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Research review paper

Recent advances in research for potential utilization of unexplored lichen metabolites

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ABSTRACT

Several research studies have shown that lichens are productive organisms for the synthesis of a broad range of secondary metabolites. Lichens are a self-sustainable stable microbial ecosystem comprising an exhabitant fungal partner (mycobiont) and at least one or more photosynthetic partners (photobiont). The successful symbiosis is responsible for their persistence throughout time and allows all the partners (holobionts) to thrive in many extreme habitats, where without the synergistic relationship they would be rare or non-existent. The ability to survive in harsh conditions can be directly correlated with the production of some unique metabolites. Despite the potential applications, these unique metabolites have been underutilised by pharmaceutical and agrochemical industries due to their slow growth, low biomass availability and technical challenges involved in their artificial cultivation. However, recent development of biotechnological tools such as molecular phylogenetics, modern tissue culture techniques, metabolomics and molecular engineering are opening up a new opportunity to exploit these compounds within the lichen holobiome for industrial applications. This review also highlights the recent advances in culturing the symbionts and the computational and molecular genetics approaches of lichen gene regulation recognized for the enhanced production of target metabolites. The recent development of multi-omics novel biodiscovery strategies aided by synthetic biology in order to study the heterologous expressed lichen-derived biosynthetic gene clusters in a cultivatable host offers a promising means for a sustainable supply of specialized metabolites.

1. Introduction

Lichens are a complex, self-supporting and stable microbial assemblage formed by the interaction of an exhabitant mycobiont (fungal) and one or more inhabitant photobiont as photosynthetic partner and several other microbial partners (Culberson, 1970; Hawksworth and Grube, 2020). Initially, lichen was considered a binary symbiosis of mycobionts and photobionts, however, some recent literature highlighted the involvement of three or more partners (tripartite lichens) and as such lichens can be characterised a multi-symbiosis (Grimm et al., 2021). This work challenged the notion that lichens only encompass a single unique fungus (Spribille et al., 2016). Lichen symbiosis is discussed as a structured consortium that has a deep evolutionary past with unexpected complexity, depth and which have been interrogated with recent advancement in molecular biology (Mark et al., 2020; Nash, 1996). Some recent reports also indicated the occurrence of viruses and protists in association with lichens microbiome (Petrzik et al., 2019; Wilkinson et al., 2015). However, the identity of the lichens is considered based on the fungal partner and to date the predominant records of lichens that have been identified are ascomycetes in nature. In this regard it has been reported that lichen-forming compatibility is adopted by about 46% of *ascomycota* (Stocker-Wörgötter, 2008).

The robustness of lichens across a long period of time and subsequently evolution phases highlight the significance of this symbiosis. In this association, both partners are mutually benefited as the photobiont partner generates carbohydrates *via* photosynthesis for the fungal partner whereas the mycobiont provide shelter and mineral nutrients to the photobiont and aids it to survive in extreme environment (Elix et al., 1984). This tendency of lichens to endure the extreme environmental influences can be correlated with the production of both a unique and diverse range of metabolites known as lichen substances (Calcott et al., 2018; Nguyen et al., 2013b). The majority of the bioactive compounds are exclusively produced by the mycobiont partner however there are some instances where the photobiont, particularly cyanobacteria are

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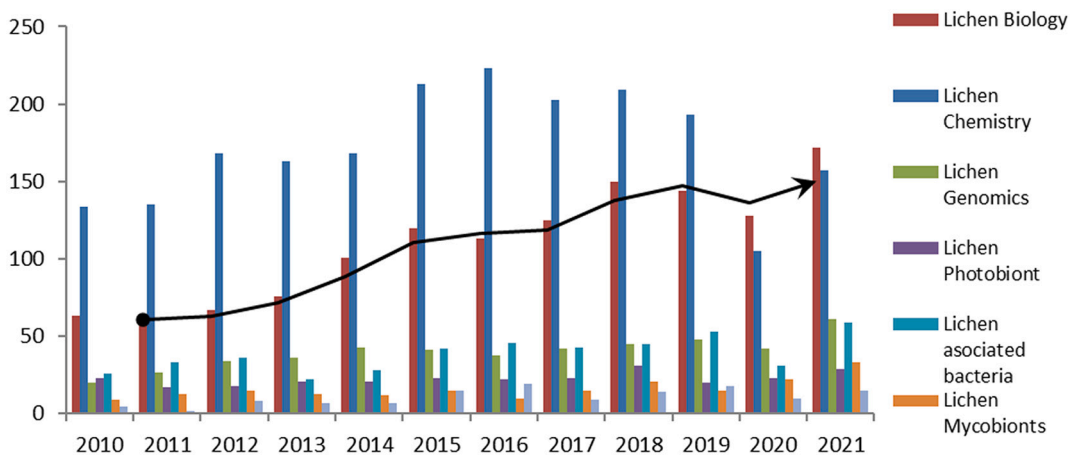

Fig 1. PubMed publication trend analysis, demonstrating increased scientific interest in lichen derived chemistry, and drug discovery. The data were retrieved with PubMed (https://www.ncbi.nlm.nih.gov/pubmed) on 12th May 2022, and cover the time period 2010-2021. As indicated, the used search keywords were lichen biology, lichen chemistry, lichen genomics, lichen photobiont, lichen associated bacteria, lichen mycobiont, endolichenic and the total number of PubMed publications per year was retrieved

also found to be engage in the production of some key secondary metabolites (Cox, 2005). Despite decades of intensive studies, tendency of lichens to provide unique and novel metabolites renewed the attention of pharmaceutical industries and researchers. The role of these compounds in the lichen symbioses is still poorly understood (Calcott et al., 2018; Hager et al., 2008; Kampa et al., 2013), however, prospective pharmacophores of these metabolites are associated with a number of bioactivities. These metabolites exhibit a diverse array of biological activities such as antioxidant (Kalra et al., 2021a; Mandal et al., 2011), anticancer (Cardile et al., 2017), anti-inflammatory (Khader et al., 2018), analgesic (Okuyama et al., 1995), anti-microbial (Goel et al., 2021; Kosanic et al., 2013; Goel et al., 2011), neuroprotective activity (Fernández-Moriano et al., 2017); UV-protectant (Nguyen et al., 2013b), allelopathic (Goel et al., 2014; Kalra et al., 2021b), antiprotozoal and

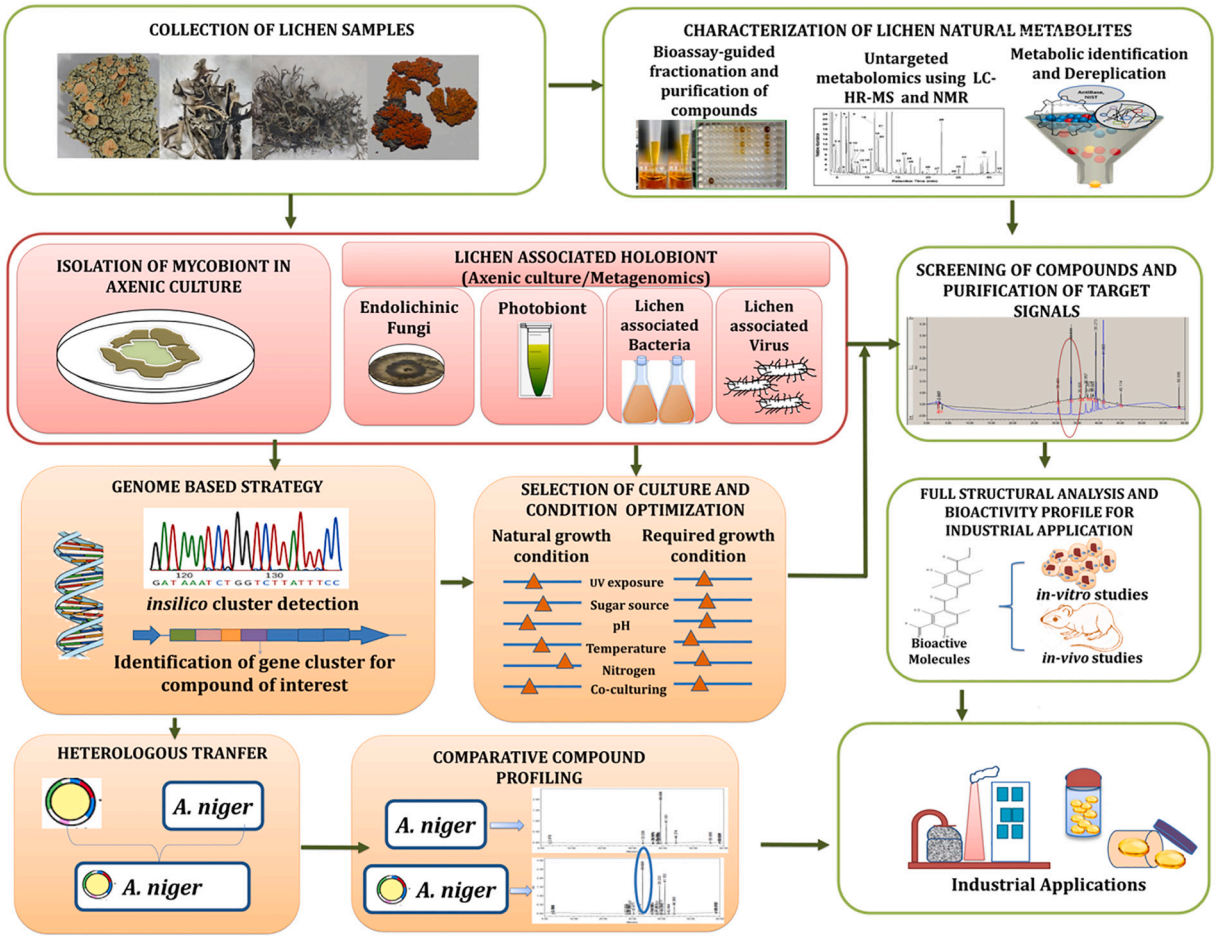

Fig 2. Integrated approaches and multiple strategies for the discovery of lichen secondary metabolites

insecticidal activity (Muhoro and Farkas, 2021). Only a handful of them have been scientifically validated and documented for pharmacological and agriculture use. A large fraction of these biologically active metabolites is likely to be unexplored due to slow growth rate of lichen. Their slow growing behaviour may limit the capacity for studying their metabolites and may make it challenging to extract these compounds in sufficient quantities. Moreover, obtaining a sustainable amount of the bioactive metabolites from these sources can be problematic.

Metabolites from natural sources have played a noteworthy role either as the foundation for new chemical entities introduced as medicinal drugs. The discovery of penicillin from the mould fungus *Penicillium notatum* (Fleming, 1928) drew the attention of drug discovery industries towards microbial sources and instigated the microbial drug era. A wide range of therapeutic applications coupled with unprecedented chemical diversity and potency towards biological activity are noted as the driving forces for considering microbes as a key source of novel bioactive molecules (Demain and Fang, 2000). However, developing resistance to existing antibiotics, chemotherapeutic agents and pesticides demand the need for novel bioactive molecules with useful properties to be sought (Lee et al., 2018). Due to the fast reproducibility of the bacterial sources, especially *Actinomycetales,* they have been the main focus of screening programmes to date (Genilloud, 2017). However, as eukaryotes, it is apparent that the mechanism of fungal metabolites can be conserved in higher eukaryotes and may respond similarly to mammalian system (Crittenden and Porter, 1991). Considering their capacity to produce a wide array of bioactive compounds with therapeutic significance, symbiotic microbes have a role in future drug discovery approaches. The microbiome interactions in a symbiotic system aid unique biochemistry and biomachinery that drive the production of bioactive secondary metabolites (Zhang et al., 2015). These symbiotic systems present an inherent complexity and can provide diverse bioactive metabolites. Above all, lichens are the most productive structure and as such demands further research attention.

Taking into consideration the significance of lichen metabolites and growing scientific interest in understanding the complex behaviour of lichen symbiosis, a rapid upsurge in the number of scientific studies addressing this research area has been observed in the last two decades (Fig. 1, Pubmed Search). This shows that lichen research is gaining interest and momentum, where this renewed scientific curiosity in lichen-derived metabolites is paralleled with major technological and scientific advances in relevant research fields including better understanding of the symbiosis mechanism, advanced screening, analytical methods and gene mining strategies. Thus, the assessment of the structural features of the bioactive metabolite, development of the strategies to focus on the detection of these metabolites and then linking the metabolic profile with genomic and metabolomics tools with culturing and synthetic strategies could open new pathways for the better utilization of the molecules. This review presents an array of basic and advanced technologies that are currently used to discover unexplored potential of lichen metabolites (Fig. 2) and discussed the advantages of each strategy in addressing the above-mentioned difficulties. The article aims at proposing an overview of the recent research in culturing of symbionts and genone mining methods. Furthermore, more traditional studies such as metabolomics studies and on isolation of lichen secondary metabolites have also been cited. The structure of the study is divided into major four sections; the first one presenting the lichen secondary metabolites, the second one describing the roles of metabolomics in detection of lichen metabolites. The third part focuses on the use of genome mining to discover lichen natural products. The last one deals with the production of lichen-associated holobiont's metabolites. This review focuses on the need of the integration of these valuable tools required for the unearthing of novel natural products from lichens.

2. Lichen secondary metabolites

The uniqueness of lichen metabolites made the study of their chemistry was of great interest to organic chemists. These metabolites are not only distinctive but occasionally are abundant, thus attracted the attention of scientific community and leads to evolution of "concept of lichen chemistry"(Shibata, 1974). Also, edible lichens that have been used as ethnic food in different societies possess multiple health benefits owing to various active substances from lichens (Zhao et al., 2021; Yusuf, 2020). The diversity of phenolic compounds isolated from lichens mainly belongs to three biosynthetic pathways, the polyketide, shikimic acid and mevalonic acid (Stocker-Wörgötter, 2008). Most of the metabolites are fungal originated phenolic polyketides and are structurally unique, with only a few of them are found in other fungi and higher plants. The core chemistry of these polyketide family compounds is relatively uniform across all described lichen species and mainly formed from the polyketide synthase (PKS) derived monoaromatic subunits, joined by various carbon-carbon, ester and/or ether linkages (Culberson and Elix, 1989). Additionally, these basic structures undergo various post-biosynthetic modifications to yield wide range of compounds (Elix et al., 1984; Nash, 1996). Depsides and depsidones are the most abundant classes of lichen metabolites (Ranković and Kosanić, 2015) along with dibenzofuran-like compound such as usnic acid (Millot et al., 2016). Importantly, some authors have described the presence of dibenzofurans such as porphyrillic acid from lichens (Erdtman and Wachtmeister, 1953). Although lichen metabolites are considered exclusively of fungal origin, the metabolic interaction between photobiont and mycobionts is found to be crucial for the production of these unique metabolites (Brunauer et al., 2007) since the mycobionts grown without a photobiont does not produce the same array of metabolites (Fazio et al., 2009). Beyond lichenized fungi, recent research has highlighted the presence of several other associated organisms such as endolichenic fungi (Singh et al., 2017), cyanolichen (Kaasalainen et al., 2012), epilichenic and bacteria associated with lichens (Lee et al., 2014; Parrot et al., 2015; Suzuki et al., 2016). These associated organisms further increase the profile of lichen chemical diversity but currently the impact of these associated organisms on the complete biochemical profile of a lichen species is unclear. An integrated approach utilising genomics and metabolomics is required to study theses complex systems. Further the uptake of advanced analytical techniques and next-generation computational tools brought the breakthrough in lichen chemistry and resulted in identification of various novel compounds.

3. Role of metabolomics in detection of lichen metabolites

Until recently, the identification of lichen and the discovery of their metabolites was largely an empirical process employed in natural product chemistry. However, in recent years, the advent of advanced analytical techniques and approaches have played a pivotal role in the chemotaxonomical identification of novel species, separation and structural analysis of more complex metabolites (Xu et al., 2017). A number of recent studies focussing on lichen chemistry highlighted the use of a range of hyphenated technology for profiling of lichen metabolites and thus their commercial value. Several examples are presented here to highlight the use of these advanced metabolomics and computational studies involving modern analytical techniques and data mining software in studying the chemotaxonomy and to search for novel bioactive molecules using untargeted metabolomics approaches.

3.1. Global metabolic profiling in chemotaxonomy

Mass spectrometry (MS) due to its sensitivity (Krug and Muller, 2014) and Nuclear Magnetic Resonance Spectroscopy (NMR) (Kim et al., 2010) coupled with chromatographic techniques has been recognized as key technologies to study the untargeted/global metabolomics. There are several recent studies which shows the power of MS or NMR in conjugation with chemometric analysis for specimen discrimination. The concept of modern lichenology is also engaging the idea of these advanced technologies in lichen chemotaxonomy (Xu et al., 2016; Xu

et al., 2017). Kai and his co-workers (Kai et al., 2017) highlighted the use of direct injection electron ionization–mass spectrometry (DI-EI-MS) in studying the chemical fingerprints of lichen species which are considered as a unique ID of a particular sample (Dettmer et al., 2007). Similarly, employing High performance Liquid chromatography-Diode Array detector-Mass (HPLC–DAD–MS) analysis and Electron spray Ionization-Mass HESI–MS–MS fragmentation patterns, a novel depside from the lichen *Everniopsis trulla* along with 32 known compounds were identified (Castro et al., 2017). This highlighted the advantage of using MS based metabolomics tools for rapid characterization of metabolites that can be employed in chemotaxonomy of this species along with indication of novel metabolites.

Also, Mittermeier et al., 2015, recognized NMR-based non-targeted metabolic profiling as a useful tool in the chemo-taxonomy of lichens and segregated different lichen samples up to species level on the basis of metabolite profile generated by NMR analysis. Beyond the chemo-taxonomical identification, the same approach could also facilitate the study of chemical behaviour of different lichen samples. For instance, by using this approach the group was able to find out that Ecuadorian lichens are a rich source of polar metabolites (Mittermeier et al., 2015).

3.2. Dereplication

Although developments in separation approaches along with data mining tools enable the possibility to study the architecture of natural products, the data generated is often very complex to interpret. As a result, dereplication *i.e.*, a process to identify and eliminate already known compounds at the early stage of the screening process, is a crucial step to accelerate novel natural products isolation process (Pérez-Victoria et al., 2016). As isolation of bioactive metabolites from a complex extract is considered as a time and resource consuming process rediscovering known metabolites is wastage of resources (Nielsen and Larsen, 2015). Also, the phenomenon known as the "cocktail effect" and decomposition of compounds during isolation pose a significant obstacle for the identification of responsible novel metabolites (Bills and Gloer, 2016). Blending of a dereplication strategy with advanced analytical and spectroscopic techniques help to develop novel multifaceted approaches that can guide the analysis towards the parent molecule responsible for the bioactivity.

A pioneering approach using soft ionization *via* fast atom bombardment and MS/MS to fully characterise a lichen crude extract was done by Holzmann and Leuckert (Holzmann and Leuckert, 1990). Musharraf, 2015 emphasized the importance of diagnostic structure-fragmentation relationships for the putative identification of compounds. In this study, negative ion mode electrospray quadrupole time-off fight mass spectrometry (ESI-Q-TOF-MS/MS) was used to study structure-fragmentation relationship of ten metabolites belonging to key classes of secondary metabolites (Musharraf, 2015). Similarly, a sensitive and rapid method using ultra high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QQQ-MS) instrument was developed for efficient separation of lichen standards in less than six minutes. More recently, Salgado et al, studied the phytochemistry of *Usnea* species using ultrahigh resolution liquid chromatography orbitrap MS analysis (UHPLC-ESI-OT-MS-MS) and identified total of 86 metabolites, out of which 76 were identified for the first time in *Usnea* species and 13 metabolites were considered as novel as they were not identified previously (Salgado et al., 2017).

Dereplication based strategies has also been employed to study metabolites obtained from endolichenic fungi. Endolichenic fungi is a group of asymptomatic fungi that is similar to plant endophytes and discovered flourishing inside healthy lichen thalli. In a recent study, diverse species of endolichenic fungi has been reported from lichen *Usnea.* Further, a dereplication based study using LC–MS-based metabolomics on these endolichenic fungi led to identification of five antibacterial metabolites from *Xylaria venustula*. Compared to all metabolites, methyl xylariate C was found to be the most significant for its antibacterial activity (Santiago et al., 2021).

To facilitate dereplication in lichen chemistry, an open access MS/MS based library with 250 metabolites known as the Lichen DataBase (LDB) was published by Olivier-Jimenez and team. To aid this area of research the MetaboLights database was generated (https://www.ebi.ac.uk/metabolights/MTBLS999) which contains the MS spectra of metabolites attained from a range of collision energies. Complementing this the GNPS platform (CCMSLIB00004751209 to CCMSLIB00004751517) contains the merged spectra of these metabolites within a metadata file. Such fundamental database empower research on lichen chemistry by reorganization and prioritizing the novel metabolites (Olivier-Jimenez et al., 2019).

3.3. Computational approaches to assist data-mining in metabolomics

In silico approaches in natural product discovery have mainly focussed on two major tasks: dereplication and bioactivity prediction. Recent progress in chemical databases such as PubChem, AntiMarin, ChemSpider, ChemBank, ChEMBL, Dictionary of Natural Products, KEGG, mzCloud and Norine has seen a step forward in drug discovery from natural sources. In an integrated approach studying spatial molecular organization of the composite microbial assemblages present in *Peltigera* lichen, Garg *et al.,* employed matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) imaging mass spectrometry and orthogonal tandem MS (MS/MS)-based molecular networking techniques to characterize the spatial distribution of compounds and annotate microbial chemistry with their biological origin respectively. Mass spectrometry-based techniques complemented with metagenomic sequencing data assist to describe the taxonomic composition and link compounds with their biosynthetic gene clusters. This type of multi-pronged strategies helps to identify various novel metabolites and also increase the understanding to study a complex biological system (Garg et al., 2016).Similarly, MS/MS-based molecular networking and extensive spectroscopic analyzes involving GIAO (Gauge-Independent Atomic Orbital) NMR shift calculation and biosynthetic gene clusters (BGCs) analyzes led to isolation and identification of novel bianthrones (quinoid pigments) from the lichen *Nephroma laevigatum* (Lagarde et al., 2021).

All these strategies discussed rely on the detection of metabolites from environmental lichen sample collected directly from wild location. However, to fully explore the chemistry of lichens, detection of the lichen holobiont: distinct axenically and/or co-culture is needed. Thus, to highlight the metabolic capacity of lichen, the chemistry of all the symbiotic partners cultivable in laboratory conditions needs to be fully explored.

4. Genome mining for the discovery of lichen natural products

In addition to the direct detection of lichen metabolites, a genome mining strategy is the pragmatic approach that assists in the detection of the metabolites and exposed the potential of biosynthetic pathways for the production of these secondary metabolites (Ziemert et al., 2016). These gene clusters are known to regulate all the genes required in a biosynthetic pathway such as biosynthesis of precursor, assemblage of the compound scaffold, tailoring of the compound scaffold and transport of substrates and/or products (Keller et al., 2005). An increasing understanding of high-quality genome sequencing and genome mining techniques coupled with the introduction of powerful computational toolkits facilitates the process of connecting these gene clusters with key compounds (Li et al., 2016).

One of the unique classes of metabolites produced by lichens are polyketides and are synthesised from a small collection of polyketide synthase (PKS) derived monoaromatic subunits (Calcott et al., 2018). The majority of lichen metabolites have not had their biosynthetic mechanism elucidated or linked to BGCs and can be referred as orphan metabolites. Understanding the genetic components leading to the biosynthesis of these metabolites provides an opportunity to exploit

their commercial utilization by employing synthetic biology approach. While classical genetic approach helps to connect many biosynthetic genes with a known molecule, new millennium genetic studies also helps to discover the unexplored potential hidden in fungal genome (Brakhage and Schroeckh, 2011). In non-lichenized fungi, various approaches such as gene knockout, heterologous expression experiments, awakening of silent gene clusters or CRISPR/Cas-based genome engineering to repurpose this information to construct artificial gene cluster are routinely applied to establish a definitive gene-metabolite link (Scharf and Brakhage, 2013). The following section of this review will discuss the various challenges in the genome mining strategy of lichen metabolites, genetic make-up of lichen metabolites, approaches to identify key gene clusters and related metabolites which can be applied for future research.

4.1. Challenges in genome sequencing studies of lichens

Lichens and their axenic mycobiont cultures are extremely slow growing, having very low metabolic turnover, are not genetically tractable, and thus are under-characterized in genome sequencing studies (Stocker-Wörgötter, 2008; Stocker-Wörgötter, 2015). These features present unique challenges to gaining a true insight into the biosynthetic system as a number of techniques that are routinely applied to other organisms are not applicable in lichen symbionts specifically mycobionts. Significant efforts have been put together to elucidate the biosynthetic pathway underpinning of metabolites present in lichens by employing axenic culture techniques however as lichen is comprised of an obligate symbiont, it is difficult to isolate and maintain axenic cultures (Honegger, 1993; Spribille et al., 2016). There are some studies which highlighted the presence of depsides and depsidones produced by mycobionts grown axenically under appropriate conditions, but most of the time these cultures are chemically silent or incapable of replicating parent metabolic profiles (McEvoy et al., 2007). Taking into account the challenges involved in lichen culturing most of the work on the lichen genomics has relied upon the environmental samples (metagenomic DNA) (Meiser et al., 2017). Nonetheless, proper sampling and advanced culturing techniques may result in the isolation of a pure culture and consideration of various factors that may affect the consistency of genomic expressions will aid this approach. To better utilize of these strategies, it is important to have a detailed understanding of their genetic makeup.

4.2. Identifying biosynthetic gene clusters in genome sequences

4.2.1. In-silico approaches

Investigating the gene clusters involved in the biosynthesis of secondary metabolites not only reveals the expression of clusters that are only present in lichens but also gives an indication of silent clusters whose products are unknown because they are not normally expressed in laboratory conditions (Gerke et al., 2012). The latest sequencing technologies complemented with the advances of computational tools and genomic research is highly informative to understanding the biosynthetic gene clusters (Blin et al., 2019; Zarins-Tutt et al., 2016). Identification of gene clusters was performed by analyzing the list of genes encoded up- and downstream as a query sequence using software like BLAST or PSI-BLAST and HMMer. These computational tools can be divided into two categories: high-confidence/low-novelty and low confidence/high-novelty. Software such as antiSMASH (Medema et al., 2011), NP.searcher (Li et al., 2009), SMURF, CLUSTSCAN (Starcevic et al., 2008) and SBSPKS (Anand et al., 2010) use rule-based approaches, *i.e.,* the specific search for recognized enzymes or enzymatic domains and thus falls under the category of high-confidence/low-novelty computational tool (Weber and Kim, 2016). Importantly, gene cluster coded for canonical pathways such as PKS and NRPS are captured using tools such as SMURF and CLUSTSCAN. AntiSMASH is the widely used and continuously improving tool among this category and currently exists in its third version having 44 known classes of biosynthetic gene clusters of secondary metabolites. The implementation of *in silico* screening tools such as AntiSMASH for prediction of PKS genes and BLAST2GO program for the prediction of the polyketide biosynthesis cluster gene was discussed in nine lichen-forming fungi genomes. This bioinformatic investigation resulted in the prediction of 15 metabolites unique to the lichens and their findings indicated the presence of emericellin as the most widely produced metabolite in these lichens (Erken et al., 2021). Similarly, PRISM was also introduced with novel algorithms capable of predicting type II polyketides, starter units and deoxygenated sugars together with type I polyketides making it a comprehensive genome-guided chemical structure prediction engine (Skinnider et al., 2015).

Computational tools based upon low confidence/high-novelty algorithms also known as rule-independent methods are more efficient in predicting gene clusters from unknown classes of enzymatic domain. These genes may encode for molecule with entirely new chemical scaffolds and thus requires more sophisticated algorithm approach. Computational tools falling under this category follow motif- independent protocols and include, MIDDAS-M, MIPS-CG, ClusterFinder and EvoMining (Cruz-Morales et al., 2016). For instance, MIPS-CG employs the principle of comparing the DNA sequence for similar order of genes in two genomes. It has also been found that these algorithms sometimes fail to detect important information for example genes interweaved in superclusters (Wiemann and Keller, 2014).

4.2.2. Phylogenetic approach

Amplification of PKS gene fragments from genomic DNA templates using degenerate primers is the most common method used for determining PKS genes in lichenizing fungi (Miao et al., 2001). Specific PKS phylogenetic clades or functionalities can be targeted using degenerate primers (Nicholson et al., 2001). Homologous genes present in a similar fungal species or genera can be linked using their phylogenetic profiles which interpret the patterns of presence or absence across a set of genomes (Zheng et al., 2002). These comparative studies then can be utilized to discover gene cluster responsible for the synthesis of specific bioactive metabolites (Adamek et al., 2019). Further, phylogenetic assessment of PKS genes from environmental symbionts helps in identification of genes specific to symbiosis, for instance Schmitt et al. (2005) discussed a novel fungal PKS clade which is seemingly unique to fungi living in lichen symbioses (Schmitt et al., 2005). Likewise investigating only fungal keto synthase (KS) phylogenies also allows predictions of the polyketide structure (Kroken et al., 2003). Application of simple PCR screening techniques for investigation of KS domains and their associated gene clusters and their function is useful particularly in complex metagenomic DNAs and non-cultivable organisms such as symbionts like lichen and environmental samples (Piel et al., 2004). Calchera et al. employed similar approach of comparative genomics and genome mining to predict the biosynthetic gene clusters along with the regulators in the genomes of two lichen-forming fungi *i.e., Evernia prunastri* and *Pseudevernia furfuracea* (Calchera et al., 2019).

Similar work has also been employed to study the lichen associated bacterial community to correlate the production of novel metabolite. Whole genome sequences of two new *Amycolatopsis* strains (CA-126428 and CA-128772) isolated from tropical lichens has been compared with 41 publicly available *Amycolatopsis* genomes. Beyond providing taxonomic position of these strains on the basis of the different phylogenetic localisation, this study also allowed the assessment of the potential role of lichen associated bacteria and their contribution to biosynthetic pathways in the secondary metabolism of lichens (Sanchez-Hidalgo et al., 2018).

4.2.3. Mass spectrometry guided gene mining

Kersten *et al.,* has introduced the concept of MS based approach to link up chemotypes of peptide natural products with their biosynthetic gene clusters (Kersten et al., 2011). This novel strategy is defined as a

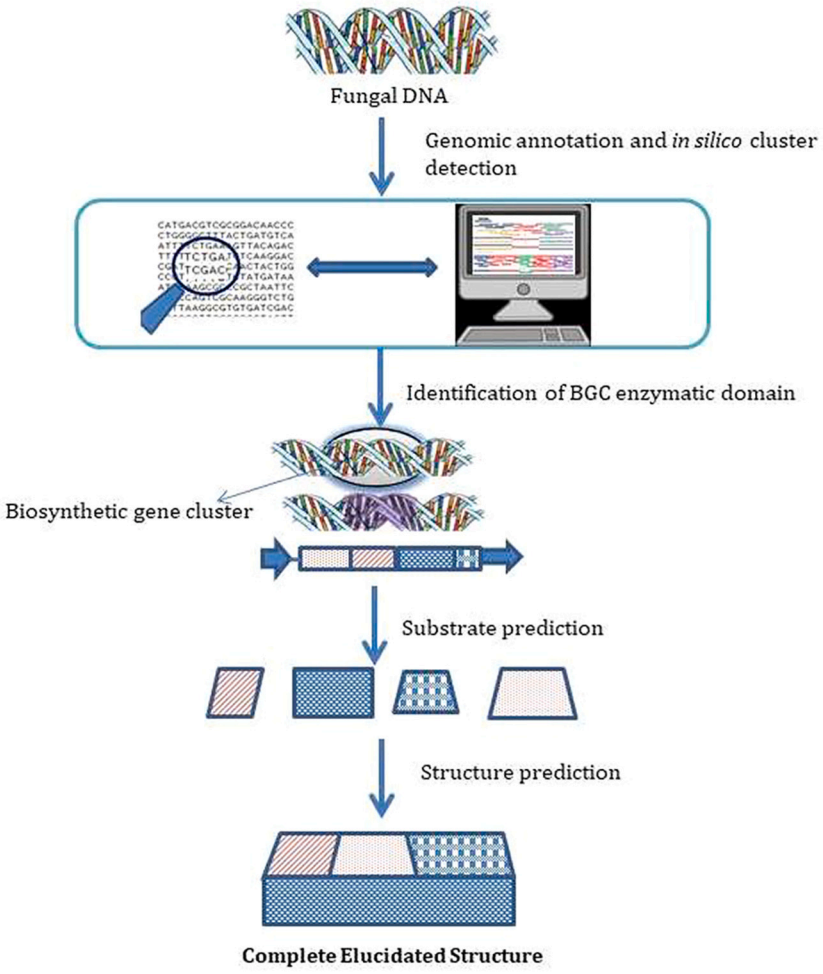

Fig 3. Metabologenomics based approach for the prediction of lichen secondary metabolites based upon identified biosynthetic gene cluster

natural product peptidogenomics (NPP) approach and helps to link the genomics-based structures obtained from biosynthetic logic with their *de novo* tandem MS (MSn) structures using an iteratively matching method. Following this Nguyen and his co-workers also discussed the concept of integration of the metabolomics profile generated with the help of mass spectrometry with the gene clusters responsible for the production of secondary metabolites. They also suggested the characterisation of molecular families (MFs) and gene cluster families (GCFs) which consequently empowers the molecular analysis of unsequenced organisms. Using this strategy, genomes from a structurally related organism can be used to connect these MFs with their corresponding GCFs (Nguyen et al., 2013a). A software tool named MetaMiner has discovered 31 known and seven unknowns RiPPs from diverse microbial communities using this approach that also includes lichen microbiome (Cao et al., 2019).

4.3. Gene to chemistry

Subsequent to identification of BGCs that encrypt the enzymatic pathways for compound production, the next key step in natural product bioinformatics is the prediction of their substrate and corresponding product. Success of the genome-guided natural product discovery is performed with methodologies to translate BGC into chemical information. With BGCs in hand, structure prediction such as the class of compounds they encode and, in some cases, the precise structures can be performed more efficiently by employing the combination of experimental characterization and computational approach (Helfrich et al., 2014; Jensen, 2016). Numerous algorithms have been developed to predict and identify the specific substrate binding sites such as NRPS adenylation domains and PKS acyltransferase domains in gene cluster. Algorithms such as NRPS-PKS (Ansari et al., 2004), NP.searcher (Li et al., 2009), ASMPKS (Tae et al., 2007), antiSMASH (Blin et al., 2019; Medema et al., 2011), SEARCHPKS (Yadav et al., 2003), suggest the structural features by predicting the individual monomers and then combining these different precursor units to give a rough idea of the core scaffold of the molecule of interest (Fig. 3). PRISM implemented novel algorithms, which improves the shortcoming associated with other methods accounted for the uncertainty inherent in biological systems and extend the precision of structure predictions (Skinnider et al., 2015).

Further, to expedite the process of organized deposition and retrieval of data on biosynthetic gene clusters, Medema and Fischbach (2015) has presented the minimum information required for Biosynthetic Gene cluster (MIBiG) data standard (Medema and Fischbach, 2015). Recently, (Kim et al., 2021) using the Big-SCAPE program and MIBiG database for identification of highly syntenic BGCs found exclusively in atranorin producing lichens. The heterologous expression of identified BGC known as as atr1 (putative atranorin PKS gene) produced a precursor compound known as 4-*O*-demethylbarbatic acid and suggested an intermolecular crosslinking activity for atranorin synthesis. The group later introduced several tailored enzymes into the heterologous host which then yielded atranorin.

Table 1
Few examples of effects of media composition and growth condition on synthesis of secondary metabolites in Lichen.

Lichen	Fungi	Media condition	Compounds identified	Altered media condition	Novel compounds identified	References
Cetrelia sp.	*Aspergillus* sp.	Starch as C source	Chlorodiaporthin analogues	Dextrose and Starch as C sources	Isocoumarindole A	Chen et al., 2019
Cladonia sp.	*Apiospora montagnei*	Solid rice media	Diterpenoid, pyridine alkaloid, xanthone derivatives	Addition of Na, Cl, N in media	(E, E)-4- hydroxymethyl-4,6-octadien-2,3-diol and lachnellin B; 6-O-demethylbostrycin and bostrycin	Wang et al., 2017
Cetraria islandica	*Myxotrichum* sp.	Starch and dextrose both as C sources	Austdiol, citromycetin and fulvic acid	Only starch as C source	Myxotritones A-C, 7, 8-dihydro-7R, 8S-dihydroxy-3, 7-dimethyl-2-benzopyran-6-one	Yuan et al., 2016
Everniastrum sp.	*Nodulisporium* sp.	Starch as sole C source	Nodulisporisteroids A and B, demethoxyvirdin	Starch and dextrose both as C sources	Nodulisporivirdins A-H	Zhao et al., 2015
Everniastrum sp.	*Ulocladium* sp.	Starch as C source	Polyketides	Presence of Na, K, S, Mg, Cl and Fe with Sucrose as C source	Mixed terpenoids – Tricycloalternarenes F-H	Wang et al., 2013a
Everniastrum sp.	*Ulocladium* sp.	Starch as C source	Polyketides	Dextrose and starch both as C sources	Sesterterpenes – Ophiobolins P-T	Wang et al., 2013b
Heterodea muelleri		[Sabouraud-2%–Glucose–Agar 25° C	Diffractaic and barbatic acid	4° C and exposure to UV-C	60x increase in barbatic acid amount	Hager et al., 2008
Lecanora rupicola		Mannitol as sole C source	Sordidone, eugenitol	Sucrose, dextrose and starch as C sources	Atypical novel substances, Absence of eugenitol and sorbitol	Brunauer et al., 2006
Lobaria spathula (mycobiont)	*nutrient medium*	In association with photobiont	Low quantities of methyl orsellinate, lecanoric acid and gyrophoric acid	Axenic culture	Methyl orsellinate, lecanoric acid, gyrophoric acids, thelephoric acid, terphenylquinone	Stocker-Wörgötter and Elix, 2002
Usnea orientalis (mycobiont)		Malt-yeast extract	Usnic and salazinic acid	Addition of mannitol and sorbitol	large quantity of extracellular dibenzofurans, hypostrepsilic and isostrepsilic acids	Kon et al., 1997

4.4. Knock-out of target genes

After identification of gene cluster for compounds of interest, the process of gene knock out provides a means to characterize its products by stopping or reducing its expression. In the studies, selective induction of silent fungal biosynthesis genes as a result of fungal-bacterial interactions is typically monitored. With the help of gene knockout studies, it has been observed that cryptic PKS gene codes for the polyketide synthase (PKS) required for the biosynthesis of the representative polyketide orsellinic acid, the cathepsin K inhibitors F-9775A and F-9775B and the typical lichen metabolite lecanoric acid (Schroeckh et al., 2009).

4.5. Heterologous host transfer

Heterologous expression of biosynthetic gene clusters in a non-natural host or model system expedites natural product discovery, elucidation, and mass production. Heterologous gene expression helps in ascertaining the significance of individual enzymes or gene cluster in certain metabolite production and thus provides a link between a cryptic cluster and secondary metabolite product (Schmidt-Dannert, 2015).

Aspergillus nidulans and *Neurospora crassa*, are the experimentally well-developed strains, and are considered as the potential hosts for the expression of lichen DNA. It has also been recognised that *A. nidulans* as being a native polyketide producer can use lichen promoters and correctly splice out introns in lichen genes thus considered as a useful host for the heterologous expression of lichen genes (Miao et al., 2001). Sinnemann et al. (2000) has also confirmed the possibility of using *A. nidulans*as as a preferable host for studying lichen genes by complementing a lichen pyrG gene in a mutant host (Sinnemann et al., 2000). Similarly, attempts were made to elucidate the cloning and sequence characterization of a non-reducing polyketide synthase gene (NRPKS) from the lichen *Xanthoparmelia semiviridis* (Chooi et al., 2008). Similarly, some more recent attempts succeeding to pave the way toward the utility of these techniques in exploring lichen genomics for sustainable supply of their specialized metabolites

5. Detection and/or identification of lichen associated holobiont's metabolites

Holobiont is a term coined in 1991 by Lynn Margulis to define the theory of symbiosis linked to miniature ecosystems. It includes the host; together with associated communities of microorganisms linked in a symbiotic assemblage that contributes towards the function of whole system (Margulis and Fester, 1991). Lichens are ideal systems to study the underlying mechanisms at the community level particularly in term of biochemical interactions and contribution of each partner toward the production of secondary metabolites. Recent development of molecular and visualizing techniques in lichenological research revealed the existence of highly diversified microbiota in lichen symbiosis (Grube et al., 2015; Hodkinson and Lutzoni, 2009; Sigurbjörnsdóttir et al., 2015; Spribille et al., 2016). These findings allow all researchers to better study and decipher the importance of each partner towards biochemical complexity and functional networks that persist under this complex system. The photobionts (most frequently green algae) produce the primary source of carbohydrates (e.g. ribitol, erythritol, mannitol by algae; mostly glucose by cyanobacteria), which are used further during primary and secondary metabolic ways in fungi (Elix and Stocker-Wörgötter, 2008). The next section of the review will highlight the contribution of each partner towards the production of potential lichen secondary metabolites.

5.1. Mycobiont- lichenized fungi

Mycobionts contribute toward the bulk of the lichen thallus and approximately (98%) these mycobionts are identified as an ascomycete fungus except for a few cases (0.3%) identified as basidiomycete fungi (Zambare and Christopher, 2012). The vast majority of the metabolites originating from lichens to date are fungal in origin in the symbiotic or aposymbiotic state (Ranković and Kosanić, 2015). However, unless otherwise specified, lichen metabolites when studied as a whole thallus are mostly considered as metabolites having fungal origin (Elix et al., 1984). One of the approaches that has recently gained momentum aiming at a sustainable harvestable source is the aposymbiotic axenic

culture of mycobionts (Stocker-Wörgötter, 2008; Yamamoto et al., 1987). Although, axenic culture of lichen mycobionts were found to be a source of novel lichen metabolites (Hamada and Ueno, 1990; Miyagawa et al., 1994; Takenaka et al., 2003), obtaining mycobiont with high purity is a challenging task. However, the advancement of molecular techniques allows for the identification of lichen fungus in culture and in native lichen using DNA analysis. Comparison of the DNA obtained from the isolated and native environmental samples confirms the identity of isolated mycobiont as the lichenized fungi.

It has been reported that there are a number of factors such as collection season, storage periods and storage temperatures contributing to the frequency of spore discharge from the apothecia (Yamamoto et al., 1998). Further the rate of spore germination varies a lot among different species (Pyatt, 1969), lichen type (Mathey and Hoder, 1979), media composition and growth condition (Sangvichien et al., 2011). A high level of success has been achieved in the culturing and production of secondary metabolites in axenic culture by different research group using modification of growth condition, improved media composition and availability of carbon and nitrogen sources and/or co-culturing containing photobiont and mycobiont (Table 1) (Brunauer et al., 2006; Hager et al., 2008). The variable growth conditions used include exposure to high light intensities of UV-C, temperature stress (Hager et al., 2008), desiccation of the medium (Stocker-Wörgötter and Elix, 2002) and osmotic stress (Kon et al., 1997) all having shown promising results for the production of lichen metabolites in culture that are representative of those found in natural lichens. There are also metabolic pathways that are normally suppressed in the lichenized form can expressed by isolation and cultivation of lichen mycobiont in appropriate media (Brunauer et al., 2007; Takenaka et al., 2000). Detailed studies of the appropriate culture conditions for mycobiont growth have led researchers to make the important conclusion that environmental factors, together with physiological stress, work as a prerequisite for the induction of secondary metabolic pathways and consequently their production in laboratory culture (Hamada and Ueno, 1990; Stocker-Wörgötter, 2001).

To investigate the functionality and effect of concentration and type of the carbon source on the fungal biomass yield, development stages and metabolites profiling, aposymbiotic mycobiont of *Caloplaca erythrantha* have been investigated. Results of the studies indicate mannitol as an efficient carbon sources than sucrose to increase the yield of emodin and 7-chloroemodin particularly from *Caloplaca erythrantha* (Fazio et al., 2012). In a similar study to highlight the effect of stress conditions on metabolite production, mycobiont was isolated from a thallus of *Parmotrema reticulatum* and cultured axenically on different media and desiccation treatments. Colonies over the age of 5 and 10 months on solid Lilly & Barnett (LB) medium were able to produce atranorin, a major cortical lichen depside. This study also revealed the fact that mycobionts switch to yield fatty acids and acylglycerides when the biosynthetic pathway for the production of lichen phenolics is not initiated (Fazio et al., 2009).

Several studies have emphasized the effect of culture conditions and media composition on the mycobiont biomass yield, secondary metabolite profiling, metabolite concentration and expression of PKS genes in culture. In a recent study, conducted on *Ramalina dilacerata* by Timsina and colleague looked at the effect of different growth media at four different pH levels in mycobiont culture was correlated with colony growth and expression of two PKS genes (non-reducing (NR) and 6-MSAS-type PKS). A negative correlation was observed between number of secondary metabolites and colony growth. Also, expression of both genes was found to be affected by pH level as pH 6.5 with glucose malt agar was found to be favourable for expression of NR PKS gene, alternatively, pH 8.5 with malt agar was found to be suitable for expression of the 6-MSAS-type PKS gene (Timsina et al., 2013). Recently, Jeong et al., studied the effects of six carbon sources and light on metabolite biosynthesis in the *C. metacorallifera* mycobiont (Jeong et al., 2021). The finding highlighted the production of red pigments in fructose-supplemented medium under fluorescent light conditions. Similarly, several other studies have discussed the effect of carbon source, vitamins and micronutrients on mycobiont growth(Hariharan et al., 2016). Along with effect of carbon and nitrogen sources, effect of light/dark cycle and temperature on growth of lichen mycobionts and metabolite production has also been observed (Gogoi et al., 2008; Molina et al., 2015; Ramos and Said, 2011).

5.2. Cyanolichen photobiont

Cyanobacteria have been identified as a promising source of novel bioactive secondary metabolites which is further supported by the presence of high numbers of biosynthetic clusters in their genome (Tan, 2007). Several reports of the presence of NRPS/PKS gene clusters in the cyanobacterial genome highlighted its potential for the discovery of new natural products. Among various other cyanobacteria, Nostocales is one of the order noted to be a rich source of NRPS and PKS gene clusters and also identified as the most common cyanobiont in lichen (Kampa et al., 2013). It has been suggested by numerous studies including those focussed on lichen that secondary metabolites produced by symbiotic bacteria are unique when compared to their non-symbiotic counter-part (Helfrich et al., 2014; Kaasalainen et al., 2012). As research into the axenic culture of lichen symbionts is in its juvenile stage, only very few studies have investigating lichen cyanobiont as a source of secondary metabolites (Calcott et al., 2018).

Implementation of metagenomic and LC/MS/MS based approach to study Nostoc *sp.*, a lichenized cyanobacterium isolated from *Peltigera membranacea* led to identification of nosperin, the first pederin scaffold and microcystins (Kaasalainen et al., 2009; Kampa et al., 2013). These metabolites have been reported for various bioactivities including anticancer (Kampa et al., 2013)

5.3. Endolichenic fungi

Along with the mycobiont and other known fungal associates of lichens, lichen holobionts harbor several *Ascomycota* microfungi (primarily *Pezizomycotina* or *Euascomycetes*) that also inhabit the lichen thallus asymptomatically. These fungi are known as endolichenic fungi (ELF) (Suryanarayanan and Thirunavukkarasu, 2017). Analogous to endophytic fungi, these ELF are recognised as an excellent reserve of structurally novel and biologically active compounds (Agrawal et al., 2020). According to Singh et al., 2017, researchers have still explored only 2% of the chemical diversity encompassed by ELF biome which might be considered merely the “tip of the iceberg” hence, considerable attention is needed to explore the hidden potential of these microbiome (Singh et al., 2017).

It is important to note that most of the studies to date have focussed on the bioactivity of these ELF extracts but endolichenic fungal chemistry is still understudied. To illustrate this, Cheon et al. (2013) investigated 571 endolichenic fungi for their antifungal properties against *Candida albicans*. Several active fractions were obtained as a lead for their antifungal property; though, metabolites responsible for the activity have not been explored fully (Cheon et al., 2013). The vast majority of metabolites obtained from ELF belongs to a broad range of chemical classes such as steroids, terpenoids, quinones, xanthones, peptides and chromenones. Further, metabolites with novel skeletons such as phaeosphaerins A–F (Li et al., 2012), pericoterpenoid A (Wu et al., 2015), nodulisporiviridins A–H (Zhao et al., 2015), coniothiepinols A and B (Wang et al., 2010a), 6-hydroxy-8-methoxy-3a-methyl-3a,9b-dihydro-3Hfuro[3,2-c]isochromene-2,5-dione (Wang et al., 2012), and conioxepinols A–D (Wang et al., 2010b) have also been reported from ELF. Substantiate evidence showed the presence of novel biosynthetic pathways present in ELF is proficient for producing novel bioactive products. One of the ultimate perks in exploring microbial resources such as ELF or similar other sources of natural product are the degree of biosynthetic plasticity, they possess in providing novel

metabolite (Kellogg and Raja, 2016).

Depending upon the triggering of defence reaction and colonization of environment, microorganisms modulate the synthesis of their metabolites. This principle commonly termed as "one strain, many compounds" (OSMAC) approach and lately draw attention to the fact that change in growth environment can completely modulate the profiling of metabolites under laboratory conditions (Singh et al., 2017). The strategy employing OSMAC approach has also been implemented on research targeting ELF metabolites and lichen-associated bacteria (discussed in Section 5.4). In a recent study employing OSMAC strategy in *Myxotrichum* sp., which is an ELF obtained from lichen *Cetraria islandica* led to identification of new metabolites myxotritone A-C along with a benzopyran (Yuan et al., 2016). A congruent approach of the OSMAC strategy in ELF culturing and gene mining strategy together can help in undermining the complete genetic profile and/or silent gene clusters whose production can be improvised in specific laboratory condition.

5.4. Lichen-associated bacteria

Implementation of novel "omics" techniques in order to better understand lichen symbiosis has revealed the presence of various bacterial communities other than cyanobionts in lichens (Cardinale et al., 2008; Grube et al., 2015). The presence of these bacterial populations specifically, Alphaproteobacteria are believed to be responsible for their functional contribution in lichen symbiosis. Several attempts at exploring lichen associated bacteria in order to obtain the bioactive compounds from bacterial origin have resulted in varying degrees of success. Except for *Alphaproteobacteria*, the most common bacterial communities associated with lichens belong to phyla *Actinobacteria, Proteobacteria* and *Firmicutes*. The effects of environmental factors (extrinsic parameters) and chemical composition of their host lichen (intrinsic parameters) on the diversity and abundance of these bacterial communities has also been observed (Parrot et al., 2012). Grube and his co-workers revealed the presence of approximately 800 bacterial species associated with lichen *Lobaria pulmonaria*. With the help of metagenomic studies, group tried to determine the significance of these bacterial communities in sustaining the symbiosis (Grube et al., 2015). Similarly, some other bacterial species namely *Firmicutes, Actinobacteria, Betaproteobacteria, Gammaproteobacteria* and *Deltaproteobacteria* have also been found to be associated with lichens and are known to be a prolific producer of specialized metabolites (Bjelland et al., 2010; Hodkinson et al., 2012).

Recently, (MALDI-TOF) imaging mass spectrometry technique was used to characterize the distributions of metabolites within the microbial community present in *Peltigera* lichen. With the help of this MS based network, it was concluded that 15.5% of the detected compounds are fungal in origin whereas 17.8 % are bacterial originated which have not been explored yet (Garg et al., 2016). Similarly, Gonzalez *et al.,* isolated total 337 bacterial isolates from lichen species and some of these are rare genera that could only be obtained from lichen specimens. High detection level of PKS-I, PKS-II and NRPS systems in their genome indicate the biosynthetic potential of these bacterial group and thus supports the perception emphasizing these bacterial communities as a source of bioactive compounds (González et al., 2005). In a similar attempt, Parrot and co-workers have isolated 51 Actinobacteria associated with two marine lichens *viz. Lichina confinis* and *L. pygmaea;* one littoral lichen *viz. Roccella fuciformis*; and the terrestrial lichen *Collema auriforme* and explored them for probable bioactive metabolites production. Actinobacteria are mostly considered as the reservoirs of microbially derived bioactive molecules. The actinobacterial families obtained includes *Micrococcaceae, Brevibacteriaceae, Mycobacteriaceae, Cellulomonadaceae, Sanguibacteraceae, Gordoniaceae, Nocardioidaceae, Pseudonocardiaceae, Promicromonosporaceaen* and *Streptomycetaceae.* Screening of genes coding for polyketide synthases types I and II further highlighted the littoral lichens are a source of diverse potentially bioactive Actinobacteria (Parrot et al., 2015). Similarly, Parrot et al., have isolated novel metabolites from two actinobacterial strains. Furthermore, two compounds cyaneodimycin and cyaneomycin containing rare methacrylate moieties were obtained from *Streptomyces cyaneofuscatus*, a bacterial culture isolated from marine lichen *Lichina confinis.* Along with these compounds, this was the foremost report of unpredictably obtaining lichen specific compound "usnic acid" from bacterial species. Importantly the authors suggest a thought-provoking possibility of horizontal gene transfer among lichen symbionts (Parrot et al., 2016b).

Actinobacterium *Nocardia ignorata* obtained from the lichen *Collema auriforme* yielded six known metabolites including indolecarboxaldehyde and two new brominated diketopiperazines (Noël et al., 2017). Recently, bioactive non-ribosomal peptides categorized under skyllamycin class of peptides were isolated from *Streptomyces* sp. KY11784 obtained from a sample of the New Zealand lichen *Pseudocyphellaria dissimilis*. The potential of this strain to yield skyllamycins was recognised on the basis of whole genome sequencing and biosynthetic gene cluster genetic analysis coupled with GNPS LCMS/MS molecular networking. Two new compounds skyllamycins D and E were obtained along with the previously reported skyllamycins A–C. skyllamycins D was found to have enhanced activity against *B. subtilis* E168 as compared to previous skyllamycins (Bracegirdle et al., 2021). Similarly, lichen-associated *Amycolatopsis sp*. YIM 130642 yielded 11 metabolites known as amycophthalazinone A (phthalazinone derivative), 7-O-methyl-5-O-α-L-rhamnopyranosylgenestein (isoflavonoid glycoside), and 7-O-α-D-arabinofuranosyl daidzein (isoflavonoid glycoside) with 8 known metabolites (Zheng et al., 2019).

Several other reports have discussed bioactive metabolites obtained from actinomycetes associated with lichens. Few examples include, coumabiocins (aminocoumarins), uncialamycin (an enediyne), 2′-O-demethylherbicidin F, 9′-deoxy-8′-oxoherbicidin B, 9′-deoxy8′,8′-dihydroxyherbicidin B, 8′-epimer of herbicidin B (herbicidin congeners), antipain, lichostatinal, JBIR-88 (angucycline), JBIR-89 (butenolide) (Motohashi et al., 2010; Parrot et al., 2016a).

As discussed above in Section 3.3, OSMAC strategy has also been employed to study the effect of different culture conditions on secondary metabolites composition. Noel et al., 2020 have studied lichen-associated *Actinobacterium*, *Nocardia sp* that demonstrated interesting cytotoxic activities. Findings of their study validated the effect of different culture conditions on the chemical profiling and cytotoxic activity of different extracts (Noël et al., 2020).

Metabolites obtained from these bacterial associates show significant low structural overlap with metabolites from free living bacterial colonies. With no doubt, exploring these communities as a source novel scaffold further add the diversity of molecules obtained from lichens.

6. Critical insights

Although the historical approach of natural product discovery is considered suitable and dedicated process to get an insight of natural products but inadequate productivity exacerbated by substantial resources requirement has led to de-emphasis of this approach and focus has been placed toward a more technology based processes.

Furthermore, in case of metabolomics based approach, more advanced, dedicated and enrich databases are required and sharing MS/MS data dedicated for lichen compounds should be encouraged. Significant advances are needed in terms of enrichment of database for lichen metabolites together with the standardization of generation of data. Standardization of MS databases is a crucial step to get maximum benefits from these databases. Variability of data is mainly based upon the method of generating the data and type of analytical tool used in the MS analysis. For the purpose of data to be utilizing judiciously these methods need to be standardised congruently. In addition, development of bioinformatics and computation tools are much needed.

Genomic strategies dealing with transcription of a gene cluster involving insertion, deletion or mutation of DNA sequence is one of the

Table 2
Few examples showing differents strategies or combination of strategies used for the isolation/ detection of metabolites and putative identification of gene responsible for lichen metabolites discovery.

Major strategy (Bold) and additional strategies (+)	Metabolite(s) Discovered/identified	Lichen species (Lichen names follow www.indexfungorum.org)	Reference
Traditional approach for isolation	• 4-O-methyloxocryptochlorophaeic acid	*Ramalina subfraxinea*	(Culberson et al., 1990)
	• (-)-usnic acid • Fumaroprotocetraric acid • 9'-(O-Methyl)protocetraric acid	*Cladonia foliacea (syn.: C. convoluta)*	(Bezivin et al., 2004)
	• Elatinic Acid • Methyl Barbatate	*Haematomma ochrophaeum*	(Culberson et al., 1986)
	• Anziaic Acid	*Hypotrachyna sp.*	(Cheng et al., 2013)
	• Parmosidone A-E • Parmoether A-B	*Parmotrema tsavoense*	(Duong et al., 2015)
	• Pygmanilines A • Pygmanilines B	*Lichina pygmaea*	(Mahajan et al., 2017)
Bioactivity Guided Isolation	• Atranorin • (+)-usnic acid • 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	*Notoparmelia erumpens (syn.: Parmelia erumpens)*	(Aravind et al., 2014)
	• (±)-isousnic acid • (±)-protolichesterinic acid • atranorin • evernyl • ethyl hematommate • ethyl orsellinate • methylhematommate(3-formyl-2,4-dihydroxy-6-methylbenzoic acid methyl ester) • 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid • 1-hydroxy-3,6-dimethoxy-8-methyl-xanthen-9-one • baeomycesic acid • Salazinic acid	*Parmotrema reticulatum (syn.:Parmelia reticulata)*	(Goel et al., 2011)
	• Roccellic acid • Everninic acid	*Roccella montagnei*	(Mishra et al., 2017)
Untargeted metabolomics/Dereplication			
UHPLC-DAD-Orbitrap-ESI–MS–MS	• 32 compound identified • 1 new compound tentatively identified	*Everniopsis trulla*	(Castro et al., 2017)
UHPLC-PDA-Q/Orbitrap/MS/MS	• 22 compound identified	*Ramalina terebrata*	(Cornejo et al., 2016)
Direct-injection electron-ionization–mass spectrometry	• Based upon detected metabolites, technique is useful for discriminating between subspecies.	*Cladonia species*	(Kai et al., 2017)
UPLC MS (HR MS/MS and MRM)	• 47 compounds identified including nine new compound	*Parmotrema species*	(Kumar et al., 2018)
LDI-MS + Dereplication strategy	• Wider range of lichen compounds was detected by LDI-MS	*Cladonia portentosa* *Nephromopsis nivalis (syn.: Flavocetraria nivalis)* *Lecidella asema Pertusaria amara* *Ramalina siliquosa* *Tephromela atra Usnea filipendula* *Vulpicida pinastri*	(Le Pogam et al., 2015)
NMR-based non-targeted metabolic profiling	• Two-dimensional NMR profiling and fingerprinting helps in directly identify lichen-specific metabolites	*Set of Ecuadorian lichens*	(Mittermeier et al., 2015)
Gene Mining strategy			
+ Axenic culture of mycobiont + Phylogenetics + SMART RACE (DNA amplification) + HPLC-DAD detection	• Parietin	*Xanthoria elegans*	(Brunauer et al., 2009)
+ Phylogenetics + Heterologous host tranfer +transcriptomics + HPLC-DAD detection	• β-orcinol depsidones	*Xanthoparmelia semiviridis*	(Chooi et al., 2007)
+ Co-cultivation + Gene-knock	• lecanoric acid,	*Not mentioned*	(Schroeckh et al., 2009)
+ Axenic culture of mycobiont + Phylogenetics + in-silico (bio-informatics tools) + HPLC-DAD detection	• Usnic acid	*Cladonia uncialis*	(Abdel-Hameed et al., 2016)
+ Phylogenetics + in-silico (bio-informatics tools)	• 6-Hydroxymellein	*Cladonia uncialis*	(Abdel-Hameed et al., 2016)
+ Heterologous host tranfer	• Expression of the Solorina crocea PKS1 gene in heterologous host • Not linked to metabolites production	*Solorina crocea*	(Gagunashvili et al., 2009)
+ Phylogenetics	• Pentaketide or tetraketide polyketide	*Usnea longissima*	(Wang et al., 2012)
+ Phylogenetics +transcriptomics	• Usnic acid	*Nephromopsis pallescens*	(Wang et al., 2018)

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Table 2 (*continued*)

Major strategy (Bold) and additional strategies (+)	Metabolite(s) Discovered/identified	Lichen species (Lichen names follow www.indexfungorum.org)	Reference
+ LCMS-IT-TOF + Axenic culture of mycobiont + metagenomic DNA + bio-informatics tools + transcriptomics	• galectin-like protein	*Peltigera membranacea*	(Miao et al., 2012)
+ Phylogenetics + bio-informatics tools (MIBiG database) + Comparative genomics	• Highlighted the untapped biosynthetic potential	*Evernia prunastri* *Pseudevernia furfuracea*	(Calchera et al., 2019)
+ Metagenomic DNA + Cyanobacterial gene study + Phylogenetics + LC-MS/MS + bio-informatics tools (MUSCLE algorithm) + Purification of crude extract	• nosperin	*Peltigera membranacea*	(Kampa et al., 2013)
Symbionts metabolic capacity			
Axenic culture of mycobiont	• Usnic acid	*Cladonia imperialis*	(Stocker and Elix, 2002)
	• Subnudatones A and B • 1-(2-hydroxy-1,2,6-trimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)ethanone • libertalide C • aspermytin A • 6,7-dimethoxy-4-hydroxymellin	*Pseudopyrenula subnudata*	(Duong et al., 2020)
+ modification of growth media	• Usnic acid	*Usnea ghattensis*	(Behera et al., 2006)
+ modification of growth media + Thallus re-synthesis	• Lecanoric acid • Haematommic acid • Orsellinic acids • Sordidone • Eugenitol • Atranorin	*Lecanora rupicola*	(Brunauer et al., 2006)
+ modification of growth media + Thallus re-synthesis	• Physcion • Emodin • Physcion-bisanthrone • Teloschistin monoacetate and derivatives.	*Xanthoria elegans*	(Brunauer et al., 2007)
+ modification of growth media	• Sekikaic acid and satellite compounds	*Ramalina sp.*	(Cordeiro et al., 2004)
+ modification of growth media + modification of growth condition (dessication stress)	• Atranorin	*Parmotrema reticulatum*	(Fazio et al., 2009)
+ modification of growth media + Purification of crude extract	• 3-epi-petasol • Dihydropetasol • sarcographol • Six known eremophilanes and ergosterol peroxide	*Sarcographa tricosa*	(Le et al., 2013)
+ modification of growth media + Thallus re-synthesis + HPTLC analysis	• Atranorin • Norstictic acid • Baeomycesic acid	*Buellia subsororioides*	(Shanmugam et al., 2016)
+ Modification of growth media + Modification of growth condition (pH stress) + Gene mining	• Unidentified metabolites detected by TLC and HPLC • Expression of the NR PKS gene highlight polyketide synthesis	*Ramalina dilacerata*	(Timsina et al., 2013)
Culturing of endolichenic fungus			
+ Bioactivity guided purification	• Ophiofuranones A – B • Ophiochromanone • Ophiolactone • Ophioisocoumarin • One sesquiterpenoid ophiokorrin • Nine known compounds.	*Physciaceae* *Physcia*	(Li et al., 2019)
+ Purification of crude extract	• 19 compounds	*Usnea sp.*	(Basnet et al., 2019)
+ Purification of crude extract	• Isocoumarindole A	*Cetrelia sp.*	(Chen et al., 2019)
+ Bioactivity guided purification +OSMAC	• Ophiobolins P–T • 6-epi-21-O-dihydroophiobolin G • 6-epi-ophiobolin G • 6-epi-ophiobolin K	*Everniastrum sp.*	(Wang et al., 2013b)
Culturing of Cynobacterial photobiont			
+ Phylogenetics + LC-MS/MS	• Microcystins	*Pannaria pezizoides*	(Oksanen et al., 2004)
+ Phylogenetics + LC-MS/MS	• Microcystins	*Peltigera leucophlebia*	(Kaasalainen et al., 2009)

(*continued on next page*)

Table 2 (*continued*)

Major strategy (Bold) and additional strategies (+)	Metabolite(s) Discovered/identified	Lichen species (Lichen names follow www.indexfungorum.org)	Reference
Culturing/ phylogenetic networking study of lichen associated bacteria			
+ Purification of crude extract	• Coumabiocins A-F	*Cladonia gracilis*	(Cheenpracha et al., 2010)
+ Purification of crude extract	• Herbicidin Congeners	*Platismatia glauca*	(Chai et al., 2014)
+Affinity Crystallography	• Antipain • lichostatinal	*Stereocaulon sp.*	(Aguda et al., 2016)
+ Flash chromatography	• Five diketopiperazines - Cyclo (l-Pro-l-OMet) • Cyclo (l-Pro-l-Tyr) • Cyclo (d-Pro-l-Tyr) • Cyclo (l-Pro-l-Val) • Cyclo (l-Pro-l-Leu) • One auxin derivative - indole-carboxaldehyde • Two brominated diketopiperazines- Cyclo (d-Pro-l-Br-Tyr) • Cyclo (l-Pro-l-Br-Tyr) (7).	*Lathagrium auriforme (syn.: Collema auriforme)*	(Noël et al., 2017)
+ Gene-mining + Comparative genomic analysis + Phylogenetic + Bio-informatics tools	• Assess to biosynthetic Pathways (PKS class)	*Arboricolous lichens*	(Sanchez-Hidalgo et al., 2018)

promising strategies for natural product discovery and resulted in characterization of various novel metabolites. However, high frequency of non-homologous end joining in a fungal genome make the process more meticulous. The advent of numerous Site-Specific Nucleases (SSN) mediated genome editing tools such as TALENs/CRISPR/Cas9 suitable for working with fungal, bacterial and plant genome has now provided state-of-the-art platform for future efforts in discovery of novel metabolites from these resources. Importantly the plasticity in the CRISPR-Cas system makes the process more operational for the awakening of gene clusters. The first successful attempt involving CRISPR-Cas9 mediated genetic manipulation in gene cluster of fungal genome was performed in 2013. Therefore, this technology has been accepted by several scientist working on fungal genome as a novel and efficient approach for global or specific transcriptional modulation owing to its unparalleled fidelity and accuracy in genetic editing. Interestingly, the majority of data recommend that every partner of lichen is significant in context of metabolites production. It is clear that every symbiont is important having functional as well as biosynthetic role in symbiosis. Systematic screening of culturable partners together with optimization of media conditions and composition might result in obtaining pure axenic culture of individual components and discovery of novel scaffolds. Implementation of advanced molecular and analytical techniques coupled with advanced culturing strategies such as OSMAC and co-culturing could open up the real potential of this complex assemblage. Synergistic operation of all these novel tools is crucial to analyse the functional chemical and genomic composition and metabolic potential of metagenomic and/or axenic culture obtained from lichens similar to free living fungi.

7. Conclusion and future prospect

The complex interactions of different organisms in harmony with lichen made them an ideal candidate to study symbiotic system for exploration of their potential to produce specific chemodiversity. Table 2 illustrates few examples employed different strategies and combination of different strategies as discussed in Fig. 1 for isolation/ detection of metabolites and putative identification of gene responsible for lichen metabolites discovery. As discussed in this review, conventional isolation strategies in conjugation with cutting-edge untargeted metabolomics techniques and computational strategies are used to characterize the unique metabolites originated from the symbiotic assemblages, axenic culture of mycobiont, endolichenic fungal partners, cyanobacterial photobionts, and variable cohort of bacterial partners. Further discovery of novel metabolites and sustainable harvesting of unique metabolites can be explored with the help of a robust genome mining strategy. This may be due to fact that a large percentage of hidden biosynthetic and metabolic capacity that is not usually expressed in laboratory conditions can be exposed only by studying their whole genome. Also, discovery of biosynthetic gene cluster of these unique metabolites has unlocked the opportunities designed for biosysnthesis of these molecules in heterologous system. Further, development of super-precise novel gene edition tools such as CRISPR provided sceientist more efficient system to simplify and accelerate the process to study the genome of this complex system. The advent of novel genetic engineering tools also provided a significant oppurtunity to the biosynthesis of a wide-range of lichen metabolites employing artificial gene clusters approach that further enhance the wide spread application of these metabolites.

Additionally, recent approaches in obtaining the axenic culture of lichen symbionts have further enabled the lichnologists to overcome the bottlenecks associated in obtaining the genomic DNA of high purity and integrity, which is a prerequisite for successful genomic studies. Implementation of the OSMAC approach for lichen mycobionts and endolichenic cultures enables assessment of entire metabolic capacity through awakening of cryptic gene clusters. To conclude, the implementation of integrative approaches employing conventional natural product discovery approach coupled with multi “Omics” tools such as metabolomics and genomics affords the full characterisation of chemical diversity of lichens. Based on this there is a great need for the development and standardization of data mining software, bio-informative tools and computational libraries to handle the ‘big data’ that would be generated by employing all these approaches in lichen research.

Author contributions

RK contributed towards conceptualization, original drafting, writing and editing of the article, MG and XA contributed towards the critical revision and final approval of the article.

Declaration of Competing Interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

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