# Antioxidant status of *Penaeus monodon* fed with *Dunaliella salina* supplemented diet and resistance against WSSV

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**Abstract**

The present study investigates the protection of shrimp *Penaeus monodon* against white spot syndrome virus (WSSV) using *Dunaliella salina* algal cells which contains an antioxidant betacarotene for the shrimp non-specific immunity. To determine the antioxidant activity, the shrimp were treated in vivo (orally with feed) methods at the concentration of 0.5 %, 1.0 % and 2 % *D. salina* incorporated with pellet feed, respectively. In the present study, anti-WSSV activity of *D. salina* incorporated diet by in vivo methods showed strong antioxidant activity and the immunological parameters such as proPO, SOD,catalase were higher in the WSSV-infected shrimp treated with *D. salina* incorporated diet when compared to control groups. These results strongly indicate that in vivo of *D. salina* incorporated diet enhances immunity of the shrimp. Based on the present data and the advantages of harvesting *D. salina* at low price, we believe that oral administration of *D. salina* live cells along with the pellet feed is a potential prophylactic agent against WSSV infection of shrimp to some extent.

*Keywords: Penaeus monodon*; *Dunaliella salina*; antioxidant activity.

## 1. Introduction

The White Spot Syndrome Virus (WSSV) continues to be one of the most serious disease problems faced by the shrimp farming industry worldwide (Flegel, 1997; Karunasagar *et al.,* 1997; Hsu *et al.,* 1999). White spot syndrome virus (WSSV) is spread by cannibalism of sick or dying shrimp or via contaminated water. Many other animals (crabs, lobsters, shellfish) may harbor and spread the disease (Chang *et al*,1998 Sahul Hameed *et al*., 2003).The quick spread of the disease among the shrimp population and the broad host range, make the control of this virus almost impossible, thus novel strategies to control WSSV are needed. Various

studies have been carried out to obtain the performance of immunostimulants for enhancing immune response and reduction of disease impacts. Several diseases were controlled by oral administration of immunostimulation.

Immunostimulants are suitable for boosting immature immune system and effective against a number of opportunistic and secondary pathogens (Felix *et al.,* 2004). Thus modern shrimp hatchers are now trying to resolve this problem of WSSV infection by using immunostimulant incorporated shrimp feed. Studies have been carried out to obtain the performance of immunostimulants for enhancing immune response and reduction of disease impacts. Certain carotenoids obtained from algal sources like astaxanthin and β-carotene induces immune system in shrimp and will certainly continue to play an important role in disease control in intensive shrimp culture (Karin van de, 2002). In order to know the role of this immunostimulant of carotenoids obtained from algae on resistance of shrimp against WSSV and its consequences on the immune condition of shrimp, the present study was carried out to determine the effect of betacarotene obtained from *Dunaliella salina* incorporated with different concentrations on prophenol oxidase, superoxide dismutase, catalase (SOD) production during the course of the WSSV challenge to assess the non-specific immune status of the shrimp.

## 2. Materials and Methods

### 2.1. Preparation of betacarotene from *D. salina*

Ten litre of Dewalnes medium were prepared with 35 ppt and inoculated with 100 ml of pure *Dunaliella salina* culture. The cultures were kept in out door condition for a period of 20 days. On $15^{th}$ day orange stage is attained. Then the cultures were harvested and centrifuged at 10,000 rpm for 10 min. The obtained biomass were lyophilised and stored in the refrigerator until they were used for the bioassay test.

### 2.2. Preparation of test diets

One litre of fresh water was taken in a vessel and boiled on a heater. The ingredients were cooked as described below. The rice bran was added at first and mixed thoroughly, followed by the addition of fishmeal, coconut cake, squid meal, wheat flour powder and mixed thoroughly, until the feed became a paste and cooled to room temperature and added the ingredients such as fish oil, vitamin mix and tapioca. To this different percentages of harvested biomass of *D. salina* cells (dry wt) such as 0.5 %, 1.0 % and 2 % were added separately and mixed thoroughly. Then the paste was taken pressed and squeezed through 2 $mm^2$ pores and collected the shreds on a polythene sheet. The shreads without *D. salina* served as control. The shreds were allowed to dry at room temperature for 2 days kept in plastic bags and sealed properly until given to shrimp.

### 2.3. Collection and maintenance of experimental animals

A total of 100 *Penaeus monodon* of 4-5 g body weight, were obtained from the shrimp pond at Meenjur, near chennai and maintained in 1000 L fibre glass tank with air- lift biological filters at room temperature (27 - 30°C) with salinity between 20 and 25 %. The experiment was conducted at the Aqua Nova Hatcheries, Kanathur, Chennai. The seawater was pumped from the Bay of Bengal and kept for 24 h and removed the sand and other suspended particles at the bottom. Then the seawater was initially chlorinated with 25 ppm sodium hypochlorite followed by dechlorination by vigorous aeration, passed through sand filter and

used for the experiment. Temperature, pH and salinity (Aqua fauna, Japan) were recorded on the spot. This experiment was carried out for a period of 30 days.

### 2.4. Feeding trial on *Penaeus monodon*

The experimental shrimp, *Penaeus monodon* was fed with normal diet (control) for a period of 2 days and then taken for the experimental study. The animals were divided into four groups of each 10 shrimps in 100 L seawater in fibre tanks at room temperature (27-30°C) with salinity between 20 and 25 ppt. Group I animals were fed with the normal pellet feed without *D. salina* (control). In Group II, the shrimp were fed with 0.5 % *D. salina* incorporated feed, Group III fed with 1.0 % *D. salina* incorporated feed, and Group IV fed with 2 % *D. salina* incorporated feed for a period of one month. The animals were fed twice daily at 09:00: and 17:00 h for a period of 30 days. The amount of feed given was 5 % of animal body weight. Every alternate days, 50 % volume of the water along with the excreta of animals and other waste were removed and compensated with fresh seawater.

### 2.5. Determination of immunological and antioxidant activity

The uninfected shrimp fed with different concentrations of *D. salina* incorporated diet were sacrificed weekly for a period of one month for immunological assay such as prophenoloxidase assay (proPO), superoxide anion assay, superoxide dismutase activity and catalase assay were analyzed for different groups.

### 2.6. Haemolymph collection

Haemolymph of 300 µl per shrimp was collected directly from the heart of animal every week for a period of one month. The haemolymph was collected by using a 23-guage needle and 1.0 mL syringe contained 300 µl (4°C) precooled 10 % sodium citrate solution as anticoagulant in glass distilled water.

### 2.7. Estimation of protein

The estimation of protein was carried out by following the method of Lowery *et al*., 1951. One hundred µL of haemolymph samples were made up to 1 mL separately with glass distilled water. To this 4.5 mL of alkaline copper reagent was added shaken well and allowed to stand for 10 minutes. To this 0.5 mL of Folin's reagent was added and kept at room temperature at 37°C for 20 minutes. The absorbance was read at 660 nm using UV visible spectrophotometer (UV- 1601 Shimadzu). Total protein expressed as µg/mL in haemolymph.

### 2.8. Phenoloxidase activity

The Prophenol Oxidase assay was carried out by following the method of Pascual *et al*., 2003. The 0.1 mL Haemolymph sample was made up to 1.0 mL with glass distilled water and centrifuged at 3000 rpm at 4° C for 10 minutes. The supernatant was discarded and the pellet was collected, rinsed and resuspended in sodium cacodylate buffer and centrifuged again. The pellet was once again resuspended with 200 µL of sodium cacodylate buffer. To 100 µL of the sample 50 µL trypsin (1 mg/mL) was added and kept for 10 minutes at 25° C, then 50 µL of L- DOPA was added followed by the addition of 800 µL cacodylate buffer after 5 minutes. Phenoloxidase activity was measured under ELISA reader by recording the formation of dopachrome from L-

DOPA (Leonard *et al.,* 1985). The data were recorded every one minute interval for a period of 5 minutes at 490 nm.

**2.9. Assay of Super oxide dismutase**

SOD assay was carried out in Haemolymph protein samples of the experimental animals as described by Misra and Fridovich , 1972. To 0.1 mL of haemolymph protein 0.75 mL ethanol and 0.15 mL chloroform (chilled in ice) were added separately and centrifuged at 10,000 rpm at 4°C for 10 minutes. To 0.5 mL of supernatant 0.5 mL of EDTA (0.6 mM) solution and 1mL of carbonate bicarbonate buffer (0.1 M) pH 10.2 were added. The reaction was initiated by the addition of 0.5 mL of substrate (Epinephrine 1.8 mM) and the increase in absorbance was recorded at 480 nm at every 30 seconds for 3 minutes. The values are expressed as 50 % inhibition of epinephrine auto oxidation /min /mg protein.

**2.10. Catalase Assay**

The Catalase assay was carried out by following the method of Takaharat *et al*., 1960. To 1.2 mL of phosphate buffer (0.05 M, pH 7), 0.2 mL haemolymph of protein samples was added separately and the catalase enzyme reaction was initiated by addition of 1 mL of substrate $H_2O_2$ (0.03 M in phosphate buffer). The decrease in OD at 240 nm was recorded at every 30 seconds for 3 minutes. The enzyme blank was run simultaneously with 1mL of glass distilled water instead of $H_2O_2$. A standard contained catalase was carried out simultaneously and expressed as µmoles of $H_2O_2$ decomposed /min/mg protein.

**2.11. In vivo determination of antioxidant activity of healthy shrimp challenged with WSSV infected shrimp**

The shrimps fed with normal pellet feed and three different concentrations of *D. salina* incorporated feed were challenged by WSSV infected tissues (200 mg) of *P. monodon* by oral route obtained from the Central Institute of Brackish Aquaculture, Chennai, were divided into four groups of 10 shrimp per group and each trial was conducted in triplicates. In Group I, shrimp fed with normal feed for 2 days and then on 3rd day alone it was fed twice a day orally with WSSV infected shrimp treated as positive control showed 100% mortalitily within 48 hrs p.i. with gross signs including lethargy, reduced feed consumption, reddishness on dorsal side and appearance of white spots in cephalothoracic region. In Group II , shrimp fed with *D. salina* incorporated diet without any challenge towards WSSV (negative control) showed 100 % survival till the end of the study period (up to 10th day). In Group III, shrimp fed with 0.5% *D. salina* incorporated diet for 2 days and fed with WSSV infected shrimp on third day alone twice a day and from fourth day fed with normal pellet feed, similarly feed was given in Group IV, shrimp fed with 1.0 % *D. salina* incorporated diet challenged with WSSV infection and Group V, 2.0 % *D. salina* incorporated diet challenged with WSSV infection. In all the groups, the shrimps were fed twice daily. Initially 5–7% of body weight of feed was fed to shrimp and thereafter it was adjusted according to the feeding response of the shrimp in each tank. Uneaten food and waste matter were removed before feeding. The experimental animals were examined twice per day for gross signs of disease, and the number of deaths was recorded. For the analysis of immunological and hematological

parameters, samples of haemolymph were taken from live infected animals on 1, 3, 6 and 9 days. The procedures for the estimation of the assays were described earlier. The immunological results were compared with the experimental animals. All the live and dead animal samples were analyzed for PCR using the primer meant for WSSV.

**Statistical analyses**

The experimental data were tabulated and analyzed using one-way ANOVA by the Agres statistical software package (Agres, 1994). The least significant difference (LSD) analysis was performed to group the treatment mean values.

## 3. Results

### 3.1. Immunostimulant and antioxidant activity of healthy *P. monodon* fed with *D. salina* test diets

#### 3.1.1 Prophenoloxidase assay

The shrimp fed with the diet without *D. salina* (control) showed the proPO levels of 0.11, 0.11, 0.11, and 0.11 U/min/mg of protein on 1st, 2nd, 3rd and 4th weeks (Fig. 1), respectively. The above values were less than 64 %, 66 %, 68 % and 70 % to the animal fed with 1.0 % *D. salina* incorporated diet. Among the three different concentrations of *D. salina* incorporated diets, the animal fed with 1.0 % diet showed maximum proPO of 0.38 U/min/mg of protein on 4th week. Whereas the shrimp fed with 0.5 % and 2.0 % *D. salina* incorporated diet showed 0.3 and 0.28 U/min/mg of protein, respectively on 4th week.

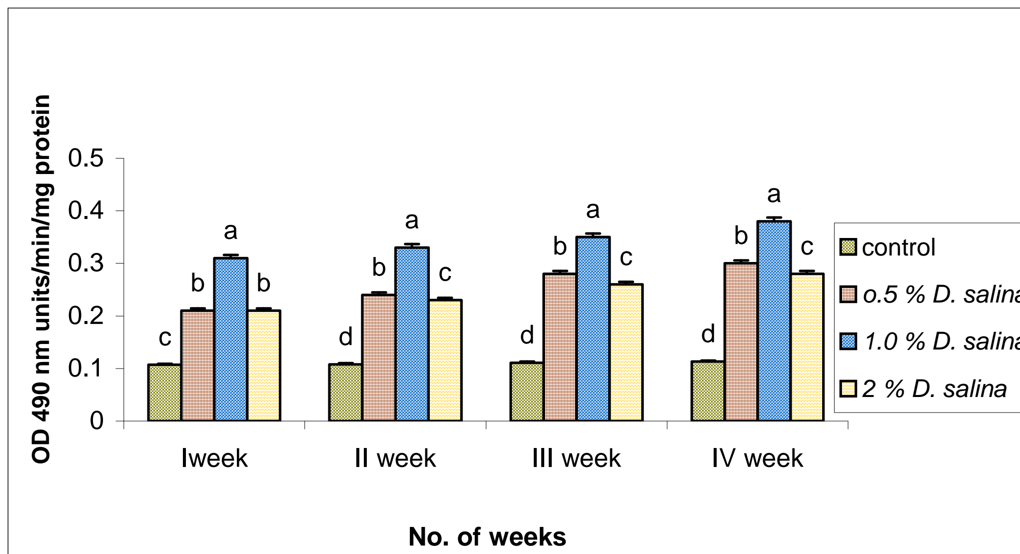


Fig. 1. Prophenol oxidase assay of *P. monodon* fed with *D. salina* incorporated diets

### 3.2. Superoxide dismutase assay

#### 3.2.1. Superoxide dismutase assay of Haemolymph

The SOD levels of the haemolymph of animals when fed with the diet incorporated *D. salina* showed enhanced values to that of control (diet without *D. salina*). The shrimp fed with 1.0 % *D. salina* incorporated diet showed 1.72, 1.9, 1.4 and 1.14 Rapid auto oxidation production (RAP) (Fig. 2), which were more than 28 %, 35 %, 12 % and 7 % to that of the control on 1st, 2nd, 3rd and 4th weeks respectively. Whereas the shrimp fed

with 0.5 % *D. salina* incorporated diet showed 1.2, 1.32, 1.2 and 1.04 (RAP) and 2 % *D. salina* incorporated diet showed 1.6, 1.21, 1.2 and 1.07 (RAP) on 1st, 2nd, 3rd and 4th weeks, respectively.

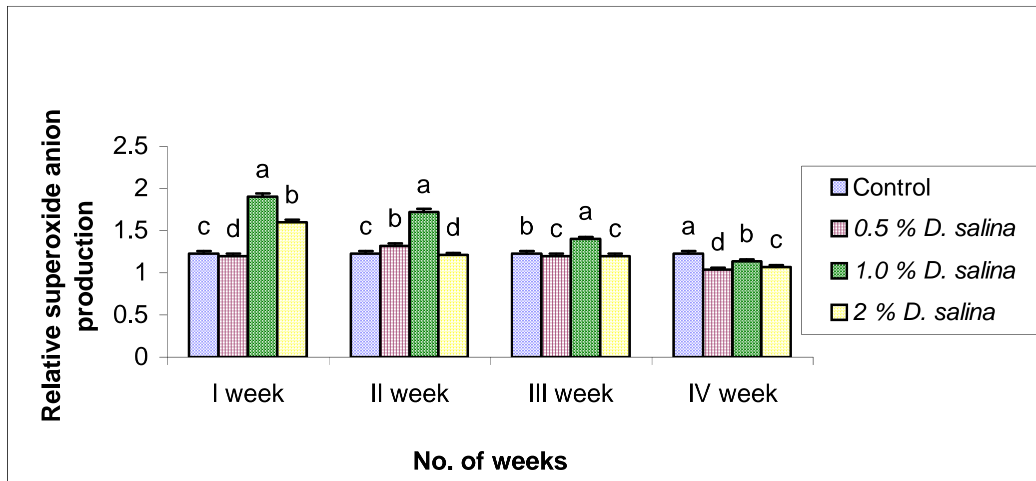


Fig. 2. Superoxide dismutase assay of hemolymph of *P. monodon* fed with *D. salina* incorporated diets

### 3.3. Catalase assay

#### 3.3.1. Catalase assay of haemolymph

The catalase activity of haemolymph in control ranged from 15.46 to 19.23 µmoles $H_2O_2$ decomposed -/min/mg of protein. The shrimp fed with 1.0 % *D. salina* incorporated diet showed the maximum catalase activity of 31.69, 36.69, 25.31 and 15.34 µmoles $H_2O_2$ decomposed /min/mg of protein (Fig. 3) which were more than 51 %, 55 %, 28 % and 20 % to that of the control on 1st, 2nd, 3rd and 4th weeks respectively.

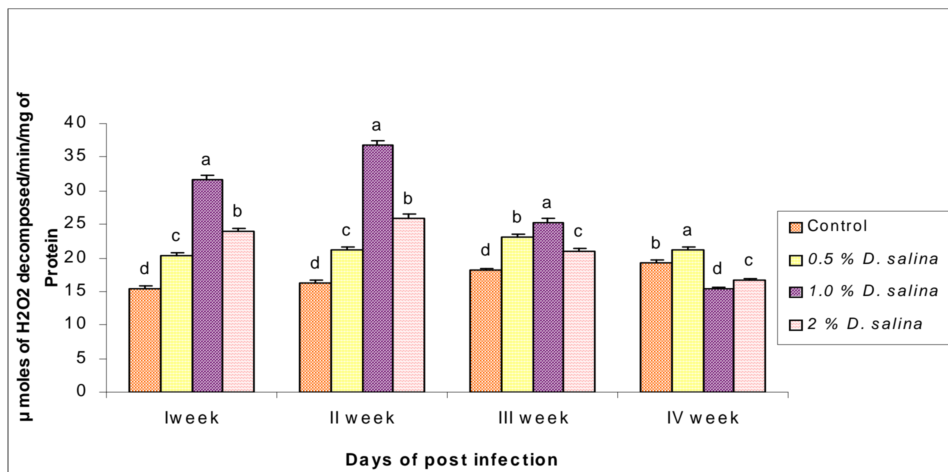


Fig. 3. Catalase assay of hemolymph of *P. monodon* fed with *D. salina* incorporated diets

### 3.4. Challenge test

The shrimp, *P. monodon* fed with three different percentages (0.5 %, 1.0 % and 2.0 %) *D. salina* incorporated diets for a period of 30 days were challenged with WSSV. The animal *P. monodon* fed with *D. salina* incorporated diet without any challenge towards WSSV (negative control) showed 100 % survival till the end of the study period (up to 10th day). Whereas the animal challenged with WSSV infection (positive control) fed without the diet *D. salina* showed 100 % mortality within 48 h. However, the animals fed with 1.0 % *D. salina* incorporated diet showed 40 % mortality on 5th day and 100 % mortality on 10th day. Similarly,

the animals fed with 0.5 % *D. salina* incorporated diet showed 50 % mortality on 5th day and 100 % mortality on 10th day and 2 % *D. salina* incorporated diet showed 55 % mortality on 5th day and 100 % mortality on 10th day.

### 3.5. PCR amplification of genomic DNA of WSSV *P. monodon*

The live animals in treated and negative control groups were WSSV negative by PCR whereas moribund animals from positive group showed WSSV positive by PCR. The animals kept in the tank without WSSV survived during the study period (Data not shown).

### 3.6. Immunostimulant and antioxidant activity of WSSV infected *P. monodon* fed with *D. salina* incorporated diets

#### 3.6.1. Prophenoloxidase assay

The WSSV infected shrimp fed with 1.0 % *D. salina* incorporated diet showed proPO levels of 0.26, 0.31, 0.33 and 0.28 (unit/min/mg of protein) on 1st, 3rd, 6th and 9th day of post infection (Fig. 4) respectively. The above values were less than 16 %, 6 %, 5 % and 26 % respectively, to that of the negative control (without infection). Whereas the infected animals fed with 2 % showed less than 26 %, 36 % and 35 % compared to the animals fed with 1.0 % *D. salina* incorporated diets.

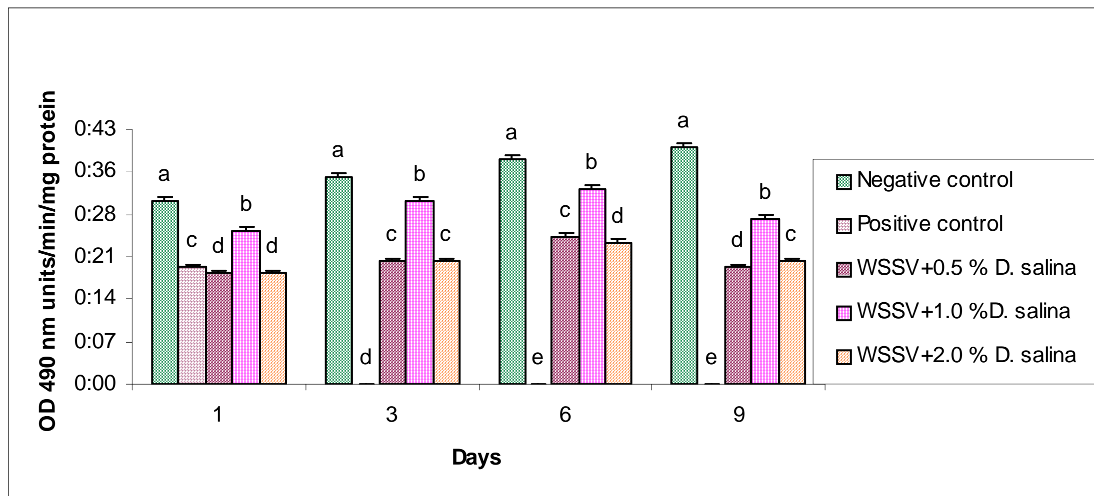


Fig. 4. Prophenol oxidase assay of WSSV infected *P. monodon* fed with *D. salina* incorporated diets

### 3.7. Superoxide dismutase assay

#### 3.7.1. Superoxide dismutase assay of haemolymph

The SOD levels in the haemolymph of WSSV infected shrimp fed *D. salina* at different concentrations revealed that the shrimp fed with 1.0 % *D. salina* incorporated diet showed the SOD activity of 2.46 RAP on 3rd day (Fig. 5), which was less than 12 % to that of negative control (without WSSV infection) (2.82 RAP). Whereas the SOD levels of the infected shrimp fed with 0.5 % and 2 % *D. salina* incorporated diet showed less than 29 % and 56 % when compared to negative control.

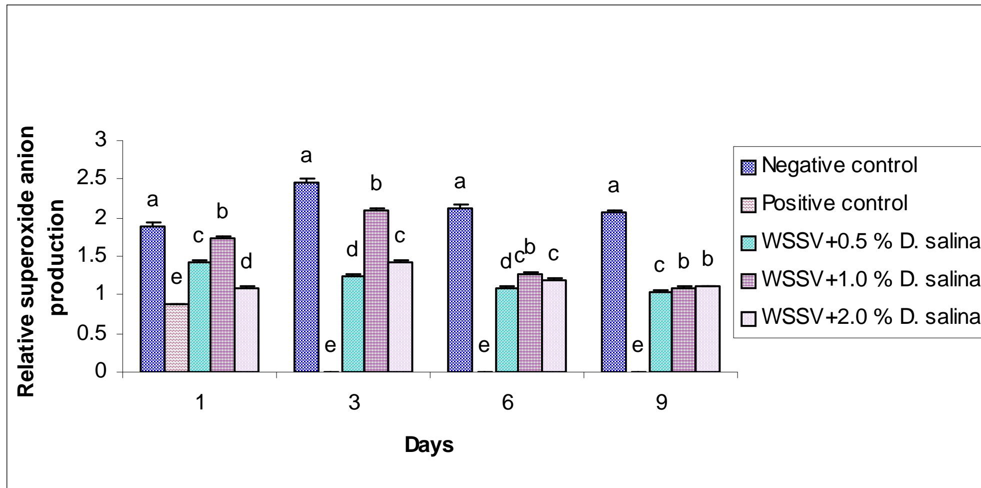


Fig. 5. Superoxide dismutase assay of haemolymph of WSSV infected *P. monodon* fed with *D. salina* incorporated diet

### 3.8. Catalase assay

#### 3.8.1. Catalase assay of haemolymph

The catalase activity in haemolymph of WSSV infected shrimp fed with 1.0 % *D. salina* incorporated diet showed the catalase level of 25.46 µmoles $H_2O_2$ decomposed /min/mg of protein on 6$^{th}$ day (Fig. 6) which was more than 35 % and 43 % of the animals fed with 0.5 % and 2 % *D. salina* incorporated diets, respectively. Whereas the animals without WSSV infection (negative control) fed with *D. salina* incorporated diet showed 31.69, 36.69, 25.31 and 15.34 µmoles $H_2O_2$ decomposed /min/mg of protein on 1$^{st}$, 3$^{rd}$, 6$^{th}$ and 9$^{th}$ day, respectively.

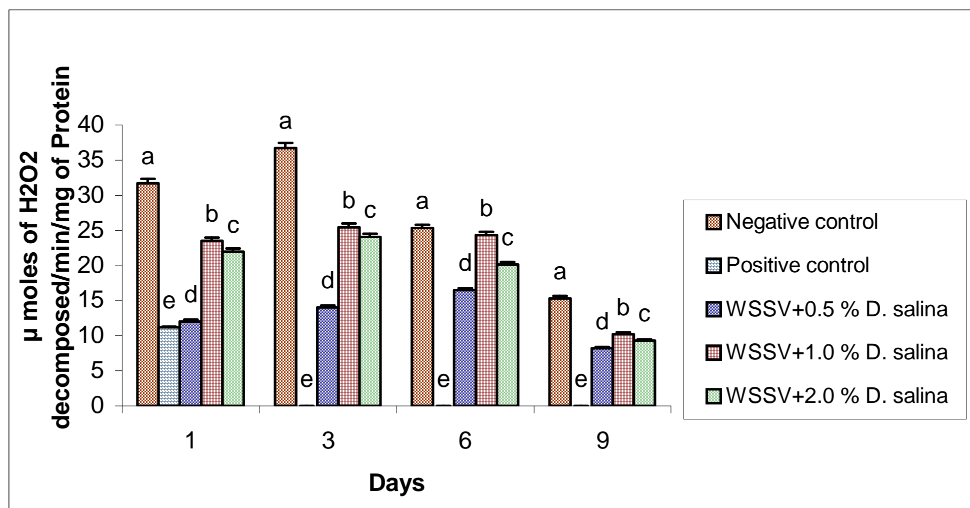


Fig. 6. Catalase assay of haemolymph of WSSV infected *P. monodon* fed with *D. salina* incorporated diet

## 4. Discussion

The color desired by the consumer for shrimp in aquaculture is by feeding the diet supplemented with carotenoids. Irrespective of whether the shrimp is fed with astaxanthin or β-carotene, main carotenoids accumulated is astaxanthin in free and esterified form. Boonyaratpalin *et al.,* (2001) stated that *P. monodon* has the metabolic ability to convert β-carotene into astaxanthin. Since β-carotene acts as an antioxidant, it is used in the feed supplement of shrimps and fishes in aquaculture. Hence in this study, *Dunaliella salina* of three

different concentrations viz: 0.5 %, 1.0 % and 2.0 % when incorporated in the diet and fed to shrimp showed that the growth of *P. monodon* was found similar among the three concentrations. When compared to the control (without *D. salina* diet) 1.0 % diet showed better growth. Similarly, Boonyaratpalin *et al.,* 2001 reported a higher survival rate and growth in *P. japonicus* fed with astaxanthin-supplemented diets than supplement of β-carotene or algal meal.

Carotenoids pigments are involved in antioxidants activities in aquatic animals and moreover carotenoids are known to enhance immune function and disease resistance in higher animals as stated by (Hunter, 2000, Supamattaya *et al.,* 2005). Phenoloxidase (PO), the key enzyme in the synthesis of melanin, occurs in haemolymph as an inactive proenzyme prophenoloxidase (proPO). ProPO is activated to form PO (phenol oxidase) when it reacts with zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ions, trypsin, or heat (Soderhall *et al.,* 1986). Hence an attempt was made on the present study with *P. monodon* fed with β-carotene producing *D. salina* incorporated diets for a period of 30 days, and also challenged against WSSV infection. The animal without WSSV infection but fed with *D. salina* incorporated diet showed 100 % survival till the experimental study indicated its non-toxic effects. It is worthwhile to mention that the control animals (fed without *D. salina* but with WSSV infection) showed 100 % mortality within 48 h. However, the animal fed with 1.0 % *D. salina* incorporated diet challenged with WSSV showed 40 % mortality on $5^{th}$ day followed by 100 % mortality at the end of $10^{th}$ day. Thus indicating that the possible role of *D. salina* for protecting the animals to WSSV to certain extent.

Screening of *Penaeus monodon* shrimp for WSSV is one of the effective ways to check the vertical transmission of disease through the hatcheries (Hsu *et al*., 1999). Henceforth, efforts were initiated to check the presence of WSSV through PCR amplification in *P. monodon*. In the present study all the dead animals showed amplification with the primer corresponded to WSSV confirmed that their mortality was due to WSSV infection. Instead the animals kept in the tank without WSSV survived well during the study period.

In the present study, WSSV treated with three concentration of *D. salina* incorporated diet by in vivo methods showed that proPO, O2 _ were all significantly ($P < 0.05$) higher than those of the WSSV infected and control groups. This was especially true for the treated shrimp group where values were greater than those of the control groups. Albores *et al.,* (1993) stated that phenoloxidase (PO) is the terminal enzyme in the proPO system of the arthropod defence system and acts as both recognition and effector component, by promoting cell-to-cell communication and subsequently eliminating pathogens. The active materials formed during the activation of proPO stimulate several cellular defence reactions, including phagocytosis, nodule formation, hemocyte locomotion, non-self recognition and other immune reactions (Johanson *et al.,* 2000). Activated phenoloxidases generate high cytotoxic quinines that can inactivate viral pathogens (Ourth *et al.,* 1993). In vivo administration of 1.0 % *D. salina* live cells incorporated diet enhances immunity of tiger prawns which showed a significantly ($P < 0.05$) higher proPO concentration than the control and WSSV infected groups. After viral challenging, the shrimp with 1.0 % feed showed value initially increased and reached the maximum level at 24 h p.i. and $6^{th}$ day of in vivo experiment respectively. WSSV infected shrimp groups showed increased levels of proPO levels; 48 h p.i. the group showed increased levels of proPO which were high in the level seen in healthy shrimp. These results indicate that a high level of proPO enhances resistance in shrimp against WSSV. Our experiment, *D. salina* live cells incorporated diet treated group PO seems to act as a promoter of the shrimp immune system by enhancing the pigmentation, increasing the O2 _ production and SOD activity, and then

postponing the dead of WSSV Shrimp to some extent. Similar findings were observed by Felix *et al*., 2004 noticed highest proPO activity was observed in WSSV infected *P. monodon* fed with *Sargassum wightii* (seaweed). A gradual increase of proPO activity was observed up to $6^{th}$ day after challenging with *Vibrio parahemolyticus* thereafter there was a gradual decrease in the activity. Balasubramanian *et al*., (2008) reported that increased levels of proPO assay were observed in shrimp fed with plant derived antiviral compound from *Cyanodon dactylon.*

Super oxide dismutase (SOD) is one of the main antioxidant defence enzymes generated in response to oxidative stress. Sarathi *et al*., (2007) and Mohankumar and Ramasamy (2007) observed the activity of SOD was significantly lowered in WSSV-infected *F. indicus*. Also in the present study, the activity of SOD was significantly lowered in the WSSV-infected hemolymph of *P. monodon,* whereas *D. salina* incorporated diet - treated in vivo shrimp significantly recovered when compared with control animals. These results concur with the findings of Lin and Chang *et al*., 2003 who have found that SOD decreases in WSSV infected *P. monodon*. In the present study, SOD level in the Haemolymph of shrimp fed with three different concentrations of *D. salina* incorporated diet showed high levels of SOD when compared to control (without *D. salina*). Nakano *et al.,* (1999) observed for the first time that astaxanthin (algal pigment) supplementation in diet fed to rainbow trout influenced liver function and increased SOD defensive potential against oxidative stress. Similarly, Chang *et al.,* 2003 observed that the shrimp fed with b-glucan (BG) diets showed significantly higher levels of $O_2$ concentration than the BG free group as observed in shrimp treated with *C. dactylon* plant extract. Holmblad and Soderhall (1999) observed that SOD is related to immunity in crustacean. The high level of $O_2$ in *P. monodon* fed with *D. salina* incorporated diets indicated that the alga may be the potential immunostimulant.

Mohankumar and Ramasamy, (2007) observed that hydrogen peroxide is toxic to cells and catalase is a major primary antioxidant defense component that catalyses the decomposition of $H_2O_2$ which is produced by the action of superoxide dismutase to $H_2O$ The present study revealed that the catalase assay of haemolymph of *P. monodon* fed with three different concentrations of *D. salina* incorporated diets showed increased levels of catalase when compared to control. In the present study, the activity of catalase in haemolymph of WSSV infected *P. monodon* fed with 1.0 % *D. salina* incorporated diet showed less than 30 % to that of the negative control.

In conclusion, this study suggests that increasing proPO activity, the superoxide anion production and catalase production of WSSV infected *P. monodon* showed lower activities than the healthy shrimp (without WSSV infection). Whereas the SOD of Haemolymph of WSSV infected *P. monodon* fed with *D. salina* incorporated diet showed a slightly less activity when compared to the shrimp fed with *D. salina* without WSSV infection. The above results indicated that the shrimp fed with *D. salina* incorporated diet showed a slightly enhancement in immune resistance towards WSSV infected *P. monodon*

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