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Research Article

Extracellular Synthesis of Silver Nanoparticles Using Actinomycetes and their Antibacterial Activity

Raja Prabu M, Janani P R, Prabakaran P*

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Corresponding Author:
*** Prabakaran P**
Department of Biology,
Gandhigram Rural Institute – Deemed University,
Gandhigram-624302,
Dindigul(DT).
India.

e-mail: rajaprabumass@gmail.com

ABSTRACT

In the present study, isolate and identify the pigment producing actinomycetes, to identify the bioactive compounds and biologically synthesis silver nanoparticles against pathogenic bacteria. A total of seven actinomycetes were isolated from soil samples and tested against pathogenic bacteria. Out of seven isolates, three isolates [A1, A3 and A7] showed better result against pathogenic bacteria by primary screening. Out of three isolates, A1 and A7 were subjected to secondary screening, identification, growth study (temperature and pH), mass multiplication and synthesis of silver nanoparticles methods. The potential isolates was identified by morphological and biochemical tests (Streptomyces sp.). The optimum temperature as room temperature 28oC and favorable pH was 7. The cell free extract of potential isolate was assessed for the synthesis of silver nanoparticles. The biologically synthesized silver nanoparticles showed major peak at 420 nm. Synthesized nanoparticles also exhibited antibacterial activity against bacterial pathogens.

Key-words: Actinomycetes, silver nanoparticles, antibacterial activity.

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Department of Microbiology, Periyar University, Salem-636 011.

INTRODUCTION

Microorganisms are the main colonizers of the earth, bestowed with inherent physiological and functional diversity and have found applications in agriculture, medicine, industry and environment. Among the various industrially important microorganisms, actinomycetes are of prime importance and are primarily recognized as organisms of academic curiosity and producers of antibiotic compounds and biologically active secondary metabolites. Actinomycetes population has been identified as one of the major group of soil population, which may vary with soil type. Apart from soil, they are found in marine and terrestrial environments and also exist in symbiotic association with plants and other living organisms (Balagurunathan and Radhakrishnan, 2010). The bioactive secondary metabolites produced by microorganisms is reported to be around 23,000 of which 10,000 are produced by actinomycetes, thus representing 45% of all bioactive microbial metabolites discovered. Among actinomycetes, approximately 7,600 compounds are produced by Streptomyces species. Several of these secondary metabolites are potent antibiotics. As a result of which streptomycetes have become the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Berdy 2005). The usage of antimicrobial drugs for prophylactic or therapeutic purposes regularly in human, veterinary and agricultural purposes were favoring the survival and spread of resistant organisms. Due to this multidrug resistant pathogenic strains were causing substantial morbidity and mortality especially in elderly people and immune compromised patients (Gebreselema et al 2013).Antibiotic resistance is a complex, continually evolving problem which is often difficult to put into perspective (Levy, 2002). The resistance problem is due to increased use of antibiotic repeatedly and the presence resistance gene in the microbial strains. Hence there is a need for development of new drugs that act on new targets and those that block resistance mechanisms (Levy, 2004).

To overcome the present situation, there is an interest to discover novel antibiotics and new therapeutic agents by continuous screening of secondary microbial products produced from potential bacterial taxa (Gebreselema, 2013).

Microorganisms are found as nanofactories that are eco-friendly. Even they have many applications in biological field. Among various metals, silver has been used as effective antimicrobial agents for the treatment of disease caused by pathogenic microorganisms. Silver nanoparticles have been widely used as a novel therapeutic agent and their used as antibacterial, antifungal, antiviral, anti-inflammatory and anti-cancerous agents (Navin *et al*. 2010). Conventional synthesis of silver nanoparticles, such as chemical reduction of silver ions in aqueous solution (Liz marzan and Lado-Tourino, 1996), and radiation chemical reduction (Hengelin, 1993, 1998, 2001). These methods are more expensive and hazard to environment (Jae and Boem, 2009). Biological methods of silver nanoparticles synthesis by using microorganisms (Klaus *et al*., 1999 and Nair and Pradeep, 2002; Konishi and Uruga, 2007) and their secondary metabolites such as enzyme (Willner *et al*., 2006) have been suggested as possible eco-friendly alternatives to chemical and physical methods.

The current study was designed to describe the isolation of pigment producing actinomycetes from soil samples which generates an antimicrobial compounds. Isolation, characterization and identification of the isolate were reported. The primary bioactive substance was isolated by fermentation and extraction process, its biological activities were determined. The active metabolites extracted from the isolate were subjected to MIC against the Gram positive and Gram negative bacteria. The antimicrobial potential of culture filtrates and synthesized nanoparticles of actinomycetes was studied against medically important bacterial pathogens.

MATERIALS AND METHODS

SAMPLE COLLECTION

The soil samples were collected from different localities of Gandhigram University. Soil samples were collected from the surface of about 10 cm deep as a mixture for the isolation of actinomycetes. Soil samples were collected with the sterile spatula and transferred to polythene bags which are labeled and transported to Microbiology laboratory (Department of Biology, Gandhigram Rural Institute-Deemed University) and stored at 4°C for further studies.

ISOLATION AND MAINTENANCE OF ISOLATES

One gram of each soil sample was poured into 100 ml of sterile distilled water in 250 ml conical flask and it set as dilution factor 10^{-2}. 1ml was taken from 10^{-2} dilution factor and transfer it into tube containing 9ml of blank and it set as dilution factor 10^{-3}. Further 1ml of aliquots was diluted upto 10^{-6}.Prepare the Starch casein

nitrate agar medium and sterilize medium under 121^{0}C for 20 minutes at 15lbs. Take 0.1ml of aliquots from dilution 10^{-2}, 10^{-3} and 10^{-4} serially were by spread plate technique on starch – casein nitrate (SCN) agar plates. The SCN agar plates incubated at 37°C for one week. After one week of incubation, the typically, pigmented, dry, powdery colonies were observed. These selected colonies were subcultured in SCN medium. Slants containing pure cultures were stored at 4°C until further examination. For every 30 days the cultures were sub-cultured freshly in starch casein nitrate slant for better bioactive production.

CHARACTERIZATION OF ISOLATES

The isolates were characterized by morphological (Gram staining), cultural and biochemical studies (Catalase test, Citrate utilization test, Starch hydrolysis test, Casein hydrolysis, Gelatin hydrolysis).

BACTERIAL STRAINS

In the present study, gram positive bacterial pathogenic organisms such as *Staphylococcus aureus, Klebsiella pneumonia* and Gram negative bacterial pathogenic organisms such as *Escherichia coli and Pseudomonas aeruginosa* were used. These selected bacterial pathogens resistant to some antibiotics.

MASS MULTIPLICATION

Prepare 100 ml of Starch casein nitrate broth medium in 250 ml Erlenmeyer flask. A loop-full of culture taken from isolated actinomycetes slant culture and inoculate in 250ml Erlenmeyer flask containing Starch casein nitrate broth medium. The cultured flasks were incubated at 28ºC for 7 days under shaking condition in rotary shaker for multiplication and pigment production.

PRIMARY SCREENING OF ISOLATES

During the primary screening, isolates were screened against selected bacterial strains by agar well diffusion method. Antagonistic activity of isolates against Gram positive and Gram negative bacteria was primarily screened by using well diffusion method. In this method, nutrient agar media was prepared and spread 24 hours culture of the tested bacterial culture on agar surface by using sterile L-rod. After few minutes, make the well in agar plate with help of cork-borer under aseptic condition and the crude culture of each isolates were added to the separate well. Allow the plates to diffusion of crude extract of isolates in to agar medium. The plates were incubated at 37^{0}C for 2-3 days for screening of isolates against selected bacterial for detect the antagonistic activity of isolates culture. The zone of inhibition (mm) was measured.

INFLUENCE OF pH ON ACTINOMYCETS GROWTH

Prepared Starch casein nitrate broth in four different tubes with pH ranges; 6, 7, 8 and 10. Respectively the pH ranges of media was brought about by the addition of 0.1N Hcl and 0.1N NaOH. The medium sterilized at 121^{0}C for 20 minutes at 15 lbs. Then potential isolated culture was inoculate into each tube containing SCN broth and incubated at room temperature 28^{o}C. Absorption spectrum of isolates from each tube was measured at 520 nm at specific time interval for isolates culture growth rate and pigment production by isolates.

INFLUENCE OF TEMPERATURE ON ACTINOMYCETS GROWTH

Starch casein nitrate broth was prepared and inoculated with potential culture of isolates in test tubes. These tubes were incubated under various temperatures like 30^{0}C, 37^{0}C and room temperature 28^{o}C. After incubation, absorption spectrum of isolates from each tube was measured at 520 nm at specific time interval for isolates culture growth rate and pigment production by isolates.

ISOLATION OF CELL FREE EXTRACT

Inoculate a loop-full isolate culture into 100 ml starch casein nitrate broth in 250 ml Erlenmeyer flask and incubate at 28^{0}C for 7 days for production of secondary metabolites and pigments. After 7 days incubation 5 ml of isolate broth culture was taken centrifuge tube. The tube centrifuged at 3000 rpm for 5 min. The supernatant was taken as cell-free extract. The cell free extract was used to secondary screening. The cell-free extract containing secondary metabolites of isolates such as pigments, enzymes and other bioactive compounds like antibiotics.

SECONDARY SCREENING OF ISOLATES

In secondary screening method, prepare nutrient agar medium and 24 cultures of selected bacterial strains were spread on surface of nutrient agar with the help of sterile L-rod by aseptic method. Then make wells by using sterile cork borer. Add broth culture and cell-free extract of potential isolates into the well and allow the cell-free extract to diffuse in agar plate. Incubate the plates at 28^0C for 24 hours and the zone of inhibition around cell-free extract and broth culture of isolates were compared.

SYNTHESIS OF SILVER NANOPARTICLES

Preparation of 1mM solution of silver nitrate

Dissolve 0.016987 g of silver nitrate in 100ml of distilled water.

The isolate culture was inoculated into 100ml of starch casein nitrate broth, incubated at 28^0C for 7 days under shake condition in rotary shaker. The broth culture was centrifuged at 3000 rpm for 5-8 minutes and the supernatant was taken. Prepare 100 ml of 1mM $AgNO_3$ solution. 95 ml of $AgNO_3$ (1mM) solution was added to cell-free supernatant obtained from isolates in 250ml conical flask at room temperature. The flask was incubated in dark condition. It was monitored continuously for color change. Absorption spectrum of the reaction mixture was measured between 200 to 600 nm by using UV-spectrophotometer. The isolate culture without silver nitrate solution was used as control. After 2 days incubation, the reaction mixtures were examined in fluorescence study.

RESULTS

ISOLATION OF ACTINOMYCETES FROM SOIL

Actinomycetes were isolated from the soil in and around Gandhigram Rural Institute-Deemed University, Gandhigram, Tamil Nadu, and India. Based on the morphological and microscopic observation, seven actinomycetes were selected for the primary screening, the cultures were maintained in starch casein nitrate agar.

PRIMARY SCREENING

Among the seven isolates, 2 isolates showed maximum antibacterial activity against tested bacteria. In agar well diffusion method, the results revealed that the isolates A1 and A7 had broad spectrum activity against tested bacteria. The A1 isolates showed potential activity against *Klebsiella pneumoniae* and *Escherichia coli.* The A7 isolates showed activity against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* (Table 1).

Table 1: Primary screening of actinomycetes isolates by using agar well diffusion method against tested bacteria (zone of inhibition in mm)

Tested Bacteria	Culture Isolates/Zone of inhibition (mm)					
	A1	**A2**	**A3**	**A4**	**A6**	**A7**
Klebsiella pneumonia	14	NA	NA	NA	NA	12
Pseudomonas aeruginosa	NA	NA	NA	NA	NA	6
Staphylococcus aureus	NA	NA	NA	NA	NA	13
Escherichia coli	10	NA	4	NA	NA	14

NA: No activity

INFLUENCE OF PH

The isolates A1 and A7 were inoculated in to starch casein nitrate broth in different pH range like 6, 7, 8 and 10. The pigmentation and cell growth of isolates was monitored by UV-spectra analysis at 520nm. Both these two isolates showed maximum growth and pigmentation were observed in pH 7 (Table 2).

Table 2: Influence of pH on Actinomycetes isolates growth UV-spectra at 520 nm

NO OF DAYS	pH	A_1	A_7
3	6	0.241	0.472
	7	0.378	0.682
	8	0.286	0.372
	10	0.192	0.201
6	6	0.537	0.992
	7	0.715	1.118
	8	0.562	0.691
	10	0.331	0.424

INFLUENCE OF TEMPERATURE

Isolates (A1and A7) incubated at various temperature conditions like 30^0C, 37^0C and room temperature. Even A1 isolate grew well and color of pigment production was higher in 30^0C and isolate A7 grow well and color of pigment production was higher in 37^0C. The pigmentation and cell growth of isolates were monitored by UV-spectra analysis at 520nm. At 30^0C can be considered as the optimum for the production of pigments by A1 and 37^0C for the production of pigment by A7 (Table 3).

Table 3: Influence of temperature on actinomycetes isolates growth UV-spectra at 520 nm.

NO OF DAYS	TEMPERATURE	A1	A7
3	Room temperature	0.42	0.67
	30^0C	0.60	0.89
	37^0C	0.53	0.97
6	Room temperature	0.86	1.34
	30^0C	1.43	1.75
	37^0C	0.92	2.02

IDENTIFICATION OF ACTINOMYCETES BY BIOCHEMICAL TEST

The potential antibiotic resistant strains (A1 and A7) were undergone biochemical test for identification. The biochemical activities of two isolates were tabulated (Table 4).

Table 4: BIOCHEMICAL ACTIVITIES OF ISOLATED CULTURE

S.NO	BIOCHEMICAL TEST	ISOLATED CULTURE	
		A1	A7
1	Gram's staining	+	+
2	Catalase	+	+
3	Citrate utilization test	+	+
4	Starch hydrolysis	+	+
5	Casein hydrolysis	+	+
6	Gelatin hydrolysis	+	+

Based on the biochemical results, A1 identified as a *Streptomyces* sp. and A7 as *Actinomyces* sp.

SECONDARY SCREENING

Cell-free extract prepared from broth culture by centrifugation methods. Further the cell-free extract was subjected to secondary screening by agar well diffusion against tested bacteria. In agar well diffusion

method, cell-free extract of isolates *Streptomyces sp.* and *Actinomyces* sp. showed potential activity against tested bacteria. Isolates *Streptomyces* cell-free extract showed activity against *E.coli* and *Klebsiella pneumoniae* and isolates *Actinomyces* cell-free extract showed activity against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* (Table 5).

Table 5: Secondary screening of antibacterial activity of cell-free extract of *Streptomyces* sp. And *Actinomyces* sp. (zone of inhibition in mm)

Tested bacteria	*Streptomyces* sp.	*Actinomyces* sp..
Klebsiella pneumonia	12	14
Pseudomonas aeruginosa	_	8
Staphylococcus aureus	_	8
E.coli	7	10

SYNTHESIS OF SILVER NANOPARTICLES

The silver nanoparticles were synthesized by mixing the cell-free extract with $AgNO_3$ (1mM) solution. Upon addition of cell-free extract to the silver nitrate solution, the solution color changed from pale white to violet. The color change of the silver nitrate solution, indicating the formation of silver nanoparticles. Consequently synthesis of nanoparticles was characterized by UV-visible spectra (300nm-480nm).The absorption spectra recorded the production of silver nanoparticles within 2 hours(Table.6). The presented spectra exhibit the appearance of an absorption peak at 420nm (Fig.3). The appearance of absorption peak at 420nm is characteristic of silver nanoparticles. In fluorescence study of reaction mixture of *Streptomyces* sp. showed sharp peak at 459nm which indicates that emission of silver nanoparticles and also slight peak appear at 490nm which indicates impurities in reaction mixtures(Fig.4). *Actinomyces sp.* reaction mixture showed sharp peak at 459nm which indicates that emission of silver nanoparticles(Fig.5).

Table 6: UV-spectra analysis of silver nanoparticles by *Streptomyces* sp. and *Actinomyces* sp.

WAVELENGTH (nm)	***Streptomyces sp.* Ag-NPs**	***Actinomyces sp.* Ag-NPs**
300	2.073	2.064
320	1.768	1.691
340	1.431	1.476
360	1.378	1.274
380	0.982	0.938
400	0.783	0.76
420	1.170	0.870
440	0.973	0.781
460	0.701	0.698
480	0.421	0.431

Ag-NPs : silver nanoparticles

Fig.3 : UV-spectra analysis of silver nanoparticles by *Streptomyces* sp. and *Actinomyces* sp.

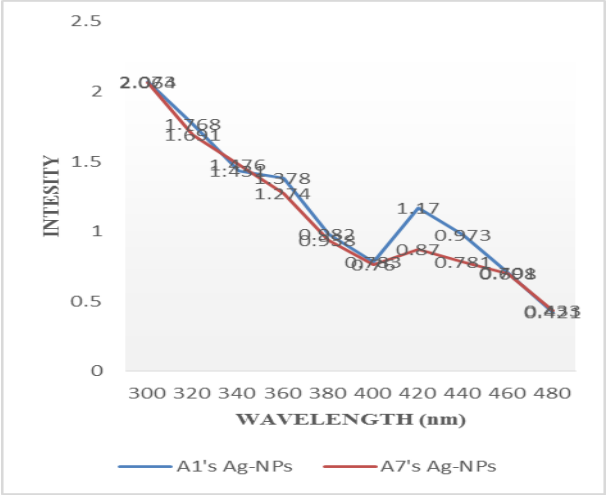


Fig. 4: FLUROSCENCE STUDY

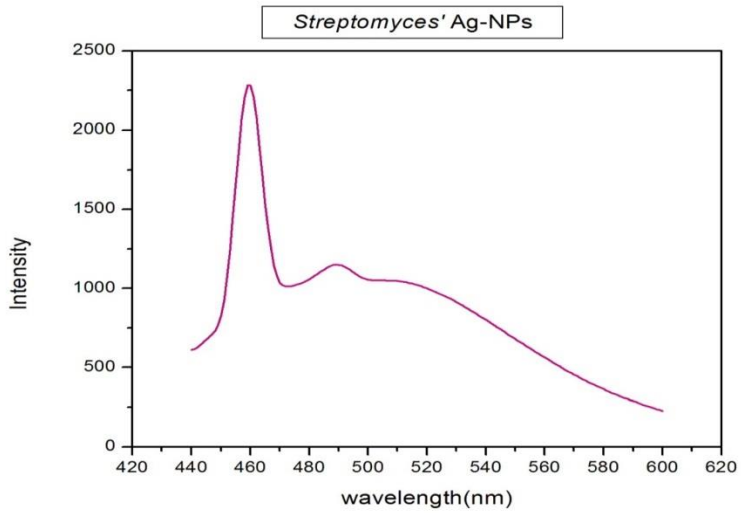


Fig.5: FLUORESCENCE STUDY

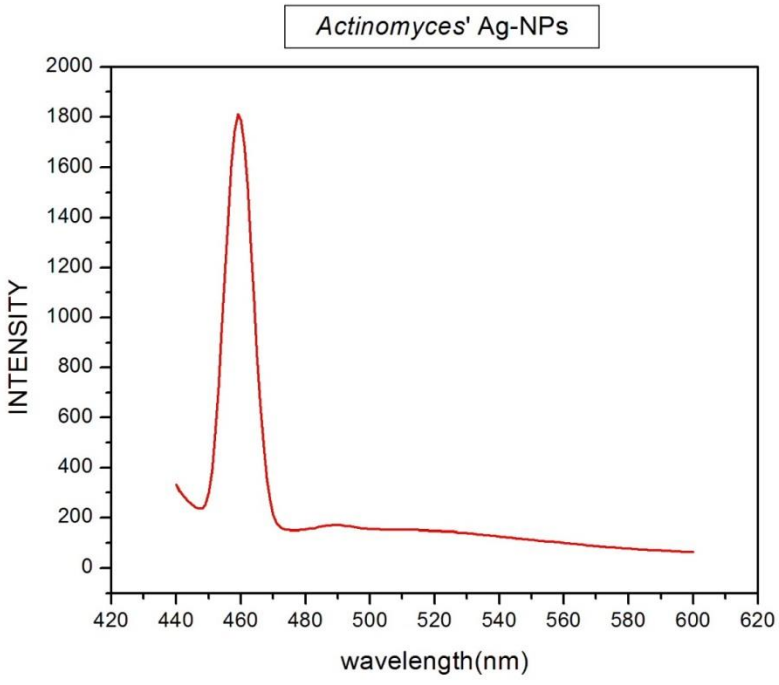


Plate 1. PURE CULTURE OF ACTINOMYCETES

Plate 2: ANTIBACTERIAL ACTIVITY OF ISOLATED ACTINOMYCETES AGAINST *Klebsiella pneumoniae*.

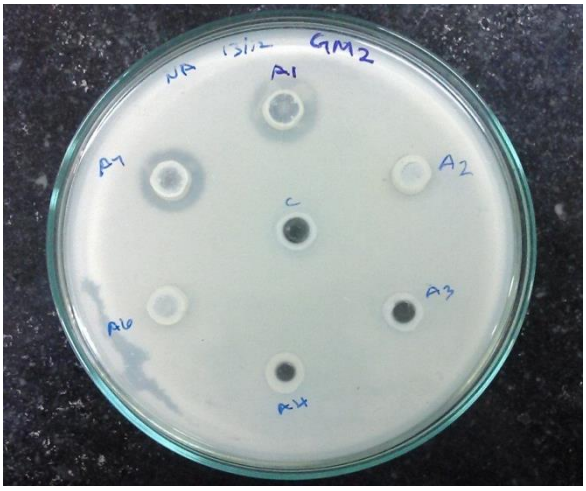


Plate 3: ANTIBACTERIAL ACTIVITY OF ISOLATED ACTINOMYCETES AGAINST *Pseudomonas aeruginosa*.

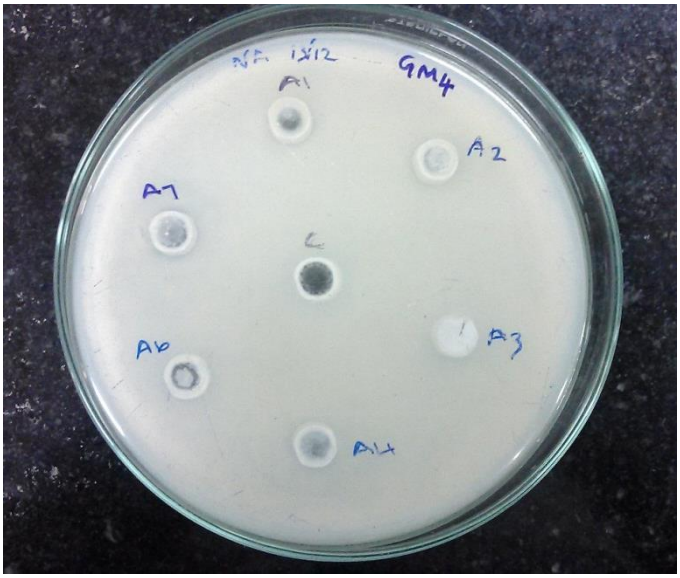


Plate 4: ANTIBACTERIAL ACTIVITY OF ISOLATED ACTINOMYCETES AGAINST *Staphylococcus aureus*.

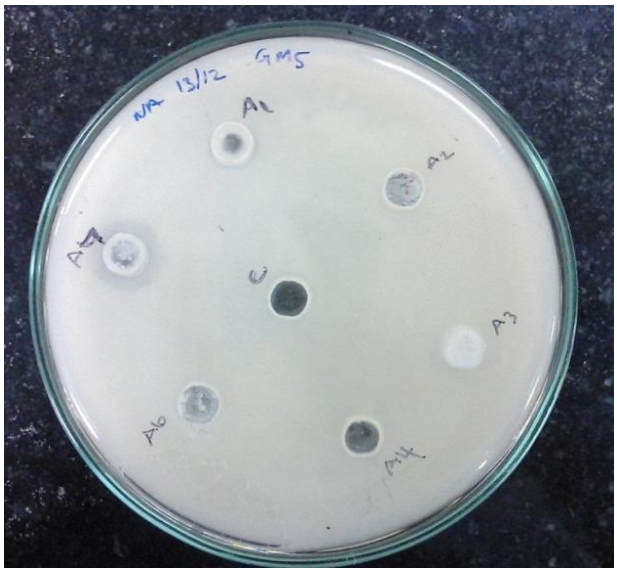


Plate 5: ANTIBACTERIAL ACTIVITY OF ISOLATED ACTINOMYCETES AGAINST *Escherichia coli*

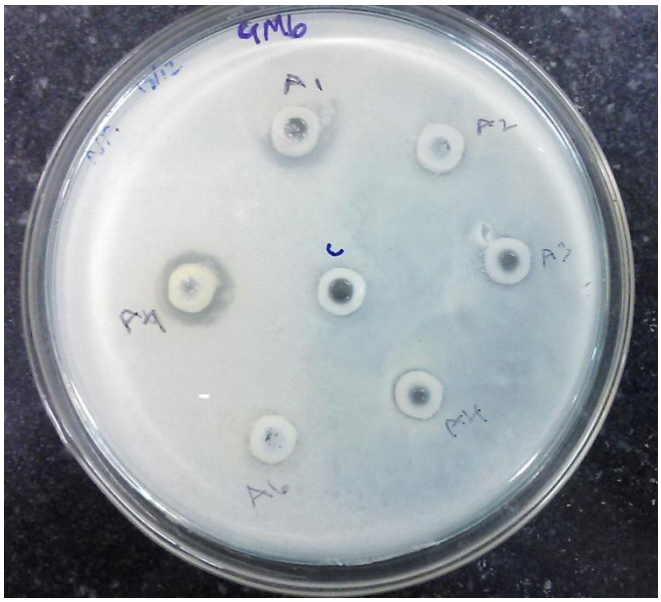
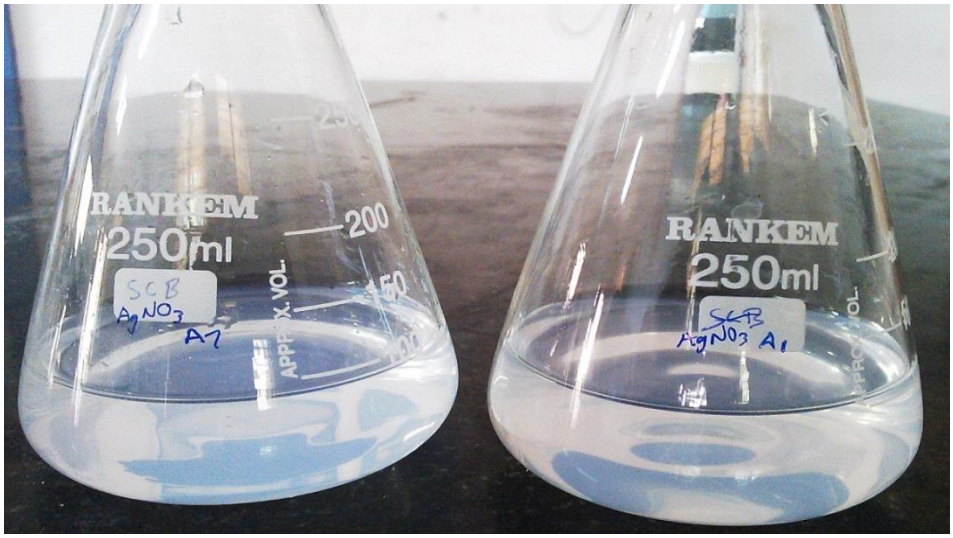
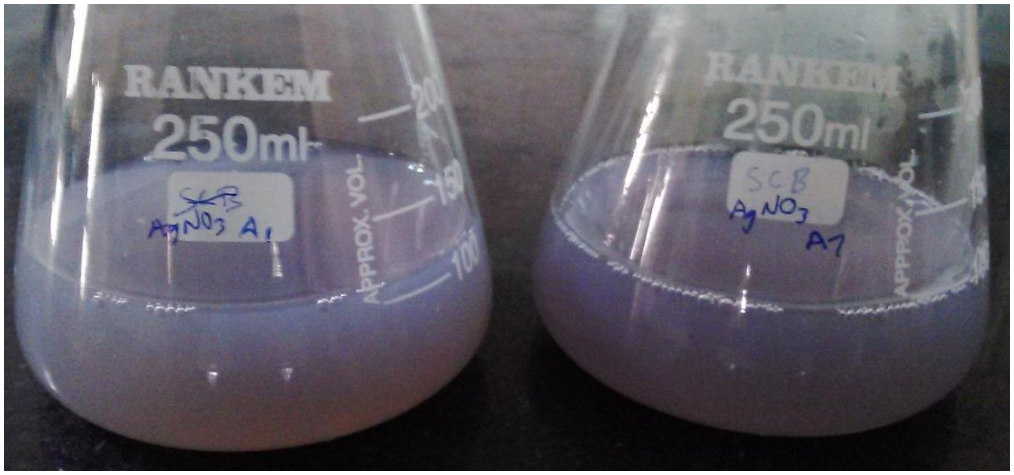


Plate 7: SYNTHESIS OF SILVER NANOPARTICLES





DISCUSSION

One of the major reason to carry out this study is that the incidence of multidrug resistant organisms are increasing and compromising the treatment of growing numerous infectious diseases. Due to this reason, there is need to develop new drugs which are effective against antibiotic resistant pathogens. Actinomycetes have been proven as a potential source of bioactive compounds and rich source of secondary metabolites (Suthindhiran *et al*., 2009).

The actinomycetes were isolated from soil showed maximum antibacterial activity against tested bacteria suggest that the isolates produces antagonistic bioactive compounds and secondary metabolites. They are used as therapeutic agents to control bacterial pathogens.

This discussion will focused on the interpretation of various studies carried out to determine antibacterial activity of isolates, influence of pH and temperature on selected isolates and extracellular synthesis of nanoparticles from noble metals such as silver.

ISOLATION AND SCREENING OF ISOLATES

In the present study, seven actinomycetes strains were isolated and Starch casein nitrate agar media were used for isolation. These seven isolates were undergone to primary screening against tested bacteria. Among those only two isolates showed potential antibacterial activity against both gram positive and gram negative bacteria in primary screening. The isolates which have the antibacterial activity against tested bacterial culture in agar well diffusion method were selected for further studies. Of these isolates A1 and A7 showed potential antibacterial activity was selected for detail studies.

The many researchers were reported that *Streptomyces* isolates appear to be highly active against gram positive bacteria when compared to gram negative bacteria (Hamid *et al.,*1980, Hussein *et al.,*1980 and Saadoun *et al.,* 1998).

BIOCHEMICAL CHARACTERIZATION

In this study, these two selected actinomycetes isolates (A1 and A7) showed positive results for catalase, Starch hydrolysis, Casein hydrolysis, Citrate utilization and Gelatin hydrolysis biochemical tests.

The results were recorded that seven actinomycetes isolates showed positive result for Catalase, Starch hydrolysis, and Casein hydrolysis and showed negative results for Indole and Triple Sugar Iron biochemical tests (Smriti *et al*., 2012).

INFLUENCE OF pH AND TEMPERATURE

Studies were carried out on the influence of pH and temperature on growth and pigment production of these two selected (A1 and A7) isolates. A1 isolates showed maximum growth and higher pigment production at pH 7 and 30°C. A7 isolates showed maximum growth and higher pigment production at pH 7 and 37°C.

The results were noted that *Streptomyces* species showed well cell growth and higher pigment production at alkaline pH ranges of 9 and 11 and 28°C (Shobha *et al*., 2014).

SYNTHESIS OF SILVER NANOPARTICLES

In this study, silver nanoparticles were biologically synthesized in extracellular by isolated culture's (*Streptomyces sp.* and *Actinomyces sp.)* cell-free extract. The formation of silver nanoparticles in extracellular secretion at room temperature. This silver nanoparticles formation was revealed by UV-visible spectroscopy. In UV-visible spectra analysis, appearance of peak at 420nm which is responsible for synthesis of silver nanoparticles. They can be used as therapeutic agents.

The results were recorded that the silver nanoparticles synthesized in extracellular by *Streptomyces rochei*. The silver nanoparticles exhibited tremendous potential antibacterial activity against common pathogenic bacteria (Selvakumar *et al*., 2012). The results were reported that synthesis of silver nanoparticles by extracellular components of *Streptomyces albogriseolus* and they exhibited antibacterial activity against bacterial food-borne pathogens (Samundeeswari *et al*., 2012)

CONCLUSION:

The importance of this study is that the antibacterial activity is high in silver nano particles when compared to the test bacterial pathogens. So, in this study the silver nano particles acts an important role.

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