PRODUCTION OF PROTEASE ENZYME AND ANTIMICROBIAL ACTIVITIES FROM MARINE ACTINOBACTERIUM OF *STREPTOMYCES SP.*

Running title: PROTEASE ENZYME AND ANTIMICROBIAL ACTIVITIES FROM MARINE *STREPTOMYCES SP*.

ABSTRACT

Introduction:

Actinobacteria are Gram-positive bacteria that have traits of both bacteria and fungus. They are found in both terrestrial and aquatic habitats and aid in the breakdown of deceased species organic matter. Marine actinobacteria have extraordinary antibacterial, anticancer, antiviral, insecticidal and enzyme inhibitory activities. The biggest genus of Actinobacteria is *Streptomyces*. They create more than two-thirds of all natural antibiotics that are therapeutically important (*e.g.* Neomycin, Cypemycin, Grisemycin, Bottromycins and Chloramphenicol). Alkaline proteases are generated by a variety of microorganisms, including yeast, bacteria, actinomycetes, fungus and plants. The process of destroying or suppressing disease-causing bacteria is referred to as antimicrobial activity. This is accomplished using a variety of antimicrobial drugs having antibacterial properties.

Aim: To find out the antimicrobial activity of protease enzymes produced by the *Streptomyces* sp. of marine actinobacterium.

Materials and methods: A sediment sample was collected and marine Actinobacteria were isolated in Kuster's Agar Medium (KUA) and were identified based on their chemotaxonomical characteristics.

Results: Screening for protease was carried out to assess enzyme production and enzyme assay was carried out to assess the enzymatic activity. The effect and pH on enzyme production was observed. Then the antimicrobial potential of protease enzymes was tested against few bacterial strains at optimum temperature and pH.

Conclusion: From the above study, we conclude that the protease enzyme obtained from *Streptomyces sp.* shows a potential antimicrobial (antibacterial) effect.

Keywords: Protease Enzyme, Effect of pH, Antibacterial potential, Novel drug

INTRODUCTION:

Actinomycetes is a Gram-positive filamentous bacteria with a fungal morphology. They are members of the phylum Actinobacteria and have a complex life cycle (Ozcan et al. 2012). They are found in terrestrial ecosystems, especially in soil, where they play an important role in the recycling of industrial wastes and biomaterials by decomposing complex polymeric structures in dead plants, animals, and flora (S, Ayswarya, and S 2020). Actinobacteria, especially *Streptomyces sp.,* are known for producing a wide range of biologically active compounds that are used as antibacterials, antifungals, antivirals, antithrombotics, immunomodulators, anti-tumor drugs, and enzyme inhibitors in a variety of fields, including medicine (S, Ayswarya, and S 2020; Sivasankar et al. 2020). Due to the emergence of multi-resistant microorganisms to almost all available antibiotics, many researchers are now concentrating their efforts on discovering novel antimicrobials from a variety of natural resources, including those developed by actinomycetes, especially those isolated from previously unknown or understudied environments. Many metabolic pathways, primarily organised by polyketide synthases (PKS) and non-ribosomal peptide synthetases, generate antimicrobials (NRPS) (Handayani et al. 2018). Actinobacteria have a high prevalence due to presence of these biosynthetic genes (Handayani et al. 2018; Maiti and Mandal 2021) (Vignesh et al. 2019) (Pranati et al. 2019).

Streptomyces are bacteria that form mycelium and have a complex developmental life cycle that involves sporulation and programmed cell death (Undabarrena et al. 2017a). Their unparalleled versatility in secondary metabolic pathways have made them important suppliers of bioactive molecules, accounting for two-thirds of all antibiotics recognised (Undabarrena et al. 2017a; Park and Kwon 2018). Genome mining has become a powerful method for revealing *Streptomyces* species. Its biotechnological potential allow researchers to identify biosynthetic gene clusters (BGCs) and even predict the chemical core structure of molecules (Undabarrena et al. 2017a; Park and Kwon 2018; "Antimicrobial Metabolites from Streptomyces Sp. SN0280," n.d.). *Streptomyces* have linear chromosomes, unlike other bacteria, and their genomes are among the largest in the bacterial world (Undabarrena et al. 2017a; Park and Kwon 2018; "Antimicrobial Metabolites from Streptomyces Sp. SN0280," n.d.; Enany 2018). Because of their exceptional value in production of antibiotics, soil microorganisms from the *Streptomyces* genus have sparked a lot of interest, but their marine equivalent has got less attention (Chen et al.

2020). Extreme abiotic stress factors and enormous biological diversity characterise the marine environment. Despite all of the isolation studies on marine actinobacteria, little is known about the molecular mechanisms that enable bacteria to adapt to their surroundings (Chen et al. 2020; Park and Kwon 2018). It is thought that marine actinobacteria have adapted by developing specific biological traits, leading to the speculation that novel organisms from previously unknown environments may contain unique bioactive cofactors. Chile has a long, largely unexplored coastline in the South Pacific. In Valparaiso Central Bay and the Comau fjord in Northern Patagonia, bioprospecting of actinobacteria for the discovery of novel marine-derived natural products, specifically antibiotics, was carried out (Undabarrena et al. 2017b). Both sites yielded a plethora of novel actinobacteria organisms with antimicrobial properties. The genome of a selected marine antimicrobial-producer, *Streptomyces* strain from the marine sediment sample was sequenced in this context (Sivaperumal, Kamala, and Rajaram 2015).

Proteases are present in prokaryotes, fungi, plants, and animals and are essential for their survival. Serine, cysteine, and metalloproteases are commonly found in many pathogenic parasites, which play crucial roles in immune evasion, nutrient acquisition for growth and proliferation, dissemination facilitation, and tissue damage during infection (Karthik et al. 2014). Several clinical trials have shown their benefits in oncology, inflammatory disorders, blood rheology management and immune regulation attracting their use in medicine (D et al. 2019). Proteases are widely employed in leather, textiles, medicines and detergents, accounting for more than 60% of the global enzyme market (Mourão and Schwartz 2013). Alkaline proteases are generated by a variety of microorganisms including yeast, bacteria, actinomycetes, fungus and plants (Karuppiah, Sun, and Li 2016). Antimicrobial activity is accomplished using a variety of antibacterial drugs (Kasanah and Triyanto 2019) (Mohapatra et al. 2020). Many plants are known for their antibacterial, antifungal, and antiviral properties (Aafreen et al. 2019) (Das et al. 2019) (Das et al. 2019; A et al. 2019). Further, our team has extensive knowledge and research experience for high quality publications (Rajeshkumar et al. 2018; Nandhini, Rajeshkumar, and Mythili 2019; Vairavel, Devaraj, and Shanmugam 2020; M. Gomathi et al. 2020; Rajasekaran et al. 2020),(Santhoshkumar et al. 2019),(Raj R, D, and S 2020),(Saravanan et al. 2018),(Gheena and Ezhilarasan 2019),(Ezhilarasan, Sokal, and Najimi 2018),(Ezhilarasan 2018),((Vairavel, Devaraj, and Shanmugam 2020; A. C. Gomathi et al. 2020; Dua et al. 2019),(Ramesh et al. 2018; Arumugam, George, and Jayaseelan 2021; Joseph and Prasanth 2021; Ezhilarasan, Apoorva, and

Ashok Vardhan 2019; Duraisamy et al. 2019), (Gnanavel, Roopan, and Rajeshkumar 2019),(Markov et al. 2021). The aim of this study was to find out the antimicrobial activity of protease enzymes produced by the *Streptomyces sp*. of marine actinobacterium.

MATERIALS AND METHODS :

Sample collection

The sediment sample was collected from the Parangipettai area, Tamilnadu. The collected sample was sun dried for 48 hrs and turned into fine powder by mortar and pestle.

Isolation of actinobacteria

Isolation and enumeration of actinobacteria were carried out in Kuster's agar medium (KUA) supplemented with 0.5% (W/v) NaCl. To minimize the fungal and bacterial contamination, KUA medium was supplemented with cycloheximide (10 µg/ml) and nalidixic acid (10 µg/ml) respectively (Kathiresan et al., 2005). Collected sediment samples were serially diluted and inoculated on KUA medium and incubated at 36°C for 7 days. The colonies were counted and the population density has been expressed as colony forming units per gram (CFU/g) of sediments. Morphologically distinct colonies were selected and pure cultures were obtained.

Identification of marine actinobacteria

Aerial mass colour: The colour of the mature sporulating aerial mycelium was recorded in naked eye. When the aerial mass colour fell between two colours series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then ,both the colour series were noted. The media used were Yeast Extract-Malt Extract Agar and Inorganic-Salt Starch Agar.

Melanoid pigments: The grouping was made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The strains were grouped as melanoid pigment produced (+) and not produced (-). In few cases, the production of melanoid pigments was delayed or weak, and therefore, it was not distinguishable. This is indicated as variable (V). This test was carried out on the media ISP-1 and ISP-7 (Appendix I), as recommended by the International *Streptomyces* Project (Shirling and Gottlieb, 1966).

Reverse side pigments: Reverse side pigment production of the isolate was determined on ISP7 medium. The pigment production was noted as distinctive (+) and not distinctive or none (-). In case, a colour with low chroma such as pale yellow, olive or yellowish brown occured, it was included in the latter group (-).

Soluble pigments: Soluble pigment production of isolate was observed on ISP7 medium. The diffusible pigment production other than melanin was considered positive (+) and not produced (-). The colour was recorded (red, orange, green, yellow, blue and violet).

Spore chain morphology: Spore morphological characters of the strains were studied by inoculating a loopful of one week old cultures into solidified agar medium contained sterile glass slide. The cultures were incubated at 28+20 °C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology.

Chemotaxonomical characteristics

Hydrolysis

Hydrolysis was done for releasing amino acids. Harvested cells of each strain weighing 20 mg (fresh) were placed in an AMPO bottle and 1 ml of 6 N HCl was added and sealed with an alcohol blast burner. The samples were kept at 121°C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28+20°C. Hydrolysis was also done for releasing sugars. Harvested cells of each strain weighing 50 mg (fresh) were placed in an AMPO bottle and 1 ml of 0.5N HCl was added and sealed with alcohol blast burner. The samples were kept at 1100 °C for 2 h. The bottles were then cooled by keeping them at a room temperature of 28+20 °C.

Thin Layer Chromatography (TLC): Spotting of the whole cell hydrolysates was made carefully on TLC plate using a microliter pipette. Spots were 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by a hand dryer.

Amino acids: Each sample (3 µl) was applied on the baselines of the TLC plate (20 cm x 20 cm). Adjacent to this, 1µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers) and 1µl of amino acetic acid (glycine) were spotted as standards. The TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H2O (5: 0.5: 0.125: 2.5 v/v). It took approximately more than 4 h for development of spots. The spots were visualized by spraying with 0.4% ninhydrin solution in water-saturated n-butanol, followed by heating at 1000 °C for 5 min. Spots of amino acids ran faster than DAP. The sample spots were immediately compared with the spots of the standards, since spots gradually disappear in a few hours.

Whole-Cell sugars: On a cellulose TLC plate (20 cm x 20 cm), 5µl of samples was spotted along with 3 µl of sugar solutions as standards on the same plates. Galactose, arabinose, xylose and madurose were the sugars, which were used as standards. The TLC plate was developed with the solvent mixture containing ethyl acetate: pyridine: acetic acid: distilled water (8: 5: 1: 1.5 v/v). The spot development time was more than 4 h. Spots were visualized by spraying with aniline phthalate reagent (3.25 g of phthalic acid dissolved in 2 ml of aniline and made upto 100 ml with water saturated n-butanol). The sprayed plate was heated at 1000 °C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots.

Assimilation of carbon source: The ability of the actinobacterial strain in utilizing various carbon compounds as source of energy was studied, following the method recommended by International *Streptomyces* Project (Shirling and Gottlieb, 1966). Chemically pure carbon source certified to be free of admixture with other carbohydrates and contaminating materials were used for this purpose. Carbon sources for this test were Arabinose, Xylose, Inositol, Mannitol, Fructose, Rhamnose, Sucrose and Raffinose. These carbon sources were sterilized by ether

sterilization without heating. The media and plates were prepared and inoculated according to the convention of ISP project (Shirling and Gottlieb, 1966). For each of the carbon sources, utilization is expressed as positive (+), negative (-) or doubtful (±). In the 'doubtful ' strains, only a trace of growth slightly greater than that of the control was noticed.

Screening of protease Production

Protease activity of the strains was screened qualitatively in skim milk agar. After inoculation, the plates were incubated at 37°C for 3 days. The diameters of clear zone around colonies on skim milk agar were measured.

Determination of Enzyme activity

The medium was inoculated with 1 ml of spore suspension of a 7 days old culture and incubated in a rotary shaker (150 rpm) at ambient temperature for three days. The cell free supernatant was collected by centrifugation at 12,000 rpm for 15 min. The supernatant was the enzyme source. The substrate 2% of casein was prepared with a 50mM phosphate buffer (pH 7). 1ml of crude enzyme was added with 1ml of casein (2%) solution which incubated for 60 min at (50ºC) desired temperature. After incubation, 2ml of Trichloroacetic acid (TCA) solution was added and boiled for 15 min in a boiling water bath. Before cooling 0.5ml of folin phenol reagent was added and the color was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme that releases 1 mg of reducing sugar as tyrosine per ml per min under the assay conditions.

Bacterial Suspension: The fungal pathogens *Aspergillus flavus* and *Candida albicans* and bacterial pathogen *Pseudomonas aeruginosa* was collected from Department of Microbiology, Saveetha medical college and hospital, Tamilnadu. The bacterial pathogens were cultured in Muller –Hinton Broth for 24 hr at room temperature. From this bacterial suspension was prepared with saline and the optical density was measured at 600 nm. The concentration of microbial suspension was fixed as 106 CFU/ml. 1ml of suspension was spread over on Muller Hinton agar plate and incubated for 24hrs at ambient temperature.

Antibacterial activity: The antimicrobial activity of the enzyme protease was performed with disc diffusion method. Whatman filter paper discs (5mm) were impregnated with various concentrations (50, 100, 150, 200, 250 µg/ml) of enzyme sample with oral antibiotic tetracycline and DMSO as a negative control. The inoculated plates were incubated for 24hr at room temperature and the inhibition zones around the discs were measured. All the results were expressed from an average of three values along with standard deviation.

Minimum Inhibitory Concentration:

Minimal Inhibition Concentration of the enzyme protease was determined in 5 concentrations (50, 100, 150, 200, 250 µg/ml) with tetracycline (Standard) and DMSO (negative control) on Mueller Hinton broth). The inoculated bacteria were incubated in test tubes for 24hr in ambient temperature then the optical density was observed.

RESULTS AND DISCUSSION:

The rise of widespread antibiotic-resistant bacteria heightened the need to discover new antimicrobial agents. Actinomycetes, especially *Streptomyces sp.*, have attracted a lot of attention because they produce a lot of useful bioactive metabolites. Isolating these species from less-explored environments may improve the chances of discovering new microbial species.

Fig-1,2 antibiotic-resistant bacteria

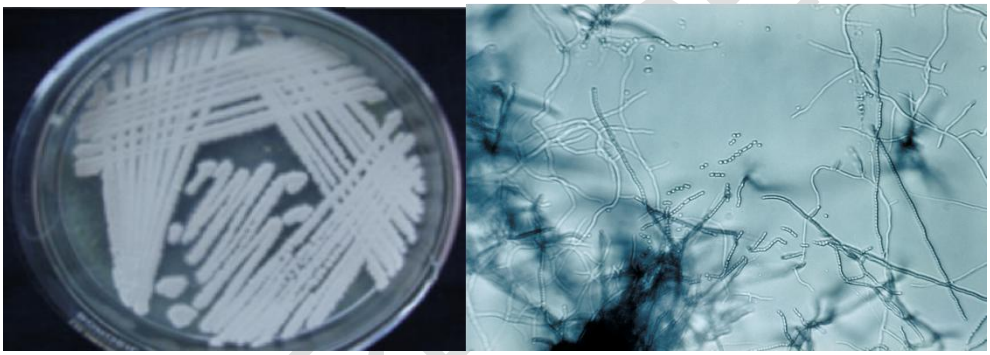

Table-1 :- Depicts the antimicrobial activity in different concentrations

Antimicrobial activity			
µg/ml	**S. mutans**	**Klebsiella pneumoniae**	**Pseudomonas aeroginosa**
0	0	0	0
50	4 +/- 2.2	5 +/- 1.3	6 +/- 2.2
100	13 +/- 3.4	11 +/- 2.6	13 +/- 2.6
150	17 +/- 2.5	17 +/- 2.1	21 +/- 2.4
200	21 +/- 2.1	22 +/- 2.4	26 +/- 2.1
250	26 +/- 2.7	27 +/- 2.9	35 +/- 2.7

Table -2 :- Depicts the minimum inhibitory concentration of protease enzyme against the bacteria.

MIC	0	10	20	30	40	50	MIC
S. mutans	+	+	+	+	-	-	30 µg/ml
Tetracycli	+	+	+	-	-	-	20 µg/ml
Klebsiella pneumoni	+	+	+	-	-	-	20 µg/ml
Tetracycli	+	+	+	-	-	-	20 µg/ml
Pseudomonas	+	+	+	+	-	-	30µg/ml
Tetracycli	+	+	-	-	-	-	10 µg/ml

This study isolated marine actinobacteria from a sediment sample and identified *Streptomyces* genus from the isolate using specific characteristics of the bacteria. Then the production of enzyme was confirmed by screening for protease and enzyme assay was carried out. Enzyme assay revealed that the total activity of the enzyme was 96.24 IU/mg in a media of 1L. The effect of the temperature and pH on enzyme production was studied on Muller Hinton broth. It was found that the optimum pH and temperature for maximum enzymatic activity was and respectively. It was also observed that as the temperature increased, the enzymatic activity increased. However at higher temperature, the rate decreased again which might probably be due to enzyme degeneration. Finally , antibacterial testing was done by incorporating the enzyme in different concentrations into the media plates containing each of the bacteria and the zone of inhibition was measured. It was observed that at the higher concentrations , *S.mutans , Klebsiella pneumoniae, Pseudomonas aeruginosa* exhibited higher susceptibility towards the antibiotic when compared to *Staphylococcus* (Ezhilarasan, Apoorva, and Ashok Vardhan 2019) (Danda, Krishna, et al. 2010) (Ramadurai et al. 2019) (Sathivel et al. 2008) (Panda et al. 2016) (P. Neelakantan et al. 2012) (Govindaraju, Neelakantan, and Gutmann 2017) (Sekhar, Narayanan, and Baig 2001)(DeSouza et al. 2014) (Nasim et al. 2010) (Danda, Muthusekhar, et al. 2010) ("Molecular Structure and Vibrational Spectra of 2,6-Bis(benzylidene)cyclohexanone: A Density Functional Theoretical Study" 2011) (Putchala et al. 2013) (Prasanna Neelakantan, Grotra, and Sharma 2013) (Suresh et al. 2014). However , the minimum inhibitory concentration of protease enzyme that prevented the growth of the bacterial strains was very close to that of the standard tetracycline.

CONCLUSION :

Actinomycetes, especially *Streptomycetes*, are still a major source of bioactive compounds used to treat various diseases such as infections, cancer, and a variety of other ailments. From the above study, we conclude that the protease enzyme obtained from *Streptomyces* shows a potential antimicrobial effect (antibacterial).

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