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PROTEIN FRACTION FROM ACTINOMYCETES ISOLATES INHIBITS PROTEASE ACTIVITY SECRETED BY *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

The pathogenic potential of *staphylococcus aureus* is attributable by the production and secretion of various toxins, pathogenic factors such as various enzymes that include proteases which collectively contribute to the virulence of the organism. The present study focused on targeting proteases that were secreted into the growth medium during the growth of *Staphylococcus aureus*. The proteases that were secreted by the *Staphylococcus aureus* were checked for their inhibition activity by the cell free culture extracts from actinomycetes isolates that were isolated from maize-rhizosphere soil. The isolation of actinomycetes from maize-rhizosphere soil was done by serial dilution plating and cellulose ester membrane isolation methods. Five isolates belonging to *Streptomyces sp, Microbispora sp, and Thermomonospora sp.* exhibited protease inhibition as assayed by well diffusion activity using skim milk agar media and observation of the extent of hydrolysis zone due to protease activity. The protein fractions with protease inhibition activity were extracted from the actinomycetes isolates by protein purification that resulted in the separation of prominent protein bands after subjecting to electrophoretic separation technique.

KEYWORDS: *Staphylococcus aureus, Proteases, Actinomycetes isolates, Protease inhibition.*

INTRODUCTION

Strategies to inhibit the pathogenic potential can be effectively considered as one of the measure to control over the pathogenic events and infections of certain pathogens. This may also give insight information to understand the molecular events occurring during the pathogenesis of infecting organisms. The pathogenic mode of certain microorganisms depends on the expression and secretion of various virulence factors ranging from toxins, surface proteins to secretions of enzymes such as proteases, nucleases and lipases.[1-3] These factors play a key role during host entry, feeding and suppression of host immune response. Among these factors, proteases are considered potentially important virulence factor as they possess the ability to degrade critical components of the host defense system such as elements of the complement system, cytokines and receptors on host immune cells.[4-6] Proteases are proteolytic enzymes that invade through the host tissue during infections. Studies have shown that these types of virulent factors are actively involved in cell disruption and tissue degradation of the host during their entry and during establishment of the infection within the host system. In the present work, *Staphylococcus aureus*, which is considered an opportunistic multidrug resistant pathogen, is known to produce proteases as one of the virulence factor during infection and diseases. Among the proteins secreted by *S. aureus* are proteolytic enzymes belonging to three distinct catalytic classes, namely metallo, serine and cysteine proteases.[7, 8] The secretion of proteases that is been considered a virulent factor, may offer a strategic exploration for targeting and inhibiting the biochemical potential of protease secretion of this opportunistic pathogen. In the present study, the secretion of proteases is achieved during the growth of *Staphylococcus aureus* in the growth media that hydrolyze the protein substituted in the growth medium. This served a model system for targeting the secreted proteases and its inhibition. For targeting the protease for its inhibition activity, we speculated the role of actinomycetes and its protein components to inhibit the protease activity exhibited by *Stapylococcus aureus*. Actinomycetes are a group of gram positive bacteria with high G+C contents. These organisms serve an important source for biotechnologically important bioactive compounds such as enzymes, antibiotics and various other compounds for practical applications.[9-11] The enormous therapeutic potential of actinomycetes is closely related to its ability to produce secondary metabolites for its survival.[12] In the present study, a simple method of casein hydrolysis with measuring the zone of hydrolysis due to protease activity that was secreted by *Staphylococcus aureus* and its inhibition by the cell free culture extract of the actinomycetes isolates was analyzed. The positive isolates with protease inhibition activity were further grown in the production media of starch casein nitrate broth and the cell free culture supernatant was subjected to protein purification by precipitating with ammonium sulphate. The protein concentration was estimated after dialysis. The dialysate

protein fraction were separated and analyzed by SDS-PAGE. The protein fraction obtained from each of the actinomycetes isolates were assayed for enzyme activity and inhibition by spectrophotometer and well diffusion method. For identifying the actinomycetes isolates, with performing staining and microscopic observation of spore arrangement were identified belonging to *Streptomyces sp., Microbispora sp*. and *Thermomonospora sp.* The identified actinomycetes isolates were subjected further for biochemical characterization.

MATERIALS AND METHODS

All the chemicals and reagents used were of high grade obtained from Himedia chemicals. Antibiotics streptomycin and griseofulvin were obtained from Sigma chemicals, Bangalore India.

Isolation of actinomycetes

Actinomycetes isolates were isolated from the rhizosphere-soil of maize cultivation in Chikka Aluvara, Kodagu. The rhizosphere soils were collected in a polythene bag aseptically and brought to the laboratory for isolation. Actinomycetes isolates were isolated by employing two methods, i) Plating technique using starch casein agar medium (pH 7.2) that was supplemented with streptomycin and griseofulvin to prevent bacterial and fungal growth. The media was inoculated with $10^{-2,}$ 10^{-4} and 10^{-6} serially diluted soil sample. The inoculated plates were incubated at 30 ± 2^{0}C for 6 days. ii) Cellulose ester membrane filter method where the selective isolation of actinomycetes isolates from mixed population was achieved by overlaying cellulose ester membrane filter of 0.45 micron pore size above the starch casein media and the surface of the filter was inoculated with soil sample. During incubation the branched mycelia of the actinomycetes penetrated the filter pores to the underlying agar medium, whereas growths of non-actinomycetes bacteria were restricted to the filter surface. The membrane filter was removed and the agar medium was re-incubated to allow the development of the actinomycetes colonies.

Selection and maintenance of actinomycetes colonies

Based on the different morphological characteristics of the colonies, actinomycetes isolates were selected considering cottony, gummy growth grown over the media. The pure cultures of actinomycetes isolates were maintained by sub-culturing and preserved maintaining the viability of the isolates until further use.

Preparation of cell free culture extracts of actinomycetes isolates.

Actinomycetes isolates were inoculated and grown in Starch casein nitrate broth at 30^{o}C for six days. The media along with culture were centrifuged at 10000 rpm for 10 min at 4^{o}C. The culture supernatant was separated and used for screening protease inhibition activity against the enzyme proteases secreted by *Staphylococcus aureus.*

Extraction of crude protease enzyme from *Staphylococcus aureus*

Staphylococcus aureus was inoculated into nutrient broth (pH- 7.0) and incubated for 24 hour at 37^{o}C. After incubation, the culture broth was centrifuged at 10000 rpm for 20 min. The secreted enzymes from the culture supernatant were extracted using phosphate buffer.

Protease inhibition assay by well diffusion method

Skim milk agar media (pH 7.5) was prepared and about 20 ml was poured onto petri-dishes. Wells of 6.0 mm diameter wells were made within the media for adding 0.03 ml of enzyme extract of *S.aureus* and 0.03 ml of cell free culture extract of actinomycetes isolates. In another well 0.03ml of cell free culture extracts of *S.aureus* and 0.03ml of phosphate buffer was added. Phosphate buffer was loaded as control. The enzyme inhibition activity was analyzed after incubation at 37^{o}C for 24 hours.

Production and purification of protein fraction from actinomycetes isolates.

The actinomycetes isolates were inoculated into 50 ml starch casein production medium and incubated in rotary shaker at 120 rpm for six days at 30°C. After growth, actinomycetes mycelium from the broth was separated by centrifugation at 10000 rpm for 10 min at 4°C. Actinomycetes isolates which showed protease inhibition activity was selected as potential isolates for further studies. The mycelium free culture extract were further subjected to salt precipitation and dialysis.

Ammonium sulphate precipitation

Cell free culture extract (50.0 ml) obtained from actinomycetes isolates were pre-chilled at 4^{o}C. The protein fraction present in the culture extract was precipitated with gradual addition of ammonium sulphate to obtain a final concentration of 60% saturation (13.1 g/50 ml solution). The solutions were stirred at 4^{o}C for 30 min and kept overnight at 4ºC for complete precipitation. The precipitated solution was centrifuged at 9000g for 5 min at 4^{o}C. The supernatant was separated, the pellet were re-dissolved in 0.01M phosphate buffer of pH 7.6 and kept in refrigeration.

Dialysis

The dialysis membrane was pre-treated by immersing in 2% sodium carbonate, 1mM EDTA solution and boiled for 10 min. The membrane was rinsed with double distilled water thoroughly. The distilled water was decanted and the precipitated protein sample was filled to the dialysis tube and tied both the ends. This set was placed in an external chamber containing distilled water. The dialysis set-up was kept for 72 hr with the external distilled water changed at every interval of two hour.

Determination of protein content

After dialysis, protein content in the sample was determined by Lowry's method using Bovine Serum

Albumin as standard. The absorbance was measured at 750nm.

Protease inhibition assay

The enzyme assay and its inhibition were determined using spectrophotometer. The activity of the protease enzyme was assayed by taking protease enzyme (0.1ml) and using casein as a substrate in a reaction tube. For inhibition assay, the protease enzyme was pre-treated with partially purified, dialyzed cell free culture extract (0.1ml) obtained from actinomycetes isolates and incubated at 37^0C for 30 min. After incubation the reaction was initiated by adding 0.5 ml of 1% casein solutions and further incubated at 37^0C for 30 min. Absorbance was measured at 280nm by using *Labman* spectrophotometer.

SDS PAGE

The dialyzed protein fractions obtained from positive actinomycetes isolates were subjected to SDS-PAGE technique for separation and analysis. For this, 10% poly-acrylamide gel was prepared and around 50 µg of each of the protein samples were loaded. After running, the gels were stained with Coomassie brilliant blue staining and destained with methanol-glacial acetic acid solution.

Identification and Characterization of Positive actinomycetes isolates.

Actinomycetes isolates which showed protease inhibition activity against *S.aureus* proteases were selected for further characterization. Actinomycetes isolates were selected and grown on Starch casein nitrate agar medium and their characteristic growth pattern was analyzed. Staining techniques such as Gram staining, Acid fast staining, Biochemical tests such as Catalase test, Hydrolysis of gelatin, Starch hydrolysis, Hydrogen sulfide production test, Carbohydrate fermentation test were performed.

Slide culture method and microscopic analysis for characteristic spore arrangement:

Starch casein nitrate agar media was prepared, cooled and poured into sterile petri-plates to obtain 1mm thickness and cut into small pieces of one square cm. Small piece of media (1 sq cm) was placed on the sterile glass slide. The culture of actinomycetes isolates was inoculated over the small media piece and cover-slip was placed above it. Then it was incubated at 30^0C for 6 days. After incubation the cover-slip was taken, placed on the glass slide and stained with crystal violet. The spore arrangement patterns were observed under oil immersion for identifying the actinomycetes isolates.

RESULTS AND DISCUSSION

Isolation and maintenance of actinomycetes

Actinomycetes isolates were isolated from the rhizosphere-soil region of maize cultivation since maize-cultivation is prominent in the study area surrounding the institution. The collected soils were serially diluted using distilled water and plated on starch-casein agar media. The specific method of isolating actinomycetes was performed using cellulose ester membrane filter of 0.45 mm pore size. The prominent colonies were selected based on their colonial growth, pigmentation and mycelial appearance. The isolates were sub-cultured to obtain pure cultures and a set of pure cultures were preserved at -20^oC for further use.

Screening of actinomycetes isolates for inhibiting the proteases secreted by *Staphylococcus aureus*

The screening of actinomycetes isolates for protease inhibition activity was performed using skim milk agar media. When grown on skim milk agar media, human pathogen such as *Staphylococcus aureus* utilizes the protein casein by secreting the enzyme proteases. Protease secretion is indicated by formation of hydrolysis zone around growth within the media. The protease enzymes secreted by *Staphylococcus aureus* was obtained after centrifugation of the growth medium and the culture supernatant was added into the wells within the media that diffused exhibiting protease activity against the protein casein. The protease enzyme activity and its inhibition by actinomycetes isolates were analyzed. The extent of diffusion and hydrolysis due to protease activity was checked for inhibition by addition of cell free culture extract of actinomycetes isolates. As shown in Fig 1, Out of twelve actinomycetes isolates, five isolates showed protease inhibition activity. The actinomycetes isolates AC 6 and AC 10 showed significant protease inhibition activity against the *Staphylococcus aureus* protease enzyme followed by the isolates AC 5, AC 9, and AC 7 showed intermediate inhibition activity and no inhibition activity was detected by the remaining isolates. The hydrolysis of casein due to protease enzyme activity of *Staphylococcus aureus* and its inhibition by the cell free culture extract of the actinomycetes isolates were measured as shown in graph 1.

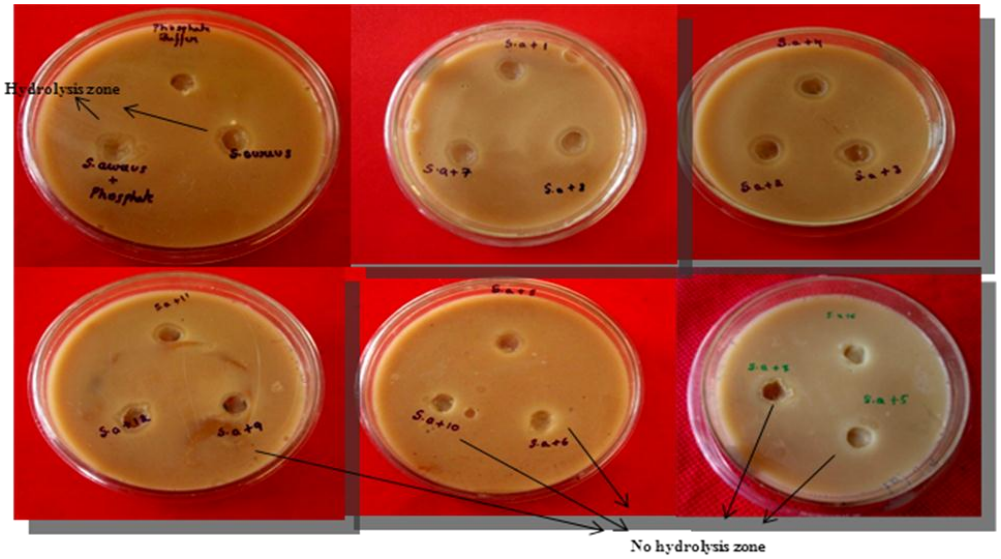
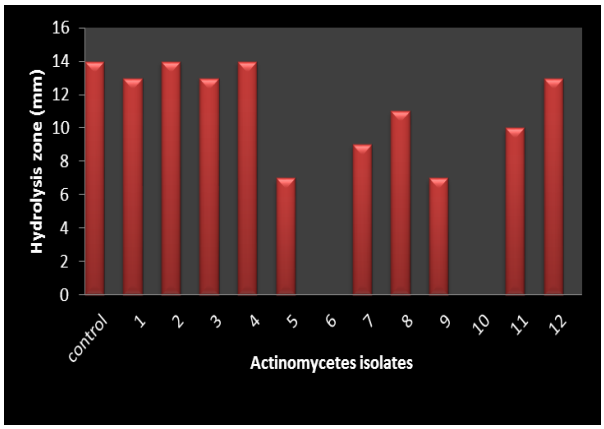
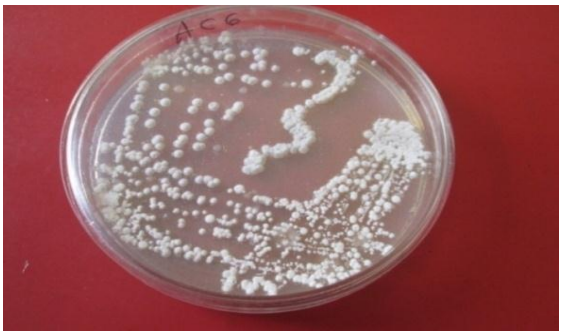


Fig 1: Well diffusion enzyme assay and protease inhibition activity against *Staphylococcus aureus* protease enzyme.

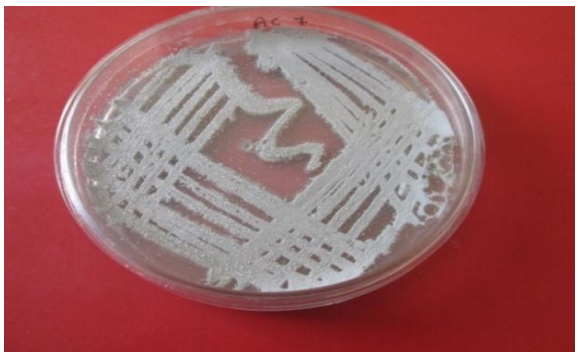
Hydrolysis zone due to protease activity on casein as shown, the numbers indicate actinomycetes isolates cell free culture extract added in addition to the proteases obtained from *Staphylococcus aureus*, out of twelve isolates, five isolates showed minimum or no hydrolysis zone due to protease inhibition activity.



Graph 1: Protease inhibition activity of Actinomycetes isolates against *Staphylococcus aureus proteases.*



Actinomycetes isolate (AC 6)



Actinomycetes isolate AC 7

Actinomycetes isolate (AC 5)

Actinomycetes isolate AC 9

Actinomycetes isolate AC 10

Fig 2:- Actinomycetes isolates with positive protease inhibition activity

Production and protein purification of cell free culture extracts from actinomycetes isolates.

The actinomycetes isolates which showed protease inhibition activity were grown in production media of Starch casein nitrate broth for 6 days at 30°C. The cell free culture extract were obtained after centrifugation.

The cell free culture extract was subjected for protein purification where the protein components were precipitated by ammonium sulphate achieving 60 % saturation and the salts further removed from the precipitated protein by dialysis. The concentration of protein in the sample was determined by Lowry's method using Bovine serum albumin as standard.

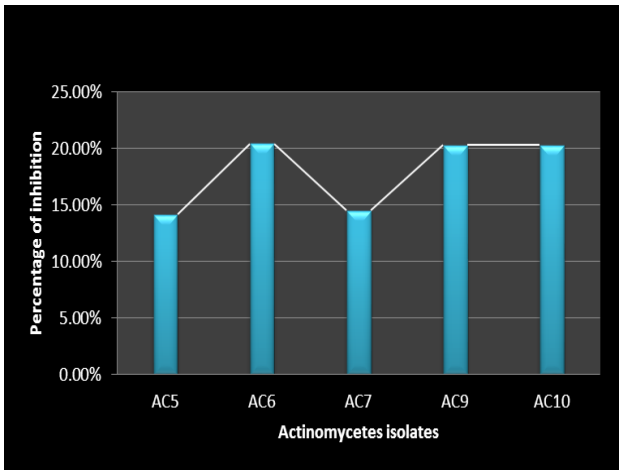
Table 1: Protein estimation of the protein fraction obtained from actinomycetes isolates

Partial purification of Cell free culture extract of	Concentration of protein (mg/ml)
Actinomycete isolate 5	0.6
Actinomycete isolate 6	0.76
Actinomycete isolate 7	0.7
Actinomycete isolate 9	0.6
Actinomycete isolate 10	0.7

Table 2: Estimation of Protease enzyme inhibition.

The absorbance value is the average of three independent experiments.

Actinomycetes isolate	Vol. of protease Enzyme (ml)	Vol. of phosphate buffer (ml)	Incubation at 37°C for 5 min	Vol. of cell free culture extract of isolates	Incubation at 37°C for 30 min	Vol. of substrate (ml)	Incubation at 37°C for 30 min	Absorbance at 280 nm
	0.1	0.1		-		0.5		1.405
AC 5	0.1	0.1		0.1		0.5		1.206
AC 6	0.1	0.1		0.1		0.5		1.118
AC 7	0.1	0.1		0.1		0.5		1.201
AC 9	0.1	0.1		0.1		0.5		1.120
C 10	0.1	0.1		0.1		0.5		1.120



Graph 2: Percentage of Protease Inhibition activity by actinomycetes isolates against *Staphylococcus aureus* proteases.

The results as depicted in the graph 2, illustrates that the actinomycetes isolates inhibits protease activity as assayed spectrophotometrically. The hydrolysis of substrate after enzyme treatment was taken as 100% activity. In comparison, the enzyme activity after treating with dialyzed sample of each actinomycete isolate inhibited the proteases and subsequent hydrolysis of casein that can attributable to the inhibition of protease enzyme. As shown in the graph, the AC 5 and AC 7 isolates showed around 14.17% and 14.52% of protease inhibition activity respectively. The isolate AC 6, showed around 20.43%, and AC 9 and AC 10 showed around 20.29% of protease inhibition activity.

Characterization of positive actinomycetes isolates.

The mycelial and morphological features of actinomycetes were analyzed by microscopic observation and colony characterization. For the microscopy, the isolates were grown by slide culture method. The growth pattern of mycelial structure, and spore arrangements on the mycelia were observed under microscope. The observed morphological characteristics of the isolates were analyzed in comparison with the morphological characteristics provided in Bergey's manual of determinative bacteriology for the identification of the isolates.

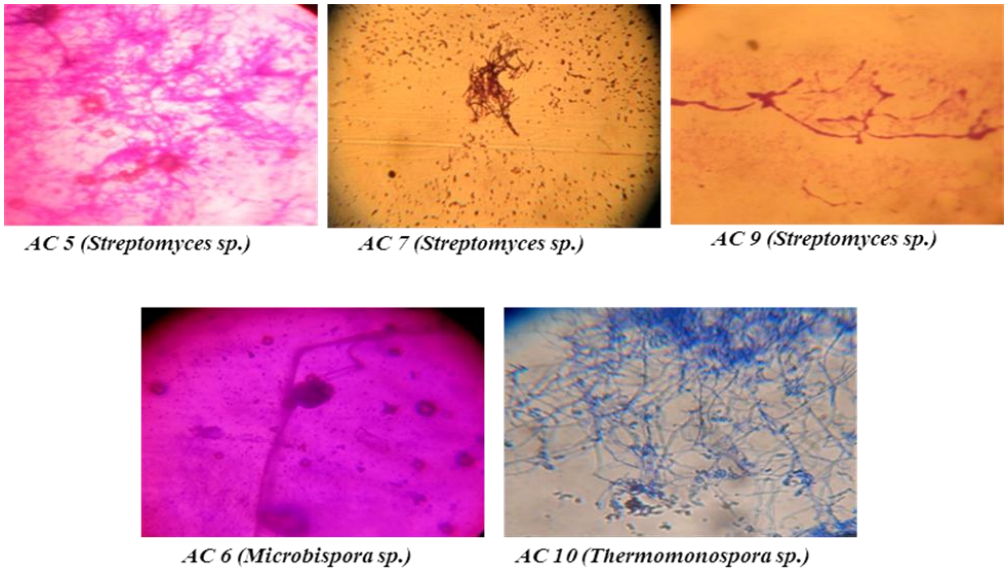


Fig 3: Slide culture technique and staining with crystal violet for visualizing the spore arrangement.

Table 3: Colony Morphology of Actinomycetes Isolates

Isolates	Microscopic observation			Macroscopic observation		
	Gram's reaction	Acid fast reaction	Spore arrangement	Colony morphology	Pigmentation (back view)	Identified as
AC 5	+	-	Hairy spores, Flexible.	Pinkish white, large, Cottony.	Absent	*Streptomyces sp.*
AC 6	+	-	Straight filamentous, Smooth spores.	White glistering, Cottony, irregular	Absent	*Microbispora sp.*
AC 7	+	-	Spiny	Large, grayish green, Flat.	Absent	*Streptomyces sp.*
AC 9	+	-	Rectus	Yellowish, smooth,large	Absent	*Streptomyces sp.*
AC 10	+	-	Long rods, short chain of conidia	Whitish brown, irregular,dry.	Creemish yellow	*Thermomonospora sp.*

The characteristic features and microscopic examination of actinomycetes isolates showed that they were gram positive and acid fast negative. AC 10, identified as *Thermomonospora sp.* exhibited creamish yellow pigmentation and the other isolates identified as *Streptomyces sp.* and *Microbispora sp.* were non-pigmented. Based on the above studies, and as depicted in the Table 3, the actinomycetes isolates AC 5 AC 7 and AC 9 that were isolated from the maize-rhizosphere were determined belonging to *Streptomyces sp.* while AC 6 belonging to *Microbispora sp.* and Ac 10 to be *Thermomonospora sp.*

Table 4: Biochemical Characterization of the Isolated Actinomycetes

Isolates	Identified as	Catalase	H_2S	Gelatin	Starch	Carbohydrate fermentation			
						Glucose	Maltose	Sucrose	Lactose
Ac 5	*Streptomyces sp.*	+	-	+	+	Acid (+)	Acid (-)	Acid (-)	Acid (-)
Ac 6	*Microbispora sp.*	+	-	-	-	Acid (+)	Acid (+)	Acid (+)	Acid (+)
Ac 7	*Streptomyces sp.*	+	+	+	+	A cid (+)	Acid (+)	Acid (+)	Acid (-)
Ac 9	*Streptomyces sp.*	+	-	+	-	Acid (+)	Acid (+)	Acid (-)	Acid (+)
Ac 10	*Thermomonospora sp.*	+	+	+	-	Acid (+)	Acid (+)	Acid (+)	Acid (-)

As shown in table 4, the identified isolates were further subjected to biochemical characterization which revealed that isolates AC 5 *Streptomyces sp.*, AC 6 *Microbispora sp.* and AC 9 *Streptomyces sp.* were negative for H_2S production test and AC 7 *Streptomyces sp* and AC10 *Thermomonospora sp.*were positive for H_2S production. All isolates showed positive for catalase test. *Microbispora sp.* showed negative for gelatin hydrolysis and while remaining isolates showed positive for gelatin hydrolysis. The isolates AC 6 *Microbispora sp*, AC 9 *Streptomyces sp.*, AC10 *Thermomonospora sp* showed negative for starch hydrolysis test, while Ac 5 Streptomyces *sp.* and Ac 7 *Streptomyces sp.* showed positive for starch hydrolysis.

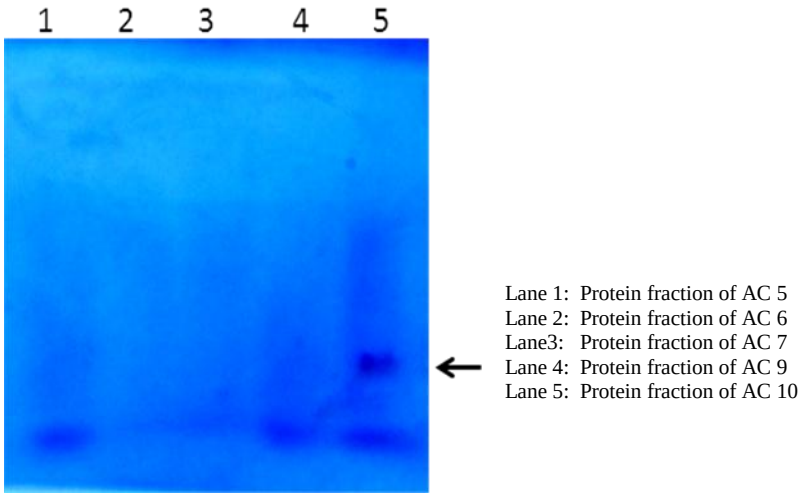


Fig 4: Analysis of protein fractions obtained from actinomyetes isolates by SDS-PAGE technique.

The dialysate protein fractions obtained from the actinomycetes isolates were analyzed by SDS- PAGE separation. As shown in Fig 4, prominent bands of low molecular weight were observed in the dialysate sample obtained from all the five actinomycetes isolates. Among the protein bands observed, acinomycetes isolate belonging to *Thermomonospora sp*.exhibited a prominent protein band as indicated. The protein fraction from each of the actinomycetes isolates thus separated contributes a significant role in inhibiting the proteases activity secreted by *Staphylococcus aureus.*

Thus, proteases that is considered one of the potential virulence factor secreted by *Staphylococcus aureus* was analyzed for its inhibition activity by the protein components extracted from actinomycetes isolates viz., belonging to *Streptomyces sp*, *Microbispora sp*, and *Thermomonospora sp.* Various studies have shown the production of protease inhibitors from actinomycetes belonging to *Streptomycetes sps.* [13-16] In the present work, starch casein nitrate broth was used as a production medium for the growth of actinomycetes isolates that exhibited positive protease inhibition activity. Further, the cell free culture extract of these isolates were subjected for protein purification protocols by ammonium sulphate precipitation and dialysis, and the protein components were separated and analyzed by SDS-PAGE technique. Prominent protein bands were observed from protein fraction obtained from the five actinomycetes isolates belonging to *Streptomyces sp*, *Microbispora sp*, and *Thermomonospora sp*.

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