OPTIMIZING GROWING CONDITIONS FOR THE TROPICAL MUSHROOMS, *Clitopilus* species AND *Agaricus subrufescens*

KRITSANA JATUWONG

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EXAMINATION COMMITTEE

....CHAIRPERSON

(Assoc. Prof. Kevin D. Hyde, Ph. D.)

....ADVISOR

(Pattana Kakumyan, Ph. D.)

....EXAMINER

(Sunita Chamyuang, Ph. D.)

....EXTERNAL EXAMINER

(Prof. Saisamorn Lumyong, Ph. D.)

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Author	Kritsana Jatuwong
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ABSTRACT

The aim of this study was to study taxonomy and phylogeny, optimal condition for mycelium growth, antibacterial, antioxidant, and enzyme activities of *Clitopilus doimaesalongensis* strain MFLUCC13-0806 and *C. chalybescens* strain MFLUCC13-0809 and to investigate the optimal conditions for the cultivation of *Agaricus subrufescens* hybrid strains in northern Thailand.

Collection of *Clitopilus* were made in the rainy season during the period 2012-2013 in northern Thailand. They were studied in terms of taxonomy and phylogeny. Two collections were identified as *Clitopilus chalybescens* and another as a new species, *C. doimaesalongensis*. *Clitopilus doimaesalongensis* (MFLUCC13-0806) and *C. chalybescens* (MFLUCC13-0809) were determined for optimal growth conditions; type of media, temperature and pH. The optimal conditions for *C. doimaesalongensis* were cultivation on malt extract agar (MEA), supplemented with sucrose and tryptone, pH can range from 5-9 at a temperature range from 27-29˚C. Yeast extract agar (YEA), a pH of 5-8 and temperature range of 20-29˚C were optimal conditions for *C. chalybescens* and this mushroom can use fructose, sucrose, starch, malt extract, yeast extract, tryptone and beef extract as carbon and nitrogen sources. The antibacterial activity from culture mycelium extracts and broth cultured extracts of

C. doimaesalongensis inhibited the growth of Gram positive bacteria; *Bacillus subtilis* and *Staphylococcus aureus* and Gram negative bacteria; *Pseudomonas aeruginosa*. This activity, however, was not observed on dried mycelial extracts. *Clitopilus chalybescens* could inhibit the growth of Gram positive bacteria; *B. subtilis* and *S. aureus*, but not Gram negative bacteria. Antioxidant assay indicated that the crude extracts had noticeable scavenging activity on 2,2-diphenyl-1-picryl-hydrazyle (DPPH) radical about 21.41% and 26.45% DPPH scavenging activity for *C. chalybescens* and 29.14% and 40.62% for *C. doimaesalongensis* at 40 and 50 mg/mL, respectively. Cellulase, xylanase and amylase activities were detected in *C. doimaesalongensis*.

Agaricus subrufescens is an edible mushroom, one of the most important medicinal mushroom with high potential to treat many diseases. Several strains of *A. subrufescens* have been cultivated throughout the world, especially in Brazil. The *Agaricus subrufescens* hybrid strain used in this study has bred at INRA, France and successfully cultivated. The optimum conditions for the cultivation of *A. subrufescens* hybrid strains in northern Thailand were investigated in this study. Suitable temperatures and pH for mycelium growth were established on compost extract agar. The optimum temperature and pH for mycelial growth ranged from 25-30˚C and pH 7-9. The four hybrid strains could be grown and produced fruiting bodies on rice straw compost. *Agaricus subrufescens* hybrid of Thai and Brazilian CA 918-076 × CA 454-4 (T2×B) produced the highest yield (56.92 g/kg of compost) among the studied strain.

Keywords: *Clitopilus*/ optimal condition/ antibacterial activity/ antioxidant activity/ *Agaricus subrufescens* hybrid strain/ compost/ mushroom cultivation

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The basidiomycetes are very large group and popular classified because of their diversity and properties. Mushrooms are macro fungi belonging to Basidiomycota called for their characteristic sexual reproductive structures; they have the basidiospores that are produced on basidium (plural, basidia). Their basidiocarps are formed completely of secondary mycelium (Raven & George, 2002). They are abundant in the forest ecosystem around the world (Pushpa & Purushthama, 2012). Most of the mushrooms grow in humid conditions whether in the soil, on dead logs and trees (Bankole & Adekunle, 2012). These include many type of fleshy fungi such as bracket fungi, fairy clubs, toadstools, puffballs, stinkhorns, earthstars, bird's nest fungi and jelly fungi (Vyas, Chaubey & Dehariya, 2014). Some mushrooms are edible/medicinal and many of them are poisonous species (Firenzuoli, Gori & Lombardo, 2008). Mycelium mushrooms have been isolated and can be cultured in broth or agar. Fruit bodies contain biologically active polysaccharides that are the best known as most potent mushroom-derived substances with antitumor, immunomodulation and anti-cancer properties (Mizuno et al., 1996; Mizuno et al., 1999; Borchers, Stern, Hackhman, Keen & Gershwin 1999; Ooi & Liu, 1999; Wasser & Weis, 1999; Tzianabos, 2000; Reshetnikov, Wasser & Tan, 2001). The family *Agaricacae* is one of the most diverse and large of Basidiomycetes. Many of them have been introduced based on molecular and morphological studies (Vellinga, de Kok & Bruns, 2003; Thongklang, Nawaz et al., 2014; Chen et al., 2015). Their diversity are important role in evolution and ecosystem. Various studies on morphological and molecular characterization are reveals several a new genera, new species and new record were discovered such species of genus *Agaricus, Auricularia, Macrolepiota, Lactarius, Rusula* (Vellinga et al., 2003; Vellinga, 2004; Nascimento & Alves, 2014; Thongklang, Nawaz et al., 2014; Chen et al., 2015;

Liu et al., 2015; Bandara, Chen, Karunarathna, Hyde & Kakumyan, 2015). Although, genus *Clitopilus* has received little study in Thailand but it is a widespread in several country.

1.2 General Characteristic of Genus *Clitopilus*

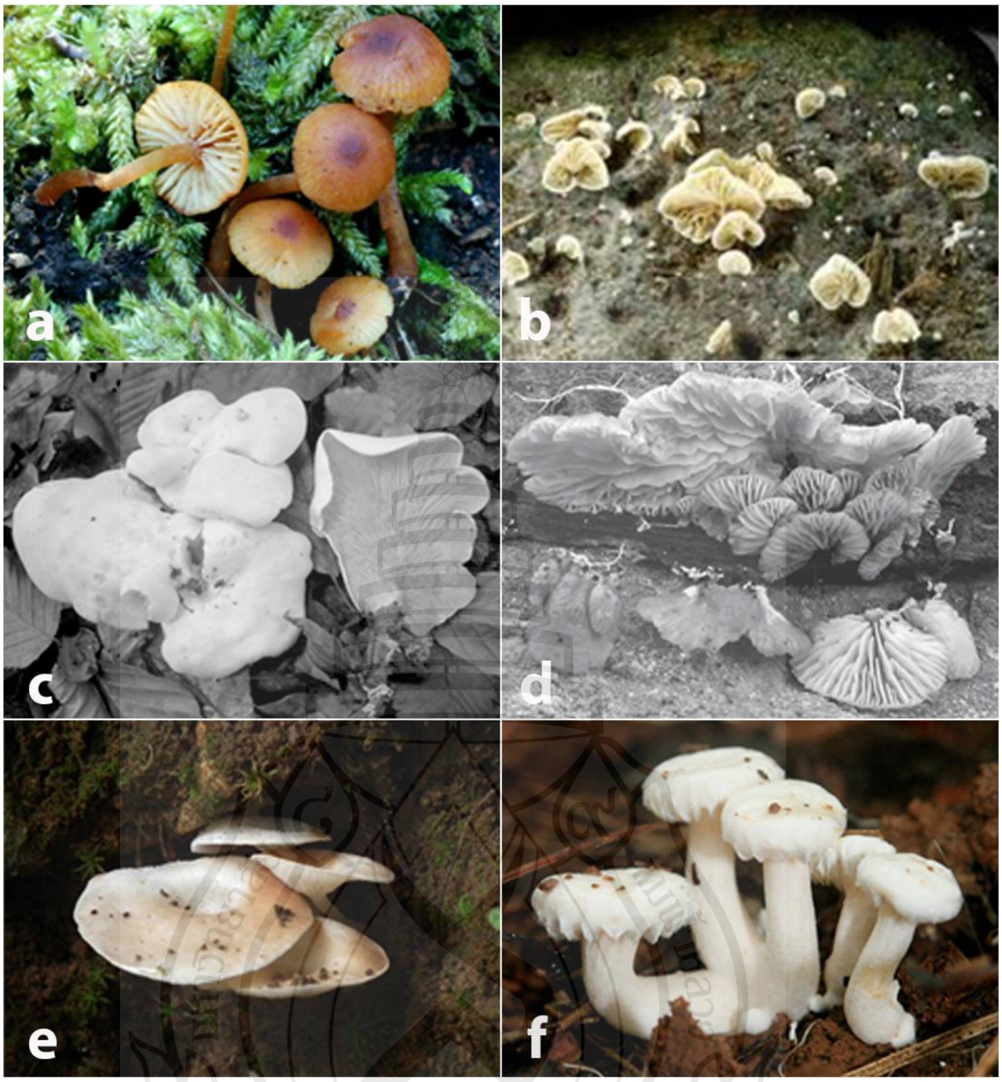
Clitopilus (Fr. Ex Rabenh.) P. Kumm is a small genus of fungi belonging to the family of the *Entolomataceae* in the order Agaricales within the division Basidomycota. The classification of genus *Clitopilus* is shown in Figure 1.1. A type species of this genus is *Clitopilus prunulus* commonly known as the miller or the sweetbread mushroom (Co-David, Langeveld & Noordeloos, 2009; Vyas et al., 2014).

This is a genus having mostly collybioid or clitocyboid basidiomata (Moreno, Contu, Ortega, Platas & Peláez, 2007; Takahashi & Degawa, 2011). It is traditionally included saprotrophic or mycoparasitic species (Vizzini, Musumeci, Ercole & Contu, 2011). It is separated into two groups: one is more or less clitocyboid with central stipe and decurrent lamellae, and the other group is often small pleurotoid basidiocarps with lateral and reduced stipe (Noordeloos, 2008) (Figure 1.2). Recently, the genus *Clitopilus* have been recorded of 326 species in the Index Fungorum including nine sections and four subgenus (Table 1.1).

Kingdom	:	Fungi
Division	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Agaricales
Family	:	Entolomataceae
Genus	:	*Clitopilus*

Source Index Fungorum (2015)

Figure 1.1 The scientific classification of genus *Clitopilus*



Note. (a) *Clitopilus rubroparvulus*, (b) *Clitopilus kamaka*, (c) *Clitopilus chrischonensis*, (d) *Clitopilus byssisedoides*, (e) *Clitopilus chalybescens* (this study), (f) *Clitopilus doimaesalongensis* (this study)

Source Noordeloos, Co-David & Gminder, 2010; Vizzini, Dähncke & Contu, 2011; Vizzini, Musumeci et al., 2011; Cooper, 2014

Figure 1.2 Basidiomes of *Clitopilus* species

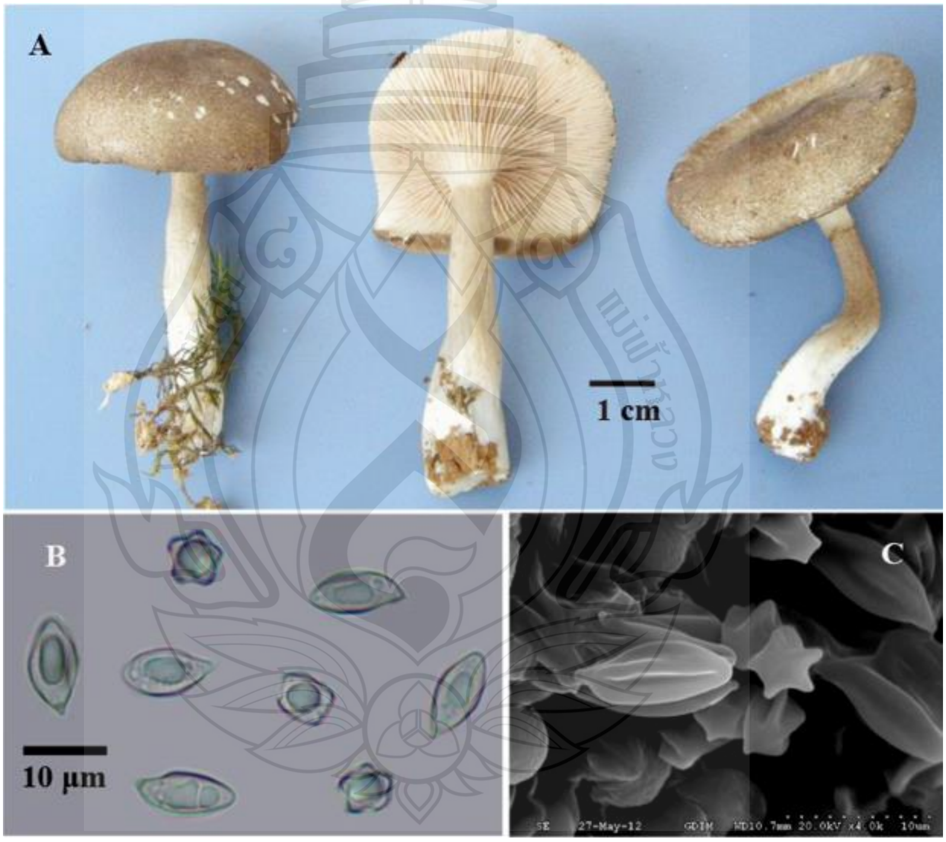
Table 1.1 Record of section and subgenus of genus *Clitopilus*

Section and subgenus	References
Clitopilus sect.* *Clitopilus* (Fr. ex Rabenh.)	Kumm (1871)
Clitopilus sect. *Omphaloidei*	Moreno et al., (2007)
Clitopilus sect. *Clitopilopsis* (Maire)	Contu (2009)
Clitopilus sect. *Claudopodes*	Contu (2009)
Clitopilus sect. *Crepidotoides* (Singer)	Contu (2009)
Clitopilus sect. *Decurrentes* (Konrad & Maubl.)	Contu (2009)
Clitopilus sect. *Rhodocybe* (Maire)	Contu (2009)
Clitopilus sect. *Rufobrunnei* (Singer ex T.J. Baroni)	Contu (2009)
Clitopilus sect. *Tomentosi* (T.J. Baroni)	Contu (2009)
Clitopilus subgen.** *Clitopilus* (Fr. ex Rabenh.) P.	Kumm (1871)
Clitopilus subgen. *Decurrentes* (Konrad & Maubl.)	Contu (2009)
Clitopilus subgen. *Rhodocybe* (Maire)	Contu (2009)
Clitopilus subgen. *Rhodophana* (Kühner)	Contu (2009)

Note. * sect = section, ** subgen = subgenus

Source Index Fungorum (2015)

Clitopilus species could be easily recognized by farinaceous odor and with the distinct characters of pink or brownish pink spores and its peculiarly shaped spores with longitudinal ribs, with five to twelve in number and appearing angular in polar view (Singer, 1986; Noordeloos, 2008). The pileus usually depressed or umbilicate, whitish to grayish when it dry and sometime slimy surface and a wavy margin. The gills are decurrent attached with the stipe, spaced together rather closely. The stipe is fleshy or fibrous, thick, white and ellipsoid in profile but rounded in polar view, without volva or annulus (Kauffman, 1981; Baroni, Desjardin & Hywel-Jones, 2001; Yang, 2007; Vizzini, Musumeci et al., 2011) (Figure 1.3).



Source Deng, Li & Shen (2012)

Figure 1.3 *Clitopilus ravus* (a) Basidiomata, (b,c) Basidiospores

1.3 Characteristics and Taxonomy Tools

The genera *Clitopilus* has been confused on the taxonomic and nomenclatural. It was earlier included within *Rhodocybe* Maire and *Entoloma* (Fr.) P.Kummer (Matheny et al., 2006). This genus was suggested about 30 species in 2008 then, has been published and redefined to include many species former *Rhodocybe* species by using based on sequence data in 2009 (Co-David et al., 2009).

This genera *Clitopilus* was updated in Index Fungorum and MycoBank as follows:

Clitopilus (Fr. ex Rabenh.) P. Kumm., Führ. Pilzk. (Zerbst): 96 (1871)

= *Agaricus* subgen. *Clitopilus* Fr. ex Rabenh., Deutschlands Kryptogamenflora 1: 507

= *Hexajuga Fayod*, Annales des Sciences Naturelles Botanique 9: 389 (1889)

= *Orcella Kuntze* ex Earle, Bulletin of the New York Botanical Garden 5: 430 (1909)

= *Paxillopsis* J.E. Lange, Flora Agaricina Danica 5: Vl (1940)

The classification of mushrooms are very important. They are identified through observing of their characters with naked eye, using hand lens, microscopes and molecular analysis is recently used. Therefore, the technique of *Clitopilus* can be classified based on morphological and molecular analyses.

1.3.1 Morphological Analysis

The taxonomic studies are very important for mushroom identification. Like other mushrooms, various studies have been reported based on macro and microscopic descriptions of *Clitopilus* species (Baroni et al., 2001; Moreno et al., 2007; Yang, 2007; Noordeloos et al., 2010; Vizzini, Dähncke & Contu, 2011; Takahashi & Degawa, 2011). The specimens are photographed in the field with the description of the macromorphological characteristic of the basidiomes. The specimens are dried under an electric drier and deposited in the herbaria.

Micromorpological characters are also studied from fresh and dried materials with standard methods, using sections mounted in 3-5% KOH, ammoniacal Congo red, Melzer's reagent, Cotton blue, Phoxin and Cresyl blue (Baroni, 1981). Microscopic structures such as spores, basidia and cystidia were observed under microscope.

1.3.2 Molecular Analysis

The molecular marker is widely used for mushroom identification and characterization. DNA typing relies on standard molecular methodologies such as PCR DNA amplification and Restriction Fragment Length Polymorphism (RFLP) mapping that may be easily applied to the taxonomic classification of any living system. The nucleotide sequencing of *Clitopilus* species use genomic DNA form either dried material or mycelium. DNA is amplified using a primers ITS1/ITS4 for the ITS region amplification (White, Bruns, Lee & Taylor, 1990; Gardes & Bruns, 1993) and primers LR0R/LR6 (Vilgalys & Hester, 1990) for the LSU. Thus, the amplification of the ITS and LUS was successful for the *Clitopilus* species (Moreno et al., 2007; Hartley et al., 2009; Vizzini, Musumeci et al., 2011; Crous et al., 2012, Cooper, 2014).

1.4 Ecology and Distribution

The genera *Clitopilus* is traditionally encompasses saprotrophic or mycoparasitic. Basidiomata is solitary or scattered on soil and probably can grow on decayed wood in primary forests in broadleaved or coniferous (Pinus) forests and in grasslands or on mosses in the rainy season or autumn (Yang, 2007; Noordeloos et al., 2010; Vizzini, Dähncke & Contu, 2011). and on its food source (Zhang, Geng, Shen, Wang & Dai, 2014). It is a widespread distribution in Europe, America, Oceania, North Africa, Southern Africa and Asia (Baroni et al., 2001; Moreno et al., 2007; Yang, 2007; Deng, Li & Shen, 2010; Noordeloos et al., 2010; Vizzini, Dähncke & Contu, 2011; Vizzini, Musumeci et a., 2011; Takahashi & Degawa, 2011; Deng et al., 2012; Crous et al., 2012; Noordeloos & Gates, 2012; Deng, Heng & Li, 2013; Cooper, 2014), especially in northern temperate areas and it has also been found in tropics and subtropics temperate area such as Taiwan, Thailand and China (Baroni et al., 2001, Yang, 2007; Deng et al., 2010; Deng et al., 2012; Deng et al., 2013) (Figure 1.4).

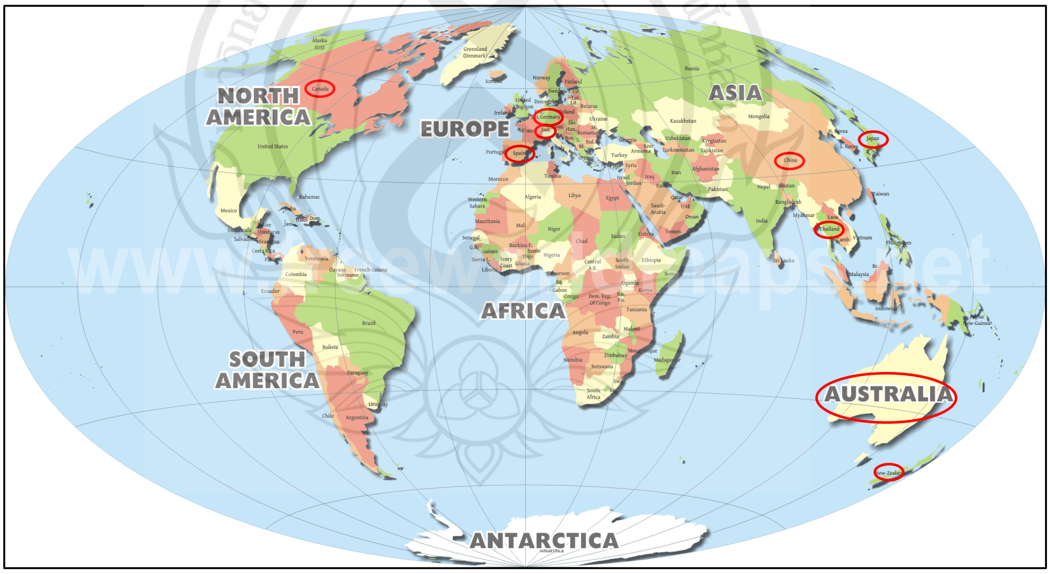


Figure 1.4 Distribution of *Clitopilus* in the world

Three species of *Clitopilus* were found and have been reported in Thailand including *C. prunulus*, *C. apalus* and *C. chalybescens* (Chandrasrikul et al., 2011) (Table 1.2). Moreover, several species from Canada, Switzerland, Spain, Japan, and Australia such as *C. fuscogelatinosus C. giovanellae, C. vernalis, C. austroprunulus* and *C. canariensis* were reported as a new species (Redhead & Baroni, 1986; Moreno et al., 2007; Vizzini, Dähncke & Contu, 2011; Vizzini, Musumeci et al., 2011; Takahashi & Degawa, 2011 Crous et al., 2012)

Table 1.2 Distribution of *Clitopilus* species in Thailand

Scientific name	Location	References
Clitopilus apalus (Berk. & Br.) Petch	Kek river, Phetchabun	Duengkae (2006)
Clitopilus chalybescens T.J. Baroni & Desjardin	Khao Yai National Park, Nakhon Nayok	Baroni et al. (2001) Jones and Hyde (2004)
Clitopilus prunulus (Scop. ex Fr.) Kummer	Deciduous Dipterocarp Forest, Mixed Deciduous Forest and Dry Dipterocarp Forest in Northeast	Museum of medicinal mushrooms (2008)

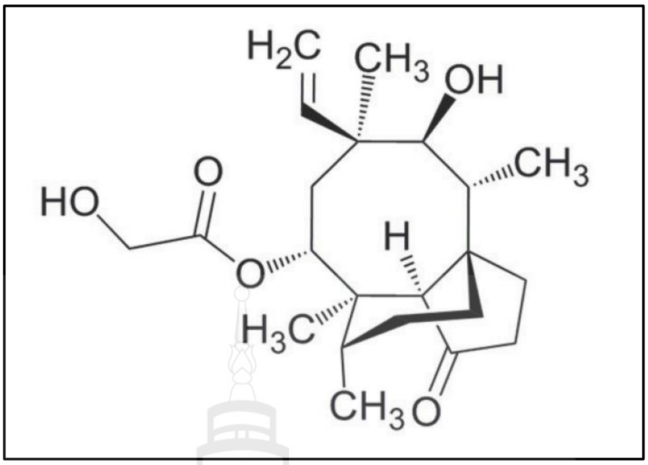
Source Chandrasrikul et al. (2011)

1.5 Bioactive Compounds

Mushrooms have long history as nutritionally food source and a source of physiologically beneficial medicines to use in therapies (Wasser & Weis, 1999; Wasser, 2002). They need antibacterial and antifungal compounds to survive in their environment. Mushroom cell wall consists of polysaccharides (glucans) derived from both fruiting bodies and mycelium and it is well-known to have a property against bacteria and viruses (Wasser, 2002; Barros et al., 2007). In fact, many mushrooms have been established in traditional medicines for various therapeutic properties such as immunological and anti-cancer. Many medicinal functions are produced by mushrooms and fungi and are referred to other potentially important health benefits including antioxidants, anti-hypertensive and cholesterol-lowering properties, liver protection, as well as anti-inflammatory, anti-diabetic, anti-viral and anti-microbial properties (Yu et al., 2009; Zhang, Cui, Cheung & Wang, 2007; Finimundy et al.,2013). In addition, mushroom extracts have been exhibited to provide health promoting benefits (Marshall & Nair, 2009). Several pure compounds from mushrooms such as *Ganoderma lucidum* (Lingzhi), *Laetiporus sulphureus* (Chicken mushroom) and *Lentinus edodes* (Shiitake) were reported to have antioxidant, anti-tumor, anti-inflammatory and antimicrobial activities (Hong, Dunn, Shen & Pence, 2004; Wong, Chao, Chan, Chang & Liu, 2004; Turkoglu et al., 2007; Joseph et al., 2011; Kao, Jesuthasan, Bishop, Glucina & Ferguson, 2013; Klaus et al., 2013).

1.5.1 Antibacterial Activity

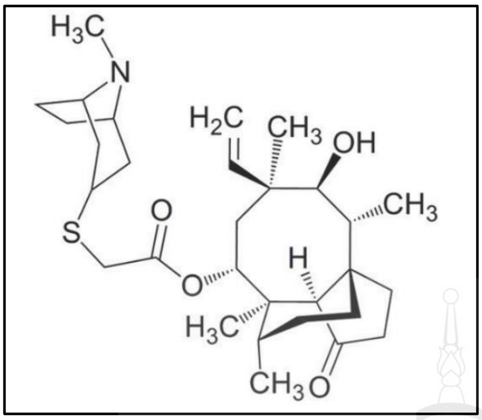
Some *Clitopilus* species produce bioactive compound known as a pleuromutilin (Figure 1.5). This compound processed a strong antimicrobial activity (Kavanagh, Hervey & Robbins, 1951; Kilaru, Collins, Hartley, Bailey & Foter, 2009). and has been used in veterinary medicine for over 20 years (Stewart, 1986; Papa, Zulaybar & Raymundo, 2006). Pleuromutilin was first discovered and isolated by Kavanagh et al. (1951) from *Pleurotus passeckerianus* and *Pleurotus mutilis*, which is now referred to *C. passeckerianus* and *C. scyphoides*, respectively. After that *C. insitus*, *Drosophila subatrata* and several species of genus *Pleurotus* have also been reported to produce pleuromutilin (Kavanagh, Hervey & Robbins, 1952; Knauseder & Brandl 1976; Stewart, 1986).



Source Stadler & Hoffmeister, 2015

Figure 1.5 Chemical structure of pleuromutilin

The activity of the pleuromutilin is primarily against gram-positive bacteria, such as *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Bacillus subtilis* (Kavanagh et al., 1951; Kilaru et al., 2009). According to Poulsen, Karlsson, Johansson and Vester (2001) who reported that pleuromutilin derivatives; tiamulin and valnemulin, were used as veterinary medicine to treat swine dysentery and enzootic pneumonia. A novel semisynthetic pleuromutilin was discovered by GlaxoSmithKline as Retapamulin {mutilin 14-(exo-8-methyl-8-azabicyclo[3.2.1]oct-3-yl-sulfanyl)-acetate}(Pankuch et al., 2006). Retapamulin, a derivative of the fungal secondary metabolite pleuromutilin, is active against *S. aureus* and *Streptococcus pyogenes.* The first pleuromutilin derivative has been formulated as a topical antibacterial agent to use as human antibiotic for treating human skin infections (Boyd & Castaner, 2006; Butler & Buss, 2006; Jones, Fritsche, Sader & Ross, 2006; Daum, Kar & Kirkpatrick, 2007; Odou, Muller, Calvet & Dubreuil, 2007). Then, in 2007 a 1% retapamulin ointment was approved by the Food and Drug Administration and the European Medicines Agency (Parish et al., 2006) which the product named ALTABAX® (Figure 1.6) to use for the treatment of impetigo caused by *S. aureus* and *S. pyogenes* (Coutinho, 2007).



Note. (a) Chemical structure of retapamulin, (b) Retapamulin (ALTABAX®)

Source Coutinho, 2007; Stadler & Hoffmeister, 2015

Figure 1.6 Natural product from retapamulin which the product named ALTABAX®

1.5.2 Anti-tumor and Immunomodulation Properties

Many mushroom polysaccharides and polysaccharide-protein complexes have been used as a source of biological activities. Many mushroom species such as *Agaricus blazei*, *Auricularia auricular*, *Ganoderma Lucidum* and *Lentinus edodes* are known as the most potent mushroom derived substances with antitumor and immunomodulating properties (Chihara, Hamuro, Maeda, Arai & Fukuoka, 1970; Ukai et al., 1983; Miyazaki & Nishijima, 1981; Mizuno, Wasa, Ito, Suzuki & Ukai, 1992; Hobbs, 2000). Antitumor polysaccharides have been reported to obtain in fruit bodies, cultured mycelium and culture broth of basidiomycetes (Zhang et al., 2007). The antitumor activity of extracts form *C. abortivus*, *Boletus edulis* and other mushrooms were first reported by Byerrum et al. (1957). In 1996 the anti-tumor substances from *C. caespitosus* were identified as polysaccharides isolated from fruiting body (Liang, Miao & Zhang, 1996).

1.5.3 Antioxidant Activity

Antioxidant is an inhibitor of the process of oxidation reactions. Phenolic compounds (phenolic acids and flavonoids), carotenoids, tocopherol and ascorbic acid found in vegetables, fruits, eggs, legumes and nuts (Zhang & Hamauzu, 2003; Hamid, Aiyelaagbe, Usman, Ameen & Lawal, 2010) are a good sources of antioxidants. They contain many different antioxidant components which are important protective agents for human health (Block, Patterson & Subar, 1992). Some mushrooms have currently shown potential antioxidant activity (Palacios et al., 2011). Several studies have investigated and found that some species of wild edible mushrooms are having antioxidant properties such as *Agaricus bisporus* (white and brown), *Lentinula edodes* (shiitake), *Pleurotus eryngii,* (king oyster) and *Pleurotus ostreatus* (oyster) (Reis, Martins, Barros & Ferreira, 2012; Gan, Nurul Amira & Asmah, 2013). While, Grangeia, Heleno, Barros, Martins and Ferreir (2011) reported the antioxidant property of *C. prunulus* on DPPH radicals scavenging activity.

1.5.4 Enzyme Activity

Enzyme are extracts in all living things, including in several plants, animals, microorganisms and mushrooms (Pandey et al., 2000; Sundar, Liji, Rajila & Suganyadevi, 2012). They are polypeptides which speed up chemical reaction in the cell. It has also been used as commercial applications for the development of a range of pharmaceutical and agrochemical agents such as anti-bacterial agents, anti-fungal agents, anti-viral agents, insecticides and herbicides (Bugg, 2004). In plants, agricultural residues and wastes consists of lignocellulosic (cellulose, hemicellulose and lignin content) which is needed to convert into simple sugars either enzymatic processes by (hemi)cellulolytic or chemical process by sulfuric or other acids (Kapu et al., 2012; Maitan-Alfenas, Visser & Guimaraes, 2015). Most of the mushrooms are saprophytic which mean they growing on wood, litter decomposition of organic matter (Miles & Chang, 1997). During their growth of mycelia and development into mature fruiting bodies, enzymes are secreted extracellularly to degrade the plant polymers lignin, hemicellulose and cellulose into simple and soluble molecules that are utilized by intracellular enzymes within the mushroom. The three group of enzymes consist of cellulases, hemicellulases and ligninases are involved in the breakdown of lignocellulosic biomass degradation. They includes the oxidative (laccase, manganese peroxidase and lignin peroxidase), which are involved in lignin degradation (Hofrichter, 2002; Shah & Nerud, 2002). and hydrolytic enzymes, xylanase and cellulase, which are involved in hemicellulose and cellulose degradation, respectively (Gan, Allen & Taylor, 2003; Hu, Arantes & Saddler, 2011). Consequently, enzymes are important role in mushroom development; in addition, they also affect the food nutrient, flavour and shelf life (Scarse, 1995; Rashad, Abdou, Mahmoud & Nooman, 2009; Yang, Dai, Ding & Wyman, 2011). Both mushroom fruiting bodies and mycelia contain some important enzymes such as cellulase, amylase, xylanase, glutamate dehydrogenase, laccase, isocitrate lyase and cytochrome (Lee et al., 2007; Jonathan & Adeoyo, 2011). Amylases and cellulases are important enzymes that can be utilized for various biological activities. They have been screened and studied for their commercial utility (Wang, 1989; Diez & Alvarez, 2001). Basidiomycetes have also been reported for the production of extracellular hydrolytic enzymes that are useful in biotechnological applications (Goud, Suryam, Lakshmipathi & Charya, 2009). Among these Basidiomycete, some species of *Clitopilus* such as *C. prunulus* was screened for the extracellular hydrolytic enzymes and promoting amylase activity (Goud et al., 2009) but very little known about their hydrolytic or oxidative extracellular enzyme. Therefore, this study was to investigate the production of amylase, cellulase and xylanase activity in *Clitopilus doimaesalongensis*.

1.6 History of Edible Mushrooms Cultivation

The diversity of mushrooms are over 200 genera including both edible and poisonous species and have been part of the fungal diversity for around 300 million years (Chang & Miles, 2004). The wild edible mushrooms have been collected and consumed by human for thousand years because edible mushrooms are showing several bioactive substances for health benefits (Cheung, 2010; Villares, Mateo-Vivaracho & Guillamón, 2012). rich in minerals, protein and fiber. They also contain low fat, calories, carbohydrates, calcium and good vitamins (Firenzuoli et al., 2008). Edible mushrooms have been popularly consumed and have potential nutrient; they were produced as medicinally effective products that their medicine values can benefit the immune system and provide health benefits for human for example anti-tumor, anti-microbial, wound-healing and hypocholesterolemic (Chang, 1999; Dai, Yang, Cui, Yu & Zhou, 2009; Roupas, Keogh, Noakes, Margetts & Taylor, 2012). Several species has a long history for cultivation such as *Auricularia auricula* which was the first mushroom that was cultivated around 600A.D. in China and latter *Flammulina velutipes* was also cultivated in China around 800-900A.D. then, between 1000-1100A.D. *Lentinula edodes* was cultivated for the first time. Furthermore, *Volvariella volvacea* and *Tremella fuciformis* were firstly cultivated around 1700 and 1800 in China, respectively (Chang & Miles, 1987).The historical record of the intentional cultivation of several important edible mushroom are shown in Table 1.3.

Table 1.3 Historical recorded of edible mushrooms cultivation

Species	Date first cultivation	Earliest record	References
Auricularia auricula	600 AD	659	So Jing (So Gung) 659
Flammulina velutipes	800-900AD	Late T'ang Dynasty (618-907)	Han O (interpreted by Shou Cheng, 1981)
Lentinula edodes	1000-1100 AD	1313	Wang Cheng (interpreted by Shou Cheng, 1981)
Agaricus bisporus	1600 AD	1650	DeBonnefons (cited by Atkins, 1979)
Volvariella volvacea	1700 AD	1822	Yuen (1822)
Tremella fuciformis	1800 AD	1866	Fung & Chih 1983 (cited by Sze Yue, 1983)
Pleurotus sajor-caju	1974 AD	1974	Jandaik (1974)
Pleurotus ostreatus	1900 AD 1930 AD	1910	Falck (cited by Zadrazil)
Calocybe indica	1972	1974	Purkasyatha & Chandra (1974)

Source Chang & Miles, 1987

In general, there are several methods for mushrooms cultivation including cultivation of saprotrophic species in composted from waste material, cut logs or sawdust and inoculation of mushroom mycelium to the roots of living trees for mycorrhizal species (Obodai, Cleland-Okine & Vowotor, 2003; Royse & Sanchez-Vazquez, 2003; Jesus, Kohori, Andrade & Minhoni, 2013). Mushrooms occur widely around the world in difference favorable climatic, regions, seasons and habitats (Crous et al., 2006). Several species of genera such as *Agaricus, Auricularia, Pleurotus Lentinus* and *Volvariella* have become to wildly cultivate in the world (Martínez-Carrera et al., 2000).

Agaricus is a large genus with a gilled mushroom belongs to family Agaricacea (Agaricales Basidiomycetes) over 400 members worldwide containing both edible and poisonous species (Thongklang, Nawaz et al., 2014). *Agaricus bisporus* is the most cultivated edible mushrooms under controlled conditions with the based compost formulations (Baysal et al., 2007; de Andrade, Zied, de Almeida Minhon & Filho, 2008; Jesus et al., 2013). Moreover, *Agaricus subrufescens* is well known as a medicinal mushroom also cultivated at commercial level in Brazil and some Asian countries base on compost by using the local material as substrate and casing mixtures (Llarena-Hernández, Largeteau, Ferrer, Regnault-Roger C & Savoie, 2014). This species, however, has not been successfully cultivated in Thailand. Therefore, study of cultivation condition should be investigated.

1.7 Research Objectives

1.7.1 To collect the species of *Clitopilus* in northern Thailand and observe their characterization based on morphology.

1.7.2 To determine the optimization condition for mycelial growth of two tropical mushrooms, *Clitopilus* species and four hybrid strain of *A. subrufescens*.

1.7.3 To investigate the antimicrobial, antioxidant and enzyme activities of *Clitopilus doimaesalongensis*

1.7.4 To cultivate *Clitopilus* species hybrid strains of *A. subrufescens* on rice straw based compost media.

CHAPTER 2

TWO SPECIES OF *Clitopilus* FROM NORTHERN THAILAND

2.1 Introduction

Clitopilus is a small genus in the family Entolomataceae, order Agaricales traditionally including saprotrophic or mycoparasitic species, characterized by basidiomes with omphaloid, clitocyboid to pleurotoid habits, subcurrent to decurrent lamellae, pink spore prints and basidiospores distinctively furrowed by 3-12 longitudinal ribs (Singer, 1986; Noordeloos, 2008). *Clitopilus* species can easily be recognized by a characteristic farinaceous odor. The type species of *Clitopilus* was described by J. A. Scopoli in 1772 as *Agaricus prunulus* Scop., but in 1871, P. Kummer transferred it to the new genus *Clitopilus* and designated the type as *Clitopilus prunulus* (Scop.) P. Kummer.

The genus *Clitopilus* has a widespread distribution especially in northern temperate areas and was earlier included within *Rhodocybe*. Although in 2008 estimate suggested that there are about 30 species in the genus (Kirk, Cannon, Minter & Stapers, 2008) a more recent publication in 2009 using molecular phylogenetics suggests that the genus should be redefined to include many former *Rhodocybe* species (Co-Devid et al., 2009).

In Thailand, there have been very few studies on *Clitopilus*, only *C. apalus*, *C. prunulus* (Chandrasrikul et al., 2011) and *C. chalybescens* (Baroni et al., 2001) were found and isolated. This study introduced a novel species of *Clitopilus* from Thailand and a collection of *C. chalybescens* with morphological character and ITS rDNA sequence data.

2.2 Materials and Methods

2.2.1 Sample Collection

Three specimens of *Clitopilus* were collected during numerous field excursions between 2012 and 2013 in Northern Thailand (Chiang Rai and Lampang provinces). The basidiomata were photographed in *situ* and cleaned (Figure 2.1). Specimens were placed in a plastic box after encasing with aluminium foil to prevent damage and then taken to the laboratory for macromorphological studies. The specimens were dried in a standard food dryer at 40˚C for 24-48 hours then, sealed in Zip lock plastic bags containing dehydrated silica gel to control humidity. All herbarium specimens are deposited in the Mae Fah Luang University Herbarium (MFLU HERB), Chiang Rai, Thailand. Faces of Fungi and Index Fungorum numbers were obtained from Subashini, Ariyawansa, Liu, Jones & Hyde (2015) and Index Fungorum (2015). The collections used in this study were shown in Table 2.1.

2.2.2 Taxonomy

2.2.2.1 Macro-morphological characteristic examination

Significant information about the specimens and macromorphological characteristics of specimens were annotated in the laboratory. All freshly collected specimens were morphologically characterized based on macrocharacters such as plieus, lamella and stipe. The colour terminology used for macromorphological identification is from the Methuen handbook of colour (Kornerup & Wanscher, 1978).

Table 2.1 *Clitopilus* species used in this study

MLFUCC Code	Scientific name	Collecting site
MFLUCC13-0806	*C. doimaesalongensis*	Doi Mae Salong, Mae Fah Luang District, Chiang rai
MFLUCC13-0808	*C. chalybescens*	Maecham Village, Chaeson district, Lampang
MFLUCC13-0809	*C. chalybescens*	Maecham Village, Chaeson district, Lampang

Note. Fruiting bodies on soil (a)-(b) *Clitopilus chalybescens*, (c) *Clitopilus doimaesalongensis*. Scale bars: (a)-(b) = 25 mm, (c) = 10 mm.

Figure 2.1 Basidiomata of *Clitopilus* species in the field

2.2.2.2 Micro-morphological characteristic examination

Microscopic work was carried out with the dried specimens by taking free hand sections under a dissecting microscope (Motic SMZ-171). Slide preparation was implemented with 3-5% KOH and 1% Congo red. A Nikon Eclipse 80i microscope was used for microscopic evaluation of basidiospores, basidia, and pileipellis. Image acquisition was made with a Canon 550D digital camera. All these evaluations were carried out with a Nikon Eclipse 80i microscope at 200x, 400x and 1000x magnification. In order to chronicle basidiospores (spore quotient), Q is used to mean “length/width ratio” of a basidiospore in side view. Basidiospore dimensions are based on the measurements of at least 25 basidiospores made in side view and measured excluding the hilar appendix or apiculus at 1000x magnification.

2.2.3 Phylogenetic Analysis

2.2.3.1 DNA extraction, PCR and sequencing

Genomic DNA was extracted from dried specimen using the Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, P.R. China). The primer is designed base Internal Transcribed Spacer (ITS4) region. The PCR reaction was 25 µl of total volumes containing 1.0 µl template DNA, 9.5 µl double distilled water, 1.0 µl of each primer and 12.5 µl of 2x power Taq PCR Master Mix [A premix and ready to use solution, including 0.1 Units/µl Taq DNA Polymerase, 500 µm dNTP Mixture each (dATP, dCTP, dGTP, dTTP), 20 mM Tris-HCL pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, stabilizer and enhancer)]. The reaction was carried out with 35 cycles using the following conditions: denaturation (95ºC, 30 s), annealing (52ºC, 30 s), extension (72ºC, 1 min) and final extension (72ºC, 10 min). The primers used for sequencing were the same as those for amplification. Amplified products were confirmed with 1% agarose gel electrophoresis stained with ethidium bromide. Sequencing was performed on DNA analyser at Kunming Institute of Botany, Kunming, China.

2.2.3.2 Sequence alignment and molecular-phylogenetic analysis

Sequenced data were checked and assembled using BioEdit 7.0.9.0 (Hall, 1999) and blasted with those available in GenBank. The taxa information and GenBank accession numbers used in the molecular work are listed in Table 2.2. Sequences for each strain were aligned using Clustal X (Thompson, Gibson, Plewniak, Jeanmougin & Hugging, 1997). Alignments were manually adjusted to allow maximum sequence similarity. Alignment gaps were treated as missing data. Phylogenetic analysis were performed using PAUP* 4.0b10 (Swofford, 2002). Ambiguously aligned regions were excluded from all analyses. Trees were inferred using the heuristic search option with tree-bisection-reconnection (TBR) branch swapping and 1,000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Clade stability of the trees resulting from the parsimony analyses were assessed by bootstrap analysis with 5,000 replicates, each with 100 replicates of random stepwise addition of taxa (Felsenstein, 1985). Trees were figured in Treeview (Page, 1996) and formatted using PowerPoint 2013.

Table 2.2 Taxon information used for molecular analyses

Taxon	GenBank accession numbers (ITS)
Clitopilus amarus	KC885963
C. abortivus	FJ770404
C. austroprunulus	KC139084
C. brunnescens	HQ222033
C. chrischonensis	HM623128
C. crispus	JQ281489
C. cystidiatus	HM623130
*C. fallax**	AF357018
C. giovanellae	EF413030
C. hirneolus	KC710132
C. hobsonii	FJ770402
C. kamaka	KJ461903
C. passeckerianus	FJ770406
C. prunulus	EU273512
C. scyphoides	FJ770390
C. scyphoides	FJ770401
C. scyphoides	KC176282
*C. truncate**	FJ770393
C. chalybescens	KP938184 (this study)
C. chalybescens	KP938185 (this study)
C. doimaesalongensis	KP938183 (this study)
Lyophyllum decastes	AF357060

Note. * in GenBank these taxa are placed under the genus *Rhodocybe*

2.3 Results and Discussion

2.3.1 Taxonomy

2.3.1.1 *Clitopilus doimaesalongensis* Jatuwong, Karun. & K.D. Hyde **(**Figure 2.2)

Etymology: The species epithet "doimaesalongensis" refers to the place where the fungus was collected.

Faces of fungi number: FoF 00712

MycoBank: MB 811415

Holotype: MFLU13-0519

Pileus 11-19 mm in diameter, depressed with incurved margin; surface white to chalk white (A1), without staining, dull and sticky, winkle cover with the slightly matted silky-fibrillose squamules. Lamellae decurrent, 0.7 cm wide, crowded, narrow, white (A1) when young. Stipe 17-25 × 7-4.5 mm, central, cylindrical, solid, white (A1) throughout surface, smooth with silky-fibrillose squamules, basal part with white (A1) cottony mycelium. Odour strong farinaceous.

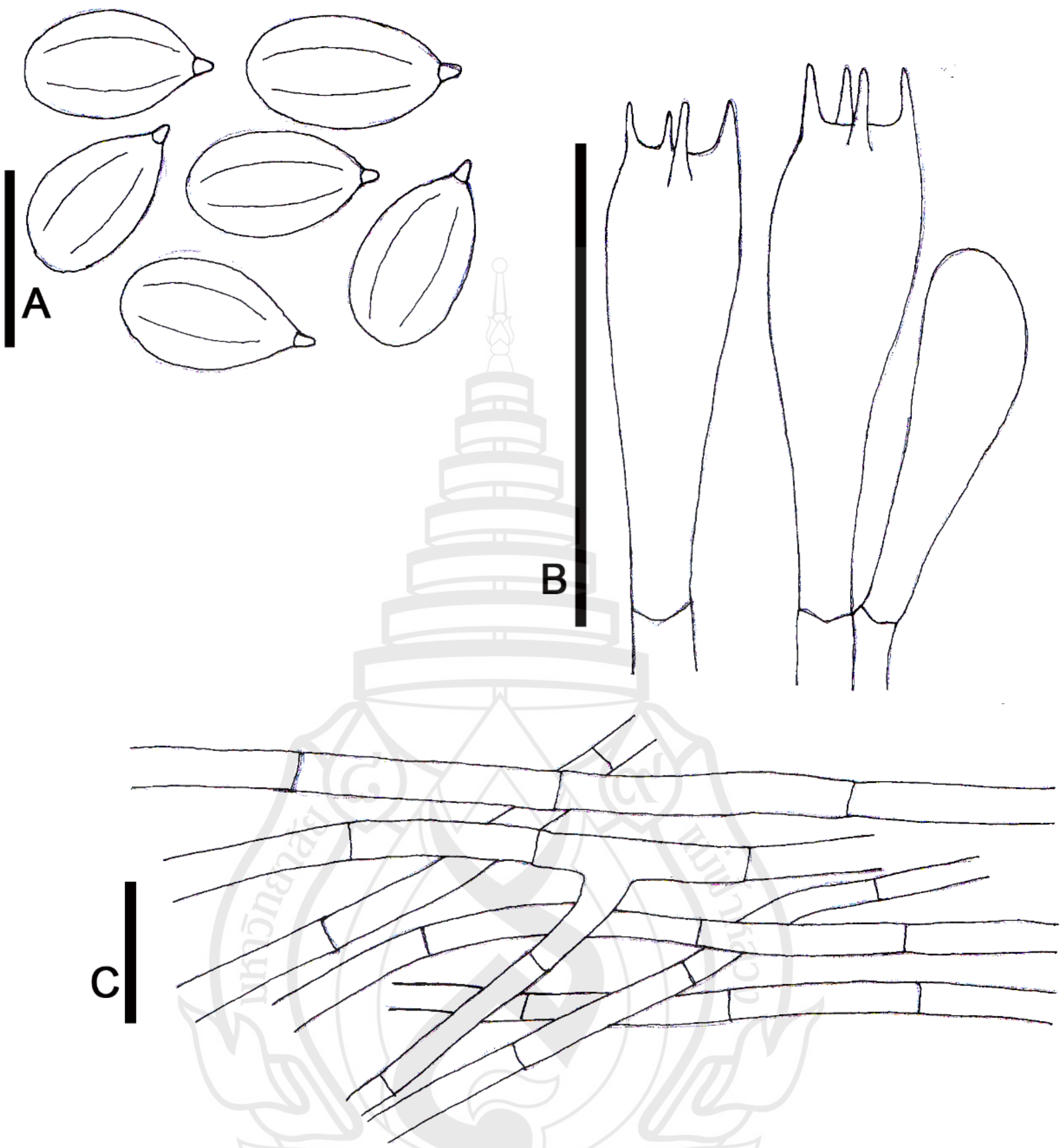
Basidiospores 6.8-9.2 × 4.1-5.5 µm (Q=2.1-3.9, Qav=2.9, n=30), hyaline, ellipsoid in polar view, amygdaliform to limoniform in side view. Basidia 9.1-21.1 × 3.3-7 µm, clavate, 4-spored. Pileipellis a layer of loosely arranged, hyaline hyphae. Cheilocystidia absent. Clamp connections absent in all tissues.

Habitat and distribution: Gregarious on soil as small groups, only known from Thailand.

Material examined: Thailand, Chiang rai Province: Doi Mae Salong, 26 June 2013, Samantha C. Karunarathna (MFLU13-0519, holotype); ex-type living culture, MFLUCC 13-0806

Notes: *Clitopilus doimaesalongensis* is characterized by its white to chalk white, small basidiomata, with an eccentric stipe, absence of pleurocystidia and cheilocystidia and amygdaliform to limoniform basidiospores.

In section *Clitopilus*, *C. chalybescens* was described from Thailand and differs from *C. doimaesalongensis* by its dull, dry, opaque, silky-felty surface, pure white to pale greyish blue pileus, the central stipe, 5.5-7.5 × 3.6-4.8 µm ellipsoid basidiospores, and cylindrical hyphae in the pileipellis (Baroni et al., 2001). The other closely related species to our new species are *C. chrischonensis* Musumeci Vizzini et Contu, *C. cystidiatus* Hauskn & Noordel and *C. austoprunnulus* Morgado, G.M. Gates & Noordel. *C. chrischonensis* was described from Switzerland, and differs from *C. doimaesalongensis* in its smooth cap surface, which is subglobose then irregularly convex and finally expanded without an umbo, cylindrical, 1-4 µm wide, 6-7 evident longitudinal ribs with 25-30 × 8-13 µm size basidiospores and, suprapellis consisting of cylindrical 1-4 µm wide hyphae (Vizzini, Musumeci et al., 2011). *Clitopilus cystidiatus* was described from Austria, and differs significantly in the slightly grey or greyish ochre tinges of the pileus, efibuiata with 4-spored, ellipsoid or oblong of basidiospores and encrusted pileipellis hyphae (Noordeloos, 2008). *Clitopilus austoprunnulus* differs from *C. doimaesalongensis* by its uniformly pale grey pileus which is convex when young then expanding to concave or infundibuliform and narrow, sometimes with a slight brown tinge at the centre and the pileipellis hyphae is cylindrical, 4-8 µm wide with dark brown walls (Crous et al., 2012).



Note. (A) Basidiospores, (B) Basidia, (C) Pileipellis. Scale bar: (A) 10 µm, (B) 20 µm, (C) 10 µm.

Figure 2.2 *Clitopilus doimaesalongensis*

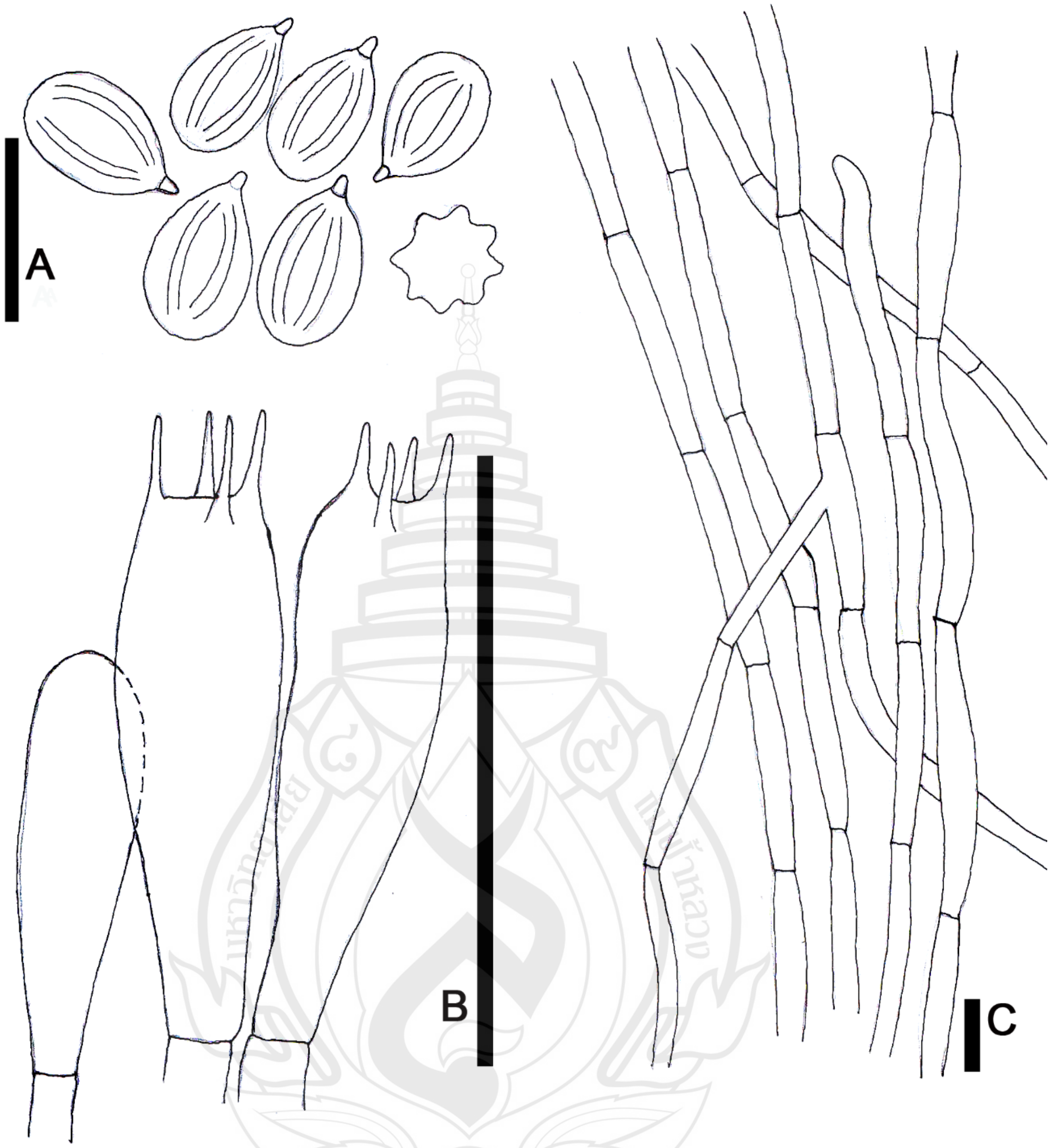
2.3.1.2 *Clitopilus chalybescens* T.J. Baroni & Desjardin, in Baroni, Desjardin & Hywel-Jones, Fungal Divers. 6: 13. 2001 (Figure 2.3)

Pileus 21-26 mm in diameter, infundibuliform, depressed centre with undulate to lobed margin, splitting with age; surface dry, dull, silky-fibrillose; pure white, often changing colour to yellowish white (2A2) in age. Lamellae decurrent, heavily crowded, narrow, white when young becoming pinkish white (10A2) with age. Stipe 20-25 mm, central, cylindrical, glabrous, dry, brittle, hollow, white overall, becoming yellowish white (2A2) with age. Odour strong farinaceous.

Basidiospores 5.3-7.1 × 3.7-5.0 µm, (6.2 × 4.2 µm, Q=1.3-2.2, Qav=1.6; n=30), hyaline, or pale pinkish in mass, ellipsoid in profile and face views, with moderately prominent longitudinal ridges, angled in polar view. Basidia 15-21 × 5.1-7.7µm, 4-spored, clavate. Lamellar trama of parallel or interwoven, hyaline, cylindrical hyphae. Pileipellis made up of loosely arranged, non-encrusted, hyaline, cylindrical hyphae. Clamp connections absent.

Habitat and distribution: Gregarious on soil, only reported from Thailand.

Material examined: THAILAND, Lampang Province: Chaeson, Maecham Village 18˚51′58.16″N, 99˚27′45.34″E, alt.1010m, 13 June 2013, Kritsana Jatuwong & Thasanee Luangharn (MFLU13-0520); 13 June 2013, Kritsana Jatuwong & Thasanee Luangharn (MFLU13-0521)



Note. (A) Basidiospores, (B) Basidia, (C) Pileipellis. Scal bar: (A) 10 µm, (B) 25 µm, (C) 10 µm.

Figure 2.3 *Clitopilus chalybescens*

2.3.2 Phylogenetic analysis

For the understanding on the phylogenetic relationships of our novel species *C. doimaesalongensis*, with other known *Clitopilus* species, we retrieved ITS sequence data from GenBank. The *Clitopilus* dataset comprised 21 collections, with 18 from GenBank, including the type species of the genus, *C. prunulus*, and the three *Clitopilus* collections from northern Thailand. *Lyophyllim decastes* (Fr.) Singer was chosen as the outgroup taxon. Of the 825 total characters, 429 characters are constant, of which 172 variable characters are parsimony-uninformative and 224 characters are parsimony-informative. The Maximum parsimony (MP) tree was produced after 6,188,809 rearrangements and the score of best tree found was 997. This most likely MP tree is presented and it was chosen to represent the phylogenetic position of *C. doimaesalongensis*.

Sequences were aligned and the alignment was subjected to Maximum parsimony analysis using PAUP to build the phylogenetic tree shown in Figure 2.4. The new species is supported by 100% bootstrap value and is closely related to *C. chalybescens* which was described from Thailand. The two sequences of *C. chalybescens* obtained from the two collections in Thailand and *C. doimaesalongensis* which was grouped in a monophyletic clade with 100% bootstrap support; new species was distinct in having unique morphological characters (Table 2.3).

According to the morphological and phylogenetic analyses of the three *Clitopilus* collections from northern Thailand, one collection was identified as a distinct new species in the genus *Clitopilus*, with 100% bootstrap support and distinct morphological features. The new species was closely related to *C. chalybescens* which was also described from Thailand. The comparison of macro- and micro-morphological characters and eight base pair differences between two sequences showed *C. doimaesalongensis* differs from *C. chalybescens* even though the phylogenetic analyses showed 100% bootstrap support. *C. chrischonensis*, *C. cystidiatus* and *C. austoprunnulus* are also phylogenetically closely related to *C. doimaesalongensis*, but according to the morphological features new species was distinct among the species in the genus.

Two of the three Thai collections were identified as *C. chalybescens* which was previously found at Khao Yai National Park, based on the macro- and micro-morphological features was described by Baroni et al. (2001). Thus we added sequence data of *C. chalybescens* to GenBank.

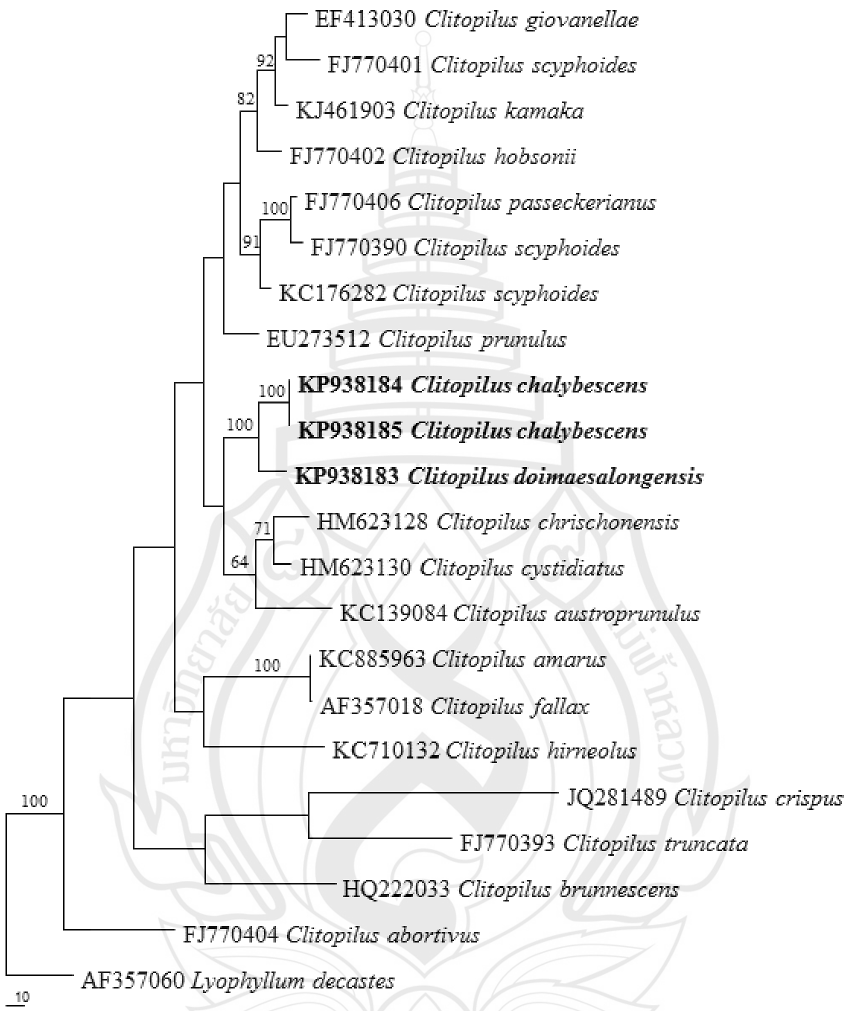


Figure 2.4 Maximum parsimony phylogram showing the phylogenetic position of *C. doimaesalongensis* sp. nov. with some selected *Clitopilus* species from GenBank based on ITS rDNA sequence data. Data were analyzed with random addition sequence, unweighted parsimony and gaps were treated as missing data. Values above the branches are parsimony bootstrap (≥ 50%). The tree is rooted with *Lyophyllum decastes*

Table 2.3 Comparison of *C. doimaesalongensis* with the closely related species

Scientific name	Basidiomata	Basidia	Basidiospores	Pileipellis
Clitopilus doimaesalongensis	- 11-19 mm in diameter - depressed with incurved margin - winkle cover with the slightly matted silky-fibrillose squamules	- 9.1-21.1 × 3.3- 7.0 µm size - clavate - 4 spored	- 6.8-9.2 × 4.1-5.5 µm size - ellipsoid in polar view amygdaliform to limoniform in side view - hyaline	- loosely arranged - hyaline
C. chrischonensis	- 40 mm diameter - smooth surface - first subglobose then irregularly convex - fially expanded without umbo	- cylindrical - 1-4 µm wide	- 9.5-11.5 (-13.5) × 5.6 µm - hyaline with 6-7 evident longitudinal ribs	- suprapellis consisting of cylindrical - 1-4 µm wide - hyphae

Table 2.3 (continued)

Scientific name	Basidiomata	Basidia	Basidiospores	Pileipellis
C. cystidiatus	- 20-70 mm diameter - slightly grey or greyish ochre tinges of the - pileus mealy-smelling context wider (4-7.5 µm) - encrusted	- 4 spored - efibuiata	- 9.5-13.5 × 5.0-6.5 µm - ellipsoid or oblong	- a cutis of cylindrical - 4.0-7.0 µm wide hyphae
C. austoprunnulus	- 40-90 mm diameter - convex when Young expanding to concave or infundibuliform - involute margin	- 20-30 × 4-8 µm - 4 spored	- (8-)9-11 × 4.5-6 µm - occasionally - amygdaliform distinctly ribbed lengthwise with 5-8 longitudinal ribs	- a cutis of densely packed - narrow - cylindrical - 4-8 µm wide hyphae with dark brown colored walls

Table 2.3 (continued)

Scientific name	Basidiomata	Basidia	Basidiospores	Pileipellis
C. chalybescens	- 15-45(-90) mm diameter - infundibuliform	- 17.8-21 × 6.4-8 µm - 4 streigmate - clavete sterigmata	- 5.5-7.5 × 3.6-4.8 µm - ellipsoid in profile and face view	- Hyaline - layer of loossy - entangled - cylindrical - hypha
C. crispus	- 20-70 mm diameter - convex to applanat - white to chalk white	- 20-30 × 8-10 µm - subclavate - 4 spored rarely 1, 2, or 3 spored	- (5.5-)6.0-7.5 × (4.0)4.5-5.5(-6.0) µm - ellipsoid to ellipsoid in side view - angular in apical view - 8-11 longitudinal	- absent

2.4 Conclusion

In this work, we isolated three collection of *Clitopilus* species in northern Thailand (Chiang Rai and Lampang). Based on morphological and molecular analyses. Two of the three collections were identified as *C. chalybescens*. This species was previously found at Khao Yai National Park, Thailand and one of these species was identified as *C. doimaesalongensis* which was a new species observed in Thailand.

CHAPTER 3

OPTIMAL CONDITIONS FOR GROWING MYCELIUM OF *Clitopilus doimaesalongensis* AND *Clitopilus chalybescens*

3.1 Introduction

Several mushrooms are highly nutritionally and pharmaceutically important. They have long been used as medicines and edible mushroom are used for foods because they contains high levels of proteins, fibbers, carbohydrates and low fat (Grangeia et al., 2011; Luangharn, Hyde & Chukeatirote, 2014; Rizal, Hyde, Chukeatirote & Chamyuang, 2015). Thus, edible mushrooms have also been consumed and cultivated in many countries including Thailand. They have been reported about 22 species as commercially grown in Thailand (Thawthong et al., 2014).

Mushrooms are popular used to treat various human diseases such as cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis and have demonstrated many interesting biological activity (Valverde, Hernández-Pérez & Paredes-López, 2015). There are several mushrooms compounds such as Lentinan from *Lentinus edodes* (Chihara et al., 1970) Schizophyllan from *Schizophyllum commune* (Tabata, Ito, Kojima, Kawabata & Misaki, 1981) and Krestin from *Coriolus versicolor* (Ng, 1998) were used as antitumor agents. Some species of *Clitopilus* have been reported to use as food and medicine (Boa, 2004) and many species are possessed biological activities (Kavanagh et al., 1951; Kilaru et al., 2009). Optimization the condition for mycelial growth may improve the production of mycelium and increase quality of mycelium (Papa et al., 2006). In addition, several study have been investigated to obtain optimal condition for mycelial growth from several mushrooms such as *Ganoderma lucidum* which was optimized for growth conditions using different culture media and physical parameters (pH, aeration, illumination and temperature) (Magday Jr, Bungihan & Dulay, 2014). Furthermore, *A. polytricha* (Xu & Yun, 2003), *Phlebopus portentosus* (Thongklang, Hyde, Bussaban & Lumyong, 2011), *Macrolepiota deters* (Rizal, Hyde, Chukeatirote, Kakumyan & Chamyuang, 2014), *L. tigrinus* (Dulay, Cabrera, Kalaw & Reyes, 2012), and many species of *Pleurotus* (Klomklung, Karunarathna, Chukeatirote & Hyde, 2014; Hoa & Wang, 2015), have also been investigated for their optimal growth condition of different environmental and nutritional factors. Although several study have been attempted extensively to obtain optimal culture conditions for mycelial growth from several mushroom but it has not been demonstrated extensively for *Clitopilus* species except *C. passeckerianus* (Vicente, Raymundo & Quimio, 2006). Thus, this study aims to determine the suitable conditions for mycelial culturing in solid media of *C. doimaesalongensis* and *C. chalybescens* which were isolated in this study and have not been carried out in Thailand so far.

3.2 Materials and Methods

3.2.1 The Sample Collection and Isolation

Two collections of *Clitopilus* strains including *C. doimaesalongensis* (MFLUCC 13-0806) and *C. chalybescens* (MFLUCC 13-0809) were collected from Chiang Rai and Lampang province, respectively. Internal tissues from the mushroom fruiting bodies were transferred to potato dextrose agar (PDA) and incubated at 25˚C for 10 days. Then, re-subculture until obtain axenic culture. The axenic cultures were maintained on PDA slants tubes and in 15% glycerol. The cultures were deposited at the culture collection of Mae Fah Luang University (MFLUCC) and coded as *C. doimaesalongensis* strain MFLUCC 13-0806 and *C. chalybescens* strain MFLUCC 13-0809.

3.2.2 Effect of Culture Media

A plug of mycelium with a 5 mm diameter was separately grown on five different culture media including potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), sabouraud dextrose agar (SDA) and yeast extract agar (YEA).

The culture plates were incubated in darkness at 25˚C. The growth of mycelium was evaluated by determination of the colony radial growth and mycelial dry weight in three replicates after inoculated for 12 days. The mycelial dry weight was processed by placed agar mycelial in a beaker, then the medium was melted and washed away with hot water. Mycelium was dried at 40˚C for 24 hrs and evaluated using analytical balance (Mettler Toledo ML204).

3.2.3 Effect of Temperatures

The optimal media obtained from previous experiment (3.2.2) was used to determine the best temperature. The plug of mycelium was centrally placed on the media plates and incubated in the dark at different temperatures; 20, 25, 26, 27, 28, 29 and 30˚C for 12 days. The growth of mycelium was determined by measuring the colony diameter and mycelial dry weight as previously describe in 3.2.2.

3.2.4 Effect of pH

The optimal pH of optimal media was evaluated. The media was adjusted separately to pH 4, 5, 6, 7, 8 and 9 with 1N NaOH or 1N HCl using a digital pH meter before sterilization. The medium were poured into Petri dish. The media plates were inoculated with a mycelium plug. All cultures were incubated at the optimal temperature. The growth of mycelium was determined by measuring the colony diameter and mycelial dry weight as previously describe in 3.2.2.

3.2.5 Supplement of Carbon and Nitrogen Source

Three different carbon sources including fructose, sucrose and starch was supplemented separately in the optimal media to study their effect on mycelial growth. Nitrogen sources including beef extract, tryptone and KNO_3 were used to study their effect on mycelial growth. Malt extract and yeast extract were used as control for the optimal media. The media were sterilized at 121˚C for 15 min. The mycelium plug was transferred to the centre of studied media, then all plates were incubated at 28˚C for 12 days. The growth of mycelium was determined by measuring the colony diameter and mycelial dry weight as previously describe in 3.2.2.

3.2.6 Cultivation of *Clitopilus doimaesalongensis*

3.2.6.1 Spawn preparation

The spawn of *C. doimaesalongensis* strain MFLUCC 13-0806 was prepared using sorghum as a substrate. The sorghum was firstly washed and soaked in distilled water for 24 hours, then washed 1-2 times before boiled for 15 minutes and the excess water was drained. Fifty grams of sorghum were placed into glass bottles and autoclaved at 121˚C for 15 minutes. After cooling to room temperature, the substrate was inoculated with the mycelium of *C. doimaesalongensis* from agar culture. All the bottles were incubated at 28˚C for 14 days or until completely colonized by mycelia.

3.2.6.2 Compost preparation

Various studies have been studied to find out the suitable variables whether composition of compost, type of casing layer, environmental conditions and methods of cultivation which are important variables for the mushroom cultivation to obtain yields, earliness and quality of mushroom production (Zied et al., 2011; Zied et al., 2012). Thus, the present study was to determine potential for cultivation of *C. doimaesalongensis*. Three compost formulation were prepared using rice straw as main substrate for composting and growing the mushroom. The substrate was supplemented with various ingredient by adding organic and inorganic fertilizers depended on the formula; Formula 1compost, rice straw was mixed with calcium carbonate, urea, rice bran, ammonium sulfate, calcium sulfate and water. Formula 2 compost was prepared by using rice straw mixed with pig manure, superphosphate, calcium ammonium nitrate, rice bran and water. Formula 3 compost was prepared by mixing rice straw mixed with chicken manure: rice hull (1:1), calcium sulfate, urea and water. Each compost formulation was fermented and periodically turned for 20-30 days, then pasteurized for 6 hours by maintaining the temperature at 55-60˚C. After cooling down, the 5 kg of formula 1 and 2 were transferred to plastic tray (35×25×20 cm) then, inoculated with 2% (w/w) of inoculum and incubated in dark room at the temperature approximately 22˚C with relative humidity at 85%. The completely colonized compost was then covered with the pasteurized casing. The formula 3 compost was placed into polypropylene bags (6.50×12.50 inch), sterilized at 121˚C for 30 minute. After sterilization, the compost bags were cooled down to room temperature, inoculated with inoculum and incubated at 28˚C.

3.2.7 Statistical Analysis

Experimental values were given as mean ± standard deviation (SD). The data of the experiment was analysed by using one way analysis of variance (ANOVA) in SPSS version 11.5 program for windows and treatments mean compared using Duncan' test ($p \leq 0.05$) followed by post-hoc tests.

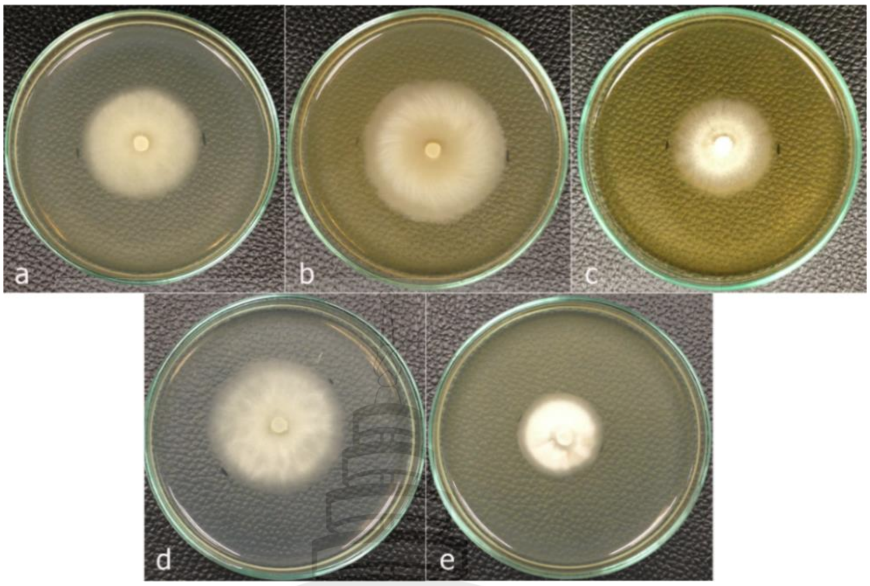
3.3 Results and Discussion

3.3.1 Mushrooms Morphology

Basidiocarps of *C. doimaesalongensis* and *C. chalybescens* occurred in gregarious soil were collected. Fresh fruiting body of *C. doimaesalongensis* was occurred with white to chalk white of surface, depressed with incurved margin with pileus size 11-19 mm in diameter. Fresh fruiting body of *C. chalybescens* was the umbillicate when young then infudibuliform (funnel-shaped) with white to yellowish white surface. Basidiocarps with pileus in diameter size 21-26 mm.

3.3.2 Effect of Culture Media

Five different culture media were used to examine a suitable media for mycelial growth of *C. doimaesalongensis* strain MFLUCC 13-0806 and *C. chalybescens* strain MFLUCC 13-0809. After 12 days of incubation at 28˚C, the result of *C. doimaesalongensis* was occurred with an average 5.30 cm of colony radial on MEA and CMA agar plate followed by PDA, YEA and SDA (Figure 3.1). However, the highest yield of mycelial dry weight was occurred on MEA medium with an average 107.03 mg (Table 3.1). For the *C. chalybescens* the highest mycelial dry weight of mycelial was occurred on MEA medium followed by YEA media with an average of 69.97 mg and 62.27 mg, respectively (Table 3.1). The fastest growth rate of this *C. chalybescens* on the agar plate with an average 8.63 cm of colony radial growth was observed in YEA media followed by CMA, MEA, SDA and PDA, respectively (Figure 3.2). Papa et al. (2006) and Vicente et al. (2006) reported a mycological agar was selected as a suitable agar medium for *C. passeckerianus*. However, this study found that MEA and YEA could promoted the growth of both *C. doimaesalongensis* and *C. chalybescens* and was selected for the further studies.



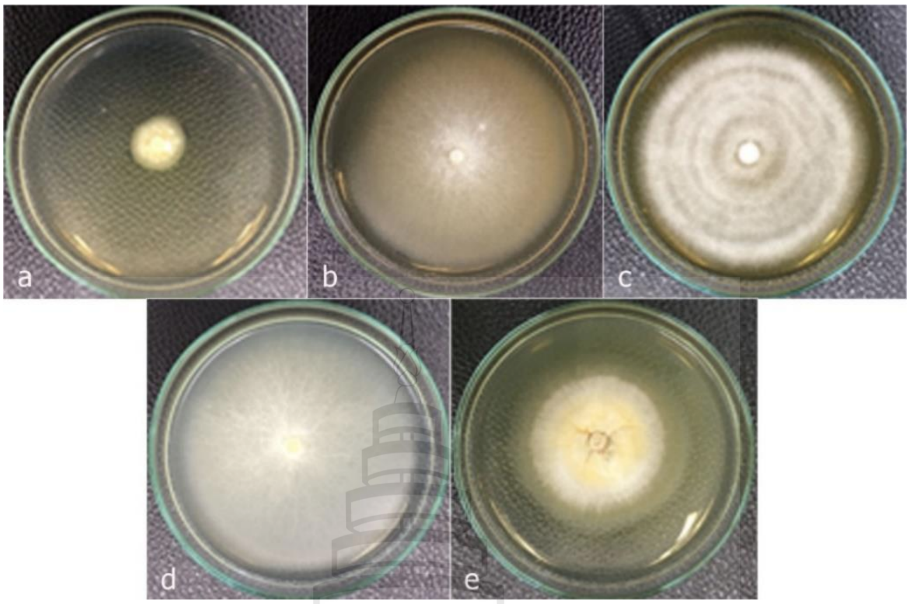
Note. (a) potato dextrose agar (PDA), (b) malt extract agar (MEA), (c) yeast extract agar (YEA), (d) corn meal agar (CMA), (e) sabouraud dextrose agar (SDA)

Figure 3.1 Mycelial growth of *C. doimaesalongensis* strain MFLUCC 13-0806 inoculated on different solid media at 28˚C for 12 days

Table 3.1 Mycelial growth and mycelial dry weight of *C. doimaesalongensis* strain MFLUCC 13-0806 and *C. chalybescens* strain MFLUCC 13-0809 inoculated on different solid media at 28˚C for 12 days.

Culture media	***C. doimaesalongensis***		***C. chalybescens***	
	Colony diameter (cm)	**Mycelial dry weight (mg)**	**Colony diameter (cm)**	**Mycelial dry weight (mg)**
PDA	4.63±0.07^{b}	64.44±4.68^{b}	2.27±0.50^{d}	14.03±2.30^{c}
MEA	5.30±0.10^{a}	107.03±13.68^{a}	7.90±0.36^{b}	69.97±2.78^{a}
YEA	3.83±0.17^{c}	23.57±10.05^{c}	8.63±0.21^{a}	62.27±6.48^{ab}
CMA	5.30±0.00^{a}	52.17±6.53^{b}	8.47±0.06^{ab}	54.23±7.62^{b}
SDA	3.23±0.07^{d}	53.77±6.06^{b}	6.63±0.42^{c}	56.63±2.04^{b}

Note. Values with the same letter are not significantly different ($p \leq 0.05$) by the Duncan's test.



Note. (a) potato dextrose agar (PDA), (b) malt extract agar (MEA), (c) yeast extract agar (YEA), (d) corn meal agar (CMA), (e) sabouraud dextrose agar (SDA)

Figure 3.2 Mycelial growth of *C. chalybescens* strain MFLUCC 13-0809 inoculated on different solid media at 28˚C for 12 days

3.3.3 Effect of Temperature

Different mushrooms have different optimal temperature such as the growth rate of *C. passeckerianus* was optimal at 24˚C (Vicente et al., 2006), *Pleurotus eryngii* and *Veriticillium fungicol* was at 25˚C (Zervakis, Philippoussis, Ioannidou & Diamantopoulou, 2001; Siwulski, Sobieralski, Górski, Lisiecka & Sas-Golak, 2011), *Pleurotus ostreatus* and *Pleurotus pulmonarius* has maximum growth at 30˚C and *Volvariella volvaceae* can grow at 35˚C (Zervakis et al., 2001). The optimum temperature is one of the most important factors in mushrooms cultivation, metabolic production and sporulation (Chang & Mile, 2004). Therefore, study of effect of temperature on the mushroom growth should be investigated. In this study, *C. doimaesalongensis* and *C. chalybescens* mycelia were grown under different temperatures (20-30˚C). The result showed that both of mushrooms were able to grow in the wide range of temperature from 20-30˚C. Nevertheless, the statistical analysis indicated that the temperature 27-29˚C were optimal temperature for the mycelial growth of *C. doimaesalongensis* and 20-29˚C for *C. chalybescens*. (Table 3.2).

Table 3.2 Mycelial growth and mycelial dry weight of *C. doimaesalongensis* strain MFLUCC 13-0806 and *C. chalybescens* strain MFLUCC 13-0809 cultivated at different temperature for 12 days.

Temperatures (˚C)	*C. doimaesalongensis*		*C. chalybescens*	
	Colony diameter (cm)	Mycelial dry weight (mg)	Colony diameter (cm)	Mycelial dry weight (mg)
20	3.53±0.25^{d}	35.5±5.13^{d}	9.00±0.00^{a}	48.7±18.71^{ab}
25	4.97±0.06^{b}	84.0±7.99^{bc}	9.00±0.00^{a}	56.2±8.03^{a}
26	4.43±0.06^{b}	76.7±3.69^{c}	9.00±0.00^{a}	46.0±1.12^{ab}
27	5.57±0.06^{a}	101.2±10.76^{abc}	9.00±0.00^{a}	42.2±3.69^{ab}
28	5.53±0.15^{a}	115.1±13.77^{a}	9.00±0.00^{a}	51.8±1.43^{a}
29	5.47±0.12^{a}	106.3±18.27^{ab}	8.73±0.25^{a}	40.9±6.35^{ab}
30	1.03±0.12^{e}	2.1±0.30^{e}	6.77±0.31^{b}	25.8±4.37^{b}

Note. Values with the same letter are not significantly different ($p \leq 0.05$) by the Duncan's test.

3.3.4 Effect of pH

The effect of initial pH for mycelial biomass of *C. doimaesalongensis* and *C. chalybescens* was studied under different initial pH (4-9). The result showed that the optimal pH for *C. doimaesalongensis* was pH 7 with a maximum colony radial growth rate 6.00 cm and pH 5, 6 and 7 for *C. chalybescens* with the colony radial growth 9.00, 8.70 and 8.73 cm, respectively. Mycelial dry weight, 61.8 mg was obtained at pH 7 after inoculated for 12 days (Table 3.3). Gbolagade, Fasidi, Ajayi and Sobowale (2006) reported the optimal pH of mycelial growth of *Lentinus subnudus* was pH 5-5.5 with the acidic medium and Kuforiji and Fasidi (1998) observed an optimal pH for mycelial growth of *Pleurotus tuberregium*, *Phellinus japonica* and *P. linteus* was at 5-7, 6-7 and 7, respectively. Eventhough they are not the same mushroom and cannot compared in this study, many results indicated that almost mushroom can grow in acidic pH range.

Table 3.3 Mycelial growth and mycelial dry weight of *C. doimaesalongensis* strain MFLUCC 13-0806 and *C. chalybescens* strain MFLUCC 13-0809 cultivated at different pH for 12 days.

pH	***C. doimaesalongensis***		***C. chalybescens***	
	Colony diameter (cm)	**Mycelial dry weight (mg)**	**Colony diameter (cm)**	**Mycelial dry weight (mg)**
4	2.67±0.15[d]	18.00±3.92[b]	5.30±0.26[d]	15.33±8.02[d]
5	4.90±0.10[c]	33.90±1.40[a]	9.00±0.00[a]	61.80±5.66[a]
6	5.53±0.25[b]	38.73±2.74[a]	8.70±0.52[a]	45.47±3.55[bc]
7	6.00±0.10[a]	42.67±1.67[a]	8.73±0.25[a]	54.76±5.20[ab]
8	5.43±0.29[b]	38.00±11.77[a]	7.23±0.06[c]	41.07±7.46[c]
9	5.27±0.15[b]	43.47±3.70[a]	8.20±0.17[b]	37.03±10.08[c]

Note. Values with the same letter are not significantly different ($p \leq 0.05$) by the Duncan's test.

3.3.5 Supplement of Carbon and Nitrogen Sources

Mushrooms require a different source of nutrition for promoting their mycelial growth. Generally, carbon and nitrogen sources play a significant role in mushroom cell proliferation and metabolite biosynthesis (Ribeiro et al., 2008). The effect of carbon sources for the mycelial growth of two selected isolates were determined in this study. *Clitopilus doimaesalongensis* showed the largest colony with 8.47 cm when sucrose was supplemented in the medium but the highest yield of dry weight, 98.73 mg were obtained when malt extract was used. Using tryptone as nitrogen source gave the largest colony diameter (6.87 cm) but yeast extract gave the highest cell biomass with 89.50 mg of cell dry weight (Table 3.4) while *C. chalybescens* mycelium grew in a wide range of carbon sources including malt extract, fructose and sucrose (Table 3.4). Among the studied nitrogen sources, tryptone, yeast extract and beef extract, all supplemented media gave the same colony diameter (9.0 cm) but not with KNO_3 supplemented media (Table 3.4). There were no significant differences in the mean growth rate of the *C. chalybescens* mycelium in the media with different carbon and nitrogen sources. Glucose was reported as a good carbon source for *Ganoderma applanatum* (Jeong, Jeong, Yang, Islam & Song, 2009) and promoted mycelial growth of *Lignosus rhinoceros* (Lai, Siti Murni, Fauzi, Abas Mazni & Saleh, 2011). While, Jayasinghe et al. (2008) repoted that dextrin was the best carbon source for mycelial growth of *Ganoderma lucidum*. For the best nitrogen source for mycelial growth of *Lignosus rhinoceros* was KNO_3 (Lai et al., 2011) and mycelial growth rate of *Tricholoma terreum* was maximum when yeast extract was supplemented (Kibar & Peksen, 2011). Therefore, favorable sources for the mycelial growth of selected mushrooms are necessary for their mycelial growth.

Table 3.4 Mycelial growth and mycelial dry weight of *C. doimaesalongensis* strain MFLUCC 13-0806 and *C. chalybescens* strain MFLUCC 13-0809 cultivated on different carbon and nitrogen sources for 12 days.

Culture media	*C. doimaesalongensis*		*C. chalybescens*	
	Colony diameter (cm)	Mycelial dry weight (mg)	Colony diameter (cm)	Mycelial dry weight (mg)
Carbon source				
Fructose	7.43±0.06[b]	34.30±2.43[b]	9.00±0.00[a]	80.67±14.67[a]
Sucrose	8.47±0.06[a]	33.53±1.00[b]	9.00±0.00[a]	31.40±6.73[b]
Starch	7.57±0.12[b]	23.60±1.82[c]	8.73±0.46[a]	94.27±8.18[a]
Malt extract	6.40±0.10[c]	98.73±0.65[a]	9.00±0.00[a]	95.80±12.82[a]
Nitrogen source				
Yeast extract	5.83±0.06[c]	89.50±8.88[a]	9.00±0.00[a]	67.33±13.98[a]
Tryptone	6.87±0.06[a]	72.73±2.56[b]	9.00±0.00[a]	55.55±10.72[ab]
Beef extract	6.56±0.06[b]	74.30±11.64[b]	9.00±0.00[a]	38.43±5.62[b]
KNO_3	2.13±0.06[d]	2.40±0.17[c]	0.00±0.00[d]	0.00±0.00[c]

Note. Values with the same letter are not significantly different ($p \leq 0.05$) by the Duncan's test.

3.3.6 Cultivation of *C. doimaesalongensis*

Clitopilus species have not been successfully cultivated in Thailand. In this study, we attempt to grow the *C. doimaesalongensis* using rice straw as the main substrate. The compost was inoculated with 2% of *C. doimaesalongensis* inoculum. The result show that the mycelium could grow on the formula 1 compost and completely colonized after inoculated the inoculum for 40 days, then cover with the sterilized casing layer. However, the mycelial did not grow well on casing surface (Figure 3.3(a)-(c)). The mycelium could slightly grow on compost formula 2 after incubated for 10 days and contaminated by other fungi (Figure 3.3(d)-(e)). As the result from the compost formulas mentioned above indicated that cultivation in the plastic tray could not succeed because of contamination by fungal disease. The environmental conditions are most important to the growth of mushroom. Thus, the formula 3 compost was developed and cultivated in the polypropylene bags for controlling the system. The result showed that mycelium could slightly grow after inoculated for 7-14 days at 28˚C. However, it still contaminated by other fungi (Figure 3.3(f)-(h)). Several factors such as substrate, temperature, humidity, water, ventilation, level of ingredient supplement (organic and inorganic) etc. are important factors for mushroom production (Duc, 2005; Zied et al., 2011). The future work may carry out the improvement of the compost formulation, observing for suitable conditions for vegetative growth and improving the casing method or formula to get the fruiting body.

Note. Mycelial production of *C. doimaesalongensis* (a) compost formula 1 after inoculated for 7 days, (b) after inoculated for 40 days, (c) casing, (d)-(e) compost formula 2 after inoculated for 10 days, (f)-(g) compost formula 3 after inoculated for 7 days, (h) after inoculated for 14 days

Figure 3.3 Mycelial production of *C. doimaesalongensis* on different compost formula.

3.4 Conclusion

The data on optimal condition for mushroom growth is important for a possibility to grow the mushroom *in vitro*. In this study MEA was optimal medium for *C. doimaesalongensis* strain MFLUCC 13-0806 and YEA was optimal medium for *C. chalybescens* strain MFLUCC 13-0809 compared with PDA which is a general medium and widely used for fungal cultivation. The optimal pH for mycelial growth of *C. doimaesalongensis* was pH 7 and this strain can grow in a wide range of temperature between 27-29˚C. Sucrose and tryptone were a suitable carbon and nitrogen source, respectively while *C. chalybescens* can grow in a wide range of temperature (20-29˚C), pH (5-7) and also carbon and nitrogen sources. Even though we do not focus to obtain fruiting bodies of *C. doimaesalongensis* in vitro, the data on optimal condition in this study are expected to possibly achieve this aim.

CHAPTER 4

ANTIBACTERIAL, ANTIOXIDANT AND ENZYME ACTIVITIES OF *Clitopilus doimaesalongensis* AND *Clitopilus chalybescens*

4.1 Introduction

Mushrooms have long been used as food and traditional medicines around the world, especially in Japan, Korea and China (Park et al., 2012). Medicinal mushrooms play an important role in several aspects of human activity and are used in the prevention and treatment of many diseases (Roupas et al., 2012). Many published data indicates that both of fruiting body and the mycelium of mushrooms have various biological functions such as antitumor, antioxidant, anti-aging, and immunological properties (Wasser, 2002; Daba & Ezeronye, 2003; Roupas et al., 2012; Zhong, Liu, Xie, Zhao, Song & Zhong, 2013). Many mushrooms and fungi have been isolated and developed to produce many important antibiotics, and could be beneficial for humans (Yamaç & Bilgili, 2006). Mushrooms produce a wide range of extracellular enzymes such as cellulolytic, xylanolytic, fibrinolytic and lignocellulolytic enzymes (Buswell et al., 1996; Lee, Lee, Lee & Kim, 2011; Praveen et al., 2012; Liu et al., 2014). The extracellular enzymes is enable mushroom that degrade complex organic matter into soluble substances and thereafter absorb them as food (Yang et al., 2011). Moreover, enzymatic activity such as amylases and cellulases are important enzymes that can be utilized for various biological activities (Jonathan & Adeoyo, 2011). Cellulases, xylanases, and ligninase have also been used in the brewing, baking, starch-processing, leather, and textile industries (Gurung, Ray, Bose & Rai, 2013).

Some *Clitopilus* species was reported to produce a biologically activity compound which processed on antitumor activity, antioxidant activity, antimicrobial activity and enzyme activity (Kavanagh et al., 1951; Liang et al., 1996; Kilaru et al., 2009; Wasser, 2002; Hartley et al., 2009; Goud et al., 2009; Grangeia et al., 2011). Consequently, the aims of this study were to evaluate antimicrobial and antioxidant activities of the mycelial extracts from *C. doimaesalongensis* strain MFLUCC 13-0809 and *C. chalybescens* strain MFLUCC 13-0806 and to screen the enzyme activity including, amylase, cellulase and xylanase activities of *C. doimaesalongensis* which are the enzymes that might be involve ability of degrading substrates.

4.2 Materials and Methods

4.2.1 The Sample Isolation

The process to obtain an axenic culture of the mushroom is the first stage for mushroom isolation. The isolation of *C. doimaesalongensis* strain MFLUCC 13-0806 and *C. chalybescens* strain MFLUCC 13-0809 were isolated from inside of mushroom fruiting bodies and transferred to potato dextrose agar (PDA), incubated at 25˚C for 10 days and re-subcultured until obtain axenic cultures. The axenic cultures were maintained on PDA slants and deposited at the culture collection of Mae Fah Luang University (MFLUCC).

4.2.2 Preparation of Crude Extracts

4.2.2.1 Culture mycelium extraction

The mycelium of *C. doimaesalongensis* and *C. chalybescens* were grown on malt extract agar (MEA) and yeast extract agar (YEA), respectively at 28˚C for 30 days. The mycelium was macerated with ethyl acetate (EtOAc) and soak overnight at the room temperature for the first extraction, and eight hours each for the second and third extraction. The combined supernatants were concentrated and dried to afford crude extract. The samples were stored at 4˚C for further use.

4.2.2.2 Culture broth and dry mycelium extraction

The mycelium of *C. doimaesalongensis* was grown in malt extract broth (MEB). The starter mycelium was prepared in Erlenmeyer flasks with 30 mL of liquid medium and incubated in the incubation shaker at 150 rpm, 28˚C for 1 week. Then, it was transferred to 100 mL of fresh medium and incubated 1 week prior up scaling to 400 mL of fresh medium and incubated for 2 weeks at the same condition. The culture broth was filtrated through a Whatman filter paper no 1 to separate the mycelia from the liquid using aspirator bump. The obtained mycelia were washed several times with sterilized distilled water and then freeze-dried in freeze-dryer. The freeze-dried mycelium was kept in glass vacuum desiccators and filtrate sample was kept at -20˚C until use for extraction.

The extraction process was carried out both from the filtrate and freeze-dried mycelium. The filtrate was extracted with an equal volume of ethyl acetate (EtOAc). The freeze-dried samples were macerated and soaked in EtOAc at a ratio of 1:20 (w/v) overnight at the room temperature for the first extraction, and eight hours each for the second and third extraction. The supernatants from all extraction were concentrated and dried to afford crude extracts. The samples were stored at 4˚C for further use.

4.2.3 Test Microorganism

Test microorganisms including Gram positive bacteria; *Bacillus subtilis* TISTR 008, *Staphylococcus aureus* TISTR 1466 and Gram negative bacteria; *Escherichia coli* TISTR 780, *Pseudomonas aeruginosa* TISTR 781 were used for antibacterial activity by the disc diffusion method. Tested microorganisms in this study were obtained from the TISTR Culture Collection of the Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand.

4.2.4 Antibacterial Activity Assay

Antibacterial activity of the mushroom extracts was carried out by the disc diffusion method against test microorganism. Bacterial suspensions were inoculated into nutrient broth (NB) at 37˚C to obtain approximately 3×10^{8} CFU/mL (O.D = 0.5 McFarland).

Crude extracts were prepared at 20 mg/mL concentration in HPLC grade methanol. A 20 µL of each crude extract was pipette to a 6 mm diameter sterilized paper discs prior bioassays. The Petri dish (90 mm diameter) containing Nutrient Agar (NA) was inoculated with 1000 µL of bacterial suspension. The prepared extract discs were placed on the bacterial loan with methanol as a negative control and amoxicillin as a positive control. Inoculated plates were incubated at 37˚C for 24 hrs. Three replicate experiments were carried out. Inhibition zone was recorded after 24 hrs of incubation. An average inhibition zone was calculated for 6 replicates.

4.2.5 Antioxidant Activity Assay

The scavenging activity of the DPPH free radical was assayed according to the method of Brand-William, Cuvelier and Berset (1995) with slight modification. The range of crude extract used included 5 to 50 mg/mL. The assay was performed in 96-well microtiter plates. The reaction mixture in each of the 96-wells was included 30 µL of the crude extract and 220 µL of methanolic solution of DPPH. The mixture was incubated in the dark at room temperature and measured absorbance at 517 nm every 30 minutes for 2 hrs. All measurements were performed in triplicate. Standard antioxidants of Butylated hydroxytoluene (BHT) was used as positive control by varying the concentration to 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL. The DPPH radical scavenging activity percentage was calculated using the following formula:

$$\text{Scavenging effect (\%)} = ([A_{blank} - A_{sample}]/A_{blank}) \times 100$$

Where,

A_{blank} = Absorbance of the control solution, DPPH solution without the tested sample.

A_{sample} = Absorbance of the test extract, DPPH solution with the tested sample.

4.2.6 Enzyme Activity Assay

The isolated of *C. doimaesalongensis* mycelium was screened for the enzyme activity of amylase, cellulase and xylanase. Carboxymethyl cellulose (CMC); soluble cellulose, was used as a substrate for screening of cellulase. Starch media was used for screening of amylase and wood xylan agar was used for xylanase activity. A mycelium plug was inoculated into the center of media. All the plates were incubated at 28˚C for 4 days, then the plates were strained by flooding with iodine solution and zone of clearance around the colony was measured. The relative index (RI) of enzymatic activity was calculated using the following formula:

$$\text{Relative index (RI)} = \frac{\text{Zone of clearance (mm)}}{\text{Colony size (mm)}}$$

4.2.7 Statistical Analysis

Experimental values were given as mean ± standard deviation (SD). The data of the experiment was analyzed by using one way analysis of variance (ANOVA) in SPSS version 11.5 program for windows and treatments mean compared using Duncan' test ($p \leq 0.05$) followed by post-hoc tests.

4.3 Results and Discussion

4.3.1 Antibacterial Activity of Crude Extract from Culture Mycelium Extraction of *C. doimaesalongensis* Cultivated in MEA and *C. chalybescens* Cultivated in YEA

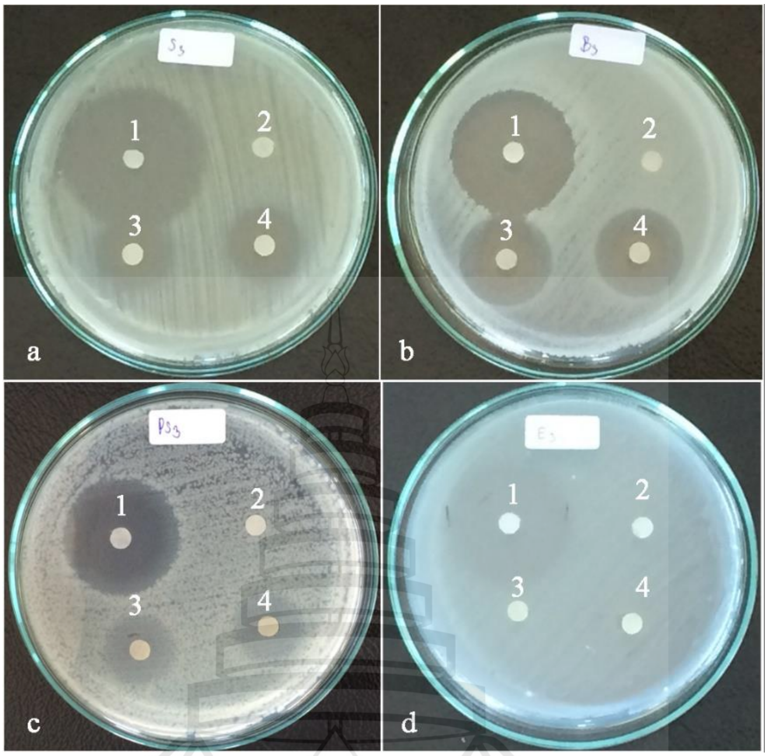
In this study an antibacterial activity of *C. doimaesalongensis* and *C. chalybescens* from cultured mycelial extract were screened using disc diffusion method. The crude extracts were tested against four species of bacteria. The results were shown in Table 4.1. The crude extracts obtained from *C. doimaesalongensis* showed activities against *S. aureus, B. subtilis* and *P. aeruginosa* but did not inhibit the growth of *E.coli* (Figure 4.1). It showed the highest activity against *S. aureus* (21.3 mm) followed by *P. aeruginosa* (16.8 mm) and *B. subtilis* (16.0 mm), respectively. Crude extract obtained from *C. chalybescens* showed activities against Gram positive but did not against bacterial Gram negative (Figure 4.2). Interestingly, the result in this study showed that crude extract of *C. doimaesalongensis* inhibited growth of some Gram negative bacteria which was not found in *C. chalybescens.*

The result of inhibition was consistent with the previous study that can be found from several Basidiomycete extracts such as *Agrocybe perfecta*, *Hexagonia hydnoides, Irpex lacteus, Nothopanus hygrophanus, Pycnoporus sanguineus and Tyromyces duracinus* (Rosa et al., 2003; Alves et al., 2012). According to Kavanagh et al. (1951) and Kavanagh et al. (1952) who reported a biologically active compound; pleuromutilin produced by *C. passeckerianus* (*Pleurotus passeckerianus*), *C. scyphoides* (*Pleurotus mutilus*) and other species of the genus *Clitopilus* mainly inhibited Gram positive bacteria such as *S. aureus*, *Streptococcus haemolyticus,* and *B. subtilis* but not with Gram negative bacteria.

Table 4.1 Antibacterial activities of crude extracts from culture mycelium extraction of *C. doimaesalongensis* and *C. chalybescens*

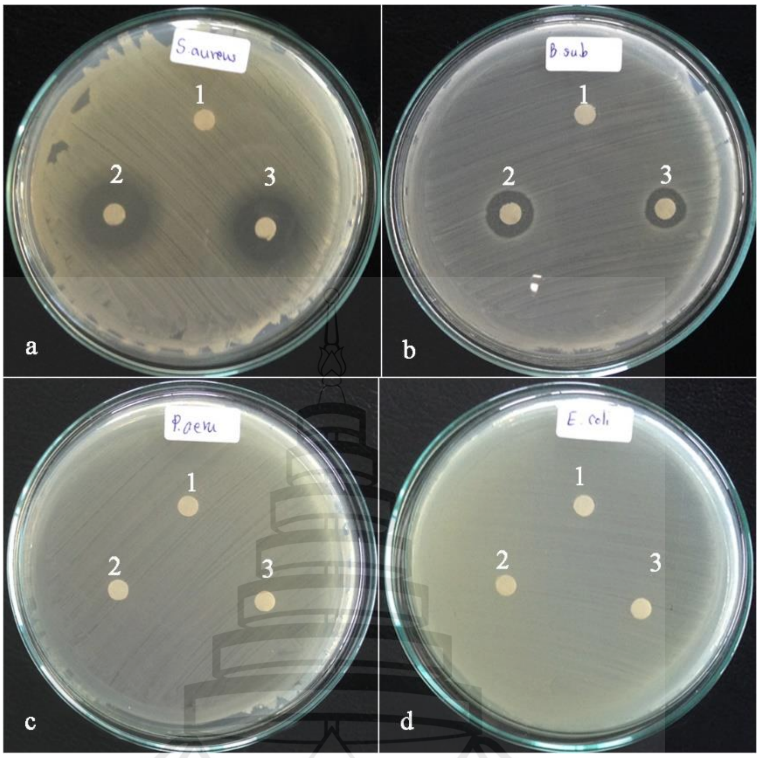
Bacteria	**Inhibition zone (mm)**	
	C. doimaesalongensis	***C. chalybescens***
Bacillus subtilis (+)	16.0±0.00[b]	13.2±0.58[b]
Staphylococcus aureus (+)	21.3±1.76[a]	19.0±1.32[a]
Pseudomonas aeruginosa (-)	16.8±0.76[b]	N/O*
Escherichia coli (-)	N/O*	N/O*

Note. N/O* means no inhibition zone detected



Note. The zones of inhibition that indicate antibacterial activity against bacteria strains (a) *S. aureus*, (b) *B. subtilis*, (c) *P. aeruginosa* and (d) *E. coli* with 1 methanol as negative control, 2 amoxicillin as positive control and 3-4 samples of crude extract of *C. doimaesalongensis*.

Figure 4.1 Antibacterial activities of *C. doimaesalongensis* on different bacteria



Note. The zones of inhibition that indicate antibacterial activity against bacteria strains (a) *S. aureus*, (b) *B. subtilis*, (c) *P. aeruginosa* and (d) *E. coli* with 1 methanol as negative control, 2-3 samples of crude extract of *C. chalybescens*.

Figure 4.2 Antibacterial activities of *C. chalybescens* on different bacteria

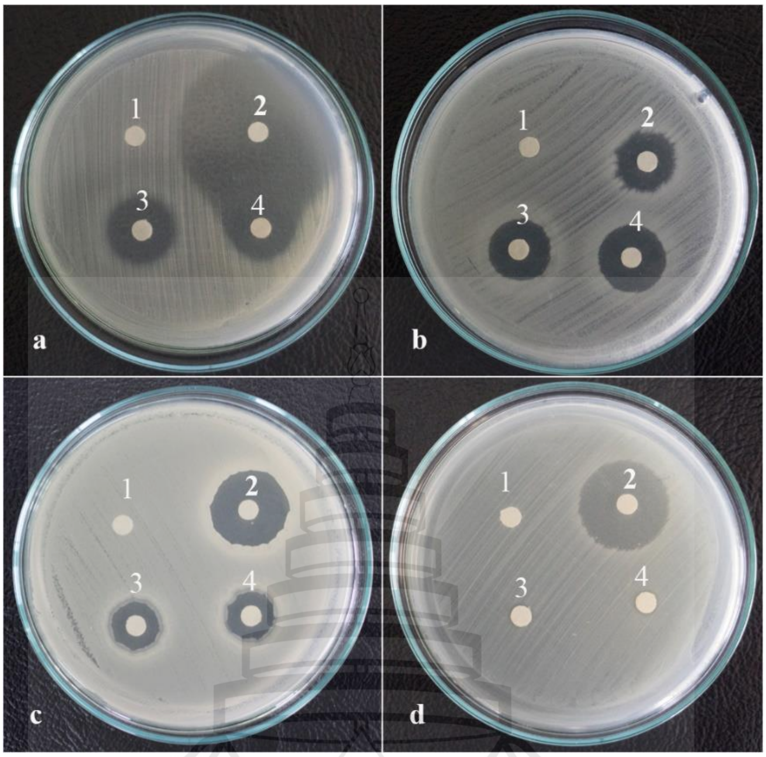
4.3.2 Antibacterial Activity of Crude Extract from Culture Broth and Dried Mycelium Extraction of *C. doimaesalongensis* Cultivated in MEB

The crude extracts of *C. doimaesalongensis* from cultured MEB media were screened against four species of bacteria. The result was shown in Table 4.2. The crude extracts obtained showed activities against *S. aureus*, *B. subtilis*, and P. *aeruginosa* but did not inhibit the growth of *E.coli* (Figure 4.3). The inhibition against all bacterial strain were not observed from dried mycelial extract. The result was consistent with the previous study that antimicrobial activity against bacteria and yeast can be found from several Basidiomycetes (*Agrocybe perfecta, Hexagonia hydnoides, Irpex lacteus, Nothopanus hygrophanus, Pycnoporus sanguineus and Tyromyces duracinus*) (Rosa et al., 2003). The extracts of *Agaricus bisporus* has been shown activity against *B. subtilis, S. aureus,* but not activity against *E. coli* (Akyuz & Kirbag, 2009). According to Kavanagh et al. (1952) and Hartley et al. (2009) reported a biologically active compound, pleuromutilin is also produced by *C. passeckerianus* (*Pleurotus passeckerianus*), *C. scyphoides* (*Pleurotus mutilus*) and other species of the genus *Clitopilus* inhibited gram positive bacteria such as *S. aureus, S. haemolyticus*, and *B. subtilis* and had no effect on *E. coli* (Kavanagh et al., 1951).

Table 4.2 Antibacterial activities of crude extracts from cultured broth and dried mycelial extraction of *C. doimaesalongensis*

Bacteria	Inhibition zone (mm)					
	Cultured broth extract	**Amoxicillin**	**Methanol**	**Freeze-dried mycelial extract**	**Amoxicillin**	**Methanol**
Bacillus subtilis (+)	17.7 ± 0.82[b]	17.0 ± 0.00[c]	N/O*	N/O*	16.7 ± 0.58[d]	N/O*
Staphylococcus aureus (+)	21.0 ± 3.40[a]	50.7 ± 2.89[a]	N/O*	N/O*	47.0 ± 0.00[a]	N/O*
Pseudomonas aeruginosa (-)	15.3 ± 0.52[c]	22.0 ± 1.73[bc]	N/O*	N/O*	24.3 ± 0.58[c]	N/O*
Escherichia coli (-)	N/O*	26.7 ± 3.06[b]	N/O*	N/O*	29.0 ± 1.73[b]	N/O*

Note. N/O* means no inhibition zone detected



Note. The zones of inhibition that indicate antibacterial activity against bacteria strains (a) *S. aureus*, (b) *B. subtilis*, (c) *P. aeruginosa* and (d) *E. coli* with 1 negative control, 2 positive control and 3-4 sample of crude extract.

Figure 4.3 Antibacterial activities of crude extract of *C. doimaesalongensis* on different bacteria

4.3.3 Antioxidant Activity

The mycelial extract was subjected to evaluate antioxidant activity. The DPPH free radical scavenging method was used for the analysis. It is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. DPPH exhibits a deep purple color with absorption maximum at 517 nm. In DPPH radical scavenging assay, an antioxidant compound can donate a hydrogen free radical to DPPH radical molecule, which is discoloration from purple to yellow (Kedare & Singh, 2011). Therefore, the antioxidant activity of a substance can be evaluated as its ability on scavenging the DPPH free radical (Duh, Tu & Yen, 1999; Chang, Yang, Wen, & Chern, 2002).

Scavenging effects of *C. chalybescens* and *C. doimaesalongensis* mycelial extract at different concentrations; 5, 10, 20, 30, 40, 50 mg/mL were shown in Figure 4.4. The DPPH radical scavenging activity effect of this were 21.41% and 26.45% at 40 and 50 mg/mL, respectively for *C. chalybescens* and *C. doimaesalongensis* were 29.14% and 40.62% at 40 and 50 mg/mL, respectively.

Huang (2000) found the methanolic extracts from mycelia of *Antrodia camphorata* and *Agaricus blazei* exhibited high antioxidant activities of 87.7% and 93.6%, respectively at concentrations as low as 0.5 mg/mL. Tsai (2002) found methanolic extract from *Ganoderma tsugae* mycelia exhibited a poor antioxidant activity of 7.6-19.3% at 0.5-20 mg/mL. Grangeia et al. (2011) were reported DPPH radical scavenging activity of *Clitopilus prunulus* shown EC_{50} values of 1.75±0.13 mg/mL. Obviously, different mushroom extract were showed different exhibition for the antioxidant activities.

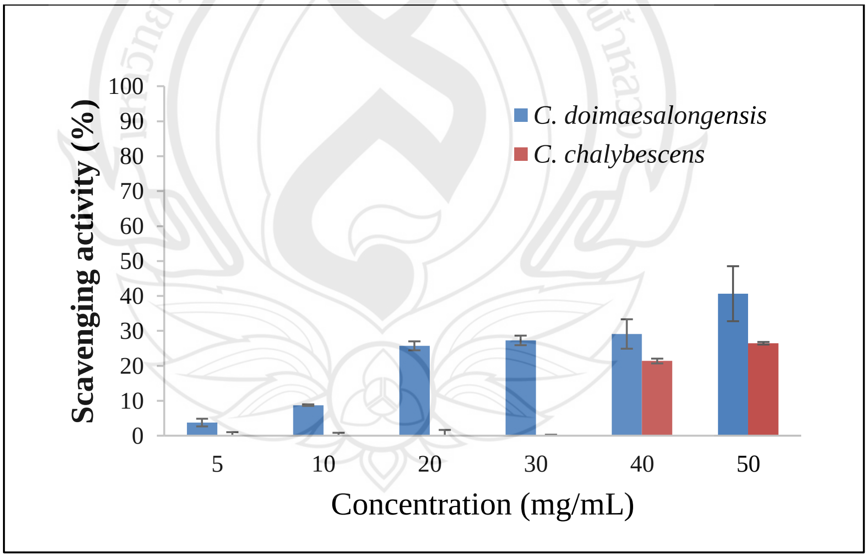


Figure 4.4 Scavenging effect of *C. doimaesalongensis* and *C. chalybescens* mycelium extraction on DPPH radicals. The results were representative of three separated experiments

The scavenging effect of BHT at different concentration (3.125, 6.25, 12.5, 25, 50, 100 and 200) was shown in Figure 4.5. The scavenging effect of BHT was higher than *C. doimaesalongensis* and *C. chalybescens* mycelial extracts at the same concentration, this might due to the degree of purity of used sample.

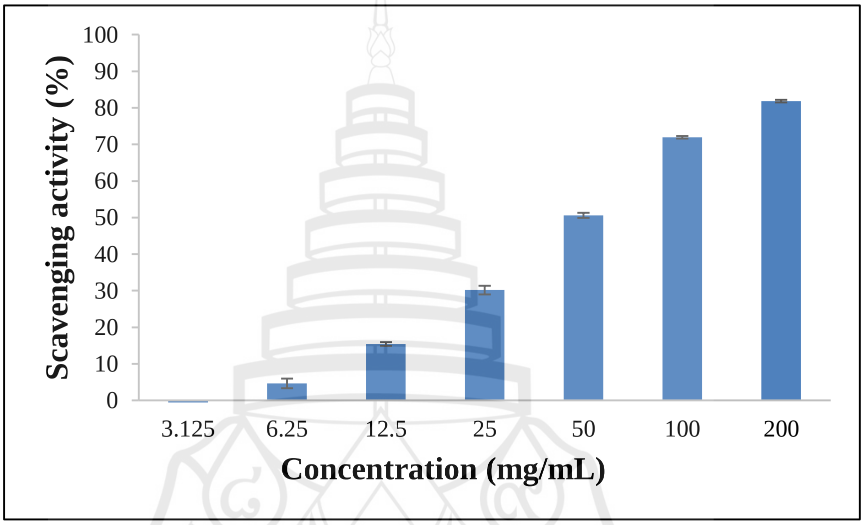


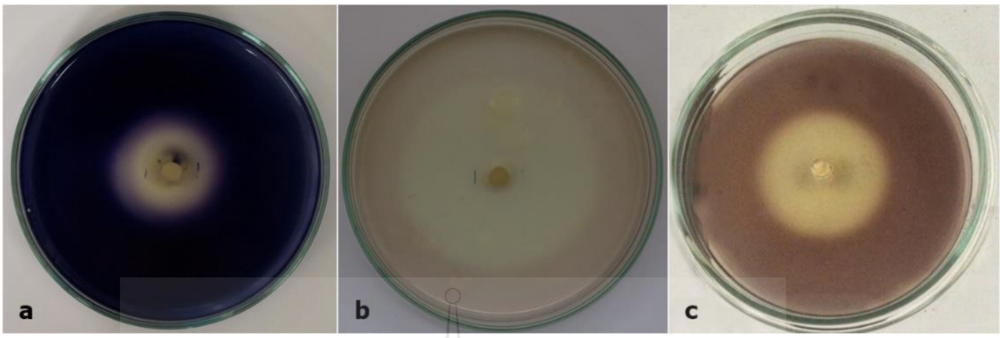
Figure 4.5 Scavenging effect of BHT on DPPH radicals. The results were representative of three separated experiments

4.3.4 Enzyme Activities

Many wild mushrooms were screened for enzymatic activities and they were detected enzymatic activities such as laccase, xylanase, protease, lipase and urease (Elisashvili, Chichua, Kachlishvili, siklauri, Khardziani, 2003; Jonathan & Adeoyo, 2011; Krupodorova, Ivanova & Barshteyn, 2014). Krupodorova et al. (2014) who reported the enzymatic activity of several macrofungi that showed the presence of different enzyme activities such as *Auriporia aurea*, *Cordyceps sinensis*, *Hericium erinaceus*, *Hohenbuehelia myxotricha*, *Lepista luscina* and *Pleurotus ostreatus* were show amylase activity; *Crinipellus schevczenkovi*, *Auriporia aurea*, *Hypsizygus marmoreus*, *Lyophyllum schimeji*, *Oxyporus obducens*, and *Spongipellis litschaueri* were produced the enzyme activity of laccase. Therefore, the *C. doimaesalongensis* was selected to observe activities of enzymes amylase, cellulase and xylanase by detecting a clear zone of hydrolysis after flooding plates with iodine solution in this study. The result showed that *C. doimaesalongensis* produced the highest level of cellulase activity followed by xylanase and amylase activities as the results in Table 4.3 and Figure 4.6. As the result in this study was occurred cellulase produce the highest level which was showed similar to previous study by Jonathan and Adeoyo (2011) who reported the high cellulolytic activities in *Coriolus versicolor* and *Pleurotus tuber-regium*.

Table 4.3 Relative index (RI) value after flooding plates with iodine solution

Enzyme	zone of clearance (mm)	Relative index (RI)
Amylase	38.5±1.00[c]	2.24±0.17[c]
Cellulase	60.5±0.71[a]	3.80±0.38[a]
Xylanase	40.7±1.15[b]	1.54±0.41[b]



Note. (a) Amylase production on starch agar, (b) Cellulase production on CMC agar, (c) Xylanase production on xylan agar.

Figure 4.6 Clear zone of hydrolysis after flooding plates with iodine solution

4.4 Conclusion

The assay of antibacterial and antioxidant activities *in vitro* demonstrated that crude mycelial extract of *C. chalybescens* exhibited antibacterial activities against Gram positive while crude extract from cultured broth of *C. doimaesalongensis* exhibited the cell growth of Gram positive and some Gram negative bacteria. However, crude extract from dried mycelial extraction did not inhibit all bacterial strains. Crude mycelial extract of *C. doimaesalongensis* and *C. chalybescens* showed scavenging activity of DPPH radical. Moreover, *C. doimaesalongensis* was demonstrated to produce cellulase, amylase, and xylanase activities which may be useful for the further experiment to select the substrate for growing the mushroom. Cellulase is important enzymes that can be utilized for various biological activities and in this study the result demonstrated cellulase was produced. Therefore, the optimization condition of growth condition for enzymes production may be useful for the further study for their commercial utility in biotechnological applications (Goud, Suryam, Lakshmipathi & Charya, 2009). The fermentation of *C. doimaesalongensis* and *C. chalybescens* in culture media could be used for the production of antibacterial metabolites. This alternative way instead direct extraction from mushroom fruiting body might attribute the presence of novel bioactive metabolites from different growth stages of the mushroom.

CHAPTER 5

OPTIMIZATION CONDITION FOR CULTIVATION OF *Agaricus subrufescens* HYBRID STRAINS

5.1 Introduction

Agaricus subrufescens Peck. was cultivated in the late 1800s in eastern north America and described by Peck C.H. in 1893 (Kerrigan, 2005) and firstly isolated in the 1960s in Brazil (Farnet et al., 2013). This mushroom has main characters as chocolate brown basidiospores, a hemispherical to convex to plano-convex shape of pileus, an almond-like or aniseed odor, positive Schäffer's and KOH reactions, a yellow discoloration, the surface is dry and covered by fibrillose squamulose. It is a saprobe and inhabits rooting leave, it often grows in clusters or scattered occasionally singly on soil. This mushroom was discovered in north America, south America and also has been found in Hawaii, Taiwan, Philippines and Thailand (Kerrigan, 2005; Wisitrassameewong et al., 2012). The first test for production of *A. subrufescens* has been cultivated as basic of cultivation of *A. bisporus* (Kopytowski-Filho, Minhoni & Rodriguez Estrda, 2006; Largeteau, Llarena-Hernández, Regnault-Roger & Savoie, 2011) then, the process of composting for *A. subrufescens* has been wildly practiced to improve yield (Zied et al., 2011; Colauto et al., 2011; Zied et al., 2012). *Agaricus subrufescens* is popular as a medicinal food having a potential to treat many common diseases such as arteriosclerosis, cancer, diabetes, cronic hepatitis and hyperlipidaemia (Mizuno et al., 1990; Hobbs, 2002; Firenzuoil et al., 2008; Liu et al., 2008; Jumes et al., 2010). It is a popular market mushroom as extensively cultivated in Brazil and in oriental countries such as Japan or China (Farnet et al., 2013). Studies of genetic breeding of this mushroom have been investigated to achieve high yield and productivities (Thongklang, Hoang et al., 2014). The aim of hybridization is to combine desirable characteristics from different strains. Thongklang, Hoang et al. (2014) could obtain the hybridization among *A. subrufescens* isolates from Brazil, France and Thailand. Straw is the agricultural waste that remains after harvested the grain and chaff such as barley, oats, rice, rye and wheat are produced worldwide (Tran, 1997; Bushuk, 2002). Their compositions which are consist of hemi-cellulose, cellulose and lignin which can be utilized for energy, animal feed, building materials, paper production and compost production to be used as an organic fertilizer for mushroom cultivation (Rashad, 2013; Yang, Guo & Wan, 2013). Rice and wheat straw have been used as basal substrate for various cultivated mushrooms for the example oyster mushroom (*Pleurotus ostreatus*) (Yang, et al., 2013), paddy straw mushroom (*Volvariella volvacea*) (Thiribhuvanamala, Krishnamoorthy, Manoranjitham, Praksasm & Krishnan, 2012). In general, each mushroom species are required different nutrition for their growing so the optimal conditions need to investigate for growing mycelium or cultivation of fruiting body (Luangharn, Karunarathna, Hyde & Chukeatirote, 2014; Magday Jr et al., 2014).

The commercial compost based on wheat straw is commonly used for cultivation of *A. bisporus* and production of *A. subrufescens* was firstly done on basis of *A. bisporus* cultivation. Thongklang, Hoang et al. (2014) have also been used commercial compost and successfully cultivated *A. subrufescens* hybrid strains of Thai × France, Thai × Brazilian and France × Brazilian in France and the result showed that Thai × France and Thai × Brazilian hybrid strain produced the highest yield of fruiting body. As we would like to introduce the cultivation of these mushroom in Thailand, in this study *A. subrufescens* of Thai × France and Thai × Brazilian hybrid provided by N. Thongklang (Thongklang, Hoang et al., 2014) obtained from INRA, France were firstly optimized and cultivated under growing condition in Chiang Rai, Thailand using compost prepared base on rice straw as the main substrate.

5.2 Materials and Methods

5.2.1 Strains of Mushroom

Agaricus subrufescens hybrid strains was obtained between Thai strain CA918 and Brazilian CA454, Thai strain CA918 and French strain CA487 (Table 5.1). All strains were maintained on potato dextrose agar (PDA) and transferred to a fresh PDA as master plate for inoculation.

Table 5.1 Cultivars used in this study

Strains	Code	Type
CA918-075 × CA454-4	Hybrid of Thai and Brazilian	T1×B
CA918-076 × CA454-4	Hybrid of Thai and Brazilian	T2×B
CA918-075 × CA487-35	Hybrid of Thai and French	T1×F
CA918-076 × CA487-35	Hybrid of Thai and French	T2×F

5.2.2 Optimization Condition for Growth of Mycelium

5.2.2.1 Effect of temperature

The mycelial growth of the four hybrid strains of *A. subrufescens* were studied on a different range of temperature. All pure culture isolates were grown on PDA, then a 0.5 mm-diameter plug from the edge of growing mycelium was transferred to compost extract media in triplicate and incubated at 16, 20, 25, 30 and 35˚C. Mycelial growth was evaluated by determination of dry weight and colony radius every 2 days for 10 days. For determination of dry weight, the agar-mycelial was removed by placed in a beaker, and then the medium was melted and washed out with hot water.

5.2.2.2 Effect of initial pH

The optimal pH for mycelial growth was determined by using compost extracted agar. The initial pH of the media was adjusted separately by 1N HCl or 1N NaOH to pH 4, 5, 6, 7, 8 and 9 and then autoclaved at 121˚C for 15 minutes. A 5 mm diameter plug of mycelium was inoculated at the center of plate. All culture media were incubated at 30˚C. Mycelial growth was evaluated by determination of dry weight and colony radius every 2 days for 10 days. Dry weight of mycelium was determined by the method described above.

5.2.3 Spawn Production

The sorghum were washed and soaked in distilled water for 24 hours, then washed 1-2 times before boiled for 15 minutes. After draining the excess water, the grains were sterilized, cooled down to room temperature, then inoculated with the mycelium and incubated at 25˚C for 14 days or until completely colonized.

5.2.4 Mushroom Cultivation

The compost media based on rice straw as main substrate was mixed with calcium carbonate, urea, rice bran, di ammonium phosphate, gypsum and water, followed by pasteurization for 6 hours by maintaining the temperature at 55-60˚C. The compost was cooled down to room temperature and inoculated with 2% (w/w) of the prepared spawn. A 5 kg of the mixture was placed in plastic tray (35×25×20 cm). The inoculated compost was incubated at 25˚C with relative humidity at 91-95% for the beginning of colonization. The completely colonized compost was then covered with the pasteurized casing (2.5 inch thickness). The fresh weight of fruiting bodies were recorded after casing for 18 days. The experiments were performed with five replicated per strain and cultivation condition.

5.2.5 Statistical Analysis

The mycelial growth and mycelial dry weight were verified by calculating the average of triplicate measurements in centimeter (cm) of mycelium diameter and milligram (mg) of mycelial dry weight and yield of fresh weight mushroom using statistical analysis and significant differences were determined by Tukey's test with a significance level $p \leq 0.05$ followed by post-hoc test in a one-way ANOVA analysis using SPSS version11.5 program.

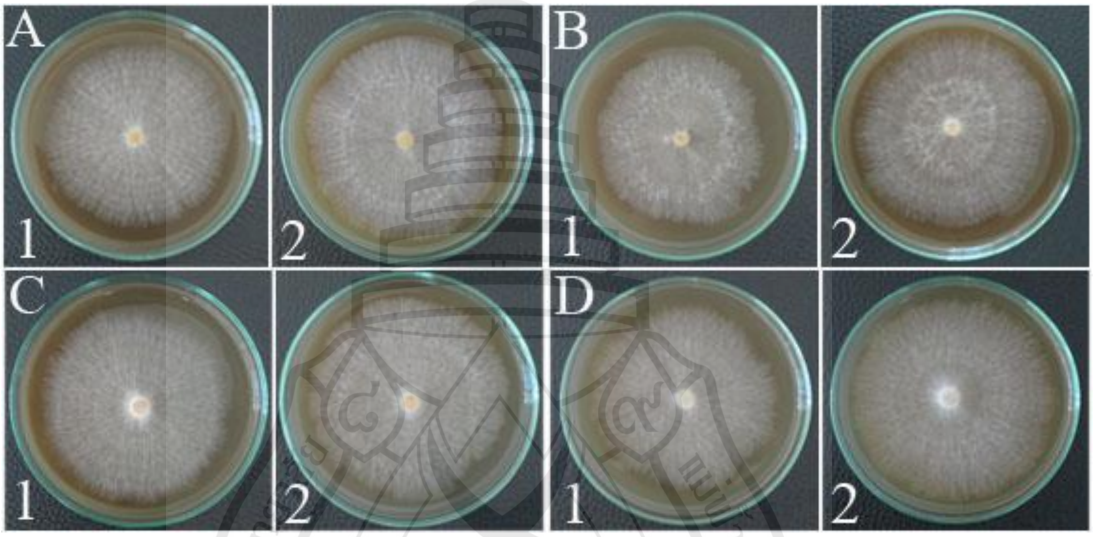
5.3 Results and Discussion

5.3.1 The Effect of Temperature on Mycelial Growth

Agaricus subrufescens grew on compost extracted agar and inoculated in the range temperatures at 16, 20, 25, 30 and 35˚C. In this study, the optimal temperature for mycelial growth was found at 25 and 30˚C (Table 5.2), the highest dry weight of mycelial was also occurred at 25 and 30˚C (Table 5.3) (Figure 5.1). Four strains of *A. subrufescens* in this study showed optimal temperature between 25-30˚C for mycelial growth which was relatively to previous reported by Chang (2008) at 23-37˚C, Colauto et al. (2008) 28-31˚C, Huang (1997) 20-33˚C, Neves et al. (2005) 25-30˚C, Quimi et al. (1990) 28-30˚C, Mendonca, Kasuya, Cadorin and Vieira (2005) 25-30˚C, and Zied et al. (2011) 25-29˚C. Therefore, the suitable temperature for mycelium growth should be maintained from 25-30˚C.

5.3.2 The Effect of Initial pH on Mycelial Growth

The mycelial growth was evaluated in the culture media with initial pH values from 4 to 9. The result form this study showed that optimal pH for mycelial growth was range between 7 to 9 for *A. subrufescens* hybrid strain Thai × Brazilian (T1×B and T2×B) and pH range between 6 to 9 for Thai × France strain (T1×F and T2×F) (Table 5.2), the mycelial dry weight for all strains are also shown in Table 5.3. All strains used in this study were able to grow at pH 4-9. The optimal pH of *Agaricus bisporus* on compost cultivation was reported as pH 7.5 by Gerrits (1988) and Rinker (1993). The optimal pH for the substrate reaction range between 6-7, strong acid or basic condition inhibited mycelium growth. Colauto et al. (2008) reported the optimal mycelial growth on malt extract agar of *A. brasiliensis* was between 5.5 and 6.0. While, Kopytowski Filho, Minhoni, Andrade and Zied (2008) and Calvalcante et al. (2008) reported that the optimal pH for compost substrate and casing soil for *A. subrufescens* was pH 7.0-7.5. This result indicated that neutral pH range was optimal pH for *A. subrufescens* hybrid strain.



Note. (1) 25˚C and (2) 30˚C. (A) strain T1×B, (B) strain T2×B, (C) strain T1×F and (D) strain T2×F.

Figure 5.1 Mycelium growth on compost extracted media when incubated at 25˚C and 30˚C for 10 days

Table 5.2 Average mycelial growth rate of *A. subrufescens* cultivated on compost extract agar under various conditions for 10 days

Condition	**Growth rate (cm of mycelial radius)***			
	T1×B	**T2×B**	**T1×F**	**T2×F**
Temperatures (˚C)				
16	2.37±0.06[c]	1.83±0.06[c]	2.87±0.15[c]	2.57±0.21[c]
20	3.00±1.00[b]	2.53±0.06[b]	4.00±0.27[b]	3.90±0.10[b]
25	7.37±0.06[a]	7.23±0.06[a]	7.33±0.06[a]	6.57±0.38[a]
30	7.50±0.10[a]	7.37±0.12[a]	7.77±0.21[a]	7.00±0.17[a]
35	1.47±0.06[d]	1.57±0.06[d]	1.43±0.06[d]	1.33±0.06[d]
pH				
4	4.23±0.21[c]	4.47±0.06[c]	4.77±0.06[b]	3.93±0.06[b]
5	5.03±0.06[bc]	4.63±0.06[c]	4.83±0.15[b]	3.97±0.06[b]
6	5.10±0.00[b]	5.40±0.46[b]	6.07±0.40[a]	6.17±0.87[a]
7	5.83±0.21[ab]	5.97±0.15[ab]	7.03±0.45[a]	6.63±0.83[a]
8	5.93±0.23[a]	6.00±0.10[a]	6.77±0.68[a]	6.50±0.50[a]
9	6.53±0.55[a]	5.87±0.15[ab]	6.05±0.44[a]	6.43±0.55[a]

Note. * Within a column, values followed by the same letter are not different at $p \leq 0.05$ by Tukey's test.

Table 5.3 Dry weight of mycelium grown on compost extract agar under various conditions for 10 days.

Condition	Dry weight (mg)*			
	T1×B	T2×B	T1×F	T2×F
Temperature (˚C)				
16	3.00±0.707^{d}	3.87±0.15^{b}	10.10±1.51^{b}	7.47±0.42^{d}
20	9.23±1.16^{c}	7.27±2.28^{b}	15.77±1.11^{b}	14.73±3.14^{b}
25	32.57±2.77^{b}	51.23±2.43^{a}	68.03±2.41^{b}	32.30±2.23^{b}
30	48.67±2.37^{a}	54.80±1.22^{a}	85.67±4.18^{a}	56.33±4.54^{a}
35	4.33±0.42^{d}	3.50±0.10^{b}	3.00±0.61^{d}	5.10±0.10^{d}
pH				
4	14.60±0.92^{b}	14.57±0.91^{c}	15.57±1.14^{b}	11.73±1.50^{c}
5	16.63±1.07^{b}	15.90±1.51^{c}	20.l0±0.53^{b}	12.53±2.20^{c}
6	17.17±1.43^{b}	27.60±1.50^{ab}	76.27±2.50^{a}	29.70±2.92^{b}
7	24.53±1.31^{a}	30.17±2.81^{a}	77.03±2.15^{a}	39.50±4.30^{a}
8	23.37±1.00^{a}	25.13±1.55^{b}	76.07±2.79^{a}	38.30±2.91^{ab}
9	27.67±3.27^{a}	27.86±1.88^{a}	75.37±2.45^{a}	38.50±4.10^{a}

Note. * Within a column, values followed by the same letter are not different at $p \leq 0.05$ by Tukey's test.

5.3.3 Mushroom Cultivation

Thailand is an agricultural country and agricultural wastes is easy to find and use as substrates for cultivating mushrooms such as rice straw. In this study, *A. subrufescens* hybrid strains presented in Table 5.1 were used for cultivation on rice straw based material of compost. The production of fruiting bodies on compost were shown in Figure 5.2 and the result shown in Figure 5.3 indicated that the hybrid of Thai × France strain type T2×F produced the highest yield of fruiting bodies with 56.92±13.30 g/kg compost while hybrid of Thai × Brazilian stain type T2×B produced 42.35±1.47 g/kg compost followed by hybrid of Thai × Brazilian strain type T1×B and hybrid of Thai × France strain type T1×F which produce the yield of fruiting bodies 34.07±2.87 and 9.86±0.04 g/kg compost, respectively.

These *A. subrufescens* hybrid strains showed a potential to grow under studied condition at the temperature 25˚C with 91-95% relative humidity in northern Thailand and produce the fruiting bodies after casing for 18 days. The hybrid strains of *A. subrufescens* from two homokaryotic single-spore isolates, CA454-3 from the Brazilian stain and CA487-100 from the French strain were provided by E. Huangand & P. Callac. They have been cultivated by Llarene-Hernandez et al. (2013) and they obtained the fruiting production 109.1 g/kg compost after casing for 30 days at 23 ± 0.5˚C with 98 ± 2.0% relative humidity. Mushroom requires suitable conditions such as temperature and humidity to produce fruiting bodies (Dias, Abe & Schwan, 2004; Mantovani et al., 2007; Dias, 2010). Iwade and Mizuno (1997) reported the suitable humidity during the formation of primordia of *A. blazei* was 60-75% and 70-85% relative humidity for development of fruiting bodies. While, Wang, Li, Li and Han (2010) reported relative humidity was kept at 85–90% for mycelium growth and the relative humidity should be at 95–99% for fruiting body formation. Llarene-Hernandez et al. (2013) and Thongklang, Hoang et al. (2014) have been successfully cultivated *A. subrufescens* hybrid strains on wheat straw based compost and obtained higher yield of mushroom production comparing to this study. This might be affected by difference type of straw as we used rice straw instead of wheat straw to prepare the compost in this study. Therefore, the results from this study mentioned that standard conditions including temperature, humidity, pH, type of waste materials, additive of compost formulation and casing are important factors for mushroom cultivation and have to be concerned.

Figure 5.2 Fruiting bodies of 4 hybrid strains of *Agaricus subrufescens* cultivated on rice straw based compost media.

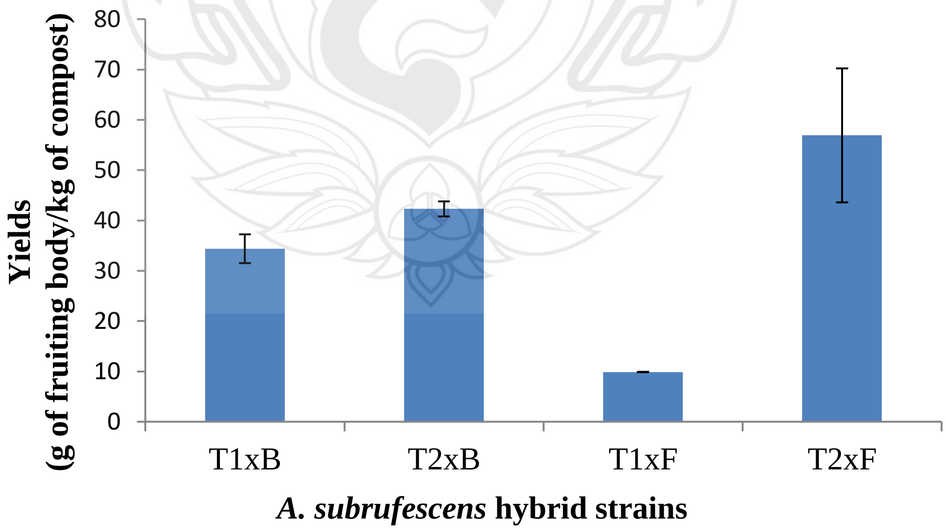


Figure 5.3 Comparison of biomass production in each strain

5.4 Conclusion

The results from this study demonstrated that mycelium of *A. subrufescens* able to grow at the temperature range between 25-30˚C and initial pH range between pH 6-9. Fruiting body of these strains can be produced by using rice straw compost prepared in Chiang rai, Thailand. The yield of the production, however, should be improved to reach a commercial scale.

CHAPTER 6

CONCLUSION

In conclusion, *Clitopilus* is a genus belonging to the family of the *Entolomataceae.* It is commonly known as the miller or the sweetbread mushroom. It is a widespread distribution especially in northern temperate areas. Some species had a potential of antibacterial, antioxidant and enzyme activities. However, it has been very few studies in Thailand. Therefore, we focus to isolate the genus *Clitopilus* and study on taxonomy and phylogeny, optimization condition for mycelial growth, antibacterial, antioxidant activities and enzyme activity. From the study many fruiting body samples of *Clitopilus* were collected from Lampang and Chiang Rai province throughout the wet seasons of 2013. The collections of *Clitopilus* were identified based on their morphological characters then, the pure culture was obtained from sterile tissue inside mushroom fruiting bodies that was grown on PDA and incubated at 25˚C. The specimens were dried in a standard food dryer at 40˚C. The pure cultures and dried specimens were deposited at Mae Fah Luang University Culture Collection (MFLUCC) and Mae Fah Luang University Herbarium (MFLU HERB), respectively.

Base on the study of taxonomy, morphological characters and ITS rDNA sequencing data, we could identify two species from three collections. The first two collections collected from Lampang, Thailand were identified as *C. chalybescens*. Another one collection collected from Chiang Rai was identified as a new species in Thailand and given name as *C. doimaesalongensis*. These two species were *C. chalybescens* strain MFLUCC13-0809 and *C. doimaesalongensis* strain MFLUCC13-0806, respectively.

The most suitable substrate and physical conditions or environmental factor are very important for mycelial and fruit bodies growth (Chihara et al., 1970; Ukai et al., 1983; Miyazaki & Nishijima,1981; Mizuno, 1992; Hobbs, 2000; Zhang et al., 2007). To get information about the growth conditions, the effect of different culture media, temperature, pH and carbon/nitrogen sources on the mycelial growth were investigated. We found that both species were able to grow on different culture media. The optimal condition for mycelial growth of *C. doimaesalongensis* was observed in MEA supplemented with sucrose as carbon source and tryptone as nitrogen source, pH 7 and incubated at the temperature range from 27-29˚C while the optimal condition for *C. chalybescens* was YEA, pH 5-8 at the temperature range from 20-29˚C and this mushroom can use wide range of carbon sources including fructose, sucrose, starch or malt extract and also various nitrogen sources including yeast extract, tryptone or beef extract.

As we can isolate a new species in this study, the study of bioactive compounds such as antibacterial and antioxidant activities, biomass degrading enzymes from this new species should be investigated. The results of the antibacterial activity from crude extract obtained from culture mycelium and cultured broth of *C. doimaesalongensis* showed their ability to inhibit the growth of Gram positive bacteria (*B. subtilis* and *S. aureus*) and Gram negative bacteria (*P. aeruginosa*), but it could not be found this effective activity from the crude extract obtained from dried mycelial sample. Interestingly, the result of crude extract from *C. doimaesalongensis* was different from the result obtained from the crude extract of *C. chalybescens* which only inhibited against Gram positive bacteria (*B. subtilis* and *S. aureus*). The crude extract of culture mycelium from *C. doimaesalongensis* and *C. chalybescens* were used to observe the antioxidant activity and the result showed that these two species slightly exhibited antioxidant properties. As during growth and fruiting of mushrooms species on a particular waste material produce a wide range of extracellular enzymes that extracellular enzyme production during growth produce the enzymes essential to degrade the major components of the waste substrate and to degrade complex organic matter into soluble substances, and absorb it as food (Leatham, 1985; Tan & Wahab, 1997). For the result of enzyme activities test, *C. doimaesalongensis* was demonstrated to produce cellulase, amylase, and xylanase activities which may be useful for the further experiment to select the substrate for growing the mushroom.

Some *Clitopilus* species are potentially edible, and cultivatable, but studies have not been reported to cultivate *Clitopilus* (Thawthong et al., 2014). The genus *Clitopilus* is also important for medicinally important secondary metabolites. The result in this study demonstrated that the biological active components from the mycelium and broth culture extract of *C. doimaesalongensis* showing their mode of action. Thus, future research should focus on the cultivation and secondary metabolite analyses of our new species *C. doimaesalongensis*.

Several mushrooms have various biological functions. Therefore, they have long been collected and used as food and traditional medicines for thousands of year. Many edible mushrooms have been widely cultivated in many countries around the world such as *Agaricus bisporus, Auricularia auricular*, *Lentinula edodes* and *Volvariella volvacea*, however, about 22 species have been reported in Thailand as commercially grown (Thawthong et al., 2014). *Agaricus subrufescens* is one of the popular mushroom consume as a medicinal food. It was first cultivated in eastern north America and has been introduced to cultivate in many countries such as Brazil and Japan (Kerrigan, 2005). *Agaricus subrufescens* was successfully developed a new hybrid strain from Thai and French strains (Thongklang, Sysouphanthong et al., 2014). In this study four *A. subrufescens* hybrid strains obtained from INRA, France were firstly optimized and cultivated under growing condition in Chiang Rai, Thailand. From this study, it was found that these hybrid strain can grow at the temperature range from 25-30˚C and initial pH range between pH 6-9. The fructification of *A. subrufescens* hybrids strain can be done using rice straw compost under growing condition at 25˚C and 95% humidity in Chiang Rai, Thailand. However, the yield obtained were lower than previous study by Llarence-Hernandez et al. (2013) and Thongkalng, Sysouphanthong, et al. (2014) who successfully grew these mushroom in France. Suggestion for further work by changing compost formulations, casing type, and also condition for cultivation, improving quality and yield of these mushrooms as a commercial scale in Thailand.

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APPENDICES

APPENDIX A

MEDIA AND CHEMICAL PREPARATION

1. Preparation of chemical reagent using for this study

Potassium Hydroxide (KOH): 3-5% aqueous solution (Lagent et al., (1977)

Potassium hydroxide	3 (-5)	g
Water	97 (-95)	ml

3-5% KOH was used to revive the hyphae of dried specimens.

Congo Red: 1% aqueous solution (Lagent et al., (1977)

Congo Red	1	g
Water; filter the excess dye	99	ml

1% Congo Red was used to observe walls hyphae and spores

Sodium hydroxide (NaOH): 1N solution

Sodium hydroxide	4	g
Water to male volume	100	ml

Hydrochloric acid (HCl): 1N solution

Hydrochloric acid	8.985	ml
Water to male volume	100	ml

2. Preparation of media

Potato dextrose agar (PDA), CRITERION

1 liter of media including	Potato infusion	4.0	g
	Dextrose	20.0	g
	Agar	15.0	g

Malt extract agar (MEA), CRITERION

1 liter of media including	Malt extract	20.0	g
	Dextrose	20.0	g
	Peptone	6.0	g
	Agar	16.0	g

Sabouraud dextrose agar (SDA), CRITERION

1 liter of media including	Dextrose	40.0	g
	Casein peptone	10.0	g
	Agar	15.0	g

Corn meal agar (CMA), CRITERION

1 liter of media including	Corn meal infusion from solids	2.0	g
	Agar	15.0	g

Yeast extract agar (YEA), CRITERION

1 liter of media including	Yeast extract		
	Agar	15.0	g

Preparation of carbon and nitrogen source

Malt extract	20.0	g
Dextrose	20.0	g
Peptone	6.0	g
Agar	15.0	g
Distilled water	1	litter

Above formula media was supplemented by fructose, sucrose, starch, yeast extract, beef extract, tryptone and KNO_3

Preparation of Composted

Rice straw	200	kg
Calcium carbonate	2	kg
Urea	2	kg
Rice bran	10	kg
Ammonium Sulfate	4	kg
Calcium sulfate	6	kg
Water		

Rice straw was used as the main substrate of compost ingredients. The compost was fermented for 20 days. Material were mixed and periodically turned following schedule

Day 1: Rice straw was mixed with calcium carbonate and water

Day 3: urea and rice bran were added to composting

Day 5: Ammonium phosphate was add to composting

Day 7: The stack was turn to keep an aerobic

Day 10: Calcium sulfate was added to composting

Day 14: The stack was turn and added calcium sulfate

Day 15: Second free turn

Day 18: Third free turn

Day 20: Fourth free turn

Compost extract media

Glucose	10	g
Agar	20	g
Water	200	ml

3. Preparation of bacterial

1. Each bacteria strain from stock was separately streak on Nutrient Agar (NA) plate

2. Incubated at 37 ˚C for 18-24 h

3. 3-5 single colonies were inoculated to 10 mL of sterile nutrient Broth (NB) to obtain 0.5 MaFarland

APPENDIX B

OPTIMAL CONDITION FOR MYCELIAL GROWTH

1. Effect of culture media for mycelial growth

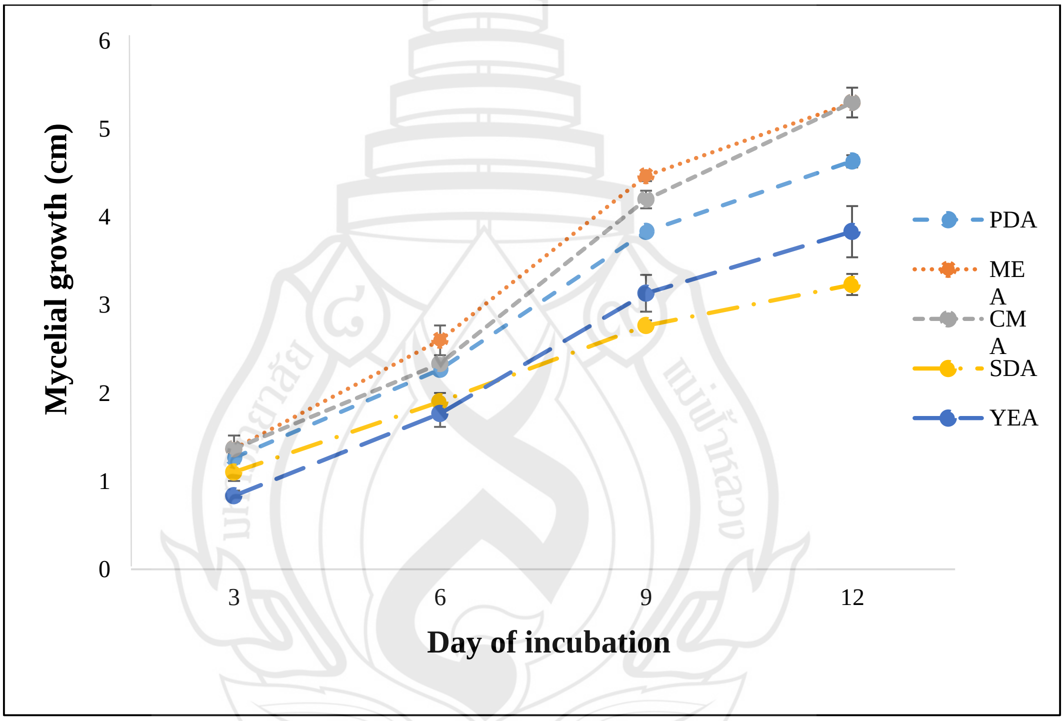

Figure B1 Effect of culture media for mycelial growth of *Clitopilus doimaesalongensis*

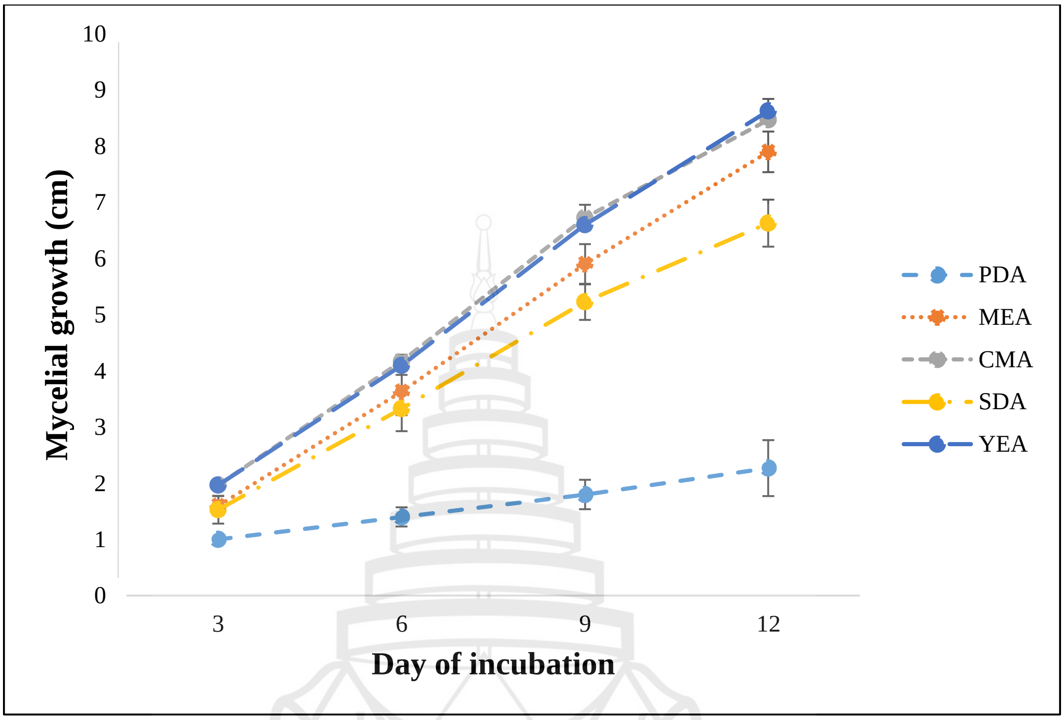

Figure B2 Effect of culture media for mycelial growth of *Clitopilus chalybescens*

2. Effect of temperature for mycelial growth

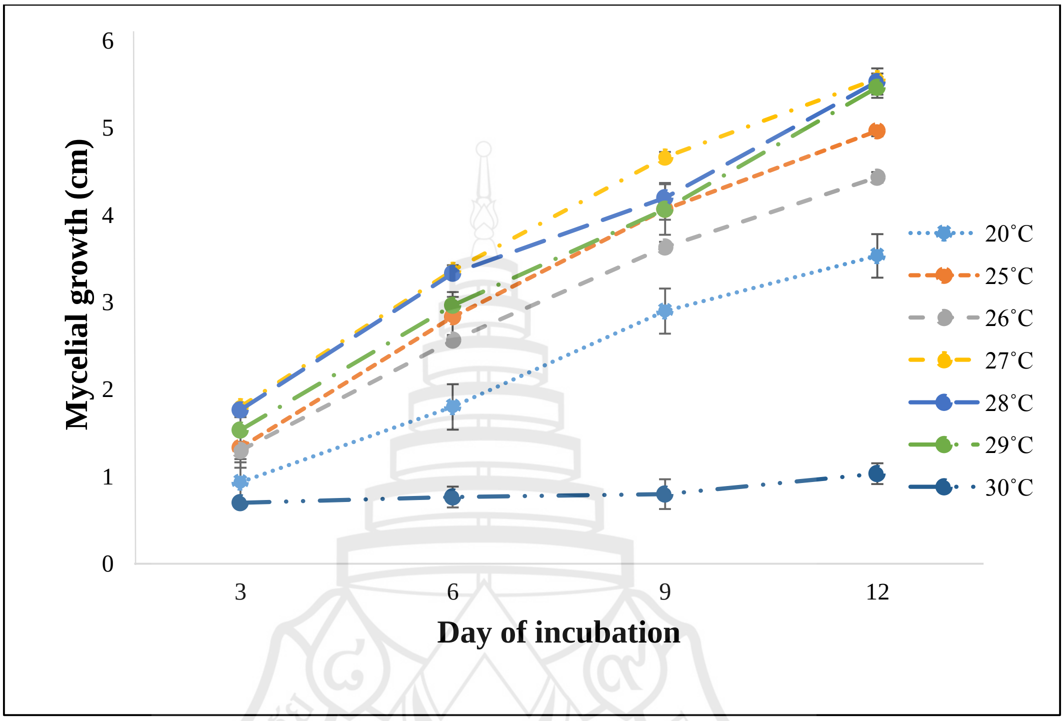

Figure B3 Effect of temperature for mycelial growth of *Clitopilus doimaesalongensis*

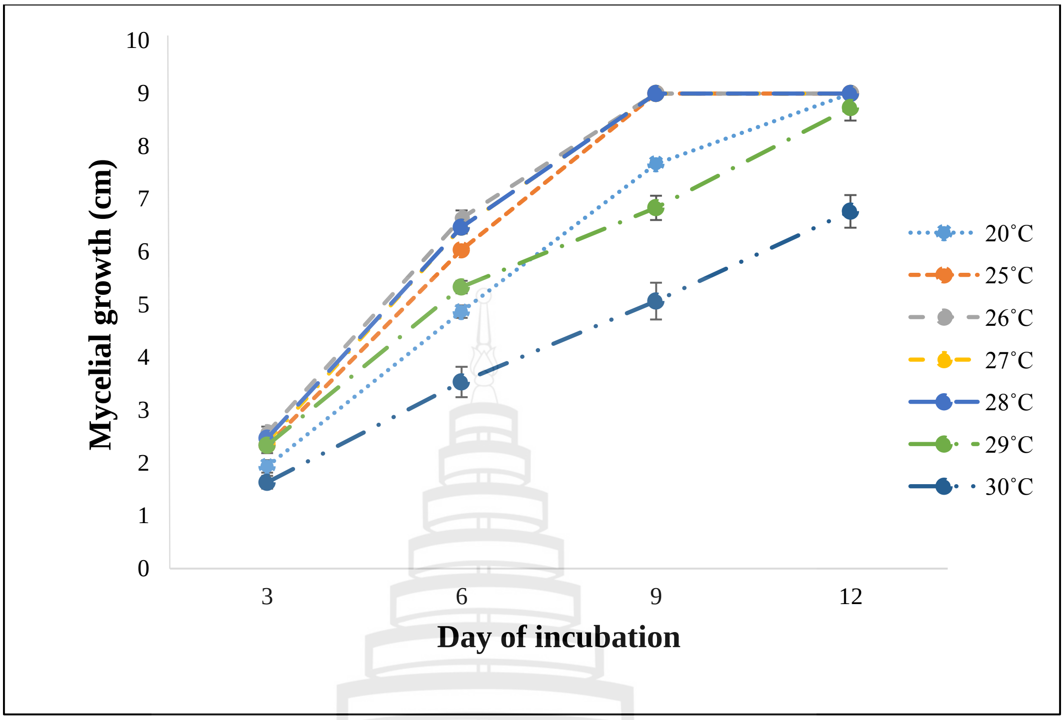

Figure B4 Effect of temperature for mycelial growth of *Clitopilus chalybescens*

3. Effect of initial pH for mycelial growth

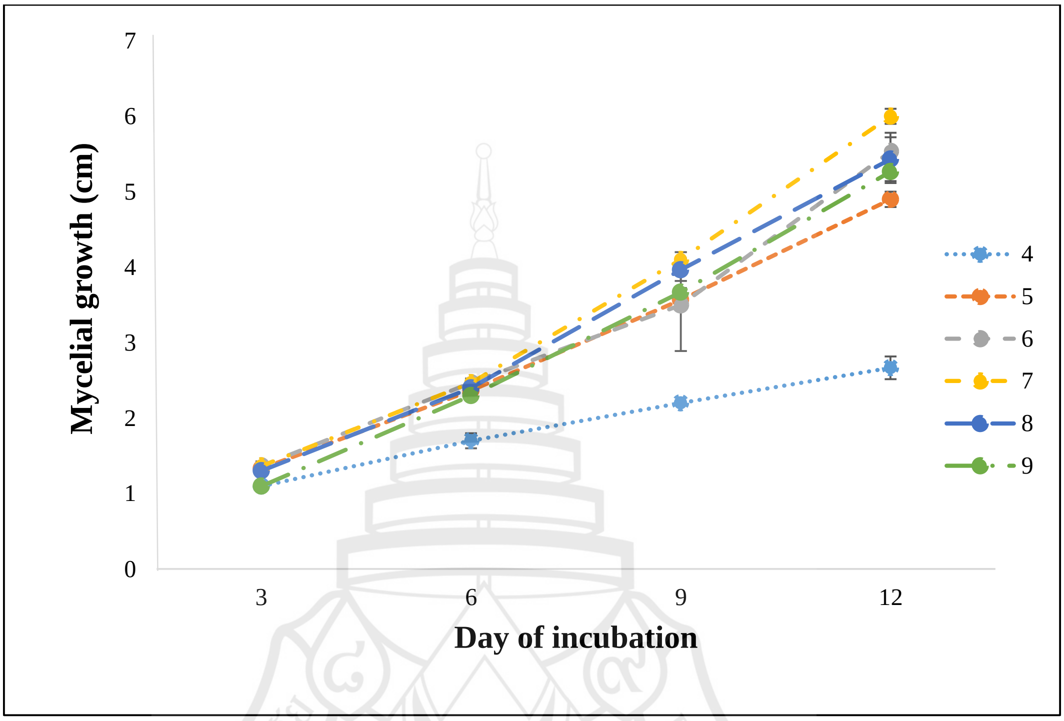

Figure B5 Effect of initial pH for mycelial growth of *Clitopilus doimaesalongensis*

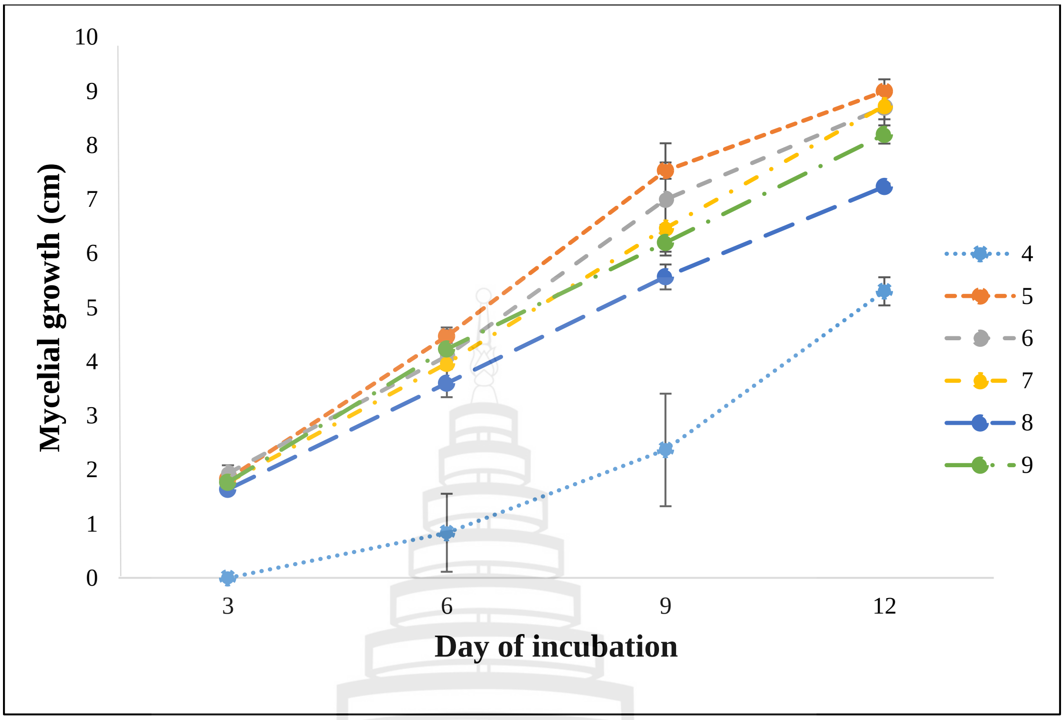

Figure B4 Effect of initial pH for mycelial growth of *Clitopilus chalybescens*

4. Supplement of carbon and nitrogen sources for mycelial growth

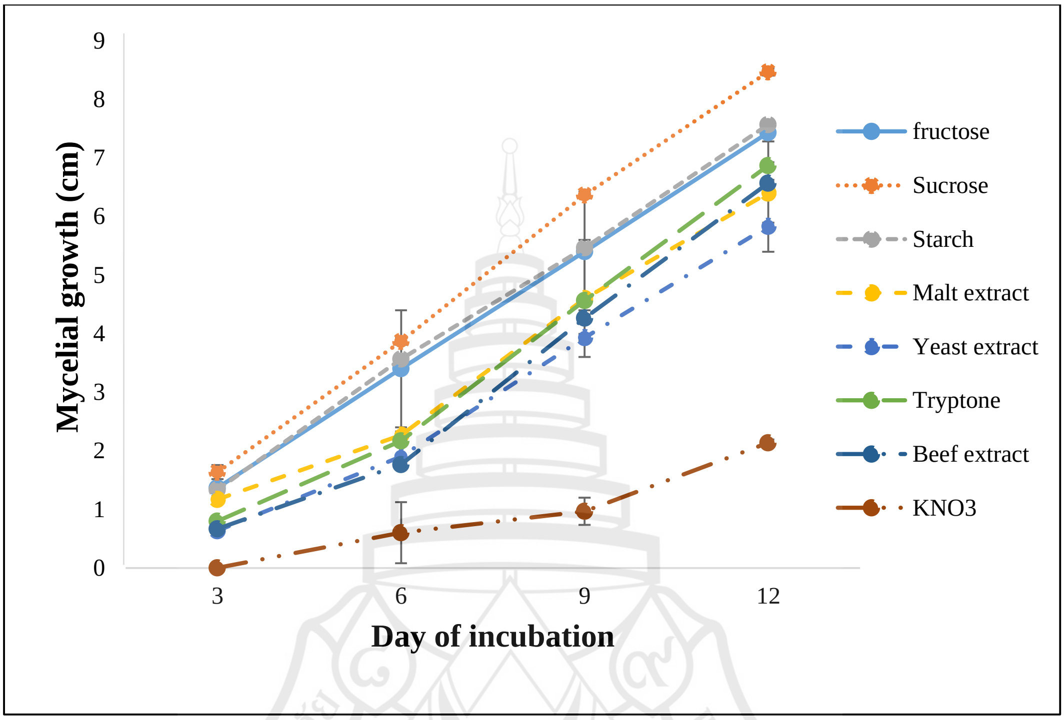

Figure B7 Supplement of carbon and nitrogen sources for mycelial growth of *Clitopilus doimaesalongensis*

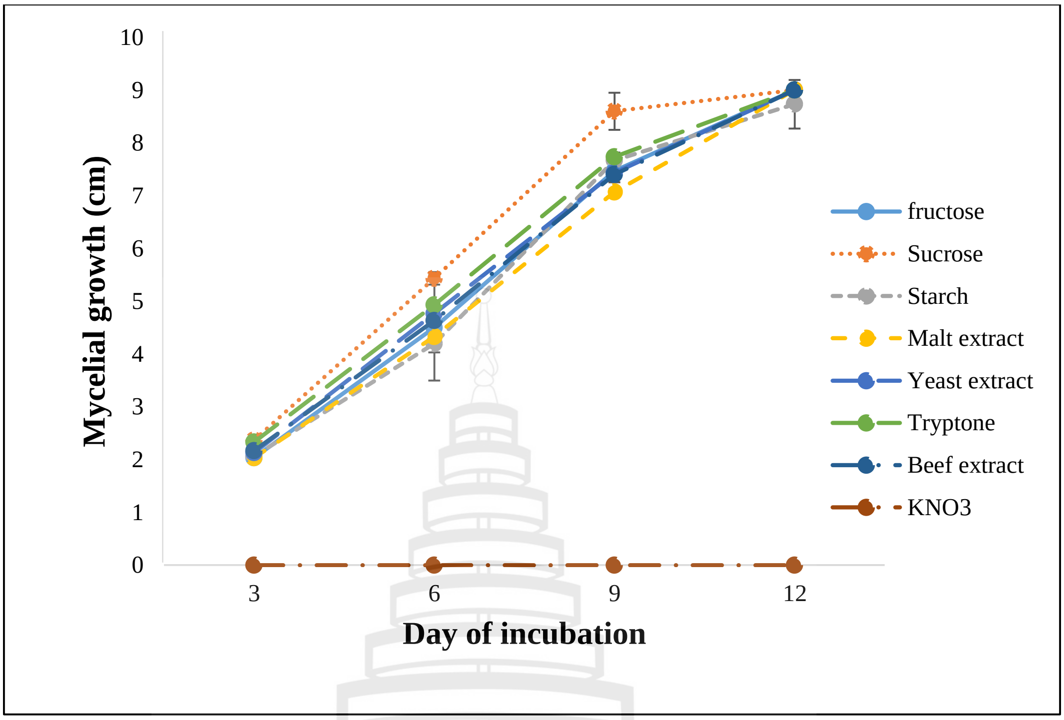

Figure B8 Supplement of carbon and nitrogen sources for mycelial growth of *Clitopilus chalybescens*

APPENDIX C

TLC CHARACTERIZATION OF *Clitopilus doimaesalongensis* AND *Clitopilus Chalybescens*

Figure C1 Identification of the *Clitopilus* extraction: a-b) Mycelium extraction of *C. doimaesalongensis* b-c) Mycelium extraction of *C. Chalybescens* e) Culture broth extraction of *C. doimaesalongensis* f) Culture broth extraction of *C. Chalybescens*

APPENDIX D

PROCEEDINGS AT CONFERENCES

Optimization condition for cultivation of *Agaricus subrufescens* hybrid strains

Kritsana Jatuwong[1,2], Pattana Kakumyan[2,*], Sunita Chamyuang[2], Ekachai Chukeatirote[1,2], Kevin D. Hyde[1,2]

[1]*Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[2]*School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*
**e-mail: pattana.kak@mfu.ac.th*

Abstract

Agaricus subrufescens is edible mushroom, one of the most important medicinal mushroom with high potential to treat many diseases. Several strains of *A. subrufescens* have been cultivated throughout the world, especially in Brazil. *A. subrufescens* hybrid strain in this study has been bred by the INRA, France. This study investigated the optimum conditions for the cultivation of *A. subrufescens* hybrid strains in Northern Thailand. The suitable conditions for mycelium growth on compost extract agar were established in terms of temperature and pH. The results revealed that the optimum temperature and pH for mycelial growth were 25–30˚C and pH 7–9, respectively. Two hybrid strains of *A. subrufescens* CA 918–076 x CA 454–4 and CA 918–076 x CA 487–35 were selected and cultivated on compost media. The ingredients of the compost were consisted of rice straw (200 kg), rice bran (10 kg), urea (2 kg), ammonium sulfate (4 kg), calcium carbonate (2 kg), calcium sulfate (6 kg) and water. The result showed that two hybrid strains, could be cultured using this rice straw compost. Fruiting bodies were produced after 2 months with a yield of 56.92 and 42.30 g /kg of compost, respectively.

Keywords: *Agaricus subrufescens*, compost, hybrid strains, mushroom cultivation

Introduction

Agaricus subrufescens Peck. (1893) (synonyms = *A. blazei, A. brasiliensis*), is a gilled mushroom belongs family Agaricacea (Agaricales Basidiomycetes). It is also called almond mushroom due to its almond odor (Firenzuoil et al., 2008; Zied et al., 2012). *A. subrufescens* was first cultivated in the late 1800s in Eastern North America and described by Peck C.H. in 1893 (Kerrigan, 2005). It was firstly isolated in the 1960s in Brazil (Farnet et al., 2013). This is recognized as the main character with chocolate brown basidiospores. This mushroom grows with a hemispherical to convex to plano-convex shape in mature stage. The surface is dry and covered by fibrillose squamulose. It is a saprobe and inhabits rooting leave, it often grows in clusters or scattered occasionally singly on soil. It was discovered in North America, South America and also has been found in Hawaii, Taiwan, Philippines and Thailand (Kerrigan, 2005; Wisitrassameewong et al., 2012). In addition, *A. subrufescens* is popular as a medicinal food having a potential to teat many common diseases such as arteriosclerosis, cancer, diabetes, cronic hepatitis and hyperlipidaemia (Hobbs, 1991) Mizuno et al., 1990; Firenzuoil et al., 2008; Liu et al., 2008; Jumes et al., 2010). It is a popular mushroom market as extensively cultivated in Brazil and in oriental countries such as Japan or China (Farnet et al., 2013). Llarena-Hernandez et al. (2013) could obtain the hybrid strain between the Brazilian and European strains after investigated the hybridization. The hybrid strains were improved in term of yield and quality for developing new cultures under European growing conditions and also a new hybrid named H1X1 was patented in USA (Kerrigan and Wach, 2008). In this study *A. subrufescens* hybrid strains provided by N. Thongklang (Thongklang

FerVAAP2015 The 6th International Conference on Fermentation Technology for Value Added Agricultural Products

Optimization conditions, antibacterial and antioxidant activities of *Clitopilus chalybescens*

Kritsana Jatuwong[1,2], Kevin D Hyde[1,2], Sunita Chamyuang[1,2], Kenji Matsui[3,4] and Pattana Kakumyan[1,2*]

[1] *Institute of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[2] *School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*
[3] *Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan*
[4] *Departments of Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi University, Yamaguchi, Japan*
* *Corresponding author: pattana.kak@mfu.ac.th*

Abstract

The aim of this work was to study the effect of media, temperature and pH on the mycelial growth of *Clitopilus chalybescens* strain MFLUCC 13–0809 collected from Lampang, Thailand. The antioxidant and antibacterial activities from the crude mycelial extracts were evaluated. The study indicated that the optimal medium, pH and temperature were observed on yeast extract agar (YEA) pH 5 to 7, and 20–29˚C, respectively. For antibacterial activities, crude extracts from mycelium slightly inhibited the growth of Gram positive bacteria (*Bacillus subtilis, Staphylococcus aureus* and *Micrococcus luteus*), but no activities were observed on Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). Antioxidant assay indicated that the crude extract had noticeable scavenging activity on 2,2-diphenyl-1-picrylhydrazyle (DPPH) radical. The results suggest that the crude extract from this mushroom mycelium deem to have a potential for further development on antibacterial and antioxidant applications.

Keywords: *Clitopilus*, optimal condition, antibacterial activity, antioxidant activity.

1. Introduction

Many published data indicate that fruiting body of mushrooms has various biological functions such as antitumor, antioxidant, anti-aging, and immunological properties (1). Few reports are so far available in the antioxidant properties of mycelial extracts.

Clitopilus is a small genus of fungi in the family Entolomataceae (Basidiomycota, Agaicales). The distinct characteristic which could be easily recognized for this genus is farinaceous odor, pink or brownish pink spores and peculiarly spores shaped with longitudinal ribs (2, 3, 4). The cap is whitish to grayish when dry and then slimy surface and sometime a wavy margin. The gill is decurrent attached with the stipe, spaced together rather than closely. The stipe is thick and white and ellipsoid in profile but rounded in polar view (5, 6, 7). This genus has a widespread distribution, especially in temperate areas or subtropics such as Spain (7), Taiwan (8), Thailand (9) and China (8, 10).

The basidiocarp of many *Clitopilus* sp. is reported to produce a biologically activity compound. This study aims to determine the culture condition for mycelium growth and evaluate the antibacterial and antioxidant activities of *C. chalybescens* strain MFLUCC 13–0809.

2. Materials and Methods

2.1 The sample collection and fungal isolation

CURRICULUM VITAE

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NAME	Miss Kritsana Jatuwong
DATE OF BIRTH	6 March 1987
ADDRESS	383 Mae Yao, Muang district, Chiang Rai Thailand 57100
EDUCATIONAL BACKGROUND	
2006 - 2009	Bachelor of Science Program in Agriculture (Plant Pathology), Maejo University, Chiang Mai, Thailand
WORK EXPERIENCE	
2010 - 2011	Sale of agricultural product, Agriculture product department Doi Tung Development Project Co-ordination Centre Royal Initialtive Project of Her Royal Highness the Princess Mother
2012-2014	Research assistance Institute of Excellence in Fungal Research, Mae Fah Luang University

PUBLICATION

Jatuwong, K., Kakumyan, P., Chamyuang, S., Chukeatirote, E. & Hyde, K. D. (2014) **Optimization condition for cultivation of *Agaricus subrufescens* hybrid strains. Proceeding of the 26th Annual Meeting of the Thai Society for Biotechnology and International Conference** (P. 244-251).

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