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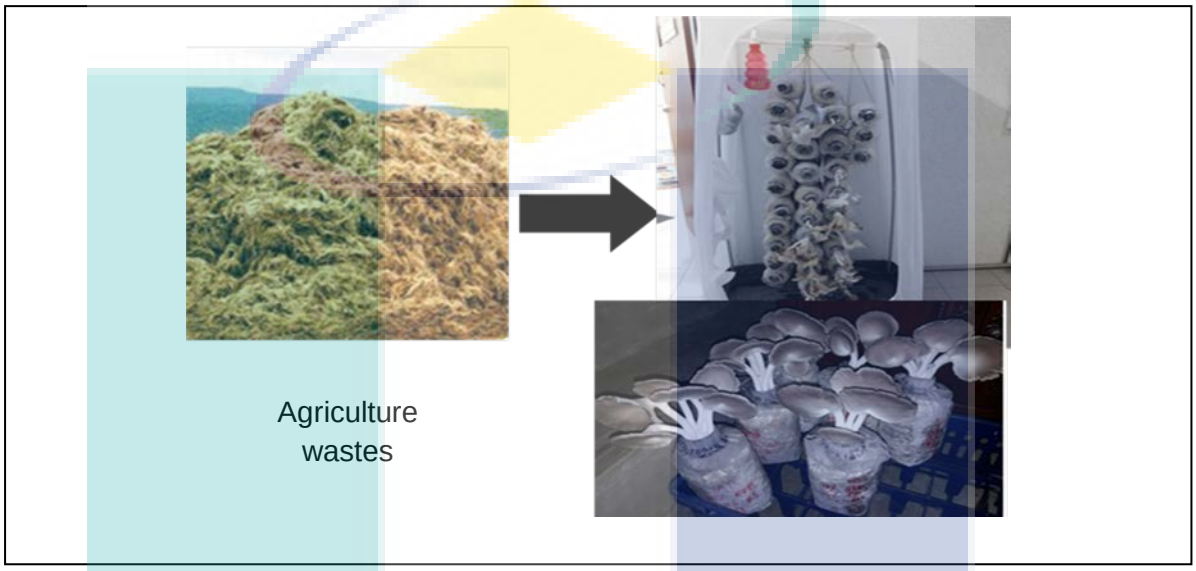

Mechanism of Pleurotus Ostreatus (Oyster Mushroom) Cultivation on Various Lignocellulosic Wastes using Empty Fruit Bunch, Palm Press Fibre and Corncob

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ABSTRACT

The utilization of low value agro-industrial waste as valuable end product has become a key research priority in the recent years. Currently, the commercial cultivation of Pleurotus ostreatus using sawdust from rubber tree as the base substrate in Malaysia has been reported. However, the price and demand of the rubber tree sawdust (RS) have been increasing, affecting the overall production cost. Thus, a new inexpensive alternative substrate yet as effective as rubber tree sawdust is needed to replace the RS as the base substrate for the cultivation of P. ostreatus. It is found that the growth and yield performance of *P.otreatus* are dependent on C/N ratio of the substrate. The substrates that contain empty fruit bunch (EFB), palm pressed fibre (PPF), sugarcane bagasse (SGB), corn cob (CC) in the ratio of 0.25 w/w and 0.5 w/w with rubber tree sawdust (RS) gave better the mycelium rate, fastest pinhead and fruiting bodies formations as well as the yields and BE (%) than 0.75 w/w and 1 w/w with RS and resulting the optimum C/N ratio for mushroom to growth well. The substrate of 25% PPF + 25%SGB + 50% (RS) resulting the best formulation in term of growth performance, yield and BE (%) are at 35 days for first harvest, 318.88 g/kg substrate and 79.72% respectively compared to other formulations of the substrates. However, the other combinations of the substrates also have a tremendous potential to be as an alternative substrate for mushroom cultivation in Malaysia. The particle size, C/N ratio, pH, moisture content significantly affects the growth of *P. ostreatus* and mineral content on the substrates. The advantages of using the biomass generated from the palm oil mills which is available throughout the year at little and no cost, and sugarcane bagasse that abundance in the rural and urban areas in Malaysia will not only solve the environmental pollution problem, but it can also offer an economically promising way to convert low quality biomasses into a valuable high protein food for human and also can help further income generation to the mushroom growers.

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Pleurotus ostreatus also known, as oyster mushroom belong to the class Basidiomycetes and family Agaricaceae and it is the second widely cultivated mushroom worldwide after *Agaricus bisporus* (Aida et al., 2009*)* . (Haimid et al., 2013) had reported that the *P. ostreatus* is the most cultivated mushroom contributing to 90.89 % of the total mushroom cultivated in Malaysia. It has high nutritional values as an important source of protein, carbohydrates, vitamins, calcium, and iron (Dündar et al., 2010). *P. ostreatus* is a fungus that can be cultivated on various lignocellulosic substrates due to its lignocellulolytic enzymes which degrade the lignocellulosic matters into useful carbohydrates for the fungi (Ali et al., 2018). Therefore, any types of organic matters that consist of lignocelluloses such as hemicellulose, cellulose and lignin can be used for oyster mushroom substrates, and this includes almost all agro-industrial wastes in Malaysia.

At present, Malaysia accounts for an overwhelming contribution to world's oil palm production and export which is 39% and 44%, respectively (A. S. A. F. Alam et al., 2015). Though, palm oil industry has boosted the national economy in Malaysia, it also generated abundant of oil palm biomass, including empty fruit bunches (EFBs) and palm pressed fibres (PPFs). For every tonne of palm oil produced from fresh fruit bunch (FFB), approximately 1 tonne of oil palm empty fruit bunch (EFB), 0.7 tonnes

oil palm fibers, 0.3 tonnes of palm kernels and 0.3 tonnes of palm shells were generated (S. H. Chang, 2014). In particular, EFB is the residual fruit bunch generated after fruits are removed from the FFB (Ahmad Naim et al., 2017) while the PPF is a form of recovered fibrous residue from palm fruit during palm oil extraction and accounts for about 11% of the fresh fruit bunch (Riansa-ngawong et al., 2011). The fruit fiber has been shown to possess high potential to be used as mushroom growing substrate without any further treatment (Abd Razak et al., 2013). The preparation of the substrates can be directly inoculated with little pre-treatment, whereas in some cases, they required microbiologically and physically pre-treatment (Abd Razak et al., 2013). (Wood, 1989) explained that microbiological pre-treatment of substrate generally comprises some form of controlled bulk composting process, while physical pre-treatment may include sterilization by autoclaving.

Besides solid wastes from palm oil mill industries, the sugarcane bagasse (SGB) and corn cob (CC) also are available in abundance both in rural and urban areas, in Malaysia. All these kinds of wastes generally have high content of hemicellulose, cellulose and lignin that can be upgraded to higher value-added products instead of being disposed consequently generating green houses and global warming.

Presently, in Malaysia, the commercial cultivation of *P. ostreatus* utilises rubber tree sawdust (RS) as the base medium. However, the high demand of RS leads to increasing price and has become a serious problem to the mushroom growers in particular *P. ostreatus* that requires carbon, nitrogen and other inorganic compounds for its nutritional sources. PPF, EFB, SGB and CC have high contents of hemicellulose, cellulose and lignin for carbohydrates or carbon source in order to ensure the *P. ostreatus* grows well (Mohd Tabi et al., 2008). Therefore, the EFB, PPF and SGB acquire great potentials to be an alternative substrate in the cultivation of *P. ostreatus* in the future and at the same time, able to solve the solid waste abundance and environmental issues in Malaysia.

In the present study, four locally available organic wastes; PPF, EFB, SGB and CC will be utilized for the cultivation of *P. ostreatus*. The influence of these agricultural wastes on the mycelium growth, yield, biological efficiency and nutrient compositions of the fruit bodies will be evaluated.

1.2 Problem Statements

In Malaysia, large volumes of unused lignocellulosic agricultural wastes can be found. These agricultural wastes are left to rot in the field or are disposed of through burning. Oil palm is one of the main important commodity products that become as transformation agent to the scenario of agricultural sector and economy in Malaysia (DOSM, 2016). The presence of wastes from oil palm plantation has created a major disposal problem such as open burning and in situ dumping. Currently, most of EFB are used as soil mulching as organic fertiliser to the plantation, otherwise, dumped in the same manner as palm oil mill effluent (POME) (Ng et al., 2012) while most of PPF are used as fuels to generate steam and electricity for the palm oil mills (Mohd Tabi et al., 2008). Indiscriminate dumping of EFB causes additional methane emission into the atmosphere (Mohd Tabi et al., 2008). An alternative application of agricultural wastes is needed in order to minimize the waste generation and protect the environment from being polluted.

Sugarcane bagasse (SGB) is another agricultural residue created after juice extraction from sugarcane industries in Malaysia. SGB creates the environmental nuisance due to direct disposal on the open lands and forms garbage heaps in that area (Abdulkadir et al., 2014). Besides, the sugarcane bagasse also is used as fuel source (Mosisa et al., 2015). In general, one metric ton of sugarcane bagasse generates 280kg of bagasse, the fibrous by-product remaining after sugar extraction from sugarcane (Sun et al., 2004). To reduce the environmental burden, the usage of agricultural wastes ought to be looked into.

Corncob (CC) also is another residue in Malaysia that are an abundant waste which are easily available throughout the year. In 2012, the production of corn in Malaysia was 52,481 tons, and in the subsequent year, it increased by about 5% to 55,000 tons (ShariffAzizIsmiza Ismail et al., 2016). In 2013, Malaysia ranked 113 out of 165 corn producing countries (Factfish, 2015). Therefore, the CC waste will increase per year and it will pollute the environment if it is not being controlled.

There is a high demand for oyster mushroom in Malaysia (almost 50 t/day) due to its nutritional values and antioxidant property (Mohd Zaffrie et al., 2014). However,

the current production rate of mushroom is about 24 tonnes/day (Mohd Zaffrie et al., 2014). In order to increase the yield and quality of mushroom, it is hypothesized that charcoal can act as a growth supporting material to boost up the growth and quality of mushroom for this study. Since in the charcoal has more carbon, it will be expected to increase the carbon content in the substrates, so that, the ratio of the carbon to nitrogen will be expected to be more optimum for oyster mushroom to grow.

Moreover, series of problems also arises in mushroom industries whereby generation and management of spent mushroom substrate (SMS) become a big challenge to the farmers. SMS can be defined as leftover of biomass generated by commercial mushroom industries after harvesting period of mushroom. Additionally, the medium-scale mushroom industry is capable of producing approximately 13.6 million tonnes per year of spent mushroom substrate (SMS) after the harvest cycle (Phan et al., 2012). The current practices substrate which is sawdust is not suitable to be used as animal feedstock and fertilisers, and it end up dumped and burned in situ (Park et al., 2012) due to the low nutrient composition. Hence, to minimize the problem, sawdust substrate should be substitute with lignocellulosic substrates such as corn cob, sugarcane bagasse, palm wastes, or crop wastes, as to minimize the wastes from mushroom industry. Mushroom cultivation using agricultural wastes promises a good quality of SMS for producing beneficial products such as animal feeding and fertilizers (Mohd Hanafi et al., 2018).

There are another two major problems faced by our local mushroom growers. Firstly, the limited supply of sawdust and lead to increasing price mostly due to the competition from other industries. Second, sawdust supplies are often mixed with chemicals used in the processing industry. The tainted supply of sawdust affected mushroom growth such as low yield, high percentage of contamination and unsynchronized flushing patterns. Therefore, it is imperative that other sources of substrates be utilized for mushroom cultivation. Mushroom commercial industries in Pekan, Pahang experienced approximately 15% contamination rate in every 1000 beds per production using the commercial substrates which is RS. Besides, *Pleurotus spp.* is categorized as fungi and very prone to contamination. Contamination occurs probably because of the techniques and handling methods used are unhygienic. Therefore,

assessments from other substrate need to be done to overcome the shortage and contamination of RS.

Most of the studies conducted in Malaysia only emphasized on a single output, for instance, mushroom cultivation by using agricultural wastes (Ali et al., 2013). There are limitations in research especially on the use of agricultural wastes as mushroom substrate and the percentage of waste recovered from the harvesting period of mushroom.

1.3 Objective of the Study

The main aim of this study is to formulate the composition of mushroom substrate by using four different agricultural wastes such as EFB, PPF, SGB and CC. The following are the objectives to achieve the research aim:

a) To characterize the sample of agricultural wastes such as empty fruit bunch (EFB), palm pressed fibre (PPF), sugarcane bagasse (SGB), corncob (CC), rubber tree sawdust (RS) and spent mushroom substrates (SMS) and sample of charcoal.

b) To study the effect of substrates and mass ratio of various agricultural wastes including EFB, PPF, SGB, CC and RS for *Pleurotus ostreatus* cultivation by comparing the mycelium growth and total yield of fresh mushroom.

1.4 Scope of the Study

In order to achieve the mentioned objectives, the following scopes are defined:

a) The EFB, PPF, SGB, CC, RS, SMS and charcoal are characterized to determine their carbon, nitrogen, hydrogen and sulphur using CHNS analyzer. The thermal stability of EFB, PPF, SGB, CC and RS will be done using Thermogravimetric Analysis (TGA). The elemental analysis of the EFB, PPF, SGB, CC and RS will be done using X-ray Fluorescence (XRF).

b) The effect of different media mass ratio of all these agricultural wastes to RS are done in a range of 100:0, 75:25, 50:50, 25:75 and 0:100. The mixtures are packed in a plastic bag and sterilised prior to spawning process. Then, the baglogs are incubated. The mycelium growth rate, the number of days for pin head formation, fruiting body yield and harvest time, physical characteristics such as the cap diameter and thickness, the stipe length and thickness and the total fruiting body yield per kg of an alternative substrate of *P. ostreatus* are determined. The percentage of EFB, PPF, SGB and CC waste recovered are calculated.

c) The nutrient composition of *P. ostreatus* fruiting bodies of these five experimented substrates; RS, EFB, PPF, SGB and CC are compared in order to determine the most nutritious mushroom using proximate analysis.

d) The mineral content of *P. ostreatus* fruiting bodies of these five experimented substrates; RS, EFB, PPF, SGB and CC are compared in order to trace any heavy metals using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

e) The moisture content, pH, substrates (RS, EFB, PPF, SGB and CC) and additional nutrients used in cultivation (wheat bran and limestone) and *P. ostreatus* spawn will be constant variables along the study.

1.6 Rationale and Significance of the Study

The significance of this study is to utilize abandoned waste from palm oil plantations, sugarcane and corn field as mushroom substrate in order to reduce the generation of agricultural wastes from burning and open dumping. Besides, mushroom cultivation using agricultural wastes promises a good quality of spent mushroom substrate (SMS) for producing beneficial products such as animal feeding and fertilizers (Mohd Hanafi et al., 2018).

Currently, Malaysia contributes about 30% of production and 37% of world exports contributing to the growth of gross domestic product (GDP) (Kushairi, 2017). The oil only contributes about 10% of the total dry matter of the palms; the remaining 90% being oil palm biomass (OPB) (Soh Kheang et al., 2013). About 80 million tonnes of OPB has been generated in 2010 but it is expected to increase to 100 million tonnes in year 2020 (Malaysia, 2013). The OPB includes empty fruit bunch (EFB), oil palm trunk (OPT), oil palm frond (OPF), oil palm kernel shell (OPKS), and mesocarp fibre, which are not fully utilised as a renewable source (Theo et al., 2017).

Besides solid wastes from palm oil mill industries, the SGB and CC also are available in abundance both in rural and urban in Malaysia. All these kinds of wastes generally have high content of hemicellulose, cellulose and lignin that can be upgraded to higher value-added products instead of being disposed consequently generating green houses and global warming.

Mushroom cultivation also is one of the immense potential agricultural activities in Malaysia. High valued crop in terms of both food and medicine aspects with low cost production technology can bring high return within short time interval. Mushroom farming in Malaysia is suitable due to its climatic conditions. The attractive factor towards mushroom farming is the short time period between cultivation and harvesting and it can be grown with locally available resources (Poudel, 2014).

Moreover, under the Economic Transformation Program (ETP), biomass from oil palm industries have been highlighted as the nation's premier niche National Key Economic Areas (NKEAs) (Jabatan, 2013). The utilization of palm biomass is increasing significantly over time, which creates a symbiotic situation where the "previous waste" serves as the input for other industries, leading the palm oil industry to a zero-waste path (Ng et al., 2012). Interestingly, the Malaysian government has declared mushroom farming as one of the eleven under agriculture business opportunities under the National Key Economic Area (PEMANDU, 2011) .

Therefore, mushroom cultivation is the easiest way to reduce the abundant of agricultural wastes in Malaysia. From the previous study, the result indicated that high yield of mushroom can be produced by using agricultural wastes as mushroom substrate (Ali et al., 2018). Furthermore, the mushroom cultivation can generate the fast income for the farmers and the country as mushroom has a short growth time and the can grow local available sources (Crosby, 2016).

Moreover, this study will provide an alternative to recycle and recover abundant of agricultural waste to another beneficial products. Reuse and recovering agricultural wastes in mushroom life-cycle will be concluded as promoting a zero-waste discharge; hence, this is important to the farmers to practice in agriculture sector. In addition, this study will not only help the country in controlling environmental pollution through the zero waste initiatives, but it can also will offer an economically promising way to convert low quality biomasses into a valuable high protein food for human and also can help further income generation to the mushroom growers.

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CHAPTER 2

LITERATURE REVIEW

2.1 Agricultural Wastes

Agricultural wastes are basically unusable substances which may be either liquid or solid produced as result of cultivation processes such as fertilizers, pesticides, crop residues and animal waste (Shehrawat et al., 2015). Generally, solid agricultural waste residues are consisted of cellulose, hemicelluloses and lignin and also pectin, starch and other polysaccharides and are insoluble in water (Thomsen, 2005). The wastes derived from agricultural activities are cost effective, renewable, abundant produced and can be used as a resource for sustainable production of food and value-added food products. Expensive treatments or disposal is required if these wastes are not recycled or used to generate a value-added product (Wang et al., 2007). According to (Elly Sabiiti, 2011), in line with the increasing in the world's population, the demand for food supplies has significantly increase and this has eventually resulted in the production of large amounts of agricultural wastes.

2.1.2 Oil Palm Wastes

Malaysia currently accounts for 39% of the world's palm oil production and 44% of the world's exports, being one of the biggest producers and exporters of palm oil and palm oil products (Tan et al., 2018). The palm oil industry is capable of generating a vast quantity of palm biomass, in line with the growth of the industry. Basically, oil palm biomass can be generated from two different sources, namely from plantations and the mills. Biomass from the plantation is mainly in the form of trunks and fronds while biomass from the mills consists of empty fruit bunches (EFB), palm pressed fibre (PPF), palm kernel shell and palm oil mill effluent (POME). These biomasses are produced daily

throughout the year. In general, the PPF are burnt to generate steam for the mills and the EFB normally be returned to the plantations (Abas et al., 2011). On average, for every tonne of Fresh Fruit Bunches (FFB) processed, of 230-250 kg of Empty Fruit Bunch (EFB), 130-150 kg of fiber, 60-65 kg of shell and 55-60 kg of kernel and 160-200 kg of unrefined oil were produced (Latif Ahmad et al., 2003).In short, oil palm plantation has the potential to yield a very large amount of biomass which is very rich in plant nutrient to be used for the production of renewable products. The large quantity of biomass produced by oil palm plantation each year and their high nutritional values make these wastes a very promising source of substrate for mushroom production.

In Malaysia, sawdust is currently used as the main substrate for cultivation of edible and medicinal mushroom. EFB and PPF are of equivalent standard as sawdust to be used in mushroom cultivation, based on their percentage of cellulose, hemicellulose and lignin content as shown in Table 2.1. Table 2.2 shows the carbon, hydrogen, nitrogen and sulphur content in sawdust, EFB and PPF.

Table 2.1: Cellulose, hemicellulose and lignin content in sawdust, PPF and EFB

Composition	**Cellulose, Hemicellulose & Lignin Content (%)**		
	Sawdust[a]	PPF[b]	EFB[c]
Cellulose	42-49	30.2	40
Hemicellulose	23-34	23.2	23
Lignin	20-26	22.9	16
Extractives	3-8	-	-
Ash	0.2-0.8	-	-

Source: a; (Manaila et al., 2016), b; (Ponthein et al., 2011), c; (Intasit et al., 2019)

Table 2.2: Carbon, hydrogen, nitrogen and sulphur content in sawdust, PPF and EFB

Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulphur (%)
EFB[a]	45.29	5.06	1.20	0.35
PPF[a]	45.47	5.73	1.29	0.36
Rubber Tree Sawdust[b]	43.27	6.83	0.39	-

Source: a; (Sulaiman et al., 2014),b; (Shaaban et al., 2013)

2.1.2 Sugarcane Bagasse (SGB)

Sugarcane bagasse is a residue produced in large quantities by sugar industries. In general, one metric ton of sugarcane bagasse generates 280kg of bagasse, the fibrous by-product remaining after sugar extraction from sugarcane (Sun et al., 2004). Production of sugar cane is concentrated in the Northwest of peninsular Malaysia in the states of Perlis and Kedah. This area has a distinct dry season needed for cost-efficient

sugarcane production. However, the sugarcane plantation has been stopped in 2011 and replaced with palm oil tree plantation.

In the sugar industry, sugarcane bagasse is more related as the by-product in sugarcane mills. After the sugarcane is pressed to remove sucrose or known as table sugar, the residue is called as sugarcane bagasse which contains highly fibrous residue. After the sucrose removal, the bags are generated and its accumulation presents as a waste problem for sugar industry. A portion of the sugarcane bagasse produced in the sugar manufacturing process is commonly burned in boilers as a fuel source (Talha et al., 2016). Some factories utilize natural gas or gasoline as their fuel (Mehryar et al., 2017). Sugarcane bagasse also has been used in boards, pulp and paper production, and animal feeding (Mehryar et al., 2017).

Growing mushrooms on a substrate of sugarcane bagasse is one of the solutions to transform these inedible wastes into accepted edible biomass of high market and food of high nutritional value. In the process of utilizing these wastes, environmental pollution from the disposal of these wastes will be reduced. Table 2.3 and 2.4 shows the characteristics of sugarcane bagasse for oyster mushroom cultivation.

Table 2.3: The characteristics of sugarcane bagasse (Anukam et al., 2014)

Analysis	**Amount in sugarcane bagasse (%)**
Proximate analysis	
Moisture content	1.14
Ash	1.42
Volatile matter content	69.99
Fixed Ccrbon	16.39
Elemental analysis	
Carbon	44.1
Nitrogen	0.2
Hydrogen	5.70
Sulphur	2.30
Oxygen	47.70

Table 2.4: The lignocellulose content in sugarcane bagasse (Rocha et al., 2011)

Analysis	Amount in sugarcane bagasse (%)
Lignocellulose	
Cellulose	45.5
Hemicellulose	27
Lignin	21.1
Extractives	4.6
Ashes	2.2

2.1.3 Corncob (CC)

In Malaysia, corn residues are an abundant waste which are easily available throughout the year. In 2012, the production of corn in Malaysia was 52,481 tons, and in the subsequent year, it increased by about 5% to 55,000 tons (ShariffAzizIsmail et al., 2016). For every 1 kg of dry corn grains produced, about 0.15 kg of cobs, 0.22 kg of leaves and 0.50 kg of stalks are produced (Sokhansanj et al., 2002). Usually, the corn wastes such as corn cob and corn stovers are left on the ground of the farm or found littering the streets of the stall or market (Ogunjobi et al., 2013). Table 2.5 shows the characteristics of corncob for oyster mushroom cultivation.

Table 2.5: The characteristics of corncob (ShariffAzizIsmail et al., 2016)

Analysis	Amount in corn cob (%)
Proximate analysis	
Moisture content	7.15
Ash content	1.05
Volatile matter content	87.76
Fixed carbon	11.19
Elemental analysis	

Carbon	43.81
Nitrogen	6.54
Hydrogen	0.77
Sulphur	0.69
Oxygen	48.19
Lignocellulose	
Cellulose	45.88
Hemicellulose	39.4
Lignin	11.32
pH	5.44

2.2 Spent Mushroom Substrates (SMS)

Spent mushroom substrate (SMS) is a bulky biomass byproduct of mushroom commercial industries and produced abundantly (Oei et al., 2007). Approximately, 5 kg of SMS is produced for each kilogram of fresh mushroom fruiting bodies (Lin et al., 2014). The huge amount of SMS in the mushroom farm is the biggest challenge for disposal management (Aziera et al., 2015). Conventionally, majority of SMS is either spread on the farmland as fertilizer, disposed of in situ or incineration (Williams et al., 2001). Based on the literature, many researchers attempted in finding the solution for minimizing SMS generation by using them as ruminant feeding (Gimeno et al., 2015; Rezaei et al., 2015)

2.3 World Mushroom Production

Mushroom production fits in very well with sustainable farming as it uses agricultural waste products, high production per surface area can be obtained, and the

spent substrate is still a good soil conditioner (Rao et al., 2018). There are three categories of mushroom available in industries which are edible mushrooms, medicinal mushroom products, and wild mushrooms (S. T. Chang, 2006). There are six major types (genera) of mushroom which contribute around 90% in total world production (Figure 2.1). Lentinula is the most widely grown mushroom accounting for over 2 million tons. The second most widely grown mushroom is Pleurotus spp. accounting around 0.4 million tons. Auricularia spp. is a close third, with six cultivated species; accounting 73840 tons constitute 18% of the world's total output while Agaricus bisporus contributes around 15% accounting 11076 tons. The other two genera, Flammulina and Volvariella are responsible for 11% and 5% of the world's total output, respectively. Among the six Lentinula, Pleurotus and Agaricus are cultivated worldwide while the other three are grown almost exclusively in Asia (D. Royse et al., 2017; D. J. Royse, 2014)

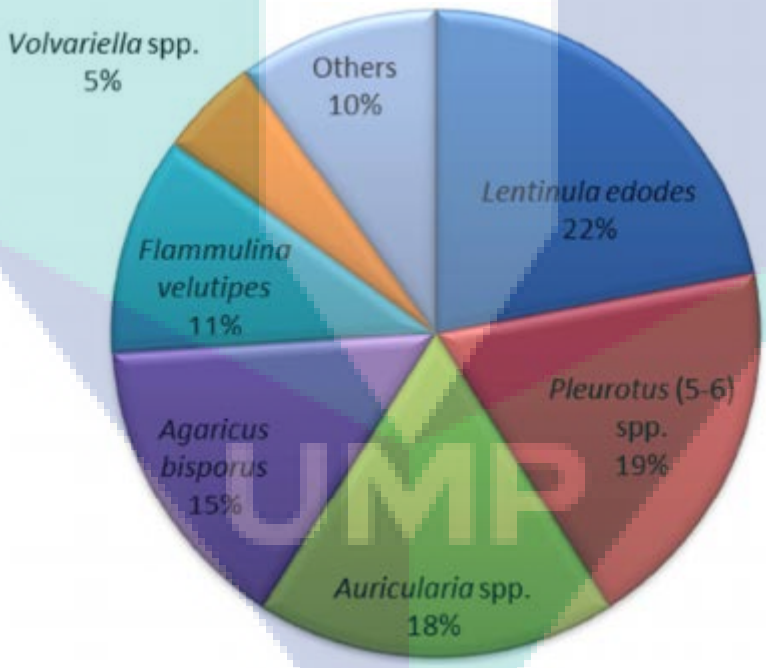

Figure 2.1: World edible mushroom production (% of world's total output) by genus (2013) (Raut, 2019)

According to (D. J. Royse, 2014), world production has increased more than 18-fold in the last 32 years, from about 350,000 metric tons in 1965 to about 6,160,800 metric tons in 1997 and consumption of mushrooms has increased at a rapid rate, especially since the mid-1990s. Not only has production and consumption increased as

the world's population has increased, but per capita consumption of mushrooms has increased as well. Over a 15-year period (1997 to 2012), per capita consumption of mushrooms increased from about 1 kg/year to over 4 kg/year.

Asian countries produce more than 74.64% of world mushroom markets followed by Europe (19.63%) respectively in 2014 (FAO, 2015) (Figure 2.2). In recent years, about 40% of total world mushroom are exported from China as the world's biggest producer of mushroom. However, 95% of the total China production is for domestic consumption, In 2013, Shiitake had the best demand for mushroom consumption in China about 22.5%, followed by Grey Oyster mushroom 18.9% and Wood ear mushroom 16.8% (M. Li et al., 2014). With the largest markets, mushrooms widely cultivated only by small-scale farmers (M. Li et al., 2014). The demand for mushrooms has been phenomenal – production to meet the growing demand is a performance seldom duplicated in agriculture today.

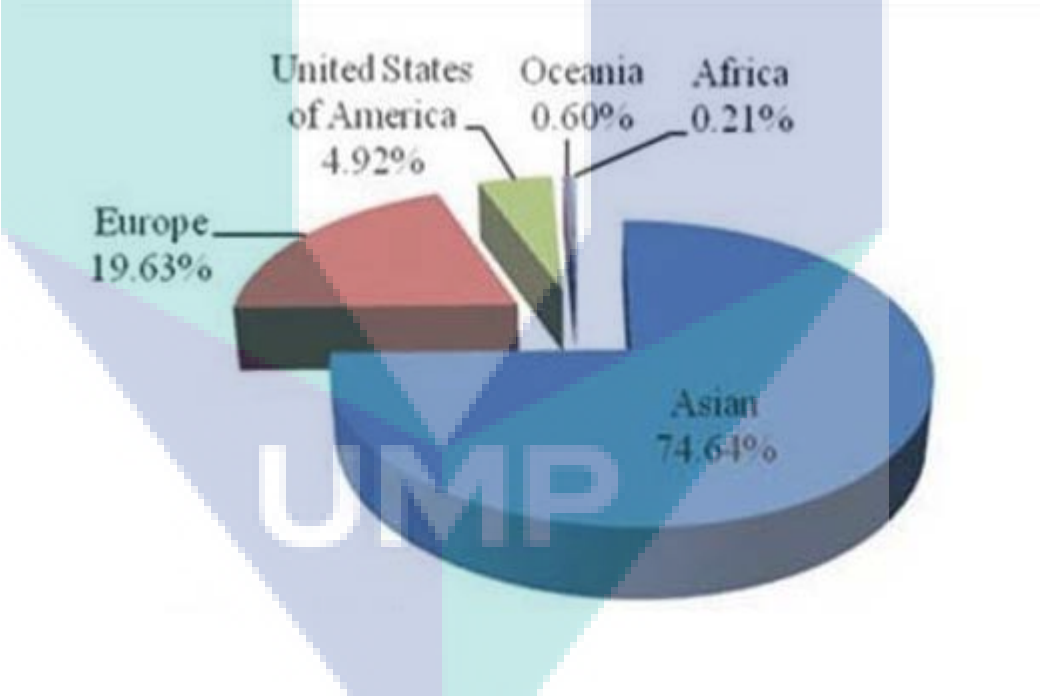

Figure 2.2: World mushroom production in 2014 (Food and Agriculture Organization, 2015)

Recent scenario revealed that oyster mushroom (*Pleurotus* spp.) production increased from time to time. China, South Korea, Japan, and Indonesia are the major producers. At present, *Pleurotus* spp. has become the second most important cultivated mushroom of the total world production. A considerable shift has occurred in the

composite of genera that constitute the mushroom supply. China is the biggest producer of mushroom in the world and record in 2005 showed that China produced 11.6 million tons of fresh mushrooms, of about 75% of the world's total production (Oei et al., 2007). Whereas, according to (Mohd Zaffrie et al., 2014), mushroom demand in Malaysia is approximately about 50tons/day every day.

2.4 Description and Classification of *Pleurotus ostretaus*

Oyster mushroom (*Pleurotus ostreatus*.) belonging to Class Basidiomycetes and Family Agaricaceae and grows naturally in the temperate and tropical forests on dead and decaying wooden logs or sometimes on dying trunks of deciduous or coniferous woods (Randive, 2012). It may also grow on decaying organic matter. This is because they lack of chlorophyll so they cannot prepare their own food. The fruit bodies of this mushroom are distinctly shell or spatula shaped with different shades of white, cream, grey, yellow, pink or light brown depending upon the species.

Mushroom can be classified into saprotrophic, mycorrhizal, parasitic and endophytic. *Pleurotus ostreatus* is categorized as saprotrophic. Saprotrophic mushrooms are decomposers. They release acids and enzymes that break down dead tissue into smaller molecules they can absorb. Thus, decaying wood, plants, and even animals can become food for a saprotroph.

The plant body of mushroom contains two major parts which is mycelium (underground part) and fruiting body (reproductive part or basidiocarp). Mycelium is the underground vegetative part of mushroom which consist of the mass of muscle-branched hypae. It absorbs the food materials from organic matters where it grows. The fruiting body is the main body of the mushroom above the ground and it contain several part which are pileus (umbrella-like structure that protects growing basidiospores), stipe (give support for pileus), annulus (structure formed when pileus separates from stipe as mushroom grow up) and gills (the place where

the basidiocarp grows). Figure 2.3 show the morphology of the mushroom.

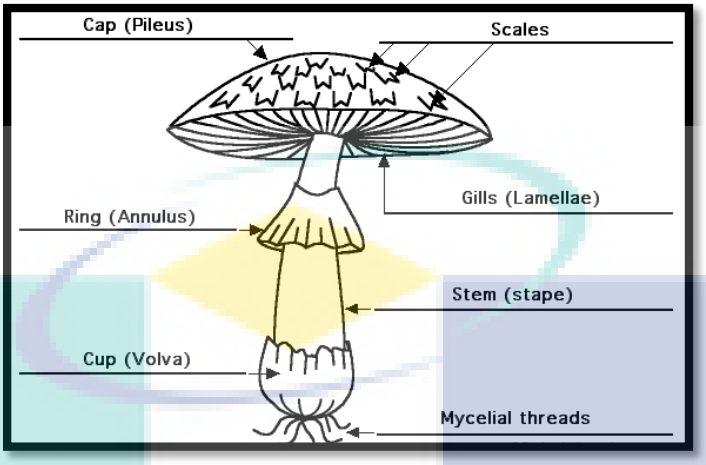

Figure 2.3: The structure of oyster mushroom

2.5 Nutritional Requirement for *Pleurotus ostretaus* Growth

The ability of a fungus to synthesize enzymes to degrade the substrate is influenced by the strain, substrate composition and nitrogen concentration in the cultivation medium (Elisashvili et al., 2008; Stajić et al., 2006).The substrate on which the mushroom is grown should supply specific nutrients required for oyster cultivation and the main nutritional sources for oyster mushrooms are cellulose, hemicelluloses and lignin (Kang, 2004). Cellulose and hemicelluloses which are the main sources of carbohydrates for oyster mushrooms are often incrusted within lignin, which forms a physical seal around cellulose and hemicelluloses and the proportion of these three structural components along with nitrogen content of residues affect mycelia growth, mushroom quality and crop yield (Philippoussis et al., 2011). The strategy of the oyster mushroom is to decompose the lignin in wood so as to gain access to the cellulose and hemicelluloses embedded in the lignin matrix (Philippoussis et al., 2011). Oyster mushrooms require more carbon and less nitrogen, however most of the substrates must be supplemented with nitrogen source to reach optimal C/N ratio for the mushroom

(Philippoussis et al., 2011). For oyster mushroom cultivation, the initial C/N ratio ranges from 80 to 100/1 when carried out under natural conditions (de Carvalho et al., 2012).

Common additional nutrients used in cultivation are lime hydrated agriculture ($CaCO_3$), and rice/wheat bran as a nutrient supplement on the dry weight basis of the substrates (Lalithadevy V et al., 2014; Pathmashini et al., 2009). The percentage of rice bran used significantly affected the fruit body yield of *Pleurotus spp.* (Chae et al., 2013). $CaCO_3$ was added as to adjust the pH value of substrates. The optimal substrate pH value for mycelial growth is 6.5 until 7, though mycelium can survive between pH 4.0 and 7.0. The mycelium grows slowly as the pH lowers and stops growing at pH 4. If the pH is higher than the optimal value, mycelial growth accelerates but produces an abnormal structure. Optimal pH for primordial induction and fruiting is 5 until 5.5 though it is possible at 5.5 until 7.8.

According to (S. T. Chang et al., 2004), the appropriate moisture in the substrate should encompass a range between 50% and 75% in the substrate, enabling the satisfactory growth of *Pleurotus spp.* Moisture above 70% makes the development of diseases and competing molds possible (Bellettini et al., 2019). Meanwhile, the relative humidity of the culture room should be maintained at 80–85% by spraying water three times per day (Júnior et al., 2016). Then, the substrates were sterilized at 100°C for about 8 hours. Based on the literature, suitable sterilization period is being identified by many studies which used period of 100°C for in 8 hours. Then, the substrates were placed in an incubation room maintained at 25°C and relative humidity at 85% until mushrooms can be harvested (Marlina et al., 2015).

2.6 *Pleurotus ostreatus* Cultivation Techniques

There were four stages of mushroom growth which are spawn run, primordial formation, and harvesting (X. Li et al., 2001). Mushrooms grow on decayed organic matters rich in lignin, cellulose, and other complicated carbohydrates. The oyster mushroom, *Pleurotus ostreatus* is characterized by its rapid growth on agro-wastes such

as olive cake, tomato stuff, pine needles, wheat straw, banana leaves, a leaf of hazelnut, cotton waste, maize stover, palm oil and other wastes. Substrates that are used in cultivating mushrooms have an effect on the chemical, functional, and organoleptic characteristics of mushrooms. Mixing agro wastes at different ratios enhances the productivity of oyster mushroom. Protein content and other nutrients were found to vary in the mushroom fruiting bodies when grown on different agrowastes (Alananbeh et al., 2014).

Cellulose, lignin, and carbon-nitrogen ratios are found to be positively correlated with mycelium growth and yield for *Pleurotus spp.* respectively (Alananbeh et al., 2014). Cultivation of mushroom really needs an intensive care by farmers in order to minimize the contamination rates of mushroom beds. Several techniques were applied by mushroom industry in order to control the mushroom beds from contamination. Table 2.6 shows the techniques for mushroom cultivation using agricultural wastes in different countries.

Table 2.6: Techniques and ratios involved in mushroom cultivation

Mushroom species	**Substrates**	**Ratio**	**Technique/Method**	**Country**	**Reference**
Pleurotus sp.	Sawdust and EFB	50:50	Chopped, shredded, soaked (one night to gain 75% moisture content)	Indonesia	(Sudirman et al., 2011)
	EFB	100	Cut into smaller pieces, dried, shredded		(Marlina et al., 2015)
	Paddy straw	100	Soaked, add 2% molasses, cover with a plastic sheet for 5 days	Philippines	(Alfredo B. Villaceran Jr. et al., 2006)
	Cottonseed hull and tea waste	50:50	Used directly after being extracted in hot water	China	(Yang et al., 2015)

Pleurotus florida	Fruit and vegetable peel, sawdust	50:50 100 fruits and vegetable wastes	Cleaned thoroughly, chopped, sundried, stored in airtight and sterilized gunny bags	India	(Lalithadevy V et al., 2014)
Pleurotus ostreatus	Weed plants and paddy straw	50:50	Dried the weed plants, soaked and drained off the excess water from paddy straw		(Das et al., 2007)
	Sawdust and EFB	50:50 100	Cut, dried, shredded	Malaysia	(Ali et al., 2013)
	Sawdust and PPF	50:50 100	Dried, shredded		(Ali et al., 2013)

2.7 Combination of Agricultural Substrates used for *Pleurotus ostreatus* Cultivation

Every mushroom substrate has different nutrient content depending on type of substrates and additional materials. Most abundant wastes from cereal crops such as corn cob, wheat, paddy, sorghum, oat, and barley are high in protein and suitable for mushroom growth (Phan et al., 2012). In addition, *Pleurotus spp.* are able to colonize various types of agricultural wastes which contain cellulose, hemicelluloses, and lignin (Ali et al., 2013).

Several agricultural residues have been used in mushroom cultivation. Most of the agricultural residues such as sugarcane bagasse, maize straw, maize cob, palm products and sawdust are wasted, while majority constitute an environmental hazard. One of the best uses is to use them as growth media for mushroom production (Chukwurah et al., 2012).

Many studies have shown that mushroom cultivation is one of the easiest and convenient ways to reduce agricultural wastes and resulted in edible fresh mushrooms compared to using rubber trees sawdust (Carabajal et al., 2012; Dündar et al., 2008; Mohd Tabi et al., 2008). Mushroom beds residue from lignocellulosic biomass such as paddy straw, wheat straw, EFB and many others have potential to be used as other product such as, animal feedstock, organic fertilizers, marsh gas, to improve physical structure of the soil, to produce plant hormone and many others (Oei et al., 2007). Table 2.7 presents the combination of agricultural substrates applied in *Pleurotus spp.* cultivation.

Table 2.7: Combination of agricultural substrates applied in *Pleurotus spp.* cultivation

Substrates	Mushroom types	References
Waste paper, peat, chicken manure and rice husk; weed plants; palm kernel cake+maize cob; palm kernel cake+sawdust; maize straw+sawdust; EFB+PPF	*Pleurotus ostreatus*	(Chukwurah et al., 2012; Das et al., 2007; Mohd Tabi et al., 2008)
Rice straw, wheat straw, sugarcane bagasse, tea waste	*Pleurotus sajor caju*	(Pant et al., 2006; Yang et al., 2015)
Coffee pulp and wheat straw; maize wastewate	*Pleurotus spp.*	(Loss et al., 2009)
Rice straw, wheat straw, sugarcane bagasse	*Pleurotus pulmanarius*	(Pant et al., 2006)
Rice straw, wheat straw, sugarcane bagasse; corncob; fruits and vegetables peels (sweet lime, watermelon, banana)	*Pleurotus florida*	(Lalithadevy V et al., 2014; Pant et al., 2006)
Rubber trees sawdust and rice straw	*Pleurotus eryngii*	(Moonmoon et al., 2010)

2.8 Composition of Mushroom Fruiting Bodies

Mushroom contains water, crude protein, crude fat, carbohydrates, glycogen, amino acids such as glutamic acid, aspartic acid, lysine and arginine, ash and minerals such as potassium (K), magnesium (Mg), nitrogen (N), iron (Fe), calcium (Ca), and zinc (Zn) (Jonathan et al., 2006; Ouzouni et al., 2009; Reis et al., 2012). (A. S. A. F. Alam et al., 2015) reported that mushroom such as *Pleurotus ostreatus*, *Pleurotus sajor-caju*, *Pleurotus florida* and *Calocybe indica* were rich in proteins (20-25%) and fibers (13-24%), carbohydrates (37-48%), lipid (4-5%) and ash content (8-13%). These findings proved that mushrooms are rich in nutritional value and beneficial to human body.

Besides, *Pleurotus spp*. are very popular and have been proved rich in proteins (20-25%) and fibers (13-24% in dry samples) and contained a lower amount of lipid (4-5%) (N. Alam et al., 2008). Medicinal purposes of mushroom have been confirmed through many researchers conducted worldwide (Aida et al., 2009). Edible mushroom contains vitamin B, vitamin C, and ergosterol (Wisitrassameewong et al., 2012). *Wild sp.* is usually used for medicinal purposes and less energetic than the commercial species. Furthermore, it possesses a high content of phenols but a lower content of ascorbic acid, compared to commercial species (Barros, et. al., 2008).

Mushrooms also low in calories and fat, rich in amino acids, protein, vitamin, chitin, minerals, and high amount of aminobutyric acid, ornithine, ascorbic acid, thiamine, niacin, riboflavin, and folic acid (G. Ramanathan et al., 2013). These nutrients are very crucial for mushroom growth process as a function for mycelia development, substrates performance and yield production. Most abundant wastes from cereal crops such as corn cob, wheat, paddy, sorghum, oat, and barley are high in protein and suitable for mushroom growth (Phan et al., 2012).

Nutrients composition in *Pleurotus spp.* are found very suitable for people with hypertension, obesity, and diabetes, since it possesses low sodium, potassium, starch, fat and calorific value meanwhile folic acid presented in *Pleurotus spp*. is suitable for curing anemia (Randive, 2012).

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter describes the methods, materials, chemicals, instruments/equipments and apparatus to achieve the objectives in this study. Before carrying out the study, it is crucial to have some general understanding of the important aspects that involved in the study process. The variables, scope, study area and suitable technique that were required for the analysis process are identified; hence, the research can be conducted in a systematic and orderly manner. The experiments were conducted in Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang. Detailed procedures that were illustrated which focus on cultivation process, techniques, types of instrument/equipment used, data collection and data analysis.

3.2 Materials and Chemicals

Materials and chemical used throughout the study were tabulated in Table 3.1. Empty fruit bunch (EFB), palm press fibers (PPF), sugarcane bagasse (SGB), corn cob (CC) and spent mushroom substrate (SMS) are processed accordingly prior cultivation.

These materials were dried under the sun at least one (1) weeks prior grinding. The ground materials were sieved to obtain uniform particles size (1-2 mm) using a sieve tray for better mushroom performances; yield, bioconversion efficiency and substrates performance (Zhang et al., 2002)

Table 3.1: List of materials and chemicals

Materials/Chemicals	Source/Supplier	Description
Empty fruit bunch (EFB)	Felda Lepar Hilir, Kuantan, Pahang	Main substrate
Palm press fibers (PPF)		
Sugarcane bagasse (SGB)	Local farmer in Kuantan, Pahang	
Corn cob (CC)	Night market in Kuantan, Pahang	
Spent mushroom substrate (SMS)	Pekan Agro Farm, Pekan, Pahang	
Rubber tree sawdust (RS)		
Wheat bran		Purposely to balance macronutrients and micronutrients in the substrate
Lime hydrated agriculture (CaCO3)		Balancing the acidity of the substrate
46 days - Oyster mushroom strains		Culturing purpose of *Pleurotus ostreatus*
Charcoal	Local shop in Kuantan	Additional supplement
Acetone, AR, 2.5 L, Bendosen*	Orioner Hightech Sdn. Bhd.	As an organic solvent to extract the oil contents in EFB, PPF, SGB and CC.
Tap water	UMP, Gambang, Pahang	To increase the moisture content of the substrate and watering of the bags in order to avoid withering of the growing mushrooms.
Distilled water	FTKKP Laboratory	To wash the beaker, measuring cylinder and etc.

3.3 Instruments/Equipments

The lists of instrument/equipment that were used in this study including the descriptions of each instrument were presented in Table 3.2.

Table 3.2: The list of instruments/equipments including its description

Instrument/Equipment	Brand	Availability	Description
Industrial Grinder	SIMA	FTKKP, UMP	To grind a material such as EFB, PPF, SGB and CC
CHNS	Elementar, Germany	Central Laboratory, UMP	To obtain the total content of carbon, hydrogen, nitrogen and sulphur in agricultural wastes
TGA	Thermogravimetric analysis (TGA) STA-7000, Perkin Hitachi	CARIFF, UMP	To determine biomass cellulosic and thermal degradation profile
XRF	Rigaku WDXRF ZXS Primus II	CARIFF, UMP	To determine the metal and metal oxide compositions of agricultural wastes
Moisture content analyzer	Yueping, China	Central Laboratory, UMP	To determine the moisture content of the substrates
pH meter	Mettler Toledo, USA	Central Laboratory, UMP	To determine the pH value of the substrates

Steamer	Nil	Pekan Agro Farm, Pekan, Pahang	To sterilize the mushroom baglos
Soxhlet extractor	500 ml	FTKKP, UMP	To determine oil content in the biomass
Rotary evaporator	Nil	FTKKP, UMP	To recover solvent

3.4 Apparatus

All apparatus that was used for the study were tabulated in Table 3.3.

Table 3.3: List of apparatus

Apparatus Name	Size
Analytical weight balance	1 kg, 5 kg
Digital Caliper	Nil
Paper	Nil
Storage box	Nil
Glove	S, M, L
Polyethylene bag	1200 mL, 1000 g
PVC Necks	Ø: 20mm
Cap 4 in 1	Ø: 20mm
Net Cap 4 in1	Ø: 20mm
Resting cover	Ø: 20mm
Beaker	50 ml, 250 ml, 500 ml
Measuring cylinder	50 ml, 100 ml
Syringe	5 ml
Parafilm	4" x 125

3.5 Research Methodology

The overall research flow diagram of this study on mushroom cultivation using various agricultural waste was simplified in Figure 3.1. To limit the scope of this study, materials for mushroom substrate were primarily chosen based on the main agricultural activity in Malaysia. There were empty fruit bunch (EFB) palm press fibers (PPF), sugarcane bagasse (SGB), corn cob (CC) and spent mushroom substrate (SMS).

Prior the mushroom cultivation process, the agricultural wastes such as EFB, PPF, SGB, CC, RS and SMS were characterized by using a CHNS analyzer, Thermogravimetric Analysis (TGA) and X-ray Fluorescence (XRF). Oil contents determination in the EFB, PPF, SGB and CC were carried out via Soxhlet Extraction process in order to investigate the effect of oil content on mycelium growth rate and mushroom growth.

There were five major steps in mushroom cultivation such as preparation of substrates, sterilization, inoculation, incubation period and harvesting process. Firstly, the dry of RS, EFB, PPF, SGB, CC and SMS with uniform particle size (1mm - 2 mm) were used as a fruiting body of *P. ostreatus* either incorporating with RS or in a sole medium. All of substrate formulations were supplemented with the wheat bran and limestone with the ratio of 100:5:1.5 in w/w. After that, the charcoal application either in the sole substrates or in the substrates that incorporating between the RS and agricultural wastes were prepared. A 2% of charcoal from 100% of dry weight of the substrate was added in the substrates. Spent mushroom substrates (SMS), a spent substrate were recycled, were also used. The SMS either in sole or incorporating with the RS, EFB, PPF, SGB and CC that supplemented with wheat bran and limestone were prepared. All substrates were placed in polyethylene bag with 15cm height. The moisture content, pH, substrates (RS, EFB, PPF, SGB and CC) and additional nutrients used in cultivation (wheat bran and limestone) and *P. ostreatus* spawn were constant variables along the study.

Secondly, all the substrate was sterilized using a sterilization chamber at

100^{o}C for eight hours. After the sterilization was done, all the substrates were left to cool down at room temperature prior to the inoculation of *P. ostreatus* process.

Thirdly, a 10 g of spawn was inoculated in every mushroom bed. All the baglogs were subsequently then kept for incubation process at 23°C to 25°C with the relative humidity approximately at 85% until all the baglos were fully colonized by mycelium. Finally, all the mushroom bags were transferred to the mushroom house and the covered of the mushroom bags were opened in order to induce the first fruiting body. The water was sprayed using an automatic sprinkler twice a day. The pin head formation was seen in 2 to 3 days after the mushroom bag cover was opened. The mushroom was matured between 2 to 3 days after the tiny pin head formation were formed. The fresh mushroom was harvested. After the first flush, the mushroom bag was closed for another 7-10 days to induce the second flush. A 3 harvests were collected in this study. The days mycelium growth, the days for pin head and fruiting bodies formations, the total weight of fruiting bodies from three flushes were obtained.

The pH and moisture content of all the mushroom baglogs were analyzed using a pH meter and moisture content analyzer. The morphological parameters, nutrient composition and mineral content of mushroom fruiting bodies by comparing five substrates; RS, EFB, PPF, SGB and CC obtained by using proximate analysis and Inductively Coupled Plasma Mass Spectrometry (ICPMS). The percentage of EFB, PPF, SGB and CC waste recovered also was calculated.

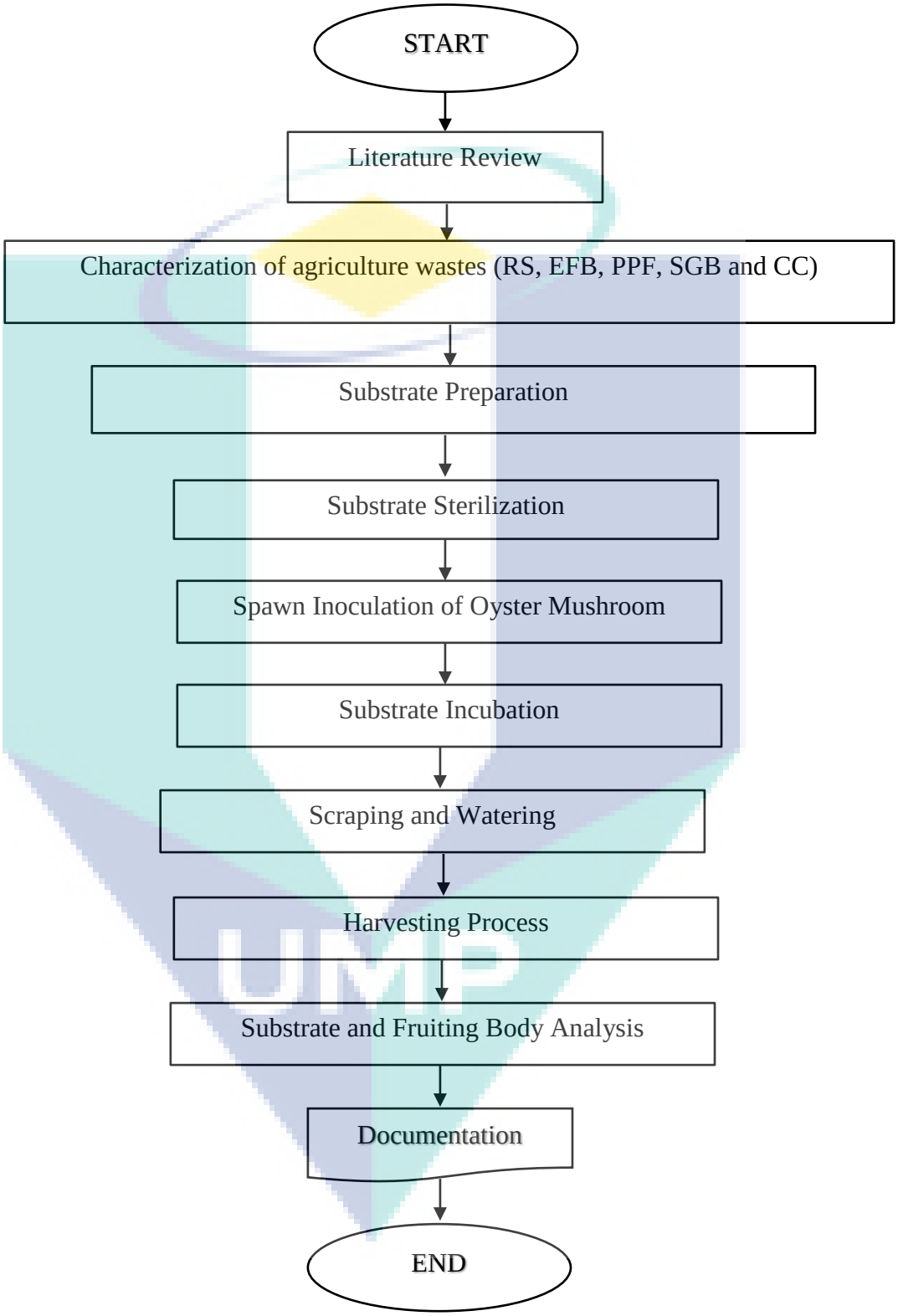

Figure 3.1: Research methodology process flow

3.6 Characterization of Agricultural Wastes

The sample of each type of agricultural wastes were characterized using CHNS analyzer in order to obtain carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) content in EFB, PPF, SGB, CC and RS. Thermal degradation analysis was carried out using Thermal Gravitmetric Analyser (TGA) in order to determine biomass cellulosic and thermal degradation profile. The metal composition analysis of various agricultural wastes was done using X-ray Fluorescence (XRF) analysis. The oil content analysis in EFB, PPF, SGB and CC were performed using soxhlet extraction process.

3.6.1 Ultimate Analysis

The ultimate analysis was conducted to determine the elements present in the agricultural wastes of EFB, PPF, SGB, CC and RS. The CHNS Elemental Analyzer (Elementar, Japan) at Central Laboratory, UMP was used for the analysis according to ASTM D5373-02. The CHNS Elemental Analyzer provides a means for determination the total of carbon, hydrogen, nitrogen and sulphur in organic matrices and other types of material. The CHNS Elemental Analyzer was capable of handling a wide variety of samples, including solids, liquids, volatile samples, in the field of pharmaceuticals, polymers, chemicals and food. The CHNS required high temperature in an oxygen-rich environment. In the combustion process, carbon was converted to carbon dioxide, hydrogen to water, nitrogen to nitrogen oxides and sulphur to sulphur dioxide.

3.6.2 Thermogravimetric Analysis (TGA)

Thermogravimetric analyzer (TGA) (STA-7000, Hitachi, Japan) was used to determine the thermal stability of the sample by observing the behavior of the weight change at elevated temperature. The sample temperature was normally increased in air or inert gas, such as nitrogen, flow. The TGA was a normal test, where the degradation temperature, moisture content and the decomposition points of organic and inorganic substance was carried out, to determine the characteristics of material.

About 5 to 10 mg of EFB, PPF, SGB, CC and RS was placed in the aluminium pan. The analysis was set using nitrogen at 50mL/min and heating rate at 10^{o}C/min from room temperature to 900^{o}C. Then, the air was used at 900^{o}C for 30 minutes.

3.6.3 Analysis of Metal Composition

Analysis of metal composition for EFB, PPF, SGB, CC and RS was done using X-ray Fluorescence (XRF) analyser. The binder was used to compress the agricultural waste samples into pallet shape. After that, the sample was put in X-ray Fluorescence (XRF) sample case and arranged in the prescribed positions in the machine, then the sample case was located in analysis hole and the result of the metal composition of each sample was displayed after 1 minute on a monitor of a computer interfaced with the machine. This analysis was carried out at CARIFF, UMP.

3.6.4 Determination of Oil Content in EFB, PPF, SGB and CC

The sample of EFB, PPF, SGB and CC was sun dried in order to remove the water content. Then, the sample of EFB, PPF, SGB and CC was grinding into small particle size using the blender. The sample was weighed by using an analytical weight balance.

The extraction of EFB, PPF, SGB and CC was performed using soxhlet extraction method (Figure 3.2). A 15-20 grams of samples (EFB, PPF, SGB and CC) was placed in the porous cellulose thimble. Then, the thimble was placed in the extraction chamber, which was hold above the round bottom flask containing 250 ml of acetone as solvent. The extraction was done at 56°C for 45-60 minutes.

After the extraction process completed, the separation of oil and solvent was carried out using rotary evaporator (Figure 3.3). The mixture of solvent and oil were heated at 56°C until solvent was fully recovered. The obtained oil was then weighted using an analytical weight balance and kept in the sample bottle. The oil content was determined as:

$$Oil\ content\ (\%) = \frac{Oil\ weight\ (g)}{Sample\ weight\ (g)} x\ 100\% \qquad (3.1)$$

Figure 3.2: Soxhlet extraction

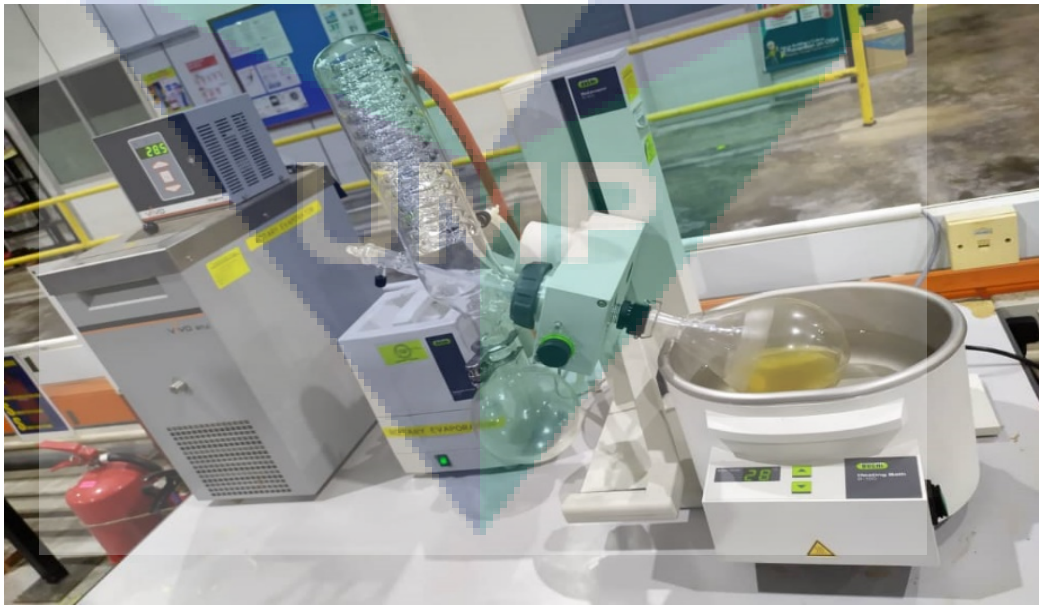

Figure 3.3: Rotary evaporator

3.7 Experimental Procedure

3.7.1 Preparation of the Substrates

The EFB, PPF, SGB and CC that used in the investigation were shown in Figure 3.4. The EFB and PPF were collected from Kilang Sawit Lepar Hilir, Gambang, Pahang, while the SGB was collected at Local Farmers in Kuantan, Pahang and the CC was collected by weekly at Pasar Malam, Gambang, Pahang, respectively. These wastes were collected in massive quantity and dried under the sun to remove water content. The samples were then ground and sieve to obtain 1-2mm samples' size.

(a) EFB (b) PPF (c) SGB (d) CC

Figure 3.4: The collection of EFB, PPF, SGB and CC

The mushroom cultivation process was practiced according to Ali et al., (2013). Cultivation process involve several steps including mixing and packaging, sterilization, inoculation, incubation, scraping, watering, and harvesting.

Dry materials such as sawdust, EFB, PPF, SGB and CC, wheat bran, and lime hydrated agriculture ($CaCO_3$) were mixed to the mass ratio of 100: 5: 1.5. All materials were combined and mixed thoroughly in a storage box until no lumps of mixture are found and no calcium carbonate are visible. The tap water (80% of the total weight of mixture) was then added into the mixing substrate to increase the moisture content until all the water was absorbed. About 600-700g of each substrate was placed in polyethylene bag with 15 cm height and a volume of 1,178 cm^3. The bags of substrate were then compressed and closed with PVC-necks and a cap.

Five different of the substrates with various mass ratio were prepared and tabulated in Table 3.4. Substrate of 100% RS was used as a control sample. The effect of charcoal application substrate preparation mixture was presented in Table 3.5. Moreover, the spent mushroom substrate (SMS) was also recycled and the substrate composition was presented in Table 3.6.

Table 3.4: Composition of different mass ratio of the substrate

Material combination	**Dry Material**	**Supplement**	
EFB + RS	25% EFB + 75% RS, 50% EFB + 50% RS, 75% EFB + 25% RS, 100% EFB	Wheat bran (5% from dry weight used)	Limestone ($CaCO_3$) (1.5% from dry weight used)
PPF + RS	25% PPF + 75% RS, 50% PPF + 50% RS, 75% PPF + 25% RS, 100% PPF		
SGB + RS	25% SGB + 75% RS, 50% SGB + 50% RS, 75% SGB + 25% RS, 100% SGB		
CC + RS	25% CC + 75% RS 50% CC+ 50% RS, 75% CC + 25% RS, 100% CC		

Table 3.5: Charcoal addition in substrate mixture composition

Dry Material	**Supplement**		
100% RS	Wheat bran (5% from dry weight used)	Limestone ($CaCO_3$) (1.5% from dry weight used)	Charcoal (2 % from dry weight used)
100% EFB			
100% PPF			
100% SGB			
100% CC			
50 % EFB + 50% RS			
50% PPF + 50% RS			
50% SGB + 50% RS			
50% CC + 50% RS			

Table 3.6: Spent mushroom substrate (SMS) for mushroom production

Dry Material	Supplement	
100% SMS	Wheat bran (5% from dry weight used)	Limestone ($CaCO_3$) (1.5% from dry weight used)
75% RS + 25 % SMS		
50% RS + 50% SMS		
25% RS + 75% SMS		
50% EFB + 50% SMS		
50% PPF + 50% SMS		
50% SGB + 50% SMS		
50% CC + 50% SMS		

3.7.2 Bags Sterilization

In this study, the substrates were placed in the sterilization chamber and sterilized at 100^{o} C for eight hours. Then, all the substrates bags was left to cool at room temperature prior to inoculation process.

3.7.3 Inoculation/Spawning

In this study, inoculation process was organized in the clean room under aseptic condition to prevent contamination during inoculum growth.

The PVC cap of each substrate bag was opened and about 10g oyster mushroom spawn was filled in the mushroom beds. Then, the baglog was closed with the cap.

3.7.4 Incubation

The baglogs were subsequently placed vertically in designated incubation area at 23°C to 25°C, relative humidity approximately at 85% and allowing only 10% sunrays. Adequate ventilation was required to prevent the increase of carbon dioxide (CO_2) (Das & Mukherjee 2007).

3.7.5 Scraping and Watering

Mycelia colonization was completed in the incubation period of maximum 46 days, the baglog were placed horizontally on a growing chamber. The PVC cover was unfolded to induce the fruiting process. The excessive spawn was scraped out in an attempt to allow growth of fruiting bodies. All bags were then transferred to the mushroom house. Racks in the mushroom house was cleaned and wiped with 70% alcohol to reduce contamination before placing the fruiting bags. Water was sprayed using a sprinkler in the form of a fine mist to maintain the moisture, stabilizing the temperature and to trigger the growth of mushroom fruiting bodies for 10 minutes in the morning and evening every day. The duration of primordia (pin-head) and mushroom formation were observed. Yield of mushroom produced in first, second and third flush were determined.

3.7.6 Harvesting Process

The baglog must be handled carefully to ensure the quality of mushroom growth. At the initial stage, a tiny pinhead was formed on the surface of the

substrates after 3 days of scrapping and watering process. In two to three days, these pinheads were grown into the full size of mushrooms fruiting bodies and meant to be harvested. The fruiting bodies were harvesting manually by twisting to clock wise or anti-clock wise direction by hand without lifting stubs on the substrates at one time for uniform harvesting to the next flush. The bags that already cropped were closed for another 7-10 days until the next harvest. In total, three harvests were done in this study. The duration of mycelium growth, pin head and fruiting bodies formations were obtained. The total weight of fruiting bodies from three flushes were recorded.

The number of mushroom flushes was considered ended once mushroom bed expired or contamination occurred. Contamination in this study referred to when mushroom bed being impure or changes in color. Furthermore, the mushroom bed was considered expired after 90 days or no mushroom formation was observed.

3.8 Experimental Analysis

3.8.1 Substrates Analysis

Percentage of water content was the most significant parameter in the mixing process of the substrate. Moisture content in all materials used in the mushroom substrate was determined using moisture analyzer.

The pH of the substrate was determined using a pH meter.

3.8.2 Fruiting Body Analysis

3.8.2.1 Mycelia Growth and Pin Head Determination

After spawning, a line was drawn across the bags using a permanent marker at where the spawns have settled to serve as a reference point for the measurement of the rate of mycelia formation. A measuring tape was used to measure the distance travelled by the mycelia in the transparent bags at three day intervals. The rate of mycelia formation was then calculated by subtracting the new measurements from the previous measurements at each three day intervals.

The colonization of the substrate by the mycelia within the bags was monitored by measurement at three days intervals. The duration of mycelia fully colonized the substrate after the day of spawning where white mycelia formed throughout the substrates in the bag was also determined After the bags are slit open, the formation of primordia was observed every two days intervals. The duration of the first primordia formation was observed and recorded.

3.8.2.2 Fresh Mushroom Yield and Morphology

The mushroom yield was determined where the fruit bodies were weighed immediately after harvest using digital analytical weight balance for every flush.

Mushrooms growing on various substrate was harvested at maturity. The length from the base of the stipe to the pileus mushroom was measured using digital caliper. The diameter of the mushroom cap was also determined using digital calliper.

3.8.2.3 Biological Efficiency (B.E)

Biological efficiency was a formula to determine the ability of mushroom strain to convert substrate materials into fresh mushroom. At the end of the third flush, biological efficiency of fruiting substrate was calculated as equation 3.2 (Chang et al. ,1993).

$$Biological\ efficiency\ (\%) = \frac{Total\ fresh\ weight\ of\ mushroom\ produced}{Dry\ weight\ of\ substrates} x\ 100\% \quad (3.2)$$

3.8.2.4 Fruiting Bodies Composition Analysis

Sample of fresh cultivated *P. ostretaus* from the first harvest that grown on various composition of the substrate is subjected to proximate analysis. The test was conducted according to the Association of Official Analytical Chemists (AOAC, 1995). These include the determination of protein, total fat, moisture content, ash, crude fiber, total carbohydrate, energy and crude fiber. This analysis was done at UNIPEQ, Universiti Kebangsaan Malaysia.

The mineral content and heavy metal trace were analyzed in fruit bodies of cultivated *P. ostreatus* from the first flush by using Inductively Coupled Plasma source with a mass spectrometer (ICPMS) at Central Laboratory, Universiti Malaysia Pahang.

UMP

CHAPTER 4

FINDINGS

4.1 Agricultural Wastes Characterization

4.1.1 CHNS Analysis

P. ostreatus requires carbon, nitrogen and inorganic compounds as its nutritional sources with the main nutrients being carbon sources such as cellulose, hemicellulose, and lignin. The elemental analysis and the carbon to nitrogen ratio (C/N) of RS, EFB, PPF and SGB are presented in Table 4.1.

Table 4.1: Element Analysis of RS, EFB, PPF and SGB

Element	RS	EFB	PPF	SGB
C	38.7	42.62	38.57	44.5
H	4.97	4.66	4.78	4.21
N	0.39	1.29	1.69	0.39
S	0.33	0.69	0.5	0.84
C/N ratio	7.79	9.15	8.07	10.57

Table 4.1 showed that the SGB had the highest C/N ratio compared to PPF and EFB. Meanwhile, the lowest C/N ratio was observed for RS (7.79). The *P. ostreatus* needs the optimum C/N ratio to grow well on substrates. The SGB contains more

carbon than EFB, RS and PPF. (Naraian et al., 2008) relate that the development of mycelium growth and pinhead formation is dependent on C/N ratio.(Philippoussis et al., 2011) reported that oyster mushrooms required more carbon and less nitrogen, however most of the substrates must be supplemented with nitrogen and carbon source to reach the optimal C/N ratio for the mushrooms to grow. Therefore, the types and the formulation of the substrates for *P. osteratus* cultivation should contain a balance content of carbon and nitrogen in order to achieve optimum C/N ratio. Table 2 tabulates the pH and moisture of block for various substrates compositions.

4.2 Effect on Different Substrates Compositions

Figure 4.1 represents the average days for completion of spawn running, first pinhead formation and first fruiting body formation for different substrates formulation. Beside substrate of 100% RS, 50% EFB + 50% RS, 50% PPF + 50% RS and 25% EFB + 25% PPF + 50% RS, other substrates managed a complete mycelium growth in less than 30 days. From the previous study, the mycelium growth rate increased gradually with the amount of RS (Ali et al., 2013). However, in this study, the substrates that contained SGB amount either 25% or 50% showed the fastest mycelium growth, attributable to the blending of RS and PPF to SGB which helped at optimizing the C/N ratio and the carbon sources in the substrates. Moreover, the SGB has higher sugar contain that can influence the rapid growth of mycelium as compared to others that are not incorporating with SGB. The substrate composition of 50% PPF + 50%RS displayed the lowest mycelium growth, followed closely by the 50% EFB + 50% RS , 100% RS and 25% EFB + 25% PPF + 50% RS substrates which was in contrast with (Hoa et al., 2015) that stated the combination of 50% RS and 50% SGB was slightly lower of mycelium growth than 100% RS. This is due to the difference in total C, total N of substrate formulas hence the difference in C/N ratio. C/N ratio had more effects on the mycelium growth, the formation and development of fruiting body (Hoa et al., 2015). Moreover, as the amount of RS decreases in the substrates formulation, slower mycelium growth was observed due to the larger particles size of the EFB and PPF in the substrates. The particle size of EFB and PPF should be sufficient enough in order to

mix well with RS. However, if the particles sizes are too small, the wet substrate can become over compact and hence, reducing the porosity and aeration available (Ali et al., 2013).

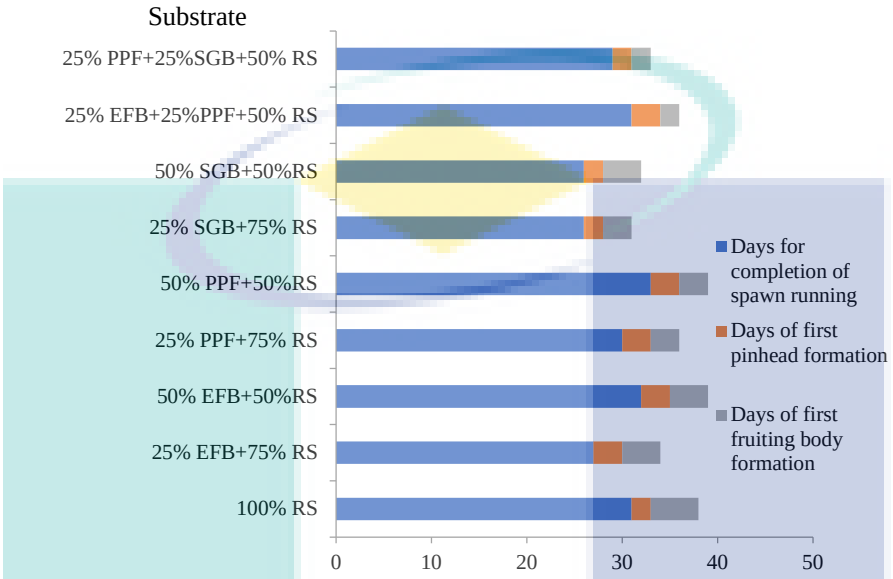

Figure 4.1: Average days for completion of spawn running, first pinhead formation and first fruiting body formation

Figure 4.1 also shows the time for first pinhead formation and time for first fruiting bodies formation and crop. Different pinhead and fruiting times were recorded, depending on the composition of the substrates. The substrates that contained SGB show the fastest pinhead and fruiting bodies formation i.e. only within 28-32 days. On the other hand, decreasing the amount of RS in the substrates reduced the pinhead and fruiting bodies formation rate, for example the substrates of 50% EFB+50% RS and 50% PPF+50% RS. The nitrogen rich source substrate speeded up the mycelium growth and pinhead formation (Jawad Ashraf et al., 2013). The fruiting body formation from almost all these substrates took between 2-4 days after pinhead formation (Figure 4.1). Fast growth of the mushroom also was influenced by addition of supplement (wheat bran mixed) (Das et al., 2007)

The yields of fruiting bodies were recorded from three replicates and calculated as an average as shown in Table 4.2. Theoretically, the yield was gradually decreased over

the flush. The highest mushroom yields were mainly from the first flush, except those from the 100% RS substrates which recorded the highest yields from the second flush. The highest total yield and biological efficiency, BE (%) was from the 25% PPF+25%SGB+50% RS substrate, while the lowest total yield and BE (%) was obtained from 50% PPF+50%RS. However, in this study, the 50% EFB+50% RS and 25% EFB+25% PPF+50% RS had resulted in the third flush being higher than second flush might be due to the environmental factor such as temperature and relative humidity that not easy to be maintained and can be affected the second flush yield. All the substrates that contained SGB resulted the higher total yield and BE (%) compared with other substrates due to higher sugar contain in SGB itself. In general, the BE(%) in the present study was far lower in comparison with other studies. (Bhattacharjya DK, 2014) observed that BE (%) of *P. ostreatus* grown on different RS substrates ranged from 187% to 213.2%. However, the results of this study were similar to (Zeng-Chin Liang, 2011) who found that BE(%) of *P.pulmonarius* between 39.55 to 58.33% that was grown on the stalks of three grass plants in Taiwan.

Table 4.2: Yield for every flushes and biological efficiency of different substrates

Substrates	Average yield (g/kg substrate)				Total yield (g/kg substrate)	Biological efficiency (%)
	1st flush	2nd flush	3rd flush	4rd flush		
100% RS	55.67	62.53	48.14	36.43	202.77	67.59
25% EFB+75% RS	107.58	55.8	46.62	40.82	250.82	62.71
50% EFB+50%RS	88.17	39.12	45.72	41.67	214.68	53.67
25% PPF+75% RS	75.23	58.16	53.38	52.11	238.88	59.72
50% PPF+50%RS	80.67	66.38	30.00	16.95	194.00	48.50
25% SGB +75% RS	92.86	64.90	48.86	43.57	250.19	62.55
50% SGB+50%RS	100.29	61.10	46.57	42.86	250.82	62.71
25% EFB+25%PPF+50% RS	111.05	30.83	57.05	42.67	241.60	60.40
25% PPF+25%SGB+50% RS	123.60	72.83	64.62	57.83	318.88	79.72

4.3 Substrate Analysis

These are two known contributing factors on the growth of *P. ostreatus* (Bellettini et al., 2019). The highest pH substrate (pH 9) was obtained from substrate of 25% EFB + 25%PPF + 50%RS, followed by 25% SGB + 75% RS, 25% EFB + 75% RS, 25% PPF + 25%SGB + 50% RS and the lowest pH was obtained by the composition of 50% SGB + 50%RS which was around pH 7.5. These result concurs with (Nwokoye et al., 2010) where the substrates should in the pH range of 3 – 10 for mushroom to grow in the tropics and thus the ability of the mycelia to tolerate the temperature of 28^{o}C. Besides, oyster mushroom can grow at moderate temperatures, ranging from 18-30^{o}C (Jaramillo Mejía et al., 2013). On the other hand, water is also one of the main factors that influence the success mushroom to grow well. The highest moisture content was obtained from the substrate compositions of 50% SGB + 50% RS which was around 78% and the lowest is from the 25% SGB + 75% RS substrates which was close to 69% as shown in Table 4.3. According to (Wetzel et al., 2004), the high moisture content in the substrate would result in difficult breathing for the mycelium, inhibiting perspiration, rendering the development of fruiting body impossible and resulting the development of non-desired organism such as bacteria and nematodes. However, the low moisture content may also result in death of the fruiting body (Bellettini et al., 2019). It is reported that the suitable moisture content should be in the range between 50% and 75% for substrate (S. T. Chang et al., 2004)

Table 4.3: Chemical properties of substrates compositions

Substrates	**pH**	**Moisture content (%)**
25% EFB + 75% RS	8.5	76.47
25% SGB + 75% RS	8.75	68.74
50% SGB + 50%RS	7.5	78.35
25% EFB + 25%PPF + 50% RS	9.3	74.89
25% PPF + 25%SGB + 50% RS	8.2	70.92

4.4 Fruiting Body Analysis

The nutritional analysis of the first fruiting bodies of 100 % RS and 50% RS that combine either EFB, PPF and SGB substrates was shown in Table 4.4. There was non-detectable fat content of the mushroom from all the substrate 50%, except the substrates from 50% PPF + 50% RS that only had the small amount of the fat content (0.09). The first flush fruiting bodies from the 50% PPF +50% RS had higher protein content compared to other substrates. (Ali et al., 2018) reported that the substrate that has higher C/N ratio contributes to the higher protein content (Ragunathan et al., 2003) found the protein content of mushroom depended on the C/N ratio and the chemical composition of the substrates. The result in the present study in contrast with the finding from (Bellettini et al., 2019) that stated increasing protein content in the mushroom resulted in low fat content.

Table 4.4: Nutritional contents (%) and Energy (kcal/100g) of P. ostreatus for first flush

Substrates	Energy (kcal/100g)	Nutritional contents of *P. ostreatus* (%)					
		Protein (%)	Total Fat (%)	Total Carbohydrate (%)	Crude Fiber (%)	Moisture (%)	Ash (%)
100% RS	42.5	2.90	0	7.67	6.19	88.78	0.66
50% EFB+50%RS	50.5	2.77	0	9.81	6.07	86.42	1.00
50% PPF+50%RS	48.5	3.82	0.09	8.19	6.06	87.09	0.82
50% SGB+50%RS	30	2.06	0	5.43	6.15	91.78	0.73
25% EFB+25%PPF+50% RS	35	3.14	0	5.63	4.90	90.27	0.97
25% PPF+25%SGB+50% RS	35.5	2.32	0	6.58	6.59	90.33	0.79

The mushroom that grows from 50% EFB + 50% RS had the highest total carbohydrates (9.81%), followed by 50% PPF + 50% RS, 100% RS, 25% PPF + 25% SGB + 50%, 25% EFB + 25% PPF + 50% RS. Meanwhile, the lowest content of total carbohydrates was in mushroom that grow from the substrates of 50% SGB + 50%RS. (W. Li et al., 2015) found that the low C/N ratio resulted in high carbohydrate content. The mushroom from 25% EFB + 25% PPF + 50% RS substrates has the lowest crude

fiber content and the others substrates were not statistically different in term of crude fiber content. It can be seen in Table 4.4 that the moisture content of mushrooms from all substrates almost similar to each other's which is in the range of 92 - 87%. The higher moisture content in the mushroom from the 50% SGB + 50% RS substrate is probably due to the greater water-holding capacity of the combination of SGB and RS itself. The highest ash composition obtained from the 50% EFB + 50% RS substrate and the lowest 100% RS which is 1 and 0.66 respectively.

Substrates are not only affect on the protein, carbohydrate, and fat content in mushroom, but also influenced on total energy of *P. ostreatus* as shown in Table 4.4. Total energy contribution of the samples ranged between 30-50.5 kcal/100 g dry weight for *P. ostreatuss*. Thus, it can be deduced that *P. ostreatus* was low in calorie food because they provided almost zero content of fat. This healthy food is one of appropriate food to be consumed for those who are watching on diet.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

It is found that the growth and yield performance of *P. otreatus* are dependent on C/N ratio of the substrate. The substrates that contain SGB in the ratio of 0.25 w/w and 0.5 w/w gave better the mycelium rate, fastest pinhead and fruiting bodies formations as well as the yields and BE (%) due to high sugar contain in sugarcane bagasse and resulting the optimum C/N ratio for mushroom to growth well. The substrate of 25% PPF + 25%SGB + 50% RS resulting in the best formulation in term of growth performance, yield and BE (%) are at 35 days for first harvest, 318.88 g/kg substrate and 79.72% respectively, as compared to other formulations of the substrates. However, the other combinations of the substrates also have a tremendous potential to be as an alternative substrate for mushroom cultivation in Malaysia. The particle size, C/N ratio, pH, moisture content can significantly affect the growth of *P. ostreatus* and mineral content on the substrates. The advantages of using the biomass generated from the palm oil mills which is available throughout the year at little and no cost, and sugarcane bagasse that abundance in the rural and urban areas in Malaysia will not only solve the environmental pollution problem, but it can also offer an economically promising way to convert low quality biomasses into a valuable high protein food for human and also can help further income generation to the mushroom growers.

5.2 Recommendation

There are several suggestions that recommended to be implemented in recovering the agricultural wastes for future studies:

a) Recovering other agricultural wastes such as kenaf, cocoa, pineapple, fruits and vegetable wastes.
b) A further recommendation to enhance this study is to increase the temperature during a substrate sterilization up to 121^{o}C by using an autoclave.
c) This study could be expanded to another phase of sustainable development which is using polypropylene plastic bottle instead of polyethylene plastic bag in mushroom cultivation. Thus, minimize the wastage in the production of mushroom.
d) The SMS produced in this study should be applied practical ruminant feeding in Malaysia.

CHAPTER 6

ACHIEVEMENT

6.1 Name of articles/ manuscripts/ books published

[1] Fathie binti Ahmad Zakil, Mohd Shafiq bin Mohd Sueb and Ruzinah Isha. Growth and Yield Performance of *Pleurotus ostreatus* on Various Agro-Industrial Wastes in Malaysia. Proceedings of the 2nd International Conference on Biosciences and Medical Engineering (ICBME2019) AIP Conf. Proc. 2155, 020055-1–020055-7; https://doi.org/10.1063/1.5125559 Published by AIP Publishing.

[2] F Ahmad Zakil, K H Muhammad Hassan, M S Mohd Sueb and R Isha. Growth and Yield of *Pleurotus ostreatus* using Sugarcane Bagasse as an Alternative Substrate in Malaysia. IOP Conference Series: Materials Science and Engineering, 736 (2020) 022021, doi:10.1088/1757-899X/736/2/022021.

6.2 Title of paper presentations (international/ local)

[1] Growth and Yield Performance of *Pleurotus ostreatus* on Various Agro-Industrial Wastes in Malaysia. 2nd International Conference on Biosciences and Medical Engineering (ICBME2019).

[2] Growth and Yield of *Pleurotus ostreatus* using Sugarcane Bagasse as an Alternative Substrate in Malaysia. Engineering Security and Chemical Engineering Congress (ESChE) 2019.

6.3 Human Capital Development

[1] Nor Sasila binti Jasmi- On going Master

[2] Fathie binti Ahmad Zakil-On Going PhD

6.4 Awards/ Others

[1] Silver Medal in CITREX 2019. Waste to wealth: Low Cost Fruiting Body Substrate for Pleurotus sotreatus Cultivation using Various Agro-Industrial Wastes in Malaysia. Organized by Universiti Malaysia Pahang.

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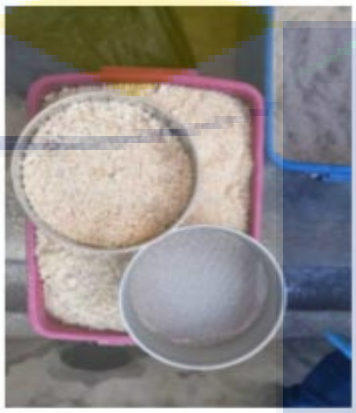
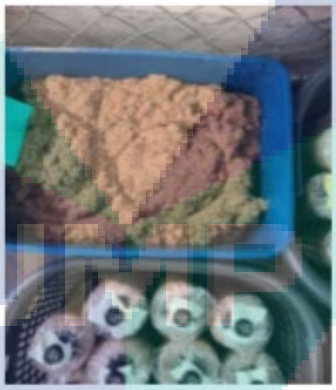
APPENDIX A - Photos of oyster mushroom growth



Sieves the substrates



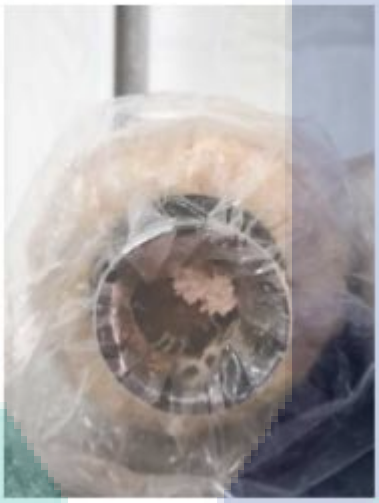
Mixed substrates with different ratio and packed them in the transparent plastic bag (each baglog is 15cm)

Sterilization at 100°C for 8 hours

Oyster mushroom spawns

Mushroom spawns are injected into the baglogs

Incubation



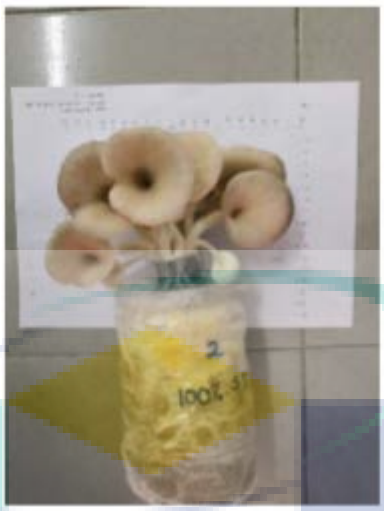
Pin head formed

Mushroom growth

Young oyster mushrooms growth

Mature oyster mushrooms formed and ready to harvest

Data collection



Oyster mushroom that growth on 100% RS

BUDGET

NO	VOTE	AMOUNT APPROVED (RM)	TOTAL EXPENSES (RM)	PERCENTAGE (%)
1	V11000	18,000.00	17,998.50	99.99%
2	V14000			
3	V21000	10,000.00	9,890.57	98.91%
4	V22000			
5	V23000			
6	V24000			
7	V26000			

8	V27000	28,834.00	28,832.00	99.99%
9	V28000			
10	V29000	13,905.00	13,904.72	99.99%
11	V35000	6,800.00	6,800.00	100%
TOTAL		**77,539.00**	**77,425.79**	**99.85%**