imperative to determine the amount of histamine present in its meat to predict the quality during different storage period and temperature before human consumption and export worldwide. Previously it was expected that histamines are formed above 7-10^{o}C temperatures allowing mesophilic bacteria such as *Morganella morganii*, *Raoultella planticola* and *Hafneia alvei* to grow to high levels and form histamine in toxic concentrations above 500 to 1000 ppm (Lehane and Olley, 2000; Taylor, 1986). However, recent studies of histamine fish poisoning in Japan and Denmark showed the psychrotolerant histamine producing bacteria *Morganella psychrotolerans* and *Photobacterium phosphoreum* to cause more histamine fish poisoning than the well-known mesophilic bacteria (Dalgaard et al., 2008). There are many methods are available to test the histamine formation and its level in sea foods. Among them, identification of histamine forming bacteria in fish and fishery products using molecular methods during processing is considered as appropriate one. Hence, the study aimed to find out the total heterotrophic bacteria, histidine decarboxylating bacteria, histamine formation in mackerel muscle during different storage condition, histamine production by psychrotolerant and mesophilic bacteria and molecular characterization of psychrotolerant and mesophilic histamine producing bacteria by 16 s rRNA analysis.

MATHODOLOGY

Collection of samples

Indian mackerel, *R. kanagurta* (Cuvier, 1817) samples were collected from Thondi, Manamelkudi and Rameswaram sea, coastal waters and fish landing centres of Southeast coast of India and brought to the laboratory under controlled temperature in sterile polythene bags (Himedia, India) and subjected to microbial analysis. The body surface of the fish was cleaned with sterile seawater to remove extraneous dirt and sand. One gram of the gill, gut and muscle with skin from the freshly captured fish and fish purchased from the market were aseptically dissected out and placed in separate polythene bags. Fishes were stored at 5^{o}C for 2 days, 0^{o}C for 3 days and -20^{o}C for 7 days to find out the Total heterotrophic bacteria (THB), Histidine Decarboxylating Bacteria (HDB) and psychrotolerant bacterial population.

ISOLATION AND ENUMERATION OF TOTAL HETEROTROPHIC BACTERIA (THB)

To enumerate the Total Heterotrophic Bacteria (THB), one ml of serially diluted homogenates (gills, gut and skin with muscle) diluted using sterile 50% seawater. One ml of sample was pipetted out in sterile petriplates. To this, 10 ml of sterilized Zobell marine agar was added and rotated clockwise and anticlockwise for thorough mixing. For each sample, duplicate plates were maintained and incubated in an inverted position at room temperature (28±2^{o}C) for 72 hrs. The bacterial colonies developed on Zobell marine agar medium after the incubation periods were counted and the population density has been expressed as log CFU g^{-1}. Colonies with different morphological features were picked up from the petriplates, streaked and re-streaked in Trypticase Soy Agar (TSA) three times, before establishing pure cultures in agar slants. Isolated strains have been identified by Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994).

ISOLATION OF HISTIDINE DECARBOXYLATING BACTERIA (HDB)

One ml of serially diluted homogenates (gills, gut and skin with muscle) were poured on to petriplates containing Niven's medium (Niven *et al*., 1981) and modified Niven's medium (Yoshinaga and Frank,

1982). To this, 5-10 ml layer of molten agar was poured over to prevent the swarming of some spreading colonies. The colonies showing positive results (purple halo around in Niven's medium and pink halo around in modified Niven's medium) were counted, after 24 hrs and 48 hrs of incubation at 28±2 ^{o} C. Duplicates plates were maintained.

Isolation and pure culture

The positive colonies in Niven's medium and Modified Niven's medium were picked up and streaked on Trypticase soy agar (TSA) plates and supplemented with 0.1% amino acid histidine. The isolated colonies were maintained as pure cultures in duplicate slants of the same composition for further confirmation of their histidine decarboxylase activity.

(i) Moeller decarboxylase agar

To isolate the histamine formers, Moeller decarboxylase agar medium supplemented with the amino acid histidine. Selected colonies were streaked on Trypticase Soy Agar (TSA) (Hi-MEDIA, Mumbai) for pure culture. For initial screening these strains decarboxylase activity was checked using Moeller decarboxylase broth (Hi-MEDIA, Mumbai) supplemented with L-histidine hydrochloride at 0.5% level (W/V). Medium without the amino acid severed as control. To check the decarboxylase activity, 18 hrs old cultures of purified strains were inoculated in to these media and inoculated at 37^{o}C for 72-96 h (Jeyasekaran and Jeya Shakila, 2003). The isolate, which shows positive reaction in decarboxlyase activity were considered as histamine forming bacteria.

(ii) Inoculation in histidine decarboxylase broth containing inverted Durham's tube

The ability of bacterial strains to decarboxylate histidine was tested by inoculating a loopful of purified culture in the medium containing tryptone (5.0 g), yeast extract (5.0 g), L-histidine (20.0 g), NaCl (10.0 g), final pH (6.3 ±0.2). A control tube with only basal medium i.e. without histidine was maintained simultaneously. The tubes were incubated at 30^{o}C for 24 to 48 hrs. The tubes were filled with 10 ml of

medium and a Durham's tube (5 x 20 mm) was put in inverted condition in each tube, after removing the air bubbles within the Durham's tubes and they were sterilized at 121°C for 15 minutes in an autoclave. After incubation, air bubble formation inside the Durham's tube was ensured for the positive reaction.

(iii) Stab culture in histidine decarboxylase agar medium

Ability of the bacterial strains to decarboxylate histidine was tested by stab inoculating the strain in test tubes containing 5 ml of sterilized Niven's medium (MNM) (Yoshinaga and Frank, 1982) at 37° C incubation for 18-24 hrs. One control tube without histidine was also maintained for each strain. The change of colour to deep red was considered as positive reaction.

(iv) Stab culture in histidine decarboxylase medium

Ability of the cultures to decarboxylate histidine was tested by stab inoculating them to the medium by a straight wire. Change of colour of the medium to purple or deep purple was taken as positive after 18-24 hrs of incubation at 35° C (Smith et al., 1982).

Stock culture

Trypticase Soy Agar supplemented with 0.1% L-Histidine –HCl (TSAH) and pH adjusted to 6.0 was used to prepare the slants. The bacterial strains were maintained in the slants and sub-cultured periodically for the maintenance of strains.

CHEMOTAXONOMIC ANALYSIS

Isolated bacterial strains were identified by Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994).

Indole test

The peptone broth was prepared and sterilized in an autoclave and 5 ml of the broth was dispersed into five test tubes indicating the duplicates for each culture. One set of uninoculated test tubes were maintained as control. From the culture broth 0.1 ml culture was inoculated into the respective tubes and incubated at 37^{o}C for 24 hours and then two drops of Kovac's reagent were added. Formation of cherry red ring is considered as positive and a colour change in to yellow is considered as negative. The absence of red coloration demonstrates that the substrates tryptophan was not hydrolyzed and indicates an indole-negative reaction

Methyl red (MR) test

Methyl Red-Voges Proskauer broth is prepared and sterilized using automated autoclave (Equitron, Mumbai, India) at 121^{o}C for 10 minutes. From this 5 ml of broth is dispersed into five test tubes and duplicates were maintained for each culture. Uninoculated test tube served as control. Then 0.1 ml of the culture is inoculated into the MR-VP broth and incubated at 37^{o}C for 24 hours. After incubation five to six drops of methyl red indicator is added to the MR-VP broth tubes. The indicator turns red considered as positive reaction. Changing colour into yellow is considered as negative.

Voges Proskauer (VP) test

Methyl Red-Voges Proskauer broth was prepared and sterilized using automated autoclave (Equitron, Mumbai, India) at 121^{o}C for 10 minutes. The broth, five ml was taken in to each test tube including duplicates. Uninoculated test tube served as control. Bacterial both culture 0.5 ml was inoculated and incubated for 37^{o}C for 24 hours. After incubation 0.1 ml of 40 w/v Potassium hydroxide and 3ml of 5 w/v

α-napthol was added. Deep rose colour develops in the culture tubes within 15 minutes indicating the positive reaction and absence of pink or deep rose colour in the medium indicates the negative reaction.

Citrate utilization test

Simmon citrate agar slants were prepared and the cultures were inoculated into the tubes by stab and streak methods. Later the slants were incubated for 24-48 hours at 37^{o}C. Changes of green to Prussian blue colour indicate positive reaction. If the media remain green is considered as negative.

HISTAMINE PRODUCTION BY BACTERIA

Histamine producing ability of the bacterial strains were tested in mackerel fish infusion broth (MFIB) by the enzymatic assay method as described by Lerke et al. (1983) and Lopez-Sabater et al. (1994) with slight modifications. Briefly, each 10 ml bacterial culture was centrifuged (REMI, CPR 30 PLUS, India) at 3000 rpm for 10 min at 4^{o}C. The volume of the supernatant was made up with 100 ml of 0.4N perchloric acid. From the supernatant 5 ml was transferred to the filtration unit and the filtrate was taken into a separating funnel, 5 ml of 1N NaOH, 10 ml of deionised water and 2.0g of NaCl were added. The supernatant was extracted in 4 times consecutively with 25 ml of n-butanol. The butonolic phases were again washed with 10 ml of 1N NaOH saturated with NaCl. The histamine was then extracted 5 times with 10 ml of 0.1N HCl and the volume adjusted to 50 ml. The histamine extract was finally derivatized with O-opthaldehyde to minimize the interference of other components already present in samples (Vidal-Carou et al., 1990).

The culture extract and standard histamine dihydrochloride 0.5 ml was transferred in to test tube. One ml of phosphate buffer (0.0075 M KH_2PO_4 and 0.075 M Na_2HPO_4, pH 6.8), 0.5 ml of diamine oxidase (0.35 U I /ml) and 0.1 ml of leucocrystal violet (0.5mg/m1 in 0.5% HCl) were added. The mixture kept on the thermostatic water bath at 37^{o} C for 30 min. Finally, the coloured test solutions were measured at

596 nm using a UV-VIS, PC Scanning spectrophotometer UVD-2960 (Labomed, Inc., USA). The histamine concentration in mackerel infusion broth samples were calculated using the following formula.

$$\text{Histamine (mg/100ml)} = \frac{\text{OD of sample x concentration of standard}}{\text{OD of standard}}$$

= histamine mg/100ml of bacterial culture

MOLECULAR CHARACTERIZATION

The presumptive histamine forming isolates were identified on the basis of morphology, Gram-stain and biochemical reaction. The identification of histamine forming isolates were further confirmed by amplifying 1400 bp of the 16S ribosomal DNA (rDNA) for bacteria (Kuhnert, et al., 1996; Kuhnert, et al., 2000). Amplification of the 16S r DNA of histamine-forming bacteria was performed using the Universal primers UNI-L (5^1-AGAGTTTGATCATGGCTCAG-3^1) and UNI-R (5^1–GTGTGACGGGCGGTGTGTAC-3^1) (Kuhnert et al., 1996 and Kuhnert, et al., 2000). Bacterial cells were cultured overnight in 2 ml of TSB at 35° C and then centrifuged at 7000g for 10 min. The cell pellet was washed and resuspended in 0.5 ml of TE-buffer (10 mM Tris-HCL, 1 mM ETDA; pH 8.0), and then lysed by 20% sodium dodecylsulfate (SDS). After the solution was boiled for 20 min and the cellular debris was discarded following centrifugation at 13,000g for 3 min. The total DNA in the supernatant was precipitated with 70% ethanol and used as template DNA for PCR.

PCR amplification was performed in 20µl reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM concentration for each of the four deoxynucleotide triphosphate, 0.5 µg of Taq DNA polymerase (Applied Biosystems) with an initial denaturation at 94° C for 4 min and a final extension at 72°C for 7 min (Kuhnert et al., 1996 and Kuhnert et al., 2000). Amplicons were detected by electrophoresis on 1.5 % agarose gel staining with ethilium bromide. They were purified using a QIA quick PCR purification Kit (Qiagen, Valencia, CA, USA) eluted in tris-HCL (10 mM, pH 8.5) prior to sequencing, the amplified DNA was directly sequenced with the

ABI Taq Dye Deoxy Terminator cycle sequencing kit and ABI Model 377 automated DNA sequencer (Applied Biosystem). The sequences were analysed with the BLAST (NCBI) for identification of histamine forming bacteria. Neighbour joining tree (Saitou and Nei, 1987; Lane et al., 1985) was employed to display the relationships among the 16 S r RNA sequenced bacteria.

HISTAMINE ANALYSIS IN EVISCERATED AND UNEVISCERATED INDIAN MACKEREL

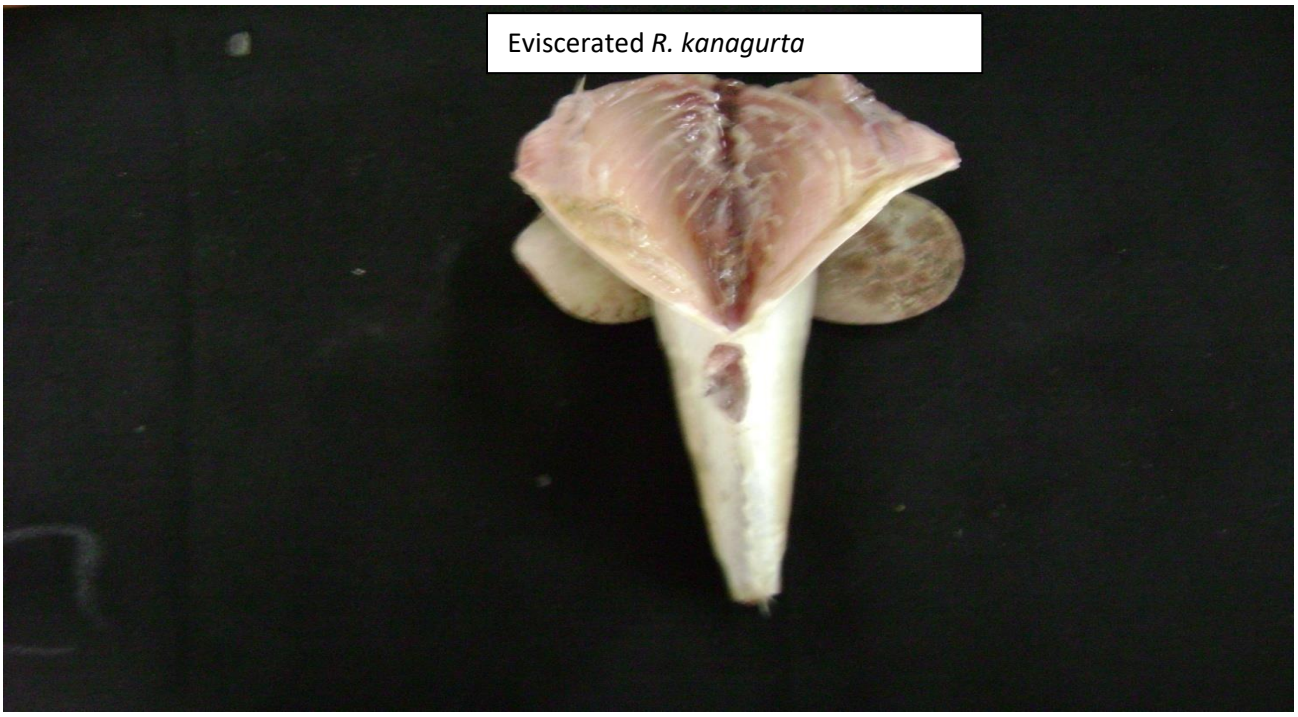
Fish sample collection

Freshly caught, *R. kanagurta* samples were collected from Thondi sea and coastal waters (Latitude: 9^{o} 44' N and Longitude: 79^{o} 00E), Palk Bay, Southeast coast of India and brought to the laboratory under controlled temperature in sterile polythene bags. The fishes were cleaned with sterile seawater to remove extraneous dirt. Fish samples were immediately transferred to laboratory. Some of the fishes were eviscerated (Gill, gut fins and scales were removed) and other fishes were left uneviscerated (whole fish). These fishes were kept in a separate polythene covers, sealed and stored at -20^{o}C (7 days), 0^{o}C (2 days), 10^{o}C (1 day), 20^{o}C (15 hrs) and 30^{o}C (10 hrs) in aseptic condition (Plate I).

Histamine contents of Indian mackerel fish muscle samples were estimated by the enzymatic assay method as described by Lerke et al. (1983) and Lopez-Sabater et al. (1994). Briefly, fish muscle 5 gram each were homogenized twice with 50 ml of 0.4N perchloric acid after that it was centrifuged (REMI, CPR 30 PLUS, India) at 3000 rpm for 10 min. The volume of the supernatant was made up with 100 ml of 0.4N perchloric acid. From the supernatant 5 ml was transferred the filtration unit and the filtrate was taken into a separating funnel, 5 ml of 1N NaOH, 10 ml of deionised water and 2.0g of NaCl were added. The supernatant was extracted in 4 times consecutively with 25 ml of n-butanol. The butonolic phases were again washed with 10 ml of 1N NaOH saturated with NaCl. The histamine was then extracted 5 times with 10 ml of 0.1N HCl and the volume adjusted to 50 ml. The histamine extract was finally derivatized with o-opthaldehyde to minimize the interference of other components already present in fish muscle samples (Vidal-Carou et al., 1990).

Plate - I

Rastrelliger kanagurta



Eviscerated *R. kanagurta*

Sample extract and standard histamine dihydrochloride 0.5 ml was transferred in to test tube. One ml of phosphate buffer (0.0075 M KH_2PO_4 and 0.075 M Na_2HPO_4, pH 6.8), 0.5 ml of diamine oxidase (0.35 U I /ml) and 0.1 ml of leucocrystal violet (0.5mg/m1 in 0.5% HCL) were added. The mixture kept on the thermostatic water bath at 37^o C for 30 min. Finally, the coloured test solutions were measured at 596 nm using a UV-VIS, PC Scanning spectrophotometer UVD-2960 (Labomed, Inc., USA). The histamine concentration in fish muscle samples were calculated using the following formula.

$$\text{Histamine (mg/100g)} = \frac{\text{OD of sample x concentration of standard}}{\text{OD of standard}}$$

= histamine mg/100g of fish muscle

pH measurement

For the measurement of pH, 10g of eviscerated and uneviscerated Indian mackerel (*R. kanagurta*) muscle samples were homogenized in sterile blender with 10 ml of double deionized water to make thick pasty like slurry. The pH of this slurry was measured using a pH meter (Eutech Instruments, Malaysia).

Determination of total volatile base-nitrogen (TVB-N)

The TVB-N content of Indian mackerel (*R. kanagurta*) muscle was measured by the Conway's dish diffusion method (Cobb et al., 1973). The TVB-N extract of fish muscle in 6% trichloroacetic acid (TCA) (Merck, Germany) was absorbed by boric acid and then titrated with 0.02N hydrochloric acid. The content of TVB-N was calculated and expressed as mg/100g of muscle sample.

Statistical analysis

All the determinations were done in triplicate. The correlation analysis among the variables (Storage temperature, month, bacterial population and storage time) and difference among the parameters were considered significant for a confidence interval at 95% level ($p< 0.05$). All the statistical analysis were performed using the software Statistical Packing for Social Sciences (SPSS) Version 14 (SPSS, Chicago, IL, USA). Standard deviation was performed by MS -Excel for statistical analysis.

RESULTS

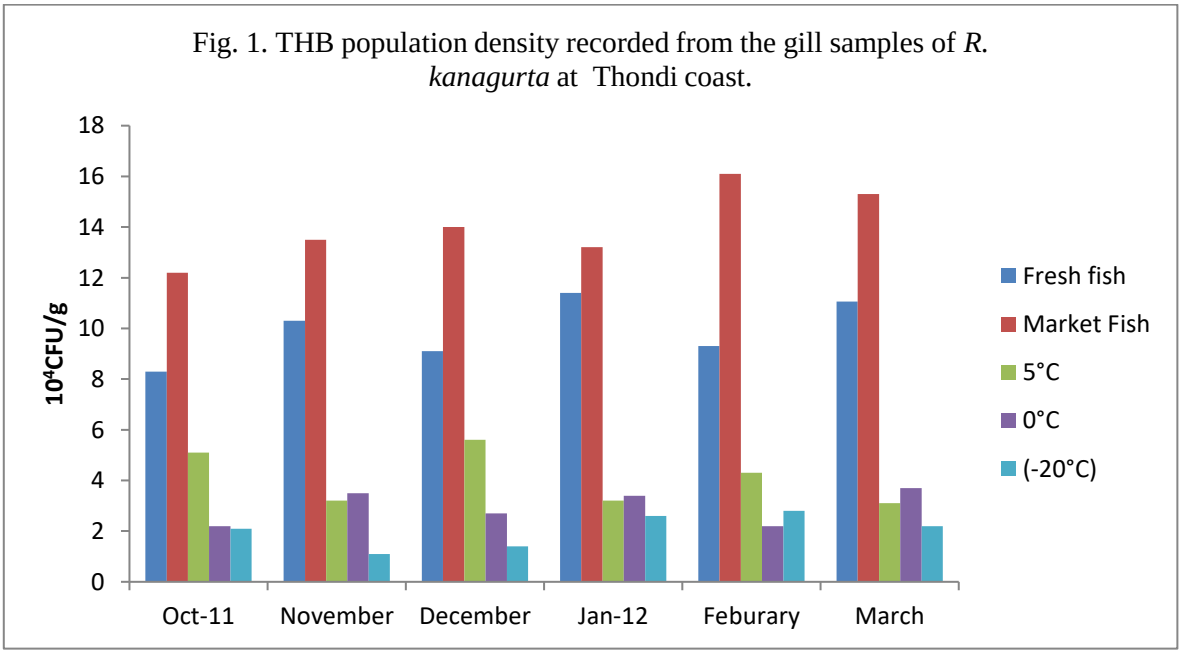
1. ISOLATION OF TOTAL HETEROTROPHIC BACTERIA (THB)

Rastrelliger kanagurta fresh fish, fish purchased from fish market and fishes stored at different temperatures (5°,0° and -20°) were showed varying populations of THB in different parts of their body such as gill, gut and muscle tissues with skin. In the present study three fish landing centres such as Thondi, Manamealkudi and Rameswaram were selected from the Palk Bay, India region to assess the THB populations from October 2011 to March 2012.

Population density of THB
Thondi coast

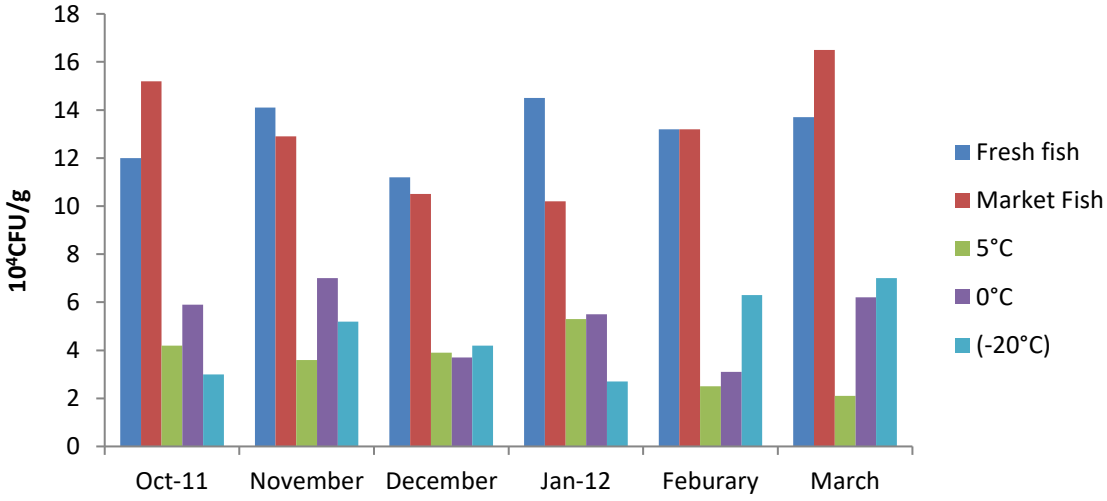
At Thondi coast the Total Heterotrophic Bacterial (THB) load varied from 8.3×10^4 to 11.6×10^4 CFU/g in gills. Minimum 8×10^4 CFU/g was recorded during October and the maximum 11×10^4 CFU/g was recorded in March. Secondary peak 12.6×10^4 CFU/g each were recorded during November and March in the fresh samples of *R. kanagurta*. Maximum 15.3×10^4 CFU/g was found during March 2012 from the fish samples collected from market. THB population was low at 5°, 0° and -20° C (Fig.1).

Fig. 1. THB population density recorded from the gill samples of *R. kanagurta* at Thondi coast.



THB population ranged from 11.2 x10^4 to 16.2 x10^4 CFU/g. The minimum (11.2x10^4 CFU/g) was recorded during December and the maximum 13.6×10^4 CFU/g was observed during March in Thondi coast from fresh fish gut samples. In the case of fishes purchased from market, the low (10.2×10^4 CFU/g) bacterial load was noticed during January and the high (16.5 × 104 CFU/g) bacterial colony counts were noticed during March. Samples stored at different temperatures exhibited the maximum colony counts 5.3×10^4 CFU/g at 5ºC, 6.2×10^4 CFU/g at 0ºC and 1.1×10^4 CFU/g at -20°C (Fig.2).

Fig. 2. THB population density recorded from the gut samples of *R. kanagurta* at Thondi coast.



At Thondi coast THB counts in muscles with skin varied from 7.3×10^4 to 11.7×10^4 CFU/g. Minimum 7.3×10^4 CFU/g were recorded during October. The maximum 11.7×10^4 CFU/g was recorded in February. Fish samples purchased from markets exhibited the population between 12.1×10^4 CFU/g and 17.6 ×10^4 CFU/g during November and March respectively. Very low population density (5.1×10^4 CFU/g) were noticed during November and high (9.5×10^4 CFU/g) were noticed in March at 5º C. At 0ºC the THB population was ranged from 3.2×10^4 CFU/g and 5.2×10^4 CFU/g during February, 2012. The THB population ranged from 2×10^4 CFU/g to 4.1×10^4 CFU/g. The minimum (2×10^4 CFU/g) was recorded

during October, 2011 and March, 2012 and the maximum (4.1×10^4 CFU/g) was recorded during December, 2011 (Fig.3).

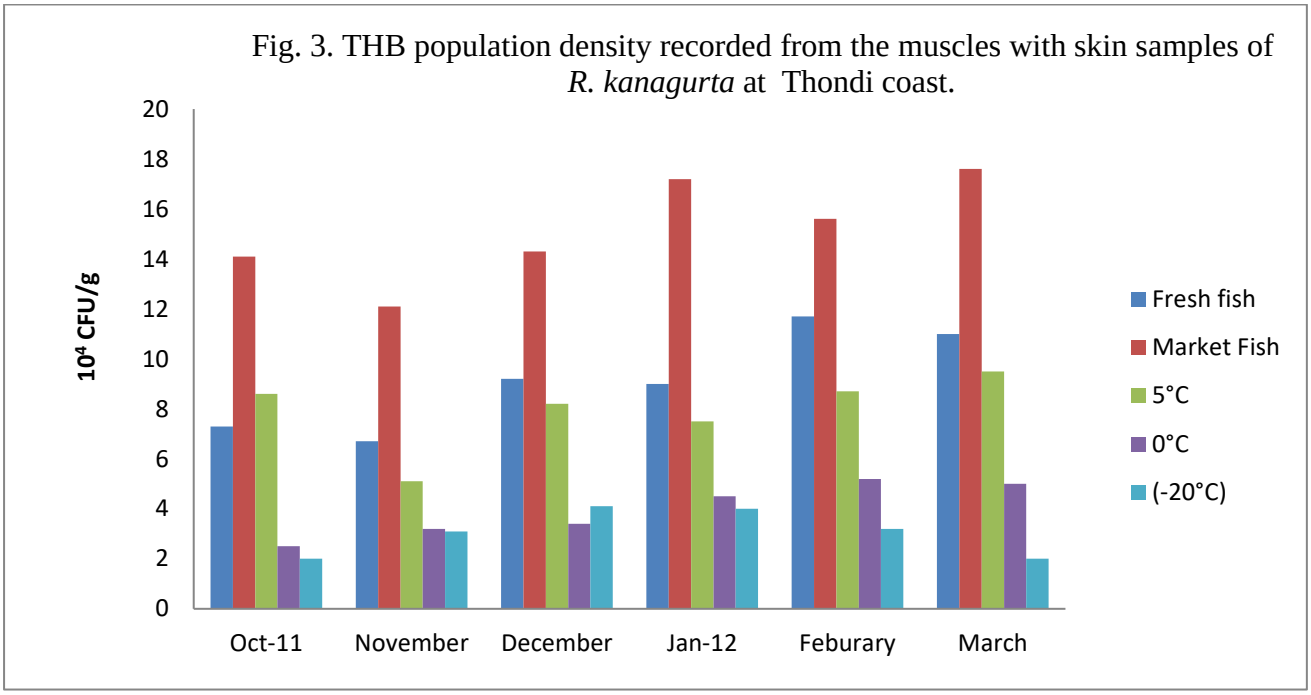


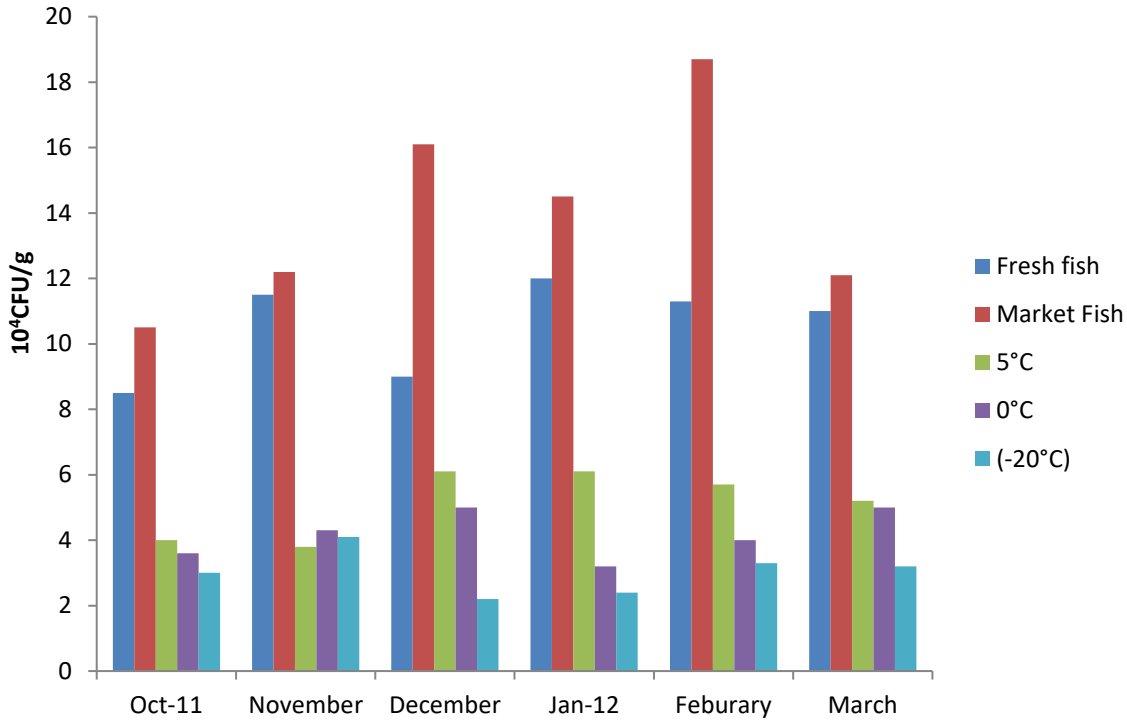
Fig. 3. THB population density recorded from the muscles with skin samples of *R. kanagurta* at Thondi coast.

Manamelkudi coast

R. kanagurta fresh fish samples were collected from Manamelkudi coast and examined to assess the THB population in gills. The THB population density varied from 8.5×10^4CFU/g to 11.3×10^4 CFU/g. The minimum (8.5×10^4 CFU/g) was recorded in October and the maximum (11.3×10^4 CFU/g) in February from the fresh fish gill samples. THB population was low (10.1×10^4 CFU/g) in October, 2011 and the highest density (18.7×10^4 CFU/g) was noticed during February, 2012.

R. kanagurta fish samples stored at different temperatures showed the minimum density (3.8×10^4 CFU/g) during November and the maximum (6.1×10^4 CFU/g) were noticed during December and January at 5^{o}C. Fishes stored at 0^{o}C and -20^{o}C showed less population density than the fish stored at 5^{o}C (Fig. 4).

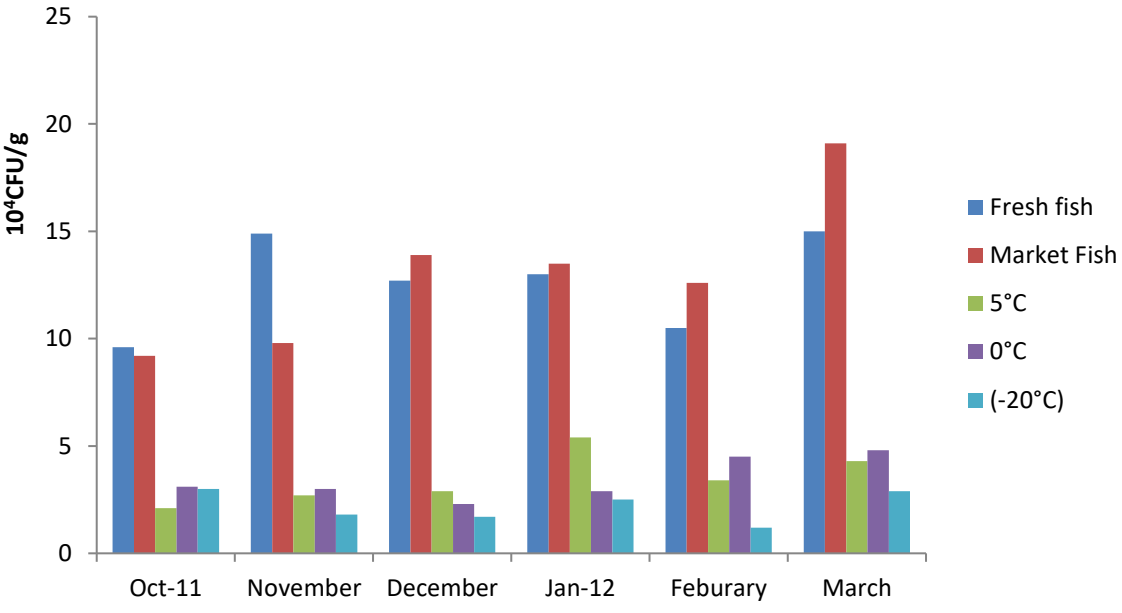
Fig. 4. THB population density recorded from the gill samples of *R. kanagurta* at Manamelkudi coast.



Occurrence of THB population density in fresh fish gut samples indicates that gut region harbours higher density (15.0×10^4 CFU/g) during March and the lowest (9.6×10^4 CFU/g) bacterial load in October. In the case of fishes purchased from retail market contains the minimum population density 12.4×10^4 CFU/g during October and maximum 19.1×10^4 CFU/g during March.

At 5^{o}C the THB population density ranged from 2.1×10^4 CFU/g to 5.4×10^4 CFU/g. The minimum (2.1×10^4 CFU/g) was recorded in October and the maximum (5.4×10^4 CFU/g) in January. Less THB population density were noticed from the fresh fish samples stored at 0^{o}C and -20^{o}C temperatures (Fig.5).

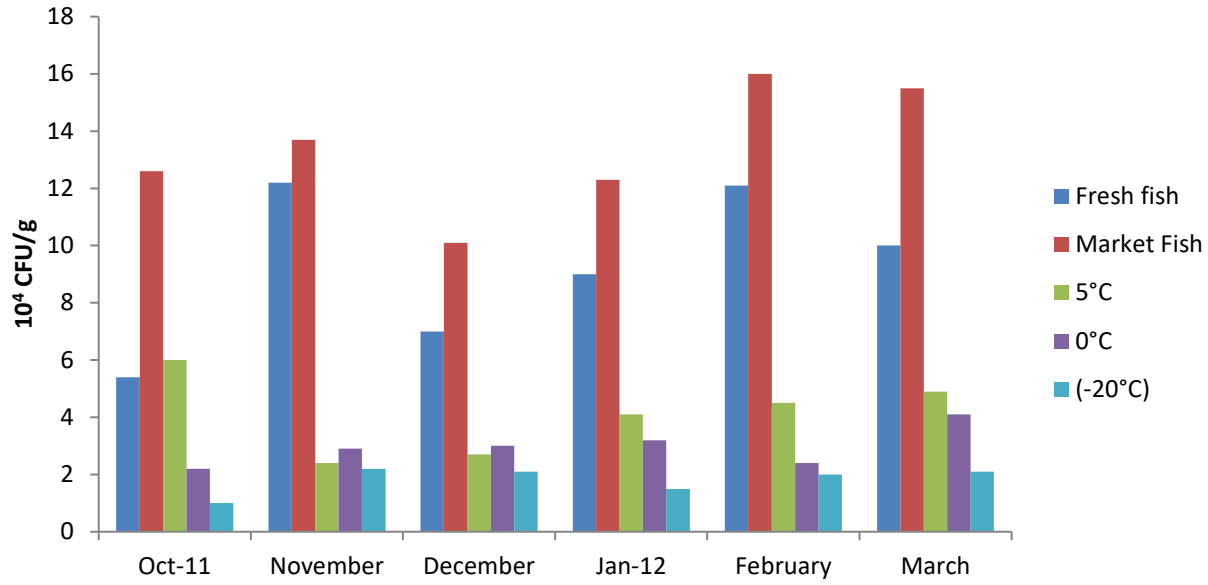
Fig. 5. THB population density recorded from the gut samples of *R. kanagurta* at Manamelkudi coast.



Distribution of THB density in the fresh fish muscle with skin samples at Manamealkudi coast varied from 5.4×10^4 CFU/g to 12.2×10^4 CFU/g during October and November respectively. THB population density was minimum 14.8×10^4 CFU/g and maximum 16.6×10^4 CFU/g in February and November respectively. Very low population density of THB recorded from the fresh fishes stored at 5°C. The minimum 2.7×10^4 CFU/g of THB recorded from the fresh fish muscles with skin during December and the maximum 4.9×10^4 CFU/g were found during March.

At 0°C the THB population density was ranged from $2.2\text{x}10^4$ CFU/g to $4.1\ \text{x}10^4$ CFU/g. The minimum ($2.2\ \text{x}10^4$ CFU/g) THB population was recorded during October and the maximum ($4.1\ \text{x}10^4$ CFU/g) was recorded during March. The least THB load $1.0\text{x}10^4$ CFU/g was noticed from the fishes stored at -20°C during December and the maximum $2.2\text{x}10^4$ CFU/g was recorded during November (Fig.6).

Fig.6. THB population density recorded from the muscle with skin samples of *R. kanagurta* at Manamelkudi coast.

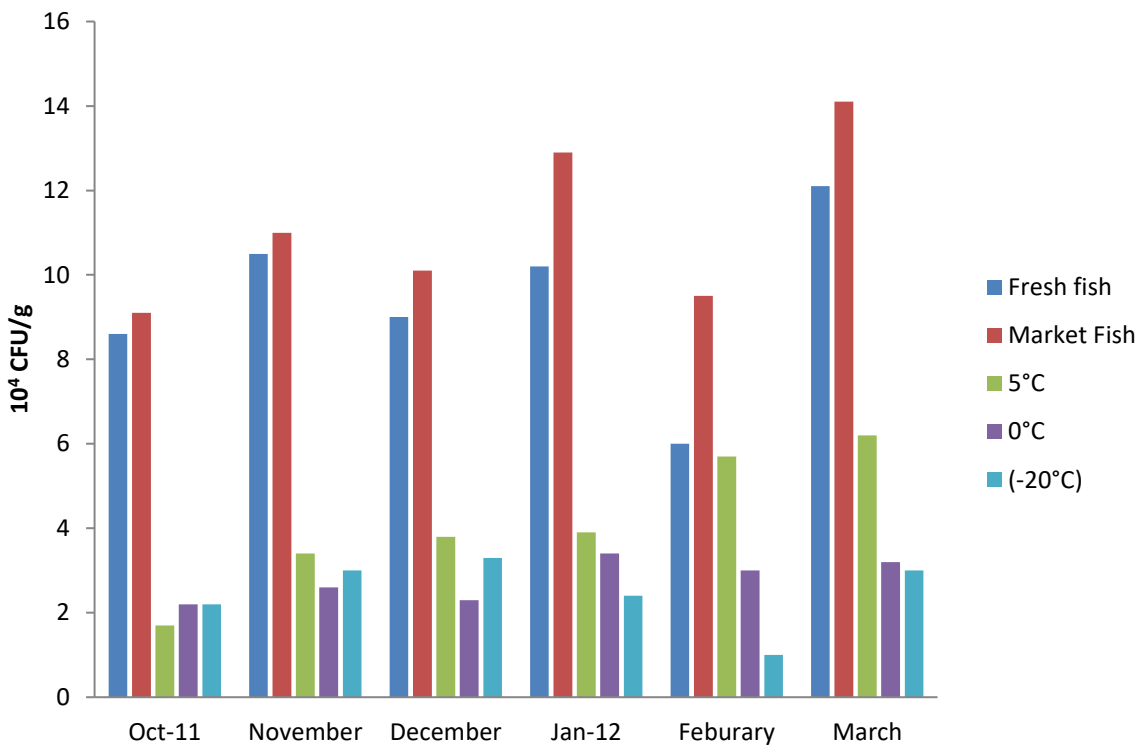


Rameswaram coast

THB population varied from 8.6×10^4 to 12.1×10^4 CFU/g from the fresh fish gill samples. The minimum (8.6×10^4 CFU/g) recorded during October, 2011 and the maximum (12.1×10^4 CFU/g) was recorded in March, 2012 in the fresh fish samples. The density of THB in the market fish samples varied from 9.1×10^4 CFU/g to 14.1×10^4 CFU/g. Low density (9.1×10^4 CFU/g) was recorded from October, 2011 and the maximum ($14.1\times10^4\times10^{-4}$ CFU/g) was recorded during March, 2012.

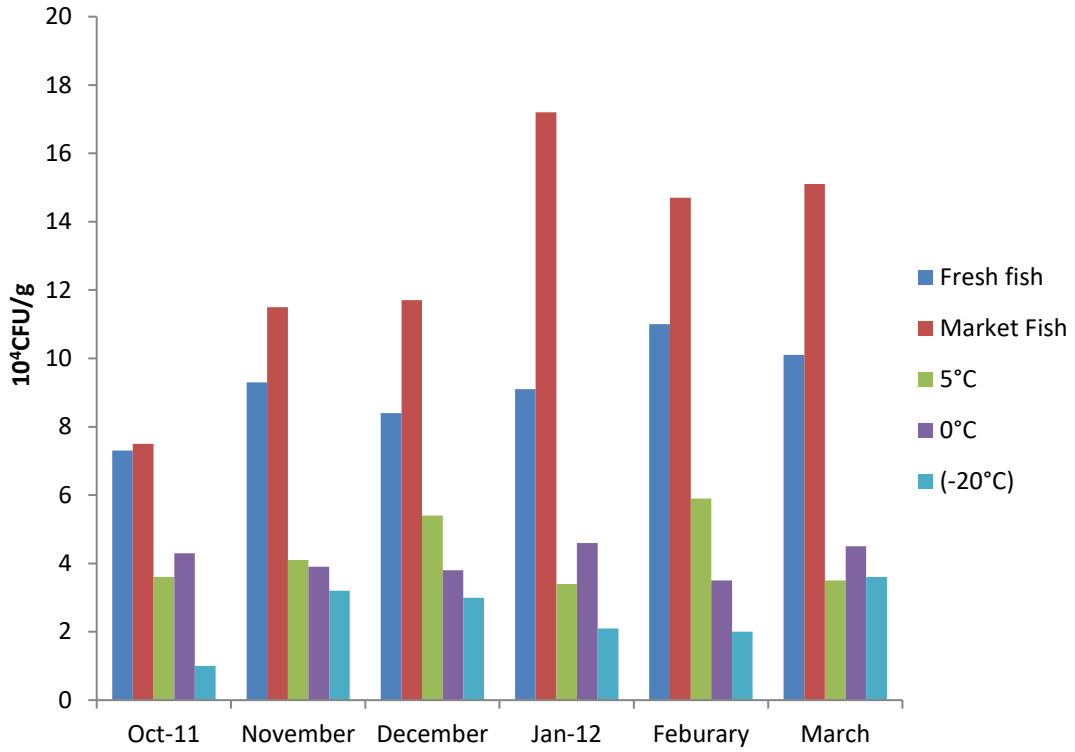
At 5°C the THB population density was minimum (1.7×10^4 CFU/g) during October and the maximum 6.2×10^4 CFU/g during March. At 0°C the THB population density ranged between 2.2×10^4 CFU/g to 3.4×10^4 CFU/g during October and January respectively. Very low number of bacterial colony was detected during February at -20° (Fig.7).

Fig. 7. THB population density recorded from the gill samples of *R. kanagurta* at Rameswaram coast.



At Rameswaram coast *R. kanagurta* fresh fish gut samples THB density ranged between 7.3 $\times 10^4$ CFU/g and 10.1$\times 10^4$ CFU/g. The minimum (7.3 $\times 10^4$ CFU/g) recorded during October and the maximum 10.1 $\times 10^4$ CFU/g was recorded in March. In the case of market fish samples, the minimum (7.5 $\times 10^4$ CFU/g) was recorded during February and the maximum (15.1 $\times 10^4$ CFU/g) was found in March. At 5^o C, the THB density varied from 3.4 $\times 10^4$ CFU/g to 5.9 $\times 10^4$ CFU/g. The minimum (3.4 $\times 10^4$ CFU/g) was recorded in January and the maximum (5.9 $\times 10^4$ CFU/g) in January. Low THB population densities were noticed at 0° and -20° C (Fig.8).

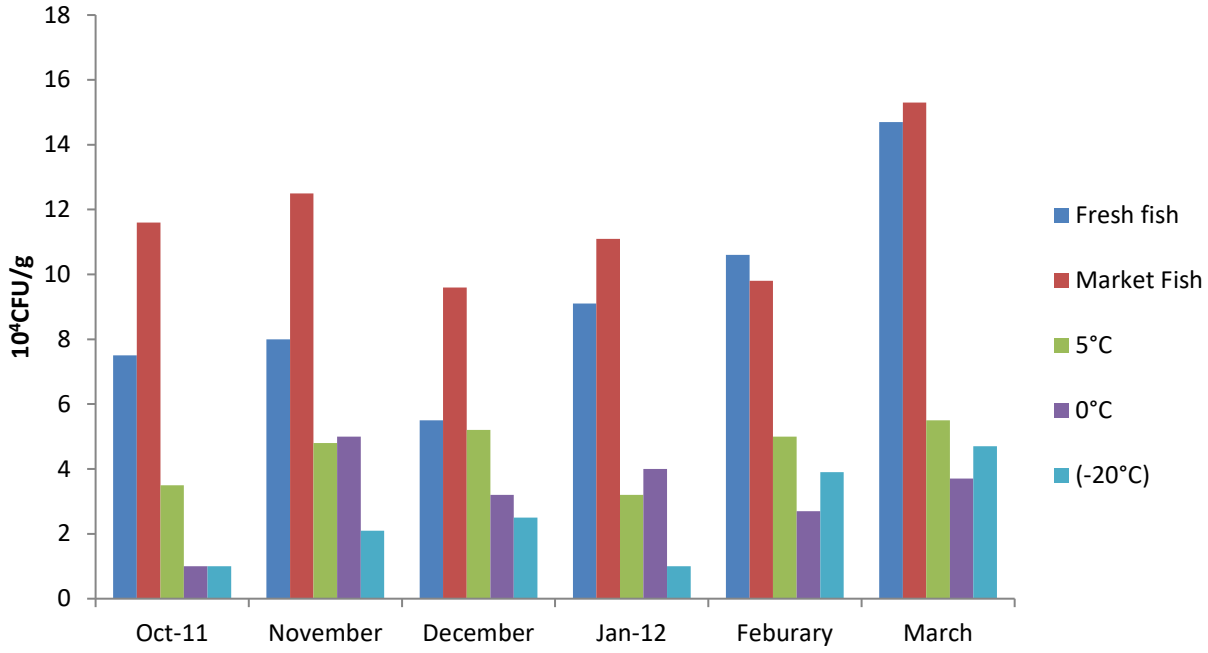
Fig. 8. THB population density recorded from the gut samples of *R. kanagurta* at Rameswaram coast.



THB density ranged from 5.5×10^4 CFU/g to 14.7×10^4 CFU/g. The minimum (5.5×10^4 CFU/g) was recorded during October, 2011 and the maximum (14.7×10^{-4} CFU/g) was recorded during March from the fresh *R. kanagurta* fish muscle with skin samples at Rameswaram coast. Fish purchased from the Rameswaram market showed the THB population in muscles with skin, minimum (9.6×10^{-4} CFU/g) during December and the maximum (15.3×10^{-4} CFU/g) was recorded during March.

THB density varied from 3.2×10^4 CFU/g to 5.5×10^4 CFU/g in the muscle with skin samples of Fresh *R. kanagurta* stored at 5°C. The minimum (3.2×10^4 CFU/g) was recorded during January and the maximum 5.5×10^4 CFU/g at March. At 0°C the fish muscles with skin contains the lowest THB population (1×10^4 CFU/g) during October and the high density 3.7×10^4 CFU/g March. The lowest density (1×10^4 CFU/g) was recorded during October and January and the maximum (4.7×10^{-4} CFU/g) was recorded during March (Fig.9).

Fig. 9. THB population density recorded from the muscle with skin samples of *R. kanagurta* at Rameswaram coast.



In general, in the present study, fresh *R. kanagurta* exhibited higher histidine decarboxylating bacteria in gut than the gill and muscle tissues. In the case of fishes purchased from the market showed higher bacterial density both in skin and gut at ambient temperatures. Whereas fishes stored at low and freezing temperature, the bacterial growth found low.

A total of 159 strains of THB were isolated from *R. kanagurta* (fresh, stored and collected from market) gill, gut and muscle with skin (Table.1). They belonged to ten genera viz. *Pseudomonas, Enterobacter, Clostridium, Aeromonas, Micrococcus, Klebsiella, Proteus, Vibrio, Citrobacter and Bacillus* (Fig.10). During the present study, Gram-negative bacteria were recorded more than the Gram-positive bacteria (Plate II (a).

Plate-II (a)

Total Heterotrophic Bacteria

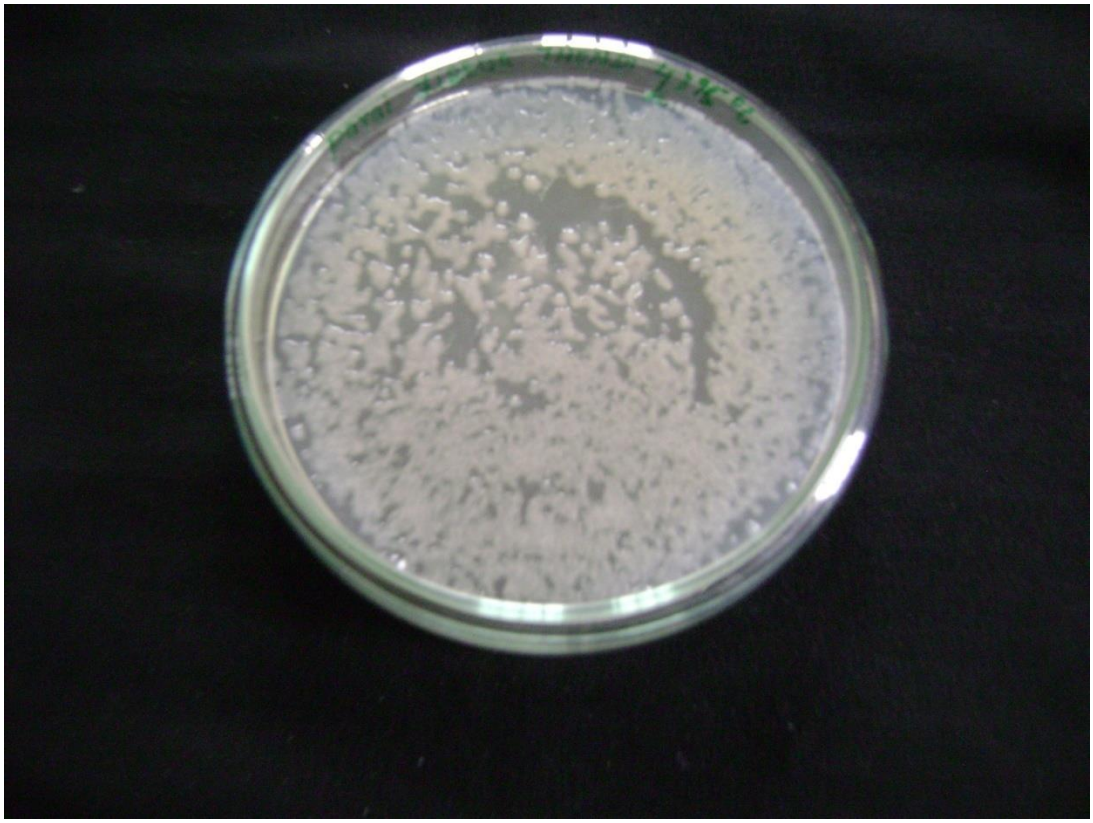
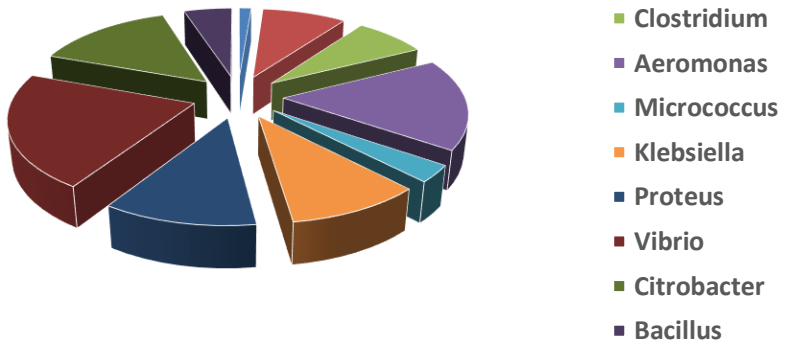


Table 1. Total Heterotrophic Bacteria (THB) isolated from the gills, gut and muscle with skin of *R. kanagurta*.

S. No	Name of the bacteria	Total no. of isolates
1	*Pseudomonas* spp.	02
2	*Enterobacter* spp.	14
3	*Clostridium* spp.	12
4	*Aeromonas* spp.	27
5	*Micrococcus* spp.	05
6	*Klebsiella* spp.	16
7	*Proteus* spp.	18
8	*Vibrio* spp.	34
9	*Citrobacter* spp.	23
10	*Bacillus* spp.	08
	Total	159

Fig.10. Generic composition of THB recorded from the gill, gut and muscle with skin samples of *R. kanagurta*



Generic composition of psychrotolerant THB

A total of 41 strains of THB were isolated from gill, gut and muscle with skin of fresh fish stored at 5^{o}C. They belonged to ten genera viz. *Pseudomonas, Enterobacter, Clostridium, Aeromonas, Micrococcus, Klebsiella, Proteus, Vibrio, Citrobacter and Bacillus.* A total of 17 and 15 strains were isolated from the fishes stored at 0^{o}C and -20 ^{o}C respectively. Among the above mentioned genus, except *Aeromonas* and *Citrobacter* strains all the strains survived at 0^{o}C and -20 ^{o}C. During the present study period, Gram-negative bacteria were recorded more than Gram-positive bacteria (Table.2).

Table.2. Psychrotolerant Total heterotrophic bacteria (THB) isolated from the gills, gut and muscle with skin of *R. kanagurta* fish stored at 5^{o}, 0^{o} and -20^{o}C.

S. No	Name of bacteria	No. of isolates		
		5^{o}C	**0 ^{o}C**	**-20 ^{o}C**
1	*Pseudomonas* spp.	3	1	1
2	*Enterobacter* spp.	4	2	2
3	*Clostridium* spp.	3	2	2
4	*Aeromonas* spp.	4	-	-
5	*Micrococcus* spp.	3	2	1
6	*Klebsiella* spp.	2	1	2
7	*Proteus* spp.	5	3	1
8	*Vibrio* spp.	9	4	4
9	*Citrobacter* spp.	1	-	-
10	*Bacillus* spp.	7	2	2
	Total	41	17	15

2. ISOLATION OF HISTIDINE DECARBOXYLATING BACTERIA (HDB)

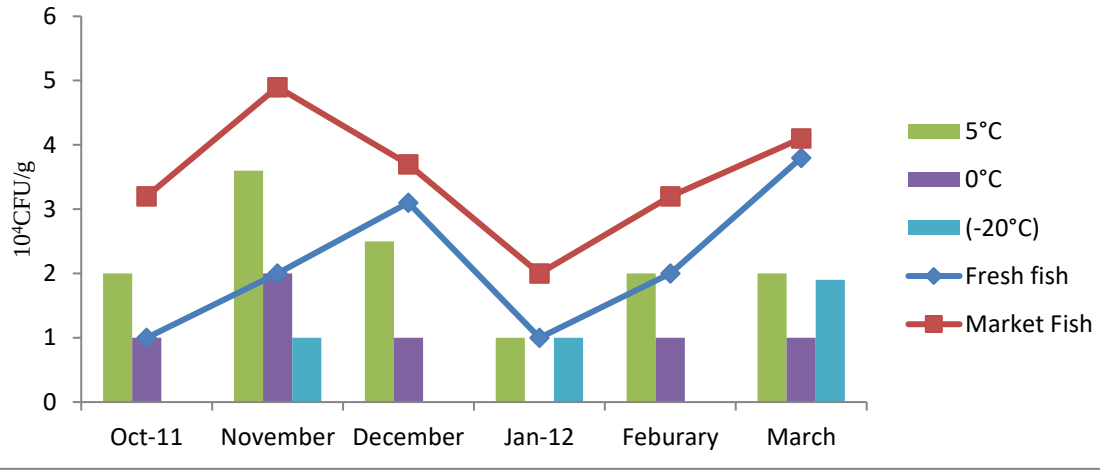
Population density of HDB

Thondi Coast

Population density of HDB varied from 1×10^4 CFU/g to 3.8×10^4 CFU/g. The minimum (1×10^4 CFU/g) recorded during October and January and the maximum (3.8×10^4 CFU/g) were recorded during March from the gill samples of fresh *R. kanagurta.* HDB population density ranged from 2×10^4 CFU/g to 4.9×10^4 CFU/g. The lowest (2×10^4 CFU/g) HDB population was recorded during January and the high (4.9×10^4 CFU/g) was recorded during November.

At 5°C, the HDB population density was minimum (1×10^4 CFU/g) during January and maximum (3.6×10^4 CFU/g) during November in the fresh fish gill. Minimum 1.0×10^4 CFU/g was recorded during October, December, February and March and the maximum (2.0×10^4 CFU/g) of HDB was recorded during November at 0°C. At -20°C the minimum 1.0×10^4 CFU/g was recorded during November and January and the maximum HDB density (1.9×10^4 CFU/g) was recorded during March (Fig.11).

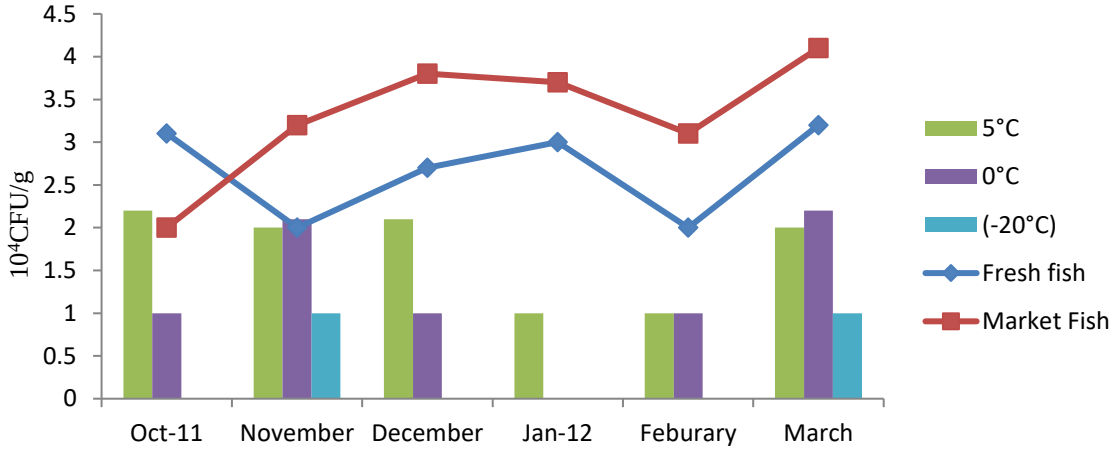
Fig. 11. HDB population density recorded from the gill samples of *R. kanagurta* at Thondi coast.



The HDB population was minimum (2×10^4 CFU/g) during November and the maximum (3.2×10^4 CFU/g) during March in the gut samples of fresh *R. kanagurta.* The HDB population was ranged from 2×10^4 CFU/g to 4.1×10^4 CFU/g. The lowest (2×10^4 CFU/g) population was recorded during October and

the highest (4.1×10^4 CFU/g) was recorded during March. At 5^{o}C the minimum HDB population (1×10^4 CFU/g) was recorded during January and February and maximum (2.2×10^4 CFU/g) was recorded during October. At 0^{o}C, the minimum HDB was recorded as 1.0×10^4 CFU/g during January and February and the maximum 2.2 ×10^4 CFU/g was recorded during March. No HDB was recorded during October, December, January and February at -20^{o}C and the maximum 1×10^4 CFU/g was recorded during March and November (Fig.12).

Fig. 12. HDB population density recorded from the gut samples of *R. kanagurta* at Thondi coast.

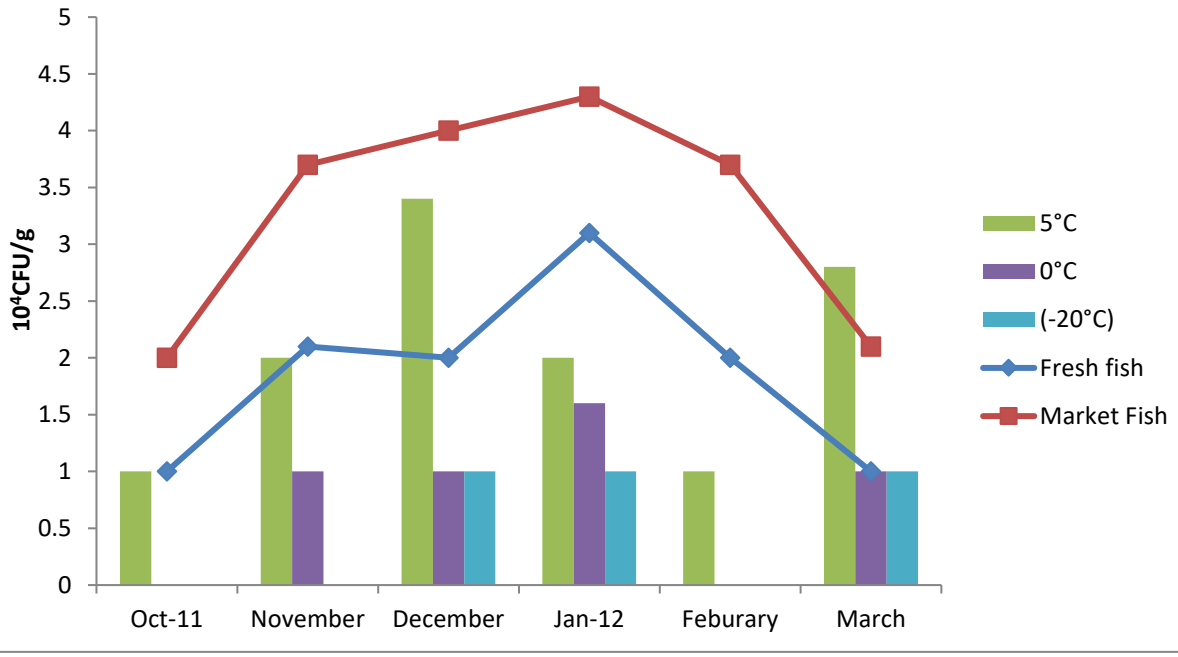


From the muscle with skin of *R. kanagurta* samples showed the HDB population density variation between 1 ×10^4 CFU/g to 3.1 ×10^4 CFU/g. The minimum (1×10^4 CFU/g) was recorded during October and March and the maximum (3.1×10^4 CFU/g) was recorded during January. In the case of fish samples purchased from the market showed the HDB population between 2 ×10^4 CFU/g to 4.3 ×10^4 CFU/g. The minimum (2×10^4 CFU/g) was recorded during October and the maximum (4.3 ×10^4 CFU/g) was recorded during January.

Fishes stored at 5^{o}C showed the HDB population ranging from 1 ×10^4 CFU/g during October and February to 3.4 ×10^4 CFU/g during December. The maximum 1.6×10^4 CFU/g and 1.0×10^4 CFU/g was

recorded respectively at 0°C during January and November, December, and March and at -20°C during December, January and March (Fig.13).

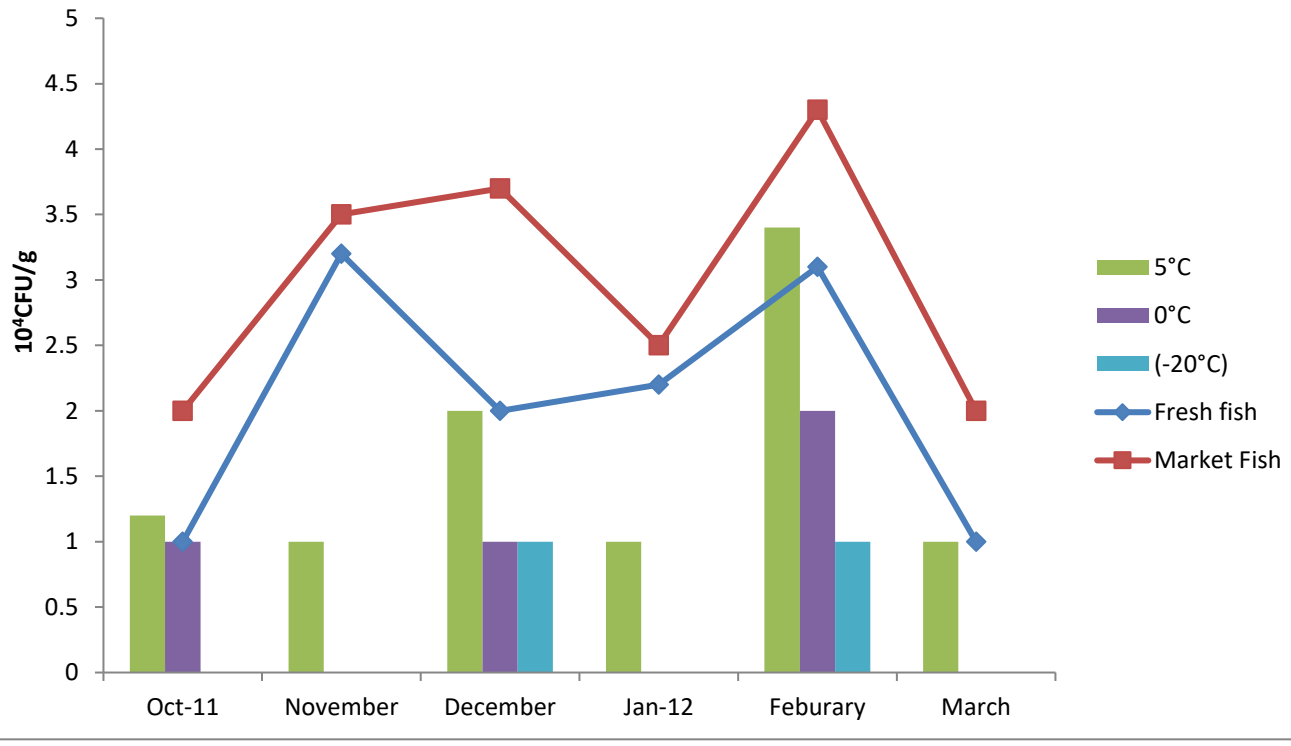
Fig. 13. HDB population density recorded from the muscle with skin samples of *R. kanagurta* at Thondi coast.



Manamelkudi coast

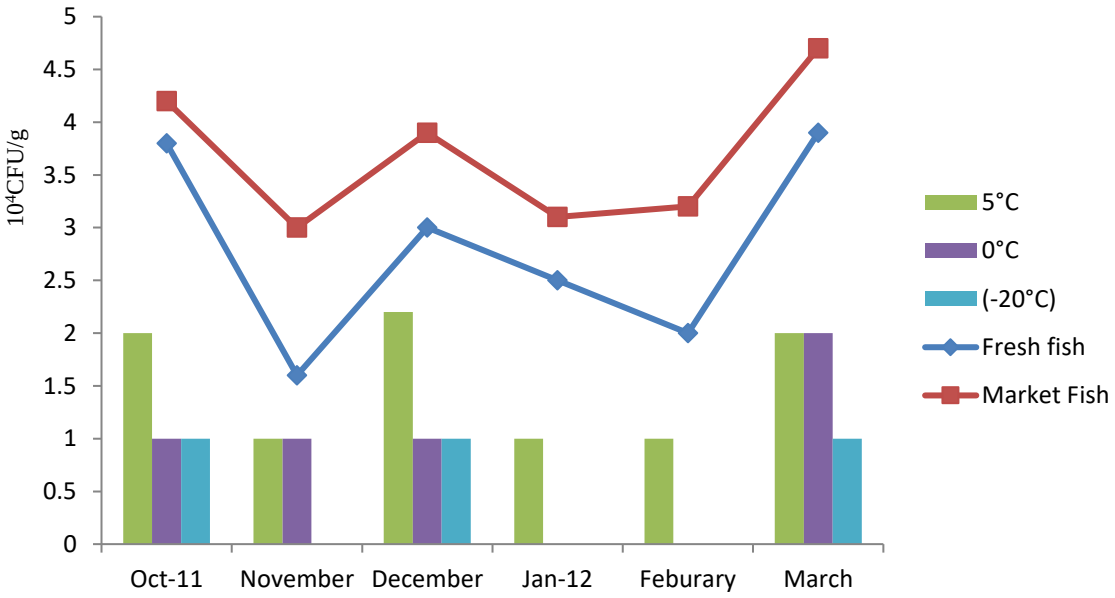
At Manamelkudi the fresh *R. kanagurta* gill samples showed HDB population from 1×10^4 CFU/g to 3.1 $\times10^4$ CFU/g during October, March and February respectively. Fish samples purchased from the fish market recorded the HDB population, minimum (2×10^4 CFU/g) during October and March and the maximum (4.3 $\times10^4$ CFU/g) during February. The HDB load was ranged from 1.0×10^4 CFU/g to 3.4 $\times10^4$ CFU/g. The minimum (1.0×10^4 CFU/g) was recorded during November January and March at 5°C and the maximum (3.4 $\times10^4$ CFU/g) was recorded during February. Very low HBD counts were recorded at 0 and -20°C (Fig.14).

Fig.14. HDB population density recorded from the gill samples of *R. kanagurta* at Manamelkudi coast.



In the gut samples of *R. kanagurta*, the HDB population was ranged from 2 $\times 10^4$ CFU/g to 3.9 $\times 10^4$ CFU/g. The minimum (2×10^4 CFU/g) was recorded during February and the maximum (3.9×10^4 CFU/g) was recorded during March. In the market fish samples, the minimum (3 $\times 10^4$ CFU/g) was recorded in November and the maximum (4.7 $\times 10^4$ CFU/g) during March. At 5^{o}C, the HDB population minimum (1×10^4 CFU/g) was recorded during November, January and February and the maximum (2.2 $\times 10^4$ CFU/g) recorded during December. Minimum HDB load 1.0 $\times 10^4$ CFU/g was observed during October, November and December and the maximum (2.0×10^4 CFU/g) was recorded during March at 0^{o}C and 1×10^4 CFU/g was recorded during October, December and March at -20^{o}C (Fig.15).

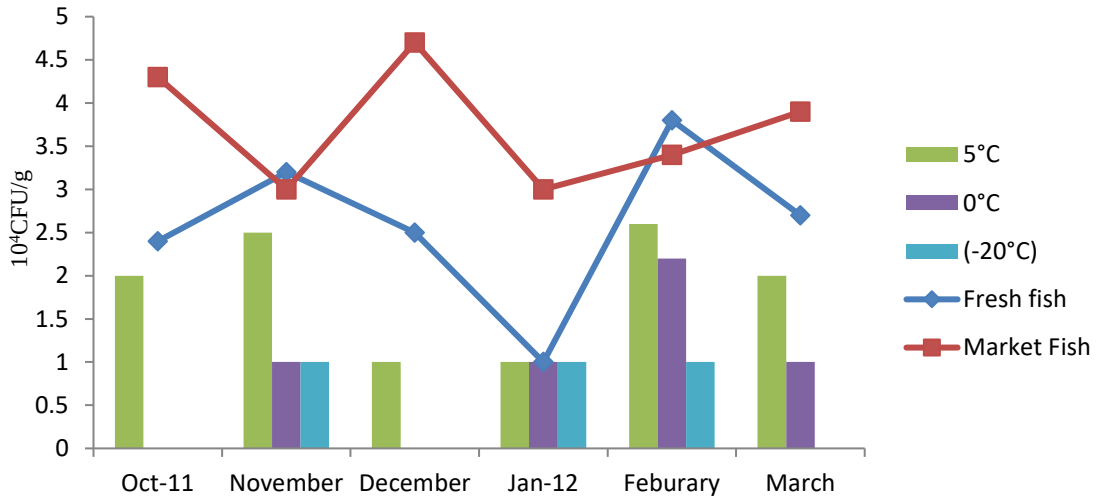
Fig.15. HDB population density recorded from the gut samples of *R. kanagurta* at Manamelkudi coast.



At Manamelkudi, the HDB population density varied from 1×10^4 CFU/g to 3.8×10^4 CFU/g in the muscle with skin samples. The minimum (1×10^4 CFU/g) was recorded during January and the maximum (3.8×10^4 CFU/g) during February. The *R. kanagurta* fish samples purchased from market showed the HDB population density between 3×10^4 CFU/g to 4.7×10^4 CFU/g. The minimum (3×10^4 CFU/g) was recorded during November and January and the maximum (4.7×10^4 CFU/g) was recorded during November.

Very low HDB population density i.e. 1×10^4 CFU/g to 2.6×10^4 CFU/g was recorded during December and January, and February respectively. At 0^oC the HDB load was ranged from 1.0×10^4 CFU/g during November, January and March to 2.2×10^4 CFU/g during February. Lowest HDB growth was observed at -20^oC storage (Fig. 16).

Fig. 16. HDB population density recorded from the muscle with skin samples of *R. kanagurta* at Manamelkudi coast.

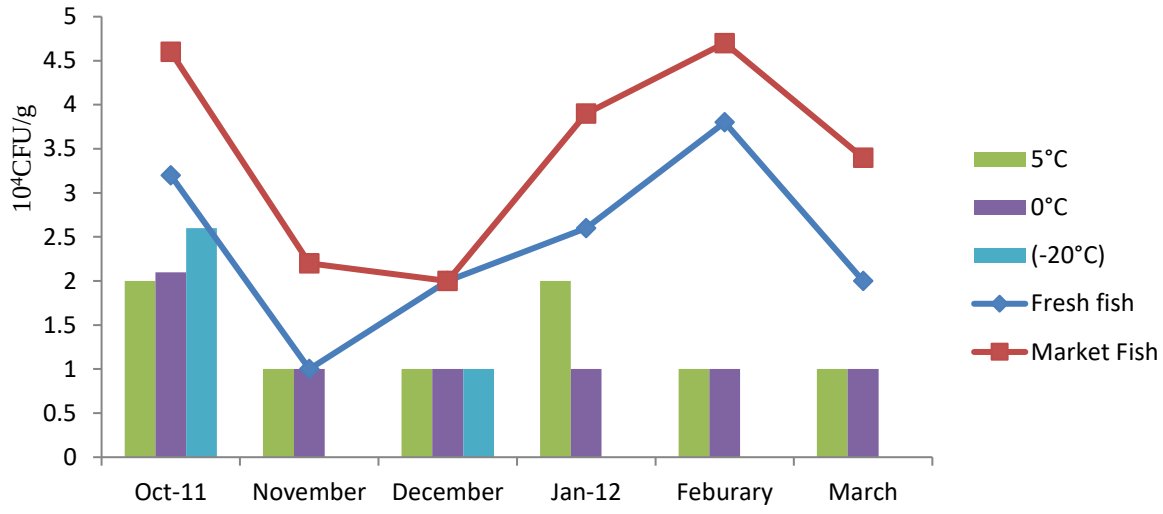


Rameswaram Coast

Population density of HDB varied from 1×10^4 CFU/g to 3.8×10^4 CFU/g. Minimum (1×10^4 CFU/g) was recorded in November and the maximum (3.8×10^4 CFU/g) in February. But the fishes purchased from fish market showed higher population density than the fresh fish samples. The minimum (2×10^4 CFU/g) was recorded in December and the maximum (4.7×10^4 CFU/g) in February.

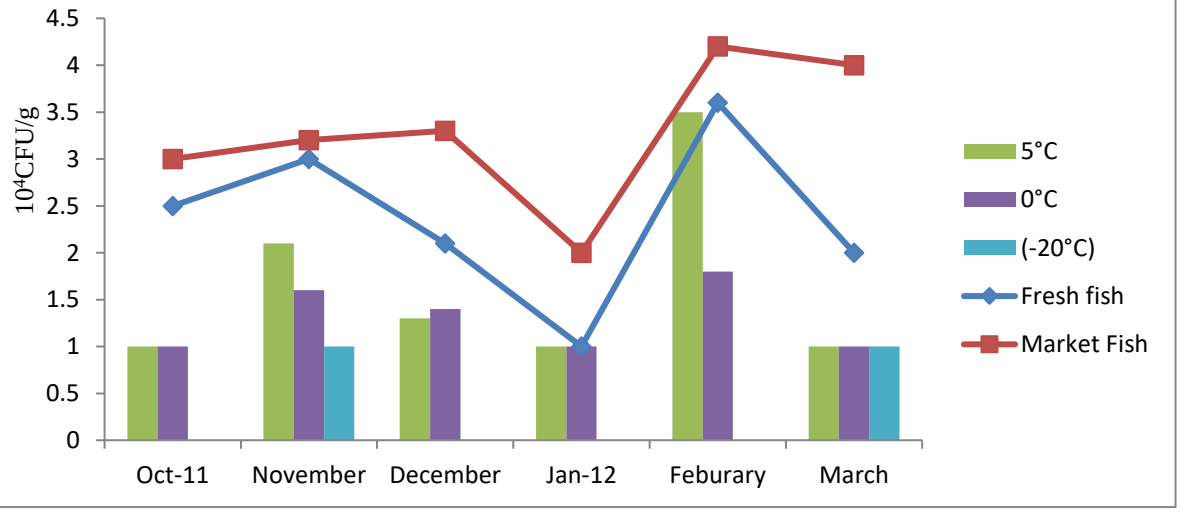
Fishes stored at different storage temperatures were showed different population density of HDB. At 5°C the HDB population was 1×10^4 CFU/g to 2×10^4 CFU/g. The low (1×10^4 CFU/g) population density was recorded during almost all months except October and January. In those months the population density was higher (2×10^4 CFU/g). At 0°C the HDB load varied from 1.0×10^4 CFU/g to 2.1×10^4 CFU/g. The minimum 1.0×10^4 CFU/g was recorded during all the months studied except October. In October the maximum HDB density 2.1×10^4 CFU/g was recorded. At -20° C, no HDB were recorded except December (1×10^4 CFU/g) and October (2.6×10^4 CFU/g) (Fig. 17).

Fig. 17. HDB population density recorded from the gut samples of *R. kanagurta* at Rameswaram coast.



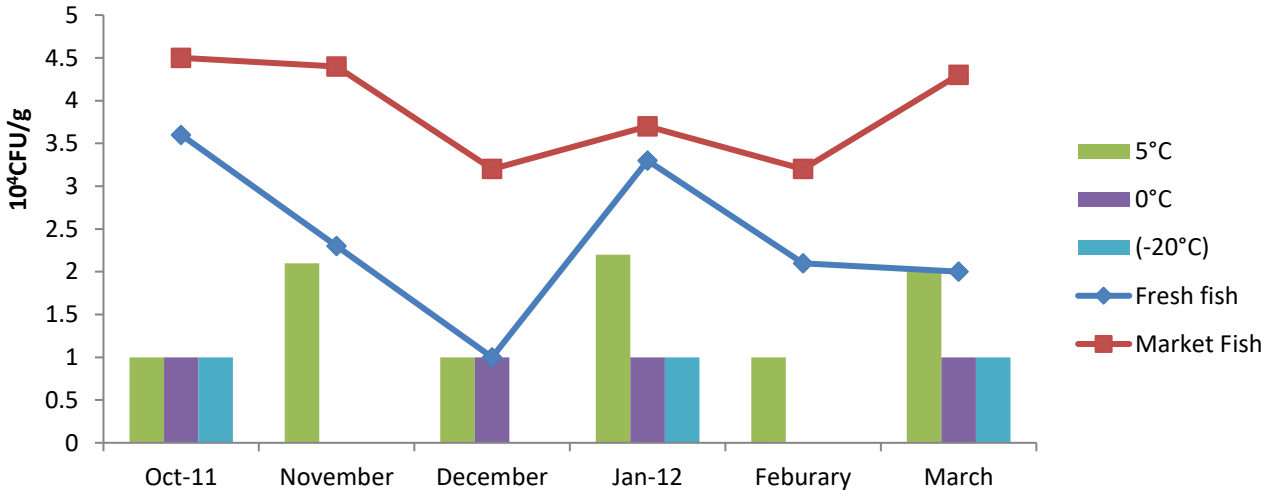
In the gut samples of fresh fishes, the HDB population density ranged from 1.0×10^4 CFU/g to 3.6×10^4 CFU/g. The minimum (1×10^4 CFU/g) was recorded in January and the maximum (3.6×10^4 CFU/g) in February. Market fish samples showed the minimum (2.0×10^4 CFU/g) population density in January and the maximum (4.2×10^4 CFU/g) in February. The population density varied from 1×10^4 CFU/g to 3.5×10^4 CFU/g at 5°C. The minimum (1.0×10^4 CFU/g) was recorded in October and January and the maximum (1.8×10^4 CFU/g) in February at 0°C. Lowest HDB was recorded at -20°C storage (Fig. 18).

Fig.18. HDB population density recorded from the gut samples of *R. kanagurta* at Rameswaram coast.



In the muscle with skin samples, the HDB population density ranged from 1.0 $\times 10^4$ CFU/g and 3.6 $\times 10^4$ CFU/g. The minimum (1×10^4 CFU/g) was recorded in December and the maximum (3.6 $\times 10^4$ CFU/g), in October. Fish samples purchased from market showed the minimum (3.2 $\times 10^4$ CFU/g) population density during December and February and the maximum (4.5 $\times 10^4$ CFU/g) in October. Fishes stored in different temperatures showed the population density variation between 1×10^4 CFU/g to 2.2 $\times 10^4$ CFU/g at 5°C during October, December and February, and January respectively. HDB load was 1.0×10^4 CFU/g during October, December and March and 1.8×10^4 CFU/g during November and February (Fig.19 and Plate II (b).

Fig.19. HDB population density recorded from the muscle with skin samples of *R. kanagurta* at Rameswaram coast.

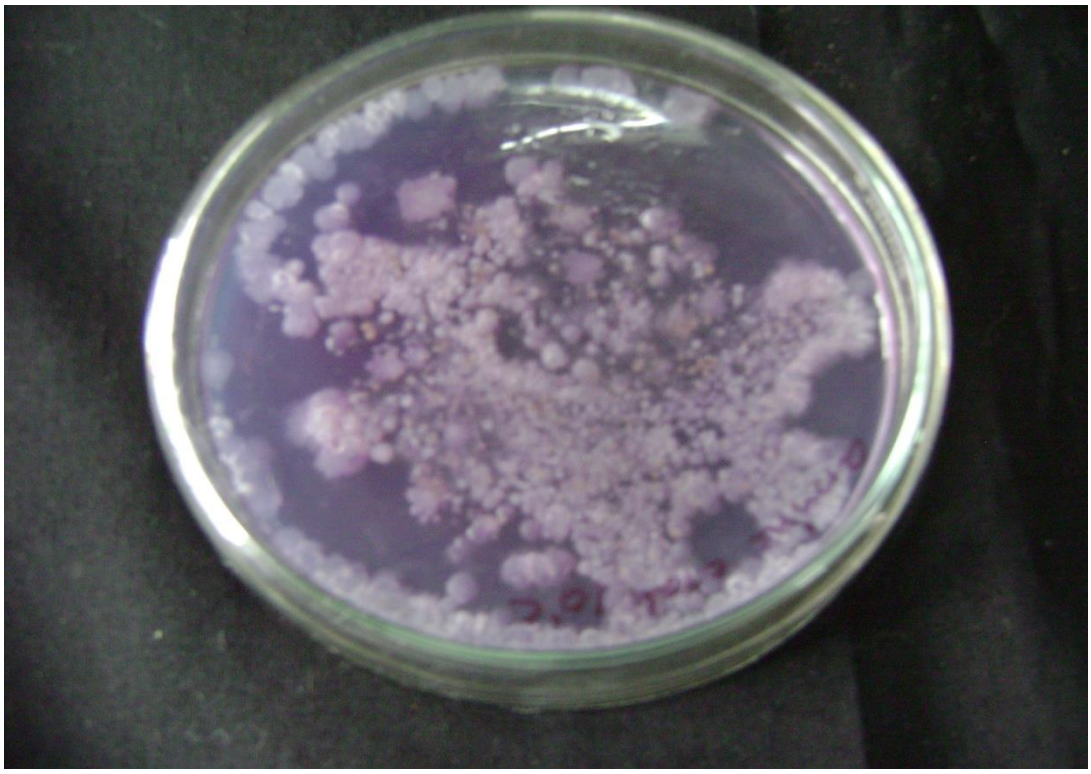


Generic composition of Histidine Decarboxylating Bacteria (HDB)

A total of 70 strains of HDB were isolated from the gill, gut and skin with muscle tissues of the *R. kanagurta* from the fresh fish, fishes purchased from fish market and fishes stored at 5 °C, 0 °C and -20°C (Table 3). They belonged to nine genera viz. *Pseudomonas*, *Enterobacter*, *Clostridium*, *Aeromonas*, *Klebsiella*, *Proteus*, *Vibrio*, *Citrobacter* and *Bacillus*. In the present study, histamine-producing Gram-negative bacteria were recorded more than the histamine-producing Gram-positive bacteria (Fig. 20).

Plate-II (b)

Histamine forming bacteria



Statistical analysis

The total heterotrophic bacteria and histamine decarboxylating bacteria showed highly significance at $P< 0.05$ level in fresh as well as marketed fish of all the station at 0, 5 and -20°C storage in October, November, December 2011, January, February and March, 2012. Negative correlation ($P>0.01$) were observed in March at 0, 5, and -20°C during storage.

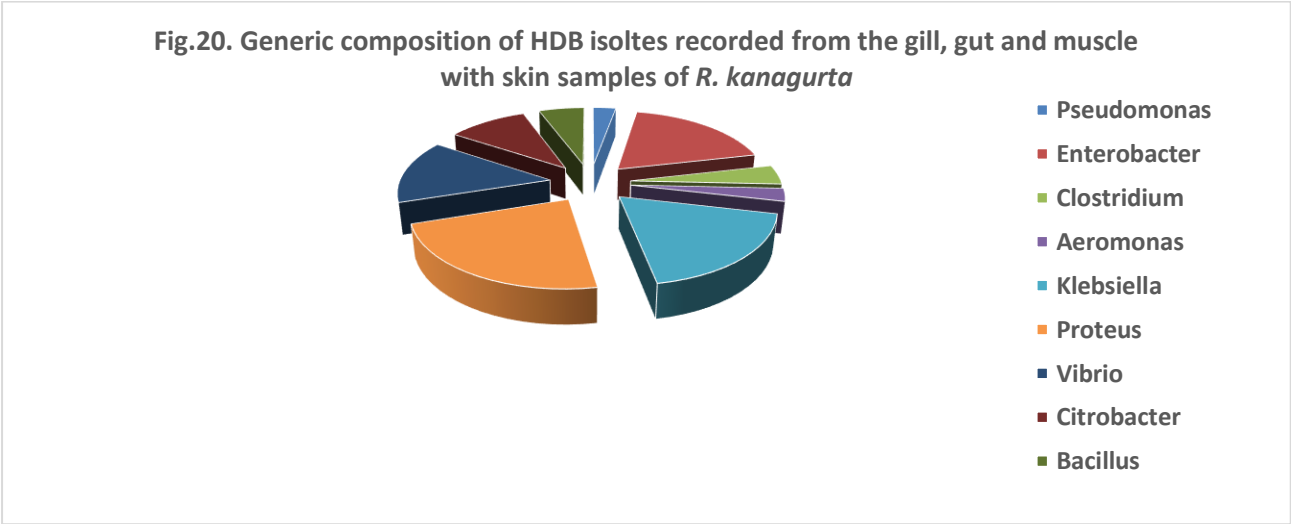


Table 3. Histidine Decarboxylating Bacteria (HDB) isolated from the gills, gut and muscle with skin of *R. kanagurta* fresh and market fish samples and stored at 0, 5, and -20°C.

S. No	Name of bacteria	No. of histidine decarboxylating bacteria isolated
1	*Pseudomonas* spp.	2
2	*Enterobacter* spp.	13
3	*Clostridium* spp.	3
4	*Aeromonas* spp.	2
5	*Klebsiella* spp.	13
6	*Proteus* spp.	16
7	*Vibrio* spp.	10
8	*Citrobacter* spp.	7
9	*Bacillus* spp.	4
	Total	70

3.IDENTIFICATION OF HISTAMINE PRODUCING BACTERIA BY CHEMOTAXONOMIC METHOD

Psychrotolerant and mesophilic bacteria

In this study both psychrotolerant and mesophilic bacterial strains were isolated from gill, gut and muscle with skin of Indian mackerel (*R. kanagurta*). The isolated strains were identified Chemotaxonomically based on gram straining, indole, methyl red, Voges Proskauer and Simmons citrate test (Table 4; Plates III (a) and III (b).

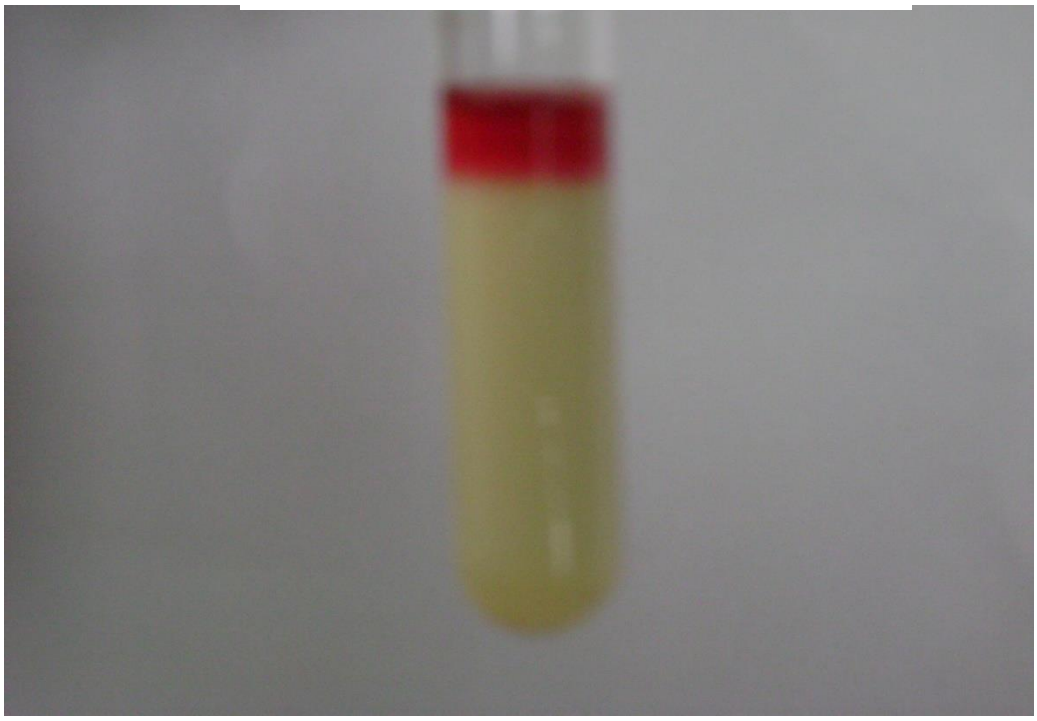
Table 4. Biochemical analysis of selected mesophilic and psychrophilic bacteria isolated from Indian mackerel fish.

Sl.no	Name of the bacteria	Growth temperature	Gram strain	Indole test	Methyl red test	Voges-Proskauer	Simmons citrate
1	*Photobacterium* sp.	15^{o}C	-	+	+	-	-
2	*Flavobacterium* sp.	15^{o}C	-	+	+	-	-
3	*Morganella* sp.	15-25^{o}C	-	+	+	-	+
4	*Klebsiella* sp.	25^{o}C- 30^{o}C	-	-	+	+	+
5	*Klebsiella* sp.	25^{o}C- 30^{o}C	-	+	-	-	+
6	*Proteus* sp.	25^{o}C- 30^{o}C	-	-	+	-	+
7	*Shigella* sp.	25^{o}C- 30^{o}C	-	-	+	+	-
8	*Salmonella* sp.	25^{o}C- 30^{o}C	-	+	+	-	-
9	*Vibrio* sp.	20-25^{o}C	-	+	-	-	-
10	*Bacillus* sp	37^{o}C	+	+	-	+	-
11	*Enterobacter* sp.	25 -30^{o}C	-	-	-	+	+
12	*Citrobacter* sp.	25 -30^{o}C	-	+	+	-	+
13	*Erwinia* sp.	25^{o}C- 30^{o}C	-	-	-	+	+

Plate III (a)

CHEMOTAXONOMIC ANALYSIS

Indole test



Methyl red

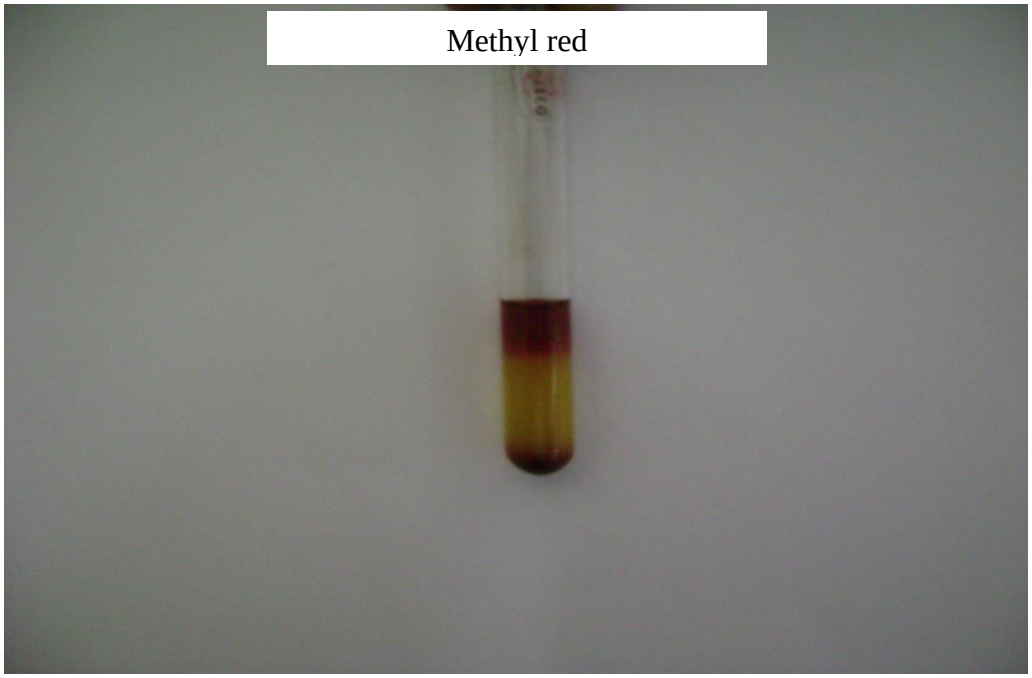
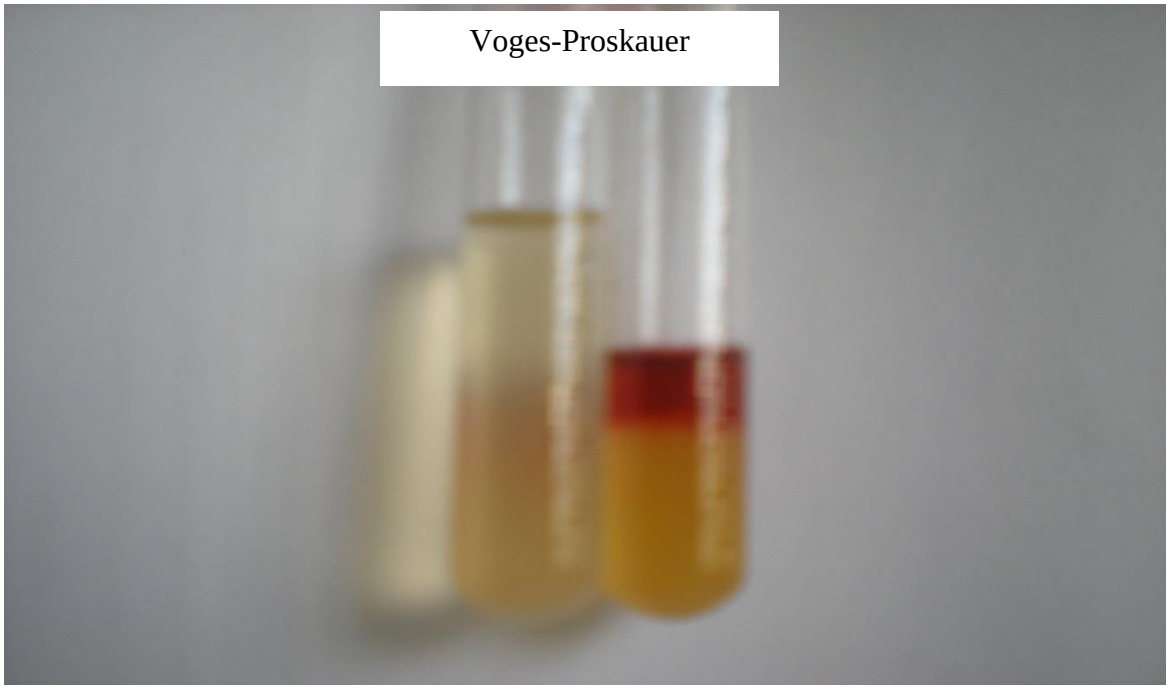


Plate III (b)



Simmons citrate agar

***Photobacterium* spp.**

Cells are rod shaped, motile by means of polar flagellum and gram negative bacteria. The colonies are cream-coloured, opaque, smooth and circular, convex, with entire margins and form on glycerol chalk medium at 30^{o}C after 24h incubation. The colonies are luminescent, facultatively anaerobic, Psychrophilic and growth occurs between 10 to 20^{o}C with an optimum at 15-20^{o}C. Grow at the pH ranges from 6.0 to 6.5. Growth occurs at 1.0 to 4.0% NaCl concentration. Optimal NaCl 2.0%. Showed positive in Indole test and methyl red test. Negative in Voges-Proskauer and Simmons citrate medium.

***Flavobacterium* sp.**

Cells are rod shaped, motile by means of polar flagellum and gram negative bacteria. The colonies are cream-coloured, opaque, smooth and circular, convex, with entire margins and form on sea water agar at 30^{o}C after 24h incubation. The colonies are facultatively anaerobic, psychrophilic and growth occurs between 10 to 20^{o}C with an optimum growth at 15-20^{o}C. Grow at the pH ranges from 6.0 to 6.5. Growth occurs at 1.0 to 4.0% NaCl concentration. Optimal NaCl concentration for growth was 2.0%. Showed positive in Indole test and methyl red test. Negative in Voges-Proskauer and Simmons citrate medium.

***Morganella* spp.**

A gram negative, motile, rod shaped, psychrophilic and halophilc bacterial strain isolated from Indian mackerel (*R. kanagurta*) from TSA agar at 30^{o}C after 24h incubation. The colony is cream coloured, smooth and circular. Non-luminescent, psychrotolarent and mesophilic bacteria. Growth occurs between 15 to 25^{o}C with an optimum temperature of 15-25^{o}C. Required pH range for growth is 6.0 to 6.8. Grow at 1.0 to 3.0% NaCl concentration and optimum growth was found at 2.0 % NaCl concentration. Positive in Indole test and methyl red test. Negative in Simmons Citrate and Voges-Proskauer.

***Klebsiella* spp.**

A gram negative, motile, rod shaped, mesophilic bacteria and colonies are punctiform, smooth circular isolated using TSA agar at 30^{o}C after 24h incubation. The colonies are non- luminescent, mesophilic growth occurs between 20 to 30^{o}C with optimum growth at 20-25^{o}C. Required pH for growth from 6.5 to 6.9. Grown between 1.0 to 3.0% NaCl concentration and optimal growth found at 1.5% NaCl concentration. Positive in Methyl red, Simmons Citrate, and Voges-Proskauer and negative in Indole test.

***Proteus* spp.**

A gram negative, motile, rod shaped, mesophilic bacteria and colonies are punctiform, smooth and circular. The colonies are non- luminescent, mesophilic growth occurs between 20 to 30^{o}C temperature and optimum temperature for growth was 20-25^{o}C. Suitable pH was 6.5 to 6.9. Tolerate at 1.0 to 3.0% NaCl concentration and gown well at 1.5 % concentration. Positive in Methyl red, Simmons citrate and negative in Indole and Voges-Proskauer tests.

***Shigella* spp.**

Gram-staining negative, motile, rod shaped, mesophilic bacteria and colonies are punctiform, smooth and circular isolated by TSA agar medium at 30^{o}C after 24h incubation. The colonies are non- luminescent, mesophilic growth occurs between 20 to 30^{O}C with optimum growth at 20 -25^{o}C. The pH ranged from 6.5 to 6.9. Grown at 1.0 to 3.0% NaCl concentration and optimal growth occurred at 1.5 NaCl concentration. Strains showed positive in methyl red and Voges-Proskauer and negative in Indole and Simmons citrate.

***Salmonella* spp.**

Gram-staining negative, motile, short rod shaped mesophilic bacteria. The colonies are smooth, low, convex, moist pink in colour, surrounding medium bright red in MacConkey agar at 25 -30^{o}C temperature. The colonies are non-luminescent, mesophilic growth occurs between 25 to 32^{o}C temperature and optimum growth was found at 25 -30^{o}C. The pH ranged from 6.5 to 6.9. Tolerate between NaCl concentrations between 1.0 to 3.0% and is optimum NaCl concentration was 1.5%. Positive in methyl red and Indole test. Negative in Voges-Proskauer and Simmons citrate.

***Vibrio* spp.**

Gram negative short or curved rod, active motile, colonies are smooth circular, green/bluish green colonies isolated from TCBS agar at 25-30^{o}C. The colonies are non-luminescent, mesophilic growth

occurs between 25 to 32^{o}C with optimum growth at 25-30^{o}C. Positive in Indole and negative in methyl red, Voges-Proskauer and Simmons citrate. The pH ranged from 6.5 to 6.9, *Vibrio* spp. Showed wide range of NaCl tolerance with 0% to 10% NaCl and growing well at 3 to 6%.

***Bacillus* spp.**

Gram-staining positive, flat, cremated to slight rhizoid colonies shape, motile, colonies are smooth circular, peacock blue colonies surrounded by a *Bacillus* agar at 30^{o}C. The colonies are mesophilic growth occurs between 30-37^{o}C. Growing between the pH 6.5 to 7.0. Tolerate between NaCl concentration at 0% to 10% and optimum growth found at the NaCl concentrations between 3 to 6%. Positive in indole test, Voges-Proskauer and catalase and negative in methyl red.

***Enterobacter* spp.**

Gram negative, motile, short rod shaped, mesophilic bacteria and colonies are smooth, low, convex, moist pinkish colour in MacConkey agar at 25-30^{o}C. The colonies are non-luminescent, mesophilic growth occurs between 25 to 32^{o}C and optimum growth was noticed at 25-30^{o}C. Required pH for growth from 6.5 to 6.9. Growing well at 1.5% NaCl concentration and tolerating between 1.0 to 3.0% NaCl concentration. Showed positive reaction in Voges-Proskauer and Simmons citrate and negative in methyl red and Indole test.

***Citrobacter* spp.**

Gram-staining negative, motile, short rod shaped, mesophilic bacteria and colonies are smooth, low, convex, moist pinkish colour colonies isolated from MacConkey agar at 25-30^{o}C. The colonies are non-luminescent, mesophilic growth occurs between 25 to 32^{o}C with optimum growth at 25-30^{o}C. Optimum pH required for growth between 6.5 to 6.9 and tolerate at NaCl concentration of 1.0 to 3.0%. Growth

found at 1.5% NaCl concentration. Positive reactions were observed at methyl red, Indole and Simmons citrate and negative reactions found at Voges-Proskauer.

***Erwinia* spp.**

Gram-staining negative, motile, short rod shaped, and colonies are smooth, low, convex, isolated in TSA agar at 25 -30^{o}C. The colonies are non- luminescent, mesophilic growth occurs at 30^{o}C. But optimum growth found at 20-30^{o}C. Required pH for growth was 6.5 to 6.9. Tolerate at 1.0 to 3.0% NaCl concentration and optimum NaCl concentration required for growth was 1.5%. Positive in Voges-Proskauer and Simmons citrate and negative in methyl red and Indole test.

4. MOLECULAR CHARACTERIZATION OF HISTAMINE PRODUCING BACTERIA USING 16 S R RNA METHOD

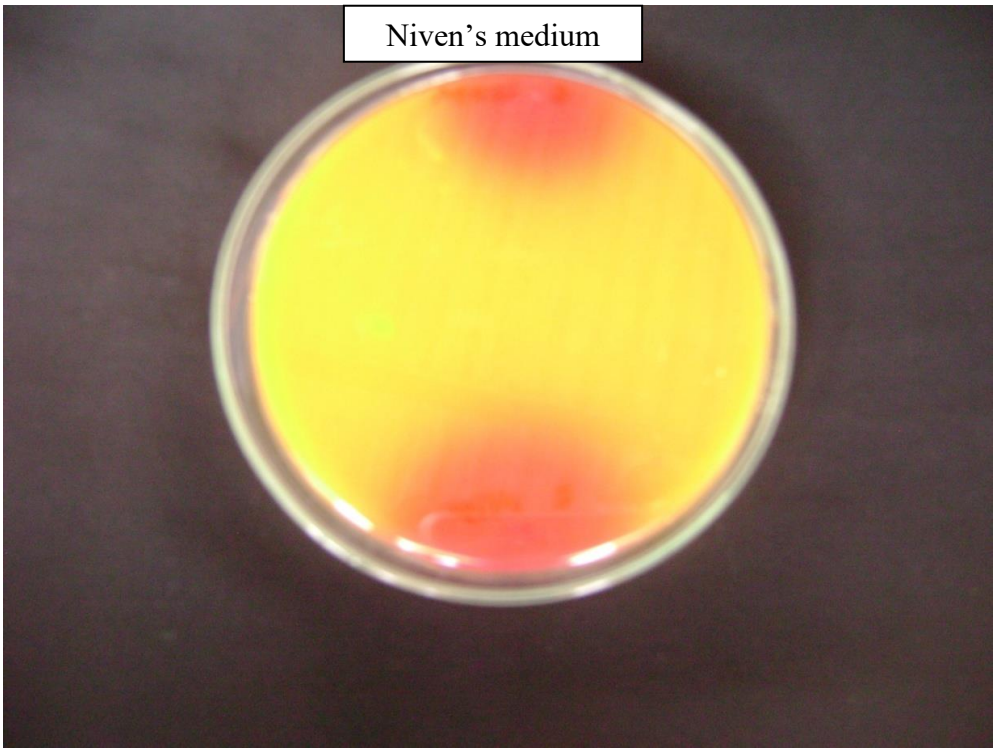
The psychrotolerant and mesophilic bacterial strains were isolated from gill, gut and skin with muscles of Indian mackerel (*R. kanagurta*) were further confirmed by 16S RNA sequencing method. A total of eight psychrotolerant and mesophilic histamine producing bacterial strains were isolated as *Photobacterium* sp. (KP689587), *Klebsiella oxytoca* (KP751412), *Klbsiella* sp. (KP689585), *Proteus mirabilis* (KP751413), *Klebsiella* sp. (KP689584), *Enterobacter* sp. (KP689583), *Proteus* sp. (KP689586) and *Klebsiella* sp. (KP689582) and tabulated (Table 5; Plates IV (a) and IV (b).

Table 5. Identification of psychrotolerant and mesophilic histamine producing bacteria by 16S r RNA based on the output results from NCBI database analysis.

S.no	Bacterial strains and GenBank accession number	GenBank accession number	Nivens Medium	Modified Nivens medium
1	*Photobacterium* sp.	KP689587	+	+
2	*Klebsiella oxytoca*	KP751412	+	+
3	*Klebsiella* sp.	KP689585	+	+
4	*Klebsiella* sp.	KP689582	+	+
5	*Klebsiella* sp.	KP689584	+	+
6	*Proteus mirabilis*	KP751413	+	+
7	*Proteus* sp.	KP689586	+	+
8	*Enterobacter* sp.	KP689583	+	+

(+) = presence of histamine; (-) = absence of histamine

Plate-IV (a)



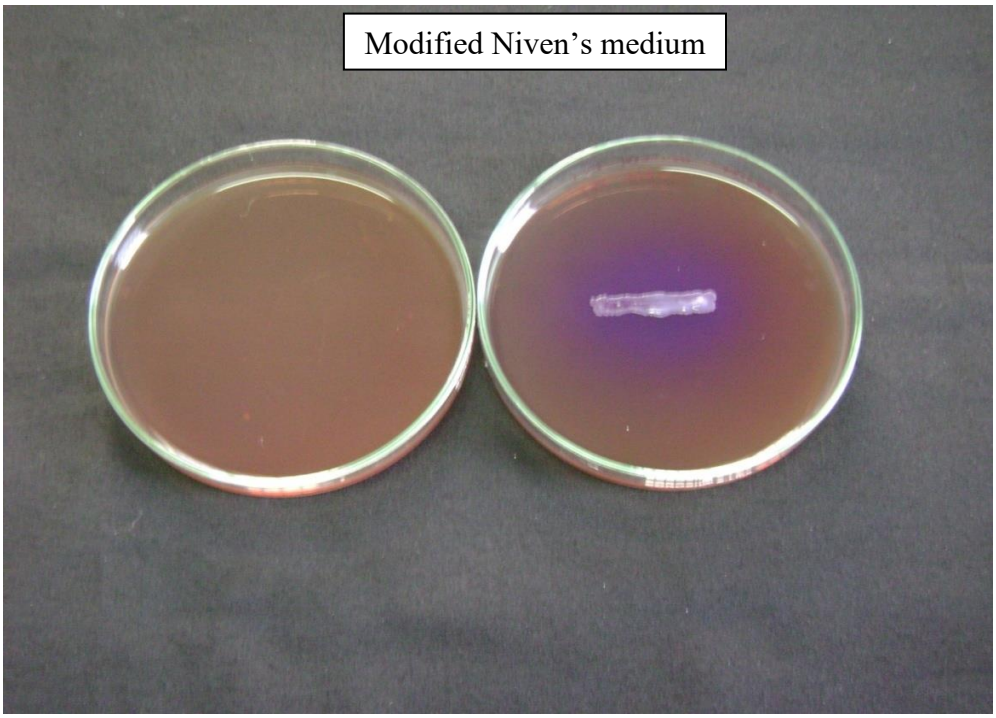
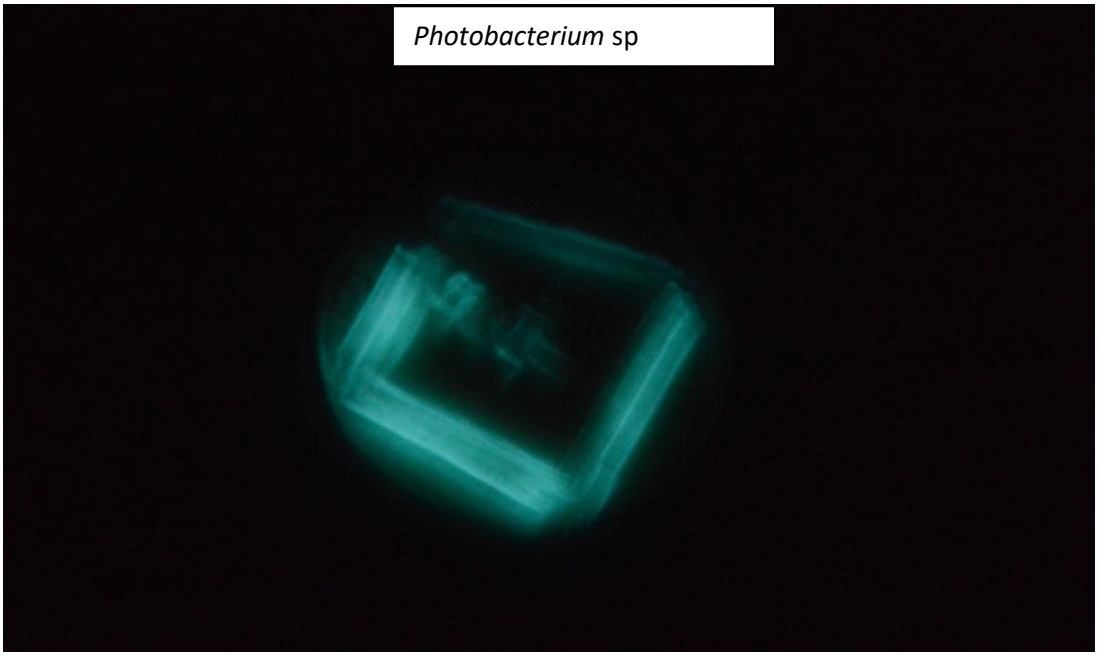
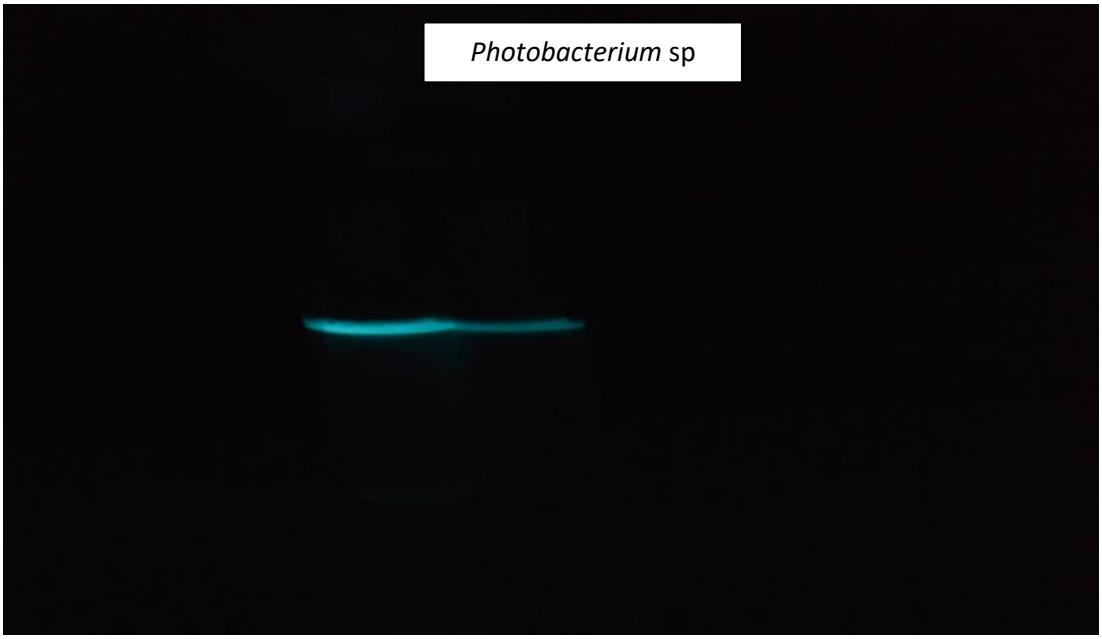


Plate-IV (b)





Phylogenetic characterization

Phylogenetic relationship between isolated strains was analysed using Geneious tree builder programme (a in-build programme of Geneious Pro, commercial version). Tamura-Nei genetic distance model was used to build neighbour-joining tree. Build tree was further resampled using Bootstrap resampling method with 1000 replicates. Sequence length of selected strains were not equal hence Cost matrix 70% similarity (IUB) used for the phylogenetic tree creation. Phylogenetic tree of isolated strains shows that *Proteus mirabilis* (KP751413) was out grouped in the single node hence it is distantly related to the other strains. Two more clusters were observed in phylogenetic tree one which has sequences with more residues (Fig.21. clade 1) and another one has sequences with fewer residues (Fig.21. clade 2). *Klebsiella* sp. (KP689582) strains was found in both the groups and shows close relationship with each other. In addition, that *Enterobacter* sp. (KP689583) and *Proteus* sp. (KP689586) strains clustered with the *Klebsiella oxytoca* (KP751412). Hence it is assumed that *Enterobacter* sp and *Proteus* sp. is closely related with *Klebsiella* sp., branch length of *Enterobacter* sp. (KP689583) and *Proteus* sp. (KP689586) shows that *Proteus* sp. (KP689586) is much closer to *Klebsiella* sp.(KP689584) compared with *Enterobacter sp.*

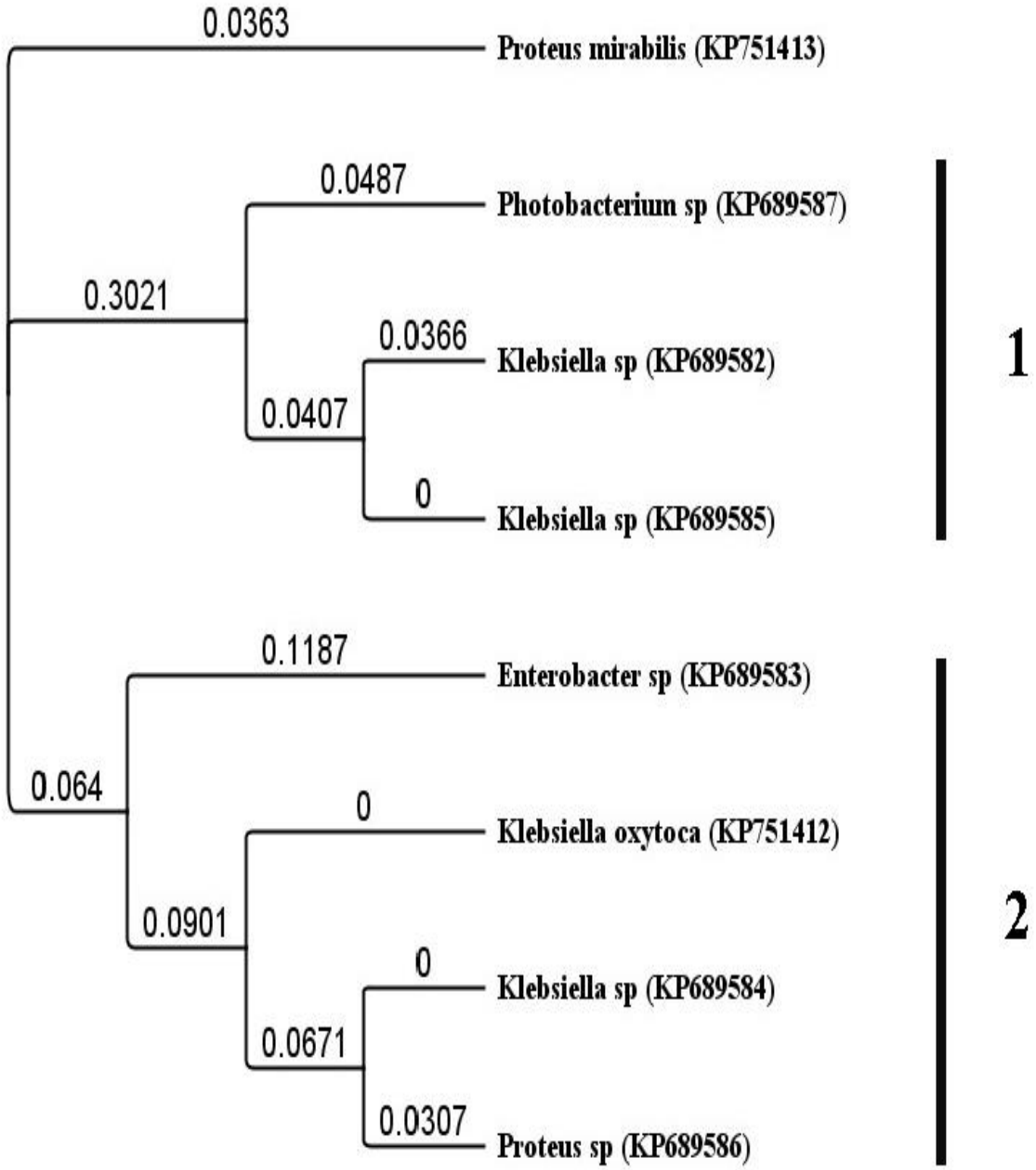


Fig. 21. Neighbour-joining phylogenetic tree of isolated strains. Branch length is expressed as the substitution per sites.

Secondary structure of r RNA

The secondary structure of histamine producing psychrotolerant and mesophilic bacterial strains isolated from Indian mackerel (*R. kanagurta*) during different storage time were predicted using RNA fold-Vienna RNA web service (http://rna.tbi.univie.ac.at/RNA fold.cgi). All the eight histamine producing bacterial strains such as *Klebsiella* sp., *Proteus mirabilis, K.oxytoca, Proteus* sp., *Photobacterium* sp. and *Enterobacter* sp. free energy and centroid energy were calculated further more total loop and stem were calculated. The highest free energy - 910.70 and centroid energy -702.28 were noted in *P. mirabilis* followed by *Klebsiella* sp., *K.oxytoca, Proteus* sp., *Photobacterium* sp., and *Enterobacter* sp. In this study *P. mirabilis* has high loop and stem than other strains (Table.6) and Fig 22-29.

Table 6. Free energy, centroid energy and secondary structure of bacterial strains identified by 16 S r RNA analysis.

Sl .No	Bacterial strains and GenBank accession number	Free energy (Kcal/Mol)	Centroid energy (Kcal/Mol)	Total loop	Total stem
1	*Proteus mirabilis* (KP751413)	-910.70	-702.28	54	36
2	*Klebsiella* sp. (KP689582)	-493.10	-372.97	67	72
3	*Klebsiella* sp. (KP689584)	-523.60	-400.25	74	65
4	*Klebsiella oxytoca* (KP751412)	-441.90	-400.93	72	66
5	*Klebsiella* sp. (KP689585)	-544.20	-421.80	82	63
6	*Proteus* sp. (KP689586)	-488.60	-421.80	73	71
7	*Photobacterium* sp. (KP689587)	-505.90	-363.84	85	65
8	*Enterobacter* sp. (KP689583)	-489.80	-285.45	58	50

Fig.22. *Proteus mirabilis* (KP751413)

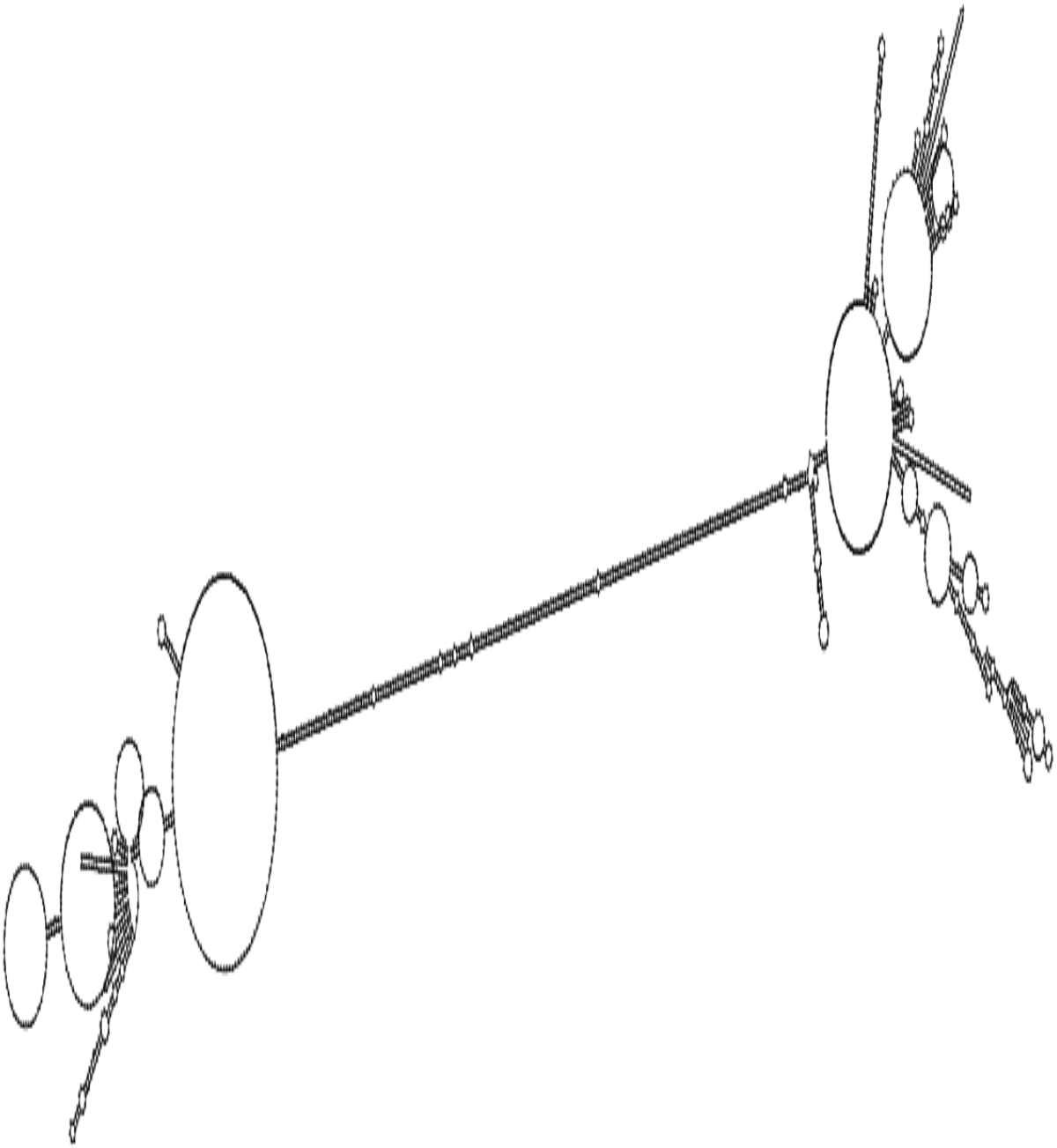


Fig.23. *Klebsiella* sp. (KP689582)

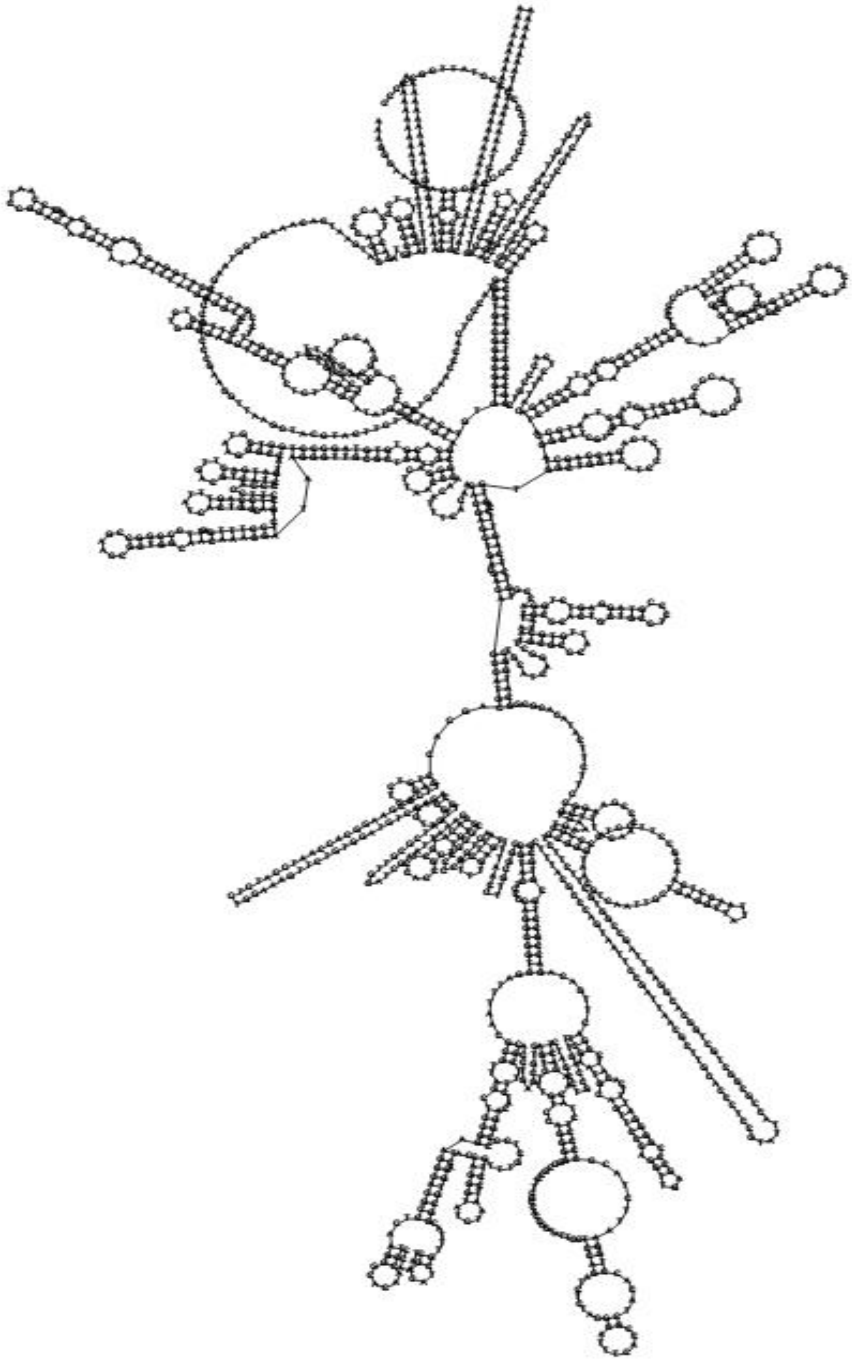


Fig.24. *Klebsiella* sp. (KP689584)

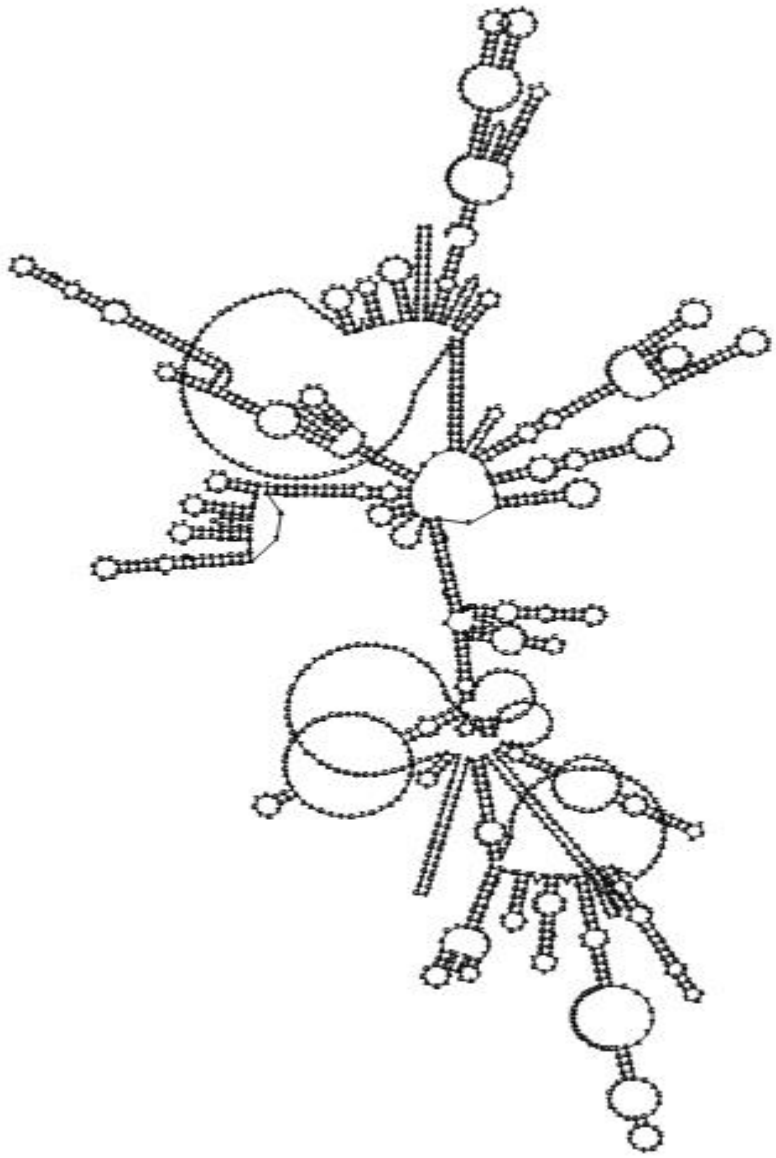


Fig.25. *Klebsiella oxytoca* (KP751412)

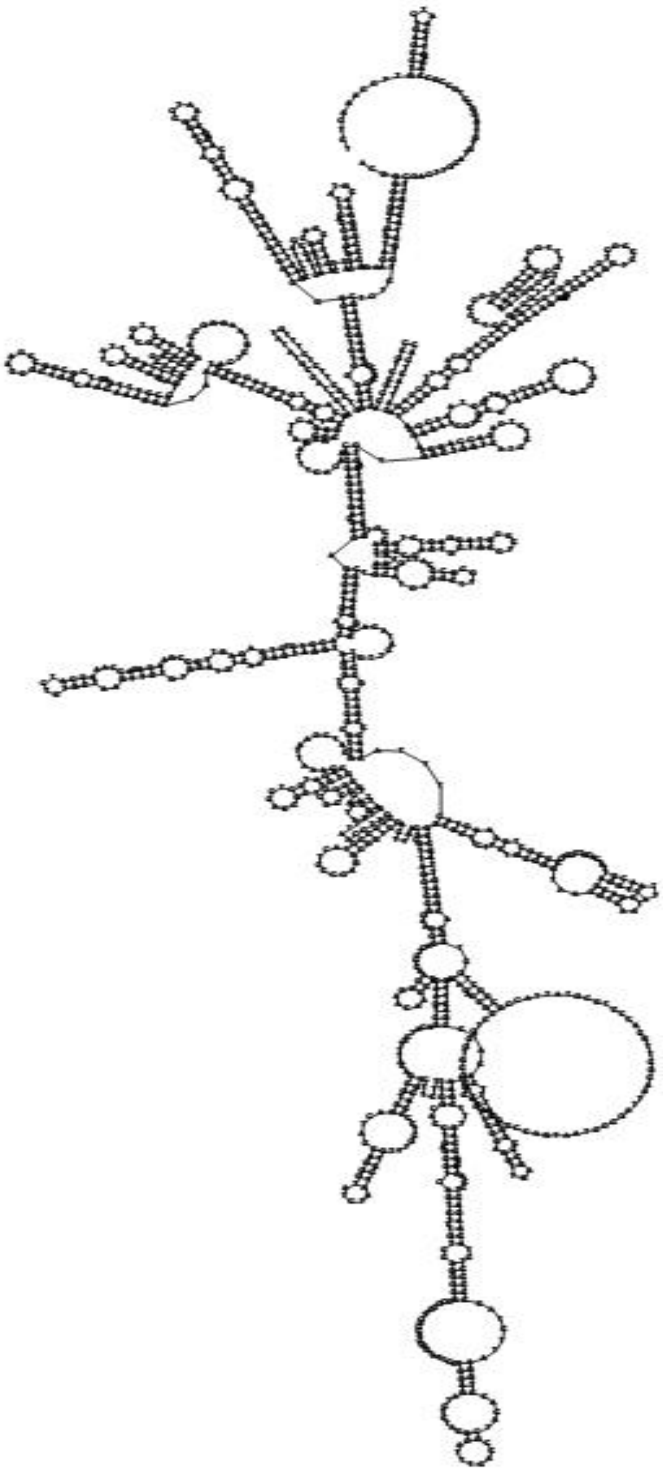


Fig.26. *Klebsiella* sp. (KP689585)

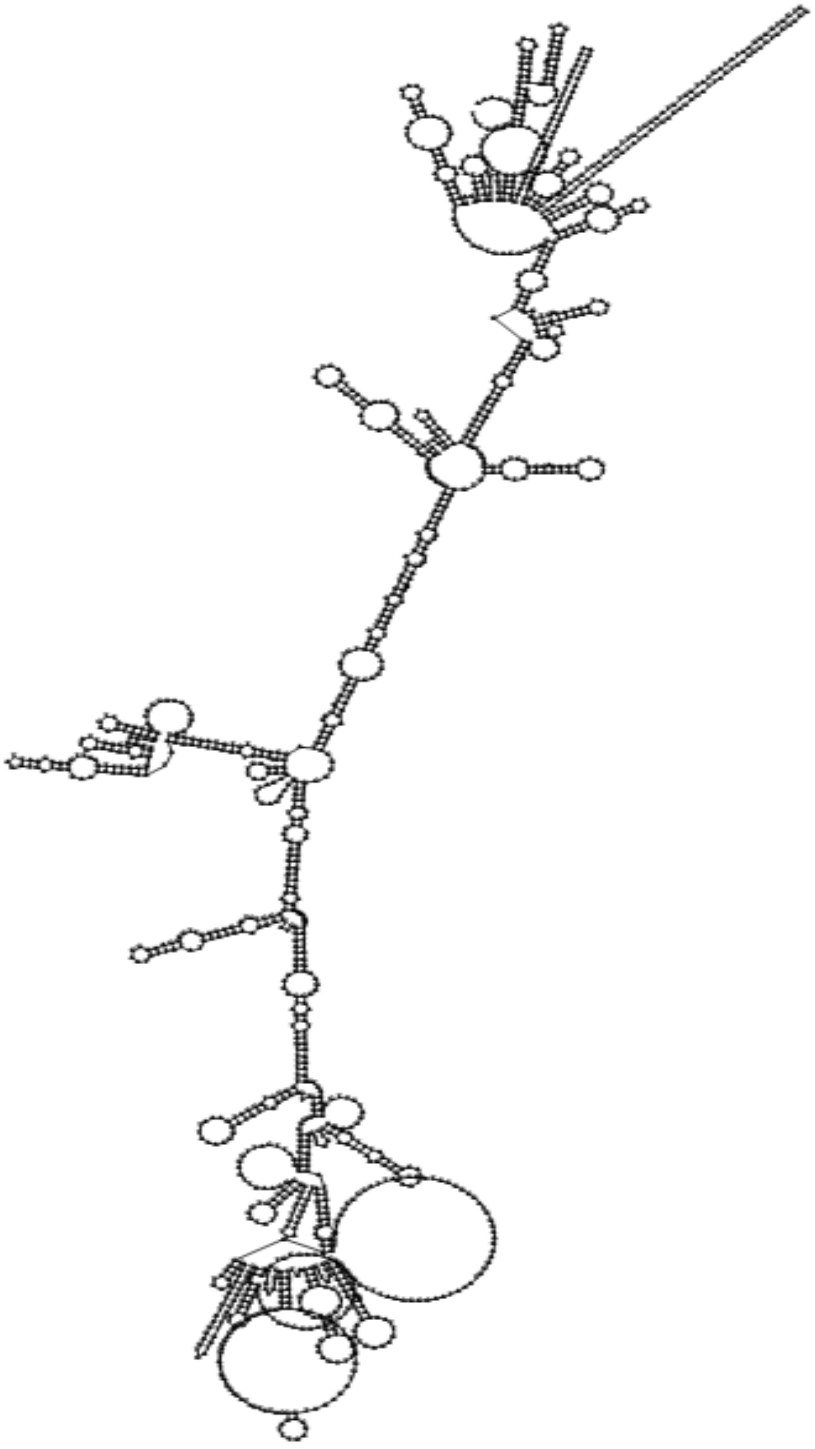


Fig.27. *Proteus* sp. (KP689586)

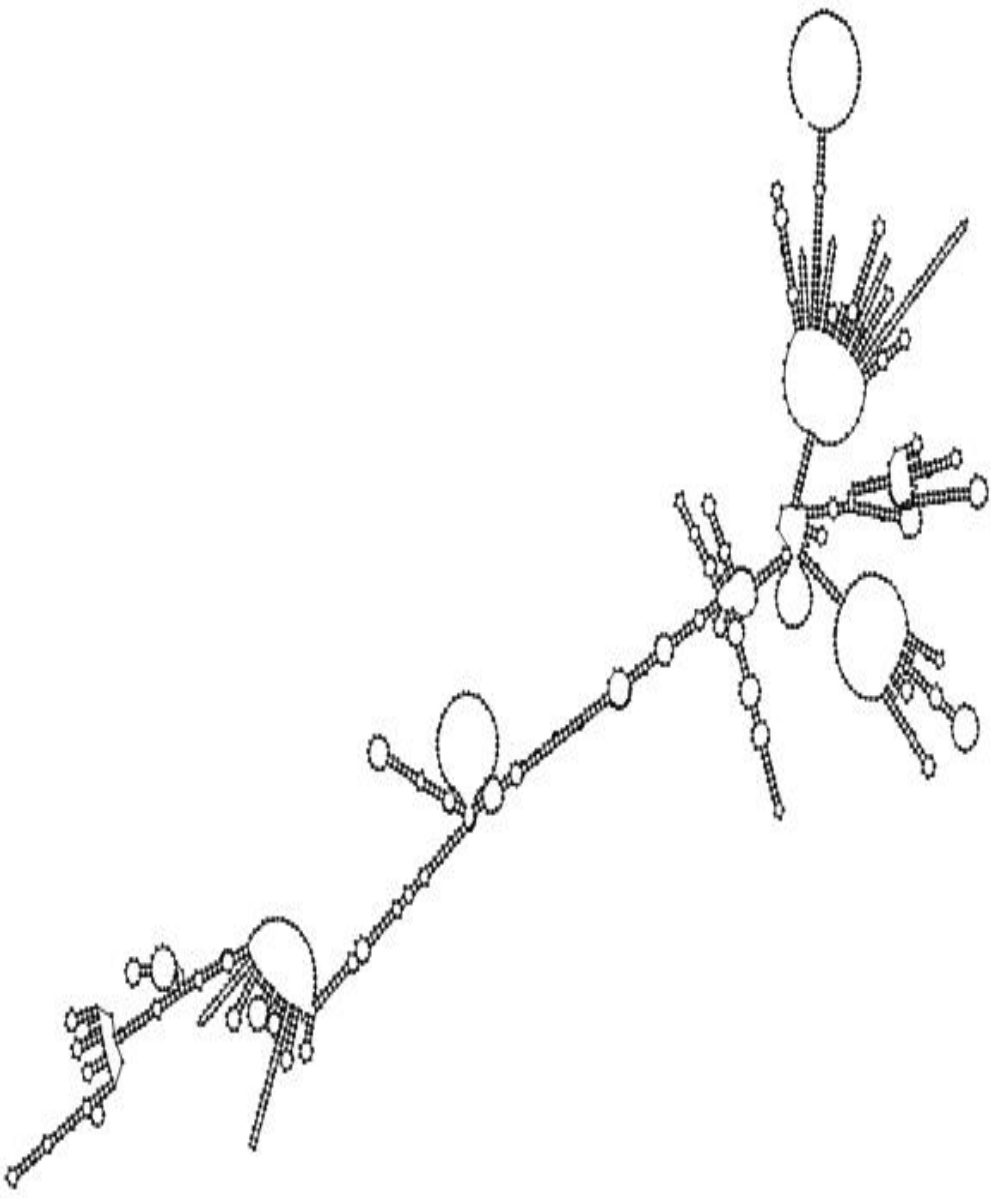


Fig.28. *Photobacterium* sp. (KP689587)

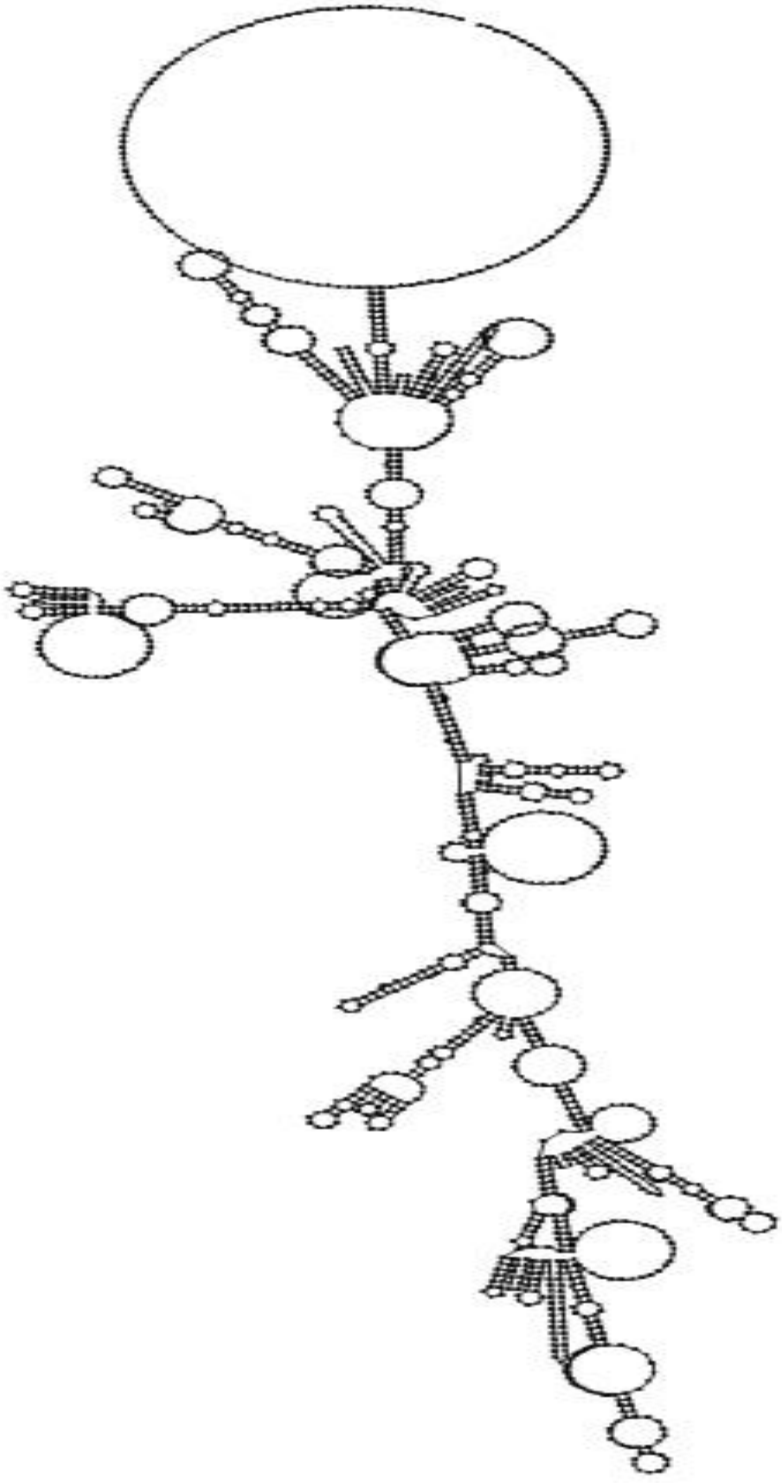
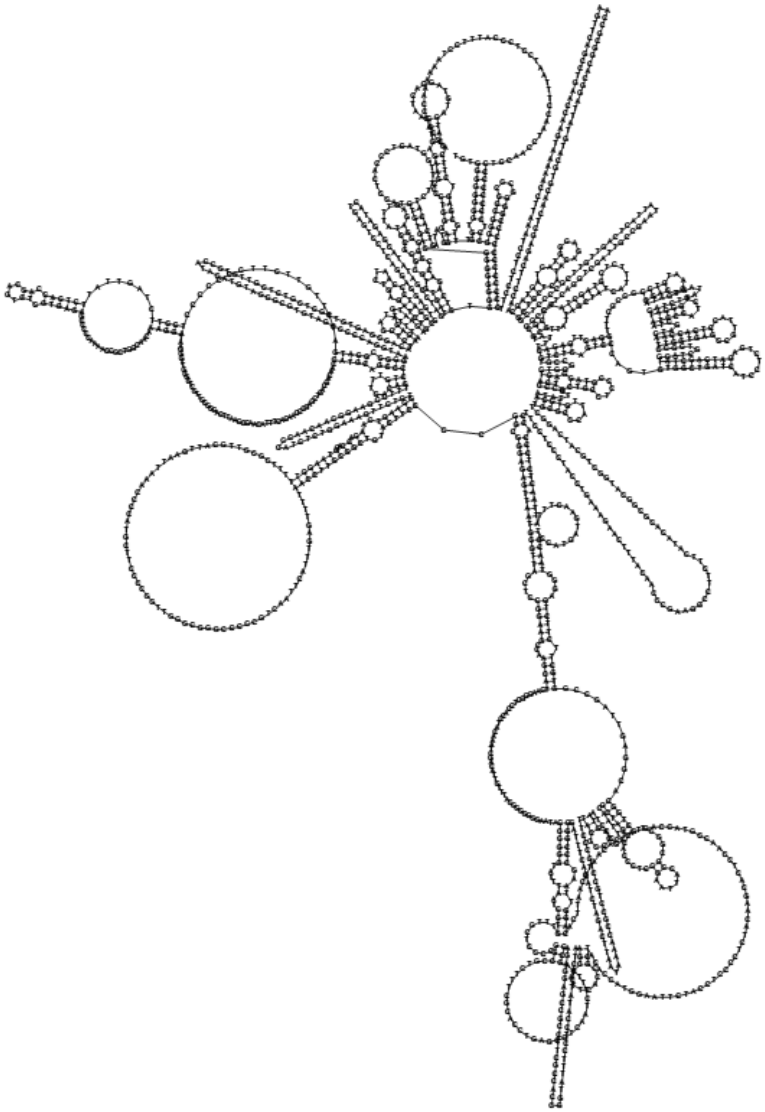


Fig.29. *Enterobacter* sp. (KP689583)



5. ESTIMATION OF HISTAMINE PRODUCTION BY BACTERIA

Histamine is mainly produced from free amino acid histidine due to the action of bacterial histidine decarboxylase (HDC). The gram positive and gram negative bacteria possess the enzyme histidine decarboxylase and convert the histidine in to histamine at ambient temperature (27^{o}C). Mackerel infusion broth was used for the first time to confirm the histamine forming ability of the bacteria such as *Photobacterium* sp., *Klebsiella oxytoca, Klebsiella* sp., *Proteus mirabilis, Proteus* sp. *Enterobacter* sp.

In this investigation a total of 70 psychrotolerant and mesophilic strains were isolated from the Indian mackerel (*R. kanagurta*). The maximum histamine 5.56±0.44 mg/100 ml was obtained from *Photobacterium* sp. followed by 4.04±0.10 mg/ml from *Erwinia* sp., 3.92±0.12 mg/100 ml from *Flavobacterium* sp., 3.5±0.26 mg/ml from *Proteus mirabilis* and 2.53±0.12 mg/100 ml from *Klebsiella* sp. (Table.7)

Table 7. Histamine producing ability of psychrophilic (20^{o}C±2^{o}C) and mesophilic (35±2^{o}C) bacteria isolated from Indian mackerel (*R. kanagurta*) tested by enzymatic assay method.

S. no	**Name of the bacteria**	**Histamine (mg/100ml)**
1	*Klebsiella* sp.	2.53±0.12
2	*Enterobacter* sp.	3.12±0.31
3	*Klebsiella oxytoca*	2.36±0.12
4	*Proteus mirabilis*	3.5±0.26
5	*Flavobacterium sp*	3.92±0.12
6	*Proteus* sp.	2.5±0.27
7	*Photobacterium* sp.	5.56±0.44
8	*Erwinia* sp.	4.04±0.10

6. ESTIMATION OF HISTAMINE CONTENT IN THE MUSCLES TISSUES OF INDIAN MACKEREL AT FROZEN CONDITION AS WELL AS IN FRESH FISHES

To find out the formation of histamine in the fresh and frozen fishes of *R. kanagurta* samples were collected from Thondi coast alone. The fishes were sold in the local market without ice in some places they are selling them with ice for local consumption. Hence the influence of the local microflora and the fish capture and handling method may influence the histamine production. Keeping this in mind the present study was carried out to find the histamine production under different storage conditions.

The change in the histamine content in Indian mackerel fish stored at -20, 0, 10, 20, and 30^{o}C were tabulated in Table 8. In our investigation, the eviscerated *R.kanagurta* fish stored at -20^{o}C for 7 days contained 2.0±0.08mg/100g of histamine and un eviscerated *R.kanagurta* fish contains 3.4±0.16mg/100g of histamine at -20^{o}C for 7 days of storage. The histamine levels in 3.56±0.28mg/100g and 4.01mg/100g of *R.kanagurta* from eviscerated and uneviscerated fish muscles stored at 0^{o}C for 2 days.

The eviscerated *R.kanagurta* fish stored at 10^{o}C for 1 day possess histamine content 7.6±0.37mg/100g and uneviscerated *R.kanagurta* fish contains 10.1±0.37mg/100g of histamine. Similarly, the *R.kanagurta* fish stored at 20^{o}C for 15h contained 10.75±0.49mg/100g and 16.56±0.57mg/100g of histamine from eviscerated and uneviscerated fish muscles respectively. *R. kanagurta* fish stored at ambient temperature for 10h contained the histamine 19.3±0.86mg/100g in eviscerated muscles and uneviscerated *R.kanagurta* fish had 22.33±1.44mg/100g of histamine during 30^{o}C storage at 10h. The higher level of histamine formation could be due to the presence of precursor amino acid histidine, histamine producing bacteria and favourable temperature in the eviscerated and uneviscerated fish muscle (Table 8).

The levels of pH in the eviscerated and uneviscerated Indian mackerel fish during different storage conditions (Table 8). The pH of the eviscerated *R.kanagurta* fish stored at -20^{o}C for 7 days was 6.45±0.18 and uneviscerated *R.kanagurta* fish contains the pH 6.72±0.05 at the same temperature and storage period.

Table 8. Histamine content at different storage temperatures in *R.kanagurta*.

Storage temperature	Storage time (days)	Histamine content mg/100g		pH	
		Eviscerated (Ev)	Uneviscerated (Uev)	(Ev)	(Uev)
-20 ^{o}C	7 days	2.0±0.08	3.4±0.16	6.45±0.18	6.72±0.05
0^{o}C	2 days	3.56±0.28	4.01±0.24	5.20±0.02	5.48±0.13
10 ^{o}C	1 day	7.6±0.37	10.1±0.37	6.57±0.04	6.72±0.08
20 ^{o}C	15h	10.75±0.49	16.56±0.57	6.82±0.05	6.87±0.06
30 ^{o}C	10h	19.3±0.86	22.33±1.44	7.01±0.08	7.18±0.04

The values are expressed as mean±SD, (n=3).

At 0^{o}C for 2 days storage, the pH was 5.20±0.02 and 5.48±0.13 in eviscerated and uneviscerated fish muscles respectively. The eviscerated *R. kanagurta* fish stored at 10^{o}C for 1 day contained the pH 6.57±0.04 and uneviscerated *R.kanagurta* fish contains the pH 6.72±0.08. Similarly, *R. kanagurta* fish stored at 20^{o}C for 15h contained 6.82±0.05 and 6.87±0.06 of pH from eviscerated and uneviscerated fish muscles. At ambient temperature *R. kanagurta* fish stored for 10h, contained the pH 7.01±0.08 in eviscerated muscles and pH 7.18±0.04 found in uneviscerated *R. kanagurta* fish muscle.

Changes in the pH of Indian mackerel muscle during the ice storage is corresponding with increasing TVB-N content. The TVB-N content of eviscerated *R. kanagurta* muscle stored at -20^{o}C for 7 days was 30.8±2.28 mg/100g and uneviscerated *R. kanagurta* fish contains 38.26±1.31 mg/100g at -20^{o}C for 7 days. The TVB-N level was 28±0.26 mg/100g and 23.24±1.39 mg/100g in both eviscerated and uneviscerated of *R. kanagurta* fish muscles stored at 0^{o}C for 2 days. The TVB-N content was 42±2.28mg/100g in eviscerated fish and 37.33±1.31mg/100g in uneviscerated fish muscle at 10^{o}C for 1 day storage. Similarly, *R. kanagurta* fish stored at 20^{o}C for 15h contained 50.4±2.28mg/100g and 60.46±23.9mg/100g of TVB-N both in eviscerated and uneviscerated conditions. At 30^{o}C storage for 10h, the TVB-N level was 32.29±3.11 mg/100g in eviscerated muscles and 27.06±3.49 mg/100g in uneviscerated muscle (Table 9).

Changes of THB count in Indian mackerel stored at different temperatures are tabulated in Table 3. This study revealed that the initial THB count in eviscerated and uneviscerated Indian mackerel were ranged from 1.54 log CFU g^{-1} to 7.02 log CFU g^{-1} during the storage at -20^{o}C. Whereas, the eviscerated and uneviscerated fish samples stored at 0^{o}C were ranged from 2.02 log CFU g^{-1} to 4.84 log CFU g^{-1}. The THB count of eviscerated *R. kanagurta* fish was 5.23 log CFU g^{-1} at 20^{o}C for 10h storage and 5.56 log CFU g^{-1} in uneviscerated fish muscle at same temperature. At ambient temperature THB rapidly increased and the count was 6.62 log CFU g^{-1} and 7.34 log CFU g^{-1} in eviscerated and uneviscerated fish muscles respectively during 30^{o}C of storage for 15h (Table 10).

Table 9. Total Volatile Base–Nitrogen (TVB-N) content in Indian macrel fish *(R. kanagurta)*.

Storage temperature	Storage time (days)	TVB-N content mg/100g	
		Eviscerated (Ev)	Uneviscerated (Uev)
-20^{o}C	7 days	14.56±0.45	17.0±0.50
		16.8±0.91	19.69±0.80
		18.85±0.69	22.12±1.95
		21.61±0.95	25.94±0.69
		23.61±0.86	26.13±0.92
		25.29±0.80	30.89±2.40
		30.8±2.28	38.26±1.31
0^{o}C	2 days	24.08±1.99	21.30±1.69
		28±0.26	23.24±1.39
10^{o}C	1 day	42±2.28	37.33±1.31
20^{o}C	15h	50.4±2.28	60.46±23.9
30 ^{o}C	10h	32.29±3.11	27.06±3.49

The values are expressed as mean±SD, (n=3).

Table 10. Changes in microbial load in Indian mackerel during different storage conditions (log CFU/g)

Temperature	Storage time (Days)														
	Eviscerated								Uneviscerated						
	h	1	2	3	4	5	6	7	h	1	2	3	4	5	6	7
-20°C	-	1.54± 0.17	2.24± 0.07	2.72 ±0.0 7	3.92± 0.11	4.49± 0.28	5.35± 0.19	6.16± 0.12	-	2.33± 0.21	2.79± 0.25	3.49± 0.22	4.78±0 .14	5.24±0 .21	6.35± 0.38	7.02±0 .10
0 °C	-	2.02± 0.02	4.15± 0.25	-	-	-	-	-	-	2.43± 0.07	4.84± 0.13	-	-	-	-	-
10 °C	-	4.24± 0.24	-	-	-	-	-	-	-	5.10± 0.16	-	-	-	-	-	-
20°C (15hr)	5.23 ±0.1 5	-	-	-	-	-	-	-	5.56± 0.37	-	-	-	-	-	-	-
30°C (10hr)	6.62 ±0.2 8	-	-	-	-	-	-	-	7. 34±0. 16	-	-	-	-	-	-	-

The values are expressed as mean±SD, (n=3). (-: sample not analysed)

The psychrotolerant, mesophilic and other enteric bacteria such as *Photobacterium* sp., *Klebsiella* sp., *Morganella* sp., *Proteus* sp., *Vibrio* sp., *Shigella* sp., *Salmonella* sp., *Enterobacte*r sp., *Citrobacter* sp. *Bacillus* sp. and *Erwinia* sp. were isolated from both eviscerated and uneviscerated Indian mackerel (*R. kanagurta*) fish samples in both Niven's medium and modified Niven's medium. Further, their histamine producing ability was confirmed by TLC analysis (Table 11and 12).

Table 11. Histamine forming bacterial strains isolated by Nivens Medium and Modified Nivens medium from *R. kanagurta*.

Sl .no	Bacterial strains and GenBank accession number	Nivens Medium	Modified Niven's medium
1	*Photobacterium* sp. KP689587	+	+
2	*Klebsiella oxytoca*, KP751412	+	+
3	*Morganella* sp.	+	+
4	*Proteus mirabilis*, KP751413	+	+
6	*Proteus* sp. KP689586	+	+
7	*Vibrio* sp.	+	+
8	*Shigella* sp.	-	-
9	*Shigella* sp.	-	-
10	*Photobacterium* sp.	+	+
11	*Salmonella* sp.	-	-
13	*Enterobacte*r sp. KP689583	+	+
14	*Citrobacter* sp.	+	+
15	*Klebsiella* sp. KP689585	+	+
16	*Klebsiella* sp. KP689582	+	+
17	*Klebsiella* sp. KP689584	+	+
18	*Bacillus* sp.	+	+
19	*Erwinia* sp.	+	+

Table 12. Confirmation of histamine formation by thin layer chromatography (TLC) from *R. kanagurta*.

Sl.no	Name of the isolates	TLC confirmation
1	*Morganella* sp.	+
2	*Proteus* sp.	+
3	*Vibrio* sp.	-
4	*Shigella* sp.	-
5	*Photobacterium* sp.	+
6	*Salmonella* sp.	-
7	*Enterobacte*r sp.	+
8	*Citrobacter* sp.	+
9	*Klebsiella* sp.	+
10	*Bacillus* sp.	+
11	*Erwinia* sp.	+

SUMMARY OF THE FINDINGS

Total heterotrophic bacteria (THB) density and total Histidine decarboxylating bacteria (HDB) density were enumerated from gill, gut and muscle tissues with skin of *Rastrelliger kanagurta*. A total of 159 THB and 70 HDB were isolated. In which 13 selected psychrotolerant and mesophilic bacterial strains were analysed chemotaxonomically.

Eight psychrotolerant and mesophilic histamine producing bacterial strains were identified as *Photobacterium* sp. (KP689587), *Klebsiella oxytoca* (KP751412), *Klebsiella* sp. (KP689585), *Proteus mirabilis* (KP751413), *Klebsiella* sp. (KP689584), *Enterobacter* sp. (KP689583), *Proteus* sp. (KP689586) and *Klebsiella* sp. (KP689582) by 16S rRNA method.

Two more clusters were observed in phylogenetic tree one which has sequences with more residues and another one has sequences with fewer residues. *Klebsiella* sp. (KP689582) strain was found in both the groups and shows close relationship with each other. In addition, *Enterobacter* sp. (KP689583) strain clustered with the *Klebsiella* sp. (KP689585). Hence it is assumed that *Enterobacter* sp. (KP689583) is closely related with *Klebsiella* sp. (KP689585).

The secondary structure of histamine producing psychrotolerant and mesophilic bacteria were predicted using RNA fold-Vienna RNA web service. The free energy and centroid energy were calculated. Furthermore, total loop and stem were calculated.

Eight psychrotolerant and mesophilic bacteria were used to analyse the histamine producing ability. The maximum histamine 5.56±0.44 mg/100 ml was obtained from *Photobacterium* sp. followed by 4.04±0.10 mg/ml from *Erwinia* sp., 3.92±0.12 mg/100 ml from *Flavobacterium* sp., 3.5±0.26 mg/ml from *Proteus mirabilis* and 2.53±0.12 mg/100 ml from *Klebsiella* sp.

The change in the histamine content in Indian mackerel fish stored at -20, 0, 10, 20, and 30^{o}C were studied. The eviscerated *R. kanagurta* fish stored at 10^{o}C for 1 day possess histamine content 7.6±0.37mg/100g and uneviscerated *R. kanagurta* fish contained 10.1±0.37mg/100g of histamine. *R. kanagurta* fish stored at ambient temperature for 10 h contained the histamine 19.3±0.86 mg/100g in eviscerated muscles. The uneviscerated *R. kanagurta* fish had 22.33±1.44mg/100g of histamine during 30^{o}C storage at 10 h. At 0 and -20^{o} C very low quantity of histamine were formed. Histamine is formed even the fishes are stored at very low temperature by Psychrotolerant histamine producing bacteria.

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Histamine-Producing Bacteria (S-96) – A Molecular Approach

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Abstract

Histamine is a biogenic amine formed through the decarboxylation of the amino acid. For the present study, a total of 126 histamine-producing bacteria (HPB) were screened from Muthupettai mangrove sediment sample. Among 126 isolates, S-96 were selected for 16s rRNA sequencing due to more histamine production. The sequencing study shows the S-96 belong to the genus of Bacillus cereus. This is probably the first report to designate *Bacillus cereus* as a histamine-producing bacterium in Muthupettai mangrove environment.

Keywords: Mangrove sediment, histamine bacteria, *Bacillus cereus*,16s rRNA

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1. INTRODUCTION

The mangroves in India are productive ecosystems that are very sensitive to environmental changes [6]. Mangroves are dominant features of the coastal areas of tropical countries and form a productive ecosystem that supports abundant life through a food chain that starts with the trees and the habitat's unique micro-biota [14]. Histidine by the enzyme histidine decarboxylase, this enzyme occurs naturally in living animals including fish and can be produced by a number of microorganisms commonly associated with marine environments [13]. Fish species such as tuna, mackerel, mahi, bonito, bluefish, and sardine, which contain high levels of histidine in muscle are more likely to contain histamine (formed by bacterial enzymatic activity) if the fish is not properly stored before consumption [3, 15]. Consumption of fish containing histamine may result in illness known as scombroid poisoning with a variety of symptoms including rash, facial flushing, sweating, vomiting, diarrhea, headache, burning sensation and metallic taste in the mouth [2].

The analysis of the 16s rRNA gene pool is a very sophisticated tool for molecularly based analysis of microbial diversity in sediment. The most powerful and recent approach to taxonomy is the study of nucleic acids because they yield considerable information about true relation among the species. PCR-based 16s rRNA profiling provides a rapid characterization of the most abundant phylotypes present within an environmental sample with or without the prerequisite of culturing the microbe in the laboratory. It has been a useful tool for monitoring microbes within a variety of environments [12]. It is a valuable tool for identifying and characterizing bacterial diversity as traditional phenotypic identification. In most prokaryotes, the ribosomal genes constitute an operon with the order 16S-23S-5S and are transcribed in a single polycistronic RNA [8]. These studies have collectively provided useful insights into the phylogenetic composition of the prokaryotic community in coastal marine sediment. The present study revealed the histamine-producing bacterium in the Muthupettai mangrove

environment by using 16s rRNA sequencing.

2. MATERIALS AND METHODS

2.1. Sample Collection

Sediment samples were collected from the Muthupettai mangroves in the southeast coast of India. The rhizosphere sediment samples were collected in the clean polyethylene bags and transported to the laboratory by keeping them in ice box and processed within three hours and microbial analyses were carried within four hours.

2.2. Screening and Identification of Histamine-Producing Bacteria (HPB)

The bacterial strains were isolated from the sediment sample of Muthupettai mangrove environment by using the Zobell marine agar medium. The different morphological colonies were frequently subcultured for getting the pure colonies. For getting the histamine-producing bacteria, the colonies were streaked in Niven's medium and modified Niven's medium and incubated at 32 ^{o} C for 18 to 24 hrs. The colonies with a purple halo in modified Niven's medium were selected as positive (i.e., histamine-producing bacteria)

2.3. Genomic DNA Isolation

Bacterial cells were harvested in mid-logarithmic growth phase. The bacterial cell culture was transferred to a sterile plastic centrifuge tube and the cells were pelleted by centrifugation at 4 °C at 1000 g for 10 min. The culture medium was then removed and the cell pellet was drained well. The samples were added directly to 200 µl Prep Man^{TM} sample preparation reagent (Applied Biosystems, Foster City, CA) and then boiled for 2 min, cooled for 2 min and micro-centrifuged for 2 min. Finally, 5 µl of the supernatant was transferred to the assay for subsequent analysis.

2.4. PCR Amplification of the 16s rRNA Gene

PCR amplification targeting bacterial 16s rRNA genes was performed with the 27F-5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r- 5'-CGTTACCTTGTTACGCTT-3'. Each PCR was performed in a total volume of 50 µl in 0.2 ml micro tubes. Reaction mixtures were contained 1 x PCR buffer with 1.5 mM MgCl2, deoxynucleoside triphosphate solution (2 mM each dATP, dCTP, dGTP and dTTP), primers 27F and 1492r-Ph (50 µM each) and 2 U of Taq DNA polymerase. The total amount of genomic DNA added to PCR mixtures was 10 ng and thermocycling. In each PCR reaction, the sample was initially denatured at 95 ^{o} C for 30 s followed by annealing at 60 ^{o} C for 30 s, extension at 72 ^{o} C for 45 s for 30 cycles and final extension at 72 ^{o} C for 10 min in an Applied Biosystems Gene Amp 9700 system. PCR products were purified with a QIAquick PCR cleanup kit (Qiagen, Germany). Amplified products were separated by electrophoresis on agarose gels (1.5% agarose gel, including 0.5 µg ethidium bromide) to quantify the amplicon and to verify that the correct size products was produced.

2.5. Sequencing of the 16s rRNA Gene

Cycles sequencing of the 16s rRNA amplification products were carried out using Ampli Taq FS DNA polymerase and dRhodamine dye terminators. Two primers (27F and 1492r) were used for sequencing. Excess dye-labeled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column. The

sequencing products were collected by centrifugation, dried under vacuum and frozen at $-20^{o}C$ until ready to load. Samples were electrophoresed on ABI Prism 377 DNA sequencers and the data were analyzed using Applied Biosystems DNA editing and assembly software. Identifications were assigned using MicroSeq analysis software and sequence database. Phylogenetic trees were constructed based on pair-wise algorithms.

3. RESULTS AND DISCUSSION

3.1. Top Ten Matches to the Microseq™ Database

Totally different 126 histamine-producing isolates were screened from the sediment sample. One of the best HPB isolates S-96 was selected for 16s rRNA study. Genetic relationships have been expressed in the form of percent genetic differences (% GD). This is the difference between any two sequences when aligned in a way to minimize sequence gaps. A species-level match was assigned if the %GD between the unknown and the closest match was less than the approximate average %GD between species within that particular genetic family, which was usually 1% (Table I).

Strain	Representative Species	% Genetic Difference
S-96	*Bacillus cereus*	0.00
S-96	*Bacillus thuringiensis*	0.84
S-96	*Bacillus mycoides*	0.93
S-96	*Bacillus atrophaeus*	7.01
S-96	*Bacillus mojavensis*	7.10
S-96	*Bacillus amyloliquefaciens*	7.20
S-96	*Brevibacterium (Bacil) halotolerans*	7.29
S-96	*Bacillus subtilis*	7.29
S-96	*Bacillus oleronius*	7.73
S-96	*Bacillus flexus*	7.84

Table I. Genetic Difference of Partial 16s rRNA Sequence of the Isolate S-96 to the Partial 16s rRNA Sequence of the Other Representative Members of Bacteria.

Phylogenetic trees were utilized to approximate the average inter species %GD which according to literature reports had an average of about 1% and ranges from 0.5% to 3.0% [9, 10]. Thus, a species-level match was assigned only if the %GD between the unknown and the database entry was less than 3% and less than the average %GD between species related to it. A genus-level match was assigned when the sequence did not meet the requirements for a species-level match but still clustered within the branching of a well-defined genus.

Distances were estimated by summing horizontal differences. Neighbor joining tree [11] was employed to display the relationships between the top ten matches and the unknown.

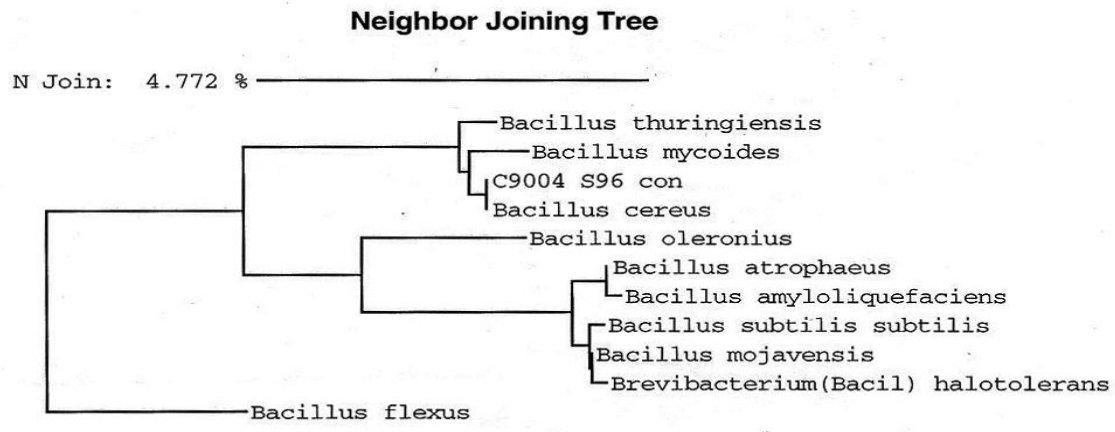

Fig. 1 Neighbor Joining Tree (Saitaonu and Nei, 1987) Based on 16s rRNA Gene Sequences Showing Relationships between the Strain S-96 and 10 Bacterial Species.

For a long time, bacterial taxonomy was considered one of the dullest fields in microbiology but with the advent of rRNA sequencing the whole sphere of research has not only been revolutionized but also it has become the most preferred discipline of young scientists. Genotypic methods are those that are directed toward DNA or RNA molecules. Of all the other genotypic methods, rRNA homology study is now generally accepted as it is present in all bacteria and is functionally constant and is composed of highly conserved as well as more variable domains. Sequencing of 16s rRNA with conserved primers and reverse transcriptase was a very important advance in bacterial phylogeny and resulted in a spectacular increase in 16s rRNA sequences [1, 7). A study encompassing multilocus enzyme electrophoresis (MEE) to compare the virulence and the potential of horizontal gene transfer within *Bacillus cereus* group has been reported by Helgasaon [5]. Detection of *Bacillus cereus* by amplifying the enterotoxin-encoding genes has also been reported by Hansen and Hendriksen [4].

In the present study, chemotaxonomic analyses revealed the fact that the isolate belongs to the genus *Bacillus*. But the most reliable method for determining species affiliations is 16s rRNA sequence homology as it provides with a phylogenetic framework which serves as a backbone for modern microbial taxonomy. In the present study, 16s rRNA of S-96 was sequenced with conserved primers, edited, assembled and identifications were ascertained using Microseq analysis software and sequence database. Identification was based on pair-wise algorithms and the phylogenetic tree was constructed using neighbor joining method, which confirmed with 0.00% sequence divergence that S-96 is *Bacillus cereus* (Fig. 1). On the basis of the phenotypic, chemotaxonomic and phylogenetic data, it is logical to conclude that the new isolate is *Bacillus cereus* and this is probably the first report to state that *B. cereus* is a histamine-producing bacterium in Muthupettai mangrove sediment.

4. CONCLUSIONS

The present study concludes that the molecular identification is a prominent tool for identification of marine environmental microbes. The sequencing study shows that the S-96 belongs to the genus of *Bacillus cereus*. This is probably the first report to designate *Bacillus cereus*

as a histamine-producing bacterium in Muthupettai mangrove environment. The histamine-producing bacterium was associated in the mangrove environment. This infects the fish community and thus reduces the economic value in the fish marketing

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Inhibitory Activity of Ink and Body Tissue Extracts of Euprymna Stenodactyla and Octopus Dollfusi Aganist Histamine Producing Bacteria

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Abstract: To assess the antimicrobial activity of the ink and body tissue extracts of the cephalopods, *Euprymna stenodactyla* and *Octopus dollfusi* against histamine producing bacteria. Methods: *Euprymna stenodactyla* and *Octopus dollfusi* were extracted using methanol, ethanol, acetone and chloroform and tested against selected human pathogenic and histamine producing Bacteria (HPB) Escherichia coli, Salmonella typhi, Klebsiella oxytoca, *Klebsiella pneumonia, Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus sp.* by agar well diffusion method. Results: Highest inhibitory activity was observed against *Pseudomonas aeruginosa* in methanol ink extracts of *E. stenodactyla* and ethanol extracts of *Salmonella typhii.* Ethanolic extracts of *O. dollfusi* showed highest inhibitory activity against can be used in the seafood processing industries to enhance the shelf life of sea foods and control the histamine fish poisoning.

Key words: Antibacterial activity · Cephalopod ink · *Euprymna stenodactyla* · Histamine producing bacteria · *Octopus dollfusi*

INTRODUCTION

Sea food is considered as one of the good source of animal protein for its high content of Poly Unsaturated Fatty Acids (PUFA). Consumption of a variety of fish is associated with an increasing number of human intoxication and diseases, which pose public health problems worldwide. This is mainly because of the highly perishable nature of the seafood species, aided by the unhealthy mode of handling and improper preservation leading to histamine fish-poisoning. Scombroid fishes and some non-scombroid fishes are able to produce histamine due to improper preservation and handling. These fishes have high amount of free L-histidine in their body tissues, which is the precursor amino acid for histamine formation [1]. Histamine toxicity occur from several minutes to several hours after ingestion of toxic fish and the illness typically lasts few hours but may continue for several days which include cutenous rash, urticaria, burning itching, edema, gastrointestinal inflammation, nausea, vomiting, diarrhea, hypertension and neurological headache [2]. Due to the growing consumer demand for seafood products it is imperative to use natural antimicrobials and antioxidants as preservatives [3]. Cephalopods are well known for their defense mechanism to escape from their enemies by changing colour and inking to confuse them [4]. Cephalopods such as Octopus, squid and Loligo ink possess the antimicrobial, retroviral and anticancer properties [5].

Several studies pertaining to biomedical properties of the ink of cephalopods had been reported previously [5-9]. Effect of natural and traditional preservatives such as spices and oils have already been tested against the histamine producing bacteria [10,11]. But research related to the antibacterial activity of

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cephalopod ink and body tissue extract against histamine producing bacteria were not available. In Cephalopods such as squid, only the mantle, fins and arms with tentacles were consumed and the other internal organs such as ink gland, gut and accessory nidamandal glands were discarded.

The objective of the present study was to single out the natural antibacterial agent from the cephalopod ink and body tissue extract which is potential against histamine producing bacteria, that may enhance the shelf life and preserve the fishery products without adversely affecting its quality and delicacy.

MATERIAL AND METHOD

Cephalopod Sample Collection: Cephalopods *Euprymna stenodactyla* (Orbigny, 1848) and *Octopus dollfushi* (Ropsin, 1929), were collected from Thondi coast, Palk Strait, Southeast coast of India. They were identified by using the Central Marine Fisheries Research Institute (CMFRI) Bulletin No. 37. Identify of common cephalopods of India [12] and [7].

Bacterial Strains: In the present study seven species of Gram negative histamine producing bacterial strains including Escherichia coli, Salmonella typhi, Klebsiella oxytoca, *K. pneumoniae Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and two Gram positive histamine producing bacterial strains *Bacillus cereus* and *Staphylococcus species* were tested. The bacterial strains obtained from the Microbial culture collection from Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parngipettai, Tamilnadu, India.

Extract Preparation: *E. stenodactyla* and *O. dollfusi* were washed with sterile water. The abdomen of Cephalopods was cut open and the ink glands and mantle tissue were carefully removed and air dried. These samples were kept at room temperature in a glass bottle with Methanol, Ethanol, Acetone and Chloroform for 7 days. Then it was filtered using Whatman No. 1 filter paper and the filtrate was evaporated. The solvent extract were collected in petriplates and allowed to evaporate the residual ethanol, methanol, acetone, chloroform solvents [7].

Antibacterial Assay: Antibacterial activity of the sample was assayed by the standard Nathan agar well diffusion method [13, 14]. Sterile cotton swab was dipped into the bacterial suspension. Then the swab was used to spread the entire dried surface of the Mueller Hinton Agar (MHA) (Himedia, Mumbai) plates. A constant amount of 0.7 mg of the dried extract in 50 µl solvent was placed on to each well. The well at the center without the extract served as control. After 24 hrs of incubation at 37°C zone of inhibition around the well was measured.

RESULTS

The crude ink extract of *E. stenodactyla* showed the highest inhibitory activity 6 mm against *P. aeruginosa* in methanolic extract and chloroform extract. E. coli growth was inhibited up to 3 mm in chloroform extract and 2 mm in ethanolic extract. *S. typhi* growth was inhibited up to 4 mm in the ethanolic extract. *B. cereus* growth was inhibited up to 2 mm in chloroform extract. Mantle tissue extract of E. stenodactyla inhibited the growth of E. coli up to 2 mm in methanolic extract *P. aeruginosa* and *A. hydrophila* inhibited at 2 mm in acetone extracts. *B. cereus* growth was inhibited up to 4 mm in chloroform extract and the *Staphylococcus sp.* growth inhibited up to 2 mm in ethanol extract.

The ink of *O. dollfusi* was inhibited the growth of *V. parahaemolyticus* at 5mm in ethanol extract, the *B. cereus* growth at 4 mm in acetone extract and *P. aeruginosa* up to 3 mm in acetone extract. Ink extract showed very less activity in different solvents against other organisms tested whereas the body tissue extracts of *O. dollfusi* showed the highest activity against *P. aeruf* up to 4 mm and 3 mm against *A. hydrophila* in methanol and acetone extracts respectively (Table 1).

DISCUSSION

Marine environment is highly interesting for its many hidden resources till now unknown to man. Those resources were not fully explored and utilised so far. Now a days more attention has been paid by the researchers to explore the potential drugs from the marine sources. Several bio active molecules extracted from marine invertebrates, including molluscs, possesses broad spectrum antibacterial activity that inhibit the growth of bacteria, fungi and yeasts [15]. The main objective of

Table 1: Antibacterial activity of the crude ink and body tissue extract of *Euprymna stenodactyla* and *Octopus dollfusi* against the histamine producing bacteria (inhibition zone mm)

	Euprymna stenodactyla								*Octopus dollfusi*							
	Ink				Body tissue				Ink				Body tissue			
Microorganisms	E	M	C	A	E	M	C	A	E	M	C	A	E	M	C	A
E. coli	2	1	3	T	1	2	1	-	1	T	2	1	T	-	1	1
S. typhi	4	1	T	1	1	T	-	T	1	T	2	1	T	T	1	1
K. oxytoca	T	1	1	1	T	1	T	T	1	1	1	T	T	1	T	2
V. parahaemolyticus	1	T	1	T	1	-	1	1	5	-	1	1	1	1	T	T
A. hydrophila	T	T	-	1	T	T	1	2	1	T	2	T	-	T	2	3
P. aeruoginosa	2	6	2	1	1	1	1	2	2	1	2	3	2	4	1	T
K. pneumonia	T	1	1	T	-	T	1	T	1	1	2	1	1	1	1	1
B. cereus	1	T	2	1	T	T	4	1	2	T	1	4	2	T	-	T
Staphylococcus sp.	1	1	T	T	2	T	1	T	1	T	1	T	T	T	1	T

(T: Trace), (-: Nil), (E: Ethanol), (M: Methanol), (C: chloroform), (A: Acetone), (1-6: Zone of inhibition in mm)

the present study was to compare the ability of Ethanol, methanol, chloroform and acetone extract against the growth of histamine producing bacteria. The result of the present study indicates that the ink extracts and body tissue extracts were inhibited the growth of histamine producing bacteria in methanol and ethanol extracts.

Antibacterial activity has been reported from many mollusc species viz. Oyster (*Crossastrea virginica*), mussel (*Mytilus edulis* and *Geukensia demissa*), murcid mollusc (*Dicathais orbita*) and sea hare (*Dolabella auricularia*) [16-18]. The broad spectrum of antibacterial activity for *Loligo duvaucelii* and *Sepia pharaonis* aqueous extract of the ink against human bacterial pathogens [19]. Inhibitory activity of *Nerita albicilla* and *Nerita oryzarum, Chicoreus virgineus, Chicoreus ramosus* against biofilm forming bacteria from Tuticorin coast [20]. The antimicrobial activities of *Sepia prashadi* methanolic extract against human pathogenic bacteria and antibacterial activity of marine bivalves *Meritrix casta* and *Tridacna maxima* with different solvents were investigated [9, 21].

Only meager studies have been carried out on antibacterial activity of cephalopod ink and cephalopod body extract with ethanol, methanol, chloroform and acetone. Antibacterial activity of sea hare *Aplysia extraordinaria*, *Dolabella auricularia* and the cephalopod *Sepioteuthius lessoniana* and *Sepia pharaonis* ink extracts against 40 biofilm forming bacteria. The ink extract of two gastropods and two cephalopod screened antibacterial activity [5]. The methanolic extracts of *Aplysia extraordinaria* and *Dolabella auricularia* ink shown activities against some biofilm forming bacteria. Antibacterial activity of the extracts of the ink samples of cuttle fish, *Sepia pharaonis* against selected human pathogens such as *Salmonella paratyphi* B, *Pseudomonas aeroginosa* and *Shigella dysentriae* 5mm each in toluene extract. Diethyl ether colum purified fractions showed prominent activity 6.5 mm against *Klebsiella pneumoniae* and *Staphylococcus epidermis* 5mm [5].

The *in vitro* antimicrobial activity of the crude methanolic extracts of six species of cephalopods, *Sepia lcobiensis, Sepiella inermis, Sepioteuthis lessoniana, Octopus aegina, Octopus aerolatus, Octopus dollfusi* were screened against clinically isolated human pathogenic bacteria viz. *Vibrio chlorae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Vibrio alginolyticus*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Streptococcus* sp., *Salmonella* sp. and *E.coli* [22]. The body tissue methanolic extract against *Bacillus cereus* was 4 mm and against *E. coli* 2mm. The antibacterial activity of the crude diethy ether ink extracts of *Sepia pharaonis* against *Salmonella paratyphi* and *Klebsiella pneumoniae* exhibited an inhibitory zone of 5mm and 4mm respectively [5]. The inhibitory activity of methanolic extract of *Sepia prashadi* body tissue at 100 % concentration observed against organisms *K. pneumoniae* 8mm, *V. Parahaemolyticus* 10 mm, *Salmonella* sp. 7 mm and *E. Coli* 11 mm. Activity has been observed from *Streptococcus* sp. There is very few studies were carried out to inhibit the histamine producing bacteria by actinomycetes, among the 41 strains of actinomycetes tested *S. aureofasciculus, S. chattanoogenesis* and *S. hawaiiensis* strains showed good inhibitory activity [23].

CONCLUSION

On comparing it was found that, *E. stenodactyla* and *O. dollfusi* ink extract showed moderate activity against the histamine producing bacteria. The ink and body tissue of *E. stenodactyla* and *O. dollfusi* extract has shown promising antibacterial activity against histamine producing bacteria. The growth of *P. aeruginosa*, *S. typhii, E. coli* and *B. cereus* were inhibited at different level by *E. stenodactyla* ink extracts. This study concluded that ink and body tissue extract showed inhibitory effects on Gram positive and Gram negative histamine producing bacteria. These findings are giving an idea that cephalopods may be considered as seafood preservative to enhance the shelf life of the fishery products. Further attempts have to be made thereby purification and characterization of these extracts is necessary to discover the efficacy of potent compound against the fish spoilage bacteria.

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Research Article

Change in Histamine Levels and Microbial Load in the Eviscerated and Uneviscerated Indian Mackerel Fish (*Rastrelliger kanagurta*) at Different Storage Temperatures

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Abstract

Histamine is a toxic compound produced by bacteria at higher temperature in seafoods. It not only causes heath risk to the consumer, but also causes economic loss to the seafood exporters if it exceeds the maximum permissible limit. In this study, effect of storage temperature (-20°C, 0°C, 10°C and 30°C) on histamine formation in eviscerated and uneviscerated Indian mackerel (*Rastrelliger kanagurta*) were tested with different time intervals (1-10 days) using enzymatic methods. Maximum level of histamine formed in eviscerated fishes was 2.0 mg/100g at – 20°C for 10 days, 3.56 mg/100g at 0°C for 4 days, 7.6 mg/100g at 10°C for 2 days, 10.75 mg/100g at 20°C for 2 days and 19.3mg/100g at 30°C for 1day of storage. Whereas, in uneviscerated fish the histamine level was 3.4 mg/100g at – 20°C for 10 days, 6.73mg/100g at 0°C for 4 days, 10.1mg/100g at 10°C for 2 days, 16.56mg/100g at 20°C for 2 days and 22.33 mg/100g at 30°C for 1 days of storage. Bacterial growth at – 20°C and after 7 days of storage exceeded the maximum acceptable level of 7 CFU/g set by the International Commission on Microbiological Specification for Foods. *Klebsiella* sp and *Erwinia* sp were identified as strong histamine producer from *R. kanagurta* fish muscle. This study revealed that the freshly caught fish had shown negligible level of histamine in its muscle. After the fish stored at different temperatures for l day it has reached a deplorable level. The present findings would be very helpful to control the histamine formation in *R. kanagurta* and other pelagic fish species during ice storage and in fish processing industry.

Keywords: Histamine; Indian mackerel; TVB-N; Enzymatic assay

1. Introduction

Indian mackerel (*Rastrelliger kanagurta*) is an important fishery resource in India, especially along

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the southwest coast of India as well as an important feed item for seer fishes and tuna varieties Vivekanandan et al. (2009). *R. kanagurta* contains the free amino acid histidine in its muscle and that may be liberated through proteolysis. The presence of microorganisms with amino acid decarboxylase which induces decarboxylase activity facilitates the accumulation of histamine (Zarei et al. 2011; Bjornsdottir et al. 2011; EFSA, 2011).

Histamine poisoning is a chemical intoxication caused by consumption of food containing toxic levels of histamine (Ababouch et al. 1991: An et al. 1998). Fresh fish meat contains negligible histamine. But improper handling and storage of fish products could increase histamine formation by the bacteria that possess the enzyme histidine decarboxylase Rawles et al. (1996). The *Enterobacteriaceae* are mainly involved in histidine decarboxylase activity (Kim et al. 2003 & Tsai et al. 2005). Other bacterial species including *Clostridium* spp*., Vibrio* spp*, Acinetobacter* sp*, Pseudomonas* spp*,* and *Photobacterium* spp have been reported as histamine producers Chen et al. (2010). Histamine formation in fish meat during storage is mainly affected by fish species, pre processing and storage condition. Since, histamine producing bacteria such as *Morganella morganii, Citrobacter braakii, Hafnia alvei* and *Raoultella planticola* are mesophiles, storage time and temperature had influence on histamine production by these bacteria Jaing et al. (2013). Storage at a temperature lower than 4^{o} C and avoidance of prolonged handling at ambient temperature could minimize histamine formation in fishery products Economou et al. (2007). In general, freshness of fishes is highly affected by the length of storage conditions Park et al. (2010).

R. kanagurta is generally marketed in fresh form and stored in ice. Bacteria that enter into fish body through gill and presence of food particles in intestine can favour to spoil the fish and produce histamine after death. Information pertaining to the formation of histamine in eviscerated and uneviscerated *R. kanagurta* fish muscles during different time of storage is meagre. Therefore, the present study has been made to test the histamine production in an eviscerated and uneviscerated *R. kanagurta* under different storage conditions along with a change of the pH and TVB-N to evaluate their potential relationship.

2. Materials and Methods

2.1. Chemicals and reagents

Histamine dihydrochloride, Leucocrystal violet, Diamine oxidase (DAO), Horse radish peroxidise (HRP) and *o*-phthaldialdehyde with high purity (98%) was obtained from Sigma Aldrich (USA). Other higher grade chemicals and reagents were purchased from Merck (Germany) and Hi Media (India). Triple deionised water was used for all reagents, media throughout the study chemical preparation. All glasswares and plasticwares were given triple wash with deionised water.

2.2. Collection of fish samples

R. kanagurta fish samples were collected from Thondi coast, (Latitude: 9^{o} 44’ N and Longitude: 79^{o} 00E), Palk Bay, Southeast India and brought to the laboratory under controlled temperature (0^{o}C) in sterile polythene bags. The fishes were cleaned with sterile seawater to remove extraneous dirt and immediately transferred to laboratory. Some of the fishes were eviscerated and other fishes

were left uneviscerated. These fishes were kept in separate polythene covers, sealed and stored at -20°C (10 days), 0°C (4 days), 10°C (2 day), 20°C (2 days) and 30°C (1 day) in aseptic condition.

2.3. Histamine analysis

Histamine content from *R. kanagurta* muscle samples were estimated by the enzymatic assay method as described by Lerke et al. (1983) and Lopez-Sabater et al. (1994). Fish muscle 5 gram each were homogenized twice with 50 ml of 0.4N perchloric acid followed by centrifugation (REMI, CPR 30 PLUS, India) at 3000 rpm for 10 min. The volume of the supernatant was made up 20 ml with 100 ml of 0.4N perchloric acid. From the supernatant, 5 ml was transferred into the filtration unit and the filtrate was taken into a separating funnel 5 ml of 1N NaOH, 10 ml of deionised water and 2.0g of NaCl were added. The supernatant was then extracted 4 times consecutively with 25 ml of n-butanol. The butonolic phases were again washed with 10 ml of 1N NaOH saturated with NaCl. The histamine was then extracted 5 times with 10 ml of 0.1N HCl and the volume was adjusted to 50 ml. The histamine was finally derivatized with o-opthaldehyde to minimize the interference of other components already present in fish muscle samples Vidal-Carou et al. (1990).

Sample extract and standard histamine dihydrochloride 0.5 ml was transferred into a test tube. One ml of phosphate buffer (0.0075 M KH_2PO_4 and 0.075 M Na_2HPO_4, pH 6.8), 0.5 ml of diamine oxidase (0.35 U I /ml) and 0.1 ml of leucocrystal violet (0.5mg/ml in 0.5% HCL) were added. The mixture was incubated in a thermostatic water bath at 37° C for 30 min. Finally, the coloured test solutions were measured at 596 nm using a UV-VIS, PC Scanning spectrophotometer UVD-2960 (Labomed, Inc., USA). The histamine concentration in fish muscle samples were calculated using the following formula.

$$\text{Histamine (mg/100g)} = \frac{\text{OD of sample x concentration of standard}}{\text{OD of standard}}$$

= histamine mg/100g of fish muscle

2.4. pH measurement

For the measurement of pH, 10g of eviscerated and uneviscerated *R. kanagurta* muscle samples were homogenized in sterile blender with 10 ml of triple deionized water to make thick paste like slurry. The pH of this slurry was measured using a pH meter (Eutech Instruments, Malaysia).

2.5. Determination of total volatile base-nitrogen (TVB-N)

The TVB-N content of *R. kanagurta* muscle was measured by the Conway's dish diffusion method Cobb et al. (1973). The TVB-N extract of fish muscle in 6% trichloroacetic acid (TCA) (Merck, Germany) was absorbed by boric acid and then titrated with 0.02N Hydrochloric acid. The content of TVB-N was calculated and expressed as mg/100g of muscle sample.

2.6. Enumeration of total heterotrophic bacteria (THB) and histamine producing bacteria

Samples were taken from the anterior dorsal portion of *R. kanagurta*. Ten gram of each fillets were individually transferred aseptically into the stomacher bag and 90 ml physiological saline (0.85% Nacl, W/V) with 0.1% peptone (W/V) was added. The mixture was homogenized for 50 to 60

seconds with stomacher. The supernatant was decimally diluted and 100µl was spread onto Tryptone Glucose Agar (TGA) (Hi-media Mumbai, India) plates for enumeration of THB. From the fish sample stored at different temperatures viz., -20, o, 10, 20, and 30^{o}C, 100 µl was spread on to the TGA agar plates and incubated for 24hrs at 30^{o}C for 2 days Radtong et al. (2005). Enumeration of histamine producing microorganisms was done in triplicate and the population density was expressed in CFU. g^{-1}.

2.7. Isolation of histidine decarboxylating bacteria (HDB)

One ml of serially diluted homogenate (fish muscle), was individually poured onto the petriplates containing Nivens medium Niven et al. (1981) and modified Nivens medium Yoshinaga and Frank, (1982). These plates were incubated at 28±2^{o}C for 2 days. Colonies that exhibited purple halo around in Nivens medium and pink halo in modified Nivens medium were considered as positive. Based on the results, positive colonies were randomly picked and inoculated onto trypticase soya agar (TSA) plate for purification and phenotypic identification Papadopoulos et al. (2003). The histamine producing ability of the positive colonies were further confirmed by thin layer chromatography (TLC) as described by Garcia-Moruno et al. (2005).

2.8. Statistical analysis

The effects of storage time and temperature on formation of histamine and TVB-N content were analyzed using the Pearson correlation test and significance ($P<0.01$ and $P<0.05$) was assessed by using SPSS version 16.0 (SPSS inc., Chicago, IL., USA).

3. Results and Discussion

The change in histamine content in muscle of *R. kanagurta* fish stored at -20, 0, 10, 20, and 30^{o}C are presented in Table 1. In the present study, low at level of histamine (2 mg/100g) was found in eviscerated Indian mackerel fish stored at -20^{o}C for 10 days (Table 1). Similar to our findings, Chytiri et al. (2004) reported low level of histamine (0.16 mg/100g) in Rainbow trout during 18 days of storage at 0^{o}C. In contrast, histamine was not detected in fresh Rainbow trout, Herring, Sea bass and Sardine at 0^{o}C during 0 days of storage (Chytiri et al. 2004; Ozogul et al. 2002; Paleologos et al. 2004 and Ozogul and Ozogul, 2006). In this study higher level of histamine was found in eviscerated fish (19.33 mg/100g) and uneviscerated fish (22.33 mg/100g) at 30^{o}C. The higher level of histamine formation could be due to the presence of precursor amino acid histidine, histamine producing bacteria and favourable temperature in the eviscerated and uneviscerated fish muscle of *R. kanagurta*. In contrast to the present study, Jiang et al. (2013) have reported highest level (2080 mg/kg) of histamine in eviscerated mackerel (*Pneumatophorus japonicus*) after 48h storage at 25^{o}C. Whereas, higher level of histamine of 2.50mg/100g during 0^{o}C storage, 14.50 mg/100g during 6 h storage, 19.50 mg/100g during 12hr storage and 75.00mg/100g during 24hr storage was reported from uneviscerated mackerel Patange et al. (2005).

In the present study, maximum histamine content of 22.33±1.44 mg/100g was found in uneviscreated fish muscle and this value has exceeded the maximum permissible limit of 5 mg/100g set by FDA, (2011). The histamine levels well correlated with storage time and temperature ($r =0.9802$, $P<0.01$) (Table 5). In contrast, Bita et al. (2013) reported that the level of histamine increased to 70.37µg/g in fresh uneviscerated orange-spotted grouper (*Epinephelus coioides*) muscle during the 12th day of storage at 0^{o}C. In this study, the histamine level increased to

22.33±1.44 mg/100g after 1 day of storage at 30°C. Similarly, Joshi et al. (2011) documented histamine level in uneviscerated mackerel fish muscle between 20-30 mg/100g. This level was much higher than the guidelines (5 mg/100g) set by USFDA, (1998). But, Afilal et al. (2006) reported high histamine content of 77.7 mg/100g in eviscerated Sardine (*Sardina pilchardus*) during 24hr of storage at 30°C. The present study revealed that histamine content was maximum in uneviscerated *R. kanagurta* muscle at different storage temperatures than the eviscerated samples (Table 1).

Table 1 Histamine content and pH at different storage temperatures in R. *kanagurta*.

Storage temperature	Storage time (days)	Histamine content mg/100g		pH	
		Eviscerated fish	Uneviscerated fish	Eviscerated fish	Uneviscerated fish
-20 °C	10 days	2.0±0.08	3.4±0.16	6.45±0.18	6.72±0.05
0°C	4 days	3.56±0.28	6.73±0.24	5.20±0.02	5.48±0.13
10 °C	2 days	7.6±0.37	10.1±0.37	6.57±0.04	6.72±0.08
20 °C	2 days	10.75±0.49	16.56±0.57	6.82±0.05	6.87±0.06
30 °C	1 day	19.3±0.86	22.33±1.44	7.01±0.08	7.18±0.04

pH in the eviscerated fish was minimum of 5.20 at 0°C and maximum of 7.01 at 30°C. The minimum pH was found to be 5.48 at 0°C and maximum was 7.18 at 30°C in the uneviscerated *R. kanagurta* muscle (Table 1). pH well correlated with storage temperature ($r = 0.99419$, $P<0.01$) in both eviscerated and unevicerated fish muscles. Similarly, pH and histamine significantly correlated ($r = 0.66408$, $P<0.05$) (Table 5). Increase in pH during storage is mainly due to the increase in total volatile base nitrogen, ammonia, primary and secondary amine formation of fish muscle of *R. kanagurta*. Li et al. (2013) reported that increase in pH during storage also increases TVBN, ammonia and trimethylamine produced by either microbial and/or endogenous enzymes. Similarly, Chen et al. (2010) reported low level of pH 6.57 to 6.60 in uneviscerated Marlin fillet (*Makaira nigricans*) stored at 4°C. Afilal et al. (2006) reported pH 6.0 to 6.3 from sardine and this pH favoured the formation of histamine by metabolic pathway of free amino acid decarboxylase. pH of uneviscerated orange spotted grouper (*Ephinepelus coioides*) reported by Bita et al. (2013) was lower value than our present finding. Similarly, Widiastuti et al. (2013) have reported low level of pH ranging from 5.91 to 6.01 from uneviscerated Loin tuna (*Thunnus albacores*) during 15 days of chilled storage. Yongjin et al. (2007) have documented that low level of pH could accelerate the accumulation of amino acids and it could prompt biogenic amine formation. Ozyurt et al. (2009) have suggested that the level of pH associated with the content of ammonia derived compounds and furthermore increase in the microbial activity during the storage results in food spoilage. Benjakul et al. (2003) highlighted that post-mortem change in fish muscle occurs owing to both endogenous and exogenous enzymatic activity and bacterial spoilage.

The highest TVB-N content 60.46 mg/100 was recorded in *R. kanagurta* muscle at 20°C during 2 days of storage (Table 2). In the present study, TVB-N was statistically significant with histamine r=0.94497, *P*<0.01. TVB-N level also correlated well with bacterial load (CFU/g) r =0.98598, *P*<0.01 (Table 4). The TVB-N is generally associated with the growth of bacteria and can be used as indicators of fish spoilage. Rodriguez et al. (2005) reported that high level of TVB-N content of 84 mg/100g after 8 days of flake ice storage from the horse mackerel muscle. This is higher than the maximum acceptable level of 35mg/100g of TVB-N in fish muscle Ruiz-Capillas and Moral, (2001). Afilal et al. (2006) have reported high level of TVB-N content of 161.28 mg/100g in eviscerated sea bass after 24hr of storage at 30°C. But, lower TVB-N content of 31mg/100g was recorded in horse mackerel after 22 days of storage in slurry ice Rodriguez et al. (2005).

Table 2 Total Volatile Base –Nitrogen (TVB-N) content in Indian mackerel fish *(R. kanagurta)*.

Storage temperature	Storage time (days)	TVB-N content mg/100g	
		Eviscerated	Uneviscerated
-20°C	10 days	ND	ND
		16.8±0.91	ND
		19.69±0.80	ND
		22.12±1.95	14.56±0.45
		25.94±0.69	17.0±0.50
		26.13±0.92	18.85±0.69
		30.89±2.40	21.61±0.95
		38.26±1.31	23.61±0.86
		46.86±1.97	25.29±0.80
		47.3±0.92	30.8±2.28
0°C	4 days	14.96±0.75	19.26±1.39
		16.3±0.77	22.63±1.73
		21.30±1.69	23.24±1.39
		23.24±1.39	28±0.26
10°C	2 days	32.4±1.72	41.5±1.23
		37.33±1.31	42±2.28
20°C	2 days	46.73±1.83	50.4±2.28
		51.26±1.31	60.46±23.9
30 °C	1 day	27.06±3.49	32.29±3.11

Data are expressed Mean±S.D for all triplicate determinations.

Changes in THB count in *R. kanagurta* stored at different temperatures are given in Table 3. The present study showed that the initial THB of eviscerated *R. kanagurta* ranged from 1.54 log CFU g^{-1} to 9.2 log CFU g^{-1} and uneviscerated fish was 2.33 log CFU g^{-1} to 11.03 log CFU g^{-1} during storage at -20°C. This value has reached 7 log CFU/g^{-1}, the upper limit set by the International Commission on Microbiological Specification for Foods (ICMSF, 1986) for raw seafoods. Storage at – 20°C, the THB count increased after 7 days of storage and reached above 10.9 log CFU/g after 10d ays of storage in eviscerated fish and 11.03 log CFU/g during 10 days of storage in uneviscerated fishes of *R. kanagurta*. Whereas, the eviscerated and uneviscerated fish muscle samples stored at 0°C, 10°C, 20°C and 30°C showed rapid increase in THB count (Table 3). Similarly Jiang et al. (2013) documented that the higher bacterial count rapidly increased to 8.0 log CFU/g^{-1} on 48 hr storage in mackerel *P. japonicus*.

Table 3 (a) Changes in microbial load in Indian mackerel (*R. kanagurta)* during different storage conditions (log CFU/g)

R.kanagurta stored at -20°C for 10 days

Days of storage/temperature-20°C	Eviscerated (log CFU/g)	Uneviscerated (log CFU/g)
1	1.54±0.17	2.33±0.21
2	2.24±0.07	2.79±0.25
3	2.72±0.07	3.49±0.22
4	3.92±0.11	4.78±0.14
5	4.49±0.28	5.24±0.21
6	5.35±0.19	6.35±0.38
7	6.63±0.20	7.02±0.10
8	7.7±0.23	8.6±0.76
9	8.23±0.38	9.73±0.79
10	9.2±0.40	11.03±0.52

The THB counts was 6.62 log CFU g^{-1} in eviscerated fish muscle and 7.34 log CFU g^{-1} in uneviscerated fish muscle after 1 day of storage at 30°C. If the bacterial population reach this level (i.e. 6-7 log CFU. g^{-1}) it could possibly produce 50 µg of histamine (USFDA, 2001; Al Bulushi et al. 2009). Widiastuti et al. (2013) reported that, the level of TPC varied from 2.0 to 3.5 log CFU/g^{-1} during 15 days of storage of *Thunnus albacores* fish muscle. Likewise, Bita et al. (2013) reported that mesophilic bacteria ranged from 2.0 to 4.61 log CFU/g^{-1} during the ice storage of *Epinephelus coioides* and remained low.

Table 3(b) Total heterotrophic bacteria (THB) count of *R. kanagurta* stored at different storage temperature (-20, 0, 10, 20 and 30°C)

Days of storage	Storage time (Days)							
Temperature	Eviscerated				Uneviscerated			
	1	2	3	4	1	2	3	4
10 °C (2days)	3.6±0.08	4.24±0.24	5.6±0.2	6.8±0.0	4.03±0.12	5.10±0.1	7.6±0.1	8.13±0.16
20°C (2days)	4.03±0.26	5.23±0.15			5.56±0.37	6.43±0.3		
30°C (1 day)	6.62±0.28				7. 34±0.16			

Mean±S.D for all triplicate determinations.

Table 4 Correlation coefficients among TVB-N level in evicrerated and uneviscerated *R. kanagurta* during -20°C storage condition.

	Evicrerated TVB-N	Uneviscerated TVB-N	Eviscerated CFU	Uneviscerated CFU
Evicrerated TVB-N	1			
Uneviscerated TVB-N	0.94497[a]	1		
Eviscerated CFU	0.96142[a]	0.9569[a]	1	
Uneviscerated CFU	0.97266[a]	0.98598[a]	0.97959[a]	1

[a] Significant correlation (*P<0.01*)
Ev-Eviscerated; Unev: Uneviscerated

Table 5 Correlation coefficients among histaminine level and pH of evicrerated and uneviscerated *R. kanagurta* during different storage tempereture.

	Evicrerated histamine	Uneviscerated histamine	Eviscerated pH	Uneviscerated pH
Evicrerated histamine	1			
Uneviscerated histamine	0.9802[a]	1		
Eviscerated pH	0.66408[b]	0.63645[b]	1	
Uneviscerated pH	0.64406[b]	0.59765[b]	0.99419[a]	1

[a] Significant correlation (*P<0.01*) [b] Significant correlation (*P<0.05*)

It has been suggested by Kim et al. (2009) that biogenic amine forming bacteria mostly belong to psychrophilic and mesophilic bacterial groups. The growth of mesophilic bacteria accelerate in

moderate temperature (20-40^{o}C) and produce high histamine and other biogenic amines. The present study agree with the previous findings of Bita et al. (2013), Jiang et al. (2013) and Kim et al. (2009) that the growth of psychrophilic bacteria accelerated faster than mesophilic bacteria and they play a considerable role in fish spoilage.

Table 6 Histamine forming bacterial strains isolated by Nivens Medium and Modified Nivens medium from *R. kanagurta*.

S.no	Name of the bacterial strains	Nivens Medium	Modified Nivens medium	TLC method
1	*Morganella* sp	+	+	+
2	*Proteus* sp	+	+	+
3	*Vibrio* sp	+	+	-
4	*Shegella* sp	+	+	-
5	*Photobacterium* sp	+	+	+
6	*Salmonella* sp	+	+	-
7	*Entrobacte*r sp	+	+	+
8	*Citrobacter* sp	+	+	+
9	*Klebsiella* sp	+	+	+
10	*Bacillus* sp	+	+	+
11	*Erwinia* sp	+	+	+

+ Positive; -Negative

In the present study, mesophilic and other enteric bacteria such as *Morganella* sp, *Proteus* sp, *Vibrio* sp, *Shigella* sp, *Photobacterium* sp, *Salmonella* sp, *Entrobacte*r sp, *Citrobacter* sp, *Klebsiella* sp, *Erwinia* sp, *Enterococcus* sp, and *Staphylococcus* sp were isolated from both eviscerated and uneviscerated Indian mackerel fish samples (Table 4). Similarly, Jiang et al. (2013) have isolated histamine forming bacteria such as *Morganella morganii* and *Raoutella planticola* using Modified Nivens medium in *P. japonicus*. Joshi and Bhoir (2011) reported that *Bacillus* sp, *Pseudomonas* sp, *Vibrio* sp, *Staphylococcus* sp, *Morganella* sp, *Enterobacter* sp and *Klebsiella* sp were isolated and confirmed by Nivens medium in uneviscerated Indian mackerel (*R. kanagurta*). Bjornsdottir-Bulter et al. (2011) have identified histamine producing bacteria such as *Photobacterium damselae*, Plesiomonas *shigellloides*, *Shewanella* sp and *Morganella morganii* from tuna and blue fish.

4. Conclusion

Histamine formation in fish muscles during storage and at different temperature is mainly affected by the fish species, storage condition and processing. In this study, varying pH affect the water holding capacity that leads to fish spoilage. The level of TVB-N content increased due to autolysis and enzymatic change in fish muscle during storage conditions. Histamine content in both eviscerated and uneviscerated fish muscles have reached beyond the level for human consumption i.e. 5 mg/100g USFDA, (2001) after storage at 20^{o}C and 30^{o}C for considerable durations. Therefore, this study brings to light that adequate storage facility with rapid cooling system is necessary after

catch, during processing, till it reaches the consumer to avoid fish spoilage, histamine accumulation in fishes and subsequently the ill effects to humans.

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Conflict of interest

All the authors declare that they have no conflict of interest.

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