CHAPTER 1

INTRODUCTION

1.1 INTIMIDATION OF EMERGING INFECTIOUS DISEASES

Emerging infectious diseases (EIDs) are diseases of contagious origin whose occurrence in humans has increased within the current, past and intimidate to boost in close proximity future. These contain both new, previously indeterminate diseases and old diseases with new descriptions. More than over thirty new infectious agents which cause severe diseases have been identified worldwide in the last three decades in which 60% of these are from zoonotic origin and more than two-thirds of these have originated from the wildlife. In addition to health, emerging infections graves economic, developmental and security challenge. SARS, (Severe Acute Respiratory Syndrome) is the first severe infectious disease emerged in twenty-first century. Having emerged in as a new corona virus, it spreads rapidly all over to 30 countries across the world with a total of 8,439 cases and 812 deaths, within 7 to 8 months. It exist as a serious threat to global health security. Because of the emergence of this disease, the economic losses to countries in Asia were estimated to several billion. Similarly, Chikungunya fever caused by the Chikungunya virus was first reported in Tanzania in 1953. Even though deaths are not known to occur, the morbidity and disability caused due to chikungunya are huge in number (Krishnamoorthy et al 2009). Likewise,

the outbreak of *Neisseria meningitidis* occurred during flu season caused the overwhelming a hospital emergency and set hurdles triage (Osterholm 2001).

The world wide annual deaths with regard to emergence of infectious diseases account for 26%. The load of morbidity and mortality connected with infectious diseases falls most a lot on people in developing countries particularly on infants and children about three million children die each year from malaria and diarrhoeal diseases alone (Fauci 2001). Developing countries such as India experience suspiciously from the load of infectious diseases. India, the second most populous country in the world is in the midst of a triple burden of infectious diseases, the fragmentary agenda of communicable diseases, non communicable diseases linked with lifestyle changes and emergence of new pathogens and overstretched health infrastructure. Many infections are coupled with pitiable hygiene, spoiled provisions, insufficient personal cleanliness or admittance to protected water and deficient basic health services setting frequent to large parts of India.

The risks on emergence of infectious diseases and emphasizes are needed to improve preparedness at national and international levels for future pandemics. It is clear that new pathogens are likely to continue to emerge and spread across countries for a variety of reasons and challenge public health as never before. It represents a serious burden, disrupting trade, travel and negatively affecting the economy. The epidemiologic triangle model developed for studying health problems and understands the spread of infectious diseases is shown in Figure 1.1.

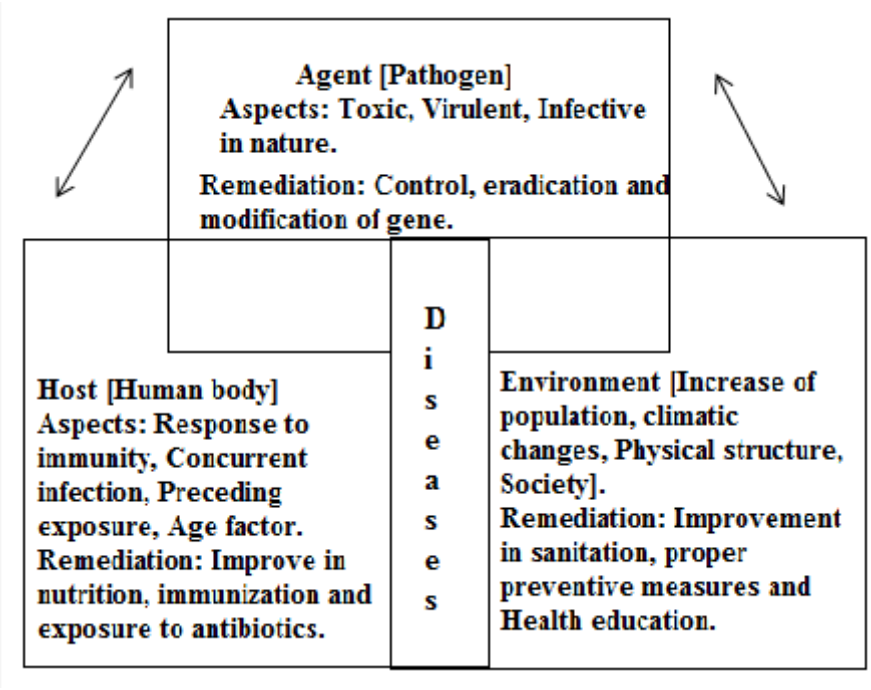

Figure 1.1 Epidemiologic triangle model for Infectious diseases

1.2 STRATEGIES TO COMBAT THE RESISTANT PATHOGENIC STRAINS

In modern times, the increase of antimicrobial resistance lead to the synthesis of new secondary metabolites (antibiotics) and new therapies needed to combat the resistant pathogens. Scientists use molecular and structural biology techniques to understand microbial pathogenesis. The information about the prediction of pathogenesis of a pathogen can improve approaches for discovering novel classes of drugs (secondary metabolites or antibiotics) that block pathogenic processes. The study and understanding of pathogens has been transformed by genomics and proteomics in the fields of molecular biology conveys on the whole functions and regulation of the genes and proteins of an organism in an environment. These technologies allow scientists to identify disease causing agents and systematically distinguish microbial pathogenic mechanisms in a limited time than in previous decades. Development of more rapid determination of antimicrobial resistances and

application of therapies. Areas of basic research must be enhanced to complement the more common agent-specific programs. Discovery of potent and reliable drugs (secondary metabolites or antibiotics) from microorganisms play an important role in mitigating disease severity, human suffering and infectious agent transmission. The future lies in the discovery of novel targets and mechanisms active against a broad spectrum of agents from unexplored marine environment which possess robust novel metabolites. The clear understanding of pathogen and host relationships such as how the host is acquainted with an invading pathogen, how pathogens avoid host defences, how hosts and microbes interrelate in non pathogenic relationships (symbiosis) and how host immune response to pathogens can be modulated by drugs.

1.3 ECO FRIENDLY MICROBES- A BOON TO BIONETWORK

All life originate in the oceans about 3.5 billion years ago, among that microbes were the only form of life for two thirds of the planet's existence and the significance of microbes is enormous. The term 'microorganism' encompasses a wide and various groups of organisms such as bacteria, viruses, protists and fungi which reveal widely different morphological, ecological and physiological characteristics. Pasteur postulated that microorganisms were essential for human life (De Kruif 1926). Microorganisms are considered as miniatures of chemical factories. The discovery of penicillin in 1940 initiated the researchers for the utilization of microorganisms for the production of secondary metabolites which revolutionized the field of microbiology (Demain & Fang 2000). Ilya Metchnikoff established the connotation of microbes and their interactions between host and itself required for the functioning of normal life (Metchnikoff 1908, Metchnikoff 1910).

A Nobel Prize winner stated that microbes play an important role in human well-being. Prominently, while several vital attempt made to confirm or prove the claimed health benefits of microbes and have gained more consideration on the composition of the normal microflora in humans in addition to the ways in which microorganisms take part in human life. Several microbial communities were reported as originator of soils (Rajendhran & Gunasekaran 2008) and many ecosystem forces were linked to terrestrial ecosystems, including safeguarding of drinking water, plant production are strongly correlated to microbial activities and their functional characters (Torsvik & Øvreas 2002). In addition to macroscopic plants and animals, microbes are the foremost primary components of biological systems on this earth. Microbes ubiquitously present in soil, water, air, inside our bodies and that of other animals and plants. They were present still at sites where no other life form could probably subsist - sites such as deep inside the geysers (thermal vents) where the temperature may be as high as 100 °C, deep in the soil, under the layers of several metres thick snow and in extremely acidic environments. When making an allowance for the human body, it occupies 10-100 trillion living on it and throughout the world it plays several important roles in their ecosystems.

Regardless of being tremendously small in nature, the sheer number of microbes existing on the planet has large effects on the cycling of nutrients and compounds, both essential for the survival of all living creatures. To stay alive in so many types of surroundings, microbes have progress an immense number of mechanisms to find its energy, assimilate food and to make a replica. Scientists employ these skills in a number of ways for the welfare of the humans including medicine, energy production, agriculture and warfare by the broad knowledge of modern biotechnology and genetic engineering

principles. With the work of Louis Pasteur, the advent of microorganisms in medicine began in the mid-19th century. Currently, the use of microbes is not restricted to the use in vaccinations. Microorganisms were diverse in population and widely used in modern medicine (Robert 1986, Dobell 1960, Tiner JH 1991). The application of microorganisms such as *actinomycetes*, bacteria, fungi and viruses possesses potential, to advance the medical field at an even faster rate than it is currently progressing. By combining the continuing understanding of microbiology with the increased knowledge about illnesses throughout the world, many diseases and other ailments seem to be on the verge of being relieved, cured, or even eradicated. In the last 50 years, more than 23,000 bioactive metabolites of which 17,000 antibiotics were discovered from the microorganisms (Berdy 2005). They have specific structures and complex chemical structures with interesting group of diverse and unique functional groups. Studies on secondary metabolites revealed the fact that the microbial secondary metabolites have unique molecular skeleton which makes the scientist incapable to synthesize more than 40% of the metabolites (Feher & Schmidt 2003).

1.4 UNIQUENESS OF MARINE MICROBES IN BIOSYNTHESIS OF SECONDARY METABOLITES

Ocean harbours in excess of 80% of all life on earth, it remains our most unexploited natural resource and the future world population depends on this environment for its food and other life saving wonder drugs (Mary et al 1998). Hence, the consumption of marine resources for developmental purpose has gained significant attention in recent times. The natural diversity of the marine environment offers enormous scope for the discovery of novel natural products, several of which are potential targets for biomedical development (Austin 1989, Jiang et al l997). The growth and preservation of

all other forms of life depend completely on the precedent and present performance of marine microbes. The rate of detection of novel metabolites from terrestrial microorganisms is decreasing (Bentley et al 2002). Discovery and recognition of novel sources of natural products from marine environment, plays an important role in the unravelling of novel drug candidates and drug development processes. Marine organisms are competent of existing and mounting in habitats of limits in addition to elevated salinity, numerous microbes have to tolerate high hydrostatic pressure and low or high temperature. Much of the biota in tropical seas possesses particular characters for sustained survival in extremely competitive habitats. In order to live and cultivate in an exceedingly competitive habitat, countless organisms ought to compete for the limited resources. A variety of unpleasant and self-protective mechanisms have evolved to allow organisms to expand discriminating gain and to survive with competitors.

The physiological demonstration of these defence abilities of marine organisms is in the form of bioactive metabolites. Nearly all groups of marine microbes produces a diversity of bioactive compounds with sole structural description and frequently exhibits processes different from those of the terrestrial environment. The bioactive metabolites are outstanding candidates for a range of applications in the pharmaceutical, agricultural and food industries. Among the massive amount of varied organisms in the marine environment; marine *actinomycetes* especially *Streptomyces* rise out as radiant basis of lots of useful metabolites. It is well implicit that marine microorganisms have been basically unknown as a source of bioactive agents for industrial applications. Figure 1.2 Shows extraction of extracellular secondary metabolites from marine *Streptomyces* sp. and its biological activities.

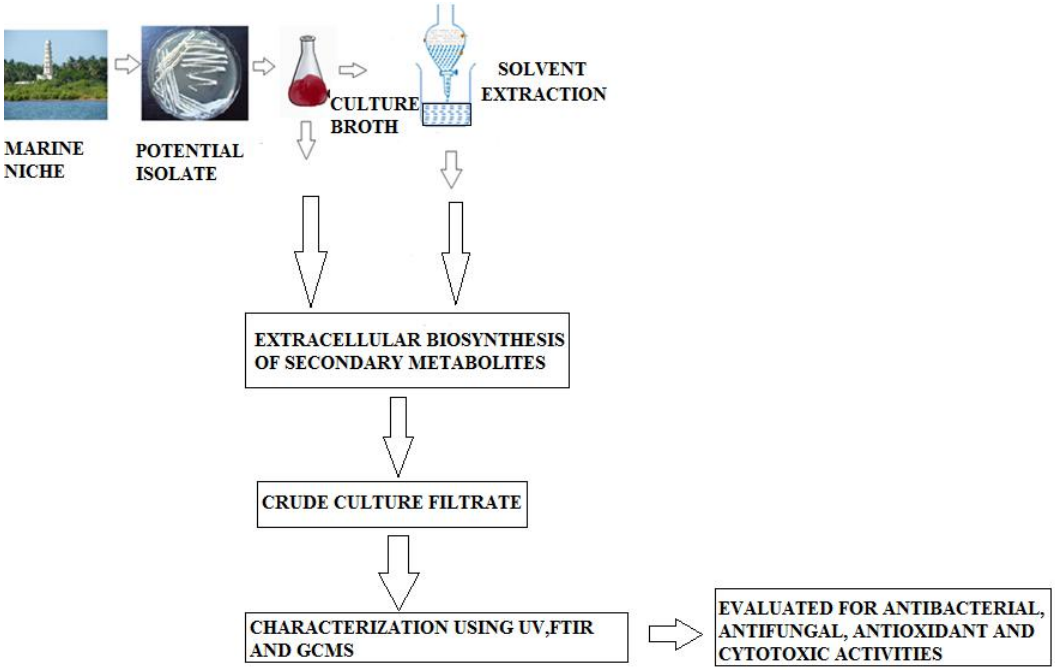

Figure 1.2 Extraction of extracellular secondary metabolites from marine *Streptomyces* sp. and its biological activities

1.5 ACTINOMYCETES - A VALUABLE PROKARYOTE

Actinomycetes are gram positive, aerobic, spore forming bacteria, belonging to the order *Actinomycetales* characterized with the presence of substrate and aerial mycelium growth (Lechevalier & Lechevalier 1981). The high (G+C) ratio of the DNA, phylogenetically associated with the indication of 16S ribosomal classification (Goodfellow & Williams 1983, Korn-Wendisch & Kutzner 1992). They are more abundant in soil and form thread like filaments in the soil. They are accountable for the earthy odour of newly turned healthy soil and rise as hyphae like fungi (Sprusansky et al 2005). Primarily they are soil inhabitants (Kuster 1968) and also found extensively spread in a varied range of aquatic ecosystem, as well as sediments obtained from deep sea (Walker & Colwell 1975, Colquhoun et al 1998) even from greatest depth of Mariana Trench (Pathom-aree et al 2006).

Actinomycetes are the most cost-effective and biotechnologically expensive prokaryotes. Half of the exposed bioactive secondary metabolites were accountable from *actinomycetes* (Berdy 2005, Balagurunathan & Radhakrishnan 2010), remarkable antibiotics (Berdy 2005, Strohl 2004), antitumor agents (Cragg et al 2012), immunosuppressive agents (Mann 2001) and enzymes (Oldfield et al 1998, Pecznska-Czoch 1988). Because of the outstanding path evidence of *actinomycetes* in this regard, a noteworthy quantity of attempt has been determined on the thriving segregation of novel *actinomycetes* from terrestrial sources for drug screening programs in the past fifty years. Among various genera of *Actinomycetes*; *Streptomyces*, *Saccharopolyspora*, *Amycolatopsis*, *Micromonospora* and *Actinoplanes* are the most important producers of commercially important bio molecules (Solanki et al 2008). The living conditions of marine *actinomycetes* had to accustomed during development range from tremendously high pressure (with a maximum of ~1100 atmospheres) and anaerobic environment in temperatures immediately under 0 °C on the deep sea bottom to elevated acidic conditions (pH as low as 2.8) at temperatures in excess of 100 °C close to hydrothermal vents at the mid-ocean ridges. It is almost reproduced in their genetic and metabolic diversity of marine *actinomycetes*. Certainly, the marine environment is nearly unexploited on the basis of novel *actinomycetes* diversity (Bull et al 2005, Stach et al 2003) as a result of new metabolites (Jensen et al 2005, Fiedler et al 2005, Magarvey et al 2004). Conversely, the distribution of *actinomycetes* in the sea is mostly unknown and the occurrence of original marine *actinomycetes* in the oceans remnants indefinable.

Among the *actinomycetes* of different origin, the deep-sea habitat stay on untouched for a long time and species isolated from this area are predominantly with novel effective sources of antibiotics. Colquhoun et al in (1998) isolated a large number of mycolata *actinomycetes* from sea sediments. An *actinomycetes* strain, isolated by Imada & Okami in (1995)

was found to be the inhibitor of beta-glucosidase. *Actinomycetes* specially *Streptomyces* species are recognized as industrially significant microorganisms as they are a rich source of several valuable bioactive natural products with potential applications (Korn-Wendisch & Kutzner 1992, Williams et al 1989, Williams et al 1983, Lakshmipathy & Kannabiran 2009) and also they are considered as the remarkable prolific producers of secondary metabolites (Curl et al 1983, Atta & Ahmad 2009). The metabolites from *actinomycetes* were reported to possess antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti- algal, anti-helmintic, anti-malarial and anti-inflammatory, toxins, pesticides, other curatives, and animal and plant growth factors (Ravikumar et al 2011, Kekuda et al 2010, Demain & Fang 1995). *Actinomycetes* have showed their capability to produce variety of bioactive secondary metabolites and for this basis, the detection of novel antibiotic and non-antibiotic directed molecules through microbial secondary metabolite screening is becoming increasingly important. *Actinomycetes* population is largest in surface layer soils and slowly decreases with as depth increased (Takahashi & Omura 2003). The remarkable group of compounds from *actinomycetes* forms a diverse group of biologically active molecules with different structures and modes of action. The *actinomycetes* are considered as vital not only in the field of pharmaceutical industries and also in the agriculture. *Actinomycetes* also have the capacity to synthesize many different biologically active secondary metabolites such as cosmetics, vitamins, nutritional materials, herbicides, anti-parasitic and enzymes like cellulose and xylanase used in waste treatment (Ogunmwonyi et al 2010). The different biological activities of bioactive secondary metabolites isolated from *actinomycetes* is shown in the Figure 1.3.

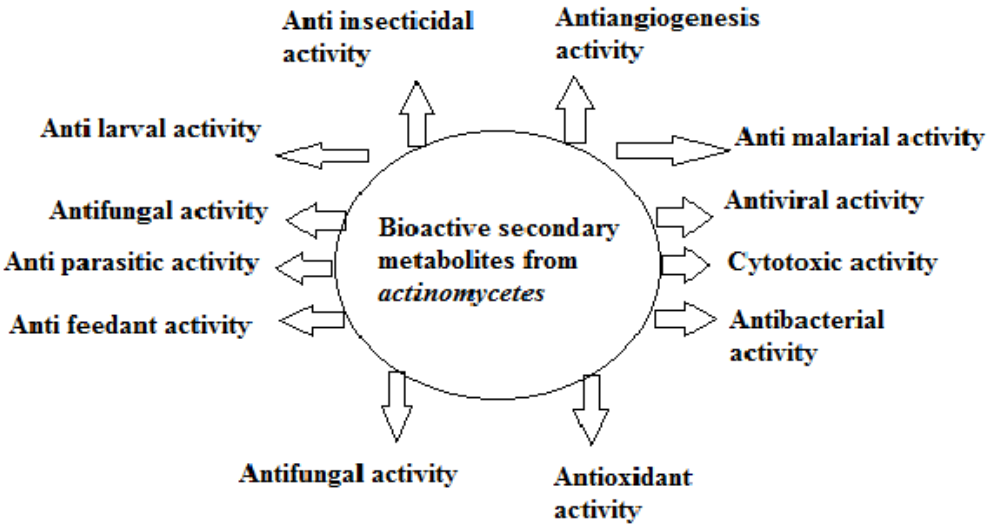

Figure 1.3 Biological activities of bioactive secondary metabolites from *actinomycetes*

1.6 INTRODUCTION TO MICROBIAL METABOLISM AND ITS METABOLITES IMPORTANCE- GENERAL

The word "**Metabolism**" is the sum of life-upholding biochemical reactions take place within the cells of living organisms catalyzed by enzyme reactions which allow the organisms to grow and reproduce and maintain their structures in order to respond to their environments. The microbial metabolism for cellular structure biosynthesis depicted in the Figure 1.4. There are two main types of metabolism in an organism. They are as follows:

1. Anabolism- Set of biochemical reactions that "**build up**" molecules involve energy requiring or endergonic process.

2. Catabolism- Set of biochemical reactions that "**break down**" molecules involve energy releasing or exergonic process.

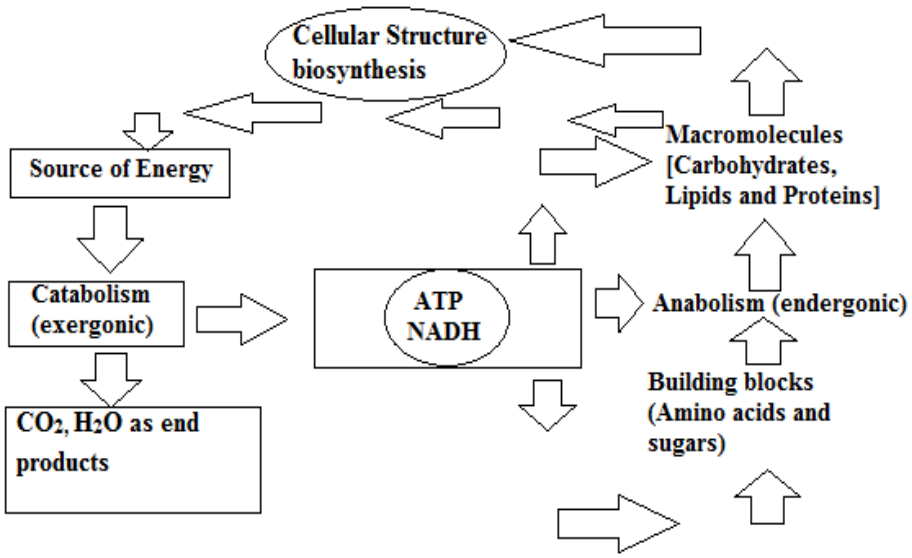

Figure 1.4 Microbial metabolism for cellular structure biosynthesis

1.6.1 Aspects of Metabolism

1. Chemical conversion of substances for tissue synthesis and operation of the cells.

2. Conversion of chemical energy provided by nutrients into energy utilizable in cells for vital functions.

Metabolism in an organism involves two major pathways. They are as follows:

- Primary metabolic pathways
- Secondary metabolic pathways

1.6.2 Primary Metabolism

Primary metabolites are basic mystery portion for all microorganisms. They result as the end products of catabolism, which forms

primary intermediates such as amino acids, nucleotides, vitamins, carbohydrates, and fatty acids. The biosynthetic intermediates are further gathered into the complex and essential metabolites which provide structure and biological function to the organism. Primary metabolism of an organism follows three potential pathways such as Embden Meyerhof-Parnas pathway, Entner-Dourdorof pathway and Hexose monophosphate pathway. Fermentation products of primary metabolism of an organism such as acetic acid, ethanol and lactic acid. The significant contribution to the food and beverage industries is obtained from the fermentation products of primary metabolism. With the consideration of current trends in products price, the primary products from microorganism occupy a vital role and will no doubt be exploited to their full potential.

1.6.2 Secondary Metabolism

Secondary metabolites are produced from the small groups of organisms which are not required for growth and development of the microorganism. They are produced in mixtures and formed at low specific growth rates with diverse structures. The most important part of secondary metabolites is antibiotics. Secondary metabolites are formed from the precursors of primary metabolites. They share a common division then branches to the synthesis of primary metabolites on one hand and to secondary metabolites on the other. The production of economically feasible significant metabolites is one of the major goals of several bioprocess industry.

Alkaloids, terpenoids, nucleosides, phenazines, antibiotics, naphtalenes, peptides, quinolines are the secondary metabolites obtained as the products from the secondary metabolism of an organism of interest. The major advantage of a single microbial type is the production of different biotechnologically important metabolites. Most of the secondary metabolites

are produced by the families with closely related compounds. Figure 1.5 shows the inter relationship of primary and secondary metabolism.

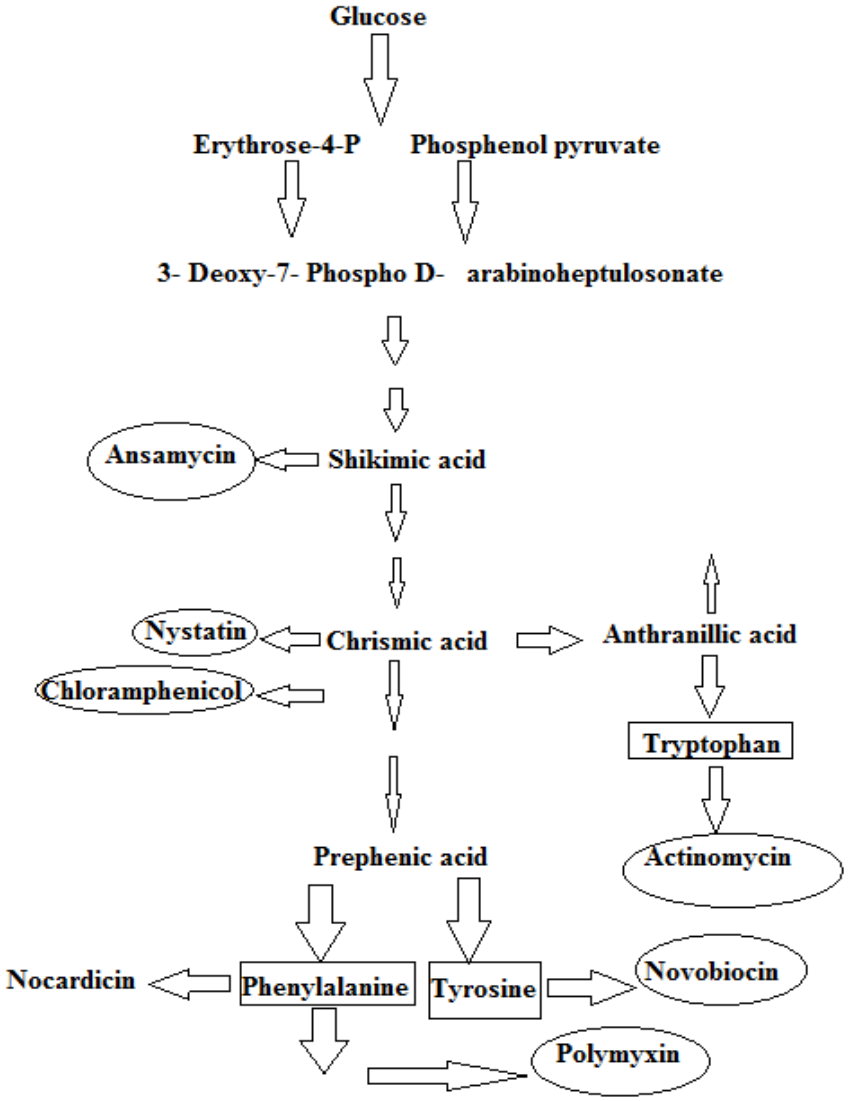

Figure 1.5 Inter relationship of primary and secondary metabolism in microbes

1.7 SIGNIFICANCE OF SECONDARY METABOLITES AND *ACTINOMYCETES*

With the emergence of new diseases up to date, screening of novel microorganisms for the production of new antibiotics has rapidly increased

during recent years. Comparing to the other group of microbes, the majority of studies are focused on the exploitation of antibiotics from *actinomycetes*, which are competent of producing secondary metabolites with widely different chemical structures. The word “Secondary metabolites” tendency to be small organic molecules, as a natural consequence of their functions (Jarvis 1995). Secondary metabolism results with the synthesis of new compounds that are not vital to the cells survival and its metabolism but are additional value for the entire organism (Vining 1992, Berdy 2005, Yarbrough et al 1993). Secondary metabolism usually occurs at the late log growth phase of the producing microorganisms. The sequential character of secondary metabolism is undoubtedly genetic in nature but expression can be inclined significantly by ecological conditions. Consequently, secondary metabolism is frequently brought on by tiredness of nutrient or addition of an inducer or by a decrease in growth rate (Bibb 2005). The generation of signals which cause a flow of regulatory actions ensuing in chemical (secondary metabolism) and morphological differentiation (morphogenesis) of the microbial secondary metabolite producers.

The occurrence of multifaceted biosynthetic pathways for production of complex antibiotics recommend that they must have an vital role in microbial survival, either as inhibitors of other rival organisms or as regulatory effectors during some stage of the cell differentiation process because susceptible organisms need to evolve only a single enzyme to inactivate most antibiotics (Demain 1999). Relatively a small number of microbial types generate the majority of secondary metabolites. Secondary metabolites are produced in cells when the cell is not under optimum conditions, when the prime nutrient source is depleted. Secondary metabolites are produced for a limited period in cells that are no longer undergoing balanced growth. The chemical composition and their activities envelop an extensive variety of possibilities including ergot alkaloids, naphtalenes,

nucleosides, peptides, phenazines, quinolines, antibiotics, terpenoids and some complex growth factors.

The production of cost-effective important metabolites such as antibiotics by microbial fermentation is one of the most important activities of the bioprocess industry. The regulation of secondary metabolic pathways is interconnected in multifaceted ways to primary metabolic regulation (Doull & Vining 1990). Secondary metabolites are produced as metabolic products that are not essential for vegetative growth of the producing organisms but they are considered as differentiation compounds conferring adaptive roles. For example, functioning as defence compounds or acting as the signalling molecules in ecological communications. They are produced at the end of the exponential growth phase and their synthesis greatly depends on the growth conditions of the microorganisms. Regardless of this enormous diversity, microbial secondary metabolites are produced from only some precursors in pathways with a relative small number of reactions which branch from just a limited number of reactions of the primary metabolism (Demain & Fang 2000, Malik 1980, Martin & Demain 1980). Microbial production of secondary metabolites is highly sensitive to environmental factors or culture conditions. The in vitro production of majority of antibiotics depends on the composition of the culture medium in which the producer organism is grown. Hence, the medium optimisation has been the standard route for optimising antibiotic production. Secondary metabolites from *actinomycetes* play a significant role in medical preparations due to their activity as antimicrobials used in the cure of microbial persuaded infections. Other than from these activities, secondary metabolites from *actinomycetes* also own other pharmacological activities useful in the medicinal field.

Microbial secondary metabolites have been in the boundary in detection of novel antimicrobial agents in pharmaceutical industry with

potential therapeutic applications to be discovered from secondary metabolites particularly those produced by *actinomycetes*. The production of secondary metabolites improves the survival of *actinomycetes* through the synthesis of sophisticated structures, mechanisms of action, complex and energetically expensive pathways. Among prokaryotic bacteria, secondary metabolites are produced by restricted group: spore-forming bacteria, bacilli and a*ctinomycetes* (Zähner & Maas 1972). *Actinomycetes* are inexhaustible producers of secondary metabolites with biological activities (Marinelli & Marcone 2011). Exploitation of less and unknown ecosystems for *actinomycetes* is significantly essential for the innovation of novel bioactive metabolites.

Among the several described *actinomycetes* genera, merely a small number are accountable for the over 10,000 bioactive compounds in medical use. The main outstanding characteristic of the *actinomycetes* is their capability to create a large diversity of secondary metabolites. During the normal life cycle of *actinomycetes*, sporulation occurs when their growth is impaired by the supply of oxygen, nutrients and other environmental factors. It is at this point of the life cycle that secondary metabolites start to be produced. A characteristic feature of secondary metabolism is that any given organism usually produces a group of compounds belonging to the same class (Willams & Wellington 1982). Normally these are relatively low molecular weight compounds (Maplestone et al 1992, Bevan et al 1995). An additional feature of this nature of metabolism is that huge quantity of products comes up from comparatively only some intermediates in primary metabolism.

Primary metabolites are either building blocks for macromolecules, intermediates in reactions generating energy-rich compounds, coenzymes and vitamins. Secondary metabolites have no such vital roles in metabolism but still may play an important role in the life cycle of the organism. When the

actinomycetes stops growing and enters a resting phase, accumulation of primary metabolites could occur. This is potentially harmful and it has been speculated that the cells avoid this by starting to produce secondary metabolites (Malik 1980, Vining 1992). The general biosynthesis mechanism of secondary metabolites in *actinomycetes* is shown in Figure 1.6. The production of the secondary metabolites from *actinomycetes* is synchronized by pathway-specific regulatory genes that establish the beginning of antibiotic production.

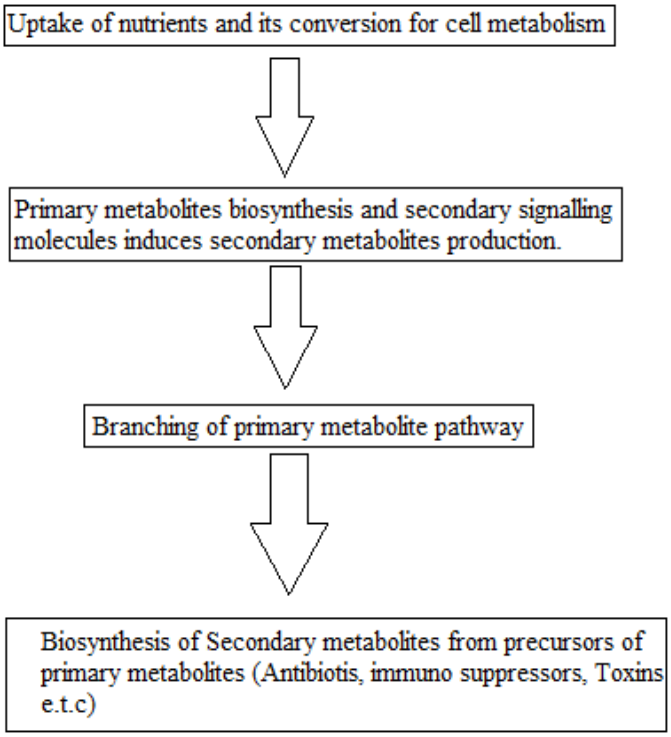

Figure 1.6 Steps involved in biosynthesis of secondary metabolites in *actinomycetes*

1.8 STREPTOMYCES – A RESERVOIR OF BIOACTIVE METABOLITES

The genus *Streptomyces* was proposed by Waksman and Henrici. The genus *Streptomyces* belong to the family *Streptomycetaceae*, Order *Actinomycetales*, Phylum *Actinobacteria*, Domain Bacteria and includes the

following species: *Streptomyces achromogenes*, *Streptomyces ambofaciens*, *Streptomyces aureofaciens*, *Streptomyces avermitilis*, *Streptomyces clavuligerus*, *Streptomyces coelicolor*, *Streptomyces felleus*, *Streptomyces ferralitis*, *Streptomyces filamentosus*, *Streptomyces griseus*, *Streptomyces hygroscopicus*, *Streptomyces iysosuperficus*, *Streptomyces lividans*, *Streptomyces noursei*, *Streptomyces scabies*, *Streptomyces somaliensis*, *Streptomyces thermoviolaceus*, *Streptomyces toxytricini*, *Streptomyces tsukubaensis*, *Streptomyces venezuelae*, *Streptomyces violaceoruber* and ~500 additional species (Kampfer 2006). They have linear chromosome, complex morphological differentiation and have an ability to produce many bioactive secondary metabolites containing significant compounds for pharmaceutical and agrochemical uses. Approximately 80% of world's antibiotics are produced by *actinomycetes*, mostly by the genus *Streptomyces* and *Micromonospora* (Pandey et al 2004). It is one of the most renowned groups of microbial secondary metabolite producers authenticated to be widespread gram positive soil bacteria of the genus *Streptomyces*. They are recognized as a rich source of antibiotics and bioactive molecules and are thus considered to be a well-off biotechnological resource. These bacteria are abundant residents in soil and exhibit outstanding metabolic diversity.

In spite of their leading role over the "golden years" of antibiotic research, only a partial number of thousands of isolated bioactive compounds proved pharmacologically effective and not too toxic for human use (Arakawa et al 2005, El-Gendy et al 2008a). In Table 1.1 listed some of the most important secondary metabolites with biological activities produced by *Streptomyces* sp. *Streptomyces* derived metabolites uphold a vital role in the medical treatment of bacterial infections and various types of cancer. Some of the reported biotechnologically important bioactive secondary metabolites of *Streptomyces* sp. is shown in Figure 1.7.

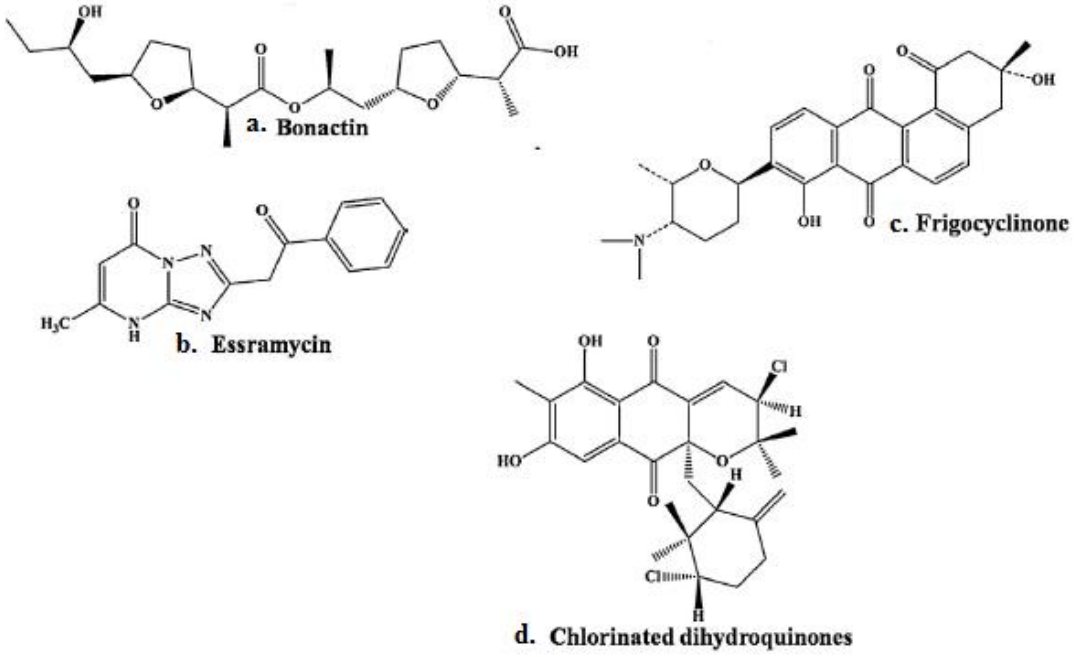

Figure 1.7 Bioactive secondary metabolites structures of Bonactin, Essramycin, Frigocyclinone, Chlorinated dihydroquinones from *Streptomyces* sp.

Table 1.1 Some of the most important secondary metabolites with biological activities produced by *Streptomyces* sp. (Lam 2006)

Bioactive compound	Source	Biological activity
Actinofuranones A and B	*Streptomyces* sp.	Cytotoxic activity
Amphotericin B	*Streptomyces nodosus*	Antifungal
Altemicidin	*Streptomyces sioyaensis*	Anticancer
Asplasmomycin	*Streptomyces griseus*	Antibiotic
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer
Bonactin	*Streptomyces* sp.	Antibacterial; antifungal
Bialaphos	*Streptomyces hygroscopicus*	Herbicide
Caprolactones	*Streptomyces* sp.	Anticancer

Table 1.1 (Continued)

Bioactive compound	Source	Biological activity
Chinikomycins	*Streptomyces* sp.	Anticancer
Chlortetracycline	*Streptomyces aureofaciens*	Antibiotic,Growth promotant
3,6-disubstituted indoles	*Streptomyces* sp.	Anticancer
Daryamides	*Streptomyces* sp.CNQ-085	Anticancer, antifungal
Doxorubicin HCl	*Streptomyces peucetius*	Antitumor
Erythromycin A	*Streptomyces erythreus*	Antibiotic
Frigocyclinone	*Streptomyces griseus*	Antibacterial
Glaciapyrroles	*Streptomyces* sp.	Antibacterial
Gutingimycin	*Streptomyces* sp.	Antibacterial
Himalomycins	*Streptomyces* sp.	Antibacterial
Istamycin	*Streptomyces tenjimariensis*	Antibiotic
Komodoquinone A	*Streptomyces* sp.	Neuritogenic activity
Lajollamycin	*Streptomyces nodosus*	Antibacterial
Marinone	*Streptomyces* sp.	Antibiotic
Piericidins C7 and C8	*Streptomyces* sp.	Anticancer
Piperazimycins	*Streptomyces* sp.	Anticancer
Pyrostatins A and B	*Streptomyces* sp. SA-3501	N-acetyl-betaglucosaminidase inhibition
Pyrizinostatin	*Streptomyces* sp. SA-2289	Pyroglutamyl peptidase Inhibition
Salinamides A and B	*Streptomyces* sp.	Antibacterial, anti-inflammatory
R-10-methyl-6-undecanolide (6R,10S)-	*Streptomyces* sp. B6007	Phytotoxic, anticancer

Table 1.1 (Continued)

Bioactive compound	Source	Biological activity
Resistomycin	*Streptomyces corchorusii* AUBN(1)/7	Antiviral
Resistoflavine	*Streptomyces chibaensis* AUBN(1)/7	Anticancer, antibacterial
Streptokordin	*Streptomyces* sp. KORDI-3238	Anticancer
Tetracenomycin D	*Streptomyces corchorusii* AUBN(1)/7	Anticancer, antibacterial
Trioxacarcins	*Streptomyces* sp.	Antibacterial; anticancer; antimalarial

Streptomyces secondary metabolites are biosynthesized through microbial fermentation. The biosynthesis of secondary metabolites in *Streptomyces* is brought by exhaustion of a nutrient, biosynthesis or addition of an inducer or by a growth rate decreases and occurs at the late growth phase (Bibb 2005). The indication is frequently a low molecular weight inducer which acts as harmful control by necessarily inactivating a regulatory protein (repressor protein) which usually prevents secondary metabolism and morphogenesis during rapid growth and nutrient sufficiency (Ohnishi et al 2005). Nutrient/growth rate/inducer signals presumably activate a “master gene” which either acts at the level of translation by encoding a rare tRNA, or by encoding a positive transcription factor. Formation of bioactive compounds regulated by nutrients (nitrogen, phosphorous and carbon source), metals, growth rate, feedback control and enzyme inactivation (Sanchez & Demain 2002). For the synthesis of secondary metabolites, nearly 5% of the *Streptomyces* genome (between 23 and 30 gene clusters) is devoted (Ikeda et al 2003). The indication of biosynthesis is brought by environmental indication including oxygen, phosphate, presence of carbon and nitrogen sources as well as variables like pH, temperature and light (Omura et al 2001,

Martín 2004). The high complex physiological adaptability life cycle of *Streptomyces* and capability to produce enormous diversity of secondary metabolites is a sign of their genome size. The genomes sizes of *Streptomyces griseus* (8.54 Mb), *Streptomyces coelicolor* (8.7 Mb) and *Streptomyces avermitilis* (9.03 Mb) are among the largest genome sequences found in the microbial world (Ohnishi et al 2008, Weber et al 2003). Since the majority of bioactive products of *Streptomyces* are generated by secondary metabolism, the part of the metabolic machinery of the *Streptomyces* have no essential role in the development of the producing organisms, but express advantages to the important species concerning its durable survival in the biological community and environment (Williams et al 1989, Vining et al 1995, Luckner et al 1977, Oleskin 1994, Vining 1992). An inverse correlation is usually observed between specific growth rate and the formation of antibiotics. An utmost invention rate of antibiotics, pigments, alkaloids, mycotoxins, enzyme inhibitors has frequently been observed when growth-promoting substrates were depleted from the medium (Demain 1992). This phenomenon is called "catabolite regulation". This may be one of the reasons for the phase-dependency of biosynthesis of many microbial drugs. Some of the secondary metabolites obtained from *Streptomyces* sp. is shown in Figure 1.8.

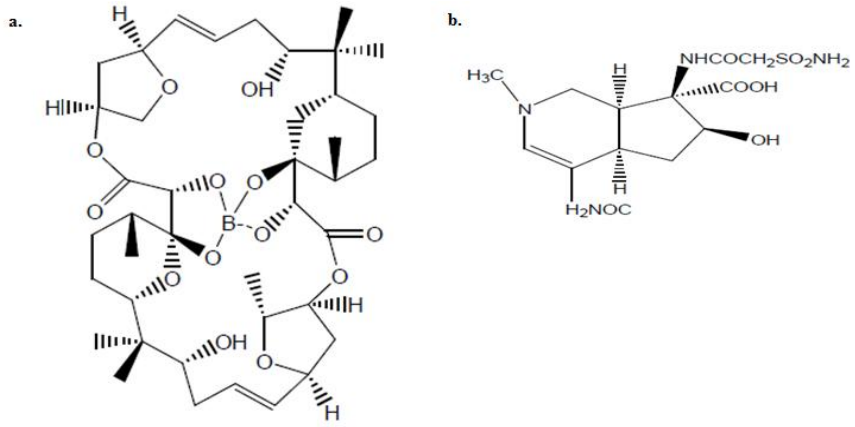

Figure 1.8 Bioactive secondary metabolite compounds reported in marine *Streptomyces* sp. possessing antibiotic and anticancer activity (Takahashi et al 1989, Nakamura et al 1977)

1.9 NEED FOR FINDING NOVEL POTENT RELIABLE METABOLITES THAN EXISTING

The prevailing increase in resistance to antibiotics has become a threat to public health worldwide. Regardless of the huge selection of reported compounds, there is constantly increasing need for novel pharmacologically active molecules and potential drug leads. The urge in finding the alternative potent metabolites (antibiotics) is to overcome the adverse clinical effects which compromise the use of many drugs usage in human therapeutic treatments. The prime goal of the research is to provide alternative treatment caused by the resistant pathogenic microorganisms such as virus, bacteria, fungi which continue as the leading cause of death and drain of resources world-wide. After the introduction of antibiotics, the future without the effective antibiotics is now facing the problem. Only a small division of the bioactive compounds established in nature have been applied in commercial purposes in medicine, pharmacology or agriculture (Newman & Cragg 2007) natural products especially secondary metabolites (antibiotics) continue to uphold a central role in the development of new drugs. Together, developed and developing countries are growing with antibiotic resistance has became phenomenon nowadays. The following relevant reasons are as follows:

- The uncontrolled, open and frequent improper utilization of the majority of metabolites (antibiotics) is the foremost reason why antibiotics are rapidly losing their efficiency.
- Improper review of antibiotic recommendation practices by the clinicians.
- Presence of majority of the prescriptions is unreasonable as they are based by the information gathered from representatives of pharmaceutical companies, especially for the innovation of newer antibiotics.

- The awareness of the issues involved in the antibiotic therapy is very deprived.
- There is also a direct cost to environmental society apart from the medical consequences of antibiotic resistance.
- Economic load of antimicrobial resistant organisms on health care expenses in developing countries (India) is not known.
- Development of antibiotic resistant genes against the existing pathogens.

1.10 IMPORTANCE OF OPTIMIZATION OF PROCESS PARAMETERS FOR THE PRODUCTION OF SECONDARY METABOLITES

Bio processing in its many forms involves a huge number of multifaceted enzyme catalysed reactions within definite microorganisms and these reactions are critically dependent on the physical and chemical conditions that exist in their environment. Successful bio processing will occur only when all the essential factors are brought together. To recognize and organize a fermentation process, it is essential to be familiar with how the organism responds to a set of quantifiable environmental conditions. The optimization of physicochemical conditions is inevitable in any fermentation process and it is more often than not performed by altering the levels of one self-determining variable while fixing other variables at a certain level. Conventional method is laborious and time consuming and frequently interaction effects are ignored, which demands the requirement of additional foremost technique by which numerous variables can be optimized in quite only some experiments.

Optimization of process parameters is an essential prerequisite to get higher productivity using any microbial strain and depends on nutrients supply. Consequently it is imperative to identify the appropriate nutrients and cultural conditions requisite to accomplish elevated productivity (Singh et al 1989). Designing a suitable fermentation parameters and conditions is of crucial importance to improve the efficiency and productivity of bioactive microbial metabolites fermentation process, since it can significantly affect the product production, yield and cost of downstream product separation (Wang et al 2008). The physical and chemical parameters like pH, temperature, incubation period, agitation rate, salinity, carbon and nitrogen sources and amino acid plays a major role on the production of bioactive compounds and antimicrobial agents (Gunasekaran & Poorniammal 2008). Optimization of bioprocess parameters to achieve high titre is a prerequisite for commercial production of any metabolite.

The optimization of fermentation variables using traditional approaches such as one variable at a time (OVAT) is not only time consuming and ignores the interactive effects amongst the variables when a number of variable are concerned. Response Surface Methodology (RSM) is a powerful technique for testing multiple process variables because less experimental trials are essential and compared to the study of one variable at a time (Box et al 1978). The interactions between variables can be recognized, acknowledged and quantified by using this RSM technique. The conventional technique of optimization involves changing one parameter at a time and observing the others constant. This repeatedly does not carry about the consequence of interaction of variety of parameters as compared to factorial design (Montgomery 1997). RSM is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carry out a limited number of experiments (Box & Draper 1987). In this study RSM was applied especially central composite design (CCD), to further

attempt to enhance the yield of secondary metabolites (g) production. The CCD is carried out in the most favourable vicinity to establish the accurate optimum values of the manifold variables. The optimization of environmental parameters (pH, temperature, incubation period, salinity and agitation rate), which have been predicted to play a very significant role in enhancing the production of secondary metabolites. Hence the use of experimental factorial design and RSM was already successfully applied in other fields (Popa et al 2007, He et al 2004, Sathyanarayanan et al 2011, Mann et al 2010) was well suited to study the main and interaction effects of the factors on the production of the secondary metabolites. Selection of the best optimum parameter ranges were conducted by the classical methods involves changing one independent variable while maintaining all others at fixed level. This selection was conducted after optimization of parameters using RSM.

1.11 SIGNIFICANCE OF CHOOSING EXTRACTION METHOD FOR ISOLATION OF SECONDARY METABOLITES

Extraction, as the term used pharmaceutically, involves the separation of bioactive secondary metabolites from microorganism, animal or plant by using selective solvents in standard extraction procedures. Extraction is the method of taking out or withdrawal of secondary metabolites from source of interest. The most common approach for the extraction of secondary metabolite compounds in microorganisms from aqueous samples (culture broth) is liquid - liquid extraction. The solvent is chosen in such a way it is not or only partial miscible with the liquid. The active agent (metabolites) present in the liquid (culture medium) transfers into the solvent is the basic principle of this method. The separation of two phases (aqueous and organic phase) by gravity or centrifugal forces. Since the procedure of extraction based on use of solvents, liquid extraction is also called solvent extraction method.

Chloroform, ethyl acetate, hexane, isooctane, petroleum ether, diethyl ether, dichloromethane are used as common water-immiscible organic solvents used for separation of bioactive metabolites. The separation of two phases occurs after separation, first phase is aqueous (often the denser or heavier phase) and the other phase is an organic solvent (the lighter phase). The base of the extraction process is that the polar hydrophilic compounds prefer the aqueous (polar) phase and the non-polar hydrophobic compounds prefer the organic solvent. Liquid/liquid extraction is frequently used as the preliminary step in the development of a reaction, prior to final purification of the product by recrystallization, distillation or sublimation.

A variety of approaches are employed in the recovery of microbial metabolites depending on whether the compounds of interest are completely or partially excreted by the cells into the medium or present within the cells. Microbial metabolites are often produced in low yields but yield a complex mixture of compounds. The purification of the compounds in the intracellular metabolite synthesis method is tedious and it is a major disadvantage while extracellular metabolites method of synthesis scores advantage in easier purification. The separation of microbial cells from the liquid medium was carried out prior to secondary metabolites extraction. Clarification is achieved by filtration or centrifugation depending on the physical properties of the medium, morphology and size of the cells. Clarification was performed and extraction of metabolites associated with the cells was established for the effective isolation of the extracellular metabolites. Although two distinct approaches for liquid – liquid extraction are possible (discontinuous and continuous liquid – liquid extraction), the equilibrium is reached only in discontinuous liquid – liquid extraction method. The most common approach in discontinuous extraction is by achieved using separating funnel. The preliminary removal of physical ‘‘impurities’’ (e.g., cells, cell debris, insoluble medium components) is the major advantageous if the metabolites

of interest are primarily extracellular (Funayama et al 1989, Cao et al 2002). Since most of the antibiotics are synthesized in extracellular method along with liquid- liquid extraction for the retrieval antibiotics, extracellular method is chosen to be the best for extraction of the antibiotics (Hacene 2000).

1.11.1 Certain Advantages for Selecting Liquid – Liquid Extraction for Secondary Metabolites Extraction

- Handling of great capacities are possible with less energy.
- Processing of heat sensitive metabolites in ambient temperature.
- Cost effective in nature.
- It separates even small amount of metabolites from aqueous phase (culture medium).

1.12 APPLICATIONS OF SECONDARY METABOLITES FROM *STREPTOMYCES* sp.

1.12.1 Antibiotics

Regardless of the achievement in the discovery of antibiotics and progress in the process of their production, infectious diseases leading cause of death worldwide and bacterial infections cause approximately 17 million deaths annually, affecting population. The discovery of Streptothricin in 1942 made the history of antibiotics derived from *Streptomyces* and with the discovery of Streptomycin two years later, scientists exaggerates the search for antibiotics within the genus. Since *Streptomyces* are an economically significant group of organisms among *actinomycetes* and they are the crucial source for several biologically active compounds (Berdy 2005). About three quarters of all the identified therapeutically useful antibiotics

(Kieser et al 2000, Cundlife 1989) and several agriculturally important compounds (Okami & Hotta 1988) were obtained from the *Streptomyces*.

1.12.2 Antibacterial Activity

An antibacterial substance inhibits the growth of bacteria or kills the bacteria. The prevalence of resistance is higher in the present antibiotics, finding of newer alternatives is highly essential. There are several reports extensively studied for antibacterial activity in *actinomyces* especially *Streptomyces* sp. A compound isolated from *Streptomyces* sp. BD21-2 obtained from a sea sediment samples showed antimicrobial activity against Gram positive and Gram negative cells (Schumacher et al 2003). Similarly, Frigocyclinone, a new angucyclinone antibiotic isolated from *Streptomyces griseus* strain NTK 97, showed antibacterial activities against Gram-positive bacteria (Bruntner et al 2005).

1.12.3 Antifungal Activity

Numerous antibiotics have been isolated from diverse microorganisms; still studies are being carried out to identify novel antibiotics which are effective against pathogenic fungi (Atlas & Bartha 1993). A group of *Streptomyces* isolates isolated from the rhizosphere of sixteen medicinal plants was assessed for in vitro antagonistic activity against six plant pathogenic fungi such as *Alternaria brassicicola, Alternaria porri, Colletotrichum gloeosporioides, Fusarium oxysporum, Penicillium digitatum* and *Sclerotium rolfsii*. The extract confirmed to show the maximum activity against the test organisms, with a minimum inhibitory concentration (MIC) of 0.781 to 6.250 mg/ml (Sutthinan Khamna 2009). A *Streptomyces* sp. ERI-04 isolated from Western Ghats showed antifungal activity against plant pathogens and dermatophytes. The minimum inhibitory concentration of the organic extract of ERI-04 against *Aspergillus niger* was 62.5 and 125 mg/ml

for *Trichophyton rubrum*, *Epidermophyton floccosum*, *Magnaporthe grisea* and *Trichophyton Simii* (Arasu et al 2010). N- (2-hydroxyphenyl)-2-phenazinamine, a new antibiotic isolated from *Nocardia dassonvillei*, showed effective antifungal activity against *Candida albicans*, with a minimum inhibitory concentration of 64 µg /ml with a greater cancer cell cytotoxicity against HepG2 (Human hepatic carcinoma cells), HCT-116 (Human colon carcinoma cells) and COC1 (Human ovarian cancer) cells (Gao et al 2012).

1.12.4 Anticancer Activity

The series of molecular events which alter the growth and properties of the normal cells results in cancer. The normal control systems are arrested which in turn lead to over growth of the normal cells and invasion of the tissues are disabled. These altered cells which grow and divide in the presence of signals that inhibit the growth of the normal cells; therefore, the requirement of specific signals is not required to induce the cell division and growth. The change in the cell structure, production of new enzymes and decrease in the cell adhesion are developed with the induction of cancer. A marine *Streptomyces* sp. KS1908 isolated from a marine sediment sample was reported for the anticancer activity against HEp-2 (Laryngeal carcinoma cells), HeLa (Human cervical cancer cells), HL-60 (Human promyelocytic leukemia cells) and MCF-7 (Human Breast adenocarcinoma cancer cells) (Kadiri et al 2013). A *Streptomyces* sp. isolated from marine environment was tested for in vitro cytotoxicity assay on human HeLa cancer cell line shown that the secondary metabolite had the strongest cytotoxicity with IC_{50} (50% of inhibitory concentration) 21.50 µg/ml (Chidambara Rajan et al 2012). A *Streptomyces* sp. from marine sediment of Todos Santos Bay, Mexico showed cytotoxic activity of IC_{50} of 69.0 to $\leq$ 0.076 µg/ml against colorectal cancer cells HCT-116 (Amayaly et al 2012).

1.12.5 Cytotoxic Activities

The crude extract of *Streptomyces avidinii* SU4, isolated from marine sediment showed cytotoxic activity against Hep2 cell line, 250 µg/ml in VERO (Green monkey kidney) cell line. This value is very close to the criteria of cytotoxicity activity for the crude extracts, as recognized by the American National Cancer Institute is in IC_{50} < 30 µg /ml (Sudha et al 2012). A bioactive *Streptomyces* sp. isolated from marine sediment samples of Bay of Bengal yielded a potent cytotoxic activity against Gastric adenocarcinoma and Hepatic carcinoma cell lines (Gorajana et al 2007). 5-(2,4-dimethylbenzyl)pyrrolidin-2-one (DMBPO) from marine *Streptomyces* VITSVK5 sp. revealed cytotoxic activity on HEp2 and HepG2 (Human hepatic carcinoma cells) cell lines with the IC_{50} value of 2.8 µg/ml and 8.3 µg/ml, respectively, as compared to Vero cell line (Saurav and Kannabiran, 2012). Two new cytotoxic antibiotics, piericidins C7 and C8 were isolated from a marine *Streptomyces* sp. (Hayakawa et al 2007). The isolated compounds showed selective cytotoxicity against adenovirus E1A gene with IC_{50} of 1.5 nM and 0.45 nM, respectively, inhibited the growth of Neuro-2a cells IC_{50} of 0.83 nM and 0.21 nM, respectively without cell death.

1.12.6 Antioxidant Activities

Scientific evidence proposes that antioxidants decrease the threat for chronic diseases including cancer and heart disease. The chief attribute of an antioxidant is its capacity to entrapment of free radicals. Elevated level of reactive free radicals and oxygen species are present in biological systems may oxidize nucleic acids, proteins, lipids or DNA (Deoxyribonucleic acid) and can begin degenerative diseases. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and inhibit the oxidative mechanisms that lead to degenerative diseases (Dekkers et al 1996). *Actinomycetes* isolated from

three different garden soil samples of VIT University exhibited antioxidant potential of the crude extract was found maximum at the concentration of 20 mg/ml with 60% inhibition (Devi et al 2013). *Streptomyces* sp. AM-S1 strain isolated from forest humus soil in Gyeongsan, South Korea, revealed that the culture filtrate of *Streptomyces* sp. AM-S1 successfully scavenged DPPH (2,2-diphenyl-1-picrylhydrazyl) with IC_{50} 90.2 µl/ml and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) with the IC_{50} 13.2 µl/ml radicals in a concentration dependent manner (Sowndhararajan & Sun Chul Kang 2013). Marine *Streptomyces* sp. LK-3 showed prospective antioxidant activity against the antioxidant studies. Similarly a marine *Streptomyces* sp. VITTK3 own greater DPPH free radical scavenging up to 96% at 5 mg/ml. The compound DMBPO from *Streptomyces* sp. VITSVK5 showed 59.32% DPPH scavenging activity and it showed cytotoxic to cancer cells (Thenmozhi et al 2010).