2. CONSUMPTION ASSESSMENT QUALITIES

2.1. INTRODUCTION

Next to fin fishery, crab fisheries generates more income due to its delicacy, nutritional richness, large size and copiousness muscle, are fixing the lofty price and export potential at global market. As reported by Choo and Williams, (2003) fish products provide excellent protein due to their balanced amino acid profile and protein digestibility. Crab is highly nutritious and healthy owing to its content of essential amino acids, proteins, unsaturated fatty acids and minerals (Adeyeye, 2002; Skonberg and Perkins, 2002; Gokoglu and Yerlikaya, 2003; Celik et al., 2004; Musaiger and Al-Ruaidh, 2005; Vilosoa-Martinez et al., 2007; Chen et al., 2007; Kuley et al., 2007 and Adeyeye., 2008).

Recently, urbanization, industrialization, globalization etc., have directly or indirectly disturbed the balance of the coastal environment. So the coastal waters are contaminated which in turn provides the favourable environment for the multiplication of undesirable microorganisms in the marine environment as suggested by Vaidya et al., (2001) and Sugumar et al., (2008). The fishes and crabs are the major marine food resources caught from such contaminated areas are the carrier of disease causing microbes (Jithendran et al., 2010).

The live and hard shelled mud crabs are always in greater demand both in national and international markets (Kathirvel, 1993), particularly in USA and EU countries. In India, the soft shelled crabs are discarded as such at the landing centre itself, as people are unaware of its nutritional status and also the presence of minimal adsorbed bacterial flora. Several studies were carried out on the proximate composition
(Skonberg and Perkins 2002; Naczk et al., 2004; Chen et al., 2007; Adeyeye, 2008 and Vilosoa Martinez et al., 2009), amino acid composition (Naczk et al., 2004 and Chen et al., 2007), total heterotrophic bacteria and related diseases on hard shelled mud crabs (Anderson et al., 2000; Chakraborty et al., 2002; Vijayalakshmi, 2007; De Pedro et al., 2008; Shanmuga Priya, 2008; Jithendran et al., 2009 and Sivakumar and Krishnakumar, 2011) and nutritional aspect (Prasad and Neelakantan, 1989; Nair and Mathew, 2000; Zafar et al., 2004; Thirunavukkarasu, 2005; Rameshkumar et al., 2009 and Sudhakar et al., 2009) in different crab species in various parts of the world.

Though the soft shelled mud crab has been profit-making target species at international market, they were poorly studied at nutritional point of view and associated bacterial flora. As these two major factors reflect the consumption qualities, the present work is designed to analyse the seasonal variation of total proximate composition, total bacterial count, pathogenic bacteria such as Vibrio and Coliforms, and pH of hard and soft shelled mud crabs.
2.2. MATERIALS AND METHODS

2.2.1. Collection of sample

The samples of raw hard crabs were collected from the sampling site and transferred to a bamboo basket. From this some alive and active crabs were taken for analysis. Newly moulted crabs were also taken and placed in sterile polythene bags and transported to the laboratory for further analysis.

2.2.2. Acclimatization of wild *S. serrata*

The collected wild crabs *S. serrata* were kept in the individual plastic boxes (30cm length × 15cm width × 30cm height) to avoid mortalities due to aggression and cannibalism. The boxes were floated at surface level of pond using plastic floats and empty oil barrels. Each box was covered with lid to prevent the crabs from escaping. The crabs were fed twice a day with low cost dry fishes and pellets. The pond water salinity was maintained between 25‰ and 35‰ throughout the study period. The water salinity was recorded daily by using Salinometer and the water temperature was also recorded by using a Thermometer. Crab’s moulting was checked thrice a day (6am, 3pm and 11pm), as suggested by Quinitio and Noe Lwin, (2009). The moulted crabs were immediately transported to the laboratory for further studies. During transport, the crabs were covered with wet cloth to maintain the softness and to minimize the body temperature. (Plate 2, showing the newly moulted soft shelled mud crab with empty shell).
2.2.3. Biochemical Estimation:

For biochemical estimation, raw, healthy crabs were selected from the bamboo baskets, washed several times in fresh water followed by sterilized sea water to remove sand and dust particles. The shell was removed carefully using sterilized knife and scalpels. The thoracic and chelate leg’s muscles were dissected out and kept in a sterile petridish and cut into small pieces using sterilized instruments. The tissue was dried at 56°C in an oven to remove all the moisture content. After removing the moisture content, the tissue was made into fine powder using a mortar and pestle. The pulverized sample was used to estimate the amount of total carbohydrate, protein and lipid by adopting the standard procedures.

2.2.3.1. Carbohydrate

Carbohydrate was estimated following the method of Shiefter et al., (1950).

100 mg of the crabs’ tissue was homogenized in a mortar with 2ml of 10% TCA and 8ml of distilled water. The homogenate was centrifuged at 3000rpm for 10 minutes. The supernatant was collected and measured and used for the estimation of total carbohydrate. 0.5ml of the supernatant was taken in a clean test tube. To this, 4.5ml of anthrone reagent was added and mixed well and this mixture was kept at room temperature. The developed colour was read at 620nm in UV – VIS Spectrophotometer – 118 model against a standard reagent blank.
2.2.3.2. Protein

The protein content of the tissue was estimated following the method of Lowry et al., (1951).

100mg of crab tissue was homogenized with 2ml of 10% TCA and centrifuged at 3000 rpm for 10 minutes. After decanting the supernatant the residue was dissolved in 1ml of 0.1N Sodium hydroxide and kept in a water bath at 60 – 70°C for 10 minutes. After filtering, from this, 0.5ml of solution was pipetted out into a clean test tube. To this, 4ml of carbonated copper solution (50ml of 2% sodium carbonate + 0.5ml of 1% CuSO₄ + 2% Potassium tartarate) was added mixed well by lateral shaking. After this, the test tubes containing the solution were kept undisturbed at room temperature for 15 minutes. To this, 0.5ml of Folin ciocalteau phenol reagent was added. The test tube was shaken well for uniform mixing and kept in the room temperature for another 30 minutes. The resultant blue colour was read at 540 nm against a reagent blank in a UV-VIS Spectrophotometer -118 model.

2.2.3.3. Lipid

Lipid was estimated following the method of Bragdon (1951).

100mg of the sample was homogenized well with 5ml of chloroform and the solution was centrifuged at 3000rpm for 15 minutes. The supernatant was collected in a test tube and evaporated to dryness by keeping it in an oven. To the dry test tube 3 ml of 3% Potassium dichromate was added which was followed by 3 ml of distilled water. The developed colour was read at 640 nm against a reagent blank in a UV – VIS Spectrophotometer -118 model. The standard curve was obtained by using cholesterol and the lipid was expressed as mg lipid / g in dry weight tissue.
2.2.3.4. Amino acid

About 2g sample was defatted using Chloroform/methanol mixture (AOAC, 1995). About 30mg of the defatted sample was weighed into glass ampoules. Seven millilitres of 6MHCL were added and oxygen expelled by passing nitrogen gas into samples. The glass ampoules were sealed with a Bunsen flame and put into an oven at 105°C for 22hrs. Then the ampoule was allowed to cool, filtered. The filtrate was then evaporated to dryness at 40°C under vacuum in a rotary evaporator. Each residue was dissolved with 5ml acetate buffer (pH= 2.0) and stored in a plastic specimen bottle and kept in the deep freezer. Aminoacid analysis was done by Ion Exchange Chromatography (FAO/WHO, 1991) using the Technicon Sequential Multisample Aminoacid Analyser.

2.2.4. Analysis of Bacterial flora

Analysis of bacterial load was carried out the following method of USFDA Bacterial Analysis Manual (BAM).

2.2.4.1. Total Plate Count (TPC)

Preparation of diluent – Normal Saline (NS)

1.7 gram of Sodium chloride (NaCl) was taken in a 500ml conical flask followed by adding 200ml distilled water and dissolved well. 95ml of this solution was taken in another conical flask and transferred to ten test tubes with a quantity of 9.5ml each.
**Preparation of Tryptone Glucose Agar (TGA)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Beef Extract (Lab lemco)</td>
<td>0.3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Agar agar</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Weighed ingredients were taken into 500ml conical flask and dissolved by heating on a water bath. The pH was adjusted to 7.1±0.1. The prepared solution was poured into the conical flask and sterilized by autoclaving. Then the solution was poured in the required number of sterile petri dishes and allowed for settling.

25 gm of crab muscle was transferred aseptically to a sterile stomacher bag, then 90ml of the diluents was added and blended using stomacher blender for 30 seconds. The resulting dilution at this stage was noted as $10^{-1}$. From this solution, 1ml of supernatant was pipetted out and added to the 9ml diluents and mixed well, this dilution was $10^{-2}$, and likewise $10^{-3}$, $10^{-4}$ and $10^{-5}$ dilutions were prepared. Then the inoculation was done by using a sterile bent glass rod on the surface of the respective agar plates. Before that, the rod was sterilized by dipping in alcohol and flaming between spreading operations of each plate. After half an hour, the plates were incubated at $37^\circ$C for 48 hours. After 48hrs of incubation, the developed colonies in each plate were counted using a Qubec Colony Counter. Only those plate counts which fall between 25 and 250 were considered for the present study.
TPC / g was calculated by the following formula.

\[
\text{TPC per gram} = \frac{\text{Number of colonies} \times \text{Dilution}}{\text{Weight of the sample}} = \text{cfu / gm}
\]

The counts shall be expressed in colony forming units (c.f.u per gram).
2.2.4.2. Coliforms:

Preparation of MacConkey Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>17 g</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>3 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10 g</td>
</tr>
<tr>
<td>Bile salt No.3</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Neutral red</td>
<td>3 ml of 1% neutral red (0.03 g)</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>1 ml of 0.1% crystal violet (0.001 g)</td>
</tr>
<tr>
<td>Agar</td>
<td>13.5 g</td>
</tr>
<tr>
<td>pH</td>
<td>7.1±0.1</td>
</tr>
</tbody>
</table>

The above ingredients were soaked in 1 litre of distilled water for 15 minutes and after soaking, the solution was sterilized by autoclaving at 15 lbs/sq. inch (121\(^0\)C) for 15 minutes.

MPN method was used to detect Coliform bacteria in water or food and normally the plating method has limitations to detect low numbers of bacteria in water or food because the inoculums size is small. Pathogenic bacteria may not be present in sufficient large numbers in water or food to be detected by plating methods. Usually all the media used were liquid.

25g of crab sample was aseptically taken and homogenized with 225ml of physiological saline to obtain 10\(^{-1}\) dilution, like that 10\(^{-2}\) and 10\(^{-3}\) dilution and were also prepared. 1ml each from 10\(^{-1}\), 10\(^{-2}\) and 10\(^{-3}\) crab samples were inoculated in separately in to 3 sets of test tubes filled with MacConkey broths. The contents of the tubes were mixed by shaking gently and then the tubes were incubated at 37\(^0\)C for 48 hours. The presence of Coliform was confirmed after detecting the production of gas and acid in the
MacConkey Broth tubes in each dilution. The result was compared with the standard MPN Table and the MPN values for presumptive total coliforms were noted.

2.2.4.3. Vibrios

Preparation of Thiosulphate-Citrate-Bile salts Sucrose (TCBS) Agar

<table>
<thead>
<tr>
<th>Ingredient (Formulation)</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extracts</td>
<td>5 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20 g</td>
</tr>
<tr>
<td>Sodium Thiosulphate (Na$_2$S$_2$O$_3$2H$_2$O)</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium Citrate (Na$_2$C$_6$H$_3$2H$_2$O)</td>
<td>10 g</td>
</tr>
<tr>
<td>Sodium Chlorate</td>
<td>3 g</td>
</tr>
<tr>
<td>Oxgall</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>10 g</td>
</tr>
<tr>
<td>Ferric Citrate</td>
<td>1 g</td>
</tr>
<tr>
<td>Bromothymol Blue</td>
<td>0.04 g</td>
</tr>
<tr>
<td>Thymol Blue</td>
<td>0.04 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

The above ingredients were dissolved in one litre of distilled water and boiled for 2 minutes, and then the solution was poured into the petri dishes.

Aseptically weighed 25 gram of composite crab sample was cut into small pieces with sterile scissor and knife. Then the pieces were transferred into a long necked flat bottomed flask of 250 ml capacity. To this, 225 ml of Alkaline Peptone Water was added, and then this broth was incubated overnight at 37±1°C (Primary Enrichment). A full loop of the above incubated peptone water broth suspension was transferred to the surface of TCBS plated media and the plates were incubated for a period of 18 to 24 hrs
at 37±1º C. For secondary enrichment, four loops full of the primary enriched broth suspension was inoculated into a test tube containing 10 ml of alkaline peptone water and incubated at 37±1ºC for 6-8 hrs. A loop full from secondary enriched tube streaked on to another dried TCBS plate and incubated at 37±1ºC for 18 to 24 hrs.

**Presumptive Tests KIA (45) / TSI (28) Reaction**

Each suspected colonies (large, smooth, yellow and slightly flattened with opaque centre and translucent peripheries) were picked up and inoculated into the separate tube containing TSI (28) and KI Agars (45) by streaking the slant and stabbing the butt and incubated overnight at 37±1ºC.

Strains were confirmed by the biochemical confirmation tests and by slide agglutination test with *Vibrio cholerae* polyvalent antisera.

**2.2.5. pH**

Ten gram of sample was homogenized with 20ml of distilled water. To arrest the changes in pH of muscle, neutral 0.01 N sodium iodoacetate was added in distilled water. The pH of the homogenate was measured by using pH Meter and corrected to Zero dilution applying following formula.

\[
\text{pH (0)} = \text{pH (D)} - 0.068 \ D^{0.5}
\]

Where pH (0) = pH at zero dilution
Where pH (D) = pH determined on the homogenate

D = dilution ratio

The pH of tissue below 7.7 is considered as good quality, between 7.7 and 7.9 are acceptable and tissue having pH above 7.9 are considered as spoiled (Reshma Zamir et al., 1998).
2.3. RESULT

The result of biochemical components such as carbohydrate, protein, lipids and aminoacids, total heterotrophic microbial load, pathogenic bacteria such as Coliforms and *Vibrio* and pH from the muscle of wild and acclimatized newly moulted soft crabs are given in the figure (1-6). The result of amino acid profile of newly moulted crab is given in the figure (7) and Table 1 and 2.

2.3.1. Biochemical composition

Among the major biochemical components, protein was higher than carbohydrate and lipids in both hard and soft shelled mud crabs. The maximum value (18.91g/100g) of protein was recorded in hard crabs during monsoon season (September 2010) and minimum value (16.82g/100g) was during winter (January 2011) season. In soft shelled mud crabs, maximum value (9.75g/100g) was found during summer (March 2010) and minimum (8.74g/100g) was also during winter season (February 2011).

Totally, 19 amino acids (Inclusive 11 essential and 8 non-essential amino acids) were detected in newly moulted soft shell crab. The contribution by essential amino acid was higher with 52.52% and among the essential amino acid arginine was maximum (13.05%), while tryptophan was minimum (0.72%). Comparatively lower contribution (47.48%) of non-essential amino acids was found in soft shell crab. Amongst, Glutamic acid was the highest (14.14%) and Alanine was the lowest (2.75%).

2.3.2. Bacterial analysis

Higher (7.3 × 10^6 cfu/gm ) bacterial load was observed in hard crabs during winter (November 2010) season and lower (1.23 × 10^6 cfu/gm ) during monsoon
(August 2010) season. Whereas in soft shelled crabs, maximum \(3.31 \times 10^6\) cfu/gm bacterial load was noted during winter (January 2011) season and minimum \(1.7 \times 10^6\) cfu/gm was found in monsoon (July 2010) season.

The pathogenic bacteria mainly the Coliforms was found to be higher \(9.4 \times 10^2\) cfu/gm in hard shell crab during monsoon season (October 2010) and lower \(3.6 \times 10^2\) cfu/gm in soft shell crabs during summer season (April 2010).

In hard shell crab the *Vibrio* was maximum \(69\) cfu/gm during monsoon (October 2010) season and minimum \(27\) cfu/gm during summer (May 2010) season. Whereas in soft shell crabs, *Vibrio* was found to be higher \(36\) cfu/gm in winter (November 2010) season and lower \(8\) cfu/gm during summer & monsoon (March & June 2010).

2.3.3. pH

The monthly variations of pH did not show notable trend. The maximum pH 7.67 and 7.17 is found in the muscle of hard crabs and soft shelled mud crabs respectively during monsoon (July) season.

2.4. Statistical Analysis

ANOVA test has been applied to test the level of significance on THB, Coliforms, *Vibrio sp*, carbohydrate, protein, lipids and pH between the soft shell and hard shell crabs. The result showed that the difference in sample is significant at 1% level (Table 3 to 9).
2.5. DISCUSSION

The result of the present study showed seasonal variations of biochemical components in hard as well as the soft shelled crabs. This could be correlated with size of the animal, stage of maturity, temperature and availability of food etc., as reported by Mayuree Chaiyawat et al., (2009). Love (1970) reported that variation of biochemical composition of fish flesh may also occur within the same species depending upon the fishing ground, fishing season, age and sex of the individual and reproductive status. The spawning cycle and food supply might be the main factors responsible for this variation.

The protein content in the muscle of both hard and soft shelled crabs showed higher values than carbohydrate and lipids. This observation was further confirmed with the study of Mathai and Devi (1993). Prasad and Neelakantan (1989) reported that the protein content of the body meat and claw meat was 20.11% and 18.54% respectively. Zafar et al., (2004) reported that the protein values in the males & females of S. serrata were 17.69% and 19.39% respectively. Moronkola et al., (2011) reported that the higher amount of protein content in chest meat, walking legs and tissue was 19.89%, 19.29% and 28% respectively in the hard shelled Callinectes amnicola living on the shore of Ojo river, Nigeria. Das (1978) also noticed that high values of protein with the spawning season when the gonads were ripe and the decline with post spawning period. The present study revealed that the lower percentage of biochemical composition in soft shelled crabs could be correlated with the stage of maturity as reported by Mayuree Chaiyawat et al., (2009).

The current results showed that mud crab tissues are good source of essential aminoacids such as glutamic acid, arginine, glycine, histidine, aspartic acid etc., however
with varied concentration. This could be due to a variety of factors such as the species, size, seasonal conditions and geographical locations as suggested by Wesselinova (2000). Comparison between the amino acid content in mud crabs and the FAO (1985) amino acid reference values for mankind showed that most of the amino acids would meet the recommended range of amino acid requirement for both children and adults.

In the present study, the amount of lipid value is lower than carbohydrate and protein and slightly varied in both soft and hard crabs seasonally. Seasonal differences in the availability of food and changes in the reproductive cycle have considerable effect on the tissue biochemistry of the fish particularly fat (Bumb, 1992). Lipids act as an extra energy reserve to assist recovery during maturation of gonads and spawning as suggested by Selvaraj (1984) in Indian shad. Lipids are the very good high efficient source of energy and they contain more than twice the energy of carbohydrates and proteins (Okuzumi and Fujii, 2000). Sudhakar et al., (2009) reported that the protein, carbohydrate and lipid contents were higher in hard shell crab than that of soft shell crab (*Portunus sanguinolentus*) and the present finding could be correlated with these findings.

Seasonal variation of microbial flora found both in wild and acclimatized newly moulted crab *S. serrata* could be correlated with the flora present in the environment. Masayo Okusumi and Masaishi (1982) reported that the seasonal variation causes changes in bacterial population in the marine environment. Chandrasekaran (1985) and Tamilselvi (2008) have also reported that animals in the natural environment carry a bacterial flora which is a reflection of the flora in the environment. Fuhrman (1999), Karner et al., (2001) and Orphan and Victoria (2001) recorded the bacteria are found in all portions of the sea water column, the sediment surface, and the sediment themselves.
The result of the present study showed that a higher bacterial load was observed in the wild caught *S. serrata* collected from the study area. Usually, the crab prefers muddy bottom as a habitat to live and the nature of substratum also plays an important role in the retention of bacteria. The muddy and clayey sediments provide high nutrient content for the bacterial population and compactness promotes greater adsorbance of bacteria as reported by Kaper et al., (1979), Marty et al., (1980) and Marshall (1985). Levine and Griffin (1993) and Sugumar et al., (2008) have stated that dumping of domestic sewage also increases the bacterial population density in the coastal waters. The present observation with high bacterial load was also further confirmed with the findings of Lalithadevi (1985), Najiah et al., (2010), Vjayalakshmi (2007), Sivakumar and Krishnakumar (2011) on mud crabs.

The acclimatized – soft shelled crabs harboured lesser density of bacteria than wild crabs collected from the study area. It could be due to the fact that the crabs confined at the controlled environment, with stabilized and controlled water salinity and temperature. The present observation was also supported with the findings of Liong (1993), Celia and Lobert (2004) and Krishnakumar and Sivakumar (2011).

As the pH value was below 7.7, this pH value could be considered to be maintaining the good quality of muscle of wild and soft shelled crabs. The dried fish having pH in between 6.0 and 6.9 are considered to be of very good quality (FAO, 1981). Many physical and chemical factors are involved in formation, stability and rheology of the protein emulsions. Emulsification varies with pH, ionic strength, temperature etc., as reported by Ramanathan et al., (1978). The emulsifying properties are known to be significantly influenced by pH. Therefore, pH 6.72 – 7.07 might have increased the good quality of product.
To conclude, the soft shelled crabs harboured minimal bacterial load and unacceptable limit of *Vibrio* sp and Coliforms than hard shelled crabs. Further, the microbial load should be reduced to extend the shelf life of soft shelled mud crabs. Hence, the modified existing techniques could be adopted to eradicate the pathogenic bacteria from the study animal to improve the quality of crab.