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Review

A review on different approaches to isolate antibiotic compounds from fungi

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Abstract
Fungal secondary metabolites are promising grounds of many antibiotic compounds; this happens because of the unique biosynthetic capabilities of the organism in adaptation with various environments. Some of the potential environmental conditions or habitats stimulate fungi to produce bioactive compounds; these include various stress factors like temperature, osmotic changes and pollution. Traditional approaches used to isolate fungal antibiotics are mainly mono-culture-based and it trails behind the ever-expanding needs of the clinical world. A recent progress made in the culture-based approach is the co-culture of microbes, which creates a competing environment for the fungi resulting in the induction of hidden biosynthetic pathways. The revolutionizing impacts of the post-genomic era also aided these search in the form of various omics-based and biosynthetic approaches. These approaches not only facilitate the invention of all-new compounds but contribute in the modification of existing compounds through which the compounds can serve as better drug candidates.

Keywords
Co-culture, novel antibiotics, omics-based, secondary metabolites, mutasynthesis

1. Introduction
Microbial metabolites have proved as rich reservoirs of antibiotic compounds with potential clinical applications (Sanglier et al., 1996). Isolation of bioactive compounds from microbial cultures remains far advantageous than chemical synthesis and the method has provided numerous antibiotic compounds with promising clinical applications till date (Shlaes, 2010). Fungal metabolites draw much attention in this regard, and whose history dated back to the discovery of penicillin in 1929 (Fleming, 1929). Filamentous fungi own many unique metabolic pathways and produce a huge share of bioactive compounds possessing clinical use; hence, filamentous fungi serve as the potential producers of antibiotic compounds (Kuck et al., 2014).

As the usage of antibiotics became common, the problem of drug resistance emerged. For example, once an antibiotic drug is introduced into clinical practice, it has a limited period of success as selection takes place among the target bacteria with intrinsic or acquired resistance mechanisms (Chandra and Kumar, 2017). Consequently, it is essential to find out novel compounds that may be able to suppress drug resistant bacteria. Traditional approaches are mainly focused on finding natural bioactive molecules from cultured microbes (Berdy, 2012). Even though this culture-based approaches remained as the backbone of the golden antibiotic era, it stayed behind and usually failed to identify

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novel lead compounds that could neutralize drug resistant strains. Moreover, the rate of incidences of reporting and re-isolation of known compounds has been more and more frequent (Corley and Durley, 1994). Hence, there is a need for a paradigm shift in the approaches of drug discovery processes, which include identifying unexplored microbial reservoirs, novel culture approaches, omics-based approaches, etc. (Kealey et al., 2017). Here we review the different methods for antibiotic compound isolation from filamentous fungi, and focus on emerging trends like searching for fungal strains from previously unexplored sites, exploring various culture-based methods, omics-based and biosynthetic approaches.

2. Potential habitats for fungal isolation

Antimicrobial compounds are synthesized by fungi as secondary metabolites (Keller et al., 2005) and are not inevitable for their vital life processes (Madigan et al., 1997). These compounds are normally generated at the end of their life cycle or in stressful conditions, which necessitate them to fight against the odds of nature including both abiotic and biotic stresses (Davies, 1985). Hence, unusual environments are promising storehouses of novel metabolites generating microbes (Park et al., 2009). Antibiotic compounds isolated from filamentous fungi belonging to some of the stressed habitats are described below. Recently discovered novel antibiotic compounds (from 2000 onwards) from fungi along with their chemical structure are provided in Supplementary Table 1.

2.1. Regions with temperature extremes

By the 1950s the exploration of microorganisms from extreme conditions began to hurry up (Yogabaanu et al., 2017). Cold adapted fungi are actually a huge repository of novel antibiotics. In these extreme conditions, they are highly prone to many stresses such as high UV, low nutrients, and extremely low freezing temperatures (Nishiyama, 1977; Montiel, 2000). The occurrence and survival of fungal communities in the extreme cold temperature in Antarctica favoured their unique biochemical pathways to synthesize new bioactive metabolites (Santiago et al., 2012). Various species of *Penicillium, Aspergillus, Cadophora, Pseudogymnoascus, Paraconiothyrium, Purpureocillium, Toxicocladosporium* are prominent among these (Santiago et al., 2012; Vieira et al., 2018). Recent studies reported a vast array of antibacterial and antifungal compounds from fungi inhabiting Arctic and Antarctic zones (Lo Giudice and Fani, 2016) such as geomycin (Li et al., 2008) chanoclavine I, griseofulvin, roquefortine C and D, elymoclavine, mycelianamide, fulvic acid (Frisvad et al., 2004), amphotericin (Svahn et al., 2015) etc. from polar soils. Godinho et al. (2015) investigated the diversity and bioactive capabilities of fungal communities in the oligotrophic soil sample collected from Ellsworth Mountains, Antarctica. Two species of *Penicillium* viz. *Penicillium allii-sativi* Frisvad, Houbraken & Samson and *P. brevicompactum* Dierckx exhibited production of antibiotics penicillin and mycophenolic acid respectively. Li et al. (2008) obtained a pure culture of Antarctic ascomycete, *Pseudogymnoascus* sp. from the soil samples collected from Field Peninsula of King George Island, Antarctica and they isolated geomycins B and C. These compounds demonstrated antifungal properties towards *Aspergillus fumigatus* Fresen. and antibacterial action towards *Staphylococcus aureus, Escherichia coli* and *Streptococcus pneumoniae*. Yogabaanu et al. (2017) conducted a study on the antimicrobial activity by 40 cold friendly soil fungi from both poles (Arctic and Antarctic). About 45% of fungal cultures produced antimicrobial compounds in culture and they exhibited antimicrobial action towards at least one of the five bacterial pathogens investigated. The fungal strains which exhibited prominent bioactivity were again subjected to temperature manipulation studies and the studies revealed the roles of culture conditions on bioactivity. Likewise, studies were also reported from high temperature regions like deserts, for example, Awaad et al. (2012) isolated two new antibiotic compounds, which were butyrolactone I derivatives, produced by *Aspergillus terreus* Thom from desert soil.

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2.2 Mangroves

Mangrove ecosystem is another stressed one, found at land-sea interface having a variety of stresses such as temperature, salinity, anoxia, UV, etc. (Kathiresan and Bingham, 2001). They are unique ecosystem composed of diverse microbes, which act as potential agents for the isolation of novel pharmaceutically active compounds. This is because, the microbiota in mangrove ecosystem are constantly adapting to the changing environmental conditions through the biosynthesis of various secondary metabolites (Thatoi et al., 2013). The microbial communities of the mangrove ecosystem remain largely unexplored (more than 99%) and those which are chemically characterized accounted only 5% of the known ones (Xu, 2015). Liu et al. (2018) conducted a study at South China Sea, and isolated two novel compounds ergosterdiacids A and B from *Aspergillus* sp. DM29 from the rhizospheric soil of *Aegiceras corniculatum,* a mangrove plant. These compounds demonstrated antibacterial activity towards *Mycobacterium tuberculosis*. Likewise, He et al. (2019) cultured *Penicillium pinophilum* Thom SCAU037 from the mangrove sediment collected from the roots of *Rhizophora stylosa* at Techeng Isle, China and isolated novel compounds pinophilone A-E, funicone derivatives. The compounds exhibited growth inhibition of *Mycobacterium smegmatis* and *S. aureus.*

2.3. Polluted sites

Many of the past studies were focused on natural extreme environments, but a recent trend is to focus on sites that have become stressed due to unchecked human interventions. Acid mine drainage is one among these characterized by heavy metal contamination, highly acidic soil and many other pollutants, which make an arena of competition between endemic microbes, resulting in the generation of novel antimicrobial compounds (Johnson and Hallberg, 2003). Park et al. (2009) isolated a novel metabolite glionitrin A, as they co-cultured bacterial strain *Sphingomonas* KMK-001 and fungal strain *A. fumigatus*, KMC-901, derived from abandoned coal mine of Young-dong, Korea. This compound exhibited notable antibacterial activity towards a series of bacteria including methicillin resistant *S. aureus* and weak antifungal activity towards some pathogenic strains. Noticeably, the monoculture of both these microbes failed to produce this bioactive compound revealing the potential of stressful interactions in the production of novel compounds.

Heavily antibiotic contaminated sites have also recently become a field of interest (Kristiansson et al., 2011). The bacteria thriving in such an environment developed antibiotic resistance and thus it became inevitable for the endemic fungi to resort to novel compounds. Svahn et al. (2012) assessed the antimicrobial potential of fungal isolates sampled from river sediment from Isakavagu stream in Andhrapradesh, India, located downstream to the outlet of a waste water treatment plant receiving waste waters from drug manufacturers, with high antibiotic contamination including fluoroquinolone antibiotics. They identified 61 strains many of which displayed activity against multidrug resistant bacteria. They also identified gliotoxin, a toxin produced from *A. fumigatus*. Even though this compound exhibited significant antimicrobial activity, its toxicity towards mammalian cells hinders its development as a potential drug candidate. Bharadwaj et al. (2017) characterized six fungal isolates belonging to four genera viz. *Aspergillus, Fusarium, Penicillium* and *Rhizopus* having antibacterial activity from soil sample collected from a pharmaceutical site and provided optimum conditions for fermentation. The bioactivities of the fungal isolates were compared with established antibiotics and much better antibacterial activities were exhibited by crude extracts from the fungal isolates towards the test pathogens.

3. Different approaches in antibiotic compound isolation

Various approaches employed in antibiotic compounds isolation are discussed below and briefly outlined (Fig. 1).

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3.1. Culture-based approaches

This method involves the culture of desired fungal strains in laboratory conditions. Culture-based approaches include monoculture and co-culture depending on whether the fermentation is maintained as the culture of a single strain or the culture of two interacting microbes (Gomez-Flores et al., 2017).

3.1.1. Monoculture approach

This is the traditional culture-based approach followed in the fermentation of many isolated fungal strains for the extraction of desired product. Most of the antibiotic isolation protocols from microbes during the past century followed this approach. The pure culture of fungus is inoculated into the fermentation medium, providing optimum conditions for the production of secondary metabolites (Hauser, 2006).

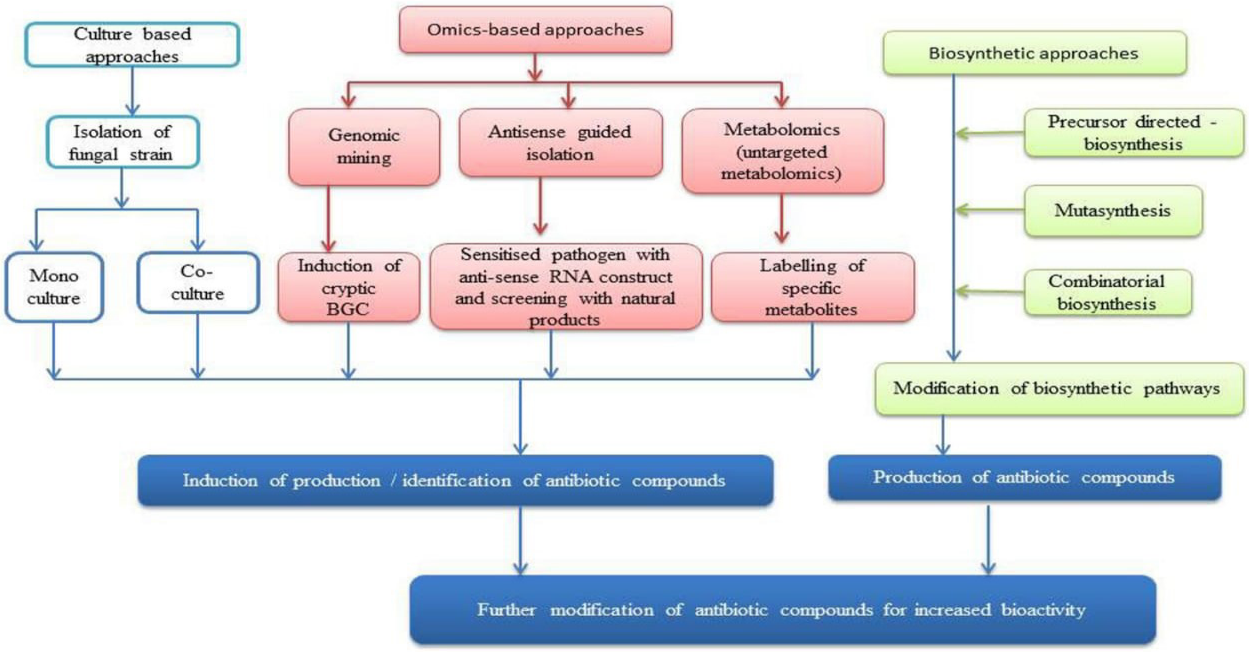

Fig. 1 – Different approaches in antibiotic discovery from filamentous fungi. The drug discovery pipeline starts with the traditional culture-based approaches and advances towards the recent omics-based and biosynthetic approaches. Each approach and its subsections are indicated with the same coloured boxes. The pipeline ends with the discovery of novel antibiotic compounds with further modifications for increased bioactivity.

3.1.2. Co-culture approach

Microbial monocultures suffer a limitation of lacking interaction between microbial communities as in the natural environment. As a result, many biosynthetic gene clusters (BGCs) in fungi stand cryptic, thus transcriptionally inactive under the standard conditions in laboratories (Scherlach and Hertweck, 2009). A solution to this is the co-cultivation of microbes, which has already attained success in isolating many novel secondary metabolites (Netzker et al., 2015; Molloy and Hertweck, 2017; Netzker et al., 2018). One of the first initiatives in this regard was reported by Fenical laboratory at the Scripps Institute of Oceanography. They co-cultured the marine fungus *Pestalotia* sp. CNL 365 (an epiphyte on the brown alga *Rosenvingea* sp. from Bahamas Islands) with the marine bacterium CNJ 328. The fungal strain is cultured in Fernbach flasks having sea water based fermentation medium with shaking. Ten millilitres of the culture of marine bacterium in the same medium is poured into the Fernbach flasks after 24 hours of initiation of fungal culture, followed by a mixed fermentation period of 6 days. The purified culture extracts showed the occurrence of pestalone, a novel antibiotic

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compound. The monoculture of both could not produce the novel compound suggesting the role of bacterial competition in the induction of the antibiotic biosynthetic pathway (Cueto et al., 2001). In the same manner, Degenkolb et al. (2002) communicated the isolation of antibiotic compounds acremostatins A, B and C from the co-culture of *Acremonium* sp. Tbp-5 (an endophytic fungus of *Taxus baccata*) and *Mycogone rosea* Link DSM 12973. The co-culturing of microbes creates a tension of competition between microbes which induce them to produce compounds, which are not usually produced when left undisturbed (Park et al., 2009). In the same way, *Emericella* sp. produced emericellamides A and B in competing co-culture with *Salinispora arenicola* (a marine actinomycete). Both these compounds exhibited modest inhibition towards methicillin resistant *S. aureus* (Oh et al., 2007). Schroeckh et al. (2009) described the synthesis of orsellinic acid from *Aspergillus nidulans* (Eidam) Winter as the fungus was co-cultured with *Streptomyces rapamycinicus*. The mixed culture conditions were able to activate a silent gene cluster of fungi. Same kind of finding was also conveyed by Konig et al. (2013) in which *S. rapamycinicus* activated a silent gene cluster of *A. fumigatus* producing fumicyclines. This compound is proposed to be involved in fungal defence mechanism as they exhibited moderate inhibition of *S. rapamycinicus*. Meng et al. (2015) also successfully isolated novel compounds, citrifelin A and B, which are citrinin adducts having tetracyclic ring structure from the co-culture of two fungi, *Penicillium citrinum* Thom and *Beauveria felina* (DC.) J.W. Carmich. Monocultures of both fungi failed to generate the novel compound and it exhibited inhibition of several pathogenic strains. Another latest innovation of co-culture materialized the production of novel compounds berkeleylactones A-H, 16-membered ring macrolide antibiotic obtained from the co-cultivation of two fungi *Penicillium fuscum* (Sopp) Biourge and *Penicillium camemberti* Thom/ *clavigerum* Demelius by Stierle et al. (2017). The culture also yielded known antibiotic compounds A26771B, citrinin and patulin. Recently, Liu et al. (2020) reported a new antifungal compound from *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson against the plant pathogen *Botrytis cinerea* Pers., as they co-cultured two fungi. The culture plates showed clear inhibition towards the pathogen and the new bioactive compound was identified using Matrix-assisted laser desorption/ionization (MALDI) time-of flight (TOF) - imaging mass spectroscopy (IMS) as lipopeptaibol (leucinostatin Z).

Co-culture not only induces novel bioactive compound production, but enhances the productivity of metabolites. The metabolic productivity of *P. pinophilum* was reported to be enhanced during the co-culture with *Trichoderma harzianum* Rifai (Nonaka et al., 2011). Along with the increased production of known compounds like stromemycin, penicillide, pestalasin A, a novel compound secopenicillide C was also isolated.

3.2 Omics-based approach

Culture-based approaches are inefficient in the sense having only limited access to the vast biosynthetic capabilities of the genome for the reason that they are not usually displayed under the undisturbed lab environment (Gross, 2009). The development of omics-based techniques has contributed much to the revitalization of novel antibiotic discoveries by providing an insight to the cryptic biosynthetic gene clusters (BGCs) encoding secondary metabolites (Palazzotto and Weber, 2018). Here we discuss some relevant omics-based approaches employed in the antibiotic discovery from fungi.

3.2.1. Genomic mining

In the post-genomic era, genomic mining has proved to perform a crucial position in the identification of untold lead compounds for clinical use. Thousands of microbial genome sequences were made available, and provided large grounds for this exploration. Along with this, various computational tools and methods have been devised to guide this resource hunting (Ziemert et al., 2016).

The core of genome mining lies in its ability to predict genes that can code for novel bioactive compounds by *in silico* approaches (Machado et al., 2017). Among the huge genome data, BGCs, which

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encode secondary metabolites, are potent resources of innovative antibiotic molecules (Cimermancic et al., 2014; Tracanna et al., 2017). BGCs also represent elements that can enhance the production of both already known natural compounds and engineered compounds (Olano et al., 2014). The genome mining can effectively predict the putative structure of the metabolites from BGCs. This approach is helpful for screening and targeting potential compounds from a huge data; thus avoiding the tedious task of screening every cryptic BGC (Zerikly and Challis, 2009).

In dealing with fungi, many of the enzymes involved in their secondary metabolite biosynthesis are large and complex with multiple modules and domains (Walsh et al., 2001). Furthermore, the number of BGCs is much greater than anticipated; this is because of the cryptic cluster of genes that remain hidden under the laboratory conditions. Bok et al. (2006) performed LaeA (Loss of aflR-expression A - a regulatory protein) based genome mining in *A. nidulans* and thus deciphered its secondary metabolome. This work involved a unique way to evaluate the gene regulation of secondary metabolism. Cain et al. (2020) sequenced the genome of the Ascomycete sp. F53 and performed mining for the purpose of rigorously exploring the biosynthetic capabilities of this endophytic fungus. 35 putative BGCs were revealed in the study, along with a distinctive azaphilone BGC which paved the discovery of lijiquinone, an azaphilone polyketide. The compound showed antifungal activity towards *Cryptococcus albidus* (Saito) Skinner and *Candida albicans* (C.-P.Robin) Berkhout. The various ways to improve the manufacture of already known compounds also came under the targets of genome mining. Van den Berg et al. (2008) sequenced the genome of *Penicillium chrysogenum* Thom and recognized various genes relevant to penicillin formation, consequently provided the potential of genomics-driven manipulation of metabolites. Wang et al. (2016) sequenced the genome of *P. lilacinum* and through comparative genomics strategy using bioactive characters; they identified 20 genes concerned with leucinostatin biosynthesis. Through various bioassays, leucinostatins revealed bioactivity against *Phyotophthora capsici* and *P. infestans*, by inhibiting their growth. Thus, the potential of *P. lilacinum* as a biocontrol agent was revealed and it was effectively used for the same.

Different techniques employed in the genome mining included bioinformatic methods, gene inactivation method, heterologous expression, use of transcription activators and inactivation of inhibitors (Scheffler et al., 2013). In the bioinformatics approach, entire genome or gene of interest is sequenced, after that gene prediction is done by various tools such as BLAST, GOLD, HMMER, SBSPKS, NORINE, antiSMASH, etc. (Nikolouli and Mossialos, 2012).

The gene inactivation method deals with the manipulation of the organism using a gene knock out approach. Here the product of a knocked out gene is unknown. This could efficiently overcome the difficulty arising from inaccurate gene predictions using bioinformatics tools (Gross et al., 2007). An example of this method involves the study conducted by Chiang et al. (2008) for the elucidation of emericellamide biosynthetic pathway. They studied six genes of *A. nidulans* using six strains with one of the genes knocked out in each strain. Only one knocked out exhibited a change from wild type indicating the silent nature of other genes. Furthermore, the gene of the concerned strain was found to be implicated in the biosynthesis of bioactive compounds emericellamides A and C-F.

As opposed to gene inactivation, heterologous expression involves the transfer of a cryptic gene from an organism to a host that can express the gene (Gross, 2007). The product of the cryptic gene (transgene) is expressed in the heterologous host. LC/MS analysis is performed and is used to compare the fermentation broth of transgenic host, with the broth of the host lacking this transgene. Metabolites present in the fermentation broth of the transgenic host with target BGC; but it is absent in the broth of the host lacking this BGC. It is assumed to be the products of the cryptic BGC (Challis, 2008). Bailey et al. (2016) heterologously expressed pleuromutilin gene cluster from *Clitopilus passeckerianus* (Pilát) Singer in the host *Aspergillus oryzae* (Ahlburg) E. Cohn. This was the initially reported in heterologous expression involving cross-phylum transfer of a gene cluster from basidiomycete to ascomycete host. The study was conducted for strain improvement and increased antibiotic production.

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Another method involves transcription activators, particularly used when the gene of interest is unable to be transferred to a host cell. In order to turn on these cryptic genes, activator genes already present in the cell are employed and thus the product is induced to form (Challis, 2008). Using this approach, Bergmann et al. (2007) successfully induced a cryptic BGC in *A. nidulans*, which generated in the formation of new polyketide synthase–nonribosomal peptide synthetase (PKS-NRPS), a hybrid metabolite. As a means to activate the cryptic BGC, an inducible activator gene, *apdR* (Aspyridones cluster regulator) in this cryptic gene cluster was identified and overexpressed. They amplified the activator and cloned into pAL4 (phenylalanine Ammonia-lyase 4), an expression vector with inducible promoter, alcAp (alcohol dehydrogenase promoter) of *A. nidulans.* The inducing conditions resulted in PKS-NRPS hybrid synthetase activation and the induced strains produced aspyridones A and B.

Another technique of the genome mining employs the expression of a cryptic gene by removing the inhibitor, which repress the product formation from the target gene. The product formed can be isolated and identified. This method suffers from a limitation; it is useful only in instances where an inhibitor is present in the organism, which repress the gene and the target gene can be activated when the inhibition is removed. In most cases, an inhibitor is not the reason, but the lack of activators. Therefore, this method is least employed in the discovery of bioactive compounds (Scheffler et al., 2013).

3.2.2. Antisense-guided isolation

Revolutionizing improvements in the area of microbial genomics have paved the way for detection of novel antibiotics based on antisense-based screening strategies. In this approach, essential genes are identified in microbes as target for antisense-based inhibition (Bai et al., 2010). The major target genes include those involved in bacterial growth, multiplication, metabolite synthesis and virulence (Woodford and Wareham, 2009).

The antisense RNA is made to express selectively, and it binds to the mRNA of the target gene resulting in its degradation, thus a reduction in the corresponding gene product. These weakened strains are exposed to an inhibitor against the targeted gene product usually by screening a natural compound library (Young et al., 2006). Ondeyka et al. (2006) employed antisense-based strategy to search for cell-permeable inhibitors of type II fatty acid synthesis pathway (FASII). They conducted a two plate assay screening over 25,000 fermentation broths of natural compounds, and discovered phomallenic acid A-C from *Phoma* sp. as potent inhibitors of β-ketoacyl-[acyl carrier protein (ACP)] synthase II(FabF)/and III)(FabH) enzymes of FASII pathway (Ondeyka et al., 2006; Young et al., 2006). Zhang et al. (2009) also employed this approach, targeting ribosomal protein S4 in the search for novel antibiotics. This protein is synthesized by the gene encoding ribosomal protein S4 (*rpsD)* in bacteria and screening is done by two-plate assay using *S. aureus,* engineered with *rpsD* antisense RNA construct. Natural compound extract from an endophytic fungus of *Pleosporales* MF7028 showed promising results and the compound was identified as pleosporone. Likewise, Parish et al. (2009) also employed the strategy of antisense-mediated isolation in identifying the inhibitors of SecA (Type II secretary pathway – a system responsible for the secretion of proteins through cell membrane – it is a membrane associated ATPase motor), an integral part of secretary machinery concerned with protein translocation. Secretary pathways are previously unexplored for antibacterial compounds, thus providing opportunity for the development of drugs with novel structural scaffolds thus offering some advantage from drug resistance. A manipulated strain of *S. aureus* with an inducible *secA* antisense RNA construct is used to screen over 115,000 natural compounds in a two plate assay. An extract from the fungus *Pseudogymnoascus pannorum* (Link) Minnis & D.L. Lindner showed promising results and an active compound is isolated and is named as pannomycin.

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3.2.3. Metabolomics

Metabolomics, a relatively new domain of omics-based method, includes the systematic characterization and quantification of the entire metabolites in a given biological sample (Idle and Gonzalez, 2007) and it has established as a successful method in the discovery of antibiotic compounds from microbes (Wu et al., 2015). This approach can be divided into targeted and untargeted metabolomics (Schrimpe-Rutledge et al., 2016).

Targeted metabolomics - It is generally involved in the identification and quantification of known compounds and the data it handles are limited (tens to hundreds of compounds) (Schrimpe-Rutledge et al., 2016). These compounds are previously defined, for example, biochemically annotated and chemically characterized (Roberts et al., 2012) and thus less employed in the discovery of novel compounds.

Untargeted metabolomics - As opposed to the targeted metabolomics, untargeted metabolomics aims at inspecting as much metabolites as possible (Patti, 2011). Untargeted metabolomics look for a fingerprint of the metabolome of the target organism with nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) data (Smedsgaard and Nielsen, 2004). Its advantage lies in its ability to produce high resolution data with the aid of MS and NMR techniques (Kind and Fiehn, 2010; Neumann and Bocker, 2010). Analytical strategies employed in this approach along with dereplication tools and software are briefly discussed (Hautbergue et al., 2018).

Analytical techniques employed in labelling of metabolites with a stable isotope called stable isotope labelling (SIL), helped the marking of fungal secondary metabolites on a complex metabolome. A labelled substrate is used to label fungal metabolites, thus effectively marking it from the background and contaminants. This technique was successfully utilized by Klitgaard et al. (2015) while studying the metabolite synthesized from phenylalanine by labelling phenylalanine in the feed of *A. nidulans*. The compounds of relevance were analysed using MS by comparing with the non-labelled control; they isolated fungisporin and nidulanin A analogues.

Another key step in metabolomics approach for novel bioactive compound detection is the screening out of already known compounds from the metabolome called dereplication. It employs the computer aided strategy in marking known compounds and directs the discovery process towards unknown compounds by submission of NMR or MS data to databases (Gaudencio and Pereira, 2015). Different databases are available based on chemical formulae, mass, liquid chromatography (LC) values, etc. for the exact characterization of molecules. Fungal specialized databases, for example, AntiBase (Laatsch, 2014), and Dictionary of natural products (http://dnp.chemnetbase.com) are based on exact mass and chemical formula. Another widely used tool based on MS/MS spectral data is Global Natural Product Social Molecular Networking (GNPS). It has about 220,000 MS/MS spectra, which represent more than 18,000 metabolites from the NIST (National Institute of Standards and Technology), ReSpect -RIKEN tandem mass spectral database (Sawada et al., 2012), MassBank (Horai et al., 2010) and is based on DEREPLICATOR algorithm (Mohimani et al., 2017).

Data mining tools: Several tools help in the process of data mining from complex metabolome such as XCMS (Smith et al., 2006), GNPS, etc. GNPS allows the grouping of compounds on the assumption that compounds with similar MS/MS spectral properties also share similar structures (Mohimani and Pevzner, 2016). On the other hand MS2LDA tool helps in describing compounds with similar MS/MS spectra (Van der Hooft et al., 2016). In dealing with structure elucidation tools of these natural metabolites, various aids such as NRPquest, which compares multiple peptide compounds on the basis of MS/MS spectra, are created (Mohimani et al., 2014).

Oppong-Danquah et al. (2020) performed untargeted metabolomics study in six sea foam-derived fungal strains included in the genera *Penicillium, Plectosphaerella*, *Emericellopsis* and *Cladosporium.*

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They employed ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry based molecular networking (UPLC-QToF-MS/MS based MN) in their investigation. Furthermore, they also tested the antimicrobial activity of organic extracts of these fungal isolates. The study identified a *Penicillium* strain as the most productive among them and also identified novel antimicrobial derivatives such as analogues of fungisporin. Albright et al. (2015) used the technique of untargeted metabolomics in a study conducted on *A. nidulans* to track the changes in small molecule metabolites secreted from the fungus after creating reduction in histone deacetylase activity (HDACi). Along with metabolites from known and unknown pathways, the study reported the presence of fellutamide, whose production is enhanced up to ~ 100 fold by HDACi.

3.3. Biosynthetic approaches

Fungi provide a large repository of secondary metabolites, many of which have potential clinical applications. Some of them cannot be utilized directly due to toxic activities or suboptimal bioactivity. To circumvent these limitations, non-natural derivatives from these natural compounds via biosynthetic routes are proposed (Boecker et al., 2016). The different strategies in this regard are outlined here.

3.3.1. Precursor –directed biosynthesis (PDB)

This approach makes use of the processing of precursor analogues created through chemical synthesis into novel bioactive compounds, since the natural biosynthetic machinery is flexible enough to accommodate synthetic substrate analogues and process them to modified intermediates. The application of PDB in creating derivatives of existing molecules has started since 1950s. Brandl and Margreiter (1954) supplemented the cultures of *P. chrysogenum* with phenoxyacetic acid and successfully isolated acid stable, orally applicable penicillin V. Nilanonta et al. (2002) reported the biosynthesis of beauvericin analogues from *Paecilomyces tenuipes* (Peck) Samson using PDB approach. Enhanced production of beauvericin A, beauvericin B, beauvericin C along with beauvericin was resulted on feeding L-isoleucine or D-alloisoleucine while the feeding of D-isoleucine or L-alloisoleucine produced allobeauvericin A, B and C. One of the limitations of this method is the competition between natural substrates and supplemented analogues (Boecker et al., 2016).

3.3.2. Mutasynthesis

This is considered as an advanced approach of combinatorial biosynthesis, and it involves the creation of mutants lacking some responsible genes concerned with the production of the natural precursor in the biosynthesis (Weist and Süssmuth, 2005; Kirschning and Hahn, 2012). Such lacking precursors are complemented by precursor analogues called mutasynthons, which are chemically synthesized and supplied. One of the limitations of PDB has been rectified, as it avoids competition between mutasynthons and natural precursor. Moreover, the isolation of the engineered product also becomes easy as the natural product formation is hindered (Sussmuth et al., 2011). Xu et al. (2009) identified the gene coding for the enzyme ketoisovalerate reductase (KIVR). This enzyme is accountable for the production of D-hydroxyisovalerate (D-Hiv), which acts as the precursor for cyclooligomer depsipeptides (COD) including bassianolide, beauvericin and enniatins which has antibiotic, antifungal and many other bioactivities. They mutated KIVR genes, thus facilitating the biosynthesis of beauvericin from mutasynthons. From the results, they found that the precursor analogues D-2-hydroxybutyrate (D-Hbu) efficiently produced beauvericin G_3 while DL-2- hydroxyl-3-methylvalerate (DL-Hmv) produced beauvericin C and also provided a strategy for a library of unnatural CODs of beauvericin type.

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3.3.3. Combinatorial biosynthesis

The genetic manipulation of biosynthetic machineries naturally present in the cell for the production of altered, novel compounds with novel bioactivities is combinatorial biosynthesis (Hopwood et al., 1985). The manipulations range from combining of enzymes from different pathways to the modifications in the enzymes themselves (Floss, 2006). Majority of fungal secondary metabolites are formed mainly by three enzymes viz. Non-Ribosomal Peptide Synthetases (NRPS), Polyketide Synthases (PKS), Terpene Synthases (TPS) with accompanying tailoring enzymes. The natural architecture of these key enzymes are modified through insertion, deletion, exchange of domains, etc. all aiming at the generation of some modified compounds with increased bioactivity (Boecker et al., 2016). In the context of rush in search for novel antibiotics, Non-Ribosomal Peptide Synthetases are drawing attention because they are engaged in the synthesis of many clinically important peptide antibiotics. A study done by Zobel et al. (2016) aimed at one of its subgroups, cyclodepsipeptide (CDP) synthetases which are a highly similar group of enzymes implicated in the synthesis of cyclo-hexadepsipeptide and cyclo-octadepsipeptide. For this, they selected depsipeptide synthetases, beauvericin synthetase (BSYN) for synthesizing beauvericin from *Beauveria bassiana* (Bals.-Criv.) Vuill., enniatin synthetase (ESYN) for producing enniatin from *Fusarium oxysporum* Schlecht. Emend. Snyder & Hansen and PF1022 synthetase (PSYN) for synthesizing PF1022 from *Rosellinia abscondita* Rehm. Engineering of hybrid synthetases were carried out by fusing module 1 of PSYN with the module 2 of BSYN and ESYN. These hybrid synthetases were heterologously expressed in *Aspergillus niger* van Tieghem and *E. coli* resulting in the formation of hybrid hexadepsipeptides.

3.3.4. Combinatorial libraries

In the search for novel bioactive compounds, combinatorial libraries are gaining ground by allowing a wide array of compounds to be screened for activity. Depending on the central structure around which the library is built, it can be divided into biased and generic libraries. In biased library, the lead compound is either from a natural compound, previously characterized by structure activity relationship (SAR) studies or a proven compound from the generic library. It has the advantage of generating a large number of analogues for a given compound in a suitable time frame. In contrast, generic libraries aim on a much different strategy of building the bioactive candidate around a pharmacophore model, which is totally unrelated to any proven compound. Thus, it is also less prone to be prevailing over by the multiple drug resistant strains (Houghten et al., 1991; Pinilla et al., 1992). Blondelle & Lohner (2000) built a biased synthetic combinatorial library (SCL) based on an 18-mer lytic peptide formed of lysine and leucine residues (YKLLKKLLKKLKKLLKKL-NH_2) named YLK (Blondelle and Houghten, 1992; Blondelle et al., 1995; Perez-Paya et al., 1996). YLK exhibited antibacterial action towards gram-positive and gram-negative bacteria as well as limited antifungal activity towards *C. albicans* (Blondelle et al., 1996). A huge set of YLK analogues (130,321 analogues) were created in the main SCL, many of which exhibited improved activity against a given organism than the parent peptide. It is generally involved in the identification and quantification of known compounds and the data it handles are limited (tens to hundreds of compounds) (Blondelle and Lohner, 2000).

4. Conclusion

The search for the novel antibiotic drug candidates is never-ending, as the resistance to almost all known antibiotics is evolving in the target groups. Screening performed among natural products served much better than that in chemical compound library as nature created more than that of human imagination. Thus, a wide array of fungal metabolites is potential targets of drug discovery methods. However, this search is very tedious like finding a needle in a haystack. Traditional monoculture based culture methods are trailing behind the expanding needs of the clinical world. For this purpose,

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potential habitats have to be explored and novel methods have to be devised. Isolation of fungal strains from various stressed environments has reported success due to their unique secondary metabolites through which they get through these various pressures. Also the emerging trends of co-culture, omics-based and biosynthetic approaches are rejuvenating this search from rapid induction or detection of potential biosynthetic pathways by combining or modifying existing pathways to generate novel lead compounds.

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