BIOACTIVE SECONDARY METABOLITES: AN OVERVIEW

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Abstract

Human mind has been successfully controlling every component of ecosystem, whether it is biotic or abiotic. The components which are not been even visible, the microbes have been exploited by science.

In present sciences, there is no field in which microbially obtain products are not used from medicine to agriculture, food additives to even fuels, agrochemicals. Many advances have been developing in antibacterial drugs. With development in scientific techniques there has been great improvement in methods of production in microbial metabolites.

Keywords: Abiotic, Microorganisms, Food Additives, Antimicrobial Drugs, Metabolite.

1. INTRODUCTION

Secondary metabolites include the substances with sophisticated and variable chemical structures, synthesized by certain variety of strains of microbial species. Apart from the commonly known antibiotics, there is other enormous range of SM with many other biological activities. This makes them economically important for related industries. These are mainly synthesized with the aim of either providing protection to the microbes from any other biological stimulus or harm; including plants, insects, humans, even other microorganisms, or regulating many biochemical pathways of higher organisms. Some of the important substances, apart from antibiotics, are other medicinals, toxins, biopesticides and animal and plant growth factors [**1**].

1.1 Microbial Secondary metabolites

In nature, microbial secondary metabolites are important to these organisms by performing following functioning as: sex hormones, ionophores, competitive weapons against other bacteria, fungi, amoebae, insects and plants are agents of symbiosis, and effectors of differentiation. Outside the cell these microbially produced secondary metabolites are extremely important for human health and nutrition [**2**]. These important classes of highly valuable compounds play wide range of roles, as drugs [antibiotics, anti-cancer agents, immunosuppressants], agrochemicals [pesticides, insecticides, antifeedants], biofuels [squalene, oleoresin] and food additives [carotenoids, flavonoids, essential oils]. However, these compounds are usually produced in very low amounts [or not at all] under typical laboratory conditions in the species from which they originate.

1.2 Bioactivity of Microbial Secondary Metabolites

It has always been the most obscure area in whole field of microbiology and biochemistry, to elucidate the exact definition, real position and function[s] of secondary metabolites. They are mostly needless for the producers, having no apparent function in their life cycle. There incredible array of exquisite chemical structures, very rare occurrence and versatile biological actions are their most characteristic features [**3**]. The biological activity may be

studied at in vivo level, or may appear at the in vitro molecular level, in the whole organism. But consideration is taken for the possible differences and specific features are manifested in these varying levels.

The secondary metabolites isolated from microbes exhibits either antimicrobial; which includes-antibacterial, antifungal, antiprotozoal action; antitumor or antiviral activities, earlier known as antibiotics. With the aid of our recent knowledge, the term “antibiotic” is more or less an obsolete conception, whenever there is discussion regarding the bioactive Secondary Metabolites. Its actual definition should include all of those microbial secondary metabolites which perform the following functions:

- Growth processes
- Replications
- Exhibit regulatory, inhibitory, or stimulating actions at biochemical level to the prokaryotic or eukaryotic cells at minimal concentration

The practical importance of antibiotics and other SMs is incredible. They have wide applications in the human therapy and veterinary therapy, agriculture, scientific research and in numerous other areas.

In general, the microbial metabolites may be practically utilized in three different ways:

- **Commercialization**: Applying the natural/fermentation product directly in the medicine, agriculture, or in any other fields.
- **Derivatization**: Using as starting material for subsequent chemical or microbiological modification.
- **Rational drug design**: Lead compounds for chemical synthesis of new analogs or as templates in the RDD studies.

1.3 Distribution of Bioactives

Antibiotics and similar microbial products, being secondary metabolites can be produced by many strains of the different classes of microorganisms.

In the prokaryotic group, unicellular bacteria *Bacillus* and *Pseudomonas* species are the most recurrent producers. In the recent years *Myxobacteria* and *Cyanobacteria* species have joined these distinguished organisms as productive species. *Mycobacteria*, Mycoplasmatales and Spirotheces are also the frequent producers. Among the entire pool of bioactive compounds known, 17% constitute those of microbial origin. Over 10000 bioactive compounds are derived from the filamentous actinomycetales species produces, 7600 from *Streptomyces* and 2500 from the rare actinomycetes species.

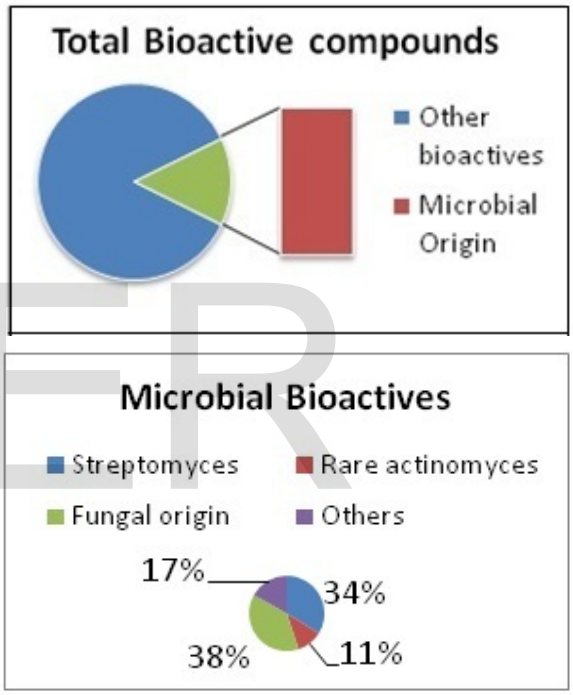

Figure 1:- [Above] Distribuiton of all Bioactive compunds derived from all biological sources. [Below] Distribution of major contributors of microbial bioactives.

Practically most of the presently known non-antibiotic bioactivities may be classified as:

- Medical or Pharmacological-biochemical activity
- Agricultural applications
- Regulatory, biophysical and other activities

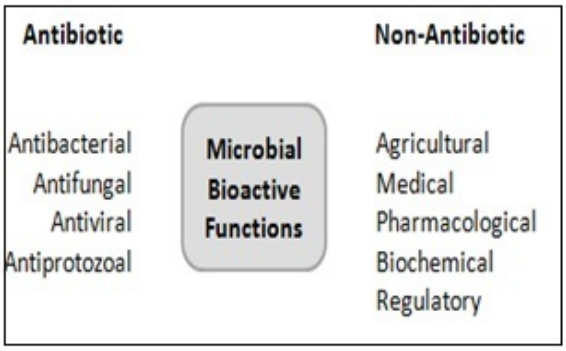

Figure 2: - The various bioactive functions of microbial SMs

After the antibiotic function, the second most important function of the SMs, applicable in the medical field, is the enzyme inhibition and related activities. Presently over 3000 compounds are discovered to possess inhibitory activity against about 300-350 various enzyme systems. These enzyme inhibitory compounds are used in agriculture also. Apart from these, there are certain immunological functions also. There are about 800 immunoactive, immunosuppressive, immunostimulatory compounds, hundreds of compounds with diverse regulatory functions; inhibitory, agonist and antagonist, anti-inflammatory/antioxidative, hypocholesterolemic, antimetabolite and various toxic action. The detected biochemical activities include the tubulin [microtubule] assembly inhibition, interferon induction, antimitotic/antimitogenic activity, DNA damage, antimutagenic effects, apoptosis induction, angiogenesis inhibition, etc. . There is a large variation in the assays employed for detecting these activities, and the range of the final physiological, biological, biochemical, phytochemical and microbiological effects include around 1000 bioactivities. The medicinal, pharmacological or immunological compounds discovered by these specific methods may be called differently as “biopharmaceutins” [**4**].

Besides the health care, feeding billions of people is one of the most urgent needs for humankind. The increase in the agricultural production, including the animal husbandry, along with environmental and economic requirements to be taken into consideration at the same time, leads to the idea of diverging towards microbial compounds over the synthetic chemicals. Most of the environment friendly agricultural compounds are the microbial products or their derivatives. The microbial compounds used for the so called agricultural and related activities include mainly:

- **Pesticides**: Insecticide, miticide, larvicide, repellents, deterrents, antifeedants, antiparasitic, anthelminthic agents, cestocides, acaricides, nematocides, antiworm compounds, algicides, other biocontrol agents
- **Herbicides**: Phytoactive agents: phytotoxins, phytohormones, plant growth regulators, germination inhibitors, allelochemicals, chlorosis inducers
- **Feed additives**
- **Preservatives**
- **Growth promoters/ permittants**

2. VARIOUS BIOACTIVE SECONDARY METABOLITES

Microbial SMs include antibiotics, enzyme inhibitors, antitumor agents, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, immune-modulating agents, receptor antagonists, pesticides and growth promoters of animals and plants. They have a key effect on the health as well as commercialization of our society. They are unique for having unusual discrete structures and their synthesis is regulated by amount of available nutrients, growth rate of the microbe, feedback mechanism, enzyme inactivation and stimulation. Regulation of production of SMs is influenced by the unique low molecular mass compounds, inducers, transfer RNA, sigma factors and gene products formed during the phase after exponential development. The main enzymes of secondary metabolism i.e. synthases, are often coded by genes clustered on chromosomal DNA and rarely on plasmid DNA. Contrasting the primary metabolism, the biochemical pathways of secondary metabolism are still unrevealed to a great extent and thus give opportunities for generalized investigations of regulatory actions, enzymology, control and differentiation.

2.1 Antimicrobial agents

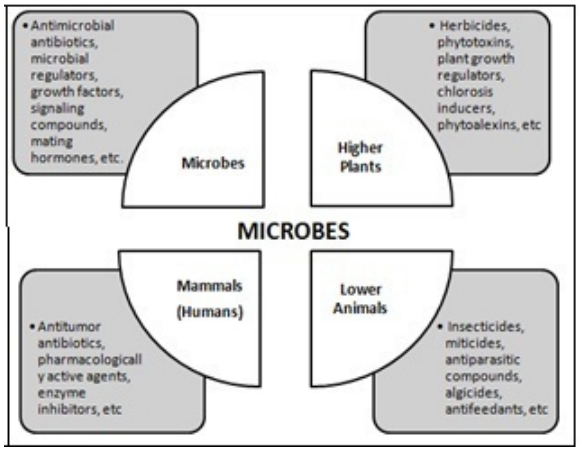

Figure 3:- Various applications of Microbial secondary metabolites depending on the type of target organism

1. Compound had microbial origin
2. Direct interaction with any other micro-organism

These key properties are reflected in the definitions in three science dictionaries. Problems with this definition arose when semi-synthetic and synthetic "antibacterial" compounds came into existence. Therefore fluoroquinolones are not antibiotics by definition. Two consequences of this was that the term antibiotic was redefined including synthetic and semi-synthetic compounds, and the term antimicrobial was used to capture non-natural compounds, including those active against microbes other than bacteria or fungi [**5**].

Antibiotics are generally used in the past for antimicrobials. However, it is now more often used to quote the antibacterials and is understood commonly in this way. Exclusively now, when people talk about antibiotic resistance, they are pointing towards antibacterial resistance.

Antibacterials are divided into two groups according to their speed of action and residue production:

A) **Non-residual producing**: The first group contains those that act rapidly to destroy bacteria or causative organism, but quickly disappear, either by evaporation or breakdown and leave no active residue behind. Examples of this type are the alcohols, chlorine, peroxides, and aldehydes.

B) **Residue producing**: The second group consists mostly of newer compounds that leave long-acting residues on the surface to be disinfected and thus have a prolonged action. Common examples of this group are triclosan, triclocarban, and benzalkonium chloride.

2.1.2 *Advances in Antibacterial drugs*

Many new antimicrobal agents with new target sites recently marketed and still awaiting FDA approval includes the following:

- **Macrocyclic antibiotics**: One of the newly introduced drugs Fidaxomicin is a new class of bioactive molecule, having narrow spectrum of activity and active against CDI. It is a substitute of vancomycin and metronidazole.
- **Newer Cephalosporins**: One of the novel broad-spectrum antibiotic effective against MRSA, VISA, and VRSA is this Ceftaroline [**6**]. Another newly discovered cephalosporin Ceftobiprole is a broad-spectrum antibiotic, active against MRSA [**7**].
- **Novel dihydrofolate reductase inhibitors**: Iclaprim, a selective inhibitor of enzyme dihydrofolate reductase, synthetic by origin. It shows activity against *Staphylococcus pneumoniae* and *Staplylococcus aureus*.
- **Oxazolidinones**: In the last 30 years, oxazolidinones are considered to be the first new class of antibacterial drugs. During 2009, linezolid is the sole oxazolidinone present in the market for treatment of gram-positive infection, including MRSA or VRE. Radezolid and torezolid are the two novel oxazolidinones under research and passes the clinical trial.
- **Lipopeptides**: These are the new class of antimicrobials, with daptomycine as its first member. It is a cyclic lipopeptide antibiotic derived from *Streptomyces roseosporus*. It has a unique action mechanism by inserting the lipophilic tail in the cell membrane without entering the cytoplasm of gram-

positive bacteria. This leads to channel formation dependent on calcium, further leading to leakage of intracellular K^+ ions causing disruption of cell membrane and cell death [**8**].

2.2 Herbicides & Weedicides

The application of microbial SMs obtained from soil microbiota for weed control represents an innovative means to manage bothersome weeds. These compounds kill or prevent the growth of weeds so that valuable plant species can gain a viable advantage. A major section of microorganisms present in our environment is largely untapped, and there is need to reveal their potential, discover novel chemical moieties, so as to substitute them with the conventional chemical herbicides. This replacement would help reduce soil erosion, degradation caused due to tillage.

Invasion of weeds lead to threaten the productivity of agricultural lands and natural areas; however, for many weeds adequate, cost-effective control measures presently are not available [**9**]. Introduction of biological controls for preventing the invasion of unwanted plants represents an alternative way to slow the spread of these weeds using natural, least harmful tools [**10**]. The recent advances in genomics, genome mining and molecular techniques will help in improving the understanding of the wealth of genetic diversity and potential in the soil and to better exploit the plant-microbe interactions. The development of biocontrol agents would reduce the need for chemical herbicides and provide greater and better options for weed management. Microbes have a place in integrated, ecologically based weed management and their potential is being realized and explored.

Biological controls for weeds are generally classified into:

1. **Classical**: The classical approach involves the introduction of a control agent into an new area where it was unused, and where the agent eventually sustains itself. An example is the release of *Puccinia chondrillina* to control rush skeletonweed [*Chondrilla juncea*] [**11**].
2. **Augmentative or inundative**: This method refers to repeated application of a foreign agent with the intention to lower the weed densities to a level where beneficial plant species can compete. An example of such a control is *Colletotrichum gloeosporioides* for the control of sicklepod [*Senna obtusifolia L.*][**12**].
3. **Cultural weed**: This control includes crop rotation, fallow periods, and hygiene to prevent the introduction and spread of weed seeds, and maintain the soil fertility to produce healthy crop plants.

2.3 Anti-cancer drugs

The increase in resistance to conventional anticancer therapies in patients with advanced solid tumors has lead to the development of novel cancer therapies that are selective for cancer cells with limited toxicity to normal tissues is a challenge for oncology researchers. Microorganisms, such as viruses with selectivity for tumor cells or tumor micro-environments, have been investigated as potential arsenals for decades. Genetically-modified, non-pathogenic bacteria have begun to emerge as potential antitumor agents, either to provide direct tumoricidal effects or to deliver tumoricidal molecules. Attenuated *Salmonella*, *Clostridium* and *Bifidobacterium* are capable of multiplying selectively in tumors and inhibiting their growth, representing a new approach for cancer treatment. Because of their selectivity for tumor tissues, these bacteria would also be ideal vectors for delivering therapeutic proteins to tumors [**13**].

Due to their selectivity for tumor tissues, these bacteria and their spores also serve as ideal vectors for delivering therapeutic proteins to tumors. Bacterial toxins too have emerged as promising cancer treatment strategy. The most potential and promising strategy is bacteria based gene-directed enzyme pro-drug therapy. Although it has shown successful results in vivo yet further investigation about the targeting mechanisms of the bacteria are required to make it a complete therapeutic approach in cancer treatment.

Experimental studies have shown that pathogenic species of the anaerobic clostridia were able to proliferate

preferentially within the necrotic [anaerobic] regions of tumors in animals as compared to normal tissues thus resulting in tumor regression but was accompanied by acute toxicity and most animals became ill or died [**14 , 15**].

2.3.1 *Bacterial Toxins*

Bacterial toxins have to some extent already been tested for cancer treatment. Bacterial toxins can kill cells or at reduced levels alter cellular processes that control proliferation, apoptosis and differentiation. These alterations are associated with carcinogenesis and may either stimulate cellular aberrations or inhibit normal cell controls. Cell-cycle inhibitors, such as cytolethal distending toxins [CDTs] and the cycle inhibiting factor [Cif], block mitosis and are thought to compromise the immune system by inhibiting clonal expansion of lymphocytes. In contrast, cell-cycle stimulators such as the cytotoxic necrotizing factor [CNF] promote cellular proliferation and interfere with cell differentiation [**16**]. CDTs are found in several species of Gram-negative bacteria, including *Campylobacter jejuni* and *Salmonella typhi* while Cif is found in enteropathogenic [EPEC] and enterohaemorrhagic [EHEC] *Escherichia coli*. The anti-tumor effect of toxins is probably with reduced side-effects compared to traditional tumor treatment. Bacterial toxins per se or when combined with anti-cancer drugs or irradiation could therefore possibly increase the efficacy of cancer treatment [**17**].

2.3.2 *Immunotherapeutic Agents*

Immunotherapy for cancer offers great promise as an emerging and effective approach. Since tumors are immunogenic, the immunotherapeutic strategy employs stimulation of the immune system to destroy cancerous cells. But the major hurdle is the ability of tumors to escape the immune system due to development of tolerance as they are weakly immunogenic and sometimes body takes them as self antigens. Thus one of the novel immunotherapeutic strategies employs bacteria to enhance the antigenicity of tumor cells [**18**]. Attenuated but still invasive, *Salmonella typhimurium* has been reported to infect malignant cells both in vitro and in vivo, thereby triggering the immune response. Attenuated *Salmonella typhimurium* has demonstrated successful invasion of melanoma cells that can present antigenic determinants of bacterial origin and become targets for anti-Salmonella-specific T cells. However, better outcomes were achieved after vaccinating tumor bearing mice with *Salmonella typhimurium* before intratumoral Salmonella injection [**19**]. Genetically engineered attenuated strains of *Salmonella typhimurium* expressing murine cytokines have exhibited the capacity to modulate immunity to infection and have retarded the growth of experimental melanomas.

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2.4 Enzyme inhibitors

Enzyme inhibitors have received increasing attention as useful tools, not only for the study of enzyme structures and reaction mechanisms but also for potential utilization in pharmacology and agriculture. Specific and selective protease inhibitors are potentially powerful tools for inactivating target proteases in pathogenic processes of human diseases such as AIDS, cancer, arthritis, pancreatitis. Amylase inhibitors are also useful for control of diseases such as diabetes and obesity. The enzyme inhibitors obtained from microbial origin are preferable than those obtained from animals and plants, as these are low molecular weight compounds obtained from hydrolysis of macromolecules.

Streptomyces is proven source of microbial enzyme inhibitors. The extraction of these chemical moieties can be done from microbial population surviving at both terrestrial and marine habitats.

Amino-protease inhibitors

Marinostatins and monastatin are the serine and cysteine protease inhibitors, respectively, isolated from marine

bacteria, *Alteromonas* species. Marinostatins find application in the elucidation of pancreatitis pathogenesis due to their intrinsic inhibition of serine proteases. Monastatin is a novel glycoprotein, exhibiting activity against proteases from fish bacteria pathogen such as *Aeromonas hydrophilial* and *Vibrio anguillarum.*

3. PRODUCTION OF MICROBIAL SECONDARY METABOLITES-CONVENTIONAL METHODS

Microorganisms are essential for many reasons, particularly because they produce things that are of immense importance to us [**21**]. These could be either the large molecules of proteins, nucleic acids, or carbohydrate polymers, or the smaller. But broadly these molecules are divided into metabolites which are essential for vegetative growth i.e. primary and those that are insignificant as SMs. The later are usually produced in small amounts. Inimitable regulatory mechanisms are evolved inside the unicellular or multicellular microorganism that enables a strain to avoid excessive production of its metabolites so that it can compete efficiently with other forms of life and survive in nature. On the contrary, the industrial microbiologist screens for a 'thriftless' strain that will overproduce a particular compound that can be isolated in large amounts and marketed. After a desired strain has been found, a development strategy is initiated to improve the concentrations by modification of culture conditions, applying mutation and recombinant DNA techniques. The main reason for this intense research on microorganisms to produce compounds that can otherwise be obtained from plants and animals, or designed by chemists, is the ease of increasing the production by genetic and environmental manipulation. Approximately 1000-fold increases have been recorded for these SMs [**22**].

The search for new antibiotics continued with the increasing demands to:

- Combat evolving pathogens, naturally resistant bacterial and fungal strains and previously susceptible microbes that have developed resistance
- Enhance pharmacological properties
- Combat tumors, viruses and parasites
- Discover safer, more potent and broader spectrum compounds for agriculture

In the search for new antibiotics, the older natural antibiotic structures are modified by the chemical method of semi-synthesis.

The non-antibiotic agents were also discovered and synthesized for overproduction. Major part of therapeutics for non-microbial parasitic diseases in animals was dependent on the screened compounds followed by molecular modification. Despite the testing of thousands of synthetic compounds, only a few capable structures were found. As it became more and more difficult to find the new lead compounds, the more there came a shift towards the microbial broths to fill the void and microbial products increased in importance in the therapy of non-microbial diseases [**23**].

3.1 Directed biosynthesis

In order to enhance the SM production, the foremost technique used in any development program was the manipulation of the culture media by testing hundreds of additives as possible limiting precursors of the desired product. Occasionally, a precursor that multiplies the production of the SM is found which direct the fermentation towards the formation of one specific desirable product. This process is termed as directed biosynthesis. Examples include benzylpenicillin fermentation by using phenylacetic acid and actinomycins production by specific amino acids. There were also many stimulatory precursors such as methionine against cephalosporin C formation and valine against tylosin production.

The regulatory mechanisms of SM synthesis are genetically determined; mutations had a major effect on them. In fact, it is the main factor responsible for the 100–1000 times

increase observed in the production of antibiotics from their initial discovery till present date. These tremendous increases in fermentation productivity and the subsequent decrease in costs have come about mainly by random mutagenesis and screening for higher-producing microbial strains. Mutation has also served to shift the proportion of metabolites produced in a fermentation broth to a more favorable distribution, elucidate the pathways of secondary metabolism and yield new compounds [**24**].

3.2 Random mutagenesis

Random mutagenesis using chemical mutagens or UV light has been employed to generate strains optimized for industrial production. The producer is subjected to rounds of mutagenesis involving either UV or chemical mutagens, with screening of the surviving clones evolved with improved activity. For example, yields of clavulanic acid from *Streptomyces clavuligerus* [**25**] and rapamycin from *Streptomyces hygroscopicus* [**26**] were both improved through random mutagenesis. Again, the effects of mutagenesis and multiple rounds of screening are unpredictable. Although this approach can be applied to enhancing yields of compounds produced at low levels, it is not a suitable screening platform as it cannot be easily adapted to high throughput.

3.3 Application of RDT

The revolutionary exploitation of microbial genetic discoveries in the 1970s, 1980s and 1990s depended heavily upon the solid structure of industrial microbiology. The major microbial hosts for production of recombinant proteins are *Escherichia coli* [**27**], *Bacillus subtilis*, *Sacharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha* and *Aspergillus niger*. The use of recombinant microorganisms provided the techniques and experience necessary for the successful application of higher organisms, such as mammalian and insect cell culture, and transgenic animals and plants as hosts for the production of glycosylated recombinant proteins. The major drive of RDT has been in the fine area of rare mammalian peptides, such as hormones, growth factors, enzymes, antibodies and biological response modifiers [**28**]. Among those genetically engineered products that have been approved for use by human are insulin, human growth hormone, erythropoietin, antihemophelia factor, granulocyte-colony stimulating factor, granulocytemacrophage colony stimulating factor, epidermal growth factor and other growth factors, interleukin, interferons and bovine somatotropin.

3.5 Combinatorial biosynthesis

RDT has made a significant impact on the production secondary metabolites. Most microbial biosynthetic pathways are encoded by clustered genes, which facilitate the transfer of an entire pathway in a single manipulation. Even in fungi, pathway genes are sometimes clustered, such as the penicillin genes in *Penicillium* or the aflatoxin genes in *Aspergillus*. For the discovery of new or modified secondary products, recombinant DNA techniques are being used to introduce genes for the synthesis of one product into producers of other antibiotics or into non-producing strains, which is the basis of combinatorial biosynthesis. It is a strategy using the non conventional molecules as precursors or substrates against the microbial biosynthetic enzymes. These enzymes can be modified or mutated in such a way so as to increase their affinity for the synthetic substrates. The unique feature is that the different activity modules of an enzyme can be manipulated by genetic engineering to obtain a strain that produces novel characterized antibiotics [**29**].

3.6 Ribosomal engineering

One strikingly successful interruption in the regular procedure of SM production had been developed through the fact that resistance to antibiotics enhances the yields of some obscure secondary metabolites. In particular, resistance to antibiotics, such as streptomycin, paromycin and gentamicin, target the ribosomal protein or resistance to rifampicin via RNA polymerase mutations have proved to be effective [**30**]. The effects of these mutations can be joined for increased effects on secondary metabolism and

have been demonstrated by developing stepwise resistance against a number of ribosomal antibiotics [**31**]. The mechanism of this fascinating effect is not entirely apparent [**32**] but involves the up regulation of pathway-specific regulators [**33 , 34**]. One possible explanation that has been advanced is that the alteration of ribosome function mimics the stringent response, up regulating the production of ppGpp, which is known to increase the production of some secondary metabolites [**35**]. Regardless of how ribosome engineering actually works at the molecular level, this approach to strain improvement is advantageous in that there is no requirement for genetic engineering. Libraries of resistant mutants have been successfully screened, resulting in the discovery of novel piperidamycins [**36**].

CONCLUSION

The total number of bioactive secondary metabolites been isolated till now is around half a million. The nature still consists of enormous amount of such undiscovered compounds which are beneficial to deal with hundreds of routine issues of mankind and other living beings. Plants and microbes are the main sources of obtaining these structurally as well as functionally diverse chemical entities. Since microbes are more easy to adapt to any change in their environment, there genetic material is easy is manipulate and they create really good deal of compounds which are unique in their own way, microorganisms are more preferred choice for extracting these compounds. There is still big potential in searching for many unknown species.

Earlier methods of isolating compounds from microbes were time consuming, tedious, required repetition of experimentation and methods proving extreme levels of sterile conditions. But nowadays, the screening methods are more suitable, effective, sensitive, highly specific and automated and diminutive high throughput assay method. The high throughput screening (HTS) techniques allow screening of limitless samples within a few minutes and as fast as possible. Not even this, data also does not seem to be tedious as bioinformatics system help the rapid selection of hits for lead compounds and identification. Definitely in the future new methods will be introduced.

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