EXOPOLYSACCHARIDE FROM MYCELIUM OF *Ganoderma lucidum* AS A POTENTIAL ADDITIVE FOR JUVENILE RED HYBRID TILAPIA (*Oreochromis* sp.)

JANATHU FERDAUS BT PAJARU RAHMAN

FACULTY OF SCIENCE

UNIVERSITI MALAYA

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EXOPOLYSACCHARIDE FROM MYCELIUM OF *Ganoderma lucidum* AS A POTENTIAL ADDITIVE FOR JUVENILE RED HYBRID TILAPIA (*Oreochromis* sp.)

ABSTRACT

Functional feed additives are added during feed preparation to improve the growth performance and cellular defense for the fishes. Interaction of cellular biomolecules and dysfunction of red hybrid tilapia (*Oreochromis* sp.) could be avoided by strengthening the antioxidant defense with antioxidant rich sources. As for this, a bioactive polysaccharide of *Ganoderma lucidum* was found to have its antioxidant bioactivities. Thus the potential of exopolysaccharide (EPS) extracted from the mycelium of *G.lucidum* as feed additive for red hybrid tilapia was evaluated in this research. In this experiment, one hundred twenty (120) juveniles were randomly divided into four groups and designated with experimental diets of EPS at 1.0 g/kg, 2.0 g/kg, 3.0 g/kg and control group with 0 g/kg of EPS. Growth performance was assessed in the term of weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), feed intake (FI) and survival rate (SR). Antioxidant activity of Glutathione S-transferase (GST) and Catalase (CAT) were determined from liver samples collection of red hybrid tilapia. Fatty acid profile was expressed as fatty acid methyl esters (FAME). The results of the present study indicate that the growth performance on WG, SGR, FCR, FI, and SR of red hybrid tilapia were positively impacted by the different inclusion of EPS from mycelium *G. lucidum*. GST activity decreased in red hybrid tilapia fed with 1g/kg and 2g/kg of EPS as feed additive. In contrast, CAT activity was lower in the control group, which might attribute due to the presence of beta glucan in EPS feed. Experimental diet of 1 g/kg and 2 g/kg of EPS shows conceivable fatty acid content in red hybrid tilapia.

In conclusion, EPS from the mycelium of *G.lucidum* can be taken as feed additive on red hybrid tilapia without enforcing any negative impact on the growth performance, antioxidant activity and fatty acid profile. Briefly, the present study indicated 2g/kg sufficiently acceptable as a feed additive as it shows improved growth performance as well as exhibit an antioxidant effect with an improved fatty acid profile of the red hybrid tilapia.

Keywords: *Oreochromis* sp, *Ganoderma lucidum*, growth performance, antioxidant activity, fatty acid profile.

EKSOPOLISAKARIDA (EPS) DARI MISELIUM *Ganoderma lucidum* SEBAGAI POTENSI TAMBAHAN MAKANAN PADA IKAN JUVANA TILAPIA HIBRID MERAH (*Oreochromis* sp.)

ABSTRAK

Aditif makanan fungsian ditambah semasa penyediaan makanan untuk meningkatkan prestasi pertumbuhan dan pertahanan selular untuk ikan. Interaksi biomolekul sel dan disfungsi tilapia hibrid merah (*Oreochromis* sp.) dapat dielakkan dengan menguatkan pertahanan antioksidan dengan sumber kaya antioksidan. Bioaktif polisakarida *Ganoderma lucidum* didapati mempunyai bioaktif antioksidan tersendiri. Oleh itu, penyelidik telah mengekstrak eksopolisakarida (EPS) dari miselium *G.lucidum.* Kajian ini bertujuan untuk menilai potensi EPS dari miselium *G.lucidum* sebagai aditif makanan pada tilapia hibrid merah. Dalam eksperimen ini, seratus dua puluh (120) ikan juvana dibahagikan secara rawak kepada empat kumpulan dan ditetapkan dengan diet eksperimen EPS pada 1.0 g/kg, 2.0 g/kg, 3.0 g/kg dan kumpulan kawalan dengan 0 g/kg EPS. Prestasi pertumbuhan dinilai dalam aspek pertambahan berat badan (WG), kadar pertumbuhan tertentu (SGR), nisbah penukaran makanan (FCR), pengambilan makanan (FI) dan kadar kelangsungan hidup (SR). Aktiviti antioksidan 'Glutathione S-transferase' (GST) dan 'Catalase' (CAT) ditentukan dari pengumpulan sampel hati tilapia hibrid merah. Profil asid lemak telah dinyatakan sebagai metil ester asid lemak (FAME). Keputusan kajian ini menunjukkan bahawa prestasi pertumbuhan pada WG, SGR, FCR, FI dan SR memberi kesan positif kepada tilapia hibrid merah. Aktiviti GST dalam 1g/kg dan 2g/kg EPS menurun dalam tilapia hibrid merah. Sebaliknya, aktiviti CAT lebih rendah dalam kumpulan kawalan, hal ini mungkin kerana kehadiran beta glukan dalam EPS. Diet eksperimen 1 g/kg dan 2 g/kg EPS menunjukkan asid lemak

yang baik dalam tilapia hibrid merah. Kesimpulannya, EPS dari miselium *G.lucidum* boleh diambil sebagai aditif makanan pada tilapia hibrid merah tanpa memberi kesan negatif terhadap prestasi pertumbuhan, aktiviti antioksidan dan profil asid lemak. Secara lanjut, kajian ini menunjukkan 2g/kg EPS boleh diterima sebagai aditif makanan kerana ia menunjukkan prestasi pertumbuhan yang lebih baik serta menunjukkan kesan positif antioksidan dan profil asid lemak yang baik pada tilapia hibrid merah.

Kata kunci : *Oreochromis* sp , *Ganoderma lucidum,* prestasi pertumbuhan, aktiviti antioksidan, profil asid lemak.

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LIST OF SYMBOLS AND ABBREVIATIONS

^oC	:	Degree Celsius
g	:	Gram
kg	:	Kilogram
µl	:	Microlitre
mg	:	Miligram
ml	:	Mililitre
min	:	Minute
%	:	Percentage
s	:	Seconds
AA	:	Arachidonic acid
ALA	:	Alpha-linolenic acid
ANOVA	:	Analysis of variance
AOAC	:	Association of official analytical chemist
ARA	:	Arachidonic acid
CAT	:	Catalase
CM	:	Corn meal
DCP	:	Di-calcium phosphate
DHA	:	Docosahexaenoic acid
DO	:	Dissolved oxygen
EFA	:	Essential fatty acids
EPA	:	Eicosapentaenoic acid
FA	:	Fatty acid
FAME	:	Fatty acid metyl ester
FAO	:	Food and agriculture organization

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Aquaculture could be a potential dynamic segment within the worldwide, which has been growing rapidly and characterized to be a significant venture in numerous locales of in Malaysia It has been distinguished as one of the vital divisions within the coming a long time, as a portion of a worldwide arrangement to the quick exhaustion within the world. In spite of the fast development of the aquaculture industry in Malaysia, the government is still looking to expand and enhance aquaculture practices in Malaysia.

According to the Fisheries Research Institute Malaysia (2019), red hybrid tilapia (*Oreochromis* sp.) has been increasing in popularity among the producers. Inevitably, they contribute approximately 90% of the total tilapia production in Malaysia. Concurring to Venugopal (2019), the point is to boost tilapia production by more than 70 percent within the next four years. As a result, the production of tilapia can be predicted as the division with the most prominent potential to meet the request in Malaysia. Yusoff et al. (2018) report that due to the outbreak of bacteria and pollution, the government had banned the locals from purchasing and consuming tilapia.

Naturally, living animals are equipped with defence systems against free radical damage, including oxidative enzymes and chemical compounds (Kruger et al., 2003), which includes aquatic living organisms. Thus, living animals have anti-oxidative compounds that help them to ensure against responsive oxygen species (ROS), which also could interact with cellular biomolecules and lead to cell injury or even death (Calabrese et al., 2005). Aging , infections and the multi contamination from the encompassing environment may diminish their anti-oxidative status, and this seems to result in a

significant loss in total production and economy of the aquatic and terrestrial animals (Ahmed et al., 2014).

Functional feed additives have become an alternative to antibiotics, and it had been used to improve growth, immune response, induce the physiological functions and health performance of the fishes over the standard feed additives (Antony et al., 2019). There are strict regulations and controls on the application of antibiotics and chemotherapeutics in aquafeeds due to bioaccumulation (Lim et al., 2013). Research has proved that many chemicals manufactured and utilized nowadays enter the environment, disperse, and persist in the environment for much longer initially anticipated (Kolpin, 2002).

Ganoderma lucidum polysaccharides (GLPs) is one of foremost examined biologic compounds and has its antioxidant bioactivities (Kan et al., 2015). According to Stajić et al. (2013), phenolic compounds, polysaccharides, proteins, organic acids, alkaloids, and nucleotides have been reported to possess antioxidant activities. Usage of mushrooms as functional feed additives in aquaculture has improved significantly due to their properties (Spolaore, 2006). Besides, in a study by Vamanu (2012), exopolysaccharides (EPS) showed a higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity than the samples from intracellular polysaccharides (IPS), and the researcher believed this could certainly be explored as a new potential antioxidant. Evidently, EPS eliminates ROS that are formed through diverse metabolic reactions, and it exhibits antioxidant activity (Pérez-Ramos et al., 2016).

On the other hand, synthetic antioxidants have been used to decrease lipid oxidation during the processing and storage of fish products (Jooyandeh & Aberoumand, 2019). In any case, the use of synthetic antioxidants has raised questions concerning food safety

and toxicity (Chaijan et al., 2005). Thus, the researcher needs to find antioxidant comprising feed to inhibit oxidation and the formation of ROS and FR occurring in the fish.

However, to date there are no scientific studies conducted on EPS of *G. lucidum* has the potential to serve as a feed additive for red hybrid tilapia. Hence, this has driven to the purpose of this study as a preliminary study to determine the potential of EPS from the mycelium of *G.lucidum* as feed additive on red hybrid tilapia. Thus, the researcher suggests EPS from the mycelium of *G.lucidum* produced could be a potential sustainable feed additive on red hybrid tilapia.

1.2 Objectives of the study

1. To examine the growth performance of the red hybrid tilapia by using the feed additive of EPS from the mycelium of *Ganoderma lucidum*.

2. To investigate the antioxidant activity of red hybrid tilapia by using the feed additive of EPS from the mycelium of *Ganoderma lucidum*.

3.To determine fatty acid profile of the red hybrid tilapia by using the feed additive of EPS from the mycelium of *Ganoderma lucidum.*

1.3 Hypothesis of the study

The feed additive of EPS from the mycelium of *Ganoderma lucidum* can be taken as feed additive on red hybrid tilapia diet without enforcing any negative impact on the growth performance, antioxidant activity and fatty acid profile of the red hybrid tilapia.

CHAPTER 2: LITERATURE REVIEW

2.1 Tilapia (*Oreochromis* sp.)

Tilapia (*Oreochromis* sp.) is one of the most adaptable and successful aquaculture species cultured worldwide (Figure 2.1). Additionally, it is known to be high tolerance to crowding, disease resistance and a wide range of salt tolerance (Yusoff, 2018).

In a review by the World Fisheries and Aquaculture (2012), according to Food and Agriculture Organization (FAO) statistics, global tilapia production grew to approximately 3.5 million tonnes in 2010 and increased to more than 3.7 million tonnes in 2012. The statistics affirmed tilapia as one of the most popular fish cultivated in the world with China as the largest producer of 1.35 million tonnes production in 2012. Thus, this indicates tilapia has been the most abundant and commercially vital species in aquaculture.

In another source, "Fisheries Research Institute Malaysia" (2019) has stated that red tilapia has been the second-largest fish production after catfish in Malaysia. However, the production of tilapia has been found to decrease each year, from 51, 554 mt (2012) to 35, 996 mt (2016). Thus, the decrease in tilapia production has reviewed activities and recalled actions to improve fish management strategies and tilapia culture practices.

Figure 2.1 : Tilapia (*Oreochromis* sp.)

2.1.1 Previous research on supplementary diets in aquaculture

Probiotic microbial feed supplements are gaining wide and may be connected to aquaculture generation frameworks. Probiotics are living microbial cells acting as nutrient sources, providing enzymes for better digestion, modulating the immune system and increasing the immune response against pathogenic bacteria (Williams, 2017). In research by Shelby et al. (2006), probiotic microorganisms show no significant difference is observed in growth and development, total serum immunoglobulin, specific anti-streptococcal antibody levels, or mortality due to infection. The results also suggest that these commercially accessible probiotic microorganisms do not provide any beneficial effects in juveniles Nile tilapia.

In the context of fungi, Mohd Din et al. (2012) have stated that mushroom (*Pleurotus sajor-caju*) supplementation as a prebiotic compound into the super worm meal based diets for red tilapia (*Oreochromis* sp.) and its effect on growth performances had shown a positive result. The researcher also stipulates that 10% supplementation level of mushroom stalk meal as a prebiotic for tilapia could be used in the insect-based diet, *Zophobas morio* for red tilapia (*Oreochromis* sp.).

Similar to the result, Kamilya et al. (2008) claim that consideration of mushroom (*Pleurotus florida*) glucan in catla diets enhanced the catla non-specific immunity and resistance to a pathogenic bacterial challenge. However, the researchers also state that such enhancement might be temporary and dependent on the feeding period and consideration level of the fish.

There are few research, which includes Muin et al. (2014) who have replaced mushroom as rice bran to prepare feed for Nile tilapia and Vetvicka et al. (2004) who expressed glucan was evaluated as an immunostimulant in common carp. Mostly, the in-vivo antioxidant properties of mushrooms were conducted using animals other than fishes.

With vitamin E supplement diet, 200 mg of vitamin E/kg diet was found to be similar to those of the control group. Supplementation of Nile tilapia (*Oreochromis niloticus*) diets with vitamin E at 100 and 150 mg of vitamin E/kg diet improves carcass quality by increasing the polyunsaturated fatty acids (PUFA): saturated fatty acids (SFA) ratio and Omega 3and Omega 6 levels. (Hasnat et al., 2013).

2.2 *Ganoderma lucidum*

A few decades away ago, medicinal mushrooms, particularly *Ganoderma lucidum* have been used as feed supplements to improve various human health parameters in Asia (Oei, 2003). Figure 2.2 shows the fruiting body of *G. lucidum* which is known as *Lingzhi* in Chinese Pharmacopoeia (Lv et al., 2012). Numerous studies have found that the extricates from fruiting bodies and mycelia of *G. lucidum* were found to possess in vitro antioxidant activity (Jones & Janardhanan, 2000).

This has also been supported by Ajith & Janardhanan (2007) who claim that medicinal mushrooms occurring in South India namely *Ganoderma lucidum*, *Phellinus rimosus*, *Pleurotus florida* and *Pleurotus pulmonaris* possessed a profound antioxidant and antitumor activities which indicates that these mushrooms would be profitable sources of antioxidant and antitumor compounds. However, in the same studies, Ajith & Janardhanan (2007) argues that intensive and extensive investigations are needed to exploit their valuable therapeutic use, as there is a various exploration that can still be done.

In another context, Hsu et al. (2017) reported that beta-glucan (beta-1,3-glucan) is one of the major components found in fungal cell wall for the development of fungi, which is well recognized as a bioactive polysaccharide. Hasnat et al. (2013) states antioxidative effect are due to increase of specific enzyme activity, which is from the arrangement of a β-glucan-protein complex, and antioxidant effect is due to the increase in the rate of β-glucan activity.

Beta-glucan of *G. lucidum* in the diet of grass carp juveniles has increases in the terms of survival rate, weight gain, feed intake and specific growth rate. Additionally, extraction from the mycelium of *G. lucidum* contains polysaccharides, proteoglycans, triterpenoids, and other active compounds, which have been used to treat numerous diseases and illnesses (Ma et al., 2015).

In recent paper by Taufek et al. (2020), common mycelial biomass and extraction of exopolysaccharide from the pre-grown Malaysian *G.lucidum* mushroom might be a potential dietary supplementation in the future as no toxicity effect have been observed in zebrafish embryo. Thus, this indicates *G.lucidum* could be a potential feed addictive in aquaculture.

Figure 2.2 : Fruiting body of *Ganoderma lucidum* (Photo source from Supramani et al., 2019)

2.2.1 *Ganoderma lucidum* polysaccharides (GLP) in aquaculture

In the aquaculture research, dietary administration of polysaccharides has proven to stimulate the immune response and reduce pathogens load which leads to a better survival and growth performance of fish (Mohan et al., 2015).

In a previous study, by Chithra et al. (2016), the administration of 1.0 g/kg of GLPs shows a significant improvement in the fish survival, weight gain, length gain, feed intake, and specific growth rate. In this way, this indicates that GLPs can promote the feeding, followed by better survival and growth performance of fish.

Mohan et al. (2016) also stated that dietary supplementation of GLPs showed significant influence on digestive enzymes' secretion which in turn increases nutrients utilization, followed by better growth, muscle biochemical composition, amino acid and fatty acid profiles with elevated alkaline phosphatase (AKP) and acyl carrier protein (ACP). Besides, the insignificant alteration in antioxidant and metabolic enzymes' activity indicates the good health status of prawns.

2.3 Antioxidant

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells (Steinbaum, 2017). Antioxidants are molecules in cells that prevent free radicals from taking electrons and causing damage. Szalay (2016) stated that antioxidants are able to give an electron to a free radical without becoming destabilized themselves, thus stopping the free radical chain reaction, supported by Tan et al. (2018) who have

proven that antioxidant cause autoxidation by interrupting the propagation of free radicals.

Naturally, organisms are protected with several antioxidants against oxidative environments, including catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) (Harris et al., 2015). Both Glutathione S-transferase (GST) and Catalase (CAT) provides the inner cell first defense system for clearing the free radicals. (Chen, 2009).

2.3.1 Catalase (CAT)

Catalase (CAT) is accountable for reducing H_2O_2 to H_2O, commonly found in peroxisome and is responsible for catalysing the decomposition of hydrogen peroxide (H_2O_2), producing water and molecular oxygen in addition to protect the cell against oxidative harmful effect (Sun, 1990). The large-molecule antioxidants are enzymes such as CAT, and GSHPx with sacrificial proteins (albumin) that absorb ROS and prevent them from attacking other essential proteins (Nimse & Pa, 2015).

In previous research by Metwally (2009), the comes about appeared that, by feeding garlic as supplementary on Tilapia *Nilotica*, catalase (CAT) has a positive impact on the treatment group as compared to the control group. The researcher has concluded that the addition of garlic in any form to the fish diet can increase the antioxidant activity in fish. In another studies, fructooligosaccharide has also shown a positive effect on the fish antioxidant activity (Zhang et al., 2014).

Next, Atli & Canli (2007) reported that CAT activity increased in liver of *O. niloticus* due to an effective antioxidant defense system acting against oxidative stress caused by metal exposures and/or compensating for the decrease in other antioxidant enzymes.

It has also been suggested that the supplementation of 1.0 – 2.5 g kg^{-1} GLPs did not produce any toxic effects to *M. rosenbergii.* (Mohan et al., 2015). To date, studies related to antioxidant enzymes in fish nutrition is still lacking especially in determining CAT activities. Thus, in order to ensure that the feed materials given to the fish do not contain any harmful substances, studies in determining CAT activities in the nutritional analysis is essential.

2.3.2 Glutathione S-transferase (GST)

Glutathione S-transferase (GST) plays a vital role in detoxifying endogenous toxic metabolites and numerous foreign contaminants (Dasari et al., 2018). The same researcher also said GST helps to detoxify toxins emerging from oil, pesticides, and other hydrocarbon components. Kannan & Venkatachalam (2015) observed that the significant alterations in the GST activities specifically reflect the metabolic disturbances and cell damage in particular organs of aquatic animals.

In the other context, increased GST activity was observed in the liver of *O. niloticus* exposed to 5 mg/L cadmium exposure for 30 days (Basha & Rani, 2003), 3 mg/L for 5, 10, and 20 days (Xu & Bai , 2007), and 2 mg/L for 8 days (Lin et al., 2011) and they indicated that the increased level of GST in liver showed a possible shift toward a detoxification mechanism.

Naturally, glutathione is an imperative antioxidant which presents in every microorganisms, plants, and animals. The function is to protect the cells damage induced by ROS including lipid peroxides, peroxides, free radicals, and heavy metals (Pisoschi & Pop, 2015).

2.3.3 Antioxidant polysaccharides of *Ganoderma lucidum*

Cor et al. (2018) reveals that *G. lucidum* is capable in radical scavenging abilities of polysaccharides and polysaccharide-complex isolated from different parts of the crude *G. lucidum*. It is now confirmed that *G. lucidum* induces a self-triggered immune response and is a very powerful antioxidant.

According to Mahendran et al. (2012) the total antioxidant capacity of EPS crude extracts of *G. lucidum* was found to be maximum in malt medium (82.30 ± 1.2 %). Sanodiya et al. (2009) argues chloroform extracts of *G. lucidum* showed significant superoxide scavenging activity (IC50: 144.6 ± 1.5 μg/ml) and is of potential interest as a source of strong natural antioxidants in the food industries.

2.4 Fatty Acid In Aquaculture

Fish fatty acid profiling is important for human wellbeing, particularly in reducing the occurrence of heart diseases, strokes, and various inflammatory injuries (Maina et al., 2003). Fatty acid molecules are classified based on the presence of the number of bonds: saturated fatty acids (SFA) have single bond, and monounsaturated fatty acids (MUFA) have one double bond; polyunsaturated fatty acids (PUFA) have two or more double

bonds. The number and position of the double bonds determine the physical and chemical properties with functional characteristics of fatty acids.

Results indicated that the wild tropical freshwater fish studied are not good sources of n-3 HUFA fatty acids (Suloma et al., 2008). The researcher also states that, tilapia lipid appears to be intermediate in nutritional quality with low proportions of DHA and EPA. According to Williams CA (2000) states that linoleic acid (LA) sources are from soy and maize oil while arachidonic acid (AA); the main n-3 alpha-linolenic acid (ALA) source is from meat. The researcher also states linoleic acid (LA), an n-6 fatty acid, can be converted into longer chains fatty acids, and ALA can be converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Studies have also shown that the content of n-3 Polyunsaturated fatty acids (PUFAs) in Nile tilapia fillets increased when the fish was fed plant oils enriched with 18:3n-3 (Carbonera et al., 2014). In another study by Ng & Teoh (2019) claims replacing fish oil into vegetable oil has decrease the health beneficial omega-3 long chain PUFA content and it is considered a major challenge. In general, total or partial replacement of vegetable oil has been reported to have no deleterious effect on the growth performance of a variety of warm water fish species as long as their essential fatty acid requirements are met.

CHAPTER 3: METHODOLOGY

3.1 Exopolysaccharide Production

3.1.1 Fungal Source

Ganoderma lucidum (Strain: QRS 5120) were obtained from Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Universiti Malaya (UM).

3.1.2 *Ganoderma lucidum* Mycelium Cultivation

After undergoing aseptic preparation by washing with 99.9% ethanol (Sigma-Aldrich,Dorset, UK) for 10 seconds, *G.lucidum* were dried in a laminar flow. After the drying process, the dried *G.lucidum* were scratched using a scalpel and the content were removed by using forceps to twist its body. The mycelium growth were taken place on the content called as tissue, on malt extract agar (MEA) (Sigma-Aldrich,Dorset,UK) under a controlled temperature (room temperature). Next, the generation of mycelium undergone the same culturing method in a fresh new MEA medium. After obtaining, the pure mycelium, it were cultured in a potato dextrose agar (PDA) (Sigma-Aldrich,Dorset,UK) and slightly slanted at 4° for further inoculum production.

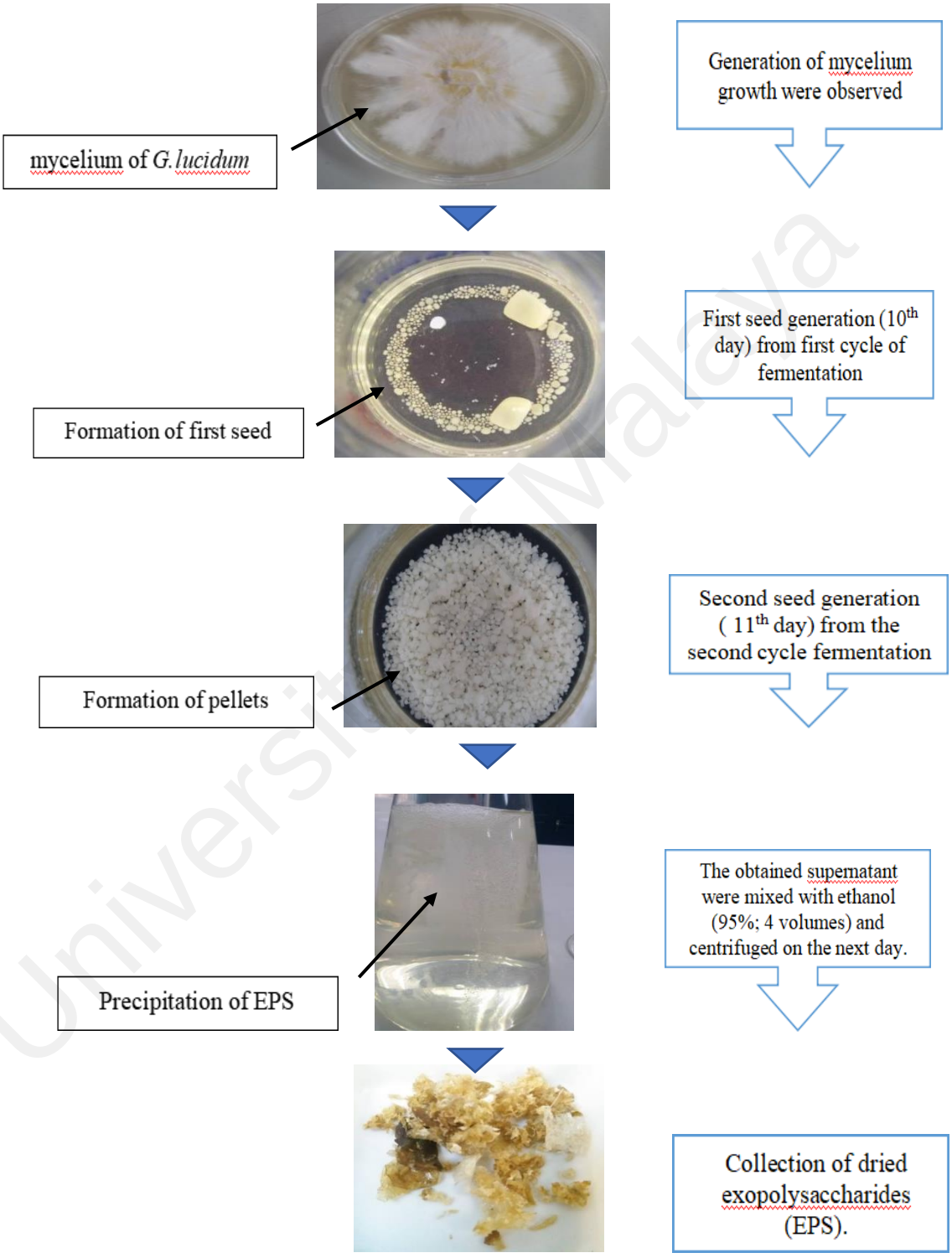
3.1.3 Exopolysaccharide Extraction

In this study, Submerged-Liquid Fermentation (SLF) method (Supramani et al., 2019) were used to enrich the growth of the microorganism. Supported by, Supramani et al. (2019) stated that SLF is efficient, reliable and less time consuming than Submerged-Solid Fermentation. The mycelium of *G.lucidum* undergone fermentation by using 500 ml Erlenmeyer flask in anaerobic condition with optimal media composition.

Two set of cycles were carried to obtain the maximum EPS production. The first seed generation was ended at 10^{th} day to upscale the growth of the pellets, while second seed generation was ended on the 11^{th} day of the process, all the samples were filtered using a Buchner funnel filter to help the separation of EPS from the mycelial biomass. The obtained supernatant were mixed with ethanol (95%; 4 volumes), and stirred well and later were left overnight at a controlled temperature (4°C). On the next day, the mixture were centrifuged at 10,000 rpm for 15 mins by using 50ml Falcon tube. The obtained precipitation were filtered out and dried to obtain the dried EPS. The dried EPS was collected and stored for the next procedure. The EPS yield were prior to the proximate analysis.

3.1.4 Flowchart of the production of EPS

Following are the flowchart of the production of EPS from the mycelium from the *G.lucidum* adapted from Supramani et al., (2019):



3.2 Diet formulation, preparation and proximate

Four different experimental diets were formulated for the supplementation of EPS at 1.0 g/kg, 2.0 g/kg, 3.0 g/kg and control group with 0 g/kg of EPS. Mini pelleting machine (KCM, Y1 32M-4) was used to produce standard sized of pellets with 0.3 cm in diameter for experimental diets and control group feed. The ingredients and proximate composition of control and experimental diets were formulated using WinFeed 2.8 version software (Table 3.1). After preparing the feed, it was dried in an oven at 60ºC for 24 hours to prevent fungal development. Later, it was been packaged and stored in a cool room (4°C). Proximate analysis of the formulated diet process were conducted at the Fish Nutrition Laboratory, Fisheries Research Institute, Glami Lemi, Jelebu, Negeri Sembilan. Samples from each diet were finely grounded for chemical test which incorporates protein, lipid, fibre, moisture and ash following method of Association of Official Analytical Chemist methods (AOAC 2012). Results of proximate composition of ingredients (Table 3.2) and experimental feed (Table 3.3) were tabulated.

Table 3.1: Formulation of the experimental diets (g/kg)

Ingredients	Control	1g/kg	2g/kg	3g/kg
FM (g/kg)	300.0	300.0	300.0	300.0
CM (g/kg)	193.9	193.5	193.1	192.7
RBM (g/kg)	199.3	198.9	198.6	198.2
SBM (g/kg)	236.8	236.6	236.3	236.1
EPS (g/kg)	0.0	1.0	2.0	3.0
Lysine (g/kg)	10.0	10.0	10.0	10.0
Methionine(g/kg)	5.0	5.0	5.0	5.0
Vitamin Premix(g/kg)	2.0	2.0	2.0	2.0
Mineral Premix(g/kg)	3.0	3.0	3.0	3.0
DCP (g/kg)	10.0	10.0	10.0	10.0
Fish Oil(ml)	40.0	40.0	40.0	40.0
Total	1000	1000	1000	1000

[1]Preparation of feed for 1000g per diet. FM: Fishmeal:CM:Cornmeal:RBM:Rice bran meal: SBM:Soya bean meal:DCP: Di-calcium phosphate: EPS: Exopolysaccharides

[2]The vitamin premix supplied the following per 100g diet. Vitamin A, 500IU; Vitamin D3,100IU;Vitamin E,0.75; Vitamin K, 0.02mg; Vitamin B1, 1.0 mg; Vitamin B2, 0.5mg:Vitamin B3, 0.3mg; Vitamin B6, 0.2 mg; Vitamin B12, 0.001 mg;Vitamin C, 0.1 mg Niacin, 0.2mg Folic Acid, 0.1mg, Biotin, 0.235mg; Panthothenic acid, 1.0mg Inositol, 2.5mg

[3]The mineral premix supplied the following per kg diet

Selenium,0.2mg; Iron,8mg;Manganese, 1.0mg; Zinc, 8.0mg; Copper, 0.15mg;Potassium Chloride, 0.4mg ; Magnesium Oxide, 0.6mg; Sodium bicarbonate, 1.55mg: Iodine, 1.0mg Cobalt, 0.25m

Table 3. 2: Proximate analysis of ingredients (%)

Components	Protein(%)	Fibre(%)	NFE(%)	Ash(%)	Lipid(%)
EPS	17.67	10.38	69.59	2.08	0.28
Fishmeal	54.27	14.54	5.60	23.16	2.41
Corn	6.64	9.81	79.23	1.73	2.60
Rice bran	11.23	19.40	55.30	5.30	8.76
Soy Meal	43.01	9.64	40.04	5.16	2.14

[1]Analysed in duplicate using the standard method for dry matter, moisture, crude protein, lipid and ash content. (AOAC, 2012).

[2] EPS: Exopolysaccharides, NFE : Nitrogen Free-Extract

[3] NFE = 100 - (% crude protein% crude fat+ % crude fibre+ % crude ash

Table 3.3: Proximate analysis of experimental feed (%)

Feed	Protein (%)	Fibre (%)	NFE (%)	Ash (%)	DM (%)	Lipid (%)	GE (kJ/g)
Control	37.86	1.63	42.22	12.00	87.25	6.29	18.98
1g/kg	36.30	1.70	42.85	12.86	66.63	6.29	18.72
2g/kg	37.02	1.92	43.82	12.40	71.48	4.84	18.49
3g/kg	35.19	1.76	43.93	12.45	72.05	6.67	18.80

[1]Analysed in duplicate using the standard method for dry matter, moisture, crude protein, lipid and ash content. (AOAC, 2012).

[2] NFE: Nitrogen Free-Extract, DM: Dry Matter, GE: Gross Energy

[3] GE = crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g

3.3 Proximate analysis

The experimental diet and ingredients were analysed for proximate composition according to the Association of Official Analytical Chemist method (AOAC,2003).

3.3.1 Crude protein

Kjeldahl method was used as a technique of quantitative determination for the crude protein during this research experiment. Titration indicator was prepared by dissolving 100 mg of bromo cresol green in 100ml methanol first then 70 ml methyl red solution was dissolved in 100ml methanol before both solutions were mixed together as a titration indicator. Later,150 mg of sample was weighed and later added into Kjeldahl digestion tube with 1 tablet of 100mg Selenium Kjeltabs Catalyst. Next, 6 ml of concentrated sulphuric acid (H_2SO_4) were also added into the digestion tube. Tubes were left to be digested in FOSS Tecator Digestor Auto at 420^oC for an hour. Then, it was allowed to cool down for 15 minutes before the distillation process. After the cooling process, 80 ml of deionized water (H_2O) and 50 ml of sodium hydroxide (NaOH) were added to each

digestion tube,and mixed thoroughly, then distilled with 25 ml of 4% boric acid as well as titration indicator. This was done in the programmable unit of Vapodest50 equipped with titrator with an automatic addition of H_2O, NaOH and boric acid. For the titration process, hydrochloric acid (HCl) with a concentration of 0.01 M was used to titrate the product of distillation in the flasks and titre them accordingly. The value was recorded. All blanks and samples were tested in duplicate. Data was recorded and crude protein was calculated according to the following formula:

The protein content of the sample and blanks calculated as :

Percentage of Nitrogen (%) = The protein content of the sample and blanks calculated as:

$$\text{Percentage of Nitrogen (\%)} = \frac{(S-B)\,(N)\,(14.007)\,(100)}{(g\,of\,sample)(1000)}$$

Where, S = titrate of HCL

B = Titrate HCL for blank

N= Normality of HCL => 0.0996

Percentage of Protein (%) = Percentage of Nitrogen (%) x 6.25

3.3.2 Crude lipid

Crude lipid was determined using the Soxhlet method with petroleum ether extraction to measure crude lipid content of prepared diets. Each sample was weighed around 4g and been added into the cellulose timber. Later, it was placed in a beaker with 80ml of petroleum ether and covered with aluminium foil. The extraction process performed for 1 day. Next day, the solution which includes petroleum ether and lipid were poured into a round bottom flask and further evaporated using a Buchi Rotavapor R-200 with Buchi heating Bath B-490 (Temperature: 45ºC) to separate lipid from petroleum ether completely. The cycle was repeated till a clear solution was observed and obtained during extraction process. After obtaining pure lipid, it was transferred into a weighed beaker and dried in oven at 80ºC until a constant weight obtained. All the samples were analysed in duplicate.

$$\text{Percentage of Lipid (\%)} = \frac{(W3-W2)}{(W1)} X \ 100$$

Where, W1 = Sample weight (g)

W2 = Initial weight of beaker (g)

W3 = Final weight of beaker (g)

3.3.3 Crude fibre

The crude fibre was determined after an alkali and acid digestion by using defatted samples from crude lipid extraction analysis. Fibre capsules with the lids were weighed together using an analytical balance. About 50 mg of samples were weighed and added into the fibre capsules and seal them with the lids. Extraction vessel with 350ml of 1.25% H_2SO_4 was placed on a hot plate and heated till it boils. The capsule tray with fibre capsules containing samples was placed in the carousel and put on the stopper to lock the capsules in place. The extraction of the carousel was partially lowered into the boiling reagent sufficient to immerse the samples. Gentle boiling was carried out for 30 minutes and after 5 minutes of boiling, the carousel was removed from the extraction vessel. The extraction carousel was washed with boiling water 3 times with fresh hot water each time. Then, the extraction vessel was filled with 350ml 1.25% NaOH on the hot plate and boiled. The same procedure as the H_2SO_4 was repeated and the washing procedure were performed 3 times. Later the samples were washed once in 1% HCl and finally in boiling H_2O. The capsules were dried in an oven at 130^{o}C for 2 hours. They were then cooled off in a desiccator and weighed. The weighed capsules were placed in pre-weighed and pre-dried crucibles for the ashing procedure at 600^{o}C for 4 hours. They were cooled off in a desiccator before reweighing to determine the crude fibre content. Data was recorded and crude fibre was calculated according to the following formula:

$$\text{Percentage of Fibre (\%)} = \frac{W3-(W1\ X\ C)-(W5-W4-D)}{(W2)}\ X \quad 100$$

Where, W1 = Weight of empty capsule and its cap(g)

W2 = Weight of sample (g)

W3 = Weight of capsule (g) after 130°C

W4 = Weight of empty crucible (g)

W5 = Weight of crucible after 600ºC

C = Black correction for capsule solubility, given 0.9995

D = Ash of empty capsule (g) given, 0.007.

3.3.4 Dry matter, moisture & ash

Dry matter was calculated from weight loss after 24 hours drying process at 105ºC. Ash was determined by drying in an oven at 600ºC for 4 hours. Crucible was weighed and added with 2.0 g of sample. The sample was then dried in an oven overnight at 100ºC. The crucible was cooled in a desiccator for 30 minutes before being weighed. Then the crucible was put into a furnace and heated at 600ºC for 4 hours. Once cooled, the crucible was weighed again. The following formula:

$$\text{Percentage of Dry matter (\%)} = \frac{(W3 - W1)}{(W2 - W1)} X \quad 100$$

Percentage of Moist (%) =100 - % dry matter

$$\text{Percentage of Ash (\%)} = \frac{(W4 - W1)}{(W3 - W1)} X \quad 100$$

Where,

W1 = Weight of empty crucible (g)

W2 = Weight of crucible + sample (g)

W3= Weight of crucible + sample (g) after 105°C

W4 = Weight of crucible + sample (g) after 600ºC

3.3.5 Nitrogen free extract

Nitrogen free extract (NFE) or carbohydrate were calculated as the following (Taufek et al., 2016):

NFE = 100 - (% crude protein% crude fat+ % crude fibre+ % crude ash).

3.3.6 Gross energy

Gross energy for every diet were calculated using the following factors: crude protein = 23.9 kJ/g, crude lipids = 39.8 kJ/g and NFE = 17.6 kJ/g (Schulz et al., 2005).

3.4 Experimental fish

Red hybrid tilapia was purchased from the local commercial farm situated at Sungai Buloh, Selangor and transported to the Freshwater Aquarium Laboratory located at the Institute of Biological Sciences, Faculty of Science, Universiti Malaya. One hundred twenty (120) mixed sex of juveniles red hybrid tilapia were randomly divided into four treatment groups, each treatment were carried out in duplicates as justified by Wan et al. (2020) with fifteen (15) fishes per tank with an average weight of 10 - 20g. All the fishes were acclimatized to natural conditions for 2 weeks prior to the feeding trials and were fed with a commercial diet, this is for the juveniles to adapt of their new surroundings and also to eliminate mortality fishes due to stress during transportation. Feeding was done twice per day at 0900h and 1500h during the acclimatization and experiment period. During the experimental period, fishes from each tank were observed and recorded if any mortality. In every weekend visit, water quality was monitored regularly following the Standard Method for Examination of Water and Wastewater APHA Method (1992).

Water temperature was maintained at 28–29 ^{o}C, pH at 6..5–7.5, dissolved oxygen (DO) above 6.5-8.0, ammonia <0.80 mg/L, and nitrate <1.9 mg/L.

3.5 Experiment set-up

Eight plastic tanks (32cm x 62cm x 34 cm) with a closed re-circulation system were used for feeding activities. The tanks were set up at Freshwater Aquarium Laboratory located at the Institute of Biological Sciences, Faculty Science, Universiti Malaya. The tanks were equipped with a top filter pump for the circulation of dissolved oxygen. Cover net was used to keep the fishes safe in the tank. For water replacement, tap water was treated with anti-chlorine before diffusing in. In addition, the water was replaced by 30% once every two days to maintain good water quality. Hebdomadally, 80% water were replaced for the better condition for the fishes. The water was maintained at the level of 3/4 of the height of the tank throughout the experiment.

3.6 Experimental Procedure

Feed additive of EPS at 1.0 g/kg, 2.0 g/kg, 3.0 g/kg and control group with 0 g/kg of EPS were formulated and packaged according to Table 3.1. Feed was given in the rate of 3% of their body weight (BW) ratio twice per day as for the first two weeks and later was reduced to 2.8% and 2.6% in the interval of two weeks due to insufficient EPS production and time constraint. The level of feed was adjusted according to the body weight after weighing them once every two weeks. The data for the growth was recorded and tabulated for further growth analysis. The feeding trials were conducted over 42 days as minimum recommendation by Caldini, N. N., et al., (2011) as a pioneer experiment. Fishes

condition were monitored regularly along the feeding period. At the end of the experiment, all fishes were weighed and proceed for further analysis.

3.7 Growth Performance Analysis

Each fish were weighed, measured and recorded fortnightly to compute growth performance throughout this study. The juveniles were individually weighed by using a sensitive weighing balance with measurement unit of grams (g). Weight gain (WG), feed intake (FI), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR) were calculated by using the data obtained from the experiment. Growth performance parameters such as weight gain, specific growth rate, feed conversion ratio and survival rate were adapted from Taufek, N. M. (2016) and calculated according to the following formulas:

1. WG (g) = final weight (g)−initial weight (g)
2. FI (g $fish^{-1}$) = total feed for the feeding period (g)/num of fish alive
3. SGR (%) = logw2−logw1/t×100

where,

w1 = initial weight

w2= final weight (g), and

t = duration of the experiment in days

4. FCR= feed intake (g)/weight gain (g)
5. SR (%)= final num of fish alive/initial num of fish alive × 100

3.8 Antioxidant Analysis

3.8.1 Sample preparation

After 42 days of feeding trials, five fishes were randomly selected from each tank and sacrificed for its liver sample collection. Liver sample collection were pooled together according to each treatment. A total of 1.0 g of liver were measured from the pooled liver sample and homogenized in 10ml buffer containing 25mM sodium phosphate buffer (pH 7.4), 0.1 mM protease inhibitor, 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) and 0.1 phenylthiourea (PTU). The sample was homogenized using a laboratory homogenizer at 150 rpm for 2 minutes in concern with achieving a distributed uniform liquid. Later, homogenates were centrifuged using 100,000 g (Beckman Coulter Optima l-100k Ultracentrifuge) at 4ºC for 30 minutes and the supernatant was separated into a centrifuge tube and later been stored at -80ºC for further analysis.

3.8.2 Liver Protein determination

Liver protein concentration was determined using Bradford assay which includes Coomassie Brilliant Blue G reagent, for standard, bovine serum albumin (BSA) was used (Bradford 1976). The total protein concentration of a test sample was determined by comparison to that with a series of linear absorbance profile produced by BSA as a protein standard.

For the preparation of Coomassie Brilliant Blue G reagent, 100 mg of Coomassie Brilliant Blue G-250 were weighed using weighing balance and dissolved in 50 ml of 95% ethanol. Next, 100 ml of 85%(v/v) phosphoric acid were added with the addition of

950 ml of distilled water to make the total volume of 1 litre of the solution. Once dissolved, the solution was filtered using Whatman No.1 filter paper and left overnight before using it. Approximately, 2 mg of BSA was added into 1 ml of distilled water for the preparation of BSA stock solution with a concentration of 2 mg/ml. From the stock solution, 6 standards solutions were prepared with a range of 50 to 300 µg/µl.

The absorbance of each sample was measured using a microplate reader at 595 nm. Later, the absorbance obtained of each BSA standard as a function of its theoretical concentration was plotted in a standard curve. The total protein concentration of the sample was calculated based on the equation obtained from the standard curve.

3.8.3 Glutathione S-transferase

Glutathione S-transferase (GST) was analysed by measuring the activity towards 1-Chloro-2,4-dinitrobenzene (CDNB) at 340 nm as described by the method from Habig et.al (1974). Firstly, 94µL of 100 mM sodium phosphate buffer with pH 7.6, followed by 2µl of 60 mM glutathione (GSH) were added into 96-well plate assay. Later, 18.7mg of GSH was dissolved in 1mL of sodium phosphate buffer. Next, 2µl of dilute samples and 2µl of 60 mM of 1-di-2,4-dinitrobenzene (CDNB) were dissolved in ethanol and added into the well. Each sample was monitored in triplicate. Data was recorded. One unit of GST activity calculated as the amount of enzyme catalyzing the conjugation of 1µ mol of CDNB with GSH per minute at 25°C (ε_{340}nm = 5.3mM^{-1} with pathlength of 0.552cm). The result expressed as nmol/min/mg/protein. Formula for the calculation as below (GST Assay Kit Technical bulletin 2007):

Enzyme activity: $$\frac{(A340nm/min)\ x\ (sample-blank)\ x\ 3\ x\ df}{5.3(0.552)\ x\ sample\ (ml)}$$

Specific activity (nmol): Enzyme activity / protein concentration of sample

3.8.4 Catalase

Catalase (CAT) activity was analysed according to the method of Claiborne (1985). Sodium phosphate buffer with the molarity of 50 mM and pH 7.0 at 25°C were prepared using sodium phosphate buffer. In 1.25ml of cuvette, 240µL of sodium phosphate buffer, 100µl of diluted samples and 100µl of H_2O_2 were mixed and added into the cuvette. Following the method from Taufek et al. (2016), the reaction was quantified at 25°C by observing the change in absorbance of H_2O_2 at 240 nm within 5 minutes. CAT activity was reported in terms of nmol H_2O_2 consumed nmol/min/mL (ε_{240} = 0.0436 mM/cm). Data was recorded and calculated with the following formula :

Enzyme activity: $$\frac{(A240\,nm/\min)\ x(sample-blank)x3xdf}{0.0436\ xsample(ml)}$$

3.9 Fatty Acids Analysis

3.9.1 Sample preparation

Seven fishes were randomly selected from each tank and carried further for fatty acid analysis. The internal organs including the respiratory organs (gills) were removed and cleared. After removing the internal organs, all the seven fishes were washed and dried. Drying process takes place in the drying oven at the temperature of 60°C for 48 hours. After the fishes had been completely dried, it was made into a powder form using a blender. After getting it into a powder form, 10 g of powder from each treatment were placed into a cellulose timber and 100 ml of petroleum ether was poured together. This process was repeated until a clear solvent is obtained. The solvent was brought into lipid extraction by using rotatory vapour Buchi Rotavapor R-200 with Buchi heating Bath B-490 (Temperature: 45ºC). Once the lipid has been extracted out, it was kept at room temperature for further analysis.+

3.9.2 Lipid Extractions

Lipid extractions were performed using the Soxhlet methods with petroleum ether according to AOAC (1990). A brief discussion was made on crude lipid (3.3.2).

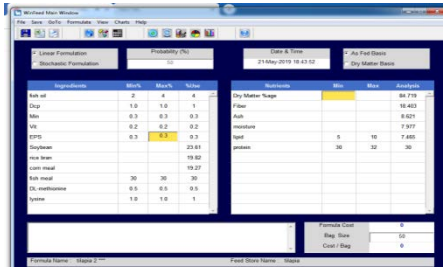
3.9.3 Gas Chromatography- Mass Spectrum(GC-MS)

The fatty acid analysis was done using gas chromatography Agilent Techonologies 6890N gas chromatograph powered with a HP Innowax 30m x 0.025mm x 0.25micron (Agilents Technologies). The determination of fatty acid was done by comparing the relative retention times of fatty acid metyl ester (FAME) peaks of samples with standard from Nanotechnology & Catalysis Research Centre, Universiti Malaya. Retention time and peak areas were identified with chromatography and library software (MS Library Search (NIS T98.L), operating at 70eV). Data was recorded by identifying the best hits from the result.

3.10 Statistical Analysis

All the data were subjected to one-way analysis of variance (ANOVA) using statistical package for the social sciences (SPSS) version 25.0 (SPSS Inc., Chicago IL, USA). The difference between means were compared using Duncan's post hoc test at 5% ($P < 0.05$) probability level. Data were presented as means ± standard error of mean.

3.11 Experimental design flowchart



The feed were formulated using WinFeed 2.8 version software

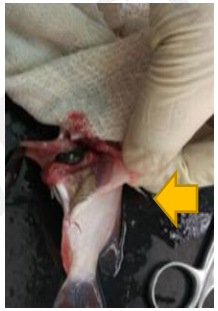
The ingredients were mixed and pelleted

1g/kg, 2g/kg and 3g/kg of EPS feed and control group feed were prepared and stored

Each treatment were carried out in duplicate during the experimental period

Fifteen fishes were segregated in each tank

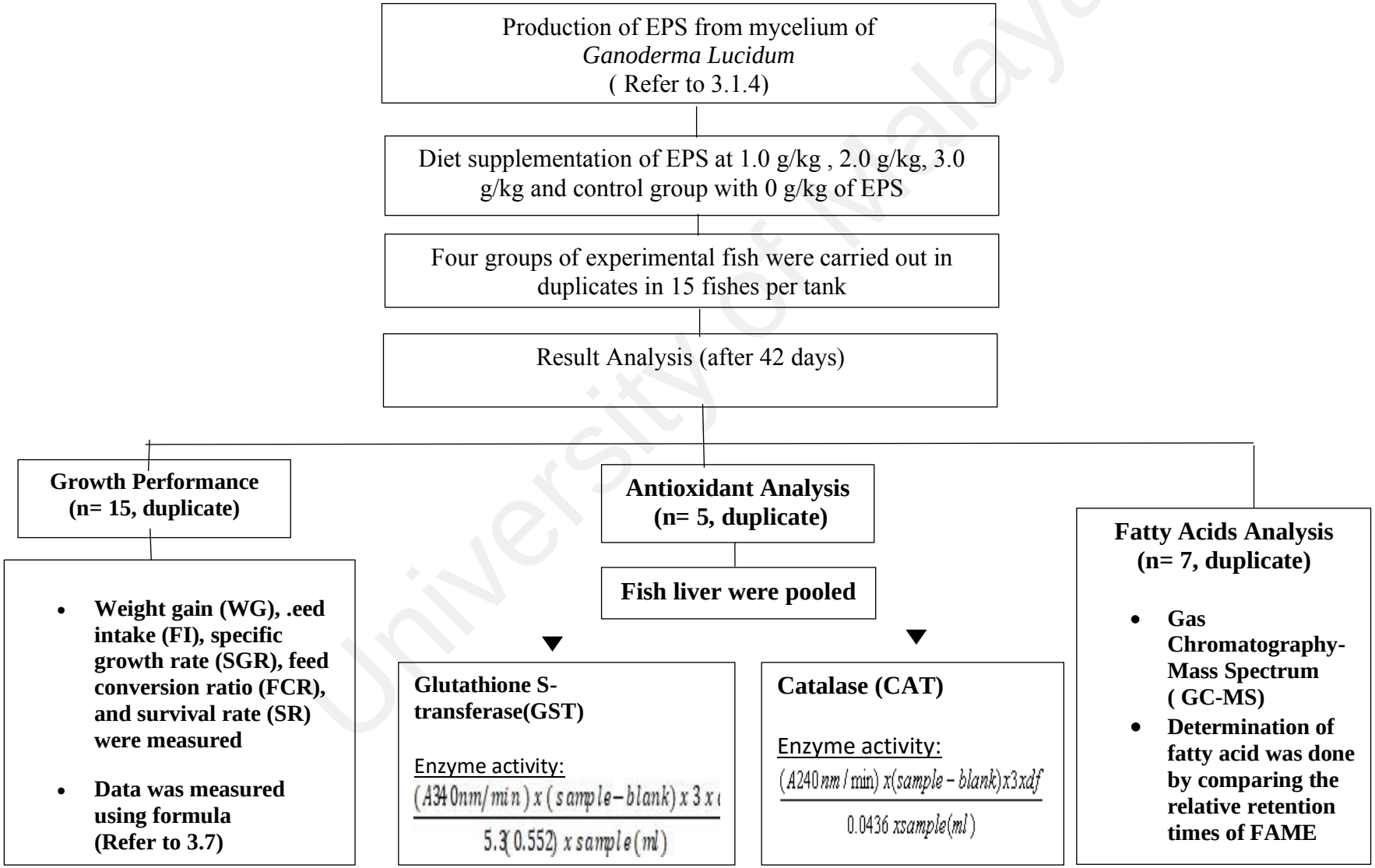
Fish weight gain were measured in every two weeks



Fish liver were removed and analysed further for antioxidant effect

Fish oil were extracted for further lipid analysis

3.12 Experimental Schematic Diagram



CHAPTER 4: RESULTS

4.1 Growth Performance of red hybrid tilapia (*Oreochromis* sp.) fed with different level of exopolysaccharides (EPS) as feed additive

Table 4.1 shows the growth performance of the red hybrid tilapia by using the feed additive of EPS from the mycelium of *G.lucidum* . The fish was fed with EPS from the mycelium of *G.lucidum* as a feed additive in the diet (Figure 4.1). Throughout the experimental period, the researcher has obtained approximately 12g - 15g of dry EPS from the mycelium of *G.lucidum.*

Water quality was monitored and the parameters were maintained for its temperature at 28–29 ^{o}C, pH at 6.5–7.5, dissolved oxygen at 6.5-8.0 , ammonia <0.80 mg/L, and nitrate <1.9 mg/L. Growth performance such as weight gain (WG), feed conversion ratio (FCR), feed intake (FI), survival rate (SR) and specific growth rate (SGR) were analysed. The growth of fish was obtained by measuring the weight gain for every two weeks.

Higher body weight gain was shown at 3.0 g/kg (18.75±0.03) diet followed by 2.0 g/kg (18.37±0.80) and 1.0 g/kg (16.55± 0.21) compared to control (12.35± 1.25) diet fed fish. The data obtained has no significant difference in 1.0g/kg, 2.0 g/kg and 3.0 g/kg of EPS supplemented diet. In contrast, 2.0 g/kg and 3.0 g/kg is significantly higher than control. Furthermore, SGR shows 3.0 g/kg supplemented diet (1.89%) and 2g/kg (1.88%) significantly improved compared to control (1.28%) and other supplemented diet.

The FCR was observed to be lower in the experimental group when compared to control with no significant difference ($P > 0.05$). Briefly, 3.0 g/kg (1.31±0.14) shows the

lowest FCR numerically compared to the other experimental diets, especially with control (1.98±0.43). Additionally, the control group has the least feed intake compared to other experimental diets with no significant difference (P > 0.05). Finally, the survival rate of control and 2.0 g/kg of supplemented diet shows a total of 100% compared to 3.0 g/kg supplemented diet (97%) followed by 1.0 g/kg (93%) supplemented diet with no significant difference (P > 0.05).

In general, the order of WG and SGR of red hybrid tilapia was 3g/kg>2g/kg>1g/kg>control feed. The results of the present study indicate that the growth performance of red hybrid tilapia was positively impacted by the different diet of feed additive of EPS from the mycelium of *G. lucidum*.

Table 4.1: Growth performance of the red hybrid tilapia by using the feed additive of EPS from the mycelium of *G. lucidum*

Performance details	Control	1g/kg	2g/kg	3g/kg
Initial Weight (g/fish)	17.45 ± 1.48[a]	16.20±0.14[a]	15.40±1.41[a]	15.65±1.91[a]
Final weight (g/fish)	29.8 ± 0.28[ab]	32.75±0.35[ab]	33.77±2.21[ab]	34.40 ±1.88[b]
WG(g/fish)	12.35± 1.77[a]	16.55± 0.21[ab]	18.37±0.80[b]	18.75±0.03[b]
SGR (%)	1.28±0.23[a]	1.60±0.11[ab]	1.88±0.06[b]	1.89±0.16[b]
FCR	1.98±0.43[a]	1.72±0.23[a]	1.32±0.06[a]	1.31±0.14[a]
FI(g/fish)	24.05±1.87[a]	26.25±0.98[a]	24.24±2.13[a]	24.61±2.60[a]
SR (%)	100[a]	93[a]	100[a]	97[a]

[1]The results represent mean± S.D of 15 fishes per tank (duplicate)

[2]Means in the same row with different letters are significantly different (P < 0.05)

[3]WG:Weight Gain, SGR:Specific Growth Rate, FCR:Feed Conversion Ratio, SR : Survival Rate, FI : Feed Intake

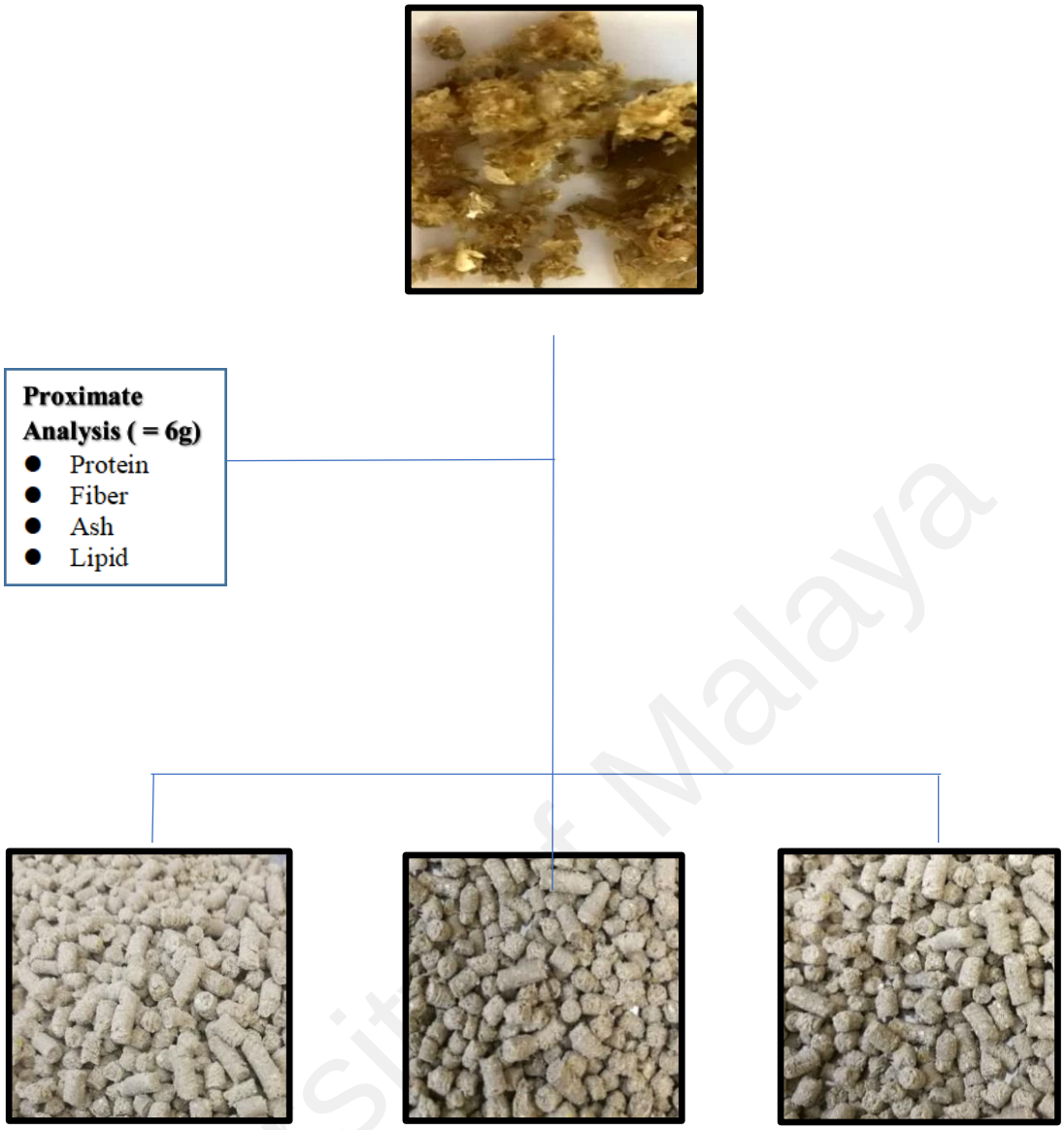


Figure 4.1: Total production and the use of exopolysaccharides (EPS) from the mycelium of *G.lucidum*

4.1.1 Weekly Growth Performance

Figure 4.2 shows the weekly weight gain (WG) of red hybrid tilapia (*Oreochromis* sp.) fed with different levels of EPS as feed additive from mycelium of *G. lucidum* diets for 42 days. The highest mean fish body weight (34 g) was observed at week 6 in the dietary supplemented with 2 and 3 g/kg of EPS while the lowest weight was observed in the initial week at 15 g at dietary supplemented with 2 g/kg, compared with control (17 g) as control feed. Figure 4.3 shows weekly weight gain of red hybrid tilapia fed with different levels (1g/kg, 2g/kg and 3g/kg) of EPS from the mycelium of *G. lucidum* observed at Week 0 (initial), 2, 4, and 6. The weight of each treated tilapia has no significant difference between each treatment in Week 0,2,4 and 6.

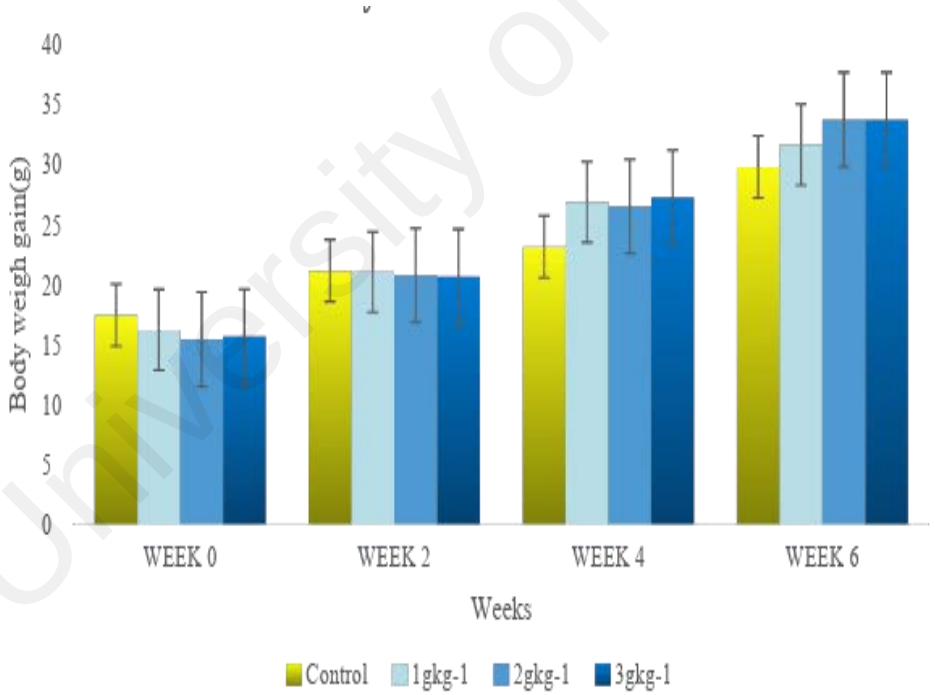


Figure 4.2: Weekly body weight gain of red hybrid tilapia fed with different level of EPS as feed additive of mycelium of *G. lucidum*. Error bars represent standard errors

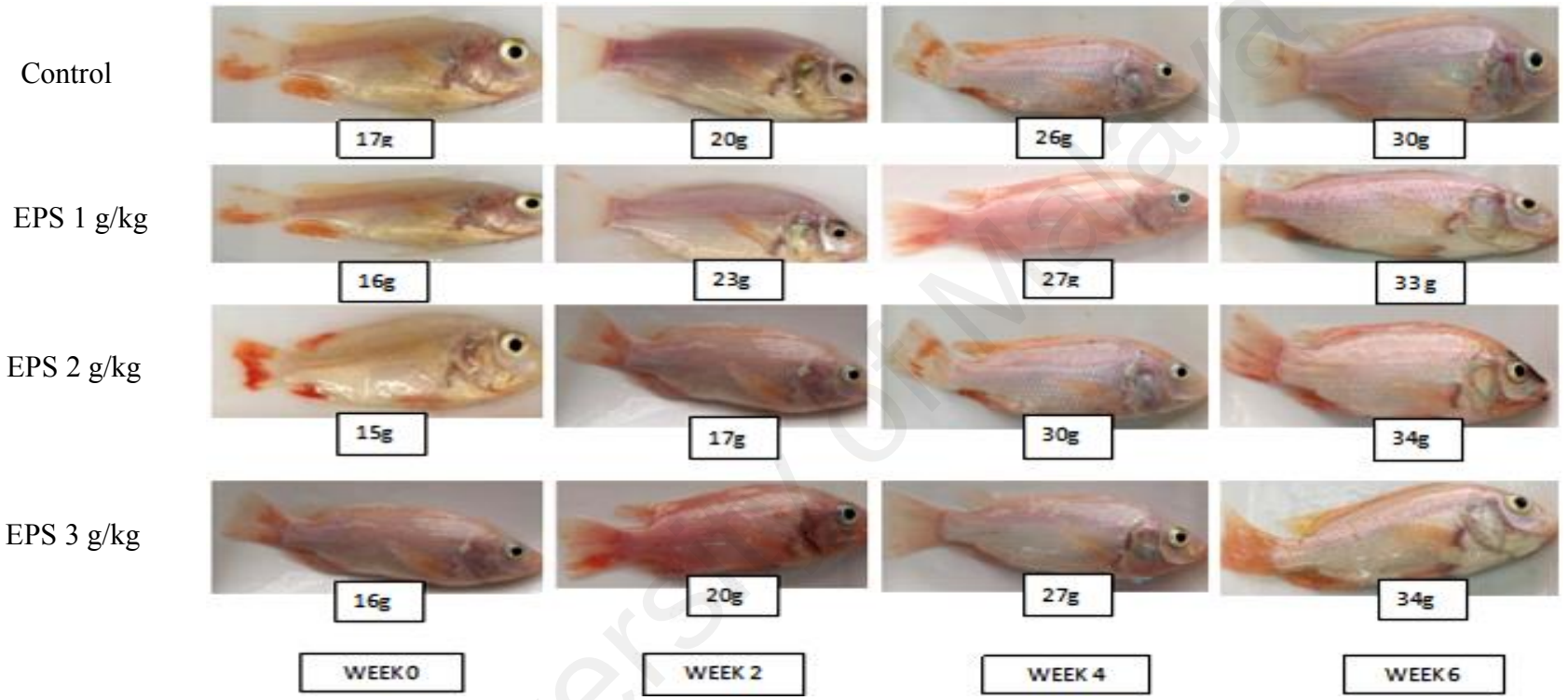


Figure 4.3: Weekly weight gain (WG) of red hybrid tilapia fed with different level of EPS from mycelium of *G. lucidum*

4.2 Antioxidant activity of red hybrid tilapia fed with different levels of EPS as feed additive.

Table 4.2 presents GST and CAT activities, which were analysed from the liver of red hybrid tilapia fed with different levels of EPS from the mycelium of *G.lucidum*. As presented, GST activity in the liver of red hybrid tilapia fed with 1g/kg EPS and 2g/kg EPS supplemented diet has a lower value of GST activity with no significant difference ($P > 0.05$) compared to control. While, 1g/kg and 2 g/kg of EPS has significant difference ($P < 0.05$) to 3g/kg experimental diet. Highest GST activity was observed at 3 g/kg EPS, and the lowest was observed at 1g/kg EPS.

Based on the observation of CAT activity, control has a significantly lower value ($P < 0.05$) compared to other experimental diets. The highest CAT activity was found on 3g/kg (230.51±13.04) with significant difference ($P < 0.05$) to other diets. However, the lowest CAT activity was obtained at 1g/kg (176.73±7.99) compared to other EPS supplemented diet. However, there was no significant difference ($P > 0.05$) between 1g/kg and 2g/kg of EPS supplemented diet.

Table 4.2: Antioxidant activity of red hybrid tilapia by using the feed additive of EPS from the mycelium of *G. lucidum*

	Control	1g/kg	2g/kg	3g/kg
GST	20.77±0.99[a]	11.18±1.14[a]	16.91±1.03[a]	38.17±6.85[b]
CAT	117.81±7.24[a]	176.73±7.99[b]	225.90±2.17[b]	230.51±13.04[c]

[1]The results represent mean± S.D of 5 fishes per tank (duplicate)

[2]Means in the same row with different letters are significantly different ($P < 0.05$)

[3]GST: Glutathione S-transferase (nmol/min/mg/protein, CAT:Catalase (nmol/min/mL)

4.3 Fatty acid profile of red hybrid tilapia fed with different levels of EPS as feed additive.

Table 4.3 shows the fatty acid profile of the red hybrid tilapia. A total of 10 fatty acids were identified on the experimental fishes fed with the control diet, 1 g/kg, 2 g/kg, and 3 g/kg supplementation of EPS. Six fatty acids are saturated fatty acid (SAFA) and four unsaturated fatty acid were present, which includes three monounsaturated fatty acid (MUFA) and one polyunsaturated fatty acids (PUFA). The most dominant SAFAs were palmitic acid and myristic acid. Among the unsaturated fatty acid, oleic acid and linoleic acid were relatively present in all the experimental groups.

Oleic acid, elaidic acid, and palmitoleic acid belong to MUFA which has one double bond bonded with their C-H atom. Linoleic acid has two double bonds of the C-H atom in its structure. Thus, linoleic acid belongs to the PUFA group. On the other hand, stearic acid, tridecanoic acid, pentadecanoic acid, nonadecanoic acid, palmitic acid, and myristic acid are saturated fatty acids with no double bond bonded between their C-H hydrocarbon atom.

Oleic acid, linoleic acid, palmitic acid and myristic acid are the common fatty acid which are present in all diets. Elaidic acid is present in the experimental diet but not on the control diet. In contrast, nonadecanoic acid is only present on the control diet. In comparison, pentadecanoic acid is present on control and 1g/kg but absent in 2g/kg and 3g/kg EPS diet. On the other hand, stearic acid and palmitoleic acid is present in 1g/kg and 2g/kg diet but not at 3 g/kg and control. Last but not least, tridecanoic acid presents only at 3g/kg diet.

Table 4.3: Fatty acid profile of red hybrid tilapia by using the feed additive of EPS from the mycelium of *G. lucidum*

Fatty acid	Control	1g/kg	2g/kg	3g/kg	Level of saturation	CD
Oleic acid	*	*	*	*	MUFA	18:1
Elaidic acid	-	*	*	*	MUFA	18:1
Palmitoleic acid	-	*	*	-	MUFA	16:1
Linoleic acid	*	*	*	*	PUFA	18:2
Stearic acid	-	*	*	-	SFA	18:0
Tridecanoic acid	-	-	-	*	SFA	13:0
Pentadecanoic acid	*	*	-	-	SFA	15:0
Nonadecanoic acid	*	-	-	-	SFA	19:0
Palmitic acid	*	*	*	*	SFA	16:0
Myristic acid	*	*	*	*	SFA	14:0

[1](*)signify fatty acid is present ; (-) signify fatty acid is absent
[2]MUFA : Monounsaturated fatty acid, PUFA : Polyunsaturated fatty acid , SFA : Saturated fatty acid,
[3]Carbon atoms of the fatty acid, and the number of double bonds in it (CD)
[4]The results represent mean± SEM of 7 fishes per tank (duplicate)

CHAPTER 5: DISCUSSION

5.1 Growth performance of red hybrid tilapia fed with different levels of EPS as feed additive.

To date, there is no reliable study on exopolysaccharides (EPS) from the mycelium of *G. lucidum* as a feed additive for red hybrid tilapia (*Oreochromis* sp.). Hence, the present study was conducted to evaluate the growth of fish when given different levels of EPS as a feed additive in comparison to 0 % of EPS in the fish feed which acts as control feed.

Evidently, Song et al. (2014) claim that the feed additive of prebiotics has shown a great interest in aquaculture due to its positive growth performance and increasing resistance of fish to pathogens. In this case, EPS is a non-soluble compound and commonly used as prebiotic fibre, which could be used as a functional food (Mahapatra & Banerjee, 2013). Thus, indicating this research could possibly lead to many beneficial research in the future.

In the current studies, higher weight gain was shown at 3.0 g/kg followed by 2.0 g/kg and then 1.0 g/kg while the lowest is observed at control diet fed fish. Briefly, fishes fed with 2.0 g/kg and 3.0 g/kg of EPS has significantly higher weight gain compared to control group fishes. Furthermore, the experimental diet shows a higher value of SGR with a significant difference ($P< 0.05$) compared to control. Weight gain elevates substantially high in EPS supplemented diet compared to control which leads to the results of higher SGR value in the experimental diet. Thus, this indicates that EPS have the ability to promote the growth rate of the fishes.

Variation in growth performance observed might due to differences within the quality of supplemental diets terms of nutrient composition (Workagegn, 2014). This might occur due to the composition of EPS supplemental diets. According to Russo et al. (2012), the availability of d-glucose and EPS as sugar sources retarded the entry into a stationary phase of the strains suggesting a synergistic effect in promoting metabolic processes. Thus, this indicates EPS as a feed additive is better in the term of nutrients composition, which suggested that the feed promotes the fish body growth.

Comparatively, a study by Takeuchi et al. (2009) examined the growth and body composition of juvenile tilapia fed raw Spirulina obtained body weight of 1.59 ± 0.26 g respectively. In another study by Din et al. (2012), it was found that feed additive of the prebiotic compound showed a positive growth performance on red hybrid tilapia with the level of 10% mushroom inclusion exhibiting the highest weight gain which is 6.72 g ±0.29.

Muin et al. (2013) states there was no significant difference in body weight gain (BWG) and SGR which indicates mushroom meal (*Pleurotus florida*) did not affect on African catfish (*Clarias gariepinus).* The highest BWG was obtained at 544.779 % ± 19.187 and SGR at 4.435 % ± 0.710 in their studies. Thus, this concludes in this study, significant difference is observed between the experimental and control group in WG and SGR, which indicates EPS has affected red hybrid tilapia in terms of WG and SGR.

Feed intake in the control diet was observed to have a lower feed consumption compared to experimental feed. This might be attributed to the taste of the control feed without any supplementation. This is supported by Lim et al. (2011) which claimed that the taste properties of the feed have a high stimulating implication on feed intake and

growth. In contrast, FCR was observed to be lower in the experimental group when compared with control. A lower value of FCR indicates high quality of feed produced as FCR plays an indicator for the feed utilization efficiency of fishes. Tilapia commonly has the FCR 1.5 (Dennis et al.,2009). It is important to scale back those components in the diets using different techniques to extend their potential as a fish feed (Workagegn, 2014).

The highest FCR was detected at 1.98±0.43 where belongs to control while the lowest is 1.31±0.14 belongs to 3.0 g/kg. The FCR values of all diets have no significant difference ($P>0.05$). Similar to the report from Razak & Sabaratnam (2015), FCR values of all diets were not significantly different shows mushroom supplementation did not affect red tilapia. Comparatively, Muin et al., (2015) has stated the best result obtained for FCR is 3.351±0.093 with no significant difference with control for Nile tilapia (*Oreochromis niloticus)* when fed with 30% of *Pleurotus sajur caju.* In conclusion, EPS supplementation did not affect the red hybrid tilapia in the term of FCR.

At the end of the experiment, survival rate of control and 2.0 g/kg of supplemented diet shows a total of 100% compared to 3.0 g/kg supplemented diet (97%) followed by 1.0 g/kg (93%) supplemented diet. In spite of that, there are various factors which could affect the rate of survival in fishes. Fundamentally, water quality management and fish handling practices during the experimental period may affect the survival rate of fish. Similarly, Dawood et al. (2020) states increasing the level of white button mushroom in the tilapia diet could enhance the growth performance of tilapia in terms of WG, SGR, and FI.

The outcomes of growth performance assessment shown in Table 4.1 manifested that EPS from the mycelium of *G.lucidum* could enhance the growth performance of the red hybrid tilapia and it may result in promising productivity in various aquaculture in future.

5.2 Antioxidant activity of red hybrid tilapia fed with different level of EPS as feed additive.

Antioxidant activity has been an important measure to detect fish well-being. GST constitutes a complex family of proteins that play dominant roles in both normal cellular metabolism and in the detoxification of a wide variety of xenobiotic compounds. They are predominantly cytosolic defence systems responsible for protecting cellular components against various toxic effects and oxidative stress (Sen & Semiz, 2007). The ROS can be detoxified by an enzyme defense system, comprising catalase (CAT), while organic peroxides can be detoxified by the activity of GST. Thus, in this study, GST and CAT have been tested on the liver of red hybrid tilapia to investigate the antioxidant activity of the fish.

In the present studies, GST activity was measured at 16.91±0.73 at 2g/kg while the lowest was detected at 11.18±0.81, which belongs to 1g/kg feed additive of EPS compared to control (20.77±0.70) with no significant difference. The presented results showed that liver GST activity decreased in red hybrid tilapia fed with 1g/kg and 2g/kg of EPS along the experimental period (42 days).

According to the presented results, GST could play a role in being an oxidative stress tolerance and could assist in protecting red hybrid tilapia against damages to the cellular components. This indicated that EPS possesses antioxidant potentials that might effectively reduce ROS production and its adverse effect, owing to the fact that red hybrid tilapia in this experiment was raised in a control substrate and was not fed with any materials exposed to harmful chemicals especially to their prior exposure to EPS.

This is also supported by Grassi et al. (2018), which states that liver actively performs biosynthetic and detoxifying activities, which needs a high amount of energy supply provided by oxidative metabolism of the liver. According to the presented results, GST could play a role in being an oxidative stress tolerance and could assist in protecting red hybrid tilapia against damages to the cellular components. Therefore, the result demonstrated that EPS 1g/kg and 2 g/kg could be a potential dietary supplement that could exhibit a refinement antioxidant activity of red hybrid tilapia.

Notwithstanding with CAT activity, the control diet has an average of 117.81±7.24 of CAT activity in the fish liver. In a study by Ahmed et al. (2014) who stated that 0.5% and 1% supplementation of hot water extract (HWE) of waste mushroom stalk significantly ($P<0.05$) increased the activity of CAT (nmol/ml/min) comparing with commercial diet and base diet. Higher increasing of CAT activity was found in 0.5% of HWE supplementation in liver, kidney, and blood. The researcher also concludes that this may also be the presence of beta glucan.

Comparatively, in this study CAT activity in EPS diet is higher compared to control with a significant difference. The highest average of CAT activity was detected at EPS 3g/kg while the lowest is detected at EPS 1g/kg among the EPS diet. Evidently, beta 1,3–

1,6 glucan is a complex, high molecular-weight polysaccharide that is present in the cell wall of *G. lucidum* (Wu et al., 2016).

Additionally, due to high molecular masses, they can be likened to a massive stimulus possessing diverse functions to stimulate receptors when enter into the body (Chen, 2009). This indicates that high CAT activity are most likely due to the presence of beta glucan in the feed additive. The outcome suggested that CAT activity is related to the increased growth response due to a high metabolic rate (Taufek et al., 2016).

In conclusion, despite the minor effect of high CAT activity in experimental feed, EPS from the mycelium of *G.lucidum* could be an antioxidant potential for red hybrid tilapia, especially for 1g/kg and 2g/kg of EPS. Therefore, the result demonstrated that EPS 1g/kg and 2 g/kg could be a potential dietary supplement that could exhibit a refinement antioxidant activity of red hybrid tilapia.

5.3 Fatty acid profile of red hybrid tilapia fed with different level of EPS as feed additive.

Tilapia (*Oreochromis* sp.), like other fish species and vertebrates, cannot biosynthesize essential fatty acids (EFA) as polyunsaturated fatty acids (PUFA), linoleic acid (18:2 omega-6) or linolenic acid (18:3 omega-3) by their own (Oğuz Taşbozan & Mahmut Ali Gökçe, 2017). Hence, fishes obtain them by consuming as their feed, usually algae or plankton (Tocher, 2010). In this study, the researcher has used EPS from mycelial of *G.lucidum* to determine the fatty acid composition of red hybrid tilapia.

Studies have shown that tilapia fatty acid composition could fluctuate depending on various factors such as biological, physical, and chemical properties of that environment

(De Silva et al., 2002). In a study by Ma et al. (2015) stated that in a controlled system other factors could also play a role in affecting the fatty acid metabolism which includes feeding frequency, starvation, and water temperature ($25^{o}C$-$30^{o}C$). The researchers claim the colder the water temperature, the more efficient the fishes are in converting saturated fatty acids into monounsaturated and polyunsaturated fatty acids. This might be due to the ability of providing a greater membrane fluidity for PUFAs at a lower temperature.

Specifically, at lower temperatures, it has the ability to elongate and desaturate shorter chain polyunsaturated fatty acids into longer-chain polyunsaturated fatty acids (Stoneham et al.,2018). In addition, another study saying starvation rate could also affect the utilization of fatty acids in the liver as an energy source (De Silva et al., 1997). As the activity involved in the synthesis of omega-3 PUFA series are also low in this experiment. This is probably due to starvation of the fishes before sacrificing it. In another context, Kyi et al. (2014) state desirable DHA majorly found on fishes increased with age compared to the juveniles. This could be related with this finding that the experimental fishes were all juveniles and they might be unable to elongate and desaturate shorter chain PUFA into DHA.

In a study by Lv et al. (2012) state that palmitic acid, linoleic acid, oleic acid, stearic acid are the main fatty acids which are present in *G. lucidum.* In this current study, only fish supplemented with 1 g/kg and 2 g/kg EPS contain these four fatty acids. In this experiment, stearic acid are present in both 1 g/kg and 2g/kg of EPS while palmitic acid is present in all diets. Hardy et al. (2003) states that stearic acid and palmitic acid are pro-apoptotic agents which does the activation of apoptosis in the cell. This is clearly due to the EPS feed as the polysaccharides from *G.lucidum* executes the anti-cancer actions through pro-apoptotic, mechanisms (Gao et al., 2005). This is also supported by Wachtel-

Galor et al. (2011), who stated that anti-cancer properties of *G. lucidum* are primarily attributed to its polysaccharides.

Tilapia requires n-6 fatty acids compared to n-3 fatty acids in their diets (Takeuchi et al., 2009). Lim et al. (2011) states that a high level of n-3 fatty acid is reported to decrease growth performance in tilapia. Thus, due to the better performance in growth there might be less linolenic acid detected in this experiment. Olsen et al. (1990) reported tilapia (*Oreochromis niloticus*) has the ability to convert linoleic acid to the longer chain of highly unsaturated fatty acid (HUFA) which includes arachidonic acid (20:4n-6, ARA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) via a series of fatty acid conversion and elongation. Thus, it requires further study to confirm that EPS has the ability to convert linoleic acid to EFA.

In a study by Sergio Lopez et al. (2010), epidemiological studies suggests that a higher proportion of oleic acid, in the diet is linked with a reduction in the risk of coronary heart disease. Oleic acid (18:1) is considered to be highly stable to oxidation and also can enhance the activity of antioxidants (Ernesto, 2016). Thus, it shows that oleic acid is essential to human health.

Typically, fish have an optimum dietary lipid requirement and above the optimum dietary lipid has the tendency to cause growth suppression (Du et al., 2005). Evidently, there is no growth reduction observed in this experiment for EPS diet. Thus, this shows that fatty acid composition makes EPS a good potential source of feed additive for red hybrid tilapia diets since it provides sufficient energy for growth. Overall, the experimental diets 1 g/kg and 2 g/kg of EPS presented in this study show promise as a feasible option for enriching beneficial fatty acids content in tilapia.

5.4 Comparison of the current study with the literature study

The current study is conducted to determine the potential of EPS from the mycelium of *G.lucidum* as feed additive on red hybrid tilapia. To the best of the author's knowledge, there is no report on EPS from the mycelium of *G.lucidum* as feed additive. Thus, comparison of significant data WG and SGR were done with other literatures (Table 5.1). As presented EPS from the mycelium of *G.lucidum* as feed additive had obtained highest WG compared to other fungi studies. Thus this shows that EPS from the mycelium of *G.lucidum* has the potential to be improved feed additive in future.

Table 5.1: Comparison of the current study with the literature study

Type of mushroom	Fish used	Aquaculture system	Weight gain (g)	Specific growth rate	References
Ganoderma lucidum	Red hybrid tilapia (*Oreochromis* sp.)	Low-density tank	18.75 ±0.02	1.89±0.12	Current study
Pleurotus sajor-caju	Red hybrid tilapia (*Oreochromis* sp.)	Low-density tank	6.72 ±0.29	1.74±0.09	Mohd Din et al.,(2012)
Pleurotus sajor-caju	Nile tilapia (*Oreochromis niloticus*)	Low-density tank	5.92 ±0.47	3.81±0.21	Muin et al., (2015)
Pleurotus florida	African catfish (*Clarias gariepinus*)	Low-density tank	5.44 ± 19.18	1.67 ± 0.03	Muin et al.,. (2013)
Pleurotus mushrooms	Rohu fish (*Labeo rohita*)	Low-density tank	0.73 ±0.18	0.64±0.07	Deborah et al., (2011)
Pleurotus mushrooms	Buenos Aires tetra (*Hemigrammus caudovittatus*)	Low-density tank	0.44 ±0.03	1.41±0.16	Deborah et al., (2011)

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This research aims to determine the potential of exopolysaccharides (EPS) from the mycelium of *G.lucidum* as feed additive on red hybrid tilapia (*Oreochromis* sp.). It is believe that the feed additive of EPS from the mycelium of *G.lucidum* can be taken as feed additive in red hybrid tilapia diet without enforcing any negative impact on the growth performance, antioxidant activity and fatty acid profile of the red hybrid tilapia.

Based on the findings presented from the current study in this study, the following conclusions can be made:

- In the context of growth study, EPS as feed additive at various levels (1g/kg, 2g/kg and 3g/kg) in red hybrid tilapia showed that experimental group exhibits a positive growth response compared to control. Briefly, 3g/kg shows improved growth performance in the aspect of WG (18.75±0.03), SGR (1.89±0.16) , FCR (1.31±0.14) and FI (24.61±2.60) with no significant different with 2g/kg in the terms of WG (18.37±0.80), SGR (1.88±0.06), FCR(1.32±0.06) and FI (24.24±2.13). Additionally, 2g/kg experimental fishes have survived 100% throughout the feeding period (42 days). From observation during feeding time, the fish accepted all the experimental diets without any rejection.

- In the context of antioxidant activity, GST activity decreased in red hybrid tilapia fed with 1g/kg (11.18±1.14) and 2g/kg (16.91±1.03) of EPS as feed additive. In contrast, CAT activity was observed to be lower in control group, which might be attribute due to the presence of beta glucan in EPS feed. These facts demonstrated that EPS

could be a potential feed additive to red hybrid tilapia in a formulated diet despite the minor effect in high CAT activity in EPS experimental diet.

- The presented study indicated that 1 g/kg and 2 g/kg of EPS has beneficial fatty acids, and the main fatty acid content found in *G.lucidum* which are palmitic acid, linoleic acid, oleic acid, stearic acid. Even though less fatty acid are present in the overall experiment due to biological and chemical factor but 1 g/kg and 2 g/kg of EPS shows conceivable fatty acid content in red hybrid tilapia.

- Taking all factors into considerations, 2 g/kg EPS from the mycelium of *G. lucidum* shows a better potential as feed additive in red hybrid tilapia diet without enforcing any negative impact on the growth performance, antioxidant activity and fatty acid profile.

6.2 Recommendations

The current findings add substantially to a growing knowledge of fish feed additive, particularly in finding a better solution to exhibit a positive performance on growth, antioxidant activity, and lipid profile. Further studies were recommended to:

1. To diagnose the fish in the terms biochemical and pathological tests such as in blood, organs, and tissues. This is to have clear evidence for the suitability of EPS to be included as feed additive for red hybrid tilapia.

2. In the aspect of growth performance, the future researcher could include the growth performance in the term of length gain of the fish using the measuring scale. As in this research, the data was not included. Furthermore, the effect of the digestive enzyme should also be determined in order to confirm the acceptance of EPS as feed additive to red hybrid tilapia.

3. To investigate with other antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH) to determine the oxidative stress of red hybrid tilapia, as this current study has only observed the enzyme activity of GST and CAT as this current study has only studied on the inner cell first defense system. Additionally, in future research, it is also recommended to test other parts of the organs such as muscle, heart, and kidney to have a clear understanding of antioxidant activity in fish.

4. In this study, the researcher has only concluded the qualitative data on the fatty acid profile. Thus, quantitative research could be done in future work to conclude the amount

of fatty acid present in each fatty acid. This is to gain a clear insight into the fatty acid profile of red hybrid tilapia.

5. Another limitation of this research is the period of this study as this is a preliminary study. Thus, in future work, it is recommended to have a large sample size to obtain more data for the fish. Besides, the feeding trial could be carried out batch by batch, this data could identify the correlation between weight gain and the antioxidant activity. In addition, this is also to gain more evidence for the potential of EPS as feed additive on red hybrid tilapia.

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