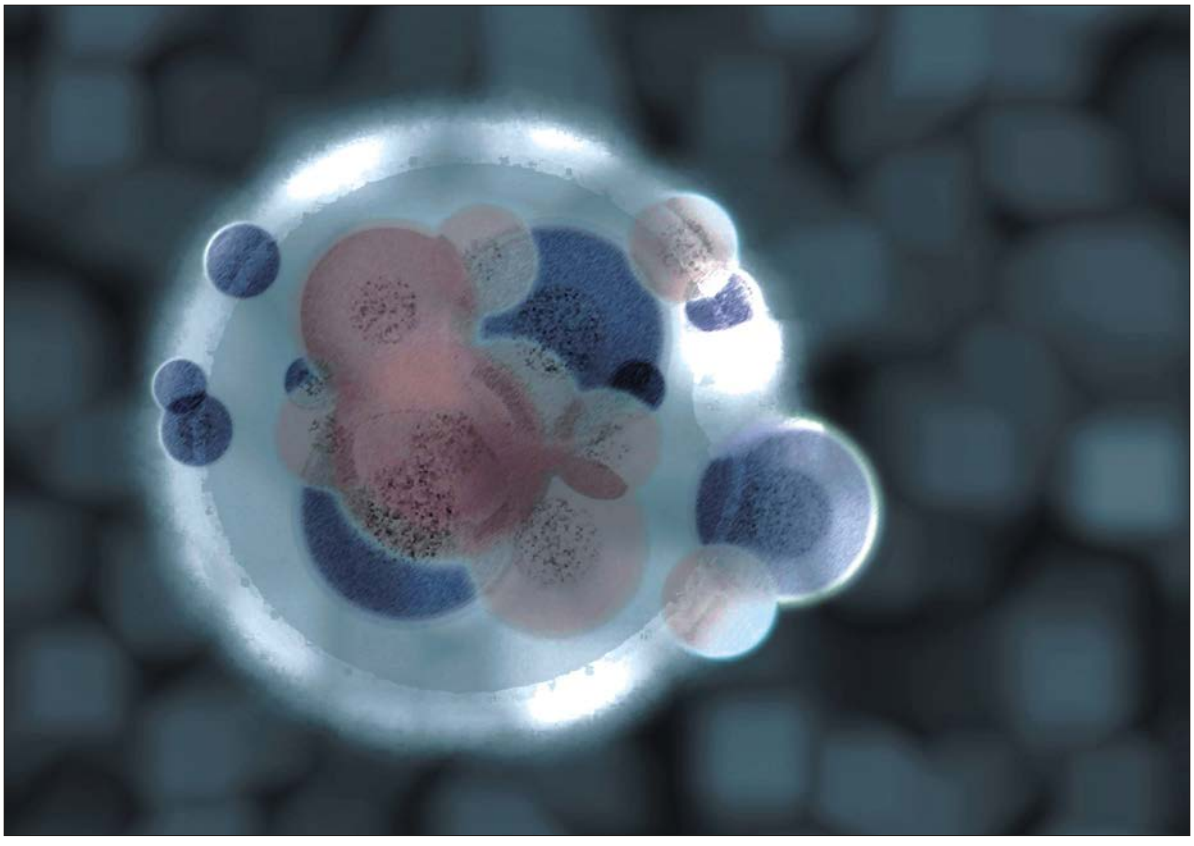

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Isolation, Characterization, Screening and Antibiotic Sensitivity of *Actinomycetes* from Locally (Near MCAS) Collected Soil Samples

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Abstract: In the present study, the soil samples were collected from Corn and Soya fields; near MCAS. *Actinomycetes* strains were isolated in specific medium using Arginine-Glycerol-Salt (AGS). These *Actinomycetes* were screened with regard to potential against Gram-positive and Gram-negative bacteria. The purified *Actinomycetes* strains were performed in biochemical tests such as, Fermentation of citrate, Starch hydrolysis, Vogus-proskauer, Triple sugar iron test etc. The positive isolate was immobilized using sodium alginate beads and the immobilized cells were cultivated in specific fermentation liquid medium for 120 h. Antibacterial compound was purified from the filtrate by solvent extraction method. A comparative study on the total antibiotic sensitivity of the free cells and immobilized cells showed that the immobilized strains were found to be effective against the tested microorganisms. The immobilized cell of *Actinomycetes* was found to be more efficient for the production of secondary metabolites with batch fermentation. The *Actinomycetes* strains were prepared in crude protein and the crude protein was determined by using SDS PAGE method. From the results, we are concluded that the positive immobilized *Actinomycetes* strain showed high antibacterial activity against tested pathogens.

Key words: Soil sample, *Actinomycetes*, antibacterial activity, immobilization, fermentation, protein profile

INTRODUCTION

Actinomycetes are Grams-positive bacteria frequently filamentous and sporulating with DNA rich in G+C from 55-75% (Ho *et al*., 2002). The name *Actinomycetes* derived from Greek aktis (a ray) and mykes (fungus) was given to these organisms from initial observation of their morphology. *Streptomycetes* is the dominant among *Actinomycetes*. The non-*streptomycetes* are called rare *Actinomycetes*, comparing approximately 100 genera numbers of the *Actinomycetes*, which live in marine environment, are poorly understood and only few reports are available pertaining to *Actinomycetes* from mangroves (Kumar, 2001). *Actinomycetes* are the most fruitful source for production of bioactive secondary metabolites. *Actinomycetes* a total 7899 (100%) compounds has been identified up to 1988; 67% from *Actinomycetes*, 12% from bacteria and 20% fungi. Japanese and American scientists (Tanaka and Omura, 1990) contributed greatly to the discovery of *Actinomycetes* products, 286 compounds are produced by *Streptomyces hygroscopicus*, 189 by *S. griseus,* 129 by *S. lavendulae,* 383 by *Micromonospora* app., 278 by *Nocardia* sp. The isolation and characterization of *Actinomycetes* were performed in different biochemical methods (Dhanasekaran *et al*., 2009). Morphological examination of the *Actinomycetes* was done by using cellophane tape and cover slip-buried methods (Williams and Cross 1971). The mycelium structure, color and arrangement of conidiophores and arthrospore on the mycelium were examined under oil immersion (1000X). The observed structure compared with Bergay's manual of Determinative Bacteriology, Ninth edition (2000) for identification *Streptomyces* sp., under the group of *Actinomycetes*. Different biochemical tests were performed to characterize the *Actinomycetes*. The tests generally used are starch hydrolysis, Triple Sugar Iron (TSI) agar test, citrate utilization test, indole test, methyl red test, vogus-proskauer (Acetone Production) test, Catalase test (Holt, 1989). The *Actinomycetes* were originally considered an intermediate group between bacteria and fungi then were recognized as prokaryotic organisms. *Actinomycetes* species synthesize a numerous natural metabolites with diverse biological activity such as antibiotics. Antibiotics of *Actinomycetes* origin evidence a wide variety of chemical structure, including amino glycosides, anthracyclines, β-lactams, nucleosides, peptides, polyenes, actinomycins and tetracycline (Barrios-Gonzalez *et al*., 2005). Generally, antibiotics are

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non-growing associated secondary metabolites. It is therefore very difficult to produce them by continuous cultivation of microorganisms. Application of immobilized growing cells may overcome these problems by controlling cultivation conditions the growth phase of cells will be maintained in preference to antibiotic production and also culture fluids will not contain heavy cell mass. In recent years, much interest has been focused on the use of immobilized microbial cells for producing useful bioactive compounds (Manjula *et al*., 2009). The antibiotics are widely produced by fermentation using free cell cultures to enhance the productivity. Among another strategies adopted, the whole cell immobilization appears to be more effective for antibiotic production. Immobilized cells have been used in a wide spectrum of application such as production of ethanol, Biosensors and production of oxytetracycline. The method of cell immobilization includes nonspecific adsorption, covalent attachment and entrapment. Entrapment of living cells with natural polymers such as agar, agarose, alginate etc., is principally carried out by ionotropic or thermal gelatin used to increase the yield of antibiotics. Some of the methods demonstrated the advantages of use of immobilized growing cells for the continuous production of antibiotics. For example, the immobilization of *Streptomyces rimoses* leads to increase in the production of oxytetracycline (Yang and Yueh, 2001), the enhanced production of neomycin by immobilization of *Streptomyces marinensis* on calcium alginate matrix (Srinivasulu and Ellaiah, 2005). The present study was to compare the efficacy of immobilized and free *Actinomycetes* for their antibiotic production and its antimicrobial property against pathogens. Proteins are polypeptides, which are made up of many amino acids linked together as a linear chain. The structure of an amino acid contained amino group, a carboxyl group and an R group, which is, usually carbon based gives the amino acid its specific properties. These properties determine the interactions between atoms and molecules, which are vander waals force between temporary dipoles, ionic interactions between charged groups and attractions between polar groups. The *Actinomycetes* strains were prepared in crude protein. Then, the crude protein was determined by using SDS PAGE method.

MATERIALS AND METHODS

Isolation of *Actinomycetes* from soil sample: The soil samples were collected from near my institute (2009), Muthayammal College of Arts and Science (MCAS) in corn and Soya fields and air dried and used. A total soil sample was collected from two areas, collected in sterile plastic bags and brought into sterile condition. *Actinomycetes* were isolated by soil dilution plate technique using Arginine-Glycerol-Salt (AGS) medium, (El-Nakeeb and Lechevalier, 1962). The organism was screened by purification method, streaking on AGS medium and incubated at 30°C for 4 days.

Biochemical characterization of *Actinomycetes*: The potent *Actinomycetes* were characterized by morphological methods consist of macroscopic and microscopic methods. The mycelium structure, color and arrangement of conidiophores and arthrospore on the mycelium were observed through the oil immersion (100X). The observed structure was compared with Berbey's manual of Determinative Bacteriology and the organism was identified. Various biochemical tests performed for the identification of potent isolates are as follows: Starch hydrolysis, fermentation of citrate, nitrate reduction and IMVIC tests.

Fermentation process: The *Actinomycetes* were cultured at 30°C for 120 h in a jar fermentor containing 1 L of a medium containing of maltose 4%, sodium glutamate 1.2%, K_2HPO_4 0.01%, $MgSO_4$ 0.05%, $CaCl_2$ 0.01% and $FeSO_4$ 0.005% with or without sodium alginate beads by the ionotropic method. At the end of fermentation cycle, the sodium alginate beads were aseptically separated from the fermentation broth by filtration using a sterile Bunchner funnel (Pandey *et al*., 2001).

Isolation of antibacterial metabolites: Antibacterial compound was purified from the filtrate by solvent extraction method; Ethyl acetate was added to the filtrate in ratio of 1:1 (v/v) and shaken vigorously for 1 h complete extraction. The ethyl acetate phase contains antibiotic substances separated from the aqueous phase. It was evaporated to dryness in water bath and the residue obtained was weighed (Westley *et al*., 1979). The obtained compound thus used to determine the antibacterial activity.

Determination of antibacterial activity: The *actinomycete* isolates often encounted show antibiotic activity on agar but not in liquid culture. The results of screening method were that most of the active isolates were active against Gram-positive and Gram-negative pathogen. Antibacterial activity was tested *in vitro* against pathogenic bacteria: *Escherichia coli* MTCC 50, *Pseudomonas aeruginosa* MTCC 424 and *Bacillus subtilis* MTCC 441. Antibacterial activities were performed by disc-diffusion assay and effectiveness was measured by zone of inhibition on bacterial culture plates.

Determination of protein profile from *Actinomycetes*

Protein profile: For the determination of protein profile from *Actinomycetes* using SDS-PAGE method as described by Laemmli (1970).

Preparation of bacterial sample: The actinomycete was grown in their respective broth at 30°C for 54 h. The cells were harvested by centrifugation at 8000 rpm for 1 min. The pellet was washed with 1 M Tris HCl buffer (pH = 6.8) and resuspended in 10 mL of the same buffer and vortexed. Then 80 mL of the sample buffer (1 M Tris HCl, pH = 6.8, 20% SDS, 20% glycerol, 10% b-mercaptoethanol and 0.005% Bromophenol blue) and 6.5 mL of b-mercaptoethanol were added to the preparations and boiled immediately in water bath at 100°C for 5 min. After boiling, the samples were placed on ice for 5 min and centrifuged at 8000 rpm for 1 min.

Preparation of SDS-PAGE: For SDS-PAGE 12.5%, separating gel and 6.5% resolving gel were prepared. A volume of 30 μL of each sample was loaded on gel and was run on mini gel electrophoresis at 100 V for 2 h and stained in a solution containing 0.1% (by mass per volume) Coomassie blue, 10% (by volume) acetic acid and 40% (by volume) methanol. De staining was performed in a solution containing 10% (by volume) acetic acid and 45% (by volume) methanol. The protein molecular mass marker (100 kDa) was used as standard.

RESULTS

Out of three *Actinomycetes* subjected for primary screening process and subjected for purification method by streak plate method (Fig. 1a-c). The identification of the potent antibiotic producing strains reveal strain belongs to the genus *streptomyces*. The biochemical tests were performed in positive isolates of *Actinomycetes*. The isolated microorganism is Gram-positive, branching and filamentous bacteria, it has also shown positive results in methyl red, indole test, starch hydrolysis, vogus-proskauer test, catalase test and triple sugar iron test and shown negative result in fermentation of citrate, the results were represented at Table 1.

Out of three isolates, the one isolate was showed in positive result. The selected isolate was performed in whole cell immobilization and free cells. The isolates were

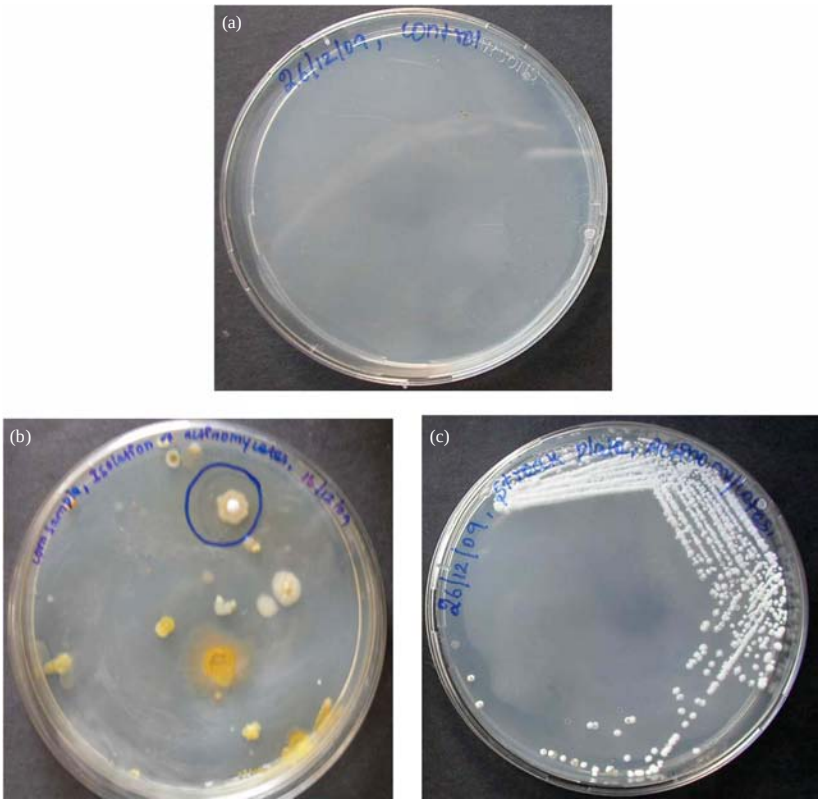

Fig. 1: (a-c) Isolation and screening of microorganism

Table 1: Bio chemical characterization of *Actinomycetes* strains from soil sample

Test	Results
Gram's staining	Positive
Methyl red	Positive
Indole test	Positive
Vogus-proskauer	Positive
Triple sugar iron test	Alkaline slant, acid bud
Fermentation of citrate	Negative
Starch hydrolysis	Positive

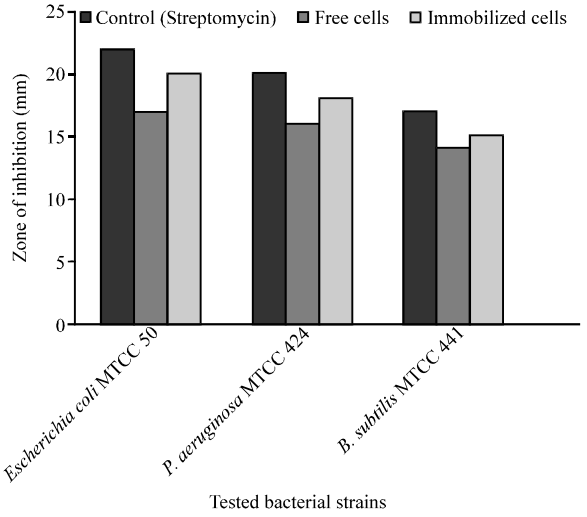

Fig. 2: Antibacterial activity for free and immobilized cell extract

Fig. 3: Isolation of crude protein from *Actinomycetes*. L1: Marker (100 kDa); L2 to L7: Crude protein from *Actinomycetes*

selected for fermentation based on their broad spectrum of activity and largest zone of inhibition. The isolates were cultivated in specific fermentation liquid medium for 120 h. After fermentation process, the Antibacterial compound was purified from the filtrate by solvent extraction method. The isolate showed the activity against tested organisms. The determination of antibiotic activity of selected strain was determined using *Escherichia coli* MTCC 50, *Pseudomonas aeruginosa* MTCC 424 and *Bacillus subtilis* MTCC 441. The comparative studies on the total antibiotic production with free and immobilized cells are shown in Fig. 2. It was found that, the immobilized cells in sodium alginate were more efficient for the production of antibiotics, which was conformed by zone of inhibition on bacterial culture plates.

The positive *Actinomycetes* strains were prepared in crude protein and the protein profile were determined by SDS-PAGE method. The results were identical to that of the same genous from various species. The crude protein separated has different molecular weight from lane 2 to 7 (Fig. 3).

DISCUSSION

The composition of an arginine-glycerol-salt medium (AGS), Suitable for the selective isolation of aerobic *Actinomycetes*, was given. When soil samples were treated with calcium carbonate and plated on the AGS medium, higher total and relative plate counts of *Actinomycetes* were obtained than when other media and methods were used (El-Nakeeb and Lechevalier *et al*., 1962). In this present study, we are using AGS medium for isolation of *Actinomycetes* from soil sample. The results were observed as filamentous, branching bacteria with a fungal type of morphology. Various biochemical tests were performed, to identify but it was unable to identify the *Actinomycetes* up to species level due to the lack of other tests. Apart from proper identification of genera and species of *Actinomycetes*, besides morphological and physiological properties (Kuster, 1972). In this study, we use various biochemical tests such as Starch hydrolysis, Triple sugar iron test, Fermentation of citrate were performed for the identification of the potent isolates (Abbas, 2006). Some of the *Actinomycetes* are characterized by the production of various pigments on natural or synthetic media. Then, the isolates were cultivated in fermentation liquid medium.

To demonstrate use of immobilized cells of *S. rimosus* for the continuous production of oxytetracycline it would be improved with calcium alginate immobilization in submerged fermentation compared with free cells. The results showed that in 1 mL culture broth, free cells produced 121 to 124 μg of oxytetracycline; where as immobilized cells produced 153 to 252 μg (Yang and Yueh, 2001). In this study, we are using the whole cell immobilization technique in an efficient way to overcome the difficulty in producing antibiotics by continuous fermentations with free-cells, since the growth and metabolite yields, have been immobilized various microbial species on different support matrices for antibiotic

production. The *Actinomycetes* isolates, which are often encounted, show antibiotic activity on agar but not in liquid culture.

In this present study, six *Actinomycetes* strains were isolated from soil available in the local area and screened with regard to their potential against Gram-positive and negative bacteria. Among the six isolated strains (A1, A2, A3, A4, A5 and A6) three isolates A2, A3 and A5 were found to be effective against tested organisms. These three strains were further cultivated in fermentation liquid medium for 120 h and immobilized using sodium alginate (Manjula *et al*., 2009). A comparative profile on the total antibiotic sensitivity of the free cells and immobilized cells showed that the immobilized strains were found to be effective against the tested microorganism then the free cells. Further, the most potent of the producer strain was selected and identified, based on the cultural and physiological characteristics. From the results, it was concluded that the three isolates showed very promising activities against tested multi-drug resistant bacteria.

The immobilized cells of *Actinomycetes* were found to be more efficient for the production of antibiotics with batch fermentation. The possible routes of antibiotic synthesis and its biological efficiency are currently under investigation (Pandey *et al*., 2001). In the present study also we are using the same methods, the results of screening method was that most of the active isolates were active against Gram-positive and Gram- negative pathogen (*Escherichia coli* MTCC 50, *Pseudomonas aeruginosa* MTCC 424 and *Bacillus subtilis* MTCC 441). The morphological differences between these microorganisms show that different sensitivity occurred. The reason is that Gram-negative bacteria have an outer polysaccharide membrane carrying the structural lipo polysaccharide compounds. This makes the cell wall impermeable to lipophilic solutes.

The Gram-positive should be more susceptible and only an outer peptide glycon layer which is not an effective permeability barrier. The *Actinomycetes* isolates are often encounted and show antibiotic activity on agar but not in liquid culture. The results of screening method was that most of the active isolates were active against Gram-positive and Gram-negative pathogen (*Escherichia coli* MTCC 50, *Pseudomonas aeruginosa* MTCC 424 and *Bacillus subtilis* MTCC 441).

Due to the morphological differences between these microorganisms's different sensitivity occurred. The reason was that a Gram-negative bacterium has an outer polysaccharide membrane carrying the structural lipopolysaccharide compounds. This makes the cell wall impermeable to lipophilic solutes. The Gram-positive should be more susceptible having only an outer peptide glycon layer which is not an effective permeability barrier. From the results, it is concluded that the immobilized cells of *Actinomycetes* are more efficient for the production of antibiotics. The possible routes of antibiotic synthesis and its biological efficiency are currently under investigation. The crude protein samples were determined by using SDS-PAGE method (Laemmli 1970).

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