



**CYTOCHEMICAL STUDIES IN MALE MUD CRAB SCYLLA SERRATA: ESTIMATION OF PROTEIN, CARBOHYDRATE AND LIPIDS AFTER INOCULATED WITH KLEBSIELLA PNEUMONIA AND BACILLUS CEREUS**

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**ABSTRACT**

The rise in the protein levels in *Scylla serrata* during first 12 hrs after bacterial inoculation might be associated with the release of other humoral defense factors involved in clearance of bacteria. In the present study it was observed that the protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 4hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs gradually in case of male crabs challenged with Gram positive and Gram Negative bacteria. The quantitative estimation of total carbohydrates was made in the crab, *Scylla serrata* at 2hrs, 4hrs, 8hrs, 12hrs, 16hrs, 20hrs and 24hrs in control, control injured and male crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus*. Basing on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 24hrs and the percentage of decrease is highest 26% in male crab challenged with *Klebsiella pneumonia*. The depletion of total carbohydrate may be due to its rapid utilization to meet the energy demands under the impact of bacterial inoculation. The lipid content increased gradually from 2 hrs to 12 hrs and decrease from 12 hrs to 24hrs in male crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus*. The percent of increase from 2hrs to 24 hrs is 18% in male crab challenged with *Klebsiella pneumonia* and 14 % in male crab challenged with *Bacillus cereus*.

**KEYWORDS:** Cytochemical studies, *Scylla serrata*, *Klebsiella pneumonia* and *Bacillus cereus*.

**INTRODUCTION**

The mud crabs are cosmopolitan in distribution and have exploited every aquatic niche thereby considerably contributing to the food web in its habitat. The species is an excellent research model to understand the molecular and physiological basis of stress in aquatic animals due to its high adaptability to stress tolerance.<sup>[1-4]</sup> In addition, mud crabs have a direct role in maintaining marine as well as fresh water ecology. Mud crabs in particular act as burrowers; as a result they help in aeration, mixing and nutrient flow in the soil. Being burrower mud crabs enhance aeration and nutrient flow in the soil.<sup>[5, 6]</sup> Consequently, soil fertility is augmented that helps in facilitating the survival of other plants and soil dwelling animals. In addition, bioturbation structures created by crabs trap sediments and mangrove seeds.<sup>[7]</sup> and significantly contribute to the conservation of mangrove plants. One of the important roles of crabs particularly in mangrove environment is the production of millions of meroplanktonic larvae, which serve as potential food source for a large number of planktophagus organisms including rich number of edible fishes. Crabs stabilize the complex food web in the mangrove ecosystem. Despite its high commercial value, information on its biochemistry and physiology especially in relation to free radical mediated metabolism is scanty and needs

attention for its use in aquaculture. Finally, it can be concluded that crustaceans like crabs may become susceptible to various stresses when they cross over the threshold value of several environmental factors.<sup>[8,9]</sup> Therefore, *S. serrata* are rightly known for its wide range of environment stress tolerance and attenuation of physiological homeostasis particularly with respect to changing salinity.<sup>[10,11]</sup> and temperature.<sup>[12]</sup> Different mechanisms on how these marine invertebrates adapt either at physical or physiological level to avoid the environmental stress are being explored by various workers.<sup>[13]</sup> As evidenced from the above, studies made in past on mud crabs were basically addressed to their characterization, distribution and taxonomy. However, not much work has been done on the physiology particularly on Biotic stress related changes in cellular and biomolecular levels like proteins, lipids of mud crabs. This metabolism is an important aspect of aerobes.<sup>[14]</sup> A fluctuation in metabolism due to changes in biochemical parameters may have adverse effect on the metabolism of aquatic organisms by pushing them to stress condition known as "Biotic stress". Although there is some information available on Biotic stress and antioxidant defence system in invertebrates particularly in molluscs and insects, not much information is available on crabs.

## METHODOLOGY

### Cytochemical Studies

For the present work, male healthy adult animals ( $6 \pm 1$  cm carapace width) were used. Healthy adult crabs (5.5 cm mean carapace width, 105 gms wt. males and 6 cm mean carapace width 120 gms wt. females) were purchased from regular animal supplier kept in the laboratory in disinfected plastic tubs, the water in the tubs was changed every day and fed with minced chicken. The crabs were acclimatized in the laboratory for 7 days.

### Bacterial strains and culture

Two different strains of bacteria, *Klebsiella pneumonia* (Gram -ve), *Bacillus cereus* (Gram +ve) were used for inoculation during the study. *Klebsiella pneumonia*, Gram Negative bacteria do not retain crystal violet dye. The cells are typically rod shaped, facultative anaerobic bacteria can live on wide variety of substrates. The optimal growth is at  $37^{\circ}\text{C}$ . *Bacillus cereus*, Gram Positive coccal bacterium retains crystal violet dye during Gram staining. The bacterium frequently found in human respiratory tract and on the skin and causes common skin and respiratory diseases.

The Bacterial strains were obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India. Small amount of bacterial culture, Gram negative (*Klebsiella pneumonia* MTCC-4030) and *Bacillus cereus* (MTCC 430) were taken from the Glycerol stock and spread on to the Luria- Bertani agar Plates. The Agar plates were incubated for 24 hours at  $37^{\circ}\text{C}$ . Pure colonies were picked from the Overnight culture and inoculated into an autoclaved broth for 12 hours and incubated at  $37^{\circ}\text{C}$  on a rotor shaker at 150 rpm. 1 ml of this culture was introduced for 3 hrs culture and incubate for 3 hrs at  $37^{\circ}\text{C}$  in shaker and 1 ml of 3 hrs culture was taken, centrifuged at 2000rpm for 10 min at  $4^{\circ}\text{C}$  and the pellet was taken. 100 ml Milli Q water was added to the pellet and subjected to serial dilutions (9 times) and 1 ml of  $10^3$  cultures was used for inoculation.

### LB Media Composition and Preparation

2.5gms Peptone, 1.25gms Yeast, 2.5gms NaCl and make up to 250 ml Milli Q Water and autoclave at 15 Psi for 25 minutes and cool it to room temperature

### Nutrient Agar Media (NAM) Preparation

2.5gms Peptone, 1.25gms Yeast, 2.5gms NaCl, 3.75gms Agar and make up to 250 ml and autoclave at 15 Psi for 25 minutes and cool it to room temperature.

### Experimental Groups

1. Group I: Control crabs without any bacterial inoculation.
2. Group II: Control injured crabs, these crabs were injected with saline/ 0.9% NaCl.
3. Group III: Male Crabs challenged with *Bacillus cereus*, Gram +ve bacteria.

4. Group IV: Male Crabs challenged with *Klebsiella pneumonia*, Gram -ve bacteria.

### Collection of Hemolymph

Hemolymph was collected from unsclerotized membrane from the ventral side with Insulin syringe and each crab was subjected to a single bleed amounting to 1–2 ml of Hemolymph at different time intervals 2h, 4h, 8h, 12h, 16h, 20h and 24 hrs. The collected hemolymph was immediately diluted with 1:1 ice cold anticoagulant solution for further biochemical studies.

### Anticoagulant Preparation

For 100 ml of Anticoagulant 9.8 ml of 1M NaOH + 18.6 ml of 1MNaCl + 17 ml of 0.01 M EDTA + 41 ml of 0.01 M Citric acid was taken mixed together and pH was adjusted to 4.5 then final volume was made up to 100 ml and autoclaved at 15 PSI( Pounds per square Inch) for 20 min.

The hemolymph was collected and diluted with anticoagulant and other chemicals (1ml Hemolymph + 1ml Anticoagulant + 10  $\mu\text{l}$  Phenylthiourea + 10  $\mu\text{l}$  of Aprotin) and centrifuged at 2000 rpm for 15 min at  $4^{\circ}\text{C}$  and the supernatant was used for assessment of biochemical parameters (G. Rameshkumar et al., 2009). In the citrate-EDTA buffer used, citric acid serves to delay cell break down while EDTA inhibits prophenoloxidase (proPO) activation and prevents the clotting reaction, (Hall et al., 1999), and this buffer at low pH, in combination with citrate, glucose and NaCl, provides a medium optimal for maintenance of cell integrity without significant loss of cell viability.

**Biochemical Parameters:** The hemolymph was collected and diluted with anticoagulant and other chemicals (1ml Hemolymph + 1ml Anticoagulant + 10  $\mu\text{l}$  Phenylthiourea + 10  $\mu\text{l}$  of Aprotin) and centrifuged at 2000 rpm for 15 min at  $4^{\circ}\text{C}$  and the supernatant was used for assessment of biochemical parameters.

### Estimation of Total proteins (Bradford method)

The total protein was estimated by the method described by Dr. Marion Bradford in 1976 (Bradford, M. (1976). Proteins bind to coomassie brilliant blue G-250 to form a blue coloured complex having greater extinction coefficient than the free dye. In the acidic environment of the reagent, protein binds to the coomassie dye. This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465nm) to the blue form of the dye (absorbance maximum at 610nm). The difference between the two forms of the dye is greatest at 595nm (Bradford, M. (1976). Acidic coomassie-dye reagent changes color from brown to blue in proportion the amount of protein present in the sample. Protein determinations are made by comparison to the color response of protein assay standards, usually prepared as a series of known dilutions of Bovine Serum Albumin (BSA). The total protein was estimated in control and challenged crabs as per the protocol given in Bradford

method, using Bovine Serum Albumin (BSA) as standard. 250  $\mu$ l of Bradford reagent was added to 5  $\mu$ l of hemolymph and incubated for 10 minutes at room temperature and absorbance was measured at 595 nm.

#### Estimation of Total Carbohydrates (Anthrone method)

The total carbohydrates were estimated by the method described by Anthrone. Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm. The total carbohydrate was estimated in control and challenged crabs as per the protocol given in Anthrone method by using standard Glucose as standard.

#### Total Lipids (Zaks method)

The total carbohydrates were estimated by the method described by Zaks. The lipids present in the sample are first precipitated by adding  $\text{FeCl}_3\text{-CH}_3\text{COOH}$  reagent. The lipid free filtrate is treated with conc.  $\text{H}_2\text{SO}_4$ . In the presence of conc  $\text{H}_2\text{SO}_4$ , cholesterol present in the sample gets dehydrated to form cholesterol 3, 5 diene in presence of excess and by the  $\text{H}_2\text{SO}_4$ . catalytic action of  $\text{Fe}^{3+}$  ions a red coloured complex is formed. The intensity of red colour is measured at 560 nm.

The total lipid was estimated in control and challenged crabs as per the protocol given in Zaks method. Pipette out 1-5 ml of standard solution in a series of test tubes. The volume in each test tube is made upto 5ml  $\text{FeCl}_3\text{-}$

$\text{CH}_3\text{COOH}$  with reagent. 3ml of conc.  $\text{H}_2\text{SO}_4$  is added to all the test tubes and mix well. Standards are incubated for about 20-30 minutes at room temperature. The intensity of standards is measured at 560 nm against blank.

5 ml of reagent,  $\text{FeCl}_3\text{-CH}_3\text{COOH}$  3ml  $\text{H}_2\text{SO}_4$  of are taken in a test tube, mixed well and used as a blank. In the centrifuged tube 0.1ml of serum and 10ml of Ferric chloride-acetic acid reagent reagents are taken, mixed well for 5 minutes and then centrifuged. 5 ml of supernatant is collected and added with 3ml of  $\text{H}_2\text{SO}_4$ . Test is incubated at room temperature to 20-30 Intensity is measured at 560nm against blank.

## RESULTS AND DISCUSSION

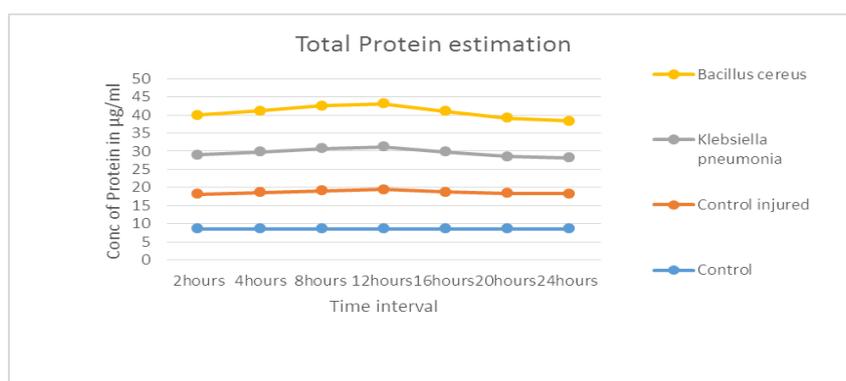
### Quantitative Estimation of Total Protein:

Quantitative Estimation of Total Protein in control, control injured and Gram positive and Gram Negative bacteria challenged crabs were made at different time intervals of 2hrs, 4hrs, 8hrs, 12hrs, 16hrs, 20hrs and 24hrs. The results indicated that the total protein content was observed to be slightly more at all time intervals in Bacterial challenged crabs than control and control injured crabs (Fig 1).

It was observed that the protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 4hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs gradually in case of male crabs challenged with Gram positive and Gram Negative bacteria (Table 1).

**Table 1: Total protein in the haemolymph of control and bacterial challenged male crabs.**

	2hours ( $\mu\text{g/ml}$ )	4hours ( $\mu\text{g/ml}$ )	8hours ( $\mu\text{g/ml}$ )	12hours ( $\mu\text{g/ml}$ )	16hours ( $\mu\text{g/ml}$ )	20hours ( $\mu\text{g/ml}$ )	24hours ( $\mu\text{g/ml}$ )
Control	$8.57 \pm 0.532$	$8.57 \pm 0.532$	$8.57 \pm 0.532$	$8.57 \pm 0.532$	$8.57 \pm 0.532$	$8.57 \pm 0.532$	$8.57 \pm 0.532$
Control injured	$9.57 \pm 0.286$	$10.12 \pm 0.421$	$10.48 \pm 0.832$	$10.88 \pm 0.395$	$10.24 \pm 0.423$	$9.89 \pm 0.183$	$9.77 \pm 0.294$
<i>Klebsiella pneumonia</i>	$10.84 \pm 0.745$	$11.12 \pm 0.439$	$11.68 \pm 0.184$	$11.76 \pm 0.327$	$10.96 \pm 0.628$	$10.12 \pm 0.274$	$9.89 \pm 0.324$
<i>Bacillus cereus</i>	$10.96 \pm 0.634$	$11.34 \pm 0.438$	$11.76 \pm 0.258$	$11.88 \pm 0.385$	$11.22 \pm 0.284$	$10.55 \pm 0.318$	$10.11 \pm 0.843$



**Fig 1: Total protein in the haemolymph of control and bacterial challenged male crabs.**

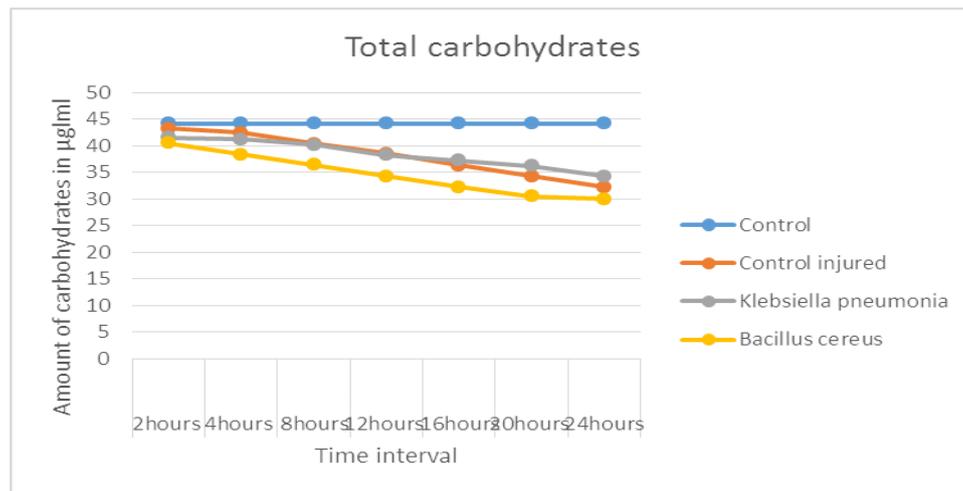
**Estimation of Total Carbohydrates**

The quantitative estimation of total carbohydrates was made at 2hrs, 4hrs, 8hrs, 12hrs 16hrs, 20hrs and 24hrs in control, control injured and male crabs challenged with Gram negative (*Klebsiella pneumonia*) and Gram

positive (*Bacillus cereus*) bacteria. Basing on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 24 hrs and the percentage of decrease is highest, i.e 20% in male crab challenged with *Klebsiella pneumonia* (Fig 2 and Table 2).

**Table 2: Total carbohydrate in the haemolymph of control and bacterial challenged male crabs.**

	2hours ( $\mu\text{g/ml}$ )	4hours ( $\mu\text{g/ml}$ )	8hours ( $\mu\text{g/ml}$ )	12hours ( $\mu\text{g/ml}$ )	16hours ( $\mu\text{g/ml}$ )	20hours ( $\mu\text{g/ml}$ )	24hours ( $\mu\text{g/ml}$ )
Control	44.231 $\pm$ 0.384	44.231 $\pm$ 0.384	44.231 $\pm$ 0.384	44.231 $\pm$ 0.384	44.231 $\pm$ 0.384	44.231 $\pm$ 0.384	44.231 $\pm$ 0.384
Control injured	43.245 $\pm$ 0.286	42.465 $\pm$ 0.768	40.425 $\pm$ 0.452	38.535 $\pm$ 0.768	36.282 $\pm$ 0.463	34.265 $\pm$ 0.768	32.245 $\pm$ 0.468
<i>Klebsiella pneumonia</i>	41.567 $\pm$ 0.436	41.265 $\pm$ 0.768	40.210 $\pm$ 0.637	38.264 $\pm$ 0.768	37.270 $\pm$ 0.453	36.267 $\pm$ 0.634	34.250 $\pm$ 0.364
<i>Bacillus cereus</i>	40.538 $\pm$ 0.272	38.34 $\pm$ 0.457	36.450 $\pm$ 0.342	34.265 $\pm$ 0.768	32.260 $\pm$ 0.648	30.460 $\pm$ 0.320	29.960 $\pm$ 0.358

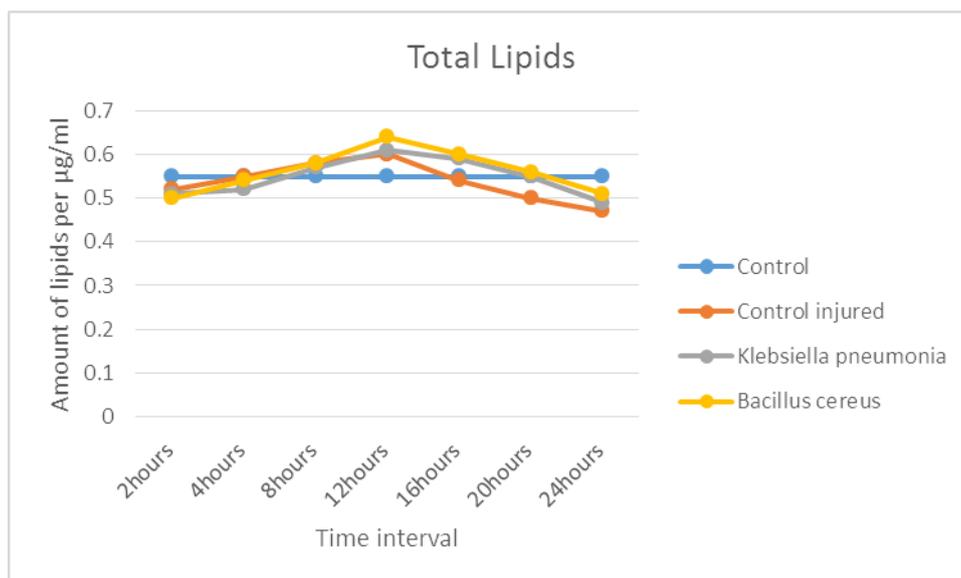
**Fig 2: Total carbohydrate in the haemolymph of control and bacterial challenged male crabs.****Estimation of Total Lipids**

The total lipid content was estimated at 2hrs, 4hrs, 8hrs, 12hrs, 16hrs, 20hrs and 24hrs in control, control injured and male crabs challenged with Gram negative (*Klebsiella pneumonia*) and Gram positive (*Bacillus cereus*) bacteria.

Basing on the results it was observed that the Lipid content increased gradually from 2 hrs to 12 hrs and decrease from 12 hrs to 24 hrs in male crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus*. The percent of increase from 2hrs to 12 hrs is 12% in male crab challenged with *Klebsiella pneumonia* and 15% in male crab challenged with *Bacillus cereus* (Fig 3, Table 3).

**Table 3: Total Lipid in the haemolymph of control and bacterial challenged male crabs.**

	2hours ( $\mu\text{g/ml}$ )	4hours ( $\mu\text{g/ml}$ )	8hours ( $\mu\text{g/ml}$ )	12hours ( $\mu\text{g/ml}$ )	16hours ( $\mu\text{g/ml}$ )	20hours ( $\mu\text{g/ml}$ )	24hours ( $\mu\text{g/ml}$ )
Control	0.55 $\pm$ 0.043	0.55 $\pm$ 0.043	0.55 $\pm$ 0.043	0.55 $\pm$ 0.043	0.55 $\pm$ 0.043	0.55 $\pm$ 0.043	0.55 $\pm$ 0.043
Control injured	0.52 $\pm$ 0.022	0.55 $\pm$ 0.054	0.58 $\pm$ 0.036	0.60 $\pm$ 0.048	0.54 $\pm$ 0.046	0.50 $\pm$ 0.061	0.47 $\pm$ 0.024
<i>Klebsiella pneumonia</i>	0.51 $\pm$ 0.032	0.52 $\pm$ 0.034	0.57 $\pm$ 0.034	0.61 $\pm$ 0.026	0.59 $\pm$ 0.035	0.55 $\pm$ 0.068	0.49 $\pm$ 0.052
<i>Bacillus cereus</i>	0.50 $\pm$ 0.015	0.54 $\pm$ 0.064	0.58 $\pm$ 0.042	0.64 $\pm$ 0.042	0.60 $\pm$ 0.018	0.56 $\pm$ 0.029	0.51 $\pm$ 0.038



**Fig 3: Total Lipid in the haemolymph of control and bacterial challenged male crabs.**

### CONCLUSION

In this work, protein content increase was moderate in the hemolymph of challenged *Scylla serrata* during first 12hrs and started decreasing from 12hrs to 24 hrs in case of male crabs challenged with Gram positive and Gram Negative bacteria. Basing on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 24hrs and the percentage of decrease is highest 26% in male crab challenged with *Bacillus cereus*. The lipid content increased gradually from 2 hrs to 12 hrs and decrease from 12 hrs to 24hrs in male crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus*.

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