

## Characterization of the defense properties of healthy and diseased coral mucus

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### Abstract

The surface mucus layer of corals aids in feeding, silt removal, and defense against pathogens. However, first line of defense of secreted mucus of corals against tissue damage generated through pathogen or environmental factors is poorly understood. Hence, we used various methods such as a well diffusion assay and tests for quorum quenching, free radical scavenging, antioxidant enzyme expression and phenoloxidase (PO) activity to determine the mucus defense properties using mucus of healthy and diseased *Porites* sp. and *Acropora* sp. Interestingly the coral mucus showed antimicrobial activity against coral pathogens such as bacteria and protozoan ciliates. Inhibition of the N-Acyl homoserine lactone (AHL) molecule suggests quorum quenching. Free radical scavenging of mucus was screened using hydrogen peroxide, hydroxyl radical, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) methods, which was found significantly more in diseased corals ( $p > 0.05$ ). Antioxidant enzymes superoxide dismutase (SOD), catalase, and peroxidase activity were observed in both the diseased and healthy coral mucus. The presence of serine and metalloproteases was also detected in coral mucus. Further, phenoloxidase (PO) activity was highest in diseased coral mucus affected by pink line syndrome and white patch *Acropora* sp. disease than the healthy coral mucus. Thus, the present study of antimicrobial properties, antioxidant enzymes, and quorum quenching properties in coral mucus may aid in understanding the corals defense and survival against pathogens and any abiotic stress.

**Keywords:-** Coral, Mucus, Oxidative stress, antioxidants, antimicrobial, Quorum quenching

## Introduction

Coral reefs are unique, diverse, economically important, and productive ecosystems, hence their description as the “rainforests” of the ocean (Rosenberg et al., 2007). However, corals that form reefs are under threat due to various biotic and abiotic stressors such as disease, UV-radiation and temperature rise. These stressors also influence the dissociation of the symbiont from the host coral which leads to coral bleaching, a response against oxidative stress (Downs et al., 2002). The generation of such oxidative stress takes place when the production and accumulation of reactive oxygen species (ROS) become uncontrollable by the host. These ROS damage the cells macromolecules such as lipids, DNA, and proteins (Schieber and Chandel, 2014) and act as damaging or signalling molecules (Sharma et al., 2012). Besides, due to the expelled endosymbiont, the host coral becomes weak and immunocompromised. This allows primary and secondary pathogens to infect the coral tissue causing disease that leads to coral mortality.

Several studies have described the role of bacteria and ciliates as primary and secondary pathogens influencing the disease progression in immunocompromised corals (Sweet and Sere, 2015; Ravindran et al., 2020). Therefore, understanding the various responses of coral reefs to disease and bleaching events is critical for their natural resilience. Furthermore, the possible evolutionary adaptation of corals to various stress conditions enhanced the stress tolerance level of individual corals, implying that corals could remember and respond to their previous environmental experiences (Hackerott et al., 2021). This supports the past reports of experimental data showing that some Scleractinian corals have adaptive-like immune responses in addition to innate immunity (Rosenberg et al., 2007). This phenomenon is known as experience-mediated tolerance, and it occurs when corals that were previously susceptible to bleaching caused by a specific bacterial disease may develop immunity to it. For example, it has been established that the coral *Oculina patagonica* can become resistant to reinfection by *Vibrio shilonii* after being severely impacted by bleaching events caused by this bacterium (Reshef et al., 2006).

Coral mucus is a complex mixture of protein, carbohydrates, and lipids produced by mucocytes in the epidermal layer and utilised by the coral as an adaptation mechanism against various stress conditions (Brown and Bythell, 2005). As a result, coral surface tissue that secretes the surface mucus layer (SML) serves as a first line of defense as well as a physiochemical and physiological barrier between animal tissue and external environment stresses (Brown and Bythell, 2005, Geffen et al., 2009, Bakshani et al., 2018). The bioactive

compounds in coral mucus have been experimentally proven to have antimicrobial activity in the defense against infectious disease by inhibiting *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Vibrio* species and *Serratia marcescens* (Brown and Bythell, 2005, Ritchie, 2006, Bakshani et al., 2018). Besides, Mycosporine-like amino acids (MAA), which act as a sunscreen that absorbs UV radiation (Teai et al., 1998), and phenoloxidase (PO) and melanin activity in mucus of healthy and diseased *Pseudodiploria strigosa* (Rivera-Ortega and Thome, 2018) have also been described. Prophenoloxidase (ProPO) is a major enzyme of coral innate immunity (Cerenius et al., 2008) which gets upregulated due to oxidative stress. During proteolysis, ProPO gets converted into phenoloxidase (PO) by serine proteinase activity which finally stimulates the melanin synthesis pathway (Mydlarz et al., 2009), an indicator for corals to respond against pathogens (Palmer et al., 2011). The antioxidants, phenols, and flavonoids found in coral mucus that play an important role in free radical scavenging activity and the quorum quenching characteristic that inhibits infections and promotes coral health have not been studied or reported. It is believed that such defensive substances in mucus are derived as secreted from coral tissue to mucus (Fleming, 1922; Ritchie, 2006). However, no study was described for the presence of antioxidants and other defense substances reported from coral mucus.

Coral disease outbreaks due to bacterial infections have increased significantly. Especially, various *Vibrio* sp. involved in causing numerous coral diseases, namely *Vibrio rotiferanus*, *V. harveyi*, *V. alginolyticus*, *V. proteolyticus* together have been described as a consortium that causes Yellow blotch/band disease (YBD) (Cervino et al., 2008); *V. parahaemolyticus*, as the causative agent of Porites ulcerative white spot disease (PUWS) (Arboleda and Reichardt, 2010), a proline-rich P-toxin of *V. shilonii* induced bleaching of *Oculina patagonica* (Banin et al., 2001); *V. coralliilyticus*, the causative pathogen of white syndrome (WS) and bleaching in the coral *Pocillopora damicornis* (Ben-Haim et al., 2003). *V. carchariae*, *V. harveyi* causing white band in *Acropora* spp. (Ritchie and Smith, 1998). Also, the role of lysogenic prophages in the conversion of non-pathogenic to virulent strains has been described in a wide range of species, including *Vibrio cholerae*, *Staphylococcus aureus*, and *Escherichia coli* (Fortier and Sekulovic, 2013) and especially in the coral pathogen, *V. coralliilyticus* (Weynberg et al., 2015).

The coral pathogen, *V. parahaemolyticus* and *S. aureus* as control pathogen has been taken in the present study for their inhibition and suppression of virulence if any by coral mucus. Besides, the coral surface mucus layer that represents the primary defense against

pathogens has been characterized for their defense properties such as antimicrobial, quorum quenching, antioxidants (for DPPH, hydroxyl radical and hydrogen peroxide scavenging, and metal chelating and reducing power activities) and antioxidant enzymes (Superoxide dismutase, catalase, peroxidase, phenol oxidase) which may aid in understanding coral survival. and resilience under various biotic and abiotic stress conditions.

## **2. Materials and methods**

### **2.1 Sample collection and preparation of extract**

Coral mucus samples were collected from the Big Manali island within a 5-meter radius transect, habitats at N 09°13'15.6" E 079°08'38.5", at depth 1-1.5 meter, Temperature 29 °C, Gulf of Mannar, India. Healthy coral mucus was collected *in situ* with a sterile syringe from the surface of the individual healthy coral colonies as earlier collected by Ravindran et al., (2020). Similarly, diseased *Acropora* sp. (white patch) and *Porites* sp. (pink line syndrome) mucus were collected along the disease margins from every single individual colony. The collected fresh mucus samples were stored below 4 °C and examined for all the experimental analysis that was done in triplicates.

### **2.2 Mucus antimicrobial activity against pathogens.**

The disc diffusion method was performed to determine the antimicrobial activity of mucus against coral pathogens *Vibrio parahaemolyticus* and *Staphylococcus aureus* isolated from diseased corals. Different concentrations of *Acropora* sp. and *Porites* sp. mucus were used (20 µl, 30 µl, 50 µl, 70 µl, and 100 µl). The *S. aureus* and *V. parahaemolyticus* were spread-plated on Muller-Hinton agar plates and varied concentrations of mucus were placed in discs aseptically over the pathogen spread agar plates and incubated at 28 °C for 24 hours.

### **2.3 Coral mucus inhibition of ciliates growth**

Adult ciliates with an equal and known number i.e., 15 or more ciliates of *Holosticha diademata* and *Cohnelimbus verminus* isolated from diseased corals (Ravindran et al., 2020) were used in mucus inhibition assay. Four treatments were tested with the above ciliates such as ciliates with only seawater, only coral mucus (with bacteria), only coral mucus 0.22 micron filtered (without bacteria) and mucus with seawater. Plates were incubated at 28 ° C. The number of ciliates was counted and observed every 8 hours, 24 hours, and 48-hour time intervals under a microscope. All the experiments were carried out in triplicates.

## 2.4 Quorum quenching analysis

N-Acyl homoserine lactone (AHL) -molecule quorum quenching by coral mucus was carried out by well diffusion plate assay (Tait et al., 2010). Quorum sensing (QS) regulates the violacein pigment production of *Chromobacterium violaceum* and is exploited for inhibitors of QS (McLean et al., 2004). Briefly, *C. violaceum* culture 100 µl was spread plated on Luria-Bertani (LB) agar plates. Wells were pricked in the spread agar plates and 100 µl of mucus was added to the well. The Plates were incubated for 24 hours at 30 °C. After incubation, 0.7 % of nutrient soft agar containing 200 µl of *C. violaceum* per 10 mL soft agar was poured over the colonies. All the plates were further incubated at 30 °C for 24 hours and plates were monitored.

Violacein pigment production was tested based on the earlier method (Choo et al., 2006). Briefly, in 1.8 ml Tryptic soy (TS) broth, 0.1 ml *C. violaceum* culture and 0.1 ml mucus were added. *C. violaceum* was grown on TS broth at 30 °C on the shaker. After incubation, to precipitate the violacein pigment, 1 ml culture was centrifuged at 13,000 rpm for 10 minutes. Then 1 mL of DMSO was added to the pellet and resuspended well. Optical density was checked at 585 nm. The control was made without the addition of mucus. All tests were performed in triplicates. Percentages of violacein pigment production are calculated by the following formula:

$(\text{Absorbance of tested concentration at 585 nm} / \text{absorbance of the control at 585 nm}) \times 100.$

The AHL lactonase enzyme activity on Penicillin G was done by incubating the reaction i.e., 200 µl Penicillin G 20 mg/ml, 200 µl 0.1 % starch, 200 µl mucus sample, and 200 µl water at 40 °C for 15 minutes (Mukherji and Prabhune, 2013). The lactonase enzyme is of the beta-lactamase superfamily that acts on the beta-lactam ring of Penicillin. Because of the high temperature used during the assay, some amount of Penicillin gets degraded. So, to avoid false readings, results were observed immediately after the addition of iodine. Controls were made without penicillin and enzyme, and only without enzyme. The exact mechanism behind the assay was that the AHL lactonase hydrolyzes penicillin into penicilloic acid. After the production of penicilloic acid, iodine is not free to interact with the 0.1 % starch and has no production of the blue colour, which confirms the presence of quorum quenching molecule in coral mucus. Thus in control, without coral mucus there is no formation of penicilloic acid and the iodine reacts with the starch and forms blue colour. Thus, the formation of the blue color depends on the interaction of free iodine with 0.1% starch. The color formation was noted within 10 seconds of the 10 % Iodine reagent addition.

## **2.5 Characterization of antioxidant properties**

### **2.5.1 Total soluble phenol content assay**

The total soluble phenolic content of coral mucus was estimated by using our earlier lab protocol (Ravindran and Naveenan 2011). To estimate total soluble phenol, the positive control used was gallic acid. A 100  $\mu$ l extract of the mucus sample was mixed with 2 ml of sodium bicarbonate (2 %) and kept for 2 minutes of incubation at room temperature. After incubation, 100  $\mu$ l of Folin-Ciocalteu reagent was added to the mixture and placed in the dark for 30 minutes. The samples were vortexed and absorbance was measured at 725 nm spectrophotometrically.

### **2.5.2 Total flavonoids content assay**

To quantify the total flavonoid content of coral mucus, quercetin was used as the standard (Ravindran and Naveenan 2011). The 100  $\mu$ l of the coral mucus was mixed in 300  $\mu$ l of distilled water and 30  $\mu$ l of sodium nitrite (5 % NaNO<sub>2</sub>). Tubes were incubated for five minutes. To this mixture, 30  $\mu$ l of Aluminium chloride (10 % AlCl<sub>3</sub>) was added, and mixed well and the reaction mixture was placed for 5 minutes at room temperature. Then 200  $\mu$ l Sodium hydroxide (1 mM NaOH) was added to the reaction and the final volume was made up to 1 ml by adding distilled water and mixed well. Optical density was observed at 510 nm.

## **2.6 Antioxidants scavenging assays**

### **2.6.1 Hydrogen peroxide scavenging assay**

The hydrogen peroxide scavenging assay was determined using the method of Ravindran and Naveenan (2011). Mucus (25  $\mu$ l, 50  $\mu$ l, and 75  $\mu$ l) were taken and 50 mM of hydrogen peroxide (HiMedia Mumbai, India) was mixed in a ratio of 1: 1 (v / v). The reaction mixture was incubated for 30 minutes, at room temperature. After 30 min of incubation, 10  $\mu$ l of HPLC grade methanol, and 0.9 ml FOX reagent (ferrous oxidation–xylenol orange reagent ) were added to the 90  $\mu$ l hydrogen peroxide solution (Sigma-Aldrich). The mixture was thoroughly mixed and placed at room temperature for 30 minutes. Blank was made by adding all the reagents except the sample. The ferric-xylenol orange complex formation was measured at 560 nm. The scavenging activity was measured based on inhibition of the complex formation of ferric-xylenol orange. To calculate scavenging activity, the following formula has been used:

$$\% \text{H}_2\text{O}_2 \text{ scavenging} = [(\text{Control absorbance} - \text{sample absorbance}) / \text{control absorbance}] \times 100.$$

### 2.6.2 Hydroxyl radical scavenging

The hydroxyl radical scavenging assay of coral mucus was carried out using the earlier method (Ravindran and Naveenan 2011; Ravindran et al., 2012). The activity is based on spectrometric estimation of the breakdown product of 2-deoxyribose by condensation with Thio-barbituric acid (TBA). The hydroxyl radicals were generated by the  $\text{Fe}^{3+}$  ascorbate EDTA  $\text{H}_2\text{O}_2$  system i.e. Fenton reaction. The reaction mixture consisted of 1 ml of 20 mM phosphate buffer (pH-7.4) containing 2.8 mM deoxyribose, 100  $\mu\text{M}$  ferric chloride, 1 mM  $\text{H}_2\text{O}_2$ , 100  $\mu\text{M}$  EDTA, and different concentrations of *Porites* sp., *Acropora* sp. mucus (25  $\mu\text{l}$ , 50  $\mu\text{l}$ , and 75  $\mu\text{l}$ ). Ascorbic acid (100  $\mu\text{M}$ ) was added at last followed by incubation for 1 hour at 37 °C. Then, 1 ml of 1 % Thio-barbituric acid and 2.8 % Trichloroacetic acid were added and the reaction was incubated for 15 minutes at 90 °C for color development. After cooling the tubes, optical density was measured at 532 nm. Mannitol was used as a control. The scavenging activity was measured by the following formula:

$$\% \text{ Hydroxyl radical scavenging} = [1 - (\text{sample absorbance} / \text{blank absorbance})] \times 100.$$

### 2.6.3 DPPH scavenging assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was performed as per earlier demonstrated methods (Ravindran and Naveenan 2011; Ravindran et al., 2012) by adding 800  $\mu\text{l}$  of 1mM DPPH (Sigma-Aldrich Steinheim, Germany) solution prepared in 95 % ethanol with mucus (25  $\mu\text{l}$ , 50  $\mu\text{l}$ , and 75  $\mu\text{l}$ ). The reactions were vortexed well and kept at room temperature for 30 minutes in the dark. Control was made by using 1 mL ethanol. Samples were centrifuged at 13,500 rpm for 5 minutes at RT. Optical density was measured at 517 nm. Butylated hydroxyanisole (BHA) (Merk Mumbai, India) was used as a reference compound.

$$\% \text{ DPPH scavenging activity} = [(\text{Absorbance of the blank} - \text{Absorbance of the sample}) / \text{Absorbance of the blank}] \times 100.$$

### 2.6.4 Metal chelating assay

The metal ion chelating capacity of the coral mucus was quantified by the modified protocol (Dinis et al., 1994; Ravindran and Naveenan 2011; Ravindran et al., 2012). The experiment was performed in HEPES buffer (pH 7.2, 20 mM). Different concentrations of mucus (25  $\mu\text{l}$ , 50  $\mu\text{l}$ , and 75  $\mu\text{l}$ ) were mixed with 12.5  $\mu\text{M}$  ferrous sulphate solution. Into the reaction, 75  $\mu\text{M}$  ferrozine solution was added and shaken vigorously. The mixture was kept for 20 mins and incubated at room temperature. After 20 minutes of incubation, the optical density

was measured at 562 nm. EDTA was used as a positive control. The chelating capacity percentage was calculated by using the formula:

% Chelating activity = [(Absorbance of the blank - Absorbance of the sample) / Absorbance of the blank] X 100.

### **2.6.5 Reducing power determination**

The reducing power of the mucus samples was determined by the method of (Oyaizu, 1986) with slight modification. Various concentrations of mucus (25  $\mu$ l, 50  $\mu$ l, and 75  $\mu$ l) extracts (25  $\mu$ l, 50  $\mu$ l, and 75  $\mu$ l) 0.5 ml were mixed with an equal volume of phosphate buffer (0.2 M pH-6.6) and 0.5 ml of 0.1 % potassium hexacyanoferrate. This reaction mixture was incubated at 50 °C for 20 minutes in a boiling water bath. After incubation 0.5 ml of 10 % Trichloroacetic acid was added to stop the reaction and 1 ml of the upper portion of the reaction mixture was mixed with 1 ml of distilled water, 0.1 ml of 0.01 % Ferric chloride (FeCl<sub>3</sub>) followed by incubation at room temperature for 10 minutes. Optical density was measured at 700 nm. An increase in absorbance indicates increased reducing power.

## **2.7 Coral mucus antioxidant enzyme spectrophotometric assays**

### **2.7.1 Superoxide Dismutase Activity.**

An assay of superoxide dismutase activity was done using the earlier method of our laboratory (Ravindran et al., 2012). Coral mucus extract was diluted with 0.3 mM sodium cyanide, 0.2 mL EDTA (0.1 M) solution, and 0.1 mL NBT (1.5 mM). Immediately 0.05 mL riboflavin (0.12 mM) was added and incubated for 12 minutes in light. Blank was made with distilled water in the place of coral samples. Optical density was measured at 560 nm. SOD activity was expressed as U/mg protein. The enzyme causing half the maximum inhibition reduction in NBT is known as one unit of SOD.

### **2.7.2 Catalase activity**

Catalase activity was determined by using our earlier lab protocol (Ravindran et al., 2012). 20  $\mu$ l of healthy and diseased coral *Acropora* sp. and *Porites* sp. mucus extract was mixed separately into the reaction mixture of 30  $\mu$ l phosphate buffer (50 mM) (pH 7.0) and 50  $\mu$ l H<sub>2</sub>O<sub>2</sub> (50 mM). The activity was measured at an optical density of 240 nm. Control was made without coral mucus and tissue samples. Catalase activity was calculated using the molar extinction coefficient for H<sub>2</sub>O<sub>2</sub> at 240 nm (Anderson et al., 1995).

### **2.7.3 Peroxidase assay**

Peroxidase activity assay was done by using the protocol developed by (Kiran et al., 2012). Mucus extract, 200 µl was mixed with 3.5 ml phosphate buffer pH 6.5 (100 mM) and added to 100 µl guaiacol (20 mM) (Sigma-Aldrich) in 10 mM phosphate buffer (pH 6.5) in the cuvette. The reaction was initiated by adding 200 µl of 200 mM H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich), which was prepared in 10 mM phosphate buffer (pH 6.5). Spectrophotometric reading was measured at 430 nm. The change in absorbance per min was measured from the linear phase. The blank was prepared with water. Peroxidase enzyme activity was expressed in units per mg protein.

### **2.7.4 Phenol Oxidase assay**

Phenol oxidase activity was measured by the procedure of Mydlarz and Palmer (2011) with slight modification. Dilutions were made by adding 30 µl of the coral mucus extract with 40 µl of 50 mM phosphate buffer, pH 7.5. To this reaction mixture, 25 µl of 0.1 mg/mL trypsin (HiMedia Mumbai, India) was added and kept for 10 mins incubation. After incubation, 30 µl of the guaiacol (10 mM stock concentration, 2.6 mM final concentration, Sigma-Aldrich) was added and the developed coloured product was measured at an optical density of 470 nm for 15 minutes. Values are represented as units per mg of protein.

## **2.8 Electrophoretic assay**

### **2.8.1 Mucus protein extraction**

Coral mucus 20 ml was centrifuged for 10 minutes, at 10,000 rpm (Thermo scientific) to allow all the cell mass and impurities to settle down. Into the mucus supernatant, the desired amount of Ammonium sulphate i.e. 9.55 gm to get desired saturation, for protein precipitation at 25 °C (HiMedia, Mumbai, India) was dissolved by continuous stirring on a magnetic stirrer for 3 hours till the Ammonium sulphate crystal formation, followed by 15 minutes of centrifugation at 10,000 rpm to pellet out the protein. Pellets were dissolved in phosphate buffered saline (PBS) to analyze proteins. Protein samples were dialyzed out of the Ammonium sulphate with phosphate buffer saline pH 7.4.

### **2.8.2 Superoxide dismutase activity**

Superoxide dismutase (SOD) enzyme activity of healthy and diseased coral mucus of *Acropora* sp. and *Porites* sp. was checked in native-PAGE gel. SOD activity was visualized on the 10 % gel by the activity staining procedure (Ravindran et al., 2012). After electrophoresis, the gel was washed with distilled water 2-3 times. Then it was treated with 0.1% NBT (Sigma-

Aldrich) on the shaker for 15 minutes in the dark. The gel was washed 2-3 times with distilled water. Riboflavin (Sigma-Aldrich) solution was added to the gel in the dark with shaking for 15 min. Again, the gel was washed 2-3 times with distilled water. Immediately the gel was exposed to light until the appearance of SOD activity bands on the gel.

### **2.8.3 Catalase activity assay**

Catalase activity staining was performed on 6 % native-PAGE gel by the earlier method (Ravindran et al., 2012). After electrophoresis, the gel was washed two times with distilled water. The gel was then rinsed with 50 mM phosphate buffer containing H<sub>2</sub>O<sub>2</sub> 0.01 M, and for 20 minutes gentle rocking was given. Further, the gel was kept in 30 ml of 2 % potassium ferric cyanide (Sigma-Aldrich) and 30 ml of 2 % ferric chloride (HiMedia Mumbai, India) and mixed well until the appearance of yellow-colored bands over the light box. Mucus catalase activity was also demonstrated on a clean glass slide against 3 % hydrogen peroxide as a substrate. 10 µL mucus was added to the substrate and the production of air bubbles was observed to confirm the presence of catalase enzymes.

### **2.8.4 Gelatin zymography for protease detection in coral mucus.**

The gelatin zymography was done to characterize and profile the proteases on a 12 % resolving polyacrylamide gel with 0.1 % gelatin and 0.15 % SDS following the protocol of Subramanian et al. (2007). The classes of protease present in the coral mucus were determined using specific inhibitors such as phenylmethylsulphonyl fluoride (5mM), benzamidine hydrochloride (5mM), EDTA (5mM), 1 mg/ml N-ethylmaleimide, 10 µg/ml pepstatin and o-phenanthroline (5 mM). All chemicals were used of make by HiMedia Mumbai, India except BHT which was of make by Merk Mumbai, India.

## **2.9 Statistical analysis**

All the experiments were carried out in triplicates. One-way ANOVA was used to compare means of the antioxidant assays and enzyme activities of healthy and diseased coral mucus (healthy *Acropora* sp. mucus, white patch *Acropora* sp. mucus, healthy *Porites* sp. mucus, and pink line syndrome *Porites* sp. mucus) samples by using SPSS software. The coefficient of determination  $r^2$  was calculated using Microsoft Excel.

### 3. Results

#### 3.1 Coral mucus antimicrobial activity against bacteria

The healthy and diseased *Acropora* sp. and *Porites* sp. mucus was observed for significant activity by inhibiting the coral pathogens of *S. aureus* and *V. parahaemolyticus* (Fig. 1A). The filtered mucus of *Porites* sp. and *Acropora* sp. were observed for potential antimicrobial activity with an increased concentration against *S. aureus* and *V. parahaemolyticus* (Fig. 1B). Thus, the zone of inhibition was increased with the increased concentration of mucus, suggesting the antagonistic ability of the coral mucus against the coral bacterial pathogens are concentration dependant.

#### 3.2 Antimicrobial activity of coral mucus against ciliates

Growth and proliferation of both the ciliate such as *Cohnelimbus* sp. (Fig. 2) and *Holosticha* sp. (Fig. 2) were observed to be inhibited by *Acropora* sp. mucus. Wherein, *Cohnelimbus* sp. exhibited more sensitivity to *Acropora* sp. mucus compared to *Holosticha* sp. While seawater alone showed support for both the ciliate growth. Seawater mixed with mucus showed intermediate growth. The ciliate inhibition rates were measured by an increase in incubation time is directly proportional to a decrease in ciliate numbers.

#### 3.3 Quorum quenching property of coral mucus

A zone of clearance was observed in a *C. violaceum* spread plate assay around the well-containing diseased and healthy mucus (Fig 3A). This reveals that mucus can to interfere with QS produced by coral host pathogens. The Violet colour of the violacein pigment with quorum sensing property was seen in the negative control wells. The inhibition of quorum sensing by mucus was investigated by the use of violacein pigment of *C. violaceum* that was estimated by spectrophotometric assay. Violacein pigment, a quorum sensing molecule of *C. violaceum* production was inhibited by healthy and diseased coral mucus (Fig. 3B). The healthy *Acropora* sp. mucus, healthy *Porites* sp. mucus, and *V. harveyi* showed maximum inhibition of violacein pigment production due to the abundance of quorum quencher molecules. Whereas less inhibition was seen in PLSM (pink line syndrome *Porites* sp. mucus) and white patch *Acropora* sp. diseased coral mucus. Control only with *C. violaceum* showed maximum production of violacein. Therefore, a fall in the violacein concentration suggests the inhibition of quorum sensing.

The healthy and diseased *Acropora* sp. and *Porites* sp. mucus were observed to be positive for lactonase enzyme presence (Fig C). The AHL lactonase of the coral mucus

hydrolyzes penicillin into penicilloic acid, confirming the presence of quorum quenching molecule in coral mucus (Fig C).

### **3.4 Total antioxidant contents of coral mucus**

The quantitative determination of total phenolic content was determined by the use of Folin–Ciocalteu reagent. Gallic acid was used as a reference compound for phenol content while the quercetin standard curve was plotted as concentration against absorbance and slope values were determined for flavonoid determination. Diseased *Acropora* sp. mucus showed significantly ( $p > 0.0004$ ) more concentration of 3.67  $\mu\text{g/ml}$  of total phenol than healthy *Acropora* sp. mucus which was 2.30  $\mu\text{g/ml}$ . The determined total soluble phenol (Fig. 4) concentration was found to be significantly more ( $p > 0.00002$ ) in diseased *Porites* sp. mucus 14.54  $\mu\text{g/ml}$  compared to the healthy *Porites* sp. mucus 9.86  $\mu\text{g/ml}$ . However, only diseased coral mucus was observed to significantly produce a larger repertoire of non-enzymatic antioxidant compounds as compared to healthy coral mucus as far as the levels of total soluble phenol were concerned. The determined flavonoid (Fig. 4) concentration of *Acropora* sp. mucus was significantly ( $p > 0.0002$ ) more in the diseased mucus 18.3  $\mu\text{g/ml}$  than in healthy *Acropora* sp. mucus 7.9  $\mu\text{g/ml}$ . The flavonoid concentration of diseased *Porites* sp. mucus was 16.09  $\mu\text{g/ml}$  which was significantly ( $p > 0.0001$ ) more than healthy *Porites* sp. mucus 9.55  $\mu\text{g/ml}$ .

### **3.5 Coral's antioxidant scavenging activity.**

#### **3.5.1 Hydrogen peroxide scavenging activity**

Diseased mucus actively scavenged hydrogen peroxide and showed maximum activity (Fig. 5A). The healthy coral mucus of both *Acropora* sp. and *Porites* sp. coral species exhibited poor hydrogen peroxide scavenging properties than the diseased coral mucus. One-way analysis of variance (ANOVA) determined a significant increase in (more 12.75 % ( $p > 0.05$ )) hydrogen peroxide scavenging activity in the diseased *Porites* sp. mucus than in healthy mucus (8.91 %). *Porites* sp. mucus displayed approximately 2-fold more hydrogen peroxide scavenging than healthy *Acropora* sp. 7.65 % and diseased *Acropora* sp. 8.91 % mucus at 50  $\mu\text{l}$  concentration. While the diseased *Porites* sp. mucus exhibited a maximum activity of 14.57 % at a 75  $\mu\text{l}$  concentration.

#### **3.5.2 Hydroxyl radical scavenging activity**

Coral mucus hydroxyl radical scavenging capacity was analyzed and compared with standard mannitol (Fig. 5B). The hydroxyl radical scavenging activity of *Acropora* sp. mucus

and *Porites* sp. mucus was subsequently found high in diseased corals. Hydroxyl radical scavenging of *Acropora* sp. diseased mucus at 75  $\mu$ l concentration was statistically significant ( $p > 0.03$ ) in diseased mucus (5.40 %) than in healthy mucus (3.85 %). Similarly, *Porites* sp. mucus also revealed statistically significant ( $p > 0.05$ ) scavenging activity in diseased mucus compared to healthy mucus at all three concentrations (25  $\mu$ l, 50  $\mu$ l, and 75  $\mu$ l). The scavenging activity at 25  $\mu$ l concentration, varies from 0.98 % in healthy *Acropora* sp. mucus to 5 % in pink line syndrome affected *Porites* sp. mucus. As compared to *Acropora* sp. mucus, *Porites* sp. mucus displayed 2-2.5-fold more activity in healthy and up to 3.5-fold more activity in diseased mucus to scavenge the hydroxyl radicals.

### 3.5.3 DPPH scavenging activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was demonstrated based on the ability of DPPH to react with proton donors such as phenols (Fig. 5C). The DPPH scavenging activity was checked for the *Porites* sp. and *Acropora* sp. mucus and the butylated hydroxy anisole (BHA) was used as the reference compound. Wherein, the reaction mixture changed colour from purple to yellow. At 25  $\mu$ l concentration of mucus, healthy *Acropora* sp. showed (19.49 %) DPPH scavenging, while *Porites* sp. mucus displayed significance ( $p > 0.03$ ) in diseased mucus (23.13 %) than healthy mucus (20.94 %). Similarly, at 75  $\mu$ l concentration, DPPH scavenging was found more ( $p > 0.01$ ) in diseased *Porites* sp. mucus (27.69 %) than in healthy *Porites* sp. mucus (25.68 %). Thus, the ability of coral mucus to scavenge the DPPH radicals was observed predominantly more in diseased coral mucus.

### 3.5.4 Metal chelating assay

The metal ion chelation capacity of coral *Acropora* sp. and *Porites* sp. mucus was analyzed (Fig. 5D). EDTA was used as a reference compound and the metal ion chelation was observed in all the coral mucus. Diseased *Acropora* sp. mucus at 75  $\mu$ l concentration showed statistically significant ( $p > 0.05$ ) metal chelation than *Acropora* sp. healthy mucus. *Porites* sp. diseased mucus at 25  $\mu$ l ( $p > 0.02$ ) and at 75  $\mu$ l concentration ( $p > 0.01$ ) showed significantly more activity (8.47 % and 10.12 %) than healthy *Porites* sp. mucus (7.4 % and 8.96 %). The capacity of mucus to capture and bind reactive metal ions was found high in *Porites* sp. diseased mucus. Thus, coral mucus interferes with the formation of the Fe  $2^+$  -ferrozine complex and captures the ferrous ion before ferrozine.

### 3.5.5 Reducing power assay

There were significant linear correlations ( $r^2 = p < 0.05$ ) between total antioxidants and the reducing ability of coral mucus (Fig. 5E). The potential of mucus to convert harmful reactive oxygen species into less harmful substances was analyzed at various concentrations of mucus. In both *Porites* sp. and *Acropora* sp., the reducing power of mucus was observed high in diseased corals than in healthy ones. There was a significant difference ( $p > 0.05$ ) between the reducing power of healthy and diseased *Acropora* sp. mucus and *Porites* sp. mucus. In diseased *Acropora* sp. mucus reducing power was 0.11 unit and whereas in *Porites* sp. diseased mucus it was found 0.12 unit which was recorded maximum in mucus.

## 3.6 Coral mucus antioxidant enzymes

### 3.6.1 Peroxidase activity

*In vitro* peroxidase activity of coral mucus was demonstrated spectrophotometrically and expressed as U/mg of protein (Fig. 6). The peroxidase activity was significantly higher ( $p > 0.0005$ ) in pink line syndrome-affected *Porites* sp. mucus (2.12 U/mg of protein) than in healthy *Porites* sp. (1.11 U/mg of protein). *Acropora* sp. mucus also showed significantly ( $p > 0.0008$ ) more activity in white patch diseased mucus which was higher (1.62 U/mg of protein) than healthy mucus (0.52 U/mg of protein). Among different mucus, peroxidase activity was observed more in pink line syndrome-affected *Porites* sp. Maximum activity was seen in pink line syndrome *Porites* sp. mucus as far as mucus peroxidase activity is concerned.

### 3.6.2 Phenol oxidase

The presence of phenol oxidase enzyme has been checked in healthy and diseased mucus (Fig. 6). One-way analysis of variance (ANOVA) demonstrated that pink line syndrome affected *Porites* sp. mucus (1.03 U/mg of protein) was statistically significantly different ( $p > 0.05$ ) and more from healthy *Porites* sp. mucus (0.34 U/mg of protein) as far as phenol oxidase activity was concerned. Phenol oxidase activity was also observed significantly more ( $p > 0.05$ ) in diseased *Acropora* sp. mucus (0.85 U/mg of protein) than in healthy *Acropora* sp. mucus (0.27 U/mg of protein). PO activity observed in *Porites* sp. mucus was 2-fold more than in *Acropora* sp. mucus. Phenol oxidase activity noticed was 2-3-fold higher in diseased mucus than in healthy mucus of both the coral species.

### 3.6.3 Superoxide dismutase activity

*Acropora* sp. and *Porites* sp. healthy and diseased mucus exhibited high SOD activity which was expressed in U/mg of proteins (Fig. 6). Maximum activity was observed in *Porites* sp. pink line syndrome mucus (1.43 U/mg of proteins) which was significantly more ( $p > 0.05$ ) than the activity found in healthy *Porites* sp. mucus (0.95 U/mg of proteins). Superoxide dismutase enzyme activity levels were less when compared with that of healthy *Acropora* sp. mucus (0.80 U/mg of protein) than in diseased *Porites* sp. mucus (1.37 U/mg of proteins) which was significant ( $p > 0.05$ ).

The superoxide dismutase enzyme activity of mucus was confirmed by developing a zymogram on electrophoresis by specific staining. SOD reacted with the reaction mixture's substrate nitro-blue tetrazolium (NBT). White-colored SOD bands had been detected on a blue background in a native-PAGE gel (Fig. 7A).

### 3.6.4 Catalase activity

Catalase activity of *Acropora* sp. and *Porites* sp. mucus was performed by spectrophotometric assay for quantitative analysis. The activity was 2-fold more in diseased mucus compared to healthy (Fig. 6). Catalase activity was high in diseased *Porites* sp. mucus (1.35 U/mg of protein) than in healthy *Porites* sp. mucus (1.12 U/mg of protein). Similarly, less activity was noticed in healthy *Acropora* sp. mucus (1.07 U/mg of protein) than in diseased *Acropora* sp. mucus (1.15 U/mg of protein). Maximum catalase activity was observed in pink line syndrome of *Porites* sp.

Further, the catalase enzyme activity of mucus was analysed qualitatively on native page gel by specific staining, by providing hydrogen peroxide as a substrate. Yellow coloured catalase enzyme activity bands (Fig. 7B) had formed against a blue-green background and Ferricyanide precipitation causes negative staining of the catalase enzyme. Besides, the presence of catalase in mucus was reconfirmed on the slide with the cleavage of hydrogen peroxide into water and oxygen bubbles (Fig.7C).

### 3.6.5 Detection of protease in coral mucus

In this study the metalloproteases were observed in healthy and diseased mucus of *Acropora* sp., while the *Porites* sp. mucus exhibited both serine and metalloprotease (Fig. 8). The cysteine and aspartic protease were not observed in both the coral mucus (Fig 8). The healthy and diseased mucus of *Porites* sp. exhibited the presence of metalloprotease by the inhibition activity of EDTA and O-phenanthroline, compared with the control or no inhibitor

treatment. The serine protease inhibition was achieved in the presence of PMSF (Fig. 8 A & B) as an inhibitor in both the diseased and healthy mucus of *Porites* sp. Unlikely, the diseased mucus of *Acropora* sp. displayed a lower level of inhibition of metalloprotease by O-phenanthroline alone. However, the healthy mucus of *Acropora* sp. exhibited the presence of metalloprotease by both the inhibitors such as EDTA and O-phenanthroline. The presence of other proteases such as cysteine and aspartic protease inhibition was not achieved in both the coral mucus during the treatment with N-ethylmaleimide and Pepstatin respectively.

#### 4. Discussion

*Acropora* sp. and *Porites* sp. mucus exhibited antimicrobial properties against various known coral pathogens such as *S. aureus* and *V. parahaemolyticus* (Fig. 1B). The present study shows that the coral mucus has activity not only against pathogenic bacteria but also against coral diseased lesions associated protozoan ciliates such as *Cohnelimbus* (Fig. 2B) and *Holosticha* (Fig. 2C). Thus, the antimicrobial activity suggests the possible mucus role in the first line of defense that favours the corals health and resilience. This preliminary defense property of mucus prompted us to screen the various mucus defense components that have been much studied in other organisms such as fish mucus but not in coral mucus. The results revealed the antioxidants and antioxidant enzymes of diseased coral mucus for maximum activity of free radical scavenging than the healthy mucus, which may be due to signals/factors involved in the activation of the corals natural defense signals against any biotic or abiotic stress.

In this study, the quorum quenching property of the healthy and diseased *Acropora* sp. and *Porites* sp. mucus (Fig. 3A, B, C) suggests that it may contain enzymes such as lactonase, which competitively inhibits the AHL molecule by binding with AHL receptors. Thus, the AHL lactonase enzyme in the coral mucus, which inactivates penicillin's beta-lactam ring, a member of the beta-lactamase superfamily (Huma et al., 2011), that converts penicillin into penicilloic acid was elucidated. This coral mucus was also confirmed for its inhibition of violacein pigment production, a quorum sensing molecule produced by *C. violaceum* (Fig. 3A, B), suggesting the vital role of the mucus components in inhibiting pathogens that cause infections in corals.

*Acropora* sp. and *Porites* sp. mucus exhibited the presence of antioxidants, which have free radical scavenging properties, reducing power, and metal ion chelating capacity. This antioxidant activity was expressed more in diseased mucus than in healthy. However, the activity levels differed in *Acropora* sp. and *Porites* sp. mucus. This variation may be due to the

*Symbiodinium* type that has been associated (McGinty et al., 2012; Krueger et al., 2014). Also, excess hydrogen peroxide ( $H_2O_2$ ) radicals cause the dissociation of zooxanthellae from the coral host by damaging holobionts cells or triggering cell signaling pathways of the symbiont (Weis, 2008). Hence  $H_2O_2$  scavenging antioxidants and catalase enzymes were analysed and found to be maximum in diseased coral mucus suggesting that diseased corals express effective defense capability to fight against  $H_2O_2$  which helps in preventing bleaching (Fig. 5A). Hydroxyl and DPPH radicals are the major free radicals that cause lipid peroxidation and severe cell damage (Aurand et al., 1977) which affects the thylakoid membrane, phospholipids (Lesser, 1996; Tchernov et al., 2004) and the photosynthesis ability of endosymbionts. The mucus of the present study showed effective scavenging activity against DPPH and diseased mucus of *Porites* sp. was observed with maximum activity (Fig. 5C). A significant amount of reducing agents in mucus reveals that it can effectively reduce the harmful oxidants by the addition or removal of hydrogen (Fig. 5D). Activity of the chelating agent was also observed in mucus (Fig. 5E). These sequestering agents can prevent corals from oxidative damage by reacting with free unstable metal ions and reduce their harm by forming a stable compound.

The presence of free radical-scavenging antioxidant enzymes in the coral mucus was also tested. Catalase inactivates this  $H_2O_2$  into oxygen and water. The SOD and catalase enzyme expression was observed more in diseased coral mucus as compared to healthy mucus, which might be due to the high oxidative stress in diseased mucus (Fig. 6, 7). Further, peroxidase enzymes protect the coral from the fungal pathogen, which has been reported in coral disease aspergillosis caused by *Aspergillus sydowia* (Mydlarz and Harvell, 2007). Peroxidase activity of the coral mucus was observed to be the highest in the *Porites* sp. diseased mucus (Fig. 6). Earlier findings on antioxidant enzyme cascade of the coral tissues have also reported the upregulation against ROS generation due to thermal and salt stress (Almeida and Mascio, 2011). This supports our present results on the upregulation of SOD, catalase, and peroxidase enzymes in diseased mucus (Fig. 6, 7).

The presence of phenoloxidase activity in coral mucus of the present study (Fig.6) was also reported in some cnidarian mucus such as healthy and black band disease of *Pseudodiploria strigosa*, healthy and bleached *Cassiopea xamachana* and *Exaiptasia pallida* mucus (Rivera-Ortega and Thome, 2018; Palmer et al., 2011). Further, the involvement of proteases in coral mucus is least explored and in the case of fish mucus, these proteases play a vital role in innate immune functions (Ingram, 1980). Based on the nature of the chemical catalysis group they are classified into cysteine, serine, aspartic and metalloproteases (Hartley,

1960). They are known for pathogen damage through pathogenic bacterial protein cleavage and function in indirect production and activation of antimicrobial peptides, complement and immunoglobulins (Cho et al., 2002a, b). In this study, the coral mucus as well displayed the presence of proteases such as serine and metalloproteases in *Porites* sp. and metalloproteases in *Acropora* sp. (Fig 8). Thus, this result was observed to be the first report in the case of coral mucus and the specific function of these proteases needs further detailed investigations.

## 5. Conclusion

The presence of antioxidants including phenoloxidase and other enzymes such as peroxidase, superoxide dismutase, metalloprotease, serine protease, and catalase in healthy and diseased *Acropora* sp. / *Porites* sp. mucus is first documented in this work. Quorum quenching and antibacterial activity of the coral mucus supports its potential as an antimicrobial agent against coral-associated microbial infections. The differential expression of antioxidants in healthy and diseased mucus explores the signals/factors involved in the activation of the immune signals against the invasion of pathogenic stress. Thus, the present study supports the presence of biomolecules in coral mucus involved in coral defense against various biotic and abiotic stresses. In addition, these biomolecules of the coral mucus act as biomarkers to determine the corals health status. Therefore, further study on the mechanism of synthesis, source, and involvement of these enzymes and other components in coral mucus may help in understanding the coral's natural defenses against pathogenic infections.

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## Figure Legend

- Fig. 1.- A.** Healthy and diseased *Acropora* sp. and *Porites* sp. **i.** *Acropora* sp. healthy, **ii.** *Porites* healthy, **iii.** *Acropora* sp. white patch disease, **iv.** *Porites* sp. pink line syndrome disease. **B.** Antimicrobial activity of mucus against coral pathogens- **i.** *Acropora* and *Porites* mucus activity against *S. aureus* **ii.** *Porites* mucus activity against *S. aureus*, **iii.** *Acropora* and *Porites* mucus activity against *Vibrio parahaemolyticus*, **iv.** Filtered *Acropora* and *Porites* mucus against *S. aureus*.
- Fig. 2.** Antimicrobial activity of mucus against eukaryotic protozoan ciliates. **A.** Ciliates associated with diseased corals **i.** *Cohnelimbus* sp. **ii.** *Holoscticha* sp. **iii.** *Uronema* sp. **iv.** *Euplotes* sp. **B.** *Acropora* sp. mucus inhibits the growth of *Cohnelimbus* sp. **C.** *Acropora* sp. mucus inhibits the growth of *Holosticha* sp. (SW- Seawater, MF- mucus filter and MF+SW- mucus filter and seawater).
- Fig. 3.** AHL quorum sensing molecule inhibition assay. **a.** **(i)** Positive control *V. harveyi* inhibiting violet color by degrading AHL molecule of *C. violaceum*. **(ii)** In wells CA4-*C. amphilecti* inhibited the AHL molecule and produced a clear zone of inhibition. **(iii)** Healthy and diseased *Acropora* and *Porites* mucus showing zone of AHL inhibition. **b.** Iodometric assay to determine the hydrolyzing activity of mucus AHL lactonase. Wells containing positive control penicillin without test samples showing blue color formation. Healthy *Acropora*, white patch *Acropora*, healthy *Porites* and pink line syndrome disease *Porites* mucus shows no color formation. **c.** Inhibition of violacein production by coral mucus. Violacein production was measured spectrophotometrically as described in the materials and methods. Data are presented as absorbance at 585 nm. (HAM- Healthy *Acropora* sp. mucus, DAM/WPAM-White patch *Acropora* sp. mucus, HPM- Healthy *Porites* sp. mucus, PLSM- Pink line syndrome *Porites* sp. mucus)
- Fig. 4.** Total antioxidants in coral mucus- Total soluble phenol and flavonoid concentration of coral mucus. Values are mean  $\pm$  SD (n = 3). HAM- Healthy *Acropora* mucus, DAM- White patch *Acropora* sp. mucus, HPM - Healthy *Porites* mucus, PLSM- Pink line syndrome *Porites* sp. mucus.
- Fig. 5.** Antioxidant assay of coral mucus- Antioxidant properties of diseased and healthy coral mucus. **a.** Hydrogen peroxide free radical scavenging activity at increasing concentrations of mucus. **b.** Hydroxyl radicals scavenging capacity observed in corals. Mannitol is used as a reference compound. **c.** 2,2-diphenylpicrylhydrazyl (DPPH) scavenging activity present in coral mucus. BHA- Butylated hydroxy anisole is used as standard. **d.** Metal ion chelation capacity of corals, EDTA- Ethylenediamine tetra-acetic acid. **e.** Relationship between reducing the power of coral mucus and total antioxidant. BHT -Butylated hydroxytoluene is used as a reference compound. Values are mean  $\pm$  SD (n = 3). All the experiments were carried out in triplicates. HAM- Healthy *Acropora* mucus ( $r^2=0.9987$ , p-value=0.02), DAM-White patch *Acropora* mucus ( $r^2=0.9997$ , p-value=0.009), HPM- Healthy *Porites* mucus ( $r^2=0.9926$ , p-value=0.54), PLSM- Pink line syndrome *Porites* mucus ( $r^2=0.9849$ , p-value=0.7).
- Fig. 6-** Antioxidant enzyme assay of coral mucus - Superoxide dismutase, catalase, phenoloxidase, and peroxidase enzyme activity assay of coral mucus of healthy and diseased *Acropora* sp. and *Porites* sp. Data represented with three technical replicates mean  $\pm$  SD (n = 3). HAM- Healthy *Acropora* sp. mucus, DAM- Diseased *Acropora* sp. mucus, HPM- Healthy *Porites* sp. mucus, PLSM- pink line syndrome disease *Porites* mucus.
- Fig. 7.** Native Page gel examination of SOD and CAT – **A.** Superoxide dismutase enzyme activity on 10 % native-PAGE gel with an equal concentration of protein 50  $\mu$ g (well 1 to 4) HAM- healthy *Acropora* mucus, WPAM- white patch *Acropora* mucus protein,

HPM- healthy *Porites* mucus, PLSM-Pink line syndrome *Porites* mucus. **B.** Catalase enzyme activity on 6 % native PAGE gel in well 1. control, 2. healthy *Porites* mucus, 3. pink line syndrome *Porites* mucus, 4. healthy *Acropora* mucus, 5. white patch *Acropora* mucus protein, and 6. *V. harveyi*. **C.** Catalase test of mucus against 3 % hydrogen peroxide. All corals mucus i.e. HAM-healthy *Acropora* mucus, WPAM-white patch disease *Acropora* mucus, HPM-healthy *Porites* mucus, and PLSM-pink line syndrome disease *Porites* mucus showing positive catalase test.

**Fig. 8.** Zymography to determine the presence of protease types in the mucus of *Acropora* sp. and *Porites* sp. **A-** *Porites* sp. healthy mucus with the presence of serine and metalloproteases. **B-** *Porites* sp. diseased mucus with the presence of serine and metalloproteases. **C-** *Acropora* sp. healthy mucus with the presence of metalloprotease. **D-** *Acropora* sp. diseased mucus with the presence of metalloprotease. Whereas the other proteases such as cysteine and aspartic proteases were not observed in both the healthy and diseased coral mucus. (EDTA- ethylenediamine tetra-acetic acid, PMSF- phenylmethylsulfonyl fluoride).

Figure 1

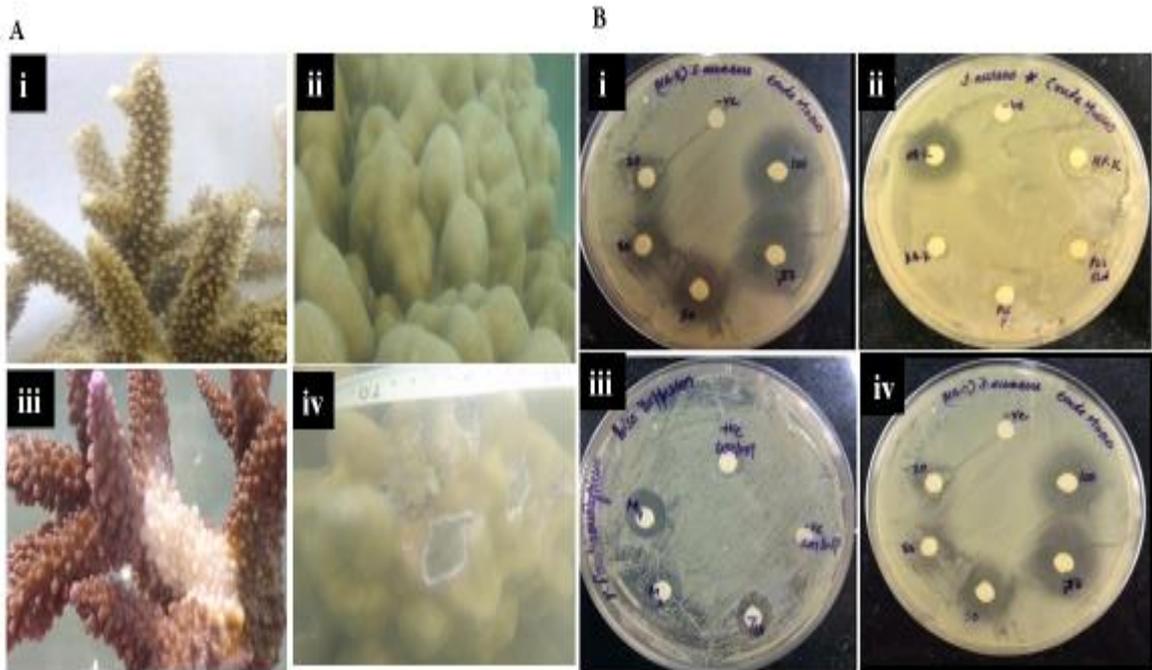


Figure 2

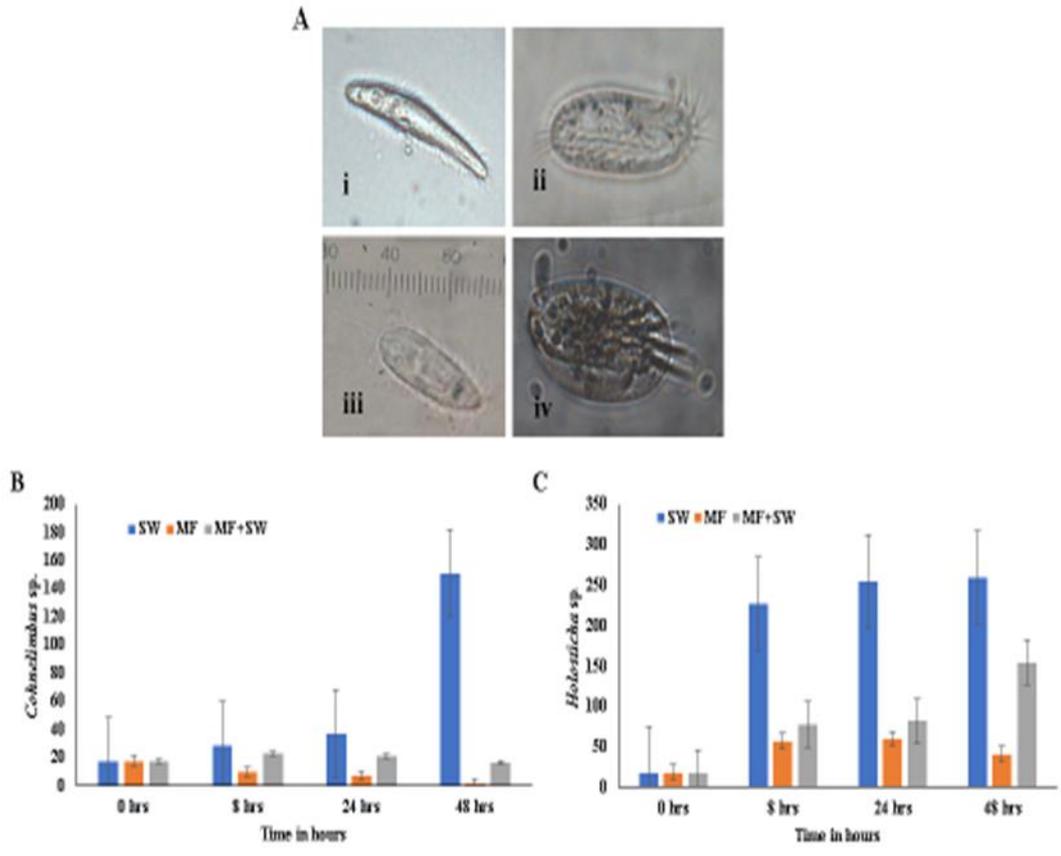


Figure 3

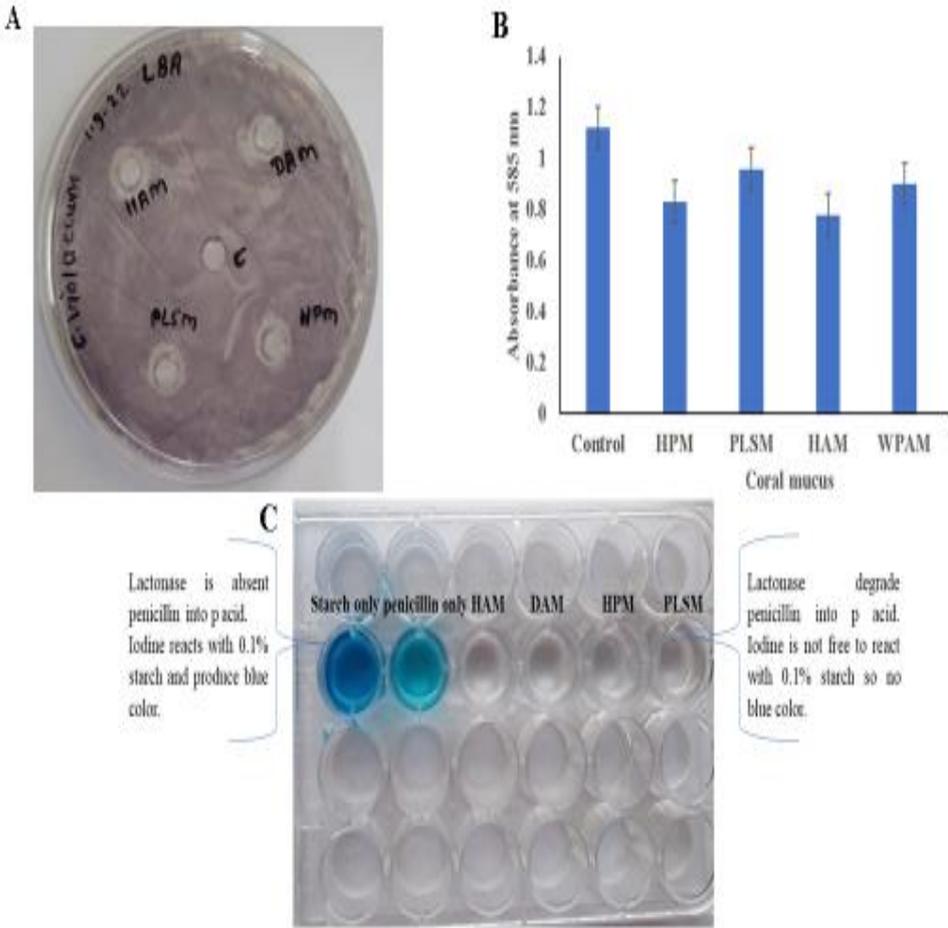


Figure 4

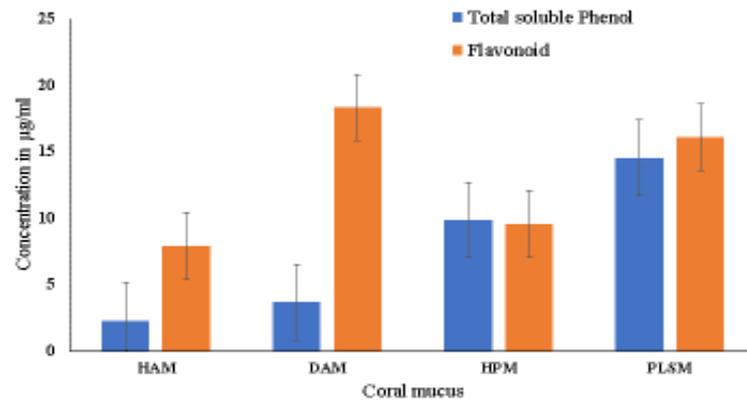


Figure 5

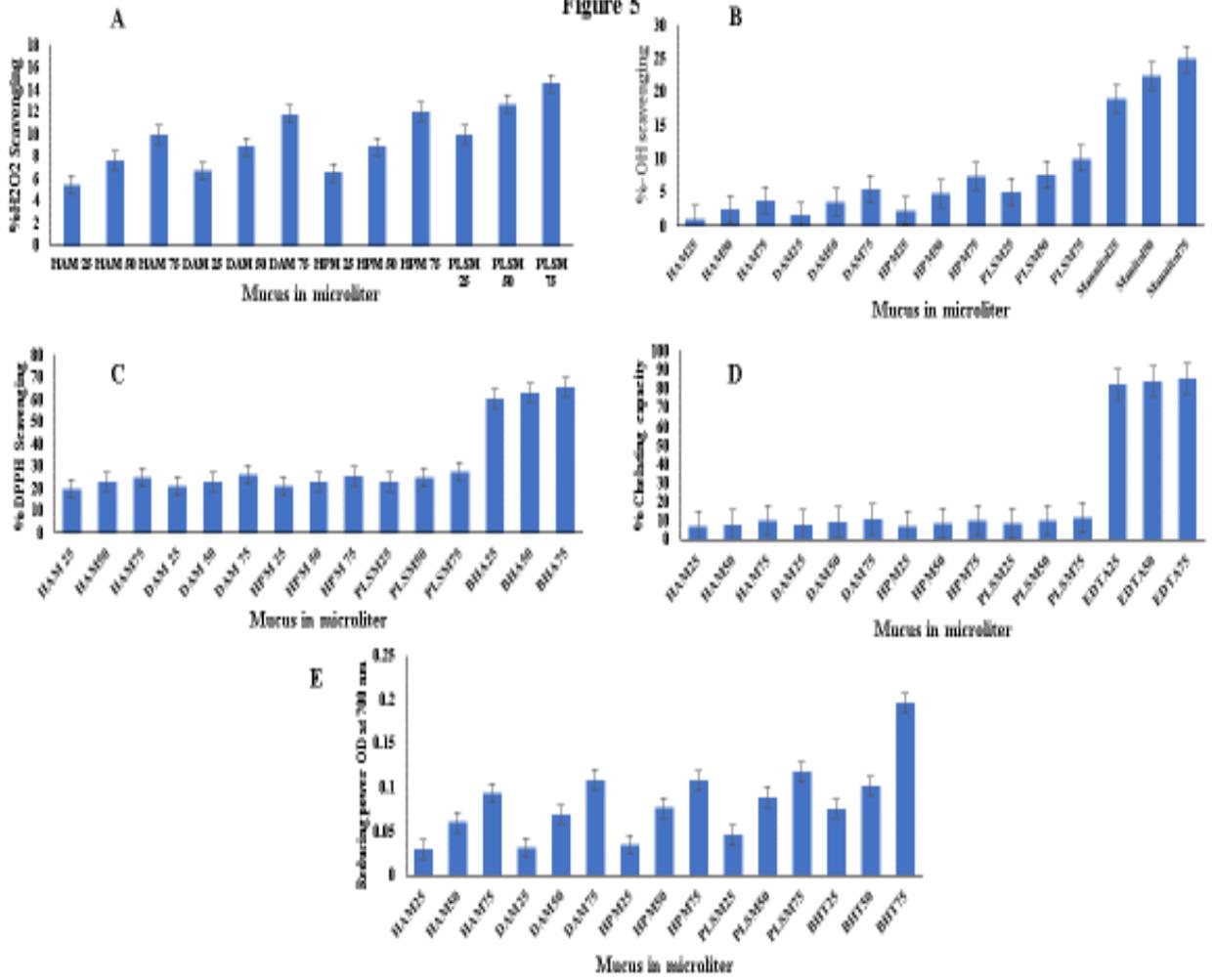


Figure 6

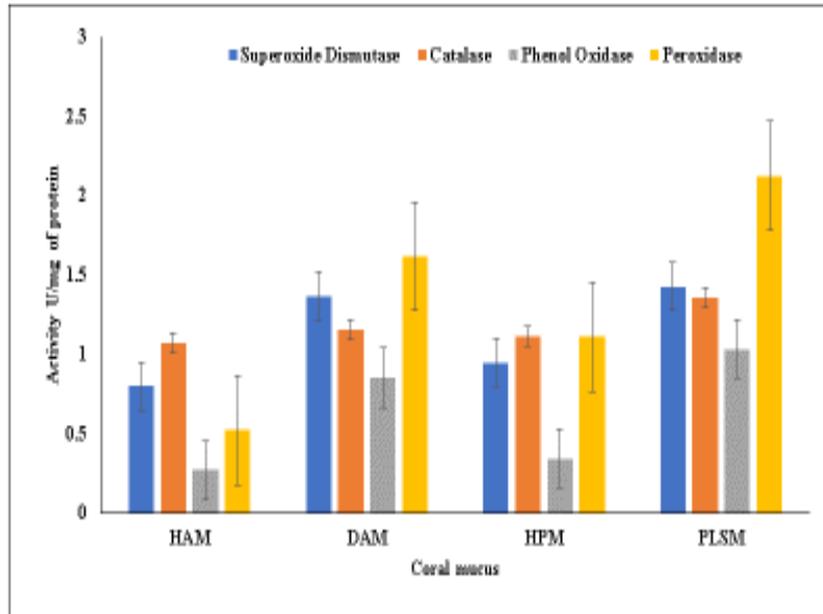


Figure 7

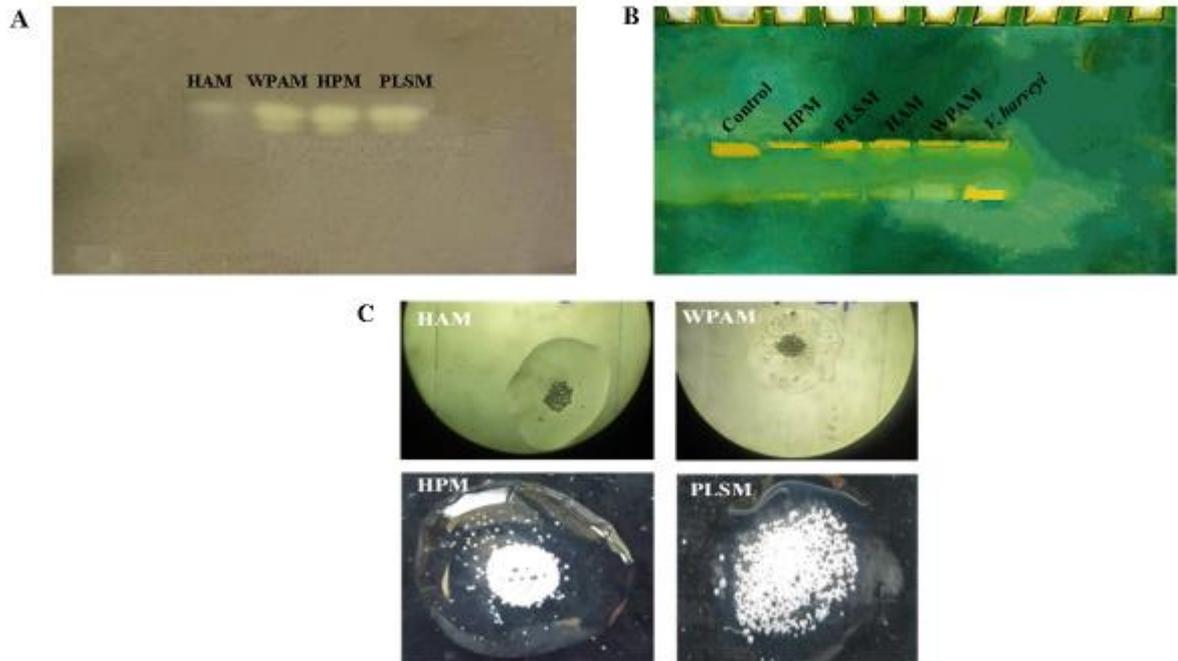


Figure 8

