IDENTIFICATION OF SELECTED CORTICOLOUS LICHENS FROM PENANG HILL, GUNUNG JERAI AND CAMERON HIGHLANDS AND *IN VITRO* CULTURE OF THEIR MYCOBIONTS

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by

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LIST OF ABBREVIATIONS

BA	6-Benzyl aminopurine
BBM	Basal Bold Medium
cm	Centimetre
C.S	Cross section
CZA	Czapek Dox agar
g/L	Gram per litre
$HgCl_2$	Mercury chloride
IBA	Indole-3-butyric acid
IKI	Iodine Potassium iodide
KI	Potassium iodide
LBM	Lilly Barnett medium
mL	Millilitre
mm	Millimeter
MIC	Minimum Inhibitory Concentration
KOH	Potassium hydroxide
Pd	Paraphenylene diamine
PDA	Potato dextrose agar
PD0	Potato dextrose agar without sucrose
RCC	Rate of contaminated culture
RGC	Rate of growing colonies
rpm	Rotation per minute
NA	Nutrient agar
SAM	Sabouraud agar medium

SDA	Sabouraud dextrose agar
TLC	Thin Layer Chromatography
µg	Microgram
°C	Degree Celsius
µL	Microlitre
R_f	Retention factor
%	Percentage
JA	Jasmonic acid
TIBA	2,3,5-triodobenzoic acid

LIST OF PUBLICATIONS

STANLY, C. and CHAN, L. K. (2011). Initiation of Mycobiont Cultures of Crusotse Lichen Species and Studies on Growth Patterns of the Mycobiont Using *Graphis* species. In: 2nd Symposium of USM Fellowship 2011, 23-24 November 2011, Penang. Penang: Universiti Sains Malaysia. p. 169 (Appendix 10)

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PENGENALPASTIAN LIKEN KORTIKOLUS TERPILIH DARI BUKIT BENDERA, GUNUNG JERAI DAN CAMERON HIGHLANDS DAN KUTUR *IN VITRO* MIKOBIONNYA

ABSTRAK

Liken adalah hasil interaksi simbiotik antara alga dan kulat. Kajian liken di Malaysia adalah terhad. Sebanyak 53 spesies liken telah dikutip dari tiga lokasi iaitu Cameron highlands, Gunung Jerai dan Bukit Bendera. Ciri-ciri morfologi, anatomi dan kimia bagi spesies liken ini telah dikaji. *Graphidastra multiformis*, *Coenogonium nepalense* dan *Malmidea inflata* dilaporkan sebagai spesies baru di Malaysia. Mikobion daripada species liken terpilih telah berjaya diasingkan dengan teknik penyebaran spora. Kepekatan sukrosa yang optimum untuk pertumbuhan koloni mikobion *Graphis arbusculaeformis* and *Glyphis scyphulifera* adalah 30 g/L. Suhu optimum untuk pertumbuhan mikobion *G. arbusculaeformis* adalah 25°C dan untuk *G. scyphulifera* adalah 20-25°C. Biojisim tertinggi diperoleh daripada mikobion *G. arbusculaeformis* yang dikultur dalam medium MY30 [1.36 ± 0.08 g (jisim basah) and 219.0 ± 11.07 mg (jisim kering)] dan medium agar dekstrosa ubi yang ditambah dengan 30 g/L sukrosa (PD30) [1.41 ± 0.11 g (jisim basah) and 232.14 ± 9.63 mg (jisim kering)]. Biojism tertinggi diperolehi apabila mikobion *G. scyphulifera* dikultur dalam media MY30 [2.10 ± 0.15 g (jisim basah) and 375.71 ± 36.32 mg (jisim kering)], PD30 [1.89 ± 0.11 g (jisim basah) and 369.42 ± 9.31 mg (jisim kering)] dan medium agar Sabouraud (SAM) [2.03 ± 0.13 g (jisim basah) and 368.28 ± 16.55 mg (jisim kering)]. Mikobion *G. arbusculaeformis* dan *G. scyphulifera* menghasilkan biojisim yang lebih tinggi apabila dikulturkan dalam keadaan agitasi berbanding dengan keadaan pegun. Jism basah dan jisim kering mikobion *G. arbusculaeformis* yang dikulturkan dalam keadaan agitasi adalah 3.14 ± 0.14 g and

296.43 ± 19.74 mg masing-masing manakala untuk species *G. scyphulifera*, adalah 7.07 ± 0.31 g dan 567.0 ± 26.93 mg masing-masing. Apabila mikobion *G. arbusculaeformis* disubkultur secara berterusan dalam medium cair MY30, pengurangan biojisim didapati berlaku. Manakala, mikobion *G. scyphulifera* menunjukkan peningkatan di biojisim dalam pengsubkulturan pertama tetapi berkurangan pada hujung kitar pengsubkulturan keenam. pH medium yang sesuai untuk mikobion *G. arbusculaeformis* dan *G. scyphulifera* adalah pH 4 hingga pH 7 dan pH 5 hingga pH 7 masing-masing. Glukosa dan sukrosa menggalakkan pertumbuhan mikobion berbanding fruktosa dan maltosa. Apabila disubkulturkan dalam medium pepejal MY30, mikobion *Sarcographa labyrinthica* menghasilkan koloni berpigmen merah. Ekstrak aseton *S. labyrinthica* menunjukkan aktiviti antimikrobial terhadap *Bacillus subtilis* (MIC-2500 µg/mL), *Escherichia coli* (MIC-2500 µg/mL) dan *Pseudomonas aeruginosa* (MIC-1250 µg/mL).

IDENTIFICATION OF SELECTED CORTICOLOUS LICHENS FROM PENANG HILL, GUNUNG JERAI AND CAMERON HIGHLANDS AND *IN VITRO* CULTURE OF THEIR MYCOBIONTS

ABSTRACT

Lichens are symbiotic associations between algae and fungi. In Malaysia, studies on lichens are limited. Fifty three lichen species were collected from three different locations, namely Cameron Highland, Gunung Jerai and Penang Hill. Their morphological, anatomical and chemical characteristics were studied. *Graphidastra multiformis*, *Coenogonium nepalense* and *Malmidea inflata* were reported as new to Malaysia. Isolation of mycobionts from selected lichen species were successfully carried out by spore discharge technique. The optimum sucrose concentration for the growth of the mycobiont colonies of *Graphis arbusculaeformis* and *Glyphis scyphulifera* was 30 g/L. The optimum temperature for the growth of mycobionts of *G. arbusculaeformis* was 25°C and that of *G. scyphulifera* was 20-25°C. Highest biomass was obtained when the mycobionts of *G. arbusculaeformis* cultured in MY30 medium [1.36 ± 0.08 g (fresh) and 219.0 ± 11.07 mg (dried)] and potato dextrose agar medium supplemented with 30 g/L sucrose (PD30) [1.41 ± 0.11 g (fresh) and 232.14 ± 9.63 mg (dried)]. When the mycobionts of *G. scyphulifera* were cultured in different media high biomass could be obtained on MY30 [2.10 ± 0.15 g (fresh) and 375.71 ± 36.32 mg (dried)], PD30 [1.89 ± 0.11 g (fresh) and 369.42 ± 9.31 mg (dried)] and Sabouraud agar medium (SAM) [2.03 ± 0.13 g (fresh) and 368.28 ± 16.55 mg (dried)]. Mycobionts of *G. arbusculaeformis* and *G. scyphulifera* produced significantly higher biomass when cultured under agitated condition than those cultured under stationary mode. The fresh and dried biomasses of mycobionts of *G. arbusculaeformis* cultured under agitated condition were 3.14 ± 0.14 g and

296.43 ± 19.74 mg respectively and those of *G. scyphulifera* were 7.07 ± 0.31 g and 567.0 ± 26.93 mg respectively. When the *G. arbusculaeformis* mycobionts were subcultured continuously in liquid MY 30 medium it showed decrease in biomass. However, the mycobionts of *G. scyphulifera* showed increase in biomass in the first subculture and the biomass decreased at the end of the sixth subculture cycle. The suitable initial pH of the medium for the culture of mycobionts of *G. arbusculaeformis* and *G. scyphulifera* was from pH 4 to 7 and pH 5 to 7 respectively. Glucose and sucrose promoted the growth of mycobionts as compared to fructose and maltose. When cultured on solid MY30 medium, mycobionts of *Sarcographa labyrinthica* produced red pigmented colonies. Acetone extracts of *S. labyrinthica* showed antimicrobial activity against *Bacillus subtilis* (MIC-2500 µg/mL), *Escherichia coli* (MIC-2500 µg/mL) *and Pseudomonas aeruginosa* (MIC-1250 µg/mL).

CHAPTER ONE

INTRODUCTION

Lichens are wonders of the natural world. It is a mutualistic relationship between fungi and algae. The union between fungi and algae has produced thousands of lichen species around the world. The complexity of the lichen symbiosis has made it very difficult for those who study them. Since lichens are combination of fungi and algae, they cannot be considered as fungi or algae. Neither mycologists nor phycologists want to take responsibility of the lichens. Thus a new group of researchers and scientists have emerged studying just lichens and they become known as lichenologists. Lichenology not only includes taxonomic study but also genetics, developmental study, physiological study of the thallus and its isolated partners, influence of environmental factors on symbionts, study of the biological activities as well as secondary metabolite production and investigation of factors responsible for symbiosis (Ahmadjan, 1993).

An expedition to a pristine rainforest often guarantees an encounter with the luxuriant growth of lichens. Many conspicuous and large cyanolichens add to the aesthetic touch of a forest which attracts botanists and naturalists (Richardson and Cameron, 2004). Lichens occur in different shapes, sizes and colours. They often appear as colourful patches or spots on rocks and tree trunks (Hill, 2001). Lichens often add a colourful touch to their habitat when they grow abundantly (Boustie and Grube, 2005). The brilliant colors of lichens offer aesthetic value (Howe and Armitage, 2003). They appear in different shades of green, white, black, yellow, orange, red and brown colours. The different colours of lichens are due to the deposition of diverse pigments which are known as ‘lichen substances’ in their

thallus (Brodo *et al*., 2001; Stocker-Wörgötter, 2008). For example, *Cryptothecia rubrocinta* is a foliose lichen with a bright red thallus which easily catches the attention of lichenologists exploring its habitat. Their red colour is due to the presence of chiodectonic acid and this lichen is popularly called Christmas lichen (Stocker-Wörgötter, 2010).

The flora and fauna tend to show high species diversity in the tropics (Aptroot, 2001). Incredibly species rich tropical ecosystems are home to major terrestrial biota as well as a broad range of interactions among organisms such as lichens. Although fungi are ubiquitious in tropical environments, the understanding of the mycobiota and its role in the tropical ecosystems are largely unknown (Aime and Brearley, 2012). It has been indicated by recent studies that the diversity of lichenized fungi is higher in the tropical regions of the world (Nelson, *et al*., 2007; Baloch and Grube, 2009). According to Aptroot (2001), a single *Elaeocarpus* tree from Papua New Guinea was a host for about 173 lichen species. Lücking and Matzer (2001) reported the inheritance of 49 lichens and one lichenicolous fungi on a dicot leaf from Costa Rica and 46 lichens and one lichenicolous fungi from a similar leaf from Ecuador. These findings indicate the high lichen species diversity in the tropical countries. Lichens in the tropics especially crustose species are poorly explored (Feuerer and Hawksworth, 2007).

Wolsley and Hawksworth (2009) concluded that there were many species remained to be discovered and described in the tropics. Every year new lichen species are discovered from different parts of the world. Jia (2011) reported a new species of lichen, *Graphis paradussi* from Hainan Province of Southern China. Jørgensen *et al*. (2012) used molecular studies to determine the new lichen species *Bryoria rigida* from the Himalayan region of China. Cáceres *et al*. (2013) reported

new lichen species *Byssoloma catillariosporum* and *Porina isidioambigua* from North Eastern Brazil. Dal-Forno and Eliasaro, (2010) reported four new *Graphis* species from Southern Brazil. These studies agree with the prediction of existence of many new tropical lichen species (Sipman and Aptroot, 2001; Lucking, 2012).

Known as one of the mega-diversity countries in the world, Malaysia is home to several unique flora and fauna (Hafidh, *et al*., 2009; Eswani, *et al*., 2010). The tropical climate of Malaysia is suitable for the growth and development of diverse lichen species (Din *et al*., 1998). Studying the species richness and taxonomy of the tropical lichens is important as it is beneficial for other areas of research such as evolutionary study through molecular approach and conservation (Hunter Jr. and Webb, 2002; Lucking *et al*., 2009). However, lack of keys and reference specimens necessary for the description of the species in the Malesiana region had halted researchers intending to specialize in the taxonomy of the lichens and also in the biodiversity conservation (Coppins and Wolsley, 2002). Similarly, monographs on numerous tropical genera and families are absent which make it difficult to identify the lichen species (Wolsley and Hawksworth, 2009). Hence, the information on Malaysian lichens is scanty. Moreover, replacements of forest cover by rubber and oil palm plantations have affected the growth of many endemic lichen species in Malaysia. Thus, it is important to study the tropical lichen biodiversity by collecting, describing, sequencing and culturing the specimens as much as possible before these precious species become extinct due to habitat loss and environmental pollution (Wolsley and Hawksworth, 2009). Lichen diversity around the world including in Malaysia are often threatened by human activity such as logging, mining, agriculture, urbanization and environmental pollution (Motiejûnaite and Faiitynowicz, 2005). There is also need to establish of lichen cultures as it will provide enough biomass

necessary to explore the potential biological activities such as antioxidant, antimicrobial, cytotoxic and antiviral activities of extremely small crustose lichen species. Thus the present study focuses on the following research objectives.

1.1 Research Objectives

1. To study the morphology, anatomy and chemical characteristics of lichen specimens from three sampling sites.
2. To isolate mycobiont cultures of selected lichen samples and to study the culture characteristics of the *in vitro* mycobionts
3. To test the anti-microbial activity of the selected mycobiont cultures

CHAPTER TWO

LITERATURE REVIEW

2.1 Characteristics of lichens

Lichens are special class of non-vascular plants formed by the association of heterotrophic fungi and photosynthetic algae or cyanobacteria. Fungi through millions of years of evolution have learned to coexist with the algae resulting in the formation of amazing phenomenon called lichens (Hill, 2001). In the early ages, lichens were considered as a type of fungus because of the dominance of the fungal partner in the symbiotic association (Muggia *et al*., 2009). In this modern age lichens are considered as composite organisms rather than separate entities because symbiotic mechanism is a very complex process (Dayan and Romagni, 2001). Lichen symbiosis represents a major livelihood for fungi and the lichen forming fungi represents about one-fifth of all known fungal species. Symbiosis defines the thallus structure of individual lichen species as well as the lichen communities. While lichens could be defined as a small and stable ecosystem rather than considering as simple organisms (Rikkinen, 2002). Lichen symbiosis is considered to be one of the successful associations in nature (Lutzoni and Miadlikowska, 2009).

Fungi that form lichens are called mycobionts which have nutritionally specialized adaptations to form symbiosis with minute algal cells called photobionts (Honeggar, 1991). Lichenized fungi do not differ from the non-lichenized group except in its ability to form a mutualistic relationship with the photobiont cells (Honeggar, 1998). Lichens are dominant and important part of the terrestrial ecosystems. The success of the lichen symbiosis depends on factors such as the ability of the mycobiont to select appropriate photobionts based on its compatibility

and the ability of both partners in successfully establishing a lichen thallus (Lucking *et al*., 2009).

In lichens, mycobionts are considered to be dominant host which controls the photosynthetic partner. The lichenized fungi and algae both get advantages from symbiosis. Photobionts produce necessary carbon compounds for the mycobiont nutrition. On the other hand, photobionts gets protection from intense solar radiation, water loss and from animals that feed on the algae by the interwining fungal hyphae (Ahmadjian, 2004; Sillet and Antoine, 2004; Veermann, *et al*., 2007). Lichenization leading to the formation of an incredible lichen thallus that houses the algae can be considered as an important and fascinating lifestyle of fungus (Stock-Worgotter, 2002). Recent studies have indicated that a wide range of bacteria and non-lichenized fungi co-exist within the lichen thalli (Cardinale *et al*., 2006).

The taxonomic name of lichen often follows the name of lichen mycobiont, although the photobionts have their own scientific names (Rikkinen, 2002; Lutzoni and Miadlikowska, 2009). For example, the lichen *Cladonia cristatella* follows the name of the dominant fungal partner which forms symbiosis with green algae *Trebouxia erici* (Ahmadjian, 2004). Lichens are often seen growing among bryophytes. These non-vascular plants together form an important community which contributes to the health and wealth of a forest (Sillet and Antoine, 2004). In a favourable and suitable environment, lichens grow abundantly forming species rich and densely crowded communities that often form complex biotic interactions (Seaward, 2008). Lichens are extremely slow growing organisms whereby they grow few millimetres per year. They also have the ability to survive thousands of years due to their ability to survive in hostile environments (Denton and Karlen, 1973; Armstrong, 2004).

According to Yuan *et al*. (2005) fungus and the algae might have started their symbiotic relationship some 600 million years ago. They discovered fossils of lichen like associations in marine phosphorites in South China which was estimated to be 551 to 635 million years old. Taylor *et al*. (1995) reported the discovery of ancient fossil lichen from the early Devonian period. The fossil lichen *Winfrenatia reticulata* an association between zygomycete like fungus and a cyanobacterium (nostoc) was collected from Rhynie Chert, near Aberdeen, Scotland and was about 400 million years old. However, fossils of lichens are rarely recorded (Taylor *et al*., 1997; Karatygin *et al*., 2009).

Lichens are poikilohydric in nature which helps them to survive extreme environmental conditions such as drought, cold and heat (Honegger, 1991; Kranner *et al*., 2008). The poikilohydric nature of lichens means that their metabolic activities such as photosynthesis and respiration depend on the availability of moisture in the environment. They do not have epidermis and cuticle like higher plants which helps them to preserve water. In extremely dry conditions lichens can halt their metabolic processes and can remain dormant for a prolonged period of time which makes them as extremely slow growing organisms (Kappen, 1993). They receive water from nature through dew formation, high humidity and rain (Souza-Egipsy *et al*., 2000).

When lichens uptake water, it stimulates carbon-dioxide absorption and the thalli resume the photosynthetic process (Lange *et al*., 1986). They also do not have roots like higher plants to absorb water and nutrients. Lichens absorb water and nutrients directly through the thallus surface (Skye, 1979). Due to the poikilohydric nature, lichens are accustomed to undergoing stressful conditions such as high irradiation and desiccation (Kranner *et al*., 2005). The extent of desiccation tolerance in lichens depends on the environment and habitat they are growing. Desiccation

tolerance in lichens enables them to thrive in places where higher plants cannot survive (Kranner *et al*., 2008). The mycobiont and photobiont cells are even known to survive in space (Sancho *et al*., 2007).

When the lichen symbionts are cultured separately in a laboratory, it did not show any morphological similarity to the intact lichen thallus. The photobionts often form little green colonies on synthetic medium. Meanwhile the mycobiont produces mounts of fungal hyphae in the absence of the photobionts (Brodo *et al*., 2001). When the cultured lichen partners were allowed to resynthesize, the resulting artificial thalli does not resemble the natural intact lichen (Sanders, 2002). The symbiotic phenotype produced as a result of the intimate relationship between the algae and fungi is extra-ordinarily unique and innovative (Honeggar, 1998). In some lichens, the same mycobiont can form two morphologically different phenotypes due to the association with different photobionts (Stocker-Worgotter, 2002). These suggest that lichenized fungi are capable of showing considerable flexibility to adapt to the environmental changes (Henskens *et al*., 2012).

Several studies have been conducted to evaluate the effect of air pollution on lichens (Nimis *et al*., 1990; Silberstein *et al*., 1996; Estrabou *et al*., 2004). Lichens are highly sensitive to pollutants such as sulphur dioxide which causes immediate death of the lichens. Because of the high sensitivity of lichens towards environmental pollution, they have been used as pollution indicators of an ecosystem. Degeneration of lichen occurs when their habitat conditions are changed. Lichens absorb heavy metals in their thallus (Garty, 2001; Grube, 2010). The lifespan of the lichens could be reduced when they are growing in industrial environments where they are constantly exposed to acidity and heavy metal stress. Exposure of lichens to polluted

environments could cause ultra-structural damages to mitochondria and chloroplast (Tarhanen, 1998).

2.2 Lichenological history

Theophrastus, a Greek philosopher, used the term 'lichen' for the first time in his work *Historia plantarum.* He mentioned lichens in *Historia plantarum* to describe certain outgrowths which he observed on the bark of olive trees (Keck, 2006). Acharius was acknowledged as the father of lichenology as he pioneered the study of lichens by collecting and identifying many lichen species (Hale, 1967; Froden, 2009). Lichens were initially considered as a single organism. However, it was Swiss botanist Schwendener who recognized lichens as double organisms composed of algae and fungi. He describes lichens fungi as parasites and its algal partner as slaves. The dual nature of lichens as proposed by Schwendener was rejected by many lichenologists but some of the eminent scientists such as De Bary accepted it (Honeggar, 2000). Schwendener's discovery later becomes the paving stone of modern lichenology (Keck, 2006).

2.3 Distribution and ecology of lichens

Lichens have worldwide distribution. According to Huneck (1999), there are approximately 17,000 lichen species worldwide. However, Boustie and Grube, (2005) reported that there are approximately 18,500 species of lichen around the world. There are endemic lichens found in tropical mountains that are isolated, coastal deserts with fog formation and few isolated pacific and Atlantic islands such as Galapagos Island. Endemism in lichens are often limited to certain lichen families and genera (Aptroot and Bungartz, 2007). Ott and Sancho (1993) reported that

Caloplaca coralligera is a lichen endemic to Antarctic regions and it has undergone morphological, anatomical and physiological changes to adapt to extreme conditions. During the course of evolution, numerous chemical and morphological adaptations were incorporated in the thallus for successful symbioses of the ascomycete fungi and algae (Sanders, 2001; Blanco *et al*., 2006).

Lichens can grow in cold and dry habitats. They grow in the arctic regions as well as tropical regions and in the tallest mountains as well as the plain terrains (Muller, 2001). Some are sun loving species while others are shade loving lichens. These ecological characteristics are important part of lichenology (Wirth, 1995). Lichens are able to colonize almost all kinds of substrates. They can grow as patches on stones and rocks, on soil crusts, as epiphytes on tree trunks and shrubs as well as on glass surface (Hawksworth, 2000; Lisci *et al*., 2003). Lichens are even seen growing on graveyard stones, tiles and on statues and monuments made of stones. They affect the appearance of these structures by causing biodegradation induced by lichen acids (Wirth, 1995; Adamo and Violante, 2000). They are also found growing in the rocky seashores (Kohlmeyer *et al*., 2004) and about 700 lichen species are known growing in the coastal rocks. Lichens also grow on the edges of rivers and lakes and about 200 species were known from these areas (Hawksworth, 2000). Lichens are also known to grow in post-industrial areas where high concentrations of toxic elements are present. Certain species of *Cladonia* have been found to be colonizing these environments (Osyczka and Rola, 2013a). Lichens are generally considered as harmless. However, Aptroot (2011) reported that the lichen *Arthonia orchidicida* as a pest on the leaves of orchids.

According to the substrate it grows, lichens are classified as corticolous (growing on bark), terricolous (growing on soil), saxicolous (growing on rocks) and

lignicolous (growing on dead woods), follicolous (growing on leaves) and muscicolous (growing on mosses) (Seaward, 2008). Corticolous lichen species do not show equal distribution on all tree species. This may be due to different physio-chemical properties such as pH of the bark of the tree species (Wirth, 1995). Lichens with ascomycetes as fungal partners are most noticeable in the arctic and alpine regions and are found growing extensively on the ground and on rocks as brightly coloured spots (Aptroot, 2001). Lichens are important members of all ecosystems. They are capable of absorbing mineral nutrients from the atmosphere and releasing back to the environment. They are the first colonizers of a barren land and they prepare the habitat for the succession of other plants and animals. Lichen acids cause the rocks to breakdown to soil which serves as a suitable substrate for plants to grow (Dayan and Romagni, 2001). Due to the sensitivity of lichens towards air pollution and their distribution is scarce near industrial areas and big cities (Lisci *et al*., 2003).

2.4 Anatomy and structure of lichens

Generally lichens have a very complex structure. The fungal tissues of lichen are made of plectenchyma. In lichens, the fungal hyphae are loosely interwoven like a mesh which could be conglutinated or interconnected by intercalary connections. It is impossible to distinguish individual hyphae as it is closely interconnected. When the plectenchyma have resemblance to the parenchyma of higher plants, the cellular arrangement of fungal tissues is known as pseudoparenchymatous or paraplectenchymatous. When the fungal cells are conglutinated strongly which eventually gives the appearance of collenchyma of higher plants, the tissue is referred as prosoplectenchyma. In certain gelatinous lichens such as *Leptogium*, the hyphal cells on the surface of the thallus form isodiametric cells which are one layer

thick (Jahns, 1973). Cross section of lichen thallus reveals the presence of tissues resembling the higher plants. However, these are not true tissues like the higher plants (Armitage and Fowe, 2006).

The thallus is the vegetative body of the lichen. A typical lichen thallus in cross section reveals an upper cortex made of thick walled mycobiont cells. The cortex functions as a light filter protecting the photobiont layer from ultraviolet rays (Boustie and Grube, 2005). The cortex could be colourless or pigmented (Brodo *et al*., 2001). In some lichens belonging to the Parmeliaceae family, there is a thin layer of polysaccharide layer above the upper cortex. This layer is called epicortex which can be with pores or without pores (Büdel and Scheidegger, 2008). Below the cortex layer is the photobiont layer. The photobiont layer is followed by loosely arranged medullary layer which forms the major part of the lichen thallus. Medulla can be white, yellow, orange or pink depending on the species (Brodo *et al*., 2001). As compared to other tissues, medulla has large capacity to hold water and is the place for storing food (Jahns, 1973). Below the medulla is the lower cortex which can be white or pigmented in colour. Lichens with stratified thallus organization are called heteromerous lichens. For those lichens which contain cyanobacteria as the photobiont, there is no differentiation of the thallus into stratified layers. Instead the photobiont is uniformly distributed among the medullary hyphae. Such lichen thalli are called homoiomerous (Jahns, 1973; Büdel and Scheidegger, 2008).

In some lichens, the same mycobiont can form two morphologically different phenotypes due to the association with different photobionts. This phenomenon in lichens is called photosymbiodemes which is common in genera such as *Peltigera, Lobaria*, *Nephroma, Sticta* and *Pseudocyphellaria* whereby the part of the thallus containing cyanobiont would be dark in colour and the part of the thallus containing

green algae will be green in colour. These different morphotypes may combine together to form a compound thallus or grow separately. These lichens with three symbiotic partners are also called as tripartite lichens. Bipartite lichens are those which have one lichen forming fungi and one photobiont (Rai and Bergman, 2002; Rikkinen, 2002). When lichens have green algae as the main photosynthetic partner, they are called chlorolichens (Rai and Bergman, 2002; Kranner *et al*., 2008; Gauslaa and Coxson, 2011). Those lichens that contain cyanobacteria as the main photosynthetic partner or as a secondary photobiont in addition to the primary green algae are called cyanolichens (Rikkinen, 2002).

Lichens have structures resembling roots called rhizines which is found on the lower part of lichens. They are usually black in colour and are formed by bundles of conglutinated hyphae. They help in attaching the lichen to the substrate. Rhizines can be branched or unbranched. Tomentum are structures which are soft or cottony mat like loosely arranged mycobiont strands. Tomentum occurs in the lower cortex in certain members of Collemtaceae, Peltigeraceae and Stictaceae. In certain genera like *Erioderma* tomentum occurs in upper surface of the thalli. Cillia are appendages which are black in colour occurring along the margins of the lichen thallus. Cillia is a common feature of many foliose lichens (Hale, 1967).

Cyphellae are round depressions found in the lower surface of the thallus. The lower cortex forms a rim around these depressions. Cyphellae are characteristics of the lichen genus *Sticta* (Jahns, 1973; Brodo *et al*., 2001). Pseudocyphellae is smaller as compared to cyphellae. It is often seen as an opening in the cortex which is formed by the protrusion of the medullary hyphae. Pseudocyphellae can occur either in the upper or lower cortex and can be large and conspicuous as in the foliose genus *Pseudocyphellaria*. Cyphellae and pseudocyphellae are believed to function as

structures of gaseous exchange and aeration of the thallus (Büdel and Scheidegger, 2008).

Cephalodia are structures considered as centers of nitrogen fixation in lichens containing green photobiont (Büdel and Scheidegger, 2008). They appear as dark patches or spots on the thallus surface and are the place where packets of blue- green algae *Nostoc* are found. They can also occur inside the thallus which is referred to as internal cephalodia. Some of the foliose genera that contain cephalodia are *Peltigera*, *Lobaria* and *Nephroma*. However, cephalodia is not found in all the species of these genera. Cephalodia are formed when small colonies of *Nostoc* come in contact with the thallus surface which eventually gets incorporated into the thallus (Hale, 1967).

Isidia are finger like structures formed on the upper surface of the certain lichen thalli and it contain photobiont, medullary tissue and cortex. They are scattered on the upper surface of thallus. Isidia are vegetative diaspores of lichen that can grow into new thalli. They are of different shapes such as cylindrical, coralloid, globose or scale shaped. Isidia are usually fragile in nature and can easily detach from the thallus surface. Presence of isidia increases the total surface area of the lichen which eventually is believed to be increasing the assimilative ability of the lichen. Isidia are found in crustose, foliose and fruticose lichens as well as certain gelatinous lichens (Jahns, 1973).

Soredia are powdery and granular mass of vegetative diaspores produced abundantly in certain lichen species. They are capable of germinating into a new thallus when encountering a suitable substratum. They are minute spherical structures composed of a handful of photobionts enveloped by mycobiont hyphae (Jahns, 1973). They are originated from the algal layer and the medullary layer. They are formed as a result of the overpopulation of photobiont cells in the thallus which is

pushed towards the thallus surface along with the medullary hyphae through the pores and cracks of the cortex. Masses of soredia occurring as a separate clumps on the thallus surface is known as soralia and have different orientation on the thallus surface which characterizes certain lichen genera. They could be laminal or marginal and are common in foliose and fruticose lichens (Hale, 1967). Soredia are easily detached from the thallus which is easily dispersed by insects, water or wind (Lisci *et al*., 2003).

2.5 Growth forms of lichens

There are three basic forms of lichen growth such as foliose, fruticose and crustose.

2.5.1 Crustose lichens

Crustose lichens form a crust on the substratum and the thallus is firmly attached to the surface and it is often difficult to remove them without damaging the thallus. Crustose lichens do not have a lower cortex. They are also the slowest growing lichens as compared to foliose and fructicose lichens (Adamo and Violante, 2000; Armstrong and Bradwell, 2010). There are several types of crustose thalli. Some of the types are endolithic, endophloeodic, leprose and squamulose. Crustose lichen thalli can be homoiomerous or heteromerous. Rock dwelling crustose lichens are often endolithic. Endolithic lichens lacks upper cortex and the mycobiont and photobiont penetrate the rock. These lichens are often detected by the presence of ascocarps on the rock surface. These lichens help in the weathering of rocks (Hale, 1967; Büdel and Scheidegger, 2008). Endolithic lichens have special oil cells in their medullary hyphae (Kushnir *et al*., 1978). *Lecidea auriculata*, an endolithic lichen

colonizing the boulder surfaces of Storbreen glacier foreland of Norway enhanced the weathering of rocks (Mathews and Owen, 2008).

Those crustose lichens that penetrate wood are called endophloeodic lichens. These lichens are also recognized by the fruiting bodies on the bark (Jahns, 1973). Majority of the crustose lichens are epilithic and epiphloeodic. Epilithic lichens are those growing on the surface of the rocks and epiphloeodic lichens grows on the bark surface. *Rhizocarpon* is lichen genus growing on rocks and is characterized by the presence of a prothallus. Prothallus is composed of mycobiont only and could be white or black which can be seen at the margins of the lichens. Squamulose lichens consist of small lobes which are known as areoles at the margin which are partially detached from the substrate. These are the most complex of crustose lichens (Büdel and Scheidegger, 2008).

Many *Graphidaceae* members possess thalli with whitish grey colour due to the presence of calcium oxalate crystals. Some species can be corticated or ecorticated. Corticated species have a thallus with firm and smooth appearance. However, the ecorticated species have a matt like rough appearance (Lucking *et al*., 2009a). The genus *Lepraria* is characterized by the presence of simple crustose thallus consisting of powdery crust of loosely associated mycobiont and photobiont. The mycobiont and photobiont do not form any distinct thallus layers. Such lichens are referred to leprose lichens (Elix, 2009).

2.5.2 Foliose lichens

Foliose lichens have the appearance of a leaf and have a flattened thallus. The thallus of the foliose lichen can be distinguished into upper and lower surfaces. They have structures resembling roots called rhizines on the lower side of the thallus to

attach to the substrate and are easily removable from the substrate (Brodo *et al*., 2001). Some of them have structures such as cilia and tomentum on the thallus (Büdel and Scheidegger, 2008). Majority of the foliose lichens have lobes and the size and the shape of the lobes are important features in identification of many species (Brodo *et al*., 2001). Foliose lichens can be either heteromerous or homoiomerous. Heteromerous foliose lichens have radially arranged lobes. Two types of growth forms are observed in foliose lichens. They are laciniate and umbilicate growth forms. Laciniate foliose lichens are highly variable in size and shape. The lichen genera *Leptogium, Collema* and *Physma* are examples of foliose lichens having gelatinous and homoiomerous thallus (Büdel and Scheidegger, 2008). When these lichens get wet they have a translucent and blue green jelly like appearance (Brodo *et al*., 2001).

The lichen genus *Parmotrema* has broad lobes and they may or may not have cilia at the thallus margins (Jayalal *et al*., 2013). The foliose lichen genus *Menegazzia* has flat thallus lobes with perforations on it (Aptroot *et al*., 2003). Some of the lichens belonging to the genera *Lobaria, Pseudocyphellaria and Sticta* are large foliose lichens. They usually occur as epiphytes on trees along with bryophytes (Sillet and McCune, 1998; Goward and Arsenault, 2000). Umbilicate lichens are almost circular in shape. They are attached to the substratum only in the centre by an umbilicus. An umbilicus is a thick conglutinated hyphae lacking photobionts (Büdel and Scheidegger, 2008).

2.5.3 Fruticose lichens

Fruticose types are three dimensional lichens which have appearances like a hair or a shrub or a moss or hanging thread (Dayan and Romagni, 2001; Lisci *et al*.,

2003). Fruticose lichens have a radial anatomy with thick outer cortex, thin photobiont layer and medullary layer and central part which may be hollow or with a dense central cord. Thallus could be round as in *Usnea* species or flattened as in *Ramalina* species. Fruticose lichens have either highly branched or unbranched thallus. The genus *Cladia* has perforated thallus and in genus *Stereocaulon* the thallus is called pseudopodetium (Hale, 1967). Fruticose lichens such as *Usnea* and *Ramalina* attach themselves to the substrate by thick hyphal appendage called holdfast (Kashiwadani and Kalb, 1993; Gauslaa, 1997). Fruticose lichens belonging to the genus *Cladonia* species show thallus dimorphism. These lichens have a horizontal primary thallus consisting of squamules and secondary erect thallus called podetia (Osyczka and Rola, 2013).

2.6 Fruiting bodies in lichen

Most of the lichen fungi belonging to the class Ascomycota produce fruiting bodies called ascoma or ascocarp. A cross section of a typical ascoma reveals different layers of tissues. The different layers are hymenium, epihymenium, hypothecium, exciple and rim of the thallus. The fertile layer of an apothecium is called thecium or hymenium. In the hymenium, there are special cells called asci which produce the ascospores. The asci are sac like structures or club shaped structures which are intermingled with sterile thread like hyphal structures called paraphyses. The paraphyses can be branched or unbranched and it gives support to the ascus. There are hundreds of asci arranged vertically inside the hymenium layer (Perlmutter, 2009). When oil droplets are found distributed on the hymenium, it is referred to as inspersed hymenium. Hymenium is inspersed in many *Graphidaceae* members. Each ascus contains varying number of ascospores. Most of the lichens

have eight spores per ascus. Many *Graphis* species have eight ascospores per ascus (Lucking, *et al*., 2009a). However, species of *Pertusaria* have spores ranging from one to eight per ascus (Archer, 2004).

The ascospores are of different shapes and sizes depending on the genus and species. The ascospores could be hyaline or pigmented. The spores may be with or without septation and with or without gelatinous sheath around them (Brodo *et al*., 2001). Presence of septation is one of the important characteristics in identifying lichen spores. When the spores do not have septa and are unicellular, it is called as simple spores. Simple spores are typical characteristic of the genus *Lecanora.* When the spores elongated and have transverse walls make it multicellular, it is called as transversely septate spores. Many *Graphis* species have transversely septate spores. When the spores have many cells formed by transverse and longitudinal septa, they are called muriform spores (Hale, 1967). Muriform spores are generally large as in the case of certain *Thecaria* and *Laurera* species (Aptroot *et al*., 2008). When the spores have two cells at opposite ends which is connected by the thin connection is called polarilocular spores (Hale, 1967). The ascospores could be oval shaped or round shaped or elongated or with tapered ends. The size of the spores varies from few microns to 200 µm long and could be upto 75 µm wide (Brodo *et al*., 2001).

The topmost part of the hymenium is known as epithecium or epihymenium. Epihymenium is formed by the tips of the paraphyses. This layer contains pigments and granules which are responsible for the colour of the apothecial disc (Brodo *et al*., 2001, Perlmutter, 2009). Epithecium can be brightly coloured as in genus *Haematomma*. The apothecia of *Haematomma* species are bright red or orange-red coloured which makes it the most noticeable genus (Nelson *et al*., 2006). The granules deposited on the surface of the epihymenium are called pruina. Such

ascomata with pruina is described as pruinose. The asci and paraphyses are formed on a layer of dense fungal tissues called as hypothecium which can be coloured or colourless. The layer of fungal tissues that form the margins of the apothecia is called excipulum or exciple. The exciple is surrounded by a layer of fungal and photobiont tissues which form the rim of the thallus. The exciple could be slightly pigmented or colourless. The exciple which is black and carbon like is called carbonized exciple (Perlmutter, 2009). The exciple could be partially to completely carbonized as in the case of genus *Graphis* (Lucking *et al*., 2009).

Many lichens produce asexual propagules known as pycnidia. Pycnidia are also known as spermagonia which are flask shaped structures which may be immersed in the thallus or occur as superficial structures on the thallus. They have resemblance to perithecia and produce small bits of mycobiont hyphae known as microconidia. Microconidia are extremely small and are usually few microns in length (Hale, 1967). Pycnidia occur as tiny black dots on the thallus surface (Brodo *et al*., 2001). There are two types of ascocarp development in lichens. They are ascohymenial and ascolocular type. In ascohymenial type, ascomata develop from a true hymenium which is developed from ascogenous hyphae. Apothecia and perithecia are formed through ascohymenial development. In ascolocular type, the ascomata is formed from a stroma. The resulting ascocarps are usually pseudothecia (Hale, 1967).

There are two main types of ascomata. They are apothecia and perithecia. Apothecia are open reproductive structures with the spore bearing layer exposed on the surface. They are cup shaped or disc shaped. There are two basic types of apothecium which are lecanorine and lecideine. In lecanorine apothecia the thallus tissue including the photobionts forms the exciple and the margin of the apothecia.

Lecideine apothecia do not have a thalline margin and only contains the tissues of apothecia. The proper exciple forms a layer outside as well as the underside of the apothecia and forms a margin. The margin can be prominent or inconspicuous and can have the same colour as the apothecial disc or different colour (Perlmutter, 2009). Biatorine apothecia have same features like lecideine type of apothecia. However biatorine apothecia differ from lecideine form by having exciple which is pale and not carbonized. The biatorine apothecia lacks thalline margin like the lecideine type. They usually have a soft and waxy texture. Biatorine apothecia become convex when mature (Sheard, 2010).

When the apothecia are long, it is called lirellate apothecia. Lirellae can be short as well as long, branched as well as unbranched as in the genus *Graphis.* The emergence of apothecia mainly the lirellate forms in lichens are of four types. They are immersed, erumpent, prominent and sessile. In immersed lirellae, the upper surface of the apothecium is more or less in equal level with the thallus surface or slightly above the thallus level. In erumpent type, the upper part of the apothecium is above the thallus level and the lower part of the apothecia is below the thallus level. The prominent lirellae are completely above the thallus level and the sessile ones are also completely above the thallus but have a constricted base (Lucking *et al*., 2009). Lichens under the order *Caliciales* have special ascocarp which is called mazaedium. In a mazaedium, asci undergo disintergration and the spores are found liberated in the hymenium which appear as powdery mass (Hale, 1967).

Perithecia are closed fruiting bodies which conceal the spore producing layer. There is a hole or an opening called ostiole usually at the top of the perithecia. Ascospores are directly liberated from the ascus through the ostiole or in a jelly drop in the perithecia which is exuded through the ostiole. The wall of the perithecia

which constitutes the excipulum is usually brown to carbon black. Sometimes the excipulum is surrounded by a protective layer known as involucrellum (Brodo *et al*., 2001). The tissues projecting into the perithecial cavity and occurring between the asci are called hamathecium. Perithecia can be found solitary or in group. Lichens with perithecium as fruiting bodies are called pyrenocarpous lichens. Examples of some pyrenocarpous lichens are *Pyrenula*, *Porina*, *Polymerdium* and *Trypethelium* species. Members of the crustose genus *Pyrenula* possess brown spores (Aptroot *et al*., 2008). In pyrenocarpous lichens such as *Pyrenula* the ascomata are pseudothecia. These pseudothecia are closed with evenly carbonized wall. The pseudothecia can be globose or conical in shape. The thickness of the pseudothecial wall usually depends on the substratum on which it grows (Aptroot, 2012).

Basidiolichens produce fruiting bodies called mushrooms or basidiocarps which are same like the non-lichenized ones. Basidiospores are formed on club shaped cells called basidia which are present all over the gills of the mushrooms. These basidiocarps are produced occasionally and large amount of basidiospores are produced to ensure the effective dispersal of basidiolichens (Oberwinkler, 2012). Mature basidiospores are liberated from the basidia and are scattered in between the gills which are eventually dispersed by the wind (Brodo *et al*., 2001).

2.7 Mycobionts and photobionts

The term mycobiont was derived from the Greek word ‘mykes’ which means light and ‘bios’ which means life (Chapman and Waters, 2004). According to Dayan and Romagni (2001), it is the mycobiont that shapes the morphology of the lichens. There are about 16,750 ascomycetes, 200 Deuteromycetes and 50 Basidiomycetes. Majority of the mycobionts in lichens belong to the class Ascomycetes. Only few

members of Deuteromycetes and Basidiomycetes (a phylum which represents mushrooms) form lichens. *Multiclavula ichthyiformis* and *Omphalina foliacea* are examples of basidiolichens described from Costa Rica (Galloway, 1992; Nelsen *et al*., 2007). Within Ascomycetes, there are three classes, namely Sordariomycetes, Lecanoromycetes and Eurotiomycetes to which the lichenized fungi belong. Among the three classes, Lecanoromycetes contain most of the lichen forming fungi (Tehler and Wedin, 2008).

The term photobiont was derived from the Greek word 'photos' which means light and 'bios' which means life (Chapman and Waters, 2004). The photobionts of lichen are usually eukaryotic unicellular green algae or filamentous green algae or prokaryotic cyanobacterium (Richardson, 1999). There are about 40 known species of photobionts that had exhibited lichenization capacity (Dayan and Romagni 2001). Most of the photobionts in lichens are green algae which are mainly included in the major genera of *Chlorella, Coccomyxa, Myrmecia, Pleurococcus Trebouxia, Trentepohlia.* These photobionts belong to the class Chlorococcales and forms about 83% of the photobionts. Minority of the photobionts are included in the class Ulotricales (9%) and cyanobacteria are commonly called as blue green algae (*Nostoc, Gloeocapsa, Scytonema, Calothrix*) forms about 8% of the photobionts (Ahmadjian, 1993; Huneck, 1999). *Trebouxia* and *Trentepohlia* are the most common photobionts in lichens (Lutzoni and Miadlikowska, 2009). *Nostoc* and *Scytonema* are the most common cyanobionts in lichens which are important nitrogen fixing organisms (Lucking *et al*., 2009a). There is an exception with the intertidal marine lichen, *Verrucaria tavaresiae,* which is the only lichen reported to have brown algae as its photosynthetic partner (Sanders *et al*., 2002). Some members of fresh water

Verrucaria species also form symbiosis with yellow-green xanthophycean algae *Heterococcus* species (Thus *et al*., 2011).

Photobionts do not have sexual reproduction within the lichens. The algae in the photobiont layer multiply through the formation of aplanospores. When an algae gets mature, its protoplast divides and the many protoplasts formed subsequently produce a cell wall by itself. Eventually the aplanospores are released by the breaking the wall of the mother photobiont cell. Various stages of development of aplanospores in the photobiont layer could be observed when a section of the lichen thallus is made. Sexual reproduction through the development of zoospores cannot be observed in the thallus. Zoospore formation can only be seen in the photobiont cultures. The thickness of the photobiont layer differs according to the genera (Jahns, 1973).

Lichen families such as *Arthoniaceae, Graphidaceae, Pyrenulaceae, Thelotremataceae* and *Trichotheliaceae* are represented by Trentepohlialean photobionts. These lichens are found growing in highly humid and shaded tropical environments. Macrolichens such as *Parmeliaceae, Ramalinaceae, Usneaceae* and some crustose families such as *Bacidiaceae* and *Pertusariaceae* are represented by *Trebouxia* photobiont and are found growing in tropical regions with alternating wet and dry seasons. Lichens with cyanobacteria alone or with green photobionts such as *Lobariaceae* and *Collemtaceae* are found in moist and humid ecosystems (Wolsley and Hawksworth, 2009).

The photobiont ensures the success of the lichen symbiosis relationship (Galloway, 1992). The photosynthetic yield of the lichens is considered to be the world's highest due to their widespread distribution (Armitage and Howe, 2007). Different lichenized fungi can form symbiotic thallus with same photobiont (Lutzoni