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Value Added Secondary Metabolites from Microorganism

Anjani Kumar Upadhyay [1], Binita Bhattacharyya [1], Lopamudra Ray [1&2]*

[1] School of Biotechnology, KIIT University, Bhubaneswar 751024, Odisha, India
[2] School of Law, KIIT University, Bhubaneswar 751024, Odisha, India

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Abstract: Metabolites, the intermediate products formed as a result of metabolic reactions catalyzed by different enzymes. Metabolites are usually classified into two Primary metabolites and Secondary metabolites. Secondary metabolites released from microorganisms are generally low molecular mass products and are not essential for the growth of the microorganism. However, these can have diverse important functions in nature. A secondary metabolite is called "secondary" merely because it has no apparent involvement in the vegetative growth of the producing culture and, not because it is produced after growth. Probably the most important use of microbial secondary metabolites has been as anti-infective drugs or antibiotics. The secondary metabolites are usually formed during the late growth phase of the producing microorganisms. Synthesis of secondary metabolites can be greatly influenced by manipulating the type of nutrients and their concentration in the formulation of culture media. Actinomycetes particularly Streptomyces are major sources of novel secondary metabolites with a range of biological activities that may ultimately find application as anti-infective, anti-cancer agents, or other pharmaceutically useful compounds. Though the nature of secondary metabolism is dependent on genetic information but its expression can be influenced greatly by environmental manipulations**.** Thus, secondary metabolism occurs by the exhaustion of a nutrient, or addition of an inducer and/or by a decrease in growth rate. Growth rate control appears to be important in secondary metabolism and may be the overriding factor in the cases where the nutrient limitation is needed for the production of secondary metabolites. The delay often seen before the onset of secondary metabolism was probably established by evolutionary pressures or selection pressure (environmental conditions). Many secondary metabolites have antibiotic activity and could kill the producing culture if made too early. Most of the secondary metabolites are formed via enzymatic pathways rather than by a ribosomal mechanism. Secondary metabolism is highly regulated by various factors, particularly carbon and nitrogen sources. Regulation by the carbon source – Glucose, which is an excellent carbon source for the growth of many bacteria, interferes with the formation of many secondary metabolites. In media containing a mixture of a rapidly used carbon source and slowly used carbon sources, the rapidly used carbon source is used first to produce cells but very little or no secondary metabolite is formed. Regulation by the nitrogen source – It has been seen that many secondary metabolic pathways are negatively affected by nitrogen sources though they were seen to be favorable for the growth of the microorganisms. e.g. ammonium salts. Thus, these value-added secondary metabolites can be obtained from the microorganism by regulation of the metabolic pathways.

Keywords: Secondary metabolites, microorganism, regulation.

Introduction

Metabolites, the intermediate products formed as a result of metabolic reactions catalyzed by different enzymes. Metabolites are usually classified into two Primary metabolites and Secondary metabolites. Secondary metabolites

*Corresponding author (Lopamudra Ray)
E-mail: < lray@kiitbiotech.ac.in >

released from microorganisms are generally low molecular mass products and are not essential for the growth of the microorganism. However, these can have diverse important functions in nature. A secondary metabolite is called "secondary" merely because it has no apparent involvement in the vegetative growth of the producing culture and, not because it is produced after growth [21]. Probably the most important use of microbial secondary metabolites has been as anti-infective drugs or antibiotics. The secondary metabolites are usually formed during the late growth phase of the producing microorganisms [26].

The proficiency to manipulate a biological process can be of great importance in many aspects. The effects of manipulation on secondary metabolism may be harder to predict. Many microbial products termed as secondary metabolites have been and will be of great economic, scientific, and medical importance. Many mutations have been done into strains responsible for the production of secondary metabolites to enhance the yield of a particular compound [8].

Synthesis of secondary metabolites can be greatly influenced by manipulating the type of nutrients and their concentration in the formulation of culture media [26]. Actinomycetes particularly Streptomyces are major sources of novel secondary metabolites with a range of biological activities that may ultimately find application as anti-infectives, anti-cancer agents, or other pharmaceutically useful compounds [19]. Though the nature of secondary metabolism is dependent on genetic information but its expression can be influenced greatly by environmental manipulations [28]. Thus, secondary metabolism occurs by exhaustion of a nutrient, or addition of an inducer and/or by a decrease in growth rate [26]. Growth rate control appears to be important in secondary metabolism and may be the overriding factor in the cases where the nutrient limitation is needed for the production of secondary metabolites. The delay often seen before the onset of secondary metabolism was probably established by evolutionary pressures or selection pressure (environmental conditions) [15]. Many secondary metabolites have antibiotic activity and could kill the producing culture if made too early [14].

Apart from the commonly known antibiotics, there are other huge ranges of secondary metabolites with many other biological activities Most secondary metabolites are formed via enzymatic pathways rather than by a ribosomal mechanism. The enzymes occur as individual proteins, either free or complexed [12].

Secondary metabolism is highly regulated by various factors, particularly carbon and nitrogen sources.

Regulation by the carbon source-Glucose, which is an excellent carbon source for the growth of many bacteria, interferes with the formation of many secondary metabolites [15]. In media containing a mixture of a rapidly used carbon source and slowly used carbon sources, the rapidly used carbon source is used first to produce cells but very little or no secondary metabolite is formed [23].

Regulation by the nitrogen source-It has been seen that many secondary metabolic pathways are negatively affected by nitrogen sources though they were seen to be favorable for the growth of the microorganisms. e.g. ammonium salts [20].

Thus, these value-added secondary metabolites can be obtained from the microorganism by regulation of the metabolic pathways.

A scientific approach towards the production of secondary metabolites

Microorganisms take a major role in the production of antibiotics and other drugs for treating certain diseases. With less than 1 % of the microbial world having been explored, the advances in techniques for microbial cultivation and extraction of nucleic acids from the soil and marine habitats allow access to a vast untapped reservoir of genetic and metabolic diversity [26].

Biosynthetic pathways are also involved in the production of secondary metabolism [15]. The main pathways are those in which aromatic compounds, isoprene, oligosaccharides, peptides, polyketides, and β-lactam rings are formed [26].

Secondary metabolites are formed via enzymatic pathways that occur via individual proteins, free or complexed, or through parts of

large multifunctional polypeptides carrying out a multitude of enzymatic steps and peptide synthesis. The genes encoding for enzymes of secondary metabolism are usually chromosomal. Secondary metabolism usually occurs at the late growth phase of the producing microorganisms. The secondary metabolism is often brought on by exhaustion of a nutrient or addition of an inducer and or by a decrease in growth rate [10]. Among all the nutrients, the carbon source is effective for the production of secondary metabolism. All the microorganisms need to alter and interconvert a huge number of organic compounds to enable them to live, grow and reproduce. Microorganisms need to supply themselves with energy in the form of ATP, and supply of building blocks to construct their tissues. An integrated enzyme-mediated and regulated chemical reactions are created for the purpose which is also called metabolism. Now the cells using this metabolism process, makes nutrient molecules and maintain a living state. A characteristic feature of secondary metabolites is that the metabolites are generally not produced during the trophophase, but are produced during the production stage i.e. idiophase (Fig. 1). Production of secondary metabolites starts when there is a limitation in growth due to the exhaustion of one major nutrient source like, phosphate, carbon, or nitrogen. It has already been shown that secondary metabolites are produced by pathways which are ramified from primary metabolism [25]. And this subdivision of secondary metabolism usually occurs at a relatively small number of points which are categorized into the following:

1. Metabolites obtained from aromatic amino acids.
2. Metabolites obtained from amino acids
3. Metabolites obtained from Acetyl-CoA and related compounds
4. Metabolites obtained from sugars

Regulation of secondary metabolism

Secondary metabolites are often produced in a limited range of culture conditions [24]. The essential requirements to observe their production are-

· The particular synthetase enzymes present in the secondary metabolites are in an active state.

· The supply of precursors is appropriate.

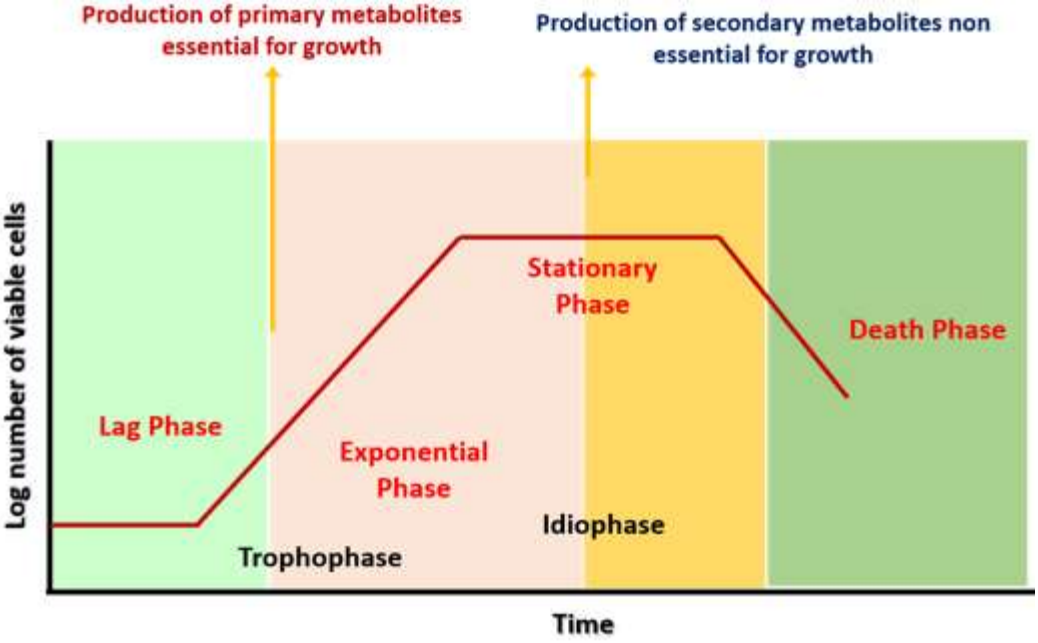


Fig. 1. Various phases of bacterial growth and production of metabolites. The primary metabolite production generally occurs at the late lag phase and middle of the exponential phase. The secondary metabolite production occurs at the end of the stationary phase and during the persistent phase. (Source: Gokulan K, Khare S, Cerniglia C. 2014)

· The export for the compounds which accumulate extracellularly.

The regulation of secondary metabolites is the same as the regulatory mechanism of primary metabolites. Depending upon the following points the regulation of secondary metabolites occurs.

· Regulation by medium composition.
· Biochemical regulation.
· Genetic regulation.

Regulation by medium composition

The effects of environmental parameters such as temperature and pH are usually evaluated during the early screening procedures or they are initially set at the values which give optimum growth of a secondary metabolite producer. After more detailed studies it is observed that the effect of growth medium composition on product formation has taken place. The media components are mentioned below.

Carbon source

It is very difficult to assess even with a well-studied process that the regulation process of secondary metabolites is stimulated or inhibited using carbon as a component in the medium. For example, to direct effects of carbon source on the synthesis of the enzymes involved or the way that its catabolism affects the concentrations of precursors for secondary metabolism or the influence of the osmotic strength of the culture medium on the producer cell [25]. That's why the choice of carbon source is undirected till now. But there are some observations mentioned below.

Growth rate

There is a relationship between growth rate and product formation. After biomass production in batch culture media the rapid growth of secondary metabolites is observed. On the media where the initial growth rate is very slow, the production of metabolites can become growth associated. For that some alterations like carbon source are needed such as the direct influence of the carbon source such as carbon catabolite repression and others are more general and refer to the "balance" of metabolism. This growth rate has been shown to affect some metabolic parameters in the cell such as the size of the intracellular pools of primary metabolites and gene dosage as well as some morphological characteristics such as cell size. The 'balance' of the metabolism determines the production of precursors for secondary metabolite synthesis. Many observations have indicated that this is the case and two processes-

· The production of hydrogen cyanide
· The production of gramicidin S

Hydrogen cyanide is made by a wide variety of microorganisms. In the case of both fungi and bacteria the direct precursor is the amino acid glycine. In batch culture media which supports the rapid growth of metabolites, if cyanogenic microorganisms are grown cyanide is first detected during the early stationary phase of batch cultures. The time at which cyanide production can be observed reflects the composition of the culture medium and the growth of cyanogenic show mould basidiomycete in media with glucose as the major source of carbon results in cyanide being only produced during the stationary phase [1]. When the media is supplemented with glycine it results in cyanide production during late log and early stationary phase.

Gramicidin S is first detected in media during late log and early stationary phase cultures and the synthases can be detected and are active before the activity of gramicidin S appears [9]. In this system, under a variety of was produced in continuous culture and this phenomenon demonstrated that the synthases were present but they did not appear to generate gramicidin S.

In the case of penicillin production, rapid growth on one carbon source, to generate biomass, may be followed by a period of secondary metabolite synthesis utilizing another carbon source.

Carbon catabolite regulation

The generality of repression or inhibition of enzymes by catabolism of rapidly utilized carbon sources is well illustrated in the case of secondary metabolism. Although glucose is often an excellent carbon and energy source for microbial growth, it is utilized infrequently as the major carbon and energy source in secondary metabolite fermentations. But glucose and other rapidly

metabolized carbon sources suppress the production of certain metabolites like, coumermycin, neomycin, penicillin, siomycins, ergot alkaloids, etc.

Catabolite repression

Although the preferred medium for actinomycin biosynthesis contains glucose and galactose, antibiotic synthesis is delayed until the glucose supply is depleted. If additional glucose is added before the initiation of actinomycin synthesis, severe repression of antibiotic synthesis occurs. Several carbon sources cause catabolite repression of N-acetyl kanamycin amidohydrolase in Streptomyces kanamyceticus. This enzyme, presumably the last enzyme which is repressed by glucose, fructose, lactose, mannose, and maltose in kanamycin biosynthesis [16]. Carbon catabolite repression is also important in the interconversion of members of an antibiotic family.

Catabolite inhibition

In some cases, glucose or other carbon sources appear to cause inhibition rather than repression of some enzyme of secondary metabolite biosynthesis [17]. Neomycin, siomycin, and penicillin cells actively producing antibiotics are inhibited by glucose addition. Unfortunately, it is difficult to decide whether the effect is indeed a result of catabolite inhibition or instead to catabolite repression of an enzyme that is rapidly being degraded and resynthesized [16].

Nitrogen source

For optimal growth the carbon-nitrogen balance in a culture medium must be correct. A large of secondary metabolites contain nitrogen atoms, frequently more than one. Nitrogen-containing compounds have stimulatory effects on certain secondary metabolites processes related to morphological changes. There is a link between nitrogen assimilation and secondary metabolism. For example, β-lactam production by Streptomyces clavuligenes declines when the glutamine synthetase activity is lowered; chloramphenicol production by *Streptomyces venezuelae* is strongly influenced by the type of nitrogen source and finally, ethylene biosynthesis from methionine by *Escherichia coli* has been implicated in nitrogen salvage by the producer cells. From the previous investigations of nitrogen source manipulations can indicate which type of genetic changes might contribute to increased production of a secondary metabolite.

Phosphate and trace element

Phosphate and trace elements are involved in so many areas of secondary metabolism. Phosphate is involved in DNA, RNA and protein biosynthesis, carbohydrate metabolism, respiration, energy charge, and transport in the producer cell. Similarly, trace elements are also involved in many of the above cases.

Other factors

The other factors which can influence the metabolic process are- temperature, pH. In low temperatures it is observed that the production of secondary metabolites is high. Also, itaconic acid production by Aspergillus terreus is highly dependent on the pH of the culture medium.

Biochemical regulation

There are many mechanisms of biochemical regulation found in primary metabolism and they also occur in secondary metabolites. The induction or derepression of enzymes involved in the biosynthesis of secondary metabolites is important but there are often some difficulties in determining whether a stimulatory response is due to true induction or an increased availability of precursors.

Regulation by end-product inhibition or repression appears to be a fairly common control mechanism in secondary metabolism. Primary metabolites share the same biosynthetic origins as certain secondary metabolites may limit the production of the secondary metabolite by feedback inhibition and repression at earlier stages in the pathway. Alternatively, the secondary metabolites themselves may regulate their synthesis.

Genetic regulation of secondary metabolite production

The groups of genes which are controlling antibiotic production are divided into five classes.

They are-

· Structural genes which code for the enzymes involved in the biosynthesis of secondary metabolites.

· Regulatory genes will determine the onset and extent of repression of the structural genes for biosynthesis.

· Genes which will determine the resistance of the producing organism to the product.

· Genes that will control the permeability to the compound (entry, exclusion, excretion)

· Regulatory genes which will control primary pathways [7].

The genetic regulation is highly complicated because of so many factors that are affecting production and may be very difficult to effect specific alterations, and the effects of mutation may not be readily predicted.

The regulatory mechanism encountered in secondary metabolism has been developed to manipulate the metabolism of organisms to desired ends.

Induction of secondary metabolites

Secondary metabolism is occurred by either exhaustion of a nutrient, or by addition of an inducer, or by a decrease in growth rate. These events generate signals which usually affect a stream of regulatory events that results in chemical differentiation of secondary metabolism [6]. The conditions which generally induce secondary metabolite production may include the use of particular growth conditions or compounds which harmonize the primary and secondary metabolism of a microorganism. The compounds such as primary metabolites or nutrients are used which start the induction of secondary metabolite production. Primary metabolites taken for induction include vitamins, peptides, carbohydrates, amino acids, etc. also primary metabolites may include nucleic acids, metal ions. Additionally, the conditions to induce secondary metabolism may include altered growth media. Nutrient limitation may affect the secondary metabolism of microorganisms and may induce the production of secondary metabolites. Compounds that have the capability of inducing the production of secondary metabolites may be added directly to the microorganism or as a substrate in their growth media upon which they are cultured. Certain environmental conditions may also be included to induce the production of secondary metabolism. These environmental conditions may include subjecting the microorganisms to radiation (electromagnetic radiation or ionizing radiation), temperature, or pressure variations. The length of time for which microorganisms may be exposed to radiation may vary depending on the microorganisms used.

While nutritional factors have been shown to have a significant impact on the metabolism profile of microorganisms, altering the culture medium has been proven the effective method for secondary metabolite production. The addition of organic compounds have been shown to affect the antibiotic production by actinomycetes, by increasing the production of secondary metabolites.

A variety of chemical discomfort has been used for the activation of a silent gene within streptomycetes to induce secondary metabolite production. According to a research article, scandium, a rare earth element dispersed throughout the earth's crust, was evaluated for its ability to induce the production of secondary metabolites in various models of *Streptomycetes*. It has also been found out that adding low concentrated DMSO to the culture medium increases the production of certain antibiotics (secondary metabolites) two to three times when compared to control culture.

Another research has confirmed the production of secondary metabolites by the use of chemical additives before the cultivation of organisms to induce genetic mutations. It has been shown that bioactive compounds that interact with the ribosome may alter the genome of the host-microbe that results in altered gene expression probably as the result of unrestrictive transcription of genes which ultimately results in the production of secondary metabolites.

Strategies for overproduction of secondary metabolites

The production of secondary metabolism is regulated by feedback control, nutrients, growth

rate, enzyme inactivation, etc. To produce secondary metabolites to a large extent, one can look for the manipulation of microorganisms. Many microbial products are classified as secondary metabolites of great economic, scientific, and medical importance. To manipulate a process, the main problem one can face is the complex nature of biological systems.

Quorum sensing is the communication between cells through the release of chemical signals when cell density reaches a threshold concentration. The quorum-sensing signals differ in different microbial systems. There are various physiological activities of microbes that are regulated by the quorum-sensing which is of great potential for industrial exploitation. Filamentous fungi are the main microbial source for the production of secondary metabolites. Precursors often stimulate the production of secondary metabolites either by increasing the amount of limiting precursor, by inducing a biosynthetic enzyme or both. Precursors are usually amino acids and other small molecules that function as inducers. The well-known precursors are the auto-inducers that includes butyrolactones (butanolides) of the actinomycetes, N-acyl-homoserine lactones of Gram-negative bacteria, and so on [13].

Overproduction of secondary metabolites based on genetic engineering which is regulated by the structural genes, that directly participates in the biosynthesis, regulatory genes. Now the methods for genetic engineering are broadly divided into two groups: classical genetic methods and molecular genetic improvement methods.

Classical genetic methods

Mutation and random selection

Mutant strains are the most widely used strategy for enhancing the yield of secondary metabolites. Depend upon mutation, followed by random screening and then careful tests are performed and new modified mutants are selected. Physical mutagens like UV-light or chemical toxins are used in this method.

The advantages of classical genetic methods are simplicity, no requirement of sophisticated equipment, minimal use of specialized technical skills, effectiveness. The only snag is that this technique is labor-intensive.

Mutation and rational selection

The design of these methods requires some basic understanding of the product metabolism and the regulation of the pathway through which the product is formed.

Recombination methods

Recombination by fusion between related species results in high productivity of the secondary metabolites from the strains.

Molecular genetic improvement methods

The knowledge and tools required to perform molecular genetic improvement includes, identification of the biosynthetic pathway, effective transformation protocols. The main strategies being used in molecular genetic improvement of secondary metabolite producing are:

Amplification of secondary metabolites biosynthetic genes

This strategy is divided into two approaches – Targeted gene amplification – identification of a site where genes can be inserted without altering the fermentation properties of the strain.

Amplification of the whole pathway

Inactivation of competing pathways

Block the pathway that competes for common intermediate precursors.

Disruption or amplification of regulatory genes

Amplification of regulatory genes may be led to 3-5 fold overproduction of certain secondary metabolites. Even disruption of negatively acting regulatory genes may cause 10-15 fold overproduction of secondary metabolites.

Combinational biosynthesis

Producing novel antibiotics, using progressive compounds as a substrate for the enzymes of microorganisms. These can be modified in a way to increase their affinity for unnatural substrates.

Since the recombinant DNA technology has already arrived, genetic engineering of cells or particularly microorganisms, has been successfully practiced for the improvement of strains that are capable of overproducing small

molecules, secondary metabolites, etc. For the latter, strategies beyond simple genetic engineering i.e. metabolic engineering are often required. Metabolic engineering is nothing but the modification of the cellular metabolism using molecular biological techniques and recombinant DNA technology. Metabolic engineering is advantageous over simple genetic engineering, as it allows specified engineering of the cell and also avoids unnecessary changes to the cell. Many secondary metabolites and their precursors found in natural organisms are sometimes difficult to synthesize chemically. Now, metabolic engineering plays an important role here by enhancing the production of these secondary metabolites and this is achieved by accompanying new metabolic pathways that lead to the product formation [22].

Effect of secondary metabolites on food products and food borne illness

The secondary metabolites unveil many beneficial effects in pharma, cosmetic, food, agriculture, and animal food industries, but there are few secondary metabolites cause detrimental effects in humans and animal and also destroy certain food types. Several pathogenic micro-organisms have evolved with synthesizing and secreting toxic secondary metabolites in the immediate environments. The secreted toxic secondary metabolite contaminate the foods, food products, and water that enter the food chain. Another route of toxin contamination is poorly packed canned foods, packed meats, and dry products, in which certain bacteria grow anaerobically and secrete toxins.

The consumption of toxins through contaminated food, food products and water causes severe illness. The toxins secreted may either kill the host or interferes with normal cellular functions. These toxins are classified into endotoxins, which are not secreted by the bacteria but are part of the cellular component of bacteria and exotoxins, that are usually secreted by the bacteria.

Streptomyces species produces macrolide antibiotic bafilomycin A and streptozotocin that cause glucose intolerance in human, resulting in type I diabetes. These secondary metabolites enter the human body through tuberous vegetables, in particular, potatoes and beets. Bafilomycin and streptozotocin are toxic to human pancreatic islet cells that lead to the secretion of low levels of insulin, and the outcome is a type I diabetics.

Bacillus cereus is a Gram-positive bacterium, that usually causes foodborne illness right after the consumption of spores or vegetative cells. This bacteria secretes secondary metabolites that cause diarrhea, nausea, and vomiting. *Bacillus cereus* causes illness in two different ways: one is by the secretion of heat-labile peptide by multiplying bacteria in the small intestine, and the other way is by ingestion of heat-labile peptide cereulide (toxin). This secondary metabolite is synthesized by non-ribosomal peptide synthase (NRPS). The cereulide toxins have been detected in several rice dishes, dairy-based desserts, and cheese [18].

In the natural environment, secondary metabolites produced by microorganisms are important for themselves by performing the following functions, releasing competitive weapons against other bacteria, fungi, and insects also they sometimes produce ionophore which helps in the transportation of ions, etc. Outside the cell these microbial secondary metabolites are extremely important for human health. These important metabolites of a high value play a wide range of roles, as food additives [carotenoids, essential oils], agrochemicals [pesticides, insecticides], biofuels and drugs [antibiotics, anti-cancer agents]. The shelf life of foods and food materials depends upon several factors, including microbial growth. Microbial proliferation helps in the modification of food products, which is not recommended for consumption. To help in bacterial reductions antibacterial agents like bacteriocins are used in food products. Bacteriocins are used as food additive agents to reduce the bacterial load and to improve the quality and safety of foods.

Applications

It is believed that presently only the biological activity like antimicrobial, antitumor, antiviral, pharmacological, and similar activities is the only guiding line that connects the bioactive microbial

secondary metabolites which distinguishes them from other inactive metabolites. The presently known secondary metabolites, exhibit several diverse and versatile biological effects, first of all, antimicrobial activities. In the scientific literature there are hundreds of different pathogenic and other microbes which are referred to as test organisms in the direct screenings. The most frequently used test organisms for antimicrobial screening are *Bacillus subtilis, Staphylococcus aureus, Micrococcus lutea, E.coli,* etc. Bacteria and fungi have found to be used in medicinal field such as in chemotherapies and treating auto-immune disorders. With ongoing studies of microbes, molecules that are associated with it, and their targets, a growing understanding of microbial secondary metabolites have emerged that announces its role in a complex biological system. The basic application of microbial secondary metabolites is as antibacterial, antifungal, and antitumor effect, but with newer inventions they are also being applied for animal and plant growth stimulation, immunosuppression, pharmacological activities and used against parasites and insects. Microbial secondary metabolites are now increasingly being used against diseases previously treated only by synthetic drugs, *e.g.* as anti-inflammatory, hypotensive, antitumor, anticholesterolemic, uterocontractants, etc. Moreover, new microbial metabolites are being used in non-medical fields such as agriculture, major herbicides, insecticides, plant growth regulators, and environmentally friendly herbicides and pesticides as well as antiparasitic agents [1].

Secondary metabolites have economic importance for related industries. They protect the microbes from any other biological stimulus and regulate many biochemical pathways of higher organisms.

Bioactivity of microbial secondary metabolites

The secondary metabolites which are isolated from microbes exhibit either antimicrobial; which includes-antibacterial, antifungal, antiprotozoal action; antitumor or antiviral activities, earlier known as antibiotics. There are some following functions-

· Growth processes
· Replications
· Exhibit regulatory

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

· Commercialization
· Derivatization
· Rational drug design

Various bioactive secondary metabolites

Regulation of production of secondary metabolites is influenced by the unique low molecular mass compounds, inducers, transfer RNA, sigma factors, and gene products formed during the phase after exponential development [10]. 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 opportunities for generalized investigations of regulatory actions, enzymology, control, and differentiation.

Antimicrobial agents

Antibiotics are generally used in the past for antimicrobials. However, it is now more often used to quote the antibacterial and is understood commonly in this way. Antibacterial are divided into two groups according to their speed of action and residue production.

Non- residual producing

The first group contains those that act rapidly to destroy bacteria or causative organism, but quickly disappear either by evaporation or break down and leave no active residue.

Residue producing

The second group consists mostly of newer compounds that leave long-consists mostly of newer compounds that leave long-acting residues on the surface to be disinfected and thus have a prolonged action [27].

Antibacterial drugs

Many new antimicrobial agents with new target

sites recently marketed and still waiting FDA approval includes the following-

· Macrocyclic antibiotics
· Newer cephalosporins
· Novel dihydrofolate reductase inhibitors
· Lipopeptides

Anti-cancer drugs

The increase in resistance to conventional anticancer therapies in patients with advanced solid tumors has led 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.

Genetically-modified, non-pathogenic bacteria have begun to emerge as potential antitumor agents, either to provide direct tumoricidal effects or to deliver tumoricidal molecules [4]. 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 a promising cancer treatment strategy. The most promising strategy is the bacteria-based gene-directed enzyme pro-drug therapy.

Some other applications of secondary metabolites

Pharmacological action of microbial secondary metabolite

Recent advances have shown that investigators have discovered varied activities of pharmacological in microbial secondary metabolites but are unwilling to screen the activities of fermentation broth due to the below reasons:

i) The screening can only be done in living organisms and investigators are against administering broths to animals.

ii) They seem to believe that microbial metabolites are only useful in treating microbial problems, which is untrue.

Rather than treating only microbial disease these metabolites were also detected to have some other activities, which came as a surprise. They were detected since they had antifungal and antibacterial properties even if they were not used as antibiotics. For example, Cyclosporine A is used as immunosuppressive agents in human organ transplantation.

Some other pharmacological activities are as follows: -

a) Anti-inflammatory activity
b) Hypocholesteremic activity
c) Hyperlipidemic activity
d) Hypotensive activity
e) Vasodilator activity

Enzyme inhibitors

Discoveries showed that some pharmacological agents inhibit enzyme activities and some diseases are also associated with enzyme activities which led to suggesting the application of enzyme assays to detect inhibitory compounds in the microbial broths that were believed to show some pharmacological activities.

Some of the microbial inhibitors mentioned below:

i) Inhibitors of 3-hydroxyl-3methylglutaryl-CoA reductase- It was used as an assay to isolate hypocholesterolemic agents, e.g.- monacolin K (mevinolin).

ii) Inhibitors of complement- It is also known as K-76 monocarboxylic acid inhibits chemotactic factor formation for polymorphonuclear leukocytes in human complement serum.

iii) Inhibitors of intestinal glycosidases- These inhibit amylase and invertase which is useful to patients who have restricted consumption of carbohydrates to avoid hypoglycemia and patients having increased levels of triglycerides in adipose tissues, liver, intestinal wall like in people suffering from diabetes and obesity.

iv) Inhibitors of pancreatic esterase- Esterasin, inhibitors of pancreatic esterase, suppresses delayed-type hypersensitivity, and antibody formation.

v) Inhibitors of cholinesterase- *Aspergillus terreus* produces a compound that inhibits cholinesterase. Some discovered synthetic insecticides are inhibitors of cholinesterase [11].

Future aspects of secondary metabolite production

The general requirements of the human beings are continuously elevating and we need new compounds that may be useful for the human

society. More amount of food, drugs, and other necessities are highly required for the benefit of humankind. The ever-expanding scientific and technical possibilities are increasing parallelly with the continuous broadening needs of human therapy, veterinary, and agriculture. This new era has been driven by modem strategies to find microbial secondary metabolites. Earlier, whole-cell assay methods, like bioassays, are being replaced by new and sophisticated, target-directed, mode-of-action screens. In this way, culture broths of new isolates are tested in key enzymatic reactions or as antagonistic or agonistic of particular receptors. This new approach relies on the knowledge of the biochemical and molecular details of different diseases or physiological processes [2].

On the other hand, secondary metabolites, particularly antibiotics have been a powerful tool against microbial infections. However, several clinically important microbial species are resistant to many available antibiotics that increase steadily. The reason behind it is due to the extensive use of antibiotics in the clinical research and existence of microorganisms in unfavorable conditions. Now the major advantage of extensive, highly sophisticated, and automated screening methods is that new biologically active secondary metabolites also increase and at the same time there will be no exploitation of the capabilities of the strains that are producing secondary metabolites. To enhance the production of secondary metabolites in future the currently available non-producing and producing strains of microorganisms can be later improved with the help of classical mutagenesis and selection, by genetic engineering or strains physiology can also be optimized. Genetic engineering methodology can use several approaches for the increased production of active secondary metabolites like the introduction of additional copies of the gene coding for the enhanced expression of secondary metabolites.

Possible future trends in the production of secondary metabolites

New advancements in chemical separations and characterization technologies have notably increased the rate of microbial secondary metabolite discovery. Now it is possible to spot new active compounds at very very low concentrations.

Investigation of many compounds with antibiotic activities which were further found to exhibit additional biological activities.

Biotransformation of already existing secondary metabolites may lead to compounds that exhibit more favorable properties, like higher biological activity, higher solubility in water, lower toxicity, etc [29].

Case study

Regulation of secondary metabolism in streptomycetes

Streptomycetes and other related actinomycetes are the profile sources of novel secondary metabolites with a wide range of biological activities that may find application as anti-infectivity's, anti-cancer agents, or other pharmaceutical compounds. The production of secondary metabolites by the streptomycetes usually occurs at the same time or slightly before the development of aerial hyphae in surface grown cultures. The genes responsible for the production of several secondary metabolites made by the strain.

Intracellular signals for secondary metabolism

A role for ppGpp in starting secondary metabolism under conditions of nitrogen-limitation

The role of highly phosphorylated guanosine nucleotide (p)ppGpp (guanosine pentaphosphate) in prompting antibiotic production in streptomycetes has received significant attention. The ribosome-allied ppGpp synthetase (RelA) is needed for antibiotic production under a limited amount of nitrogen in Streptomyces coelicolor. Whether ppGpp was directly mingled in stimulating transcription of antibiotic biosynthetic genes or whether the subsequent was an indirect result of a shortage in growth rate promoted by ppGpp-mediated inhibition of rRNA synthesis was unclear. However, when a modified relA gene was applied to promote ppGpp synthesis in Streptomyces coelicolor without educing a detectable shortage in growth rate, transcription

Microorganisms involved in secondary metabolite production

Activity	Examples	Producing microorganism
Antibacterial	Cephalosporin	*Acremonium chrysogenum*
	Cephamycin	*Streptomyces clavuligerus*
	Chloramphenicol	*Streptomyces venezuelae*
	Erythromycin	*Saccharopolyspora erythrae*a
	Kanamycin	*Streptomyces kanamyceticus*
	Tetramycin	*Streptomyces aureofaciens*
	Penicillin	*Penicillium chrysogenum*
	Rifamycin	*Amycolatopsis mediterranei*
	Spectinomycin	*Streptomyces spectablis*
	Streptromycin	*Streptomycin griseus*
Anticholesterolemic	Lovastatin	*Aspergillus terreus*
	Monacolin	*Monascus ruber*
	Pravastatin	*Penicillium citrinum*
Antifungals	Amphotericin	*Streptomyces nodosus*
	Aspergillic acid	*Aspergillus flavus*
	Aureofacin	*Streptomyces aureofaciens*
	Candicidin	*Streptomyces griseus*
	Griseofulvin	*Penicillium griseofulvum*
	Nystatin	*Streptomyces nourse*
	Oligomycin	*Streptomyces diastachromogenes*
Antitumoral	Actinomycin D	*Streptomyces antibioticus*
	Bleomycin	*Streptomyces verticillus*
	Doxorubicin	*Streptomyces peucetius*
	Mitomycin C	*Streptomyces lavendulae*
	Taxol	*Taxomyces andreanae*
Enzyme inhibitors	Clavulanic acid	*Streptomyces clavuligerus*

(Source: Barrios-Gonzalez, J., Fernandez, F.J. and Tomasini, A., 2003)

of actII-orf4, the pathway-specific regulatory gene for actinorhodin (Act) production, occurred. The mechanism by which this process occurs is not known, but it is interesting to note that mutations that provide resistance to rifampicin and that by-pass the requirement for ppGpp synthesis for activation of antibiotic production occur in the b subunit of RNA polymerase (RNAP). These mutations may mimic the effect of ppGpp binding to RNAP and lock it in a conformation that permits, or favors, transcription of secondary metabolic gene clusters.

A role for phosphate in repressing antibiotic production

While RelA is required for antibiotic production in S. coelicolor under nitrogen starvation, it is insignificant under conditions of phosphate starvation, where a ppGpp-independent signaling mechanism must operate to initiate secondary metabolism an excessive level of inorganic phosphate in the culture medium prevents the production of many structurally diverse secondary metabolites and in at least some cases this reflects repression of transcription of biosynthetic gene clusters. Mutation of the two-component regulatory system PhoR-PhoP of Streptomyces lividans developed in reduced levels of alkaline phosphatase activity and phosphate transport at lesser phosphate concentrations, and a marked increase in the level of Act and undecylprodigiosin (Red) production. While this might imply a direct

or indirect role for phosphorylated PhoP in preventing transcription of antibiotic biosynthetic genes, phosphate inhibition of antibiotic production is still observed at high phosphate levels in a phoPR deletion strain, perhaps reflecting a role for intracellular phosphate itself in the inhibition of secondary metabolism. Consistent with this idea, inactivation of the polyphosphate inactivation of the polyphosphate kinase (PPK) of S. lividans, which produces polyphosphate during conditions of phosphate sufficiency, also resulted in a marked increase in Act production, and increased levels of transcrip-tion of pathway-specific regulatory genes for Act, Red and a calcium-dependent antibiotic (CDA). Under conditions of phosphate limitation, polyphosphate would normally be broken down into inorganic phosphate by exopolyphosphatases. Since this presumably cannot occur in a ppk mutant, intracellular levels of phosphate would be expected to be lower upon depletion of extracellular phosphate than in the wild-type strain.

Extracellular signals for secondary metabolism A role for A-factor, γ-butyrolactone, in both secondary metabolism and morphological differentiation

γ-Butyrolactones are produced by many, if not all streptomycetes and by several other genera of actinomycetes and are implicated in the onset of secondary metabolism in several species. The most characterized ã -butyrolactone is A-factor (2-isocapryloyl-3Rhydroxymethyl-γ-butyrolactone) of Streptomyces griseus. Unusually, A-factor is required for both secondary metabolism (streptomycin and grixazone production) and morphological differentiation. The function of A-factor, which requires afsA for its synthesis, in controlling the onset of streptomycin production has been largely solved. Binding of A-factor to its cytoplasmic binding protein ArpA releases the latter from the adpA promoter, permitting adpA transcription. AdpA is required for activation of transcription of strR, the pathway-specific regulatory gene for streptomycin production, and for expression of other members of the adpA regulon, some of which are required for morphological differentiation. adpA seems to be the only ArpA-dependent gene for both secondary metabolism and morphological differentiation, and function in modulating A-factor synthesis once the γ-butyrolactone has fulfilled its function in triggering both processes. Even though attempts to crystallize ArpA have coherently failed, the structure of the homologous CprB from S. coelicolor has provided vision into the likely conformational changes that occur upon ligand binding that result in dissociation of ArpA from the adpA promoter.

γ-Butyrolactone synthesis and quorum sensing

The exogenous addition of a γ-butyrolactone to a streptomycete culture often results in precocious antibiotic production. While this has prompted speculation that these compounds act as quorum sensors analogous to the homoserine lactones of Gram-negative bacteria, it seems just as likely that their synthesis occurs in response to an unknown physiological signal, perhaps some aspect of nutrient limitation, and that they do not simply function as indicators of population density. Perhaps their role is to co-ordinate both secondary metabolism and morphological differentiation throughout the developing mycelial colony, rather than to effect communication between dispersed members of the same species.

PI factor-a novel extracellular elicitor of antibiotic production

Although there were earlier reports of extracellular signaling molecules other than g-butyrolactones that could elicit secondary metabolism in actinomycetes, the only recent example is the identification of PI factor as an elicitor of the anti-fungal glycosylated polyene pimaricin in Streptomyces natalensis. PI factor (2,3-diamino-2,3- bis(hydroxymethyl)-1,4-butanediol) elicited pimaricin production in a mutant (npi287) deficient in PI and pimaricin synthesis, and stimulated pimaricin production in the wild-type strain. Remarkably, addition of A-factor also restored pimaricin production in the npi287 mutant, although PI could not complement an A-factor deficient mutant of *S. griseus*. Presumably *S. natalensis* possesses a γ-butyrolactone signaling system that can elicit

primaricin production and that shares some functional similarity to the A-factor cascade of S. griseus. It will be interesting to see whether PI exerts its influence on pimaricin production through a recently identified novel pathway-specific regulatory protein PimR. PimR contains an N-terminal domain corresponding to the SARP family of transcriptional activators, a central domain with similarity to the nucleotide triphosphate binding motif characteristic of the LuxR family of DNA-binding proteins, and a C-terminal domain that resembles guanylate cyclases.

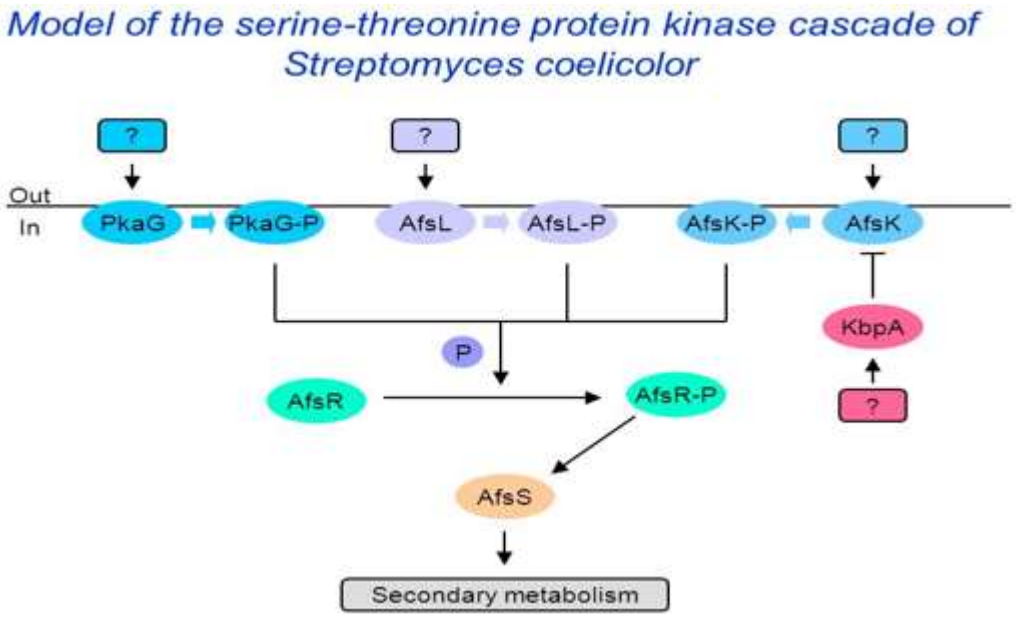
The SARP family of regulatory proteins

Pathway-specific regulators

Many of the pathway-specific regulatory proteins that control secondary metabolism in streptomycetes belong to the SARP family. These transcriptional activators contain a winged helix-turn-helix motif towards their N-termini that is also found in the OmpR family of proteins, and at least some of the SARPs appear to recognize heptameric repeats within the promoter regions of genes that they regulate. They have been found associated with secondary metabolic gene clusters that encode aromatic polyketides ribosomally and non-ribosomally synthesized peptides undecyl-prodiginines .Type I polyketides β-lactams and azoxy compounds While genes encoding phylogenetically diverse classes of bacterial regulatory proteins occur in many secondary metabolic gene clusters, the SARP family of proteins have only been found in actinomycetes, and most of them within the streptomycetes (other genera include Mycobacterium, Nocardia, Thermobifida and Lechevalieria). One member of the SARP family, CcaR, regulates the biosynthesis of both cephamycin C and clavulanic acid in Streptomyces clavuligerus CcaR binds several promoter regions within the cephamycin C gene cluster presumably activating their transcription, as well as positively regulating its own synthesis. Disruption of ccaR also abolishes expression of claR, which encodes a LysR-type regulatory protein that is required specifically for clavulanic acid production. However, CcaR does not appear to interact directly with the claR promoter.

Pleiotropic regulators of secondary metabolism

While SARPs generally appear to function as pathway specific regulatory proteins, at least one pleiotropic regulator, AfsR of *S. coelicolor*, incorporates a SARP domain. The N-terminal region of the 993 amino acid AfsR shows significant amino acid sequence identity to the SARP family of proteins, while the central region contains both A- and B-type ATP-binding consensus sequences. AfsR appears to play a key role as an integrator of multiple physiological and environmental signals that are transduced by phosphorylation cascades (Figure 2). The genome sequence of S. coelicolor A3(2) encodes at least 34 eukaryotic-like serine/threonine or tyrosine protein kinases One of these, the membrane-associated AfsK, autophosphorylates on threonine and serine residues, presumably on sensing a particular environmental signal, thus enhancing its kinase activity. The activated AfsK then phosphorylates threonine and serine residues of the cytoplasmic AfsR, greatly enhancing its DNA binding activity. AfsR-P then serves to activate transcription of afsS, which encodes a 63-amino acid protein that functions in an unknown manner to enhance production of Act, Red and CDA. While binding of ATP to AfsR is not required for binding of the protein to the afsS promoter, it is required for transcriptional activation, suggesting that the energy obtained from ATP hydrolysis may be required for isomerization of a closed complex consisting of RNA polymerase and AfsR into a transcriptionally competent open complex. In addition to AfsK, other kinases-including PkaG and AfsL-are capable of phosphorylating AfsR consistent with a role for AfsR in integrating a variety of environmental signals. Interestingly, the kinase activity of AfsK is inhibited by binding of KbpA to its N-terminal region the delayed transcription of kbpA is consistent with a role in restoring the signal cascade to its pre-stimulatory condition.



Unknown and presumably extracellular signals (shown as '?') activate the autophosphorylation of the membrane-associated protein kinases, which then phosphorylate the pleiotropic regulatory protein AfsR, permitting synthesis of AfsS, which enhances secondary metabolite production. (Source: Bibb, M.J., 2005) [5]

Fig. 2. Model of the serine-threonine protein kinase cascade of *Streptomyces coelicolor*

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