

DEVELOPMENT AND EVALUATION OF *Pleurotus
pulmonarius* MYCELIUM AS ENCAPSULATED LIQUID
SPAWN FOR CULTIVATION

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2019

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pulmonarius* MYCELIUM AS ENCAPSULATED LIQUID
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OF THE REQUIREMENTS FOR THE DEGREE OF
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**DEVELOPMENT AND EVALUATION OF *Pleurotus pulmonarius*
MYCELIUM AS ENCAPSULATED LIQUID SPAWN FOR CULTIVATION**

ABSTRACT

Pleurotus pulmonarius also known as grey oyster mushroom is the most popular edible mushroom in Malaysia. Typically, commercial cultivators use grain spawn to inoculate into sterilized fruiting substrate but contamination rate and attraction to pest during spawn running is high. Good quality spawn should be free from diseases, fast colonisation with high yield potential. Therefore, to overcome the low quality spawn problems, the application of submerged culture technology followed by encapsulation of mycelium is proposed. Growers can easily utilize the spawn using this technology without the need for an expensive spawn inoculator equipment. The aim of this study is to optimize production and delivery of *P. pulmonarius* mycelium to be used as spawn for cultivation. An optimised culture medium at initial pH of 5.5 consisting of brown sugar, 2%; baker yeast, 1%; spent grain extract, 1%; potassium dihydrogen phosphate (KH_2PO_4), 0.05%; dipotassium hydrogen phosphate (K_2HPO_4), 0.05%; magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.05% and Tween 80, 0.5% successfully supported high growth of 11.89 ± 3.80 g/L dried mycelial biomass rapidly in 60 hours at 28°C in a 2-L stirred tank bioreactor. The optimum storage time determined for soluble starch *P. pulmonarius* encapsulated mycelial broth (SS-PPEMB) was 10 days stored in sterile distilled water at 4°C (C4 condition) exhibiting 100% germination on sawdust fruiting substrate. The potential of SS-PPEMB as spawn was assessed on sawdust fruiting substrate in polyethylene bags and the spawn run rate observed was 3.80 mm/day with biological efficiency of 180.99 ± 13.16 % even after storage at 10 days. As a conclusion, this study has successfully produced a high quality spawn with low risk of contamination and high yield. This technology is also applicable to other species of mushroom cultivated by mushroom industry.

Keyword: Mushroom liquid inoculum, liquid fermentation, encapsulated mycelium, mushroom cultivation

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**PERKEMBANGAN DAN PENILAIAN MISELIUM *Pleurotus pulmonarius*
SEBAGAI BENIH CECAIR TERENKAPSULAT DALAM PENANAMAN**

CENDAWAN

ABSTRAK

Pleurotus pulmonarius juga dikenali sebagai cendawan tiram kelabu merupakan cendawan yang boleh dimakan paling digemari di Malaysia. Pengusaha cendawan lazimnya menggunakan benih gandum untuk disuntik ke bongkah yang steril tetapi menghadapi risiko pencemaran yang tinggi di samping menarik haiwan perosak semasa pengeraman. Ciri-ciri benih yang berkualiti tinggi ialah bebas dari pencemaran dan pertumbuhan yang cepat serta berpotensi mengeluarkan hasil tuaian yang tinggi. Oleh yang demikian untuk mengatasi masalah benih yang tidak berkualiti, aplikasi teknologi kultur tenggelam diikuti dengan enkapsulasi miselium dicadangkan. Pengusaha dapat menggunakan benih dengan mudah melalui teknologi ini tanpa memerlukan penyutikan benih yang mahal. Matlamat kajian ini adalah untuk mengoptimum penghasilan miselium dan pengedaran miselium untuk digunakan sebagai benih dalam penanaman cendawan. Kultur medium dengan pH awal 5.5 yang telah dioptimumkan terdiri dari gula perang, 2%; yis roti, 1%; ekstrak bijirin buangan, 1%; kalium dihidrogen fosfat (KH_2PO_4), 0.05%; dikalium hidrogen fosfat (K_2HPO_4), 0.05%; magnesium sulfat ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.05% dan Tween 80 berjaya menampung pertumbuhan yang tinggi 11.89 ± 3.80 g/L biomasa miselium kering dalam masa yang cepat 60 jam pada 28°C dalam tangki bioreaktor 2-L yang dikacau. Masa penyimpanan optimum yang ditentukan untuk kaldu miselia *P. pulmarius* terenkapsulat dengan kanji terlarut (SS-PPEMB) adalah 10 hari yang disimpan dalam air suling yang steril pada suhu 4°C (keadaan C4) dengan 100% percambahan atas medium habuk kayu. Potensi SS-PPEMB sebagai benih dinilai menggunakan medium habuk kayu dalam bag polietilena menunjukkan kadar pertumbuhan miselium sebanyak 3.80 mm/hari dan kadar kecekapan biologi $180.99 \pm 13.16\%$. walaupun selepas

penyimpanan 10 hari. Kesimpulannya, hasil kajian ini berjaya menghasilkan benih cendawan yang berkualiti tinggi dengan risiko pencemaran yang rendah dan hasil yang tinggi. Teknologi ini dapat diaplikasikan kepada penanaman spesies cendawan yang lain dalam industri cendawan.

Katakunci: Cendawan, cecair inokulum, penapaian cecair, miselium terenkapsulat, penanaman cendawan

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
±	:	Plus-minus
BE	:	Biological efficiency
BKY	:	Baker Yeast
BS	:	Brown sugar
CaCl ₂	:	Calcium chloride
CaCO ₃	:	Calcium carbonate
cm	:	Centimetre
CO ₂	:	Carbon dioxide
DNS	:	3,5-dinitrosalicylic acid
EM	:	Encapsulated mycelium
EMB	:	Encapsulated mycelium broth
<i>et al.</i>	:	And other
g	:	Gram
g/L	:	Gram per litre
h	:	Hour
hrs	:	Hours
H ₂ SO ₄	:	Sulfuric acid
K ₂ HPO ₄	:	Dipotassium hydrogen phosphate
KH ₂ PO ₄	:	Potassium dihydrogen phosphate
L	:	Litre
M	:	Molar
MB	:	Mycelial broth

mg	:	Milligram
MgSO ₄ .7H ₂ O	:	Magnesium sulfate heptahydrate
min	:	Minute
mL	:	Millilitre
mm	:	Millimetre
NaOH	:	Sodium hydroxide
NO	:	Number
NS-PPEMB	:	Nutrient supplemented <i>Pleurotus pulmonarius</i> encapsulated mycelium broth
PPEMB	:	<i>Pleurotus pulmonarius</i> encapsulated mycelium broth
RB	:	Rice bran
rpm	:	Rotation per minute
SD	:	Sawdust
SG	:	Spent grain
SGE	:	Spent grain extract
SS-PPEMB	:	Soluble starch <i>Pleurotus pulmonarius</i> encapsulated mycelium broth
STD	:	Standard deviation
sp	:	Species
v/v	:	Volume per volume
w/v	:	Weight per volume
w/w	:	Weight per weight
YE	:	Yeast Extract

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CHAPTER 1

INTRODUCTION

Chang and Miles (1992) defined mushroom as a macrofungus with a distinctive fruiting body, which can be either epigeous or hypogeous, and large enough to be seen with the naked eye and to be picked by hand. Edible and medicinal mushrooms are becoming valuable horticultural crop worldwide. Only around 35 species amongst the 300 species of edible mushroom that exist can be cultivated and around 20 species are cultivated on an industrial scale (Sánchez, 2004). The popular mushroom that have been commercially cultivated worldwide are *Agaricus bisporus* (button mushroom) followed by *Lentinula edodes* (shiitake), *Pleurotus* spp. (oyster mushroom), *Auricularia* spp. (wood ear mushroom), *Flamulina velutipes* (winter mushroom) and *Volvariella volvacea* (straw mushroom) (Chang, 1999a).

Mushroom production is observed as the second most essential profitable microbial technology behind yeast (Pathak *et al.*, 2009). From Figure 1.1, the global mushroom production by Asian countries is leading producing more than 69.1% of world mushroom markets and then followed by Europe (22%). from total world mushroom in 2016 (FAO, 2016). Currently, 40% of total world edible mushroom are exported from China and by this reason, China is known as the world's biggest producer of mushroom. Nevertheless, in China 95% of the total China production is for domestic consumption (Zhang *et al.*, 2014). In China, the government strongly boost and financially supported the cultivation mushroom industry due to the benefit of mushroom as a source of quality food. (Zhang *et al.*, 2014) because of their high-quality protein; excellent unsaturated fatty acids and high vitamins content available (Marshall & Nair, 2009; Kumar *et al.*, 2014; Valverde *et al.*, 2015). The protein content in numerous mushrooms are about 19-40% (dry weight) offering twice as much protein as vegetables and four times that of oranges and can be a potential choice to meat (Jonathan *et al.*, 2012; Bashir *et al.*, 2014;

Amunke *et al.*, 2017). China major consumption is Shiitake with 22.5%, followed by Grey oyster mushroom 18.9% and Wood ear mushroom 16.8% (Li & Hu, 2014).

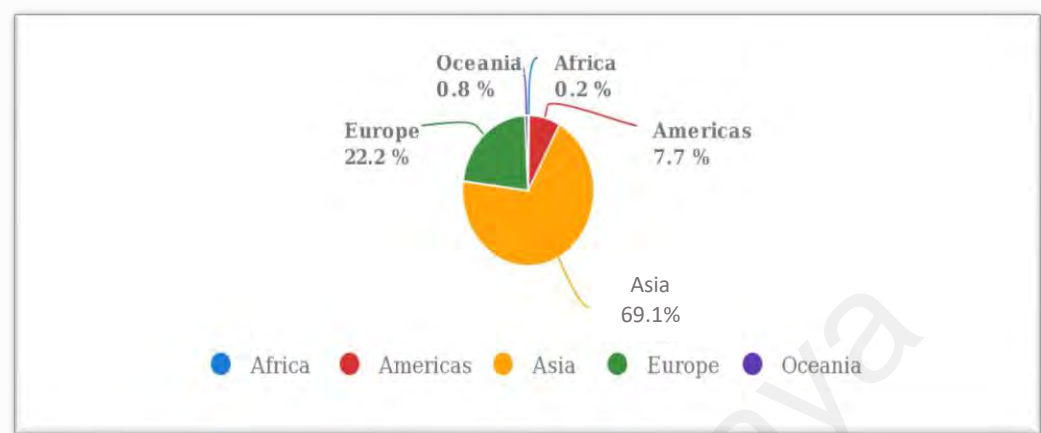


Figure 1.1: Average production share of mushroom and truffles by region from 1994-2016 (Adapted from <http://www.fao.org/faostat/en/#data/QC/visualize>). (Reprinted permission by FAO publication)

Pleurotus species are mostly cultivated particularly in south East Asia, India, Europe and Africa, (Mandeel *et al.*, 2005). Reis *et al.* (2012) stated that *Pleurotus* spp. contains high levels of proteins, fibers, carbohydrates, vitamins and minerals, whereas offering low calorie, fat and sodium levels. In Malaysia, mushroom is included as one of the seven profit crops that are cultivated comprehensively (Ministry of Agriculture Malaysia, 2011) and grey oyster is the most dominantly cultivated and marketed in Malaysia (Haimid *et al.*, 2013). Mushroom production total value in Malaysia has shown tremendous increased from RM49.1 million in 2007 to RM79.0 million in 2011. In 2014, the total value of production had further increased to more than RM110 million due to the rising number of growers, land area and productivity (Department of Agriculture Malaysia, 2015).

Mushroom production in Malaysia that has started in 1961 still shows a slow increase due to many problems faced by the growers. One of the problem is the lack of quality spawn. Spawn is defined as the substrate in which mushroom mycelium has grown

and which will be used as a “seed” in propagation for mushroom production. The genetic and cultural characteristic of the mushroom species are carried in the spawn (Chang & Miles, 2004). The quality of spawn affects the production yield and quality of mushroom commercially. Chang and Miles (2004), stated that if the spawn has not been prepared from a genetically proper fruiting culture or if a stock has degenerated or if it is too old, the yield of mushrooms will be less than optimal. The intent and purpose of inoculum/spawn is to promote the mycelium to a state of vigor where it can be launched into bulk substrates. The substrate (solid or liquid) is not only used as a vehicle for evenly spreading the mycelium at the same time it also acts as nutritional supplement (Staments, 1993).

Traditionally for mushroom production, solid inoculum using wheat grain colonized by the fungal mycelium has been used (Hesseltine, 1987; Abe *et al.*, 1992; Gunde-Cimermam, 1999). According to Confrotin *et al.* (2008) the preparation of grain spawn took longer growth period and stand higher risk of contamination compared to liquid spawn. Certain findings showed that liquid spawn is a potential replacement of the conventional grain spawn to be used in the various mushrooms cultivation (Kirchhoff & Lelley, 1991; Friel & McLoughlin, 2000; Silveira *et al.*, 2008).

However, currently it faces problems in inoculation since a sterile injector is required which is expensive. Under non-aseptic conditions it may be contaminated since its residual nutrients is a source of contamination. Meanwhile, certain mushroom such as *A. bisporus* and *L. edodes* could not colonize on several cultivation substrates by using liquid spawn (Leatham & Griffin, 1984; Friel & McLoughlin, 1999). In addition, this problem will affect the yield potential of mushroom.

The encapsulated microbial enriched the capability of microorganisms under storage or in opposing environmental conditions (Ortiz *et al.*, 2017). Throughout encapsulation process, the microorganisms are trapped to a micro-environment, this

micro-environment potential be practically developed including complete nutrients to preference the preservation or growth of microorganism (Khosravi *et al.*, 2014). Hence, this study is carried out to produce spawn in the form of encapsulated mycelium of a popular edible mushroom, *Pleurotus pulmonarius* and to evaluate the performance for the production of sporophores.

OBJECTIVES OF STUDY

- 1) To optimize medium formulation for submerged fermentation of *P. pulmonarius* mycelium
- 2) To optimize immobilization of *P. pulmonarius* mycelium to be used as liquid spawn and to assess the viability and storage life.
- 3) To evaluate the efficacy of immobilized mycelia on sawdust fruiting substrate.

CHAPTER 2.0

LITERATURE REVIEW

2.1 Overview of mushroom cultivation industry

Mushroom is treasured as a precious natural ingredient recognised for their therapeutic value, and even often used in religious rituals (Stille,1994) in the early evolution of the Greeks, Egyptian, Romans, Chinese, and Mexicans (Chang & Miles, 2004).

Traditionally, edible mushroom was found to grow and harvested wild in the forest of hilly areas, since it was initially difficult to domesticate and cultivate in temperate and subtropical regions of the world (Shah *et al.*, 2004; Zhang *et al.*, 2014). According to Van (2009), historically mushrooms have been collected to be consumed as food, as hallucinogens and remedy or for utilitarian aspect such tinder. Even in this era, particularly in southern Asia and other developing countries, picked/harvested edible mushroom from wild woodlands is still essential as a source of food and medicinal (Arora, 2008; Yang *et al.*, 2008; Fanzo *et al.*, 2012). Fasola *et al.* (2007) reported that mostly people in Nigerian depend on mushroom collected from the wild rather than commercial mushroom production. Normally mushroom hunters collect different sporophores of Nigerian mushroom and put on sale in local markets (Fasola *et al.*, 2007).

There are various techniques and species in mushroom cultivation history. Around 600-700 A.D., China was the first to use wood logs method to cultivate *Auricularia auricula* and *L. edodes* (Chang & Miles, 2004; Van, 2009). Later around 1600 year, France cultivated *A. bisporus* by using composted substrate method. Consequently, in Asia countries, mushroom species like *L. edodes* and *Pleurotus* spp. are famous and produced in large scale and these make inroads into Western markets (Chang & Miles, 2004).

Through 1990-2016, China, Italy, USA, Netherlands and Poland were the major producers of the world's mushrooms. The worldwide total production of mushrooms in 2016 was 10.790 million tonnes, which was a tremendous increase of nearly 521% from the 2.071 million tonnes of mushrooms produced in 1990 (FAOStat 1990-2016). According to the National Research Centre for mushroom, NRCM (2009), the percentage of types of mushrooms cultivated internationally based on types are as follows: button (31%), shiitake (24%), oyster (14%), black ear (9%), paddy straw (8%) and milky/ others (14%). In 2013, the highest mushrooms in demand for consumption in China were the Shiitake (22.5%), followed by grey oyster mushroom (18.9%) and the wood ear mushroom (16.8%) (Li & Hu, 2014).

The technology of artificial mushroom cultivation is an integration of non-traditional crops in the current agricultural techniques (Shah *et al.*, 2004). Through the innovation of mushroom science technology, mushroom cultivation can be implemented in different regions of various countries. Mushroom cultivation is preferred by growers due to the short period of harvest, utilisation of small plot of space and its contribution in sustainable agriculture and forestry (Shah *et al.*, 2004; Zhang *et al.*, 2014). Mostly small farmers in China, India and other developing countries gain benefits from mushroom cultivation and the processed mushroom products in terms of financial, social and health improvement (Shah *et al.*, 2004).

Mushroom cultivation involves microbiology, composting technology, environmental engineering, and marketing and management (Chang & Miles, 2004). Edible mushrooms cultivation is an important modern practice in biotechnological process that has potency for lignocellulosic organic waste recycling in order to decrease of environmental pollution and produces food of protein-rich food and superior nutritious value (Silva *et al.*, 2002; Beetz & Kustudia, 2004; Sánchez, 2010;).

Currently, mushroom cultivation industry is rising and new methods are being developed with supportable research to boost the different numbers of cultivated mushroom species production and mushroom products (Chang, 1999b; Lomberh *et al.*, 2002).

2.2 Stages of mushroom cultivation for *Pleurotus* species

In mushroom cultivation there are five stages that involves i) fruiting substrate preparation, ii) spawning/ inoculation, iii) spawn run / incubation, iv) fruiting phase, v) harvesting (Figure 2.1). On the other hand, according to Martínez-Carrera *et al.* (2000) and Wang (1999), mushroom cultivation involved three major stages: (1) inoculum (spawn) production, (2) substrate preparation, and (3) mushroom growing.

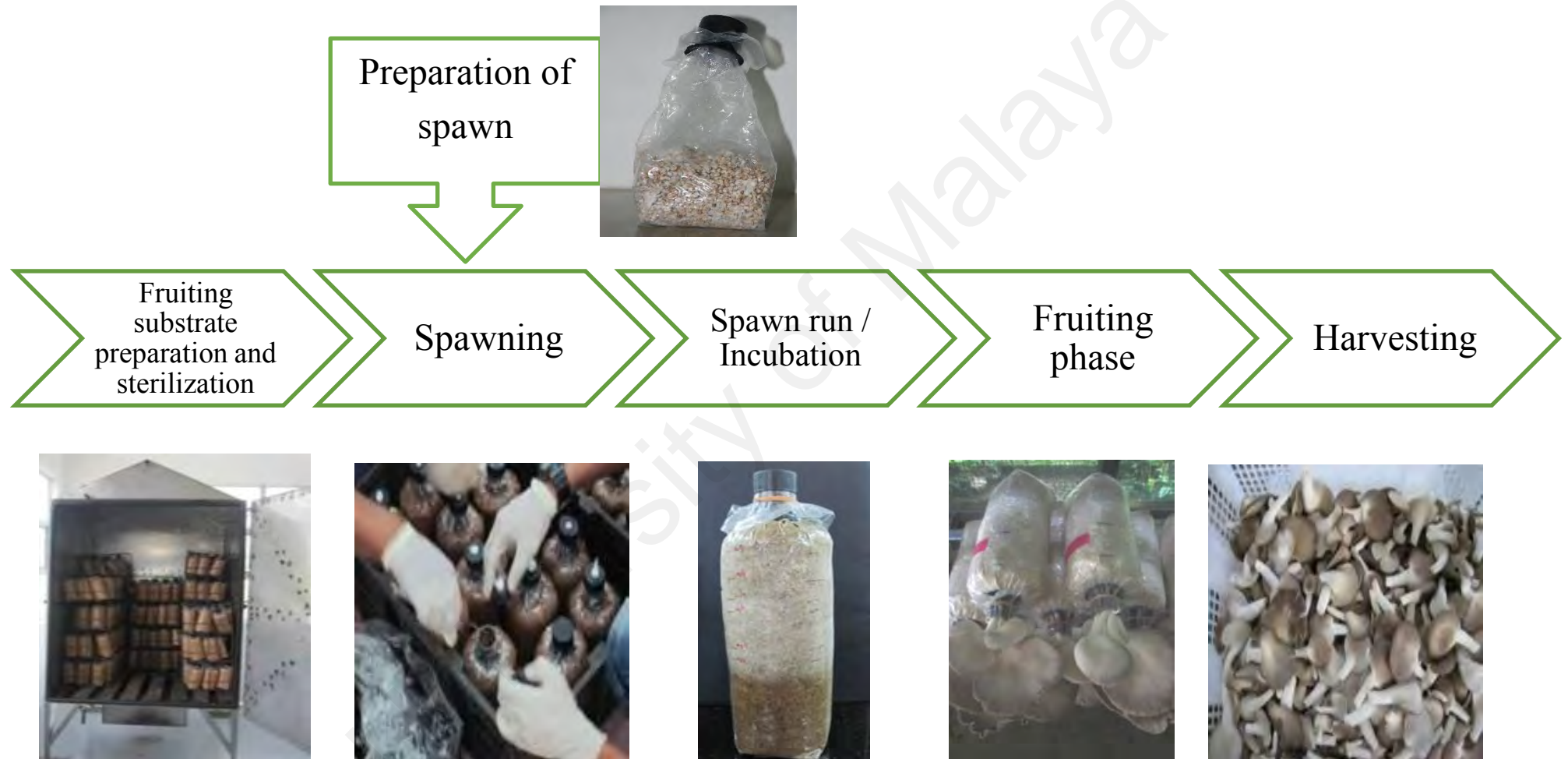


Figure 2.1: Process flow of mushroom cultivation.

2.2.1 Fruiting Substrate Preparation

In Malaysia, the bag cultivation method is the most practical technique that most mushroom growers implement in the preparation of fruiting substrates. This method results in higher biological efficiency and shorter production cycle (Mata & Savoie, 2005). Through the process of fruiting substrate preparation, the carbon source and nitrogen source are supplemented with minerals such as lime, calcium nitrate (Thevasingh *et al.*, 2005), gypsum, calcium carbonate (CaCO₃) and calcium superphosphate (Fan *et al.*, 2005). In addition, water is added and evenly mixed to raise the moisture content of the substrate to 70-85%.

The principal ingredient of a basic fruiting substrate is normally various agricultural waste such as sawdust, cottonseed hulls, wheat straw, paddy straw, coffee residue and oil palm, as carbon source. Meanwhile, addition of nitrogen source is needed such as rice bran, wheat bran, corn bran, and CaCO₃ are also included (Shu-Ting & Philip, 2004). Other sources of cellulose, hemicellulose, and lignin may also be used in various combinations with or without supplementation of nitrogen source. The prominent wood moisture of the dry weight is in the range of 50%-60%. In practice, wood with 90-100% water is acceptable, but may retard mycelial growth, which can lead to the low oxygen content in the cells (Vintila *et al.*, 1963).

In addition, Dietzler (1997) stated that the type of substrates, quantity (formulation of substrate) and supplementation may affect some substrate abilities such as water holding capacity and intensity of aeration; characteristics that consequently have an effect on mushroom yield. There may be difficulties for the mycelium to colonize in a fruiting substrate, if the substrate condition or type is too tight or too loose. Chen (2005) reported that if the substrate is too wet, the air flow in the substrate will be clogged. If the water collects at the bottom of the bag it may be due to the substrate being too wet.

After mixing substrate is packed into polyethylene or polypropylene bags, they are covered with a plastic cover and a neck ring (Figure 2.2). The sterilization process of fruiting bag is needed to remove competitive microbes and exterminate any contamination. Several methods and parameters are used for the sterilization, and the selection of these methods is dependent on several factors such as the nature of the bags, bag size and amount of the substrate (Kim, 2005).



Figure 2.2: Polyethylene or polypropylene bags were covered with a plastic cover and a neck ring.

2.2.2 Spawning

The usage of pure culture from the stock mycelium growing on an agar plate is not appropriate to be used directly as mushroom spawn. Therefore, it should be relocated for growth on a suitable culture medium that is easy to handle, distribute, inexpensive and convenient to inoculate (Chang & Miles, 2004). According to Chang and Miles (2004), spawn was defined as the substrate in which mushroom mycelium had grown and which would be used as a “seed” in propagation for mushroom production. The genetic and cultural characteristic of the mushroom species are carried in the spawn (Chang, 2001;

Chang & Miles, 2004). It is a medium impregnated with mushroom mycelium. According to Stanley and Awi-Waddu (2010), production of spawn is the first stage in mushroom production. According to Sánchez (2010), the function of grain coated with mycelium is to speed the mycelium to migrate to the specific bulk of the fruiting substrate.

This process of inoculating the spawn into the logs or cooled sterile fruiting substrate in polyethylene bags is called spawning. It is important to make sure that the mushroom spawn used is fresh, robust and not degenerated (Shu-Ting & Philip, 2004). According to Miles and Chang (1997), there are several techniques of spawning, depending on the cultivation technique. According to the method of applying a spawn on composted substrate, the spawn is shattered into small pieces by crumbling it with fingers and then spreading the pieces over the bed of the substrate surface. At the same time, it is vital to ensure that the spawn is in good contact with the substrate, which can be done by pressing it down firmly. Another technique of spawning is by using the fruiting bag, in which the spawn is injected 2-2.5 cm deep into the substrate. In spawning, the most vital aspect we should pay attention to is the amount of the spawn used per unit surface area, and the larger the amounts of the spawn used the more rapid the substrate will be full with mycelium (Miles & Chang, 1997). However, it is disadvantageous to use a large amount of spawn per unit area, as it will result in higher cost for the additional spawn.

The inoculated bag of substrate is then incubated at 20 to 25°C. This is the mycelial growth phase, in which the mycelium develops immediately after inoculation (spawning) until the substrate is completely permeated with mycelium. Spawn running (mycelial running) is the phase during which mycelium develops out from the spawn and permeates the substrate. During the phase of spawn running, the mycelium secretes enzymes to degrade complex substances in the substrate of bag and the mycelium assimilates and conglomerates the needed nutrients in sufficient amounts required for fruiting (Shu-Ting & Philip, 2004; Zadrazil *et al.*, 2004).

2.2.2.1 Grain Spawn

There are two types of spawn used in mushroom cultivation, which are the solid and liquid spawns. According to Wang *et al.* (2011), there are many types of substrates to prepare solid spawn such as:

- i) grains spawn eg rye, sorghum, wheat, millet, or crushed corn;
- ii) wood block or sawdust spawn eg hardwood sawdust especially oak, alder, cottonwood, poplar, ash, elm, birch;
- iii) straw spawn eg paddy straw or wheat straw.

Normally, mushroom growers will use solid spawns, such as the cereal grain spawn as the conventional method due to the simple equipment preparations and techniques, its competence to endorse the fruiting substrate faster and the ease of planting in this technique (Abe *et al.*, 1992; Friel & McLoughlin, 1999; Gunde-Cimermam, 1999). However, this method also needs a large space for a long incubation period (Bahl, 1988; Wang *et al.*, 2011). In Malaysia, commonly spawn is grown on grains such as wheat, crushed corn or millet. In addition, whole grain is also used since each kernel can become a mycelia capsule, a platform from which mycelia can leap into the surrounding expanse. One of the benefit from using smaller kernels of grain is that the grains offer more points of inoculation per pound of spawn (Stamets, 2000). On the other hand, the preparation of spawn for *P. ostreatus* cultivation is on grains such as wheat (Nwanze *et al.*, 2005a; Elhami & Ansari, 2008), corn (Nwanze *et al.*, 2005a; Elhami & Ansari, 2008), millet (Nwanze *et al.*, 2005a; Elhami & Ansari, 2008; Narh *et al.*, 2011), sorghum (Narh *et al.*, 2011) and other work on mixture of grain and grain straw (Muthukrishnan *et al.*, 2000; Sainos *et al.*, 2006; Pathmashini *et al.*, 2008).

The findings of Nwanze *et al.* (2005b) on grain selection showed that corn spawn stimulated highest yield of fruiting *Lentinus squarrosulus* as compared to wheat and

millet spawn. Elhami and Ansari (2008) also showed that corn spawn can contribute on highest mycelia growth of oyster mushroom species (*Pleurotus florida*, *Pleurotus citrinopileatus* and *Pleurotus ostreatus*) as compared to wheat and millet spawn. Moreover, use larger grain of corn and wheat may promote more nutrient for mycelia growth (Mottaghi, 2004).

The general method of grain spawn preparation includes grain washed with tap water to remove dust and then it is soaked overnight. Then, the grain is formulated with rice bran and calcium carbonate was added to adjust the pH and act as mineral. The grain is then distributed in the autoclavable polyethylene bag (Figure 2.3a) and sterilized by autoclaving for 1 hour at 121°C (Figure 2.3b). After the grains are cooled to room temperature, they are inoculated with the plug of mycelium (Figure 2.3c). After a period of around 2-3 weeks the grain is ready to be used as a spawn for mushroom cultivation (Figure 2.3d).

From the point of view of Stamets (2000), sawdust spawn is far better than grain spawn for the inoculation of outdoor mushroom cultivation because when grain spawn is introduced to an outdoor bed, insects, birds and slugs quickly seek out the nutritious kernels for food. By using sawdust spawn, it has the improvement of having more particles or inoculation points per pound than does the grain, with more points of inoculation and the mycelium colonization is also quicker (Stamets, 2000). Another technique involves the use of wood plug spawn. This is done by inoculating the mushroom mycelium into wedge-shaped pieces or cylindrical pieces of wood. After the mycelium has fully colonised into the pieces of wood, these wood plugs can be used as mushroom spawns. (Chang & Miles, 2004).

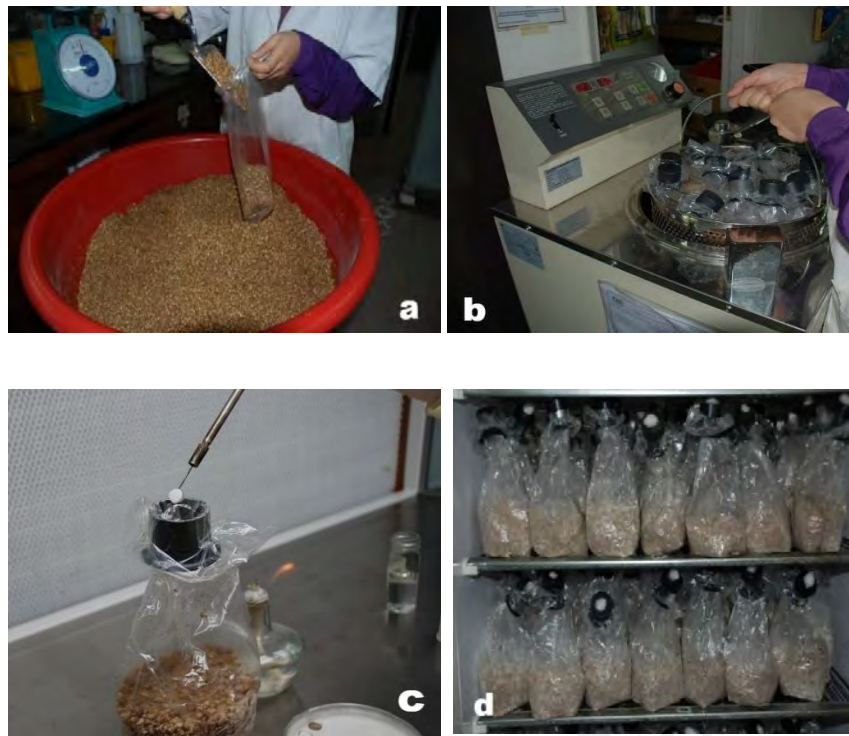


Figure 2.3 : *Pleurotus pulmonarius* spawn production process : a) grain distribution into autoclavable polyethylene bags; b) sterilization by autoclaving for 1 h at 121°C; c) inoculation of cooled grain *P. pulmonarius* mycelial plugs; d) colonization of the grain with mycelium.

Certain species of mushroom are suitable for cultivation using the straw spawn technique, such as the *V. volvacea*. In preparation of straw spawn (paddy straw), firstly the paddy straw is soaked in water for 2 to 4 h, and then the water is drained and the straw is cut into pieces of sized 2.5 to 5 cm long. Next it is mixed with 1% calcium carbonate and 1% to 2% rice bran and is placed into clean wide-mouthed quart bottles (Chang & Miles, 2004).

The problem that mushroom growers will have faced when using solid spawn is the high risk of contamination by mitosporic fungi such as *Trichoderma* spp., also known as the green mold disease (Hatvani *et al.*, 2007). Normally *Trichoderma* spp is present in the early stage of mushroom cultivation, especially during the spawning stage, but it can also contaminate during the harvesting stage, which can cause huge losses in the mushroom yield (Jandaik & Guleria, 1999). According to Ortiz *et al.* (2017), the contamination of spawn may occur because of incorrect handling during the spawn

preparation phase, or by intrinsic grain microbial contamination. Laca *et al.* (2006) and Sreenivasa *et al.* (2010) stated that the population of microbial shift among grains batches and the mitosporic fungi existing in cereals constitute a potential problem for spawn quality.

2.2.2.2 Liquid Spawn

Mycelia or liquid spawn is an alternative method for generating spawn by submerged fermentation. The implementation of liquid culture technology to the mycelia production of higher fungi afford the possibility of industrial scale application to this group of organisms (Humfeld, 1948). There are many advantages of adapting the liquid spawn compared to grain spawn, as it produces higher yield of mycelium in compact space, more uniform distribution mycelia biomass in shorter period, could lower laboratory cost, and make the inoculation procedure smooth with reduced possibilities of contamination and promote early fruiting (Chang & Miles, 1993; Eyal, 1991; Rosado *et al.*, 2002).

Liquid spawn can be inoculated directly to fruiting substrate and another method is by encapsulation of the mycelium. On other hand, liquid spawn also makes it possible for the mycelium to be propagated on solid support, encapsulation of mycelium or it can be directly inoculated in the fruiting substrate (Friel & McLoughlin; 2000). According to Kirchhoff and Lelley (1991), the use of liquid spawn for the sawdust substrate cultivation lead to a higher fruiting body yield than grain spawn. Abdullah *et al.* (2013) reported that liquid spawn of *P. pulmonarius* has the proficiency to colonise sterile sawdust substrate in shortened time suggesting that the mycelium was spread more efficiently as opposed to grain spawn. Liquid culture technology can increase the level of process control growth rates and nutritional content (Friel & McLoughlin, 1999). Based on Leatham and Griffin (1984) the process of spawn production in liquid culture production should rapidly and reliably inoculate the given substrate. In order to produce a high quality liquid culture it

should have a high density of viable inoculum particles. At the same time, the culture should be homogeneous and consists of unpeletted mycelium (Itavaara, 1993).

There are many studies that have been done on liquid spawn production using the submerged method. Kawai *et al.* (1996b) applied the liquid spawn method of *L. edodes* on a sawdust fruiting substrate. Friel and McLoughlin (2000) has done submerged method in the production of *A. bisporus* liquid spawn such as under static condition, continuous incubation under shaking at 3000 rpm and incubation by exposure to alternating period of agitation at 100 rpm to 300 rpm. Corfortin *et al.* (2008) produced *Pleurotus sajor-caju* biomass in submerged culture, combining soy protein, yeast extract and ammonium sulfate. Abdullah *et al.* (2013) produced high amounts of *P. pulmonarius* biomass in brown sugar, rice bran, malt extract and yeast extract medium using an automated bioreactor. At the same time, liquid spawn has been broadly used in the cultivation of many species of mushrooms, including oyster mushroom (Alain, 1966; Silveira *et al.*, 2008), *L. edodes* (Kirchhoff & Lelley, 1991; Pellinen *et al.*, 1987), *Pleurotus ostreatoroseus* (Rosado *et al.*, 2002), *A. bisporus* var. *hortensis*, *Tricholoma nudum*, *Morchella hortensis*, *Morchella esculenta* and *Cantharellus cibarius* (Alain, 1966).

Despite these facts, there are some disadvantages by using liquid spawn in mushroom cultivation. It is strenuous to store and transport liquid spawn and its residual nutrients may cause contamination (Kumar *et al.*, 2017). Additionally, liquid spawn of several mushroom (*A. bisporus* and *L. edodes*) could germinate on some cultivation substrate (Friel & McLoughlin, 1999; Leatham & Griffin, 1984). According to Friel and McLoughlin (2000), the liquid spawn without nutrition, could not survive in pasteurized compost and that the biomass levels were significantly lower than that of conventional grain spawn with the entrapment of both mycelium and nutrients. In order to improve the liquid spawn method, the aseptically encapsulated mycelium (EM) were implemented in spawning mushroom production.

Meanwhile, in the agriculture industry, immobilization technique has been extensively used in the preparation of inocula to improve its ecological competence (McLoughlin, 1994; Harith *et al.*, 2014; Kumar *et al.*, 2017). By using this technique, the EM had a higher growth rate in pasteurised compost than both liquid spawn and the conventional grain spawn by a shorter adaptation (lag) period. Also, EM was associated to the high biomass loading capacity of these beads and afford a high viable inoculum offering protection of the mycelium (Friel & McLoughin, 1999; Harith *et al.*, 2014).

Certain works on EM of edible mushroom have been reported. A study conducted by Rosado *et al.* (2002) on button mushrooms showed that, the EM were prepared by entrapping liquid spawn with vermiculite, hygramer, and nourishment in sodium alginate; its form was similar to that of grain spawn in the shape and yield of fruiting body. In another study performed by Wang *et al.* (2011), the *P. ostreatus* mycelium was encapsulated by using a mixture of cottonseed hull, corn core and wheat bran with a ratio of 4.5:4.5:1. The outcome of using this EM showed similar result as using the grain spawn. A study done by Friel and McLoughin (1999) show a potential of EM of *A. bisporus* in pasteurised compost. Ortiz *et al.* (2017) reported that EM of *A. bisporus*, *L. edodes*, *P. ostreatus* and *Gymnopilus pampeanus* showed no significant difference when compared to conventional spawn to germinate the mycelium on sterilized substrate. In addition, they found that the EM can be preserved frozen for six months and the mycelia remain viable in most of the species assayed.

2.2.2.3 Submerged fermentation of mycelium to produce liquid spawn and encapsulation medium

Typically, nutrient source plays an important role affecting the yield of any submerged products (Ma *et al.*, 2016). Many works reported that carbon and nitrogen source usually play a significant part because these nutrients are directly linked with cell

proliferation and metabolite biosynthesis (Fang & Zhong, 2002a; López *et al.*, 2003; Ma *et al.*, 2016; Park *et al.*, 2001; Tang & Zhong, 2002).

Commonly, mushroom mycelium grows throughout a broad range of carbon source (Yang *et al.*, 2003). According to Chang and Miles (2004), the sort of carbon sources for mycelial growth are starch, glucose, fructose, maltose, mannose, sucrose, pectin, cellulose and lignin. Chang *et al.* (2006) reported that brown sugar and lactose can stimulate mycelium formation in submerged culture. It is also stated that, although the main component of brown sugar is sucrose, the present of trace elements in brown sugar initiate on the production of mycelium and polysaccharide than sucrose. Hamachi *et al.* (2003) state that brown sugar is usually distinguished as the most readily applicable carbon source for most mushrooms cultures because it consists of approximately of 94–98.5% (w/w) sucrose and various types of non-sucrose components ranging from 1.5–6% (w/w). Furthermore, Hawker (1950) had mentioned that contribution of brown sugar being low cost and easily available in supply compared to glucose, sucrose and lactose is more suitable for the preparation of mushroom growth medium on a large scale.

Many authors stated that organic nitrogen sources frequently gave higher mycelial biomass growth in submerged culture (Yang *et al.*, 2003; Shih *et al.*, 2006; Asatiani *et al.*, 2008). The type of organic nitrogen that be used in submerged are yeast extract, peptone (Pokhrel & Ohga, 2007), yeast powder (Jr-Hui & Shang, 2006) and casein (Fang & Zhong, 2002a). Jr-Hui & Shang (2006) stated the effect of nitrogen source on the mycelia growth of higher fungi be influenced by on the species and cultivation conditions.

Brewers' spent grains (BSG) are accessible at low or no cost throughout the year and are generated in bulky quantities by both large and small breweries (Aggelopoulos *et al.*, 2013). Spent grain is rich in cellulose and non cellulosic polysaccharides (Aliyu & Bala, 2011). Therefore, spent grain needed extracellular enzymes to break down to simpler, soluble units so that it is easier to absorb nutrition for fungal hyphae growth

(Chang *et al.*, 1993). Gregori (2008), stated that the additional of high concentration of spent grain leads to a decreased enzymes activity. In addition, spent grain caused insufficient oxygen supply for maximum growth during fermentation (Nakano *et al.*, 1997). Roberts (1976) stated that use of spent grain extract in the fermenter acts as an effective antifoaming agent. According to Stein *et al.* (1973) spent grain extract may supply 30% to 60% of the biochemical oxygen demand. Furthermore, spent grain extract contribute in aerobic biological organism by breaking down organic material present in the medium and enhance the mycelial growth in submerged medium.

Tween 80 is a non-ionic surfactant that is effective in discharging fungi enzymes to the external environment (Rancaño *et al.*, 2003) and exhibits low toxicity to the cellular membrane (Giese *et al.*, 2004). Its efficacy alters the structure and the morphology of fungi and bacteria cell wall, prominent to the enhance of protein secretion (Giese *et al.*, 2004). Furthermore, effect of Tween 80 in submerged medium is it has the capability to boost the growth of the mycelia (Zhang & Cheung, 2011; Zhang *et al.*, 2012). According to Li *et al.* (2011), by adding Tween 80 in submerged medium it can achieve small pellets and to produce unicellar propagates, viscosity of broth, increase oxygen transfer and biomass. In addition, Tween 80 has the potential to hinder breakdown of mycelial cells due to the shearing forces during the shake-flask experiments by maintaining the intact structure of the mycelial pellets during fermentation (Zhang & Cheung, 2011). According to Domingues *et al.* (2000), the effect of Tween 80 on mycelial morphology could be associated in part to its surface-active properties, lowering the mycelium-liquid interfacial tension and consequently the potential or tendency of mycelia to form aggregates. By decreasing the surface tension of the medium by surfactant lowers the thermodynamic potential for the aggregation but favours the dispersion of mycelia. A work done by Zhang & Cheung (2011), used Tween 80 as stimulatory agent in the submerged

fermentation of mushroom mycelium due to its competence maintained the pH value of the fermentation broth at an acidic level.

Encapsulation medium technique was improved by integration of nutrient carriers/ supplementation such as wheat bran, milled chitin, corn cobs, fish meal, soy fibers, peanut hulls, gluten (Chien *et al.*, 2001) and casein (John *et al.*, 2010) into the biopolymers (e.g. alginate) to afford a nutrition source essential for propagation of the microorganisms (El-Komy, 2001; Woodward, 1988). Various supplementations which help in the stability and survival rate can be affected through the formation and storage of micro-beads (John *et al.*, 2011). John *et al.* (2011) reported that, the supplementation in encapsulated medium is used to preserve the viability of microorganisms or to uphold the properties of the microcapsule also have significant effect on the performing of microorganisms in microbeads. Supplementations are the ingredients that help in the maintenance and protection of the microbial cells in a formulation through storage, transport, and at the target zone (Xavier *et al.* 2004). Also based on the works by Young *et al.* (2006) reported that the encapsulated medium must be stable from manufacture to application site, it would boost the activity of the organism in the field, be inexpensive, and be practical. At the same time, besides with carrier, supplementations act a vital role in an extended survival through different phases of formulation.

Gluten is a natural polymer and the major by-product from the manufacture of wheat flour. Gluten is beneficial for practice as the matrix for supplementation in encapsulated medium of cells or cellular elements because it is biodegradable, low-cost, nontoxic and instantly accessible (Chien *et al.*, 2001). A study done by Chien *et al.* (2001), were used gluten for entrapping fungal mycelia.

A study done by Bok *et al.* (1993) are using biopolymeric gels such as potato starch, rice, rye, barley, and soybean powders for entrapped biopesticide. These is done

to hold its entomocidal activity through better protection against dehydration, sunlight, heat, and the damaging effects of UV light.

2.2.2.4 Spawn quality

Ortiz *et al.* (2017) stated that the vital factor that contributes to yield of mushroom production is spawn quality. According to Chang and Miles (2004), some aspects that need to be considered in production of quality spawn are:

- i) the genetic potentials of the fruiting culture for vegetative growth both in the spawn substrate as well as spawn running in fruiting substrate and for quality mushroom production;
- ii) the type of the spawn material because this stimulus the speed and thoroughness of mycelial growth in the spawn substrate as well as spawn running in the fruiting substrate;
- iii) spawn substrate cost and accessibility;
- iv) the ability of the spawn to survive while in storage;
- v) a good quality spawn will encourage the growth rate of the mycelia and by that the window of opportunity for contaminants is significantly restricted and yield are increased. (Stamets, 2000).

2.2.3 Spawn running / Incubation

The inoculated substrate bag is then incubated at temperature 23 to 27°C. Generally, incubation temperature runs higher than the temperature for primordia formation (fruiting phase) (Stamets, 2000). Further, the environmental condition for incubation is no direct sunlight, unfiltered or no bright light because light can be damaging or harmful to the mycelial. This phase is referred to as the mycelial growth stage, which develops immediately after inoculation (spawning) until the bag of substrate is completely permeated with mycelium. Spawn running (mycelial running) is the phase during which mycelium develop out from the spawn and permeates the substrate. During

the spawn running, the mycelium secretes enzymes to degrade the complex substance in the substrate of the bag and the mycelium assimilates and conglomerates the needed nutrients in sufficient amounts required for fruiting (Shu-Ting & Philip, 2004).

2.2.4 Fruiting phase

The formation of fruiting bodies of mushrooms is commonly in rhythmic cycles called “flushes”. Management of mushroom house is vital in this stage as it is in this stage that the mushroom development phase begins. In order to obtain primordia formation, followed by the formation of fruiting bodies, optimum environment condition is necessary. This stage of environment condition is different from those for spawn running. During this fruiting phase, we need to maintain the optimum temperature, humidity and ventilation. These factors will influence the number of flushes and total yield that will be obtained. Requirement of humidity, temperature and ventilation for fruiting is commonly different than for mycelial running. In the meantime, inappropriate aeration will contribute to an increase in carbon dioxide (CO₂) in the vicinity of the mushroom beds where the mycelium is respiring. Therefore, this may hinder formation of primordia or later mushroom developmental stages.

Based on Sánchez (2010), mushrooms are ready to be harvested almost 3 to 4 weeks after spawning, although it is influenced by the strain, amount of supplement used, and temperature of fruiting house.

2.3 *Pleurotus pulmonarius*

Pleurotus pulmonarius (Fr.) Quéf commonly known as grey oyster mushrooms has increased its popularity due to its high nutrition level and its delicious taste. The genus *Pleurotus* has been intensively studied in many different parts of the world due to many reasons: they have high gastronomic values, they are able to colonize and degrade a large variety of ligno-cellulosic residues, they require shorter growth time when compared to

other edible mushrooms, they demand few environmental controls, their fruiting bodies are not very often attacked by diseases and pests and they can be cultivated in a simple and cheap way (Jwanny *et al.*, 1995; Patrabansh & Madan, 1997). In addition, it improves the physical properties of the soil or as feed for animal by converting the substrate to be used as a fertiliser (Fox, 1993; Maziero & Zadrazil, 1994; Bononi *et al.*, 1995). This edible mushroom is also used in environmental remediation (Pérez *et al.*, 2008; Yan *et al.*, 2009), and biofuel technology (Okamura-Matsui *et al.*, 2003).

From the standpoint of its medicinal values, the compounds extracted from *P. pulmonarius* are capable to treat/cure various types of diseases, including atherosclerosis (Abidin *et al.*, 2018), hypertension (Ajith & Janardhanan, 2007) and cancer (Xu *et al.*, 2014) since it possessed significant antioxidant, anti-inflammatory and antitumor activities (Badole *et al.*, 2006). Research done by Reshetnikov *et al.* (2001) has proven that *P. pulmonarius* is rich in pharmacologically active polysaccharides such as xyloglucan and xylanprotien.

From the nutritional standpoint, this mushroom contains high quantities of proteins, carbohydrates, minerals (calcium, phosphorus, iron) and vitamins (thiamine, riboflavin and niacin) and low fat (Randive, 2012). According to Sharma and Madan (1993), the genus is characterized by its high protein content 30- 40% on dry weight basis which is twice that of vegetables. The content of niacin in oyster mushroom surpasses almost ten times than any other vegetables and the folic acid content in oyster mushroom is beneficial for conditions such as anaemia (Randive, 2012). Besides that, it has benefits for people who have hypertension, obesity and diabetes, due to its low sodium: potassium ratio, starch, fat and calorific content. The content of alkaline ash and high fibre in the oyster mushroom give an advantage for those suffering from hyperacidity and constipation and cholesterol inhibitors (Randive, 2012).

Pleurotus spp. are known to be easily and most widely cultivated in various agricultural wastes in the world (Chang, 1999a; Akyüz & Yildiz, 2008; Shauket *et al.*, 2012). It has a promising future in the tropical and subtropical countries in the range of moderate temperature from 20 to 30°C with 55-70% of humidity, because it is easily cultivated and has a relatively inexpensive cultivation technique (Chang & Miles, 2004; Randive, 2012).



Figure 2.4: *P. pulmonarius* a & b) a mature fruiting body was cultivated on sawdust substrate.

Various technique has been implemented in the cultivation of *Pleurotus* spp., such as in different bagging systems like trays, cylindrical containers, wooden or polystyrene racks, blocks and plastic bags (Quimio *et al.*, 1990). Zadrazil and Kurtzman (1982) has reported that by using plastic bag in cultivation has improve in yield of harvesting with reduced contamination rate. Commonly, growers in Europe uses large black perforated bags, however Asian growers' mostly favour the use smaller sized bags, where the technique of inoculation and harvesting are handled at one end of the bag. Meanwhile, mushroom production in Japan use polypropylene bottle technology by filling the bottles with sterilised substrates and then they are inoculated mechanically after substrate is cooled (Royse, 1995).

In addition to sawdust substrate, various types of lignocellulosic substrates can be used to cultivate *Pleurotus* spp., such as paddy straw (Chang & Quimio, 1982; Rani *et al.*, 2008; Ahmed *et al.*, 2009), leaves (Zadrazil, 1978; Shah *et al.*, 2004), cotton stalks (Balasubramanya, 1981; Balasubramanya & Khandeparkar, 1989; Hüttermann *et al.*, 2000), coffee pulp (Chang & Quimio, 1982; Guzman & Martinez, 1986), coffee residue (husks) (Fan *et al.*, 2003); rice hulls, water hyacinth leaves, coconut shell, corncobs and leaves, (Hadar *et al.*, 1992), sugarcane bagasse (Chang & Quimio, 1982; Hadar *et al.*, 1992), tree leaves wood wastes of timber work shops, cupola of nut trees, corn stalks, waste tea leaves of tea factories, and waste paper (Sivrikaya & Peker, 1999), rice straw (Hüttermann *et al.*, 2000; Cayetano-Catarino & Bernabé-González, 2008; Daba *et al.*, 2008), peanut shells, cotton waste (Philippoussis *et al.*, 2001), sawdust, oil palm fibre, dry cassava peels (Onuoha *et al.*, 2009), banana leaves (Cayetano-Catarino & Bernabé-González, 2008), banana pseudostem, sorghum stalk (Rani *et al.*, 2008), cotton seed hulls (Daba *et al.*, 2008), soybean straw (Daba *et al.*, 2008), wheat straw (Shah *et al.*, 2004; Daba *et al.*, 2008; Ahmed *et al.*, 2009), handmade paper and cardboard industrial waste (Kulshreshtha *et al.*, 2013). Based on Mikiashvili *et al.* (2006) oyster mushroom has the capability to generate lignolytic and hydrolytic enzymes and consequently can be easily cultivated for fruiting by using a type of lignocellulosic waste and by enhancing the carbohydrates availability and biomass accumulation.

2.4 Cultivation of *P. pulmonarius* in Malaysia

Mushroom production in Malaysia is concentrated on fresh mushrooms. Ninety percent of the growers concentrate in *P. pulmonarius* cultivation because of its popularity, high demand and many products that have been developed from this mushroom. According to the Department of Agriculture of Malaysia (2015) the total production of *P. pulmonarius* was recorded to be 90.89%. Meanwhile, in the hotel trade and caterers are demanding for the Shiitake and button mushrooms in the market (Haimid *et al.*, 2013).

The total value of mushrooms production in Malaysia has increased from RM49.1 million in 2007 to more than RM110 million in 2014. (Department of Agriculture of Malaysia, 2015). The remarkable improvement of production value was influenced by the increasing number of growers, land area and productivity (Department of Agriculture Malaysia, 2015). Mohd-Syauqi *et al.* (2014) stated that this increase being partly due to the growth of population and higher interests towards health. The Ministry of Agriculture (2011) predicted that the consumption of mushrooms will increased at a relative rate from 1.0 kg/ person in 2008 to 2.4 kg/ person in 2020.

According to the Department of Agriculture of Malaysia (2015), the total amount of growers in Malaysia has reportedly increased every year from 339 growers in 2007 to 428 growers in 2014. About 80% of these growers consists of small growers, with the production of fresh mushrooms of below 50 kg per hectare per day. Meanwhile, 17% of the growers consists of medium scale growers (producing 50-500 kg of mushrooms per day) and 3% is made up of big scale industries (producing more than 500 kg of mushrooms per day) (Haimid *et al.*, 2013).

A study done by Rosmiza *et al.* (2016) showed there were numerous problems and challenges were identified that could obstruct effective mushroom industry growth in Malaysia. In Malaysia we are facing a problem in getting the raw materials supply with increasingly expensive price, especially sawdust and rice bran. In the cultivation stage of mushroom, the poor quality of spawn contributes to low production yield of mushrooms and may cause green fungus diseases.

Meanwhile, the lack of skills and knowledge among the mushroom growers in mushroom cultivation may affect the yield of mushrooms. Sometimes mushroom growers cannot overcome the problems regarding mushroom contamination due to low knowledge in mushroom biology. Low skills in marketing of mushroom sales also contribute to this problem (Rosmiza *et al.*, 2016). At the same time mushroom growers are facing the issue

in marketing competition from neighbouring countries such as Thailand. Thailand can offer low price of mushroom due to low cost of raw materials that are available in their country and can produce high yield of mushroom.

Mushroom growers are also facing problems regarding mushroom marketing due to the short shelf life of fresh mushrooms (Rosmiza *et al.*, 2016). At same time, there are also factors such as the lack of awareness from local markets regarding the benefit of eating mushroom and the limitation of knowledge (recipes) in preparing mushroom dish/cooking. The lack of mushroom products in the market is also another contributing factor in the mushroom marketing problem to growers.

University of Malaysia

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Preparation of *P. pulmonarius* mycelial culture

Pleurotus pulmonarius was cultured from cleaned mushroom sporophore by wiped outer surface of the fruitbody with a clean tissue may harbor bacteria and mold spores. Then, the pileus and stipe was split to expose the interior tissue is free from contaminated organisms, assuming the sporophore is not water logged. The sporophore was placed on the table with the clean surface faced sideways. Then immediately a small fragment of tissue was firstly cut using a sterile scalpel and then pinched using a sterile forcep and transferred into the centre of Petri plate containing malt extract agar (MEA) as prepared in Appendix A, 1.0. The plate was sealed with parafilm and incubated in 25°C incubator for seven days. (see Figure 3.1).

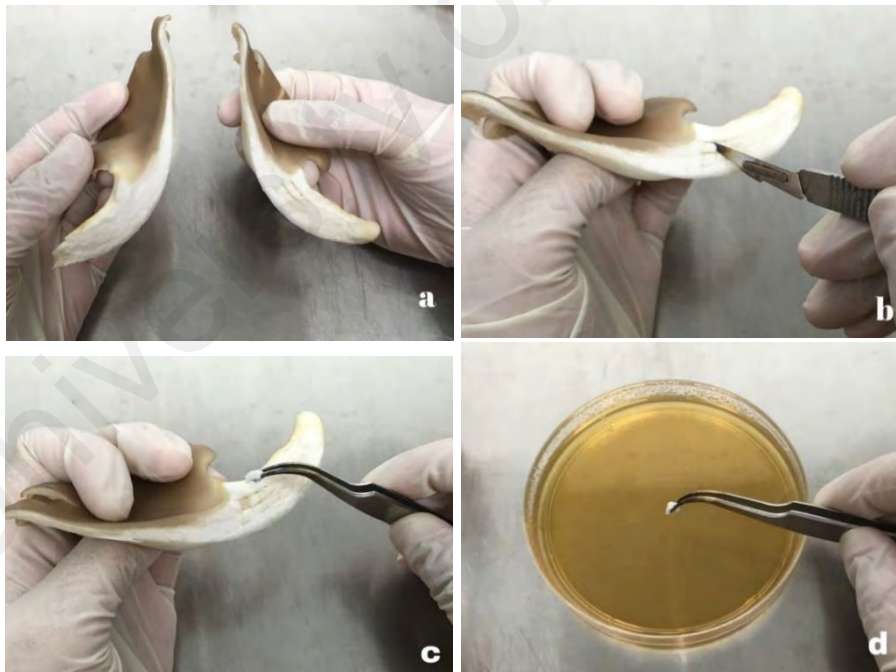


Figure 3.1: Preparation of mycelial culture by tissue transfer; a) Splitting the sporophore and stipe to expose interior tissue, b) Cutting a small fragment of tissue using a sterile scalpel, c) Pinching a piece tissue fragment using a sterile forcep, d) Transferring the tissue fragment into the centre of MEA.

The fruiting body has been authenticated by mycologist and by molecular method and was given a code KLU-M1234 and deposited in Mushroom Research Centre, University of Malaya herbarium. Pure mycelial culture was coded as KUM61119 and deposited in Mycology Laboratory culture collection. The stock culture was preserved on MEA slants stored at 4 °C.

3.2 Optimization of growth medium formulation for mycelial growth of *P. pulmonarius* by submerged fermentation

A series of experiments for fermentation condition and medium composition were done to optimise the best low-cost liquid medium for the submerged fermentation of *P. pulmonarius* mycelium using shake flasks. For the formulation of liquid medium, the components selected were brown sugar as the carbon source based on Hawker (1950). Brown sugar was also selected due to being low cost and easily accessible in supply compared to glucose, sucrose and lactose is more suitable for the in preparation of mushroom medium production in large scale. Various yeast types were tested as the nitrogen source together with the supplementation of brewery spent grain extract, minerals and Tween 80. In all the experiments, dry weight of *P. pulmonarius* mycelial biomass was measured to evaluate the mycelial yield.

3.2.1 Optimization of brown sugar concentration and yeasts concentration

This experiment was done using a total volume of 50-mL liquid medium consisting of either 0.5% or 2% of brown sugar as carbon source and three types of yeast as nitrogen source i.e Baker's yeast, brewer's yeast and yeast extract. The percentage of yeasts investigated was 0.1% and 1% (w/w) and pH for each formulation was measured using a pH meter. The combinations of brown sugar and yeasts types at various concentrations to formulate the liquid medium is depicted in Table 3.1.

Table 3.1: The percentage of carbon and nitrogen sources concentration in liquid fermentation medium.

Brown sugar (w/v) (%)	Brewer's yeast (w/v) (%)	Baker's yeast (w/v) (%)	Yeast Extract (w/v) (%)	Spent Grain (w/v) (%)	pH reading	C:N ratio
0.5	0.1	–	–	0.1	5.32	117.83
	0.1	–	–	1	5.00	29.40
	1	–	–	0.1	5.59	34.56
	1	–	–	1	5.37	21.59
	–	0.1	–	0.1	5.39	71.17
	–	0.1	–	1	5.83	25.84
	–	1	–	0.1	5.84	24.21
	–	1	–	1	5.82	19.82
	–	–	0.1	0.1	6.58	47.98
	–	–	0.1	1	6.15	24.55
	–	–	1	0.1	6.91	13.05
	–	–	1	1	6.68	12.91
2	0.1	–	–	0.1	5.27	433.75
	0.1	–	–	1	5.04	80.60
	1	–	–	0.1	5.15	99.97
	1	–	–	1	5.2	54.44
	–	0.1	–	0.1	5.19	241.85
	–	0.1	–	1	5.67	72.81
	–	1	–	0.1	5.49	46.63
	–	1	–	1	4.94	37.04
	–	–	0.1	0.1	6.14	164.90
	–	–	0.1	1	5.97	64.68
	–	–	1	0.1	6.85	27.51
	–	–	1	1	6.62	24.60

250-mL Erlenmeyer flask containing 50-mL medium was sterilised at 121 °C for 20 min and cooled to room temperature. *Pleurotus pulmonarius* culture was initially grown on MEA medium in a Petri dish for 7 days, and ten 5 mm-diameter plugs cut from

the periphery of the colony were transferred into the Erlenmeyer flasks. Inoculated flasks were incubated on a shaking incubator at $25 \pm 2^\circ\text{C}$ rotating at 150 rpm for 6 days. All medium formulation experiments were performed in triplicate flasks.

Mycelium pellets formed were filtered using filter paper (Whatman No.1) after 6 days of incubation. Mycelial dry weight was measured after repeated washing with distilled water and left to dry at 60°C overnight in oven until constant weight was achieved. All results were expressed as mean \pm standard deviation from triplicates data. Statistic significant differences were determined by One-way ANOVA Duncan test with p values <0.05 were considered as significant difference using SPSS16.

3.2.2 Effect of supplementation of spent grain extract, minerals and Tween 80 in the liquid medium

Brown sugar (BS) (2%) and baker's yeast (BKY) (1%) were selected as the best concentration for the mycelial growth. To this formulation, 1%, 50% and 100% spent grain extract (SGE) and 0.05% minerals (M) consisting of potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) were supplemented to determine the effect on mycelial growth (Table 3.2). SGE as opposed to insoluble spent grain was added to produce dense mycelium. The pH values of these media were fixed to pH 6. The combinations of concentrations tested is described in 3.2.1. Three replicates were prepared for each formulation. The medium containing the best concentration of SGE and M will be further optimised by adding Tween 80.

Further experiments were carried out using best combination of culture media (2% BS, 1% BKY, 1% SGE, 0.05% M) with additional Tween 80. In this experiment, 0.5% (v/v) Tween 80 was added to the culture media based on Li *et al.* (2011).

Table 3.2: The percentage concentration of supplementation of spent grain extract, and minerals in liquid fermentation medium.

Brown sugar (%) (w/v)	Baker's yeast (%) (w/v)	Spent grain extract (%) (w/v)	Minerals (%) (w/v)
2	1	1	-
2	1	50	-
2	1	100	-
2	1	1	0.05
2	1	50	0.05
2	1	100	0.05

3.2.3 Effect of pH of optimised growth medium

The optimised growth medium (2% BS, 1% YE, 1% SGE, 0.05% M, 0.5% Tween 80) was prepared with pH of the medium adjusted to 4.50 (± 0.05), 5.00 (± 0.05), 5.50 (± 0.05), 6.00 (± 0.05), 6.50 (± 0.05), 7.00 (± 0.05), using 1M of sodium hydroxide (NaOH) and 1 M of sulfuric acid solution (H₂SO₄). Procedure for preparing this liquid medium and data collection is as described in 3.2.1. Three replicates were prepared for each formulation. All results were expressed as mean \pm standard deviation from triplicates data. Statistic significant differences were determined by One-way ANOVA Duncan test with p values < 0.05 were considered as significant difference using SPSS16

3.3 Production of *P. pulmonarius* mycelium in optimal medium using a 2l-automated stirred bioreactor

Pleurotus pulmonarius mycelium to be used as inoculum was further scaled-up and to obtain a more uniform mycelial biomass in a shorter period of time using an automated bioreactor. Medium for the preparation of mycelial broth (MB) was prepared in a 500 mL Erlenmeyer flask containing 100 mL of 2% brown sugar, 1% baker's yeast, 1% spent grain extract, 0.5% Tween 80, 0.05% MgSO₄, 0.05% KH₂HPO₄ and 0.05% K₂HPO₄ sterilized by autoclaving at 121°C for 20 minutes. Then 20 plugs of *P. pulmonarius*

mycelium were cut from an actively growing plate on malt extract agar 7 days was inoculated into the flask medium. *Pleurotus pulmonarius* broth was cultured and incubated at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ on shaker at 250 rpm for 6 days.

Then 10% (v/v) of MB was inoculated into the 2-L stirred tank bioreactor (STR) with an operational volume of 1.5 L containing of 2% brown sugar, 1% baker's yeast, 1% spent grain extract, 0.5% Tween 80, 0.05% MgSO_4 , 0.05% KH_2HPO_4 and 0.05% K_2HPO_4 (Figure 3.2). The cultivation conditions in bioreactor were as follows: temperature (28°C), agitation speed (250 rpm), initial pH (5.5), and oxygen partial pressure (30-40%).

Reducing sugar concentrations in the MB was determined by the 3, 5-dinitrosalicylic acid (DNS) methods (Miller, 1959) and expressed as glucose equivalents (described in Appendix A, 2.0). Twenty mL samples of the MB being collected every 12 hrs for analyses up to 84 hrs. The biomass was determined after filtration of 20 mL of the broth through filter paper (Whatman No.1). The solids were washed with distilled water. The filters with mycelium were dried at 60°C oven until constant weight was achieved. Three batches of fermentation were done and data were represented as average. All results were expressed as mean \pm standard deviation from triplicates data. Statistic significant differences were determined by One-way ANOVA Duncan test with p values <0.05 were considered as significant difference using SPSS16



Figure 3.2: Two litre stirred tank bioreactor containing of 10% (v/v) of liquid inoculum in optimised medium consisting of 2% brown sugar, 1% baker's yeast, 1% spent grain extract, 0.5% Tween 80, 0.05% MgSO₄, 0.05% KH₂HPO₄ and 0.05% K₂HPO₄.

3.4 Non-supplemented encapsulation of *P. pulmonarius* mycelial broth (MB)

The objective of this study was to determine the optimum harvesting time of bioreactor for good viability of *P. pulmonarius* mycelium. MB of *P. pulmonarius* was prepared as inoculum in 500-mL Erlenmeyer flasks containing 100 mL of liquid optimised medium (w/v) medium. The sterile medium was then inoculated with 20 mycelial plugs from a six-days-old colony grown in MEA and incubated at $25 \pm 2^\circ\text{C}$ on a shaker at 150 rpm for 6 days.

MB of *P. pulmonarius* (100 mLs) was then inoculated at 10% (v/v) into the bioreactor containing sterile optimised medium. The cultivation conditions of bioreactor were set as follows: temperature (28°C), agitation speed (250 rpm), pH (6.0), and oxygen partial pressure (30-40%). *Pleurotus pulmonarius* mycelium was cultivated for 48 hrs and 60 hrs to evaluate the optimum harvesting time.

MB of *P. pulmonarius* obtained from the bioreactor was then encapsulated using encapsulation solution consisting of 500 mL of 2.5% (w/v) sodium-alginate salt (Sigma-Aldrich), 2% (w/v) malt extract and 4% (w/v) glucose autoclaved at 121°C for 20 minutes. All solutions, materials and apparatus were initially sterilized for 20 min at 121°C . After

the encapsulated solution was cooled, it was mixed with 1 litres of a MB at the ratio of 2:1 (MB: encapsulated solution). The mixture was lightly agitated until homogenous and then slowly dripped into a beaker containing 200 mL of 0.25 M calcium chloride (CaCl_2) solution using a peristaltic pump with continuous stirring as shown in Figure 3.3. The received gel beads were allowed to hardened in this solution for 15 minutes and then was washed twice with 200 mL sterile distilled water (Figure 3.3). The beads were stored at 4°C before use.



Figure 3.3: The setup for MB encapsulation.

Pleurotus pulmonarius encapsulated MB (PPEMB) was stored in vials for five days and viability was observed on day three to determine percentage germination of PPEMB. Five replicates sawdust substrates in glass petri dish (Appendix A, 3.0) was prepared. Viability of PPEMB was tested by inoculating ten pieces of PPEMB on each sterile sawdust substrate in glass petri dish. All petri dishes were double-sealed with plastic paraffin film. The data were represented as percentage of germination of PPEMB in average.

To determine the growth rate of PPEMB, one PPEMB was inoculated at the centre of each sawdust substrate in glass petri dish (five replicates). Mycelial growth in each petri dish growth rate were determined by measuring the average diameter every day for

one week. The average reading was plotted against time (day) to obtain the growth rate in mm/day.

3.5 Optimization of *P. pulmonarius* encapsulated mycelial broth (PPEMB)

3.5.1 Optimization of encapsulation solution by supplementation with mashed potato and soluble starch at various concentrations.

Mashed Potato (Appendix A, 4.0) and soluble starch grade AR (Friendemann Schmidt) were selected as additional nutrient supplementation in encapsulation solution to extend viability to allow for germination on sawdust substrate. The encapsulation solution was supplemented with different concentrations of mashed potato or soluble starch (3%, 5% and 8% w/v), to determine percentage germination upon storage of PPEMB. Procedure for preparing this encapsulation solution is as described in section 3.4. Nutrient supplemented PPEMB (NS-PPEMB) was stored in different vials for 3, 7, 14, 21, 28 days and viability was determined upon storage. Five replicates sawdust substrates in glass petri dish (Appendix A, 3.0) were prepared. Viability of NS-PPEMB was tested by inoculating ten beads of NS-PPEMB on each sterile sawdust substrate in glass petri dish. All petri dishes were double-sealed with plastic paraffin film. The data was represented as average percentage of germination of NS-PPEMB.

3.5.2 Effect of supplementation of 5% mashed potato and 5% soluble starch in encapsulation solution on sporophore yield

Based on the result obtained above, supplementation with 5% mashed potato and 5% soluble starch in the encapsulation solution that had showed highest percentage germination of *P. pulmonarius* mycelium. Thus, these NS-PPEMB (5% mashed potato and 5% soluble starch) were evaluated as spawn for cultivation by mycelial growth and sporophore yield expressed as biological efficiency on sawdust as fruiting substrate in plastic bags. Three replicate bags were prepared (Appendix A, 5.0). The procedure for preparing this encapsulation solution is described in 3.4. The NS-PPEMB were stored in

sterile distilled water for two weeks in vials. After the sterile fruiting substrate bag were cooled to room temperature, 5 pieces of NS-PPEMB were inoculated into fruiting substrate bag by using sterile spatula and done under aseptic condition.

Then, the inoculated bags were incubated at an ambient temperature of 25°C ($\pm 2^\circ\text{C}$) until full spawn run completed. Mycelia growth during spawn run was determined by measuring mycelium extension at 4 sides of the bag at 2-day intervals for 30 days. The average reading was plotted against time (day) to obtain growth rate in mm/day.

Once spawn running completed, all bags were transferred to the experimental room temperature (30-32°C) mushroom house equipped with misting system. Sufficient air flow was provided by the surrounding netting of the mushroom house. Fully colonised bags were scratched on the top of bag and subjected to an environmental relative humidity of above 85%. This was done by spraying water in the form of fine mist using a sprinkler. This was done to promote initiate sporophore formation and to provide space for sporophore to emerge. Harvesting was done as sporophore matured and was done over a period of three weeks. The yield of sporophore were recorded for two flushes was expressed as biological efficiency (BE) and was calculated as below:

$$\text{Percentage biological efficiency (BE)} = \frac{\text{Grams of fresh sporophore produced}}{\text{Grams of dry substrate used}} \times 100$$

All results were expressed as mean \pm standard deviation from triplicates data. Statistic significant differences were determined by One-way ANOVA Duncan test with p values < 0.05 were considered as significant difference using SPSS16.

3.5.3 Evaluation of viability of soluble starch (SS)-PPEMB after storage at various conditions.

The storage condition and storage life of SS-PPEMB were evaluated. The formulation of encapsulation solution with 5% soluble starch was selected due to practical

and consistency of material for preparation of encapsulation solution and further evaluated. The SS-PPEMB prepared as previous was stored in different vials for 5 days and 10 days under the following conditions:

- i. C1: Stored in vial without water at room temperature (25°C)
- ii. C2: Stored in vial without water at 4°C
- iii. C3: Stored in sterile distilled water at room temperature (25 °C)
- iv. C4: Stored in sterile distilled water at 4°C.

This study was done in four replicates glass petri dish of sterile sawdust substrate and on four replicates sterile fruiting substrate bags for each formulation. The preparation of sterile sawdust substrate in glass petri dish was the same as in the Appendix A, 3.0. After sterile substrate in glass petri dish were cooled to room temperature, 10 pieces of SS-PPEMB were put on it. The percentage of 10 pieces of SS-PPEMB that germinated on each petri dish and viability of storage life SS-PPEMB were observed. Four replicates of sawdust substrate in petri dish were done and data were represented as percentage of germination of SS-PPEMB mycelium in average.

The preparation of fruiting sawdust substrate bag was the same as in the Appendix A, 5.0. After the sterile sawdust substrate bag allowed were cooled to room temperature, 5 pieces of SS-PPEMB were inoculated into sterile sawdust bag by using sterile spatula done under aseptic condition. The length of the mycelium run was measured at 2-day intervals for 30 days in unit of millimeter (mm). The yield of sporophore was recorded after three flushes, and the biological efficiency (BE) were calculated.

As for the control used in this study, the commercialized wheat grain spawn was used. The mycelial growth of SS-PPEMB and sporophore yield were compared with grain spawn. Grain spawn was prepared using 200g wheat grains pre-soaked for two hours with hot water and then autoclaved for 1 h at 121°C. Then five plugs of mycelial *P.*

pulmonarius were inoculated and incubated in the dark for 2 weeks or until complete colonization of the surface of the grains by the mycelium. Ten grams (one spoon spatula) of mycelial colonised grain spawn was inoculated into sterile fruiting substrate in bags as above. All results were expressed as mean growth rate \pm standard deviation from triplicates data. Statistic significant differences were determined by One-way ANOVA Duncan test with p values <0.05 were considered as significant difference using SPSS16.

University of Malaya

CHAPTER 4.0

RESULTS AND DISCUSSION

4.1 Optimization of growth medium formulation for the production of *P. pulmonarius* mycelium

4.1.1 Effect of brown sugar concentration and nitrogen sources concentration

Nutrient sources affect the production of high-density mycelium in submerged fermentation. Many works has shown that the utilisation of carbon sources, nitrogen sources, and minerals elements constitutes the biochemical aspects that influence the growth rates of mycelium (Mukhopadhyay *et al.*, 2003; Yang *et al.*, 2003). By using liquid medium, the nutrient transfer was easier, the potential for contamination reduced, able to make simple growth measurement, and large volume of mushroom culture as inoculant can be used.

Table 4.1 shows the mycelial dry weight of *P. pulmonarius* grown in different concentration of brown sugar and nitrogen sources. The highest dry mycelial weight of *P. pulmonarius* obtained was 11.75 g/L when 2% (w/v) of brown sugar (BS), 1% (w/v) of spent grain (SG) and 1% (w/v) yeast extract (YE) were used. Across all experiments, the effect of brown sugar concentration is significant ($p < 0.05$) between 0.5% to 2.0%. It was observed that when the concentration of brown sugar in the medium is 2%, there was an increase in the average mycelial dry weight.

According to Hamachi *et al.* (2003), brown sugar is commonly distinguished as the most readily applicable carbon source for most mushrooms cultures because it consists of approximately of 94–98.5% (w/w) sucrose and various types of non-sucrose components ranging from 1.5–6% (w/w). Chang *et al.* (2006) reported that brown sugar and lactose induced the formation of *Ganoderma lucidum* mycelium. Hawker (1950) had mentioned that contribution of brown sugar being low cost and easily available in supply

compared to glucose, sucrose and lactose is more suitable for the preparation of mushroom growth medium on a large scale.

This was supported from earlier work done by Abdullah *et al.* (2013), who obtained results using shake flasks for nine edible mushrooms with BRMY medium containing brown-sugar (2%), rice bran (0.4%), malt (0.4%) and yeast extract (0.4%) favored highest mycelial dry weight of *Schizophyllum commune* ($14.62 \pm 2.39 \text{ gL}^{-1}$) followed by *P. pulmonarius* ($9.96 \pm 1.14 \text{ gL}^{-1}$) and *P. sapidus* ($7.56 \pm 2.03 \text{ gL}^{-1}$).

A lot of work has been done on glucose as carbon source in submerged culture medium of *Pleurotus* spp. Kim *et al.* (2002a) observed that 20 g/L glucose as the carbon source in submerged culture of *P. pulmonarius*, *P. osteratus* (NO.1 and NO.2) showed lower mycelial production with 7.17 g/L, 7.35 g/L and 6.52 g/L respectively, compared to this study. Further, Smiderle *et al.* (2012) has shown that mycelial biomass of *P. pulmonarius* obtained using glucose as carbon source was 9.07 g/L. A study done by Confortin *et al.* (2008) on optimized culture medium with 10 g/L of glucose showed that mycelial biomass *P. sajor-caju* can achieved values of approximately 5.5 g/L. Haixia *et al.* (2009) reported that biomass of *Pleurotus nebrodensis* obtained was 14.15 g/L in optimal 40 g/L glucose medium of submerged culture.

There was no work relating to the production of mycelial biomass of *P. pulmonarius* on sucrose medium as carbon source. On the other hand, Park *et al.* (2001) reported that highest dry weight of *Cordyceps militaris* mycelial was obtained in 40 g/L sucrose medium with 12.75 g/L. Kim *et al.* (2002b) reported that the highest mycelial growth of *Paecilomyces sinclairii* (12.6 g/L) was achieved when 10g/L sucrose medium in shake flask cultures was used.

Table 4.1: Effect of brown sugar concentration, nitrogen sources and concentration on mycelial dry mycelial dry weight of *P. pulmonarius* in shake-flask liquid fermentation medium.

Brown sugar (%)	Brewer's yeast (%)	Baker's yeast (%)	Yeast Extract (%)	Spent Grain (%)	pH reading	C:N ratio	Average Mycelial dry weight (g/L) ¹	
0.5	0.1	–	–	0.1	5.32	117.83	5.79 ± 1.48 ^e	
	0.1	–	–	1	5.00	29.40	0.88 ± 0.76 ^{abc}	
	1	–	–	0.1	5.59	34.56	3.90 ± 0.67 ^{def}	
	1	–	–	1	5.37	21.59	1.14 ± 0.36 ^{abc}	
	–	0.1	–	0.1	5.39	71.17	1.67 ± 0.34 ^{bc}	
	–	0.1	–	1	5.83	25.84	0.56 ± 0.12 ^{ab}	
	–	1	–	0.1	5.84	24.21	5.48 ± 0.49 ^{ef}	
	–	1	–	1	5.82	19.82	1.64 ± 1.38 ^{abc}	
	–	–	0.1	0.1	6.58	47.98	0.63 ± 0.07 ^{ab}	
	–	–	0.1	1	6.15	24.55	0 ± 3.50 ^{ab}	
	–	–	1	0.1	6.91	13.05	0.26 ± 0.24 ^{ab}	
	–	–	1	1	6.68	12.91	0 ± 5.55 ^a	
	2.0	0.1	–	–	0.1	5.27	433.75	8.99 ± 1.35 ^f
		0.1	–	–	1	5.04	80.6	9.34 ± 2.64 ^f
		1	–	–	0.1	5.15	99.97	4.97 ± 2.31 ^{ef}
		1	–	–	1	5.2	54.44	1.25 ± 0.70 ^{abc}
–		0.1	–	0.1	5.19	241.85	4.02 ± 0.85 ^{def}	
–		0.1	–	1	5.67	72.81	3.86 ± 0.76 ^{de}	
–		1	–	0.1	5.49	46.63	9.28 ± 0.30 ^f	
–		1	–	1	4.94	37.04	7.93 ± 1.40 ^f	
–		–	0.1	0.1	6.14	164.9	0 ± 0.94 ^{bcd}	
–		–	0.1	1	5.97	64.68	7.47 ± 5.85 ^{abc}	
–		–	1	0.1	6.85	27.51	0 ± 1.21 ^{cd}	
–		–	1	1	6.62	24.6	11.75 ± 1.68 ^{cd}	

Based on Kim *et al.* (2005) the omission of nitrogen in the medium significantly influences fungal growth and metabolite production. Many authors stated that organic nitrogen sources supplemented produces higher biomass mycelial growth in submerged culture (Jung *et al.*, 1997; Shih *et al.*, 2006; Asatiani *et al.*, 2008). Hence, baker's yeast and brewer's yeast are included as organic nitrogen sources. Feng *et al.* (2010) reported

that yeast powder was highly necessary to enhance the biomass of mycelial growth. To investigate the effects of nitrogen sources on mycelial dry weight, three types of organic nitrogen sources were examined in this study viz. brewer's yeast (brewery waste), baker yeast (bread yeast) and yeast extract (OXOID). It is proven that low cost carbon source ie brown sugar (2%) incorporated with baker yeast (1%) as nitrogen source can produce optimum mycelial growth of *P. pulmonarius* biomass.

Better growth of mycelia was obtained, when the higher baker's yeast concentration (1%, w/v) or the lower the brewer's yeast concentration (0.1%, w/v) were added. The effect seen between baker's yeast and brewer's yeast concentration towards growth could be attributed to the type of yeasts. In this study, baker's yeast was selected as nitrogen source due to because it is readily available in the market in a standardized form and more practical to prepare in growth medium formulation.

Table 4.1 shows, at 2% BS the effect of spent grain at high concentration (1%) in the medium promote the average mycelial dry weight of *P. pulmonarius* growth. Spent grain is rich in cellulose and non cellulosic polysaccharides (Aliyu & Bala, 2011). Therefore spent grain needed extracellular enzymes to break down to simpler, soluble units so that it is easier to absorb nutrition for fungal hyphae growth (Chang *et al.*, 1993). Gregori (2008), stated that the additional of high concentration of spent grain leads to a decreased enzymes activities. In addition, spent grain caused insufficient oxygen supply for maximum growth during fermentation (Nakano *et al.*, 1997). To enhance the solubility of spent grain constituents, further work is done on the effect of a water extract of spent grain in the medium.

4.1.2 Effect of supplementation of spent grain extract, minerals and Tween 80

Optimization was further done on the selected medium consisting of 2% of brown sugar and 1% baker's yeast. From Table 4.1 showed that when the high concentration of

spent grain (1%) supplemented to medium, *P. pulmonarius* mycelium growth was inhibited because being insoluble spent grain will likely reduce the oxygen mass transfer which in turns reduce the growth. In addition, according Roberts (1976), use of spent grain extract in the fermenter acts as an effective antifoaming agent. Therefore, in this study spent grain extract was used to replace insoluble spent grain. Hence, according to Stein *et al.* (1973) spent grain extract may supply 30% to 60% of the biochemical oxygen demand. This support that spent grain extract contribute in aerobic biological organism by breaking down organic material present in the medium and enhance the mycelial growth in submerged medium.

The effect of spent grain extract or minerals consisting of a mixture of potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) were also studied. The concentration of spent grain extract was 1% and each mineral used was 0.05% while the pH values of these medium was fixed to pH 6.

Table 4.2 reflected the average dry weight of *P. pulmonarius* mycelium on different medium formulation. From the results, brown sugar 2% (w/v), Baker's yeast 1% (w/v), spent grain extract 1% (w/v) and minerals 0.05% (w/v) showed the best mycelia growth which is 15.07 g/L. Under the effect of spent grain extract, the best mycelial growth was 14.48 g/L at a spent grain extract concentration of 100% however, it was non-significantly different ($p > 0.05$). From previous study by adding the spent grain it indicates positive effect on mycelial growth. However, study done by Hang *et al.* (1975) has indicated that mycelial yield of *Aspergillus niger* achieved was 13 g/L by using brewery spent grain extract instead of solid spent grain that is insoluble. In addition, Shannon and Stevenson (1975) reported that dry weight of mycelia of *P. ostreatus*, *M. esculenta*, *A. bisporus*, *Calvatia gigantean* obtained was in range the between 3.28 g/L and 6.28 g/L in spent grain extract medium.

The results shows supplementation of minerals (0.05%) to growth medium consisting of brown sugar 2% (w/v), Baker's yeast 1% (w/v) and spent grain extract at 50 and 100% (w/v) showed a non-significant ($p > 0.05$) decreased in mycelial growth. However, the supplementation minerals 0.05% to brown sugar 2% (w/v), Baker's yeast 1% (w/v), spent grain extract of 1% (w/v) showed higher mycelia growth of *P. pulmonarius* of 15.07 g/L. The yield is higher compared to work done by Hwang *et al.* (2003) who reported that the effect of mineral source (5 Mm KH_2PO_4 , K_2HPO_4 , MgSO_4) by submerged culture of *Phellinus linteus* with the mycelial growth of only 2.88 g/L, 2.92g/L and 2.85 g/L respectively. Further, higher mycelia growth was also obtained in our study compared to previous study by Xu *et al.* (2003). In their study on the effect of 5 mM K_2HPO_4 and 5 mM MgSO_4 on the mycelial growth of *Paecilomyces tenuipes* showed of 8.74 g/L and 8.23 g/L respectively (Xu *et al.*, 2003). The mycelial growth of *P. pulmonarius* in this study was superior compared with *Antrodia cinnamomea* which produced mycelial growth is 2.73 ± 0.50 g/L in supplementation of 0.1%, (w/v) MgSO_4 in growth medium as reported by Lin *et al.* (2006). Many studies has recognized $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as a promising for mycelial growth in liquid cultures of several basidiomycetes (Chardonnet *et al.*, 1999; Okba *et al.*, 1998; Kim *et al.*, 2005).

In order to obtain small pellets and to produce unicellular propagates of mycelium, addition of 0.5% Tween 80 to the selective medium formulation consisting of brown sugar 2%, baker's yeast 1%, spent grain extract 1%, minerals 0.05% was carried out. It showed dry biomass weight of *P. pulmonarius*, 9.64 ± 0.70 g/L was achieved (Table 4.2). Figure 4.1 shows the effect of 0.5% Tween 80 on *P. pulmonarius* mycelium in 50 mLs liquid fermentation medium in shake flasks. A study done by Giese *et al.* (2004) has proven that Tween 80 can change the structure and morphology (Figure 4.1) of fungi and bacteria cell wall.

Another remarkable effect of Tween 80 in submerged medium is it has the potency to enhance the growth of the mycelia (Zhang & Cheung, 2011; Zhang *et al.*, 2012). A study done by Zhang and Cheung (2011) has shown the effect of Tween 80 on the 5th day of fermentation by increasing the production of mycelial biomass by 51.3%. In studies performed by Hsieh *et al.* (2008) and Lim and Yun (2006), Tween 80 supplemented in medium submerged fermentation gave a positive result in enhancing mushroom mycelial biomass production. Meanwhile Tween 80 has the capability to hinder breakdown of mycelial cells due to the shearing forces during the shake-flask experiments by maintaining the intact structure of the mycelial pellets during fermentation (Zhang & Cheung, 2011).

According to Domingues *et al.* (2000), the effect of Tween 80 on mycelial morphology could be associated in part to its surface-active properties, lowering the mycelium-liquid interfacial tension and consequently the potential or tendency of mycelia to form aggregates. By decreasing the surface tension of the medium by surfactant lowers the thermodynamic potential for the aggregation but favours the dispersion of mycelia.

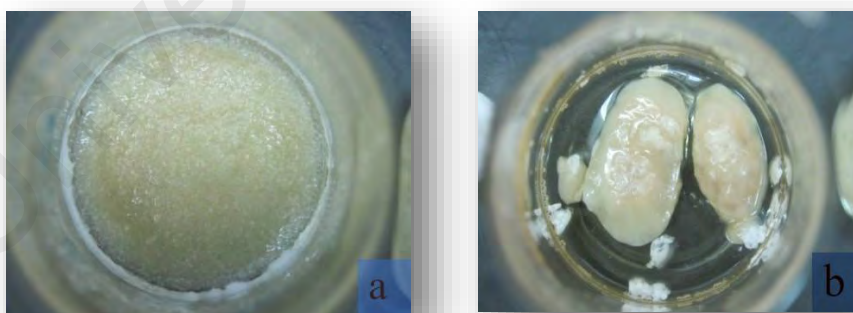


Figure 4.1: Effect of mycelial of *P. pulmonarius* supplemented a) with 0.5% Tween 80 and b) without Tween 80 in 50mL shake-flask liquid fermentation medium.

Table 4.2: Effect of spent grain extract, minerals and Tween 80 on the mycelial dry weight by *P. pulmonarius* in shake-flask liquid fermentation medium.

Brown sugar (%) (w/w)	Baker Yeast (%) (w/w)	Spent Grain Extract (%) (w/v)	Minerals (%) (w/v)	Tween 80 (%) (v/v)	Average mycelia dry weight (g/L)	pH before adjusted	pH before autoclave	pH after autoclave
2	1	1	–	–	11.48 ± 1.76 ^{ab}	5.55	6.07	5.35
2	1	50	–	–	14.04 ± 0.31 ^{ab}	4.81	6.05	5.60
2	1	100	–	–	14.48 ± 0.12 ^{ab}	4.54	6.08	5.51
2	1	1	0.05	–	15.07 ± 4.79 ^b	6.37	6.10	5.50
2	1	50	0.05	–	13.22 ± 0.48 ^{ab}	4.87	6.04	5.89
2	1	100	0.05	–	12.96 ± 3.62 ^{ab}	4.75	6.04	5.78
2	1	1	0.05	0.5	9.64 ± 0.70 ^a	5.91	6.10	5.63
2	1	50	0.05	0.5	11.97 ± 0.95 ^{ab}	5.07	6.03	5.71
2	1	100	0.05	0.5	11.64 ± 0.60 ^{ab}	4.63	6.05	5.73

Values are the mean ± STD of three replicates analyses, and alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p > 0.05$).

4.1.3 Effect of pH of growth medium

In this study, *P. pulmonarius* was cultivated in a basal medium at different values of initial pH (4.5-7.0). Table 4.3 presents the effect of initial pH of culture medium after autoclaving on mycelial dry weight (g/L). The average mycelial dry weight of *P. pulmonarius* was non-significantly ($p > 0.05$) affected by medium initial pH. Therefore, we can conclude that good mycelia dry weight of *P. pulmonarius* can be obtained with the initial pH in the range 4.5 to 5.5.

Table 4.3: Effect of initial pH on the average mycelial dry weight by *P. pulmonarius* in shake-flask medium culture.

Initial pH of medium before autoclaving	pH of medium after autoclaving	Average mycelial dry weight (g/L)
4.5	5.0	5.04 ± 0.02 ^{ab}
5.0	5.5	5.65 ± 0.03 ^b
5.5	6.0	4.38 ± 0.13 ^{ab}
6.0	6.5	4.13 ± 0.10 ^{ab}
6.5	7.4	2.97 ± 0.04 ^a
7.0	7.65	4.72 ± 0.05 ^{ab}

Values are the mean ± STD of three replicates analyses, and alphabet letters indicate the same letters in the same column are not significantly different according to Duncan ($p > 0.05$).

Several types of mushroom have tendency to perform more acidic pH that is optimal for mycelial biomass during their submerged medium cultures (Kim *et al.*, 2003; Lee *et al.*, 1989; Shu & Lung, 2004). According to Litchfield (1967) various species of mushroom mycelia will grow over a wide range of pH values but for certain species (*Agaricus blazei*, *Agaricus campestris*, *M. esculenta*, *Morchella hortensis* and *T. nudum*) were more favourable between pH range 5 to 7. Humfeld and Sugihara (1949) stated that an initial pH of 4.5 tend to inhibited the growth of bacterial contaminants. A study done by Rosado *et al.* (2003) for biomass production of *P. ostreatus* “florida” and *P. ostreatoroseus* initial medium pH was setup at pH 6. Another study by Wu *et al.* (2003) observed the medium pH of fermentation was set at pH 5.5 for mycelial growth of

Pleurotus tuber-regium (Fr.) Singer. Song *et al.* (1987) reported that *L. edodes* required a relatively acidic pH (4.3-4.8) for optimum mycelial growth. For maximum mycelial biomass growth of *G. lucidum*, 17.3 g/L was obtained at an initial pH of 6.5. Park *et al.* (2001) and Shih *et al.* (2007) reported that the optimum to produce mycelial biomass of *C. militaris* was at pH 6. A study conducted by Kim *et al.* (2002a) for mycelial growth of *G. lucidum* and *P. linteus* initial medium pH was performed at pH 5. In another work, even though generally mycelial biomass grew well at pH 5-6, the pH optimum for mycelial growth is apparently dependent on the strain (Bae *et al.*, 2000; Kim *et al.*, 2002b; Xu *et al.*, 2003). From this study, initial medium pH 6 were selected.

According to Pokhrel and Ohga (2007), a fundamental factor that regulates mycelial growth is the pH of the culture medium. Other studies claimed that different morphology of fungal mycelia under various initial pH values has vital influence in mycelial accumulation and metabolite formation (Shu & Lung, 2004; Wang & McNeil, 1995). In addition, initial pH of medium may influence cell membrane function, cell morphology and structure, the uptake of various nutrients and biosynthesis of product (Gerlach *et al.*, 1998; Fang & Zhong, 2002b; Shu & Lung, 2004).

4.2 Scale-up production of *P. pulmonarius* mycelium using a 2-L automated bioreactor

After we obtained the optimal medium composition, we tested its feasibility in a 2-litre automated bioreactor for large scale cultivation. Three batches of cultivation of *P. pulmonarius* were carried out for 72 hrs and sampling was performed every 12 hrs. The dry biomass production and concentration of reducing sugar of *P. pulmonarius* were measured as the cultivation progressed. The profile of biomass production and reducing sugar concentration are shown in Figures 4.2 and 4.4 respectively. The mycelial growth was increased onwards until 60 hrs of fermentation. The dry weight of biomass reached

a maximum level of 11.89 ± 3.80 g/L after 60 hrs of fermentation before mycelial biomass weight decreased.

In this study, the dry weight of biomass decreased after 60 hrs of fermentation possibly due to reduction of available nutrient such as sugar and nitrogen sources in the medium as indicated by the reducing sugar level which starting to decrease on 48 hrs onwards (Ma *et al.*, 2016). In addition, another contribution of decrease in yield of mycelial biomass have been caused by fungal cell lysis (Pokhrel & Ohga, 2007).

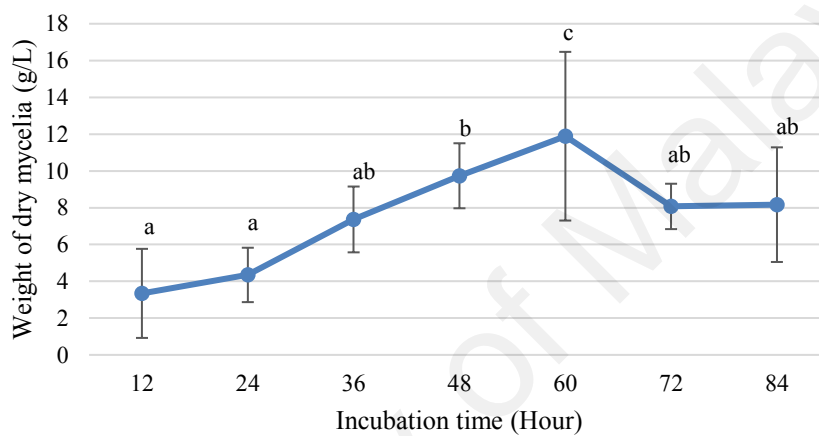


Figure 4.2: Mycelial biomass production (dry weight, g/L) of *P. pulmonarius* in 2-L automated bioreactor. Each value is expressed as mean \pm STE of three batch. Means that do not share a letter in the same condition of mycelial biomass production are significant different ($p < 0.05$).

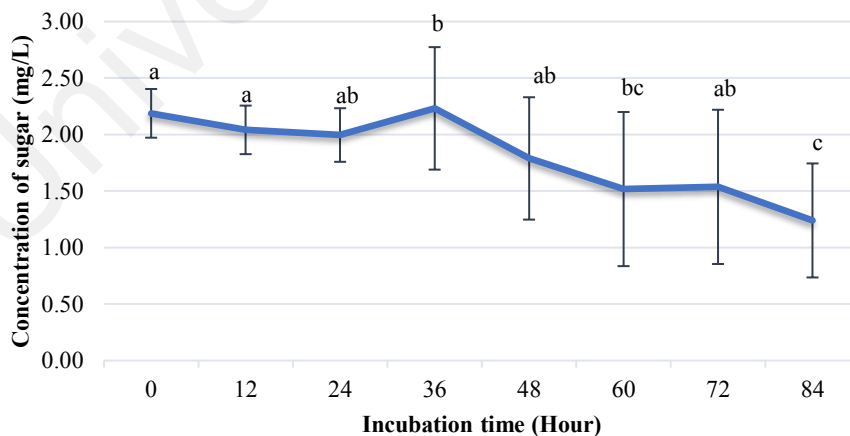


Figure 4.3: Concentration of reducing sugar (mg/L) in the medium during growth of *P. pulmonarius* in an automated bioreactor. Each value is expressed as mean \pm STE of three batch. Means that do not share a letter in the same condition of mycelial biomass production are significant different ($p < 0.05$).

As reported by Lee *et al.* (2004), mycelial growth was beneficial in an automated bioreactor than shaken flasks by virtue of conducive mixing and controlled conditions. This study showed higher dry weight biomass of *P. pulmonarius* was achieved, 11.89 ± 3.80 g/L which is higher than shake flask (9.64 ± 0.70 g/L) in our basal medium at 60 hrs. Kim *et al.* (2002a) obtained a much lower yield where dry biomass of *P. sajor-caju* grown in an automated bioreactor (5L) was estimated at 1.08 g/L in mushroom complete medium, 2.64 g/L in medium consisting of yeast malt extract and 2.84 g/L of potato malt peptone media, respectively at 5 days. Further, the biomass of *P. sajor-caju* (8.18 g/L and 5.94 g/L) culture medium using 10 g/L of glucose or sucrose as carbon source done by Corfortin *et al.* (2008) in a 5-L stirred tank bioreactor was also lower than this study. A different *Pleurotus* species studied by Papaspyridi *et al.* (2010), showed that biomass production of *P. ostreatus* at pH 6 and temperature 28°C produced 39.2 g/L of in 20-L tank bioreactor after 68 hrs.

4.3 Effect of fermentation duration/ harvesting time mycelium in a bioreactor on germination and storage time of non-supplemented PPEMB.

Even though 60 h harvesting time show the highest dry weight of mycelial on bioreactor, we evaluated the effect of 60 h and 48 h harvesting time on the based on germination and growth rate of PPEMB on sawdust substrate. In our study, 48 h showed 100% viability even after 15 days of storage exhibiting mycelial growth rate of mycelial 6.38 mm/day on sawdust substrate (Table 4.4 & Figure 4.4).

Table 4.4: Percentage of germination of non- supplemented PPEMB on sawdust substrate in glass petri dish.

Storage time of PPEMB		5 Day	10 Day	15 Day
Fermentation duration of harvesting	48 hours	100 ± 0	100 ± 0	100 ± 0
	60 hours	100 ± 0	72 ± 38.34	32.5 ± 12.58

Values are the mean \pm STD of five replicates analyses.

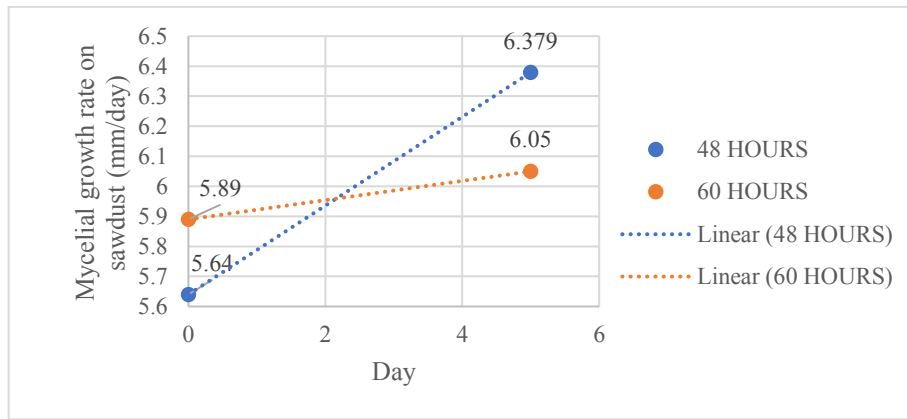


Figure 4.4: Growth rate of freshly prepared PPEMB on sawdust substrate in glass Petri dish at different harvesting times of 48 h and 60 h.

Previous studies stated that rapid mycelial growth is characteristic of good liquid spawn (Kawai *et al.*, 1996a; Friel & McLoughlin, 2000; Abdullah *et al.*, 2013). In this study, the mycelial growth rate of *P. pulmonarias* was fastest at 48 h. Therefore, we chose 48 h as the optimal harvesting time to produce PPEMB before the fungal cell lysis occurred (Wu *et al.*, 2003) and mycelial cell being too mature or inactive.

From this study, we found that PPEMB size decreased at longer storage time as the beads were shrunken (Figure 4.5). Even though the PPEMB germinated however, the mycelium did not fully cover the whole surface of sawdust as well as the base of the substrate. The shrunken of beads may due to lack of supplementation ingredients that help in the maintenance and protection of the microbial cells in a formulation thru storage (Xavier *et al.* 2004). Therefore, the encapsulation solution needed to be supplemented with nutrient for better germination and growth on sawdust substrate.



Figure 4.5: Germination of PPEMB on sawdust substrate after storage of 15 days.

4.4 Optimization of encapsulation solution to prepare PPEMB

4.4.1 Optimization of encapsulation solution by supplementation with mashed potato and soluble starch at various concentrations [Nutrient supplemented (NS)-PPEMB]

Suitable concentration of nutrients supplementation incorporated in the encapsulation solution were evaluated to prevent the PPEMB from shrinkage before mycelial germination. In addition, this study was to observe the ability of the NS-PPEMB to emerge and colonize on sawdust substrate represented by percentage of NS-PPEMB germination. The percentage of germination of NS-PPEMB when 3%, 5% and 8% of mashed potato or soluble starch was supplemented in the encapsulation solution were determined.

From our observation, the viability of fungal mycelium finds better survival condition when entrapped inside the alginate pellets and this was supported from previous works from Mauperin *et al.* (1987) and Tacon *et al.* (1985). They claimed that the fermentation of fungal mycelium and then immobilised in gel beads, granted a high viable inoculum by reason of the protection of the mycelia by the gel after associated into soil.

The encapsulation solution of PPEMB was supplemented with different concentrations of mashed potato or soluble starch (3%, 5% and 8 % w/v) and Table 4.5 shows that incorporation of mashed potato or soluble starch increase in the capacity of the mycelia to emerge from the beads. The results obtained shows that 93-100% germination of NS-PPEMB was achieved when stored for less than 7 days and decreased after 14 days. Therefore, maximum storage time for NS-PPEMB was 14 days when supplemented with optimal concentration either 5% mashed potato or 5% soluble starch. Additionally, NS-PPEMB was able to colonised on sterile sawdust as fruiting substrate (Figure 4.6).

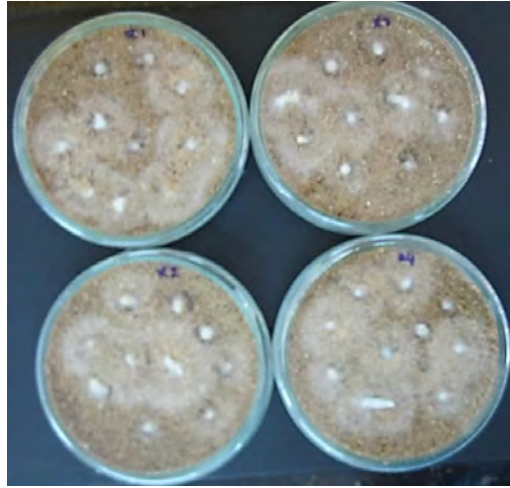


Figure 4.6: Germination and colonisation of NS-PPEMB on sawdust substrate in glass Petri dish.

A study done by Friel and McLoughlin (1999) using (1) an extract compost malt medium, (2) a suspension (soya bean meal or malt extract) or (3) particles (ground compost) to incorporated into beads showed that co-entrapped mycelial beads with supplementation had a higher colonisation. Ortiz *et al.* (2017) had verified that nutrient source added in the process of encapsulation had effected the mycelial emerging capacity. In addition, they also indicate that the encapsulated mycelium had no negative effect on the mycelium growth capacity. Abdullah *et al.* (1995) had claimed that immobilized fungi has potential as a source of viable inoculum and they also concluded that the encapsulated mycelium not only was able to maintain the fungal viability but the immobilized hyphae was also able to function well as ready-to-grow inoculum for spawn-production.

Table 4.5: Percentage of germination of NS-PPEMB on sawdust substrate in glass Petri dish plate.

Storage time	Days of germination	Percentage germination (%)					
		3% Potato	5% Potato	8% Potato	3% Soluble Starch	5% Soluble Starch	8% Soluble Starch
3 Day	1	93 ± 5	95 ± 10	95 ± 10	95 ± 5.7	100 ± 0	100 ± 0
	< 7	100 ± 0	100 ± 0	100 ± 0	95 ± 5.7	100 ± 0	100 ± 0
	>7	100 ± 0	100 ± 0	100 ± 0	95 ± 5.7	100 ± 0	100 ± 0
7 Day	1	45 ± 31.1	57.5 ± 12.6	42.5 ± 17.1	contamination	57.5 ± 15	85 ± 17.3
	< 7	100 ± 0	100 ± 0	97.5 ± 5	contamination	100 ± 0	100 ± 0
	>7	100 ± 0	100 ± 0	97.5 ± 5	contamination	100 ± 0	100 ± 0
14 Day	1	2.5 ± 5	15 ± 5.8	0 ± 0	5 ± 10	12.5 ± 9.6	0 ± 0
	< 7	2.5 ± 5	70 ± 14.1	50 ± 16.3	20 ± 20	60 ± 14.1	30 ± 16.3
	>7	5 ± 5	70 ± 12.2	52.5 ± 8.3	20 ± 20	62.5 ± 17.1	40 ± 21.6
21 Day	1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	5 ± 10	contamination
	< 7	0 ± 0	2.5 ± 5	15 ± 17.3	5 ± 5.8	2.5 ± 5	contamination
	>7	0 ± 0	17.5 ± 15	67.5 ± 9.6	10 ± 11.5	12.5 ± 18.9	contamination
28 Day	1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	< 7	0 ± 0	5 ± 5.8	22.5 ± 5	0 ± 0	0 ± 0	12.5 ± 5
	>7	0 ± 0	20 ± 21.6	22.5 ± 5	7.5 ± 5	12.5 ± 9.6	15 ± 5.8

Percentage of germination was measured based on four replicates in values are the mean ± STD

4.4.2 Effect of supplementation of 5% mashed potato and 5% soluble starch in encapsulation solution on sporophore yield

Based on the results of previous studies, 5% mashed potato and 5% soluble starch were the most optimum supplement concentration to support high percentage of germination. Therefore, this study was conducted to compare 5% mashed potato and 5% soluble starch as supplementation in encapsulation solution on mycelial germination and the potential to fruit on sawdust fruiting substrate.

Both nutrient supplementation (NS)-PPEMB have the capability to germinate on sawdust substrate and produced *P. pulmonarius* sporophores. Table 4.6 shows that 5% mashed potato and 5% soluble starch had no significant effect on mycelia growth rate ($p > 0.05$) and BE. Based on the results, supplementation of 5% mashed potato and 5% soluble starch showed comparable in BE of *P. pulmonarius* as non-significant differences was detected when 5% mashed potato or 5% soluble starch was supplemented in the encapsulation solution.

Table 4.6: Growth rate (mm/day), total sporophore yield (g) and biological efficiency (%) of NS-PPEMB.

	5 % Mashed potato	5% Soluble starch
Mycelial growth rate (mm/day) ¹	3.87 ± 0.42 ^a	3.50 ± 0.18 ^a
Total sporophore yield (g) ²	124.46 ± 7.41 ^a	128.79 ± 8.48 ^a
Biological Efficiency (%) ³	103.71 ± 6.17 ^a	107.31 ± 7.08 ^a

^{1,2,3}Values are the mean ± STD of three replicates analyses, and alphabet letters indicate the same letters in the same horizontal are not significantly different according to Duncan ($p > 0.05$).

Rosado *et al.* (2002) reported that the performance of encapsulated mycelia of button mushroom prepared by entrapping liquid spawn with vermiculite, hygramer, and nourishment in sodium alginate was comparable to that of grain spawn in the shape and yield of fruiting body. On the other hand, Friel and McLoughlin (1999) reported

that if EM spawn without supplements could not endure on pasteurized compost and the biomass levels were significantly lower than that of the conventional grain spawn. Friel and McLoughlin (1999) stated, the advantage by using supplemented nutrient EM:

- i) enhanced by 3-folds the biomass levels after cultivation in malt extract broth for 4 day
- ii) had more inocula points,
- iii) a shorter adaption (lag) period on substrate
- iv) a higher growth rate in pasteurized compost than the conventional grain spawn.

Salmones *et al.* (1999) stated that if the grain of spawn is small, the mycelial growth will have a higher number of points of inoculum by weight of spawn and as a result contribute to a higher number of colony and a faster colonization of the substrate. Therefore, we chose 5% soluble starch due to practical and consistency of material for preparation of encapsulation solution.

4.4.3 Evaluation of viability of soluble starch (SS)-PPEMB after storage at various conditions

This study was conducted to determine the suitable/appropriate storage condition and storage life of SS-PPEMB evaluated by the growth of mycelium and sporophore yield. Encapsulation solution with 5% soluble starch supplemented were evaluated. Fructification trial was done using sawdust substrate in polyethylene bags, so as to evaluate the yield of mushroom produced.

From the results shown in Table 4.7 and Figure 4.7, there was a non-significant difference ($p > 0.05$) in mycelial growth rate upon storage at all condition and storage life. In addition, the results (Table 4.7 & Figure 4.8) for sporophore yield and biological

efficiency also showed non-significant difference ($p > 0.05$). Storage life of 10 days showed higher percentage biological efficiency compared to 5 days storage of SS-PPEMB. Storage life of 10 days stored at C4 condition shows the highest of BE, $180.99 \pm 13.16\%$ in this study.

The production of *P. pulmonarius* sporophore using SS-PPEMB after 10 days was not significantly different ($p > 0.05$) from those of wheat spawn used as control. Our results was within in the range of storage suggested by Wang *et al.* (2011) who concluded that encapsulated spawn of *P. ostreatus* can be stored for at least 2 months. Another study by Ortiz *et al.* (2017), also suggested the use of EM before one month of storage at 4°C and the encapsulated spawn can be kept frozen for at least six months.

Based on this study, our SS-PPEMB showed a better BE, 180.99% compared to Wang *et al.* (2011). They use *P. ostreatus* EM with supplemented with cottonseed hull, corn core and wheat bran (4.4:4.5:1) which storage for 15 days at 20-25°C show 88.1% of *P. ostreatus* BE.

Table 4.7: Growth rate (mm/day), total sporophore yield (g) and biological efficiency (%) of SS-PPEMB in tested storage condition and storage life.

	Mycelial Growth rate (mm/day)¹	Dry weight of bags (g)	Average 1st and 2nd Harvest / Average weight of sporophores (g/bag)²	Average Biological Efficiency (%)³
Wheat Spawn	4.3 ± 1.0 ^a	80	103.14 ± 24.36	128.93 ± 30.46 ^a
5Day C1	4.5 ± 0.4 ^a	80	89.16 ± 15.02	111.45 ± 18.77 ^a
5Day C2	4.5 ± 0.8 ^a	80	107.34 ± 10.24	134.17 ± 12.80 ^a
5Day C3	4.3 ± 0.8 ^a	80	115.44 ± 49.70	144.31 ± 62.13 ^{ab}
5Day C4	4.4 ± 0.4 ^a	80	121.01 ± 10.39	151.26 ± 12.99 ^{ab}
10Day C1	4.08 ± 0.5 ^a	80	121.98 ± 19.19	152.48 ± 23.99 ^{ab}
10Day C2	4.18 ± 0.3 ^a	80	124.07 ± 15.04	155.09 ± 18.79 ^{ab}
10Day C3	3.81 ± 0.6 ^a	80	120.63 ± 34.36	150.79 ± 42.95 ^{ab}
10Day C4	3.8 ± 0.3 ^a	80	144.79 ± 10.53	180.99 ± 13.16 ^b

^{1,2,3} Values are the mean ± STD five of replicates analyses, and alphabet letters indicate the same letters in the same horizontal are not significantly different ($p > 0.05$) according to Duncan

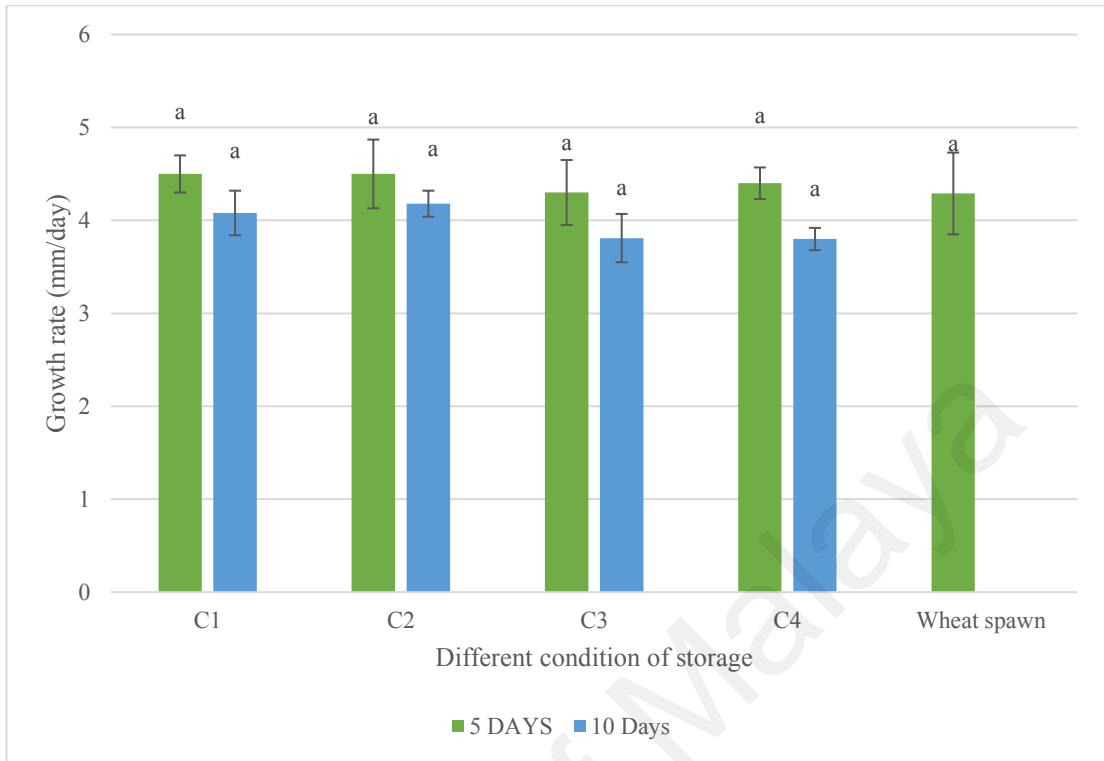


Figure 4.7: Growth rate of SS-PPEMB mycelium on sawdust substrate bag after different conditions of storage. Means that share a letter in the same condition of storage are non significant different ($p > 0.05$).

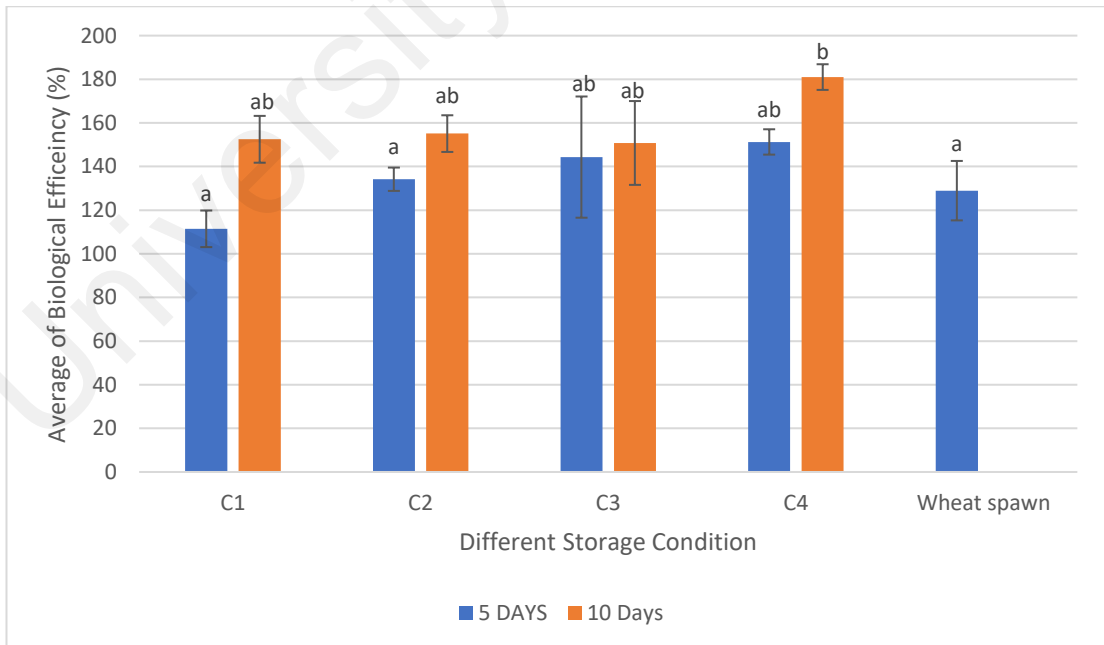


Figure 4.8: Biological efficiency of SS-PPEMB on sawdust substrate bag after different condition of storage. Means that do not share a letter in the same condition of storage are significant different ($p < 0.05$).

This study showed that SS-PPEMB stored for 10 days exhibited higher BE of $180.99 \pm 13.16\%$ when grown on sawdust fruiting substrate compared to work done by Velázquez-Cedeño *et al.* (2002) where BE of *P. pulmonarius* strain IE 226 grown in coffee pulp substrate at 138% by using sorghum spawn. Further, higher BE of *P. pulmonarius* was also obtained in our study compared to previous study by Salmones *et al.* (2005). They reported that in the cultivation of *P. pulmonarius* strain IE-225 in coffee pulp and wheat straw fruiting substrate, values of 80.5% and 77.1% of BE were obtained by using sorghum spawn, respectively. The BE of SS-PPEMB in the present study was higher with the *P. ostreatus* and *P. sajor-caju* BE at 112% and 100.2% by using wheat spawn in fruiting substrate consisting of wheat straw and wheat bran at a ratio (2:1) of as reported by Kurt and Buyukalaca (2010).

This study also proves that SS-PPEMB performed better or comparable to wheat spawn in sporophore yield production (Figures 4.9, 4.10, 4.11, 4.12, 4.13, 4.14). In another studied performed by Ortiz *et al.* (2017) encapsulated mycelium of *P. ostreatus*, *G. pampeanus*, *A. bisporus* and *L. edodes* also found that the EMB had no negative influence on the mycelial growth capacity. In addition, by using EMB achieve a very good colonization of the substrate without any contamination. Frequently mushroom growers are facing problems of contamination from spawn due to grains of cereals used for spawn production usually carry many fungi (*Fusarium* spp., *Trichoderma* spp., *Apergillus* spp., *Rhizopuss* spp., etc). According to Lee *et al.* (1986) this vital problem of mushroom spawn will reduce the profitability of mushroom production and consequently, it causes economic losses. Ortiz *et al.* (2017) reported by using EMB prevent contamination since they are produced under sterilized conditions.

Therefore, SS-PPEMB has potential to be used as spawn of *P. pulmonarius* in commercial cultivation. SS-PPEMB has shown the ability to germinate on sterile sawdust substrate and sporophore yield production as comparable or better to wheat spawn.



Figure 4.9: Mycelial run of *P. pulmonarius* using grain spawn on fruiting sawdust substrate.



Figure 4.10: Mycelial run of SS-PPEMB that when stored at C1 condition for 10 days on fruiting sawdust substrate.



Figure 4.11: Mycelial run of SS-PPEMB that when stored at C2 condition for 10 days on fruiting sawdust substrate.



Figure 4.12: Mycelial run of SS-PPEMB that when stored at C3 condition for 10 days on fruiting sawdust substrate.



Figure 4.13: Mycelial run of SS-PPEMB that when stored at C4 condition for 10 days on fruiting sawdust substrate.



Figure 4.14: Sporophore yield of SS-PPEMB on fruiting sawdust substrate inoculated with SS-PPEMB stored at C1- C4 condition for 5 days and 10 days

CHAPTER 5.0: CONCLUSION AND FUTURE RECOMMENDATION

Mushroom cultivation is an expanding industry in Malaysia. To be on par with other world mushroom producers, improvement in mushroom cultivation technique need to be done to meet increasing demand of mushroom as food. Mushroom spawn is one of the critical tool in mushroom cultivation and *P. pumonarius* is the highly produced mushroom in Malaysia. This study has successfully formulated a low-cost simple medium to produce a high amounts of *P. pulmonarius* mycelium (utilizing brown sugar 2%, baker's yeast 1%, spent grain extract 1%, minerals 0.05% and Tween 80 0.5%) using shake flasks (9.64 ± 0.70 g/L) and an automated bioreactor (11.89 ± 3.80 g/L). In addition, this study has shown that by adding Tween 80 into the medium induced the formation of small pellets producing unicellular propagates of mycelium.

Large scale production in a bioreactor shows the optimal harvesting time of mycelial biomass is at 48 hrs at cultivation conditions as follows: temperature (28°C), agitation speed (250 rpm), initial pH (5.5) and oxygen partial pressure (30-40%). The viability PPEMB was 100% even after 15 days storage with mycelial growth rate on sawdust substrate achieved was 6.38 mm/day. Further optimization of encapsulation solution with 5% soluble starch (SS-PPEMB) exhibited 65% germination after maximum storage time of 14 days increasing the proficiency of mycelium to emerge from SS-PPEMB. Storage time of 10 days with C4, store in sterile distilled water at 4 °C condition shows the highest BE of $180.99 \pm 13.16\%$.

Therefore, SS-PPEMB has potential to be used as spawn of *P. pulmonarius* in commercial cultivation. The SS-PPEMB is handy for storage, transportation and could grow on the SS-PPEMB during storage or transportation and a very attractive feature for the industry. The results obtained are considered useful for the production of *P. pulmonarius* mycelia on a large scale and can be widely applied to other mushroom species to enhance the future of the mushroom industry. Further large scale application

could be made possible by designing an automated machine to produce SS-PPEMB of mushroom in a quick, safe and efficient manner.

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