PRODUCTION OF ANTIBIOTICS BY MARINE ACTINOMYCETES INDUCED BY HUMAN PATHOGENS

PROJECT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIEMENT OF MASTER OF SCIENCE IN LIFE SCIENCE

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CERTIFICATE

This is to certify that the project report titled **"PRODUCTION OF ANTIBIOTICS BY MARINE ACTINOMYCETES INDUCED BY HUMAN PATHOGENS"** submitted by Ms Kavala Parimala to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfilment of the requirement for the degree of Master of Science in Life Science is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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This project is by far the most significant accomplishment in my life and it would not have been impossible without people who supported me and believed in my caliber.

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DECLARATION

I, Miss Kavala Parimala, M. Sc. Life Science, 4th semester, Department of Life Science, NIT, Rourkela hereby declare that my project work titled "**PRODUCTION OF ANTIBIOTICS BY MARINE ACTINOMYCETES INDUCED BY HUMAN PATHOGENS, ODISHA"** is original and no part of this work has been submitted for any other degree or diploma. All the given information is true to best of my knowledge.

(Kavala Parimala)

Date:

Place:

CONTENTS

ABBREVIATIONS

%	Percentage
Min	Minute
Mg	Milligram
µg	Microgram
g	Gram
ºC	Degree Celsius
ºF	Degree Ferhenite
m	Meter
µm	Micrometer
mm	Millimeter
ml	Milliliter
µl	Microliter
cm	Centimeter

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Cross species signal transfer mediated induction of antibiotic production by the marine actinomycetes against common bacterial pathogens was investigated in the present study. Marine actinomycetes were isolated and analyzed for their efficacy in antibiotic production against common clinical pathogens viz., *Pseudomonas sp., Escherichia coli, Bacillus subtilis, Klebsiella* and *Proteus sp.* Out of 4 actinomycetes isolates analyzed, two isolates exhibited significant antibacterial activity against *Pseudomonas and proteus sp.* Isolate designated as PAS5 *(S.echinatus)* and PAS8 (*A.mutabilis*) produced antibiotic when co-cultured with *Pseudomonas and proteus sp*. cells. Cell free extract of PAS5 (*S.echinatus*)and PAS8 (*A.mutabilis)* cultivated in the presence of heat killed *Pseudomonas and Proteus sp.* cells was subjected to solvent extraction by ethyl acetate and antimicrobial activity was evaluated by disc diffusion method. Present study on induced antibiotic production by *Pseudomonas and Proteus sp.* gives in a new insight for the discovery of pathogen targeted or specific antibiotic production which can be later commercially developed.

Keywords: co-culture, antibiotic production, *Streptomyces,* marine microorganisms

1. INTRODUCTION

Antibiotic discovery its mode of action and mechanisms of its resistance have been productive research topics in academia (Bryskier, 2005) and until recently, in pharmaceutical industry (Figure 1). As natural products, provide challenging intellectual exercises and surprises with respect to their biosynthetic pathways, chemical nature, evolution, and biochemical mode of action (Strohl, 1997: Brötz-Oesterhelt and Brunner, 2008,). The total synthesis of such natural products in the laboratory is difficult, since the small molecules are often extremely complex in functionality and chirality (Nicolaou and Montagnon, 2005). The antibiotic penicillin was discovered in 1928, but complete structure of penicillin which is relatively simple molecule was not revealed until 1949, by X-ray crystallographic studies of Dorothy Crowfoot Hodgkin (Hodgkin, 1949), and was confirmed by total synthesis in 1959 (Sheehan et al., 1959). Studies of mode of action have provided biochemical information on ligands and targets throughout antibiotic history (Gale et al., 1981: Walsh, 2006), and the use of antibiotic as “phenotypic mutants” has been a valuable approach in cell physiology studies (Vazquez et al., 1969).

Figure 1: Antibiotic

The definition of “antibiotic,” was first proposed by Selman Waksman, who was the discoverer of streptomycin and a pioneer in screening of soils for the presence of biologicals, has been seriously over interpreted; it is simply a description of a use, laboratory effect or an activity of a chemical compound (Waksman, 1973). The term “antibiotic” denotes that any class of organic molecule that inhibits or kills microbes by specific interactions with target bacterials, without any consideration of the source of the particular compound or class. The discovery of antibiotics is rightly considered one of the most significant health related events of modern times. Studies with these compounds have been often showing unexpected non antibiotic effects that indicate a variety of other biological activities; the result has been significant number of additional therapeutic applications of “antibiotics” as antiviral, antitumor, or anticancer agents the alternative applications have surpassed those of antibiotic activity in importance, such as in treatment of cardiovascular disease or use as immunosuppressive agents (Demain, 2009). The common scheme of classification for antibiotics is drawn below in the figure 2.

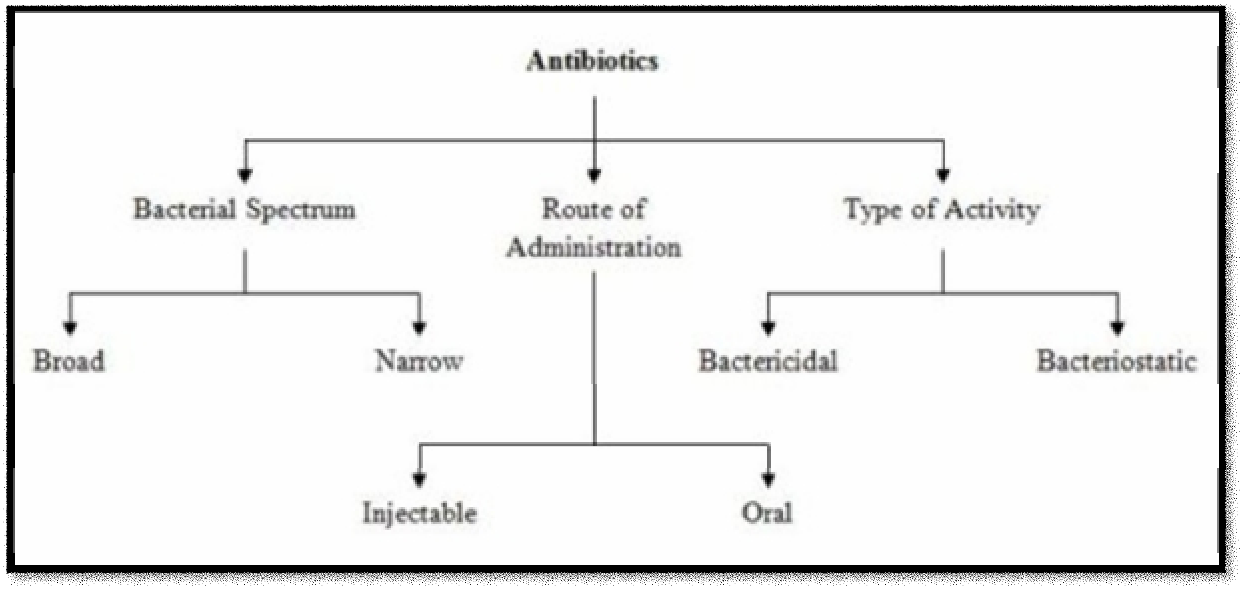


Figure 2: Classification of antibiotics

Antibiotics can also be classified based on the chemical structure. A similar effectiveness of, toxicity levels and side effects is rendered by the antibiotics having similar structural group. Antibiotics with broad spectrum are effective against a broad range of microorganisms in comparision to narrow spectrum antibiotics. Bacterial antibiotics kill the bacteria whereas bacteriostatic antibiotics halt the growth of bacteria.

During ancient times Greeks and Indians used moulds and other plants to treat infections. In Serbia and Greece, bread mould was traditionally used to treat wounds and infections. Warm soil was used in Russia by peasants to cure the infected wounds. Sumerian doctors gave the patients beer soup mixed with turtle shells and snake skins. Babylonian doctors healed the eyes using a mixture of frog bile and sour milk. Sri Lankan army used oil cake (sweetmeat) to server both as desiccant and antibacterial.

During modern times in 1640, England John Parkington recommended using mold for treatment in his book on pharmacology. In England, 1870, Sir John Scott Burdon-Sanderson observed that culture fluid covered with mould did not produce bacteria. In 1871, England Joseph lister experimented with the antibacterial acion on human tissue on what he called Penicilliun glaucium. John Tyndall in 1875 in England explained antibacterial action of the Penicillium fungus to the Royal society. In 1877, France Louis pasture postulated that bacteria could kill other bacteria (anthrax bacilli). In 1897, France Ernest Duchesne healed infected guinea pigs from typhoid using mould (Penicillium glaucium). In 1928, England sir Alexander Fleming discovered enzyme lysozyme and the antibiotic substance penicillin from the fungus Penicillium notatum. In 1932, Germany Gerhard Domagk discovered sulfoamidochrysoidine (prontosil).

Sir Alexander Fleming, a Scottish biologist, promoted new horizons for modern antibiotic with his discoveries of enzyme lysozyme (1921) and the antibiotic substance penicillin (1928). The discovery of penicillin from the fungus *Penicillium notatum* perfected the treatment of bacterial infections like Gangrene, Syphilis and Tuberculosis. He was the one who also contributed immensely towards medical sciences with his writings on the subjects of bacteriology, immunology and chemotherapy. His research and study during his military career inspired him to discover naturally antiseptic enzyme in 1921, which he named as lysozyme. This substance existed in tissues and secretions such as mucus, tears and egg-white but it did not have much effect on the harmful strong bacteria. As a result of some intelligent serendipity, six years later, he stumbled on discovering penicillin. In 1928 he observed while experimenting on influenza virus that a common fungus, *Penicillium notatum* destroyed the bacteria in *Staphylococcus* culture plate. This newely discovered active substance was effective even diluted up to 800 times. He named it Penicillin. He was knighted in 1944 and was given the nobel prize in physiology or Medicine in 1945 for his extraordinary achievements which revolutionized the medical science.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of *Staphylococcus aureus* infection that is resistant to all beta-lactam antibiotics. Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) were first recognized during 1961, one year after the methicillin antibiotic was introduced for treating *S. aureus* infections. MRSA is resistant to (unable to be killed by) all beta-lactam antibiotics. This includes all penicillins (e.g., amoxicillin) and cephalosporins (e.g., keflex).

In May 1996, the first case of Vancomycin intermediate *S. aureus* (**VISA)** was first reported in Japan. Four month old boy became infected after heart surgery, as a result of unsuccessfull treatment with vancomycin for 29 days and finally the boy was recovered with a combination of antibiotics. Later, VISA related cases were reported from France, England, United States, Hongkong, and so on. Upto date, 17 cases of VISA infection have been reported in the United States. *Staphylococcus aureus* is a major pathogen of hospital and as well community. These days VISA IS becoming a challenge for the clinicians to treat life threatening infections that are caused by Methicillin resistant *S. aureus* (MRSA). It becomes more challenging when such MRSA strains show reduced susceptibility to glycopeptides like vancomycin and telcoplanin. Till date only few cases of Vancomycin intermediate *S. aureus* (VISA) strains have been reported from Japan, France, United States, United kingdom, China, South Africa, South Korea, India, Brazil and Germany. Vancomycin Resistant *S.aureus* (VRSA) have been reported from some countries. There are some discrepancies about cutoff points to define the sextant of Vancomycin resistance present in *S.aureus*. National committee for clinical Laboratory standards defines VISA with Vancomycin MIC 8-16 µg/ml and VRSA with Vancomycin MIC > 32µg/ml. but clinical and laboratory standards Institute (CLSI, former NCCLS) set Vancomycin MIC's of 4-8 µg/ml and >16µg/ml for VISA and VRSA. There are various methods for detection of VRSA but accepted methods include NCCLS north microdilution, agar dilution and E test. Main mechanisms behind VISA isolates was Vancomycin therapy in the patients and cell wall thickening in bacteria. Whereas vanA, vanB and vanC genes of enterococci were isolated from most of the VRSA isolates with the exception of few Vancomycin resistant strains that lack these genes. The clinical significance of VRSA is difficult to determine because of its similarity with MRSA in colonisation, disease causing ability and spreading capacity.

According to the study it has been an uphill battle for scientists. Antibiotic-resistance continues to be present in different strains of bacteria, such as strep, tuberculosis and gonorrhea researchers have not made any real strides in combating the bacteria's evolution. According to a senior World Health Organization expert the fight against new antibiotic resistant strains of tuberculosis has already been lost in some parts of the world. There is a 5% rise in the number of new cases of the highly infectious disease in the UK. A team of researchers from the United States and Thailand says the growing number of cases of drug-resistant malaria being reported in Thailand and neighboring countries threatens the worldwide campaign to control and eliminate the mosquito-borne disease. Resistance is being shown by the malarial parasite in this region to the first-line malaria therapy - artemisinin combination treatment - and experts say there is a really a dangerous issue of the resistant strain of malaria moving to Africa, where malaria is most widespread.

Actinomycetes are gram-positive bacteria, with high guanine (g) plus cytosine (c) ratio in their DNA (>55 mol%), which are phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA: rDNA pairing studies (Goodfellow and Williams, 1983). The name “actinomycetes” was derived from Greek “atkis” (a ray) and “mykes” (fungus), and that has features of both Bacteria and Fungi (Das et al., 2008). Actinomycetes are soil organisms which have characteristic common to bacteria and fungi and yet possess significant distinctive features to delimit them into a distinct category in the strictly taxonomic sense. Actinomycetes are bound with bacteria in the same class of Schizomycetes but confined to the order Actinomycetales (Kumar *et al.*, 2005).

The actinomycetes are a group of bacteria which possess many important as well as interesting features. They have considerable value as producers of antibiotics and of other therapeutically useful compounds. They exhibit various ranges of life cycles which are unique among the prokaryotes and appear to play a major role in the cycling of organic matter in the soil ecosystem (Veiga *et al.*, 1983). Thus it hold a prominent position due to their diversity and proven ability to produce new compounds, because discovery of novel antibiotic and non antibiotic lead molecules through microbial secondary metabolite screening is becoming increasingly important.

They are unicellular likewise bacteria, but they also produce mycelium which is non septate (coenocytic) and more slender, like true bacteria they do not have distinct cell wall and their cell wall is without chitin and cellulose (commonly present in the cell wall of fungi). Unlike slimy distinct colonies of true bacteria which grow quickly on culture media actinomycetes colonies show slow gowth, show powdery consistency and firmly stick to agar surface. They produce hyphae and conidia or sporangia like fungi. Some Actinomycetes whose hyphae undergo segmentation resembles bacteria, both by morphologically and physiologically.

Actinomycetes are numerous and widely distributed in soil, compost etc and are next to bacteria in abundance. Plate count estimates gives value ranging from 10^4 to 10^8 per gram of soil. They are sensitive to acidity or low pH (optimum pH range is within 6.5 to 8.0) and waterlogged soil conditions. As we go deep into the soil, the population of actinomycetes increases. They are aerobic (requires oxygen), heterotrophic (cannot make its own food) and mesophilic (25-30ºC) organisms and some species are commonly present in compost and manures are thermophilic growing at 55-65ºC temperature (eg. *Thermoactinomycetes, Streptomyces*). The common genera of actinomycetes in the order of abundance in soils are streptomyces (nearly 70%), *Micromonospora* and *Nocardia* although *Actinomycetes, Actinoplanes, Streptosporangium* and *Micromonospora* are also generally encountered.

Inter-cell communication (Raina et al., 2009) are aided by released chemical signals when cell density reaches a critical concentration has been investigated for 30 years as quorum sensing. It was originally discovered in gram-negative bacteria, quorum sensing systems have been studied extensively in gram–positive bacteria and fungi (dimorphic). Microbial communities communicating through quorum sensing employ various chemical signals to supervise their surrounding environment which alter genetic expression and gain advantage over their competitors. In streptomyces, (Weber et al., 2003) a family of butyrolactones and their corresponding proteins receptor which serves as quorum sensing systems that control morphological development and antibiotic biosynthesis. Quorum sensing systems (Wang et al., 2009) of pathogens are central regulators for the expression of virulence factors. Increasing evidence shows that targeting quorum sensing system of many pathogenic bacteria is a promising therapeutic approach to control infections

2. REVIEW OF LITERATURE

Recent findings from the culture-dependent and culture independent methods demonstrated by Lam (2006), is that indigenous marine actinomycetes exist in the oceans and these are widely distributed in different marine ecosystems. There is tremendous novelty and diversity among the marine actinomycetes present in marine ecosystem. Progress is been made to isolate novel actinomycetes from samples that are collected from different marine environments and habitats. Different types of newly discovered secondary metabolites are being produced by these marine actinomycetes produces. Many of these metabolites have the potential to be developed as therapeutic agents as they possess biological activities. Marine actinomycetes are fruitful but underexploited source for the discovery of novel secondary metabolites.

A study was conducted by Devi et al. (2008), Seventy-three actinomycetes strains isolated from five areas of Bay of Bengal. Fourteen of these showed antagonistic activity against seventeen human pathogens of gram positive and gram negative bacteria and two fungal species. Six of them showed broad spectrum activity. Six broad spectrum actinomycetes were found according to morphological and biochemical characteristics that belonged to the genera *Streptomyces, Saccharopolyspora., Micromonospora, Nocardia, Actinopolyspora* and *Actinomadura*. Also by synergistic activity and heat killing activity, these six actinomycetes showed clear zone of inhibition in all selected human pathogens. The antibacterial antagonistic activities produced by these strains clearly indicated that marine environment harbours many broad spectrum antibiotic producing actinomycetes.

The study was conducted by Gopalakrishnan et al. (2010), Actinomycetes are potential sources for the production of antibiotics. Six eight actinomycete strains were isolated from marine sediment samples of Indian EEZ and were screened for the anti-vibrio activity. The pathogenic *vibrios* selected for study were *Vibrio harveyi, V. cholerae, V. proteolyticus, V. nereis, V. parahaemolyticus, V. alginolyticus, V. splendidus, V. Mediterranean and V. fluvialis.* Kirby-baur disc method was employed for testing bioactivity. Out of the 68 strains isolated, 29 strains showed anti-vibrio activity. Most of the strains (25 strains) showed activity against human pathogens such as *V. cholerae* and *V. parahaemolyticus*. Among all these strains S26 had showed maximum antagonistic activity (70%) against these *vibrios*. So the broth culture of this strain was taken and subjected to solvent extraction by Ethyl Acetate, Hexane and 1-butanol. extracts were then tested for their activity against the *vibrios* and was found that 1-butanol Extract of strain S26 inhibits all the *vibrios* used for testing, hexane extract of S26 strain showed inhibition towards *V. alginolyticus*, whereas Ethyl acetate extract of S26 strain showed inhibition against *V. cholera, V. harveyi and V. parahaemolyticus*.

A study was carried out by Adel et al. (2005), to inspect the factors affecting the antifungal production of four actinomycetes species i.e. *Streptomyces lydicus, S. antimycoticus, S.erumpens and S.ederenis.* Glycerol was used which is the best nitrogen source for antifungal production by *S.erumpens and S.lydicus*, ammonium sulphate for *S.ederensis*, while soyabean for *S.antimycoticus*. The optimum temperature for antifungal production by for *S.antimycoticus* and *S.erumpus* was 28ºC *while S.lydicus* and *S.ederensis* was 24 ºC. The optimum pH for antifungal production by the four selected species was 7.0. It was then observed that *S. lydicus, S. erumpens* and *S. antimycoticus* each produced four active components, while *S.ederensis* produced only three active components. After the chemical analysis of the culture filtrates, the species revealed the presence of 13 chemical compounds in the culture filtrates of *S. lydicus*, 12 chemical compounds in the culture filterate of each of *S. erumpens* and *S. ederensis*, 11 chemical compounds in the culture filterate of *S. antimycoticus*. It was then concluded from the experiment that actinomycetes play an important role in antagonizing both human and plant pathogenic fungi, and may be used in agricultural and medicinal scales after being further studied.

The aim of the present study by Vimal et al. (2009) was to isolate and identify the actinomycetes having antagonistic activity. Actinomycetes strains isolated from marine sediment samples were collected at the Pondicherry coast of India which showed antibacterial activity against some selected microbial pathogens. The cultural conditions and nutritional requirements for maximum growth and yield of secondary metabolites is been optimized under the shake - flask conditions. The growth and yield of the secondary metabolites was maximum when grown in ISP2 medium supplemented with sea water, pH 7.4, and incubation temperature of 28ºC, salt tolerance is 2% and incubation time is 4-7 days. Basing on the morphological, physiological, phylogenetic and biochemical characterization the strains were identified as *Nocardiops* sp. VI T SVK5 (FJ973467). The petroleum ether extract (1000g/ml) obtained from the isolate showed significant antibacterial activity against Gram negative bacteria - *Escherichia coli* (20mm), *Pseudomonas aeruginosa* (18 mm) and *Klebsiella pneumonia* (15mm) and Gram positive bacteria - *Enterococcus faecalis* (20mm), *Bacillus cereus* (13 mm) and *Staphylococcus aureus* (6mm) when compared with streptomycin (25 µg /disc). The ethyl acetate extract (1000 µg/ml) showed antifungal activity against *Aspergillus fumigatus* (23mm), *Aspergillus flavus* (15 mm) and *Aspergillus niger* (12mm) was then compared with amphotericin-B (25 µg/disc). The chloroform extract (1000µg/ml) was very much effective against yeasts, *Candida cruzi* (18mm), *Candida tropicans* (15 mm) and *Candida albicans* (14mm) was when compared to streptomycin (25µg/disc). It was then concluded that the isolated strain had broad spectrum of antagonistic activity against gram positive and gram negative bacteria and *Aspergillus* sp.

Screening of six marine sediment samples studied by Dasari et al. (2011) near NTPC of the Visakhapatnam (India) Coast of Bay of Bengal resulted in the isolation of 72 isolates of actinomycetes. Among all these, *Amycolatopsis Alba* var. nov. DVR D4 had showed broad spectrum of antibacterial activity against Gram-positive bacteria and Gram-negative bacteria; and these produced antibacterial metabolite extracellulary under submerged fermentation conditions. The chemical and physical process parameters affecting the production of the antibiotic were optimized. The maximum antibiotic activity was observed with the optimized production medium containing D-glucose, 2.0 %w/v; malt extract, 4.0 %w/v; yeast extract, 0.4 %w/v; dipotassium hydrogen phosphate, 0.5 %w/v; sodium chloride, 0.25 %w/v; zinc sulphate, 0.004 %w/v; calcium carbonate, 0.04 %w/v; with inoculums volume of 5.0 %v/v at 6.0 pH, incubated at 28°C temperature at 220 rpm and for 96 hrs.

The work was carried out by Houssam et al. (2011) in the course of a screening program for specifying the bioactive substances demonstrated inhibitory affects against microbial pathogenic from actinomycetes strains. Eighty eight actinomycete strains isolated from twelve soil samples collected from different localities in Egypt. A single actinomycete culture AZ-146 from eight cultures was found to be exhibiting to produce wide spectrum antimicrobial activities.

It was active in vitro against some microbial pathogenic viz: *Staphylococcus aureus*, NCTC; *Klebsiella pneumonia,* NCIMB 9111; *Pseudomonas aeruginosa*, ATCC 10145; *Escherichia coli*, NCTC 10416; *Candida albicans*, IMRU 3669; *S. cerevisiae* ATCC 9763; *Aspergillus niger* IMI 31276; *Fusarium oxysporum*. The 16s RNA gene (1.5 kb) nucleotide sequence of the potent strain evidenced that there is a 99% similarity with *Streptomyces rimosus* in the morphological, physiological and biochemical characteristics. Thus, it was given the name *Streptomyces rimosus*, AZ-146. The biosynthetic process of antimicrobial agent formation was controlled by parameters including different inoculums size, temperatures, pH values, incubation period and different carbon and nitrogen sources were all fully investigated.

Antibacterial activity of 107 marine actinomycetes isolated form near sea shore sediment and seawater from Konkan coast of Maharashtra was studied by Gulve and Deshmukh (2012). A total of 107 actinomycetes were subjected to primary screening by the perpendicular streak method against various test microorganisms. Out of 107 actinomycetes 07, 22, 14, 34, 14, 27, 6 and 52 number of actinomycetal isolates showed antagonistic activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris, Klebsiella aerogenes, , Aspergillus niger, Pseudomonas aeruginosa and Candida albicans* respectively. Out of 107 actinomycetes isolates 13 of the isolates showed maximum antagonistic activities that were subjected to secondary screening by agar well method. Finally 5 isolates were selected for further study on the basis of the maximum zone of inhibition and broad spectrum activity. Selected 5 isolates were inoculated in glucose soybean broth for 7 days at 30^0C and antibacterial substances were extracted with ethyl acetate. T L C of the ethyl acetate extract was carried out using N butanol: acetic acid: water (4:1:5) as a solvent system. The spots were then observed under UV light and in iodine chamber. Ethyl acetate extract's bioautograhy of selected 5 isolates were carried out using test organisms *P. vulgaris and B. subtilis*. The inhibition zones were then observed and they were associated with the purple spots at the chromatograms as detected under UV light. This may indicate that the same compound is responsible for the antibacterial activity of the actinomycetes. Finally single potent actinomycetes isolate (GA-22) was selected and it's cultural, morphological, physiological and biochemical characters were studied. It was found that biochemically GA-22 was very active marine actinomycetes and was also able to produce variety of enzymes and can utilize number of sugars.

Present study on induced antibiotic production by *E.coli*, *B.substilis*, *pseudomonas* and *klebsiella* with *Streptomyces* paves way for the discovery of pathogen targeted/ specific antibiotic production. As we know that actinomycetes strain streptomyces itself can produce antibiotics but when it is induced with the human pathogens, the cell, it produces antibiotics the secondary metabolities in large amount in same time so in less time more production of antibiotics. This may further become very useful for mankind. So lot of work has been recorded which deals with the production of antibiotic described above and not much work is done on the current topic cell induced production. So some of the following are.

Cross species signal transfer mediated induction of antibiotic production by the soil actinomycetes against human bacterial pathogens was investigated by Musthafa et al. (2010). Soil actinomycetes isolated and analyzed for their efficacy in antibiotic production against common clinical pathogens viz. *Staphylococcus aureus* (MTCC 96), *Pseudomonas fluorescens* (MTCC 103), *Escherichia coli* (MTCC 443), *Bacillus subtilis* (MTCC 441) and *Salmonella typhi* (MTCC 733). Out of 36 actinomycetes isolates that were analyzed, four isolates exhibited significant antibacterial activity against *S. aureus* cells. Cell free extract of SA25 that was cultivated in the presence of heat killed *S.aureus* cells were then subjected to solvent extraction and was partially purified. In TLC band Antibacterial compound of the strain SA25 was identified as *Streptomyces* sp. Actinomycetes are known to be producing antibiotics and this property has been exploited for years for the commercial production of antibiotics, but exposure of actinomycetes (Streptomyces) to bacterial pathogens is expected to pave for the production of novel antibiotics with high degree of specificity and huge production. It can be looked upon as an alternative way to overcome the problem of drug resistance and reemergence of the resistant pathogens.

The study was done by Selvin *et al.* (2009) on sponge-associated actinomycetes which were isolated from the marine sponge *Dendrilla nigra* and collected from the southwest coast of India. A total of Eleven actinomycetes were isolated depending upon the heterogeneity and stability in the subculturing. Out of these, *Nocardiopsis dassonvillei* MAD08 showed 100% activity against the multidrug resistant pathogens were tested. The culture conditions for *N.*

dassonvillei MAD08 was optimized under submerged fermentation conditions for enhanced antimicrobial production. The unique and significant feature of MAD08 includes extracellular amylase, cellulase, protease and lipase production. These enzymes ultimately increase the scope of optimization using broad range of raw materials which might be efficiently utilized. The extract of the cell free supernatant with ethyl acetate yielded bioactive crude extract that displayed activity against a panel of pathogens tested. Analysis of this active thin layer chromatography fraction by Fourier transform infrared and gas chromatography-mass spectrometry evidenced showed 11 compounds with antimicrobial activity. The culture supernatant ammonium sulfate precipitation was at 80% saturation which yielded an anticandidal protein of molecular weight 87.12 kDa. This was the first strain that produces both organic solvent and water soluble antimicrobial compounds. The active extract was non-hemolytic as well as it showed surface active property envisaging its probable role in the inhibition to the attachment of pathogens to host tissues, thus, blocking the host–pathogen interaction at an earlier stage of pathogenesis.

QUORUM SENSING

The study was done by Raina et al. (2009) where inter-cell communication aided by released chemical signals when cell density reaches a critical concentration; it has been investigated for over 30 years as quorum sensing. It was originally discovered in gram negative bacteria, quorum sensing systems have been studied extensively in gram positive bacteria and a dimorphic fungi. Microbial communities communicate through quorum sensing employ various chemical signals to supervise their surrounding environment that alter genetic expression and gain advantage over their competitors. These signals differ from acylhomoserine lactones to small modified or unmodified peptides to complex butyrolactone molecules.

Wang et al. (2009) made a study on quorum sensing systems of pathogens are central regulators for the expression of the virulence factors. Increasing evidence shows that targeting quorum sensing system of any pathogenic bacteria is a promising therapeutic approach to control infections. In this work they isolated 47 strains of actinomycetes from the mud sample of Jiaozhou Bay. Quorum sensing inhibitory activity was mentioned by chromobacterium violaceum CV026. As a result, the broth culture extract of actinomycetes WA-7 was 16S rDNA sequence. Further investigations have revealed that the crude extract could inhibit the quorum sensing-controlled violacein and proteases production of *C. violaceum* in concentration dependent manner.

The study was done by Weber et al. (2003) in which prokaryotic transcriptional regulatory elements has been adopted for the controlled expression of cloned genes in mammalian cells and animals, the cornerstone for drug discovery, gene-function correlations, biopharmaceutical manufacturing as well as advanced gene therapy and tissue engineering. Many prokaryotes have evolved with specific molecular communication systems known as quorum sensing to coordinate population-wide responses to physiological and physic-chemical signals. A generic bacterial quorum sensing system is based on a diffusible signal molecule that prevents binding of a repressor to the corresponding operator sites thus resulting in derepression of a regulon targeted. In streptomyces, there is a family of butyrolactones and their corresponding receptor proteins, serve as the quorum sensing systems that control morphological development as well as antibiotic biosynthesis.

3. OBJECTIVES

Present study on induced antibiotic production by *E.coli*, *B.substilis, Pseudomonas, Proteus* and *Klebsiella* in *Actinomycetes* paves way for the discovery of pathogen targeted/ specific antibiotic production. As we know that actinomycetes strain streptomyces itself can produce antibiotics but when it is induced with the human pathogens, the cell, it produces antibiotics the secondary metabolites in large amount in the same time so in less time more production of antibiotics. This may further become very useful for mankind.

- Isolation and enumeration of actinomycetes from marine environment.
- Screening for antimicrobial activity of isolated actinomycetes.
- Extraction of crude compound from the actinomycetes culture.
- Study species signal transfer mediated induction of antmicrobial compounds in Actinomycetes.
- Characterization and identification of potent actinomycetes strains.

4. MATERIALS AND METHODS

4.1. SAMPLING

A Study was done by collection of sediment soil samples from different sites of Gopalpur, Odisha. (19°19.218’N & 084° 57.73’E). (Figure 3)

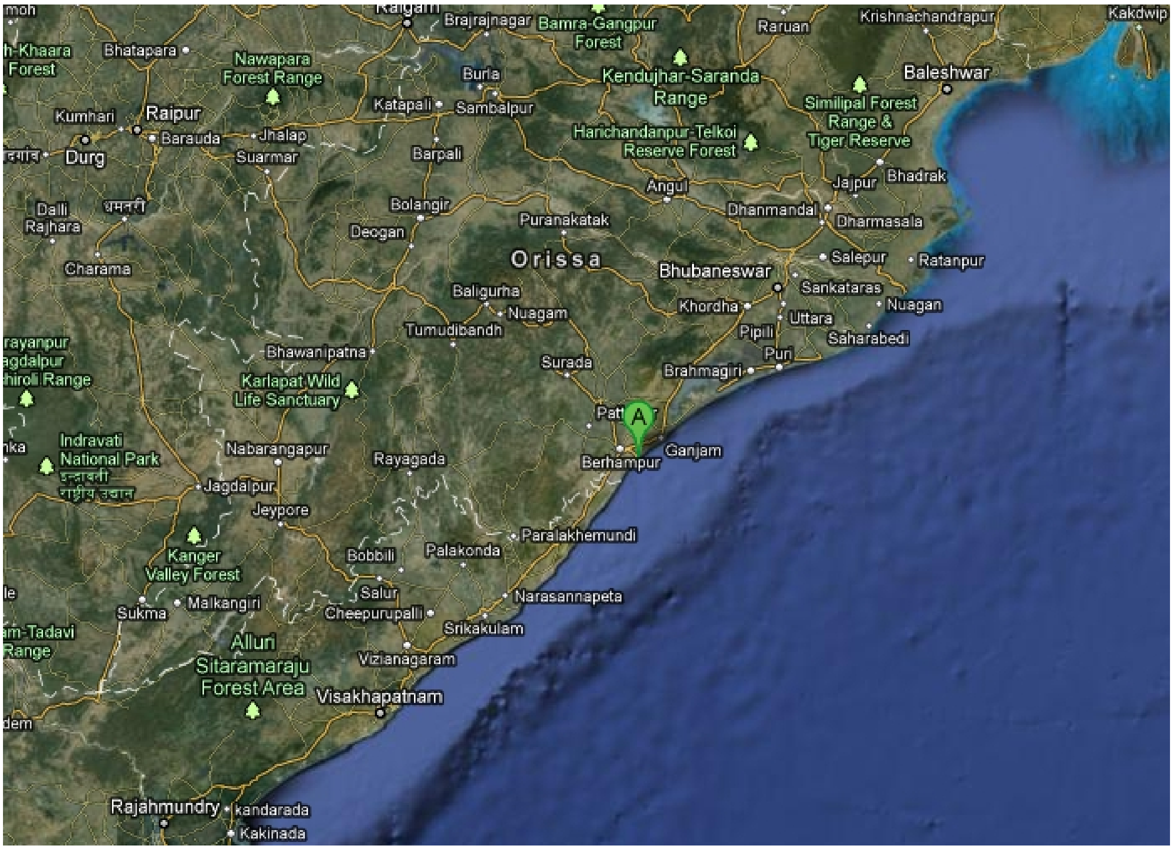


Figure 3: Site Map of Gopalpur, Odisha

4.2. SAMPLE COLLECTION

The samples were collected from top soil profile where most of the microbial activity takes place thus where most of the bacterial population is concentrated. Soil sample were collected using clean, dry and sterile polythene bags along with sterile spatula, marking pen and other accessories. The selection of the site was done by taking care of the point the soil must be fully wet, with widely varying characteristics as possible with regards to the organic matter, particle size, colour of the soil and to avoid contamination as far as possible. Samples were stored at 4ºC in the refrigerator.

4.3. ISOLATION OF ACTINOMYCETES FROM SEDIMENT SOIL SAMPLE

A pinch of wet soil sample was added to the 9 ml of autoclaved water. It was then mixed properly. 0.1ml of sample were inoculated in duplicate plates of ISP-2 media (Table no. 1) for the isolation of actinomycetes by the spread plate technique. All plates incubated at 37ºC in the incubator for 7 days. Both the pure culture technique strains were isolated. Chloramphenicol was used as antibacterial agent respectively in plates. Four pure strains of actinomycetes have been isolated by streak plate method. Then the strains were identified on the basis of their phenotypic and biological characteristics.

Table no.1: Composition of ISP2 media in g/lit

Sl.No.	Ingredients	g/lt
1	Yeast extract	4.0g
2	Malt extract	10.0g
3	Dextrose	4.0g
4	Agar	20.0g
5	Distilled water	1 lit

pH: 7.3

4.4. PHENOTYPIC CHARACTERISTICS:

The classification of actinomycetes was originally based upon the morphological observations. So morphological is still an important characteristic for the description of taxa and it is not adequate in itself to differentiate between many genera. In fact, it was the only characteristic which was used in many early description, particularly of streptomyces specie in the first few editions of Bergey’s manual. Those observations are the best made by the variety of standard cultivation media. Several of the media suggested for the international streptomyces project (Shirling and Gottileb, 1966) and by Pridham et al., (1957) have proven to be useful in our hands for the characterization of strains accessioned into the (ARS) Actinomycetes Culture Collection (Labeda *et al.,* 1985). It includes some basic tests Aerial mass colour, spore chain morphology and spore morphology.

a) Aerial mass colour

For the grouping and identification of actinomyctes the chromogenicity of the aerial mycelium is considered to be important character. The colours of the mature sporulating aerial mycelium are white, grey, red, green, blue and violet following Prauser (1964). When thee aerial mass colour falls between two colour series then both the colours are recorded. If aerial mass colour of a strain showed intermediate colour tints, then in that case both the colour series should be noted (Shirling and Gottileb, 1966).

b) Reverse side pigments

The strains are divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive (+) and not distinctive or none (-). A colour with low chroma such as pale yellow, yellowish brown or olive occurs, these are included in the latter group (-).

c) Melanoid Pigments

The grouping was made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown pigment modified by other colours) on the medium. The strains are grouped as melanoid pigment producing (+) and non producing (-) (Shirling and Gottileb, 1966). The melanoid pigment was observed by the inoculating plates which were kept under incubator for 4 to 5 days. The strains which show cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color are recorded as positive (+) and total absence of diffusible pigment, were recorded as negative (-) for melanoid pigment production.

d) Spore chain morphology

The species belonging to the genus Streptomyces are divided into three sections (Shirling and Gottlieb, 1966), namely

- Rectiflexibiles (RF)
- Retinaculiaperti (RA)
- Spirales (s)

Characteristic spore bearing hyphae and spore chains should be determined by using direct microscopic examination of the culture surface. Adequate magnification of 40x was used to establish the presence and absence of spore chains and to observe the nature of sporophores by the standard protocol of cover slip culture technique the plates were prepared and after the incubation of 7 to 10 days it is observed for the spore chain morphology shown in, simple Figure 4(a-d) and verticillate Figure 5(a-d).

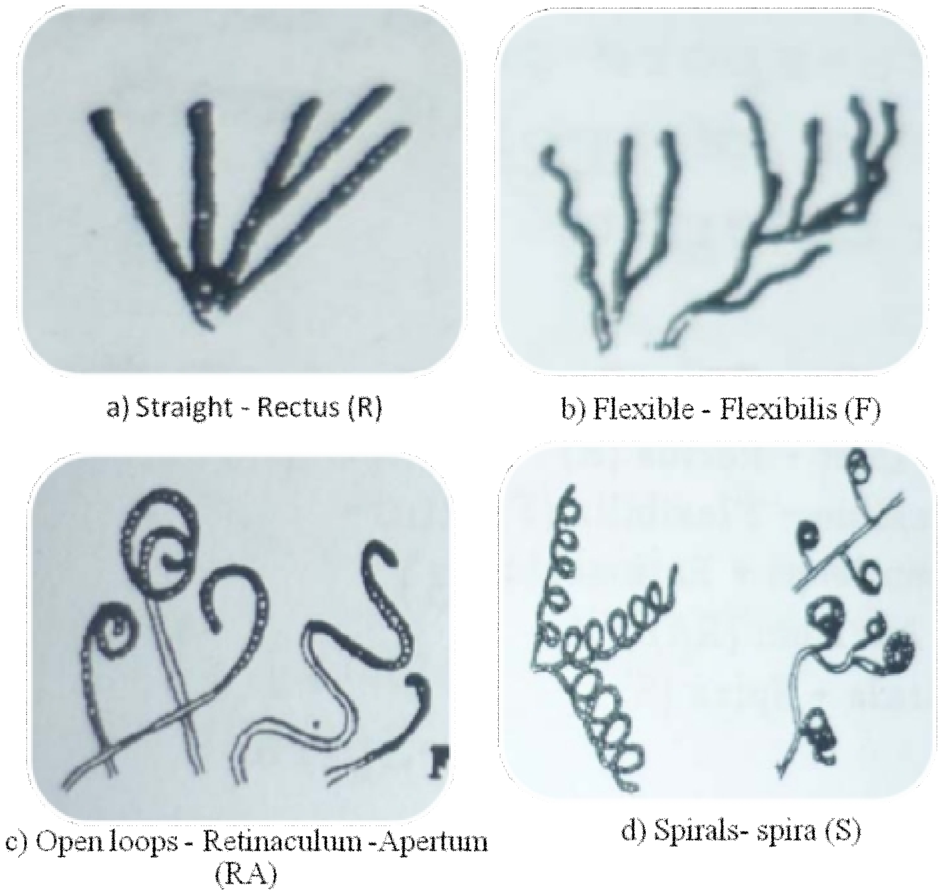


Figure 4(a – d) : Types of spore bearing hyphae.

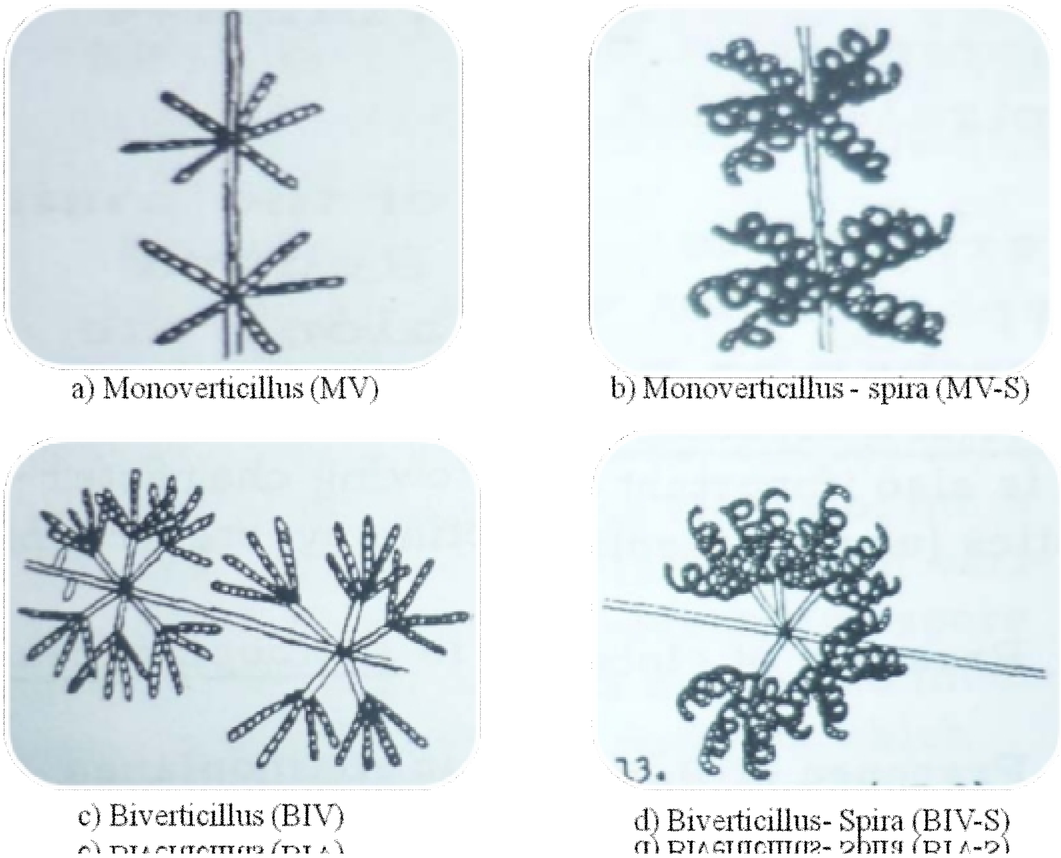


Figure 5(a-d): Types of spore bearing hyphae.

For this method to study the spore chain morphology the ISP2 media containing plates were prepared. After solidification, with the help of sharp Razor the central portion of the plate, medium is scooped out making a rectangular groove like area. Then three sterile coverslips were placed on the hollow rectangular space. Slowly actinomycetes spores have been inoculated at the edges of the coverslips touching and stick on to the medium. The plates must be inoculated at 28ºC ±2ºC for 5 days and examined periodically taking out the coverslips.

d) Spore surface morphology

Spore surface and its morphology was observed under the scanning electron microscope (SEM). Here instead of coverslip 1cm*1cm glass slide is used. The electron grid is cleaned and adhesive tape is placed on the surface of the grid. The spore structures in actinomycetes are reported (Figure 6) to be 4 types:

1. Smooth (sm)
2. Spiny (sp)
3. Warty (wa)
4. Hairy (ha)

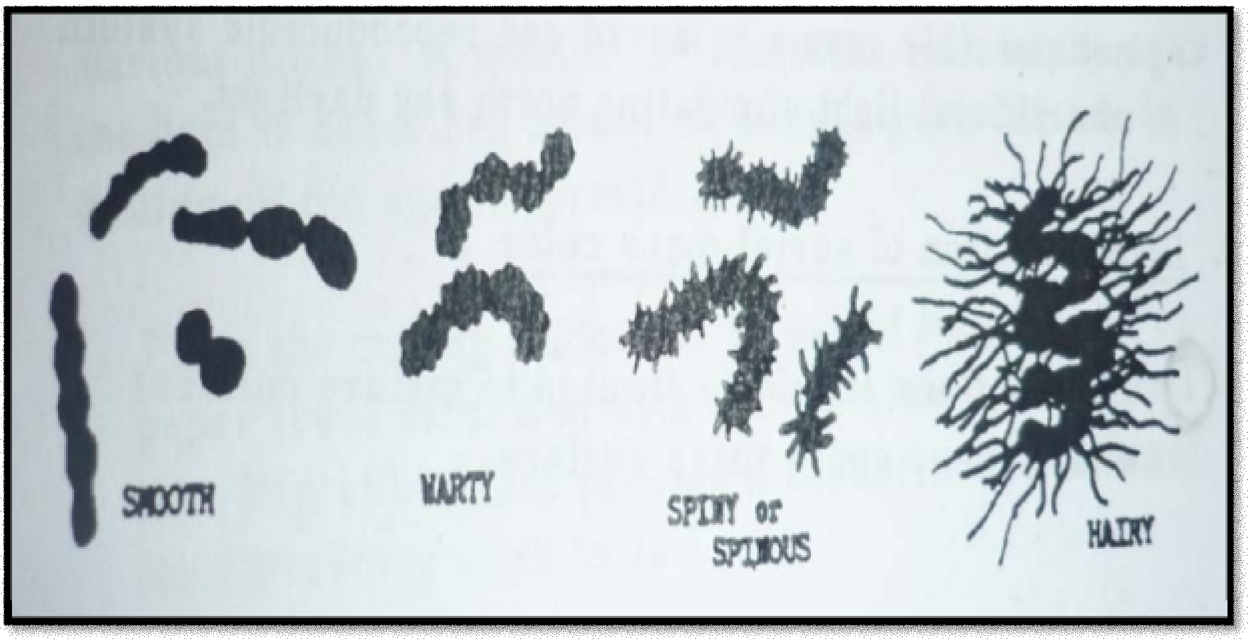


Figure 6: spore surface morphology of actinomycetes

4.5. SCREENING FOR ANTIMICROBIAL ACTIVITY

a) Cross streak method

Screening of actinomycetes is done by the antimicrobial activity, primarily studied by cross streak method against five pathogenic bacteria, the 6 isolated actinomycetes strains were streaked as parallel line on Trypton soya agar plates and incubated at 28 ºC for 7 days. After observing a good ribbon – like growth of the actinomyceteеs on the petriplates the pathogens was streaked at right angles to the original streak of actinomycetes and again incubated at

28ºC±2ºC. The inhibition zone was measured after 24 and 48 hr. A control plate was also maintained without inoculating the actinomycetes activity the strains were selected for further studies.

b) Agar well diffusion method

The second method of testing antimicrobial activity was through well diffusion agar assay (Musthafa et al., 2010). Overnight cultures of bacterial pathogens cultivated in Luria Bertani broth were used to induce antibacterial compound production in actinomycetes. Bacterial pathogens were swabbed on Tryptone soya agar plates. 100 µl of 7 days old actinomycetes cultures grown in Tryptone soya broth (pH 7.2±0.2) were spotted on the plate. Plates were incubated at 30ºC for 24 hr and the zone of inhibition against bacterial pathogens was measured. Antagonistic actinomycetes strains which produced antibacterial activity against test pathogens were cultivated in 5 ml of nutrient broth and incubated at 30ºC for 7 days in rotator shaker at a 50-rev/min. The cell free supernatant obtained through centrifugation was checked for its antagonistic activity against test pathogens through well diffusion assay.

c) Disc diffusion method:

The actinomycetes were further studied for antibiotic activity by fermentation technique in rotary shaker by using soyabean meal medium. 25 ml medium was distributed in falcons and sterilized by autoclaving at 120 ºC for 25 minutes. The spores from each strain was then transferred to 25ml medium the falcons were then incubated on a rotary shaker (250 rpm) at 28 ºC for 48 hrs.

After 96 hrs of fermentation in rotary shaker, the mycelia and culture filterate were separated by centrifugation. The broth culture was centrifuged at 4000 rpm for 10min at 10 ºC, and clear supernatant was separated and extracted twice with equal volume of ethyl acetate. Ethyl acetate was added and mixed in a shaker for about 40 minutes. This allowed any organic molecule to suspend itself in the polar solvent. The solution was then put into a separator funnel; the aqueous part of the solution was removed and discarded. The ethyl acetate phase was concentrated in vacuum at 35 ºC to get the crude extract.

This crude extract was then mixed in 0.5ml of DMSO and a mixture was prepared. The discs were prepared by punchering the Whatmans filter paper with the paper puncher and was autoclaved. The overnight cultures of bacterial pathogens were cultivated in luria Bertani broth were used to induce antibacterial compound production in actinomycetes. The 100 µl of bacterial pathogens were spread on Tryptone Soya agar plates. 500 µl of DMSO mixed crude extract of actinomycetes strains were inoculated on the autoclaved discs and these were then placed in the plates which are swabbed. The plates were then incubated at 30ºC for 48 hrs.

d) Enhancement of antimicrobial compound synthesis by cross signaling induction

The method of Spragg *et al.* (1998) was followed. The actinomycetes strains which produced antibacterial compound only in the presence of competing organisms was subjected to the co-cultivation assay. In this method the producer strain actinomycetes was co-cultivated with live as well as heat killed pathogen. 25 ml Tryptone soya medium was distributed in falcons and sterilized by autoclaving at 120 ºC for 25 minutes. The spores from each of the strains was then transferred to 25ml medium the falcons were then incubated on a rotary shaker (250 rpm) at 28ºC for 4days. The respective overnight cultures of pathogens were heat killed by placing the tubes in the hot water bath temperature maintained at 100ºC for 1 hr. The experiment was then performed by taking the actinomycetes strains where live actinomycetes strain alone was kept as control, live actinomycetes strain + live pathogen, live actinomycetes strain + heat killed pathogen. These were then incubated on a rotary shaker (250 rpm) at 28ºC for 3 days.

After 3 days of fermentation in rotary shaker, culture filterate were separated by centrifugation. The culture broth was centrifuged at 4000 rpm for 10min at 10 ºC, and clear supernatant was separated and extracted twice with equal volume of ethyl acetate. Ethyl acetate was added and mixed in a shaker for about 40 minutes. This allowed any organic molecule to suspend itself in the polar solvent. The solution was then put into a separator funnel; the aqueous part of the solution was removed and discarded. The ethyl acetate phase was concentrated in vacuum at 35 ºC to get the crude extract.

This crude extract was then mixed in 0.5ml of DMSO and a mixture was prepared. The discs were prepared by punchering the Whatmans filter paper with the paper puncher and was autoclaved. The overnight cultures of bacterial pathogens were cultivated in luria Bertani broth were used to induce antibacterial compound production in actinomycetes. The 100 µl of bacterial pathogens were spread on Tryptone Soya agar plates. Crude extract mixed with 50 µl of DMSO Actinomycetes strains were inoculated on the autoclaved discs and these were then placed in the plates which are swabbed. The plates were then incubated at 30ºC for 48 hrs.

4.6. SPECIES AFFILIATION – PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

a) Assimilation of carbon sources:

The ability of different actinomycetes strains in utilizing various carbon compounds as soure of energy was studied by following the method recommended in international streptomyces project. Carbon utilization medium (modified from Pridham and Gottlieb, 1948). Stock solution of around ten sugars i.e; D-glucose, L-arabinose, Sucrose, D-fructose, Mannitol, D-xylose, Raffinose, Cellulose, I-inositol, Rhamnose having a concentration of 10x was prepared in autoclaved water and sterilized by filtering it through 0.22 mili pore size membrane filters and stored at 4ºC. Growth of actinomycetes strain was checked by taking 1% carbon source in ISP2 media. Plates were streaked by inoculation loop by flame sterilization technique and incubated at 37ºC for 7 to 10 days. Growth was observed by comparing them with control.

Sterile carbon sources use chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials.

Carbon sources for this test are:

- No carbon source (negative control)
- D-glucose (positive control)
- L-arabinose
- Sucrose
- D-fructose
- D-xylose
- I-Inositol

- Raffinose
- D-mannitol
- Cellulose
- Rhamnose

These carbon sources were sterilized by membrane filteration without heating. Carbon source sterilized by this method was added to the basal mineral salts agar to give a final concentration of 1% stock. Stock solution was prepared of 10% and 10ml of this is added to the 90ml of basal medium.

Complete medium was prepared by sterilizing basal medium and agar separately. This was cooled at room temperature and then both were mixed. Then sterilized carbon sources was aseptically added to a concentration of approximately 1%. Mixture was agitated and poured in 25ml of medium per dish into 9cm petridishes. Each organism requires 2 petridishes with no carbon (as a negative control) plus duplicate plates for each carbon source tested. Carbohydrate utilization could be determined by growth on carbon utilization medium (ISP2) supplemented with 1% carbon sources.

Results were recorded as follows:-

- Strongly positive utilization (++), when growth on tested carbon in basal medium was equal to or greater than growth on basal medium plus glucose.
- Positive utilization (+), when growth on tested carbon was significantly better than on basal medium without carbon, but somewhat less than on glucose.
- Utilization doubtful (), when growth on tested carbon was only slightly better than on the basal medium without carbon and significantly less than with glucose.
- Utilization negative (-), when growth was same or less than growth on basal medium without carbon.

b) Salt tolerance:

Different concentrations of sodium chloride (0, 5, 10, 15, 20, 25 and 30%) solution was added to the starch casein agar medium to check the sodium chloride tolerance test. This test was very important to understand the native nature of the marine actinomycetes isolates. The isolates were streaked on the agar medium, incubated at 37 ºC for 7-15 days and the presence or absence of growth was recorded on 7th day onwards.

c) Degradation of cellulose:

1% of Carboxy methyl cellulose (CMC) was added to the ISP2 media. The plates were inoculated and incubated for 7-15 days. Control plate was used as standard to check the growth of actinomycetes after 7-15 days for cellulose degradation activity which may be visually observed.

d) Hydrogen sulphide production:

The inoculated Tryptone –Yeast extract agar (Table no. 2) slants were incubated for 7 days for this test. Observations were done on the presence of the characteristic greenish-brown, brown, bluish-black or black colours of the substrate, indicative of H2S production were recorded on 7th, 10th and 15th days. The tubes incubated were compared with uninoculated controls.

Table no.2: Composition of Tryptone –Yeast extract agar in g/lit

Sl.No.	Ingredients	g/lt
1	Casein enzymic hydrolysate	6g
2	Yeast extract powder	3g
3	Agar	12g

e) Gelatin liquefication:

Due to the absence of tryptophan Gelatin is called as incomplete protein its value in identifying bacterial species is well established. Hydrolysis of collage gives gelatin which is a protein produced by a major component of connective tissue and tendons in humans and other animals. Below temperature of 25ºC, gelatin will maintain its gel properties and exists as a solid at temperatures above 25ºC, gelatin is liquid. Liquefaction is accomplished by some microorganisms capable of producing a proteolytic extracellular enzyme called gelatinase, which acts to hydrolyse this protein to amino acids. Once the degradation occurs, even very low temperatures of 4ºC will not restore the gel characteristic. Gelatin tubes were used to demonstrate the hydrolytic activity of gelatinase.

The medium consists of nutrient supplemented with 12% gelatin this high gelatin concentration results in a stiff medium and also serves as the substrate for the activity of gelatinase. Gelatin liquefaction was studied by sub-culturing the strain on the gelatin agar medium and inoculated them at 37ºC. observation should be made after the 7 days. The extent of liquefaction should be be recorded after keeping the tubes in cold conditions (5-10 ºC) for an hour. Cultures that remain liquefied were indicative of slow gelatin hydrolysis.

Some types of gelatin test slants:-

Liquefaction configurations

- Crateriform: saucer-shaped liquefaction
- Napiform: tumiplike
- Infundibular: Funnel-like or inverted cone
- Saccate: Elongate sac, tubular, cylindrical
- Stratiform: Liquefied to the walls of the tube in the upper region

Growth without liquefaction:

- Filiform
- Beaded
- Papillate
- Villous
- Arborescent

f) Hydrolysis of starch:

Starch is a high molecular weight compound consisting of branched polymer composed of glucose molecule linked together by glycosidic bonds. At first the macromolecule requires the presence of the extracellular enzyme amylase for its hydrolysis into shorter polysaccharide, namely dextrins and ultimately into maltose molecules. The final hydrolysis of maltose, a disaccharide is done by the maltase enzyme, yields low molecular weight, soluble glucose molecules that can be transported into the cell and used for energy production through the process of glycolysis.

For this test, cultures should be grown for 5-7 days on ISP2 media. The development of clear zone around the culture streaks. When the plates were flooded with Lugol's iodine solution should be recorded as the hydrolysis of starch. The medium is composed of ISP2 media supplemented with 1% starch, which serves as the polysaccharide substrate.

g) **Coagulation of milk:**

Milk coagulation was studied with Skimmed milk (Hi media). The skimmed milk tubes were inoculated at 37ºC. The amount of coagulation was recorded on the 7^{th} and 10^{th} days of incubation. The importance of milk as a culture medium for the study of bacteria has long been recognized by workers in bacteriology; although milk is a very complex medium, it is more or less standard in its composition and the reactions produced upon it by microorganisms are so characteristic, that it had found general acceptance. The plates were incubated for the 7^{th} to 10 days after inoculation.

h) Ability to grow in different pH:

Principle: pH is defined as logarithm to the base 10 of the inverse of the hydrogen ion concentration (or preferably H+ ion activity). It is also defined as the negative logarithm to the base 10 of H+ ion activity. This test was carried out on ISP2 media. The pH of the media was adjusted to different ranges of 5, 6, 7, 8, 9 and 10. Duplicate slants were prepared for each strain of each range. After the incubation of 10-12 days readings were taken for each strain.

i) Lipolytic activity

The formation of lipase is demonstrated by adding water soluble Tweens to a nutrient medium. Appearance of a well visible halo around the colonies is indicative of the lipolytic activity which is due to crystal of the calcium salt of the fatty acid liberated by lipolysis.

There are some advantages of the use of Tween 20

- Between cells that are growing and the fatty substrate there is an optimum contact which is necessary in those cases in which the formation of lipase is adaptive.
- They can be used in studies in the specificity of the lipase.
- The results are directly visible.

This test was done by taking 1% Tween 20 (Hi media) with ISP2 media. Incubated at the temperature of 37 ºC for 7 to 10 days.

Finally, after all these experiments were done results have been matched with the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project) and the species identification was done.

5. RESULTS

Morphological and biochemical characterization

Samples were inoculated by serial dilution and 4 pure strains were isolated by streak plate method.

5.1. PHENOTYPIC CHARACTERIZATION:

Morphology is an important characteristic for the description of taxa, it is not adequate itself to differentiate between many genera. In fact, it was the only characteristic used in many early descriptions.

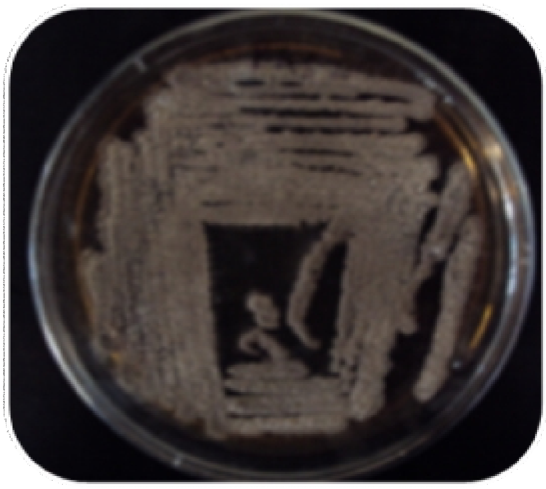
a) Aerial mass colour

The colour of the substrate mycelium was determined by observing (Table no.3) the plates after 7 to 10 days. It was reported only after seeing the heavy spore mass surface.

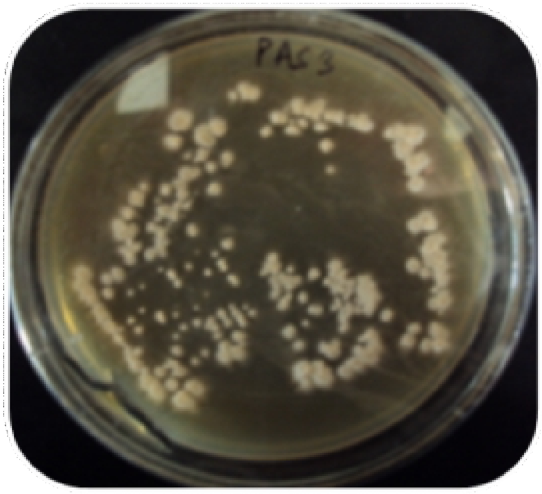
Table no.3: Reading of Aerial mass colour of actinomycetes.

Sl. No.	STRAIN	AERIAL MASS COLOUR
1	PAS2	Gy (grey)
2	PAS3	W (white)
3	PAS5	Gy (grey)
4	PAS8	WGy (white grey)

The common colours that were observed in the strains were white, grey and white grey in the figure 7(a-d).

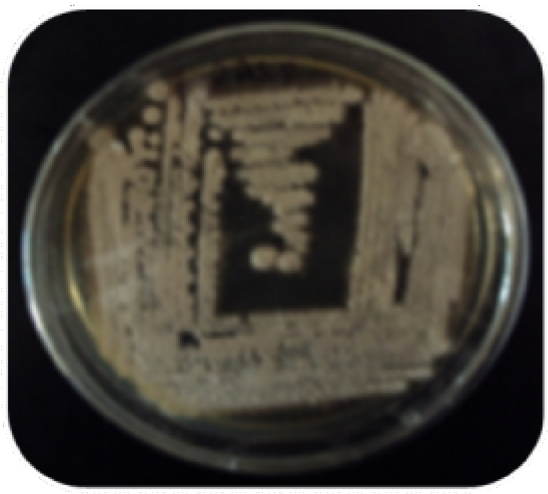


a) PAS 2



b) PAS 3

a) PAS 2



b) PAS 3

Figure 7(a –d): Aerial mass colour of actinomycetes strains.

b) Reverse side pigment, Melanoid pigments and soluble pigments

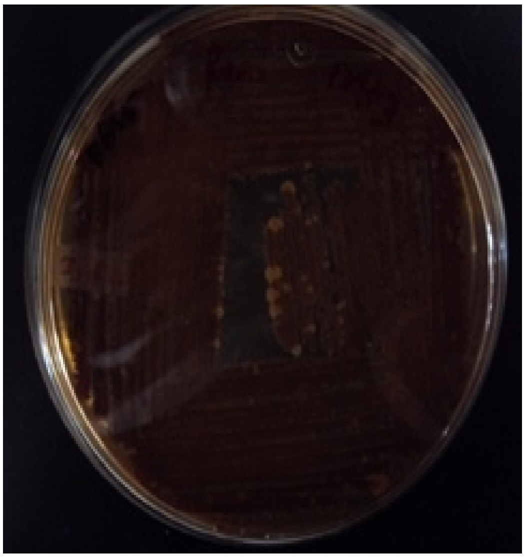
The strains were divided into two groups according to the ability of the strains to produce pigments on the reverse side of the colony, namely distinctive (1) and not distinctive or none (0) (Table no. 4) for Reverse side pigments and Melanoid pigmentation was observed by the formation of brownish black, greenish brown or distinct brown pigment.

Table No 4: Observation of Reverse side Malanoid and soluble pigment of actinomycetes.

Strain	Soluble pigments	Malanoid pigments	Reverse side pigments
PAS2	0	1	1
PAS3	0	0	0
PAS5	0	1	1
PAS8	0	0	0

Colour observed for not distinctive were pale yellow, olive or yellowish brown colour marked as 0 in the Table. Two of the strains had shown the pigment formation. The other two strains namely PAS2 and PAS5 shows the distinctive (1) character shown in Figure 8(a-b). No strains had shown melanoid formation. Soluble pigment was also not observed by any of the strains.

a) PAS2



b) PAS5

Figure 8: Result showing reverse side and melanoid pigmentation

c) Spore chain morphology

This was performed by coverslip culture technique (Figure 9). The slides were examined under microscope of 100x (Table no.5)

Table no. 5 : Results showing Spore chian morphology.

Sl. No.	STRAIN	Spore chain morphology
1	PAS2	S (Spiral)
2	PAS3	RF (Rectifexibilies)
3	PAS5	SRA (Spiral Rectifexibilies)
4	PAS8	SRA (Spiral Rectifexibilies)

All strains were examined under microscope and accordingly the spore chain morphology was observed as shown in the Figure 10(a-d).

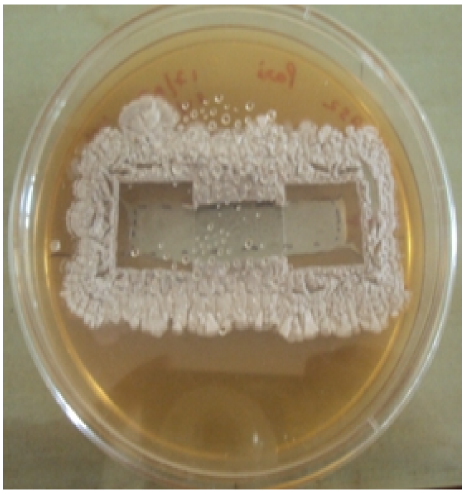
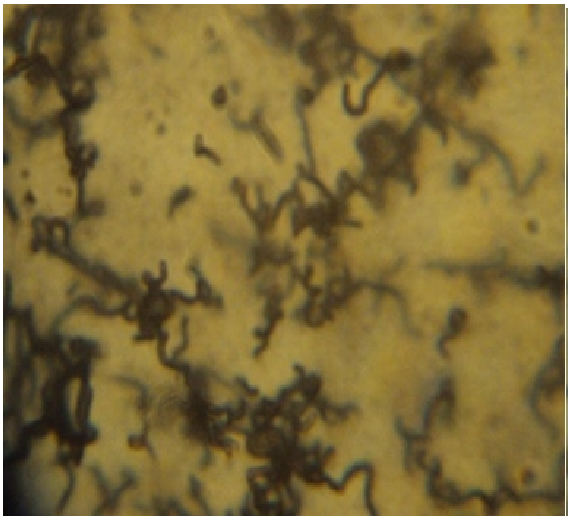
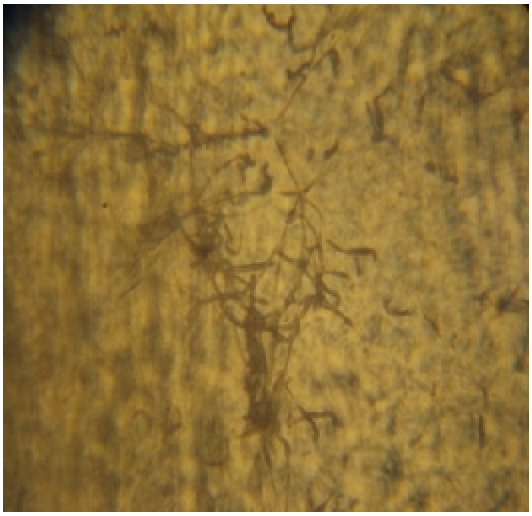


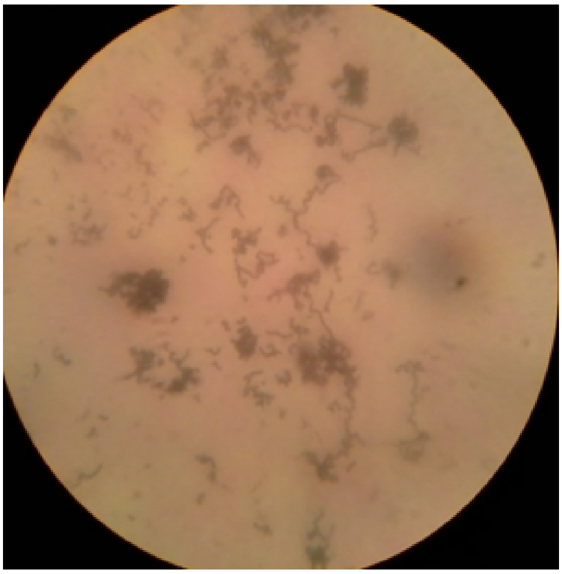
Figure 9: Plate showing the coverslip culture technique.



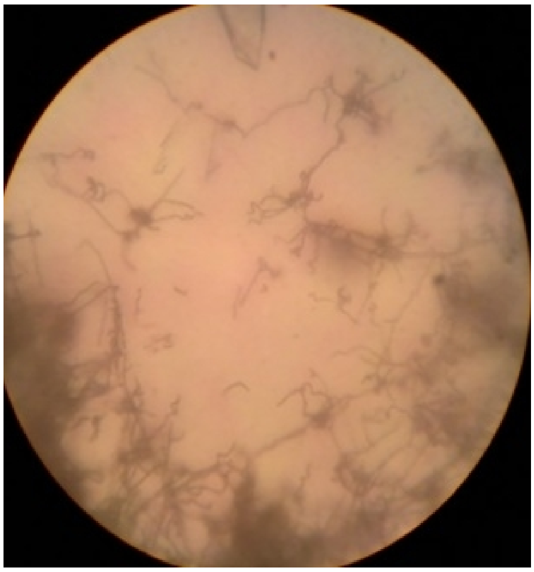
a) PAS2



b) PAS3



c) PAS5



d) PAS8

Figure 10(a – d): Results of spore chain morphology at 100x microscope.

d) Spore surface morphology

Spore surface morphology was studied under the scanning electron microscope (SEM). By the coverslip culture technique (Figure 11) but here instead of coverslip the 1cm*1cm glass slides were used and the slides were prepared for 7 to 10 days incubation. The reading were taken at 3000X magnifications observation shown in the (Table no 6).

Table no 6: Result of spore surface morphology

Sl. No.	STRAIN	Spore surface morphology
1	PAS5	Smooth

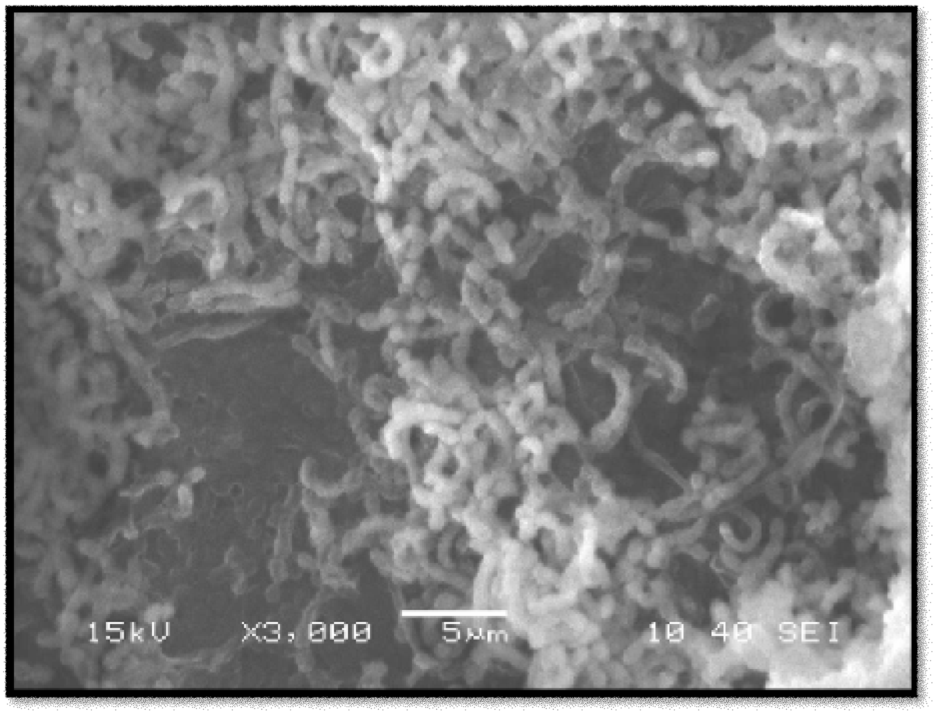


Figure 11: Spore surface morphology of PAS5

5.2. SCREENING FOR ANTIMICROBIAL ACTIVITY

a) Cross streak method

This was done by the cross streak method against five pathogenic strains of bacteria namely *E. coli, Pseudomonas, Klebsiella, Bacillus and Proteus* results shown in the Table no 7 and figure 12(a-d) .

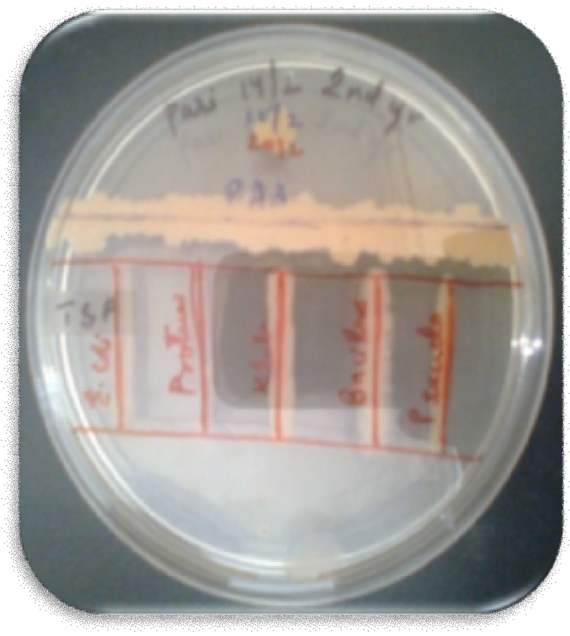
Table no.7: General observation of actinomycetes for antimicrobial activity.

Sl. No.	Strain	*Pseudomonas*	*E.coli*	*Klebsiella*	*Bacillus*	*Proteus*
1	PAS2	+	-	-	-	++
2	PAS3	±	-	-	±	-
3	PAS5	+++	-	-	+	+++
4	PAS8	+++	+	+	++	+++

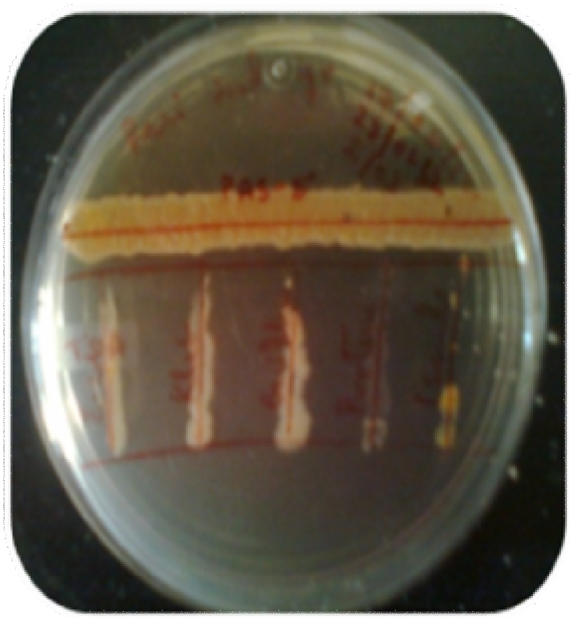
+++: very good ++: good +: positive - : negative ±: moderate

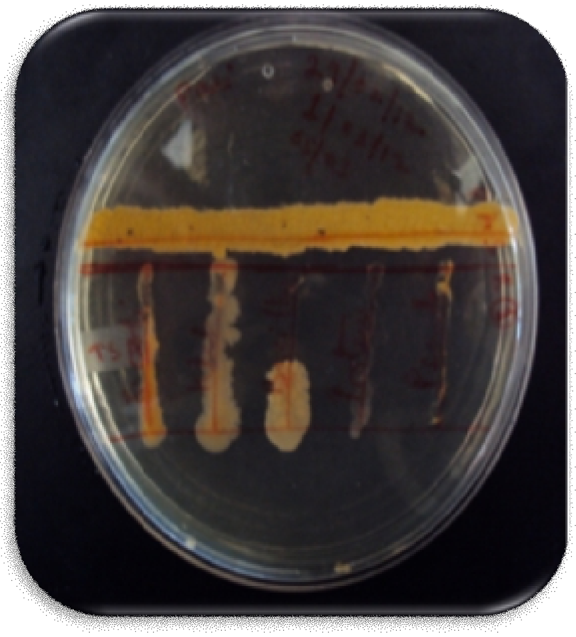
Among all the strains only PAS8 had shown goood antimicrobial activity against almost all the pathogenic strains. The pathogens *E.coli* and *Klebsiella* were least inhibited pathogenic strains. PAS5 had also ahown antimicrobial activity against three pthogenic strain except *E.coli* and *Klebsiella* sp.But both PAS5 and PAS8 shown potential activity against *Pseudomonas* and *Proteus* sp. So these two strains were given more attention on the production of the antimicrobial compounds by further fermentation in the appropriate medium.

a) PAS2



b) PAS3





c) PAS5 d) PAS8

Figure 12(a – d): Screening of antimicrobial activity by cross streak method against five bacterial pathogenic strains.

b) Agar well diffusion method

Among all the strains only PAS5 and PAS8 had shown goood antimicrobial activity against *Pseudomonas* and *Proteus* pathogenic strains which are seen in the figure 13.

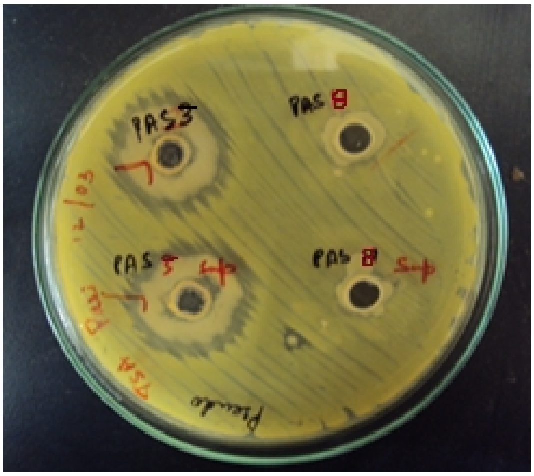


Figure 13: showing zone of inhibition PAS5 and PAS8 against *Pseudomonas* and *Proteus*

c) Enhancement of antimicrobial compound synthesis by cross signaling induction:

Two pure strains of Actinomycetes PAS5 and PAS8 has shown high antimicrobial activity, so these two strains are taken for production of antimicrobial compounds by cross signalling induction of the pathogen against whom they had shown more antimicrobial activity. As microbial production of antibiotics is an adaptive defence mechanism, which is activated in the presence of competing organisms (Patterson and Bolis, 1997).So in our present study we also fermented the two strain in their logarthimic phase with the co cultivation of the respective pathogen *Pseudomonas sp*. and *Proteus* sp. and studied the antimicrobial activity according to the methods described in the material and methods chapter. This antimicrobial activity was measured in the form zone inhibition (mm) given in the Table-8 and also is represented graphically in figure 14 and 15.

Table no 8 – Production of antimicrobial activity by marine actimycetes exposed to heat killed as well as live cells of *Pseudomonas* and *Proteus* sp. (inducer strains)

Diameter of the zone of inhibition (mm)

Actinomycetes strain	***Pseudomonas* sp.**			***Proteus* sp.**		
	Control	Heat killed cells	Live cells	Control	Heat killed cells	Live cells
PAS 5	9	14	16	8	15	19
PAS 8	7	12	17	8	15	21

In the controls only ISP/SCA broth was added.

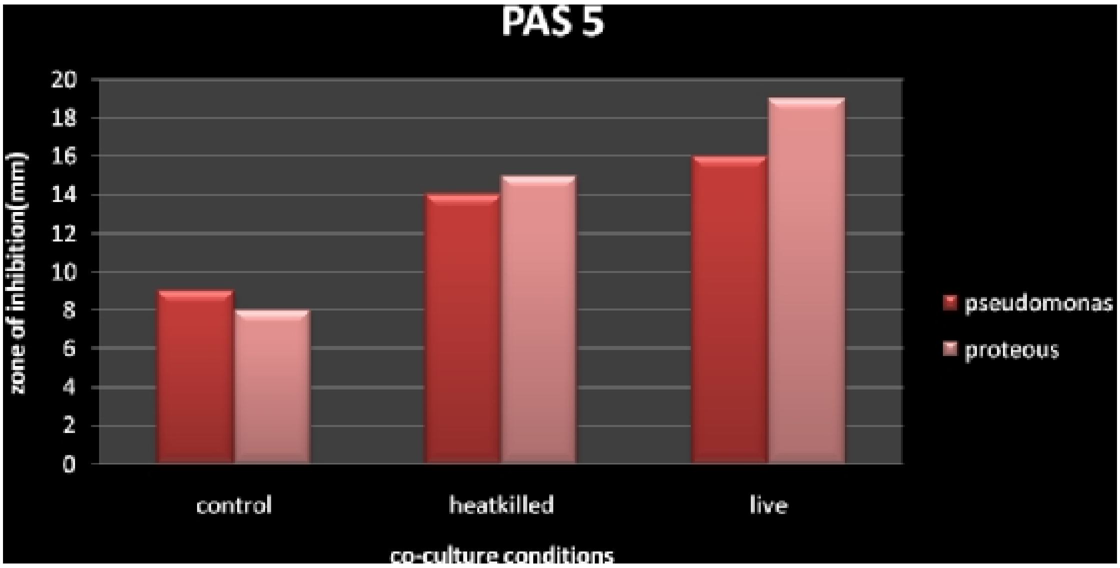


Figure 14: Production of antibacterial compound by Actinomycetes (PAS 5) in response to live and heatkilled *Pseudomonas* sp.and *Proteus* sp.

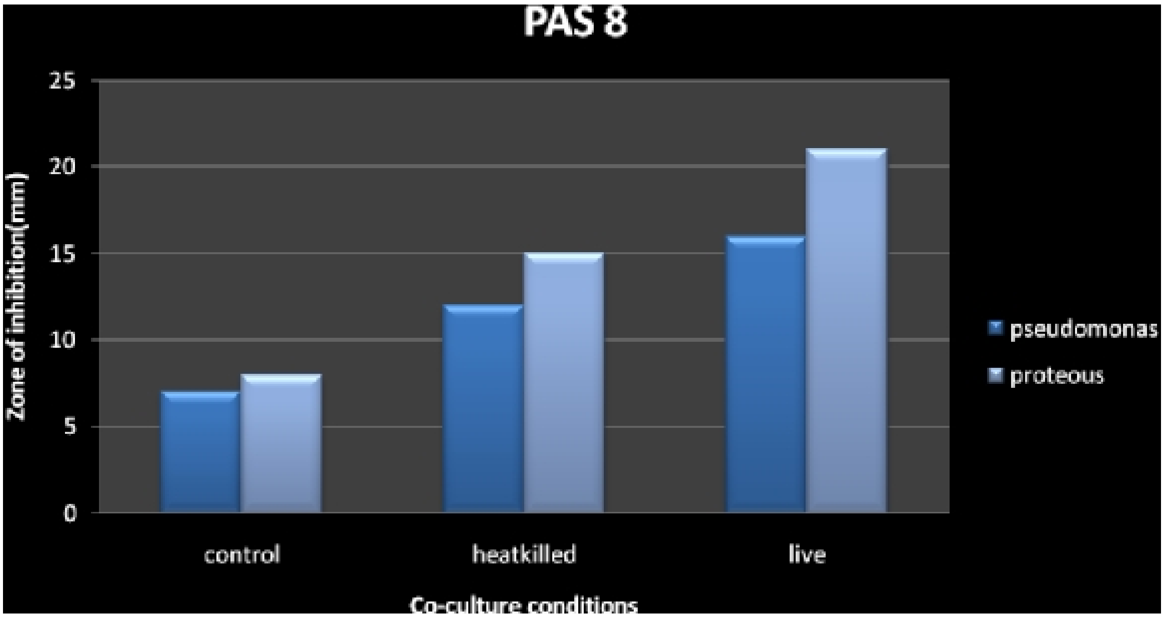


Figure 15: Production of antibacterial compound by Actinomycetes (PAS 8) in response to live and heatkilled *Pseudomonas* sp.and *Proteus* sp.

5.3. SPECIES AFFILIATION- PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

a) Assimilation of carbon sources

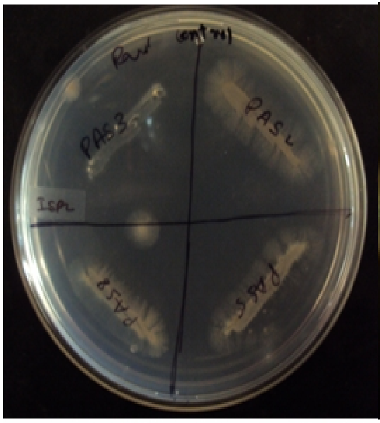
The ability of different actinomycetes strains in utilizing various carbon compounds as source of energy (Table no.9) was done by following the method recommended international Streptomyces Project (figure 16(a-r)).

Table no.9 : Results showing assimilation of carbon sources.

STRAIN	PAS2	PAS3	PAS5	PAS8
NEGATIVE CONTROL (NO CARBON SOURCE)	++	++	++	+
POSITIVE CONTROL GLUCOSE	±	+++	±	±
XYLOSE	++	++	++	+
INOSITOL	++	+++	+++	++

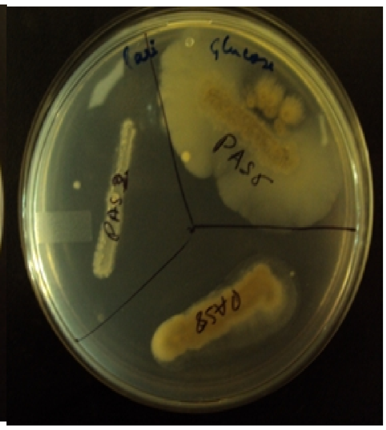
SUCROSE	++	++	+++	+
FRUCTOSE	±	+++	±	±
RHAMNOSE	++	++	++	+
MANITOL	++	+++	+++	++
RAFFINOSE	++	++	+++	+
ARABINOSE	±	+++	±	±

+++: very good ++: good + : positive - : negative ±: moderate

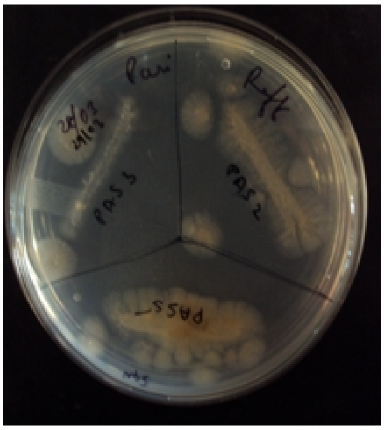


(a)

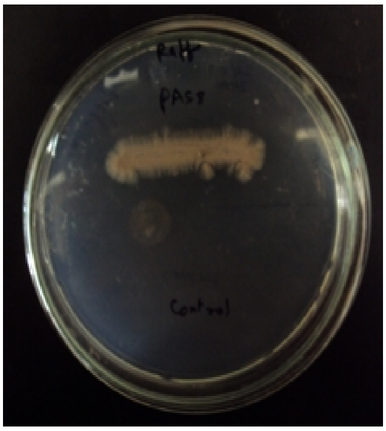
(b)



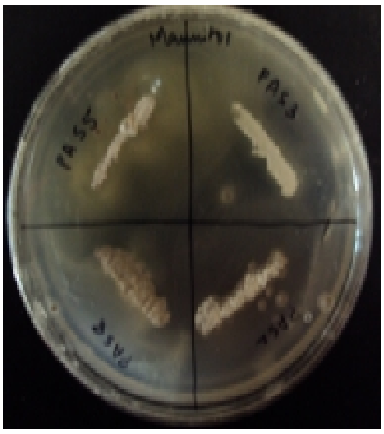
(c)



(d)

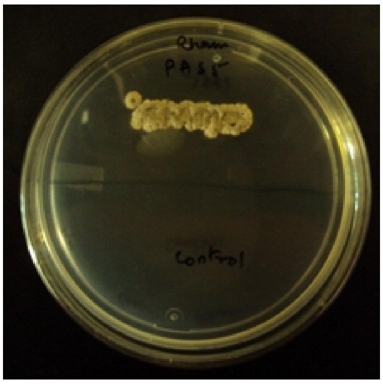


(e)

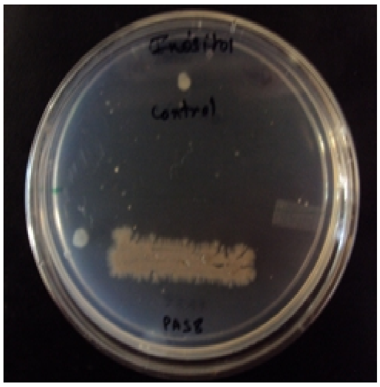


(f)

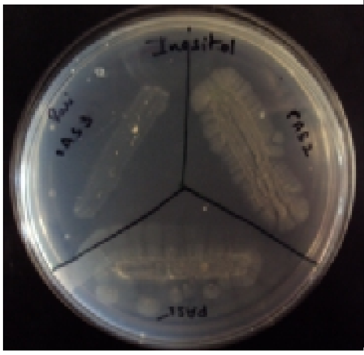
(g)



(h)

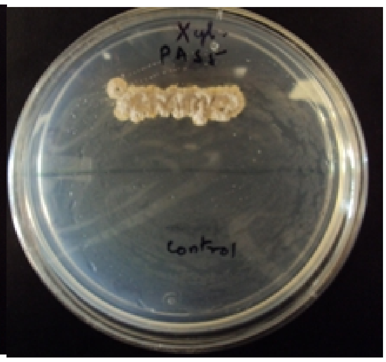


(i)



(j)

(k)



(l)

(m)

(n)

(o)

(p)

Figure 16(a-p): Assimilation of different carbon sources.

After comparing growth with negative and positive control, it was observed that fructose was the most assimilated carbon source by all strains of actinomycetes and arabinose, mannitol and raffinose were least assimilated carbon sources. Among all the strains PAS2, PAS5 and PAS8 had growth on all carbon sources. PAS3 had shown least growth utilization of the carbon sources.

b) Salt tolerance test

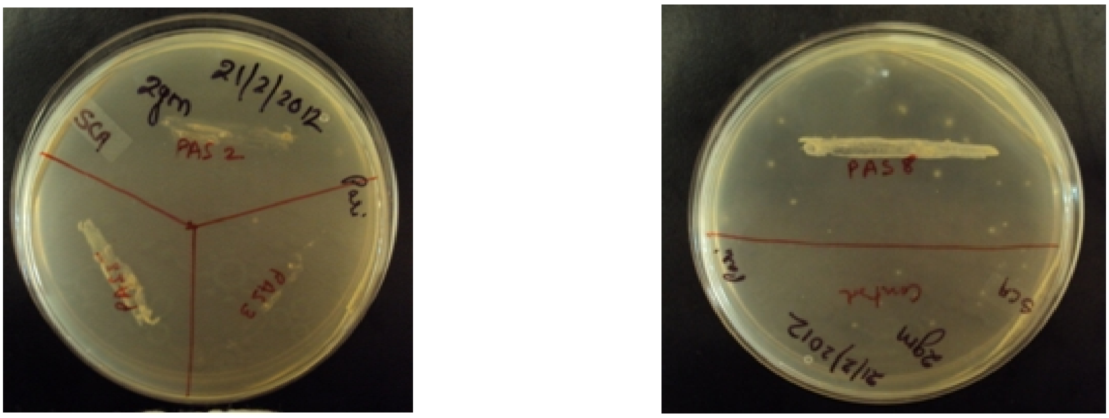
This test was very important to understand the native nature of the marine actiomycetes isolates. Test results were obtained after 7 to 15 days of incubation. Growth was compared with the control. The results obtained were shown in the Table no.10.

Table no. 10: Results of salt tolerance test at different concentrations.

Sl.no.	STRAIN	5%	10%	15%	20%	25%	30%
1	PAS2	±	-	-	-	-	-
2	PAS3	±	-	-	-	-	-
3	PAS5	±	-	-	-	-	-
4	PAS8	-	-	-	-	-	-

+++: very good ++: good + : positive - : negative ±: moderate

Some moderate results of tolerance was observed only at 5%. PAS2, PAS5 and PAS8 has shown moderate tolerance of sodium chloride shown in figure 17(a-b).



(a) (b)

Figure 17(a – b): Strains showing the sodium chloride tolerance test.

c) Degradation of cellulose

Cellulose degradation was observed to study the activity of cellulose enzyme shown in the Table no. 11

Table no. 11: Results of degradation of cellulose

Sl.no.	STRAIN	CELLULASE ACTIVITY
1	PAS2	-
2	PAS3	+++
3	PAS5	+
4	PAS8	++

+++ : very good ++: good + : positive - : negative ±: moderate

Out of 4, 3 strains have shown good degradation of cellulose. PAS2 had shown very good activity of cellulose enzyme than PAS5 and PAS8 shown in figure 18.

Figure 18: Strains showing the cellulose degradation

d) Hydrogen sulphide production

H_2S was odserved after the incubation of 7th, 10th and 15th. By comparing the presence of bluish black and black colour slants to the control slants observation were taken (Table no. 12)

Table no.12: Results showing H_2S production

Sl.no.	STRAIN	H_2S PRODUCTION
1	PAS2	+++
2	PAS3	-
3	PAS5	+
4	PAS8	+++

+++ : very good   ++: good   + : positive   - : negative ±: moderate

Among 4 strains of actinoycetes 3 strains have shown the positive result for the production of H_2S. only PAS3 did not show positive result for H2S production (figure 19)

Figure 19: Slants showing the H_2S production by actinomycetes

e) Gelatin liquefication test

This test was performed to study the activity of enzyme gelatinase. The slants were observed after the incubation of 7 to 10 days. All the results of gelatin liquefication test was negative (Table no. 13).

Table no. 13: Results of gelatin liquefication test.

Sl.no.	STRAIN	GELATINASE ACTIVITY
1	PAS2	-
2	PAS3	-
3	PAS5	+++
4	PAS8	-

+++ : very good ++: good + : positive - : negative ±: moderate

Among all the 4 strains only PAS5 has showed the positive result of gelatinase activity (Figure 20).

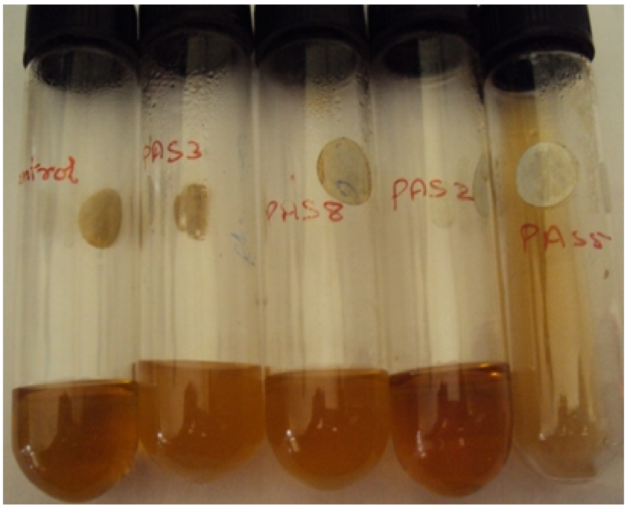


Figure 20: Slants showing the results of gelatin liquefication test

f) Hydrolysis of starch

This test was done to observe the activity of amylase enzyme. Almost all strains had shown good amylase activity is shown in the table no. 14.

Table no.14: Results of starch hydrolysis by amylase activity.

Sl.no.	STRAIN	AMYLASE ACTIVITY
1	PAS2	±
2	PAS3	-
3	PAS5	-
4	PAS8	-

+++ : very good ++: good + : positive - : negative ±: moderate

Starch hydrolysis was observed by the area of clear zone around the culture streak. Among all strains only PAS2 had shown moderate hydrolysis and other three strains did not show the clear zone around the culture streak (Figure 21).

(a) (b)

Figure 21 : a) strain showing the hydrolysis of starch and b) Strains showing no hydrolysis of starch.

g) Coagulation of milk

The test was done to study the activity of caseinase enzyme. The slants were observed after the incubation of 7 to 10 days as shown in table no.15.

Table no.15: Results showing the coagulation of milk

Sl.no.	STRAIN	CASEINASE ACTIVITY
1	PAS2	++
2	PAS3	++
3	PAS5	++
4	PAS8	++

+++ : very good ++: good + : positive - : negative ±: moderate

All the 4 strains PAS2, PAS3, PAS5 and PAS8 showed the good caseinase activity (Figure 22).

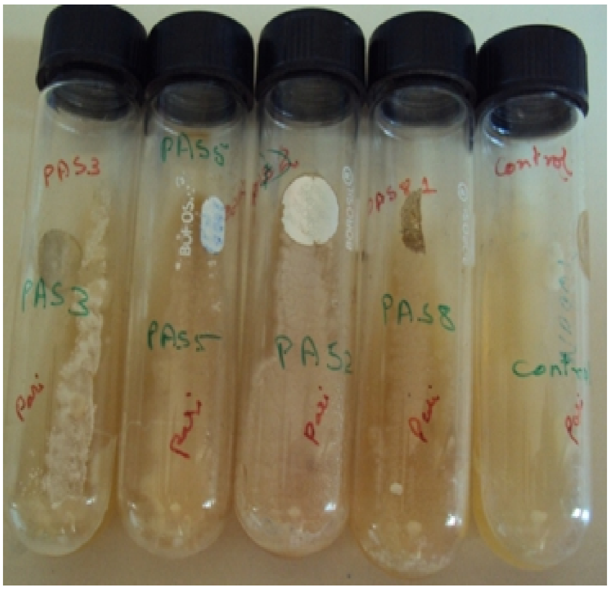


Figure 22: Slants showing the coagulation of milk

h) Ability to grow in different pH

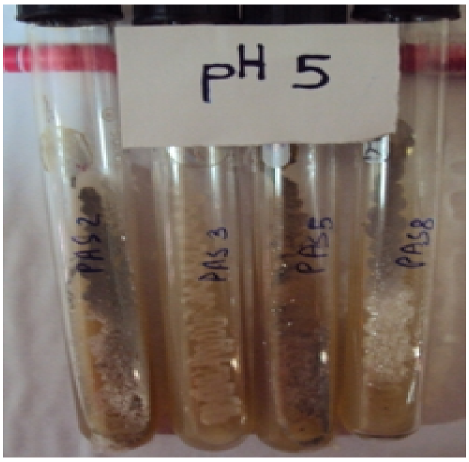
All the strains have shown pH tolerance at different ranges of pH to pH9 shown in the Table no.16.

Table no.16: Results showing the growth of actinomycetes on different ranges of pH.

Sl.no.	STRAIN	pH5	pH6	pH7	pH8	pH9	pH10
1	PAS2	+	+	+	+	+	+
2	PAS3	+	+	+	+	+	+
3	PAS5	+	+	+	+	+	+
4	PAS8	+	+	+	+	+	+

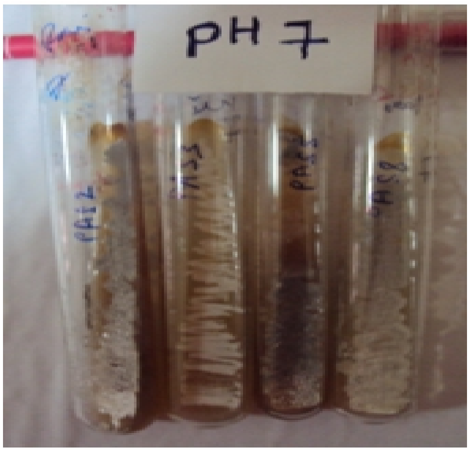
+++ : very good ++: good + : positive - : negative ±: moderate

All the strains had shown good growth on different ranges of pH those shows there tolerance to grow in acidic as well as basic conditions   shown in figure 20(a-f).



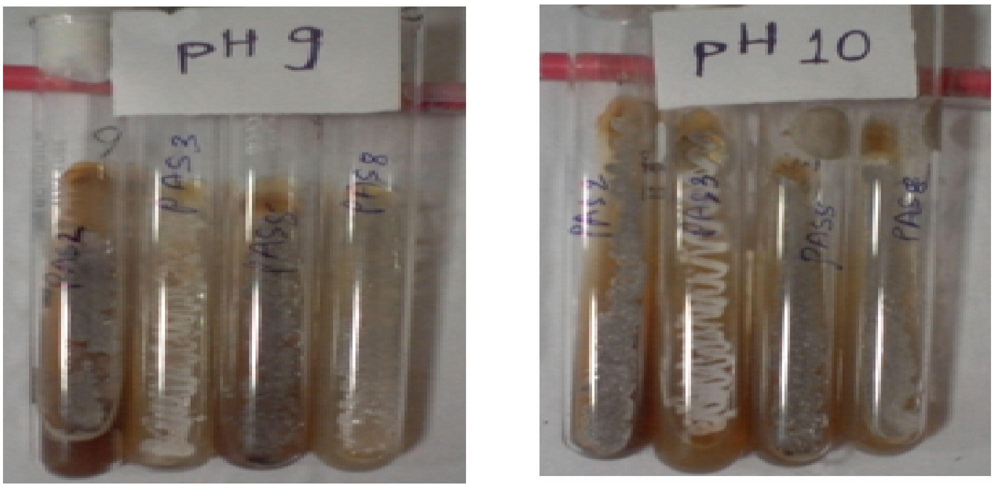
(a)

(b)



(c)

(d)



(e) (f)

Figure 23(a – f): Slants showing the growth on different pH.

i) Lipolytic activity

Lipolytic activity was studied to observe the enzymatic activity of lipase enzyme shown in the Table no.17.

Table no. 17: Results showing the lipolytic activity of actinomycetes.

Sl.no.	STRAIN	LIPOLYTIC ACTIVITY
1	PAS2	+
2	PAS3	-
3	PAS5	-
4	PAS8	-

+++ : very good ++: good + : positive - : negative ±: moderate

Among all the strains only PAS2 has shown good lipolytic activity (Figure 24).

Figure 24: Plate showing the clear hollow zone of lipolytic activity of strain PAS2.

After obtaining all the results from the experiment done were matched with the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project) and the species identification was done, the results obtained are given below. The match was reported on the basis of maximum percentage of resemblance of characteristics (Table no 18).

Table no.18: Results obtained from the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project)

Sl.no	Strain	Species name	ISP2 description	Page
1	PAS2	*S.resistomycificus*	**II**	165
2	PAS3	*A.albovinaceus*	**II**	82
3	PAS5	*S.echinatus*	**II**	106
4	PAS8	*A.mutabilis*	**II**	148

6. DISCUSSION

It has been well established that antibiotic production can be induced or enhanced by exposing producing strains with competing organisms (Patterson and Bolis, 1997). In 1998, Spragg *et al.* , showed both live and heat killed cells of *S. aureus* induced production of antibiotics in two marine strains (Mbbc1122 and Mbbc1123). They suggested the possible rationale for the enhancement of antibiotic production in bacteria, as due to the competition for space, nutrient and to deter or kill potential competitor bacteria in the environment. As the concept of the survival of the existence the actinomycetes are also compete for the nutrients and the available space for growing for which they are induced and forced to secrete some molecules that can help them for the existence of that environment and rest to eliminate. The results of the present co-cultivation study confirm that the isolate PAS5 and PAS 8 produced antimicrobial compounds more amounts in the presence of *Pseudomonas* sp. and *Proteus* sp. against whom they are reactive. Similar observations have been made by Spragg *et al*., (1998), in which surface-associated marine bacteria AMS1·6 produced antibacterial compound only in response to the presence of methicillin resistant *S. aureus*.

Results of the present co-cultivation study depict interspecies quorum-sensing pattern where *Pseudomonas* sp. and *Proteus* sp. induced antibacterial compound production in PAS5 and PAS8. There are few reports available regarding interspecies relationship between actinomycetes with other bacterial organisms in response to antibacterial compound production. Robles and Joanne (2006) reported that out of 53 bacterial isolates obtained from soil, 33 isolates including *Bacillus sp , Micrococcus*, *Stenotrophomonas* and *Lysobacter* were found to induce antibiotic production in *S. coelicolor* through molecular signals. Similarly in Slattery *et al*., in 2001 examined the impact of co-culture of marine bacteria on istamycin antibiotic production by *S. Tenjimariensis* with 53 different bacterial species and found that 12 bacterial strains induced the production of istamycin and this antibiotic inhibited growth of other competitor bacterial colonies.

In the present study, a strain of *Proteus* sp. and *Pseudomonas* sp. was introduced into PAS5 and PAS8 culture during growth phase by adopting the method described by Kanagasabhapathy and Nagata (2007), in contrast to the method of Spragg *et al*., (1998). Mixing of live/ heat killed *Pseudomonas* sp. and *Proteus* sp. with actinomycetes will facilitate the transfer of signal molecules produced by the inducer. Kanagasabhapathy and Nagata (2007) observed that, *B. licheniformis* when grown as a pure culture did not produce any activity against marine fouling bacteria but, when challenged with fouling bacteria (FB-9), it produced antibacterial compound. In the present study, strain PAS5 and PAS8 when cultured alone in a shake flask did not produce of any antibacterial compound(s). The isolate PAS5 and PAS8 produced antibacterial compound(s), only when co cultured with live and /or heat-killed cells of *Pseudomonas* sp. and *Proteus* sp.

7. **SUMMARY AND CONCLUSION**

Sediment sample was collected for the isolation of actinomycetes from the soil. Actinomycetes was isolated using ISP2 media (international streptomyces project medium). Four isolates were selected by cross streak method. Bacterial pathogens such as *Escherichia coli, Bacillus substilis, Pseudomonas, Proteus* and *Klebsiella* were used for induced antibacterial compound production in actinomycetes. Zone of inhibition was observed to evaluate the anti microbial potential of isolated Actinomycetes. The one showing maximum zone of inhibition were taken to be as potent strain for further study for production of antibiotics against that particular pathogen which it was reactive to produce antibiotics. Here PAS5 and PAS8 are the most potent strains reported against *Pseudomonas* and *Proteus*. The identification of this strain was performed by Physiological characterization, biochemical tests, microscopic observations at 100x and scanning electron microscope (SEM) analysis. Finally the strain PAS2 was identified as *Streptomyces resistomycificus,* PAS3 as *Actinomyces albovinaceus,* PAS5 as *Streptomyces echinitus,* PAS8 *as Actinomyces mutabilis*. Strain PAS5 (*S.echinatus*) and PAS8 (*A.mutabilis*) according to the identification key (ISP). When the strains PAS5 and PAS 8 cultured alone in a shake flask produced very little antibacterial compound(s). The isolate PAS5 (*S.echinatus*) and PAS8 (*A.mutabilis*) produced higher amount of antibacterial compound(s), only when it was co cultured with live and /or heat-killed cells of Pseudomonas sp. and Proteus sp. By making the actinomycetes strains more competitive.

Antibiotics holds an important position due to their diversity and proven ability to produce new compounds i.e. discovery of antibiotics. This property has been exploited for the commercial production of antibiotics; but by exposing actinomycetes to bacterial pathogens is expected to pave for the production of novel antibiotics which has high degree of specificity and can be looked upon as an alternative pathway to overcome the problem of drug resistance and reemergence of resistance pathogens.

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