UNIVERSITI PUTRA MALAYSIA

EFFECT OF TEMPERATURE ON SPOROPHORE DEVELOPMENT AND EXTRACELLULAR ENZYME PRODUCTION *IN AGARICUS BITORQUIS* (QUEL.) SACCARDO

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FSAS 2001 33

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MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA
2001

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By

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Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Science and Environmental Studies Universiti Putra Malaysia

April 2001

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

DEVELOPMENT AND EXTRACELLULAR ENZYME PRODUCTION IN *AGARICUS BITORQUIS* (QUEL.) SACCARDO

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April 2001

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This study was carried out to determine the optimum temperature of different stages of *Agaricus bitorquis* fruit body development from 2 mm to cup stage. Changes in extracellular CM-cellulase and laccase in compost during growth and fruiting were monitored in order to understand the underlying biochemical changes. Fruit bodies were produced at all sets of temperature conditions in rhythmic cycle. Effect of temperature on sporophore development and enzyme production could only be truly tested in the first flush as non-uniform condition of substrate occurred in the later flushes. Incubation of 2 to 10 mm stage at 23^0C (Sets II, III) gave an increase of 58.9% in percentage of primordia survival when compared to that at 30^0C (Set I). Exposure of 10 mm stage sporophores to 30^0C did not affect them from growing satisfactorily. Maximum yield (g of fresh mushroom weight) was attained by Set III cultures, followed by Set II cultures and Set I cultures. The highest average weight of harvested mushroom was attained by cultures kept at 30^0C throughout 2 mm to cup stage (Set I). The time taken for sporophores maturation was shortest in Set I, Set II, and Set III temperature condition, in descending order. A rise in temperature from 23^0C to 30^0C during 10 mm to cup stage

speeded up the sporophore maturation process but resulted in smaller mushroom. The production of CM-cellulase and laccase activities was closely associated with the mycelial growth, fruit bodies initiation and their subsequent development to full maturity. Laccase activity increased with mycelial growth, peaked at maximum growth and then declined rapidly at the onset of fruiting. The activity remained low during fruit body development period but increased suddenly during the flush interval. CM-cellulase activity showed the reverse pattern by remaining low throughout mycelial growth but an increase at the onset of fruiting. The activity remained high during fruit body development and declined rapidly during flush interval. Effect of temperature on the enzymes production was significant only during the 2 to 10 mm stage but not during the subsequent 10 mm to cup stage. The results revealed the extra-sensitivity of young sporophores to environmental factors during the 2 to 10 mm stage.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENGARUH SUHU KE ATAS PERKEMBANGAN BADAN BUAH DAN PENGHASILAN ENZIM EKSTRASELULAR DALAM *AGARICUS BITORQUIS* (QUEL) SACCARDO

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Kajian ini bertujuan untuk menyiasat suhu optimum untuk setiap peringkat perkembangan badan buah *Agaricus bitorquis* dari ukuran diameter 2 mm hingga peringkat cendawan matang. Meneliti perubahan-perubahan pada enzim CM-cellulase dan laccase yang dirembes ke dalam kompos semasa fasa pertumbuhan miselia dan pembuahan cendawan dapat membantu memahami perubahan biokimia yang berlaku pada cendawan ini. Dua hingga tiga pusingan pembuahan didapati berlaku pada kesemua keadaan suhu yang diuji. Pengaruh suhu ke atas perkembangan badan buah dan penghasilan enzim hanya dapat diuji dengan nyata dalam pusingan pembuahan pertama kerana ketidakseragaman kandungan media berlaku pada pusingan yang berikutnya. Pengeraman peringkat ukuran diameter 2 hingga 10 mm pada 23^0C (Set II, III) telah meningkatkan peratus primordia yang terus hidup sebanyak 58.9% berbanding dengan yang dieram pada 30^0C (Set I). Pendedahan badan buah yang berperingkat 10 mm ke atas kepada 30^0C tidak menjejaskan perkembangannya. Hasil cendawan (berat basah cendawan, g) yang paling maksimum diperolehi oleh Set III, diikuti oleh Set II dan Set I. Cendawan yang paling berat diperolehi daripada Set I. Perkembangan badan buah adalah paling cepat pada keadaan

suhu Set I, Set II, dan Set III, dengan turutan menurun. Kenaikan suhu daripada 23^0C ke 30^0C selepas badan buah mencapai peringkat ukuran diameter 10 mm telah mempercepatkan proses kematangan badan buah tetapi juga mengakibatkan penghasilan cendawan yang bersaiz kecil. Penghasilan aktiviti enzim CM-cellulase dan laccase berkait rapat dengan peringkat pertumbuhan miselia, pembuahan dan perkembangan badan buah hingga matang. Aktiviti enzim laccase adalah tinggi berbanding dengan aktiviti enzim CM-cellulase semasa fasa pertumbuhan miselia. Aktiviti ini meningkat secara berkadaran langsung dengan pertumbuhan miselia sehingga mencapai tahap maksimum pada pertumbuhan miselia yang maksimum, dan kemudian merosot dengan cepat pada permulaan fasa pembuahan. Sepanjang peringkat perkembangan badan buah hingga matang, aktiviti ini berkekalan rendah tetapi meningkat semula semasa peringkat rehat di antara dua pusingan pembuahan. Sebaliknya, corak penghasilan aktiviti enzim CM-cellulase adalah bertentangan dengan aktiviti enzim laccase sepanjang fasa pertumbuhan miselia dan pembuahan. Pengaruh suhu ke atas penghasilan enzim-enzim tersebut didapati sangat berkesan pada peringkat ukuran diameter 2 hingga 10 mm sahaja dan tidak berkesan pada peringkat perkembangan badan buah yang seterusnya dari peringkat ukuran diameter 10 mm hingga cendawan matang. Keputusan kajian ini menunjukkan bahawa cendawan ini adalah sangat sensitif kepada perubahan faktor-faktor sekeliling semasa peringkat perkembangan badan buah berdiameter ukuran 2 hingga 10 mm.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Misri Kusnan for his invaluable advice and effective guidance throughout the course of this project. I am indebted to Assoc. Prof. Dr. Tan Yee How for many helpful discussions and critical reviewing of this manuscript. My appreciation also goes to Dr. Faridah Abdullah for her useful suggestions and allowing the use of facilities. Special thank to Assoc. Prof. Dr. Vijaya for her stimulating interest in this research.

Appreciation is extended to Mr. Ganesan and Mr. Hidir Kassim for their skillful technical assistance. Thanks are also expressed to all the staff of Department of Biology for their generous cooperation and assistance during the period of the study is performed.

I would like to express my heartfelt gratitude and indebtedness to Bing for his assistance, concern, encouragement and support throughout my whole study in UPM. He shares the honour of the degree of Master of Science.

TABLE OF CONTENTS

		Page
ABSTRACT		ii
ABSTRAK		iv
ACKNOWLEDGMENTS		vi
APPROVAL SHEET		vii
DECLARATION		ix
LIST OF TABLES		xii
LIST OF FIGURES		xiii
LIST OF PLATES		xiv
CHAPTER		
I	INTRODUCTION	1
II	LITERATURE REVIEW	9
	Genus *Agaricus*	9
	Agaricus bitorquis	13
	Classification	13
	Morphology	13
	Distribution	14
	Background of Cultivation	14
	Life Cycle	16
	Breeding Work	18
	Advantages and Disadvantages of *A. bitorquis* over *A. bisporus*	20
	Advantages	20
	Disadvantages	22
	Mushroom Compost	23
	Materials and their Function in Composting	24
	Basic Materials	24
	Compost Activators	25
	Supplements	27
	Compost Formulation	29
	The Role of Microorganism in Mushroom Nutrition	31
	Spawn Production	33
	Growth Cycle of *Agaricus bitorquis*	35
	Composting	35
	Spawning and Spawn-running	37
	Casing	37
	Pinning and Cropping	39
	Effect of Temperature on Mycelial Growth and Fructification	39
	Production of Cellulase and Laccase by *Agaricus bitorquis*	42
	Cellulase	42
	Cellulosic Substrates	45
	Endocellulase as A Morphogenetic Enzyme of *A. bisporus*	46
	Laccase as A Morphogenetic Enzyme of *A. bisporus*	48

III	MATERIALS AND METHODS	51
	Source of Culture	51
	Preparation of Spawn	53
	Preparation of Rice Straw Compost	55
	Cultivation of *Agaricus bitorquis*	58
	Casing	60
	Manipulation of Temperature during Fruiting Period	64
	Harvesting Strategy	65
	Assays of Enzyme Activities	66
	Preparation of Crude Enzyme Extract	66
	Assay of CM-cellulase Activity	70
	Assay of Laccase Activity	72
	Determination of Protein	72
	Data Analysis	73
IV	RESULTS	74
	Effect of Temperature on Percentage of Primordia Survival	74
	Effect of Temperature on Mushroom Yield	77
	Effect of Temperature on Weight and Cap Diameter of Harvested Mushroom	79
	Effect of Temperature on the Time Taken for Sporophore Maturation	81
	Extracellular Enzymes Production during Mycelial Growth and Primordia Initiation	83
	Production of Extracellular Enzymes during Fruiting	87
V	DISCUSSION	93
	Effect of Temperature on Percentage of Primordia Survival	93
	Effect of Temperature on Mushroom Yield	96
	Effect of Temperature on Weight and Cap Diameter of Harvested Mushroom	98
	Effect of Temperature on the Time Taken for Sporophore Maturation	102
	Extracellular Enzymes Production during Mycelial Growth and Primordia Initiation	105
	Production of Extracellular Enzymes during Fruiting	108
VI	CONCLUSION	113
	REFERENCES	115
	BIOGRAPHICAL SKETCH	127

LIST OF TABLES

Table		Page
1	Some formulations for the preparation of compost	30
2	Components of cellulase system and their functions	44
3	Composition of controls and the test sample for assay of CM-cellulase activity	71
4	Effect of temperature manipulation at different stages of morphogenesis of *A. bitorquis* fruit bodies on percentage of survival of primordia	75
5	Effect of temperature manipulation at different stages of morphogenesis of *A. bitorquis* fruit bodies on the mushroom yield over 45 cropping days at three different sets of temperature conditions	78
6	Effect of temperature manipulation at different stages of morphogenesis of *A. bitorquis* fruit bodies on the number, weight, and cap diameter of harvested mushroom under three different sets of temperature condition	80
7	Effect of temperature manipulation at different stages of morphogenesis of *A. bitorquis* fruit bodies on the time taken for sporophore maturation	81
8	Mycelial growth and specific activities of CM-cellulase and laccase during spawn-run period of *Agaricus bitorquis* (ATCC 32675) at 30^0C cultivated on rice straw compost	85
9	Specific activities of CM-cellulase and laccase in compost during various fruiting stages of *Agaricus bitorquis* (ATCC 32675) kept at different temperatures during 2 mm to cup stage of sporophore development	88

LIST OF FIGURES

Figure		Page
1	C_1-C_X model proposed by Mandel and Reese (1964).....	43
2	Manipulation of temperature at different developmental stages of *Agaricus bitorquis* sporophores	65
3	(A) Mycelila growth and (B) specific activities of laccase and CM-cellulase produced in rice straw straw compost during spawn-run of *Agaricus bitorquis* (ATCC 32675) at 30^0C	86
4	Changes in specific activities of CM-cellulase and laccase in compost during different fruiting stages	89

LIST OF PLATES

Plate		Page
1	Fruit body of *Agaricus bitorquis* (ATCC 32675)	4
2	Subculture of *Agaricus bitorquis* grown on MEA slant	51
3	Pure culture of *Agaricus bitorquis* obtained with tissue culture method	52
4	Spawn ready to be used	54
5	Stacking of rice straw with wooden box	56
6	Covering of composting substrates with gunny sack	56
7	Comparison of rice straw before and after composting	58
8	Rice straw compost filled in polypropylene bag	59
9	Different stages of mycelium colonization process of the casing layer	60
10	Opened production bag covered with a polyethylene bag	63
11	Cup stage of *Agaricus bitorquis*	66
12	Compost bearing primordia	67
13	Compost bearing 10-15 mm young sporophores	67
14	Compost bearing closed button stage sporophores	68
15	Compost bearing sup stage sporophores	68
16	Mycelium-colonized compost	69
17	Crude enzyme extracts	70
18	Comparison of 2-10 mm stage fruit bodies incubated at 23^0C and at 30^0C	76
19	Button stage of *Agaricus bitorquis*	82

CHAPTER I

INTRODUCTION

Since thousands of years ago, mushrooms have been highly prized as a luxury food for their delicious flavour, nutritional value, and medicinal properties. The ancient Greek and the Romans valued them as the 'Food of the Gods'; while the Chinese have treasured mushrooms as a health food and an 'elixir of life' (Quimio *et al.*, 1990; Chang & Buswell, 1996). Until recently, mushrooms have come into their own as a food crop of great economic importance because of renewed interest in natural health foods.

On the aspect of biology, mushrooms are fleshy macrofungi. Their fruiting bodies containing billions of sexual spores (Alexopolous, 1979). The term *mushroom* often refers to the edible sporophore; while the poisonous species are known as *toadstools* but these terms are no longer valid. Most of the edible marcofungi belong to Basidiomycetes, only some are Ascomycetes (Chang & Quimio, 1982).

Commercial cultivation of mushrooms for human consumption is now a world-wide industry. According to Chang (1998), world production of edible mushrooms in 1994 was estimated to be 4,909.3 thousand tonnes which was valued at about 9.8 billion US dollars. He listed the ten most popular cultivated mushrooms of which the

white button mushroom (*Agaricus bisporus*) toped the list at 37.6% of production. It is the world's most popular and consumed mushroom, particularly familiar to North Americans and Europeans. It accounts for nearly 60% of the world mushroom demand (Quimio *et al.*, 1990). Since the first artificial cultivation in France in 1650, the button mushroom has been exploited to be a crop of great economic importance. Nowadays, the mushroom is commercially cultivated in more than 80 countries especially the United Kingdom, Netherlands, France, United States and China. However, the production of *A. bisporus* is limited only to the temperate and sub-temperate regions due to its low temperature requirement. The main drawback of the mushroom cultivation in the lowlands in tropical countries is its requirements for high cost cooling. Otherwise mushroom can be grown only at high altitudes. For this reason, mushroom growers in the sub-tropics and tropics focus mainly on other species of mushrooms with a higher optimum growth temperature such as the straw mushroom (*Volvariella volvacea*) and the oyster mushroom (*Pleurotus spp.*). These species, however, never achieved the same degree of popularity as that of the temperate mushroom. The blackish colour of these mushrooms greatly hinders their acceptance by consumers as a safe table delicacy, especially in Western countries.

For the above reasons, it is commonly thought that growing mushroom in a tropical country like Malaysia cannot be a big and profitable industry as the hot and humid tropical climate can cause serious limitations in the variety of cultivable mushrooms. Consequently, the potential of mushroom industry in Malaysia is often underestimated. There is relatively few research on edible mushroom cultivation so far and the mushroom industry remains in an underdeveloped state. To date, only five species of edible mushrooms are being commercially cultivated. They consist of the

oyster mushroom (*Pleurotus spp.*), shiitake (*Lentinula edodes*), straw mushroom (*V. volvacea*), temperate button mushroom (*A. bisporus*), and wood ear mushroom (*Auricularia spp.*). The total production of mushroom of 1,885 tonnes per year is not adequate to meet the demand of local consumption. Annually, more than RM 21.7 million worth of mushroom still have to be imported from abroad (Noor Auni & Lee, 1991).

Being an agriculture-based nation, Malaysia has a great potential to be successful in mushroom cultivation. There is an enormous quantity of agro-industrial wastes like paddy straw, oil palm wastes, sawdust and cotton wastes that are ideal substrate for mushroom production. With improved knowledge on proper cultivation techniques based on scientific principles, the mushroom industry should be a profitable investment. As the major problem of mushroom cultivation in this nation is the limited number of cultivated species, research on the exploitation of new species of tropical edible fungi for commercial production and their cultivation system adapted to local conditions are most urgently needed.

In the screening for heat-tolerant mushrooms, a tropical strain of *Agaricus* namely *Agaricus bitorquis* (Quel) Saccardo (Plate 1) was found to be the most promising mushroom to be introduced for cultivation in Malaysia. This edible fungus, or commonly known as the hot button mushroom, was first cultivated in the 1970's in Netherlands and is recently introduced into tropical areas like Taiwan and Thailand as a substitute for *A. bisporus*. Being a tropical fungal, *A. bitorquis* grows well under warm and humid tropical climate, at a temperature range of 28-35^{0}C and relative humidity of 80-95% (Isaac *et al.*, 1993). The most striking superiority of the fungus

over other tropical mushrooms is its morphological and edible similarity to the world's most popular mushroom, *A. bisporus*. It produces tasty and silky white fruit bodies typical of *A. bisporus* in shape, thus minimising the consumer resistance towards this mushroom. In addition, it has been proven to be resistant to most known mushroom viruses (Van Zaayen, 1972, 1976). The species is also claimed to be a higher quality mushroom than *A. bisporus* because of its longer post-harvest shelf life and bruise-resistant properties (Nicols & Hammond, 1976; Barnard, 1977; Smith *et al.*, 1993). It is therefore suitable for selling fresh and transported over long distances.

Plate 1: Fruit body of *Agaricus bitorquis* (ATCC 32675)

In Malaysia, *A. bitorquis* has yet to be commercially cultivated due to inadequate information on its cultivation technique. The first cultivation trial of the mushroom was conducted by Choi (1986). The strain tested (Ag_3) failed to produce

fruit body as a result of the severe contamination problems confronted. Teow (1990) had examined the genetic features of *A. bitorquis* but the cultivation technology was unexplored. Subsequently, the cultivation potential of this mushroom in Malaysia was initially revealed in a cultivation trial by Lee in 1997. The strain tested, ATCC 32675, succeeded in producing fruit bodies on a rice-straw compost under sterile conditions. From the study on effects of temperature on mycelial growth and fruit body initiation, two significant conclusions have been drawn. First, 30^0C was the optimum spawn-running temperature. Second, there was no necessity for a cold temperature shock to induce fruit body initiation as recommended in normal cultural practice (Quimio *et al.*, 1990). On the contrary, sporophore initiation occurred best at 30^0C, the optimum spawn-running temperature. The greatest number of primordium initials was formed at 30^0C, compared to the lower temperature of 23^0C and 18^0C. These results reflected that *A. bitorquis* might be cultivated under the natural condition in Malaysia, without the need for artificial cooling. At 30^0C, however, most of the primordia died upon the caps reaching about 2 mm in diameter and hence resulted in a low yield of cup-stage mushrooms. According to Flegg (1980, 1981), there is a sensitive 2-10 mm diameter stage during the development of *A. bisporus* sporophore. The primordium initials when reaching 2 mm in diameter requires a period of a lower temperature, about 6-8^0C lower than the spawn-running temperature. Otherwise, the high spawn-running temperature can greatly inhibit further development of the young mushrooms which subsequently may die off. This temperature requirement remains until the primordia reach about 10 mm in diameter after which the temperature should be raised back to the spawn running temperature to hasten the mushroom development process. These findings clearly indicated that there is different temperature requirements for different stages of morphogenesis for the *Agaricus* mushrooms.

It has been found that in addition to change their requirements toward temperature, the basidiomycete fungi also undergo a dramatic change in the pattern of enzyme excretion in relation to their morphogenesis. In natural or man-made environment, the fungi grow on lignocellulosic substrates comprising of lignin, hemicellulose, cellulose, and protein. In order to utilise these nutrients, the fungal hyphae excrete a range of extracellular enzymes to depolymerize such nutrients into lower molecular weight compounds which the mycelium can then assimilate and utilise for growth and reproduction (Moore-Landecker, 1982). Many cultivated basidiomycetes including *A. bisporus* have been shown to produce the following hydrolytic and oxidative enzymes: endocellulase, exocellulase, β-glucosidase, laccase, protease, xylanase, laminarinase, lipase, glucosaminidae, and N-acetyl-muramidase (Wood & Fermor, 1981). Certain of these enzymes are produced in large quantity and show large changes in activities associated with the mushroom life cycle. Laccase (EC 1.14.18.1) and endocellulase or carboxymethylcellulase (EC 3.2.1.4), for instance, are two well-studied extracellular enzymes whose activities have been found to be tightly linked to the fungal developmental stages. The first enzyme, laccase, is a type of copper-containing polyphenoloxidase which functions in lignin degradation or ligninolysis (Thurston, 1994; Temp *et al.*, 1999). It oxidizes primarily diphenols through a one-electron reaction generating a free radical and quinones as byproducts (Perry *et al.*, 1993). In the cultivated mushroom *A. bisporus* and *L. edodes*, this enzyme activity increases during vegetative growth in precise parallel with mycelial mass and peaks at maximum growth of mycelium. Shortly after the onset of fruit body formation, the enzyme undergoes a rapid inactivation. This accumulation and inactivation cycle is repeated with successive flushes of fruit body development (Wood & Goodenough, 1977; Turner, 1974; Wood, 1980a; Tan & Wahab, 1997). The other

enzyme, CM-cellulase, is a component enzyme in the complex cellulase system. It plays a role in cellulose breakdown by cleaving the cellulose chains to form a variety of smaller molecules of the dimer cellobiose and the trimer cellotriose (Wood & Bhat, 1988). In contrast to laccase activity, CM-cellulase shows the reverse pattern by remaining at a low level of activity throughout mycelial growth period until after the formation of fruit body initials. The activity then increases markedly and remains in direct proportion to the quantity of fruit body biomass produced during each flush of mushrooms (Wood & Goodenough, 1977; Wood, 1978; Claydon *et al.*, 1988).

For their close relationship with basidiomycete morphogenesis, the change in the pattern of enzyme activities is thus an ideal 'marker' to assess the mycelial colonization in growing substrate and to predict successive fruiting cycles. Laccase, for example, is often used to estimate mycelial growth in culture where direct measurements is not possible (Wood, 1979; Smith *et al.*, 1981). The practical application of this knowledge could greatly facilitate the development of a better growth media and a more reliable cultivation technique. Unfortunately, although the association between these extracellular enzymes and morphogenesis of *A. bisporus* has been well-investigated, none of such studies is conducted on *A. bitorquis*. To date, the physiology and biochemistry of vegetative growth and reproduction of *A. bitorquis* are still poorly understood, though it has been artificially cultivated for more than 20 years. This explains why even up to now, mushroom fruiting is still not reliable. Therefore, for an effective and reliable manipulation of the crop, research on the biochemical changes underlying the mycelium morphogenesis into fruit body initials and finally into mature basidiocarps is urgently in demand.

The main objective of the present study is to investigate the different temperature requirements of *A. bitorquis* fruit bodies at different stages of morphogenesis from 2 mm primordia to cup-stage or mature fruit bodies. Survival percentage of primordium initials, yield, size and weight of the harvested mushroom, and the time taken for sporophore maturity were used as parameters in determining the optimum incubation temperature for each stage of development. In order to understand the underlying biochemical changes associated with the fungal developmental stages at different temperature conditions, monitoring of CM-cellulase and laccase activities was carried out during vegetative growth and fruiting of *A. bitorquis* on a sterile rice-straw compost.

CHAPTER II

LITERATURE REVIEW

The Genus *Agaricus*

In most of the older literature, the genus *Agaricus* is known as *Psalliota* (Singer, 1961). However, under the International Code of Nomenclature, the name is no longer considered valid and *Agaricus* is the correct name (Groves, 1975). According to the classification of Ainsworth and Bisby's (Hawksworth *et al.*, 1995), the genus is classified as follows:

Phylum: Basidiomyceta
Class: Basidiomycetes
Order: Agaricales
Family: Agaricaceae

The genus can be easily identified by its dark purple-brown spores and free lamella or gills that are not adjoined to the stipe. The lamellae is white when young but becomes brown coloured when mature due to the attached spores. The pileus or cap is fleshy, white or coloured, naked or squamose, sometimes with warts, or they can be smooth dry. The stipe readily separates from the pileus (Groves, 1975). Another distinguishing property of *Agaricus* is the presence of a simple or double annular membrane around the upper and lower portion of the stipe (Singh, 1961). This, together with a characteristic pileus colour range, simplify the recognition of species in