Isolation and screening of waterborne actinomycetes for their antistaphylococcal activity

A

Thesis submitted

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By

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Date: July 16th, 2012 Amrit Kaur Hans

Certificate

This is to certify that the thesis entitled ***"Isolation and screening of waterborne actinomycetes for their antistaphylococcal activity "*** being submitted by **Ms Amrit Kaur Hans (Roll No. 301001031)** in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.

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Candidate's Declaration

I hereby declare that the work being presented in the thesis entitled ***"Isolation and screening of waterborne actinomycetes for their antistaphylococcal activity"*** in partial fulfilment of the requirements for the award of degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2012 to June 2012, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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Abbreviations

S. no	Abbreviation	Full form
1.	µg	Microgram
2.	µl	Microliter
3.	g/l	Grams per litre
4.	GPA	Glucose Peptone Agar
5.	GPB	Glucose Peptone Broth
6.	KA	Kuster's Agar
7.	MDRs	Multi drug resistant organisms
8.	MHA	Muller-Hinton Agar
9.	MIC	Minimum Inhibitory Concentration
10.	Ml	Milliliter
11.	mm	Millimeter
12.	MRSA	Methicillin resistant *Staphylococcus aureus*
13.	PDA	Potato Dextrose Agar
14.	SCN	Starch Casein Nitrate Agar
15.	TTC	2,3,5 triphenyl tetrazolium chloride
16.	VISA	Vancomycin intermediate resistant *Staphylococcus aureus*
17.	VRSA	Vancomycin resistant *Staphylococcus aureus*

Executive Summary

Due to emergence of multidrug resistance in bacteria and lower efficacy of the present antimicrobial drugs there is an urgent need to explore newer sources and to develop them as potent antimicrobials. Actinomycetes are powerhouse of the bioactive compounds e.g. *Streptomyces* genus which is known to produce 80% of the known antibiotics. The current study is focused on isolation and screening of actinomycetes from fresh water bodies for anti-staphylococcal activity. Water samples from three different locations (Harike wetland area, Bhakhra canal, Patiala and Allahabad confluence, Allahabad) were collected and subjected for the selective isolation using Kuster's agar and Starch casein nitrate media. A total of 27 actinomycetes were recovered from water samples from Harike wetland area. No actinomycetes were obtained from other two water samples. All the 27 isolates were purified using streak plate method and maintained on the PDA slants supplemented with 10% glycerol for long term preservation. All the isolates were subjected for production of secondary metabolites on GPB medium. None of the culture filtrate (aqueous fraction) showed any anti-staphylococcal activity. The culture filtrates were then extracted with ethyl acetate. The ethyl acetate fraction was screened for antistaphylococcal activity against three standard *Staphylococcus sp*. using pre screen assays like KB disc and agar well diffusion. 5 isolates out of 27 exhibited potential antistaphylococcal activity with zone size ranging from 11 to 20.33 mm in KB disc assay and 10.66 to 18.33 mm in agar well diffusion assay. The ethyl acetate fractions of all the 5 isolates were then examined to obtain MIC against test organisms. #11HKSCN showed best activity whereas #2HKSCN shows least activity. The MIC range for all five of the isolates were found to be in between 0.4 to 2.5 mg/ml. Microscopic examination showed that #2HKSCN, #3HKSCN, #6HKSCN, #11HKSCN were Gram positive and #12 HKSCN was Gram negative. Further actinomycetes were biochemically characterized for the production of enzymes like amylase, pectinase and cellulase. Four isolates (#3HKSCN, #6HKSCN, #11HKSCN, #12HKSCN) has the ability to produce amylase enzyme however none of the isolates found to possess the ability to produce pectinase and cellulase enzyme. Further purification of the crude bioactive compounds, structure elucidation and taxonomic identification is warranted to develop these bioactive compounds as pharmacore with potential anti-staphylococcal activity.

Chapter 1

Introduction

Introduction

Actinomycetes are prokaryotic organisms belonging to the subclass *Actinobacteridae*, order *Actinomycetales*. Originally they were considered as intermediate group between bacteria and fungi. The name 'Actinomycetes' has been derived from Greek words '*aktis*' (a ray) and '*mykes*' (fungus) (Dhananjeyan *et al*.,2010). They are also known as filamentous bacteria as they possess both aerial mycelium and substrate mycelium. Spores are formed at the tip of the aerial hyphae.

Actinomycetes are widely distributed in natural ecosystem such as soil, water and colonizing plants. They possess both prokaryotic as well as eukaryotic characters. Prokaryotic characteristics include lack of membrane bounded organelles such as nuclei and mitochondria and frequently susceptibility to bacteriophage while true branching mycelium and formation of motile spores represent eukaryotic characters. On the culture medium colonies of actinomycetes grow slowly exhibiting powdery consistency and stick firmly to agar surface. The actinomycetes are widely distributed in many natural environments including soil, water, organic matter habitats and colonizing plants. They are found to occur in aquatic environment; freshwater (Wohl *et al*., 1998; Rifaat 2003) and marine habitats (Valli *et al*., 2011). Actinomycetes are well known for the production of biologically active secondary metabolites. The genus *Streptomyces* produces clinically useful antibiotics such as aminoglycosides (streptomycin and its relatives), tetracyclines, chloramphenicol and macrolides (erythromycin and its relatives). *Streptomyces* derived antifungals are tend to be macrolide polyenes which includes Nystatin, was first isolated from actinobacteria *S.noursei* for development of antifungal drugs, amphotericin B and natamycin. Thus almost 80% of the antibiotics have been derived from *Streptomyces*. *Micromonospora*, other genera of the actinomycetes is also important as it also has the potential to produce antibiotics. Tigecycline drug isolated from *Streptomyces aureofaciens* is effective against tetracycline resistant organisms. The calicheamicin drugs effective against acute myeloid lymphoma has been isolated from *Micromonospora echniospora sp. Calichensis*. Several drugs isolated from actinomycetes are in clinical trials such as Elsamitrucin that shows antitumor effects and has been isolated from *Streptomyces chartreusis*. Of the predominant part of bioactive compounds discovered so far, it was observed

that 45% are of microbial origin of which over 10,000 compounds are isolated from Actinomycetales, 34% from *Streptomyces* and 11% from rare actinomycetes group. *Streptomyces*, the most frequent producer of bioactive metabolites, produce over 7600 compounds (74% of all Actinomycetes) and the 26 % part is contributed by rare actinomycetes, altogether 2500 compounds. Mostly drugs have been isolated from genus *Streptomyces.* Thus there is need to explore novel actinomycetes with antimicrobial potential (Raja and Prabakarana, 2011)

Staphylococcal aureus is one of the common human pathogen and is most frequently isolated in community and hospital acquired infections. It is facultative anaerobic, gram positive coccus which appears as grape like clusters and forms large round golden yellow colonies. It causes broad spectrum of diseases ranging from superficial lesions such as wound infections to systemic and life threatening conditions such as endocarditis, osteomyelitis, pneumonia, brain absecesses, meningitis and bacteremia. The invasive *S.aureus* infections, whether noscocomial and whether due to antimicrobial resistance, are associated with marked increase rates of mortality, morbidity and treatments costs. The mortality of *S.aureus* bacteremia remains approximately 20-40% despite the availability of effective antimicrobials (Franklin, 2003). The mortality rates in case of noscocomial infections have been found to be increased twofold to fivefold higher than those with S.*aureus* infections as compared to that of non *S.aureus* infections. A study of around 300 hospitals in U.S revealed that by 1998 noscocomial infections caused by MRSA account for 55% of all *S.aureus* infections. The increased use of antibacterial and antifungal agents has led to the development of superbugs and super resistance. The development of resistance to multiple drugs is a major problem in treatment of infectious diseases caused by pathogenic microorganisms. The term superbugs refer to microbes with enhanced morbidity and mortality due to multiple mutations endowing high level of resistance to antibiotic classes specially recommended for their treatment (Dvies and Davies, 2010).Currently 90-95% of clinical *Staphylococcus aureus* strains throughout the world are resistant to penicillin (Sakoulus and Moellering, 2008). Methicillin, semisynthetic penicillin was introduced to provide defense against the penicillinases but the appearance of methicillin resistant *S.aureus* strain in 1961 has led to the emergence of multidrug resistant

organisms (MDRs). Methicillin resistant *Staphylococcus aureus* (MRSA), first detected in hospitals now has become community acquired pathogen with enhanced virulence. MRSA is resistant to all β- lactams, cephalosporins and carbapenems. In Asian countries and in USA, the incidence of MRSA isolates is increasing significantly (Appelbaum, 2006). Lineolid and Daptomycin were introduced in 2000 and 2003 respectively to treat the infections caused by MRSA but MRSA has also developed resistance against these drugs. Currently vancomycin is used to treat infections caused by methicillin resistant staphylococci but the increased use of vancomycin has led to the emergence of vancomycin intermediate- resistant *Staphylococcal aureus* (VISA) strains and vancomycin resistant *Staphylococcal aureus* (VRSA) strains. With MRSA being so resistant to many of the best antibiotics, it makes treatment of skin infections and invasive internal infections much more problematic, resulting in many yearly deaths *and it may be a matter of time before antibiotics can no longer can be relied upon*. The pace of progress on the discovery facade of innumerable antibiotics has been lower down. All of them can't be exploited for commercial employment. It can be due to some problems posed by them that can be low potency, pharmacological profiling, safety issues and may be due to rapid emergence of resistant strains. For the revolutionary act in the antibiotic discovery pipeline, efforts has to made to improve the older compounds from natural sources which will improve the performance of the respective entities enhancing the antimicrobial spectrum and thereby reducing the side-effects as well as it will the mask the drug resistance in susceptible strains. Thus exploration of the natural system is the need of the hour to reinforce the natural antimicrobial storehouse (Mahajan *et al*., 2012).

Actinomycetes have been predominantly dwelling in the soil and these have been isolated and screened for their antibiotic/ pharmaceutical potential from different part of the globe. However discovery of bioactive compounds from actinomycetes is becoming increasingly difficult due to the isolation of common species and subsequent rediscovery of large number of previously described metabolites. Thus it is necessary to explore other natural ecosystems such as wetlands/marine systems for isolation of novel species of actinomycetes that could produce metabolic products effective against resistant pathogens. Wetlands are link between land and water and thereby are most productive ecosystems. Some common names for different type of

wetland include: marshes, swamps, moor, and fen. As wetland is the transitional area between terrestrial and aquatic environment, it possesses characteristics of both the ecosystems. Different type of wetland vegetation, organic and heavy metal pollution and xenobiotics draining from terrestrial environment affects the ecological function of wetlands. Wetlands provide great range of natural nutrients and therefore are habitat of wide variety of microorganisms (Zhao *et al.,* 2012). An appreciable number of antibiotics have been produced from bacteria (Gramicidin, Tyrocidine, Bacitracin); fungi (Penicillin, Griseofulvin) and soil actinomycetes. But only few novel secondary metabolites have been isolated from aquatic actinomycetes: Abyssomicins which possesses anti-staphylococcal activity; Mechercharmycins which has anticancer activity. Therefore aquatic actinomycetes should be explored to isolate novel antibiotics that are effective against resistant organisms. Aquatic actinomycetes are source of novel secondary metabolites as they are biologically and metabolically different from terrestrial actinomycetes. As little is known about the antimicrobial potential of freshwater actinomycetes, the present study focuses on the isolation of actinomycetes having antimicrobial potential from fresh water bodies.

Chapter 2

Review of Literature

2.1 Actinomycetes and their role in antibiotic drug discovery

2.1.1 General Characteristics of Actinomycetes

The actinomycetes are morphologically, physiologically and ecologically diverse group of bacteria (Suneetha *et al.,* 2011). They are prokaryotes with hyphal (hence fungal like) morphology (Thongchai *et al.,* 2003) and has high G+C (>55%) content in their DNA (Cwala *et al.,* 2011). Actinomycetes belong to the order, Actinomycetales, comprising of 14 suborders, 49 families and 140 genera (Mobalaji and Olubukola, 2002) that covers wide range of morphology extending from coccus and rod coccus to branched mycelium (Barakati *et al.,* 2002). *Streptomycetes* is the dominant genus of actinomycetes. The non Streptomycetes are called rare actinomycetes comprising approximately 100 genera numbers of actinomycetes (Dhananjeyan, 2010). Actinomycetes are widely distributed in natural and manmade environment. They are found abundantly in all soils both cultivated and non cultivated, fertile and non fertile and in various regions of world (Barakati *et al.,* 2002).

2.1.2 Role in Antibiotic Drug Discovery

Actinomycetes are economically and biotechnologically valuable prokaryotes responsible for the production of about half of the discovered bioactive secondary metabolites (Lam, 2006). *Streptomyces* cover around 80% of the total antibiotic product (Pandey *et al.,* 2004). Streptothricin is the first antibiotic discovered from the genus *Streptomyces* in 1942 by Selman A waksman and his group at Rutgers university. The discovery of streptomycin by Selman A Waksman and his student Albert Schatz triggered screening of antibiotics from *Streptomyces.* Waksman was awarded Noble prize in medicine for streptomycin discovery to treat tuberculosis. The commercial production of streptomycin was started by Merck and Co. Some *Actinoplanes* also produce enzymes and metabolites of medical importance such as *Actinoplanes missouriensis* produces xylose isomerase (Cwala *et al.,* 2011). Actinomycetes also synthesize different biologically active secondary metabolites which are used as cosmetics, vitamins, nutritional material, herbicides and pesticides (Valli *et al.,* 2012). A range of useful antibiotics are presented in table 1

S.no.	Antibiotic	Producer	Application
1	Erythromycin	*Sacchropolyspora erythraea*	Antibacterial
2	Amphotericin B	*Streptomyces nodosus*	Antifungal
3	Streptomycin	*Streptomyces griseus*	Antibacterial
4	Nystatin	*Streptomyces noursei*	Antifungal
5	Vancomycin	*Amycolatopsis orientalis*	Antibacterial against *Streptococcus sp.*
6	Chlorotetracyclin	*Streptomyces aureofaciens*	Antibacterial
7	Rifamycin	*Amycolatopsis mediterranei*	Antibacterial against *M. tuberculosis*
8	Miglustat	*Streptomyces lavendulae*	Enzyme replacement therapy
9	Daptomycin	*Streptomyces roseosporus*	Treats skin infections
10	Ertapenem	*Streptomyces cattleya*	Antibacterial activity

Table no. 1: Actinomycetes antibiotics for medical applications.

2.2 Actinomycetes from water bodies and their metabolites

Actinomycetes are widely distributed in natural environments including terrestrial and aquatic habitats. Aquatic ecosystem includes both freshwater and marine habitats. Recent findings from culture dependent and culture independent methods have demonstrated that indigenous marine actinomycetes exist in oceans and are widely distributed in different marine ecosystems. There is tremendous diversity and novelty among marine actinomycetes (Lam, 2006). Oceans consists of indigenous marine actinomycetes belonging to the genera *Streptomyces, Nocardiopsis* and *Verrucosispore* (Shumei *et al.,* 2007), *Micromonospora* (Hong *et al.,* 2009) and *Cellulosimicrobium* (Jiang Shumei, 2007). Novel actinomycetes have been found in Great Barrier Reef Sponges: *Rhopaloeides odorabile, Pseudoceratina clavata* and

Candidaspongia flabellate (Kim *et al.,* 2005). Table 2.1 shows some examples of novel secondary metabolites isolated from marine actinomycetes. Fig 1 shows the structure of some novel secondary metabolites isolated from marine actinomycetes.

S.No.	Compound	Source	Activity
1.	Abyssomicins	*Verrucosispora sp.*	Antibacterial
2.	Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer
3.	Chandrananimycins	*Actinomadura sp.*	Antialgal; antibacterial
4.	Diazepinomicin	*Micromonospora sp.*	Antibacterial; anticancer
5.	Framycetim	*Streptomyces fradiae*	Antibacterial
6.	Gutingimycin	*Streptomyces sp.*	Antibacterial
7.	Himalomycins	*Streptomyces sp.*	Antibacterial
8.	Komodoquinone A	*Streptomyces sp.*	Neuritogenic activity
9.	Marinomycins	*Marinospora*	Antibacterial; anticancer
10.	Neomycin	*Streptomyces fradiae*	Antibacterial
11.	Salinosporamide A (NPI-0052)	*Salinospora tropica*	Anticancer
12.	Sporolides	*Salinospora tropica*	Anticancer
13.	Streptomycin	*Streptomyces griseus*	Antibacterial
14.	Tobramycin	*Streptomyces tenebrarius*	Antibacterial

Table no.2: Novel metabolites produced by marine actinomycetes

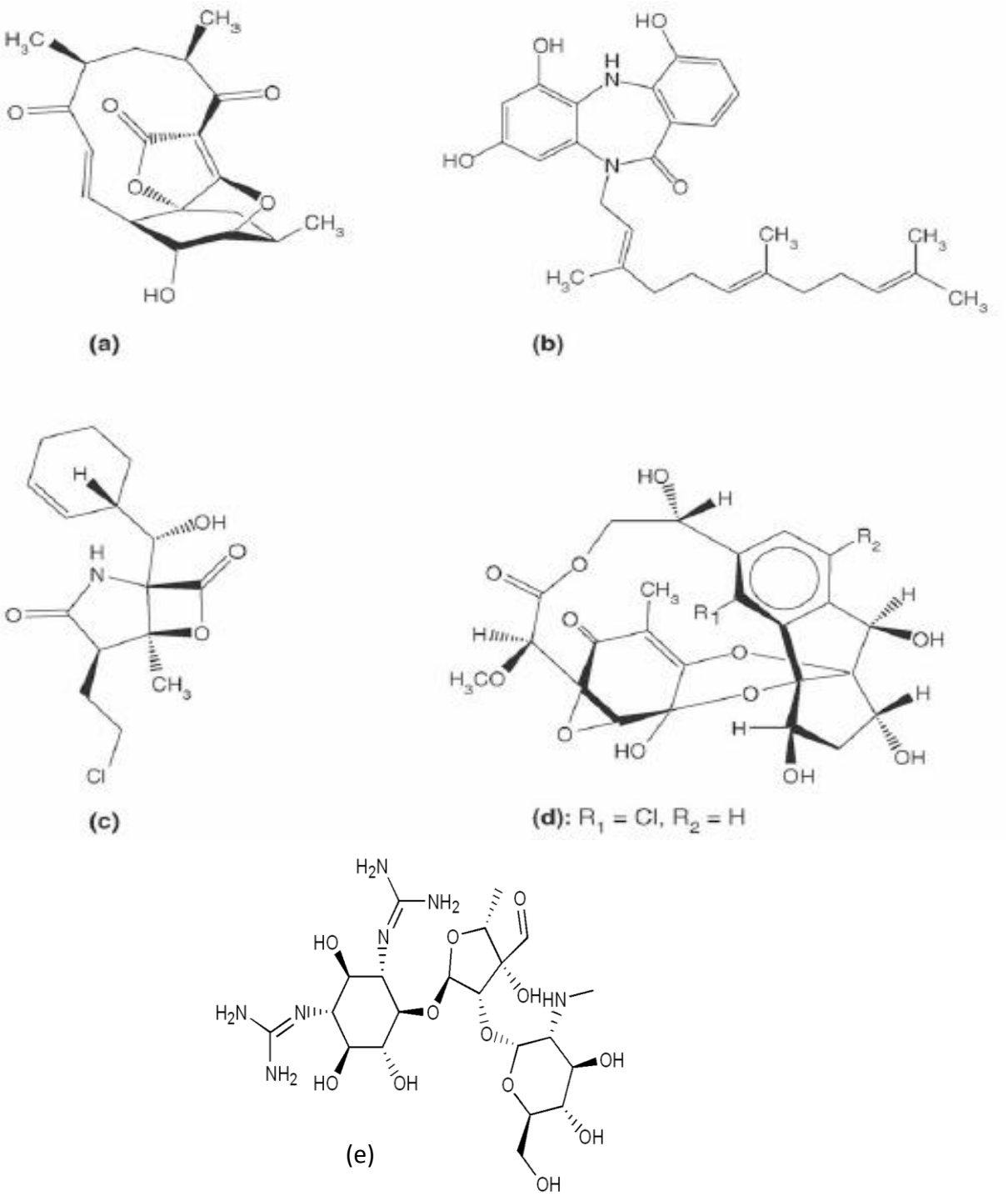


Fig 1: Structure of (a) Abyssomicin C, (b) Diazepinomicin (ECO-4601), (c) Salinosporamide A(NPI-0052, (d) Sporolide A and (e) Streptomycin

Source: Discovery of novel metabolites from marine actinomycetes, Lam, 2006.

Bioprospecting studies have been largely focused on terrestrial and more recently on marine ecosystem but freshwater habitats have been unexplored and studies on freshwater actinomycetes in India are rare (Debananda *et al.,* 2011). Only few reports are available on isolation of novel actinomycetes from fresh water bodies. Suchitra *et al.,* 2011 studied the actinomycetes diversity of Manipur sediments from various depths of Lotak Lake (the largest freshwater lake in North East India). Timothy *et al.,* 2010 has isolated actinomycetes belonging to genera *Saccharopolyspora* and *Actinosynnema* from fresh water and showed their antibacterial activities.

2.3 *Staphylococcal aureus* as a dreaded drug resistant pathogen

Staphylococcal aureus causes nosocomial and community acquired infection (Theresa *et al*., 1999). *S.aureus* has close association with humankind, it is carried as a nasal commensal in 30 % of the population and its presence causes common skin infections such as boils (Davies and Davies, 2010). *S.aureus* is pathogen of greatest concern because of intrinsic virulence, its ability to cause diverse array of life threatening infections and its capacity to adapt to different environment conditions (Franklin, 2003). The emergence of high level of penicillin resistance followed development and spread of strains resistant to semisynthetic penicillins (methicillin, oxacillin), macrolides, tetracyclines and aminoglycosides has made therapy of staphylococcal diseases a global challenge (Fred *et al.,* 2001). MRSA is resistant to all the β- lactams, penicillin, cephalosporin, carbapenems and penems. *Staphylococcus* is resistant to aminoglycosides because of aminoglycoside modifying enzymes. Since the emergence of MRSA vancomycin has been the only effective treatment for staphylococcal infection but recently vancomycin intermediate resistant *Staphylococcus aureus* (VISA) and vancomycin resistant *Staphylococcus aureus* (VRSA) strains like *Staphylococcus hemolyticus* has been isolated, this has led to the concern that *S.aureus* could also acquire vancomycin resistance. Verma *et al.,* 2000 reported that incidence of MRSA was as low as 6.9% in 1998 in India and reached to 24% and 32.8% in 1994 in Vellore and Lucknow and overall prevalance remains same in year 1996. In a study at Aligarh, India, it was shown that 35.1% of *S. aureus* and 22.5% of coagulates-negative staphylococcal isolates were resistant to methicillin (Dechen *et al.,* 2011). Thus a novel

antibiotic to cure such dreadful infections with low cost and less immunogenic response is the need of the hour.

2.4 Need of new antibiotics

S. aureus resistance has developed against every main class of antibiotic today (Walsh, 2003). Thus current armamentarium of antibiotics exhibit less efficacy against infections caused by multidrug resistant MRSA. Antibiotic resistance is caused by two main components: the antibiotic or antimicrobial drug which inhibits susceptible organisms and select resistant ones and genetic resistant determinant in microorganism selected by antimicrobial drug. Drug resistance emerges only when two components come together in environment or in host (Stuart and Bonnie, 2004). In 1941 all strains of *S.aureus* were susceptible to penicillin G but by 1944, *S.aureus* was capable of destroying penicillin by means of penicillinase. Today in excess of 95% of *S.aureus* worldwide are resistant to penicillin, ampicillin. MRSA is resistant to β-lactams, penicillin, cephalosporin (Harold, 1992). Notable examples of multidrug resistant strains (MDR) are *Mycobacterium tuberculosis, Enterrococcus faecium, Klebsiella pneumonia, Acinetobacter baumanii and Pseudomonas aeruginosa* (Stuart and Bonnie, 2004). Thus there is urgent need to explore newer antibiotics to combat the infections caused by resistant bacterial pathogens (Michael and Christopher, 2009). Table 3 shows mechanisms of resistance to antimicrobials.

Antibiotic(s)	Mechanisms	Genetic basis	Present in Pathogens	Crisis now	Future crisis
β-lactums Penicillins Cephalosporins Monobactums Carbapenems	Altered penicillin-binding proteins	Chromosomal	*S.aureus* *S.epidermidis* *S.pneumoniae* *S.sanguis* *H.influenae* *N.gonorrhoeae* *N.meningitidis* *E.coli* *P.aeruginosa*	*S.pnemoniae* *S.epidermidis*	*N.menigitidis*

	Reduced permeability	Chromosomal	*P.aeruginosa* *E.cloacae* *S.marcescens* *K.pneumoniae* *K.oxytoca*	*P.aeruginosa* *E.colacae*	*S.marcescens* *K.pneumoniae*
	β-lactamase	Plasmid and Chromosomal	*S.aureus* *S.epidermidis* *Enterococci* *P.aeruginosa* *Enterobacteriaceae* *N.gonorrhoeae* *N.meningitidis* *Moraxella* *Bacteroids*	*Xanthomonas* *Acinetobacter*	*Bacteroides* *N.menigitidis* *Enterobacteriaceae* *Salmonella* *Shiegella* *Haemophilus* *Enterococci*
Fluoroquinolones Norofloxacin Ofloxacin Ciprofloxacin Lomefloxacin	Altered DNA gyrase	Chromosomal	*S.aureus* *S.epidermidis* *Enterobacteriaceae* *Pseudomonads*	MRSA	*Pseudomonads* *Enterobacteriaceae* *Haemophilus* *N.gonorrhoeae*
	Reduced permeability	Chromosomal	*Enterobacteriaceae* *P.aeruginosa*	*Serratia* *P.aeruginosa*	*Enterobacteriaceae*
Aminoglycosides Gentamicin Tobramycin Amikacin	Decreased ribosomal binding	Chromosomal	*Streptococci*		
	Reduced uptake	Chromosomal	*Bacteroides* *Pseudomonas* *Enterobacteriaceae*	*Pseudomonas*	*Enterobacteriaceae*

	Modifying enzymes	Plasmid	*Staphylococci* *Enterococci* *Streptococci* *Enterobacteriaceae*	*Enterococci* *Pseudomonas*	*Streptococci*

Table no.3: Mechanism of resistance to antimicrobials

❖ Redrawn from: The Crisis of Antibiotic resistance, C. Neu Harold, 1992.

2.5 Isolation and Identification of actinomycetes

Actinomycetes in general are at competitive disadvantage when growing on agar media in association with other microbes. Actinomycetes are slow growers, their slow growth and inability to dominate plates rapidly has often led them overlooked. The long incubation times that actinomycetes colonies require often result in overgrowth by other bacteria and fungi or plates to be discarded before their appearance (Wohl and Vaun, 1998). Numerous methods have been used for isolating actinomycetes. The methods utilize variety of agar media usually supplemented with selective inhibitors (Masayak Hayakaw *et al.,* 1996). Porter *et al.,*1959 isolated actinomycetes by using various media: Czapek, Yeast extract and Bennett agar supplemented with antibacterial antibiotics such as tetracycline , streptomycin.

Actinomycetes form waxy, slimy, pinpoint colonies exhibiting wide range of colors (grey, cream, pink, white, orange, yellow). Shumei *et al.,* 2007 reported the isolation of 62 actinomycetes from the agriculture soils of Semongok, Sarawak exhibiting wide range of colony colors (dark grey, grey, brownish, white and yellowish white). Microscopic characteristics of actinomycetes can be studied using Gram staining (Valli *et al.,* 2011; Padma *et al.,* 2012). Actinomycets stain Gram positive (*Streptomyces, Actinomadura*) to Gram variable (*Nocardia)*.

2.6 Fermentation

The term ‘fermentation‘ is derived from latin word ‘fevere’ meaning to boil. Conventionally the fermentation is the breakdown (metabolism) of large complex molecules into small simple ones by the enzymes of microorganisms. In microbiological way fermentation is the process of production of useful products through mass culture of micro organisms where as in biochemical sense fermentation means numerous oxidation and reduction reactions in which organic

compound used as source of carbon and energy acceptor or donor of hydrogen ions. The organic compounds used as substrate give rise to various products of fermentation which accumulate in the growth medium. The various products produced during fermentation are classified as primary and secondary metabolites. Secondary metabolites are industrially important and are most exploited in biotechnology e.g. antibiotics, steroids, alkaloids, toxins. Antibiotics as secondary metabolites are produced by multistep biosynthetic pathway starting from intermediates of primary metabolism to specific moieties (Abbas *et al.,* 2010). The antimicrobial activity of marine derived actinobacteria has been revealed against pathogenic bacteria (Kumar *et al.,* 2011). Culture filtrates of *Streptomyces* VITSVK9 showed antibacterial and antifungal activity (Kumar and Kannabiran, 2010).

2.7 Solvent Extraction

Each secondary metabolite has some specific chemical characteristics which implies that specific extraction methods should be developed. For purification of secondary metabolites variety of methods are used like solvent extraction, distillation and acid and alkaline extraction. Solvent extraction is method of choice for purification of bioactive compounds as it is carried out at room temperature and thereby prevents loss of heat labile compounds.

Liquid –liquid extraction is process of transferring solute from one liquid phase to another immiscible liquid in contact with first. The two phases are chemically quite different and flows in opposite directions. The lighter solvent flows upward and heavier solvent flows downward. This leads to the separation of compound according to its distribution and partition between two phases-normally one organic and other aqueous. Various solvents like alcohol, chloroform, ethyl acetate and methanol could be used for extraction of bioactive compounds from culture filtrate of actinomycetes (Remya and Vijayakumar, 2008). Biswas *et al.,* 2012 isolated two pure bioactive compounds MB-1a and MB-1b from *Streptomyces* sp MBS-15 by ethyl acetate extraction.

2.8 Methods to assess *in vitro* antimicrobial activity

2.8.1 Kirby-Bauer Disc diffusion assay

KB test is simple qualitative assay that has been well standardized. The test is performed by applying bacterial inoculum of approximately $1X10^8$ CFU/ml to Muller Hinton agar plate. Then sterile disc of fixed concentration are placed on the inoculated agar surface. During incubation antibiotics diffuse outwards from disc creating a concentration gradient. The zone of inhibition around each disc are measured and related to the susceptibility of isolate and to diffusion rate of drug through agar medium (Bauer and Kirby, 1966).

2.8.2 Agar Well diffusion assay

This technique was initially designed by Heatley in 1944. The principle of agar well diffusion assay is as follow: Wells of 5 mm are punched with sterile cork borer. The fixed volume and concentration of extract is dispensed into the wells and finally wells are sealed. A standard concentration of inoculum is spread on surface of agar plates. The extract diffuses into the agar medium and inhibits the growth of bacteria. The zone of inhibition is measured and it is determined whether bacteria are susceptible or resistant to antimicrobial compound. (Goomber and Saxena, 2007)

2.8.3 Micro Broth dilution test

In vitro micro broth dilution test is used to determine the minimum inhibitory concentration (MIC).MIC is considered 'good standard' for determining the susceptibility of organisms to antimicrobials. The lowest concentration of antimicrobial that prevented growth of the test organisms represented the minimum inhibitory concentration (MIC). This procedure involves preparing two fold dilutions of antimicrobials in liquid growth medium and inoculation with standardized bacterial suspension. Following overnight incubation bacterial growth is examined and MIC is determined. (Gomber and Saxena, 2007).

2.9 Enzymatic Screening

Enzymatic tests are basically conducted to determine whether actinomycetes have the ability to act as degrader of organic compounds: starch, pectin and cellulose. Actinomycetes can produce amylase enzyme and can utilize starch as the sole carbon source (Abbas, 2006). Bruhlmann *et al.,* 1994 screened 79 actinomycetes isolated from soil and compost samples for the production of pectinolytic enzymes and reported that 66 isolates produce pectinolytic enzymes. Amira *et al.,* 1989 isolated *Streptomyces sp* strain AT7 having cellulolytic activity from Iraqi soil.

Chapter 3

Aim of the study

3.1 Aim of the Study

Isolation and screening of actinomycetes from fresh water bodies for their antistaphylococcal potential.

Objectives:

I. Isolation and preservation of water borne actinomycetes.

II. To produce culture filtrate and evaluate them for antistaphylococcal activity using pre screen assays.

III. To establish minimal inhibitory concentration (MIC) of the bioactive compounds from actinomycetes against *Staphylococcus aureus and Staphylococcus epidermidis.*

IV. To characterize the actinomycetes biochemically and microscopically.

Chapter 4

Materials & Methods

4.1 Isolation and culture maintenance

4.1.1 Collection of Samples

The water samples were collected from following three spots in sterile screw capped glass bottles.

- Spot 1:Harike wetland, Amritsar, Punjab
- Spot 2:Bhakhra canal ,Patiala ,Punjab
- Spot 3:Allahabad confluence, Allahabad, Uttar Pradesh

The samples were stored at 4°C till further processing.

4.1.2 Preparation of selective isolation media

Starch Casein Nitrate agar (SCN) (Hua, 1996; Suchitra *et al.,* 2011; Debananda *et al.,* 2011*)* and Kuster's agar (KA) (Kumar *et al.,* 2005; Baskaran *et al.,* 2011) were used for selective isolation of actinomycetes from collected water samples. Composition for preparation of KA and SCN per liter is given in Table no.1 and 2. Briefly describing the preparation of SCN and KA medium all the ingredients were properly weighed and dispensed in one liter double distilled water. The contents were thoroughly mixed by mild heating and final pH of the medium was adjusted to 7.2. It was then autoclaved at 121°C/15 lbs for 15mins. On cooling of the medium 10 µg/ml amphotericin and 25 µg/ml Streptomycin was supplemented to suppress the fungal and bacterial growth respectively (Remya and Vijayakumar, 2008). The medium was then poured in sterile plates (Borosil) and allowed to solidify under laminar air flow at room temperature.

S.No.	Components	Conc. (g/l)
1	L-asparagine	0.1
2	Glycerol	10
3	Casein	0.3
4	KNO_3	0.3
5	NaCl	2
6	K_2HPO_4	2
7	$MgSO_4.7H_2O$	0.05
8	$CaCO_3$	0.02
9	$FeSO_4.7H_2O$	0.01
10	Agar	15

Table no. 4: Composition of Kuster's agar medium

S.No.	Components	Conc. (g/l)
1	Starch soluble	10
2	Casein	0.3
3	NaCl	2
4	K_2HPO_4	2
5	$MgSO_4.7H_2O$	0.05
6	$CaCO_3$	0.02
7	$FeSO_4.7H_2O$	0.01
8	Agar	15

Table no. 5: Composition of Starch Casein Nitrate agar medium

4.1.3 Isolation, purification and maintenance

Actinomycetes were isolated by spread plate technique (Gurung *et al*., 2009). The water samples were serially diluted up to 10^{-3} dilutions and then from each dilution 0.1 ml aliquot was spread over SCN and KA medium under aseptic conditions. The plates were then incubated for about 10 days at 26±2°C. The isolation plates of actinomycetes contaminated with bacteria and fungi were purified by streak plate method (Rifaat, 2003; Arifuzzaman *et al*., 2010). A loopful of typically isolated colonies were streaked on Glucose and peptone Agar (GPA) and Potato Dextrose Agar (PDA) plates. The plates were incubated at 26±2°C for 4-5 days. GPA plates were prepared by dispensing 20 gm glucose, 20 gm peptone and 15 gm agar in one liter double distilled water and stirred thoroughly. This was then dispensed in 250 ml flasks and autoclaved at 121°C/15lbs for 15 min. Then under aseptic conditions about 22ml media was poured in sterile plates and allowed to solidify at room temperature. Similarly PDA plates were prepared by dispensing 39gm of PDA (Himedia) per liter double distilled water. The isolates were maintained on PDA slants and containing 10% glycerol (v/v) for long term storage.

4.2 Liquid Culture and Broth Testing

4.2.1 Production of secondary metabolites

Production of secondary metabolites was carried out on Glucose Peptone Broth (GPB). The composition of GPB is given in Table no. 6. The pH of the broth was adjusted to 7.0. The media was then autoclaved at 121°C, 15 psi for 15 min. A loop full of culture grown on PDA plates was used to inoculate 100 ml pre-sterilized medium under aseptic conditions. The flasks were then incubated at 120 rpm, 26±2°C for about 10 days for production of secondary metabolites (Nonoh *et al.,* 2010; Valli *et al.,* 2011). The growth was monitored in terms of pigmentation and loss of translucency of the medium. To remove the microbial mass, the broth was filtered using Whatman filter paper followed by centrifuging for 10 min at 12000 rpm. The cell free supernatant was stored at -20°C till further testing (Jianyou *et al.,* 2011).

S.NO.	Components	Concentration(g/l)
1	Glucose	20
2	Peptone	20

Table 6: Composition of Glucose peptone Broth (GPB)

4.2.2 Broth Testing By Agar Well Diffusion Method

Muller Hinton Agar (MHA) was prepared by dispensing 37gm MHA in one liter double distilled water and then autoclaving the medium at 121°C, 15 psi for 15min. 22.5ml of medium, cooled to room temperature was then poured in 90mm sterile glass plates and allowed to solidify in laminar air flow at room temperature. The wells of 5mm were then bored and loaded with 50µl of broth. The plates were left for 15 min so that diffusion can occur. After 15mins wells were sealed with molten MHA by dispensing 30µl in each well. The plates were incubated for 20 min at room temperature and finally swabbed with 18 hrs old McFarland culture in three different directions and along the edges of the plate. The plates were then incubated overnight at 37°C and observed for the zone of inhibition. The diameter of zone of inhibition was measured in millimeters and mean area of inhibition was calculated (Goomber and Saxena, 2007).

4.2.3 Test Organisms used in study

Three test cultures two belonging to *Staphylococcal aureus* and one to *Staphylococcal epidermidis* were used in the study to determine anti-staphylococcal activity of isolated actinomycetes.

S.no.	Culture Code	Taxonomic Identification	Repository
1	Sau NCTC 6571	*Staphylococcal aureus*	National Cell Type Culture Collection
2	Sau MTCC 96	*Staphylococcal aureus*	Microbial Type Collection Chandigarh
3	Sep MTCC 2639	*Staphylococcal epidermidis*	Microbial Type Collection Chandigarh

Table 7: Test organisms used in study

4.3 Solvent extraction of Crude bioactive metabolite

Bioactive compound was purified from the filtrate by solvent extraction method (Westley *et al.,*1979; Gurung *et al.,*2009; Cwala *et al.,*2011).The extraction of metabolites was carried out by ethyl acetate on the basis of best solubility and maximum antimicrobial activities (Biswas *et al.,*2012). Ethyl acetate was added to the culture filtrate in the ratio of 1:1 (v/v) and shaken vigorously. The aqueous phase was extracted twice with ethyl acetate. The organic phase was separated from aqueous phase by adding sodium sulphate. The organic phase was evaporated to dryness at room temperature. The residue obtained was weighed and reconstituted in methanol: ethyl acetate (3:2) and then stock solution of 1 mg/ml was prepared and stored at -20°C till further use.

4.4 *In vitro* assessment of antimicrobial activity

4.4.1 Agar well diffusion assay

The anti-staphylococcal activity of the crude ethyl acetate extracts was tested using agar well diffusion assay. The Muller Hinton Agar (MHA) was prepared by dispensing 37 gm MHA in one liter double distilled water and stirring thoroughly. The medium was then autoclaved at 121°C, 15 psi for 15 min. After autoclaving, medium was allowed to cool to room temperature and then 22.5 ml of medium was measured and poured in 90mm sterile glass plates so that uniform depth of 4 mm was formed. The plates were then allowed to solidify. 5 mm wells were bored in MHA plates. To these wells 30µl of test extract was dispensed and allowed to diffuse for 15 min at room temperature. The wells were then sealed by dispensing 30 µl of molten MHA in each well and plates were again left for about 20 min. Then 18 hr old 0.5 McFarland adjusted test cultures were swabbed in three different directions. Plates were sealed with double parafilm and incubated overnight at 37°C. After incubation the diameter of the zone of inhibition was measured and recorded in mm and mean area of inhibition was calculated (Goomber and Saxena, 2007).

4.4.2 KB Disc diffusion assay

The disc diffusion susceptibility assay (Kirby Bauer *et al.,* 1966) was also used to test anti-staphylococcal activity of the crude ethyl acetate fractions of the actinomycetes. 30 µl of the

test compound was loaded onto sterile discs (Himedia) and allowed to dry. The MHA plates were swabbed with 18 hr old 0.5 McFarland adjusted test cultures in three different directions. The dried antimicrobial discs were then placed on to the seeded plate with the help of sterile forceps. The plates were incubated at 37°C for 16- 18 hrs. The mean zone of inhibition around each disc was measured using Himedia ruler and mean area of inhibition was calculated.

4.4.3 Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by *in vitro* broth dilution assay. 120 µl of MHB was dispensed in the wells of the 96 well microtitre plate and then 50 µl of 18 hrs old 0.5 McFarland adjusted culture was dispensed to the wells. The plate was incubated for 3hrs at 37°C.Two fold dilutions were prepared for each extract. Then according to the template (Fig 2) 30 µl of extract was dispensed to each well. Again the plate was incubated at 37°C overnight. After incubation 30 µl of 2, 3, 5 triphenyl tetrazolium chloride (TTC) was added and 1-2 hr incubation was given. The appearance of pink color due to formation of formazan indicates growth and the minimum concentration at which growth was inhibited (no pink color) represents the MIC.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	C_1O_1	S_3O_1	S_7O_1	S_2O_2	S_6O_2	C_3O_3	S_4O_3	S_8O_3				
B	C_1O_1	S_4O_1	S_8O_1	S_2O_2	S_6O_2	S_1O_3	S_4O_3	S_8O_3				
C	S_1O_1	S_4O_1	S_8O_1	S_3O_2	S_7O_2	S_1O_3	S_5O_3					
D	S_1O_1	S_5O_1	C_2O_2	S_3O_2	S_7O_2	S_2O_3	S_5O_3					
E	S_2O_1	S_5O_1	C_2O_2	S_4O_2	S_8O_2	S_2O_3	S_6O_3					
F	S_2O_1	S_6O_1	S_1O_2	S_4O_2	S_8O_2	S_3O_3	S_6O_3					
G	S_3O_1	S_6O_1	S_1O_2	S_5O_2	Blank	S_3O_3	S_7O_3					
H	Blank	S_7O_1	Blank	S_5O_2	C_3O_3	Blank	S_7O_3					

O_1 ⟶ Sau NCTC 6571 O_2 ⟶ MTCC 96 O_3 ⟶ MTCC 2639 (O_1-O_3: Test organism)
S1: Concentration 1, S2: Concentration 2, S3: Concentration 3, S4: Concentration 4,
S5: Concentration 5, S6: Concentration 6, S7: Concentration 7, S8: Concentration 8,
C1: Control 1, C2: Control 2, C3: Control 3.

Fig 2: Template for MIC assay via *in vitro* broth dilution method

4.5 Identification and Biochemical Characterization

4.5.1 Morphological Characterization

The morphological method consists of macroscopic and microscopic characterization. Macroscopically actinomycetes were identified by colony characteristics e.g. color, consistency. For microscopic studies gram staining was performed (Arifuzzaman *et al*., 2010; Singh *et al.,* 2011). Smear was prepared and heat fixed .The smear was then flooded with crystal violet solution for 1 min and subsequently rinsed with water. After this mordant (Gram's iodine solution) was added. Again after 1min slide was rinsed with water, thereafter slide was rinsed with decoloriser (Gram alcohol solution) until the slide is colorless. The slide was then counter stained with safranine for about 30 seconds and finally rinsed with water and observed under microscope. The organisms appearing blue or purple were gram positive while appearing red were gram negative.

4.5.2 Starch Hydrolysis (Amylase activity)

Starch is branching polymer consisting of glucose units. Amylase enzyme is required for the hydrolysis of starch into maltose which is further hydrolyzed by maltase to yield glucose molecules used for energy production through glycolysis. Thus amylase activity was determined using starch hydrolysis test (Muvva *et al.* 2011; Abbas, 2006). The nutrient agar plates were prepared by dissolving 24gm in liter double distilled water. 1% soluble starch was added and contents were thoroughly mixed using mild heating. The medium was then autoclaved at 121°C, 15 psi for 15 min. The media was allowed to cool and then poured in sterile plates and allowed to solidify. The plates were then inoculated with loopful of actinomycetes culture and incubated for 5-6 days till visible growth was seen. After incubation plates were flooded with iodine solution (3.3 mg/ml of iodine and 6.7 mg/ml of potassium iodide) and observed for the formation of clear zone around the actinomycetes colony.

4.5.3 Pectin Hydrolysis

Pectin hydrolysis test (Bruhlmann *et al.,* 1994) was performed using nutrient agar plates consisting of 1% pectin. The plates were then inoculated with loopful of actinomycetes culture

and incubated for 5-6 days till visible growth was seen. After incubation plates were flooded with congo red dye solution (1%) and washed with 1% NaCl solution. The plates were then observed for the formation of clear zone around actinomycetes colony.

4.5.3 CMC (Carboxy methyl cellulose) utilization

CMC hydrolysis test (Ariffin *et al.,* 2006) was performed using nutrient agar plates consisting of 1% CMC. The plates were then inoculated with loopful of actinomycetes culture and incubated for 5-6 days till visible growth was seen. Following the incubation, the plates were flooded with congo red dye solution (1%) and washed with NaCl solution (1%). The plates were then observed for the formation of clear zone around actinomycetes colony.

Chapter 5

Results & Discussion

5.1 Isolation of Actinomycetes

A total of 27 actinomycetes were isolated from Harike wetland, Amritsar only. For isolation the water samples were diluted up to 10^{-3}dilutions. Out of 27 isolates, 12 were obtained from Starch Casein Nitrate (SCN) agar medium and 15 were obtained from Kuster's agar (KA) medium supplemented with 10 µg/ml amphotericin and 25 µg/ml Streptomycin by spread plate technique (Table no. 8 and 9)(Fig 3 and 4).

S.no.	Culture Code	Dilution	Medium	Place of sampling
1.	#1 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
2.	#2 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
3.	#3 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
4.	#4 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
5.	#5 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
6.	#6 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
	#7 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
7.	#8 HKSCN	10^{-1}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
8.	#9 HKSCN	10^{-2}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
9.	#10 HKSCN	10^{-2}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
10.	#11 HKSCN	10^{-2}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab
11.	#12 HKSCN	10^{-2}	Starch Casein Agar	Harike Wetland area, Amritsar, Punjab

Table no.8: showing Selective isolation of water borne actinomycetes from different water bodies on SCN medium

Fig 3. Selective isolation of actinomycetes on Starch Casein agar (SCN) medium

S.No	Culture Code	Dilution	Medium	Place of Sampling
1.	#1 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
2.	#2 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
3.	#3 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
4.	#4 HKKA	10^{-1}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
5.	#5 HKKA	10^{-1}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
6.	#6 HKKA	10^{-1}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
7.	#7 HKKA	10^{-1}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
8.	#8 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
9.	#9 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
10.	#10 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
11.	#1 H1KKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
12.	#12 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
13.	#13 HKKA	10^{-2}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
14.	#14 HKKA	10^{-1}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab
15.	#15 HKKA	10^{-1}	Kuster's Agar	Harike Wetland area, Amritsar, Punjab

Table no. 9: Selective isolation of water borne actinomycetes from different water bodies on KA medium.

Fig 4. Selective isolation of actinomycetes on Kuster agar (KA) medium

However no isolates were obtained from Bhakhra canal, Patiala and from Allahabad confluence, Allahabad. Similarly,Mohan and Vijayakumar, 2008 isolated 173 actinomycetes colonies from near shore marine environment and mangrove ecosystem at 8 different locations of Kerala, West Coast of India by spread plate technique using Starch Casein Nitrate (SCN) agar medium supplemented with 10 µg/ml amphotericin and 25 µg/ml Streptomycin to inhibit the fungal and bacterial growth respectively. KA and SCN media are suitable for isolation of actinomycetes from water samples of Vellar estuary, South Coast of India when water sample was diluted to 10^{-2}(Kumar *et al.,* 2005).

5.2 Purification and long term Storage

Colonies appearing on SCN and KA were purified by streak plate method on PDA and SCN plates and then stored on PDA slants containing 10% glycerol (v/v) for long term storage (Shumei *et al.,* 2007). All the isolates grow well on PDA slants and can be actively stored for 6 months. Similarly, 62 actinomycetes isolated from 7 soil samples collected from Agriculture Research Centre Semongok, Sarawak were purified by streak plate method on SCN plates (Jeffrey, 2008).Fig 5 shows the purified colonies maintained on PDA slants.

Fig.5. Purified actinomycetes maintained on PDA glycerol slants

5.3 Production of Secondary Metabolites

For the production of secondary metabolites actinomycetes culture was inoculated in 20 ml GPB. The growth of actinomycetes in the broth was observed in terms of turbidity and formation of pigments. After fermentation volume of the medium gets reduced. The pH of the fermented broth of some of the isolates gets reduced while in some of the isolates marked increase in pH was observed (Table no. 10). The culture filtrate of #11HKSCN strain was basic in nature as indicated by change in pH from 7 to 7.94 whereas culture filtrate of #5HKKA strain becomes acidic as pH changes from 7 to 5.94.This indicates the production of secondary metabolites. In case of isolates: #2HKKA, #13HKKA, #1HKSCN, #4HKSCN and #5HKSCN not much change in pH was observed.

S.No.	Culture Code	Final volume(ml)	pH
1.	#1HKKA	19.4	7.13
2.	#2HKKA	19.7	7.08
3.	#3HKKA	18.9	6.9
4.	#4HKKA	19.8	7.3
5.	#5HKKA	14.5	5.94
6.	#6HKKA	19.3	6.83
7.	#7HKKA	19.7	6.91
8.	#8HKKA	19.1	7.23
9.	#9HKKA	18.9	6.76
10.	#10HKKA	19.4	6.8
11.	#11HKKA	19.1	6.76
12.	#12HKKA	19.2	7.15
13.	#13HKKA	18.8	7.1
14.	#14HKKA	19.7	6.78
15.	#15HKKA	18.4	7.2
16.	#1HKSCN	13.7	7.05
17.	#2HKSCN	14.3	6.65
18.	#3HKSCN	13	6.5
19.	#4HKSCN	19	7.1
20.	#5HKSCN	18.5	7.08
21.	#6HKSCN	12	6.75
22.	#7HKSCN	14.4	7.08
23.	#8HKSCN	19.2	7.24
24.	#9HKSCN	14.2	7.22
25.	#10HKSCN	19	7.23
26.	#11HKSCN	13.7	7.94
27.	#12HKSCN	14.4	6.9

Table no. 10: Final volume and pH of culture filtrates of all isolates

5.4 Preliminary Screening for anti-staphylococcal activity

Broth Testing: The culture filtrates obtained after submerged fermentation of isolates were examined for their anti staphylococcal potential by agar well diffusion assay against standard staphylococcus aureus isolate obtained from National Cell type culture collection coded as NCTC 6571. None of the culture filtrates posses any anti staphylococcal potential. There was no zone of inhibition formed by the culture filtrates (Fig. 6). The aqueous phase has not shown any antimicrobial activity thereby solvent phase was examined for antimicrobial activity. Many antimicrobial compounds are water immiscible but have excellent solubility in organic solvents. Extraction separates the aqueous phase from organic phase and provides concentrated and purified products. Thus solvent extraction of filtrate was carried out by ethyl acetate to isolate crude bioactive compound.

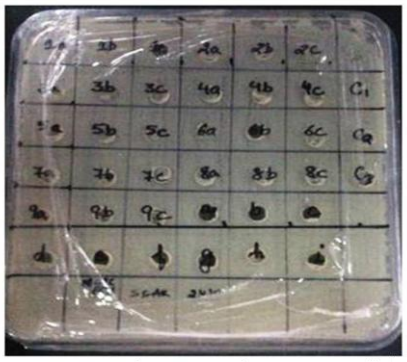


Fig. 6. Broth testing by agar well diffusion assay for antimicrobial activity

Solvent Extraction: The culture filtrates of all the isolates were extracted with ethyl acetate (Gurung *et al.,* 2009; Cwala *et al.,* 2011). Maximum yield was obtained in case of #11HKSCN and minimum was obtained in case of #1HKSCN and #9HKSCN (Table no. 11). Stock solution of extracts were prepared in methanol: ethyl acetate in concentration of 1 mg/ml and was stored at -20°C till further use.

S.No.	Culture Code	Yield (mg)
1	#1HKKA	1.1
2	#2HKKA	1.3
3	#3HKKA	0.9
4	#4HKKA	0.6
5	#5HKKA	0.6
6	#6HKKA	0.7
7	#7HKKA	1.5
8	#8HKKA	1.2
9	#9HKKA	0.9
10	#10HKKA	0.8
11	#11HKKA	0.4
12	#12HKKA	1.4
13	#13HKKA	0.6

14	#14HKKA	1.5
15	#15HKKA	1.2
16	#1HKSCN	0.2
17	#2HKSCN	0.7
18	#3HKSCN	0.9
19	#4HKSCN	0.5
20	#5HKSCN	1.1
21	#6HKSCN	0.9
22	#7HKSCN	0.4
23	#8HKSCN	0.7
24	#9HKSCN	0.2
25	#10HKSCN	0.8
26	#11HKSCN	1.7
27	#12HKSCN	1.1

Table no. 11: showing the yield of the extracted bioactive compound

Antimicrobial testing of the crude residue: The crude ethyl acetate extracts were then examined for anti-staphylococcal activity by agar well diffusion method. The crude extracts were tested against Sau NCTC 6571. Out of 27 isolates subjected to preliminary screening, visible zone of inhibition was observed in case of #2HKSCN, #3HKSCN, #6HKSCN, #11HKSCN and #12HKSCN (Table no.12). The highest anti-staphylococcal activity was shown by #6HKSCN with mean zone diameter 13.33 mm whereas #2HKSCN, #3HKSCN and #12HKSCN showed intermediate activity having mean zone diameter between 10 and 13 mm and #11HKSCN has least activity with mean zone diameter 9.33 mm. These bioactive isolates were then subjected to secondary screening.

S.No.	Culture Code	Zone of inhibition (in mm)
1	#1HKKA	no zone
2	#2HKKA	no zone
3	#3HKKA	no zone
4	#4HKKA	no zone
5	#5HKKA	no zone
6	#6HKKA	no zone
7	#7HKKA	no zone
8	#8HKKA	no zone
9	#9HKKA	no zone
10	#10HKKA	no zone
11	#11HKKA	no zone

12	#12HKKA	no zone
13	#13HKKA	no zone
14	#14HKKA	no zone
15	#15HKKA	no zone
16	#1HKSCN	no zone
17	#2HKSCN	12
18	#3HKSCN	10
19	#4HKSCN	no zone
20	#5HKSCN	no zone
21	#6HKSCN	13.33
22	#7HKSCN	no zone
23	#8HKSCN	no zone
24	#9HKSCN	no zone
25	#10HKSCN	no zone
26	#11HKSCN	9.33
27	#12HKSCN	13

Table no.12: showing anti-staphylococcal potential of crude residues

5.5 Mass Production

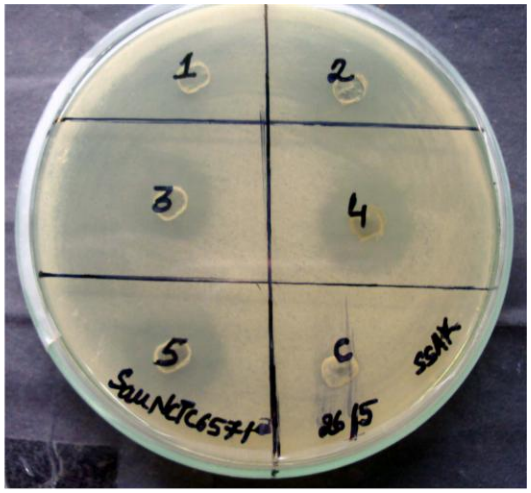
Out of the 27 isolates, the 5 isolates showing anti-staphylococcal potential were subjected to mass production of culture filtrate. The reduction in pH of culture filtrates was observed for all the strains except in case of #11HKSCN where culture filtrate becomes basic in nature with pH 7.9.The maximum reduction in pH was observed in case #12HKSCN strain having pH 5.79. The culture filtrate of all the isolates was then extracted with ethyl acetate for the recovery of crude bioactive compound. The maximum yield of residue was obtained from #3HKSCN i.e. 91 mg and minimum yield was obtained in case of isolate #6HKSCN i.e. 74 mg. (Table no. 13.).

S.No	Culture Code	Initial volume(ml)	Final volume(ml)	pH	Yield(mg)
1.	#2HKSCN	500	480	6.21	77
2.	#3HKSCN	500	470	5.97	91
3.	#6HKSCN	500	480	6.65	74
4.	#11HKSCN	500	467	7.7	80.1
5.	#12HKSCN	500	473	5.79	85.9

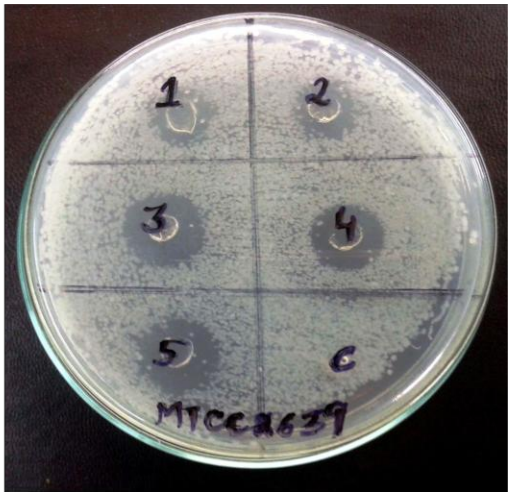
Table no. 13: Production and extraction of secondary metabolites of bioactive actinomycetes

5.6 Agar well diffusion assay

The crude bioactive compounds obtained from selected 5 isolates were tested against Sau NCTC 6571, Sau MTCC96 and Sep MTCC 2639 by agar well diffusion method. #12HKSCN and #3HKSCN possesses antimicrobial potential against all three test organisms i.e. Sau NCTC6571, Sau MTCC96 and Sep MTCC 2639 where as #2HKSCN, #6HKSCN and #11HKSCN showed antimicrobial activity only against Sau NCTC 6571 and Sep MTCC 2639 (Fig 7). The mean zone of inhibition against Sau NCTC 6571 ranges from 12.3 mm (#6HKSCN) to 18.33 mm (#11HKSCN), against MTCC 2639 ranges from 10.66 mm (#2HKSCN) to 16 mm (#12HKSCN) and against MTCC 96 it was 14.6 mm (#3HKSCN). Table no 14 and 15 shows mean zone diameter of inhibition (in mm) and area of inhibition (in mm^2) respectively. Suchitra Sanasam *et al.,* 2011 has isolated 207 actinomycetes from freshwater Loktak lake, Manipur and from these 207 isolates, crude ethyl acetate extracts of nine isolates showed antimicrobial activities with zone size ranging from 11 to 24 mm. Cwala *et al.,* 2011 studied antibacterial activities of crude ethyl extracts of actinomycetes against both Gram-negative and Gram-positive bacteria with zones of inhibition ranging between 2 and 27 mm (NB 034); 9 and 15 mm (TR 007); 8 and 13 mm (NB 069).



Well 1: #2HKSCN, Well 2: #3 HKSCN,
Well 3 #6 HKSCN, Well 4: #11 HKSCN,
Well 5: #12 HKSCN, Well C: Control.



Well 1: #2HKSCN, Well 2: #3 HKSCN,
Well 3 #6 HKSCN, Well 4: #11 HKSCN,
Well 5: #12 HKSCN, Well C: Control.

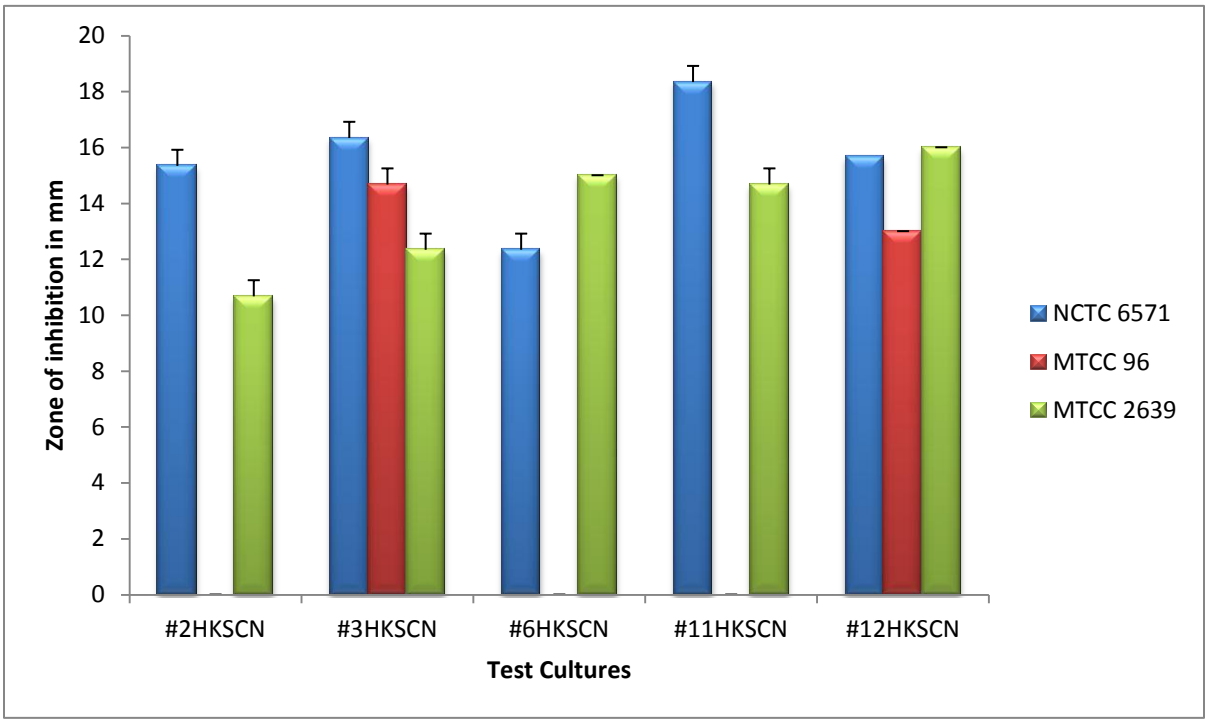
Fig. 7 Antimicrobial potential of EA extract of actinomycetes in agar well diffusion assay

Culture Code	Mean zone of inhibition in mm against test cultures		
	NCTC 6571	**MTCC 96**	**MTCC 2639**
#2HKSCN	15.33	0	10.66
#3HKSCN	16.33	14.66	12.33
#6HKSCN	12.33	0	15
#11HKSCN	18.33	0	14.66
#12HKSCN	15.66	13	16

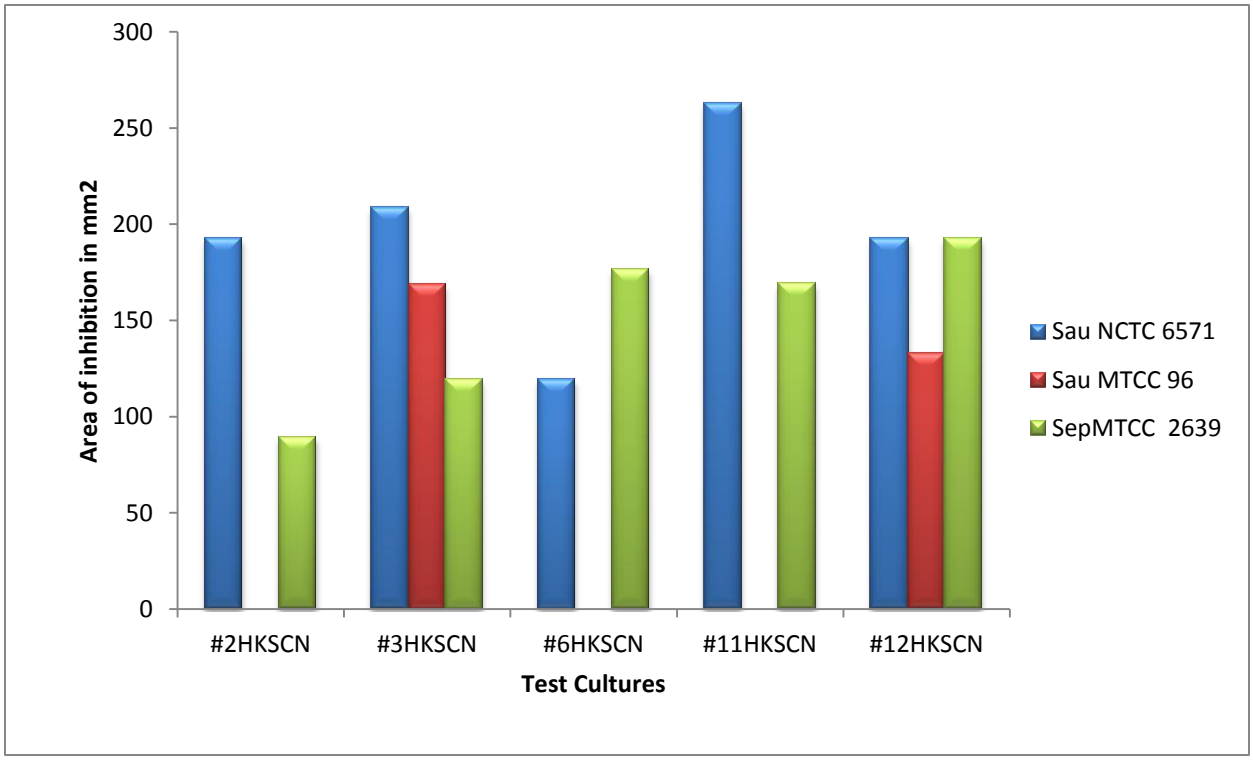
Table no. 14: Showing Zone of inhibition (in mm) in agar well diffusion assay

Culture Code	Mean area of inhibition in mm^2 against test Organism		
	NCTC 6571	MTCC 96	MTCC 2639
#2HKSCN	192.75	0	89.37
#3HKSCN	208.56	168.7	119.34
#6HKSCN	119.34	0	176.6
#11HKSCN	262.88	0	168.93
#12HKSCN	192.75	132.66	192.75

Table no. 15: Showing mean area of inhibition (in mm^2) against test organism.



Graph 1: Zone of inhibition of EA extract (in mm) in agar well diffusion assay against three test organism



Graph 2: Area of inhibition of EA extract (in mm) in agar well diffusion assay against three test organism

5.7 KB Disc diffusion assay

The anti-staphylococcal activity of crude extracts was also tested by disc diffusion assay. #3HKSCN and #12HKSCN were active against all three test organisms i.e. Sau NCTC 6571, Sau MTCC 96 and Sep MTCC 2639 where as #6HKSCN and #11HKSCN showed activity only against Sau NCTC 6571 and Sau MTCC 2639 and #2HKSCN was active only against Sep MTCC 2639. (Fig 8).The maximum zone of inhibition observed against NCTC 6571 was 13 mm (#12HKSCN), against MTCC 2639 was 20.33 mm (#6HKSCN) and against MTCC 96 was 12.33mm (#3HKSCN). Table no 16 and 17 shows mean inhibition zone diameter (in mm) and area of inhibition (in mm^2). Kumar *et al.,* 2011 studied the antimicrobial activity of marine actinobacteria by disk diffusion method and reported that actinobacteria showed antimicrobial potential with zone of inhibition between 10 and 14 mm.

Fig.8. Antimicrobial potential of EA extract of actinomycetes in KB disc assay

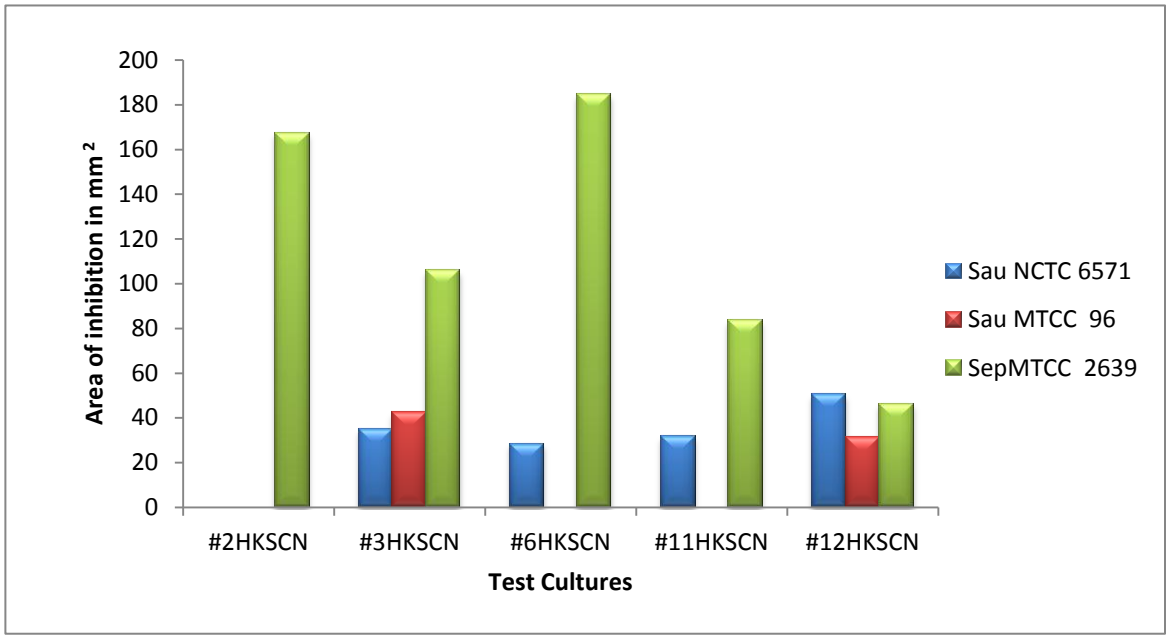
Culture Code	Mean zone of Inhibition (in mm) against test organisms		
	NCTC 6571	MTCC 96	MTCC 2639
#2 HKSCN	0	0	19.66
#3 HKSCN	11.66	12.33	16.66
#6 HKSCN	11	0	20.33
#11 HKSCN	11.66	0	15.33
#12 HKSCN	13	11.66	12.66

Table no. 16: Showing Mean Zone of inhibition (diameter in mm) against test organisms.

Culture Code	Mean area of inhibition in mm^2 against test Organism		
	NCTC 6571	MTCC 96	MTCC 2639
#2HKSCN	0	0	167.3
#3HKSCN	34.88	42.2	105.6
#6HKSCN	28.26	0	184.56
#11HKSCN	31.49	0	83.4
#12HKSCN	50.24	31.16	46.14

Table no. 17: Showing mean area of inhibition (area in mm^2) against test organisms.

Graph 3: Zone of inhibition (in mm) of EA extract of the actinomycetes against three test organisms in KB disc assay



Graph 4: Area of inhibition (in mm) of EA extract of the actinomycetes against three test organisms in KB disc assay

5.8 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) is considered to be gold standard method. MIC is the lowest concentration at which growth of the organism is completely inhibited (Goomber and Saxena, 2007). For determining the MIC two fold dilutions were prepared as shown in the template.

Template for MIC ⟶

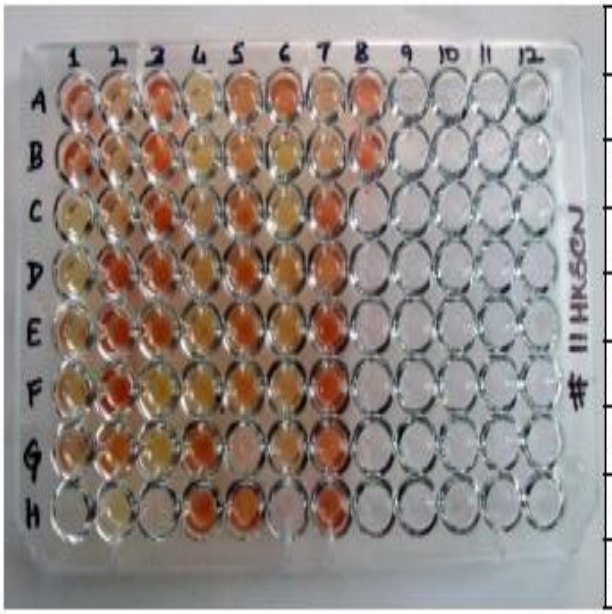
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	C_1O_1	S_3O_1	S_7O_1	S_2O_2	S_6O_2	C_3O_3	S_4O_3	S_8O_3				
B	C_1O_1	S_4O_1	S_8O_1	S_2O_2	S_6O_2	S_1O_3	S_4O_3	S_8O_3				
C	S_1O_1	S_4O_1	S_8O_1	S_3O_2	S_7O_2	S_1O_3	S_5O_3					
D	S_1O_1	S_5O_1	C_2O_2	S_3O_2	S_7O_2	S_2O_3	S_5O_3					
E	S_2O_1	S_5O_1	C_2O_2	S_4O_2	S_8O_2	S_2O_3	S_6O_3					
F	S_2O_1	S_6O_1	S_1O_2	S_4O_2	S_8O_2	S_3O_3	S_6O_3					
G	S_3O_1	S_6O_1	S_1O_2	S_5O_2	Blank	S_3O_3	S_7O_3					
H	Blank	S_7O_1	Blank	S_5O_2	C_3O_3	Blank	S_7O_3					

O_1 ⟶ Sau NCTC 6571 O_2 ⟶ MTCC 96 O_3 ⟶ MTCC 2639
O_1, O_2, O_3: Test organisms (*Staphylococcus sp*)
1: Concentration 1, S2: Concentration 2, S3: Concentration 3, S4: Concentration 4,
S5: Concentration 5, S6: Concentration 6, S7: Concentration 7, S8: Concentration 8,
C1: Control 1, C2: Control 2, C3: Control 3

8 Different concentrations were prepared from the stock solution of 44.3 mg/ml of EA extract of #11 HKSCN shown in table no. 18. Lt exhibited highest anti-staphylococcal activity with MIC in range of 0.4-0.8 mg/ml.

S.No.	Conc.	Stock (µl)	Diluent (µl)	Conc. (mg/ml)	Effective Conc. (mg/200µl)	Effective Conc. (mg/ml)	MIC in mg/ml
1	S_1	500	500	22.15	0.66	3.3	NCTC 6571= 0.4
2	S_2	500	500	11	0.33	1.6	
3	S_3	500	500	5.5	0.165	0.8	
4	S_4	500	500	2.8	0.08	0.4	MTCC 96 = 0.4
5	S_5	500	500	1.38	0.04	0.2	
6	S_6	500	500	0.7	0.02	0.1	
7	S_7	500	500	0.35	0.01	0.05	MTCC 2639 =0.8
8	S_8	500	500	0.17	0.005	0.025	

Table no. 18: Showing different concentrations of crude extract of #11HKSCN and MIC against three test organisms



	1	2	3	4	5	6	7	8	9	10	11	12
A	G	NG	G	NG	G	G	G	G				
B	G	NG	G	NG	G	NG	G	G				
C	NG	NG	G	NG	G	NG	G					
D	NG	G	G	NG	G	NG	G					
E	NG	G	G	NG	G	NG	G					
F	NG	G	NG	NG	G	NG	G					
G	NG	G	NG	G	B	NG	G					
H	B	G	B	G	G	B	G					

Fig 9: showing *In vitro* broth dilution assay for minimal inhibitory concentration of EA extract of #11HKSCN

In case of **#3HKSCN** the stock of the bioactive compound was prepared from stock solution of 53 mg/ml and then diluted to 8 different concentrations as shown in the Table no. 19. The MIC was found to be in the range of 0.49- 1.98 mg/ml (Fig 10).

S.No.	Conc.	Stock (µl)	Diluent (µl)	Conc. (mg/ml)	Effective Conc. (mg/200µl)	Effective Conc. (mg/ml)	MIC in mg/ml
1	S_1	500	500	26.5	0.795	3.98	NCTC 6571= 0.49
2	S_2	500	500	13.25	0.397	1.98	
3	S_3	500	500	6.6	0.198	0.99	
4	S_4	500	500	3.3	0.099	0.49	MTCC 96 = 0.49
5	S_5	500	500	1.6	0.048	0.24	
6	S_6	500	500	0.8	0.024	0.12	
7	S_7	500	500	0.4	0.012	0.06	MTCC 2639 = 1.98
8	S_8	500	500	0.2	0.006	0.03	

Table no. 19: showing different concentrations of crude extract of #3HKSCN and MIC against three test organisms

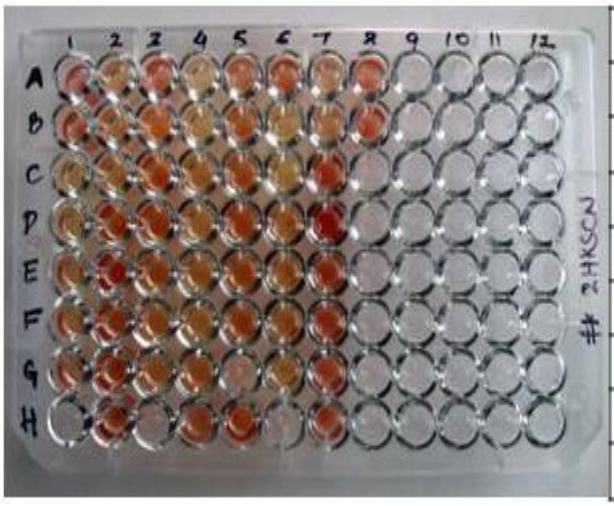
	1	2	3	4	5	6	7	8	9	10	11	12
A	G	NG	G	NG	G	G	NG	G				
B	G	NG	G	NG	G	NG	NG	G				
C	NG	NG	G	G	G	NG	G					
D	NG	G	G	G	G	NG	G					
E	NG	G	G	G	G	NG	G					
F	NG	G	NG	G	G	NG	G					
G	NG	G	NG	G	B	NG	G					
H	B	G	B	G	G	B	G					

Fig10: showing *In vitro* broth dilution assay for minimal inhibitory concentration of EA extract of #3HKSCN

In case of **#2HKSCN**, 61.1 mg/ml stock was diluted to different concentrations (Table no.20) and it showed the least anti-staphylococcal activity with MIC above 4.6 mg/ml against Sau NCTC 6571 ; 1.15 mg/ml against Sau MTCC 96 and 0.57 mg/ml against Sep MTCC 2639.(Fig 11).

S.No.	Conc.	Stock (µl)	Diluent (µl)	Conc. (mg/ml)	Effective Conc. (mg/200µl)	Effective Conc. (mg/ml)	MIC in mg/ml
1	S_1	500	500	30.55	0.92	4.6	NCTC 6571= above 4.6
2	S_2	500	500	15.3	0.46	2.3	
3	S_3	500	500	7.6	0.23	1.15	
4	S_4	500	500	3.8	0.11	0.57	
5	S_5	500	500	1.9	0.057	0.28	MTCC 96=1.15
6	S_6	500	500	0.95	0.028	0.14	
7	S_7	500	500	0.48	0.014	0.07	
8	S_8	500	500	0.24	0.007	0.035	MTCC 2639 = 0.57

Table 20: showing different concentrations of crude extract of #2HKSCN and MIC against three test organisms.



	1	2	3	4	5	6	7	8	9	10	11	12
A	G	G	G	NG	G	G	NG	G				
B	G	G	G	NG	G	NG	NG	G				
C	G	G	G	NG	G	NG	G					
D	G	G	G	NG	G	NG	G					
E	G	G	G	G	G	NG	G					
F	G	G	NG	G	G	NG	G					
G	G	G	NG	G	B	NG	G					
H	B	G	B	G	G	B	G					

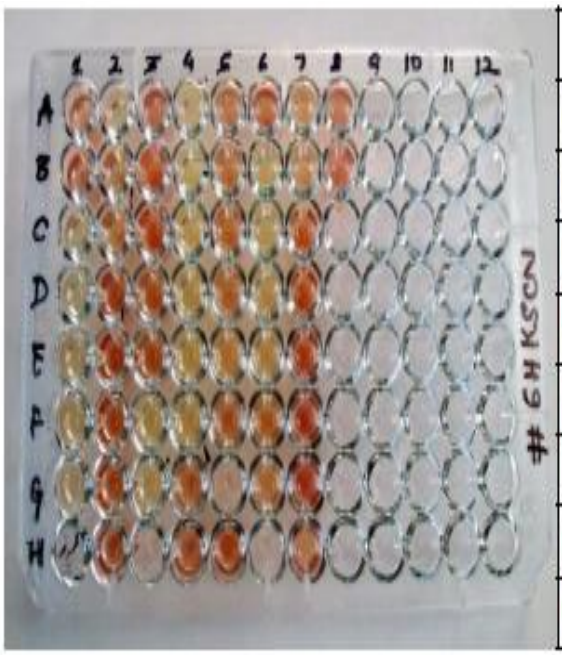
Fig. 11 *Invitro* broth dilution assay for MIC of EA extracts of #2 HKSCN.

From ethyl acetate residue of #6HKSCN, Stock concentration of 66 mg/ml was prepared as shown in Table no. 21. It was further diluted into various concentration and then subjected for MIC estimation. It exhibited intermediate activity against all three test organisms with MIC ranging from 0.5 to 2.5 mg/ml (Fig 12 and 13).

S.No.	Conc.	Stock (µl)	Diluent (µl)	Conc. (mg/ml)	Effective Conc. (mg/200µl)	Effective Conc. (mg/ml)	MIC in mg/ml
1	S_1	500	500	28.65	0.86	4.3	NCTC 6571= 0.5
2	S_2	500	500	14.3	0.4	2	

3	S_3	500	500	7.2	0.2	1	
4	S_4	500	500	3.6	0.1	0.5	MTCC 96 = 0.5
5	S_5	500	500	1.8	0.05	0.25	
6	S_6	500	500	0.9	0.025	0.125	
7	S_7	500	500	0.45	0.01	0.05	MTCC 2639 = 2
8	S_8	500	500	0.22	0.005	0.025	

Table no. 21: showing different concentrations of crude extract of #6HKSCN and MIC against three test organisms



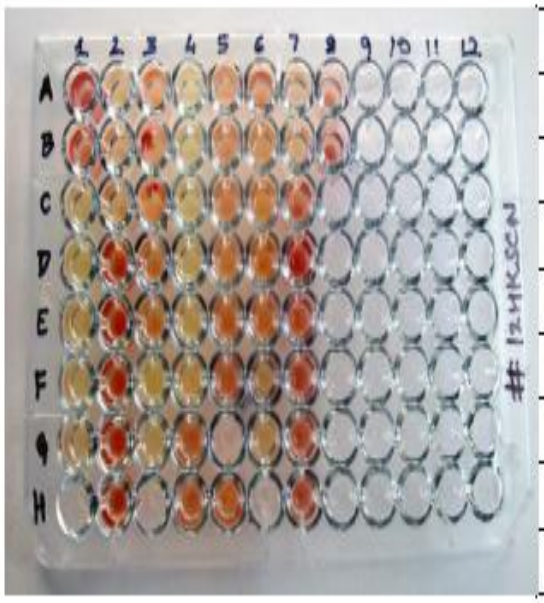
	1	2	3	4	5	6	7	8	9	10	11	12
A	G	NG	G	NG	G	G	G	G				
B	G	G	G	NG	G	NG	G	G				
C	NG	G	G	NG	G	NG	G					
D	NG	G	G	NG	G	NG	G					
E	NG	G	G	NG	G	NG	G					
F	NG	G	NG	NG	G	G	G					
G	NG	G	NG	G	B	G	G					
H	B	G	B	G	G	B	G					

Fig.12. *In vitro* broth dilution assay for MIC of EA extracts of #6 HKSCN

From ethyl acetate residue of **#12HKSCN**, Stock concentration of 57.3 mg/ml was prepared which was firther diluted into different concentration. (Table No. 22) . It exhibited intermediate activity against all three test organisms with MIC ranging from 0.5 to 2.5 mg/ml (Fig 13)

S.No.	Conc.	Stock (µl)	Diluent (µl)	Conc. (mg/ml)	Effective Conc. (mg/200µl)	Effective Conc. (mg/ml)	MIC in mg/ml
1	S_1	500	500	33	0.99	4.95	NCTC 6571= 1.25
2	S_2	500	500	16.5	0.5	2.5	
3	S_3	500	500	8.25	0.25	1.25	
4	S_4	500	500	4.125	0.125	0.6	MTCC 96 = 0.6
5	S_5	500	500	2.06	0.06	0.3	
6	S_6	500	500	1	0.03	0.15	MTCC 2639 = 2.5
7	S_7	500	500	0.5	0.015	0.07	
8	S_8	500	500	0.25	0.007	0.035	

Table no. 22: showing different concentrations of crude extracts of #12HKSCN and MIC against three test organisms



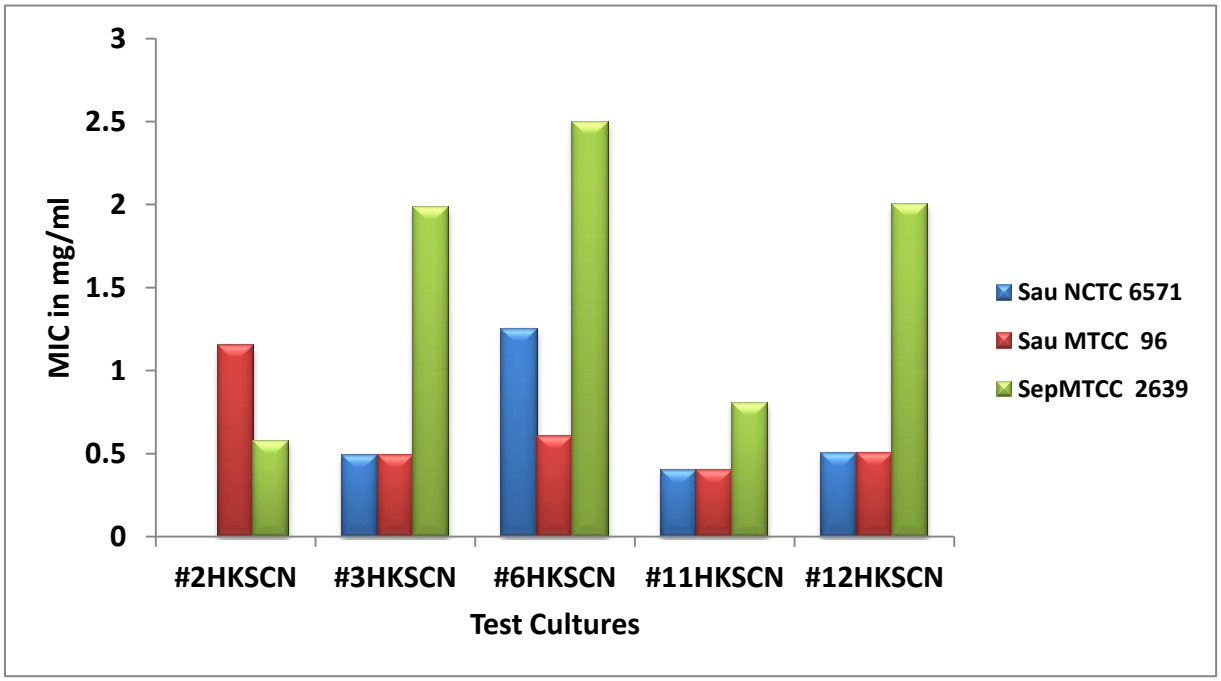
	1	2	3	4	5	6	7	8	9	10	11	12
A	G	NG	G	NG	G	G	G	G				
B	G	NG	G	NG	G	NG	G	G				
C	NG	NG	G	NG	G	NG	G					
D	NG	G	G	NG	G	NG	G					
E	NG	G	G	NG	G	NG	G					
F	NG	G	NG	NG	G	G	G					
G	NG	G	NG	G	B	G	G					
H	B	G	B	G	G	B	G					

Fig.13. *In vitro* broth dilution assay for MIC of EA extracts of #12 HKSCN

The TTC (2, 3, 5 triphenyl tetrazolium chloride salt) is redox metabolic indicator and in living cells is enzymatically reduced to pink colored formazan. After the addition of TTC, appearance of pink color indicates growth and the minimum concentration at which growth was inhibited (no pink color) represents the MIC. The MIC of the crude residues against the test organisms ranges from 0.4 to 2.5 mg/ml (Table no. 23) and the comparative MIC profile of all the crude extracts against test organisms is shown in Graph 5.

Culture Code	MIC of EA extract against the test organisms		
	Sau NCTC 6571	Sau MTCC 96	Sep MTCC 2639
#2HKSCN	Above 4.6 mg/ml	1.15	0.57
#3HKSCN	0.49	0.49	1.98
#6HKSCN	1.25	0.6	2.5
#11HKSCN	0.4	0.4	0.8
#12HKSCN	0.5	0.5	2

Table no. 23: showing the MIC of crude extract against test organisms



Graph 5: *In vitro* MIC of crude ethyl acetate extracts against test organisms.

Cwala *et al.,* 2011 reported that minimum inhibitory concentration (MIC) of the crude ethyl extracts of four aquatic actinomycetes against the test bacteria ranged from 0.039 to 5 mg/ml. Timothy *et al.,* 2010 studied that three freshwater actinomycetes showed antibacterial activity with MIC ranged from 0.078 to 10 mg/ml.

5.5 Characterization of Actinomycetes

The colony characteristics and microscopic characteristics of bioactive isolates are shown in Table no 24 (Fig 14). The colonies exhibited wide range of colors ranging from white (#11 HKSCN) to pink (#2 HKSCN and #3HKSCN), yellow (#6HKSCN) and orange (#12HKSCN).

S. No.	Culture Code	Colony characteristics	Microscopic characteristics	Inference
1	#2HKSCN	Pale pink pin point colonies	Gram positive rods	Actinomycetes, (Arifuzzaman, 2010)
2	#3HKSCN	Pink colonies	Gram positive cocci	Actinomycetes, (Arifuzzaman, 2010)
3	#6HKSCN	Yellow slimy colonies	Gram positive rods	Actinomycetes, (Arifuzzaman, 2010)
4	#11HKSCN	White colonies ,light yellow pigment produced	Gram positive rods	Actinomycetes, (Arifuzzaman, 2010)
5	#12HKSCN	Orange colonies	Gram negative rods	Actinomycetes, (Arifuzzaman, 2010)

Table no. 24. Showing Morphological and cultural characteristics of isolates

Shumei *et al.,* 2007 reported the isolation of 62 actinomycetes from the agriculture soils of Semongok, Sarawak exhibiting wide range of colony colors (dark grey, grey, brownish, white and yellowish white).

Fig 14. showing the colony color of the water borne actinomycetes

The micromorphology of actinomycetes was studied using Gram staining method. Isolates #2HKSCN, #3HKSCN, #6HKSCN and #11HKSCN showed Gram positive character whereas #12HKSCN showed Gram negative character (Fig15-19). Thus actinomycetes has been identified by the presence of colored colonies on the surface of agar plates. Valli *et al.,* 2011 identified actinomycetes macroscopically and microscopically by Gram staining. Padma *et al.,* 2012 has isolated actinomycetes from soil that stain Gram positive to Gram variable.

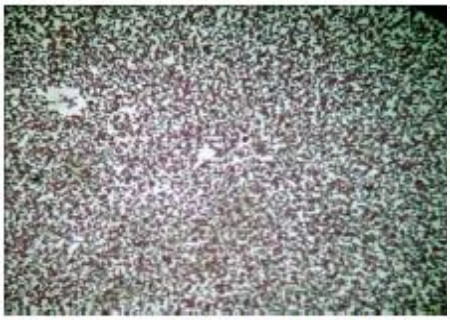


Fig 15. Gram's staining of #2 HKSCN

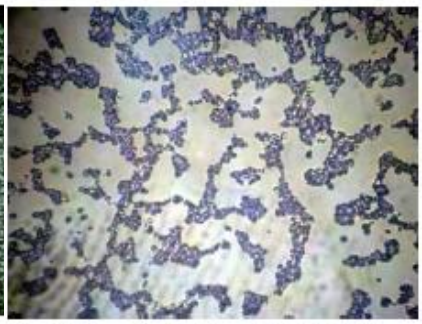


Fig 16. Gram's staining of #3 HKSCN

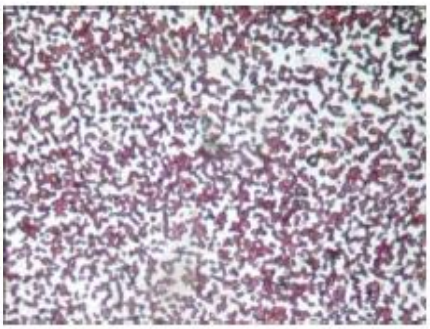


Fig17 . Gram's staining of #6 HKSCN

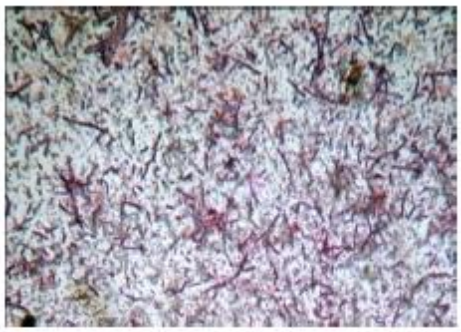


Fig 18. Gram's staining of #11 HKSCN

Fig 19. Gram's staining of #12 HKSCN

Biochemical Characterization:

Bioactive isolates were screened for the production of three enzymes amylase, pectinase and cellulase. All the bioactive isolates except #2HKSCN were found to produce amylase. #6HKSCN exhibited highest starch hydrolysis activity where as #3HKSCN showed minimum starch hydrolysis activity (Fig 20).Pectin is hydrolyzed by pectinase and CMC is hydrolyzed by cellulase enzyme, thus organisms that can hydrolyze pectin and CMC are positive for production of pectinase and cellulase enzyme respectively. None of the isolates showed pectin hydrolysis and CMC utilization activity (Table no.25) (Fig 21 and 22).

S.No.	Culture code	Starch hydrolysis	Pectin hydrolysis	CMC utilization
1	#2HKSCN	**-**	**-**	**-**
2	#3HKSCN	**+**	**-**	**-**
3	#6HKSCN	**++++**	**-**	**-**
4	#11HKSCN	**++**	**-**	**-**
5	#12HKSCN	**+++**	**-**	**-**

(++++: Maximum; +++: Good; ++: Moderate; +: Positive; - refers Negative)

Table no. 25. Biochemical Characterization for production of novel enzymes.

Abbas, 2006 has isolated 60 halototolerant actinomycetes that can degrade starch and can utilize it as sole carbon source. Bruhlmann *et al.,* 1994 screened 79 actinomycetes isolated from soil and compost samples for the production of pectinolytic enzymes and reported that 66 isolates produce pectinolytic enzymes. Salahudin *et al.,* 2012 reported that two mesophilic strains of actinomycetes produce three components of cellulase complex (endoglucanase, exoglucanase and β – glucosidase)

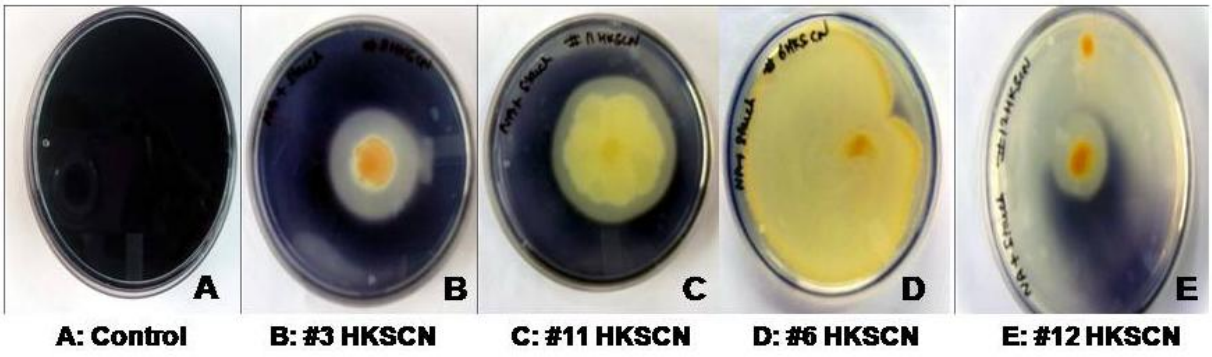


Fig. 20 Screening for amylase production by water borne actinomycetes.

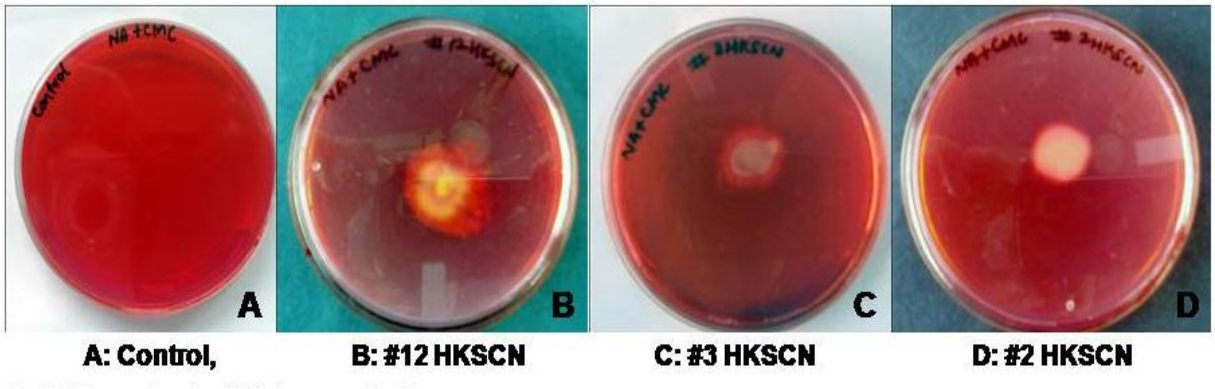


Fig. 21 Screening for cellulase production by water borne actinomycetes.

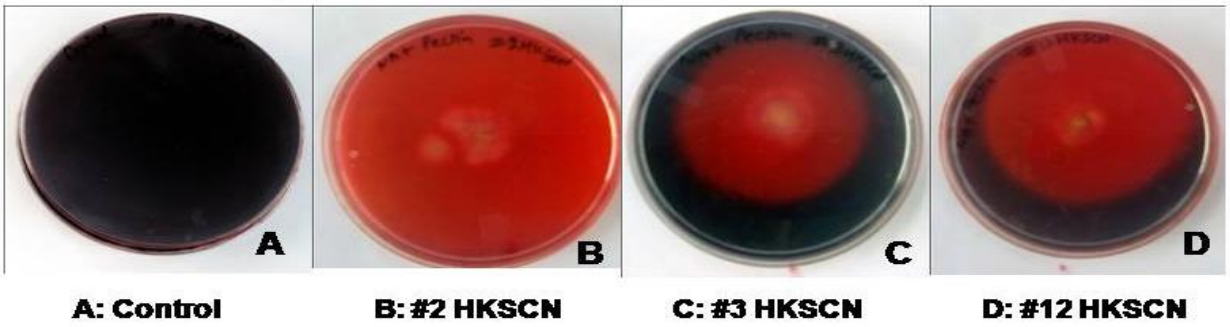


Fig. 22 Screening for pectinase production by water borne actinomycetes.

Chapter 6

Conclusion

Conclusion

27 actinomycetes isolates were recovered from Harike wetland water sample. Only 5 isolates (#2HKSCN, 3HKSCN, #6HKSCN, #11HKSCN and #12HKSCN) exhibited antistaphylococcal activity which was confirmed using agar well diffusion, KB disc diffusion and *in vitro* broth dilution assay against NCTC 6571, MTCC 96 and MTCC 2639.The MIC range of all the five isolates was in between 0.4 to 2.5 mg/ml. Further these isolates also exhibited amylase activity but do not exhibited pectinase and cellulase activity. Microscopic examination of four actinomycetes (#2HKSCN, #3HKSCN, #6HKSCN, #11HKSCN) showed that they were Gram positive with coccus and rod shape except one isolate i.e. #12HKSCN which was Gram negative.

Further studies on purification, characterization along with phylogenetic placement of the biologically active actinomycetes will open a new biome in drug development and industrial approaches.

Chapter 7

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