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RESEARCH ARTICLE

Peptide Analysis from Soil Actinomycetes Exhibiting Antimicrobial and Antiproliferative Activities

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ABSTRACT

Actinomycetes are considered a potential group of microorganisms capable of producing bioactive molecules. In the present study, the actinomycetes species belonging to the genus *Thermoactinomycetes*, *Micromonospora* and predominantly *Streptomyces* were isolated from maize rhizosphere soil and from compost soil. With anticipating the isolation of potential antimicrobial peptides from the actinomycetes isolates, the isolates were subjected for total protein extraction after growing them in starch casein nitrate media. The antimicrobial peptides are low molecular weight peptides attacking the cell membrane by ionic interactions, thus disrupting membrane integrity of invading pathogens or the abnormal cells such as cancer cells. The peptides obtained from actinomycetes were analyzed by SDS-PAGE technique after their salt precipitation and dialysis. The total peptides were checked for antimicrobial activity against the bacteria *Pseudomonas* sp, *Enterobacter sp, Klebsiella sp, Streptococcus sp, Escherichia coli* and *Staphylococcus aureus.* The peptides obtained from *Streptomyces* predominantly showed effective antibacterial activities. The compost isolated actinomycetes mainly identified belonging to *Thermoactinomycetes, Micromonospora* and *Streptomyces* also showed antibacterial activity but showed good antiproliferative effect against the proliferative yeast cultured cell lines. The morphological features of actinomycetes were characterized by gram staining, acid fast staining and slide culture techniques.

Key words: Actinomycetes; protein extracts; peptides; antimicrobial; antiproliferative.

INTRODUCTION

Exploring antimicrobial peptides from actinomycetes group of microorganisms offers many advantages of biomedical importance. Antimicrobial peptides are low molecular peptides with less than fifty amino acids, with cationic properties and capable of non-specific interaction with microbial membranes [1-3]. Antimicrobial peptide promotes the microbe rapid death and decreases the probability of resistance development [4]. These molecules interact with the membrane molecules by ionic interactions leading to formation of pores and membrane disruptions in the lipid membrane [5, 6]. Also, AMPs are capable of intracellular targeting of the pathogen [7-8] since AMPs can bind to nucleic acids and proteins [9]. In addition to these well-known and described activities and targets, a growing number of studies report a broad spectrum of cytotoxic activity against cancer cells by these peptides [10, 11]. A number of AMPs have been isolated and studied from eukaryotic organisms including invertebrates and insects and from prokaryotic organisms such as bacteria and fungi. The actinomycetes are another major group under prokaryotes which has offered as a source for antibiotics synthesis. However, the studies on the peptide repository from actinomycetes are very limited. The emergence of antibiotic resistance exhibited by infecting micro organisms propels an effort to identify novel antimicrobial molecules. In consideration of the above facts, the actinomycetes group comprising one of the major groups in soil source remains unexplored and unidentified with their unique peptide synthesis machinery from a particular ecological region. Actinomycetes are the important genus among the soil microorganisms significantly accounting for the maintenance of soil ecosystem. Actinomycetes have significant role in the turnover of complex compounds in the soil environment [12, 13]. The members among the actinomycets vary from one soil type to another and also from one region to other specific regions. Therefore, soil source from

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a particular region proves to be an excellent natural resource for isolating potential candidates of actinomycets species [14, 15]. Actinomycetes are literally considered "ray fungus" and belong to Actinobacteria, exhibiting the characteristics of both bacteria and fungus. They are gram-positive, spore bearing with aerial and substrate mycelium when cultured on suitable agar medium. At the molecular level actinomycetes are characterized to contain high GC nucleotides which imply their genetic stability in varied environments. Like bacteria, actinomycetes can be easily cultured on variety of agar medium. Their protein synthetic machinery can be manipulated by supplementing with specific nutrients in the nutrient agar medium. Actinomycetes may serve as biological factories for the production of useful biomolecules. Also other molecules of industrial importance have been produced by the actinomycetes group of microorganisms [16-20]. Currently, the inefficiency of antibiotics against microbial infections and the emergence of antibiotic resistant strains of pathogenic microorganisms has become an uncontrollable menace in the clinical field [21]. The extensive usage of nonspecific and inappropriate dosages of antibiotics is considered one of the reasons for the emergence of antibiotic resistance strains. This situation has stimulated search for specific antimicrobials and therefore, there remains to identify novel antimicrobial molecules from potential candidates such as actinomycetes species. Also, that such novel antimicrobial peptides isolated from actinomycetes can be analyzed targeting against the uncontrolled proliferation of cancer cells with specific mechanism of action. In view of the above, the present work demonstrates the isolation of actinomycetes species from rhizosphere soil of maize cultivation and vermicompost soil to examine the prevalence and selection of potential actinomycetes isolates. The selected actinomycetes colonies were subjected to protein extraction procedure by ammonium sulphate precipitation after achieving growth in suitable medium such as starch casein nitrate agar medium and glycerol yeast extract agar medium. The growth patterns in two different media were analyzed. The precipitated protein, further dialyzed, estimated for protein content by Lowry's method were checked for antimicrobial activity against the species of *Pseudomonas, Enterobacter, Klebsiella, Streptococcus, Escherichia coli* and *Staphylococcus aureus.* The extracted antimicrobial peptides from the selected actinomycetes were checked for anti-proliferative activity against actively dividing yeast cells, to obtain preliminary evidence of the extracted antimicrobial peptides to possess anticancer activity and testing against other cancer cell lines.

MATERIALS AND METHODS

All the chemicals and reagents used were of high grade obtained from Himedia chemicals.

Collection of soil samples:

The soil samples were collected from a depth of 10 cm marked within the agriculture field of maize cultivation and vermi-composted soil. The soil samples were aseptically collected using polythene bags and brought to the laboratory for isolation using media. The isolation was carried out by serial dilution of soil sample and plating on starch casein nitrate agar medium supplemented with fluconazole (50µg/ml) and streptomycin (30µg/ml).

Growth studies: The characteristic growth pattern of the actinomycetes isolates were analyzed on two different culture media viz., Starch casein nitrate agar medium and Glycerol yeast extract agar media. The growth, Pigmentation, mycelia formation, spore formation were studied.

Extraction and Protein precipitation:

Based on the effective growth on starch casein nitrate media, actinomycetes colonies were selected for total protein extraction. For this, the actinomycetes isolates were aseptically inoculated into 100 ml of starch casein nitrate broth and incubated at 30^{o}C in a shaker incubator set to 100 rpm for 6 days. The culture biomass was separated by centrifugation at 10000 rpm for 10 min at 4^{o}C and the cell free extract was obtained.

Protein precipitation by Ammonium sulfate salt:

Ammonium sulfate was added to the cell free culture extract gradually under continuous steady stirring condition to achieve eighty percent saturation. The salt precipitated protein solution was kept overnight at 4^{o}C. After complete precipitation the cell free culture extract was centrifuged at 9000rpm for 5 minutes at 4^{o}C. The obtained protein pellet was separated in phosphate buffer pH 7.4. The protein in phosphate buffer was further subjected to dialysis and total protein concentration was determined by Lowry's method.

SDS-PAGE:

After determining the protein concentration, around 50 µg of total protein that was obtained from selected actinomycetes were subjected for

SDS-PAGE separation. The separating gel of 12.5% was prepared. The protein samples were loaded along with pre-stained protein MW sample. The protein samples were electrophoresed at 100 volts followed by staining with coomassie brilliant blue and de-stained with the mixture of methanol: glacial acetic acid in water.

Antibacterial activity:

To check whether the extracted peptides possess antimicrobial activity, the total protein extracted from actinomycetes were analyzed for antibacterial activity according to the Kirbey Bauer method of well diffusion. The microorganisms such as *Pseudomonas sp, Enterobacter sp, Klebsiella sp, Streptococcus sp, Escherichia coli* and *Staphylococcus aureus* maintained in the department laboratory were subcultured for antimicrobial activity analysis. Different concentrations of protein extracts as 20 µg, 40 µg, 60 µg, and 80 µg were loaded into different wells within the spread inoculated bacterial cultures.

Determination of anti-proliferative activity using yeast cell lines:

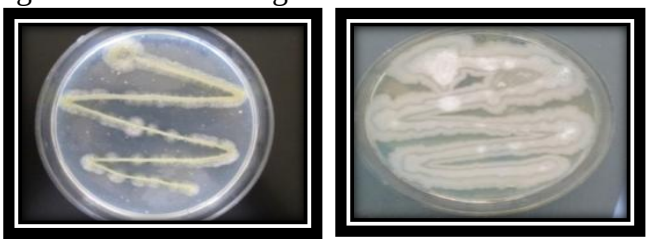
Saccharomyces cerevisiae, the eukaryotic single cell fungi was maintained in Sabouraud's dextrose agar that was incubated at 37^{o}C for 24 hr and maintained as seeded broth. About 0.5 ml seeded yeast cells was taken in 2.5 ml fresh SD broth. An aliquot containing 10 µg of the protein extracts were added to the yeast suspensions and incubated at 37^{o}C for different time intervals from 0 to 10 hr. The same set up without the protein extracts but with phosphate buffer was assayed along to observe the proliferative rate of the yeast cells.

Cell viability staining:

Yeast cell counting was performed using haemocytometer after staining with trypan blue dye. In brief, 10 µl of trypan blue dye was added to a volume of 990 µl of phosphate buffer containing 10 µl of cell suspension taken after treating with or without protein fraction. The dead and viable cell count was taken at each time intervals. Percentage of cell viability was calculated by using the formula; percentage of cell viability= total viable cell / total cell × 100

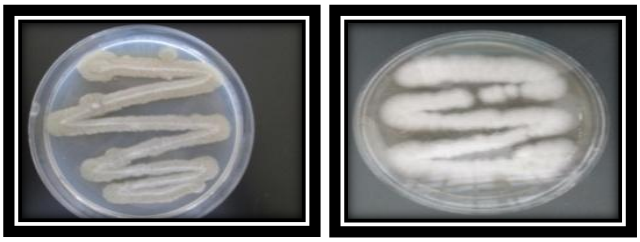
RESULTS AND DISCUSSION

Appearance of rhizoidal like growth of colonies in starch casein nitrate agar medium was achieved after serial dilution and plating of soil sample (rhizosphere soil and vermicompost). The colonies with growth characteristics of actinomycetes were subcultured into fresh medium. Pure actinomycetes isolates were maintained and were tested for gram staining reaction. Depending on the unique mycelial growth and pigmentation, around twelve different isolates were selected. Six isolates were obtained from rhizosphere soil and another six isolates obtained from vermicompost soil. Studies have shown that actinomycetes can be cultured on different media to study their morphological and cultural properties. Similarly, in this study for comparative growth analysis glycerol yeast extract agar medium was used to study the growth and morphological features of the isolated actinomycetes. Comparatively the glycerol yeast extract agar medium although supported the growth of the isolates, the growth pattern of all the six isolates from soil was shown to grow with distinct morphological features in starch casein agar medium as in Fig 1.



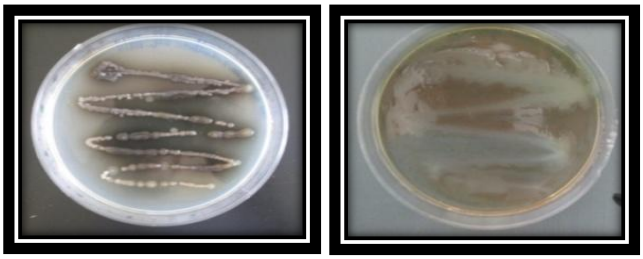
A **B**

Actinomycetes Isolate S1



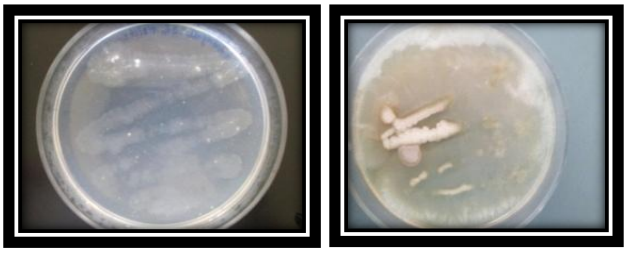
A **B**

Actinomycete Isolate S2



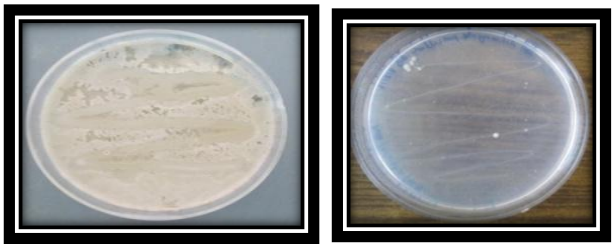
A **B**

Actinomycete Isolate S3



A **B**

Actinomycete Isolate S4



A B

Actinomycete Isolate S5

A B

Actinomycete Isolate S6

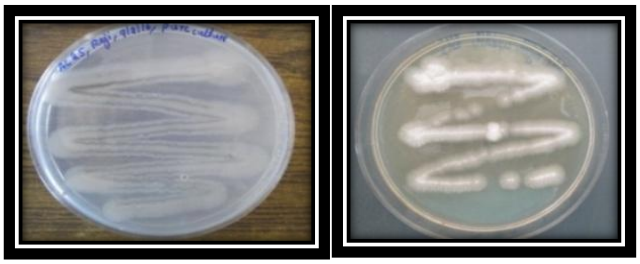
Fig 1: Characteristics growth of actinomycetes isolates on starch casein nutrient agar medium (A) and Glycerol yeast extract agar medium (B) isolated from soil sample

The density of the mycelium development, pigmentation varied among the isolates growing in two different media. The actinomycetes isolates showed following observable growth and tabulated as in table 1.

Table 1: Growth Characteristics of Actinomycetes isolates from soil source in two different media

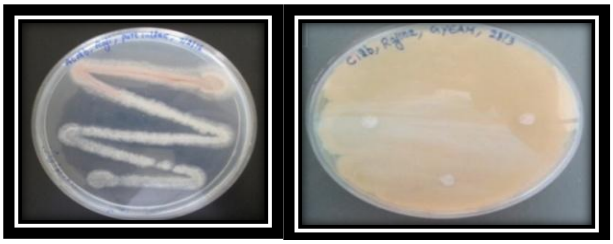
Isolate	Starch casein nitrate agar medium	Glycerol yeast extract agar medium
S1	Pale yellow colored mycelium	white colored rhizoid like growth
S2	Aerial mycelium is ash brown	White powdery aerial mycelium sporulation observed after 6 days of incubation.
S3	Gummy, produced dark pigmentation.	Pigmentation light and spreading type growth
S4	Spreading colony, gummy growth	good growth with no sporulation
S5	Dense growth	Less densely growth
S6	Colony firm, sticky	Gummy growth

As shown in Fig 2, the isolates from compost soil also showed good growth in starch casein nitrate broth compared to their growth in glycerol yeast agar medium and brief illustration as given in table 2.



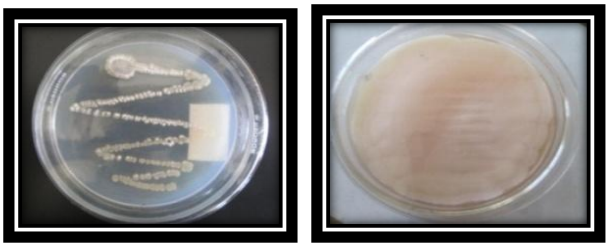
A B

Actinomycetes Isolate C1



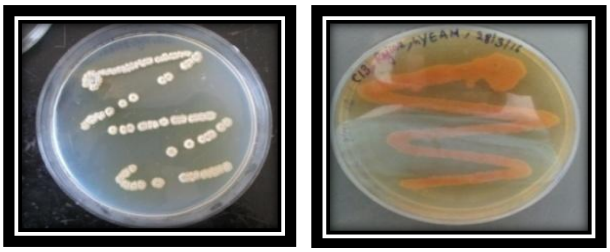
A B

Actinomycete Isolate C2



A B

Actinomycetes Isolate C3

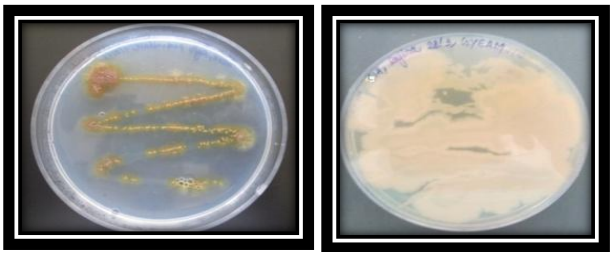


A B

Actinomycete Isolate C4

A B

Actinomycetes Isolate C5



A B

Actinomycete Isolate C6

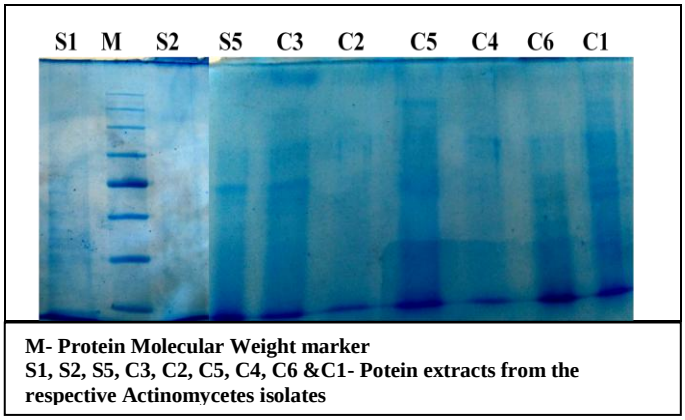
Fig 2: Characteristics growth of actinomycetes isolates on starch casein nutrient agar medium (A) and Glycerol yeast extract agar medium (B) isolated from Compost soil

Table 2: Growth Characteristics of Actinomycetes isolates from compost source in two different media

Isolate	Starch casein nitrate agar medium	Glycerol yeast extract agar medium
C1	Submerged mycelium	White aerial mycelium
C2	White colored aerial mycelium having slight pink shade	Gummy growth
C3	Colony tough and stuck firmly to the surface	White colored gummy growth
C4	Yellow colored pigmentation, tough colonies	tough colonies, Orange colored

C5	Tough, pink colored colony	growth gummy
C6	Brown colored diffusible pigmentation	Spreading type growth

Further, the starch casein nitrate broth supporting the growth of actinomycetes isolates were subjected to protein extraction. The protein extractions were carried out to analyze the total peptide composition produced constitutively or induced in the specific growth medium. This extraction procedure followed resulted in the analysis of different peptide molecules of different molecular weights. With, further screening for antimicrobial and antiproliferative activities of this effective peptide/s, basic criteria for selection and identifying therapeutic peptides was followed. Various reports have illustrated therapeutic peptides isolated from various natural sources having immense applications with target specific activities [22-25]. In continuation, for isolating potential peptides from the actinomycetes isolates, the isolates were grown in incubator shaker for seven days after inoculation into Starch Casein Nitrate broth. The maximum growth was achieved on seventh day, on subsequent day the mycelium were separated from the broth by filtration followed by centrifugation to obtain mycelium free culture extract. The total protein from culture extract was precipitated by ammonium sulfate salt. The salt and other impurities were removed by dialysis process from the protein precipitates. The protein content was determined by Lowry's method using Bovine serum albumin as standard. The total protein from actinomycetes isolates S4 and C6 was found to be 102µg/ml and 100µg/ml respectively. Around, 92µg/ml was estimated from the isolates S1, S5, and S6 actinomycetes isolates. Approximately around 50 µg/ml of total protein concentration were estimated from isolates S2, C1, C2, C3, C4, and C5. The least protein was estimated from the isolate S3 of 14µg/ml. To determine the different protein components and to characterize the peptides based on their molecular weights, the protein fraction obtained from all the isolates were analyzed after separation by SDS-PAGE technique. As shown in Fig 3, prominent bands were observed from the actinomycetes isolates. A common band of 35 kDa was observed from among the isolates. A high MW protein of around 180 kDa was separated from the isolate C3.



M- Protein Molecular Weight marker
S1, S2, S5, C3, C2, C5, C4, C6 &C1- Potein extracts from the respective Actinomycetes isolates

Fig 3: SDS-PAGE profile of the total protein extracts from the Actinomycetes isolates

The protein bands with MW of 75 and 63 kDa were separated from the isolates. Low molecular weight protein bands were also visible among the isolates. The protein extraction from C3, C5, C4, C6 and C1 resulted in the separation of major protein bands after electrophoretic separation. Likewise, the protein extraction from S1, S2 and S5 also resulted in the separation of major protein bands. These peptides were analyzed for their antibacterial activity to screen for antimicrobial peptides. The antimicrobial peptides have been isolated from various sources such as from the saprophytic fungus [26]. Accordingly, as shown in table 3, the peptides from isolated actinomycetes showed efficient antibacterial activity as corresponding to their detection on the ployacralyamide gel with respect to the number and prominent protein bands.

Table 3: Antibacterial assay by well diffusion of protein extracts from Actinomycetes isolates against the test pathogenic bacteria species.

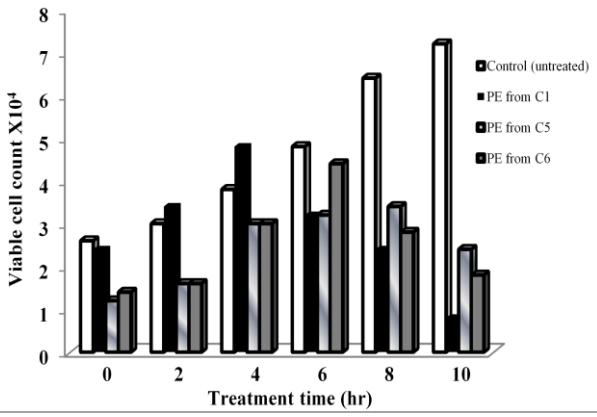
Soil Actinomycetes Isolates	**Bacteria**	**Zone of inhibition (mm) formed at different protein concentration**			**Compost Actinomycetes Isolates**	**Zone of inhibition (mm) formed at different protein concentration**		
		20 µg	40 µg	80 µg		20 µg	40 µg	80 µg
S5	*Pseudomonas* sp. *Klebsiella* sp. *E.coli* *Enterobacter* sp. *Staphylococcus aureus* *Streptococcus* sp.	2 - 2 - 5 -	7 6 4 - 8 -	10 11 10 - 10 -	**C3**	- - 5 - - -	- 2 10 - 2 -	7 6 15 - 5 -
S2	*Pseudomonas* sp. *Klebsiella* sp. *E.coli* *Enterobacter* sp.	2 2 - - - -	5 4 - - - -	6 6 - - - -	**C5**	8 3 5 - 8 -	10 8 10 3 11 -	16 10 15 6 15 -

	Staphylococcus aureus *Streptococcus* sp.							
S1	*Pseudomonas* sp. *Klebsiella* sp. *E.coli* *Enterobacter* sp. *Staphylococcus aureus* *Streptococcus* sp.	- - - 2 - -	- - - 5 - -	- - - 8 - -	**C4**	2 5 4 4 - 3	5 8 7 8 3 5	8 10 8 10 7 9
S6	*Pseudomonas* sp. *Klebsiella* sp. *E.coli* *Enterobacter* sp. *Staphylococcus aureus* *Streptococcus* sp.	- 2 - - 2 -	- 4 - - 7 -	- 6 - - 9 -	**C6**	- 3 2 - - -	7 7 4 5 - -	10 8 7 9 - -
S4	*Pseudomonas* sp. *Klebsiella* sp. *E.coli* *Enterobacter* sp. *Staphylococcus aureus* *Streptococcus* sp.	- - - - - -	- - - - - -	- - - - - -	**C2**	- - - - - -	- - - - - -	- - - 8 - -

Maximum antibacterial activity was observed comparatively by the peptides obtained from the compost soil than by the peptides obtained from the soil sample. Four of the actinomycetes isolates from the compost source exhibited efficient antibacterial activity against the test pathogenic bacteria.

The antimicrobial peptides from the actinomycetes isolates were analyzed for anti-proliferative property. The assay was carried out using the yeast cell model to assess anti-proliferative activity against the actively dividing cells. The culturing of *Saccharomyces cerevisae* using growth specific media offers a non mammalian cell line system for anticancer activity determination [27-29]. The abnormal functioning of the cell leading to the development of cancer is being targeted in various anticancer mechanisms. Hence, controlling the high rate of uncontrolled cell proliferation was analyzed preliminarily using the yeast cell culture to determine the anti-proliferative properties of the actinomycetes isolates. The trypan blue dye exclusion cell counting assay was performed to assess the viability of the yeast cells that were treated with an appropriate concentration of the protein peptides. The Yeast cells were standardized to grow in the Sabouraud dextrose agar medium for maximum growth with respect to time. The growth due to cell division was analyzed without any treatment but only with phosphate buffer. The proliferation of yeast cells were checked by cell counting using haemocytometer and staining with trypan blue. The same proliferation efficiency was tested after treatment with the protein components isolated from the actinomycetes isolates. The cell counting records as depicted in the Graph 1 shows antiproliferative efficiency of the protein components from the actinomycetes isolates C1, C5 and C6. Of the total isolates from both the soil source and from the compost soil sources, only the three isolates from compost soil exhibited antiproliferative activity.

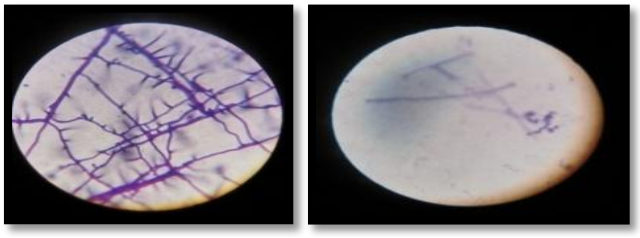
Graph 1: Determination of antiproliferative activity of the protein extracts from the Actinomyctes isolates



Graph1: The result plotted is the average of three independent experiments.

Initially, the cell division was unaffected and resulted in increased cell division up to 4 hr after treating with the protein components from C1 but, after 6 hr of treatment, resulted in the gradual decrease in the viable cell number. Similarly, slight increase in the viable cell number were recorded after treatment with the protein components from C5 and C6 isolates but resulted in the gradual decrease in the viable cell number at 8 hr of incubation.

The actinomycetes isolates designates as above that exhibited antimicrobial and antiproliferative activity were microscopically characterized by staining techniques such as gram staining and acid fast staining. The results as shown in Fig 4A and 4B confirms that all the isolates were gram positive and acid fast negative.



Isolate C1 **Isolate C2**

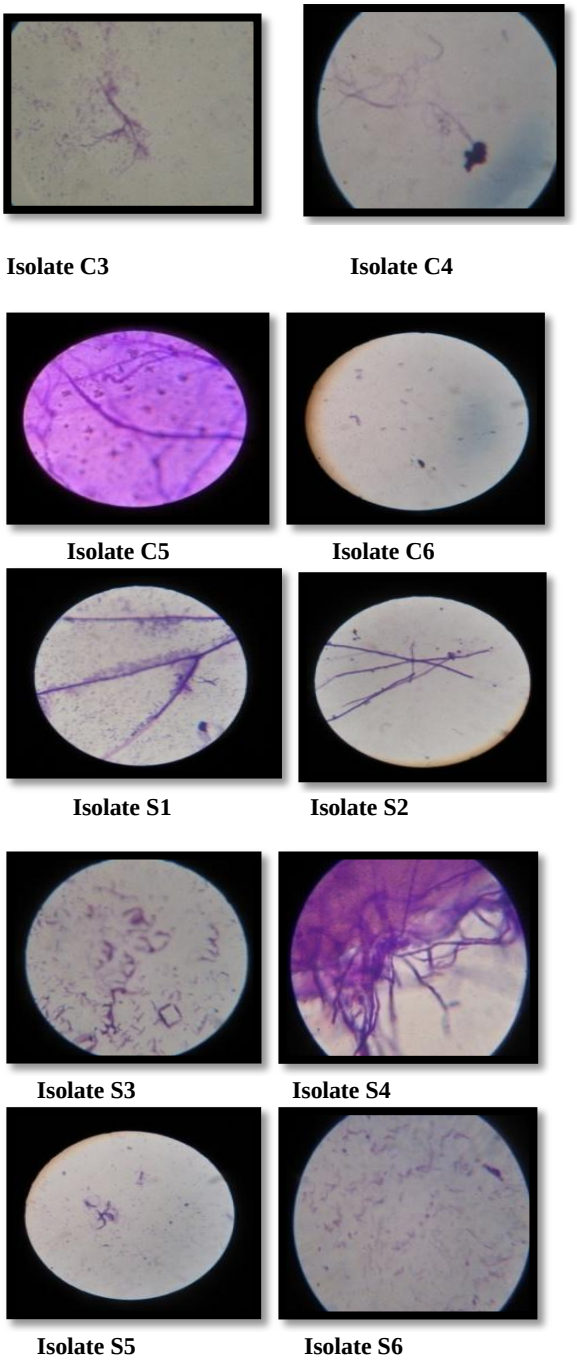
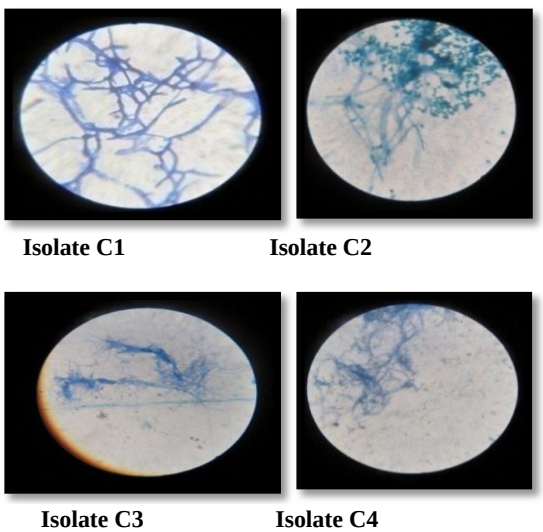


Fig.4A: Gram staining of Actinomycetes



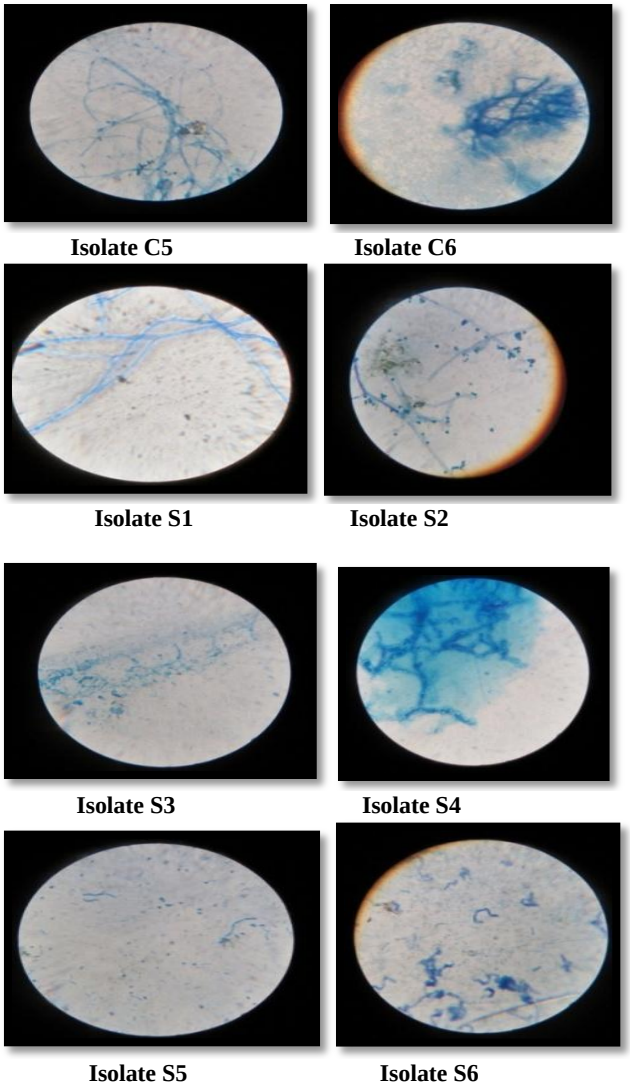


Fig.4B: Acid fast staining of Actinomycetes

The isolates were further analyzed by slide culture technique for spore structure and their arrangement on the aerial or vegetative mycelium. As shown in Fig 5A & 5B, the mycelium and different spore arrangements were analyzed for identification.

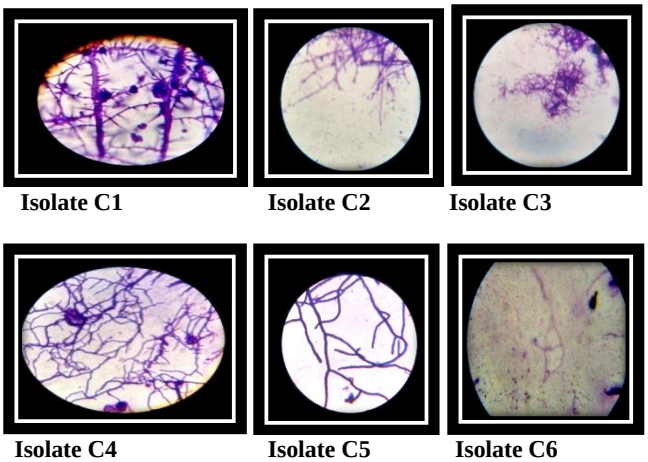


Fig 5A: Slide culture observation of Actinomycetes isolated from compost soil

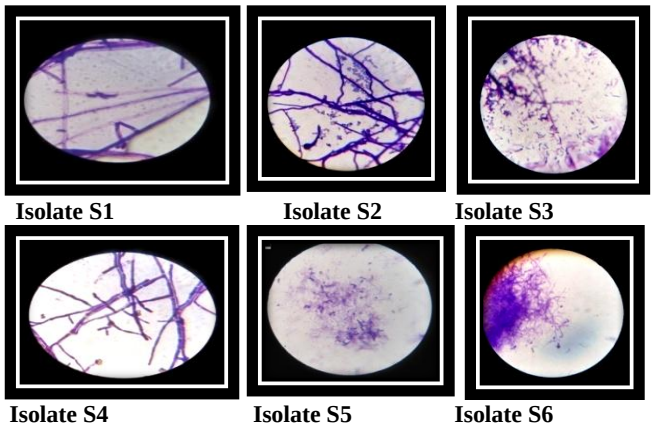


Fig. 5B: Slide culture view of Actinomycetes isolated from soil

According to the Bergey's manual of determinative bacteriology and also with reference to the Classification and identification scheme by Waksman, the designated isolates were identified on the basis of the morphological characterization of mycelium, spore observation by slide culture technique and biochemical characterization. The above designated isolates from the soil sample which showed antimicrobial activity were identified belonging to the *Streptomyces* sp., The actinomycetes isolates from the compost sample which showed antimicrobial activity were identified belonging to the *Micromonospora* sp, *Thermoactinomycetes* sp., and *Streptomyces* sp.,. Also the isolates from the compost exhibiting both antimicrobial and antiproliferative activity against proliferating yeast cell culture that were designated C1, C5 were identified belonging to *Thermoactinomycetes* sp., and C6 as *Streptomyces* sp. Thus, the present work demonstrates the isolation of actinomycetes isolates belonging to the genera *Streptomyces* from the rhizoshere soil along with *Thermoactinomycetes* and *Micromonospora* sps. from the vermicompost soil. The *Thermoactinomycetes* considered as facultative thermophiles and the *Streptomyces* from the compost soil could demonstrate antimicrobial and antiproliferative activities by their extracted peptides. Likewise the peptides from *Streptomyces* species isolated from the rhizosphere soil exhibited antimicrobial activity against the pathogenic bacteria. These peptides thus can be considered as therapeutic peptides may significantly contribute towards targeting against antibiotic resistance pathogens. Also, speculation of antimicrobial peptides as anticancer peptides may be aimed treating against various cancer types. Thus, further analysis of these peptides offers target specific antimicrobial and anticancer therapeutics.

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