



Development of bioactive feed from a marine based *Actinomycete, Bacillus licheniformis* for treating *Aeromonas hydrophila* infection in freshwater prawn, *Macrobrachium rosenbergii*

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Abstract

The bioactive compounds from *Actinomycetes* have antibacterial, anticancer, antioxidant, and antifungal properties. The antioxidant activity of ethyl acetate extract of *Bacillus licheniformis* was determined by carrying out several assays. Preparation of a bioactive feed encapsulated with sodium alginate and chitosan was carried out for treating the bacterial infection in *Macrobrachium rosenbergii*. An experimental trial with three different groups was conducted to determine the effect of crude extract of *B. licheniformis* against pathogenic bacteria, *Aeromonas hydrophila* in freshwater prawn, *M. rosenbergii*. The first group of prawns was given commercial diet. The second group of prawns challenged with *A. hydrophila* was given commercial feed. The third group of *A. hydrophila* challenged prawns was given the encapsulated *B. licheniformis* feed. The survival percentage of prawns was decreased in group 2 compared to the prawns of group 1 and group 3. The antioxidant properties were found to be significantly increased in case of group 3 prawns. Real time Reverse transcription - Polymerase chain reaction (RT-PCR) study of three different groups with three immune genes - Superoxide dismutase (SOD), Crustin and Prophenoloxidase was carried out and antioxidant enzymatic activity, and immunological parameters were recorded. Group 1 was designated as control, injected with saline. Group 2 was designated as infected, challenged with *A. hydrophila* and group 3 as treated, challenged with *A. hydrophila* and injected with crude extract of *B. licheniformis*. The results revealed that the SOD and Prophenoloxidase of gills and hemolymph showed highest gene expression in the case of treated group compared to the infected and control group. The data indicated that *B. licheniformis* showed enhanced antioxidant activities, immune response, disease resistance, and gene expression in *M. rosenbergii* challenged with *A. hydrophila*.

Keywords *Bacillus licheniformis* · Antibacterial · Antioxidant activity · Immune genes · RT-PCR · *Macrobrachium rosenbergii*

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Introduction

Actinomycetes are widely found in both marine as well as terrestrial ecosystems. Marine *Actinomycetes* are the source of various bioactive secondary metabolites like antibacterial, antiviral, and anticancer compounds, pigments, antibiotics, plant growth hormones, enzymes, and single-cell protein (SCP) feed. The bioactive compounds extracted from *Actinomycetes* have unique dynamic chemical structures, diverse functional, biological activities, and particular microbial producers (Selim et al. 2021). Till date, 83 novel species of marine *Actinomycetes* belonging to 28 genera have been reported from marine habitats. Moreover, several novel bioactive compounds were clinically investigated that demonstrated non-toxicity and no side effects (Mikami 2007; Roshan et al. 2014).

Iber and Kasan (2021) reported that aquaculture is regarded as the fastest growing industry in the past few decades having huge potential to arrest the increasing demand of shrimps by making a constant halt in overfishing. India being the largest aquaculture products producing country reported a production of approximately 10.79 million tons as per the FAO. The shrimp culture plays a pivotal role in contributing to the global economy (Sharma et al. 2023). Shrimp culturing has been considered as a traditional occupation for ages (Kumar et al. 2023). Moreover, the emerging diseases in prawns and other aquatic animals has impacted the aquaculture industry in a significant way globally. The usage of antibiotics, and other chemicals indiscriminately is causing resistance to diseases. Therefore, it has become imperative to find an alternative to antibiotics with less or no side effects for treating infections in prawns.

Abdelfattah et al. (2016) isolated a marine-derived *Actinomycetes* from the marine sediments of the Red Sea coast. They reported that the isolation of crude extract of *Streptomyces* sp. and *Nocardioopsis* sp. showed antimicrobial activity against several human pathogenic bacteria like *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Salmonella typhi*, *Staphylococcus hemolyticus* and *Candida tropicalis*. Another study by Siddharth et al. (2021) reported an antibacterial and anticancer compound, 1-acetyl-4-(4-hydroxyphenyl) piperazine, a diketopiperazine from the marine sediments of *Nocardioopsis* sp. SCA30. The ethyl acetate extract of *Nocardioopsis* showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* ATCC NR-46171 and NR-46071. Mondal et al. (2023) reported an *Actinomycete*, *Bacillus licheniformis* isolated from marine sediments. They demonstrated that the bioactive compounds extracted from *Bacillus licheniformis* were found to have good antibacterial activity against *Aeromonas hydrophila* and *Vibrio parahaemolyticus*. The production of bioactive compounds with antimicrobial properties from *Actinomycetes* can help in overcoming the problems of antibiotics resistance.

Microencapsulation by extrusion method is a technique by which the water-soluble bioactive compounds of the extract can be fixed compactly to a Sodium-alginate (Na-alginate) and chitosan biopolymer containing matrices. This novel encapsulation method is applied to prevent the bioactive compounds of the extract from oxidation and degradation. This microencapsulated method of development of bioactive products is reported to be stable, retaining their activity for longer duration (Helen Fitton 2003).

The present work focuses on determining the antioxidant activity of the ethyl acetate extract of *B. licheniformis* by carrying out 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging, metal chelating, hydrogen peroxide (H₂O₂) scavenging, hydroxyl radical scavenging, reducing power, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) scavenging and total antioxidant assays. The effect of bioactive compounds present in *B. licheniformis* was estimated. They were encapsulated in a biopolymer matrix to check their

antioxidant and antibacterial effect on *A. hydrophila*. A feeding experimental trial involving 3 different groups of *M. rosenbergii* was carried out to check the survival and mortality rate of the experimental prawns. The immunological parameters and antioxidant enzymatic activities of *B. licheniformis* at different concentrations was determined in 3 different groups of *M. rosenbergii* challenged with *A. hydrophila* infection. The expression of immune genes like crustin, SOD and Prophenoloxidase was estimated in control, treated and infected groups of *M. rosenbergii* challenged with *A. hydrophila* infection by RT-PCR. In addition, a bioactive feed containing protein macromolecule was formulated to treat the bacterial infections and serve as a feed in prawn diet.

Material and methods

Crude extract preparation

B. licheniformis was isolated from marine sediments and showed good antibacterial activity against *A. hydrophila* and *Vibrio parahaemolyticus*. The crude extract of potential *B. licheniformis* strain was prepared according to the method mentioned by (Mondal et al. 2023; Rajkumar et al. 2018). The crude extract of *B. licheniformis* was extracted and dried in hot air-oven at 40–45°C. The different stock concentrations of the crude extract were prepared at 10, 50, 100, 150, 200, 250, 300, and 350 µg/mL to carry out the antioxidant assays.

In vitro antioxidant activity of *Bacillus licheniformis*

The antioxidant activity of *B. licheniformis* was determined by carrying out using several assays.

DPPH scavenging assay

The broth culture of *B. licheniformis* isolate was centrifuged at 5 000 ×g for 10 mins. The cell free supernatant was collected to estimate the DPPH radical scavenging activity of the bacterial isolate. At first 2 ml of 0.1 mM DPPH reagent prepared in ethanol was added in each of the test tubes in the dark. Then different concentrations of the cell free supernatant – 50, 100, 150, 200, 250, 300, and 350 µg/mL were taken, and the volume was made upto 1 mL with deionized or distilled water, and added to the test tubes. The mixture was vortexed and incubated at room temperature in dark condition for 30 mins. The antioxidant activity of the DPPH is determined by the reduction of DPPH and the decrease in the absorbance. The absorbance of the control and samples was recorded at 517 nm using UV Spectrophotometer (Revathy et al. 2013). The experiment was performed in triplicates. The percentage of the radical scavenging activity of the bacterial extract was determined using the formula:

$$\text{Radical scavenging activity (\%)} = [(A_C - A_S) / A_C] \times 100$$

Where, A_C and A_S represent the absorbance of control and samples respectively.

Metal chelating assay

The chelating activity of the ferrous ions of the *B. licheniformis* extract was estimated by following the method of Dinis et al. (1994) with some modifications. 50 µL of cell free supernatant of bacterial culture at different concentrations (50 µg/mL – 350 µg/mL) was dissolved in 1.6 mL of deionized /distilled water. 0.05 ml of FeCl₂ (2 mM) was added to each of the test tubes and mixed properly. 0.1 mL of ferrozine solution (5 mM) was added later to the mixture to begin the reaction, shaken vigorously, and left standing at room temperature for 10 mins. The absorbance of the control and test samples was recorded at 562 nm using a UV Spectrophotometer (Dholakiya et al. 2017). The chelating ability of the ferrous ions was noted.

The inhibition percentage of the Fe²⁺ - Ferrozine complex in the bacterial extract was calculated as:

$$\text{Inhibition (\%)} = [(A_C - A_S) / A_C] \times 100$$

Where, A_C and A_S represent the absorbance of control and samples respectively.

Total antioxidant assay

Total antioxidant activity of the bacterial culture was estimated using the method reported of (Prieto et al. 1999). 0.3 mL of each test sample prepared at different concentrations (50 µg/mL – 350 µg/mL) was added to the 3 mL reagent solution :- sodium phosphate (28 mM), sulfuric acid (0.6 M), and ammonium molybdate (4 mM). The reaction mixture was kept in waterbath at 95°C for 90 mins for incubation. The absorbance of the control and test samples was taken at 695 nm. Ascorbic acid (100 µg/mL) was prepared at different concentrations (50 µg/mL – 350 µg/mL) and taken as standard.

H₂O₂ radical scavenging assay

The scavenging activity of H₂O₂ was determined by following the method of Ruch et al. (1989) using the *B. licheniformis* extract with some modifications. The reaction mixture containing different concentrations of 1 ml of bacterial culture (50 µg/mL – 350 µg/ml) was added to 2.5 mL of phosphate buffer (100 mM, pH – 7.4) and 400 µL of H₂O₂ (5 mM) and incubated at room temperature for 20 mins. The reaction mixture was compared with standard Ascorbic acid (mg/mL) and the absorbance was read at 610 nm using UV-visible spectrophotometer (Keser et al. 2012; Mishra et al. 2015; Patel et al. 2016).

The inhibition percentage was calculated as:

$$\text{Inhibition (\%)} = [(A_C - A_S) / A_C] \times 100$$

Where, A_C and A_S represent the absorbance of the reaction mixture without bacterial extract (control) and absorbance of the reaction mixture with bacterial extract (samples) respectively.

Hydroxyl radical scavenging assay

The reaction mixture containing 1 mL of ferrous sulphate (FeSO_4) (1 mM), 0.3 mL of Sodium salicylate (20 mM), and 0.7 mL of H_2O_2 (6 mM) was added to each of the test tubes. Aliquots of varied concentrations of bacterial culture from 50 $\mu\text{g}/\text{mL}$ – 350 $\mu\text{g}/\text{mL}$ were added to the reaction mixture. The test tubes containing the reaction mixtures with samples were incubated at room temperature (37°C) for 1 h. The absorbance of the reaction was measured at 562 nm.

The scavenging activity of hydroxyl radical was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = [(A_C - A_S)/A_C] \times 100$$

Where, A_C and A_S represent the absorbance without bacterial extract (control) and absorbance with bacterial extract (samples) respectively.

Reducing power assay

The reducing ability of the ferrous ions in *B. licheniformis* extract was assessed by the method of Oyaizu (1986). 0.1 mL of various concentrations of test samples (50 $\mu\text{g}/\text{mL}$ – 350 $\mu\text{g}/\text{mL}$) were dissolved in 1 ml of phosphate buffer (pH – 7.4), and mixed properly. 1 mL of 1 % potassium ferricyanide was added to the mixture, and incubated in water-bath at 50°C for 20 mins. The test tubes containing the reaction mixture were cooled to room temperature and mixed with 1 ml of deionized water and 1 mL of 10 % trichloroacetic acid (TCA). 0.1 mL of 0.1% ferric chloride (FeCl_3) was added, and incubated for 10 mins. After the incubation period, the absorbance was read at 700 nm (Chang et al. 2002; Karthik et al. 2013). The increase in the absorbance and the concentration of the reaction mixture indicated the greater reducing power activity.

ABTS radical cation scavenging assay

ABTS radical scavenging assay was assessed by following the method of Ser et al. (2016). The ABTS solution was prepared by adding an equal volume of ABTS (7 mM) stock solution to potassium persulfate solution (2.45 mM) (HiMedia), and incubated in dark condition for 12-16 hrs. The oxidation of ABTS stock solution with potassium persulfate after the incubation period forms ABTS cation complex. 1 mL of ABTS stock solution was added to 50 μL of varied concentrations (50 $\mu\text{g}/\text{mL}$ – 350 $\mu\text{g}/\text{mL}$) of the *B. licheniformis* extract. The reaction mixture was incubated at room temperature for 7 mins. The absorbance was read at 730 nm.

The radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = [(A_C - A_S)/A_C] \times 100$$

Where, A_C represents the absorbance of control and A_S the absorbance of test samples respectively.

Bacterial pathogen used in the study

Aeromonas hydrophila strain was obtained from Gene Bank and Microbial Type Culture Collection (MTCC 1739). The pathogenicity and treatment experiments were carried out as per the protocol mentioned by Mondal et al. (2023). It was maintained at 4°C for further experimental use. The pathogen was inoculated in nutrient broth maintaining aseptic conditions. The inoculum was kept in a rotary shaker and incubated at 37°C overnight and stored in cell frost for further use in the study.

Preparation of bacterial suspension

The bacterial suspension of *B. licheniformis* was prepared based on the protocol of Mondal et al. (2023).

Preparation of bioactive feed encapsulated with Na-alginate and chitosan

Preparation of bioactive feed by encapsulating crude extract of *B. licheniformis* in sodium alginate and chitosan was carried out using extrusion method (Trabelsi et al. 2013). 25 mL of crude extract was added to 2% of 100 mL Na-alginate. The suspension with Na-alginate mixture was kept in a magnetic stirrer overnight and mixed together using a magnetic bead. It was dropped from a distance of 1 m using a 5 mL sterile disposable syringe into 200 mL of CaCl₂ solution (0.45M). The beads were stirred for 40 mins to remove the unbound particles and collected using a sterile net strainer or Whatman filter paper.

The filtered beads were washed twice with sterile water and transferred to a beaker containing 1% chitosan (w/v) solution in 1% glacial acetic acid (w/v). The encapsulated alginate-chitosan beads were stirred for 45 mins to uniformly coat the surface of beads. The encapsulated beads were separated and washed twice with sterile water to remove the unbound chitosan. The beads were air dried and stored at 4°C.

Experimental animals

Healthy freshwater prawns, *Macrobrachium rosenbergii* with body weight averaging 25 ± 2 g, were collected from a nearby farm in Chengalpattu district near Chennai, India. The clinical symptoms of the prawns were checked and transported in a live condition maintaining continuous aeration. They were allowed to acclimatize for 1 week in laboratory conditions in fiber tanks of 5000 L capacity containing freshwater. They were fed regularly with commercial feed containing nutrients and maintained under aseptic conditions for further investigation.

Feed treatment

The pathogenicity and treatment studies of *A. hydrophila* challenged with crude extract of *B. licheniformis* were carried out according to the procedure followed by (Thani-gaivel et al. 2019; Mondal et al. 2023). A feeding trial experiment with three groups was conducted. Group 1 was fed with control feed (without bacterial suspension), Group 2

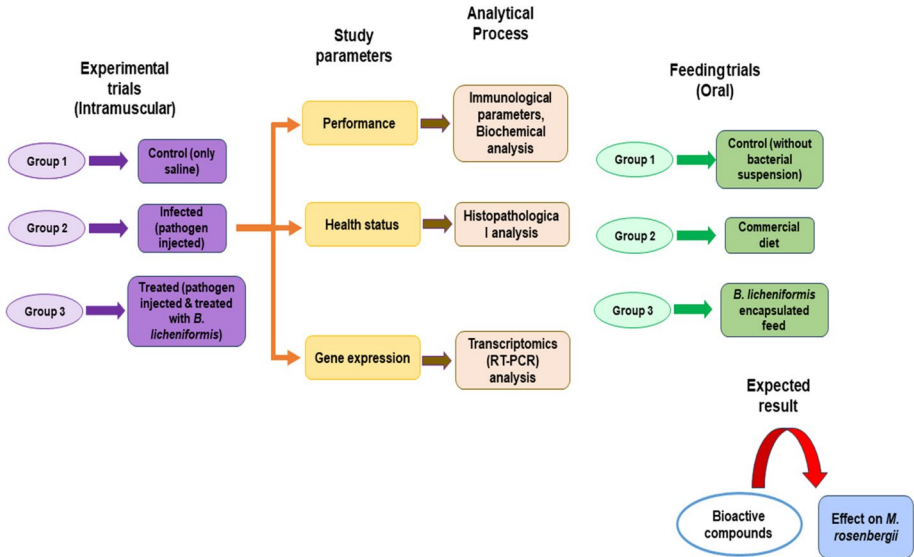


Fig. 1 Schematic representation of experimental design of the trials conducted, groups and their treatment

was given the commercial diet and Group 3 was fed with *B. licheniformis* encapsulated feed. This experiment was carried out in glass tanks and in triplicates. 9 prawns were used per group and the water was changed periodically, maintaining the water quality parameters. All the prawns were fed twice a day for 5 days. Later, group 2 and group 3 were challenged with 20 µL of *A. hydrophila* culture. The survival rate of the prawns was recorded after *A. hydrophila* injection for one month. The schematic diagram of the experimental trials, groups and treatment are represented in Fig. 1.

Antioxidant enzymatic activity and Immunological parameters of experimental prawns

Different antioxidant and immunological parameters were assessed to determine the enzymatic activities in hemolymph and various organs – gills, tissue and hepatopancreas of infected *M. rosenbergii* and compared with control and treated prawns. The prawns were sterilized with ethanol and hemolymph was drawn from the tail region or ventral sinus cavity of prawns using a 2 mL sterile disposable syringe containing an anticoagulant solution. Gills, tissue, and hepatopancreas were collected from the prawns of all 3 groups. These organs were homogenized using a sterile mortar and pestle with 1-2 mL of phosphate buffer. The homogenates were centrifuged at 6 500 ×g for 5 mins at 4°C. The supernatant was collected and stored at -20°C for further experimental study.

SOD activity

The SOD activity in the gills, tissue, hepatopancreas, and hemolymph of all the 3 groups of prawns of was interpreted according to the method of Kono (1978). The reaction mixture was prepared with 50 µL of Triton X-100 (0.6%), 250 µL of Nitroblue tetrazolium

(NBT) (96 μM), and 0.65 mL of sodium carbonate (NaCO_3) buffer (pH – 10.0). 50 μL of hydroxylamine hydrochloride (20 mM; pH – 6.0) was added to initiate the reaction. 35 μL of supernatant of all the organs were added after 5 mins. Absorbance was measured at 540 nm using UV- visible Spectrophotometer. The inhibition percentage of the superoxide radicals was determined depending on the increase in the absorbance rate.

Peroxidase (POD) activity

64 μL of Potassium buffer (0.1 M; pH – 6.0), 64 μL of the substrate pyrogallol (5%) (w/v), 32 μL of H_2O_2 (0.147 M) and 420 μL distilled water was added to make the reaction mixture. 20 μL of hemolymph, gills, tissue, and hepatopancreas was added to the reaction mixture. The sample was mixed vigorously with the reaction mixture and the absorbance taken at 420 nm using UV – visible Spectrophotometer after 20 secs (Laria Lamela et al. 2005).

Estimation of total plasma protein

The concentration of protein present in the three different groups of *M. rosenbergii* was estimated by Bradford method (Bradford 1976). 5 μL of supernatant of all the three different organs were taken in test tubes and 250 μL of Bradford reagent was added to them. Bovine Serum Albumin (BSA) was taken as a protein standard. The samples were incubated for 10 min at room temperature. The absorbance of standard and test samples was recorded spectrophotometrically at 630 nm.

The hemolymph was centrifuged at 4°C, 700 \times g for 10 mins. 5 μL of the supernatant was added to the Bradford reagent. Based on the Bradford method (Bradford 1976), the total plasma protein present in hemolymph was evaluated in 3 different groups of prawns. BSA was taken as protein standard.

Catalase (CAT) activity

The enzymatic activity of catalase for tissue, hepatopancreas, gills, and hemolymph was evaluated by the method of Xu et al. (Xu et al. 1997) with slight modifications. 10 μL of enzymatic extracts of samples was added to 3 mL of H_2O_2 prepared in phosphate buffer (pH – 7.0). The reaction mixture was mixed properly and incubated at 25°C. The change in the absorbance of H_2O_2 was recorded at 240 nm using UV – visible Spectrophotometer after 1 minute interval for 3 mins.

Reduced glutathione (GSH) activity

The reduced GSH level was estimated according to the Ellman method (Ellman 1959). 100 μL of tissue, gills, and hepatopancreas homogenates, and hemolymph was added with an equivalent volume of tri-chloro acetic acid (TCA) (5%) in 1:1 ratio. The mixture was allowed to precipitate and centrifuged at 5000 \times g at 4°C for 10 mins. 200 μL of supernatant was taken after centrifugation, and 1.8 mL of Ellman's or 5,5'-dithobis-(2-nitrobenzoic acid) (DNTB) reagent (0.1 mM) prepared in phosphate buffer (0.3 M) was added. The DNTB molecules were reduced to 2-nitro-5-benzoic acid by the thiol residues of glutathione and the absorbance was recorded at 412 nm using UV – visible Spectrophotometer.

Phenol oxidase activity

The phenol-oxidase activity of the hemolymph, tissue, gills, and hepatopancreas homogenates was measured according to the method of Liu et al. (2004) with some modifications. The hemolymph and the homogenates were centrifuged at 4°C, 700 × g for 20 mins. 8 µL of supernatant of different organs and hemolymph was added to 400 µL of ice-cooled Phosphate buffer saline (PBS) (pH = 7.4). The samples mixed with PBS were vortexed properly. 100 µL aliquot of the sample-PBS mixture was added to equivalent volume of L-dihydroxyphenylalanine or levodopa (L - DOPA) (20 mM), and incubated at 25°C for 30 mins. The amount of phenol-oxidase present in the test samples was determined by the formation of dopachrome from L - DOPA, measured spectrophotometrically at 492 nm.

Field emission scanning electron microscopy

The beads stored at 4°C were dried overnight for 24 h to remove the moisture content for analysis. The size, shape and surface morphology of the alginate and alginate-chitosan coated *B. licheniformis* extract beads were analysed by field emission scanning electron microscopy (FESEM).

The beads were gold sputtered and mounted on aluminium studs. The sample beads were loaded and viewed under the FESEM. The images of the both the exterior and interior part of the beads were observed under different magnifications.

Immune gene expression in *M. rosenbergii* using RT-PCR

Sample preparation

Tissue, gills, and hepatopancreas were taken out from 3 different set of prawns - control, infected and treated groups. 100 mg of test samples were collected and homogenized with 1 mL of anti-buffer. The mixture was centrifuged at 5 000 ×g at 4°C for 10 mins. The supernatant was collected for further experiment. Similarly, the hemolymph was drawn using a sterile syringe from all the 3 groups of prawns and anticoagulant was added.

Total RNA extraction

200 µL of test samples was submerged in 600 µL of Trizol reagent, and incubated for 5 mins. 200 µL of chloroform was mixed, shaken vigorously, and kept in either ice for 15 mins or at -20°C for 5 mins. The RNA was treated with DNase I. The mixture was spun at 12000 ×g at 4°C for 20 mins to remove the DNA and debris. The upper aqueous phase was carefully collected in a separate microcentrifuge tube without disturbing the interphase to collect the RNA. 400 µL of ice-cold isopropanol was added to the aqueous phase to precipitate the RNA and spun at 12000 ×g at 4°C for 15-20 mins. The supernatant was discarded, pellet washed with 200 µL of ice-cold 70% ethanol prepared in diethylpyrocarbonate (DEPC)-treated double distilled water, and spun at 12 000 ×g at 4°C for 10 mins. The pellet was dried, resuspended with 20 – 60 µL of DEPC-treated double distilled water.

RNA was quantified using a Nanodrop spectrophotometer (Biorad) to determine the final RNA concentration. The purity of the RNA was assessed by performing agarose gel electrophoresis.

Complementary DNA (cDNA) synthesis

cDNA synthesis was carried out on 3 µg concentration of RNA in a 20 µL of total volume using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV RT). Based on the quantified concentration, the RNA sample was added to 2 µL of oligo(dT) and random hexamer mix, and the volume was made up using Molecular biology grade water. The cDNA synthesis was carried out in 1 cycle – denaturation at 25°C for 5 mins, annealing at 42°C for 60 mins, and extension at 70°C for 5 mins. The cDNA samples were stored at -80°C.

Primer design

The primers were designed and are tabulated in the Table (supplementary file).

Immune gene expression analysis by RT-PCR

Immune genes – Crustin, Prophenoxidase, and SOD were used to determine the gene expression level of the 3 groups of different prawns. Housekeeping gene, β -actin was used first to check the gene expression level of each of the test samples.

RT-PCR was carried out in a 20 µL of total volume that included 1 µL of cDNA, 3 µL of each forward and reverse primers (25 nM each), 3 µL of molecular grade water, and 10 µL of SYBR green master mix (HiMedia). Reactions were performed in duplicates on Insta Q96 RT-PCR machine (HiMedia). The reaction was carried out in 40 cycles – initial denaturation at 95°C for 10 mins, denaturation at 94°C for 10 secs, annealing at 60°C for 45 secs, and extension at 72°C for 30 secs. The melting temperature was set according to the oligo nucleotide synthesis report given by the manufacturer. The results were obtained from MS-Excel and were analyzed. The quantification of gene expressions was evaluated using comparative C_t method. The difference between the infected and treated was compared to the control (only saline) to determine expression level of the genes for all the samples.

Statistical analysis

A statistical software GraphPad Prism version 8.0.1 was used to perform the One-way Analysis of variance (ANOVA). The results were analyzed as the means of standard deviation (S.D). The data was interpreted using one-way ANOVA. The column comparison ($P < 0.05$) for group comparison was taken as statistically significant.

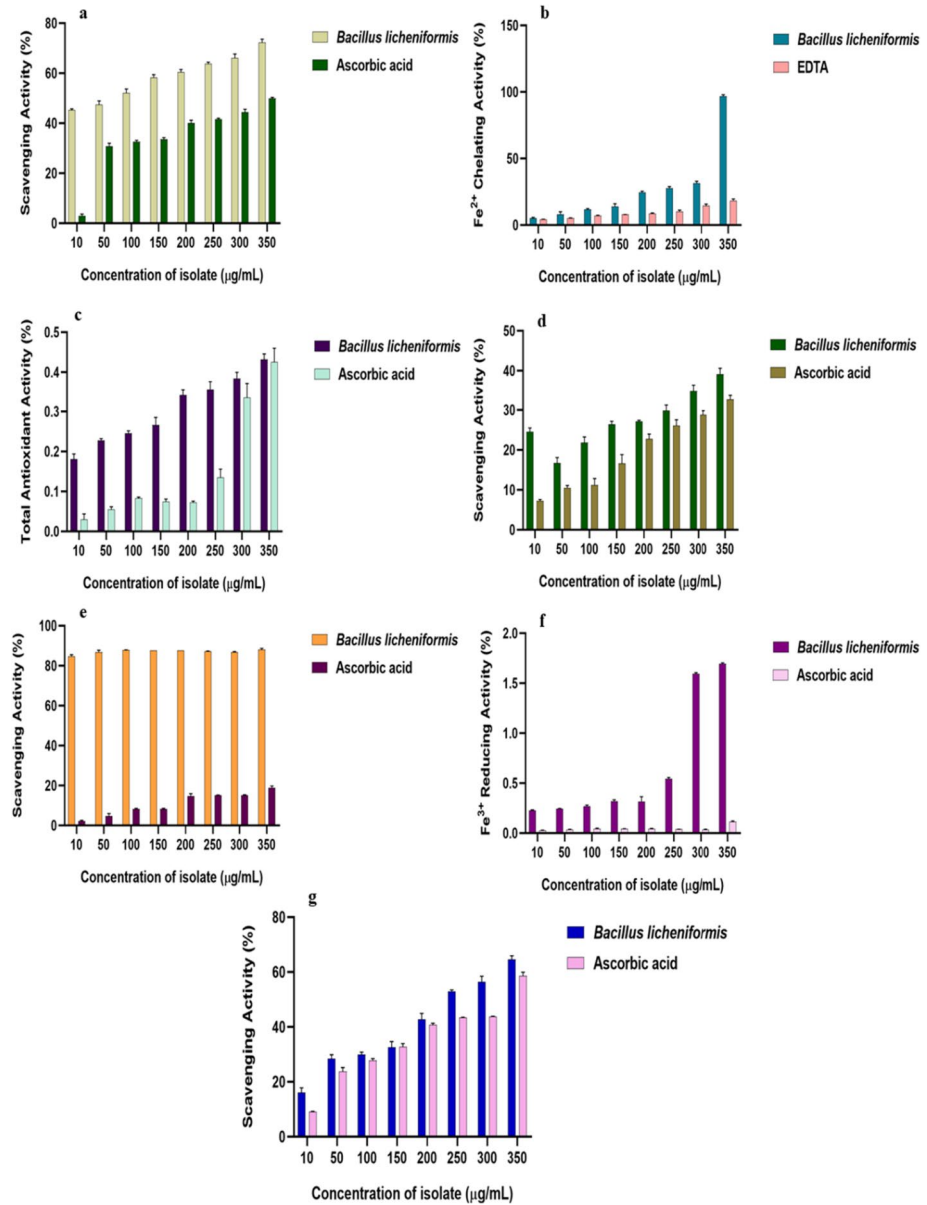


Fig. 2 Antioxidant activity **a)** DPPH scavenging assay **b)** Metal chelating Assay **c)** Total antioxidant assay **d)** Hydroxyl Peroxide Radical Scavenging Assay **e)** Hydroxyl Radical Scavenging Assay **f)** Reducing Power Assay **g)** ABTS Radical Cation Scavenging Assay of *Bacillus licheniformis* at different concentrations of extract

Results

In-vitro antioxidant activity of *B. licheniformis* extract

DPPH scavenging assay

The scavenging activity of *B. licheniformis* extract by DPPH radicals was evaluated based on the hydrogen donation. The percentage of DPPH radical scavenging activity of *B. licheniformis* extract at different concentrations was determined and shown in Fig. 2a.

The activity of *B. licheniformis* was found to be highest at 350 µg/mL compared to other concentrations. It revealed that the *B. licheniformis* showed 45.32% activity at 10 µg/mL, 47.37% at 50 µg/mL, 52.18% at 100 µg/mL, 58.25% at 150 µg/mL, 60.52% at 200 µg/mL, 63.73% at 250 µg/mL, 66.15% at 300 µg/mL, and 72.21% at 350 µg/mL.

Metal chelating assay

The chelating activity of ferrous ions in *B. licheniformis* extract at various concentrations was estimated. Fig. 2b reveals that the highest chelating activity was seen at 350 µg/mL. *B. licheniformis* showed 3.45% at 10 µg/mL, while it was 8.11%, 11.72%, 13.95%, 24.45%, 27.76%, 31.55%, and 96.63% at 50, 100, 150, 200, 250, 300, and 350 µg/mL, respectively.

Total antioxidant assay

The antioxidant activity present in *B. licheniformis* extract at various concentrations was represented in Fig. 2c. The data revealed that with increasing concentrations of *B. licheniformis* extract, there was increase in the antioxidant activities of the extract. The highest antioxidant activity was found to be 0.431 at 350 µg/mL, while *B. licheniformis* showed antioxidant activities of 0.18, 0.227, 0.245, 0.266, 0.341, 0.355, and 0.384 at 10, 50, 100, 150, 200, 250, and 300 µg/mL respectively.

H₂O₂ radical scavenging assay

The scavenging activity of the H₂O₂ of *B. licheniformis* extract evaluated at different concentrations was shown in Fig. 2d. The data suggested increase in the scavenging activities of the extract with the increasing concentrations of *B. licheniformis* extract with slight variations. The scavenging activity of *B. licheniformis* extract was found to be 24.53% at 10 µg/mL, while it was 16.675% at 50 µg/mL, 21.8% at 100 µg/mL, 26.38% at 150 µg/mL, 27.18% at 200 µg/mL, 29.875% at 250 µg/mL, 34.76% at 300 µg/mL, and 39.11% at 350 µg/mL.

Hydroxyl radical scavenging assay

The radical scavenging activity of the hydroxyl ions present in *B. licheniformis* extract at different concentrations of the extract was represented in Fig. 2e as follows: 84.61% at 10

$\mu\text{g/mL}$, 86.765% at 50 $\mu\text{g/mL}$, 87.895% at 100 $\mu\text{g/mL}$, 87.545% at 150 $\mu\text{g/mL}$, 87.52% at 200 $\mu\text{g/mL}$, 87.33% at 250 $\mu\text{g/mL}$, 86.71% at 300 $\mu\text{g/mL}$, and 88.125% at 350 $\mu\text{g/mL}$.

Reducing power assay

The reducing power is an indicator of the potential antioxidant activity due to its ability to donate electrons. It is connected with antioxidant activity and may be considered as an important reflection of antioxidant activity. Figure 2 f represents the reducing ability of *B. licheniformis* extract at various concentrations of the extract. The highest reducing power of the extract was found to be 1.694 at 350 $\mu\text{g/mL}$, while it was 0.225 at 10 $\mu\text{g/mL}$, 0.242 at 50 $\mu\text{g/mL}$, 0.267 at 100 $\mu\text{g/mL}$, 0.317 at 150 $\mu\text{g/mL}$, 0.316 at 200 $\mu\text{g/mL}$, 0.542 at 250 $\mu\text{g/mL}$, and 1.595 at 300 $\mu\text{g/mL}$.

ABTS radical cation scavenging assay

The antioxidant ability of *B. licheniformis* extract at different concentrations of the extract was determined based on the decoloration of ABTS and is represented in Fig. 2g. The extract revealed the scavenging activities of ABTS with increasing concentrations as follows: 16.18% at 10 $\mu\text{g/mL}$, 28.38% at 50 $\mu\text{g/mL}$, 29.9% at 100 $\mu\text{g/mL}$, 32.57% at 150 $\mu\text{g/mL}$, 42.71% at 200 $\mu\text{g/mL}$, 52.94% at 250 $\mu\text{g/mL}$, 56.35% at 300 $\mu\text{g/mL}$, and 64.62% at 350 $\mu\text{g/mL}$.

Enumeration of *B. licheniformis* suspension in alginate-chitosan

B. licheniformis suspension encapsulated with chitosan coated on alginate beads was taken and colonies were observed. Chitosan coated alginate beads encapsulated without *B. licheniformis* suspension were taken as control and colonies were not found in them.

Feeding experimental trial treatment

The survival percentage of group 2 and group 3 prawns when challenged with pathogen, *A. hydrophila*, was recorded after 15 days of experimental trial. The percentage of mortality was found to be very high in group 2 prawns compared to group 3 prawns. 67% mortality was seen in group 2 challenged with 10^6 concentration of *A. hydrophila* and fed with commercial diet. Several clinical symptoms were observed in group 2 prawns: lesions on carapaces and tails, broken legs, lethargy, and hypoxic. In the case of group 3, no mortality was seen upto 30 days of exposure to *A. hydrophila* in *M. rosenbergii* fed with *B. licheniformis* encapsulated chitosan coated alginate beads. After 30 days, 34% mortality was observed in group 3 prawns but there was no sign of clinical symptoms. Group 1 prawns also did not show any clinical manifestations.

Antioxidant enzymatic activity and Immunological parameters

SOD activity

SOD activity in the hemolymph of three different groups of *M. rosenbergii* was estimated and represented in Fig. 3a. Based on the inhibition percentage, the SOD activity

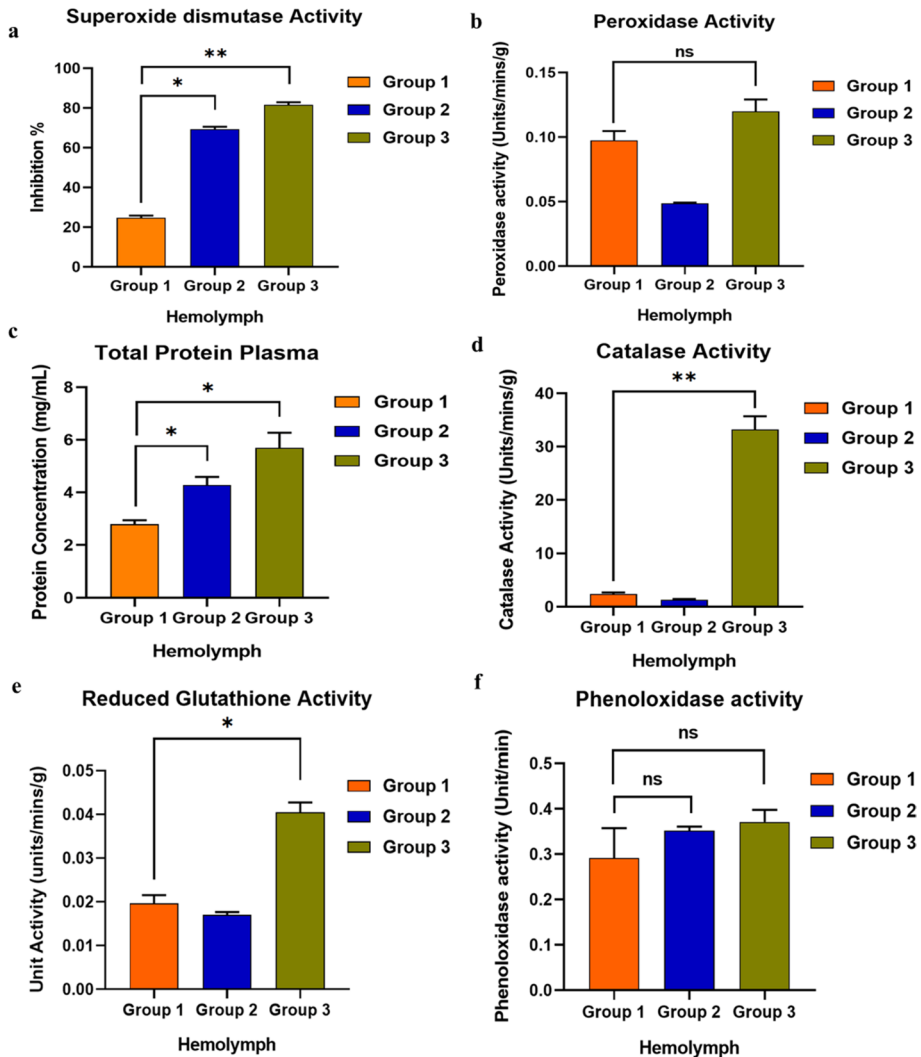


Fig. 3 Immune response and antioxidant enzymatic activity of *M. rosenbergii* fed with control feed (without bacterial suspension) (Group 1), prawns challenged with *A. hydrophila* fed with commercial diet (Group 2), and prawns challenged with *A. hydrophila* fed with *B. licheniformis* encapsulated feed (Group 3). Bar graph representing **a**) SOD activity **b**) POD activity **c**) Total plasma protein concentration **d**) CAT activity **e**) GSH activity **f**) Phenoloxidase activity of hemolymph in 3 different groups of experimental prawn, *M. rosenbergii*. Data were represented as mean \pm SD ($n = 3$) and analyzed by one-way ($P < 0.05$) ANOVA. The asterisks ** indicates a very high significant difference, * indicates a significant increase or decrease, and ns indicates no significant difference between the control compared to other experimental groups

is determined since it plays a beneficial role in crustaceans. The inhibition percentage of SOD activity was found to be much higher in hemolymph of group 3 that was fed with *B. licheniformis* encapsulated feed compared to group 1 (control) prawns. A significant difference in the SOD activity was also observed in group 2 when compared with the

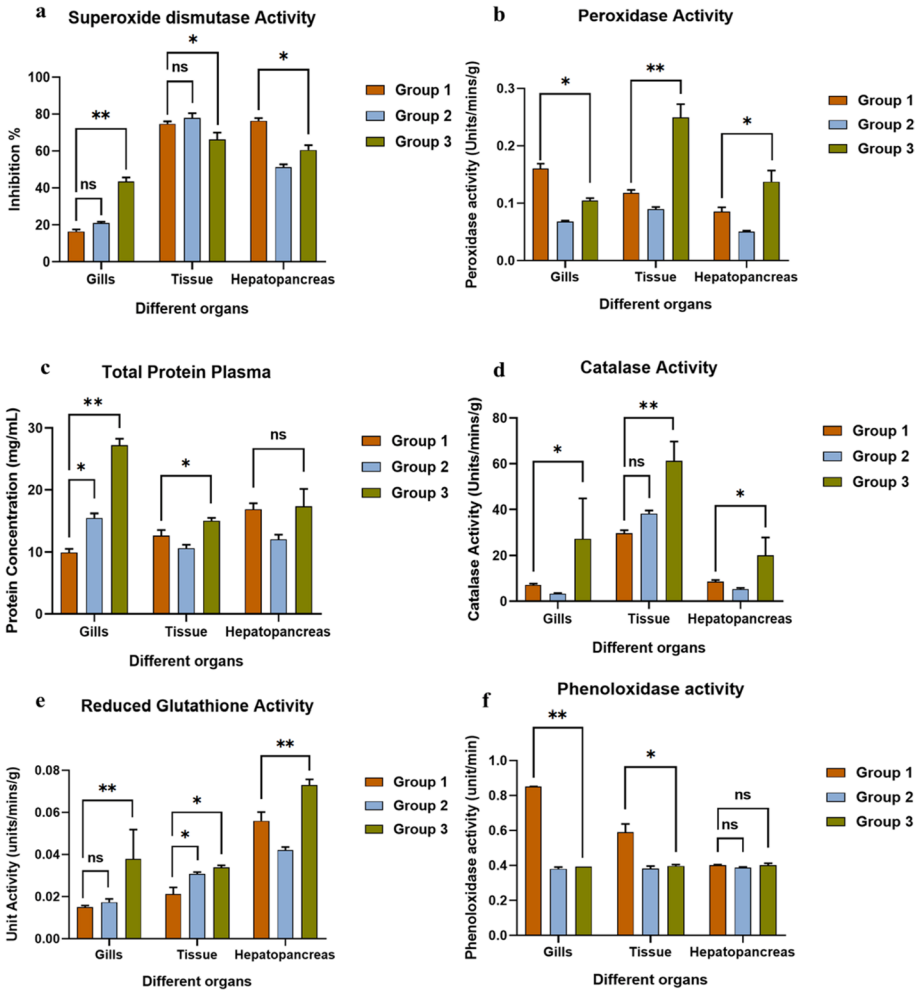


Fig. 4 Immune response and antioxidant enzymatic activity of *M. rosenbergii* fed with control feed (without bacterial suspension) (Group 1), prawns challenged with *A. hydrophila* fed with commercial diet (Group 2), and prawns challenged with *A. hydrophila* fed with *B. licheniformis* encapsulated feed (Group 3). Bar graph representing a) SOD activity b) POD activity c) Total plasma protein concentration d) CAT activity e) GSH activity f) Phenoloxidase activity of different organs in 3 different groups of experimental prawn, *M. rosenbergii*. Data were represented as mean \pm SD ($n = 3$) and analyzed by one-way ($P < 0.05$) ANOVA. The asterisks ** indicates a very high significant difference, * indicates a significant increase or decrease, and ns indicates no significant difference between the control compared to other experimental groups.

control group. The data revealed that group 3 ($P < 0.05$) prawns showed 81.64% activity, while group 1 and group 2 showed 24.74% and 69.375% activity respectively.

Similarly, SOD activity in the gills, tissues and hepatopancreas of all the 3 different groups of prawns was determined. Based on the inhibition percentage, the SOD activity was analysed and shown in Fig. 4a. The data suggested that there was no significant difference in the gills and tissues of group 1 and group 2. In contrast, there was a significant difference observed in between group 1 and group 2 in the hepatopancreas. In the gills, the

SOD activity in group 3 prawns was found to be 43.61% which is much higher compared to group 1 and group 2 prawns that showed 16.49% and 21.01% respectively. In the tissue, the SOD activity of group 3 ($P < 0.0001$) significantly decreased compared to group 1 and group 2. They revealed that group 1, group 2, and group 3 showed 74.75%, 78.03%, and 66.345% inhibition percentage respectively. In the hepatopancreas, group 2 and group 3 showed decreased activity compared to group 1. The SOD activity in group 1 was found to be 76.45%, while group 2 and group 3 showed 51.33% and 60.66% inhibition percentage respectively.

POD activity

The POD activity in hemolymph of three different group of shrimps, *M. rosenbergii* are shown in Fig. 3b. There was no significant difference between group 1 and group 3 prawns, while group 2 ($P < 0.05$) showed a significant difference compared to group 1. The peroxidase activity of hemolymph in group 1, group 2, and group 3 were found to be 0.975, 0.0488, and 0.12 units/min/g respectively.

In a similar manner, the POD activity in gills, tissues, and hepatopancreas is represented in Fig. 4b. The data indicated that there was a significant difference between both group 2 and group 3 when compared with group 1 in all the 3 different organs. The POD activity of tissue was much higher in group 3 prawns compared to group 1. In the gills, the POD activity of group 1 was found to be 0.1608 units/min/g while, group 2 and group 3 showed 0.0685 and 0.1049 units/min/g activity respectively. In the tissue, group 2 showed a slight increase in the POD activity, whereas, group 3 showed a major significant difference compared to group 1. The activity of group 1, group 2, and group 3 was found to be 0.1184, 0.0899, 0.2496 units/min/g respectively. In the hepatopancreas, group 2 showed a decrease ($P < 0.0001$) in the activity, while, group 3 showed an increase in the POD activity compared to group 1. The data revealed that group 1, group 2, and group 3 showed 0.0859, 0.0508, and 0.1376 units/min/g activity respectively.

Estimation of protein

The total protein concentration in the hemolymph in all 3 groups of prawns is represented in Fig. 3c. There was a significant difference in the protein concentration of plasma in both group 2 and group 3 ($P < 0.05$) compared to group 1 prawns. The total concentration of protein plasma in group 1, group 2, and group 3 was found to be 2.79 mg/mL, 4.285 mg/mL, and 5.695 mg/mL respectively.

The protein concentration in the gills, tissue and hepatopancreas was estimated in a similar manner and is shown in Fig. 4c. In the gills, there was a significant increase in the total protein concentration of group 2 and a more significant increase in the case of group 3 compared to control group 1. The data showed 9.95 mg/mL, 15.515 mg/mL, and 27.22 mg/mL protein concentration in group 1, group 2, and group 3 respectively. In contrast, no significant difference was observed in the protein concentration of tissues in group 2, while an increase in concentration in group 3 was found compared to group 1. The total protein concentration of group 1, group 2, and group 3 was found to be 12.655 mg/mL, 10.635 mg/mL, and 15.055 mg/mL respectively. In the hepatopancreas, no significant difference in group 3 was found, whereas, a decrease ($P < 0.0001$) in protein concentration in group 2 was observed compared to group 1. The protein concentration of group 1, group 2, and group 3 was found to be 16.865 mg/mL, 12.06 mg/mL, and 17.345 mg/mL respectively.

CAT activity

The CAT enzyme activity in hemolymph of different groups is represented in Fig. 3d. The data indicated a significant increase in the group 3 ($P < 0.05$) compared to group 1. No significant difference was found in group 2 prawns when compared with control group. The enzymatic activity of catalase of group 1, group 2, and group 3 was found to be 2.415, 1.315, 33.25 units/min/g respectively.

The CAT activity in the gills, tissues, and hepatopancreas is shown in Fig. 4d. The data showed that no significant difference was observed in the gills, tissue, and hepatopancreas of group 2 prawns when compared to group 1 (control). In contrast, a significant difference ($P < 0.0001$) was observed in all the organs of group 3 prawns compared to the control group. In the gills, the CAT activity was found to be 7.18, 3.29, and 27.285 units/min/g for group 1, group 2, and group 3 prawns respectively. In the tissue, group 1, group 2, and group 3 showed 29.755, 38.35, and 61.255 units/min/g activity, while, in the hepatopancreas, it was 8.595, 5.335, and, 20.115 units/min/g respectively.

Reduced glutathione activity

The GSH level in hemolymph of all the 3 different groups is shown in Fig. 3e. The data showed no significant difference in the reduced glutathione level in group 2 compared to the control group, while, a significant increase in the GSH level was found in group 3 ($P < 0.05$) compared to group 1. The GSH level in group 1, group 2, and group 3 was found to be 0.1965, 0.017, and 0.0405 units/min/g respectively.

The reduced glutathione level in the gills, tissues, and hepatopancreas is represented in Fig. 4e. In the gills, no significant difference was observed in the GSH level in group 2 compared to group 1. In contrast, a significant increase in the GSH level was found in group 3 compared to the control group 1 prawns. The data revealed that the GSH level observed in group 1, group 2, and group 3 was found to be 0.015, 0.0174, and 0.0379 units/min/g respectively. In the tissue, the GSH level of group 2 and group 3 showed a significant increase of 0.0307 and 0.0339 units/min/g respectively compared to group 1 (0.0213 units/min/g). The hepatopancreas showed a significant decrease in the GSH level in group 2 ($P < 0.0001$) prawns compared to the control, while group 3 showed a significant increase in the GSH level compared to group 1. The data showed that the GSH level of group 1, group 2, and group 3 was found to be 0.056, 0.0422, and 0.0731 units/min/g respectively.

Phenol oxidase activity

The phenoloxidase activity of the 3 different group of prawns in hemolymph was shown in Fig. 3f. The data revealed that the phenoloxidase activity showed no significant difference in the group 2 and group 3 ($P =$ not significant) prawns compared to the control group. The phenoloxidase activity in group 1, group 2, and group 3 was found to be 0.2915, 0.352, and 0.371 units/min respectively.

The phenoloxidase activity in the gills, tissue and hepatopancreas of all the 3 groups was shown in Fig. 4f. There was a significant decrease in the phenoloxidase activity of group 2 and group 3 ($P < 0.0001$) prawns in the gills and tissue compared to the control group. In contrast, no significant difference in phenoloxidase activity was observed in

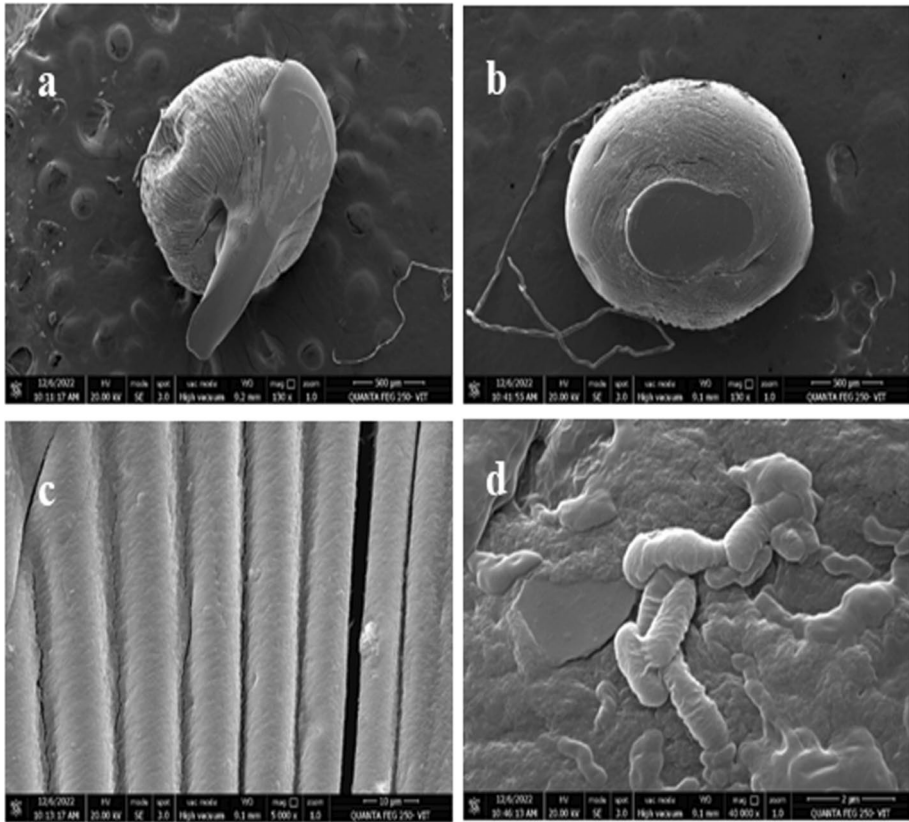


Fig. 5 a) and b) showing the field emission scanning electron micrographs of external morphology of the control (without extract) bead and *B. licheniformis* encapsulated bead, c) and d) micrographs of Internal morphology of control bead and *B. licheniformis* encapsulated bead

the hepatopancreas of group 2 and 3 compared to group 1. The phenoloxidase activity in the gills of group 1, group 2, and group 3 was found to be 0.8515, 0.381, 0.394 unit/min respectively. Similarly, in the tissue, the phenoloxidase activity of group 1, group 2, and group 3 was found to be 0.59, 0.383, 0.398 unit/min and in the hepatopancreas the activity was 0.403, 0.388, and 0.4025 units/min for group 1, group 2, and group 3 respectively.

FESEM analysis

Figure 5a and b represent the FESEM image of the dried alginate-coated chitosan beads (control) and dried *B. licheniformis* encapsulated alginate-coated chitosan beads under 130 X magnification respectively. After the beads were dried, some of the beads lost their initial shape. The image revealed the outer surface morphology of the beads. The beads were found to be irregular in the case of control but retained the spherical shape for the *B. licheniformis* encapsulated and the surface was found to be irregular.

Figure 5c and d showed the interior surface morphology of the control and *B. licheniformis* encapsulated beads respectively. The FESEM image revealed the difference between the interior surface morphology of control and *B. licheniformis* encapsulated beads under

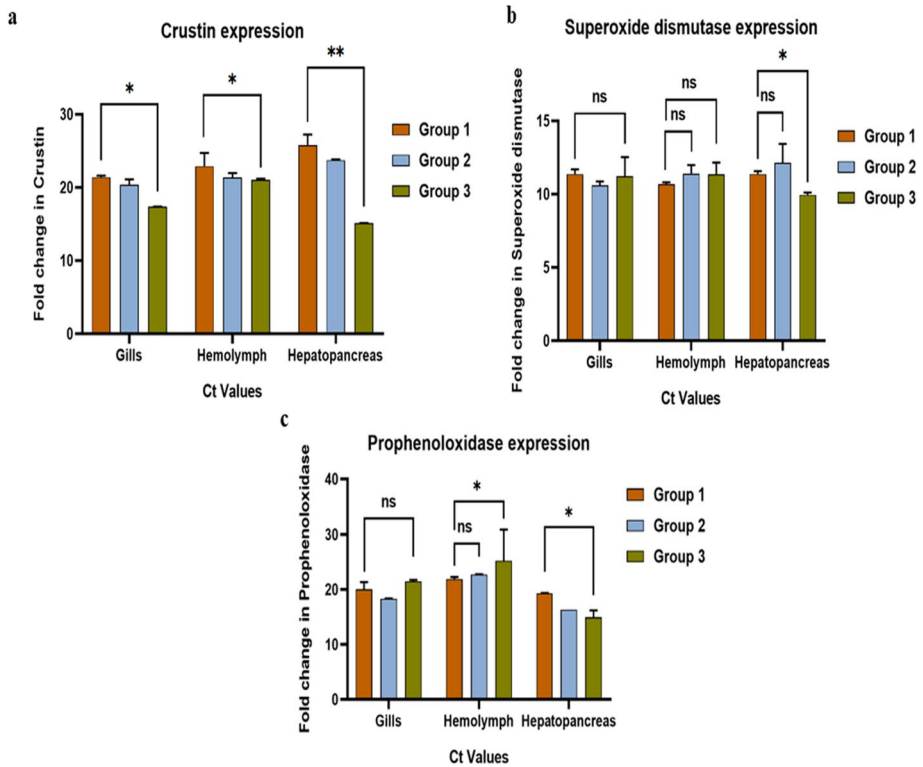


Fig. 6 Expression level of **a)** Crustin **b)** SOD **c)** Prophenoloxidase genes in hemolymph, gills, hepatopancreas in 3 different groups of *M. rosenbergii*. Group 1- *M. rosenbergii* injected with PBS (without bacterial suspension), Group 2 - prawns challenged with *A. hydrophila*, Group 3- prawns challenged with *A. hydrophila* and treated with *B. licheniformis*. The qRT-PCR was carried out in duplicates. Bar graphs were represented as mean \pm SD. The asterisks ** indicates a very high significant difference, * indicates a significant increase or decrease, and ns indicates no significant difference between the control compared to other experimental groups

different magnifications. No colonies were observed in the control bead, while *B. licheniformis* clusters were observed in the *B. licheniformis* encapsulated alginate coated chitosan bead.

Immune gene expression analysis by RT-PCR

Figure 6a shows the gene expression of crustin in hemolymph, gills, and hepatopancreas of the 3 different groups of prawns. In gills, no significant difference was observed in the crustin expression of group 2 prawns (20.375 relative gene expressions) compared to group 1 (21.44 relative gene expressions), while, there is a significant difference in the gene expressed in the case of group 3 (17.375 relative gene expressions) compared to the control group. In the hemolymph, the crustin expression in group 2 (21.42 relative gene expressions), and group 3 (21.05 relative gene expressions) showed a significant decrease ($P < 0.0001$) compared to group 1 (22.95 relative gene expressions). In contrast, the genes expressed in hepatopancreas of group 2 (23.77 relative gene expressions) showed a slight

decrease and in group 3 (15.13 relative gene expressions) it was found to be significantly decreased compared to group 1 (25.835 relative gene expressions).

Figure 6b represents the SOD expression in the gills, hemolymph and hepatopancreas of all the 3 groups of prawns. In the gills, no significant difference in the SOD expression was observed in both group 2 (10.615 relative gene expressions) and group 3 (11.235 relative gene expressions) compared to control group 1 (11.385 relative gene expressions). Similarly, the SOD expression of hemolymph in group 2 (11.42 relative gene expressions) and group 3 (11.365 relative gene expressions) prawns showed no significant difference compared to the control group (10.68 relative gene expressions). In the hepatopancreas, the data revealed that group 2 (12.145 relative gene expressions) showed no significant difference, while there was a slight decrease in the gene expression in case of group 3 (9.975 relative gene expressions) prawns compared to group 1 (11.375 relative gene expressions).

The Prophenoloxidase expression in the gills, hemolymph, and hepatopancreas is shown in Fig. 6c. The data suggested a significant decrease in the Prophenoloxidase expressed in the gills of group 2 (18.32 relative gene expressions) prawns, while no significant difference was found in group 3 (21.495 relative gene expressions) compared to group 1 (20.055 relative gene expressions). In the hemolymph, no significant Prophenoloxidase expression was observed in group 2 (22.74 relative gene expressions), whereas, a significant increase in the expression in group 3 (25.25 relative gene expressions) was found compared to group 1 (21.92 relative gene expressions). The Prophenoloxidase expression in both group 2 (16.31 relative gene expressions) and group 3 (14.965 relative gene expressions) were significantly decreased compared to the control group (19.315 relative gene expressions).

Discussion

Aquaculture is facing a challenge in dealing with the problem of resistance of aquatic pathogens towards the antibiotics. In the past few decades, numerous studies have been reported on marine *Actinomycetes* as a source of secondary metabolites. In the present study we observed antioxidant activities in *B. licheniformis* by carrying out several antioxidant assays. The data revealed that the inhibition percentage of the antioxidant activity in all the assays increased based on the highest concentration of *B. licheniformis* extract i.e., 350 µg/mL.

A study by Padma et al. (2018) reported the antioxidant activity of DPPH produced by terrestrial *Actinomycetes*. They estimated the antioxidant activity and reported 93.59% maximum activity at 650 µg/mL concentration. In the present study, DPPH activity of *B. licheniformis* extract showed maximum activity of 72.21% at 350 µg/mL. (Law et al. 2019) reported the result of a study on the chelating activity of metal ions in *Streptomyces* extract from mangrove forest. They reported a significant activity at 4 mg/mL concentration ranging from $19.84 \pm 1.53\%$ to $80.77 \pm 0.84\%$. In the current work, *B. licheniformis* extract showed a maximum chelating activity ranging from 5.345% to 96.63% at concentrations from 10 - 350 µg/mL. Tan et al. (2017) reported ABTS scavenging activity of $9.57 \pm 1.32\%$ to $61.52 \pm 30.13\%$ at 0.25 to 4 mg/ml concentration in *Streptomyces* sp. MUM212 extract from mangrove soil. Our present study reported a significant scavenging activity in *B. licheniformis* extract from 16.18% to 64.62% at 10 - 350 µg/mL concentrations. A study by Narendhran et al. (2014) reported that the ethyl acetate extract of *Streptomyces cavouresis* KUV39 exhibited a H₂O₂ scavenging activity at IC₅₀ of 42.35 ± 0.75 µg/mL. In the present work, *B. licheniformis* extract showed a maximum H₂O₂ scavenging activity activity

of 39.11% at 350 µg/mL concentration. Kumar et al. (2014) reported a study on *Streptomyces lavendulae* SCA5 exhibiting a hydroxyl scavenging activity at IC₅₀ of 617.84 ± 0.57 µg/mL. Our study showed a scavenging activity of hydroxyl ions in *B. licheniformis* extract ranging from 84.61% to 88.125% at 10 - 350 µg/mL concentration. A study on the reducing power of ferric ions in *Streptomyces* sp. NMF6 by Fahmy and Abdel-Tawab (2021) reported activity ranging from $1.91 \pm 0.14\%$ to $7.67 \pm 0.15\%$ at 25 - 150 µg/mL. In the current work, *B. licheniformis* extract exhibited reducing activity of 0.225% to 1.69 % at 10 - 350 µg/mL concentration. Similarly, the total antioxidant activity of *B. licheniformis* extract exhibited a maximum activity of 0.18% to 0.43% at 10 - 350 µg/mL concentration.

Macrobrachium rosenbergii and other species of prawns have been seriously affected due to the emerging infectious pathogens that has led to overall huge economic losses in aquaculture. Among the aquatic pathogens, *Aeromonas* sp. are the primary cause of huge mass mortalities of prawns in the aquaculture industry. A study by Vidhya Hindu et al. (2018) demonstrated the increased immunological and antioxidant activities like phenoloxidase, SOD, and total plasma protein in 3 different groups of *M. rosenbergii* challenged with *A. hydrophila* and treated with probiotic *Bacillus vireti* and seaweed *Gracilaria folifera*. Our study reported several immunological parameters and antioxidant enzymatic activities such as GSH, POD, SOD, CAT, phenoloxidase, and total plasma protein in hemolymph, gills, tissue and hepatopancreas of 3 different groups of *M. rosenbergii* challenged with *A. hydrophila* and treated with *B. licheniformis* extract. The increased immune and antioxidant activity indicated the safety of incorporating *B. licheniformis* extract in the diet of prawns. The data showed a significant increase in all the immunological and antioxidant activities in the treated group of *M. rosenbergii* in the case of hemolymph, gills, tissue and hepatopancreas compared to the control and infected group. It indicated increase in immune response and antioxidant activity which suggested that *B. licheniformis* extract can be safely incorporated in prawn diet.

For the past two decades, transcriptomic study has been a significant method to evaluate the gene expression in prawns challenged with several bacterial infections. A study reported by Soo et al. (2019) employed RNA Sequencing high-throughput technology to estimate the immune gene expression in the hepatopancreas of the marine shrimp, *Panaeus monodon* challenged with *Vibrio parahemolyticus*. In our current study, we used three immune genes – crustin, Prophenoloxidase, and SOD to determine the immune gene responses in the hemolymph, gills, and hepatopancreas of freshwater prawn, *M. rosenbergii* challenged with *A. hydrophila* by RT-PCR. The data suggested that there was a significant difference in the treated group compared to the control group. The response to SOD and Prophenoloxidase showed that there was a significant increase in the treated group of hemolymph, gills, and hepatopancreas compared to the control.

Biologically active compounds can be encapsulated by using various methods. Thanigaivel et al. (2019) prepared a microencapsulated seaweed bead using Na-caseinate and xanthum gum. They incorporated the beads with seaweed extract. Another study by Vidhya Hindu et al. (2018) reported the development of alginate-chitosan with seaweed encapsulated beads by extrusion method. In the present study, we incorporated the alginate-chitosan coated bead with *B. licheniformis* extract using extrusion method. Alginate has been reportedly used in biomedicine, drug delivery, and wound healing. Chitosan is a marine polymer that can be used in the encapsulation. The formulation of alginate, xanthum gum and chitosan is a very beneficial process to capture the biological macromolecules and proteins for the stability of polymer matrix. They have been used together to form the *B. licheniformis* encapsulated alginate chitosan beads. The active bioactive compounds of *B. licheniformis* were encapsulated in the beads. The

survival and mortality rate of the prawns in this study was found to be dependent on the bioactive compounds produced by *B. licheniformis*. The data revealed that the prawns treated with *B. licheniformis* encapsulated alginate chitosan beads showed more survival compared to the control group. 34% mortality of prawns fed with the bioactive compound encapsulated feed was observed after 30 days of the feeding trial experiment. This method is strongly recommended as an alternative to antibiotics. This may be the first study to report the treatment of bacterial infections by developing a biologically active feed from a marine natural source using *Actinomycetes*.

Conclusion

The current research work determined the antioxidant activity of bioactive compounds present in *B. licheniformis* at different concentrations. The expression levels of several immune genes with their significance were evaluated to estimate the effect of *B. licheniformis* in the experimental prawn. The data obtained from the present study suggested that the encapsulated *B. licheniformis* bead could improve the immune response and antioxidant defense of the freshwater prawn, *Macrobrachium rosenbergii* challenged with *A. hydrophila*. This encapsulated *B. licheniformis* alginate coated chitosan bioactive bead can be fed orally to prawns and can serve as an alternative to antibiotics.

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Declarations

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Ethical approval and consent to participate Not applicable

Consent for publication Not applicable

Competing interests The authors declare no competing interests.

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References

- Abdelfattah M.S.; Elmallah M.I.Y.; Hawas U.W.; El-Kaseema L.T.A.; Eid M.A.G (2016) Isolation and characterization of marine-derived actinomycetes with cytotoxic activity from the Red Sea coast. *Asian Pac J Trop Biomed* 6:651–657. <https://doi.org/10.1016/j.apjtb.2016.06.004>
- Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Chang LW, Yen WJ, Huang SC, Der Duh P (2002) Antioxidant activity of sesame coat. *Food Chem* 78:347–354. [https://doi.org/10.1016/S0308-8146\(02\)00119-X](https://doi.org/10.1016/S0308-8146(02)00119-X)
- Dholakiya RN, Kumar R, Mishra A et al (2017) Antibacterial and antioxidant activities of novel actinobacteria strain isolated from Gulf of Khambhat, Gujarat. *Front Microbiol* 8:1–16. <https://doi.org/10.3389/fmicb.2017.02420>
- Dinis TCP, Madeira VMC, Almeida LM (1994) Action of phenolic derivatives (acetaminophen, salicylate, and 5-Aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxy radical scavengers. *Arch Biochem Biophys* 315:161–169
- Ellman GL (1959) Tissue Su ~ yd ~ I Groups. 70–77
- Fahmy NM, Abdel-Tawab AM (2021) Isolation and characterization of marine sponge-associated *Streptomyces* sp. NMF6 strain producing secondary metabolite(s) possessing antimicrobial, antioxidant, anticancer, and antiviral activities. *J Genet Eng Biotechnol* 19:. <https://doi.org/10.1186/s43141-021-00203-5>
- Helen Fitton J (2003) Brown marine algae: A survey of therapeutic potentials. *Altern Complement Ther* 9:29–33
- Iber BT, Kasan NA (2021) Recent advances in Shrimp aquaculture wastewater management. *Heliyon* 7:e08283. <https://doi.org/10.1016/j.heliyon.2021.e08283>
- Karthik L, Kumar G, Rao KVB (2013) Antioxidant activity of newly discovered lineage of marine actinobacteria. *Asian Pac J Trop Med* 6:325–332. [https://doi.org/10.1016/S1995-7645\(13\)60065-6](https://doi.org/10.1016/S1995-7645(13)60065-6)
- Keser S, Celik S, Turkoglu S et al (2012) Hydrogen Peroxide Radical Scavenging and Total Antioxidant Activity of Hawthorn. *Chem J* 02:912
- Kono Y (1978) Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. *Arch Biochem Biophys* 186:189–195. [https://doi.org/10.1016/0003-9861\(78\)90479-4](https://doi.org/10.1016/0003-9861(78)90479-4)
- Kumar PS, Al-Dhabi NA, Duraipandian V et al (2014) In vitro antimicrobial, antioxidant and cytotoxic properties of *Streptomyces lavendulae* strain SCA5. *BMC Microbiol* 14:1–12. <https://doi.org/10.1186/s12866-014-0291-6>
- Laria Lamela RE, Coffigny RS, Quintana YC, Martínez M (2005) Phenoloxidase and peroxidase activity in the shrimp *Litopenaeus schmitti*, Pérez-Farfante and Kensley (1997) exposed to low salinity. *Aquac Res* 36:1293–1297. <https://doi.org/10.1111/j.1365-2109.2005.01344.x>
- Law JWF, Chan KG, He YW et al (2019) Diversity of *Streptomyces* spp. from mangrove forest of Sarawak (Malaysia) and screening of their antioxidant and cytotoxic activities. *Sci Rep* 9:1–15. <https://doi.org/10.1038/s41598-019-51622-x>
- Liu CH, Yeh ST, Cheng SY, Chen JC (2004) The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio* infection in relation with the moult cycle. *Fish Shellfish Immunol* 16:151–161. [https://doi.org/10.1016/S1050-4648\(03\)00058-5](https://doi.org/10.1016/S1050-4648(03)00058-5)
- Mikami Y (2007) Biological Work on Medically Important *Nocardia* Species. *Actinomycetologica* 21:46–51. <https://doi.org/10.3209/saj.saj210107>
- Mishra A, Patel MK, Jha B (2015) Non-targeted metabolomics and scavenging activity of reactive oxygen species reveal the potential of *Salicornia brachiata* as a functional food. *J Funct Foods* 13:21–31. <https://doi.org/10.1016/j.jff.2014.12.027>
- Mondal H, Chandrasekaran N, Mukherjee A, Thomas J (2023) Antibacterial activity of *Bacillus licheniformis* isolated from marine sediments and its effect in treating *Aeromonas hydrophila* infection in freshwater prawn, *Macrobrachium rosenbergii*. *Aquac Int*. <https://doi.org/10.1007/s10499-023-01121-8>
- Narendhran S, Rajiv P, Vanathi P, Sivaraj R (2014) Spectroscopic analysis of bioactive compounds from *Streptomyces Cavouensis* KUV39: Evaluation of antioxidant and cytotoxicity activity. *Int J Pharm Pharm Sci* 6:319–322
- Oyaizu M (1986) Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese J Nutr Diet* 44:307–315. <https://doi.org/10.5264/eiyogakuzashi.44.307>

- Padma B. J, K. S, P. A, Shiny R. A (2018) Isolation, characterization and evaluation of antioxidant activities of secondary metabolites producing actinomycetes of terrestrial origin. *Int J Res Med Sci* 6:1017. 10.18203/2320-6012.ijrms20180643
- Patel MK, Mishra A, Jha B (2016) Non-targeted metabolite profiling and scavenging activity unveil the nutraceutical potential of psyllium (*Plantago ovata* forsk). *Front Plant Sci* 7:1–17. <https://doi.org/10.3389/fpls.2016.00431>
- Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a *Anal Biochem* 269:337–341. <https://doi.org/10.1037/a0037168>
- Rajkumar T, Manimaran M TG et al. (2018) Antiviral viral compound from *Streptomyces ghanaensis* like strain against white spot 2 syndrome virus (WSSV) of shrimp. *bioRxiv*. <https://doi.org/10.1101/340265>
- Revathy T, Jayasri MA, Suthindhiran K (2013) Anti-oxidant and enzyme-inhibitory potential of marine *Streptomyces*. *Am J Biochem Biotechnol* 9:282–290. <https://doi.org/10.3844/ajbbsp.2013.282.290>
- Roshan K, Biswas K, Solanki V et al (2014) Actinomycetes: potential bioresource for human welfare: a review. *Res J Chem Environ Sci* 2:5–16
- Ruch RJ, Jun CS, Klaunig JE (1989) Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from chinese green tea. *Carcinogenesis* 10:1003–1008. <https://doi.org/10.1093/carcin/10.6.1003>
- Sandeep Kumar, Tejal Dahiya, Surender Kumar PK (2023) Best management practices in shrimp farming. In: Kahate, P.M; Gudadhe, S; Zade, R.V; Verma S (ed) *Frontiers in Life Science Volume XI*, 6th edn. Bhumi Publishing, Nigave Khalasa, Kolhapur, pp 89–109
- Selim MSM, Abdelhamid SA, Mohamed SS (2021) Secondary metabolites and biodiversity of actinomycetes. *J Genet Eng Biotechnol* 19. <https://doi.org/10.1186/s43141-021-00156-9>
- Ser HL, Tan LTH, Palanisamy UD et al (2016) *Streptomyces* antioxidants sp. nov., a novel mangrove soil actinobacterium with antioxidative and neuroprotective potentials. *Front Microbiol* 7:1–14. <https://doi.org/10.3389/fmicb.2016.00899>
- Sharma K, Gulati R, Singh S, Rani A (2023) Mineral Fortified Inland Low Saline Water for Shrimp Culture. 7:35–43. 10.31080/ASAG.2023.07.1264
- Siddharth S, Aswathanarayan JB, Kuruburu MG, Madhunapantula SRV, Vittal RR (2021) Diketopiperazine derivative from marine actinomycetes *Nocardiosis* sp. SCA30 with antimicrobial activity against MRSA. *Arch Microbiol* 10:1–9. <https://doi.org/10.1007/s00203-021-02582-2>
- Soo TCC, Devadas S, Mohamed Din MS, Bhasu S (2019) Differential transcriptome analysis of the disease tolerant Madagascar-Malaysia crossbred black tiger shrimp, *Penaeus monodon* hepatopancreas in response to acute hepatopancreatic necrosis disease (AHPND) infection: Inference on immune gene response and in. *Gut Pathog* 11:1–13. <https://doi.org/10.1186/s13099-019-0319-4>
- Tan LTH, Chan KG, Khan TM et al (2017) *Streptomyces* sp. MUM212 as a source of antioxidants with radical scavenging and metal chelating properties. *Front Pharmacol* 8:1–18. <https://doi.org/10.3389/fphar.2017.00276>
- Thanigaivel S, Chandrasekaran N, Mukherjee A, Thomas J (2019) Protective efficacy of microencapsulated seaweed extracts for preventing *Aeromonas* infections in *Oreochromis mossambicus*. *Comp Biochem Physiol Part - C Toxicol Pharmacol* 218:36–45. <https://doi.org/10.1016/j.cbpc.2018.12.011>
- Trabelsi I, Bejar W, Ayadi D et al (2013) Encapsulation in alginate and alginate coated-chitosan improved the survival of newly probiotic in oxgall and gastric juice. *Int J Biol Macromol* 61:36–42. <https://doi.org/10.1016/j.ijbiomac.2013.06.035>
- Vidhya Hindu S, Thanigaivel S, Vijayakumar S et al (2018) Effect of microencapsulated probiotic *Bacillus vireti* 01-polysaccharide extract of *Gracilaria folifera* with alginate-chitosan on immunity, antioxidant activity and disease resistance of *Macrobrachium rosenbergii* against *Aeromonas hydrophila* infection. *Fish Shellfish Immunol* 73:112–120. <https://doi.org/10.1016/j.fsi.2017.12.007>
- Xu JB, Yuan XF, LPZ (1997) Determination of catalase activity and catalase inhibition by ultraviolet spectrophotometry. *Environ Chem* 16:73–76

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