HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading doi: 10.12980/APJTB.4.2014APJTB-2014-0230

Fungi as chemical industries and genetic engineering for the production of biologically active secondary metabolites

Abid Ali Khan[1,2*], Nafees Bacha[2], Bashir Ahmad[2], Ghosia Lutfullah[2], Umar Farooq[1], Russell John Cox[3]

[1]*Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad, 22060, KPK, Pakistan*

[2]*Centre of Biotechnology and Microbiology, University of Peshawar, 25210, KPK, Pakistan*

[3]*School of Chemistry, University of Bristol, Cantock's Close, Bristol, UK, BS8 1TS*

PEER REVIEW

Peer reviewer

Dr. Johar Ali, Research Director, International, Alviarmani, International, 2680 Matheson Blvd. East, Suite 102, Mississauga, ON L4W 0A5, Canada.
Tel: 001 647-556-3703, 001 416-823-3773
E-mail: ali.johar@gmail.com
johar.ali@alviarmani.com

Comments

This is a really a valuable contribution for the new research in the field of fungal secondary metabolites. Because the author has compiled all the information in a very consistent manner. The authors have established a fine link that fungi is living organism but is used as chemical industries for the production of secondary metabolites, which I do believe is the real scientific contribution by naming fungi as chemical industries, that no one have ever used this term before for fungi.
Details on Page 867

ABSTRACT

Fungi is somewhere in between the micro and macro organisms which is a good source of producing biologically active secondary metabolites. Fungi have been used as tool for producing different types of secondary metabolites by providing different nutrients at different laboratory conditions. The fungi have been engineered for the desired secondary metabolites by using different laboratory techniques, for example, homologous and heterologous expressions. This review reported how the fungi are used as chemical industry for the production of secondary metabolites and how they are engineered in laboratory for the production of desirable metabolites; also the biosynthetic pathways of the bio-organic-molecules were reported.

KEYWORDS

Fungi, Natural products, Microbiology, Molecular genetics, Chemical biology

1. Introduction

Fungi survive in a wide ranging of habitats, such as in water, in land/soil, in air, and also in/on animals and plants, simply including both terrestrial and marine environments[1,2]. However, majority of them are terrestrial, living in/on soil or surviving on dead bodies of the multi-cellular organisms including both plants and animals, and are contributing to the natural recycling of the dead bodies into organic compounds. Besides, many of the terrestrial fungi are pathogenic to animals and plants, and potentially cause difficulty to cure fungal diseases[1-3].

The chemistry of fungi are a little more complex because they are structurally different from plants and animals but

*Corresponding author: Dr. Abid Ali Khan, Assistant Professor, Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad Campus, KPK, Pakistan.
Tel: +92 321 901 2972
E-mail: abidkhanuop@gmail.com

Foundation Project: Supported by Higher Education Commission of Pakistan (Grant PIN No. 074-1053-Bm4-207).

Article history:
Received 7 May 2014
Received in revised form 18 May, 2nd revised form 5 Jun, 3rd revised form 5 Sep, 4th revised form 24 Sep 2014
Accepted 29 Sep 2014
Available online 7 Oct 2014

they have some patterns similar to plants and animals. For example, they decompose their foods using extra-cellular digestion, and then absorb the nutrients; whereas, they follow the same biosynthetic pathways for the synthesis of secondary metabolites like terpenes and polyketides using similar starting units[1,4].

By knowing that the fungi possess the same biosynthetic pathways like plants, the fungi became important to the scientists. They are also widely used as experimental model because they can be grown easily in the laboratory conditions *i.e.*, yeast[5,6].

2. Fungi as a source of natural products

Natural products are the organic compounds which have been produced as secondary metabolites by the living organisms. Almost all of the secondary metabolites possess biological importance. The secondary metabolites show different varieties of the structurally and functionally diverse group of natural products. The diversity has led them biologically active against various chronic diseases, rendering them important and valuable for the human being[7].

Natural products are the main source of drugs. According to one survey, about 61% (535 out of 877) naturally isolated chemical compounds have been developed into drugs in the entire world in 22 years (from 1981 to 2002). Seventy eight percent of antibacterial and seventy four percent of anticancer compounds are natural products[8]. Thus natural products offers a remarkable platforms for the development of front-line medicines[9].

Significant improvement and variation in the microbial natural products discovery is confined to the management of nutrient and environmental factors which encourage the biosynthesis of secondary metabolite. While the small changes in nutrient and/or environment have the ability to affect the quantity, quality and diversity of the secondary metabolites as fermentation products[10].

The natural products from any source have been classified as alkaloids, isoprenoids, non-ribosomal peptides and polyketides. All the groups differ from one another in their structures, functions and even in biosynthetic pathways.

2.1. Alkaloids

Alkaloids are the natural organic compounds mainly of plants origin. They have at least one basic nitrogen heterocyclic ring, possessing remarkable physiological activities in human. Alkaloids are divers in their function, because some stimulate central nerves system in human, some relieve pain, while some are toxic and others cause paralysis. Most of the alkaloids are colourless crystalline solids and a few are liquids. The solid alkaloids are soluble in lipids while the liquid ones are aqueous soluble. Coniine[11], graminutee[12], papaverine and quinine are some of the common alkaloids[13,14].

2.2. Isoprenoids

Isoprenoids (also called terpenes or terpenoids) are natural products that give proper odour or flavour to plants. Isoprenoids are the oils from plants that consist of a mixture of hydrocarbons (polyene) and their oxygenated derivatives. Isoprenoids is one of the largest group of natural products with approximately 25 000 known compounds[15,16].

Otto Wallach has received Noble Prize in 1910 for working with isoprenoids and assigning so called isoprene-rule that molecules of all isoprenoids are synthesized from two or more of the isoprene units joining into head-to-tail fashion[17]. Some of the common and well known isoprenoids from plants are camphor[18], isoprene[17], limonene[19], myrcene and vitaminute-A[20,21]; whereas, microorganisms may also synthesized some important isoprenoids such as aristolochene and gibberellin-GA4[22,23].

2.3. Non-ribosomal peptides

Non-ribosomal peptides are the natural products, which is an important class of secondary metabolites, mainly produced by microorganisms including actinomycetes, bacteria and fungi. Non-ribosomal peptides are produced by multi-domain and multi-modular enzyme called non-ribosomal peptides synthetase (NPRS)[24]. These secondary metabolites are bio-synthesized in the cytoplasm by cytosolic protein outside the ribosome[25,26]. Some common NRPS are bleomycin[27], cyclosporin-A and penicillin-G[28,29].

Non-ribosomal peptides are large multi-domain protein consisting of several modules (a group of domain or segments of the NRPS's polypeptide chain) which has the potential to join the building blocks (amino acid) together, forming a long peptide chain. The mechanism, which are involved in the bio-synthesis of polypeptide chain, are the selection of amino acid activation, and finally the condensation of amino acids. As the module is a group of domains, the chemistry of domain is very essential for the function of the module. The major domains that tie up the amino acids together are the adenylation domains (A-domains) serving as catalyst for the activation of substrate. The peptide carrier protein (PCP)-domain or thiolation domain, is an important domain because it is always activated by 4-phosphopantetheinyl-transferases to start its function. This domain is a bonding

domain which links the substrate through covalent bond. The condensation domain (C-domain) is responsible for the peptide bond (Figure 1)[24].

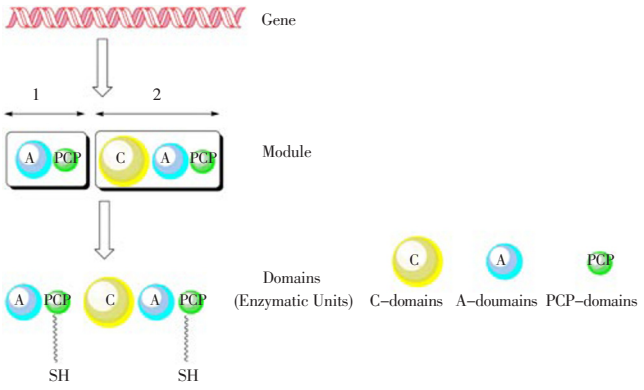

Figure 1. Gene is differentiated into modules which can be further subdivided into domains.
Domains are the enzymatic units that tie up the amino acids together to form polypeptide chain.

The NRPSs are simultaneously used as template and biosynthetic equipment, because the module will select the amino acids and also tie up all the catalytic functions. This is often a quality of the fungi that can synthesize a complete metabolite from a single NRPS.

2.3.1. A-domain

The A-domain consists of approximately 550 amino acids. The main function of this domain is to select the amino acid for making the protein and also control the primary sequence of protein. A-domains are responsible for the activation of amino or carboxylic acid substrate as amino acyl adenylate, whereas, adenosine triphosphates is consumed during the process[30,31].

So far, two crystalline structures of A-domains have been properly studied after their isolation. The crystal structure of the phenylalanine activating the A-domain of the gramicidin S-synthetase A isolated from *Bacillus brevis* explains the role of those amino acid residues which are involved in the coordination of the substrate[24,32].

2.3.2. C-domain

The C-domain consists of approximately 450 amino acids. C-domains are the essential unit of non-ribosomal peptide synthesis; their main function is to connect amino acyl substrates with PCPs of the adjoining modules through peptide bond[24,33].

2.3.3. PCP-domain

The PCP-domain comprises approximately 80-100 amino acids, which is a small domain. This domain is selective and stands for the transfer of the unit responsible for the acceptance of the activated amino acid. The PCP-domain is covalently bounded to its 4PP-cofactor as thioester. The 4PP-cofactor is then transferred to a conserved serine residue of the carrier protein which acts like a flexible arm and hence permits the travel of bounded amino acyl and peptidyl substrate between different catalytic centres[24,34].

2.4. Polyketides

Polyketides are the natural products that have been considered as the most valuable class of secondary

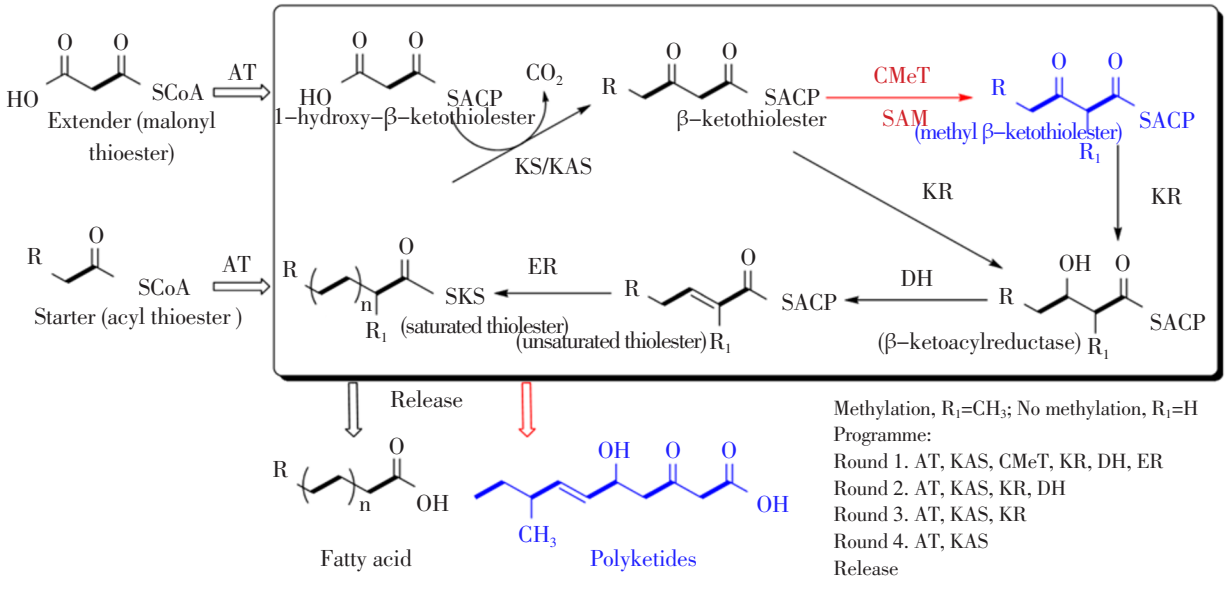

Figure 2. Different steps of enzyme catalysis in the biosynthesis of fatty acid and polyketides.
Bold bonds indicate the pattern of incorporation of the labeled acetate units.
AT: acyltransferase; KAS: β-ketoacylsynthase; SAM: S-adenosylmethionine; KR: β-ketoreductase; CMeT: C-methyltransferase; DH: dehydratase; ER: enoyl reduction; KS: β-ketosynthase; SKS: β-ketosynthase attahced to sulfur atom; SCoA: Co-enzyme A attahced to sulfur atom; SACP: acyl carrier protein attahced to sulfur atom.

metabolites. They are produced by bacteria, fungi, marine organisms as well as plants[2]. Polyketides are structurally and functionally diverse class of natural products that exhibit a variety of biological activities. Among the polyketides, aromatic polyketides have more biological potential against the microorganisms and cancer, for example, aspergiolide A, a novel anticancer compound produced as secondary metabolite by fungus[35].

Some of the important biologically active polyketides are actinorhodin, aflatoxin B_1, lovastatin and 6-methylsalicylic acid[36,37]. However structurally and functionally different polyketides have the same pattern of their assembly by the decarboxylative Claisen condensations between an acyl thioester and malonyl thioester (Figure 2)[2].

Basically enzymes are involved to catalyse the condensations between the starter and extender units. They are called as polyketide synthases (PKSs) based on the enzymes involved in the biosynthesis of fatty acids. Because the mechanism of polyketide biosynthesis are similar to the fatty acid biosynthesis, PKSs have been classified and characterized using the pattern of nomenclature for fatty acid synthases with little modifications[37-39].

The major catalytic domains like AT, β-ketoacylsynthase or β-ketosynthase (KAS or KS) and acyl carrier protein (ACP) are found in all fatty acid synthase (FAS) and in PKS[29,40,41]. They also have KR, DH and ER domains; whereas, PKS has an additional important domain *i.e.* CMeT which is responsible for the methylation of polyketides (Figure 3) has a very simple elucidation of both the biosynthetic pathways[36,42].

In round 1, the CMeT is functional, therefore it results in the formation of polyketides while its silencing in round 2 results in the formation of fatty acid by using the same raw material malonate.

ACP is used by the fatty acid synthase that carries the malonyl thiolester or malonate unit and rapidly attached with the acyl chain. This feature has a very clear homology to PKS.

Most FAS and PKS proteins also require an AT enzyme to transfer acyl groups from coenzyme A onto the KS and ACP components. During the biosynthesis of fatty acid, the newly formed β-ketothiolester is further proceeded for chemical reactions while it is attached to the terminal thiol of the ACP phosphopantetheine; first of all it is reduced to secondary alcohols by a KR. It then undergoes dehydration reaction catalyzed by DH for the formation of an unsaturated thiolester, and finally the ER results in the formation of a fully saturated thiolester. Fungal PKS has the ability to deploy all these chemical reactions; furthermore whenever the chain will be methylated, the methyl group will be provided from SAM. This probably occurs after KAS, giving a methyl β-ketothiolester.

2.4.1. History of polyketides biosynthesis

In 1893 James Collie at London University, obtained a product orcinol, ever first polyketides, by the chemical reaction of dehydroacetic acid with barium hydroxide. This simple aromatic compound became challenging for the scientists because the mechanism was based on the key polyketone intermediate (Figure 4)[43]. By knowing the chemistry and resolving the mystery of polyketone intermediate after the pioneer work of Collie, which hypothesized that acetate is the precursor of almost all polyketides, the field of polyketides were developed. Latter on the bacteria and fungi were engineered by cloning their

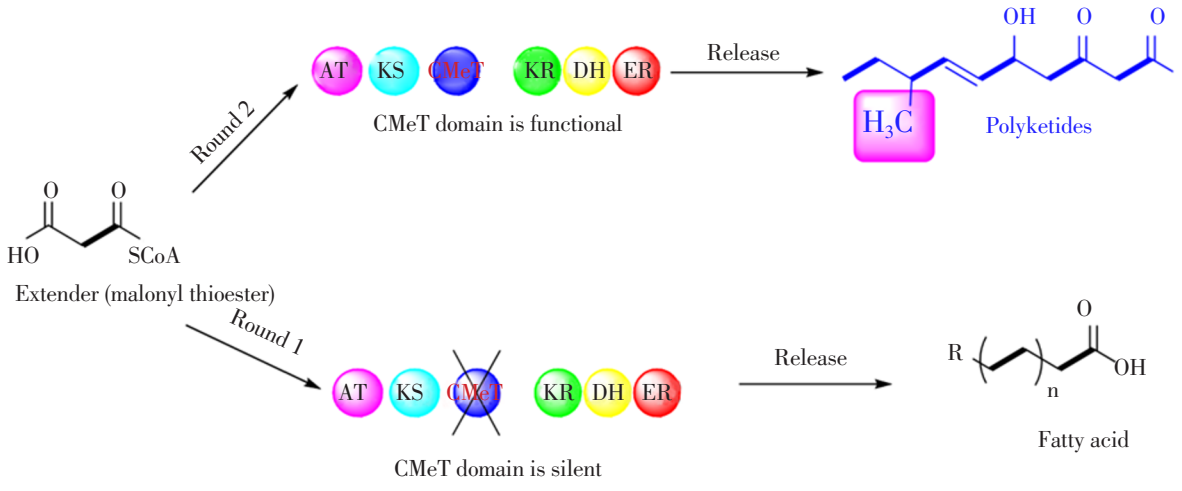

Figure 3. All the domain in the module are the same, while only the CMeT domain is silent in round 2, results in the production of fatty acid. It shows that CMeT is the only domain which can differentiate the polyketide pathways from fatty acid pathways.

enzymes to accomplish the task of the hidden pathways of polyketides[44,43].

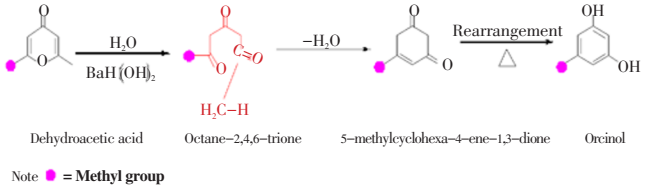

Figure 4. Collie's un-predictive synthesis of orcinol from dehydro-acetic acid[42].

2.4.2. Radioactive isotopic (^{14}C) in polyketides biosynthesis

The major interest in the field of polyketides came from impetus of Arthur Birch in 1950. He spent much of his time in Robinson's laboratory at Oxford, which was a famous laboratory for the research in bio-organic chemistry. Birch's contribution to the field of polyketides was important for two reasons. First, he suggested that polyketones (polyketides) could be produced by the repeated condensation reactions of acetate units (staring units); and second, he tested his suggestions by feeding the isotopically labelled acetate units to an organism for the production of suitable polyketides[43]. Birch selected 6-methylsalicylicacid (6-MSA), an aromatic polyketide for the confirmation of his idea, which is involved in the biosynthetic pathways of the toxin patulin and also has some biological importance (Figure 5)[46].

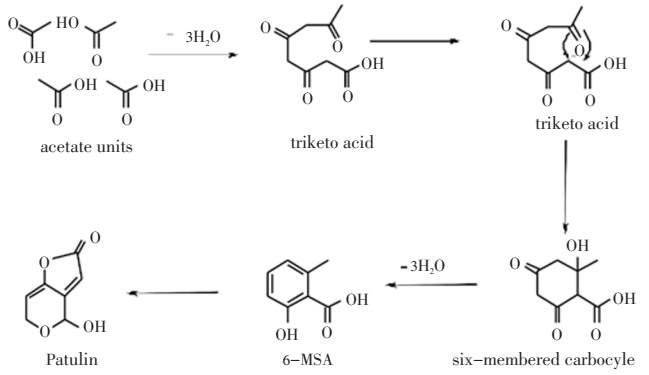

Figure 5. Sequence of reactions in the biosynthesis of 6-MSA and patulin.

Four acetate units are linking/bounding to each other by head-to-tail manner and produce a triketo acid. Then one of the keto groups in triketo acid is reduced to hydroxy group. Then different mechanistic reactions including the formation of carbanion at the β-keto residue in (35) would then allow an aldol condensation to form a six-membered carbocyle. Finally, reasonable reactions like dehydration and enolisation reactions will lead to the formation of aromatic natural products (6-MSA and toxin patulin)[45].

Birch further tested the ideas by feeding acetate labelled with [1-^{14}C] to Penicillium patulum the producer of 6-MSA (Figure 6)[43], because that was the only available technique. However, for the confirmation of the idea, it was necessary to understand the pattern of labelling in the compound. The incorporated sites in the compound 6-MSA were predicted by the degradation methods. Then the fragments were correlated with specific sites in the natural product. The three products were isolated by degradation and then these products were subjected to radioactivity measurement for the determination of their relative molar activity. The results were remained the same as predicted by Birch[43].

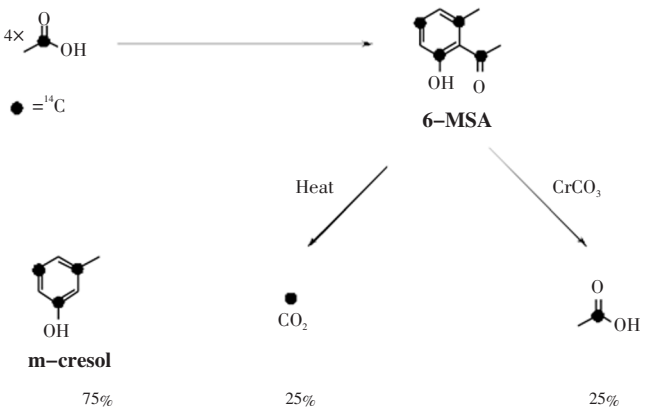

Figure 6. Birch's verifications that 6-MSA is assembled from four acetate units[42].

Although radiolabels are useful tracers in the incorporation studies, they are time consuming, tedious and difficult to handle. Therefore radiolabels have no longer been used in microbial systems for labelling studies.

2.4.3. Stable isotopic (^{13}C) in polyketides biosynthesis

The development of nuclear magnetic resonance (NMR) spectrophotometer resulted in the growth of the field of feeding isotopically labelled precursors. Till 1960, the natural product chemist depended on the degradation phenomenon to produce recognisable fragments for the structure elucidations. They used to bring all those fragments together on paper and to create an idea for the structure of compounds. That was really a very tiring job but yet the scientists developed some standard and well-tried methods of degradation and structural determination[47,48]. However, the NMR solved all these challenges at once or almost overnight. Because the stable isotopic labels (^{13}C) were open to direct detection by NMR spectroscopy. It was significant to use the isotope C, because it has a suitable nuclear spin for NMR observation like ^{1}H[36]. Therefore, the chemists turned to stable isotope (^{13}C) instead of the radioactive isotope (^{14}C).

Isotopically labelled precursors usually [1-^{13}C] acetate is administered in the standard way after the time course production for the desire metabolites and then the metabolites will be re-isolated[49]. It is then a tricky job to establish the sites of isotopic enrichment by the measurement of ^{13}C from NMR spectrum. In successful experiment, the incorporated isotope will give even an increase of 1% over the natural abundance, however for

the reliable results it is better to look for higher increase in the signal size. Luckily, maximum of the polyketides are produced by the micro-organisms especially fungi; therefore, they often takes up labelled substrates[36].

In perfect situation, each pair of coupled ^{13}C nuclei will have a unique coupling constant which helps in the confirmation of ^{13}C NMR spectrum. Labelled ^{13}C nuclei have replaced their original carbon; the signal will appear as an enriched singlet. It is essential for interpreting this easy pattern of signals to dilute the labelled precursor by unlabelled acetate; otherwise, more than one doubly-labelled isotopic precursor (acetate) will incorporate into a single molecule and this will result in a complicated spectrum by inter-unit ^{13}C-^{13}C couplings.

Additional estimation of the mechanisms of biosynthetic pathways can be obtained by studying the probability of hydrogen atoms in intermediate molecules. Different experiments have been developed to study the effect of ^{2}H or ^{3}H, either by the direct observation of ^{2}H or ^{3}H by NMR spectroscopy or indirect detection of ^{2}H or ^{3}H isotope attached on the adjacent neighbour ^{13}C labelled in the precursor. The α shift is sufficient to demonstrate extra information regarding the path of hydrogen atoms involved in the biosynthetic pathways.

For this technology, the hydrogen atoms are replaced by deuterium or tritium and the carbons are replaced with ^{13}C to which they are attached. Then the chemistry is very simple because one deuterium in molecules lead to the shifting of ^{13}C signal to upfield by about 0.3 ppm and it appears as a 1:1:1 triplet with J=20 Hz due to the coupling ^{2}H-^{13}C. Now for each additional deuterium with ^{13}C, there will be further upfield shift of the carbon and a corresponding increase in the multiplicity of the signal (Figure 7)[48].

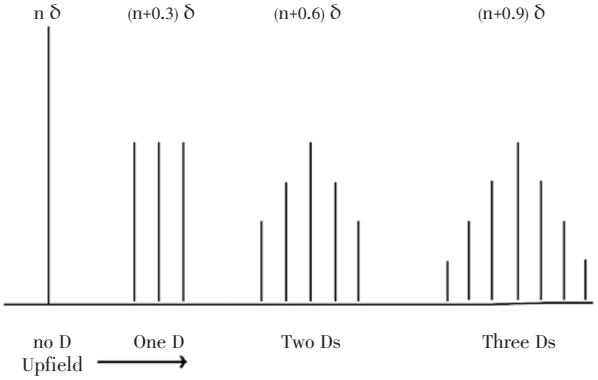

Figure 7. Imaginary ^{13}C NMR spectrum demonstrating the α-effect. D stands for deuterium. Each additional deuterium shifts the signal of carbon to upfield by 0.3 ppm along with the increases its multiplicity.

With a high field NMR spectrometer, therefore, it is obvious to determine the extent of deuterium labelling in considerable detail. It will be sufficient to say that, with the developments of various elegant scientific methods, the bio-organic chemists published a lot of data, upon which they have built a detailed story of the types of biosynthetic processes employed in polyketides pathways.

3. Fungi and biotechnology

Fungi have been used as folk medicines, *i.e.* a very common fungi *Agaricus campestris* Linn (field mashroom) will serve as tonic when used 3 to 6 g for 2 to 3 times a day and is used against inflammation, sinusitis and tuberculosis. Besides, *Laricifomes officinalis* is used against diarrhoea, night sweating. *Inonotus obliquus* is used for chronic gastritis, early tumours and ulcer. *Daedaleopsis flavida* cures jaundice dramatically by reducing the level of bilirubin and biliviridin; whereas *Ficus religiosa* Linn is used for all kind of kidney disorders[49].

It is now clear that fungi are the significant source for new and/or biologically active secondary metabolites. During the period of 1981 to 2002, 40% of the total drugs and 14% of the antifungal drugs launched were of natural products or biologically modified natural products[8]. However in agriculture, it is estimated that only about 10% of the natural products are available in the market for the protection of crops[50]. Therefore, for the development of new drug, it is necessary to test their adverse effects on humans, but during critical conditions like high fungal infections some extent of toxicity could be accepted for example the use of standard antifungal drugs like amphotericin-B, although that may cause some severe kidney infections[51].

Although investigation of new microorganism from the un-investigated areas will lead to many new biologically active secondary metabolites, only a small fraction (0.1%-1%) of all microorganisms have been exploited under laboratory conditions[52]. Because it is a challenging job to understand the huge genetic diversity, we can simply approach our target towards metabolites by the modifications in growth conditions or by genetic engineering to make a transformant organism by inserting the biosynthesis genes of uncultivable microorganisms[53-55].

Genetic approaches could be helpful techniques for estimating the biosynthetic potential of microorganisms. That can be evaluated for known biosynthesis genes and then possible suggestions that which strain can produce which kind of compounds are made. This approach has been remained successfully for the gene(s) involved in the synthesis of polyketides[56,57].

Nevertheless, it is not necessary that in-depth search for microorganisms will result in novel bioactive components, but sometimes a very easily accessible microorganism may

produce broad spectrum of diverse metabolites depending on the culturing conditions and also some additives, or chemical modifiers. Single strain may lead to an increase in metabolites and even generation of a new compounds, this approach is called one strain many compounds[58,59].

Natural products have been used by man for a long time and the plants are the most important source of medicine[60]. Due to their abundant variety and structural diversity, natural products are of great significance in biotechnology and pharmacology. They can also be used as a model for synthesis by knowing their characteristics[61,62]. The best known examples of natural metabolites available commercially are antibiotics, such as penicillin discovered by Alexander Fleming in 1928 and available in the market by Chainand Florey in 1940[63].

Natural resources, especially fungi, are a best known factory for their metabolic capacity to produce a broad diversity of bioactive metabolites. These can be extremely toxic, *e.g.*, mycotoxins, or be rather useful because they can be used as drugs for various diseases[64]. Fungi produce a vast range of secondary metabolites. Some of the metabolites are high-value products with pharmaceutical applications such as penicillins, a group of structurally related β-lactam antibiotics isolated from *Penicillium chrysogenum*. Several non-β-lactam antibiotics are also produced by fungi such as griseofulvin. Griseofulvin isolated from *Penicillium griseofulvum* has been used for several years to treat dermatophyte infections of the skin, nails and hair of humans. Some common secondary metabolites of fungal origin are listed in Table 1[45].

Table 1

Some common secondary metabolites produced commercially from fungi.

Metabolites	Fungal source	Application
Cephalosporins	*Acremonium chrysogenum*	Antibacterial
Ciclosporins	*Tolypocladium* spp.	Immunosuppressants
Fusidin	*Fusidium coccineum*	Antibacterial
Gibberellins	*Gibberella fujikuroi*	Plant hormone
Griseofulvin	*Penicillium griseofulvum*	Antifungal
Penicillins	*Penicillium chrysogenum*	Antibacterial
Zearalenone	*Gibberella zeae*	Cattle growth promoter

In recent years, marine fungi have been explored more deeply to obtain novel and biologically active compounds, because they are still less explored. Nevertheless, successful stories in marine fungi are quite significant. Cephalosporin-C which was originally isolated the first time from *Cephalosporium acremonium* isolated from a sewage outlet off the Sardinian coast have played a key role in the reduction of infectious diseases and suffering of people throughout the world since last three decades[65]. However, it was about incidental discovery and it took another 30 years until marine-derived fungi were investigated more systematically[66].

4. Genetic engineering in fungi

Genetic engineering is defined as any change in the natural genetic code of an organism for a specific function. It may be a single base pair change or a complete synthesis of a genome of an organism[40]. This work was started in early 1920 to 1940 by Muller *et al.*; it was only a modification of gene by radiations and chemicals[41,67]. However, it was developed by Jackson *et al.* in 1972 with the achievement of first recombinant DNA[68]. Latter on genetic engineering reached to its high level of success by achieving genetically engineered human insulin through cloning and expression of the gene in *Escherichia coli* by genetic engineering technology in 1978[69], this was an ever first successful targeted achievement.

Transformation is a core method for attaining the genetic modification in fungi[70,71]. One of the most useful and important method for transporting the genetic mattering into fungi is the protoplast mediated transformation (PMT) [71,72]. In this method the cell wall is removed with the help of enzyme from young mycelia while leaving the protoplast covered by cell membrane. Whereas the use of calcium ion (Ca^{2+}) enhance the penetration of DNA into the cell membrane. However sometimes some of the fungal strains do not develop their cell wall around their cell membrane[70]. Therefore, other methods like *Agrobacterium* mediated transformation (AMT) as well as biolistic, electroporation and lithium acetate mediated transformation methods were also developed in last decades for the solution of the problems happened due to PMT[70,71]. AMT depends on using a carrier organism *Agrobacterium tumefasciens* to transport the genetic material into the host[73].

Naturally, it is the bacterium that infects the whole fungal or plant's cell and then starts the genetic alteration by means of integrating a part of certain plasmid, so called Ti, into their genome. By knowing this phenomenon, the gene of interest is usually incorporated on the Ti plasmid after some modification in it. Finally, the recombined plasmid is transferred into bacterium and the result of this transformation is generally a single integration on the fungal genome[73,74].

There are two types of methods or mechanisms for the

integration to transfer the genetic materials onto the receiver fungal genome[75,76]. The first method is homologous recombination[76]. This leads to the integration of the familiarized DNA sequence onto a homologous targeting on genomic locus, which is catalysed by the RAD52 epistasis proteins group. The second method is called non-homologous end joining[76]. This results in the ligation of the transformed DNA sequence to the recipient genome without homology. This leads to ectopic integration of several copies on variable genomic sites. Approaches for the genetic engineering of fungi and other micro-organisms were developed during last decades including gene knock-out, gene silencing and gene overexpression to confirm their link with metabolites.

4.1. Gene knock out approach

Gene knockout approach is a technique of genetic engineering, in which one desired gene of an organism is made inoperative. AMT was successfully used to disorder the genes hypothesised to be linked to radicicol biosynthesis from *Chaetomium chiversii*. It was also noted that the use of AMT has achieved better homologous recombination in *Aspergillus awamori*[75]. It is not necessary that all the fungi will obey the procedure of AMT, because some fungal species like *Sclerotinia sclerotiorum* and *Aspergillus niger* are disobedient to AMT[75]. In addition, it was found that some genetic loci and some fungal species are resistant to homologous recombination[77]. This could be greatly enhanced through knocking out genes expressing system. On the other hand PMT usually results in numerous ectopic integrations which make it beneficial for both heterologous and homologous overexpression[78]. Hence, from the mentioned study, it can be concluded that there is no single method for transformation that could deal with all the genetic modification approaches.

4.2. Gene silencing approach

Recently the gene silencing techniques have been introduced and the scientists are increasingly using it for confirming gene involved in the production of metabolites. They depend on down-regulation of gene expression. These techniques do not influence gene transcriptional process. However it shows its effect by decreasing the level of expressed RNA[79]. As a result the corresponding protein level turns down and silencing of gene function succeeded. However in some cases complete blockage of expression has been achieved[80]. Gene silencing techniques are proven to be more proficient than gene knock out[81], because of the fact that these techniques did not need homologous recombination which could be inappropriate to some of the fungi[82].

Among gene silencing techniques two types of methods are the most famous. The first is the antisense RNA technique which depends on the incorporation and expression of a DNA sequence in the antisense direction to the target gene. Therefore, both the native mRNA and the antisense RNA overlap with each other, leading to the translation blockage[83].

The second method used is RNA interference[81]. Its function is the expression of short homologous double stranded RNA that starts up the mechanism for degrading the native RNA. It is a set of proteins called dicer proteins, RNA-dependent RNA polymerase, and the RNA induced silencing complex mediate RNA interference responsible for the gene silencing, but these are absent in some fungi[84].

4.3. Overexpression of gene

The gene(s) overexpression is a term that mainly depends on upgrading the level of gene(s) expression. When it is done in its original organism, it is called homologous overexpression; while when it is done in other organism, it is called heterologous overexpression. The basic theme of gene(s) overexpression is achieved by the discoveries of novel and biologically active secondary metabolites from fungi, and these discoveries are the achievements of the applied biotechnology[85,86].

4.3.1. Homologous overexpression

In the process of homologous overexpression, the gene is cloned and overexpressed in the native organisms under the control of native or non-native promoter and terminator; the promoter may either be constitutive or inducible. This process is different from the process of homologous recombination that generally occurs during gene knock out experiments[87]. While in gene knock out experiments, it is proposed that the gene or parts of the gene are cloned to recombine with the targeted gene in the chromosome to disrupt its expression. However, homologous overexpression is mainly accomplished by ectopic integration on different location(s) within the chromosome from its native copy[88,89].

4.3.2. Heterologous overexpression

In the process of heterologous overexpression, the gene is cloned and overexpressed in the non-native organisms

under the control of suitable promoter and terminator; the promoter may either be constitutive or inducible. This process is performed to provide the proposed or desired protein in suitable quantities which will be able for detection and application[90,91]. Heterologous expression can be performed in wide range, starting from simple bacteria like *Escherichia coli* to complex eukaryotic organisms like animals and plants[92,93]. While the choice of the host depends on the extent of the knowledge about the capacity of the host to express the foreign gene effectively and also the availability of suitable substrate molecules[93-95].

5. Conclusion

It is clear from the whole scenario that fungi can be used as micro-chemical industries for the production of biologically active secondary metabolites, which is a diverse group and can be isolated from any ecosystem of the universe. We have summarised various aspects of the study that fungi could be used as source of producing biologically active secondary metabolites naturally, or either it could be genetically engineered for the production of important class of secondary metabolites, by knowing the biosynthetic pathways of the registered metabolites. It is obvious that the fungi revealed diversity and hence are capable for producing the diverse class of metabolites. Thus to obtain a very clear and complete picture of the fungi used in chemical industries, we will apply the traditional cultivation methods for both its natural metabolites and/or for genetically engineered metabolites. This will help in either case because it can reproduce the production of metabolites in quite few days because of its short span of life.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are highly thankful to Higher Education Commission of Pakistan for financing the studies of Abid Ali Khan under Grant PIN No. 074-1053-Bm4-207.

Comments

Background

The fungi are as diverse as animals and plants and their diversity is reflected in the variety of their secondary metabolites. The biosynthetic pathways for the secondary metabolites are also diverse. Because these compounds have biological activity either harmful or beneficial such as antibiotics, it is quite challenging to discover novel secondary metabolites.

Research frontiers

This review paper have been written in detail which emphasizes in the field of biosynthesis and molecular genetics of fungal secondary metabolites. This provides a cutting-edge viewpoint on fungal secondary metabolism and fundamentals of molecular biology. Therefore, it is a valuable resource for researchers in the field of fungal secondary metabolites/ biology.

Related reports

This is a review paper, therefore it has been explain in detail that how the research is going on with fungal secondary metabolites with reference to the reports of other researchers. This review article also explains how the other researcher have conducted their research in the area of fungal secondary metabolites. Therefore, it will be a beneficial source for the researchers in this field.

Innovations and breakthroughs

The review has described all the aspects of fungal secondary metabolites, while in the present report the authors have demonstrated that the fungi should be used for the exploitation of secondary metabolites, because of their diversity in secondary metabolites.

Applications

From the literature survey it has been found that the secondary metabolites of different fungi with current information on their biosynthesis and molecular genetics reveal the possible application of molecular biology to directed strain improvement in great detail.

Peer review

This is a really a valuable contribution for the new research in the field of fungal secondary metabolites because the authors have compiled all the information in

a very consistent manner. The authors have established a fine link that fungi is living organism but is used as chemical industries for the production of secondary metabolites. It is the real scientific contribution by naming fungi as chemical industries, that no one have ever used this term before for fungi.

References

[1] Moore D, Frazer LN. *Essential fungal genetics*. New York: Springer; 2002.

[2] Cox RJ. Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. *Org Biomol Chem* 2007; **5**(13): 2010-2026.

[3] Ou SH. *Rice diseases*. 2nd ed. Wallingford: CAB International; 1985, p. 109-201.

[4] Griffin DH. Fungal physiology: Wiley-Liss. 1996.

[5] Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, et al. Life with 6000 genes. *Science* 1996; **274**(5287): 546, 563-567.

[6] Mewes HW, Albermann K, Bähr M, Frishman D, Gleissner A, Hani J, et al. Overview of the yeast genome. *Nature* 1997; **387**(6632 Suppl): 7-65.

[7] Zerikly M, Challis GL. Strategies for the discovery of new natural products by genome mining. *Chembiochem* 2009; **10**(4): 625-633.

[8] Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981-2002. *J Nat Prod* 2003; **66**(7): 1022-1037.

[9] Lopez SN, Ramallo IA, Sierra MG, Zacchino SA, Furlan RLE. Chemically engineered extracts as an alternative source of bioactive natural product-like compounds. *Proc Natl Acad Sci U S A* 2007; **104**(2): 441-444.

[10] Bills GF, Platas G, Fillola A, Jiménez MR, Collado J, Vicente F, et al. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *J Appl Microbiol* 2008; **104**(6): 1644-1658.

[11] Munchhof MJ, Meyers A. An asymmetric route to chiral, nonracemic 2-substituted piperidines. Synthesis of (-)-pipecoline,(+)-coniine, and (-)-coniceine. *J Org Chem* 1995; **60**(22): 7084-7085.

[12] Hanson AD, Traynor PL, Ditz KM, Reicosky DA. Gramine in barley forage-effects of genotype and environment1. *Crop Sci* 1981; doi: DOI: 10.2135/cropsci1981.0011183x002100050024x.

[13] Virag R. Intracavernous injection of papaverine for erectile failure. *Lancet* 1982; **320**(8304): 938.

[14] Brodie BB, Udenfriend S. The estimation of quinine in human plasma with a note on the estimation of quinidine. *J Pharmacol Exper Ther* 1943; **78**(2): 154-158.

[15] Gershenzon J, Dudareva N. The function of terpene natural products in the natural world. *Nat Chem Biol* 2007; **3**(7): 408-414.

[16] Sacchettini JC, Poulter CD. Creating isoprenoid diversity. *Science* 1997; **277**(5333): 1788-1789.

[17] Barkovich R, Liao JC. Review: metabolic engineering of isoprenoids. *Metab Eng* 2001; **3**(1): 27-39.

[18] Banthorpe DV, Baxendale D. Terpene biosynthesis. Part III. Biosynthesis of (+)-and (-)-camphor in Artemisia, Salvia, and *Chrysanthemum* species. *J Chem Soc Perkin 1* 1970; **19**: 2694-2696.

[19] Bouwmeester HJ, Gershenzon J, Konings MCLM, Croteau R. Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway. Demonstration of enzyme activities and their changes with development. *Plant Physiol* 1998; **117**(3): 901-912.

[20] Hughes PR. Myrcene: a precursor of pheromones in Ips beetles. *J Insect Physiol* 1974; **20**(7): 1274-1275.

[21] Wolf G. A history of vitamin A and retinoids. *FASEB J* 1996; **10**(9): 1102-1107.

[22] Govindachari TR, Mohamed PA, Parthasarathy PC. Ishwarane and aristolochene, two new sesquiterpene hydrocarbons from *Aristolochia indica*. *Tetrahedron* 1970; **26**(2): 615-619.

[23] Kawaide H, Sassa T, Kamiya Y. Plant-like biosynthesis of gibberellin A_1 in the fungus *Phaeosphaeria* sp. L487. *Phytochemistry* 1995; **39**(2): 305-310.

[24] Finking R, Marahiel MA. Biosynthesis of nonribosomal peptides. *Annu Rev Microbiol* 2004; **58**: 453-488.

[25] Sieber SA, Marahiel MA. Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. *Chem Rev* 2005; **105**(2): 715-738.

[26] Mootz HD, Schwarzer D, Marahiel MA. Ways of assembling complex natural products on modular nonribosomal peptide synthetases. *Chembiochem* 2002; **3**(6): 490-504.

[27] Umezawa H, Maeda K, Takeuchi T, Okami Y. New antibiotics, bleomycin A and B. *J Antibiot (Tokyo)* 1966; **19**(5): 200-209.

[28] Weber G, Schörgendorfer K, Schneider-Scherzer E, Leitner E. The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8-kilobase open reading frame. *Curr Genet* 1994; **26**(2): 120-125.

[29] Fleming A. On the antibacterial action of cultures of a pénicillium, with special reference to their use in the isolation of *B. influenzae*. *Br J Exp Pathol* 1929; **10**: 226-236.

[30] Stachelhaus T, Marahiel MA. Modular structure of peptide synthetases revealed by dissection of the multifunctional enzyme GrsA. *J Biol Chem* 1995; **270**: 6163-6169.

[31] Mootz HD, Marahiel MA. The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal denylation domains. *J Bacteriol* 1997; **179**: 6843-6850.

[32] Conti E, Stachelhaus T, Marahiel MA, Brick P. Structural

basis for the activation of phenylalanine in the nonribosomal biosynthesis of gramicidin S. *EMBO J* 1997; **16**(14): 4174-4183.

[33] Stachelhaus T, Mootz HD, Bergendahl V, Marahiel MA. Peptide bond formation in nonribosomal peptide biosynthesis. Catalytic role of the condensation domain. *J Biol Chem* 1998; **273**: 22773-22781.

[34] Stachelhaus T, Huser A, Marahiel MA. Biochemical characterization of peptidyl carrier protein (PCP), the thiolation domain of multifunctional peptide synthetases. *Chem Biol* 1996; **3**: 913-921.

[35] Tao KJ, Du L, Sun XQ, Cai MH, Zhu TJ, Zhou XS, et al. Biosynthesis of aspergiolide A, a novel antitumor compound by a marine-derived fungus *Aspergillus glaucus* via the polyketide pathway. *Tetrahedron Lett* 2009; **50**(9): 1082-1085.

[36] Staunton J, Weissman KJ. Polyketide biosynthesis: a millennium review. *Nat Prod Rep* 2001; **18**(4): 380-416.

[37] Chan YA, Podevels AM, Kevany BM, Thomas MG. Biosynthesis of polyketide synthase extender units. *Nat Prod Rep* 2009; **26**(1): 90-114.

[38] Hertweck C, Luzhetskyy A, Rebets Y, Bechthold A. Type II polyketide synthases: gaining a deeper insight into enzymatic teamwork. *Nat Prod Rep* 2007; **24**(1): 162-190.

[39] Shen B. Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. *Curr Opin Chem Biol* 2003; **7**(2): 285-295.

[40] Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang RY, Algire MA, et al. Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 2010; **329**(5987): 52-56.

[41] Muller HJ, Carlson E, Schalet A. Mutation by alteration of the already existing gene. *Genetics* 1961; **46**(2): 213-226.

[42] Hopwood DA. Genetic contributions to understanding polyketide synthases. *Chem Rev* 1997; **97**(7): 2465-2498.

[43] Birch A, Massy-Westropp R, Moye CJ. Studies in relation to biosynthesis. VII. 2-Hydroxy-6-methylbenzoic acid in *Penicillium griseofulvum* Dierckx. *Aust J Chem* 1955; doi: 10.1071/ch9550539.

[44] Crump MP, Crosby J, Dempsey CE, Parkinson JA, Murray M, Hopwood DA, et al. Solution structure of the actinorhodin polyketide synthase acyl carrier protein from *Streptomyces coelicolor* A3(2). *Biochemistry* 1997; **36**(20): 6000-6008.

[45] Deacon H. Towards a sustainable theory of health-related stigma: lessons from the HIV/AIDS literature. *J Community Appl Soc Psychol* 2006; **16**(6): 418-425.

[46] Shoolingin-Jordan P, Campuzano I. Biosynthesis of 6-methylsalicylic acid. In: Sankawa U, editor. *Comprehensive Natural Products Chemistry*. Vol 1. Oxford: Elsevier; 1999, p. 345-365.

[47] Simpson TJ. *Application of isotopic methods to secondary metabolic pathways*. In: Leeper FJ, Vederas JC, editors. *Biosynthesis*. New York: Springer; 1998, p. 1-48.

[48] Simpson TJ. Applications of multinuclear NMR to structural and biosynthetic studies of polyketide microbial metabolites. *Chem Soc Rev* 1987; **16**: 123-160.

[49] Vaidya JG, Rabba AS. Fungi in folk medicine. *Mycologist* 1993; **7**: 131-133.

[50] Pachlatko JP. Natural products in crop protection. *Chimia* 1998; **52**: 29-47.

[51] Fox T, Fertleman M, Cahill P, Palmer RD. Medical slang in British hospitals. *Ethics Behav* 2003; **13**: 173-189.

[52] Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Reviews* 1995; **59**: 143-169.

[53] Kaeberlein T, Lewis K, Epstein SS. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* 2002; **296**: 1127-1129.

[54] Handelsman J. Metagenomics: applications of genomics to uncultured microorganisms. *Microbiol Mol Biol Rev* 2004; **68**: 669-685.

[55] Daniel R. The soil metagenome-a rich resource for the discovery of novel natural products. *Curr Opin Biotechnol* 2004; **15**: 199-204.

[56] Bode HB, Müller R. The impact of bacterial genomics on natural product research. *Angew Chem Int Ed Engl* 2005; **44**: 6828-6846.

[57] Pelzer S, Vente A, Bechthold A. Novel natural compounds obtained by genome-based screening and genetic engineering. *Curr Opin Drug Discov Devel* 2005; **8**: 228-238.

[58] Knight V, Sanglier JJ, DiTullio D, Braccili S, Bonner P, Waters J, et al. Diversifying microbial natural products for drug discovery. *Appl Microbiol Biotechnol* 2003; **62**: 446-458.

[59] Bode HB, Bethe B, Hofs R, Zeeck A. Big effects from small changes: possible ways to explore nature's chemical diversity. *Chembiochem* 2002; **3**: 619-627.

[60] Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. *Microbiol Mol Biol Rev* 2003; **67**(4): 491-502.

[61] Demain AL. From natural products discovery to commercialization: a success story. *J Ind Microbiol Biotechnol* 2006; **33**(7): 486-495.

[62] Gullo VP, Mcalpine J, Lam KS, Baker D, Petersen F. Drug discovery from natural products. *J Ind Microbiol Biotechnol* 2006; **33**(7): 523-531.

[63] Demain AL. Microbial biotechnology. *Trends Biotechnol* 2000; **18**(1): 26-31.

[64] Li JW, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier? *Science* 2009; **325**: 161-165.

[65] Demain AL. Pharmaceutically active secondary metabolites of microorganisms. *Appl Microbiol Biotechnol* 1999; **52**(4): 455-463.

[66] Ebel R. Natural product diversity from marine fungi. In: Mander L, Liu HW, editors. *Comprehensive natural products II: chemistry and biology*. Oxford: Elsevier; 2010, p. 223-262.

[67] Kilbey BJ. Charlotte Auerbach (1899-1994). *Genetics* 1995; **141**(1): 1-5.

[68] Jackson DA, Symons RH, Berg P. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proc Natl Acad Sci U S A* 1972; **69**(10): 2904-2909.

[69] Goeddel DV, Kleid DG, Bolivar F, Heyneker HL, Yansura DG, Crea R, et al. Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc Natl Acad Sci U S A* 1979; **76**(1): 106-110.

[70] Meyer V. Genetic engineering of filamentous fungi—progress, obstacles and future trends. *Biotechnol Adv* 2008; **26**(2): 177-185.

[71] Ruiz-Díez B. Strategies for the transformation of filamentous fungi. *J Appl Microbiol* 2002; **92**(2): 189-195.

[72] Fincham JR. Transformation in fungi. *Microbiol Rev* 1989; **53**(1): 148-170.

[73] Gouka RJ, Gerk C, Hooykaas PJ, Bundock P, Musters W, Verrips CT, et al. Transformation of *Aspergillus awamori* by *Agrobacterium tumefaciens*-mediated homologous recombination. *Nat Biotechnol* 1999; **17**(6): 598-601.

[74] Michielse CB, Hooykaas PJ, van den Hondel CA, Ram AF. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Curr Genet* 2005; **48**(1): 1-17.

[75] Haber JE. DNA repair: gatekeepers of recombination. *Nature* 1999; **398**(6729): 665, 667.

[76] Timberlake WE, Marshall MA. Genetic engineering of filamentous fungi. *Science* 1989; **244**(4910): 1313-1317.

[77] Jacobs DI, Olsthoorn MM, Maillet I, Akeroyd M, Breestraat S, Donkers S, et al. Effective lead selection for improved protein production in *Aspergillus niger* based on integrated genomics. *Fungal Genet Biol* 2009; **46**: S141-S152.

[78] Meyer V, Mueller D, Strowig T, Stahl U. Comparison of different transformation methods for *Aspergillus giganteus*. *Curr Genet* 2003; **43**(5): 371-377.

[79] Chi JT, Chang HY, Wang NN, Chang DS, Dunphy N, Brown PO. Genomewide view of gene silencing by small interfering RNAs. *Proc Natl Acad Sci U S A* 2003; **100**(11): 6343-6346.

[80] Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. *Nature* 2004; **431**(7006): 343-349.

[81] Agrawal N, Malhotra P, Bhatnagar RK. siRNA-directed silencing of transgene expressed in cultured insect cells. *Biochem Biophys Res Commun* 2004; **320**(2): 428-434.

[82] Heneghan MN, Costa AM, Challen MP, Mills PR, Bailey A, Foster GD. A comparison of methods for successful triggering of gene silencing in *Coprinus cinereus*. *Mol Biotechnol* 2007; **35**(3): 283-296.

[83] Zheng XF, Kobayashi Y, Takeuchi M. Construction of a low-serine-type-carboxypeptidase-producing mutant of *Aspergillus oryzae* by the expression of antisense RNA and its use as a host for heterologous protein secretion. *Appl Microbiol Biotechnol* 1998; **49**(1): 39-44.

[84] Nakayashiki H. RNA silencing in fungi: mechanisms and applications. *FEBS Lett* 2005; **579**(26): 5950-5957.

[85] Bergmann S, Schümann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat Chem Biol* 2007; **3**(4): 213-217.

[86] Berdy J. Bioactive microbial metabolites. *J Antibiot (Tokyo)* 2005; **58**(1): 1-26.

[87] Karlsson J, Saloheimo M, Siika-Aho M, Tenkanen M, Penttilä M, Tjerneld F. Homologous expression and characterization of Cel61A (EG IV) of *Trichoderma reesei*. *Eur J Biochem* 2001; **268**(24): 6498-6507.

[88] Sims JW, Schmidt EW. Thioesterase-like role for fungal PKS-NRPS hybrid reductive domains. *J Am Chem Soc* 2008; **130**(33): 11149-11155.

[89] Collemare J, Billard A, Böhnert HU, Lebrun MH. Biosynthesis of secondary metabolites in the rice blast fungus *Magnaporthe grisea*: the role of hybrid PKS-NRPS in pathogenicity. *Mycol Res* 2008; **112**: 207-215.

[90] Weinacker A, Chen A, Agrez M, Cone RI, Nishimura S, Wayner E, et al. Role of the integrin alpha v beta 6 in cell attachment to fibronectin. Heterologous expression of intact and secreted forms of the receptor. *J Biol Chem* 1994; **269**(9): 6940-6948.

[91] Sweeney HL, Straceski AJ, Leinwand LA, Tikunov BA, Faust L. Heterologous expression of a cardiomyopathic myosin that is defective in its actin interaction. *J Biol Chem* 1994; **269**(3): 1603-1605.

[92] Pickens LB, Tang Y, Chooi YH. Metabolic engineering for the production of natural products. *Annu Rev Chem Biomol Eng* 2011; **2**: 211-236.

[93] Pfeifer BA, Khosla C. Biosynthesis of polyketides in heterologous hosts. *Microbiol Mol Biol Rev* 2001; **65**(1): 106-118.

[94] Tang L, Shah S, Chung L, Carney J, Katz L, Khosla C, et al. Cloning and heterologous expression of the epothilone gene cluster. *Science* 2000; **287**(5453): 640-642.

[95] Ahmed S, Riaz S, Jamil A. Molecular cloning of fungal xylanases: an overview. *Appl Microbiol Biotechnol* 2009; **84**(1): 19-35.