CULTIVATION OF *AURICULARIA POLYTRICHA* MONT. SACC (BLACK JELLY MUSHROOM) USING OIL PALM WASTES

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FACULTY OF SCIENCE
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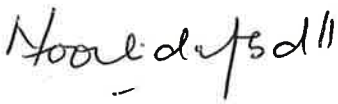
Tandatangan Saksi  Tarikh 17.4.2013

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ABSTRACT

The increasing demand for continuous and quality supply of many types of mushrooms has drawn much attention and research to find potential substrate and domesticating the cultivation several species of edible mushrooms to suit local environmental conditions and resources. One of the popular mushrooms among Malaysians due to its nutritional and medicinal values, *Auricularia polytricha* or Black Jelly, offers high income to local growers due to its ability to grow and fruit in tropical region such as Malaysia. The availability of a wide variety of agro-industrial by-products rich in lignocellulose and other materials required for mushroom growth should be explored and used as the substrate ingredients for the cultivation of *A. polytricha* as well as other mushroom species. This present study consisted of two main parts, namely the study of *A. polytricha* spawn production and formulation of fruiting substrates using oil palm wastes. The best substrate for production of spawn / inoculum was determined by comparing *A. polytricha* mycelia growth on several types of grains. It was found that crushed corn soaked for 4 hours, without adjusting its moisture content and pH and with no addition of supplement or adjustment of C:N ratio was the optimum substrate to support mycelia growth of *A. polytricha* at 6±1 mm/day growth rate. Corn (uncrushed) supported the lowest mycelia growth rate, while soybean and peeled soybean were deemed as unsuitable because of their high contamination rate. For fruiting substrate, the potential of using agricultural and industrial wastes was evaluated using several formulations of selected oil palm wastes combined with sawdust and also supplemented with selected nitrogen sources. The best substrate formulations selected were sawdust (SD) + oil palm frond (OPF) at a ratio of 90:10 supplemented with 15% spent grain (SG) and sawdust (SD) + empty fruit bunch (EFB) at a ratio of 50:50 supplemented with 10% spent grain (SG) with mycelia growth rates of 8±1 mm/day and

7±1 mm/day respectively. These two formulations were then subjected to different moisture content levels (65%, 75% and 85%). Highest total fresh mushroom yield of 43.3% was obtained on SD+OPF (90:10) + 15% SG at 85% moisture content, followed closely by SD+EFB (50:50) + 10% SG with 40.4% total yield, also at 85% moisture content. Each of the substrate formulations at 85% moisture content gave the highest biological efficiencies (BE) at 288.9% and 260.7% respectively. Both yield and biological efficiency of *A. polytricha* on these two formulations were almost three times higher when compared to sawdust substrate alone, thus proving the potential of these formulations to improve yield of this mushroom. Analysis of nutrient content of *A. polytricha* sporophores grown on SD+OPF (90:10) + 15% SG proved that this mushroom contains eight essential amino acids at levels ranging from 0.007 to 0.05%. In addition, cultivated *A. polytricha* in this study was found to be a valuable source of protein, carbohydrates, crude fibre, magnesium, potassium, manganese and phosphorus with values greater than 15% of the percentage daily portion based on RDA for each nutrient.

ABSTRAK

Permintaan yang tinggi dan semakin meningkat bagi bekalan berterusan pelbagai jenis cendawan yang berkualiti telah menarik perhatian ramai penyelidik untuk mencari substrat yang berpotensi bagi penanaman cendawan dan seterusnya mengadaptasi kaedah penanaman cendawan tersebut kepada sumber dan keadaan persekitaran tempatan. Salah satu cendawan yang semakin popular di Malaysia disebabkan oleh nilai nutrisi dan perubatannya adalah *Auricularia polytricha* atau cendawan Jeli Hitam, menawarkan pulangan ekonomi yang besar kepada penanam cendawan tempatan oleh kerana kesesuaian cendawan ini untuk ditanam di rantau tropika. Ketersediaan pelbagai jenis sisa pertanian yang kaya dengan lignoselulos dan bahan lain yang diperlukan bagi pertumbuhan cendawan perlu diterokai potensinya untuk digunakan sebagai bahan substrat alternatif untuk penanaman *A. polytricha* serta cendawan yang lain. Kajian ini terdiri daripada dua bahagian utama iaitu penghasilan benih atau inokulum dan formulasi substrat penghasilan janabuah *A. polytricha* menggunakan sisa dan produk sampingan industri pertanian terutamanya industri kelapa sawit. Substrat terbaik bagi penghasilan benih atau inokulum telah dipilih berdasarkan kadar pertumbuhan miselia *A. polytricha* di dalam beberapa jenis bijirin terpilih. Melalui beberapa siri eksperimen, didapati bahawa jagung hancur yang direndam selama 4 jam, tanpa pengubahsuaian ke atas kandungan kelembapan, nilai pH dan nisbah C:N serta tanpa ditambah dengan sumber nitrogen luaran merupakan substrat optima untuk menyokong pertumbuhan miselia pada kadar pertumbuhan 6±1 mm/hari . Jagung menyokong pertumbuhan miselia yang terendah manakala kacang soya dan kacang soya tanpa kulit didapati tidak sesuai kerana kadar kontaminasi yang tinggi. Bagi substrat penghasilan janabuah, potensi penggunaan sisa dan produk sampingan industri pertanian telah dinilai dengan menggunakan beberapa jenis formulasi sisa

industri kelapa sawit terpilih yang digabungkan dengan habuk kayu serta ditambah dengan sumber nitrogen terpilih. Formulasi substrat yang terbaik adalah habuk kayu (SD) + pelepah sawit (OPF) pada kadar 90:10 ditambah dengan + 15% bijirin terpakai (SG) dan habuk kayu getah (SD) + tandan kosong (EFB) pada kadar 50:50 ditambah dengan + 10% bijirin terpakai dengan kadar pertumbuhan miselia masing-masing sebanyak 8±1 mm/hari dan 7±1 mm/hari. Kedua-dua jenis formulasi kemudiannya diuji dengan kadar kandungan kelembapan yang berbeza-beza (65%, 75% dan 85%). Jumlah hasil cendawan segar yang tertinggi pada kadar 43.3% telah diperolehi daripada formulasi SD+OPF (90:10) + 15% bijirin terpakai (SG) pada kandungan kelembapan 85%, diikuti rapat oleh SD+EFB (50:50) + 10% bijirin terpakai juga pada 85% kandungan kelembapan dengan jumlah hasil cendawan segar sebanyak 40.4%. Kedua-dua formulasi substrat yang sama juga masing-masing telah memberikan kadar kecekapan biologi (BE) tertinggi iaitu 288.9% dan 260.7%. Hasil cendawan segar dan kecekapan biologi *A. polytricha* di dalam kedua-dua formulasi tersebut adalah hampir tiga kali ganda lebih tinggi dari substrat habuk kayu getah, sekaligus membuktikan potensi kedua-dua formulasi substrat ini untuk meningkatkan kadar pengeluaran cendawan *A. polytricha*. Analisa kandungan nutrient ke atas cendawan *A. polytricha* yang tumbuh pada substrat SD+OPF (90:10) + 15% SG membuktikan bahawa cendawan ini mengandungi lapan jenis asid amino penting pada kadar antara 0.007 hingga 0.05%. Selain itu, *A. polytricha* yang ditanam dalam kajian ini merupakan sumber berharga bagi protein, karbohidrat, serat, magnesium, potassium, manganese dan fosforus dengan kadar melebihi 15% berdasarkan RDA untuk setiap nutrient.

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Dang Lelamurni Binti Abd Razak

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	percentage
°C	:	degree Celsius
BE	:	biological efficiency
C	:	carbon
$CaCO_3$	:	calcium carbonate
EFB	:	empty fruit bunches
kg	:	kilogram
kg/cm^2	:	kilogram per square centimetre
g	:	gram
mm	:	milimetre
mg	:	miligram
mg/kg	:	milligram per kilogram
N	:	nitrogen
OPF	:	oil palm fronds
PPF	:	palm pressed fibres
SD	:	sawdust
SG	:	brewery's spent grain
SY	:	brewery's spent yeast
sp	:	species
RB	:	rice bran
UV	:	ultra violet
w/w	:	weight per weight

CHAPTER 1

INTRODUCTION

1.1 Agricultural Wastes Problem in Malaysia

All over the world, great amounts of solid wastes, residues and by-products, produced in the agriculture sector and its related industries, giving continuously increasing and serious environmental pollution problem. The scenario is not so much different in Malaysia. Malaysia as one of the world's primary producers and exporters of palm oil involves 4.17mil hectares of plantation, 397 mills and 51 refineries in 2006. Two of the largest amounts of wastes from oil palm plantation and palm oil processing being produced are EFB and OPF. PPF also are being produced in a very large quantity. All wastes are available daily throughout the year when the palms are pruned during the harvesting of fresh fruit bunches for the production of oil. From all the fibre and shell waste, only about 60% is burnt to generate electricity and steam. Because of a ban on open burning of agricultural waste in Malaysia, the remaining 40% of the waste is taken away by contractors to be put into the landfills. Given the large amount of available palm oil waste, the lack of landfill space, the ban on agricultural open burning and the large number of palm oil mills (417 as of 2009), there is good potential for these wastes to be recycled to produce beneficial products.

Apart from residues from palm oil industry, other residues include paddy straws, rice husks, wood sawdust and chips, sugarcane bagasse and many more. All wastes are very rich in plant nutrients. Without proper management, these wastes can become a problem to our country. Alternatively, these residues and by-products can be recovered

and upgraded to higher value and useful products by chemical and biological process (Wang, 1999) in order to solve the wastes abundance issue.

1.2 Mushroom Cultivation as a Potential Solution to Wastes Problems

One economically suitable solution to the waste is by producing mushrooms using agricultural wastes. Cultivation of edible mushrooms on agricultural residues / wastes, such as palm oil wastes, rice straw, rice bran and sugarcane bagasse, is a value-added process for conversion of the waste materials into food and value-added products for human consumption and benefits. It is highly regarded as one of most efficient biological ways to both recycle and reuse these wastes and by-products (Madan et.al, 1987). Furthermore, there is a problem of deficiency of protein-rich source of food around the world and especially in developing countries. Mushroom cultivation offers solution to both problems.

Fungi have the capability to colonize organic materials such as woods and forestry wastes to produce edible reproductive structures called fruit bodies or sporophores. This special ability has been exploited for centuries in Asia for the production of mushrooms like Shiitake (*Lentinulus edodes*), the oyster mushroom (*Pleurotus* spp.) and *Auricularia* spp. (Paterson, 2006). In addition, the spent substrate produced after final harvesting of the cultivated mushrooms, can be recycled into valuable fertilizer which also function as a soil conditioner for the growth of plants and animal feed (Soto-Cruz *et al*., 1999). Palm plantation has the potential to produce very large volumes of biomass that can be used for the production of renewable and beneficial products. The large quantity of OPF, EFB and PPF yielded by a plantation each year and their high nutritional values make these a highly suitable and promising source of substrate for mushroom production. Therefore, palm oil by-products is the perfect candidate as a

primary substrate for mushroom cultivation because they are available all-year-round, in abundance, cheap (if not free) and contain nutrients such as hemicellulose and lignin needed by the mushroom to grow.

One of the best mushroom candidates to be cultivated on a large scale in Malaysia using particularly palm oil residues is *Auricularia polytricha*, also known locally as 'Jeli Hitam' or Black Jelly mushroom. This mushroom ranked number four behind button mushroom (*Agaricus bisporus*), shiitake (*Lentinus edodes*) and osyter mushroom (*Pleurotus* spp.*)* in the list of most popular and most produced mushroom in the world (Mau *et al*., 2001). *Auricularia polytricha* is highly prized in Asia and can be cultivated in both temperate and tropical regions depending on its varieties. It is rich in protein, low in fat and contains variety of vitamins and minerals. Black Jelly mushroom has a long-standing reputation in Chinese traditional medicine for its ability to increase the fluidity of the blood and improving blood circulation along with other health benefits. Although listed as one of the top culinary mushroom and highly demanded in Malaysia, the production of Black Jelly mushroom by our local growers is still insufficient and inefficient. Growers are still using conventional methods for the mushroom cultivation without efficient management. Most of the dried *A. polytricha* mushroom in Malaysian market is imported from China, and fresh form of this mushroom can hardly be found.

Our local black jelly growers are using sawdust as the primary substrate. For these growers, there is a problem of shortage or limited supplies of sawdust due to competition from other industries such as fiber and wood based boards and charcoal briquet production. These industries offer higher price for sawdust supplies from its supplier causing the price of sawdust to increase. Second, sawdust supplies are often mixed up with chemicals used in the processing industry. The tainted supply of sawdust affected mushroom growth – low yield, high percentage of contamination &

unsynchronized flushing patterns. Therefore, alternative substrates to replace sawdust are desperately needed by our local mushroom industry.

The cultivation of mushrooms on agricultural wastes may offer economic incentives for agribusiness by examining these residues as valuable resources and use them to produce nutritious mushrooms. Utilization of palm oil empty fruit bunch (EFB), fronds (OPF) and pressed fiber (PPF) as alternative substrates for the cultivation of Black Jelly mushroom will provide solutions to both the glut of palm oil wastes and the problems faced in Black Jelly mushroom cultivation. All three wastes are of equivalent standard to sawdust used in mushroom cultivation, based on their carbon and nitrogen content and other nutrients beneficial for mushroom growth. A new substrate formulation using palm oil agricultural wastes developed in this project will be later introduced to our local growers.

1.3 Objectives of Study

(i). To select spawn substrate and to optimize growth conditions for the production of *Auricularia polytricha* spawn / inoculums.

(ii). To select fruiting substrate(s) utilizing oil palm wastes and to optimize growth conditions for cultivation of *A. polytricha*.

CHAPTER 2

LITERATURE REVIEW

2.1 Agriculture and Agro-industrial Wastes or By-products in Malaysia

Large amounts of agriculture and agro-industrial residues or wastes are excessively produced by the agriculture and agro-industrial activities. Generally, solid agro-industrial residues are consisted of cellulose, hemicelluloses and lignin and also pectin, starch and other polysaccharides and are insoluble in water (Thomsen, 2005). The wastes derived from agricultural activities can be used as a resource for sustainable production of food and value-added food products. According to Wang and Yang (2007), expensive treatments or disposal is required if these wastes are not recycled or used to generate a value-added product. Adverse effect on the environment would also occur if these wastes are not managed effectively and left in the waste stream.

2.1.1 Oil palm wastes

Malaysia, one of the largest global producers and exporters of palm oil is reported having 4.17 million hectares of plantation, 417 mills and 51 refineries in 2009. The largest amounts of palm oil waste being produced are empty fruit bunches (EFB), oil palm fronds (OPF) and palm pressed fibres (PPF). Wastes are available daily throughout the year and only about 60 % of all the produced fibre and shell waste are burned to generate electricity and steam. Due to a ban on open burning of agricultural waste in Malaysia, the remaining 40 % of the waste is removed by contractors to be put into landfills. According to Chang (1999), 95% of total biomass produced in coconut

and palm oil plantations is discarded without being reused. For each ton of crude palm oil produced from fresh fruit bunches, around 150 tons of waste oil palm fronds (OPF), palm oil mill effluent (POME), palm trunks, empty fruit bunches (EFB) and palm pressed fiber (PPF) become wastes and by-products. In short, oil palm plantation has the potential to yield a very large amount of biomass which is very rich in plant nutrient to be used for the production of renewable products. Currently, OPF are left rotting between the rows of palm trees to be used mainly for soil conservation, control of erosion control and eventually for nutrient recycling. The large quantity of biomass produced by oil palm plantation each year and their high nutritional values make these wastes a very promising source of substrate for mushroom production. In Malaysia, sawdust is currently used as the main substrate for cultivation of edible and medicinal mushroom. OPF, EFB and PPF are of equivalent standard as sawdust to be used in mushroom cultivation, based on their percentage of carbon sources as shown in Table 2.1.

Table 2.1: Carbon and lignin composition and content in sawdust, OPF, PPF and EFB

Carbon & Lignin Composition	**Carbon & Lignin Content (%)**			
	Sawdust/ Hardwood[a]	OPF[a]	EFB[a]	PPF[b]
Cellulose	40-55	49.8	44.2	30.2
Hemi-cellulose	24-40	33.3	33.5	23.2
Lignin	13-25	30.5	30.5	22.9

Source: a; Deraman, 1993, b; Ponthein & Cheirsilp, 2011

2.1.2 Other wastes

Apart from oil palm wastes, Malaysia also produced wastes from agriculture industry such as paddy, rubber, pineapple and many more. Nearly 600 million tons of rice is produced each year around the world (Pourali *et al*., 2009 and Sereewatthanawut

et al., 2008). About 60 million tons of rice bran, a by-product of the rice milling process is produced yearly (Pourali *et al*., 2009) and is usually used as animal feed (Renuka Devi and Arumughan, 2007). According to Rao *et al*. (1993), rice bran also has been used for the production of enzymes such as lipase by *Candida* sp. as a fermentation substrate. Other utilisation of rice bran is as a supplement to fruiting substrate in mushroom cultivation. Rice bran naturally contains proteins, fibres, vitamins, minerals and antioxidant (Pourali *et al*., 2009). Its nutritional property, which can support the growth and development of mushroom, makes it suitable as a source of supplement used in mushroom cultivation.

Relatively large amounts of wastes are generated from the brewing industry and the most common are spent grain and spent yeast (Mussatto *et al*., 2006). Spent grain contributing most of total by-products generated from the brewing industry and is available at a very low or no cost throughout the year. Mussatto *et al*. (2006) stated that brewer's spent grain rich in polysaccharide (cellulosic and non-cellulosic), lignin, and also contain protein and lipid. According to Schildbach *et al*. (1992), brewer's spent grain has been successfully used as ingredient in preparation of substrate for cultivation of several edible mushrooms, such as *Pleurotus* and *Lentinus*. Brewer's spent grain was found to favour the growth of mushroom due to its high protein and moisture content. Physical properties of spent grain such as particle size, porosity, density, volume weight and water-holding capacity are also part of the cause (Wang *et al*., 2001 and Mussatto *et al.,* 2006). Brewer's yeast is used to make beer. It is made from a one-celled fungus called *Saccharomyces cerevisiae*. Most of the yeasts are reused in the brewing processes and excess yeast is recovered by natural sedimentation at the end of the fermentation. Only small percentage of the yeast can be reused in the brewing process. Brewer's spent yeast is very high in protein and B vitamins, and may be given as a feeding supplement

to livestock such as ruminants. Spent brewer's yeast is also known for being used for yeast extract production (Tanguler and Erten, 2008) and the insoluble yeast cell wall fraction obtained was used as a raw material for the preparation of brewer's yeast beta-glucan (Suphantharika *et al.*, 2003)

2.2 Mushroom Cultivation

2.2.1 Mushroom cultivation in Malaysia

According to Chang (1999), approximately 12,000 species of fungi are considered as mushroom with around 200 species are edible. Edible mushrooms are easily cultivable and can be consumed either in fresh, dried or processed form. Mushrooms are known to be excellent sources of protein, besides having a low fat content and are free of cholesterol. Cultivation of edible and medicinal mushroom presents an economically important biotechnological industry that has developed all over the world (Fan *et al*., 2008). Significant increase in consumer awareness on the health benefit of edible mushrooms, market demand and production of mushrooms are continuously increasing (Aida *et al*., 2009). Besides being a source of food, mushrooms have also been used as medicinal resources. Both edible and medicinal mushroom production has expanded over the past decade due to the community's better acceptance of consuming both mushrooms and also their derivative products, along with strain and technical improvements (Chang 1999, 2005 and Fan *et al*., 2008). Royse (2005) and Fan *et al*. (2008) reported an estimation of more than 10 million metric ton of edible and medicinal mushrooms were produced in 2004 in various countries.

2.2.2 Mushroom cultivation using agricultural wastes or by-products

Cultivation of edible and medicinal mushrooms using agricultural wastes / by-products, such as palm oil wastes, rice straw, rice bran and sugarcane bagasse, is a value-added process for conversion of these materials into human food. As described by Sanchez (2010), cultivation of edible mushroom can be regarded as the only current process that combines the production of highly nutritious food with the reduction of environmental pollution, in which agricultural wastes and by-products are recycled.

Hundreds of billion tons of organic matter are produced per year through the photosynthetic process on the surface of our planet (Philippoussis, 2009 and Zhang, 2008). Subsequently, there is a significant emphasis on recovery, recycling and also upgrading of wastes (Philippoussis, 2009). Agricultural and agro-industrial residues and by-products mostly consisted of lignocellulosic biomass are annually generated with a massive amount (Kuhad *et al.* 1997 and Philippoussis, 2009). The chemical properties of agricultural and agro-industrial by products and wastes make them a substrate of enormous and vast biotechnological value. According to Philippoussis (2009), these lignocellulosic wastes which are often discarded as wastes, can be converted into various different value-added products such as mushrooms animal feed enriched with microbial biomass, bio-fertilizer or bio-pesticide made from compost, enzyme, secondary metabolites and many more, by solid state fermentation (SSF). Chang (2006) and Chiu *et al.* (2000) stated that, compared with other applications of solid state fermentation, mushroom cultivation is a proven tool of economic strength and is ecologically important for the sustainable management of agro-industrial residues or wastes.

Many wood decomposing fungi utilize lignocelluloses efficiently and this characteristic can be attributed to their ability to metabolize lignin (Baysal *et al*., 2003).

Huge lignocellulosic materials can be used in edible and medicinal mushroom cultivation and at the same time also protecting and regenerating the environment. Martinez-Carrera *et al.* (2000) and Chiu and Moore (2001) reported that commercial mushroom production is a relatively short but efficient biological process of food protein recovery from negative-value lignocellulosic materials by utilizing the degrading capabilities of mushroom fungi. Currently, the mushrooms are considered and universally accepted as the most profitable and environment friendly method for recycling of the vast lignocellulosic wastes. Mushroom cultivation has already generates reasonable economic growth by utilizing the non-productive agricultural wastes and by-products. However, scientific research on mushrooms especially mushroom cultivation, is in general, lags behind bacterial, plant and animal researches (Sonnenberg *et al.*, 2005) most probably due to the limited investment in the area of mushroom researches and also because the mushroom industry is still small compared with many other plant crops.

It is reported that more than 2000 species of mushrooms exist in nature, but approximately only 22 species are intensively cultivated (Manzi *et al.*, 2001) with the most cultivated worldwide species are from *Agaricus, Pleurotus, Lentinula, Auricularia, Flammulina* and *Volvariella* genus. Although these edible mushroom species have the capability to degrade lignocellulosic materials both in their natural or composted form (Rajarathnam *et al.* 1998 and Philippousis, 2009), they exhibit differences regarding the production of enzymes necessary to degrade lignocellulosic substrates (Philippoussis, 2009) and consequently different capabilities to grow and fruit on lignocellulosic-residue substrates (Chen *et al.* 2003 and Baldrian and Valaskova 2008). According to Philippoussis (2009) regarding the types of wastes, agricultural residues can be divided into crop-based residues which are generated in the field and

processing-based residues, generated during the processing of crop. Crop based residues are plant materials left behind in the field or farm after removal of the main crop produce, consisted of different sizes, shapes, forms and densities, such as straw, stalks, leaves, roots and branches. As reported by Philippoussis (2009), by-products of the post-harvest processes of crops such as cleaning, threshing, sieving and crushing are considered as processing-based agro-industrial residues in various forms such as of husk, dust or stalks. Some of the food processing wastes that come from plant materials are culls, rinds, seeds, pits, pulp, press cakes, marc, malts, hops and a variety of other by-products from mass food production processes. Some examples of these materials are coffee processing by-products, sugarcane bagasse, hulls and husks, wheat middlings, corncobs, seed meals and many more (Philippoussis, 2009). All of these wastes and by-products have the potential to be used in mushroom cultivation.

From 2001-2004, Malaysian government experienced a RM 56.93 million trade deficit for the importation of mushroom (Department of Agriculture Malaysia, 2006). According to a report by Department of Agriculture Malaysia in 2006, it is approximated that 8,100 ton of mushroom per year is needed and demanded by Malaysian consumers today and the figure will steadily increase for the years to come. However, only 5,500 ton can be produced by our local growers. It is obvious that our mushroom local industry is in desperate need to be further developed into a stable, economical, sustainable and profitable mushroom-producing industry. Cellulose, hemicelluloses, lignin, pectin, starch and other polysaccharides and other materials existed in agriculture as well as agro-industrial residues and by-products are the basic nutrients needed by mushroom to grow. Philippoussis *et al.* (2000, 2002) reported that the composition of nutrients in substrates is one of the factors affecting fungi colonization and also can affect yield of cultivated mushroom, quantitative and

qualitatively. Also, it is widely known that different type of mushrooms require different types of substrates. Therefore, scientific approaches are very much needed to obtain sufficient information on the best substrates for each cultivated mushroom species, and also to find supplement that best work with each substrates in order to support and guarantee high yield and good quality mushroom. Strong scientific research and development can definitely fill in the gap that has been slowing our mushroom industry and ultimately transformed it into a well developed industry in Malaysia and in the world.

2.3 *Auricularia polytricha* (Montagne) Sacccardo

2.3.1 Taxonomy

In scientific classification, *Auricularia* spp. belongs to the Fungi kingdom and is grouped in Basidiomycota division, in Heterobasidiomycetes class, Auriculariales order and in the family of Auriculariacea. The genus name *Auricularia* is believed to be derived from the Greek word *auricula*, meaning ear (Chang & Miles, 2004). It is reported that there are 15 to 20 species worldwide, with eight species, *A. auricula*, *A. polytricha*, *A. mesenterica*, *A. delicate*, *A. fusfocucolnea*, *A. peltata*, *A. cornea* and *A. hispida* have been reported to be identified in China alone. Among these species, *A. auricula* and *A. polytricha* is the most popular and the most cultivated around the world.

Auricularia polytricha has a variety of common names, such as Wood Ear, Tree Ear or Ear Fungus. In China, it is known as Yu er, Mu-er or Mo-er, in Japan as Kikurage or Mokurage and in Indonesia as Kuping Jamu. In Malaysia, it is locally known as Black Jelly or 'Jeli Hitam'.

As described by Stamets (1993), the spores of *A. polytricha* are produced on a club-like structure and the spore print is white in color. The mycelia of this species are longitudinally linear. As they grow older, the mycelia mat is thickening with age to form a dense cottony white mat and becoming mottled with brown discoloration. *Auricularia polytricha* fruiting bodies have no stipe and are covered by medulla of fine hairs. Its surface is smooth but wrinkled towards the center and upturned towards the outer edge. The fruiting bodies when fresh are usually brownish to reddish brown, ear-shaped and have a consistency of jelly – firmly gelatinous and rubbery (Figure 2.1). Upon drying, they are usually looks purplish brown to black, they shrink to a portion of their original size. When contacted with water, they rehydrate and enlarge true to form. Black Jelly mushroom has an atypical texture when eaten but it is not really flavorful when compare to other popular edible mushrooms (Stamets, 1993).

2.3.2 Distribution

Auricularia polytricha is worldwide in its distribution in both temperate and tropical region as well as subtropical regions. The natural habitat for this mushroom is on conifer or hardwood logs or stumps, and also commonly occurring in soils rich in wood debris. *Auricularia* exists in a mutualistic relationship with the plants and trees on which it grows, and is known as a mycorrhizal fungus. This mushroom generally favors cool weather, but can be cultivated in both temperate and tropical regions depending on the varieties. *Auricularia polytricha* is extremely popular and highly prized in Asia and is popularly used in soups especially by the Chinese community. They are marketed in both fresh and dried form, but the greatest volume of this mushroom is sold in dried form. Due to their organoleptic attributes, dried mushrooms can be used as ingredients for many types of dishes (Stachowiak & Regula, 2012).

Figure 2.1: *Auricularia polytricha* fruit body
(Source: http://fungi.sakura.ne.jp/ajiwai_kinoko/aragekikurage.htm)

2.3.3 Nutritional and medicinal properties

Most edible mushrooms are food with good nutritional value, but some mushroom also exhibit medicinal value apart from its nutritional properties. *Auricularia polytricha* is among those mushroom that contains both nutritional and medicinal properties that can be considered as functional food. The fruiting body of this mushroom contains around 8-10% protein, 0.8-1.2 % fat, 84-87% carbohydrate, 9-14% fiber and 4-7% ash (Ying, 1987). Moisture content of fresh mushroom is reported around 90%. This mushroom also contains vitamins such as thiamine, riboflavin, niacin, biotin and ascorbic acid. *Auricularia polytricha* is also a good source of essential amino acids, such as leucine, isoleucine, lysine and valine. Like other popular edible

mushrooms, *A. polytricha* also contains minerals such as potassium (K), phosphorus (P), sodium (Na), calcium (Ca) and magnesium (Mg).

Auricularia polytricha has a reputation in Chinese herbal medicine for increasing the fluidity of the blood and improving blood circulation. Research has shown that black jelly mushroom lowers cholesterol levels and lipid concentration in blood (Kaneda & Tokuda, 1966). Additionally, the methanolic extract of *A. polytricha* has antioxidant activity and thus, helps in preventing lipid oxidation, scavenging radicals and chelating metal ions in vitro (Chang, 1999). Ma *et al.* (2010) reported that polysaccharide extracts of *A. polytricha* posses anticancer activity. This finding is supported by a recent research involving in vitro test on mice conducted by Song and Du (2012) which revealed that α β-glucan polysaccharide from *A. polytricha* exhibits antitumor activity. Also, based on the tests conducted by Yuan *et al.* (1998), it is discovered that water-soluble polysaccharide fraction from *A. polytricha* had a hypoglycaemic effect on genetically diabetic mice. Furthermore, *A. polytricha* also exhibits antinociceptive effects. Recent findings by Li *et al*. (2012) suggested that isolated melanin from *Auricularia* species have the potential for application in clinical medicine and food industry.

2.3.4 Cultivation of *Auricularia polytricha*

Auricularia is described to be the very first mushroom cultivated by human beings, attributed by its long-standing popularity as food and also for its medicinal properties in China. *Auricularia polytricha* is reported as the first edible mushroom species to be cultivated (Chang and Miles, 2004). It is reported that widespread cultivation takes place in China and many other Asia countries such as Vietnam, Philippines Taiwan and Thailand. The cultivation of this mushroom is not restricted by

geography because the mushroom can be cultivated in both tropical and temperate region, also can be basically produced on a variety of sawdust substrate from many types of woods.

In Asia, the most common natural method of cultivation of this species is by using cut logs drilled with holes. The mushroom's spawn is then pack tightly into the cavities of the cut logs. On large scale, the method of cultivation for this specific mushroom varies. *Auricularia polytricha* commonly are produced on a synthetic medium consisting of sawdust, cotton seed hulls, bran, and other cereal grains as nutrient supplements, as reported by Chang & Quimio (1982) and Oei (1991). Some growers prefer using polypropylene bags and others might prefer using bottles. However, plastic bag cultivation is now a more popular option due to the scarcity of suitable logs for *A. polytricha* cultivation and also the ease with which different species of *Auricularia* can be cultivated on sawdust. Spawn are mostly made of grains with the more popular option being wheat, but millet, rye or sorghum also are being used. Alternatively, there is also a report of sawdust spawn for this species. The substrate for fruiting also varies but the most common substrate used by growers is sawdust. However, there is a report of successful usage of wheat straw as fruiting substrate for this species (Jianjung, 1991). In Philippines, the fruiting substrate used in cultivation of *A. polytricha* is sawdust with rice bran and calcium carbonate as supplements.

In Malaysia, *A. polytricha* is one of the top culinary mushrooms and is highly demanded. Interest in *A. polytricha* cultivation in Malaysia is increasing day by day due to its longer shelf life in addition to the nutritional and medicinal importance. There are a lot of information that exists about the cultivation and production of oyster mushroom in Malaysia, but very little information is available about *A. polytricha.* Its production by Malaysian growers is still inefficient with low yield due to lack of research to

domesticate the species under local conditions. Most of the dried *A. polytricha* mushroom in Malaysian market is imported from China, and fresh form of this mushroom can hardly be found. Telinga Kera growers of our local industry are using sawdust as the primary substrate, but there are problems concerning the use of sawdust for *A. polytricha* cultivation. One of the problems is limited supplies of sawdust mostly due to competition from other industries, such as fiber and wood based boards and charcoal briquet production. These industries offer higher price for sawdust supplies from its supplier causing the price of sawdust to increase. Another problem is that sawdust supplies are often mixed up with chemicals used in the processing industry. The tainted supply of sawdust affected mushroom growth – low yield, high percentage of contamination and unsynchronized flushing patterns. Therefore, alternative substrates to replace sawdust and optimized conditions for high yield mushroom growth are desperately needed by our local mushroom industry, and these alternatives should be supported by strong scientific research to generate a commercial production technology for *A. polytricha* by obtaining a good quality spawn and creating specifically-made substrates for the species, thus ensuring high yield and good quality mushroom. For local *A. polytricha* production, the fruiting substrate currently used by the growers is sawdust.

The suitable solution to find alternative substrate for sawdust is by using agricultural wastes, specifically oil palm waste. Very few researches has been reported on using palm oil by-products as ingredients in cultivation substrates for *A. polytricha* mushroom, but attempts to use them as primary substrate have never been reported so far. A research with the aim of producing high yield biomass of *A. polytricha* in sub-merged liquid medium under different physico-chemical parameters has been conducted (Jonathan *et al.*, 2009). Research on the effects of oil palm kernel meal on yield of Hed

Hu Hnu (*Auricularia polytricha* (Mont.) Sacc.) and Hed Khon Khao (*Lentinus squarrosulus* Mont.) have been conducted in Prince Of Songkla University, Thailand (Petcharat and Tongwised, 2003).

2.4 General Nutritional Requirement for Mushroom Growth

Mushroom is a macro-fungus with a unique fruiting body, can be easily seen with naked eye and large enough to be picked by hand, which can be either epigeous or hypogeous (Chang and Miles, 2004). This definition is acceptable as a working term in cultivation of edible mushroom although it is not a perfect one. Mushroom, like all fungi, cannot undergo photosynthesis because of the lack of chlorophyll and thus get the necessary nutrients from organic materials.

Mushrooms require carbon, nitrogen and inorganic compounds as their nutritional sources (Sharma *et al*., 2013). The main organic materials required by the mushroom are carbon sources such as cellulose, hemicelluloses and lignin, along with other compounds which can be easily broken down by the extensive enzyme system in mushroom to provide nutrients for mushroom growth. The enzymes involved in degradation process of lignocellulosic substrate are extracellular enzymes such as celluloses, pectinases, xylanases, oxidases and peroxidases. All these enzymes degrade the insoluble compounds to smaller and soluble micro-molecules that are then transported to the intracellular space and stored as glycogen (Letham, 1985). The organic compounds can be converted to the various sugars, polysaccharides, proteins, lipids, purines, pyrimidines and vitamins once they have entered the fungal cells, where they are required for the vital activities and structural needs of the fungi (Chang and Miles, 2004). For this purpose, types and formulation of substrates chosen in mushroom

cultivation should contain a balance content of nutritional compounds needed for the mushroom, and also a balance content of carbon and nitrogen.

2.4.1 Basic substrate

Materials that can be used as substrate that can be used for mushroom cultivation are very well diverse and abundant. A vast variety of wood and wide range of wastes or by-products from agriculture and forestry industries can be explored to be used as substrate materials because the majority of cultivated mushroom is saprophytic-typed, which exist on dead organic matter (Fan *et al*., 2008). These organic materials contain lignin and cellulose, besides other compounds that can be easily broken down by the extensive enzyme system in mushroom. The substrates chosen and used in mushroom cultivation can include both field-based residues such as oil palm frond, corn husk, rice straw and wheat straw and also processing based-residues such as sugarcane bagasse, brewers/spent grains, rice bran and palm pressed fibre. However, it is recommended that supplements containing sugars and starch as well as fats be added to the basic ingredient because these supplements are time-lasting nutrient sources and are more slowly degraded (Philippoussis, 2009). This is because the composition of nutrients of the substrate is one of the many important factors limiting fungi colonization and also affects the yield quantity and quality of cultivated mushrooms (Philippoussis *et al.* 2000, 2002).

Carbon, nitrogen, minerals and vitamins are the four basic chemical compounds needed by mushroom for growth. Therefore, to ensure success in mushroom cultivation, all four compounds should be sufficiently present in the basic substrate with emphasis on a balance content of carbon and nitrogen. This is because carbon and nitrogen play bigger roles on overall growing process of mushroom. Varied form of carbon source

such as monosaccharide, oligosaccharide and polysaccharide are essential for the growth of mycelium, especially polysaccharide such as cellulose and hemicelluloses. Most polysaccharides are hydrolyzed to produce sugar. Nitrogen sources such as acid amino, urea, ammonium and nitrate are needed by the fungus to synthesize proteins, purines, pirimidines and also help to produce chitin (Chang *et al.* 1981). Compared to other plant materials, wood has low level of nitrogen content but high in lignin. High concentrations of carbon and nitrogen sources are generally required to obtain high mycelium biomass, but in some types of mushrooms, it is observed that high glucose concentration inhibits mycelium growth. Different level of carbon and nitrogen content are required by different types of mushroom and thus require different optimum C:N ratio for both mycelium growth and fruiting body formation. For example, *V. volvacea* can be grown on plant materials with low nitrogen content but evidently some mushroom types requires high nitrogen content.

All Basidiomycetes mushroom like *A. polytricha* also need several element of mineral to stimulate their growth. According to Chang (1982), most fungi are able to synthesize its own vitamin and low concentration of vitamin resulted in optimum growth of fungi, reported in some cases. Thiamin and B1 vitamin are needed for mycelium growth, primordia formation and also fruiting bodies. Generally, higher level of vitamin is required by mushroom during primordia and fruiting body formation phase compare to vegetative phase or mycelia growth.

It is recommended by Stamets (1993) that particular attention should be given to the structure of substrate, because the structure of the fruiting substrate affects the design of the mycelium network as it is projected. Studies conducted by Royse & Sanchez-Vazquez (2000, 2001) have demonstrated the influence of substrate particle size on mushroom yield. Too small or too fine particle size can cause the loss of air

space due to compression, although fine particles encourage mycelia to grow quickly. Large particle size can cause the lack of density of substrate and subsequently resulted in the increase of mycelium cellular energy required by mushroom to generate chains of cells in order to move from one particle down to the other with a large gap in between. Therefore, a mixture of fine and large particles should be the ideal substrate structure. Chang and Miles (2004) explained that a suitable chemical condition is one that allows for the release of some nutrients from the substrate during fermentation, whereas suitable physical condition is defined as one that provides for good aeration, water-holding capacity and anchorage or support for the mushrooms.

2.4.2 Supplement

Supplements are used to enhance nutritional content, accelerate growth as well as to increase mushroom yield (Philippoussis, 2009 and Royse, 1997). There are a wide variety of protein-rich materials used in mushroom cultivation, such as rice bran, wheat bran, spent grain, spent yeast, molasses, cotton and coffee wastes and many more. According to Stamets (1993), supplementing a substrate risks competition from contaminants and insects because supplementation changes the number and the type of organisms that can be supported. This means that contamination can be easily occur if supplementation of the substrate is not done appropriately. Therefore, extra caution is required to prevent contamination and ensure success. One of the methods to achieve this is by prolonging the sterilization cycle of the substrates. Many studies have reported an increase in mushroom yield by adding supplements to the basic spawn and fruiting substrates, depending on the type of mushroom cultivated, supplement types and concentration of supplements added. Nevertheless, excessive use of supplements can resulted in the reduce effects of the substrates on mushroom production. Hadwan *et al.*

(1997) recommended supplementation of the substrate with various materials such as rice bran, spent grain and wheat grain, prior to spawning for enhancement of the yield of mushroom. To provide optimum growth medium for mushroom cultivation, Royse (2001) suggested the addition of different starch –based supplements such as wheat and rice bran, rye, millet and maize powder to sawdust to serve as major nutrients. Research conducted by Chang *et al.* (1981) and Laborde *et al.* (1985) exhibited substantial increase in the yield of fruiting bodies per unit weight by addition of supplements to wheat straw substrate in oyster mushroom cultivation. Wang (2010) also reported that supplementation of fruiting substrate resulted in a significant increase of oyster mushroom yield. This is further supported by reports by Mau *et al.* (2002), Chang (1996) and Okhuoya *et al.* (2005) which has proved that supplementation of substrate were indeed improve the production, quality, flavor and also shelf life of cultivated mushrooms. The positive effect of supplementation can be correlated with the nutrients present in those supplements. According to Fasidi and Kadiri (1993), carbohydrates, amino acids and mineral elements present in rice bran can be the triggering factor for the increase of the productivity of Shiitake mushroom.

2.5 Stages in Mushroom Cultivation

According to Wang (1999), mushroom cultivation consisted of three major stages which are spawn (inoculum) production, preparation of substrate for fruiting and mushroom growing. Basically, these stages involved inoculation propagules of the specific fungus on the substrate, followed by colonisation of the substrate by the growth of the fungal mycelium and subsequently fruiting and harvesting of fruiting bodies or sporophores. Vegetative phase (mycelium) and reproductive phase (fruiting body

formation) are the two phases of mushroom life cycle involved in this stage of mushroom cultivation. Substrates chosen for both phases should be done carefully, in order for the substrate to be able to provide suitable chemical and physical environment for good growth of mushroom. Supplementation should also be considered in both phases to enhance growth and also fruiting. The flow chart of mushroom cultivation is given in Figure 2.2.

2.5.1 Spawn production

Zadrazil *et al.* (2004) described that spawn or mycelia running phase involved the growth of mycelium through substrate following inoculation, biodegradation of the substrates ingredients by the mycelium and at the same time the mycelia supports the formation of fruiting bodies. During this phase, it is crucial to maintain the suitable temperature and humidity for the cultivated mushroom species. Mushroom spawn is described as a medium through which the mycelium of a fruiting culture has grown and which serves as the inoculum or "seed" for the substrates in mushroom cultivation (Chang, 2001 and Chang and Miles, 2004). The whole process of preparation in inoculums or spawn making should be performed under sterile condition. The phase in which the spawn is produced is called a vegetative phase of mushroom life cycle. The substrates used in inoculum /spawn manufacture may be different from the materials used in the cultivation or fruiting of the mushroom. Substrates may be used singly or in combination. Some of the popular and widely used substrates used in the spawn making include various grains such as wheat and sorghum (Chang, 2001). Production of spawn is considered as a difficult and fastidious task and is often regarded as non-practical for the common mushroom grower. Therefore it is usually produced by specialist spawn manufacturers using microbiological sterile techniques (Philippoussis, 2009).

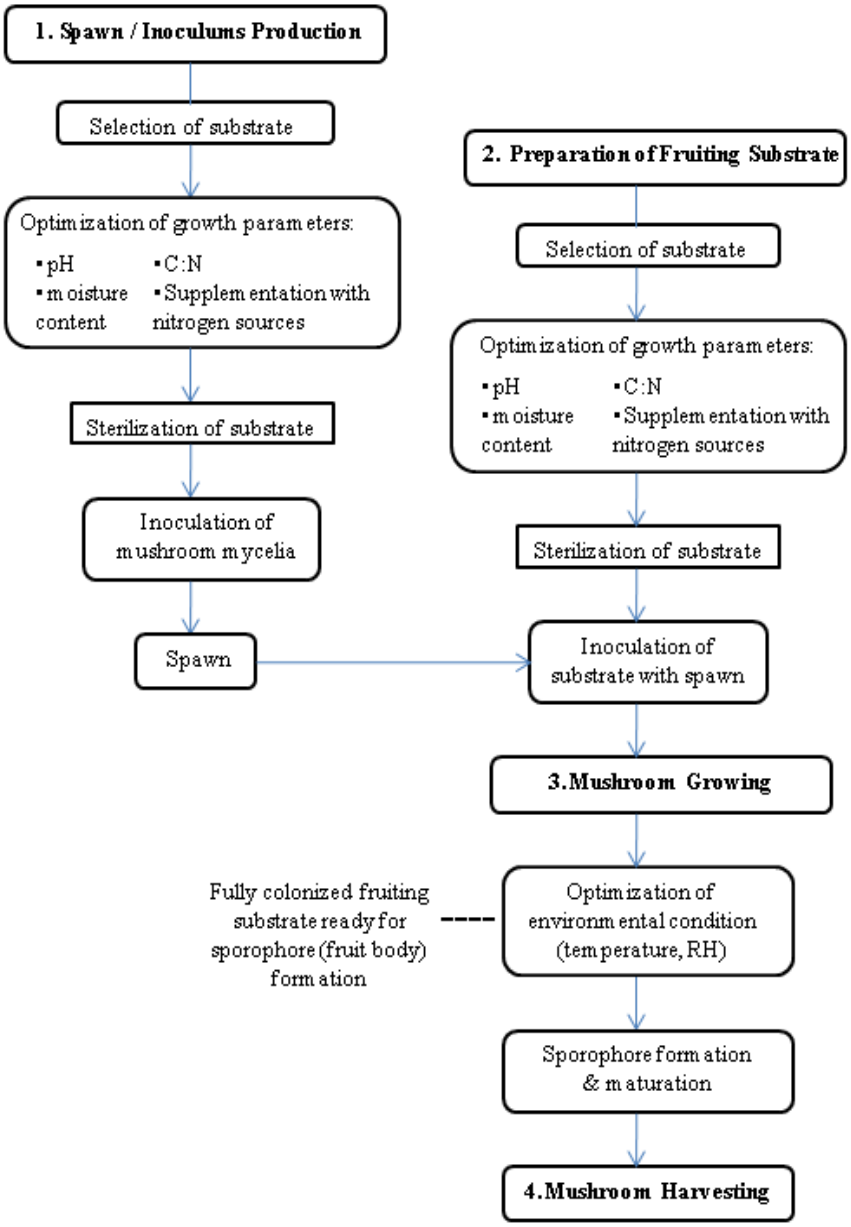

Figure 2.2 : Simplified diagram of processes in mushroom cultivation

As described earlier, inoculum or spawn is a colonized grain-mycelium mixture is grown under sterile conditions in polyethylene bags by which sufficient gas exchange is ensured. Stamets (1993) explained that the intent and purpose of inoculum / spawn is to boost the mycelium to a state of vigor where it can be launched into bulk substrates. The substrate used is not only as a vehicle for evenly distributing the mycelium, but also act as nutritional supplement. The spawn is indicated as ready to be used when the mycelium has run over the whole surface as well as permeated through the substrate. According to Klebs' principles (Griffin, 1981) the process of growth (vegetative phase) is different from reproduction (fruiting bodies formation) where, as suggested by Parbery (1996), growth may take place under a wider range of environmental conditions than reproduction, and also under conditions that inhibit reproduction.

In Malaysia, wheat is currently being used as a substrate for creating inoculum / spawn for every type of mushroom cultivated locally. Organized scientific research can provide alternatives for spawn makers so that they will have a range of option to choose their substrates from, if anything should limit or interrupt the supply of wheat. Furthermore, there is a high possibility which any grains, other than wheat, are much more suitable for specific types of mushroom.

2.5.2 Preparation of fruiting substrate

The quality and yield of mushroom as well as mycelium growth are affected by the nutrient composition as well as the physical nature of the substrate used (Kues & Liu, 2000 and Baldrian & Valaskova, 2008). Thus, the best substrate for mushroom cultivation must be chemically and physically suitable to the targeted species. As the platform from which mushroom arise, the fruiting substrate formulas are specifically designed and often added with varieties of supplements to further nourish the substrates

for specific mushroom production. Referring to Phillipoussis (2009), the first stage of mushroom production involved the preparation of growth medium which is assembly and treatment of the substrate used. The substrates used in mushroom cultivation are prepared from forest and agricultural materials or wastes by using ingredients such as sawdust, wheat and rice straws or other suitable crop residues, and are varies according to cultivated species. These substrates can be directly inoculated with little pre-treatment, whereas in some cases, they required microbiologically and physically pre-treatment. Wood (1989) explained that microbiological pre-treatment of substrate generally comprises some form of controlled bulk composting process, while physical pre-treatment may include sterilization by autoclaving. Such steps are crucial because, as explained by Chang and Miles (2004), a substrate rich in available nutrients does not necessarily constitute a satisfactory medium for growing mushroom. They need to be treated accordingly or else contamination by bacteria or molds will occur in abundance and consequently limits the growth of mycelium and ultimately the growth of mushroom. The mean of substrates preparation method relies entirely on the specific cultivated mushroom species.

Philippoussis (2009) reported that to ensure that the water activity of the final medium is optimal for fungal growth, sufficient water in the substrate is highly important and must be carefully considered during substrate preparation. Therefore, in order to allow good growth of mycelium and later, growth of mushroom, it is crucial to prepare the substrates to their optimal conditions.

2.5.3 Mycelia growth and sporophore formation

There are many factors involved in determining the success of mushroom growing. Chemical factors, such as type of substrate, composting method, if suitable and necessary as well as nutritional supplements should all be filling the needs of the fungi to grow and bear fruits. These factors should also be able to help the fungi to withstand any microbial competitors that can interrupt the whole process. Equally important is physical factors, such as pH, temperature, aeration and light should be all set to the precise requirement for specific cultivated mushroom. The formation of fruiting bodies or sporophores is critically influenced by the nutritional state as well as physiological condition of the mycelium.

When spawn running phase are completed, primordial formation will occur under suitable environmental conditions. This is followed by the production of sporophores. There are two general types of primordia. The first type is called compact primordium, which is tightly interwoven with hyphae and the other type is called diffuse primordium which develops into a hemisphere of hyphae. These primordia later develop into sporophores, given the suitable conditions that they need.

As mentioned earlier, many researchers found that the conditions that are favourable for reproduction are always less favourable for growth (Parbery, 1996). Environmental conditions appropriate for the reproductive phase are generally different from those for spawn running (vegetative phase). The beginning of fruiting body development is in correlation with nutritional deprivation of the growth substrates (Fan *et al*., 2008). According to Granado *et.al*. (1997), wounding causes outgrowth of fresh hyphae. Therefore, it is evident that mechanical injury of established mycelium can locally stimulates fruiting body development (Fan *et al*., 2008). This is why matured fruiting bags are cut either horizontally or vertically using a clean knife, to promote

primordia formation and also to provide space for mushrooms / fruiting bodies to emerge. Hydrogen ion concentration (pH), temperature, aeration and light are the environmental factors attributing to the success of primordia and fruiting body formation. The optimal pH values may differ between species and between growth and fruiting phases. The optimum temperature range for fruiting is generally narrower than for mycelia growth. Adequate aeration is required in both vegetative and reproductive phases, but the requirement is reportedly more stringent in reproductive phase. Harmful accumulation of carbon dioxide occurs when good ventilation is not provided in the mushroom house and can lead to the failure of fungi to fruit. Thus, proper management of aeration and ventilation in mushroom houses are very important to guarantee the good quantity and quality of fruiting bodies. Depending on the species, fungi can equally fruit in darkness, continuous light or alternating light and darkness. Generally, many edible fungi fruit well with alternating light and darkness.

The appearance of mushrooms is commonly in rhythmic cycles called “flushes” (Chang and Miles, 2004). Factors that determined different maturation stages which mushroom should be picked are type of species, consumer preference and market value, among others. All physical parameters should be maintained during cropping period as these factors will also affect the number of flushes and hence, total yield that will be obtained (Chang, 2001).

2.6 Biological Efficiency

Biological efficiency (BE) is an expression of mushroom yield. This formula is widely used to measure the ability of mushroom strains to convert substrate materials into fruiting bodies. BE is determined as the ratio of weight of fresh mushroom

harvested per weight of dry substrate used and expressed as a percentage (Shen and Royse, 2001). The formula states that 1 kilogram of fresh mushrooms grown from 1 kilogram of dry substrate is 100% biological efficiency. This simple formula can be easily used by the mushroom growers to determine the effectiveness of the substrate they used, whether it is economically efficient in producing their mushrooms.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Procedure for Mushroom Cultivation

3.1.1 Media preparation

Malt extract agar (Oxoid) was used as the basic medium for the growth of *Auricularia polytricha* mycelia. Twenty grams malt extract agar was weighed on the balance (Mettler Toledo) and dissolved in 250 ml distilled water. Medium was then sterilized in an autoclave (Tomy) for 20 minutes at 120°C under 1 kg/cm^2 pressure. After sterilization, the medium was poured into pre-sterilized 90 mm plastic Petri dishes and cooled in room temperature prior to use. This was done aseptically in laminar air flow cabinet (Gelman Sciences).

3.1.2 Preparation of mycelia culture

Auricularia polytricha (KUM61091) was derived from stock culture from University Malaya Culture Collection. For the propagation of the main culture, malt extract agar (MEA) was used. After sterilization in an autoclave for 20 minutes at 120°C under 1kg/cm^2 pressures, the medium was poured into 90-mm diameter pre-sterilized plastic Petri dishes. After solidification of the media, the plates were inoculated with a 9-mm diameter mycelium-agar plug of a young, actively growing margin of the colony. Prior to its use as materials for grain spawn / inoculum, the plates were incubated in a growth chamber at 25°C for 7 days. All inoculation work was done aseptically in a sterilized laminar air flow. Laminar air flow was sterilized by spraying

the cubicle with 70% denatured alcohol before it was wiped clean and radiated with ultra-violet (UV) light for 30 minutes.

Sterilization of some materials and apparatus are crucial in order to prevent contamination from occurring. Therefore, sterilization of materials and apparatus were conducted before beginning of any work. Cotton wool plugs, needle, spatula, cork borer, plastic bag necks and covers and distilled water were sterilized in an autoclave for 20 minutes at 120°C under 1 kg/cm^2 pressure. Before autoclaving, needle, spatula and cork borer were wrapped in aluminium foil while plastic bag necks and covers and cotton wool plug were packed in autoclave bag. All apparatus and materials were sprayed with 70% denatured alcohol before being transferred into laminar air flow cabinet. Before carrying out work, autoclaved cork borer and needle were dipped in alcohol and then heated with flame from oil lamp in the laminar air flow cabinet. This was also done from time to time during inoculation work.

3.1.3 Collection of fruiting substrates

Oil palm fronds (OPF) were collected at Ladang Ampar Tenang in Dengkil, Selangor. Fronds were cut into smaller parts and grounded. Empty fruit bunches (EFB) and palm pressed fibres (PPF) were collected at Seri Ulu Langat Palm Oil Mill in Dengkil, Selangor. Both EFB and PPF were dried under the sun before being grounded. All grounded materials were kept in sacks in a dry place.

Brewer's spent yeasts and spent grains were collected at Carlsberg Brewery Malaysia Berhad. After collection from the brew house at Carlsberg, spent grains were dried in the oven at 50°C approximately for 3 to 4 days until fully dried. The grains cannot be kept while still damp because they are easily contaminated by fungus and bacteria. The grains were then kept in plastic bags in a dry place. For spent yeasts, the

wastes were put into autoclave bags and then autoclaved at 120°C under 1 kg/cm^2 pressures for 5 minutes. This step is done to stop the yeasts from growing and to prevent foaming during sterilization in autoclave machine. After 24 hours, all yeasts were then transferred into Schott Duran bottles prior to autoclaving at 120°C under 1 kg/cm^2 pressure for 20 minutes to fully sterilise the spent yeasts. After cooling, the yeasts were kept in a fridge prior to use.

3.1.4 Carbon and nitrogen content analysis of substrate materials

All grains used in the production of spawn / inoculum study (corn, wheat, soybean and millet), palm oil wastes (EFB, OPF and PPF), sawdust, supplements (rice bran, spent yeast, spent grain and molasses) were subjected to carbon and nitrogen content analysis. This was done in order to determine the C/N ratio of each materials and also to determine the C/N ratio of substrate formulations used in the present study. Total carbon content was analysed using Furnace method and nitrogen content was analysed using Kjeldahl method.

3.2 Optimization of Substrates for *A. polytricha* Inoculum / Spawn Production

A series of experiments were done to find the best substrate material and suitable physical conditions for the production of *A. polytricha* inoculum in this present study. The components of this study were selection of substrate materials, moisture content, substrates' pH and supplementation of nitrogen sources. In all experiments involved in this particular study, *A. polytricha* mycelial growth rate and mycelia thickness were measured to evaluate and select the best conditions for the production of *A. polytricha* inoculum. Flow chart of the experiments involved is displayed in Figure 3.1.

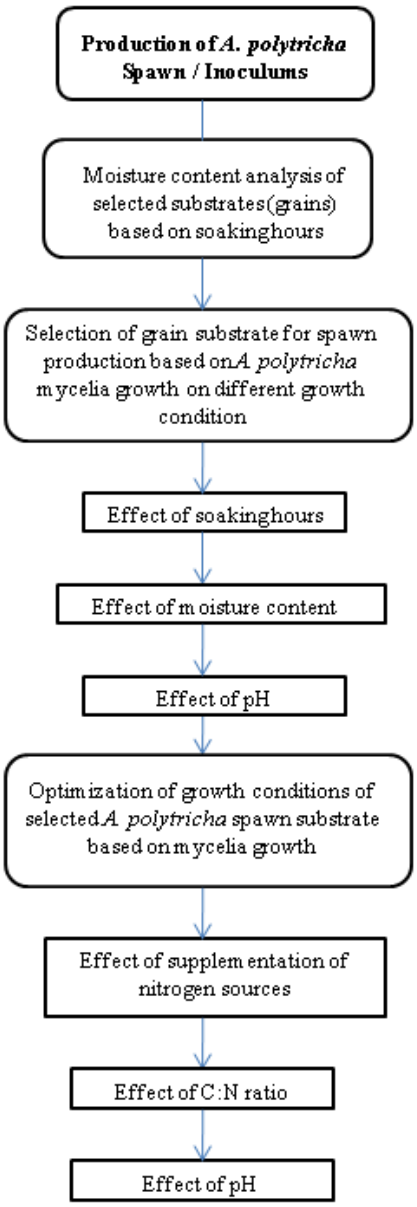

Figure 3.1 : Stages of experiments involved in production of spawn / inoculums for *A. polytricha*

3.2.1 Moisture content analysis

Several grains namely corn, crushed corn, soybean, peeled soybean, wheat and millet, used in the experiments for inoculum development were tested for their moisture content. This was done to obtain the moisture content reading of all grains used in this study for further experiments involving adjustment of the grains' moisture content. The moisture content of the grains when soaked for 2 hours, 4 hours, 6 hours, 8 hours, overnight and 24 hours were also tested. The grains were washed and rinsed repeatedly and then soaked in filtered water. All the grains were ground using a Waring blender. Moisture content analyses were done in three replicates for each sample. Moisture content was measured using AND MX-50 Moisture Analyzer. This experiment was carried out in Fermentation Lab, Biotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI) in Serdang, Selangor.

3.2.2 Selection of grain(s) as substrate for *A. polytricha* inoculums / spawn production

Grains (corn, crushed corn, millet, wheat, soybean and peeled soybean) were washed, rinsed and soaked in filtered water. Different sets of experiments were run to test the effect of (a) soaking hours, (b) moisture content and (c) pH on *A. polytricha* mycelia growth. Calcium carbonate and vinegar were used to adjust the pH of grains as needed. Moisture content of the grains was adjusted to the desired values based on the weight and moisture content (after soaking) of the grains used. The grains were prepared in 5 replicates for each experiment. For each replicate, 300 grams of grain were put in autoclavable polyethylene bag. All bags were then sterilized by autoclaving at 120°C under 1 kg/cm^2 pressure for 60 minutes to be fully sterilized. The bags were let cooled prior to inoculation.

A 7-day old mycelia plug each was put on the four sides of the bag on top of the grains in the polyethylene bag. Inoculated bags were transferred to an incubation cabinet and kept in room temperature. Mycelia growth was determined by measuring mycelia extension at 4 sides of the bag at 2-day intervals for 14 days. The average reading was plotted against time (day) to obtain the growth rate in mm/day. ANOVA were also performed using Minitab Statistical Software to determine the mean growth rate, standard deviation (SD) and homogeneity.

3.2.3 Effect of supplementation of nitrogen sources to selected spawn substrate on mycelia growth of *A. polytricha*

This experiment were done using only crushed corn as the primary substrates, while three types of nitrogen sources were added as a supplement i.e rice bran, spent yeast and spent grain. Crushed corns were soaked overnight. Percentage of nitrogen source added was 10% (w/w) and pH of the substrates was adjusted to 6.00 (±0.15) using calcium carbonate and vinegar. Procedure for preparing the inoculum bags, inoculation, incubation and data collection were as describe in 3.2.2. Five replicates were prepared for each substrate formulations.

3.2.4 Effect of carbon and nitrogen ratio (C:N) content in selected spawn substrate on mycelia growth of *A. polytricha*

Crushed corn was used as the primary substrate, while molasses and spent grains were used to adjust C:N value to targeted values, which is 5-points and 10-points lower and 5-points and 10-points higher than the natural C:N value of crushed corn, which is 77.26. Crushed corns were soaked overnight and pH of the substrates was adjusted to 6.00 (±0.15) using calcium carbonate and vinegar. Five substrates formulations

prepared for this experiment are listed in Table 3.1. C/N ratio for each formulation was calculated using a specific mathematical equation as described in Appendix B. Procedure for preparing the inoculum bags, inoculation, incubation and data collection were as describe in 3.2.3. Five replicates were prepared for each substrate formulations.

Table 3.1: Substrate formulations created according to targeted C/N values

Targeted C/N value	Actual C/N value	Formulations used to achieve targeted C/N values
87.26	85.20	95% Crushed Corn + 5% Molasses
82.26	81.02	90% Crushed Corn + 10% Molasses
-	**77.26**	**100% Crushed Corn**
72.26	70.38	95% Crushed Corn + 5% Spent Grain
67.26	64.27	90% Crushed Corn + 10% Spent Grain

3.2.5 Effect of pH of spawn substrate on mycelia growth of *A. polytricha*

Crushed corn was used as the primary substrate and was soaked overnight. pH of the substrates was adjusted to 5.00 (±0.15), 6.00 (±0.15) and 7.00 (±0.15) using calcium carbonate and vinegar. Procedure for preparing the inoculum bags, inoculation, incubation and data collection were as describe in 3.2.3. Five replicates were prepared for each substrate formulations.

3.3 Determination of the Best Fruiting Substrate Formulation(s) For *A. polytricha* Cultivation

In this present study, we tested three types of palm oil wastes namely oil palm frond (OPF), empty fruit bunches (EFB) and palm pressed fibre (PPF) for their ability to support *A. polytricha* cultivation as a sole fruiting substrate at 100% each. Then,

several formulations were prepared by combining selected palm oil waste (OPF, EFB and PPF) with sawdust according to specific percentages and compared to each substrate used at 100%. Dried and ground oil palm wastes were used in both studies. The basis of selecting the best fruiting substrate formulations in this study was the mycelia growth rate, sporophore yield and also biological efficiency (BE) of substrates. Flow chart of the experiments involved is displayed in Figure 3.2.

3.3.1 Selection of palm oil waste as fruiting substrate for *A. polytricha*

The objective of this study was to determine if the selected oil palm wastes can be solely used as fruiting substrates for *A. polytricha* cultivation. Three types of oil palm wastes namely OFB, PPF and EFB at 100% and without any addition of supplement were tested. Sawdust was used as comparison. Two groups of substrate were prepared: one with adjusted initial pH to 6.00±0.15 and the other pH was not adjusted. The initial moisture content was fixed to 80% by adding water based on the weight of the substrates used. 15 gram of each substrate was put into glass Petri dishes, compressed by pressing the substrates using fingers and autoclaved for 60 minutes at 120°C under 1 kg/1cm^2 pressures. 7- day old mycelia plugs were then inoculated in the centre of the substrate. All Petri dishes were double-sealed with plastic paraffin film. Inoculated substrates were transferred into incubation cabinet and kept in room temperature. Mycelia growth in each Petri dish was determined by measuring the average diameter of the mycelia colony every day for 2 weeks. The average reading was plotted against time (day) to obtain the growth rate in mm/day. Mycelia thickness was also observed. The growth rate of mycelia in each formulation was measured using a mathematical formula described in 3.5.1. ANOVA were also performed using Minitab Statistical Software to determine the mean growth rate, standard deviation (SD) and homogeneity.

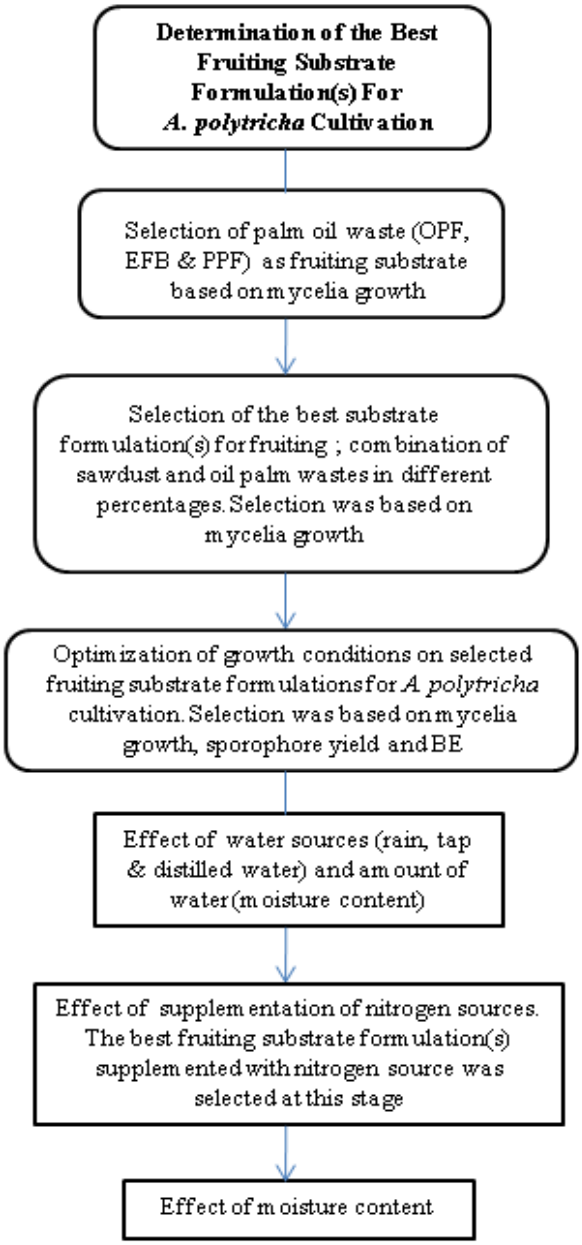

Figure 3.2 : Stages of experiments involved in determination of the best fruiting substrate formulation(s) for *A. polytricha* cultivation

3.3.2 Selection of the best substrate formulation(s) for fruiting

Based on the results obtained from previous experiment, several fruiting substrate formulations were created. The objective of this study is to find the most suitable fruiting substrate(s) for *A. polytricha* cultivation which can give a rapid mycelia run and thick mycelia. Several fruiting substrate formulations were prepared by combining selected palm oil waste (OPF, EFB and PPF) with sawdust according to specific percentages and compared to each substrate used at 100%. Dried and ground oil palm wastes were used in this study. The initial moisture content was fixed to 80% by adding water. Two groups of substrate were prepared, one with the pH adjusted to 6.00±0.15 using calcium carbonate or vinegar and the other was not adjusted. This study was done in Petri dishes. Sterilization, inoculation and incubation works are as described in 3.4.1. The formulations tested in this study are as listed in Table 3.2. Observation and data collections are as described in 3.3.1. C/N ratio for each formulation was calculated using a specific mathematical equation as described in Appendix B.

Table 3.2: Fruiting substrate formulations

No.	Formulations
1.	Sawdust + OPF (90:10)
2.	Sawdust + PPF (90:10)
3.	Sawdust + EFB (90:10)
4.	Sawdust + OPF (80:20)
5.	Sawdust + PPF (80:20)
6.	Sawdust + EFB (80:20)
7.	Sawdust + OPF (70:30)
8.	Sawdust + PPF (70:30)
9.	Sawdust + EFB (70:30)
10.	Sawdust + OPF (50:50)
11.	Sawdust + PPF (50:50)
12.	Sawdust + EFB (50:50)

3.4 Optimization of Growth Conditions on Selected Fruiting Substrate Formulations for *A. polytricha* Cultivation

3.4.1 Effect of moisture content in selected fruiting substrate formulations on *A. polytricha* mycelial growth, sporophore yield and biological efficiency

Different levels (65%, 75% and 85% v/w) of moisture content were used in substrate formulations selected based on the results of previous study. The substrate formulations used were sawdust mixed with EFB (50:50) and sawdust mixed with OPF (90:10) and compared to 100% sawdust. The formulations were evaluated for mycelia growth and sporophore or fruiting body yield of *A. polytricha* in polyethylene bags with the size of 82×322 mm. Initially, each heat-resistant polyethylene bag was filled with substrate moistened with distilled water according to the substrate's initial moisture content to achieve the respective moisture content. Five replicates were prepared for each treatment. The substrate bags were then autoclaved for 60 min at 120°C under 1 kg/1 cm2 pressures and allowed to cool. Inoculation, incubation and data collection were as described in 3.4.5.

3.4.2 Effect of water sources in selected fruiting substrate formulations on *A. polytricha* mycelial growth, sporophore yield and biological efficiency

Two selected fruiting formulations, sawdust mixed with EFB (50:50) and sawdust mixed with OPF (90:10) were subjected to test with different types of water sources. The sources used were rain, tap and distilled water. Based on the results from previous study (3.4.1), substrate bags were prepared by adding water to achieve 75% moisture content in SD+OPF (90:10) and 85% in SD+EFB (50:50) and 85% in SD (100) used as comparison, respectively. Five replicates were prepared for each treatment. All substrate

bags were then autoclaved for 60 minutes at 120°C under 1kg/1cm^2 pressures and cooled prior to inoculation. Inoculation, incubation and data collection were as described in 3.4.5.

3.4.3 Effect of supplementation of nitrogen sources to selected fruiting substrate formulations on *A. polytricha* mycelial growth

Based on the results of previous study, two substrate formulations were selected ie sawdust mixed with EFB (50:50) and sawdust mixed with OPF (90:10). Different levels (10 %, 15 % and 20 % w/w) of rice bran (RB), spent grain (SG) and spent yeast (SY) were added as nitrogen supplements to the 80–90 % of substrate formulations separately. This study was done using 90 mm glass Petri dishes. pH of substrate formulations were adjusted to 6.00±0.15 using calcium carbonate or vinegar and initial moisture content was adjusted to 80% by adding water. The growth rate of mycelia in each formulation was measured using a mathematical formula described in 3.5.1. Sterilization, inoculation, incubation and data collection works are as described in 3.3.1.

3.4.4 Effect of moisture content in selected fruiting substrate formulations with supplementation of nitrogen sources on *A. polytricha* mycelial growth, sporophore yield and biological efficiency

Different levels (65%, 75% and 85% v/w) of moisture content were used in substrate formulations selected based on the results of previous study. The substrate formulations used were sawdust mixed with EFB (50:50) added with 10% SG and sawdust mixed with OPF (90:10) added with 15% SG and compared to 100% sawdust. The formulations were evaluated for mycelia growth and sporophore or fruiting body yield of *A. polytricha* in polyethylene bags of 82×322 mm. Initially, each heat-resistant

polyethylene bag was filled with substrate moistened with distilled water according to the substrate's initial moisture content to achieve the respective moisture content. Five replicates were prepared for each treatment. The substrate bags were then autoclaved for 60 min at 120°C under 1 kg/1 cm2 pressures and allowed to cool. Inoculation, incubation and data collection were as described in 3.4.5.

3.4.5 Inoculation, incubation of substrate bags and data collection

For inoculation of spawn in the substrate bags, spawn of *A. polytricha* was prepared by inoculating mycelia colonies on sterilised crushed corn grown for 2 weeks. Spawn were transferred on top of each substrate bag using a spatula until full. This was done in sterile conditions in the laminar air flow cabinet. Inoculated bags were transferred to an incubator and kept at room temperature (27 – 28°C) to allow spawn run.

Mycelia growth during spawn running was determined by measuring mycelia extension at 4 sides of the bag at 2-day intervals for 30 days. The average reading was plotted against time (day) to obtain the growth rate in mm/day. ANOVA were also performed using Minitab Statistical Software to determine the mean growth rate, standard deviation (SD) and homogeneity.

After completion of spawn running, all bags were transferred to the experimental room temperature (30 – 32 °C) mushroom house equipped with a misting system. Sufficient air flow was provided by the surrounding netting of the mushroom house. Fully colonised bags were slit either horizontally or vertically using a clean knife. This was done to promote primordia formation and to provide space for sporophore to emerge. During this fruiting phase, relative humidity (RH) was maintained above 85%. This was done by spraying water in the form of fine mist using a sprinkler. The

sprinkler was set to spray fine mist for ten minutes every two hours. Relative humidity was measured by using humidity probe (Humidity Alert II, Extech). Appearance of primordia and sporophore formation were observed. Harvesting was done every day for three weeks. Fresh sporophore yield produced during first, second and third harvest was recorded. Total fresh sporophore yield and biological efficiency were calculated.

3.4.6 Mushroom growing and harvesting

After the completion of colonisation of substrate in the fruiting bags (end of spawn running phase) and the maturation of mycelia in the fruiting bag, the substrate-mycelia mixture on the top of the bag were removed using a spatula, in sterile condition in laminar air flow cabinet. This work was done to prevent contamination. All bags were then transferred to the mushroom house. Racks in the mushroom house were cleaned and wiped with 70% alcohol to reduce contamination before placing the fruiting bags.

Matured fruiting bags were identified by the browning of *A. polytricha* mycelia in the bags. The matured fruiting bags were slit either horizontally or vertically using a clean knife. This is done to promote primordia formation and to provide space for sporophores / fruiting bodies to emerge. During this fruiting phase, moisture was maintained up to the desired level suitable for *A. polytricha*. This was done by spraying water in the form of fine mist using a sprinkler. The sprinkler was set to spray fine mist for 10 minutes every two hours.

The time taken for the appearance of primordia (pin-head) and sporophore formation were observed. Yield of sporophores produced in first, second and third flush were measured and recorded.

3.5 Analysis of Data

3.5.1 Determination of mycelia growth rate

Mycelial growth rate in Petri dishes and during spawn running in plastic bags was determined by measuring the mycelia extension from the starting point using a ruler. Calculation of mycelia extension is as follow:

$$\text{Mycelia extension in Petri dish (mm)} = \frac{\text{horizontal length (mm) + vertical length (mm)}}{2}$$

Mycelia extension in plastic bag (mm) = mean vertical length (mm) from 4 corners of the plastic bag

3.5.2 Determination of fresh sporophore yield

Total fresh mushroom yield (from first, second and third flushes) was calculated using the following equation:

$$\text{Total fresh sporophore yield (\%)} = \frac{\text{Total weight of fresh sporophore harvested}}{\text{100 g of wet substrate used}}$$

3.5.3 Determination of biological efficiency (B.E)

Biological efficiency is a formula to determine the ability of mushroom strain to convert substrate materials into fresh sporophores. At the end of the third flush, biological efficiency of fruiting substrate was calculated using the following equation:

$$\text{Biological efficiency (\%)} = \frac{\text{Grams of fresh sporophores produced}}{\text{Grams of dry substrate used}} \times 100$$

3.6 Nutrient Content Analysis of Cultivated *A. polytricha* Sporophores

3.6.1 Proximate

Sample of fresh cultivated *A. polytricha* sporophores grown on SD+OPF (90:10) + 15% SG and from our local market produced by Vita Agrotech Sdn. Bhd. were subjected to proximate analysis. The test was conducted according to the Association of Official Analytical Chemists (AOAC, 1995). These include the determination of crude protein, crude fat, moisture content, dry matter, ash, crude fiber, carbohydrate and minerals. The minerals include sodium, potassium, calcium, phosphorus, magnesium, iron, copper and manganese. Values for, Fe, Cu and Mn were read on Atomic Absorption Spectrophotometer after standardizing with respective elements. The percentage of all the fractions (crude protein, crude fat, minerals and ash) were added together and subtracted from 100 to obtain the total carbohydrate percentage, while the nitrogen free extract (dry weight) was calculated as the percentage of the total carbohydrate and crude fibre.

3.6.2 Mineral content

Sample of cultivated *A. polytricha* sporophores grown on SD+OPF (90:10) + 15% SG and from our local market produced by Vita Agrotech Sdn. Bhd. were subjected to analysis of mineral content. The mineral content analysis was conducted according to the AOAC 985.01 and AOAC 922.02 2005 as described in AACC (Approved Methods of the American Association of Cereal Chemists) 40-70, Volume 2, 9th Edition.

3.6.3 Essential amino acid

Sample of fresh cultivated *A. polytricha* sporophores grown on SD+OPF (90:10) + 15% SG were subjected to essential amino acid content analysis. The analysis was carried out according to the method of Spackman *et al.* (1958) by ion exchange chromatography after protein hydrolysis.

3.6.4 Heavy metal trace

Sample of cultivated *A. polytricha* sporophores grown on SD+OPF (90:10) + 15% SG was subjected to analysis of heavy metal trace. The analysis was conducted according to the AOAC 986.15 and AOAC 971.21 (2005) method for mercury (Hg) and arsenic (As). For lead (Pb), cadmium (Cd) and tin (Sn), the method followed was according to the AOAC 985.01 and AOAC 922.02 2005 as described in AACC (Approved Methods of the American Association of Cereal Chemists) 40-70, Volume 2, 9^{th} Edition.

CHAPTER 4

RESULTS

4.1 Optimization of Substrates for *A. polytricha* Inoculum / Spawn Production

4.1.1 Moisture content analysis

In this study, 6 types of grains used in inoculum / spawn production which were corn, millet, wheat, crushed corn, peeled soybean and soybean were soaked for 0, 2, 4, 6, 8, 12 hours (overnight) and 24 hours and the moisture content was measured using AND MX-50 Moisture Analyzer.

From Table 4.1, all grains showed no significant difference in moisture content when soaked over 4 hours. It is also shown in the table that their initial moisture content (before soaking at 0 h) were not significantly different among the grains tested. For corn, moisture content was increasing at approximately 2.00% until 24h. As for wheat, although there was an increase in moisture content from 4h to 12h, there was no significant difference in the percentage of moisture content from 12h (overnight) to 24h soaking time.

4.1.2 Selection of the best grain(s) for inoculum / spawn production

(a) Effect of soaking hours (4 hours and 12 hours / overnight) on mycelia growth

Mushroom growers in Malaysia prepare their grains for spawn production by soaking the grains overnight or approximately 12 hours. Based on the results (Table 4.1), the changes of moisture content for grains soaked for 4 hours up until 12 hours were considered as insignificant ($p>0.01$).

Table 4.1: Moisture content of grains after soaking at different time intervals

Grains	Moisture content (%) on soaking time[1]						
	0 h	**2 h**	**4 h**	**6 h**	**8 h**	**12 h**	**24 h**
Corn	13.1 ± 0.2^{a}	26.7 ± 0.6^{b}	30.2 ± 0.6^{bc}	30.8 ± 0.4^{c}	32.8 ± 0.5^{c}	35.3 ± 0.3^{c}	37.0 ± 0.2^{c}
Millet	14.3 ± 0.5^{a}	28.0 ± 0.2^{b}	31.9 ± 0.7^{c}	31.9 ± 1.1^{c}	32.4 ± 0.67^{c}	33.3 ± 0.5^{c}	36.8 ± 0.2^{c}
Wheat	11.9 ± 0.4^{a}	40.7 ± 0.6^{c}	44.9 ± 0.6^{cd}	46.2 ± 0.6^{d}	47.8 ± 0.7^{d}	50.0 ± 1.4^{d}	50.0 ± 0.4^{d}
Crushed corn	13.9 ± 0.3^{a}	37.8 ± 0.8c	39.5 ± 0.9^{c}	40.0 ± 0.7^{c}	40.7 ± 0.6^{c}	40.9 ± 0.7^{c}	41.5 ± 0.8^{c}
Peeled soybean	11.3 ± 0.1^{a}	59.1 ± 0.4^{d}	62.5 ± 0.4^{e}	62.9 ± 0.4^{e}	62.8 ± 0.3^{e}	62.8 ± 0.4^{e}	63.0 ± 0.5^{e}
Soybean	11.5 ± 0.5^{a}	48.5 ± 1.2^{d}	54.9 ± 0.7^{d}	55.2 ± 0.7^{d}	56.1 ± 0.3^{d}	58.9 ± 0.5^{d}	59.6 ± 1.0^{d}

[1]Each value is expressed as mean ± SD (standard deviation) of three replicate analyses. Statistical analysis was done horizontally.

This study was conducted in order to find any difference in mycelia growth rate for *A. polytricha* when the grains used as substrate were soaked in sterilized water for 4 hours and overnight. Initial pH of the grains was adjusted to 6.00 (± 0.15).

From the results (Table 4.2), mycelia growth was higher in all grains soaked overnight compared to grains soaked for only 4 hours, except for crushed corn which did not show significant difference between soaked for 4 hours and overnight soaking. The results can be correlated to the moisture content the grains achieved in 4 hours and overnight. It is obvious that moisture content of corn and wheat differ considerably between the two soaking hours tested and therefore mycelia growth in the two groups showed significant variations ($p<0.01$). In both groups, *A. polytricha* mycelia grown on crushed corn exhibited the highest growth rate, at 6±0 mm/day as shown in Table 4.2 and Figures 4.1 - 4.2. On the contrary, the lowest growth rate for both groups was mycelia grown in uncrushed corn.

Table 4.2: Growth rate (mm/day) of *A. polytricha* grown on grains soaked for four hours and grains soaked overnight

Grains	Moisture content (%)		Growth rate (mm/day)[1,2]	
	Soaked for 4 hours	Soaked for overnight	Soaked for 4 hours	Soaked for overnight
Corn	30.15	35.26	2 ± 0^{a}	3 ± 0^{b}
Crushed Corn	39.46	40.93	6 ± 0^{d}	6 ± 0^{d}
Millet	31.89	33.27	3 ± 0^{b}	4 ± 0^{c}
Wheat	44.91	50.07	3 ± 0^{b}	5 ± 0^{c}

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD (standard deviation) of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

Figure 4.1: Mycelia extension on substrates soaked overnight, after 10 days. (From left; wheat, crushed corn, corn and millet)

Figure 4.2: Mycelia extension on substrates soaked for 4 hours, after 10 days. (From left; crushed corn, corn, millet and wheat)

(b) Effect of substrate moisture content on mycelia growth

This study was done using all grains (corn, crushed corn, wheat, millet, soybean and peeled soybean) initially measured for their moisture content. 60% moisture content was chosen because it is the optimum value for spawn / inoculum production using grains. Adding water to grains to achieve moisture content higher than 60% poses a risk of contamination. The objective of this study was to determine if moisture content adjusted to 60% after soaking for 4 hours supports good mycelia growth. pH of the grains were adjusted to 6.00 (± 0.15) and grains were soaked for 4 hours.

It is shown in Table 4.3 that moisture content of 60% had significant ($p<0.01$) effect on mycelia growth when corn and crushed corn were used as substrates. Mycelia growth was higher when the moisture contents of the substrates were adjusted to 60%. In both groups, *A. polytricha* mycelia grown on crushed corn exhibited the highest growth rate of 5±0 mm/day for unadjusted moisture content group and 6±0 mm/day for 60% moisture content group. It was observed that after sterilization, all grains at the bottom of the bags in this group were found to be enlarged and squashy due to the excess water that was not absorbed by the grains. The most affected by this situation were soybean and peeled soybean because of their soft natural structure. During experiments, it was detected that grains with adjusted moisture content (60%) were most affected by contamination problems, especially soybean and peeled soybean. Due to this problem, soybean and peeled soybean were regarded unsuitable as potential grains to be further developed as substrate for *A. polytricha* inoculum / spawn production and thus, were not further studied.

Table 4.3: Growth rate (mm/day) of *A. polytricha* grown on grains with adjusted moisture content (60%) and unadjusted moisture content

Grains	Moisture content after 4 hours (%)	Growth rate[1,2] (mm/day)	
		Moisture content not adjusted	Moisture content adjusted to 60%
Corn	30.15	3 ± 0^{d}	4 ± 0^{c}
Crushed Corn	39.46	5 ± 0^{b}	6 ± 0^{a}
Millet	31.89	4 ± 0^{c}	4 ± 0^{c}
Peeled Soybean	62.45	4 ± 0^{c}	4 ± 0^{c}
Soybean	54.89	4 ± 0^{c}	4 ± 0^{c}
Wheat	44.91	4 ± 0^{c}	4 ± 0^{c}

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD (standard deviation) of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

(c) Effect of substrate pH on *A. polytricha* mycelia growth

This study was conducted to compare the mycelia growth of *A. polytricha* in the grains with their natural pH value and in the grains where their pH values were adjusted to 6.00, which is the value universally accepted as the optimum pH value for mycelia growth of mushroom. Only four grains were used in this study namely corn, crushed corn, millet and wheat, where as soybean and peeled soybean were not included. Based on the results of previous studies, soybean and peeled soybean were not suitable to be developed as the substrate used in the production of spawn / inoculum of *A. polytricha* due to high contamination rate and low growth rate exhibited by the mycelia grown in the two grains.

It is shown in Table 4.4 that pH of grains have significant effect on mycelia growth rate ($p<0.01$). Mycelial growth was higher in grains with their initial pH value, except for crushed corn. Mycelial growth in crushed corn with adjusted pH value (6.00 ± 0.15) was significantly higher than crushed corn with their natural pH value. The initial pH value of crushed corn was 5.74 ± 0.02, therefore it can be speculated that a slight change of pH in crushed corn can affect mycelial growth of *A. polytricha*.

Table 4.4: Growth rate (mm/day) of *A. polytricha* grown on grains with adjusted pH value and natural pH value

Grains	pH (without adjustment)	Growth rate (mm/day)[1,2]	
		pH not adjusted	pH adjusted to 6.00±0.15
Corn	5.31	3±0[d]	2±0[e]
Crushed Corn	5.69	5±0[b]	6±0[a]
Millet	5.89	4±0[c]	3±0[d]
Wheat	5.80	4±0[c]	3±0[d]

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD (standard deviation) of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

Based on the results of the studies shown in Table 4.2 - 4.4, *A. polytricha* mycelial growth rate in crushed corn was the highest among all other grains subjected to the same studies. It was also observed that contamination rate in crushed corn was also the lowest compared to other grains tested. Therefore, crushed corn was selected as the grain to be further optimized in the succeeding studies for the production of spawn / inoculum of *A. polytricha*.

4.1.3 Effect of supplementation of nitrogen sources in selected spawn substrate on mycelia growth of *A. polytricha*

Crushed corn selected as a substrate for spawn production was further optimised by nitrogen source supplementation. Spent grain, brewer's spent yeast and rice bran were chosen as the nitrogen source for this experiment because their nitrogen content was considered high, around 2.0% and above (Appendix A), their availability (all are wastes from agriculture-related activities) and because of their shape and structure. Spent grain is sand-like in shape and dusty, while rice bran is a little bit sticky and brewer's spent yeast is a thick liquid. The objective of this experiment was to investigate whether supplementation of nitrogen source in selected grain for inoculum

production (crushed corn) can further enhance mycelial growth of *A. polytricha*. This study was considered as a screening test to see changes in mycelia growth rate when the substrate is added with available nitrogen sources, and this was the reason why 10% was chosen as the percentage used in this study. Note that the addition of nitrogen sources at 10% changed the overall ratio of carbon and nitrogen in crushed corn as given in Table 4.5.

Results from Table 4.5 and Figure 4.3 indicated that crushed corn, without addition of any nitrogen source, exhibited the highest mycelial growth rate of 6±0 mm/day. Figure 4.2 showed the growth of *A. polytricha* mycelia was consistent on crushed corn without supplementation and the scenario was contrary on crushed corn with supplementation of nitrogen sources. None of the nitrogen sources added, regardless of their shape and structure, enhances *A. polytricha* mycelia growth rate compared to crushed corn alone.

Table 4.5: Growth rate (mm/day) of *A. polytricha* grown on crushed corn supplemented with different nitrogen sources

Formulations	C:N Value	Growth rate (mm/day)[1,2]
100% crushed corn	77.26 : 1	6±0[a]
90% crushed corn + 10% spent grain	64.27 : 1	4±0[b]
90% crushed corn + 10% brewer's spent yeast	67.16 : 1	4±0[b]
90% crushed corn + 10% rice bran	71.51 : 1	4±0[b]

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD (standard deviation) of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

Figure 4.3: Mycelia growth of *A. polytricha* on crushed corn supplemented with various nitrogen sources.
(From left; crushed corn, crushed corn + 10% brewer's spent yeast, crushed corn + 10% rice bran and crushed corn + 10% spent grain)

4.1.4 Effect of adjusting carbon and nitrogen ratio (C:N) content in selected spawn substrate on mycelia growth of *A. polytricha*

In this study, spent grain was used to lower C:N value while molasses was used to increase C:N value of the primary substrate for inoculum, which is crushed corn. The experiment was done in order to observe the range of C:N values that can support good mycelial growth of *A .polytricha*. The C:N values were adjusted to 5 and 10 points lower and 5 and 10 points higher than the natural C:N ratio.

The growth rates in inoculum both with higher and lower C:N value decreased significantly when compared to the control (100% crushed corn ; 77.26 C:N value) as shown in Table 4.6. Inoculum with higher C:N values exhibited the lowest mycelia growth rates. From the observation done in this study, after sterilization, crushed corns added with spent grains to lower the C:N value became a bit dry. Crushed corn added with molasses to increase the C:N values were becoming sticky and brown in colour (Figure 4.4) , due to the structure and natural colour of molasses. This experiment also concludes that 100% crushed corn with no modification or addition of other materials,

gave the best growth rate, which is 6mm/day (Figure 4.4). Nitrogen concentration did not influence mycelia growth of *A. polytricha* on crushed corn.

Table 4.6: Growth rate (mm/day) of *A. polytricha* grown on crushed corn with adjusted C:N values, pH adjusted to 6.00±0.15 and moisture content adjusted to 60%.

Formulations	C:N Value	Growth rate (mm/day)[1,2]
100% crushed corn	77.26 : 1	6 ± 0^{d}
90% crushed corn + 5% spent grain	70.38 : 1	5 ± 0^{c}
90% crushed corn + 10% spent grain	64.27 : 1	5 ± 0^{c}
90% crushed corn + 5% molasses	85.20 : 1	3 ± 0^{b}
90% crushed corn + 10% molasses	81.02 : 1	1 ± 0^{a}

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD (standard deviation) of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

4.1.5 Effect of pH of spawn substrate on mycelia growth of *A. polytricha*

pH optimization of inoculum, using crushed corn as substrate was carried out to determine the optimum pH for *A. polytricha* mycelia growth. In this study, three pH values which was 5, 6 and 7 were tested against the natural pH value of crushed corn, which was 5.61±0.10. Calcium carbonate and vinegar were used to adjust the pH value.

From the results of this study (Table 4.7 and Figure 4.5), inoculum with pH value of 5 showed significant decrease ($p<0.01$) in mycelia growth rate among inoculum with pH value of 6, 7 and 5.6 (pH not fixed). Inoculum with pH 6 and 7 showed the same as inoculum with natural pH value. Hence, lower pH value gave lower mycelia growth rate. From this result, increasing the pH above the pH of crushed corn did not influence the mycelia growth rate while lower than this value resulted in a decrease in growth rate.

Figure 4.4: Mycelia extension in crushed corn with adjusted C:N value. (From left; control, crushed corn + 5% spent grain, crushed corn + 10% spent grain, crushed corn + 5% molasses and crushed corn + 10% molasses)

Table 4.7: Growth rate (mm/day) for inoculum (crushed corn) with different pH value

Formulations	**Growth rate (mm/day)[1,2]**
Crushed corn pH5	5 ± 0^{a}
Crushed corn pH6	6 ± 0^{b}
Crushed corn pH7	6 ± 0^{b}
Crushed corn pH not adjusted (5.6)	6 ± 0^{b}

[1]ANOVA analysis were performed using Minitab Statistical Software

[2]Each value is expressed as mean ± SD (standard deviation) of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

Figure 4.5: Mycelia extension in crushed corn with different pH values after 7 days. (From left; crushed corn with pH5, control, pH6 and pH7)

All the results from studies related to spawn / inoculum production revealed that crushed corn soaked for 4 hours and without adjusting its moisture content and pH was the optimum substrate or medium to support mycelia growth of *A. polytricha* to be used as inoculum / spawn in *A. polytricha* cultivation.

4.2 Determination of the Best Substrate Formulation(s) for Fruiting

4.2.1 Selection of palm oil waste as fruiting substrate

Three types of palm oil wastes were studied, namely oil palm frond (OPF), palm pressed fibre (PPF) and empty fruit bunch (EFB) compared to sawdust. Sawdust is currently used as the major fruiting substrate for cultivation of *A. polytricha* by local growers. This study was carried out to select the best palm oil waste to be further developed as fruiting substrate for *A. polytricha* cultivation. Selection was based on the growth rate and thickness of *A. polytricha* mycelia observed during the study. This study was done in glass Petri dishes.

Table 4.8: Growth rate (mm/day) of *A. polytricha* mycelia on different fruiting substrates

Substrate Formulations	C/N value	Initial pH	Growth rate (mm/day)[1,2]		Mycelia thickness	
			Substrate pH not adjusted	Substrate pH adjusted to 6±0.15	Substrate pH not adjusted	Substrate pH adjusted to 6±0.15
100% SD	434.9	7.14	7 ± 0^{d}	6 ± 0^{d}	+	+
100% PPF	140.4	5.88	3 ± 0^{b}	4 ± 0^{b}	+++	+++
100% OPF	87.9	6.06	2 ± 0^{a}	4 ± 0^{b}	++	++
100% EFB	214.9	5.73	4 ± 0^{b}	5 ± 0^{c}	++	++

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

In general, results listed in Table 4.8 shows that sawdust supported the best growth rate for both pH groups and the growth rates were significantly different ($p<0.01$) from all the palm oil wastes tested. This is followed by EFB, PPF and OPF for both pH groups. On the other hand, from the careful observation carried out during the study, mycelia thickness for sawdust was the lowest compared to oil palm waste substrates. Contrary to this, palm pressed fibre (PPF) showed the thickest mycelia mat. Grade of mycelia thickness is as shown in Figure 4.6.

4.2.2 Selection of the best fruiting substrate formulation(s) for *A. polytricha*

This study was conducted with the objective to select the best fruiting substrate for *A. polytricha* cultivation. The basis of selection was substrate which exhibited high mycelia growth rate and thickness. Based on the results from previous study (Table 4.8), sawdust was used as the primary substrate and oil palm wastes as secondary substrate. Similar to the previous study, this study was also carried out in glass Petri dishes and divided into two pH groups.

From the results shown in Table 4.9, as overall view in both pH groups, all formulations with 90% SD showed high mycelia growth rate, between 5±0 to 7±1 mm/day, while other formulations exhibited moderate to low mycelia growth rate, which is between 5±1 to 3±1 mm/day. SD+OPF (90:10) substrate formulation showed the highest mycelia growth rate in both pH groups. The growth rate for every formulation varied in both groups and not significantly different ($p>0.01$). 100% OPF exhibited the lowest growth rate which is 1±0 mm/day for unadjusted pH group and 2±0 mm/day for adjusted pH group. On the other hand, mycelia mat for every formulations tested were observed to be similar in both pH groups, except for 100% PPF, 100% EFB, SD+PPF (90:10) and SD+EFB (90:10).

(a) : Lowest mycelia thickness : +

(b) : Intermediate mycelia thickness :++

(c) : Highest mycelia thickness : +++

Figure 4.6: Grades of *Auricularia polytricha* mycelia thickness grown on substrate in glass Petri dish

Mycelial mat for SD+EFB (50:50) was found to be the thickest among all formulations tested, in both groups. Based on these results displayed in Table 4.9, there was no specific pattern in both growth rate and mycelial thickness that were strong enough to assert that one pH group is better than the other. However, from this study,

two formulations were selected for succeeding experiments, which were SD+OPF (90:10) and SD+EFB (50:50), based on growth rate and mycelia thickness, respectively.

4.3 Optimization of Physical Factors in Selected Substrate Formulation(s) for *A. polytricha* Cultivation

4.3.1 Effect of moisture content in selected fruiting substrate formulations on mycelia growth, sporophore yield and biological efficiency of *A. polytricha*

Two fruiting substrate formulations selected from previous study was subjected to the test for determining the suitable moisture content level for *A. polytricha* mycelial growth. This study was done in polyethylene bags. Three levels of moisture content were chosen for this study, which were 65%, 75% and 85%. 65% moisture content was considered as low level while 85% was considered as high level of moisture content. Moisture content was adjusted using distilled water. Results shown in Table 4.10 indicate that there was no significant difference ($p>0.01$) of mycelia growth rate between the formulations and among the three levels of moisture contents. All formulations tested in this study showed similar growth rate which is at 4 mm/day. However, this rate was found to be lower than the growth rates measured in both selected fruiting substrate formulations in the previous study (Table 4.9). This can be speculated to be due to the breadth and depth of the areas available for mycelia extension which was distinctively dissimilar in Petri dishes and polyethylene bags. The time for spawn run to be completed was observed to be earliest at day-30 and latest at day-36. From these results, *A. polytricha* mycelia can relatively grow in low to high level of moisture content. However, although the difference were minuscule and not significant, one level of moisture content which exhibited the highest mycelia growth rate was selected in both fruiting substrate formulation, which were 85% for SD+EFB

(50:50) formulation and 75% for SD+OPF (90:10) formulation. This level of moisture content for each substrate was used in the succeeding study.

Table 4.9: Growth rate (mm/day) and mycelia thickness of *A. polytricha* mycelia inoculated on different formulations of fruiting substrate

Substrate Formulations	C: N ratio	Initial pH	Growth Rate[1,2] (mm/day)		Mycelia thickness	
			pH not adjusted	pH adjusted to 6±0.15	pH not adjusted	pH adjusted to 6±0.15
Sawdust (100)	434.9	7.44	4±2^{b}	5±1^{bc}	+	+
PPF (100)	140.4	6.18	3±1^{b}	3±1^{b}	++	+++
OPF (100)	88.0	6.39	1±0^{a}	2±0^{ab}	++	++
EFB (100)	214.9	5.91	4±2^{b}	3±1^{ab}	+++	++
SD + PPF (90:10)	361.3	7.02	5±0^{bc}	5±1^{bc}	++	+
SD + OPF (90:10)	311.0	7.08	7±1^{c}	6±1^{c}	++	++
SD + EFB (90:10)	394.9	7.14	6±1^{bc}	5±1^{bc}	++	+
SD + PPF (80:20)	308.7	6.57	5±1^{bc}	4±1^{bc}	++	++
SD + OPF (80:20)	242.2	6.84	4±2^{bc}	5±1^{bc}	++	++
SD + EFB (80:20)	361.6	7.07	4±2^{b}	4±1^{b}	+	+
SD + PPF (70:30)	269.3	6.74	5±2^{bc}	5±1^{bc}	++	++
SD + OPF (70:30)	198.4	6.85	4±1^{b}	5±1^{bc}	++	++
SD + EFB (70:30)	333.4	6.69	4±2^{b}	4±1^{b}	++	++
SD + PPF (50:50)	214.0	6.22	4±2^{b}	3±2^{ab}	++	++
SD + OPF (50:50)	145.8	6.52	4±2^{bc}	4±1^{b}	++	++
SD + EFB (50:50)	288.2	6.55	5±1^{bc}	5±1^{bc}	+++	+++

[1]ANOVA analysis were performed using Minitab Statistical Software

[2]Each value is expressed as mean ± SD of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 (P<0.01)

The highest fresh sporophore yield resulted from this study calculated after the third flush was from SD+EFB (50:50) substrate formulation with 85% moisture content, yielded 18.7±6.64%. However, based on ANOVA analysis performed on the results, the value was not significantly different ($p>0.01$) from any other total sporophore yield from the rest of the tested fruiting substrate formulations (Table 4.10). On the other hand, the highest biological efficiency resulted from this study as displayed in Table 4.11 were from SD+EFB (50:50) with 85% moisture content, followed by SD+OPF (90:10) with 85% moisture content and SD (100) also with 85% moisture content. However, the biological efficiency percentages between these substrate formulations were not significant. The lowest biological efficiency came from SD (100) with 65% moisture content, with the percentage of 25.2±1.45%.

To measure the linear relationship between biological efficiency of *A. polytricha* grown on selected fruiting substrate formulations with different levels of moisture content and their growth rate, the Pearson correlation coefficient test was conducted and a linear regression graph was plotted using Minitab 14 statistical software (Appendix C). A very weak positive correlation coefficient (Pearson correlation = 0.161) was observed between the biological efficiency and mycelia growth rate of *A. polytricha* (Figure 4.7).

4.3.2 Effect of water sources in selected fruiting substrate formulations on mycelia growth, sprophore yield and biological efficiency of *A. polytricha*

Local mushroom growers, specifically *A. polytricha* cultivators are using different sources of water to moisten their substrates. This study was conducted with the objective to verify whether different types of water supplied to fruiting substrates give any effect on the growth of *A. polytricha* mycelia and ultimately, sporophore yield. The sources of water used were rain, tap and distilled water.

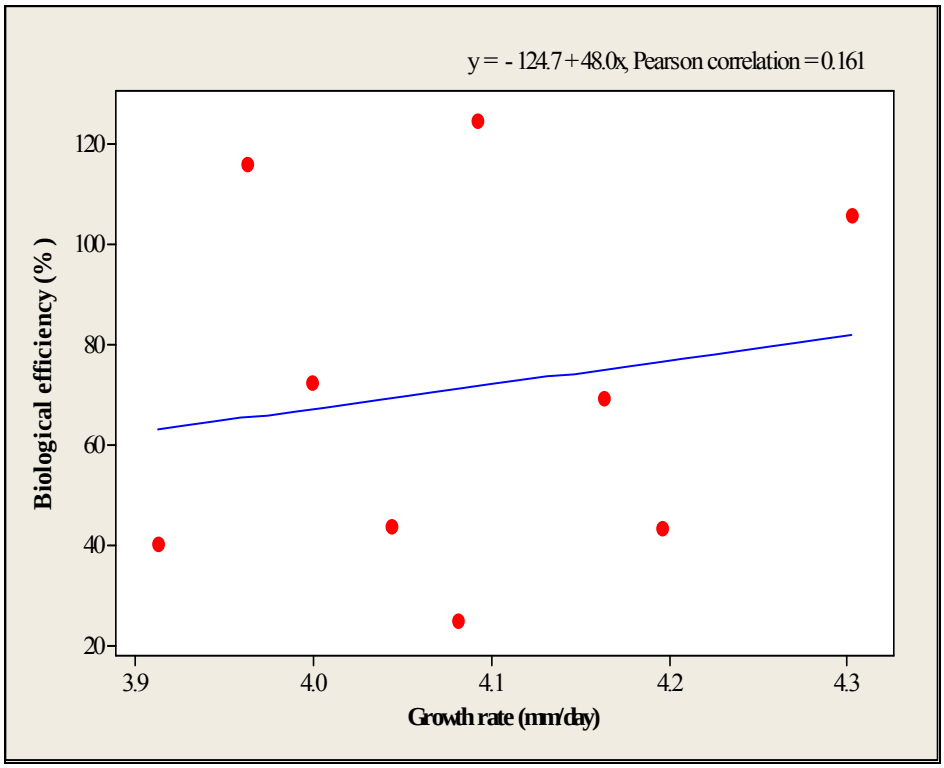

Figure 4.7: Correlation between the biological efficiency and mycelia growth rate of *A. polytricha*

Based on the mycelia growth rates displayed in Table 4.11, the average growth rate for every substrate formulations tested, regardless of their water sources, were around 5mm/day. Based on ANOVA analysis carried out on the results, the rate was not significantly varied ($p>0.01$) between formulations and water sources. However, from the observation carried out during this study, moisture supplied using rain water in fruiting substrate formulations tested exhibited higher risk of contamination. The contamination occurred as early as during the spawn run and some were becoming visible when the bags were already transferred to the mushroom house. Some bags that were not severely affected by contamination were still able to yield fresh sporophore, although the numbers were relatively small. Contaminated bags were discarded.

The time for spawn run to be completed was observed earliest at day 30 and latest at day 35 (Table 4.11). Total fresh mushroom yield was found to be relatively high in substrate formulations supplied with distilled water as moisture source for each substrate formulations tested, with the highest yield recorded was from SD+EFB (50:50) substrate formulation with 34.7±8.32% (Table 4.11 and Appendix C). Biological efficiency of substrate also followed the similar trend; with SD+EFB (50:50) substrate formulation supplied with distilled water provided the highest BE at 231.65±55.46%. However, this value was statistically not significant ($p>0.01$) when compared to other values of total sporophore yield presented by other substrate formulations tested. SD (100) and SD+OPF (90:10) substrate formulations both supplied with rain water suffered from severe contamination resulted in no acceptable data can be collected to determine their respective percentage of total sporophore yield. According to the overall results, a strong recommendation cannot be made as to which water sources are most suitably used for the substrate formulations tested, but it can be speculated that rain water was the least suitable out of the other two water sources based on the contamination rate.

4.3.3 Effect of supplementation of nitrogen sources to selected fruiting substrate formulations on mycelia growth of *A. polytricha*

In this study, two fruiting substrate formulations selected from previous study were subjected to supplementation of nitrogen sources at various levels. The nitrogen sources selected for this study were rice bran (RB), spent grain (SG) and brewer's spent yeast (SY). The aim of this study was to find out if supplementation of nitrogen source has any effect to *A. polytricha* mycelial growth at 10, 15 and 20% (w/w) respectively and consequently on the yield of fresh sporophores.

Table 4.10: Growth rate (mm/day), time for complete spawn run (day), fresh sporophore yield (%) and biological efficiency of substrates (%) for selected fruiting substrate formulations with different levels of moisture content, pH fixed to 6.00±0.15.

Substrate formulation (percentage)	Moisture content (%)	Growth rate[1,2] (mm/day)	Time for complete spawn run (day)	Fresh sporophore yield[1,2]				Biological efficiency (%)
				1st harvest (g)	2nd harvest (g)	3rd harvest (g)	Total (%)	
SD+EFB (50:50)	65	4±0^{ab}	30-35	15.5±4.8	28.5±21.7	6.2±2.7	15.4±4.9^{bc}	44.0±13.9^{ab}
	75	4±0^{ab}	31-35	17.8±12.1	32.3±23.0	9.6±4.4	18.1±5.8^{bcd}	72.4±23.4^{bc}
	85	4±0^{bc}	30-35	22.9±22.6	33.0±17.4	9.5±5.8	18.7±6.6^{bcd}	124.5±44.7^{cd}
SD+OPF (90:10)	65	4±0^{ab}	32-35	15.1±3.4	22.1±3.8	6.4±2.7	14.1±2.3^{bc}	40.2±6.5^{ab}
	75	4±0^{bc}	31-35	30.4±6.0	18.6±1.1	13.4±2.1	17.3±1.8^{bcd}	69.3±7.1^{bc}
	85	4±0^{ab}	32-36	26.1±17.5	27.1±13.8	12.5±3.1	17.4±2.9^{bcd}	116.0±19.2^{cd}
SD (100)	65	4±0^{ab}	31-35	18.3±1.9	9.9±0.5	8.1±2.4	8.8±0.5^{ab}	25.2±1.5^{a}
	75	4±0^{bc}	30-34	24.8±10.2	13.8±3.6	7.6±0.7	10.8±3.2^{ab}	43.3±12.8^{ab}
	85	4±0^{bc}	30-33	35.5±13.6	24.9±5.6	13.5±5.1	15.9±3.0^{bc}	105.9±19.7^{c}

[1]ANOVA analysis were performed using Minitab Statistical Software

[2]Each value is expressed as mean ± SD of three replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

According to the results shown in Table 4.12, generally the mycelia growth rate of *A. polytricha* were more or less the same on all formulations. Supplementation of nitrogen sources clearly lowered the C/N ratio of each substrate formulations. 15% spent grain added to SD+OPF (90:10) formulation exhibited the highest growth rate at 8±1 mm/day. However, the highest mycelia growth rate was also exhibited by supplementation of 20% spent yeast to the same formulation, which was at 7±2 mm/day. ANOVA analysis proved that the growth rates between the two supplementations were not significantly different ($p>0.01$). For SD+EFB (50:50) formulation, supplementation of 10% spent grain and 15 % spent yeast gave the highest mycelia growth rate, which were 7±1 mm/day and 7±2 mm/day, respectively and differed insignificantly. Conversely, mycelia thicknesses in SD+EFB (50:50) control and with supplementation of rice bran at all three levels were thick. For SD+OPF (90:10) formulation, mycelia mat was thickest when supplemented with 20% spent yeast, and was thinnest when supplemented with 10% spent grain. From observation during the course of this experiment, high contamination rate in formulations supplemented with spent yeast were noticed. This might be attributed to spent yeast's physical property which is in thick liquid form. High moisture content of the spent yeast encouraged bacterial blooms. Based on the results and observation, supplementation of spent grain was selected as the best supplement for both fruiting substrate formulations, at 15 % for SD+OPF (90:10) and 10 % for SD+EFB (50:50).

Table 4.11: Growth rate (mm/day), time for complete spawn run (day), fresh sporophore yield (%) and biological efficiency of substrates (%) for selected fruiting substrate formulations with different water sources, pH fixed to 6.00±0.15

Substrate formulations (percentage) & moisture content	Water source	Growth rate[1,2] (mm/day)	Time for complete spawn run (day)	Fresh sporophore yield[1,2]				Biological efficiency[1,2] (%)
				1st harvest (g)	2nd harvest (g)	3rd harvest (g)	Total (%)	
SD (100), 85%	Rain	5±0^{bc}	31-35	NA	NA	NA	NA	NA
	Tap	5±0^{bc}	30-35	35.6±15.5	25.0±5.1	7.3±6.4	15.9±5.9^{ab}	106.2±39.0^{ab}
	Distilled	5±0^{cd}	30-33	39.5±8.0	24.7±4.7	12.6±2.6	17.8±3.2^{ab}	118.5±21.5^{abc}
SD+EFB (50:10), 85%	Rain	5±0^{ab}	30-35	11.6±3.2	22.1±10.0	6.0±5.6	10.5±4.6^{ab}	69.5±30.7^{ab}
	Tap	5±0^{bc}	30-34	17.1±3.7	50.5±42.8	17.8±5.1	23.1±14.3^{bc}	155.3±93.7^{bc}
	Distilled	5±0^{bcd}	30-34	27.8±2.9	53.8±31.4	44.3±5.0	34.7±8.3^{cd}	231.65±55.5^{cd}
SD+OPF (90:10), 75%	Rain	5±0^{bc}	32-35	NA	NA	NA	NA	NA
	Tap	5±0^{bc}	31-35	22.5±3.7	20.6±7.1	9.4±1.1	15.2±1.5^{ab}	61.5±6.9^{ab}
	Distilled	5±0^{abc}	30-34	37.4±10.4	21.9±8.2	13.7±5.6	20.2±2.8^{abc}	80.6±11.2^{ab}

[1]ANOVA analysis were performed using Minitab Statistical Software

[2]Each value is expressed as mean ± SD of three replicate analyses. Value with different small letters is significantly different at the level of 0.01 (P<0.01), NA : not available

Table 4.12: Growth rate (mm/day) and mycelia thickness of *A. polytricha* mycelia inoculated in different fruiting substrate formulations supplemented with different types of nitrogen sources

Nitrogen source & content (%)		C:N ratio of formulations		Growth rate (mm/day)[1,2]		Mycelia thickness	
		SD+EFB (50:10)	SD+OPF (90:10)	SD+EFB (50:10)	SD+OPF (90:10)	SD+EFB (50:10)	SD+OPF (90:10)
Control	0	288.2	311.0	5 ± 1^{ab}	6 ± 1^{bc}	+++	++
Rice Bran	10	182.8	191.3	6 ± 1^{bc}	6 ± 1^{bc}	+++	++
	15	154.3	160.1	5 ± 1^{ab}	6 ± 0^{bc}	+++	++
	20	133.4	137.6	6 ± 0^{bc}	5 ± 2^{ab}	+++	++
Spent Grain	10	150.3	156.5	7 ± 1^{bc}	5 ± 0^{abc}	++	+
	15	110.5	121.9	6 ± 1^{bc}	8 ± 1^{cd}	++	++
	20	97.2	99.9	6 ± 0^{bc}	4 ± 0^{ab}	++	++
Brewer's spent yeast	10	171.0	179.1	6 ± 3^{bc}	5 ± 1^{abc}	++	++
	15	139.4	144.7	7 ± 2^{bc}	6 ± 2^{bc}	++	++
	20	116.1	119.9	6 ± 2^{bc}	7 ± 2^{cd}	++	+++

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

4.3.4 Effect of moisture content in selected fruiting substrate formulations with supplementation of nitrogen sources on mycelia growth, sporophore yield and biological efficiency of *A. polytricha*

Further optimization of fruiting substrate was carried out to determine the suitable moisture content level for selected fruiting substrate supplemented with certain levels of nitrogen source, which is spent grain. This study was done using polyethylene bags, so as to determine the yield of mushroom produced. Mycelia growth rate was calculated for each substrate and time for complete spawn run was also recorded. All the bags were transferred to the mushroom house to allow fruiting.

From the results shown in Table 4.13, there was a significant difference ($p<0.01$) in mycelia growth rate at 5mm/day, exhibited in SD (100) with 85% moisture content and with its natural pH value. Other than that, all tested substrates exhibited a mycelia growth rate around 4mm/day, insignificantly differed from each other, for both pH groups.

Table 4.13: Growth rate (mm/day) and time for complete spawn run (day) of selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content, pH adjusted to 6.00±0.15

Substrate formulations	Moisture content (%)	Initial pH of substrate	Time for complete spawn run (day)	Growth rate[1,2] (mm/day)
SD+EFB (50:50) +10%SG	65	5.30	31-35	4 ± 0^{bc}
	75	5.42	31-34	4 ± 0^{ab}
	85	5.49	30-38	4 ± 0^{ab}
SD+OPF (90:10) +15%SG	65	5.51	32-35	4 ± 0^{ab}
	75	5.62	30-35	4 ± 0^{ab}
	85	5.72	30-33	4 ± 0^{ab}
SD (100)	65	6.47	30-33	4 ± 0^{b}
	75	6.51	31-34	4 ± 0^{bc}
	85	6.65	29-35	4 ± 0^{bc}

[1]ANOVA analysis were performed using Minitab Statistical Software
[2]Each value is expressed as mean ± SD of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.01 ($P<0.01$)

Results for sporophore yield and biological efficiency of substrates are as shown in Table 4.14. Fruiting substrate formulation of SD+OPF (90:10) +15%SG with 85% moisture content gave the highest yield of fresh *Auricularia polytricha* sporophore with total yield of 43.3±5.06%. This is followed closely by SD+EFB (50:10) +10%SG with 85% moisture content at 40.4±12.7% total yield. Both yields were not significantly varied ($p>0.01$) from each other according to ANOVA analysis conducted on the results. To measure the linear relationship between biological efficiency of *A. polytricha* grown on selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content and their growth rate, the Pearson correlation coefficient was conducted and a linear regression graph was plotted using Minitab 13 statistical software (Appendix C). A negative correlation coefficient (Pearson correlation = -0.559) was observed between the biological efficiency and mycelia growth rate of *A. polytricha* (Figure 4.8). It was observed in this study that the biological efficiency of *A. polytricha* decreased when the mycelia growth rate increased.

Table 4.14: Fresh sporophore yield (%) and biological efficiency of substrates (%) of selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content

Substrate formulation	Mois-ture content (%)	Fresh sporophore yield[1,2]								Biological efficiency (%)[1,2]	
		pH6				pH not adjusted					
		1st harvest (g)	2nd harvest (g)	3rd harvest (g)	Total (%)	1st harvest (g)	2nd harvest (g)	3rd harvest (g)	Total (%)	pH 6	pH not adjusted
SD+EFB (50:50) +10%SG	65	39.9± 18.7	10.3± 3.4	28.6± 13.7	19.2± 8.4^{ab}	23.8± 8.7	10.0± 4.8	12.2± 7.2	12.9± 1.7^{ab}	54.8± 24.0^{a}	36.7± 4.8^{a}
	75	61.9± 20.7	17.6± 4.3	39.4± 14.3	29.7± 8.6^{bc}	50.9± 28.7	20.3± 13.5	18.8± 12.0	24.3± 9.6^{b}	118.7± 34.2^{ab}	97.0± 38.2^{ab}
	85	92.6± 26.9	14.4± 0.6	55.1± 17.1	40.4± 12.7^{c}	54.9± 22.7	15.4± 12.0	48.1± 6.9	29.3± 6.7^{bc}	260.7± 73.3^{c}	195.6± 44.6^{bc}
SD+OPF (90:10) +15%SG	65	45.7± 22.9	13.1± 6.8	20.7± 6.1	20.0± 8.5^{b}	44.8± 24.1	8.6± 4.9	13.5± 1.8	14.3± 6.7^{ab}	57.1± 24.3^{a}	40.8± 19.2^{a}
	75	59.6± 4.4	12.7± 3.2	29.1± 3.9	23.9± 1.8^{b}	39.0± 17.9	13.7± 4.2	38.4± 5.8	19.4± 4.2^{ab}	95.7± 7.2^{ab}	77.46± 16.7^{ab}
	85	106.2± 27.6	19.5± 10.2	63.1± 4.7	43.3± 5.1^{cd}	53.6± 20.1	29.9± 16.3	54.9± 24.4	27.7± 5.8^{bc}	288.9± 33.7^{cd}	184.8± 38.4^{bc}
SD (100)	65	18.3± 1.9	9.9± 0.5	8.1± 2.4	8.8± 0.5^{a}	16.8± 2.1	7.4± 2.4	8.5± 1.3	7.8± 0.8^{a}	25.2± 1.5^{a}	22.3± 2.2^{a}
	75	24.8± 10.2	13.8± 3.6	7.6± 0.7	10.8± 3.2^{ab}	22.9± 5.7	10.6± 2.2	13.5± 10.4	11.0± 2.6^{ab}	43.3± 12.8^{a}	43.9± 10.4^{a}
	85	35.5± 13.6	24.9± 5.6	13.5± 5.1	15.9± 3.0^{ab}	42.6± 8.4	14.7± 0.7	20.0± 2.8	17.0± 2.9^{ab}	105.9± 19.7^{ab}	113.6± 19.2^{ab}

[1]ANOVA analysis were performed using Minitab Statistical Software

[2]Each value is expressed as mean ± SD of three replicate analyses. Value with different small letters is significantly different at the level of 0.01 (P<0.01

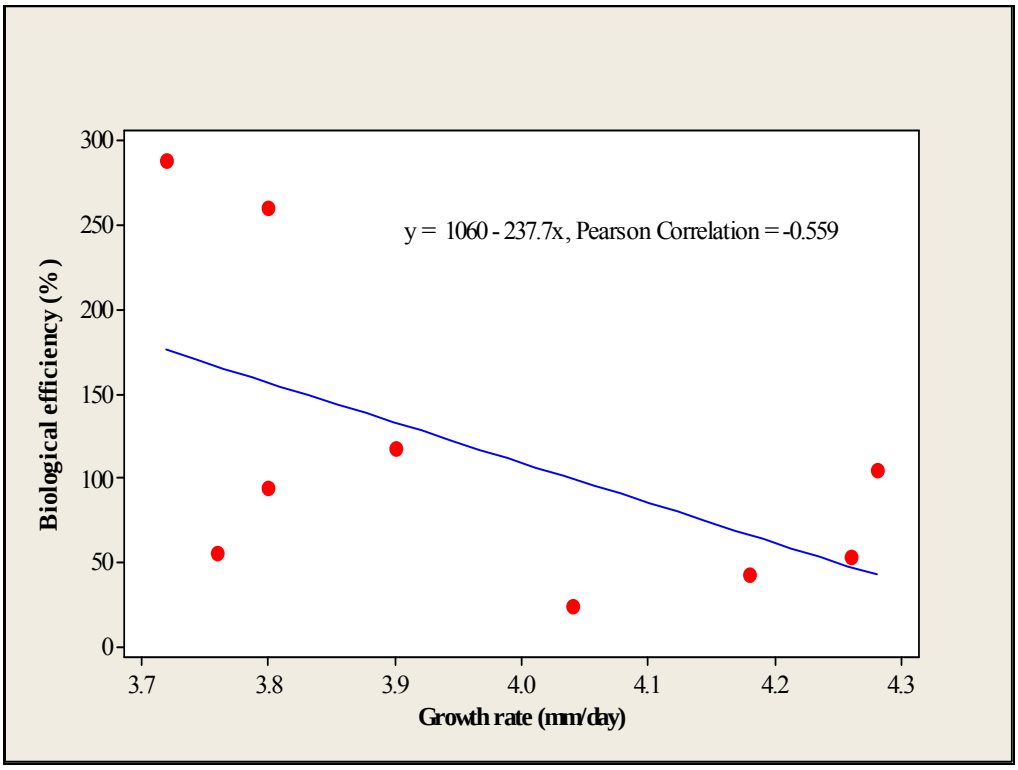

Figure 4.8: Correlation between the biological efficiency and mycelia growth rate of *A. polytricha*

4.4 Primordia and Sporophore Formation

Bags were slit in the mushroom house using a pre-sterilized knife. Primordia or basidiomata formation occurred approximately on average between 5 to 7 days after the slitting of the fruiting bags. Primordia usually formed on the exposed surface of the fruiting bags although there were some occurrences where primordia already formed before slitting the bags. These isolated cases happened when the mycelia in the substrate bags matured earlier than other bags, combined with factors triggering the primordial formation such as light and moisture. From the observation, there was no apparent or solid difference on the time of primordial formation between different substrate bags. Some primordia were formed simultaneously covering all the exposed surface of the fruiting bags (Figure 4.9), but there were also cases where the formation of primordia

only happened at certain area of the exposed surface of the fruiting bags, or primordia were not simultaneously formed along all the exposed surface. The example of this condition can be seen in Figure 4.10.

The development of sporophores can be seen in Figure 4.11 and 4.12. sporophores of *A. polytricha* were usually formed between 10 to 14 days after primordial formation. The development of sporophores took about 3 days before they can be harvested (Figure 4.13), depending largely on the relative humidity in the mushroom house. Fruiting bags placed right underneath the sprinkler in the mushroom house were observed to have a good yield of sporophore compared to the ones placed further away from the sprinkler. Without proper moisture, the development of both primordial and sporophores were retarded, sporophores appeared to be dried and shrinking. Time interval between flushes were around 10 days, and flushes can be up to 5 times. The size of sporophores were greatly varied, with the biggest observed was up to 8cm in diameter. The fresh sporophores of *A. polytricha* is as shown in Figure 4.14.

Figure 4.9: Primordia formation along the exposed surface of a fruiting bag

Figure 4.10: Primordia formation on the exposed surface at the bottom of a fruiting bag

Figure 4.11: Early development of sporophores of *A. polytricha*, approximately 5 days after primordia formation

Figure 4.12: *A. polytricha* sporophores at the middle stage of development, approximately 4 days from the early stage of sporophores development

Figure 4.13: Matured *A. polytricha* sporophores ready to be harvested, approximately 3 days after the middle stage of development

Figure 4.14: Fresh *A. polytricha* sporophore after harvest

4.5 Nutrient Content Analysis of Cultivated *A. polytricha* Sporophores

4.5.1 Proximate and mineral content analysis

Sporophoress of *A. polytricha* grown on SD+OPF (90:10) + 15% SG and from our local market were subjected to proximate and mineral content analysis in order to compare the amount of nutrient components present in the sporophores. Sporophores from local market were supplied by Vita Agrotech Sdn. Bhd.

From the results displayed in Table 4.15, it was found that dried sporophores of *A. polytricha* from the local market has almost double amount of crude fibre at 27.1 g/100g compared to 14.8 g/100g crude fibre found in the cultivated sporophores from this project. Also, there was no fat detected in sporophores from local market compared to

0.4 g/100g fat found in the sporophores from this project. On the other hand, all other components tested produced relatively the same amount between the two samples. For other edible mushrooms listed in the table, *L. edodes* showed the highest amount of energy and fat, while *A. polytricha* showed higher amount of carbohydrate and also both *P. sajor-caju* and *A. bisporus* showed higher amount of protein. *A. polytricha* sporophores harvested from this project showed higher amount of every type of mineral tested compared to sporophores from the local market. The amount of iron (Fe) and manganese (Mn) were almost doubled, at 2.63 mg/100g and 0.61 mg/100g (Table 4.16).

Table 4.15: Proximate composition of *A . polytricha* dried sporophores (mushroom) compared to *A. polytricha* sporophores from the local market and other edible mushrooms from literature

Sporophores[1]	Components[2]						
	Energy	Total fat	Carbo-hydrate	Protein	Ash	Crude fibre	Mois-ture
Cultivated[a]	349.0	0.4	78.7	7.6	3.1	27.1	88.70
Commercial[b]	365.0	ND	83.5	7.6	3.2	14.8	90.35
Lentinula edodes[c]	387.0-392.0	4.9-8.0	67.5-78.0	13.4-17.5	3.7-7.0	7.3-8.0	90.0-91.8
Pleurotus sajor-caju[d]	300.0	2.0	50.7	26.6	6.5	13.3	90.1
Agaricus bisporus[c]	328.0-368.0	1.7-8.0	51.3-62.5	23.9-34.8	7.7-12.0	8.0-10.4	78.3-90.5

[1]Sources: a: sporophores from the present study, b: sporophores from local market, c; Crisan & Sands, 1978, d; Chang et al., 1981.
[2]Units for test components are g/100g except energy (kcal/100g) and moisture were based on fresh weight, ND: not detected.

Table 4.17 showed the nutrient content of cultivated *A. polytricha* from this study and the value of Recommended Daily Allowances (RDAs) of every type of nutrient listed. RDA is the daily amount of nutrient that the Food and Drug Administration of the United States (US FDA) has established as sufficient to maintain the nutritional health of persons in various age groups and categories. Based on the percentage of daily portion based on RDA listed in Table 4.19, it is clear that *A. polytricha* can be

considered as especially valuable source for protein, carbohydrate, crude fibre, magnesium, potassium, manganese and phosphorus.

Table 4.16: Mineral content on *A. polytricha* dried sporophores compared to *A. polytricha* sporophores from the local market and from reference

Sporophores[1]	Components (mg/100g)							
	Ca	**P**	**K**	**Mg**	**Fe**	**Mn**	**Zn**	**Na**
Cultivated[a]	125.73	208.47	771.01	108.29	2.63	0.61	1.10	15.21
Commercial[b]	77.32	150.42	541.80	77.12	1.34	0.30	0.70	12.62
Reference[c]	607.00	NA	588.4	136.00	NA	1.30	1.00	858.4

[1]Source: a: sporophores from the present study; b: sporophores from local market, c: data obtained from Manjunathan *et al*., 2011, NA; not available

Table 4.17: Data analysis of nutrient content in cultivated *A. polytricha* sporophores and its recommended daily allowance (RDA)

Test parameter	Unit (dry weight basis)	Values	Recommended daily allowance (RDA)	Percentage of daily portion based on RDA (%)
Protein	g/100g	7.6	50 g	15.2*
Carbohydrate	g/100g	78.7	300 g	26.2*
Total fat	g/100g	0.4	65 g	0.6
Crude fibre	g/100g	27.1	25 g	108.4*
Iron (as Fe)	mg/100g	2.63	18 mg	14.6
Calcium (as Ca)	mg/100g	125.73	1000 mg	12.6
Magnesium (as Mg)	mg/100g	108.29	400 mg	27.0*
Potassium (as K)	mg/100g	771.01	3500 mg	22.0*
Zinc (as Zn)	mg/100g	1.10	15 mg	7.3
Manganese (as Mn)	mg/100g	0.61	2.0 mg	30.5*
Phosphorus (as P)	mg/100g	208.47	700 mg	29.8*
Sodium (as Na)	mg/100g	15.21	1500 mg	1.0

*A specific food if consumed at 100 g and provide ≥ 15% of the daily intake requirement of a substance is regarded as especially valuable source.

4.5.2 Essential amino acid and heavy metals content

Sporophores of *A. polytricha* grown on SD+OPF (90:10) + 15% SG were dried and subjected to essential amino acid and heavy metals content analysis. As shown in Table 4.18, *A. polytricha* sporophores harvested from this project has 8 types of essential amino acid content out of 9 types that were tested. There was no tryptophan detected from the sporophores. *A. polytricha* have relatively low levels of threonine, methionine and isoleucine, compared to other edible mushrooms listed in the table.

Table 4.18: Essential amino acid content of cultivated *A. polytricha* sporophores compared to other edible mushrooms

Essential amino acid	Quantity (%)				
	Auricularia polytricha	***Agaricus bisporus*[a]**	***Lentinula edodes*[a]**	***Pleurotus sajor-caju*[a]**	***Volvariella volvacea*[a]**
Histidine	0.03	0.03	0.02	0.02	0.04
Threonine	0.02	0.06	0.06	0.05	0.06
Valine	0.03	0.03	0.04	0.05	0.05
Methionine	0.007	0.009	0.018	0.019	0.011
Phenylalanine	0.04	0.04	0.06	0.05	0.03
Isoleucine	0.01	0.05	0.05	0.04	0.03
Leucine	0.05	0.08	0.08	0.07	0.05
Lysine	0.05	0.09	0.04	0.06	0.07
Tryptophan	ND	0.02	ND	0.01	0.02

Note: ND: not detected, Source: a; Chang and Miles, 2004.

Out of six types of heavy metals tested, there were two types detected in fruit bodies of *A. polytricha*. Mercury was detected at 0.01mg/kg and arsenic was detected at 0.17mg/kg. Results are displayed in Table 4.19.

Table 4.19: Selected heavy metals content of cultivated *A. polytricha* sporophores

No	Component	Quantity (mg/kg)
1.	Lead (Pb)	ND
2.	Mercury (Hg)	0.01
3.	Arsenic (As)	0.17
4.	Cadmium (Cd)	ND
5.	Tin (Sn)	ND
6.	Antimony (Sb)	ND

Note: ND: not detected

CHAPTER 5

DISCUSSION

5.1 Optimization of Substrates for *A. polytricha* Inoculum / Spawn Production

It is of highly importance that a good quality spawn is used in order to ensure the success of *A. polytricha* cultivation. In Malaysia, wheat is currently being used as a substrate for creating inoculum / spawn for every type of mushroom cultivated locally, including *A. polytricha*. While the use of a suitable substrate is an important factor in mushroom cultivation, research to find the specific spawn substrate to each type of cultivated mushroom in Malaysia is lacking. The first part of this present study was aimed to select a comparatively more suitable material than wheat as a substrate to be used in preparing inoculum for *A. polytricha* spawn production, by investigating the effect of moisture content, soaking time and pH on *A. polytricha* mycelia growth. The second part of the study involved investigations on the effect of supplementation of nitrogen sources, ratio of carbon and nitrogen and also pH of selected substrate (cushed corn) on *A. polytricha* mycelia growth. In this chapter, we will discuss the results of these experiments and its relation to the studied parameters. Related studies by other researchers will also be discussed.

5.1.1 Selection of the best substrate for *A. polytricha* inoculum / spawn production

Corn, crushed corn, soybean, peeled soybean and millet were used in this study, with wheat used as a comparison. All grains were selected based on their availability,

price, physical structure and more importantly their nutrient content. All the grains were subjected to experiments regarding moisture content and pH.

Moisture content plays critical role in the successful colonization by mushroom mycelium of sterilized grains, where specific mushroom requires specific amount of moisture in the substrate. In order to increase the moisture content of substrate to a required level, the substrate can be soaked in water for a specific amount of time or by adding water to the substrate prior before sterilization. Therefore, all the grains used in the study were subjected to moisture content analysis before experiments regarding the selection of substrate for spawn production were carried out. From the results shown in Table 4.1, it is evident that the physical structure and water holding capacity of grains affected the moisture content of the grains when soaked in water in specific amount of time tested. It was also noted that peeled soybean has higher moisture content than soybean when soaked from 2 hours up to 24 hours. This was obviously due to the absence of the soybean's peel, thus making it easier to absorb water. The same pattern was also observed in crushed corn, most probably due to its smaller physical structure that contributes to its larger surface area to volume ratio than uncrushed / intact corn.

Mushroom growers in Malaysia generally soaked the grains for 12 hours or overnight prior to producing the mushroom inoculums / spawn. However, results shown in Table 4.1 indicated that the changes of moisture content in grains soaked for 4 hours and above were not greatly significant. Based on these results, the soaking time of grains for spawn production prior to their used as spawn substrate and consequently the total production hours of spawn can possibly be shortened by the growers. The results shown in Table 4.2 indicated that there was significant difference in mycelia growth rate between grains soaked for 4 hours and overnight except for crushed corn in which *A. polytricha* showed the highest mycelia growth rate. This is most possibly due to the moisture content of crushed corn was not significantly different when soaking for 4

hours and overnight. Wheat showed the highest increment of moisture content when soaking in water from 4 hours to overnight and subsequently also showed the most significant difference in *A. polytricha* mycelial growth rate between the two tested soaking hours. Therefore, it is evident that the changes of moisture content of grains when soaked at different period of time can affect mycelia growth rate in the specific grain.

The next experiment was to determine whether adjusting moisture content to 60% by adding water support good growth of *A. polytricha* mycelia. Moisture content of 60% was chosen because it is the average value for many mushroom species can optimally grow. In this study, soybean and peeled soybean were most affected by contamination, therefore it can be concluded that these grains were the worst candidate for *A. polytricha* spawn production, among other grains tested. This might be attributed to its high water absorption and water holding capacity, which changes its form to being very squashy when water was added. Although corn and crushed corn have the same nutrient and moisture content, their physical structure is different. Crushed corn, with its natural moisture content after soaking for 4 hours (39.46%) and with adjusted moisture content to 60% still gave the highest *A. polytricha* mycelia growth rate, in both moisture content groups, compared to other grains tested. However, according to Stamet (1993), substrate moisture content for spawn run should ideally be between 60 and 75%. Higher moisture contents encourage bacterial blooms and on the other hand, moisture content below 40% promotes slow and wispy mycelia growth. This result leads to suggestion that for the growth of *A. polytricha* mycelia, the grains' physical form and structure may have superior effect than moisture.

The pH of medium is often neglected as one of the most important environmental factor but nevertheless has a remarkable influence on morphology of fungi mycelia (Jonathan *et al*., 2009 and Shu & Lung, 2004). Due to the effect of hydrogen ion

concentration, or pH on the mycelia growth of mushroom, this study was conducted. It is well known from experimental studies that the optimal pH values for fruiting may differ from those for growth. For experiments unrelated to the study of the effect of substrates pH, pH value was standardized to 6.0 because it is the average value for many mushroom species can optimally grow. Based on the results (Table 4.4), mycelia growth of *A. polytricha* was significantly the highest in crushed corn for both pH groups; unadjusted and adjusted to 6.0, followed by wheat, millet and corn. A significant effect of pH on mycelia growth on all grains tested was also observed. Mycelia growth was significantly higher in grains at their initial pH value, except for crushed corn. In this study, calcium carbonate was added to all the grains in order to adjust the pH to the desired value. According to Chang and Miles (2004), the optimal pH values for *A. polytricha* mycelia falls between 5.0 and 5.4 but the mycelia can also grow in the pH ranging from 2.8 to 9.0. This information contradicts the findings of this study, where mycelia growth was highest in crushed corn with pH adjusted to 6.0 but lower in corn with natural pH value of 5.31, which was the closest value to the reported optimal range. However, our findings can be supported by a report by Kues & Liu (2000) which indicates that mycelia growth is less affected by pH. In this study, it can be speculated that the growth of *A. polytrciha* mycelia was mostly attributed by the nutrient content in the respective substrates, rather than the pH of the substrates.

Substrate structure or particle size also plays an important role in mycelia growth of *A. polytricha*. A research conducted by Nisikado *et al*. (1941) has found that vegetative mycelia growth of shiitake was greater on large and medium-sized particles (1.5-3.00 mm) than on small particles (<1.5 mm). In this study, the clear evidence of this scenario is shown by the results of experiments involving both corn and crushed corn (Table 4.2 -4.4). Both have the same carbon, nitrogen other nutrients content and also the same C:N value, yet *A. polytricha* mycelia grown in crushed corn exhibited

higher growth rate than in uncrushed / intact corn. The smaller size of crushed corn provides a larger surface, making the nutrients becomes more accessible for the mycelia hyphae, compared to intact corn. *Auricularia polytricha* possibly favours a substrate density and complexity such as possessed by crushed corn. All of these may explain the difference in mycelia growth rate for uncrushed corn and crushed corn despite having the same nutrient content.

5.1.2 Optimization of growth conditions on selected spawn substrate

Based on the results of previous experiments, crushed corn was selected as the substrate to be used in the production of *A. polytricha* spawn. Crushed corn was subjected to experiments involving the investigations of the effect of nitrogen sources supplementation, carbon and nitrogen ratio and pH on *A. polytricha* mycelia growth.

The purpose of supplementing a substrate is to enhance growth of the mushroom mycelia. However, according to Stamets (1993), extra care is required to discourage contamination when supplementing a substrate with nitrogen source. This is because supplementing a substrate changes the number and the type of organism that can be supported. Thus, prolong sterilization of the supplemented substrate is highly recommended. A wide variety of nitrogenous materials can be used to enhance the base substrate. Royce (1997) stated that different starch based supplements, such as wheat bran, millet, rye and maize powder are suggested to be added to substrate to serve as major nutrients in growth medium for fungi. According to researches done by Tang and Zhong (2002), Kim *et al.,* (2003), Park *et al.,* (2001) and Fang and Zhong (2002), carbon and nitrogen sources in culture medium or substrate generally play a highly significant role because these nutrients are directly associated with cell proliferation and also metabolite biosynthesis. In this study, spent grain, brewer's spent yeast and rice bran are all regarded as nitrogen sources to the basic spawn substrate due to their high

nitrogen content (Table 4.15). The results showed that crushed corn without supplementation of any of the tested nitrogen sources exhibited the highest mycelia growth rate. This result contradicts the findings of a research done by Onyango *et al*. (2011) which proved that supplementation of wheat bran and rice bran to *A. polytricha* millet and sorghum grain spawn increased the speed of mycelia growth. These contradicting results can be attributed to the different types of grains being used for spawn production in both researches. The addition of supplements for this experiment was tested at 10% only. The results can be more varied if supplementation was also added at higher percentage. However, Oei (2003) reported that excessive supplementation can increase the risk of contamination, anaerobiosis, antibiosis and subsequently lower yields. According to research done by Han et *al.* (1981), there is an optimum concentration of different supplements that can enhance or stimulate mycelia growth of shiitake mushroom. This can also applies to other mushroom species including *A. polytricha*. However, Fanadzo *et al.* (2010) reported that supplementation may cause a rise in substrate temperature, which is possibly due to faster metabolic activities triggered by extra nitrogen, and this provides another perspective to be considered. Overall physical structure of spawn substrate was also modified due to the different physical structure of the supplements added. It is untimely to draw any conclusion from this study until further study is carried out.

It is widely recommended that C/N ratio for vegetative stage of mushroom growth is around 25 to 40 but this value might differ between species. Corn and crushed corn has the highest carbon content of 92.71% (Appendix A) compared to other grains tested in this study. The carbon and nitrogen content of these grains make corn and crushed corn having the highest C:N value (77.26). We carried out an experiment to investigate the effect of carbon and nitrogen ratio to *A. polytricha* mycelia growth. The means to increase C:N ratio in a substrate is by adding a carbon-rich supplement while addition

of nitrogen-rich supplement can decrease the ratio. In this study, molasses was used to increase the C:N ratio of the selected spawn substrate, which was crushed corn, while spent grain was used to lower the C:N ratio. Molasses has high carbon content (85.77%) without any nitrogen content, while spent grain has high nitrogen content (2.7%) and low carbon content (33.2%). The C:N values were adjusted to 5 and 10 points lower and 5 and 10 points higher than the natural C:N ratio. However, the conversion was not 100% exact. Based on the results (Table 4.6), the mycelia growth rates in crushed corn both with higher and lower C:N value decreased significantly when compared to the control. However, it can be suggested that *A. polytricha* can grow well in a substrate with C:N value between 64 to 78. Philippoussis *et al*. (2003) demonstrated that mycelium growth rate of *Pleurotus* spp. and *L. edodes* were positively correlated to C:N ratio. Research done by Okhuoya et al., (2000) indicated that a well balance carbon and nitrogen ratio enhances the growth and development of mushrooms while an imbalance of C:N hampers their growth. However, in this study, it is suggested that the physical structure of substrates greatly affected the mycelia growth rate, probably greater than the C:N ratio itself. The addition of molasses to the basic substrate caused structural changes to the whole substrate which became sticky and hard, thus causing difficulty for *A. polytricha* mycelia to penetrate the substrate to obtain the nutrients it needed and to move forward. This is further proven when the growth rate was the lowest when the addition of molasses was highest (10%). Addition of spent grain to lower the C:N ratio also resulted in decrease of mycelia growth rate (5 mm/day), but the rate was not far from the highest growth rate resulted in this study (6 mm/day). Suitability of C:N ratio in crushed corn without supplement and its physical structure, to *A. polytricha* might be responsible for the higher mycelial growth.

A study was conducted in order to observe the effect of pH of a selected spawn substrate (crushed corn) to the growth of *A. polytricha* mycelia. It has been reported

that for many types of ascomycetes and basidiomycetes, acidic pH was more suitable for mycelial growth and production of metabolites (Hsieh *et al*., 2005). In this study, there was no significant difference of *A. polytricha* mycelia growth rate in crushed corn substrate with unadjusted / initial pH value (5.6), 6 and 7 (Table 4.7). This agrees with the earlier report of Ma and Luo (1992) that vigorous mycelia growth of *Auricularia* occurs in substances where the pH ranges between 5.5 and 6.5, while at pH values below 5.0 and above 7.0, the mycelium growth rate markedly decreases. Ibekwe et al. (2008) suggested that the decrease of mycelia growth at lower pH could be due to the toxicity of very acidic pH to the hyphae. Bilgrami and Verma (1992) reported that mushroom mycelia are more tolerant to acidic substrate than basic substrate or media. This however cannot be verified in this study because the highest pH tested was at the value of 7. It can be suggested based on the result of this study that crushed corn can be used as substrate for spawn production of *A. polyricha* without adjusting its pH.

5.2 Determination of the Best Fruiting Substrate Formulations for *A. polytricha* Cultivation

One of the limiting factors towards domestication of native mushrooms is identification of suitable lignocellulosic substrates for cultivation. The problems associated with the disposal of oil palm wastes and high cellulosic components of OPF, EFB and PPF make these a very promising source of substrate for mushroom production. Therefore, this study was conducted to assess the suitability of selected oil palm wastes to be used as fruiting substrate for *A. polytricha* cultivation. Several experiments were conducted to select the best substrate formulations for *A. polytricha*. The selected substrate formulations were then subjected to optimization of physical parameters such as moisture content, supplementation of nitrogen sources and also

water sources. The results from this study and related research from other researchers will be compared to and discussed.

5.2.1 Selection of palm oil waste as fruiting substrate for *A. polytricha*

In this study, sawdust was found to support the highest mycelia growth rate, followed consecutively by EFB, PPF and OPF, for both pH groups (Table 4.8). As for mycelia thickness, PPF support the thickest mycelia mat, while sawdust gave the thinnest, also for both pH groups. It is also observed that mycelia growth rate increased as the C/N ratio increased, but the increment were not statistically significant. The mycelia mat was the thickest in PPF in both pH groups, presumably due to the physical structure of PPF, which, as observed during experiment, has the highest water holding capacity and most coarse. Sawdust has the finest structure among all the substrates used. The natural pH values for OPF, PPF and EFB were acidic, while pH of sawdust was neutral. However, even when the pH was adjusted to one value (6.00±0.15) for all substrates, mycelia growth rate was still the highest in sawdust.

According to Savoie (1998), although hemicelluloses are less abundant than cellulose fraction, hemicellulose might have functioned as an important source of carbon during developmental stages prior to primordial formation. The carbon and lignin content in sawdust, EFB, PPF and OPF (Table 2.1) are about the same value for each composition (cellulose, hemicelluloses and lignin), therefore it can be speculated that the content of carbon and lignin in these substrates were not the only limiting factors determining the rate of *A. polytricha* mycelial growth in this study. Martinez-Carrera *et al.* (2002) reported that wood ear mushroom has the capacity to grow on a wide variety of agriculture wastes containing cellulose, hemicelluloses and lignin, due to their lignolytic enzymes that are necessary for degradation of such substrates. This provides further explanation for the results obtained in this study by which *A. polytricha*

mycelia can grow in all substrates tested. Carbon and nitrogen ratio, pH and the physical structure of the substrates can be considered as important as the chemical content of substrates. Therefore, based on the results of this study, it is suggested that the physical structure of substrate, such as particle size and thickness, might contribute more in determining the mycelia growth rate of *A. polytricha* than any other factors.

5.2.2 Selection of the best fruiting substrate formulation(s) for *A. polytricha*

A few formulations of fruiting substrates were prepared by combining oil palm wastes (EFB, OPF and PPF) with sawdust according to several percentages. In this study, sawdust was regarded as the primary substrate, based on the results from previous study (5.3.1). Based on the results shown in Table 4.9, there was no significant difference in mycelia growth rate between substrates with adjusted pH and substrates with their natural pH. This is consistent with a report by Kues & Liu (2000) in their publication which indicated that mycelia growth is less affected by pH. The result was also supported by a report by Chang & Miles (2004) demonstrating that mycelia of *A. polytricha* can grow in a wide range of pH, from 2.8 to 9.0.

Regarding the association of mycelia growth rate with C:N ratio of substrates, the results of this study showed sufficient variation in which there is no direct correlation can be made between the C:N ratio and mycelia growth. The C: N ratio of the substrates used in this study ranges from 430 to 88, with the highest is from SD (100) with 434.9 and the lowest from OPF (100) with the value of 88. The highest mycelia growth rate of *A.polytricha* was in SD+OPF (90:10), with C:N value of 311.0 while the lowest growth rate was in OPF (100) with C:N value of 88.0. The increase on C/N ratio means that more carbon sources are available in the medium. The activity of fungal enzymes may be inhibited by the excess of carbon source above a particular level; therefore faster growth rate cannot be achieved (Griffin, 1981).

According to Naraian *et al* (2008), mycelia growth and primordial development is dependent on the lignocellulosic materials with the emphasis on the carbon and nitrogen ratio. On the other hand, Philippoussis *et al.* (2002, 2003) have demonstrated that mycelium extension rate is related to bioavailability of nitrogen and they also suggested that nutritional and porosity levels of substrates are affected by substrate formulation. Also, Royse (1997) reported that nutritional composition of substrates is a crucial factor in determining how mycelia growth and also how primordial initiation occurs. Therefore it is evident that the complexities of nutritional content in the substrate formulations, along with other factors, all together influence the growth of mycelia in the substrates. At the end of the study, two substrate formulations was selected, which were SD+OPF (90:10) due to high mycelia growth rate and SD+EFB (50:50) due to the thickness of mycelia mat.

5.2.3 Optimization of growth conditions on selected fruiting substrate formulations for *A. polytricha* cultivation

Moisture content plays a very important role in all mushroom growth phases, from spawn run to primordial formation and up until sporophores / fruiting bodies development and maturation. According to Flegg and Wood (1985), Kinugawa (1993) and Kues (2000), high humidity (90-95%) of the growth environment is favourable for pinning (primordial formation) and fruiting, but the moisture content of the substrate is more critical factor that should be taken into account. Shen *et al.* (2008) reported that optimum mycelia growth and mushroom production is dependent upon adequate moisture and gas exchange within the substrate used. Chang and Miles (2004) reported that a moisture content of the substrate between 50 and 75% support maximum growth of most types of mushrooms. In this study, we tested three level of moisture content in each substrate formulations – low (65%), medium (75%) and high (85%) and also

recorded the fresh mushroom yield and biological efficiency for each substrate formulations. For the experiment of the effect of moisture content in selected fruiting substrate without supplementation of nitrogen sources (Table 4.10), the mycelia growth rate of *A. polytricha* on all substrates formulations were almost the same at 4 mm/day, regardless of moisture content. Time for complete spawn runs also were about the same for all substrate formulations, with earliest completion at day 30 and the latest at day 36. Highest total mushroom yield for each substrate formulations were obtained from substrates with 75 and 85% moisture content, except for SD (100). The highest yield and biological efficiency in this study were obtained from SD+EFB (50:50) with 85% moisture content but this result might be partly due to nutritional content of the substrate combination itself. The results of the experiment of the effect of moisture content in selected fruiting substrate with supplementation of nitrogen sources (Table 4.13) also exhibited the same pattern, where mycelia growth rates for each substrate formulations were not significantly different in both pH group and with low to high moisture content. Fruiting substrate formulation of SD+OPF (90:10) +15% SG with 85% moisture content and adjusted pH (to 6.00±0.15) gave the highest yield of fresh *Auricularia polytricha* mushroom with total yield of 43.3±5.06% and also the highest biological efficiency at 288.9±33.73. This is followed closely by SD+EFB (50:50) +10% SG with 85% moisture content and adjusted pH (to 6.00±0.15) with 40.4±12.7% total yield and biological efficiency at 260.7±73.32. Even though there were differences in the figures, the results were not statistically different. The apparent differences in the yield of mushrooms and BE from each substrate formulations at a different rate of moisture content proved that moisture content affected the formation of mushroom. These results was supported by Kadiri and Kahinde (1999) that factors such as pH, moisture content, temperature and light intensity affect mushroom growth. Also, according to Kashangura *et al*. (2006) and Scrase and Elliot (1998), there is a balance

between too high a water content that reduces aeration and too low a water potential that provides insufficient water for mushroom development. According to Fan *et al*. (2000), biological efficiency (BE) expresses the bioconversion of dry substrate to fresh fruiting bodies and indicates the fructification ability of the fungus utilizing the substrate. In this study, biological efficiency was highest in substrate formulations with 85% moisture content. Therefore it can be suggested that *A. polytricha* mycelia can grow well with moisture content between 65 and 85%. This is supported by a report by Chang and Miles (2004) indicated that mycelia growth or vegetative phase can take place under a wider range of environmental conditions than reproduction phase or fruiting. These results were in agreement with a report by Kues and Liu (2000) indicated that the optimal water content for mushroom fruiting for wooden substrate is 35-60% and for other type of substrates is between 60-80%. However, it is clear that *A. polytricha* prefers higher moisture content to achieved higher yield and BE.

Local mushroom growers used different types of water to moisten their substrate. Some use collected rain water and some use tap water. In the lab, type of water used in conducting all experiment in this project was distilled water. Similar research specifically to *A. polytricha* cultivation has never been done before and thus this study was done with the objective to determine if the type of water supplied to the fruiting substrate can affect the growth of mycelia and subsequent growth of mushroom. With reference to the results displayed in Table 4.11, the highest mushroom yield was obtained from SD+EFB (50:50) with 85% moisture content from distilled water. The same formulation also gave the highest biological efficiency at 231.65%. The lowest fresh mushroom yield obtained from SD+EFB (50:50) with rain water, the only substrate moistened with rain water that yielded fresh mushroom. Two substrate formulations, SD (100) and SD+OPF (90:10), both moistened with rain water, failed to yield any fresh mushroom and thus BE cannot be calculated, which is due to serious

contamination. The lowest BE comes from SD+OPF (90:10) with tap water at 61.5%. However, this result was not significantly differed with BE obtained from SD+EFB (50:50) with rain water, at 69.5%. The effect of pH of the water sources used in this study cannot be considered as contributing factor to results obtained in this study because the pH of all the substrates was fixed to 6.00±0.15. Therefore, the chemical content of each type of water should be considered as the contributing factor to the growth of mycelia and *A. polytricha* sporophores. According to Petraccia *et al.* (2006), all water molecules, whether from a distiller, a water tap or rain are exactly the same physically and chemically but bear differences in the amount of minerals and also the contaminants the water might contain. It is known that tap water contains chlorine which is added in the tap water as disinfectant, and a lot of different minerals which are mainly inorganic. As for rain water, a lot of chemicals from the air are dissolved in the water as the rain falls down to earth. Distilled water, on the other hand, lost some minerals during the distillation process. Based on the results of this study, all types of water can be used to moisten the fruiting substrate, but extra caution must be made when using rain water. It is suggested that the use of rain water should involve prolonged sterilization process to lower the risk of contamination. The best choice would be using distilled water.

It is widely known that lignocellulosic materials are generally low in protein content and insufficient for the cultivation of mushroom which requires nitrogen, phosphate and potassium. Nitrogen supplementation is an important factor to be considered in mushroom cultivation because the C:N ratio plays a crucial role in spawn running and the growth of fruiting body. Jadhav *et al*. (1998) reported that supplementation of main substrates with nutrient or combination of two or more substrates increase yields of *Pleurotus sajor-caju*. Okhuoya *et al.* (2005) and

Isikhuemhen *et al.* (2000) have concluded that supplementations of substrate improved the production, quality, flavour and shelf life of cultivated mushroom, based on their research on *Lentinus squarrosulus* and *Pleurotus tuberregium* cultivation, respectively.

In this study, spent grain, spent yeast and rice bran were used as a nitrogen source supplements for the basic fruiting substrate, which comprised of lignocellulosic materials which were sawdust, EFB and OPF. They were added at 10, 15 and 20% (w/w). The results (Table 4.13) showed that for SD+OPF (90:10) formulation, 15% spent grain added exhibited the highest growth rate at 8±1 mm/day. For SD+EFB (50:50) formulation, 10% spent grain and 15% spent yeast gave the highest mycelia growth rate, which were 7±1 mm/day and 7±2 mm/day, respectively and differed insignificantly. Based on the results and observation in this study, spent grain and rice bran was deemed as a suitable supplement for the selected fruiting substrate formulations for *A. polytricha* cultivation, because the mycelia growth rates from the formulations with the supplemented spent grain and rice bran were not significantly different enough to conclude that spent grain was better than rice bran and vice versa. However, for succeeding work in the scope of this project, spent grain was selected as the best supplement. The result is in agreement with report from Schildbach *et al.* (1992) that spent grain has been successfully used as supplement for cultivation of mushroom species *Pleurotus, Lentinus and Agrocybe*. Wang *et al.* (2001) proposed that high protein content and physical properties of spent grain, such as particle size, porosity, density, volume weight and water holding capacity are the determining factors for the successful usage of spent grain for the growth of mushroom. For rice bran, it is considered as the most popular and abundantly available organic substrate additive for a growing number of edible mushrooms in Asia (Peng *et al*., 2000). Spent yeast, on the other hand, was more easily contaminated and difficult to handle due to their physical nature.

Addition of supplement to the basic substrate altered the physical property of the overall substrate used. Fanadzo *et al*. (2010) stated that mycelium will have difficulties in colonizing a substrate if the substrate is too tight or too loose, therefore this can be regarded as important factor in determining the type of supplements selected to be added to the basal substrate. The results of this study can also be attributed to nutritional variations among the substrates. The increased level of nutrient available at higher rates would provide more energy for mycelia growth and primordial formation (Yang *et al*., 2013). Research by Onyango *et al.* (2011) concluded that the complexity of substrates in terms of their cellulose content, resulting in difference in the rate of degradation by the mushroom enzymes can be attributed to the variations observed in yield of mushroom and as well as biological efficiency . Since the oil palm wastes used in the formulation of fruiting substrates in this study were supplemented with spent grain as nitrogen source, there was a definite difference of nutrient content of the substrates involved in this study. The ability of fungi to utilize nitrate, ammonium, or organic nitrogen sources determines the extent of vegetative growth (mycelia run) and, consequently, the reproduction capacity of the fungus.

According to Royse *et al*. (1991), supplementation of substrate with other nutrient bases such as rice and wheat bran is necessary in order to achieve maximum mushroom yield, as they reportedly can increase yield by two-fold. Due to low protein content, lignocellulosic materials require different supplements or additives with sufficient amounts of nutrients for better growth and yield of mushroom (Mangat *et al*., 2008). This is in agreement with previous report by Choi (2003) indicating that often an addition of a limited or small amount of supplement to the lignocelluloses-based substrates will increase yields. Research by Ayodele and Akpaja (2007) demonstrated that supplementation of sawdust with 20% oil palm fibres enhanced the mycelia growth and sporophores yield of *Lentinus squarrosulus*. Moda *et al*. (2005) stated in their report

that supplementing a substrate is a common method to increase productivity which is evaluated by biological efficiency, which evidently support our findings in this study. Further explaining the results of this study is a report by Chang and Miles (2004) which stated that mushroom species differ in their ability to utilize nitrogen sources for fruiting as well as for mycelia growth. They further explained that the minimum concentration of nitrogen necessary for fruiting body formation may be slightly greater than the concentration supporting mycelia growth or vegetative phase of mushroom growing.

As mentioned in the earlier part of this chapter, other factors such as substrate's particle size, shape and porosity can affect *A. polytricha* mycelial growth and primordia formation. It is observed during experiment that SD (100) is the most compact substrate compared to the other two substrate formulations. Reduction of BE in SD (100) in this study might be due to insufficient utilization of nutrients which because of the compactness or poor aeration of the substrates, which was suggested by Alam *et al*., 2010. Another cause suggested by Royse *et al*. (2004) is the limitation of spaces or surfaces for developing sporophores. Compared to the other two fruiting substrate formulations, SD (100) did not have a complex mosaic of substrate components as according to Stamets (1993) is beneficial during the period of fruiting body formation. It should also be noted that biological efficiency is highly affected by the quality of the spawn used in cultivation (Mandeel *et al*., 2005), but in this study, this aspect was not investigated. Substrate type, quantity and supplementation can affect substrate qualities such as water holding capacity and degree of aeration that subsequently affect mushroom yield. It should also made clear that yield response is also determined by the duration of the cropping period and also cultivation practice applied during the whole cultivation process (Obodai *et al*., 2003).

5.3 Primordia and Sporophores Formation

According to Stamets (2000), the occurrence of primordial is an affirmation of the strain's identity and is also indicative of the strains' readiness to fruit. The fruiting body of basidiomycetes, including *A. polytricha*, is completely made up of hyphae,and these hyphae are dikaryotic in most cases (Chang and Miles, 2004). Flegg and Wood (1985) described that physiological condition and nutritional state of the mycelium highly influence fruiting body formation. This is also in agreement with Kues and Liu (2000) in their report stated that onset of fruiting body development is indeed in correlation with nutritional exhaustion of the growth substrates. They further explained that environmental conditions play a crucial role in the formation of fruiting body/ sprophores, where fruiting body development is often induced after the environment conditions were drastically altered. This is consistent with a report by Chang and Miles (2004), stated that the environmental conditions that are favourable for reproduction (sporophores production) are always less favourable for growth (mycelia run).

All fruiting bags from the studies we conducted in this project were transferred to the experimental mushroom house after spawn run was completed. The environmental conditions in the mushroom house were very much different from the growth cabinet where we placed the fruiting bags for spawn run. Prior to being transferred to the mushroom house, all the fruiting bags were put in a cabinet with reduced light, but not in total darkness, except during the night. In the mushroom house, the bags were exposed to higher intensity of light that came from surrounding sunlight and alternating darkness during the night. Elliott (1984) reported that light has diverse effects on formation of reproductive structure and increasing or decreasing their number can affect the development of fruiting body in different Basidiomycetes. According to Chang and Miles (2004), many fungi are noticeably uninfluenced in reproduction by light in the

visible range. Also, in the mushroom house, the bags were watered in the form of fine mist using a sprinkler. The sprinkler was set to spray fine mist for 10 minutes every two hours. Sufficient water was applied and proper aeration was maintained in mushroom house to release CO_2 and the supply of O_2 for primordial initiation and sporophores development.

Chang and Miles (2004) stated that no changes in cytochemical properties of the cells or differentiation into specialised tissues were observed at the early stages of primordial development. Moore (1998) reported that reproductive structure or sporophores often arise when mycelial growth had been arrested, by either physical or chemical means or both. Moore further explained that only preconditioned mycelium that is beyond a particular minimum age or size, is capable of undergoing morphogenesis for sporophores development. In order to support development of reproductive structure, the mycelium should be able to accumulate sufficient supplies of reserve materials. All of these explained the development of primordia of *A .polytricha* observed during the course of this study, where the fruiting bags which were not appear "brown" have a delayed pinning compared to browning fruiting bags. The browning of the fruiting bags was the indication used to identify which fruiting bags with mycelia matured enough and ready to be wounded for sporophore development.

It was observed that primordia or basidiomata formation occurred approximately on average between 5 to 7 days after the slitting of fruiting bags. We slit the fruiting bags either horizontally or vertically using a clean knife, thus injuring the mycelia grown inside the fruiting bags. Granado *et al.* (1997) reported that mechanical injury of established mycelium locally stimulates fruiting body development due to the outgrowth of fresh hyphae caused by wounding. Earlier, Ross (1982) has reported that mycelia of basidiomycetes are not uniformly competent to differentiate, and also suggested that only young hyphae can be induced to initiate fruiting body development.

This can explain the scenario we observed on several fruiting bags, where the formation of primordia only happened at certain area of the exposed surface of the fruiting bags, or primordia were not simultaneously formed along the entire exposed surface (Figure 4.8). Therefore, it can be suggested that the outgrowth of fresh hyphae from wounding were not sufficient to further induce primordial formation.

It was also observed that sporophores of *A. polytricha* were usually formed between 10 to 14 days after primordial formation. Relative humidity affects sporophores initiation and usually conducive to initiation of fruiting (Moore, 2005 and Stamets, 2000). This is apparently true for *A. polytricha* where the the development of fruiting bodies from fruiting bags placed further away from sprinkler in the mushroom house appeared to be retarded and dried. Also, according to Kinugawa (1993), high humidity around 90 to 95% is favourable for pinning or primordial formation and fruiting. On the other hand, high moisture content in a substrate also reported by Kimenju *et al.* (2009) to cause delayed pinning. It is also observed that some primordial did not develop into fruiting body, but this specific occurrence was very rare. The same scenario has been described by Kues and Liu (2000), which at the competent stage upon environmental induction, numerous hyphal knots and primorida appear on a colony but subsequently only a few of these come to maturation. They further explained that this scenario may probably due to relocation of nutrients throughout the mycelium to favour one specific or a few selected primordial. However, it is unknown what determines these preferences in maturation of primordia. A report by Kimenju *et al.* (2009) stated that materials with high quality lignin and cellulose contents reportedly take longer time to start pinning compared to the substrates with low contents of lignin and cellulose. High nutrition in the substrates also causes mycelia to remain vegetative for a longer period of time, resulting in vigorous growth and late primordial formation.

5.4 Nutrient Content Analysis on *A. polytricha* Sporophores

5.4.1 Proximate and mineral content analysis

Based on the results of proximate analysis on *A. polytricha* sporophores cultivated in this project and from local market, also proximate compositions of other popular edible mushrooms, all in Table 4.15, it is obvious that mushrooms contains large amounts of carbohydrate, which constitutes the major part of mushroom nutrients. The moisture content of all mushrooms listed was between 78% and 91.8%. This is in line with a report by Chang *et al*. (1981) that moisture content of fresh mushrooms varies within the range of 70 - 95% but this value is highly depending upon the harvest time as well as environmental conditions. On the other hand, the moisture content of dried mushrooms is about 10 - 13%. According to Kurtzman (1997), the carbohydrates in mushrooms include polysaccharides such as glucans, mono- and disaccharides, sugar alcohols, glycogen, and chitin. According to researches by Crisan & Sand (1978) and many other research conducted on the particular subject, the amount of crude protein in mushrooms rank well above most other foods, including milk but below most animal meats. However, protein contents for both *A. polytricha* samples were relatively lower than other edible mushroom listed. This is in agreement with a report by Kakon *et al.* (2012) which stated that *Auricularia* sp. has lower protein content than other popular edible mushroom, proved by his research that found only 8.9g/100g protein in *Auricularia auricula*. According to Zadrazil (1980), addition of organic supplements e.g soybean meal, alfalfa meal and cotton seed powder to cultivation substrates increase not only mushroom yield but also proteins content of the mushrooms. This is also concurred with further report from Tshinyangu (1996) that the amount and nitrogen sources present in the substrate evidently influences the protein content of sporophores.

There was a relative difference of crude fibre content in both *A. polytricha* samples, with *A. polytricha* cultivated from this project having higher crude fibre content at 27.1 g/100g dry matter. The proximate composition and mineral content of *A. polytricha* from this study was contradicted with the proximate composition and mineral content of the same species reported by Manjunathan *et al.* (2011). In their report, the protein, fat and ash content of *A. polytricha* were higher with 37 g/100g, 0.74 g/100g and 6.87 g/100g respectively, while carbohydrate and energy content were lower with 38.48 g/100g and 274 kcal respectively. However, the substrate of *A. polytricha* used in their study was not informed in the paper, thus the contradicting results could not be further discussed. It is also found that *A. polytricha* from the present study has traces of fat, while there was no fat detected in *A. polytricha* sporophores from local market. This is presumably due to the traces of oil present in the substrates used in the study, which were palm-oil by-products, while *A. polytricha* sporophores from the local market were known to have been cultivated on sawdust. They also reported that, compared to our results, the natrium, potassium, calcium and iron content was very high in their sample with 858.4, 588.4, 607 and 16.3 mg/100g respectively, while zinc, manganese and magnesium content of their sample were about of the same amount with ours. On the other hand, our result was closer to the proximate composition of *A. polytricha* as reported by Gbolagade *et al.* (2006) with protein content at 8.5 g/100g and ash content at 5.2 g/100g. There were a noticeable high amount of potassium and phosphorus in our samples, which is supported by a report from Clinton *et al.* (1999) that higher levels of potassium and phosphorus can be found in wood-decomposing fungi. Chang and Miles (2004) reported that the composition of a given species is affected by the diversity of its genetic makeup, which leads to strain differences, and also by environmental conditions. This might explain the variation of data within the same species.

5.4.2 Essential amino acid content and heavy metal trace

An essential amino acid or indispensable amino acid can be defined as an amino acid that cannot be synthesized *de novo* by the organism (usually referring to humans), and therefore must be supplied in the diet. Essential amino acids are "essential" not because they are more important to life than the others, but because the body does not synthesize them, making it essential to include them in one's diet in order to obtain them. There are nine essential amino acids which are listed in Table 4.17. These nine essential amino acids must be present simultaneously and in correct relative amounts for protein synthesis to occur. For all five types of mushrooms (Table 4.17), the most abundant essential amino acids were lysine and leucine while the lowest levels among the essential amino acids were tryptophan and methionine. This is consistent with report by Manzi *et al*. (1999) that tryptophan was found to be the least abundant essential amino acid in several edible mushrooms and also an earlier report by Chan (1981) that mushrooms are generally rich in lysine and leucine. It is evident that cultivated mushrooms, including *A. polytricha* cultivated from this project contain all nine essential amino acid except tryptophan and thus strengthening their position as one of the most nutritious food in the world.

Noble and Gaze (1994) described in their report that the nutritive value of cultivated mushroom can be influenced by the mixture of growth substrate. They also reported that unlike protein, it is not known as to how the amino acid content or profile could change in relation with substrate mixture or quality.

In this study, the distribution of lead, mercury, arsenic, cadmium, tin and antimony was investigated in the cultivated *A. polytricha* mushroom sample. The results displayed in Table 4.18 showed that there are traces of mercury and arsenic in the mushroom at 0.01 and 0.17 mg/kg, respectively. According to a report of Joint Food and Agriculture Organization of the United Nations (FAO) / World Health Organization

(WHO) Expert Committee on Food Additives, the provisional tolerable weekly intake (PTWI) of arsenic is 15 μg/kg body weight (equivalent to 2.1 μg/kg body weight per day). PTWI for mercury is 4 μg/kg body weight. The amount of mercury and arsenic accumulated in the cultivated *A. polytricha* were 10 and 170 μg/kg dry weight respectively. Based on calculation, for a 60 kg person the maximum PTWI for mercury is 240 μg. If a 60 kg person consumed 50 g of dried *A. polytricha* per day, the daily intake of mercury by this person would be 30 μg and weekly intake would be 210 μg. For arsenic, the maximum PTWI for a 60 kg person is 900 μg. Given the same amount of average *A. polytricha* intake per day, the daily intake of arsenic by this person would be 510 μg and weekly intake would be 3570 μg. This value is far more than the PTWI for arsenic. Thus, daily consumption of *A. polytricha* at 50 g dry weight daily by a 60 kg person can be suggested as potentially hazardous. Based on these values, safe consumption of cultivated *A. polytricha* using the same fruiting substrate formulation in this study would be at lower than 10 g dry weight per day.

Various reports have indicated that mushrooms are long known to accumulate high levels of heavy metals (Alonso *et al*., 2000; Vetter, 1994 and Kalac & Svoboda, 2000).

There is no report on heavy metal accumulation in *A. polytricha* found so far. One of the popular cultivated mushrooms, *Agaricus bisporus*, has been reported to uptakes metals such as mercury, zinc and cadmium from substrate and casing material, and traces of cadmium and mercury have also been found in *Pleurotus ostreatus* (Kalac and Svoboda, 2000). Cocchi *et al*. (2006) reported that the amount of arsenic accumulated in an edible mushroom in Italy, *Sarcosphaera exemia* was 1000 mg/kg dry weight and some of *Agaricus* species such as *A. bitorquis*, *A. arvensis* and *A. albertii* had high contents of mercury within 5 to 10 mg/kg dry weight. Slekovec and Irgolic (1996) reported the presence of arsenic accumulation of about 5 mg/kg was found in some

Agaricus species. 2.99 mg/kg dry weight of cadmium was traced in *Flammulina velutipes* and 26.50 mg/kg dry weight of arsenic was traced in *Macrolepiota rhacodes* (Vetter, 1994). A research conducted by Jonathan *et al.* (2011) also concluded that *Lentinus squarrosulus* could accumulate heavy metals if grown in heavy metal polluted environment. Apart from mushrooms, vegetables and fruits are also known to accumulate traces of heavy metals. As reported by Alam *et al.* (2003) in their report, several types of vegetables grown in Bangladesh contained arsenic, such as brinjal with 0.2 μg/g dry weight and green papaya with 0.4 μg/g dry weight. Radwan and Salama (2006) reported that fruits namely apple, banana, melon and peach contained lead (Pb) in the range of 0.02 to 0.47 mg/kg dry weight, while vegetables namely lettuce, carrot, spinach and tomatoes contained cadmium between 0.005 to 0.15 mg/kg dry weight. Based on these reports on accumulation of heavy metals in mushrooms and vegetables, it is evident that the presence of certain heavy metals in food cannot be avoided.

According to Cocchi *et al.* (2006), the accumulation and concentration of heavy metals and trace elements are affected by several factors, and the concentrations of the elements are suggested to be species-dependent. Additionally, other factors influencing the accumulation of heavy metals in mushroom, which are environmental and fungal factors, was reported by Garcia *et al*. (1998). They described in their report that environmental factors such as pH, organic matter amount, metal concentration in soil/substrate and fungal factors such as mushroom species, biochemical composition and morphological part of fruiting body affect metal accumulation in mushroom. This is supported by Kalac and Svoboda (2000) when they considered in their reports that substrate composition is one of the most important factors affecting the accumulation and concentration of heavy metals in mushrooms. Based on these reports, it is highly important for mushroom growers to be thoroughly cautious when choosing the proper substrate materials for fruiting of mushrooms.

CHAPTER 6

CONCLUSIONS

The popularity of mushrooms as protein-rich food is gaining more attention in Malaysia due to the increase demand for healthier choice of food to be consumed daily by Malaysians. One of the most popular mushrooms in Malaysia is *Auricularia polytricha*, or Black Jelly mushroom. This mushroom can be found in local market mostly in dried form and imported from China. The efficiency and economics of the ultimate *Auricularia polytricha* cultivation is however still a problem from many points of view and substrate compositions play vital role in the improvement of such a process.

In this present study, the potential use of selected palm oil wastes was studied to explore their usage as alternative fruiting substrate for *Auricularia polytricha* cultivation by local growers. The study was carried out to investigate the most suitable fruiting substrate formulations, which also include selection of grain to be used to produce spawn and the usage of selected nitrogen sources consisting of agro-industrial wastes as supplements. Proximate analysis, minerals and essential amino acids contents as well as traces of heavy metals in the cultivated *A. polytricha* mushroom were also conducted. The results presented in this study are believed to be the first information on such usage of palm oil wastes for *A. polytricha* cultivation.

The earlier activity of the present study was to select the most suitable grain to be used for spawn production of *A. polytricha*. The findings suggested that crushed corn was the most suitable grain as a substrate for spawn production among other grains tested, due to the highest mycelia growth rate exhibited by *A. polytricha* when grown on crushed corn. The findings also suggested that crushed corn can be used without

addition of any supplement of nitrogen source, presumably due to their excellent nutrient content and physical structure which were most appropriate to the needs of *A. polytricha* mycelia growth. These findings can be conveyed to our local growers in order for them to have alternative other than wheat in production of spawn for the cultivation of this mushroom. Furthermore, this study also found that the mycelia growth rate of *A. polytricha* grown on wheat was lower than on crushed corn. Due to the importance of spawn quality on overall success of mushroom cultivation, it is suggested that further study should be conducted in optimization of crushed corn as spawn substrate and perhaps in finding other cheaper alternatives to be developed as *A. polytricha* spawn substrate.

Cellulose, hemicelluloses and lignin are the crucial carbon sources highly needed by *Basidiomycetes* for their growth and development of sporophores. These substances can be found in palm oil wastes used in the present study with quantity close to that of sawdust, the current material used as a fruiting substrate for *A. polytricha* cultivation in Malaysia. The oil palm wastes and by-products used in the present study which were oil palm frond (OPF), empty fruit bunch (EFB) and palm pressed fibre (PPF) were formulated with sawdust in order to develop fruiting substrate formulations for *A. polytricha* cultivation. The basis of selecting the best fruiting substrate formulations in the present study were the mycelia growth rate, sporophore yield and also biological efficiency (BE) of substrates. These materials were tested for mycelia growth rate in Petri dishes and sporophore yield and also biological efficiency using polyethylene bags. Based on the results of the study regarding this aspect, it was found that oil palm wastes and by-products can definitely be used as fruiting substrate for *A. polytricha* cultivation. The study found that sawdust + OPF (90:10) and sawdust + EFB (50:50) were the two fruiting substrates formulations that gave the highest mycelia growth rate among other 11 formulations tested. Both formulations were subjected to optimization

of moisture content and also tested for several types of water as source of moisture content. These two formulations' ability as fruiting substrate was further enhanced by the addition of nitrogen source supplements. It is found that spent grain (SG) was the best supplement for both selected fruiting substrate formulations, but at different percentages. At the end of this specific study, the two formulations found best as *A. polytricha* fruiting substrate formulations were sawdust + OPF (90:10) + 15% SG and sawdust + EFB (50:50) + 10% SG with 75% and 85% moisture content respectively. These results clearly indicate that palm oil wastes can be used and further developed for cultivation of *A. polytricha* by local growers. The addition of spent grain in the substrates proved to be beneficial in the growth and development of *A. polytricha* mycelia and sporophore.

The cellulose, hemicellulose and lignin content in palm oil wastes used in the study cannot be regarded as the most important contributing factor because the content of those substances were more or less the same between all the three palm oil wastes. It is also interesting to note that carbon and nitrogen ratio of the fruiting substrate formulations, although important, cannot be linked directly to the performance of the two selected fruiting substrate formulations. Based on these findings, it can be suggested that the more important factor in this aspect were the overall nutritional content and their balance in the substrate formulations. Another important factor that can be seen clearly affected the results of the study were the physical properties of the substrate materials and the composite of the fruiting substrate formulations when different types of substrate materials were mixed together. Moisture content indeed played a significant role, but the range of moisture content tested in this study can still be tolerated by *A. polytricha*, as proven by the results of the study. Further studies should be undertaken to precisely determine the range of the important factors involved in the growth and development of *A. polytricha* sporophore.

It is equally important to know the quality of the cultivated *A. polytricha* sporophores from this present study compared to the one in the local market and also to other types of popular edible mushroom. Therefore, the proximate analysis, mineral content and essential amino acid content nutritional content of the cultivated *A. polytricha* sporophores were conducted. It can be concluded that the cultivated sporophores from the present study was of the same nutritional quality with the same type of mushroom from the local market, except for the presence of low amount of fat (0.4%) detected in the cultivated sporophores. It is suggested that this occurrence might be due to the residual oil present in the palm oil wastes / by-products used in the present study. It is interesting to note that sporophores of *A. polytricha* from this study contains higher content of every types of mineral tested than the sporophores from local market. In addition, cultivated *A. polytricha* in this study was found to be a valuable source of protein, carbohydrates, crude fibre, magnesium, potassium, manganese and phosphorus with values greater than 15% of the percentage daily portion based on RDA for each nutrient. However, it was also found that sporophores cultivated in the present study contained traces of arsenic and mercury. From this information, it is recommended that further studies should be carried out to verify if nutritional content of sporophores is dependent on substrate used in cultivation. Information gained from the suggested study can be further used to investigate if the materials used in cultivation substrate can be manipulated to attain certain type of nutrient at the desired quantity in the sporophores.

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APPENDICES

APPENDIX A: Sample Analysis

1.0 Determination of carbon and nitrogen content

All grains used in the production of spawn / inoculum study, palm oil wastes, sawdust, supplements material were subjected to carbon and nitrogen content analysis before being used in the studies related to spawn / inoculums production and fruiting substrates development. This analysis was done to determine their respective carbon and nitrogen ration, one of the most important factors in determining the growth and development of *A. polytricha* mycelia and sporophore.

Table 1.1 Carbon and nitrogen content on selected grains, agro-industry wastes and palm oil wastes used in the production of inoculums and fruiting substrate formulations

Sample	Carbon (%)	Nitrogen (%)	C:N
Wheat	90.60	1.4	64.71:1
Corn	92.71	1.2	77.26:1
Soybean	42.45	6.0	7.08:1
Millet	87.89	1.4	62.78:1
Rice Bran	80.92	2.0	40.46:1
Spent Grain	33.20	2.7	12.30:1
Spent Yeast	25.63	2.0	12.82:1
Molasses	85.77	ND	ND
Sawdust	86.98	0.2	434.90:1
Empty Fruit Bunch (EFB)	85.95	0.4	214.88:1
Palm Pressed Fiber (PPF)	84.25	0.6	140.42:1
Oil Palm Frond (OPF)	87.98	1.0	87.98:1

ND: not detected

2.0 Determination of nutrient content

Proximate analysis was conducted to all grains used in the study of spawn / inoculum production. Supplements involved in those studies were also subjected to test.

Table 2.1: Nutrient content on selected grains and agro-industry wastes used in the production of inoculums and fruiting substrate formulations

Grains/ Susbtrate	Components[1]						
	Energy	Total fat	Carboh -ydrate	Protein	Ash	Crude fibre	Moistu -re
Wheat	351	0.7	72.5	13.7	1.6	1.4	11.51
Corn	364	3.1	76.4	7.8	1.2	1.8	11.53
Soybean	401	13.0	36.6	34.4	5.6	3.8	10.41
Millet	369	4.0	72.4	10.8	3.2	11.7	9.57
Rice bran	391	13.7	53.0	14.0	11.4	3.0	10.62
Dried spent grain	407	10.2	54.6	24.2	4.2	15.7	6.77
Spent yeast	78	ND	8.3	11.3	1.7	0.2	78.75

[1]Units for test components are g/100g except energy (kcal/100g)
ND: not detected

3.0 Determination of mineral content

All grains and supplements used in the studies of spawn / inoculum production of *A. polytricha* were subjected to mineral content analysis. A total of 8 types of minerals were applied to the analysis, which were calcium (Ca), phosphorus (P), kalium (K), magnesium (Mg), ferum (Fe), Manganese (Mn), zinc (Zn) and natrium (Na).

Table 3.1: Mineral content on selected grains and agro-industry wastes used in the production of inoculums and fruiting substrate formulations

Grains/ Susbtrate	Components (mg/100g)							
	Ca	**P**	**K**	**Mg**	**Fe**	**Mn**	**Zn**	**Na**
Wheat	34.59	224.21	314.34	102.95	5.95	3.10	2.50	24.84
Corn	6.39	153.70	223.50	59.89	1.64	0.36	1.02	17.33
Soybean	135.84	436.89	1400.64	172.54	7.10	2.15	2.67	5.78
Millet	16.25	172.71	207.94	94.02	6.17	1.26	1.82	1.22
Rice bran	43.37	1393.63	1190.50	883.89	16.19	7.22	6.54	7.93
Dried spent grain	339.11	489.88	28.13	182.79	14.41	3.99	6.64	3.79
Spent yeast	74.23	403.56	365.27	46.72	2.47	0.23	1.75	1.29

APPENDIX B : Mathematical Calculation

1.0 Mathematical formula for C:N ratio calculation

Due to the importance of carbon and nitrogen ratio and balance in mushroom growth substrate, all grains, palm oil wastes and supplements used in the present study were subjected to carbon and nitrogen content analysis to determine their quantity in respective materials. Calculations for C:N ratio of all materials used in the present study were done by using the following formula:

$$C:N = \frac{\text{Carbon content (\%)}}{\text{Nitrogen content (\%)}}$$

Example 1: C:N ratio in crushed corn

$$\text{C:N of crushed corn} = \frac{92.71}{1.2}$$

$$= 77.26\ \#$$

Example 2: C:N ratio in sawdust + OPF (90:10)

$$\text{C:N ratio in 90\% SD + 10\% OPF} = \frac{90}{100} \times \left(\frac{86.98}{0.2} \right) + \frac{10}{100} \times \left(\frac{87.98}{1.0} \right)$$

$$= \left(0.9 \times 434.9 \right) + \left(0.1 \times 87.98 \right)$$

$$= 400.21\ \#$$

APPENDIX C: Experimental and Statistical Data

1.0 Determination of total sporophore yield

1.1 The experimental data of *A.polytricha* sporophore yield between selected fruiting substrate formulations with different levels of moisture content, pH fixed to 6.00±0.15

Substrate formulations	Moisture content (%)	Replicate no.	Wet substrate weight (g)	Dry substrate weight (g)	Sporophore yield (g)		
					1st flush	2nd flush	3rd flush
100% SD	85	1	469.00	70.35	46.59	23.23	17.92
		3	463.65	69.55	39.57	20.26	14.78
		5	461.35	69.20	20.31	31.05	7.89
	75	1	412.15	103.04	23.90	15.32	6.97
		3	434.38	108.60	35.32	16.46	8.32
		6	431.03	107.76	15.02	9.67	7.47
	65	1	410.53	143.69	20.11	9.65	5.48
		3	416.02	145.61	18.43	10.45	10.31
		5	404.35	141.52	16.34	9.55	8.48
90SD+10OPF	85	2	347.09	52.06	8.34	39.78	11.99
		3	404.67	60.70	43.38	29.13	9.71
		4	376.73	56.51	26.70	12.33	15.79
	75	3	367.06	91.77	36.89	18.97	14.58
		4	355.09	88.77	25.10	19.41	11.05
		5	357.32	89.33	29.07	17.39	14.69
	65	1	321.16	112.41	15.34	17.98	5.00
		2	303.41	106.19	11.49	25.54	4.78
		4	307.44	107.60	18.31	22.73	9.55
50SD+50EFB	85	1	348.89	52.33	9.63	51.78	14.56
		3	352.85	52.93	48.95	29.82	3.10
		4	346.46	51.97	10.06	17.46	10.77
	75	2	330.81	82.70	8.91	58.26	13.88
		5	325.04	81.26	13.05	24.23	5.16
		4	332.67	83.17	31.57	14.39	9.65
	65	3	344.99	120.75	21.11	8.67	9.30
		4	333.06	116.57	12.97	51.70	4.55
		5	302.02	105.71	12.49	25.22	4.79

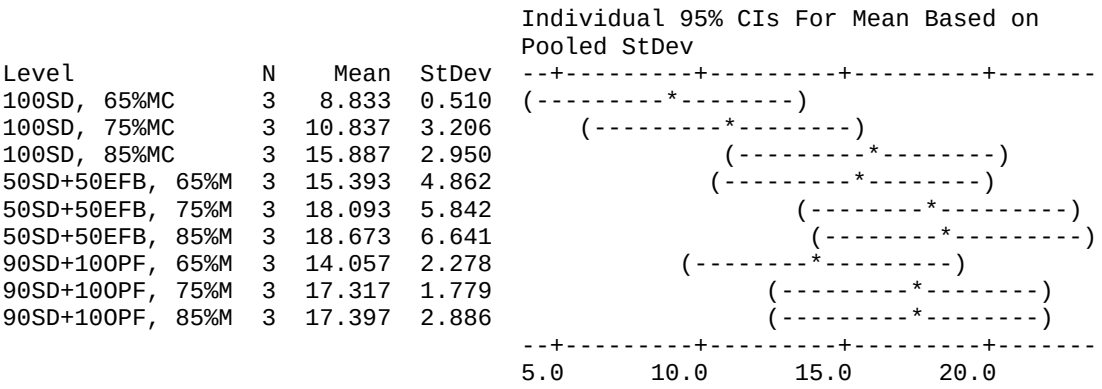
1.2 The statistical data of total mushroom yield (%) between selected fruiting substrate formulations with different levels of moisture content

```
Welcome to Minitab, press F1 for help.
```

One-way ANOVA

```
Source        DF     SS    MS     F      P
Formulations   8  273.4  34.2  2.23  0.075
Error         18  275.6  15.3
Total         26  548.9

S = 3.913   R-Sq = 49.80%   R-Sq(adj) = 27.48%
```




```
Pooled StDev = 3.913
```

Residual Plots for Total Mushroom Yield (%)

1.3 The experimental data of *A.polytricha* sporophore yield between selected fruiting substrate formulations with different types of water source, pH fixed to 6.00±0.15

Substrate formulations (% moisture content)	Water sources	Replicate no.	Wet substrate weight (g)	Dry substrate weight (g)	Sporophore yield (g)		
					1st flush	2nd flush	3rd flush
100% SD (85%)	Tapis /Distilled	2	388.68	58.30	40.11	29.88	12.41
		4	400.01	60.00	25.69	23.31	10.19
		5	434.06	65.11	39.05	20.77	15.32
	Paip / Tap	1	420.16	63.02	37.13	20.00	9.71
		3	424.32	63.65	50.28	30.12	12.08
		4	440.60	66.10	19.43	25.01	n/a
	Hujan / Rain						n/a
							n/a
							n/a
90SD+10OPF (75%)	Tapis /Distilled	1	375.65	93.91	41.57	27.17	11.65
		2	350.67	87.67	45.03	12.45	20.08
		5	360.24	90.06	25.55	26.13	9.37
	Paip / Tap	3	335.89	83.97	23.71	19.95	9.94
		4	352.93	88.23	25.49	13.74	8.20
		6	337.42	84.36	18.31	27.98	10.11
	Hujan / Rain						n/a
							n/a
							n/a
50SD+50EFB (85%)	Tapis / Distilled	3	372.24	55.84	28.63	30.08	40.46
		1	361.62	54.24	24.63	89.48	42.33
		5	355.41	53.31	30.15	41.93	49.97
	Paip / Tap	3	360.31	54.05	19.61	97.52	20.34
		1	372.54	55.88	18.79	40.09	21.22
		5	378.85	56.83	12.85	13.89	11.94
	Hujan / Hujan	1	362.43	54.36	10.31	25.11	11.02
		2	393.97	59.10	15.23	30.29	7.00
		3	391.13	58.67	9.12	10.90	n/a

n/a : not available

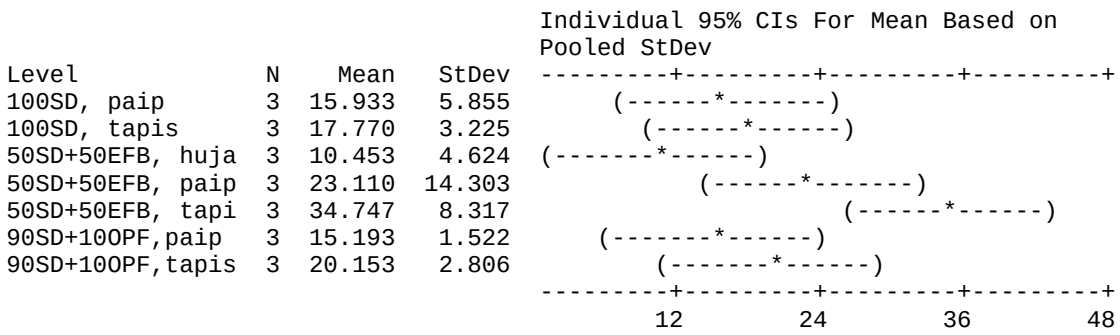
1.4 The statistical data of total mushroom yield (%) between selected fruiting substrate formulations with different types of water source

```
Welcome to Minitab, press F1 for help.
```

One-way ANOVA

```
Source        DF      SS     MS     F      P
Formulations   6  1085.8  181.0  3.62  0.022
Error         14   700.0   50.0
Total         20  1785.8

S = 7.071   R-Sq = 60.80%   R-Sq(adj) = 44.00%
```




```
Pooled StDev = 7.071
```

1.5 The experimental data of *A.polytricha* sporophore yield between selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content, pH fixed to 6.00±0.15

Substrate formulations	Moisture content (%)	Replicate no.	Wet substrate weight (g)	Dry substrate weight (g)	Mushroom yield (g)		
					1st flush	2nd flush	3rd flush
100% SD	85	1	469.00	70.35	46.59	23.23	17.92
		3	463.65	69.55	39.57	20.26	14.78
		5	461.35	69.20	20.31	31.05	7.89
	75	1	412.15	103.04	23.90	15.32	6.97
		3	434.38	108.60	35.32	16.46	8.32
		6	431.03	107.76	15.02	9.67	7.47
	65	1	410.53	143.69	20.11	9.65	5.48
		3	416.02	145.61	18.43	10.45	10.31
		5	404.35	141.52	16.34	9.55	8.48
90% SD+10% OPF+15% SG	85	4	472.22	70.83	116.03	9.22	59.51
		5	446.96	67.04	127.61	29.65	61.48
		3	388.81	58.32	75.06	19.53	68.44
	75	4	421.30	105.33	64.54	10.76	33.43
		2	418.16	104.54	56.30	10.95	25.79
		1	432.38	108.10	58.00	16.41	28.00
	65	4	399.27	139.74	65.33	20.17	26.88
		1	405.94	142.08	51.09	12.28	20.42
		6	374.66	131.13	20.55	6.70	14.71
50% SD+50% EFB+10% SG	85	1	422.08	63.31	61.57	14.45	37.49
		6	371.40	55.71	106.95	14.87	71.68
		5	424.60	63.69	109.36	13.78	55.99
	75	3	399.38	99.85	79.35	20.71	44.72
		5	397.86	99.47	67.33	12.69	50.31
		1	407.79	101.94	39.11	19.32	23.15
	65	2	421.33	147.47	36.52	12.90	26.58
		4	408.85	143.10	60.04	11.52	43.19
		6	399.58	139.85	23.10	6.43	16.03

1.6 The experimental data of *A.polytricha* sporophore yield between selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content, pH not fixed

Substrate formulations	Moisture content (%)	Replicate no.	Wet substrate weight (g)	Dry substrate weight (g)	Sporophore yield (g)		
					1st flush	2nd flush	3rd flush
100% SD	85	2	442.89	66.43	51.91	15.32	22.38
		3	459.07	68.86	40.16	13.94	20.53
		4	460.45	69.07	35.67	14.77	16.97
	75	1	440.80	110.20	28.15	8.11	25.27
		3	425.13	106.28	23.67	12.39	5.40
		4	413.97	103.49	16.92	11.31	9.87
	65	2	432.65	151.43	15.35	4.85	9.76
		4	412.69	144.44	19.22	7.66	7.13
		5	409.16	143.21	15.72	9.64	8.55
90% SD+10% OPF+15% SG	85	2	509.48	76.42	37.22	19.75	50.77
		4	498.05	74.71	47.60	21.30	81.04
		5	493.09	73.96	75.99	48.64	32.74
	75	2	461.40	115.35	56.53	18.43	31.90
		3	463.66	115.92	39.64	11.98	40.29
		4	488.24	122.06	20.85	10.56	43.12
	65	2	492.80	172.48	44.63	7.19	13.92
		3	458.02	160.31	69.04	14.07	15.00
		5	454.29	159.00	20.80	4.62	11.45
50% SD+50% EFB+10% SG	85	1	405.08	60.76	38.08	7.43	51.09
		3	407.60	61.14	80.65	29.11	40.24
		5	396.10	59.42	45.87	9.51	53.10
	75	3	386.99	96.75	79.23	35.43	20.08
		4	339.38	84.85	51.56	15.88	6.19
		5	377.74	94.44	21.81	9.46	30.13
	65	4	341.49	119.52	32.65	6.63	10.97
		1	390.38	136.63	23.44	15.45	5.69
		3	346.60	121.31	15.30	7.81	20.01

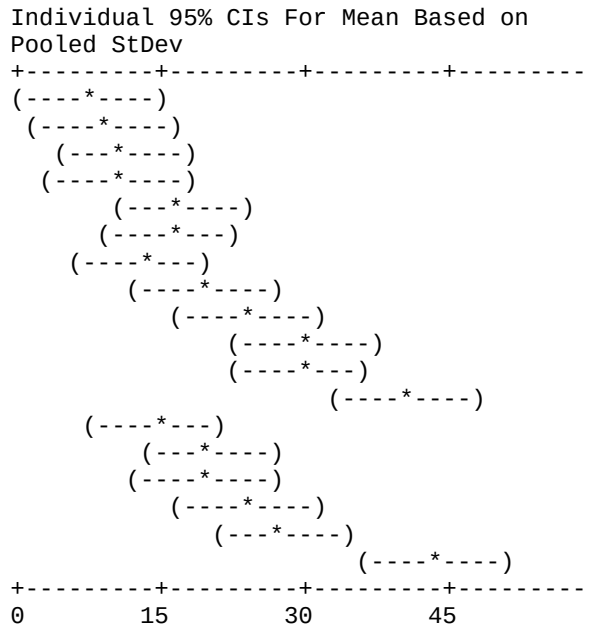
1.7 The statistical data of total sporophore yield (%) between selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content

```
Welcome to Minitab, press F1 for help.
```

One-way ANOVA

```
Source        DF      SS     MS     F      P
Formulations  17  5308.4  312.3  8.33  0.000
Error         36  1349.9   37.5
Total         53  6658.3

S = 6.123   R-Sq = 79.73%   R-Sq(adj) = 70.15%
```



Level	N	Mean	StDev
100SD, 65%MC, pH	3	7.817	0.777
100SD, 65%MC, pH	3	8.833	0.510
100SD, 75%MC, pH	3	10.970	2.604
100SD, 75%MC, pH	3	10.837	3.206
100SD, 85%MC, pH	3	17.043	2.876
100SD, 85%MC, pH	3	15.887	2.950
50SD+50EFB+10SG,	3	12.857	1.684
50SD+50EFB+10SG,	3	19.170	8.392
50SD+50EFB+10SG,	3	24.257	9.545
50SD+50EFB+10SG,	3	29.670	8.554
50SD+50EFB+10SG,	3	29.347	6.693
50SD+50EFB+10SG,	3	40.393	12.701
90SD+10OPF+15SG,	3	14.293	6.701
90SD+10OPF+15SG,	3	19.997	8.493
90SD+10OPF+15SG,	3	19.367	4.161
90SD+10OPF+15SG,	3	23.913	1.786
90SD+10OPF+15SG,	3	27.727	5.767
90SD+10OPF+15SG,	3	43.330	5.057

```
Pooled StDev = 6.123
```

2.0 Determination of biological efficiency

2.1 The statistical data of biological efficiency (%) between selected fruiting substrate formulations with different levels of moisture content

```
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```
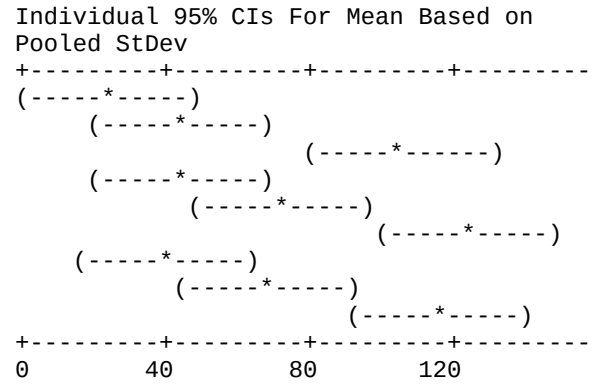

One-way ANOVA

```
Source        DF     SS    MS     F      P
Formulations   8  31948  3993  9.67  0.000
Error         18   7430   413
Total         26  39378

S = 20.32   R-Sq = 81.13%   R-Sq(adj) = 72.75%
```



Level	N	Mean	StDev
100SD, 65%MC	3	25.24	1.45
100SD, 75%MC	3	43.34	12.82
100SD, 85%MC	3	105.92	19.66
50SD+50EFB, 65%M	3	43.98	13.90
50SD+50EFB, 75%M	3	72.36	23.38
50SD+50EFB, 85%M	3	124.50	44.27
90SD+10OPF, 65%M	3	40.16	6.50
90SD+10OPF, 75%M	3	69.27	7.12
90SD+10OPF, 85%M	3	115.97	19.23

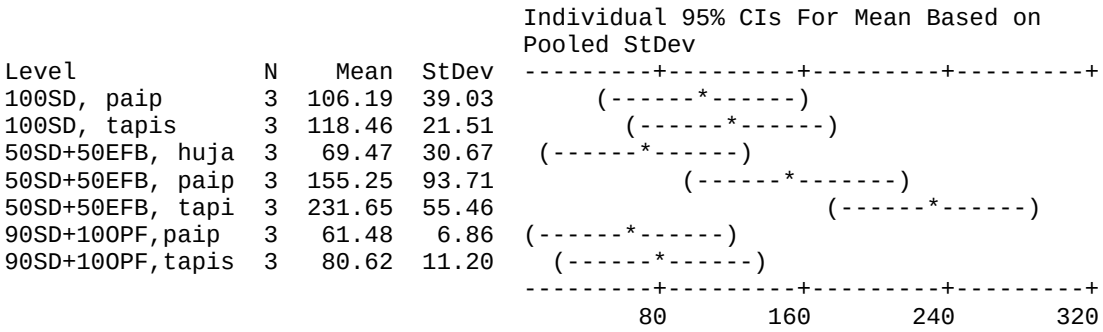
```
Pooled StDev = 20.32
```

2.2 The statistical data of biological efficiency (%) between selected fruiting substrate formulations with different types of water source

```
Welcome to Minitab, press F1 for help.
```

One-way ANOVA

```
Source        DF     SS     MS     F      P
Formulations   6  64167  10695  5.01  0.006
Error         14  29912   2137
Total         20  94079

S = 46.22   R-Sq = 68.21%   R-Sq(adj) = 54.58%
```




```
Pooled StDev = 46.22
```

2.3 The statistical data of biological efficiency (%) between selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content

```
Welcome to Minitab, press F1 for help.
```

One-way ANOVA

```
Source        DF      SS     MS      F      P
Formulations  17  324317  19077  22.12  0.000
Error         36   31051    863
Total         53  355368

S = 29.37   R-Sq = 91.26%   R-Sq(adj) = 87.14%
```

```
                                        Individual 95% CIs For Mean Based on
                                        Pooled StDev
Level              N    Mean  StDev  -+---------+---------+---------+--------
100SD, 65%MC, pH   3   22.34   2.22  (--*---)
100SD, 65%MC, pH   3   25.24   1.45  (---*--)
100SD, 75%MC, pH   3   43.89  10.40    (--*---)
100SD, 75%MC, pH   3   43.34  12.82    (--*---)
100SD, 85%MC, pH   3  113.62  19.19           (--*---)
100SD, 85%MC, pH   3  105.92  19.66          (---*--)
50SD+50EFB+10SG,   3   36.74   4.82   (---*--)
50SD+50EFB+10SG,   3   54.77  23.97     (--*---)
50SD+50EFB+10SG,   3   97.02  38.17         (---*--)
50SD+50EFB+10SG,   3  118.68  34.20           (---*--)
50SD+50EFB+10SG,   3  195.63  44.63                   (---*--)
50SD+50EFB+10SG,   3  260.69  73.32                          (--*---)
90SD+10OPF+15SG,   3   40.83  19.15    (--*---)
90SD+10OPF+15SG,   3   57.07  24.26     (---*--)
90SD+10OPF+15SG,   3   77.46  16.66       (---*--)
90SD+10OPF+15SG,   3   95.65   7.15         (---*--)
90SD+10OPF+15SG,   3  184.82  38.44                  (--*---)
90SD+10OPF+15SG,   3  288.91  33.73                            (---*--)
                                        -+---------+---------+---------+--------
                                         0       100       200       300

Pooled StDev = 29.37
```

APPENDIX D: Publications

1.0 Conferences

1.1 Abstract for poster presented at the Universiti Malaysia Terengganu 10th International Annual Symposium, 11-13July 2011, Terengganu, Malaysia

Investigation on Production of *Auricularia polytricha* (Black Jelly/ Wood Ear Mushroom) Inoculum Using Different Type of Grains

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As one of the most popular culinary mushrooms in the world, *Auricularia polytricha* can be grown on a large scale. However, its production by Malaysian growers is still inefficient with low yield due to lack of research to domesticate the species under local conditions. This study aims to investigate the possible use of different types of grains for the preparation of *A. polytricha* inoculums/spawn to be used in large scale cultivation by local growers. Wheat is the common grain currently used to prepare inoculum. In this study, we chose crushed corn, soybean, peeled soybean and millet while wheat was used as the control. Two groups of grains were prepared, both to test the effect of adjusting their moisture content and pH to *A. polytricha* mycelia run, respectively. The effect of selected nitrogen source supplementation on mycelia run was also tested. Growth rate for crushed corn was highest with 6mm/day growth rate recorded, among all other grains, regardless of pH and moisture content tested. Without addition of any nitrogen source, crushed corn gave the highest mycelial growth rate at 6mm/day. It is concluded that crushed corn is an alternative substrate for the production of *A. polytricha* inoculum, but further investigation will be carried out to optimize its potential.

2.2 Abstract for poster presented at the 7th International Conference on Mushroom Biology and Mushroom Product, 4-7 October 2011, Arcachon, France

Investigation on utilization of palm oil wastes as alternative fruiting substrate for *Auricularia polytricha* (black jelly/ wood ear mushroom) in Malaysia

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Cultivation of edible mushrooms using agricultural residues or wastes, such as palm oil by-products /wastes, is one of the most efficient biological ways by which these residues can be recycled using a value-added process for conversion of these materials into human food. As one of the most popular culinary mushrooms in the world, *Auricularia polytricha* can be grown on a large scale. However, its production by Malaysian growers is still inefficient with low yield due to lack of research to domesticate the species under local conditions. This study aims to investigate the possible use of different types of oil palm wastes for the preparation of *A. polytricha* fruiting substrates to be used in large scale cultivation by local growers. Sawdust is currently used by the local growers to prepare fruiting substrates for *A. polytricha*. In this study, we chose palm pressed fibre (PPF), oil palm fronds (OPF) and empty fruit bunches (EFB) while sawdust was used as the primary substrate and also as control. Two groups of different fruiting substrates formulations were prepared, to test the effect of oil palm wastes solely and in combination with sawdust, to *A. polytricha* mycelia run, respectively. Growth rate for 90% sawdust + 10% OPF was highest with 7 mm/day growth rate recorded, among all other substrate formulations tested. The lowest growth rate recorded for this study was 3 mm/day for 100% OPF. It is possible that oil palm wastes can be adopted and developed as alternative fruiting substrate for the production of *A. polytricha*, but further investigation will be carried out to optimize its potential.

2.0 Paper accepted for publication in Applied Microbiology and Biotechnology Journal

Comparative study of mycelia growth and sporophore yield of Auricularia polytricha *(Mont.) Sacc on selected palm oil wastes as fruiting substrate*

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ENVIRONMENTAL BIOTECHNOLOGY

Comparative study of mycelia growth and sporophore yield of *Auricularia polytricha* (Mont.) Sacc on selected palm oil wastes as fruiting substrate

Dang Lelamurni Abd Razak · Noorlidah Abdullah · Norjuliza Mohd Khir Johari · Vikineswary Sabaratnam

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Abstract The potential for using agricultural and industrial by-products as substrate for the production of the edible mushroom, *Auricularia polytricha*, was evaluated using several formulations of selected palm oil wastes mixed with sawdust and further supplemented with selected nitrogen sources. The best substrate formulations were sawdust (SD) mixed with oil palm frond (OPF; 90:10) added with 15 % spent grain (SG) and sawdust mixed with empty fruit bunch (EFB; 50:50) added with 10 % spent grain (SG) with mycelia growth rate of 8 mm/day and 7 mm/day respectively. These two substrate formulations were then subjected to different moisture content levels (65 %, 75 % and 85 %). Highest total fresh sporophore yield at 0.43 % was obtained on SD+OPF (90:10)+15 % SG at 85 % moisture content, followed closely by SD+EFB (50:50)+10 % SG with 0.40 % total yield, also at 85 % moisture content. Each of the substrate formulations at 85 % moisture content gave the highest biological efficiency (BE) at 288.9 % and 260.7 %, respectively. Both yield and biological efficiency of *A. polytricha* on these two formulations were almost three times higher when compared to sawdust substrate alone, thus proving the potential of these formulations to improve yield of this mushroom.

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Published online: 12 May 2012

Keywords Agro-residues · Sawdust · Edible mushroom · Mycelia growth · Biological efficiency · Fruiting

Introduction

Malaysia, one of the largest global producers and exporters of palm oil reported 4.17 million hectares of plantation, 417 mills and 51 refineries by 2009. The largest amounts of palm oil waste being produced are empty fruit bunches (EFB), oil palm fronds (OPF) and palm pressed fibres (PPF). Wastes are available daily throughout the year and only about 60 % of all the produced fibre and shell waste are burned to generate electricity and steam. Because of a ban on open burning of agricultural waste in Malaysia, the remaining 40 % of the waste is removed by contractors to be put into landfills. These palm oil wastes are heterogeneous water insoluble materials consisting of cellulose, hemicelluloses and lignin and to a lesser extent pectin, starch and other polysaccharides (Thomsen 2005). The problems associated with the disposal of these solid wastes and high cellulosic components of OPF, EFB and PPF make these a very promising source of substrate for mushroom production.

Mushroom cultivation is an economically important biotechnological industry that has developed globally. It is estimated that more than 10 million tonnes of edible and medicinal mushrooms were produced in 2004 in various countries (Royse 2005). Cultivation of edible mushrooms using agricultural wastes represents one of the most efficient biological ways by which these by-products can be recycled (Madan *et al*. 1987; Philippoussis 2009).

Auricularia polytricha, commonly known as Black Jelly mushroom, is in high demand in Asia due to its nutritional and medicinal properties. Also locally known as Telinga

Kera mushroom, *A. polytricha* contains around 8–10 % protein, 0.8–1.2 % fat, 84–87 % carbohydrate, 9–14 % fibre and 4–7 % ash (Ying 1987) and also contains essential amino acids, vitamins and minerals. It has long being used in traditional Chinese medicine for increasing the fluidity of blood and improving blood circulation. It is also reported that *Auricularia* spp. exhibits antioxidant and hypoglycaemic activities and lowers cholesterol levels (Chang 1999; Kho et al. 2009). Recently, Song and Du (2011) reported that β-glucan polysaccharide from *A. polytricha* exhibited antitumour activity against sarcoma-180 in mice.

This species can be cultivated in both temperate and tropical regions. In Malaysia, the principal substrate used by local growers of *A. polytricha* is sawdust. However, there are problems with shortage and tainted supplies of sawdust due to competition from other industries, such as fibre and wood-based boards and charcoal briquette production. These industries offer higher prices for sawdust from the suppliers, also causing the price of sawdust to increase. Furthermore, the production of this mushroom is still insufficient due to lack of research concerning domestication of the species under local conditions and resources. This study aims to investigate the possible use of selected palm oil wastes as fruiting substrate to be used in large scale *A. polytricha* cultivation by local growers.

Materials and methods

Selection of the best fruiting substrate formulation(s) for *A. polytricha* mycelial growth

Several fruiting substrate formulations were prepared by combining selected palm oil waste (OPF, EFB and PPF) with sawdust according to specific percentages and compared to each substrate used at 100 %. Dried and ground oil palm wastes were used in this study. Two groups of substrate were prepared, one with the pH adjusted to 6.00±0.15 using calcium carbonate or vinegar and the other was not adjusted. The initial moisture content was fixed to 80 % by adding water. A total of 15 g of each substrate was put into 90 mm glass Petri dishes and autoclaved for 60 min at 120 °C under 1 kg/1 cm^2 pressures. *A. polytricha* strain KUM61091 used in this study was obtained from University Malaya Culture Collection. A 7-day-old 1 mm mycelia plug was then inoculated in the centre of each Petri dish. Five replicates were prepared for each treatment and all Petri dishes were incubated in an incubator at room temperature (27–28 °C). Mycelia growth in each Petri dish was determined by measuring the average diameter of the mycelia colony every day for 2 weeks. The average reading was plotted against time (day) to obtain the growth rate in mm/day. ANOVA were also performed using Minitab 14 statistical software to determine the mean growth rate and standard deviation (SD). Mycelia thickness was also observed and photographed.

Effect of nitrogen sources in fruiting substrate formulations on mycelial growth of *A. polytricha*

Based on the results of previous study, two substrate formulations were selected ie sawdust mixed with EFB (50:50) and sawdust mixed with OPF (90:10). Different levels (10 %, 15 % and 20 % *w*/*w*) of rice bran (RB), spent grain (SG) and spent yeast (SY) were added as nitrogen supplements to the 80–90 % of substrate formulations separately. Formulations without nitrogen supplement added served as control. Spent yeasts and spent grains were collected at Carlsberg Brewery Malaysia Berhad, while rice bran was obtained from paddy processing factories. This study was also done using 90 mm glass Petri dishes. All raw materials and nitrogen supplements were analysed for carbon and nitrogen content using Furnace and Kjeldahl methods, respectively. The C/N ratio for each formulation was calculated based on the carbon and nitrogen content as shown in Table 2. pH of substrate formulations were adjusted to 6.00±0.15 using calcium carbonate or vinegar and initial moisture content was adjusted to 80 % by adding water. Sterilisation, inoculation and incubation works were as previously described.

Effect of moisture content of selected fruiting substrate formulations on *A. polytricha* mycelial growth, sporophore yield and biological efficiency

Different levels (65 %, 75 % and 85 % *v*/*w*) of moisture content were used in substrate formulations selected based on the results of previous study. The substrate formulations used were sawdust mixed with EFB (50:50) added with 10 % SG and sawdust mixed with OPF (90:10) added with 15 % SG and compared to 100 % sawdust. The formulations were evaluated for mycelia growth and sporophore or fruiting body yield of *A. polytricha* in polyethylene bags of 82× 322 mm. Initially, each heat-resistant polyethylene bag was filled with substrate moistened with distilled water according to the substrate's initial moisture content to achieve the respective moisture content. Five replicates were prepared for each treatment. The substrate bags were then autoclaved for 60 min at 120 °C under 1 kg/1 cm^2 pressures and allowed to cool. Spawn of *A. polytricha* was prepared by inoculating mycelia colonies on sterilised crushed corn grown for 2 weeks. Spawn were

transferred on top of each substrate bag using a spatula until full. This was done in sterile conditions in the laminar air flow cabinet. Inoculated bags were transferred to an incubator and kept at room temperature (27–28 °C) to allow spawn run. Mycelia growth during spawn running was determined by measuring mycelia extension at 4 sides of the bag at 2-day intervals for 30 days. The average reading was plotted against time (day) to obtain the growth rate in mm/day.

After completion of spawn running, all bags were transferred to the experimental room temperature (30–32 °C) mushroom house equipped with a misting system. Sufficient air flow was provided by the surrounding netting of the mushroom house. Fully colonised bags were slit either horizontally or vertically using a clean knife. This was done to promote primordia formation and to provide space for sporophore to emerge. During this fruiting phase, relative humidity (RH) was maintained above 85 %. This was done by spraying water in the form of fine mist using a sprinkler. The sprinkler was set to spray fine mist for ten minutes every two hours.

Appearance of primordia and sporophore formation were observed. Harvesting was done every day for three weeks. Fresh sporophore yield produced during first, second and third harvest was recorded. Total fresh sporophore yield and biological efficiency were calculated using the following equations:

$$\text{Total fresh sporophore yield}(\%) = \frac{\text{Total weight of fresh sporophore harvested}}{\text{100 g of wet substrate used}}$$

$$\text{Biological efficiency}(\%) = \frac{\text{Grams of fresh sporophore produced}}{\text{Grams of dry substrate use}}$$

Results

Mycelia growth rate

From the results shown in Table 1 and Fig. 1, SD+OPF (90:10) substrate formulation showed the highest mycelia growth rate in both pH groups. 100 % OPF exhibited the lowest growth rate which is 1±0 mm/day for unadjusted pH group and 2±0 mm/day for adjusted pH group. Mycelia mat for SD+EFB (50:50) was found to be the thickest among all formulations tested, in both pH groups. The degree of mycelia thickness was as

Table 1 Growth rate (mm/day) and mycelia thickness for *A. polytricha* mycelia inoculated on different formulations of fruiting substrate

Substrate formulations (percentage)	pH of formulation	Growth rate[a, b] (mm/day)		Mycelia thickness[c]	
		pH not adjusted	pH adjusted to 6±0.15	pH not adjusted	pH adjusted to 6±0.15
Sawdust (100)	7.44	4±2b	5±1bc	+	+
PPF (100)	6.18	6±1c	4±1b	++	+++
OPF(100)	6.39	1±0a	2±0ab	++	++
EFB (100)	6.46	4±2b	3±1ab	+++	++
SD+PPF (90:10)	7.02	5±0bc	5±1bc	++	+
SD+OPF(90:10)	7.08	7±1c	6±1c	++	++
SD+EFB (90:10)	7.14	6±1bc	5±1bc	++	+
SD+PPF (80:20)	6.57	5±1bc	4±1bc	++	++
SD+OPF (80:20)	6.84	4±2bc	5±1bc	++	++
SD+EFB (80:20)	7.07	4±2b	4±1b	+	+
SD+PPF (70:30)	6.74	5±2bc	5±1bc	++	++
SD+OPF (70:30)	6.85	4±1b	5±1bc	++	++
SD+EFB (70:30)	6.69	4±2b	4±1b	++	++
SD+PPF (50:50)	6.22	4±2b	3±2ab	++	++
SD+OPF (50:50)	6.52	4±2bc	4±1b	++	++
SD+EFB (50:50)	6.55	5±1bc	5±1bc	+++	+++

[a] ANOVA analysis were performed using Minitab 14 Statistical Software

[b] Each value is expressed as mean±SD of five replicate analyses, brought to the nearest mm. Value with different letters is significantly different at the level of 0.05 ($P<0.05$)

[c] Refer to Fig. 1 for mycelia thickness indicator

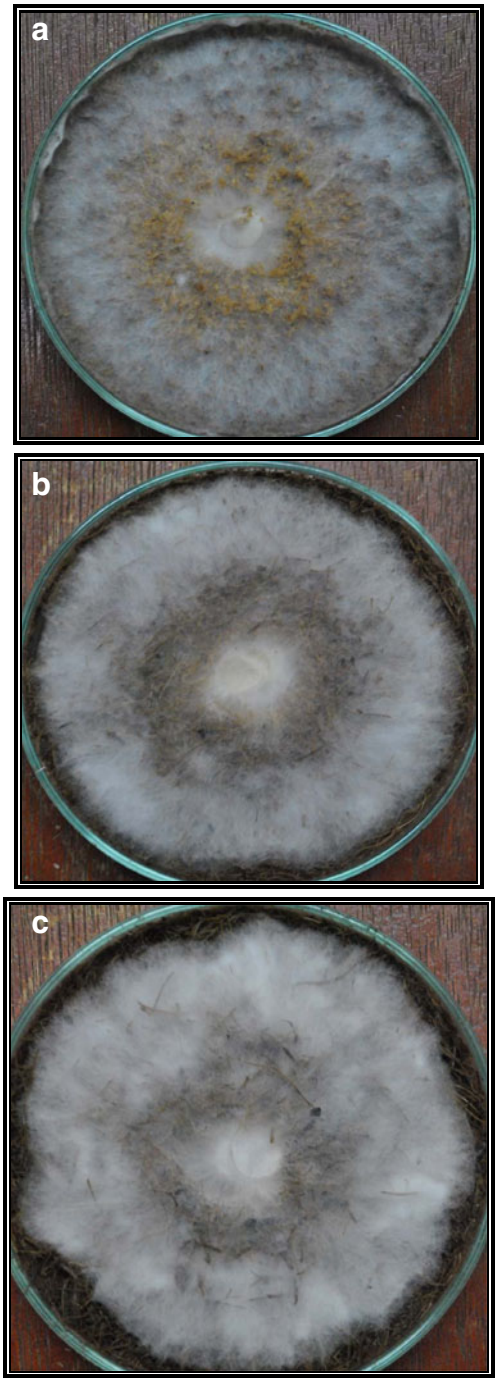

Fig. 1 *Auricularia polytricha* mycelial thickness degree on substrate in glass Petri dish. **a** The lowest degree (marked as "+") of mycelia thickness. **b** The intermediate degree (marked as "++") of mycelia thickness. **c** The highest degree (marked as "+++") of mycelia thickness

shown in Fig. 1. Selection of substrate formulations to be used in the succeeding studies was based on mycelia growth and mycelia thickness, therefore SD+OPF (90:10) and SD+EFB (50:50) were selected. Based on the result, pH substrate was not considered as critical for *A. polytricha* mycelia growth in those substrate formulations because the growth rate for both pH groups were not significantly different.

According to the results shown in Table 2, generally the mycelia growth rate of *A. polytricha* on all formulations were very close to one another. Supplementation of nitrogen sources clearly lowered the C/N ratio of each substrate formulations. 15 % spent grain added to SD+OPF (90:10) formulation exhibited the highest growth rate at 8±1 mm/day. However, the highest mycelia growth rate was also exhibited by supplementation of 20 % spent yeast to the same formulation, which was at 7±2 mm/day. ANOVA analysis proved that the growth rates between the two supplementations were not significantly different. For SD+EFB (50:50) formulation, supplementation of 10 % spent grain and 15 % spent yeast gave the highest mycelia growth rate, which were 7±1 mm/day and 7±2 mm/day, respectively and differed insignificantly. Conversely, mycelia thicknesses in SD+EFB (50:50) control and with supplementation of rice bran at all three levels were thick. For SD+OPF (90:10) formulation, mycelia mat was thickest when supplemented with 20 % spent yeast, and was thinnest when supplemented with 10 % spent grain. From observation during the course of this experiment, high contamination rate in formulations supplemented with spent yeast were noticed. This might be attributed to spent yeast's physical property which is in thick liquid form. High moisture content of the spent yeast encouraged bacterial blooms.

Based on the results and observation, supplementation of spent grain was selected as the best supplement for both fruiting substrate formulations, at 15 % for SD+OPF (90:10) and 10 % for SD+EFB (50:50).

Sporophore yield and biological efficiency

From the results shown in Table 3, there was a significant difference in mycelia growth rate at 5 mm/day, exhibited in SD (control) with 85 % moisture content and with its natural pH value. Other than that, all tested substrates exhibited a mycelia growth rate of approximately 4 mm/day, which is not significantly different from each other, for both pH groups. Generally, complete spawn runs were achieved between 29 and 38 days and primordial formation occurred on average between 5 and 7 days after the slitting of the fruiting bags. Sporophores of *A. polytricha* were usually formed between 10 to 14 days after primordial formation. The development of sporophores took roughly 3 days before they could be harvested. Results for sporophore yield and biological efficiency of substrates are as shown in Table 3.

Fruiting substrate formulation of SD+OPF (90:10) added with 15%SG with 85 % moisture content and adjusted pH (to 6.00±0.15) gave the highest yield of fresh *A. polytricha* sporophore with total yield of 43.3±5.06 %. This is followed closely by SD+EFB (50:50) added with 10%SG with

Table 2 Growth rate (mm/day) and mycelia thickness for *A. polytricha* mycelia inoculated in different fruiting substrate formulations added with different types of nitrogen sources, at pH 6.00±0.15

Nitrogen source and content (%)		C:N ratio of formulations[a]		Growth rate (mm/day)[b,c]		Mycelia thickness[d]	
		SD+EFB (50:50)	SD+OPF (90:10)	SD+EFB (50:50)	SD+OPF (90:10)	SD+EFB (50:50)	SD+OPF (90:10)
Control	0	288.2	311.3	5±1ab	6±1bc	+++	++
Rice bran	10	182.8	191.3	6±1bc	6±1bc	+++	++
	15	152.9	159.7	5±1ab	6±0bc	+++	++
	20	133.4	138.6	6±0bc	5±2ab	+++	++
Spent grain	10	150.3	157.2	7±1bc	5±0abc	++	+
	15	117.1	121.6	6±1bc	8±1cd	++	++
	20	97.2	100.5	6±0bc	4±0ab	++	++
Spent yeast	10	170.9	180.0	6±3bc	5±1abc	++	++
	15	138.1	144.3	7±2bc	6±2bc	++	++
	20	116.1	120.7	6±2bc	7±2cd	++	+++

[a] Percentage of carbon and nitrogen content: SD 86.98 and 0.2, EFB 85.95 and 0.4, OPF 87.98 and 1.0, PPF 84.25 and 0.6, RB 80.92 and 2.0, SG 33.20 and 2.7, and SY 25.63 and 2.0.

[b] ANOVA analysis were performed using Minitab 14 Statistical Software

[c] Each value is expressed as mean±SD of five replicate analyses, brought to the nearest mm. Value with different letters is significantly different at the level of 0.05 ($P<0.05$)

[d] Refer to Fig. 1 for mycelia thickness indicator

85 % moisture content and adjusted pH (6.00±0.15) with 40.4±12.7 % total yield. These yields were almost three times higher than on sawdust with the same moisture content and in the same pH value. The same trend was also observed for biological efficiency, where SD+OPF (90:10) added with 15%SG with 85 % moisture content and adjusted pH (6.00± 0.15) gave the highest BE at 288.9 %.

Discussion

Based on these results displayed in Table 1, there was no specific pattern in both growth rate and mycelia thickness strong enough to assert that one pH group is better than the other. However, an earlier report by Ma and Luo (1992) that vigorous mycelia growth of *Auricularia* occurs in substances where the pH ranges between 5.5 and 6.5, also that at pH values below 5 and above 7, mycelium growth rate markedly decreases. From this study, two formulations were selected based on growth rate and mycelia thickness, which were SD+OPF (90:10) and SD+EFB (50:50) to be used in succeeding studies. These two formulations can be used in large scale cultivation by local growers without having to adjust their pH value. Research done by Okhuoya et al. (2000) and reports from Stamets and Chilton (1983) indicated that a well balanced carbon and nitrogen ratio enhances the growth and development of mushrooms while an imbalance of C/N ratio hinders growth. In this study, good mycelia growth was observed on substrates with a C/N ratio between 120 and 150. However, this is not strong enough to become conclusive. Based on results shown in Table 2, although the supplementation of rice bran resulted in quite similar growth rate of *A. polytricha* mycelia to the supplementation of spent grain, spent grain is a better choice because high contamination rates found in substrates containing rice bran as a supplement. According to Schildbach et al. (1992), brewer's spent grain has successfully been used as substrate for cultivation of several edible mushrooms, including *Pleurotus* and *Lentinus*. It is observed during the mixing process, spent grain is more easily mixed with other materials compared to rice bran, which is often precipitated at the bottom of the mixing container due to its very fine particle. In short, the use of spent grain ensures the homogeneity of substrate mixtures especially in large scale. In contrast with rice bran, spent grain is also available all year round, making it a better choice as a supplement.

Water (moisture) plays an important role in the growth and development of *Auricularia* mushroom. Based on the results of our study shown in Table 3, high moisture content (85 %) provided in the substrates were proven to favour the growth and development of *A. polytricha* mushrooms. However, maintaining RH above 85 % in the experimental mushroom house by spraying water is a crucial step to ensure good development of *A. polytricha* sporophores. Townsley (1979) and Wang et al. (2001) reported that spent grain was found to greatly favour the growth of mushroom due to its high protein and moisture content and also its physical properties such as particle size, volume weight, density, porosity and water-holding capacity. Our results

Table 3 Time for complete spawn run (day), mycelia growth rate (mm/day), fresh sporophore yield (%) and biological efficiency of substrates (%) for selected fruiting substrate formulations supplemented with nitrogen source with different levels of moisture content

Substrate formulation (average dry weight)	Moisture content (%)	Time for complete spawn run (day)		Spawn run rate[a,b] (mm/day)		Fresh sporophore yield[a,b]								Biological efficiency (%)[a,b]	
		pH adjusted to 6±0.15	pH not adjusted	pH adjusted to 6±0.15	pH not adjusted	pH adjusted to 6± 0.15				pH not adjusted				pH adjusted to 6±0.15	pH not adjusted
						1st harvest (g)	2nd harvest (g)	3rd harvest (g)	Total (%)	1st harvest (g)	2nd harvest (g)	3rd harvest (g)	Total (%)		
SD+EFB (50:50)± 10%SG (101.60 g)	65	31–35	30–34	4±0bc	4±0ab	39.89	10.28	28.60	0.19± 0.08ab	16.76	7.38	8.48	0.13± 0.02ab	54.8± 23.97a	36.7± 4.82a
	75	31–34	29–34	4±0ab	4±0bc	61.93	17.57	39.39	0.30± 0.09bc	22.91	10.60	13.51	0.24± 0.10b	118.7± 34.20ab	97.0± 38.17ab
	85	30–38	29–34	4±0ab	4±0ab	92.63	14.37	55.05	0.40± 0.13c	42.58	14.68	19.96	0.29± 0.07bc	260.7± 73.32c	195.6± 44.63bc
SD+OPF (90:10) +15%SG (103.01 g)	65	32–35	31–35	4±0ab	4±0a	45.66	13.05	20.67	0.20± 0.08b	44.82	8.63	13.46	0.14± 0.07ab	57.1± 24.26a	40.8± 19.15a
	75	30–35	30–35	4±0ab	4±0a	59.61	12.71	29.07	0.24± 0.02b	39.01	13.66	38.44	0.19± 0.04ab	95.7± 7.15ab	77.46± 16.66ab
	85	30–33	30–34	4±0ab	4±0ab	106.23	19.50	63.14	0.43± 0.05cd	53.60	29.90	54.85	0.28± 0.06bc	288.9± 33.73cd	184.8± 38.44bc
100 % SD (106.59 g)	65	30–33	31–35	4±0b	4±0ab	18.29	9.88	8.09	0.08± 0.01a	16.76	7.38	8.48	0.08± 0.01a	25.2± 1.45a	22.3± 2.22a
	75	31–34	31–35	4±0bc	4±0bc	24.75	13.82	7.59	0.11± 0.03ab	22.91	10.60	13.51	0.11± 0.03ab	43.3± 12.82a	43.9± 10.40a
	85	29–35	30–33	4±0bc	5±0c	35.49	24.85	13.53	0.16± 0.03ab	42.58	14.68	19.96	0.17± 0.03ab	105.9± 19.66ab	113.6± 19.19ab

[a] ANOVA analysis were performed using Minitab 14 Statistical Software

[b] Each value is expressed as mean±SD of three replicate analyses. Value with different letters is significantly different at the level of 0.05 ($P<0.05$)

were supportive of this finding. According to Stamets (2000), homogeneity in particle size is important at all stages leading up to and through spawn generation. However, the fruit body formation period benefits from having a complex mosaic of substrate components and thus substrate structure comprising a mixture of fine and large particles is considered ideal. This also proves, other than chemical composition factors in substrates, why total yield and biological efficiency for formulations supplemented with spent grain were significantly higher than those of sawdust alone.

In conclusion, the present study clearly indicates that palm oil wastes can be used and further developed for cultivation of *A. polytricha* by local growers. The addition of spent grain and rice bran in the substrates proved to be beneficial in the growth and development of *A. polytricha* mycelia and sporophore.

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