CHAPTER-2
REVIEW OF LITERATURE

This chapter presents a detailed review of literature on various aspects taken under investigation in the present study. The literature is reviewed under following aspects:

2.1 Fungal diversity
2.2 Ganodermataceae
2.3 Medicinal *Ganoderma*
2.4 Systematics of *Ganoderma*
2.5 Bioactive molecules and medicinal effects
2.6 Biological activities of *Ganoderma*
2.7 Cultivation of *Ganoderma*

2.1 FUNGAL DIVERSITY

Fungi are highly diverse group that has profound impact on life. The 10\textsuperscript{th} edition of the *Dictionary of Fungi* gives a figure of 98,128 species, majority of which are terrestrial ascomycetes and basidiomycete species (Kirk et al., 2008). The total number of fungi recorded in India exceeds 27,000 species, which is considered as the largest biotic community after insects (Sarbhoy et al., 1996).

2.2 GANODERMATACEAE

Ganodermataceae (*Basidiomycota*) was described in 1948; it is distinct from other families of polypores in having peculiar double-walled basidiospores separated by inner wall pillars (Adaskaveg and Gilbertson, 1988). The inner walls (endosporium) of these spores are often pigmented, thick and usually ornamented; outer (exosporium) layer is relatively thin and hyaline (Donk, 1964; Cannon and Kirk, 2007). This family includes four genera: *Amauroderma*, *Ganoderma*, *Haddowia* and *Humphreya* (Kirk et al., 2008), with economically and ecologically important species because of their medicinal properties and their role in cycling of nutrients in ecosystems. *Ganoderma* represents the largest genus of the Ganodermataceae. Basidiocarps which are the sexual structures,
grow from a living or more commonly from a dead trunk or branch of a tree in the form of bracket, showing various morphological characteristics such as sessile, stipitate, imbricate and non-imbricate (Shin et al., 1986; Adaskaveg and Gilbertson, 1988), colour of pileus surface varies from deep red, non-laccate, laccate and light yellow to white. *Ganoderma* has a worldwide distribution in both tropical and temperate geographical regions (Arora, 1986). Many deciduous trees such as *Quercus*, *Acer*, *Alnus*, *Betula*, *Castanea*, *Corylus*, *Fagus*, *Fraxinus*, *Populus*, *Pyrus*, *Magnolia*, *Tilia* etc. has been described as its predominant hosts of *Ganoderma* species (Wasser and Weis, 1997).

### 2.2.1 Classification of *Ganoderma*

*Ganoderma* has been recognized as a medicinal mushroom for many thousands of years, and its powerful effects have been documented in ancient scripts (Wasser, 2005). Petter Adolf Karsten, a mycologist from Finland, named the genus *Ganoderma* in 1881 (Karsten, 1881), with *G. lucidum* (Curtis: Fr.) P. Karst from England as the type species (Moncalvo, 2000). *Ganoderma* is a member of domain Eukarya; Kingdom-Fungi; Phylum- Basidiomycota; Class- Basidiomycetes; Order- Polyporales and Family- Ganodermataceae (Schwarze and Ferner, 2003). It is popularly known as "Ling Zhi", in China, “Reishi, Mannentake or Sachitake” in Japan, and “Youngzhi” in Korea (Willard, 1990).
The Genus was subdivided into two sub genera: subgenus *Ganoderma* based on *Ganoderma lucidum* for the laccate species and subgenus *Elfvingia* based on *Ganoderma applanatum* for the species with a non-laccate fruit body (Moncalvo and Ryvarden, 1997).

**2.3 MEDICINAL GANODERMA**

*Ganoderma* is not edible because of its bitter taste and wooden texture owing to high fibre content. It is commonly used for pharmaceutical purposes and as health foods (Chang, 1996). Traditionally, it is prepared into various formulations including slices and powder of fruiting bodies, water and organic solvent extracts, spore products, drinks, syrups, and lotions for external use (Wachtel-Galor et al., 2011). This traditional medicinal system has served as templates for identifying new drugs. Bioactive compounds from *Ganoderma* possess an enormous structural and chemical diversity which is unsurpassable by any synthetic library. Many species of genus *Ganoderma* are reported to possess medicinal properties, these include, *G. lucidum* (Gao et al., 2011), *G. tropicum* (Liu et al., 2009), *G. tsugae* (Kuo et al., 2013), *G. boninense* (Mitchell et al., 2009), *G. capense* (Yan et al., 2013), *G. sinense* (Yue et al., 2013), *G. japonicum* (Liu et al., 2009) and *G. applanatum* (Akpera et al., 2012). Amongst all *G. lucidum* is believed to have maximum number of therapeutic properties (Barbosa-Filho et al., 2006) and a large number of experimental evidences provide scientific support to many of the ancient claims of its health benefits; for this reason *G. lucidum* has been included in the American herbal pharmacopoeia and therapeutic compendium (Chen et al., 2012). Majority of these medicinal properties are related to the antitumor, antioxidant and antimicrobial potentials (Mau et al., 2002; Akihisa et al., 2007; Keypour et al., 2008; Jia et al., 2009).

**2.4 SYSTEMATICS OF GANODERMA**

**2.4.1 Numerical Taxonomy**

Traditional identification of *Ganoderma* species has been based on morphology, physiology and developmental characters, as well as on chemical components such as secondary metabolites (Smith and Sivasithamparam, 2003a; Pilotti et al., 2004). Some
researchers used characteristics such as spore shape and size, context colour and consistency, microanatomy of the pilear crust, chlamydospore production and shape. Another important criterion includes enzymatic studies and optimum growth conditions (temperatures and pH) for differentiating *Ganoderma* species (Moncalvo, 2000; Saltarelli et al., 2009). Unfortunately, these characteristics are subjected to variations due to differences in cultivation technique and conditions, different geographical locations and the natural genetic development (e.g., mutation, recombination) of individual species. Consequently, the use of above mentioned characteristics has resulted in a large number of synonyms and a confused, overlapping, and unclear taxonomy for this mushroom (Hong et al., 2002). Owing to high phenotypic plasticity, some taxonomists consider macro- morphological features to be of limited value in the identification of *Ganoderma* species (Zhao and Zhang, 1994; Moncalvo, 2000), thus the genus is in a state of taxonomic crisis (Ryvarden, 1991).

The shape of the basidiocarp (fruit body) has been demonstrated to be greatly influenced by the environment (Chen, 1993), basidiospores by latitude and altitude (Steyaert, 1975) and in some species, context colour by latitudes (Steyaert, 1972). More recently, age and environment have been also shown to have a marked effect on the colour, size and brightness of the fruit body, and length, presence or absence of the stipe (Moncalvo, 2000). Moncalvo (1995) used characters of growth rate at variable temperatures, and number and shape of chlamydospores to characterize 29 strains of *Ganoderma* complex and classified the mushroom according to geographical origins, i.e., Asia, America and Europe. Gottlieb and Wright (1999) applied 26 characters to study relationship of 45 specimen of *Ganoderma* complex from Southern America and grouped the specimen mushroom into nine taxa based on spore character but other characters remained doubtful.

### 2.4.2 Alternate Approaches To Study Diversity of *Ganoderma*

Traditional identification parameters are becoming outdated and new identification methods such as ribosomal DNA (rDNA) sequencing (Moncalvo et al., 1995; Gottlieb et al., 2000), RAPD (Hseu et al., 1996; Rolim et al., 2011), ITS sequence analysis (Hong et al., 2002; Zheng et al., 2007), AFLP (Zheng et al., 2007) and PCR-
RFLP (Miller et al., 1999; Zheng et al., 2007), sequence related amplified polymorphism (SRAP) (Sun et al., 2006) and isoenzyme profile (Smith and Sivasithamparam, 2000b) are being rapidly investigated. Bruns et al. (1991) also reported phylogenetic analysis of N-terminal amino acid sequencing or DNA sequences to have very high resolving power in identification process. Some other approaches to solve the problem of *Ganoderma* taxonomy include near-infrared (NIR) spectroscopy combined with chemometrics (Chen et al., 2008), nuclear magnetic resonance (NMR) based metabolomics (Wen et al., 2010), and high performance liquid chromatography (HPLC) for generating chemical fingerprints (Su et al., 2001; Chen et al., 2008; Shi et al., 2008; Chen et al., 2010).

2.4.2.1 Molecular systematics of *Ganoderma* internal transcribed spacer (ITS) regions

The nuclear ribosomal genes have been extensively used for taxonomic purposes in fungal molecular systematics (Gargas et al., 1995; Zhang et al., 2009a, 2009b). The coding regions of these genes are highly conserved among fungi and show little sequence divergence between closely related species and are useful for phylogenetic studies among distantly related organisms (Swann and Taylor, 1993, 1995; Binder and Hibbett, 2002). Whereas, the internal transcribed spacer (ITS) region are highly variable (Moncalvo et al., 1995; Perlin and Park, 2001), and for this reason are useful in distinguishing between *Ganoderma* species (Moncalvo et al., 1995). Moncalvo et al. (1995) observed similar nucleotide substitutions frequency in both ITS regions but found most of the variations located in the central region of ITS 1 and close to the termini in ITS 2. They also reported that nucleotide divergence between recently diverged taxa was usually in the ITS 2 region. This was also observed by Gottlieb et al. (2000), who reported that a lower level of resolution of internal phylogenetic branches was obtained from the ITS 1 data set. Variable sequence regions in both the small (18S) and large (25S) subunits of rDNA genes have also led to numerous molecular approaches that provide rapid identification of fungal species (Perlin and Park, 2001).

Molecular taxonomy (based on the sequences of ITS and 26S rDNA) of the Ganodermataceae family was first performed by Moncalvo et al. (1995), with expectation to resolve the controversy associated with *Ganoderma* systematic to generate novel
taxonomic characters and with the use of phylogeny based classification methods. There have been many other reports published on the analysis of the ITS regions to establish taxonomic relationships within the *Ganoderma* species (Gottlieb *et al.*, 2000; Smith and Sivasithamparam, 2000a). Hseu *et al.* (1996) analysed ITS sequences of 36 strains of *G. lucidum* complex and the reported that the mushrooms could be clearly differentiated into six groups. Smith and Sivasithamparam (2000a) revealed that ITS sequence analysis could classify Australian *Ganoderma* species into five clades. Manassila *et al.* (2005) used ITS sequence analyses and PCR-RFLP to study the relationship of *Russula* species in North-East Thailand and found that the mushroom could be divided into three groups. Guzeldag and Colak (2007) used 5.8 S rRNA gene and ITS sequences for identification of *G. lucidum* from Turkey and found that 5.8 S rRNA gene sequences of the samples were absolutely identical (100%) to *G. lucidum*.

### 2.5 BIOACTIVE MOLECULES AND MEDICINAL EFFECTS

*Ganoderma* represent an inexhaustible source of bioactive metabolites. The basidiocarp, mycelia and spores of *Ganoderma lucidum* contain approximately 400 different bioactive compounds which mainly includes triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins/peptides and trace elements (Boh *et al.*, 2007; Zhou *et al.*, 2007); each has been reported to have a number of pharmacological effects.

#### 2.5.1 Polysaccharides

The quality of *G. lucidum* is evaluated through the content of polysaccharide in “Chinese Pharmacopeia” (Zhang and Yang, 2006). More than 200 polysaccharides have been isolated from the fruiting bodies (Bao *et al.*, 2002; Zhang and Lin, 2004), spores (Wang *et al.*, 2013), mycelia (Zhao and He, 2002) and cultivation broth (Kim *et al.*, 2003; Huie and Di, 2004) of *G. lucidum*. These mainly includes, β-D-glucans (Usui *et al.*, 1983; Sone *et al.*, 1985), glycans (Hikino *et al.*, 1985; Tomoda *et al.*, 1986), protein bound polysaccharides (Kim *et al.*, 1993; Cheong *et al.*, 1999; Eo *et al.*, 1999a), heterogalactans (Usui *et al.*, 1981; Miyazaki and Nishijima, 1982) and ganoderans A, B and C (Lindequist, 1995). Only few studies have been conducted on polysaccharides

### 2.5.1.1 Structure of polysaccharides

The basic structure of polysaccharides is β-1-3 D-glucopyranan with 1 to 15 units of β-1-6 monoglucosyl side chains (Mizuno, 1991). The antitumor polysaccharides differ greatly in their sugar composition and consequently in chemical structure, but one common feature is their relatively high molecular weight (Kim *et al.*, 1993). Most of the anticancerous glucans were reported to contain a branched glucan core with an average molecular weight of 1050 kDa (Bao *et al.*, 2002). It has been reported that polyglucans with a higher molecular weight (10^4-6 Daltons) tend to have greater water solubilities and therefore have a more effective antitumor activity (Mizuno, 1991).

![Figure 2.2: Possible repeating units of *Ganoderma lucidum* glucans](Hung et al., 2008)

Six water-soluble polysaccharides were extracted sequentially from the mycelium of *G. tsugae* (Peng *et al.*, 2005). They were heteropolysaccharides-protein complexes. GTM3 and GTM4 contained (1→3)-β-D-glucans and (1→4) - α-D-glucans, while GTM5 and GTM6 were mainly a (1→6) - branched (1→3) - β- D-glucan. Although some water insoluble polysaccharides in *Ganoderma* species, known as diet fibres, also have displayed antitumor activity (Wang *et al.*, 1993). Recently, Zhao *et al.* (2010) extracted
and purified polysaccharides with 1.926 kDa and 1080 kDa weight from *G. lucidum* fruiting body, and found it to inhibit human breast cancer cells.

### 2.5.1.2 Mode of action of polysaccharides

Several studies revealed the medicinal potentials of crude polysaccharides isolated from *G. lucidum* (Gao *et al.*, 2003; Shao *et al.*, 2004; Stanley *et al.*, 2005; Kuo *et al.*, 2006). Specifically the polysaccharides which occur in the form of β-D-glucan (huge sugar molecule) bound to amino acids were reported to possess immune-modulating and anticancer properties (Jones, 1992). This anticancer effect of polysaccharides arises from the enhancement of the host’s immune system rather than direct cytocidal effects (Gao *et al.*, 2000a, 2000b; Lu *et al.*, 2004; Zhu *et al.*, 2007). These intricate sugars stimulate or modulate the immune system by activating immune cells such as macrophage (Gao *et al.*, 2003, 2005; Shao *et al.*, 2004; Akramiene *et al.*, 2007; Ahmadi and Riazipour, 2007), B cells (Shao *et al.*, 2004; Manassila *et al.*, 2005), natural killer cells (Gao *et al.*, 2005; Ahmadi and Riazipour, 2007; Akramiene *et al.*, 2007; Huang and Ning, 2010), T cells (Gao *et al.*, 2003, 2005; Manassila *et al.*, 2005; Ahmadi and Riazipour, 2007). Polysaccharides are also reported to increase the immunoglobin levels to produce an elevated response to antigenic substance such as bacteria, viruses and tumor cells (Gao *et al.*, 2003) and stimulate TNF-α and IL-6 production, activate NF-kB (Kuo *et al.*, 2006) and increase hepatotoxicity activity (Manassila *et al.*, 2005).

The majority of notable polysaccharide for their ability to activate immune system is glucan and derivative of glucan (Bao *et al.*, 2002; Lu *et al.*, 2004). Bao *et al.* (2002) discovered two heteroglycan (PL1 and PL4) and glucan from fruiting bodies and six derivative of the (1, 3)-alpha-D-glucan from spore of *G. lucidum* to enhance the proliferation of T and B cells *in-vitro*. Moreover, PL 1 exhibited an immune stimulating activity in mice. The soluble glycoprotein fraction (F3) purified from water soluble extract of *G. lucidum* could enhance CD56+ NK cell cytotoxicity in cord blood (Chien *et al.*, 2004). β-glucans have been studied for their hypocholestromic effects, the mechanisms include reducing the intestinal absorption of cholesterol and bile acids by binding to glucans; shifting the liver from cholesterol synthesis to bile acid production and fermentation by intestinal bacteria to short chain fatty acids, which are absorbed and

Polysaccharides can prevent oncogenesis due to protective effect against potent genotoxic carcinogens. As an immunostimulating agent which acts through the activation of macrophages and NK cell cytotoxicity β-glucan can inhibit tumor growth, reduce tumor proliferation and prevent tumor metastasis. These also showed role in restoration of hematopoiesis (Akramiene et al., 2007). G. lucidum polysaccharides have also been shown to possess anti-angiogenic property (Cao and Lin, 2004). The polysaccharide fraction from G. lucidum has been reported to stimulate the proliferation of mouse spleen lymphocytes (Li et al., 2007), and to exhibit various other bioactivities, including anti-HIV, anti-herpetic, antiviral (Kim et al., 2000), immune regulating (Bao et al., 2002; Zhang et al., 2002) and antitumor properties (Wang et al., 1997). Bao et al. (2002) obtained a crude polysaccharide fraction from G. lucidum by hot water extraction and found that it exhibited immunostimulating activity in mice. The extract of fruiting body is reported to inhibit the growth of pre-cancerous human uroepithelial cells, medullary thyroid carcinoma (MTC-11) cells and breast cancer cells (Lu et al., 2004; Wu et al., 2006) and extract of sporoderm broken germinate spores has shown significant growth inhibition of mouse hepatoma, sarcoma S-180, reticulocyte sarcoma L II cells and malignant human breast carcinoma cells by 80-90% (Leu et al., 2002; Xie et al., 2006).

2.5.2 Triterpenoids

Triterpenoids found in Ganoderma mushroom are called ganoderic acids. The genus contains about 200 species known for the production of triterpene compounds. Many of these species have found wide applications in the prevention and treatment of various diseases due to the numerous biological activities due to triterpene components (Ofodile et al., 2005). The first triterpenes isolated from G. lucidum are ganoderic acids A and B, which were identified by Kubota et al. (1982). Since then, more than 100 triterpenes with known chemical compositions and molecular configurations have been reported to occur in G. lucidum. Among them, more than 50 were found to be new and unique to this fungus. The majority is ganoderic and lucidenic acids, but other triterpenes such as ganoderals, ganoderiols, and ganodermic acids have also been identified (Ma et
Triterpenoids are considered to be potential anticancerous agents (Min et al., 2000; Dzubak et al., 2006) and this effect seems to be related to their direct cytotoxic activity against the tumor cells (Gonzalez et al., 2002; Lin and Zhang, 2004; Kuo et al., 2006; Russell and Paterson, 2006). Ganoderic acid could induce DNA damage, G1 cell cycle arrest and apoptosis in human breast cancer cells (Wu et al., 2012). Liu and Zhong (2011) also reported that Ganoderic Mf and Ganoderic S isolated from mycelia of *G. lucidum* could induce apoptosis in HeLa cells and stimulate cysteine proteases (caspase-3, caspase-9) indicating their essential roles in apoptosis, necrosis and inflammation.

Figure 2.3: Multiple health benefits of *Ganoderma lucidum* polysaccharides and triterpenoids with possible mechanisms (Kao et al., 2013)

2.5.3 Other Compounds

Elemental analysis of *G. lucidum* fruit bodies revealed phosphorus, silica, sulphur, potassium, calcium, and magnesium to be their main mineral components and iron,
sodium, zinc, copper, manganese, strontium, lead, cadmium, and mercury were also detected in traces (Chen et al., 1998). It also contains organic germanium (Chiu et al., 2000), protein (Chang and Buswell, 1996; Mau et al., 2001), lectins (Kawagishi et al., 1997; Thakur et al., 2007), enzymes such as metallo-protease, ergosterol (provitamin D$_2$), nucleosides and nucleotides such as adenosine and guanosine (Wasser, 2005; Paterson, 2006).

2.6 BIOLOGICAL ACTIVITIES OF GANODERMA

Extract of *Ganoderma* is used as a Chinese remedy for the treatment of many illnesses. Some of these health benefits include antioxidant and antimicrobial activities.

2.6.1 Antioxidant Activity

Uncontrolled production of free radicals and lack of antioxidative defences in biological systems may lead to oxidative stress, associated with a variety of disorders such as coronary heart diseases, neural disorders, diabetes, cancer, rheumatoid arthritis, and atherosclerosis as well as in other degenerative processes associated with aging (Spiteller, 2001; Lee et al., 2001; Lakshmi et al., 2003). Therefore, the inactivation or elimination of these reactive oxygen species may be critical in preventing these diseases. Data from *in-vitro* and *in-vivo* studies have indicated that components of *G. lucidum*, in particular polysaccharides and triterpenoids, show antioxidant activity and radical-scavenging effects (Wachtel-Galor et al., 2005; Yuen and Gohel, 2008; Saltarelli et al., 2009; Wu and Wang, 2009). Protein bound polysaccharides from *G. lucidum* (Lee et al., 2001; You and Lin, 2002), methanolic extracts from *G. tsugae* (Yen and Wu, 1999) and ethanolic extracts from *G. lucidum* (Lakshmi et al., 2003) are reported to exhibit superoxide and hydroxyl radical scavenging activity. Shieh et al. (2001) reported antioxidative effect of its aqueous extract on mouse liver and kidney lipid peroxidation and extracts have also been found to reduce strand breakage in DNA caused by ultraviolet induced photolysis of hydrogen peroxide (Kim and Kim, 1999; Lee et al., 2001).
Methanolic extract of this mushroom is reported to have high antioxidant abilities in some more reports (Mau et al., 2002; Yang et al., 2002; Lakshmi et al., 2004). In particular, phenols have been identified as major constituent and naturally occurring antioxidants in its extract (Mau et al., 2002). There have been many investigations of the antioxidant activities of triterpenes from *Ganoderma* species (Zhu et al., 1999). Ethyl acetate and aqueous extracts of the mushroom is reported to possess significant superoxide radical and hydroxyl scavenging and lipid peroxidation inhibition (Jones and Janardhanan, 2000). The polysaccharides extract from fruiting body also exhibited this activity. Chen et al. (2009) found that crude polysaccharides have superoxide anion radical scavenging activity and it significantly enhanced the antioxidant enzyme (superoxide dismutases, catalase and glutathione peroxidase) activities. Tseng et al. (2008) reported that hot water extracted polysaccharides from fruiting body, primordium, mycelia and fermentation filtrate of *G. tsugae* show antioxidant activity by 78-88%, while the hot alkaline extracted polysaccharides exhibited only 55% activity. Paterson (2006) found that the antioxidant property of *Ganoderma* polysaccharide peptide decreased the oxidation of low density lipoprotein and exhibited antioxidant effect by scavenging reactive oxygen species in mice.

2.6.2 Antimicrobial Activity

Mushrooms need antimicrobial compounds to survive in their natural environment, hence are rich sources of natural antibiotics. Many of its metabolites are known to combat bacteria (Kupra et al., 1979) and viruses (Eo et al., 1999b) and fungi (Liu et al., 2009). Several species of *Ganoderma* like *G. pfeifferi* (Mothana et al., 2000), *G. lucidum* (Gao et al., 2003; Keypour et al., 2008; Jonathan and Awotona, 2010), *G. orgonense* (Brian, 1951), *G. applanatum* (Smania et al., 1999), *G. japonicum* (Liu et al., 2009) and *G. recinaceum* (Coletto and Mondino, 1991) have been demonstrated to have antimicrobial activities against several bacterial, fungal and viral pathogens.

2.6.2.1 Antibacterial activity

*Ganoderma lucidum* was reported to be best among other *Ganoderma* species that generally exhibited high antagonistic activity against many bacteria (Jonathan and
G. pfeifferi is reported to inhibit the growth of methicillin-resistant Staphylococcus aureus (Mothana et al., 2000; Liu et al., 2009). Some other bacteria like Bacillus subtilis (Sudirman and Mujiyati, 1997; Keypour et al., 2008), Staphylococcus aureus (Coletto and Mondino, 1991; Keypour et al., 2008), Escherichia coli (Ohno et al., 1998), Micrococcus luteus, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris, and Salmonella typhimurium have been reported to be susceptible to Ganoderma extracts.

Yoon et al. (1994) examined antimicrobial effect of a G. lucidum water extract against 15 species of bacteria alone and in combination with four antibiotics and found G. lucidum more effective than antibiotics against Escherichia coli, Micrococcus luteus, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris, and Salmonella typhimurium, but less effective against other tested species. This combination of G. lucidum with antibiotics resulted in an additive (synergistic) effect in most, but not all, instances, with apparent antagonism against cefazolin and ampicillin effects on Proteus vulgaris. Steroidal compounds from the basidiocarps of G. applanatum were found to have broad spectrum activities and bactericidal effects (Smania et al., 1999). Ganomycins A and B, from G. pfeifferi exhibited antibacterial activity against Gram-negative and Gram-positive bacteria (Mothana et al., 2000).

2.6.2.2 Antifungal activity

Ganoderma lucidum has been shown to have strong activities against Candida albicans (Bhosle et al., 2010), Penicillium species, Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus and Mucour indicus (Sridhar et al., 2011). Applanoxidic acid A isolated from Ganoderma annulare, showed weak antifungal activity against Trichophyton mentagrophytes (Smania et al., 2003). Wang and Ng (2006) isolated a 15 kDa antifungal protein (named ganodermin) from G. lucidum fruiting bodies. Antifungal activity of culture filtrate and fruit body extract of local strain of Ganoderma species was studied against four common contaminants of oyster mushroom beds under in-vitro conditions, culture filtrate found to inhibit 70% and 75% growth of Rhizoctonia solani and Trichoderma viride, respectively (Sharma and Thakur, 2012).
2.6.2.3 Antiviral activity

In contrast to bacterial infectious diseases, viral diseases cannot be treated by common antibiotics and specific drugs are urgently needed. As the propagation of viruses depends on the metabolic activity of the host cells (Eo et al., 1999b), objective of antiviral chemotherapy is the discovery of an agents that are specific for the inhibition of viral multiplication without affecting normal cell metabolism (Eo et al., 1999b). Polysaccharide fractions from *G. lucidum* are shown to exhibit activity against herpes simplex virus-1 (HSV-1) and herpes simplex virus-2 (HSV-2) (Oh et al., 2000; Liu et al., 2004; Pillai et al., 2010). Lanostane type triterpenes from *G. pfeifferi* have also been shown to exhibit activity against HSV-1 and influenza A virus (Mothana et al., 2003). Its low molecular weight aqueous fractions strongly inhibit multiplication of HIV-1 (Kim et al., 1997; El-Mekkawy et al., 1998) and, of triterpenes inhibiting both HIV-1 protease (Min et al., 1998) and HIV-2 protease (El-Mekkawy et al., 1998). A marked synergistic effect was reported with protein-bound polysaccharide (PBP) from *G. lucidum*, when used in tissue culture in conjunction with anti herpetic agents, acyclovir or vidarabine, and with interferon alpha (IFN-α) (Kim et al., 2000; Oh et al., 2000). The crude polysaccharides (Eo et al., 1999a) and protein bound polysaccharide from fruiting body reduced plaque formation of HSV-1 and HSV-2 to Vero cells (Pillai et al., 2010). The possible mode of action describes direct inhibition of viral enzymes, synthesis of viral nucleic acids or adsorption and uptake of viruses into mammalian cells. These direct antiviral effects are exhibited especially by smaller molecules. Indirect antiviral effects are the result of the immunostimulating activity of polysaccharides or other complex molecules (Brandt and Piraino, 2000).

2.7 CULTIVATION OF *GANODERMA*

The first mushroom to be intentionally grown (cultivated) was probably *Auricularia auricula* circa A.D. 600, followed by *Flammulina velutipes* circa A.D. 800. *Ganoderma* was cultivated outdoors in 1621, at that time spawning might involve the placing of fruiting bodies on the substrate (Chang and Miles, 2004). *G. lucidum*, an annual mushroom (Perumal, 2009) and its wild form is extremely rare in nature (Willard, 1990). Different members of the *Ganoderma* genus need different conditions for growth
and cultivation (Mayzumi et al., 1997). A period of several months is required to cultivate the mushroom and the product yield is low in soil. Being non-chlorophyllous it depends on substrate materials for nutrition (Chang and Miles, 2004). Large amounts of freely available sawdusts offer a potential alternative substrate source for Ganoderma cultivation (Grodzinskaya et al., 2003). Scientists attempted artificial cultivation of Ganoderma fruiting bodies on solid substrates in 1937 and mass production was achieved in 1970’s by Y. Naoi (Mizuno, 1997) by cultivating the spawn in sawdust containing pots.

Riu et al. (1997) assayed oak cork residues alone and with the addition of 30% wheat bran in a mixture containing 60% water for G. lucidum production, and obtained lower production rates (BE=14%) in 105 days for the first treatment, and a lower BE of 2% in 115 days for the second treatment. Many starch-based supplements such as wheat bran, rice bran, millet, rye or corn, can be added to the main ingredients to stimulate the growth (Royse, 1996; Ivan et al., 2003). A cheap substrate such as agricultural residues with little pre-treatment or enrichment can also be used to get a high yield (Wagner et al., 2003). Supplements such as sucrose, wheat and rice are generally added to the substrate to improve the yield. Gurung et al. (2012) studied the effects of Alnus nepalensis, Dalbergia sissoo, Shorea robusta and supplements of rice bran, wheat bran, corn flour and gram flour on G. lucidum growth. Berovic et al. (2012) cultivated G. lucidum on substrate of sawdust, olive oil, and mineral salts. Results showed that optimal moisture of the solid matrix was in the range of 80% to 74%, when the moisture content dropped below 57%, the growth of the mycelium and polysaccharide production stopped. Veena and Pandey (2011) cultivated G. lucidum on paddy straw in combination with sawdust (0, 22.5%, 45%, and 67.5%) and rice bran (10%). The highest biological efficiency (BE=29.9%) was observed with the combination sawdust: paddy straw: rice bran (22.5:67.5:10), followed by saw dust: paddy straw: rice bran (45:45:10) with BE 27.3%.

Matute et al. (2002) used sunflower seed hull as a substrate for growing G. lucidum in a synthetic log system and found that addition of 5% malt to the substrate improved the mushroom growth rate. Petre and Teodorescu (2009) cultivated G. lucidum on poplar and beech substrates and reported it as the longest mushroom culture (40-50
days) in comparison to *Lentinula edodes* and *Pleurotus ostreatus*. They tested various nitrogen sources (rice bran, malt extract, peptone, tryptone and yeast extract at 1% concentration) for optimal growth and found rice bran the most efficient for mycelia growth. During optimization of others cultural parameters maximum fresh fungal biomass was obtained at pH 5.5 and temperature 23°C. Erkel (2009) investigated its growth on sawdust of poplar, oak and beech and bran of wheat, rice and corn and found oak sawdust to give highest yield and wheat bran to be best among tested brans. Negi *et al.* (2008) cultivated *G. lucidum* on *Alnus nepalensis, Aesculus indica, Toona ciliate* and *Quercus leucotrichophora* in combination with 10% rice bran and got highest yield on *Alnus nepalensis* (BE of 32%). They reported temperature 32±2°C, pH 5.5-6.5, relative humidity of 85-90% optimum for fruit body yield. Song *et al.* (2007) used whey permeate for cultivating *G. lucidum* mycelia and found very high mycelial extension rate (17.6±0.4 mm/day).

### 2.7.1 Cultivation of Ganoderma in Liquid Media (Vegetative Growth)

*G. lucidum* is cultured on solid substrate; however, this production method has serious disadvantages, including an extensive culture time, the difficulty in controlling product quality and the culture’s susceptibility to environmental changes (Chang *et al.*, 2006). Propagation of mushroom mycelium in submerged culture was initially developed during the 1950’s. This method was very successful in growing lower fungi (fungi that do not form basidiocarps) in fermenters for economical production of various natural products (Yang and Liau, 1998a). Since it generally takes several months to cultivate the fruiting body of the mushroom and it is also difficult to control the quality of the product during cultivation, researchers are turning to the liquid cultivation of mycelium to obtain useful cellular material or to produce effective active substances without waiting for a full fruiting body to develop (Song *et al.*, 1998; Hatvani, 2001). Liquid culture has many advantages over solid substrate cultivation because it requires minimal space and there is less chance of contamination (Bae *et al.*, 2000). Mycelium in liquid culture can disperse within the substrate more uniformly, thus the time taken to produce the first crop may be shortened. Additionally, broth media parameters can be easily manipulated. Considering
all advantages, mycelial cultivation has received great interest as an efficient method for industrial production of valuable metabolites (Hatvani, 2001; Fang and Zhong, 2002a).

2.7.1.1 Investigation of growth parameters

Different environmental parameters and medium compositions have also been reported to strongly influence mycelial growth and the production of bioactive compounds (Fang and Zhong, 2002b; Tang et al., 2011; Zhu et al., 2010), thus, quantification of these limits is necessary in order to model and predict effects on growth of the fungus (Knudsen and Stack, 1991). Although many workers have attempted to grow *G. lucidum* in submerged culture to produce mycelium, only few have investigated the influence of environmental factors (Yang and Liau, 1998b) and the effects of the culture medium (Lee et al., 1999; Kim et al., 2002).

2.7.1.1.1 Effect of culture medium on mycelial biomass

The culture medium is extremely important as it provides the nutrients for growth of the mycelium. There have been few investigations reporting the effect that different carbohydrates have on the growth of the mycelium (Lin et al., 1973; Tseng et al., 1984). Sone et al. (1985) examined the growth of *G. lucidum* with different carbohydrates, including galactose, glucose, lactose, maltose, mannose, sucrose and xylose and found that lactose produced the highest dry weight of mycelium after seven days, and glucose and glucitol produced the least biomass, while Lin et al. (1973) reported that the mass of mycelium produced in the lactose medium was approximately 1.5 times greater than that in the glucose medium.

The presence of nitrogen in the culture medium is another important factor and it has been observed that no mycelial growth occurs in its absence (Lin et al., 1973). There is little information on the investigation of different nitrogen sources and how they affect the mycelial growth and formation of *Ganoderma* species. Culture media buffered with inorganic salts (Lin et al., 1973) and with low concentrations of ammonium phosphate (Lee et al., 1999) have also been reported to result in good mycelial growth. Researchers are investigating alternate materials including fatty acids (Yang et al., 2000) and plant oils (Schisler and Volkoff, 1977), which have been also found to have a stimulatory
effect on the mycelial growth of some mushrooms. Chaves *et al.* (2013), optimized culture medium for biomass and phenolic compounds production by *G. lucidum*. Both responses (biomass and phenolic compounds) were simultaneously optimized using 50.00 g/l sucrose, 13.29 g/l yeast extract and 2.99 g/l olive oil.

### 2.7.1.1.2 Effect of the environmental parameters on biomass production

Temperature is one of the most important environmental factors requiring careful control. *G. lucidum* isolated from different regions generally has an optimal growth temperature of 30-35°C (Mayzumi *et al.*, 1997; Yang and Liau, 1998b), which explains why *Ganoderma* species are often found in hot climates and tropical regions. *G. japonicum* has been observed to grow at an optimal temperature of 25°C (Mayzumi *et al.*, 1997) and for *G. applanatum* optimum temperature was 25°C and optimum initial pH for mycelial growth was 5.0 (Jeong *et al.*, 2009). The initial pH of the growth medium also affect cell membrane function, cell morphology and structure, the uptake of various nutrients and product biosynthesis (Fang and Zhong, 2002b). Fungi generally metabolise acids to decrease the pH (Lin *et al.*, 1973) and during the cultivation of mycelia, the media becomes acidic and when it reaches this acidic level (pH 3.0 to 4.0) the growth of the mycelia is retard (Lin *et al.*, 1973). In contrast to this, Kim *et al.* (2002) found that the pH of the liquid medium in which *Ganoderma* species was grown increased, while the other fungi tested caused a decrease in the medium pH.

It has been demonstrated that the mycelia from a number of fungal species can grow over a wide range of pH values (Lonergan *et al.*, 1993; Yang and Liau, 1998b). Yang and Liau (1998b) observed that *G. lucidum* grown in a glucose ammonium chloride medium has optimal pH 4.0 for mycelial growth, however, when it was grown in a glucose malt extract medium they observed that the optimal pH 5.0 for the same. They concluded that the optimal initial pH for the growth of the mycelium could change depending on the cultivation medium used. Fang and Zhong (2002b) also observed that the initial pH of the cultivation medium affected the production of mycelial biomass. They reported that as the initial pH of the medium decreased from 6.5 to 3.5, the production of mycelial biomass decreased. Earlier, Lin *et al.* (1973) noted the morphology and size of the mycelial pellet of *Ganoderma* species varied as the pH of the
culture medium changed. At acidic pH (4.0), colonies of *Ganoderma* species were covered with ‘feather like hairs’, whereas at pH 7.0, the colonies appeared small, with or without short hair like structures. Shear stress as a result of agitation can affect the morphology of the mycelium, which can directly reduce the cell growth and bioactive compound production (Pace, 1980). Too low agitation speed may limit the oxygen transfer in the medium, reducing mycelial biomass production, whilst too high agitation speed has been shown to increase the sheer stress on the mycelium, which in turn, also decreases biomass production (Yang and Liau, 1998a). It has been reported that a favourable agitation speed for *Ganoderma* is approximately 100 rpm (Lin et al., 1973; Yang and Liau, 1998b).

Tang and Zhong (2003) investigated the production of mycelial biomass with respect to available oxygen. They observed that a high initial oxygen supply increased mycelial biomass production. The mycelial pellet size has been reported to be another factor of importance in mycelial cultivation (Fang and Zhong, 2002a). Inoculation density has been shown to be another important factor for many cell culture processes (Johansen et al., 1998) including submerged culture process of *Ganoderma* (Fang et al., 2002). Fang et al. (2002) also observed that a large inoculation density led to a small pellet size while a low inoculation density led to a larger pellet size. Lin et al. (1973) suggested that it was best to select a three to four day old culture, when liquid culture is used as the inoculum for mycelial growth, as the fungal mycelium has just started to enter the logarithmic growth phase. Light is not necessary for the growth of mycelia in liquid cultures or on an agar medium. Submerged cultivation experiments with *Ganoderma* species and many other fungi show that strong light can inhibit the mycelial growth (Lin et al., 1973). Nasreen et al. (2005) showed that *G. lucidum* mycelia grow best in potato dextrose medium (1.59 g/100 ml) at pH 5.0 and 25ºC, followed by malt extract (0.91 g/100 ml), Kirk medium+ molasses (0.68 g/100 ml), and Kirk medium + glucose (0.38 g/100 ml) after 15 days.

For solid-state *G. lucidum* biomass cultivation and polysaccharide production, moisture content of solid substrate is considered of crucial importance. Habijani and Berovi (2000) reported a moisture fraction of 70% as a critical value. Moistures higher
than 70% promote the growth of *G. lucidum* mycelium and polysaccharide production up to 0.68 mg/g and 6.0 mg/g respectively. As the moisture fraction of the substrate drops to 55%, growth of mycelium stops. However, mycelium survives when moisture fraction drop even to 40% and it begins to grow with maximal growth rate when the substrate is remoistened in same study.

### 2.7.1.1.3 Effect of species and strain on *Ganoderma* growth

Growth and dispersal of fungi in natural environments varies among fungal genera, species and strains (Knudsen and Stack, 1991) and different strains can produce different compounds (Xing *et al.*, 2004). Consequently, the methods employed in mushroom cultivation, whether it be solid substrate or liquid cultivation, may require modification from one geographical region to another due to different environmental conditions and the presence of different species of organisms (Chang, 1999). Kim *et al.* (2002) studied a number of fungi, including two *Ganoderma* species, in liquid culture and observed that the rate of mycelial growth was different between genera and some species and both isolates which were initially thought to be the same species, were actually unique. Not only do cultivation conditions differ between species and strains but storage conditions may vary too. Mayzumi *et al.* (1997) reported black *Ganoderma* from China to have extreme intolerance to low temperatures (20°C) and red *Ganoderma* from Japan to be cold tolerant.

Growth data are dependent on the cultivation media, environmental parameters, species and the region from which the fungus is isolated. Thus, a local cultivator would need to investigate the optimal growth conditions for each species and strain they are working with, rather than simply use the conditions reported as optimal for a different environmental region.