Chapter II

A review of literature available on isolation methods and bioprospects of actinomycetes from various habitats

This chapter provides review of strategies adopted by various researchers to isolate actinomycetes and the prospecting for various biomolecules and also serves as a source of information on various methodologies with critical analysis based on reports from researchers.

2.1. Development of methods- the timeline

The earlier work on actinomycetes concentrated on development of isolation techniques and their improvement. Pioneering work on isolation of actinomycetes, especially media compositions, still finds successful application in research today. The work on improvement on isolation strategies kept modifying with the intent of research and habitats explored. This is especially true in case of the paradigm shift we talk about. In totality, the research on culturable actinomycetes from the past four decades has seen a very little change of pattern when it comes to isolation.

The favorite habitat for isolation of actinomycetes has traditionally been the terrestrial habitat. Much of the earlier work which focused on isolation of actinomycetes from soil had a far reaching applicability and served as the basis of modifications for isolation from other habitats also. The historical perspectives and the earlier pioneering works find mention in a book titled ‘The Actinomycetes’ by Selman A. Waksman (Waksman, 1959). If we look at the literature, by the middle of the 20th century, research got established in terms of technique- isolation media and plating. This acceptance and continued use of the dilution-plating based methods was due to the relative merit of the principle they employed- nutrition. However, at the same time, a challenge researchers were dealing with was the fungal and bacterial contamination in soil. This made isolation of actinomycetes a difficult task. Selective...
media were designed based on the nutritional preferences of actinomycetes. The preferred choice of Carbon and Nitrogen components in the media were selective for actinomycetes. Taking into consideration the media available and introducing some modifications, a selection of 8 defined media for characterization of actinomycetes by collaborators was grouped under the International Streptomyces Project (Gottlieb and Shirling, 1967; Pridham and Gottlieb, 1948) Several authors around the same time were working on other methods to combat bacterial and fungal contamination. These involved the pre-treatment of samples from which the actinomycetes were to be isolated and the use of antibiotics as supplements to media. Finally, the strategy that can be summed up as most successful is the one which employed multiple pre-treatment methods and more than one media to obtain a better representation of diversity of actinomycetes within a sample.

2.2. Media used for isolation of actinomycetes

The most commonly used media used for actinomycetes include Starch Casein Agar (Küster and Williams, 1964); Collidal Chitin Agar (Hsu and Lockwood, 1975); glycerol l-arginine agar (Benedict et al., 1955); glycerol-glycine medium (Lindenbein, 1952); arginine-glycerol-salt medium (El-Nakeeb and Lechevalier, 1963); humic acid-vitamin agar (HVA) and HHVA i.e., hair hydrolysate vitamin agar (Hayakawa and Nonomura, 1987). Some specialized media introduced were the media M1 through M5 (Mincer T. et al., 2002); Minimal Medium (Albarracín V. H. et al., 2005); soil extract agar medium (Hamaki et al., 2005), etc.

It is also possible that a medium may be reported to have a disadvantage from one author, but would be reported suitable for isolation of actinomycetes from another. One example, as with humic acid vitamin agar, we can cite contrasting references: in two geographically different studies: one on humus layer forest soil samples collected from Mt. Baekun, Korea (Seong C. et al., 2001) and other on soil samples from Karwar, India (Naikpatil and Rathod, 2011); HHVA was found to be the most efficient medium in isolation of rare actinomycete genera. The authors in the first study reported shortcomings of HVA. But in another study, HVA was successfully
employed in isolating *Streptomyces* sp. with antimicrobial and cytotoxic activity from Lonar soda lake of India (Kharat K. R. *et al.*, 2009).

Thus, media are modified depending on the aims of the study by researchers. The modifications include addition of soil/sediment extracts to make the media ‘habitat based’ and/or addition of selective inhibitory agents to check bacterial and fungal contaminations.

### 2.3. Selective inhibitory agents and supplements incorporated in isolation media

Isolation involving use of antibiotics as selective agents was investigated to curb the menace of fungal contamination (Beech and Carr, 1955; Phillips and Hanel, 1950). A potent antifungal agent piramicin (A-5283) was isolated and successfully employed in decontaminating fungus infested *Streptomycete* soil samples from over 521 samples worldwide. A complete decontamination and a yield of *Streptomyces* ranging from $10^4$ to $10^8$ cfu/ml was obtained with 50µg per ml concentration. Since piramicin (A-5283) is insoluble in water, it was dissolved in N, N-dimethylformaldehyde prior to use. The authors also reported the same concentration of nystatin and cycloheximide to be effective (Porter *et al.*, 1960).

A comparative study involving use of antibiotics for selective isolation revealed antifungal antibiotics nystatin and actidione gave an average yield of 165 bacterial colonies per plate. A comparative study involving use of antibiotics for selective isolation also revealed the ‘most satisfactory’ media and antibiotic combination. They tested several combinations and reported that SCA in combination with antifungal antibiotics nystatin and actidione gave an average yield of 165 bacterial colonies per plate. The researchers reported opalescence of CCA as a shortcoming, as it could not allow detection of pin point colonies. It was also pointed out that the chitin medium did not allow pigmentation from actinomycetes (Williams and Davies, 1965).
Importance of role of habitat based media in isolation of novel actinomycetes was demonstrated in a research on a Japanese forest larch sample. The sample forest soil was used to prepare soil extract agar medium supplemented with 50µg/ml cycloheximide. The strategy adopted was selection of colonies that showed obligate dependence on soil agar and could not grow on other media. Such colonies were subject to analysis and were found to be novel and were proposed to be new species within *Actinomadura*, *Streptosporangium* and *Acrocarpospora* (Hamaki *et al.*, 2005). One study has also reported employed nalidixic acid as selective inhibitory agent will be dealt with in detail later (Takizawa *et al.*, 1993).

### 2.4. The pre-treatment methods

Though habitats are different, the samples from which actinomycetes have been isolated are basically variants of ‘soil types’- from forest soil to sediments from ocean or rivers. Microbial entrapment in soil aggregates is a phenomenon which retains microbes within soil. It hence becomes crucial to use techniques which detach microbes from the soil aggregates to make them available during serial dilution. One such strategy is Dispersion and Differential Centrifugation (Hopkins *D. et al.*, 1991) which uses successive centrifugation and dilution of samples to ensure maximum dispersion. The technique has been employed in various studies successfully and has been mentioned in reviews for increase in yield of actinomycetes. In a study on cultivable bacteria in geographically diverse marine samples an ‘extreme degree of actinobacterial diversity’ was obtained (Maldonado *et al.*, 2005). In this research, community DNA analyses were used to gain a firsthand knowledge on the actinomycetes diversity in the selected samples. Based on this selective isolation procedures, including DDC were successfully employed to obtain culture based diversity using selective isolation procedures. DDC technique was also used for isolation and differentiation of *Streptomyces* sp. isolates using Fourier transform infrared spectroscopy technique at subspecies and strain level (Zhao H. *et al.*, 2006). Besides DDC, sucrose gradient centrifugation has been reported for successful isolation of genus *Nocardia* from soil (Yamamura *et al.*, 2003) and high speed homogenization has been reported for improved isolation of actinomycetes (Baecker
and Ryan, 1987). Besides dispersion, some treatments on soils have been reported to improve the yield of actinomycetes from the sample. Earlier re-treatment methods introduced were addition of some chemical selective such as yeast extract, Sodium Dodecyl Sulfate (SDS) agents

and/or heating at 40 °Celsius (Hayakawa and Nonomura, 1989). The most commonly used pre-treatment strategies employed are drying (Jiang and Xu, 1996), heat treatment (Takizawa M. et al., 1993) and dilution heating (Mincer T. et al., 2002). Less frequently used include UV radiation, Ultra Violet, super high frequency, extremely high frequency radiation as described by earlier researchers (Galatenko and Terekhova, 1990; Li et al., 2002) and cold shock by freezing at -18 °C (Bredholdt et al., 2007). The methods will be dealt with in detail when the description of various works is presented.

The literature reviewed above, served as the platform for future researches. The methodologies reported in these papers have been used directly or as modifications for most studies and have either been acclaimed or criticized.

2.5. Isolation strategies and diversity of actinomycetes reported from habitats, other than terrestrial ones

(Note 1: Critical review on the reports, if any, has been italicized)

(Note 2: Few studies involving isolation diversity and bioprospects together have been separately mentioned in the bioprospecting section of this chapter)

Researches on prevalence of actinomycetes from habitats other than terrestrial (soil) were available from mid to late 1900’s. They involved actinomycetes from lakes, rivers and streams (Kasimir, 1987; Niemi et al., 1982; L. G. Willoughby, 1966, 1969, 1971). At this time exploration of marine environment (Goodfellow and Haynes, 1984; Jensen et al., 1991) as a source of actinomycetes also got reported. Isolation and diversity of culturable actinomycetes has been reported from wide geographical locations.
Investigation on Chesapeake Bay as a source of bioactive actinomycetes falls in line with the paradigm terrestrial to other habitat shift. Sediment samples were collected from mid axis, mouths of bay and 10km offshore using Smith-McIntyre sediment grab sampler and thereafter, collecting aliquots using a syringe. For isolation of actinomycetes, SCA was chosen medium, which was further supplemented with 0.5% NaCl and nystatin and cycloheximide (25 and 10 µg/ml, respectively) to check fungal contamination. Actinomycetes in range of $1.4 \times 10^5$ to $2.8 \times 10^2$ Colony Forming Units per milliliter of the sediment were reported and they constituted 0.15 to 8.63% of the microbial community. Out of 298 actinomycetes isolates, 249 were actinoplanetes (chemotype IID), suggesting their abundance. The study made an important contribution through successful use of nalidixic acid to inhibit gram positive bacteria (Takizawa M et al., 1993). The only medium for isolation employed in this report, was SCA. No attempt was made to modify the medium to make it habitat based and using it as a strategy to isolate other rare actinomycetes. The same set of medium and antibiotics was later used to isolate actinomycetes from a heavily polluted Baltimore Inner Harbor (Ravel et al., 1998).

Research on near sea shore marine sediments of four different sites in Alexandria revealed presence of actinomycetes. Samples were collected at a depth of 0.5m in polypropelene bags and sediment samples were collected using an undefined corer. Heat pre treatment at 70 °Celsius for 60 minutes was done. Authors employed four different media for isolation (SNA, SCA, CCA and glycerol-glycine agar). The media were prepared using sea water (modification) and supplementation with antibiotics (75µg/ml cycloheximide and 25µg/ml nystatin). The count of actinomycetes was highest in upper sediment layer (0-20 cm depth). The authors reported that heat treatment could have lowered the actinomycetes count or selected only heat resistant spores. SNA medium was found to perform best. However authors mentioned that one best medium may not be possible to generalize as the best (Ghanem et al., 2000).
SNA in combination with AV medium was successfully employed in isolation of *Streptomyces, Actinomadura* and *Kitasatospora* from soil samples of Antarctica (Moncheva *et al.*, 2002).

A study on world-wide ocean sediments (Bahamas, Atlantic Ocean, Red Sea and Sea of Cortez) provided the first conclusive evidence for a taxon of actinomycetes that was exclusively marine. This formed a distinct clade within *Micromonosporaceae* and designated MAR 1. Sediment samples (top 1cm) were collected in sterile bags from depths that varied between 0-30m for stations and processed within 4 hours of collection. Two pretreatments were used: Dilution-heating (1:4 soil:sterile seawater solution heated at 55 °Celsius for 6 minutes followed by 1:4 dilution) and drying followed by stamping (samples air dried for 24hr and ground with pestle). The media (M1 to M5) were composed as: (each medium composed, per liter of natural sea water): M1 (10g of starch, 4g of yeast extract, 2g of peptone, 18g of agar); M2 (6ml of 100% glycerol, 1g of arginine, 1g of K$_2$HPO$_4$, 0.5g of MgSO$_4$, 18g of agar); M3 (6g of glucose, 2g of chitin, 18g of agar); M4 (2g chitin, 18g of agar); M5 (18 g of agar). The media were supplemented with 100 µg/ml of cycloheximide and 5 µg/ml of rifampin. Effect of sea water and tolerance to sodium chloride on isolates was also determined. For this purpose, media without antibiotics were employed and modified. The modification involved preparation of media M1, each in of the following four: natural sea water, deionised water, artificial sea water, artificial sea water with equimolar components of potassium instead of sodium and deionized water with 1M sodium chloride. Macerated mycelia of isolates were swabbed on the above media. All the actinomycetes isolated, required sea water for growth and could not tolerate 1M NaCl concentration. However, two isolates CNB394 and CNB512 could grow in deionized water media and tolerate 1M NaCl concentration (Mincer T. *et al.*, 2002).

Using the same technique and Medium M1 (Mincer T. *et al.*, 2002) a comparative study on diversity of actinomycetes in marine near shore and off shore sediments from San Diego was done revealing high diversity of actinomycetes was observed. Out of 623 actinomycetes obtained, a majority of 557 were obtained from near shore samples. The near shore samples gave 2-5 times more count of
actinomycetes than off shore samples. The researchers, based on Operational Taxonomic Units (OTU) analyses, concluded that *Micromonospora* numbers increased in off shore samples (Prieto-Davó *et al.*, 2008).

M1 medium has also been successfully employed in obtaining a good diversity of actinomycetes (dominated by genus *Streptomyces*) from North Pacific and Caribbean coasts of Costa Rica (Solano G. *et al.*, 2009).

Further M1 was the medium of choice for isolating actinomycetes from Nahoon Beach, South Africa (Ogunmwonyi *et al.*, 2010); M1-M6 were used in isolation of actinomycetes from Nicobar marine sediments (Karthik L. *et al.*, 2010).

Media M1-M4 were also employed to reveal the diversity of actinomycetes from coastal multiponds solar salterns from Tuticorin, India. From these salt salterns also, the typical *Streptomyces-Micromonospora* domination was evident. Other genera isolated were *Nocardia, Nocardiopsis* and *Saccharopolyspora*. Besides this was the first report of isolation of genus *Nonomuraea* from India (Jose and Jebakumar, 2012).

M5 was reported as the most efficient medium for selective isolation of marine sponge associated actinomycetes in the China Sea. The marine sponge *Hymeniacidon* was reported to possess the highest actinomycete diversity with seven genera (Xi L. *et al.*, 2012). Using a selective isolation strategy isolated 102 actinomycetes from Bismarck Sea and Solomon Sea off the coast of Papua New Guinea. Authors inoculated wet sediments. The medium used was NaST21Cx, prepared in artificial sea water and supplemented with trace metal solutions and 25µg/ml cycloheximide. Incubation was done for 30-90 days in a humidified chamber and colonies appearing were purified on ISP-2 prepared in sea water with antibiotic supplements (25µg/ml each of cycloheximide and nalidixic acid). 90% of the isolates belonged to the new genera, a result which was attributed to the unique isolation strategy and its selectivity. Using physiological, chemotaxonomic, distinguishing 16s rRNA gene sequences and phylogenetic studies, it was concluded that two new genera within *Micromonosporaceae* (PNG 1 clade and strain UMM518) were evident (Magarvey N. *et al.*, 2004).
A study revealing diversity of rare actinobacteria in Trondheim fjord, Norway employed ten different media and four different sediment pre-treatment methods. The pretreatment methods were Ultra Violet, super high frequency, extremely high frequency radiation and cold shock by freezing at -18 °C. A total of 2689 strains of actinomycetes were obtained which the authors termed ‘unexpected diversity’. They demonstrated that extremely high frequency irradiation favored *Nocardiopsis*, *Nocardia* and *Streptosporangium* species, super high frequency radiation for *Streptosporangium* and *Rhodococcus* species, and UV irradiation for *Nocardiopsis*, *Nocardia* and *Pseudonocardia* species. The research was important not only as a geographical mapping for diversity, but also the fact that authors had used radiation as a selective pre-treatment (Bredholdt *et al.*, 2007).

Nagasaki Prefecture in Japan found mention in a report for 800 actinomycetes strains isolated. *Streptomycetes* followed by *Nocardia* were demonstrated as the dominant culturable actinomycetes in marine samples from various sites. Other isolates obtained in this study *Glycomacetaceae, Micromonosporaceae, Nocardiaceae, Nocardioidaceae, Nocardiopsaceae, Pseudonocardiaceae* and *Thermonosporaceae* (Anzai *et al.*, 2008).

Four new marine phylotypes of actinomycetes representing two families within the order *Actinomycetales* were isolated from sediment samples of Yellow Sea, China. The medium used was Gause’s synthetic agar and potassium dichromate was used as gram negative bacterial and fungal inhibitor. The novel strains belonged to genera *Streptomyces* and *Nocardiopsis* (Zhang M.* et al.*, 2011).

In a report on isolation of actinomycetes from Kothapattanam, India, most colonies were observed on AIA, followed by Kuster’s agar and SCA; all supplemented with 100µg/ml cycloheximide (Kumar and Rao, 2012).

A study on diversity and activity of aquatic actinomycetes in lakes of the middle plateau, Yunnan province in China employed three media for isolation from fresh sediments- colloidal chitin agar, starch casein agar and glycerol-asparagine agar. An attempt was made to isolate thermophilic actinomycetes by dry-heat treatment.
followed by inoculation on three media- half strength nutrient agar, glycerol-asparagine agar and yeast extract-malt extract agar. A total of 16 genera were isolated and *Micromonospora* sp. were reported to be the most abundant, followed by *Streptomyces* sp. with some of the isolates showing unusual cell wall composition (Jiang and Xu, 1996). However the researchers though employed a variety of media did not make an attempt to modify them in any way. Observations on media which gave the best yield of actinomycetes, if the case be, were not reported. A total of 114 actinomycetes were isolated from water and sediment samples of River Nile and from Giza and Cairo sites. The selective media used were starch casein agar, malt-yeast extract agar and chitin agar. The research reported abundance of *Streptomyces* in water samples while sediments were dominated by *Micromospora* (Rifaat M, 2003).

HVA was successfully employed in isolating *Streptomyces* sp. with antimicrobial and cytotoxic activity from Lonar soda lake of India (Kharat K. R. *et al.,* 2009).

In a study on estuary sample from the mouth of Kuiragawa River, Okinawa Prefecture, Japan, the use of a specially designed medium ‘R’ resulted in the successful isolation of a novel genus and species *Ilumatobacter fluminis* gen. nov., sp. nov. (Matsumoto A. *et al.,* 2009).

SCA was successfully employed in isolation of actinomycetes from sediments of Lake Oubeira, Algeria (Ayari A. *et al.,* 2011) and sediments of lake Tana, Ethiopia (Gebreyohannes G. *et al.,* 2013). In combination with glycerol-asparagine agar and soil extract agar, SCA was also the preferred medium of choice for isolating actinomycetes from Vembandu Lake, Kottayam, India (George M. *et al.,* 2011). *Streptomyces* sp has been reported from Vellar estuary in Tamil Nadu, India, isolated on SCA (Dhanasekaran D. *et al.,* 2009), sediments of River Krishna (Elliah *et al.,* 2002) and *Streptomyces* sp. and *Micromonospora* sp. from freshwater samples of Karimnagar, India have also been reported (Mohan M. *et al.,* 2012). Some other notable studies involving culture based diversity studies include isolation of
actinomycetes from soil samples of Mount Everest region, Kalapatthar (Gurung T. et al., 2009) and Karanjal regions in Sundarbans (Arifuzzaman M. et al., 2010). The choice of an isolation medium may also depend on the intended bioprospects the researcher would like to work on. Out of many, two researches with reference to metal resistance can be cited as examples. In a research to isolate copper resistant actinomycetes from sites Tucuman, Argentina, of use of Minimal Medium (MM) was reported (Albarracín V. H. et al., 2005). Composed as (per litre of distilled water): 0.5g of L-asparagine, 0.5g of K$_2$HPO$_4$, 0.2g of MgSO$_4$.7H$_2$O, 0.01g of FeSO$_4$, 10g of glucose, 15g of agar supplemented with 16mg/l of CuSO$_4$ and 10µg/ml nalidixic acid and cycloheximide. Supplementing the medium with copper sulfate was the required modification the authors opted for in order to isolate actinomycetes with copper resistance. MM was the chosen medium in this case because the supplemented metal does not form complexes.

2.6. Bioprospecting of actinomycetes for antimicrobial, cytotoxic, enzyme production and bioremediation potential- the paradigm shift perspective

Even when the shift of focus from terrestrial to other habitats for search for novel microbes and their chemistries happened, the favorite biological activity researchers looked for was the production of antibiotics. However, with the shift of habitat focus, there has also been a shift of bioprospects taking the research on actinomycetes to new dimensions. Biomedical prospects of actinomycetes have been reported from different habitats. The general trend for the medical prospect research is: Isolation- preliminary screening on agar- liquid fermentation- solvent extraction- activity. It is in a very few cases that a metabolite is taken to the identification stage, especially in Indian research (Velho-Pereira and Kamat, 2013). Apart from medical bioprospects, there have been reports of them being potential contenders for bioremediation processes. Although the prospects are not limited to the ones mentioned here, but these are surely the exciting new possibilities that need to be explored further.
The most promising contribution to bioprospects from the paradigm shift has been from the marine habitat. The main reason for this could be, the marine habitat has been the most sought after habitat for finding novel microbes.

2.6.1. Antimicrobial and cytotoxic activities

Actinomycetes of the MAR 1 clade of the various ocean sediments showed cytotoxic, antifungal and antibacterial properties with a novel series of cytotoxic β lactones. Antibacterial activity was demonstrated in the extracts of ‘bona fide’ marine clade PNG1 and UMM518. A resin XAD-16 was used to extract active antibacterial metabolites from fermented broth. Important finding was that 80% of extracts obtained were active (Mincer T. et al., 2002). The prominent extracts were later reported showing activity against MRSA, VRE and metabolites active against vaccinia virus topoisomerase I (Magarvey N. et al., 2004). New members of highly cytotoxic Anthraquinone-γ-pyrones namely Saliniquinones A-F were isolated from a novel marine actinobacterium Salinispora arenicola (Murphy et al., 2010). The screening of crude extracts of novel actinomycetes isolates from the sediments of Yellow Sea, China, revealed diverse classes of compound with potential antibiotic and cytotoxic activity (Zhang S. et al., 2011). A novel Pyridinium compound with antimicrobial and cytotoxic activity was reported from marine Amycolatopsis alba var. nov. DVR D4 isolated from Vishakhapatnam coast, India (Dasari et al., 2012). Fermented broths (yeast extract malt extract broth) and mycelial-methanol extracts of marine Streptomyces sp., Actinosynnema sp., two Micronomonospora sp and one rare actinomycete, were reported for their anti-mycobacterial activity (Radhakrishnan M. et al., 2011). Antibacterial activity of actinomycetes from marine sediments of Parangipettai (Sathiyaseelan and Stella, 2011) and Konkan coast of India have also been reported (Gulve R. M. et al., 2012). Ethyl acetate extract of fermented broth of a marine Streptomyces sp. isolate from Kothapattanam, India showed activity against MDRSA clinical isolate (Kumar and Rao, 2012). The MIC was 1000µg/ml. The report also stated that chloroform and butanolic extracts of the broth did not show any activity.
A compound 7, demethoxy rapamycin was reported as the potent antimicrobial compound of *Streptomyces hygroscopicus* BDUS 49 from Bigeum Island, South Korea (Parthasarathi S. *et al.*, 2012). Also, a novel species of *Streptomyces* was found to produce actinomycin D as one of the components of an active antibacterial and cytotoxic crude extract from Bangladesh (Sharmin T. *et al.*, 2013). Actinomycetes from alkaline soda lake of Lonar, were reported for antibacterial and cytotoxic activity (Kharat K. *et al.*, 2009).

Antifungal activity against phytopathogenic fungi was reported from various Indian marine *Streptomyces* sp. (Kathiressan K. *et al.*, 2005); soil samples in Punjab (Sharma and Parihar, 2010) and soil samples from Riyadh (Ara I. *et al.*, 2012). A similar observation was recorded with isolates from Bay of Bengal, East coast of India (Ramesh and Mathivanan, 2009). Antibacterial activity was also reported in actinomycetes isolated from various other sources- soil of Sundarbans, Bangladesh (Arifuzzaman M. *et al.*, 2010); soil samples from Belgam, Karnataka, India (Nanjwade B. *et al.*, 2010); Banten West Coast (Sunaryanto and Marwoto, 2010).

*Streptomyces* sp. isolated from The Arctic was found to produce novel benzoazaine secondary metabolites arcticoside and C-1027 chromophore V, that inhibited *Candida albicans* (Moon *et al.*, 2014)

Interesting biological activities have been reported from actinomycetes isolated from aquatic habitats as well. The actinomycetes isolated from lakes of Yunnan China, were found to inhibit *Bacillus subtilis* and *Aspergillus niger* (Jiang and Xu, 1996).

Actinomycetes from water samples from Algeria showed broad activity against both bacterial and fungal pathogens (Kitouni M. *et al.*, 2005).

*Streptomyces* sp. isolated from river Nile (Rifaat M., 2003) and Algerian Ouberia Lake (Ayari *et al.*, 2011) were also reported to have antifungal activity.

Species belonging to *Saccharopolyspora* and *Actinosynnema* isolated from Tyume River were reported for their antibiotic production potential. Ethyl acetate extracts were obtained from liquid culture broths, dried and reconstituted in 50%
methanol. The extracts showed activity against several reference and environmental strains including *Bacillus subtilis*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Staphylococcus epidermis* and *Pseudomonas aeruginosa*. The minimum inhibitory concentrations (MIC) of the extracts were usually less than 10 mg/ml (Sibanda T. et al., 2010) A strikingly similar report is available on activity of *Streptomyces* sp. and *Actinopolyspora* sp. species isolates obtained from the same habitat (Cwala Z. et al., 2011).

The study on actinomycetes isolates from Nambul River (Ningthoujam et al., 2011), also reported their biological activity. Six of the different *Streptomyces* sp. isolated showed broad antimicrobial activity, out of which, three tested species were active against both human (*Candida albicans*) and rice pathogenic fungi. The authors however did not describe the fermentation and crude extract preparation.

Antibacterial activity of *Streptomyces* sp. and *Micromonospora* sp. isolated from freshwater samples of Karimnagar, India has also been reported (Mohan M. et al., 2012).

In a report on antibacterial activity of actinomycetes from Lake Tana in Ethiopia, it was found that more crude extract was be obtained from solid fermentation than submerged fermentation (Gebreyohannes G. et al., 2013). Both fermentation products were subject to ethyl acetate extractions with MIC and MBC values <4mg/ml. The extracts inhibited *Staphylococcus aureus* and *Escherichia coli*. The authors hypothesized that solubility of the active compound in water and extraction solvents may have influenced the obtained results. From this we can note, that the choice of fermentation medium- liquid or solid, may influence antibiotic production.

Let us now recall the noteworthy Indian contributions to actinomycetes medical research-Swalpamycin from *Streptomyces* sp. Y-84,30967 isolated from a soil sample near Pune (Sugata C. et al., 1987); Butalactin from *Streptomyces* sp. Y-86,36923 source- Sahyadri hill region near Pune (Franco et al., 1990); Alisamycin from *Streptomyces* sp. HIL Y-88,31582 source- coastal region near Alibag (Franco et
al., 1991), Maharashtra. For all the three mentioned strains, same seed medium was used to develop inoculums which were used at 4% for further inoculating production media. The composition of production media for each antibiotic however was different. In case of Butalactin, the researchers used three different media to find out the most suitable one with high yield. In one of the media used, namely medium ‘A’, butalactin activity detected was very less and subsequently, no activity was detected at 15 liter fermentation stage.

In another case, an interesting and crucial finding in relation to screenings for antibacterial properties became evident when PKS type I, II and NRPS genes were found in Nocardium sp. isolate (Trondheim fjord, Norway) which had not shown antimicrobial activity in a parallel screening test (Bredholdt et al., 2007). Hence, keeping this finding in mind and the case of Butalactin, it is crucial to note that special conditions may be required to activate genes responsible for synthesis of bioactive metabolites. Using the same approach of screening isolates for presence of PKS type I, II and NRPS genes, antibacterial activity of actinomycetes from sponges of China seas was revealed (Xi et al., 2012). This approach was also used with actinomycetes isolated from sea slug samples from Indonesia, however the authors did not describe the findings properly (Widada and Radjasa, 2009).

2.6.2. Enzyme production potential

Enzymes have been another remarkable metabolite of actinomycetes with wide applications.

A protease producing Streptomycete PS-18A was obtained from estuarine shrimp pond (Vonothini G. et al., 2008) with maximum protease production, at 3% NaCl, pH 7 and temperature 4 °C. Although the paper makes a good report, the data with respect to carbon source is contradictory. The reported best carbon source in abstract is sucrose, whereas in the results starch has been reported to yield maximum protease production. Proteases in culture supernatants of Streptomyces sp. BFI 250 and Kribbella sp. BFI 1562 were reported to detach Staphylococcus aureus biofilms (Park et al., 2012).
A study on endoglucanase production by two *Streptomyces* isolates from a university soil sample in Gujarat, India addition of surfactant tween-80 to the production media was reported to enhance yield (Chellapandi and Jani, 2008). Three actinomycete isolates from Krishna River- *Streptomyces canus*, *Streptomyces pseudogriseolus*, and *Micromonospora brevicatiana* were reported to produce chitinase (Mane and Deshmukh, 2009).

Actinomycetes isolated from Kuruva Island and Pookot lake of Kerala, India, were screened for their enzyme production potentials. Isolates were screened for production of amylase, protease, pectinase, cellulase and xylanase. It was revealed, that 33% of isolates could produce all the enzymes and all isolates were able to produce amylase and protease (Khadijeh H. *et al.*, 2012). Actinomycetes isolated from lakes of middle plateau, Yunnan were found to produce a wide range of enzymes (Jiang and Xu, 1996).

### 2.6.3. Bioremediation potential

The potential of actinomycetes in bioremediation has been revealed through reports on production of biosurfactants from them and their metal absorption capacities. This section shall find mention of the most noteworthy contributions.

#### 2.6.3.1. Biosurfactants from actinomycetes

Biosurfactants are surface active compounds that exhibit surface activities at gas-liquid and liquid-liquid interfaces respectively (Satpute *et al.*, 2010). Biosurfactants have been reported from *Streptomyces* sp., and genus *Actinopolyspora* sp. from garden soil the fermentation medium used being maltose yeast extract broth (Maniyar *et al.*, 2011). In this study highest surfactant production was reported from *Streptomyces* sp. S22 showing maximum emulsification with sunflower oil (320 EU/ml). Marine sediment actinomycetes have also been investigated as sources of biosurfactants (Kalyani *et al.*, 2014; Kokare *et al.*, 2007; Manivasagan *et al.*, 2014).

An interesting finding has been that of production of biosurfactants from a chromium resistant actinobacterium and optimization using factorial design (Colin V.
et al., 2013). In this study, authors investigated a chromium resistant bacterium *Streptomyces* sp. MC1 for biosurfactants production and found that an alkaline pH 8 and concentrations of phosphate (2.0g/L) and calcium (1.0g/L) influenced production and led to an increase of surfactant yield by 3.5%. However, the results obtained in biosurfactants production from *Streptomyces* sp. SS 20 isolated from hydrocarbon contaminated sites, it was found that maximum production and activity were both observed at neutral pH with a decline in activity in alkaline pH ranges (Hayder et al., 2014). The same observation was reported with references to a marine isolate *Streptomyces* Sp. S1 (Kokare et al., 2007). Another genus of actinomycetes that has gained importance owing to biosurfactants production potential is genus *Nocardia* (Khopade et al., 2012; Vyas and Dave, 2011). Apart from their potential applications in bioremediation, biosurfactants compounds have wide prospects in medicine (Fracchia and Cavallo, 2012; Satpute et al., 2010). Production of biosurfactants from *Bacillus* sp., *Candida* sp. and *Pseudomonas* sp. are well documented (Chandran, 2010; Ghribi et al., 2012; Padmapriya and Suganthi, 2013; Tambekar and Gadakh, 2013; Vandana and Peter, 2014).

However there are, in comparison, only a few reports that describe biosurfactants from actinomycetes (Khopade et al., 2012). The literature highlights that studies on biosurfactant production from actinomycetes have typically involved screening, fermentation and optimization of culture conditions for maximum yield.

2.6.3.2. Metal absorption and removal potential of actinomycetes

Another potential application of actinomycetes in bioremediation has been highlighted by their metal absorption abilities demonstrated through various reports. The Actinomycetes have been tested for removal of uranium and lead (Friis and Myers-Keith, 1986); cadmium and lead resistance and removal (Lebeau T et al., 2002; Saurav and Kannabiran, 2011); resistance to mercury and copper (Koushalshahi et al., 2012); zinc (Lin et al., 2011). Indigenous copper resistant actinomycetes strains have been reported from Argentina (V.H. Albarracín et al., 2005) Even in metal absorption studies, actinomycetes from marine habitat have got their potential unleashed. In a study on mercury resistance in actinomycetes isolated from Chesapeake Bay,
Streptomyces spp. CHR3 and CHR28 were found to be resistant to mercuric chloride and phenylmercuric acetate (Ravel et al., 1998). Chromium resistance and removal has been reported from Arthrobacter crystallopoietes ES 32 (Camargo et al., 2004); Streptomyces sp. MC1 (Colin et al., 2013) and Streptomyces sp. and Amycopatopsis sp. (Polti et al., 2007). While testing resistance to metals, it is important that the medium components chosen should be such that they do not affect the bioavailability of the metal against which the resistance is desired. In this respect, we can cite the example that media components, especially amino acids are known copper binders (Koch et al., 1977), which may reduce the bioavailability of copper. Hence, Minimal Medium agar and broth is the medium of choice for such studies.

2.7. Identification of actinomycetes- the molecular biological approaches

When it comes to identification of actinomycetes, molecular biology methods are being increasingly preferred for identification of isolates. The advent of Polymerase Chain Reaction (PCR) and molecular biological identification techniques using universal primers to facilitate faster and more accurate identification of microbes have revolutionized research and led to the growth of huge databases and discovery of novel taxa. The working principal of molecular identification of microbes is that there is a conserved region within their genomes which is complementary to a ‘universal primer’. However, the reports of failure of universal primers in striking complement with 16s rDNA sequences suggest they cannot be over generalized and applied to all prokaryotes (Baker et al., 2003). Thus new primers or alternate molecular biological techniques have become inevitable to allow identification of species not having sequences complimentary to the universal primers. This section shall review a few reports which have reported successful application of various molecular biology techniques to identify actinomycetes within samples or to differentiate between isolates.

Genus specific primers corresponding to nif gene (nifH and nifD) were used for identification of Frankia sp. by PCR (Simonet et al., 1991). This approach was also
used to identify *Streptomyces* sp. from environmental DNA samples through primers StrepB/StrepE and StrepB/StrepF combined with BstYI restriction endonuclease digestion (Rintala *et al*., 2001). Genus specific primers for identification of actinomycetes from genera *Nocardiopsis* and *Saccharothrix* called NspI and NspII (for *Nocardiopsis*) and StxI and StxII (for *Saccharothrix*) were designed *in silico* after procuring the specific conserved sequences within each genus based on comparison of known 16s rRNA sequence variability (Salazar *et al*., 2002).

Specific primers to amplify gyrase subunit B (gyrB) of *Microbacteriaceae* members were designed to differentiate closely related species thus revealing that gyrB could be a valuable tool to differentiate *Microbacteriaceae* members (Richert *et al*., 2005). Genus specific and primers nested PCR-DGGE techniques were for identification of genus *Gordonia* in foam samples. The primers designed were G699F and G1096R (Shen *et al*., 2007).

In *silico* restriction digestion of genus specific 16s rRNA genes of validly published actinomycetes sequences from GenBank followed by in vitro experiments using a *Streptomycetes* species specific primer and sau3AI, AsnI, KpnI and SphI restriction endonucleases was reported to generate a pattern that could easily differentiate *Streptomycetes* from other actinomycetes (Cook and Mayers, 2003).

A novel fingerprinting based approach to study the distribution of PKS and NRPS genes using wild type actinomycetes was presented as an alternative method to characterize actinomycetes. The primers designed and used were Consensus Ketosynthetase (KS) primers KS-BEF and KS-BER to detect KS domains from PKS-I systems and K2R derived from KS-BER in combination with K1F to amplify shorter fragments to detect PKS-II, KSα and KSβ. A clear cut relationship between occurrence of biosynthetic genes and production ability of related biomolecules could be established (Ayuso *et al*., 2005).

Randomly amplified polymorphic DNA (RAPD) based approach using different primers was employed to identify conserved regions within *Streptomyces* sp.
for rapid identification (Mehling et al., 1995) and for differentiation between Nocardia sp (Işık and Goodfellow, 2002).

A technique to detect actinomycetes in different environmental soil samples was designed by using group specific primer F243 and two indirect approaches: selective amplification of actinomycetes conserved sequences and subjecting the products directly to denaturing gradient electrophoresis and secondly, a nested-PCR followed by gradient gel electrophoresis (Heuer et al., 1997). Actinomycetes could be detected using both the approaches. This approach was also successfully employed to identify actinomycetes from a soil sample from The Antarctic Barrientos Island (Learn-Han et al., 2012).

Thus, at all a basic strategy had to be generalized, one would find that ‘sampling---pre-treatment---dilution plating’ has been the most used method and ‘diversity---biological activity’ has been the most favored objective. Each of the strategic component- sampling, pre-treatment and media has been subject to permutations and combinations by researchers using variants of each. This has led to the discovery of a large diversity of actinomycetes from across a range of habitats and led to numerous opportunities with their bioprospects.