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Production, purification and characterization of Metalloenzyme Isolated from Epiphytic bacteria

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Abstract
Marine bacteria especially *Bacillus* species are used in pharmaceutical industries, Food and detergents producing industries, also it is very important in making enzyme medicines to cure various diseases. Seaweeds associated bacteria can produce a valuable biocontrol agent against kinds of pathogenic organisms. In this present investigation was conducted for production and characterization of metalloenzyme from epiphytic bacteria of seaweeds. All the samples were collected in Thirumullavaram coastal area of Kollam, Kerala state of India. *Bacillus* spp. was isolated by standard laboratory techniques. Identification of *Bacillus* spp. was characterized by phenotypic and biochemically. BL-3 was to made higher production of metalloenzyme. Protein estimation on SDS PAGE and observed at 25kDa. Specific activity of metalloenzyme was observed at 0.125 U/mg by spectrophotometer. This enzyme can inhibit the clinical pathogen. Hence this metalloenzyme could be a biocontrol agent in pharmaceutical industries and used for making enzyme medicines.

Keywords: Bacillus spp.**,** Metalloenzyme, Characterization, SEM, XRD and DEAE.

1. Introduction

Enzymes therapies are becoming more prevalent in medicine today with many manufacturers targeting their advantages in disease treatment. In the last 100 years, enzymes have been increasingly used to treat various diseases. Enzymes are found throughout the natural world.

There is an increasing demand for biodiversity in the screening programmes for selecting therapeutic drugs from natural products, the marine organisms; especially seaweeds are of with immerse interest, since they are having a broad range of biological activities such as antibacterial, antifungal and antiviral. It has a decreased efficiency and resistance as of antitumorals, anti-inflammatory and antioxidants in potential sources of antibiotics substances. The production of antimicrobial was considered to be an indicator of the organisms the seaweeds to synthesize bioactive secondary metabolites [1].

Seaweeds associated epibiotic bacteria become an incredible source of new natural products [2]. The isolation of these epibionts is relatively very easy as compared to other bacteria. Thus seaweed-associated bacteria may be useful in the development and production of bioactive compounds and biocontrol agent. Several of the bacterial strains phylogenetically related, have industrial application; therefore, it is necessary to study the chemical interactions seaweed - bacteria for a better understanding of the process of production of the different enzymes produced by epibiotic bacteria.

Bacillus species is one of the important epibiotic bacteria and have been found to possess chemical compounds with anticancer activity [3]. In the recent years, the bioactive properties of marine algae and marine microorganisms have been analyzed and in both cases positive results have been Obtained [4, 5]. *Bacillus* spp. is capable of producing several antimicrobial compounds.

The metalloprotease enzyme isolated from epiphytic bacteria *Bacillus spp*. of *Sargassum* spp and *Padina* spp seaweeds. Hence this study focuses on the production and characterization of Metals like Zn containing enzymes as a biocontrol agent used to inhibit the clinical pathogens in further studies.

2. Materials and methods

2.1 Marine Seaweed Collection

Seaweeds samples of the *Padina,* and *Sargassum* were collected by handpicking during intertidal from Thirumallavaram in the District of Kollam (Kerala state), Arabian coast (8.90 26ºN, 76.56 11ºE). Collected samples were transferred in sterilized bags on ice. Seaweed species were labeled with common name and date. The algal surface was washed and a bacterial sample was taken from the surface with a sterile swab. Swab the epiphytic bacteria of seaweed were taken into the test tubes containing Zobell marine broth used as a transported media. All the test tubes were stored at 37 ºC for 24 to 48 h for incubation period [6].

2.2 Isolation of *Bacillus* spp.

MYP Agar is the media used to *Bacillus* spp. Polymyxin-B and Egg-Yolk Emulsion were additive chemicals for killing other gram positive and gram negative species on the media. Yellow color appearance of colonies and slightly agar surroundings changed to determined colonies of *Bacillus* spp. [7].

2.3 Identification of *Bacillus* spp.

Identification of *Bacillus* spp. is done by the phenotypic characterization and biochemical characterization according to Bergey's Manual of Determinative Bacteriology [8].

2.4 Production and purification of metalloenzyme

The production medium was inoculated at 1.0 % (V / V) with 24 h old (OD = 0.6) culture of *Bacillus* spp. Incubated at (15 ± 1) °C in a refrigerated incubator shaker at 80 rpm / min for 48 h. The growth cultures were then centrifuged at 4 °C (10,000 × g, 15 min) [9].The supernatant was used for further purification assay. Metalloprotease enzyme was purified from crude extraction of *Bacillus* spp. by Ammonium sulphate precipitation, Dialysis method and DEAE Cellulose column chromatography. Purification was confirmed with sodium dodecyl sulphate (SDS) gel electrophoresis.

2.5 Protein Estimation by Lowry Method [10]

Protein can be estimated by Lowry method with BSA standard solution. OD was measured at visible range 680 nm by a spectrophotometer.

2.6 SDS PAGE Analysis

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS - PAGE) was performed on a 5 % stacking and a 12 % running gel according to the method of Laemmli [11] to determine the molecular weight of metalloprotease.

2.7 Precipitation by Ammonium Sulfate

A specific weight of ammonium sulphate crystals was added to the crude enzyme Gradually in an ice bath with continuous stirling for 30 min.Crude enzyme attained the saturation percentage 40 - 80 % and centrifuged at 10000 rpm at 4 ºC for 20 min. Precipitate was taken and dissolved in minimal amount of 0.1 M phosphate buffer, It was dialyzed against the same buffer for 24 h.

2.8 Deae Cellulose Column Chromatography

After the purification of dialyzed sample, it was subjected to purification by DEAE Cellulose column chromatography. 4 g of DEAE Cellulose was suspended in 25 mM buffer and kept overnight for equilibration. The column was carefully packed and equilibrated with the buffer at a constant flow rate of 30 ml / h. 3 ml of the dialyzed enzyme extract was diluted to 15 ml and loaded onto the column. The flow through was collected as a single fraction and tested for protease activity. The column was washed with approximately 100 ml of the equilibration buffer. The washings were collected as 3 ml fractions and tested for absorption at 280 nm. Fractions were collected. The washings were checked for protease activity. The equilibration buffer with 0.1M NaCl was used for elution. The column was eluted at a flow rate of 30 ml / h and 3 ml fractions were collected. The absorbance at 280 nm was recorded. The fractions with a high absorption at 280 nm were tested for protease activity. The fractions with the highest protease activity were pooled followed by equilibration and elution.

3. Results

Marine bacterium *Bacillus* spp. was isolated from marine seaweed namely, Brown seaweed *Padina* spp and *Sargassum* spp. (Plate 1A) collected from Thirumallavaram coast in Kollam, Kerala (Plate 1) and the seaweeds are identified at CMFRI, Tamil Nadu, India.

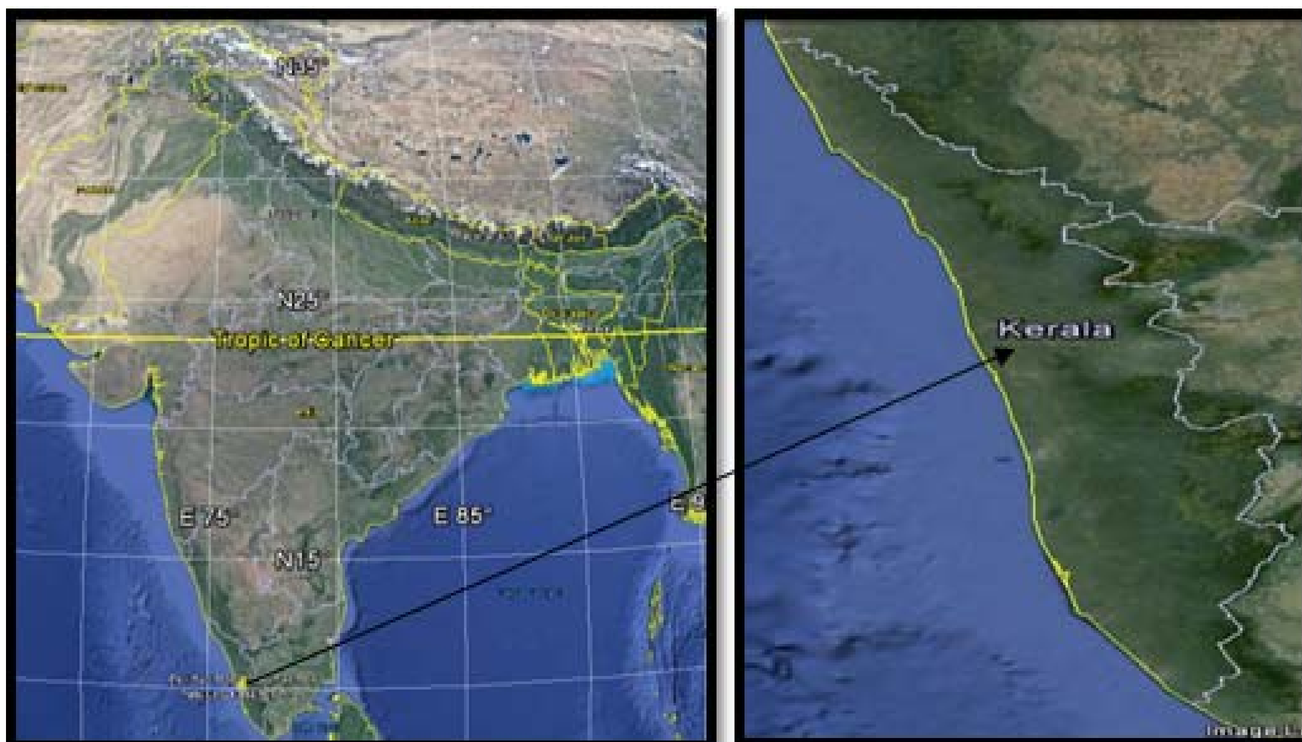

Plate 1: Satellite map showing the macro algae sample collection area

Plate 1(A): *Sargassum* spp. and *Padina* Spp.

The epiphytic bacterium was identified by biochemical characters. The bacteria belonging to Class *Bacillales* were present only in the macro algal samples that were collected during the pre-monsoon seasons. A total of 100 isolates only the 14 isolates were confirmed as epiphytic bacteria *Bacillus* spp. based on their distinct morphological characteristics.

Production of protease enzyme from *Bacillus* strains was carried out in a 'Protease Specific Medium'. The contents were then centrifuged at 5,000 rpm for 20 min at 4°C and the cell-free supernatant was used for determining extracellular protease activity.

The crude enzyme solution was used for determination of enzyme activity. Metalloprotease activity it was observed in 0.104 at 600 nm by Digital Spectrophotometer. The specific enzyme activity of the crude sample was found to be 0.125 U / mg. Total protein content of the protease was done by Folin

Ciocalteau method using bovine serum albumin (BSA) as a standard. Metallo protease was purified with ammonium sulphate precipitation, Dialysis and DEAE cellulose chromatography. The molecular weight of purified enzyme as determined by SDS-PAGE was found to be 25 kDa (Plate 2).

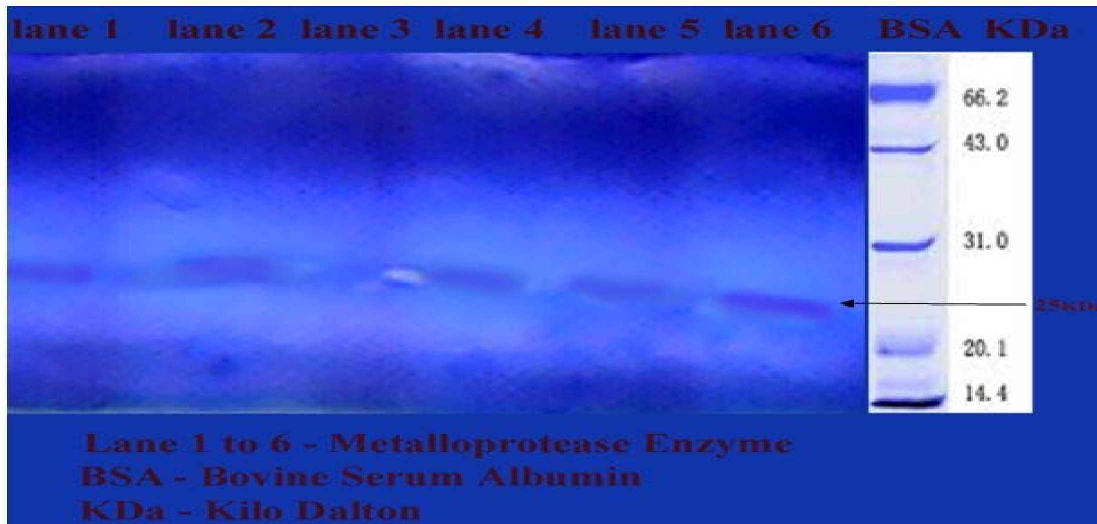

Plate 2: Molecular mass determination of metalloprotease enzyme by SDS-PAGE

The scanning electron microscopy images of Metalloprotease enzyme (Plate 3) and the micrograph showed that the particles have a spherical nature and the average size (mean ± SD) of the particles can be calculated as 144.6 nm.

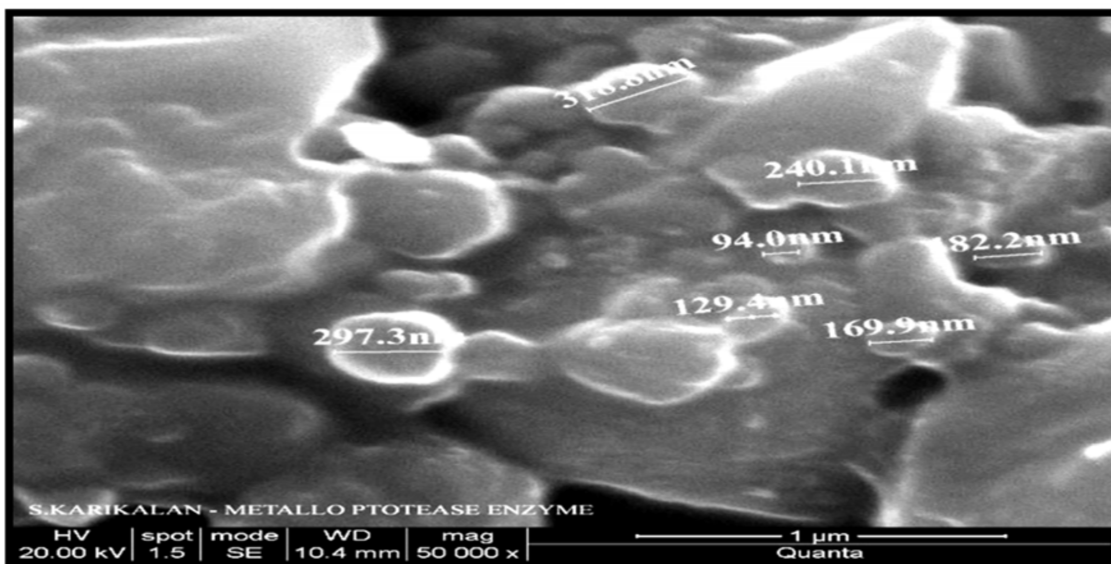

Plate 3: SEM images of metalloprotease enzyme at 50,000 x magnification

The diffraction peaks of Metallo protease enzyme were observed above 10,000 intensity at 30 (2 Theta) and moderate value nearly 5,000 at 65 (2 Theta). The final low value observed below 400 intensity at 68 (2 Theta). In the obtained spectrum, the position and their intensities were observed. The spectrum the result showed that the particles have a cubic in structure (Plate 4).

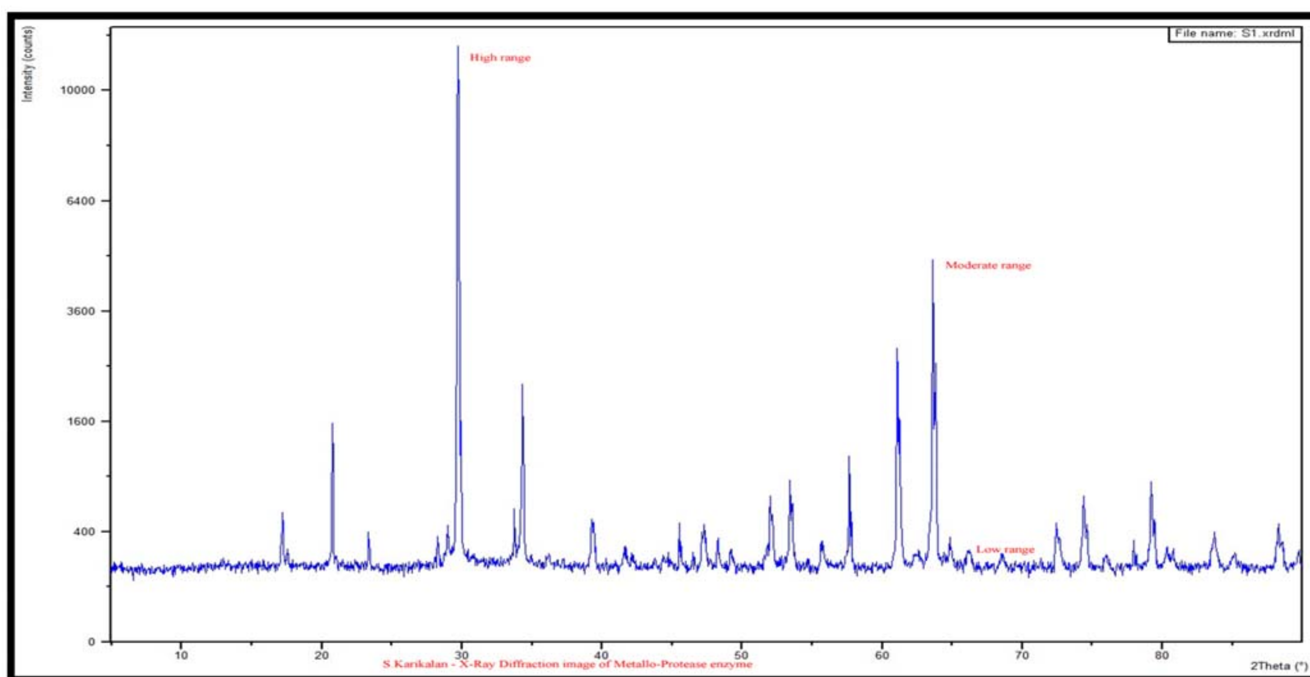

Plate 4: Spectrum of Metalloprotease enzyme by X-ray Diffraction Method

The effect of different concentrations of *p*H 3-12 on the purified metalloprotease enzyme was determined. The optimum enzyme production of *p*H was observed at *p*H 9 and the enzyme activity decreased rapidly in the range of *p*H 3. The enzyme was very stable in the range of *p*H 7 to *p*H 12 at 37°C for 1 hr. in presence of casein as a substrate (Fig 1).

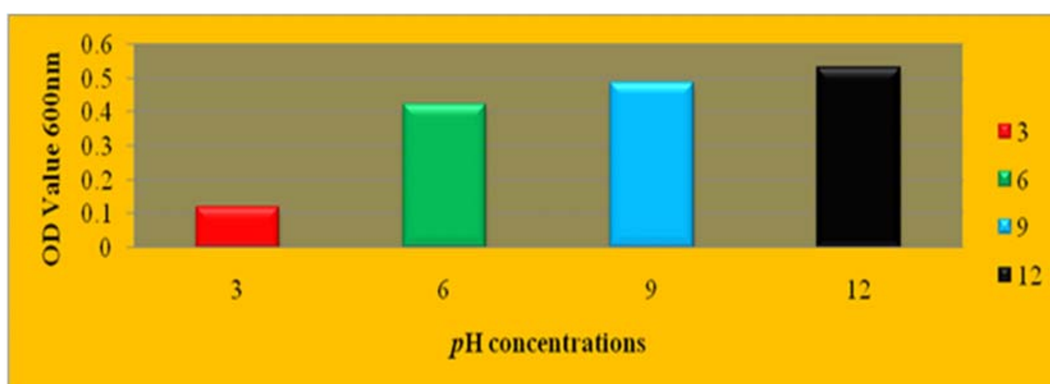

Fig 1: Effect of *p*H values on Metalloprotease

The activity of the purified enzyme was determined at different temperatures ranging from -20°C to 80°C for 1 hr. in presence of casein as a substrate. The maximum level of protease activity was observed at 37°C and minimum level of enzyme activity was observed at 55°C and 80°C. Moderate activity was observed at -20°C and 4°C. The enzyme showed the stability for an hour in 55°C and 80°C (Fig 2).

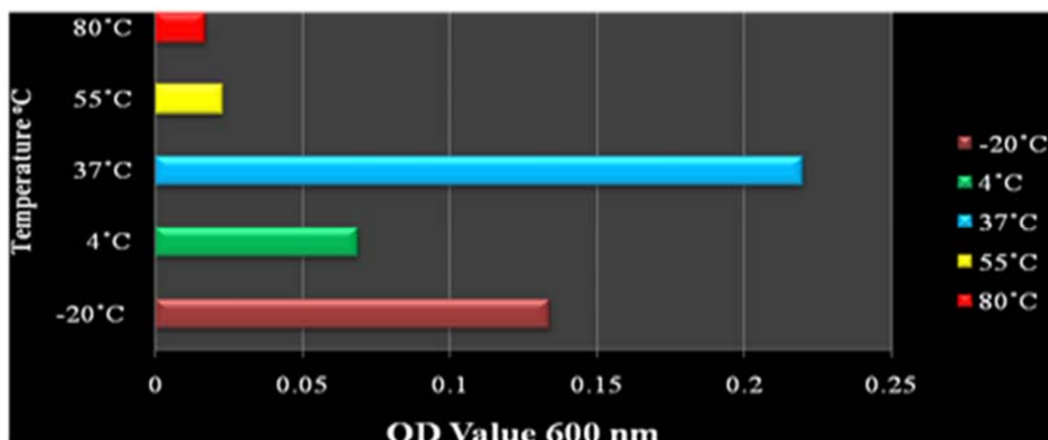

Fig 2: Effect of Temperature on Metalloprotease

The effect of different activators such as magnesium chloride, calcium chloride, manganous sulphate and ferric chloride at concentration of 0.1% was added in to 0.1 ml of the enzyme solution. It was incubated at 37°C for 1 hr. Finally the completion of incubation period enzyme activity was checked. The maximum amount of enzyme activity was observed in $MgCl_2$ and minimum amount of enzyme activity was partially obtained from $MnSO_4$, $CaCl_2$ and $FeCl_3$ in the presence of casein as a substrate (Fig 3). The enzyme solution 0.1 ml was incubated at 37˚C for 1hr in the presence of casein substrate with 0.1% of $ZnSO_4$, $AgNO_3$, $CuSO_4$, $K_4 Fe(CN)_6$ and H_2O_2 showed maximum inhibition. Enzyme activity was partially inhibited by $CuSO_4$ and $ZnSO_4$ but no inhibition was showed by $AgNO_3$ and $K_4 Fe(CN)_6$ (Fig 4).

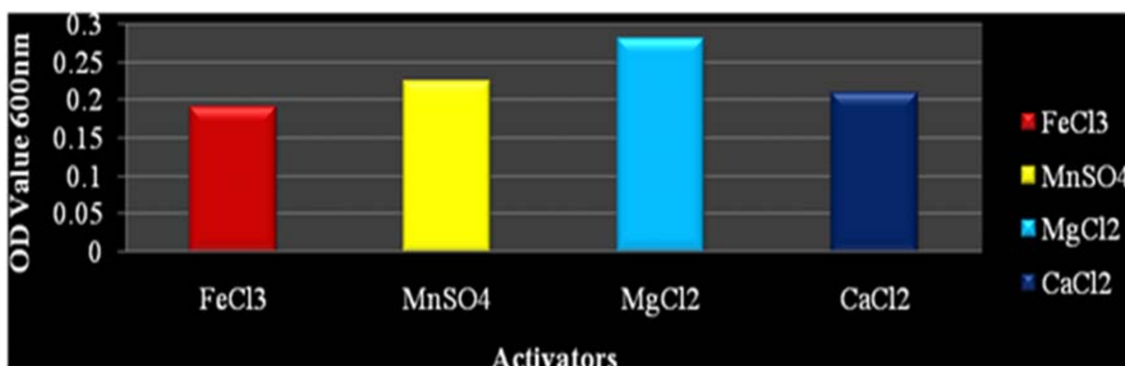

Fig 3: Effect of Activators on Metalloprotease

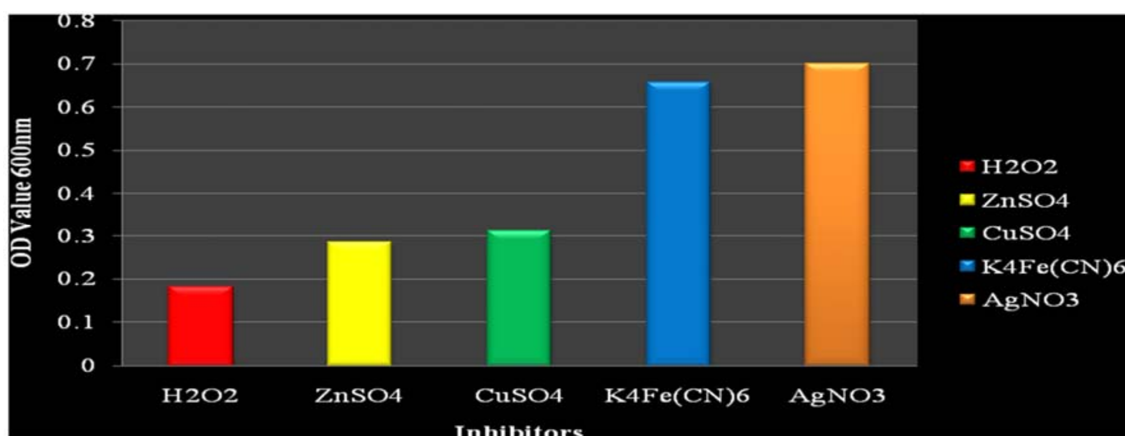

Fig4: Effect of Inhibitors on Metalloprotease

4. Discussion

Compared with this study slightly higher results were reported on *Beauveria* spp. has 29 kDa [12]. The data is similar to particularly various *p*H concentrations of protease secreted by *Bacillus* spp. ATCC 26933 [13]. The optimum *p*H range of alkaline proteases is generally found in between *p*H 9 and 11 [14]. The enzyme stability was determined in different *p*H buffers and it can be deduced from the data in that proteases were mostly stable at a *p*H *range* from 9 to 12 with the highest activity observed in the sample incubated at *p*H 12. In a related

study proved that the optimum *p*H was 10 and it was stable at alkaline *p*H ranges [15].
The optimum temperature of proteases was observed from microbes generally ranges at 50-70˚C. The temperature range 37˚C of proteases were retained, the maximum activity. It was exhibited low activity at temperatures ranges 55 and 80˚C, but in other hand, this enzyme showed stability at all temperatures respectively at -20˚C to 80˚C. The similar results were obtained that the effect of different temperatures [16]. The proteases of *B. amyloliquefaciens* was active at all temperatures (30 to 80ºC) tested, with a maximum activity recorded at 60ºC, qualifying it to be designated as a moderately thermo active proteases. A sharp decline was observed in activity at temperatures above 80ºC.
The related data of this study on activators of metalloprotease enzyme were reported that $CaCl_2$ enhanced the activity of Protease enzyme produced by *Aspergillus Niger* [17]. Some researchers have reported the role of Ca2+ in enzyme stabilization, by increasing the activity and thermal stability of alkaline proteases at higher temperatures [18]. Parallel results of this study on alkaline protease often require a divalent cation like Ca2+, Mg2+ and Mn2+ or a combination of these cations for maximum activity of alkaline protease produced by *Aspergillus* spp. [19].
Inhibitors can reduce the hydrolysis of the substrate by protease enzyme and it was agreed with this study. Similar results were reported on inhibitors that many proteases were inhibited by silver metal ions (Ag2+) [20, 21, 22]. Compared to the following reports were accompanied with Zn2+ and Cu2+ ions were found to inhibit the activity of the alkaline proteases secreted by *Bacillus polymyxa* [23]. The same result was reported on H_2O_2 slightly inhibit the proteolytic activity [24].

5. Conclusion
Metalloprotease enzyme isolated from the epiphytic bacteria *Bacillus* spp. of the marine macroalgae *Gracillaria* spp. and *Chaetomorpha* spp. It could be used for inhibiting the clinical pathogens and treatment against for various diseases in pharmaceutical industries to make an enzyme medicine.

6. References

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