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Production of Protease Enzyme and Antimicrobial Activities from Marine Actinobacterium of *Streptomyces* SP

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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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 are unusual in that they have 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 relevant (*e.g.* Neomycin, Cypemycin, Grisemycin, Bottromycins, and Chloramphenicol). Alkaline proteases are generated by a variety of microorganisms, including yeast, bacteria, 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 potential as well as antiviral 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

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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 were observed. Then the antimicrobial potential of protease enzymes was tested against a few bacterial strains at optimum temperature and pH.
Conclusion: From the above study, we conclude that the protease enzyme obtained from *Streptomyces* shows a potential antimicrobial (antibacterial) effect.

Keywords: Protease enzyme; effect of pH; antibacterial potential; novel drug.

1. INTRODUCTION

Gram-positive filamentous bacteria with a fungal morphology are known as actinomycetes. They are members of the phylum Actinobacteria and have a complex life cycle [1]. 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 [2]. Actinobacteria, especially *Streptomyces* sp., are known for producing a wide range of biologically active compounds that are used as antibacterial, antifungals, antivirals, antithrombotics, immunomodulators, anti-tumor drugs, and enzyme inhibitors in a variety of fields, including medicine [3,4,5,2,6]. 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 organized by polyketide synthases (PKS) and non-ribosomal peptide synthetases, generate antimicrobials (NRPS) [7]. Actinobacteria have a high prevalence and presence of these biosynthetic genes [7, 8,9,10]. The *Streptomyces* are bacteria that form mycelium and have a complex developmental life cycle that involves sporulation and programmed cell death [11]. Their unparalleled versatility in secondary metabolism pathways has made them important suppliers of bioactive molecules, accounting for two-thirds of all antibiotics recognized [11, 12]. Genome mining has become a powerful method for revealing *Streptomyces* species' biotechnological potential, allowing researchers to identify biosynthetic gene clusters (BGCs) and even predict the chemical core structure of molecules [11, 12]. *Streptomyces* have linear chromosomes, unlike other bacteria, and their genomes are among the largest in the bacterial world [11,12,13]. Because of their exceptional position as antibiotic producers, soil microorganisms from the *Streptomyces* genus have sparked a lot of interest, but their marine equivalent has gotten less attention [14]. Extreme abiotic selection pressures and enormous biological diversity characterize 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 [14, 12]. 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 [11]. Both sites yielded a plethora of novel actinobacteria organisms with antimicrobial properties. The genome of a selected antimicrobial-producer marine *Streptomyces* strain from the marine sediment sample was sequenced in this context [4].

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, where they play important roles in immune evasion, nutrient acquisition for growth and proliferation, dissemination facilitation, and tissue damage during infection [15]. Proteases, as a result, play a crucial role. Several clinical trials have shown their benefits in oncology, inflammatory disorders, blood rheology management, and immune regulation, so their use in medicine is gaining traction [16]. Proteases are widely employed in leather, textiles, medicines, and detergents, accounting

for more than 60% of the global enzyme market [17]. Alkaline proteases are generated by a variety of microorganisms, including yeast, bacteria, fungus, and plants [18]. The process of destroying or suppressing disease-causing bacteria is referred to as antimicrobial activity. This is accomplished using a variety of antibacterial drugs [19, 20]. Many plants are known for their antibacterial, antifungal, and antiviral properties [21, 22]. Further, our team has extensive knowledge and research experience that has translated into high-quality publications [23-32, 24, 33-41]. The aim of this study was to find out the antimicrobial activity of protease enzymes produced by the *Streptomyces* sp of marine actinobacterium.

2. MATERIALS AND METHODS

2.1 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.

2.2 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 [42].

2.3 Identification of Marine Actinobacteria

Aerial mass colour: The colour of the mature sporulating aerial mycelium was recorded in the 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 also, both the colour series was noted. The media used were Yeast Extract-Malt Extract Agar and Inorganic-Salt Starch Agar.

2.3.1 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 a few cases, the production of melanoid pigments was delayed or weak, and therefore, it was not distinguishable. This is indicated as the variable (V). This test was carried out on the media ISP-1 and ISP-7, as recommended by the International Streptomyces Project (Shirling and Gottlieb, 1966).

2.3.2 Reverse side pigments

Reverse side pigment production of the isolate was determined on the 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 occurred, it was included in the latter group (-).

2.3.3 Soluble pigments

Soluble pigment production of the isolate was observed on the 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).

2.3.4 Spore chain morphology

Spore morphological characters of the strains were studied by inoculating a loopful of one-week-old cultures into solidified agar medium containing a sterile glass slide. The cultures were incubated at 28±2°C and examined periodically for the formation of aerial mycelium, sporophore structure, and spore morphology.

2.4 Chemotaxonomical Characteristics

2.4.1 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 1210 C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28+20C. 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 an 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±2°C.

2.4.2 Thin Layer Chromatography (TLC)

Spotting of the whole-cell hydrolysates was made carefully on a 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.

2.4.3 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. 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 disappeared in a few hours.

2.4.4 Whole-Cell sugars

On a cellulose TLC plate (20 cm x 20 cm), 5µl of samples were 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 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 up to 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.

2.4.5 Assimilation of carbon source

The ability of the actinobacterial strain in utilizing various carbon compounds as sources of energy was studied, following the method recommended by International *Streptomyces* Project (Shirling and Gottlieb, 1966). Chemically pure carbon sources 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 the 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.

2.5 Screening of Protease Production

The 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 the clear zone around colonies on skim milk agar were measured.

2.6 Determination of Enzyme Activity

The medium was inoculated with 1 ml of spore suspension of a 7-day old culture and incubated in a rotary shaker (150rpm) at ambient temperature for three days. The cell-free supernatant was collected by centrifugation at 12,000rpm 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 the crude enzyme was added with 1ml of casein (2%) solution which was 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.

2.7 Antibacterial Activity

2.7.1 Bacterial suspension

The fungal pathogens *Aspergillus flavus* and *Candida albicans* and bacterial pathogen *Pseudomonas aeruginosa* were collected from the 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.

The antimicrobial activity of the enzyme protease was performed with the disc diffusion method [4]. 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 with a standard deviation.

2.7.2 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 in test tubes are incubated for 24hr at ambient temperature then the optical density was observed.

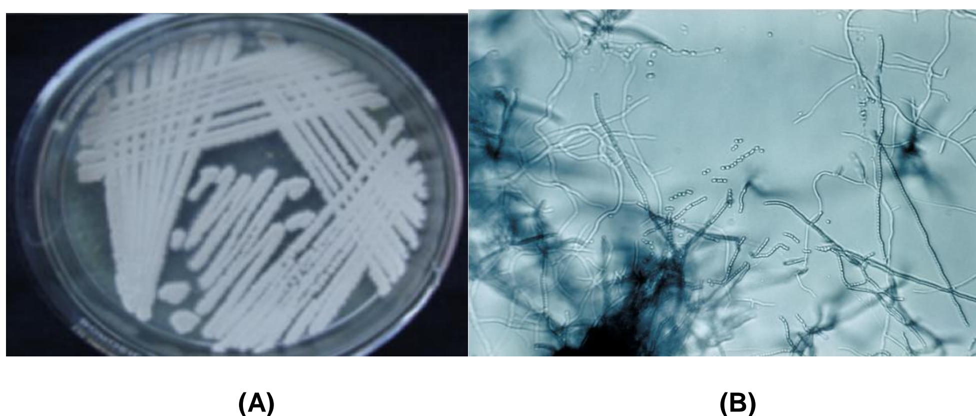
3. RESULTS AND DISCUSSION

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.

This study isolated marine Actinobacteria from a sediment sample and identified *Streptomyces* genus from the isolate using specific characteristics of the bacteria (Fig. 1 & Table 1). 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 Mueller Hinton broth. It was found that the optimum pH (7.8) and temperature (35°C) for maximum enzymatic activity from marine *Streptomyces* sp. However, at higher temperatures (50°C) the enzyme activity rate decreased again which might probably be due to enzyme degeneration.

Table 1. Conventional identification of marine actinobacterium of *Streptomyces* sp

Color of aerial mycelium	**Whitish Yellow**
Melanoid pigment	-
Reverse side pigment	-
Soluble pigment	-
Spore chain	Spiral
Assimilation of carbon source	
Arabinose	+
Xylose	+
Inositol	+
Mannitol	+
Fructose	+
Rhamnose	+
Sucrose	+
Raffinose	+



(A) **(B)**

Fig. 1. A-B: *Streptomyces* sp. pure culture (A) and Spore chain morphology (B)

Table 2. Depicts the antimicrobial activity in different concentrations of protease enzyme

Protease enzyme concentration (μg/ml)	*S. mutans*	*Klebsiella pneumoniae*	*Pseudomonas aeroginosa*
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 3. Depicts the minimum inhibitory concentration of protease enzyme against the bacteria

MIC	0	10	20	30	40	50	MIC
S. mutans	+	+	+	+	-	-	30 μg/ml
Tetracyclin	+	-	-	-	-	-	10 μg/ml
Klebsiella pnemoniae	+	+	+	-	-	-	20 μg/ml
Tetracyclin	+	-	-	-	-	-	10 μg/ml
Pseudomonas aeroginosa	+	+	+	+	-	-	30 μg/ml
Tetracyclin	+	-	-	-	-	-	10 μg/ml

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 pneumonia*, and *Pseudomonas aeruginosa* exhibited higher susceptibility towards the antibiotic when compared to *Staphylococcus* (Table 2). 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 (Table 3). In addition to that, our researchers are done the research area of biological activities from the different sources [38,43-47,7,42,48-52,3,4,53-60].

4. CONCLUSION

Actinobacteria, especially *Streptomycetes* sp. are still a major source of bioactive compounds used to treat infections, cancer, and a variety of other ailments. From the above study, we conclude that the protease enzyme obtained from *streptomyces* shows a potential antibacterial effect against different pathogens. Further, purification and characterization of protease enzyme needed and different biological properties also possible to study at *in-vitro* and *in-vivo* condition.

ETHICS APPROVAL

It is not applicable.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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