CHAPTER 3

METHODOLOGY

3.1 Process Flow Chart

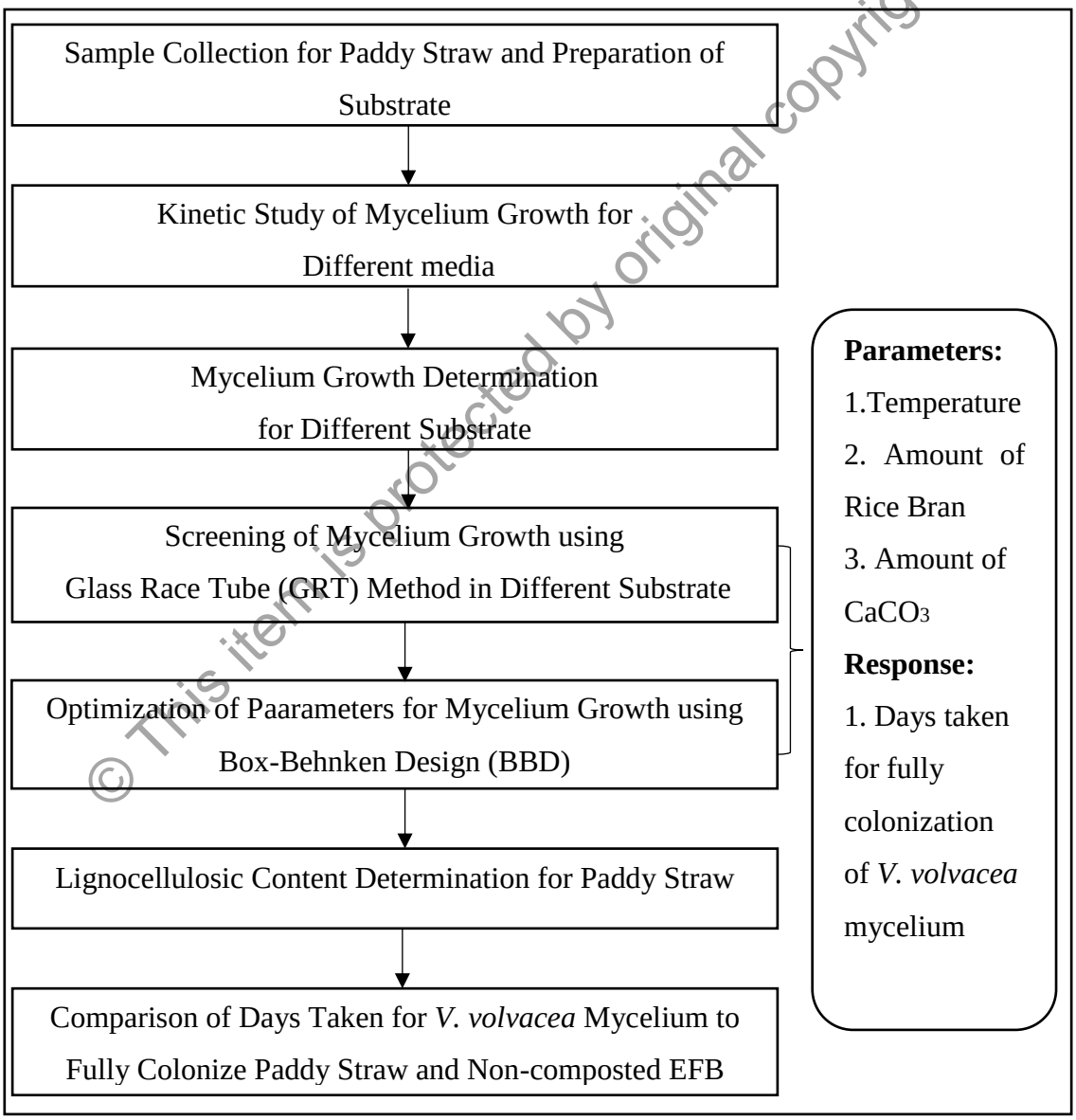

Figure 3.1: Overall process flow chart

3.2 Description of Apparatus, Chemicals and Glassware

The list containing the type of chemicals that were needed in the experiment are shown below in Table 3.1. All the materials that used in the experiment are listed in Table 3.2. A list of the equipment are also shown in Table 3.3.

Table 3.1: List of chemicals

Name	Brand
Malt extract (ME)	Merck
Nutrient agar (NA)	Merck
Potato dextrose agar (PDA)	Merck
Calcium carbonate	N/A
Sodium Hydroxide	Sigma-Aldrich
Sulphuric acid	Sigma-Aldrich
Barium Chloride	Sigma-Aldrich
Agar	Sigma-Aldrich
Glucose	Sigma-Aldrich
Benzene	Sigma-Aldrich
Ethanol	HmbG Chemicals

Table 3.2: List of materials

Name	Brand
Volvariella volvacea mushroom	N/A
Grains (paddy rice)	N/A
Paddy Straw	N/A
Rice bran	N/A
Cotton wool	Bella cotton
Plastic petri dish	IdealCare
Aluminum foil	Diamond

Tables 3.3: List of equipment

Name	Brand
Furnace	WiseTherm
Low temperature incubator	Centronics
Oven	Memmert
Soxhlet extractor	Wheaton
Weighing balance	Sartorius

3.3 Kinetic Study of Mycelium Growth

3.3.1 Preparation of Media

Malt extract agar (MEA), potato dextrose agar (PDA) and nutrient agar (NA) was prepared according to the formula as shown in Table 3.4. All media was autoclaved at 121 ˚C, 1 atm for 20 minutes and was poured into petri dish for solidification in laminar flow before storing into fridge for inoculation of mushroom tissue. The preparation of all three media was followed according to the manufacturer label.

Table 3.4: Preparation of MEA, PDA and NA media

Media	Malt extract powder (g)	PDA powder (g)	NA powder (g)	Agar (g)	Glucose (g)	Distilled water (L)
MEA	20			15	5	1
PDA		39				1
NA			10			1

3.3.2 Preparation of Pure Mother Culture

Cell and tissue culture technique was used to culture the pure mother culture from the fresh straw mushroom. The mushrooms was surface sterilized with 70 % ethanol and sliced longitudinally with approximately 3 mm each for the inner and outer part of the mushroom. A sterilized cork borer with diameter 5 mm was used to cut the sliced mushroom into cylindrical shape and transferred into 3 different media to incubate under 25 ˚C. The same steps was used to subculture the mycelium of the mushroom for the fully colonize petri dish.

Plate 3.1: Outer surface of straw mushroom

Plate 3.2: Inner heart of straw mushroom

3.3.3 Radial Growth Measurement of Mycelium Extension of *V. volvacea* in Different Culture Media

The average radial mycelium growth extensions was measured from one end to the another end at three different loci and recorded every day until the mycelium had fully colonized the petri dish. The best average radial mycelium growth extensions was chosen for preparation of inoculums.

3.3.4 Kinetic Study of Mycelium Growth on Different Culture Media

The measured radial growth of mycelium extension on different extension on different media were studied using dynamics of microbial growth as in heterogeneous cultivation techniques as shown in Equation 3.1, 3.2 and 3.3 (Panikov, 2002).

$$\frac{dR}{dt} = \mu_w W = K_r \tag{3.1}$$

And

$$R = R_0 + K_r t \tag{3.2}$$

And

$$td = \frac{\ln 2}{\mu_w} \tag{3.3}$$

Where *R* and *W* are radius of the radial growth of mycelium extension. td is the doubling time of the radial growth of mycelium extension, K_r is the radial growth of mycelium linear expansion rate in mm/h and μ_w is the mycelium specific growth rate.

3.4 Screening and Mycelium Growth Determination for Different Substrate for Mother Spawn Production

3.4.1 Preparation of Substrates

A total of 0.2 kg of composted and non-composted EFB, RSD, paddy and PS were washed with clean water for three times and soaked in distilled water for 24 hours. The distilled water was poured away after 24 hours of soaking and excess water was drained by placing into a 100 - 105 ˚C oven for 5 hours. Distilled water was reintroduced until the moisture content of each substrates reached 58 - 62 %. Then, the substrates were mixed with rice bran and $CaCO_3$ with a ratio of 200:10:1 (Zervakis *et al.*, 2001). Once done preparing the substrates, the substrates were autoclaved under 121 ˚C, 1 atm and 15 min. The cooling process of the sterilized substrates was cooled for 24 hours before ready to inoculate.

3.4.2 Preparation of Inoculums

The selected growth media with growing mycelium was cut into 9 mm cylindrical shape and transferred into the substrates. The cylindrical cut mycelium was known as mycelium plug.

3.4.3 Screening of Mycelium Growth of *V. volvacea* Mushroom Cultivation for Mother Spawn Production using GRT.Method in Different Substrate

In order to design the optimization studies, different substrate were used to find and screened out which substrate is the most suitable for *V. volvacea* mushroom cultivation.

3.5 Evaluation of the Mycelium Growth of *V. volvacea* on Different Substrates using Glass Race Tube (GRT) Method for Mother Spawn Production

3.5.1 Kinetic Study of Mycelium Growth on Different Substrates using GRT Method for Mother Spawn Production

Mycelium growth on different substrates were determined by using GRT method (1.5 cm height and 0.02 cm diameter). All the tubes were filled completely based on the volume of the substrates as mentioned in 3.4.1. After autoclaved the tubes, the tubes was allowed to cool down to room temperature before inoculating the mycelium plug into the sterilized substrates and incubated in 30 ˚C incubator. The mycelium growth rate was determined by measuring the mycelium extension form the inoculation point and by calculating the average mycelial extension at four equidistant point of circumference of the tubes. The data obtained were analysed by using Equation 3.4 (Zervakis *et al.*, 2001)

$$y = k_t x + c \tag{3.4}$$

Where k_t is the mycelium growth rate in cm/day, *y* is the distance and *x* is the time.

3.5.2 Optimization of Mycelium Growth of *V. volvacea* Mushroom Cultivation for Mother Spawn Production

Temperature, amount of rice bran and $CaCO_3$ were the parameters that was optimized to study the mycelium growth rate in this study. These parameters were optimized using Box-Behnken Design (BBD) in Design-Expert® version 10.

3.5.3 Optimization of Mycelium Growth of *V. volvacea* Mushroom Cultivation for Mother Spawn Cultivation using Box-Behnken Design (BBD)

The BBD was used to study the interaction between the parameters that affects the mycelium growth rate. Among the three screened parameters, the significant parameters were chosen to be optimized further. A total of 17 runs (if all three parameters are significant) was conducted with triplicates as in Table 3.5 and Table 3.6 below.

Table 3.5: Range of parameters for BBD design for the chosen parameters in optimization (Design-Expert® version 10)((Diamantopoulou *et al.*, 2016)

Parameters	Symbols	Range and Levels		
		-1	0	+1
Temperature (˚C)	A	25.0	30.0	35.0
Amount of Rice Bran (g)	B	0.50	0.75	1.00
Amount of $CaCO_3$ (g)	C	0.05	0.08	0.10

Table 3.6: The model design to study the effect of the chosen factors in optimization (Design-Expert® version 10)

Run	Factors: A	Factors: B	Factors: C	Response: Days
1	30.0	0.75	0.08	
2	25.0	0.75	0.05	
3	30.0	1.00	0.10	
4	30.0	1.00	0.05	
5	35.0	0.75	0.05	
6	35.0	1.00	0.08	
7	25.0	1.00	0.08	
8	35.0	0.50	0.08	
9	30.0	0.75	0.08	
10	25.0	0.75	0.08	
11	30.0	0.50	0.10	
12	30.0	0.75	0.08	
13	25.0	0.50	0.08	
14	30.0	0.75	0.08	
15	35.0	0.75	0.10	

3.5.4 Mycelium Growth Rate

The mycelium growth rate was determined by measuring the length or height (in mm) of the mycelium propagation in the spawned compost for every 2 days until the compost is fully colonized. The mycelium growth rate was based on 3 replicates and average values was calculated.

3.6 Collection and Preparation of Paddy Straw

The paddy straw were collected from Pertubuhan Peladang Kawasan Kobah Pendang, Kedah. The paddy straw was cut into smaller size, dried and shredded by using a shredder.

3.6.1 Lignocellulosic Content Determination of Paddy Straw

The analysis of the lignocellulosic content of paddy straw samples would involve five different procedures. Firstly, the ash content of PS was determined according to American Standard Test Method. Subsequently, the analysis of extractives was done by removing the extractives from PS sample. After the removal of extractives, hemicellulose content is removed from the PS sample through the dissolution of the sample in NaOH and followed by the dissolution of lignin content in H_2SO_4. Lastly, the cellulose content was calculated by using the difference of ash, extractives, hemicellulose and lignin from the total of 100 % of lignocellulosic content in PS. All analytical experiments were run in triplicates and the average values was calculated.

3.6.2 Analysis of Ash

An empty crucible and cover were ignited in a furnace at 600 °C for 20 min. After heating, the crucible was cooled down in desiccators and was weighed to the nearest 0.001 g (M_1). Then, a total weight of 2.0 g of PS was placed in the crucible and drying process was took placed at 100 to 105 °C with the crucible cover. After 1 hour, the crucible with PS sample were weighed (M_2). The drying and cooling process were repeated until the weight were constant to within 0.0001 g. Consequently, the crucible with the PS sample were placed in the muffle furnace and were heated to 10 °C/min until the final ignition reach temperature of 600 ℃ for 1 hour. Finally, the crucible with the content were cooled in desiccator for 30 min. The heating for 30 min period were repeated until the weight

after the cooling process become constant to within 0.002 g (M_3). The % ash, A_d was calculated using Equation 3.5 (Sluiter *et al.*, 2008).

$$\% \, Ash, A_d = \frac{M_3 - M_1}{M_2 - M_1} \times 100\% \quad (3.5)$$

3.6.3 Analysis of Extractives

By utilizing benzene/ethanol with ratio of 2:1 in volume, the dried biomass sample (G_o, *g*) was leached for 3 hours. The sample was introduced to air-drying and the residue was placed in an oven at 105–110 ℃ until constant weight was obtained. After the cooling of sample in a desiccator, the sample was weighed again (G_1, *g*). The extractive wt. % was calculated using Equation 3.6 (Li et al., 2004).

$$W_1 \, (\text{wt.}\%, \text{d}) = \frac{G_o - G_1}{G_0} \times 100\% \quad (3.6)$$

3.6.4 Analysis of Hemicellulose

The residue from G_1 was placed in a flask and mixed with 150.0 mL of NaOH (20.0 g/L). The sample was then boiled for 3.5 hours with distilled water. The sample was filtered and washed until Na^+ is absence in the residue using distilled water. The residue was cooled and weighed (G_2, *g*). The hemicellulose wt. % was calculated using Equation 3.7 (Li *et al.*, 2004).

$$W_2 \, (\text{wt.}\%, \text{d}) = \frac{G_1 - G_2}{G_0} \times 100\% \quad (3.7)$$

3.6.5 Analysis of Lignin

A total of 1.0 g of the residue of extractives analysis was placed in weighed flask and dried to a constant weight. After the cooling in a desiccator, the sample was weighed again (G_3, g) and 30.0 mL of H_2SO_4 (72 %) was poured into the sample. The temperature of the mixture was kept at 8 – 15 ℃ for 24 hours. The mixture then was transferred into a flask for dilution with 300 mL of distilled water. The sample then was boiled in distilled water for 1 hour. After that, the sample undergoes cooling and filtration process. The residue from cooling and filtration process was washed until sulfate ion in the filtrate is absence using 10 % barium chloride solution. Then the sample was dried to a constant weight and cooled to room temperature. Lastly, the sample was weighted (G_4, g). The lignin wt. % was calculated using Equation 3.8 (Li *et al.*, 2004).

$$\mathrm{W_3\ (wt.\%, d)} = \frac{\mathrm{G_4\ (1-W_1)}}{\mathrm{G_3}} \times 100\% \tag{3.8}$$

3.6.6 Analysis of Cellulose

The cellulose wt. % was calculated using Equation 3.9 (Li *et al.*, 2004).

$$\mathrm{W_4\ (wt.\%, d)} = 100 - (A_d + W_1 + \mathrm{W_2} + \mathrm{W_3}) \tag{3.9}$$

3.7 Comparison of the Total Days Taken for *V. volvacea* Mycelium to Fully Colonize the Substrate Paddy Straw and Non-composted EFB

The preparation of substrate PS and non-composted EFB was prepared as described in 3.4.1. Next, the substrates were loaded into a high temperature resistance plastic bag "bongkah" with height 20 cm and 7 cm width. The autoclave and cooling process was shown in 3.4.1 before inoculating the mycelium plug. After the inoculum

was placed into the substrates as mentioned in 3.4.2, some cotton wool was placed on top of the opening and sealed tightly with a cap. The total days taken for *V. volvacea* mycelium to fully colonize the substrate was observed and recorded (Apetorgbor *et al.*, 2015).