



Faculty of Resource Science and Technology

Protein Sparing Effect of Sago Starch in Diet of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

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Protein Sparing Effect of Sago Starch in Diet of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Six isoenergetic (20.25 ± 1.35 kJ/g) semi-purified experimental diets were formulated with three levels of protein, P(22%, 26% and 30% diet) and two levels of sago starch as source of carbohydrates, C(38% and 44% diet). A corn starch (C40%) diet was used as a control diet. Diets were fed to triplicate groups of tilapia ($n=20$), *Oreochromis niloticus* juveniles (mean weight 4.61 ± 0.04 g), to apparent satiation twice daily for 12 weeks. The results showed 100% survival rate for all diet groups in which fish fed on 38% sago starch diet incorporated with protein level, P22% (D1: 29.53 g), P26% (D2: 31.87 g) and P30% (D3: 32.32 g) were significantly higher ($p < 0.05$) on final body weight (FBW) than those fed on 44% diet treatments combined with P22% (D4: 23.29 g), P26% (D5: 25.65 g) and P30% (D6: 27.71 g). Whole body proximate compositions ($n=6$) were significantly ($p < 0.05$) affected by 38% and 44% of dietary sago starch with different level of protein, P22%, P26% and P30, respectively. Fish fed on 38% sago starch diets with protein level, P26% (D2: 13.64 mmol/L) and P30% (D3: 13.68 mmol/L) showed high significant different ($p < 0.05$) of glucose concentration in plasma ($n=6$) than those fish fed on diet treatments with protein level, P22%, P26% and P30% which contained 44% of sago starch. Significantly higher ($p < 0.05$) of triglyceride plasma ($n=6$) were observed in fish fed on 44% sago starch with protein level, P22% (D4: 6.52 mmol/L) compare with those fish from other diet treatments, meanwhile, total protein plasma ($p < 0.05$) were varied from 4.18 to 22.11 mmol/L. Nutrients digestibility ($n=12$) showed significantly ($p < 0.05$) high statistics on fish fed with ratio P30%:C38% (D3:80.12%), followed by P26%:C38% (D2:77.54%), P26%:C38% (D1:74.72%), P30%:C40% (D0:69.83%), P30%:C44% (D6:65.67%), P30%:C44% (D5:57.40%) and P30%:C44% (D4: 50.29%), respectively. Digestive enzyme activities ($n=12$) were significantly ($p < 0.05$) affected among all diets in which fish fed on treatment

D3 with P30%:C38% ratio were statistically higher (amylase: 6.54 Umg^{-1} , lipase: 5.68 Umg^{-1} , protease: 0.77 Umg^{-1}) compare with the other diet treatments. The carbohydrate metabolic enzyme activities (n=15) showed significant ($p < 0.05$) influenced between 38% and 44% sago starch incorporated with protein level, P22%, P26% and P30% recorded approximately from 0.76 to 0.97 Umg^{-1} (PFK), 0.73 to 0.96 Umg^{-1} (FBPase) and 0.78 to 0.95 Umg^{-1} (G6PD). Two-way ANOVA result confirmed that the interaction between different level of both protein and carbohydrate have significant ($p < 0.05$) influenced on growth performance, whole-body proximate compositions, blood plasma compositions, nutrient digestibility digestive enzyme and carbohydrate metabolic enzyme activities of *O. niloticus* juveniles. Overall, fish fed on 38% sago starch-based diets showed positive result and performed better than those fed with 44% diets. The study revealed the ability of *O. niloticus* juveniles to spare protein by dietary carbohydrate was at optimum level of 38% sago starch and 30% protein.

Keywords: Sago starch, tilapia, growth, blood plasma, digestibility, digestive enzymes

**Kesan Pengurangan Protein Menggunakan Kanji Sagu dalam Diet Tilapia Nila,
Oreochromis niloticus**

ABSTRAK

Enam diet eksperimen separa tulen (20.25 ± 1.35 kJ/g) dirumuskan dengan tiga tahap protein iaitu P(22%, 26% dan 30% diet) dan dua tahap kanji sagu sebagai sumber karbohidrat iaitu C(38% dan 44% diet). Manakala itu, kanji jagung (C40%) pula digunakan sebagai diet kawalan. Tiga kumpulan juvenil ikan tilapia ($n=20$), Oreochromis niloticus (purata berat badan, 4.61 ± 0.04 g) diberi makan sehingga kenyang menggunakan diet tersebut iaitu sebanyak dua kali sehari selama 12 minggu. Hasil kajian menunjukkan kadar kelangsungan hidup adalah 100% untuk semua kumpulan diet di mana ikan yang diberi makan kanji sagu 38% dengan gabungan tahap protein, P22% (D1: 29.53 g), P26% (D2: 31.87 g) dan P30% (D3: 32.32 g) menunjukkan jumlah berat badan akhir (BBA) yang lebih tinggi ($p < 0.05$) daripada ikan yang diberi diet 44% yang digabungkan dengan P22% (D4: 23.29 g), P26% (D5: 25.65 g) dan P30% (D6: 27.71 g). Komposisi keseluruhan badan ($n=6$) adalah ketara ($p < 0.05$) iaitu dipengaruhi oleh gabungan antara 38% dan 44% kanji sagu dengan tahap protein yang berbeza iaitu P22%, P26% dan P30%. Ikan yang diberi makan kanji sagu 38% dengan tahap protein, P26% (D2: 13.64 mmol/L) dan P30% (D3: 13.68 mmol/L) menunjukkan kepekatan glukosa ($n=6$) yang lebih tinggi ($p < 0.05$) dalam plasma berbanding dengan ikan yang diberi diet dengan tahap protein, P22%, P26% dan P30% yang mengandungi 44% kanji sagu. Signifikasi yang lebih tinggi ($p < 0.05$) pada plasma trigliserida ($n=6$) diperhatikan pada ikan yang diberi 44% kanji sagu dengan kadar protein, P22% (D4: 6.52 mmol/L) berbanding dengan ikan dari kumpulan diet yang lain manakala jumlah protein plasma bervariasi dari 4.18 hingga 22.11 mmol/L. Pencernaan nutrien ($n=12$) menunjukkan statistik signifikasi yang lebih tinggi ($p < 0.05$) pada ikan yang diberi

diet dengan nisbah P30%:C38% (D3:80.12%), diikuti dengan P26%:C38% (D2:77.54%), P26%:C38% (D1:74.72%), P30%:C40% (D0:69.83%), P30%:C44% (D6:65.67%), P30%:C44% (D5:57.40%) dan P30%:C44% (D4:50.29%) juga sebaliknya. Aktiviti enzim pencernaan (n=12) adalah ketara ($p < 0.05$) di antara semua diet di mana ikan yang diberi diet D3 dengan nisbah P30%: C38% mempunyai kadar statistik yang lebih tinggi (amilase: 6.54 Umg^{-1} , lipase: 5.68 Umg^{-1} , protease: 0.77 Umg^{-1}) jika dibandingkan dengan kumpulan diet yang lain. Aktiviti enzim metabolik karbohidrat (n=15) menunjukkan kesan ketara ($p < 0.05$) iaitu dipengaruhi oleh gabungan antara 38% dan 44% kanji sagu dengan P22%, P26% dan P30% tahap protein iaitu mempunyai rekod anggaran dari 0.76 hingga 0.97 Umg^{-1} (PFK), 0.73 hingga 0.96 Umg^{-1} (FBPase) dan 0.78 hingga 0.95 Umg^{-1} (G6PD). Hasil daripada ANOVA dua hala mengesahkan bahawa interaksi antara tahap protein dan karbohidrat yang berlainan mempunyai kesan yang ketara ($p < 0.05$) terhadap prestasi pertumbuhan, komposisi badan, komposisi plasma darah, penghadaman nutrien, aktiviti enzim pencernaan dan enzim metabolik karbohidrat bagi juvenil *O. niloticus*. Secara keseluruhannya, ikan yang diberi makan diet 38% kanji sagu menunjukkan kesan positif dan prestasi yang baik berbanding kumpulan ikan yang diberi makan diet 44%. Kajian ini juga menunjukkan kemampuan juvana *O. niloticus* untuk mengurangkan protein melalui sumber karbohidrat pada tahap optimum adalah dengan menggunakan 38% kanji sagu dan 30% protein.

Kata kunci: Kanji sagu, tilapia, pertumbuhan, plasma darah, penghadaman, enzim

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LIST OF ABBREVIATIONS

FBPase	Fructose 1, 6-bisphosphatase
FBW	Final Body Weight
FCR	Feed Conversion Ratio
FI	Feed Intake
GE	Gross Energy
G6PD	Glucose 6-phosphate dehydrogenase
HSI	Hepatosomatic Index
IBW	Initial Body Weight
IPF	Intraperitoneal Fat
NFE	Nitrogen Free Extract
PER	Protein Efficiency Ratio
PFK	6-phosphofructokinase
SGR	Specific Growth Rate
SR	Survival Rate
VSI	Viscerosomatic Index
WG	Weight Gain

CHAPTER 1

INTRODUCTION

1.1 Study Background

The increases of global population growth have boosted the consumption demand of the fish as source of protein. Therefore, the role of aquaculture sector is important and an excellent approach to be an opportunity to bridge the supply and fill up the demand gap (Xu et al., 2019). Aquaculture is well known as the fastest growing food production sector in the world and produce a variety of products, hence reducing the pressure on the capture fisheries industry (Ng & Romano, 2013; Sandrea et al., 2017). It is also developing, expanding and intensifying in almost all regions of the world. The persistent goal of new world aquaculture is maximizing the efficiency of fish production and optimizing the profitability. However, the expansion of aquaculture to meet the rising demand for food supply increases the demand for more feeds from aquaculture feed industry, especially for intensive fish culture (Zhou et al., 2011; Fortes-Silva & Sánchez-Vázquez, 2012).

Tilapia is an omnivorous fish of the family Cichlidae (Sklan et al., 2004; Qiang et al., 2014) which includes over 100 species under three genera; *Oreochromis*, *Sarotherodon* and *Tilapia* (Ridha, 2006; Wang & Lu, 2016). Tilapia is the most cultured fish in the world and has been recognized as the great economic important for many countries. Currently, tilapia is the second most farmed fish in the world after carps, with global production estimated around 6.3 million tonnes in 2018 (FAO, 2019) and expected to reach 7.3 million tonnes by 2030 (FAO, 2013). The global tilapia market is valued about 11,700 million USD in 2017 and estimated to be reach 13,400 million USD by the end of 2025 which is mounting at a CAGR of 1.8% during the period. Nile tilapia (*Oreochromis niloticus*) is the most farmed

among the tilapia species (Singh et al., 2006; El-Sayed & Kawanna, 2007) and have greater demand for larvae and juveniles (Ribeiro et al., 2018).

Global aquaculture production was 114.5 million tonnes in 2018 and was predicted to reach 109 million tonnes in 2030, an increase of 32% over the period (FAO, 2019). In aquaculture feed industry, protein is one of the most expensive and important nutrient for fish growth. In view of this, studies on the quality and optimal requirement of protein in feed formulation for *O. niloticus* are important to reduce feed production costs. Moreover, feeds containing high level of protein do not necessary indicate better growth but depend on the bioavailability of the amino acids (Gao et al., 2011). This might due to the ability of fish to utilize protein which specifically related to the existence of proteases in the digestive system that are responsible for hydrolyzing or breaking down the protein into peptides and amino acid and eventually absorbed by fish for growth (Luo et al., 2016).

In aquaculture field, the successful and sustainable are depends on the provision of nutritionally balanced, environment friendly and economically viable artificial feeds. Generally, the feed preparation diet is a principal factor that increase the growth and production of reared fish in aquaculture (Thankur et al., 2004; Liti et al., 2005; Abdel-Tawwab et al., 2007). However, the high cost and short supply of protein source in feed production is the main problem facing by majority aquaculture industry nowadays (Sayed et al., 2018). This also may become the biggest challenge to formulate more effective feeds which is very important for fish in order to reach maximum growth rate and balanced energy requirements. Consequently, the alternative and strategic approach for the issue is important because the aim is not only to reduce cost but also to make feeds become more adequate and sustainable.

The main protein source used in aquaculture feed industry is fishmeal. This is due to its high quality of protein, stable amino acids profile, essential fatty acids content, high digestibility and better palatability to fish (Wang et al., 2017). FAO (2019) reported that it was estimated about 70% of total fishmeal production was utilized for aquaculture feeds. However, due to low production and consistent increase in demand, the price of fish meal rose to USD 1,650 per tonne in 2018, the highest from the previous years (FAO, 2019). This has forced the aquaculture feed manufacturers to use cheaper alternative protein source as fishmeal replacement to sustain the developing aquaculture production. Fishmeal is commercially made from the whole fish like chapelin, herring, anchovy or menhaden white fish including the bones and offal after lipid extraction (Wu et al., 2015). Generally, the nutrient compositions of fishmeal were comprised of approximately between 66 to 71% protein, 5 to 9% lipid, 7 to 8% moisture and 12 to 19% ash (He et al., 2015; Rahmah et al., 2016).

Indeed, the total production cost have been the main issue in fish feed manufacturing. This due to dietary protein source which is considered as the main and most expensive ingredients in fish diet (Boonanuntanasarn et al., 2018). Therefore, the alternative approach is to spare the protein source in the aquaculture feed with less expensive ingredients such as lipids and carbohydrates. In the present study, the protein-sparing effect of sago starch in the diet for Nile tilapia, *O. niloticus* juveniles was conducted. Sago starch is extracted from sago palm, *Metroxylon sagu* which is also considered as a potential candidate for carbohydrate source to spare protein in aquaculture feeds production (Umar et al., 2013). To take into account, the largest supply of sago comes from South East Asia, particularly Indonesia and Malaysia (Jong, 2018). In Sarawak, the sago production mainly occurred in Mukah and it is

a popular foodstuff especially among the Melanau ethnic group in the area (Karim et al., 2008).

1.2 Problem Statement

The cost-effective development and formulation of adequate nutritional diet is important in any reared fish species. In the fish culture, practical feeds are the important aspect for source of nutrients and also the main factor which greatly influence the total yield of fish in the aquaculture farm. However, feed is often considered as one of the major challenges for aquaculture practices, as it represents the main contributor to operational costs approximately between 40 to 60% production expenses of the whole farm (Azaza et al., 2015). Therefore, the feed must be economical and sustainable besides nutritionally sufficient relevance with the demand of current aquaculture industry (Watanabe, 2002; Deng et al., 2010).

Protein is very crucial macronutrient in aquafeed as it provides major energy source, build and repair the damaged tissues and for general body maintenance. It is an essential nutrient and well known as the most expensive component in fish diet (Zhang et al., 2016). The optimum level of protein used in fish diet is crucial to prevent the amino acid from catabolized as energy rather than growth (Stone et al., 2003a). It is reported that the catabolism of protein in the diet can be minimize for energy metabolism through the adequate levels of non-protein energy sources such lipid and carbohydrate (Mohanta et al., 2007; Kamalam et al., 2017). Thus, the use of another alternative sources with reliable nutrient contents such as carbohydrate source that can reduce feed costs is important to make the fish culture become more effective and economic (Boonanuntanasarn et al., 2018).

Dietary carbohydrates are considered as less expensive energy source component in aquatic animal feeds and efficiently utilized by omnivorous and/or herbivorous warm water fish (Wang et al., 2014; Kong et al., 2019). Starch is the predominant carbohydrate in plant such as wheat, corn, field peas, grains and legumes and have been incorporated into formulated diets for fish (Lee et al., 2003). In addition, sago (*Metroxylon sagu*) starch also may considered as a nutritive and potential carbohydrates sources for aquaculture feeds (Umar et al., 2013). The application of carbohydrates in fish diets have a great benefit in aquaculture sustainability because it contributes to low cost feed formulation, increases protein retention, reduces ammonia excretion, and improves stability and floatability of pellets in extruded diets (Wang et al., 2016; Corrêia et al., 2019). However, the use of carbohydrate as a protein-sparing energy source in fish has received less attention compared to lipid while the utilization of digestible energy in the form of carbohydrate is considered as a prime importance under practical aquaculture conditions as stated by Mohanta et al. (2009).

Furthermore, a clear knowledge and better understanding on the optimal level of protein as dietary carbohydrates used is important to study the protein-sparing effect that may be essential in minimizing the cost of fish preparation diets (Abdel-Tawwab et al., 2010; Rahman et al., 2017). Boonanuntanasarn et al. (2018) mentioned that the application of dietary carbohydrate as a protein-sparing source of energy still showed fewer attention in formulated feed for cultured fish. Besides, there is little information available on the growth performance, blood plasma, nutrient digestibility, digestive and carbohydrate metabolic enzymes response in fish that utilized on starch-based carbohydrate diets (Qiang et al., 2014).

1.3 Objectives

This present research was designed to evaluate the effect of Sago (*Metroxylon Sagu*) starch in the diet for Nile tilapia, *Oreochromis niloticus* juveniles. Specifically, the objectives were:

- i. To determine the protein-sparing capacity of sago starch in promoting tilapia, *Oreochromis niloticus* juveniles growth performance.
- ii. To investigate the nutrient digestibility of sago starch based diets in *Oreochromis niloticus* juveniles.
- iii. To determine the digestive and carbohydrate metabolic enzymes of the fish in response toward the sago starch.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This section provides the information on comprehensive knowledge of previous research on the present study. Briefly, this chapter explained on challenges in aquaculture, details on *O. niloticus* (taxonomy, distribution, biology, economic importance, feeding mechanism, digestibility enzymes) and also the potential of sago starch as source of dietary carbohydrate in fish formulated diet. These will be explained further in the following subsections.

2.2 Global Aquaculture and Challenges

Aquaculture is the culture of aquatic organisms, which includes fish, mollusks, crustaceans, algae and plants (Chen et al., 2018). Aquaculture has a long history whose root can be traced back for more than 2000 years in China (Allen & Steeby, 2011; Azaza et al., 2015). The intensification of aquaculture and globalization of the seafood trade have led to remarkable developments in the aquaculture industry (Boonanuntanasarn et al., 2018). Aquaculture is the fastest growing food-producing sector in the world. It is developing, expanding and intensifying in almost all regions of the world. Since the global population is increasing, thus, the demand for aquatic food products are also increasing. Recently, the production from capture fisheries have level off and most of the main fishing areas have reached their maximum potential (FAO, 2019). Therefore, sustaining the fish supplies from capture fisheries are no longer able to meet the growing global demand for aquatic food. Thus, aquaculture is considered as a good potential to be an opportunity to bridge the supply and demand gap of aquatic food in most regions of the world.

The principal challenge of global aquaculture tends to mitigate the supply and demand of fish and fisheries products in paralleled with the outbreking population growth in the world. The FAO (2019) reported that the total aquaculture production including the aquatic plants for 2018 was 114.5 million tons, where only fish and fisheries aquaculture production was 82.1 million tons with a farm-gate value USD 263.6 billion. This inland aquaculture production was accounted at 62.5% contribution in total fisheries production (Figure 2.1). The State of World Fisheries and Aquaculture (FAO, 2019) reported that the world food fish aquaculture production was at 46% in 2018 which was increased at an average annual rate of 25.7% in the period of 2000 to 2018. During this period, the growths were relatively faster in Africa (17.9%), followed by Europe (17%), Americas (15.7%) and Oceania (12.7%). It was reported that the share of aquaculture in Asian fish production (excluding China) has reached 42.0% in 2018, up from 19.3% in 2000 (FAO, 2019). China has produced more farmed aquatic food than the rest of the world combined since 1991. Since the introduced of government policies in 2016, fish farming in China grew by only 2.2% and 1.6% in 2017 and 2018, respectively. China's share in world aquaculture production has declined from 59.9% in 1995 to 57.9% in 2018 and is expected to decrease further in the coming years. Nevertheless, comparison to the projected population by 2030, an additional 40 million tons of fish and fisheries production will be required to maintain the present per capita consumption. Therefore, this sector is going to face some challenges, which are already adopted. Presently, aquaculture is thought to be the fastest growing food producing sector and is perceived as having the greatest potential to meet the growing demand for aquatic food.

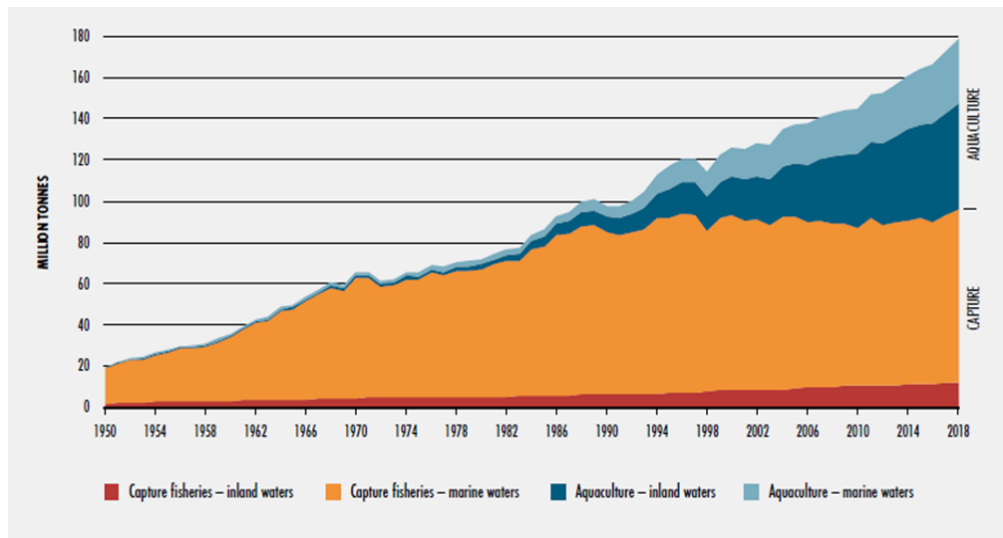


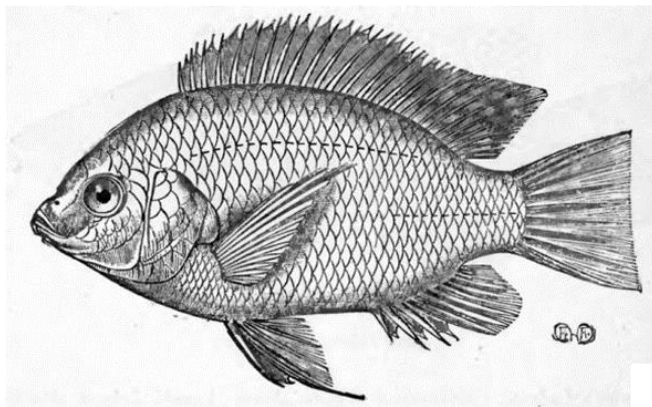
Figure 2.1: Fisheries production of both capture and aquaculture in 1950-2018. Source: The state of World Fisheries and Aquaculture, FAO (2019).

The present aquaculture is growing with special attention for maintaining the food security, mitigating the unemployment, developing the national economy including recreation. The success rate of aquaculture varies with the geographic location, market access and the affordable technology through taking some specific interventions which allow the maintain the production in a sustainable way. Besides, in order to mitigate these challenges also, the aquaculture sector must develop the capacity to build and run effective quality assurance systems to comply with increasing stringent international standards of international markets as well as extending these to the domestic markets. Similarly, it should promote efforts to improve selective feeding technologies to make economical utilization of fishes.

2.3 Taxonomy and Distribution of *Oreochromis niloticus*

Tilapia represents the most common group of the family Cichlidae (Figure 2.2). This group consists of three important genera which are *Tilapia*, *Oreochromis* and *Sarotherodon* (Trewavas, 1983). Cichlids are considered as a family of the most successful fish among

teleosts. It has been estimated that the number of their species is more than 1350 distributed in the worldwide (Xie et al., 2017; Chen et al., 2018). Cichlids are tropical freshwater fishes well known for their high rates of speciation often resulting in rapid radiation (Ndiwa et al., 2014). They exhibit remarkably high levels of genetic and morphological diversity, which affect their morphology, ecology, behaviour and genomes (Azaza et al., 2015; Chen et al., 2017). The detail scientific classification is given below:



Body size descriptions:

Juvenile: > 20 mm / >10.0 g

Adult: > 250 mm / > 200 g

Source: The state of World Fisheries and Aquaculture, FAO (2019).

Figure 2.2: Nile tilapia, *Oreochromis niloticus* used in this study.

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Cichliformes

Family: Cichlidae

Genus: *Oreochromis*

Species: *Oreochromis niloticus* (Linnaeus, 1758)

A large extent of cichlid diversity is observed in three main area of East African Lakes (Victoria, Tanganyika and Malawi) where 1800 to 2000 species (60% of all cichlid species worldwide) are present in species flocks (Nyingi et al., 2009; Ishikawa et al., 2013).

These cichlid species flocks are primarily monophyletic groups of closely related species inhabiting the same ecosystem and evolved from a single ancestral species (Chen et al., 2017). Within these three East African Lakes, some cichlid species went through rapid morphological diversification and speciation, leading to formation of many new species within a few thousand generations (Sultana et al., 2010; Aanyu et al., 2014). In Lake Victoria for instance, the radiation of monophyletic haplochromine species occurred within a geologically short time of about 750,000 years and resulted to the emergence of approximately 500 cichlid species (Kerschbaumer & Sturmbauer, 2011; Ndiwa et al., 2016). Additionally, the evolutionary older cichlid assemblages of Lake Tanganyika have shown the highest degree of morphological differentiations (Ishikawa et al., 2013; Tran-Ngoc et al., 2018).

Under the ecological theory of diversification, a strong relationship exists between speciation events and colonization of new environments, or niche diversification, which may in turn induce changes in organism's behaviour, morphology and physiology (Xiong et al., 2014; Deng et al., 2015). Generally, selection pressures that exist in new environments favour divergence of populations from their ancestral forms. A classic example is provided by the case of the three spine sticklebacks (*Gasterosteus aculeatus*) in Canada, where two sympatric forms (limnetic and benthic) emerged as a consequence of ecological divergence (Ishikawa et al., 2013; Ndiwa et al., 2016). This example among others (Azaza et al., 2015; Boonanuntanasarn et al., 2018) has provided evidence of morphological diversifications as a result of environmental variations.

However, Gagnaire et al. (2013) proposed that ecological divergence may involve multiple facets of species divergence on which divergent selections may operate, hence the

need for an integrative approach to study the process of speciation. Some of the factors proposed to influence divergence include variations in quantitative phenotypic traits, regulation in gene expression and differential transmission of particular allelic combinations (Gagnaire et al., 2013; Chen et al., 2017).

2.3.1 Native Distribution

Tilapias or tilapiine fishes are native to the African continent and well known as one of the major cultured fish in the world after carp fish (Wang & Lu, 2016; Ndiwa et al., 2016). This economically important fish has one of the largest natural distributions among African fresh-water fishes, covering the entire Nilo-Sudanian province (from Senegal to Nile basins), the Ethiopian Rift Valley province, the Kivu province, north Tanganyika province (Ruzizi) and the Northern part of the East African Rift Valley (Xiong et al., 2014). *Tilapia nilotica*, which was classified as *Oreochromis niloticus*, was apparently regarded as sacred by Egyptians some 5,000 years ago (Bezault et al., 2011; Azaza et al., 2015), where depictions in paintings have been found. The same authors indicated that fossil remains of tilapia that are some 18 million years old have been discovered in Africa.

Further, they listed dozens of species in the genera *Tilapia* and *Sarotherodon*, most of which have not been of interest to culturists. While all know tilapia species naturally occur mostly in freshwater environments, some have been found well distribute in brackish African lakes and estuaries (Chen et al., 2018). The claim has been made that tilapia evolved in the marine environment and invaded freshwater (Martins, 2017). That claim may be credible since some tilapia species and hybrids have remarkable salinity tolerances, and both *Oreochromis aureus* and *Oreochromis niloticus* occur in African brackish water lakes (Abdel-Tawwab et al., 2015).

2.3.2 Introduced Distribution

Since World War II, tilapias have been aggressively introduced throughout the world to control aquatic vegetation and insects, as bait fish for fisheries, and to facilitate aquaculture development (Canonico et al., 2005; Ishikawa et al., 2013). Consequently, tilapias are now established in subtropical and tropical waters worldwide due to its great advantages toward aquaculture such as fast growth rate, year-round reproductive season, versatility to adapt to a large range of environmental conditions and lesser susceptibility to viral, bacterial and parasitic diseases than other commonly cultured local fish (Bezault et al., 2011; Van Bers et al., 2012; Azaza et al., 2015). This ability allowing them to be introduced and distributed to many countries outside Africa such as Asia, Southeast Asia, USA and Europe (Chen et al., 2018).

Boonanuntanasarn et al. (2018) indicated that tilapia have now been introduced into some 100 countries for not only culture but also to establish capture fisheries. Interestingly, those authors indicated that 98% of tilapia production in culture occurs outside their native range. Currently, Nile tilapia (*Oreochromis niloticus*), Blue tilapia (*Oreochromis aureus*), Mozambique tilapia (*Oreochromis mossambicus*) and various hybrids including Red tilapia are the commonly produced tilapia (Martins, 2017). One of the most important tilapia species in aquaculture is the Nile tilapia (Chen et al., 2020).

Nile tilapia has been introduced to numerous countries, primarily for use in aquaculture and stock enhancement, and the fish demonstrates high degrees of establishment (García-Berthou et al., 2005; Froese & Pauly, 2011). This species has been recognized for its ability to tolerate a wide range of salinity, dissolved oxygen and temperature, high fecundity and rapid growth rate and an omnivorous mode of feeding (Romano & Kumar,

2019). Due to these attributes, the species has the ability to adapt rapidly to new environments, showing a wide range of biological responses to different environmental conditions both in culture and in nature (Grammer et al., 2012; Azaza et al., 2015). As a result of this plasticity or of the high degree of physiological tolerance found in the Nile tilapia, the species has been able to successfully establish itself in extreme environmental conditions such as temperate winter conditions, hot springs and saline waters (Nyingi et al., 2009; Froese & Pauly, 2011; Schofield et al., 2011; Grammer et al. 2012; Ndiwa et al., 2016). Consequently, Nile tilapia is one of the 10 most introduced fish species worldwide (Lemos et al., 2018) and is established in at least 60 countries (Boonanuntanasarn et al., 2018).

The first introduction of tilapia to Asia was the Mozambique tilapia which also known as *Oreochromis mossambicus*, in the 1940 (Ng & Romano, 2013). The tilapia, *Oreochromis mossambicus* was first introduced to Malaysia from Indonesia in 1943 (Lim et al., 2016). Later, *Oreochromis niloticus* was introduced in 1979 because of its fast-growing features that are suitable for aquaculture (FAO, 2013; Lim et al., 2016). However, the red hybrid tilapia (*Oreochromis* spp.) have become popular due to customer preference and become the dominant species (> 90%) cultured in Malaysia (DOF, 2019).

2.4 Biology of *Oreochromis niloticus*

Tilapia are shaped much like sunfish or crappie but can be easily identified by an interrupted lateral line characteristic of the Cichlid family of fishes (Abdel-Tawwab et al., 2010). They are laterally compressed and deep-bodied with long dorsal fins. The forward portion of the dorsal fin is heavily spine. Spines are also found in the pelvis and anal fins. Nile Tilapia (*Oreochromis niloticus*) is considered as an omnivore fish of the family Cichlidae (Qiang et al., 2014; Ndiwa et al., 2016). This mean that this species can utilize

wide spectrum of foods compare to both carnivores and from many herbivorous fish. This species has been recognized for their rapid growth, tolerance to high, stocking densities and poor water quality, high reproductive rates, and relatively few problems with diseases (He et al., 2015; Xie et al., 2017). Due to these attributes, this species has the ability to adapt rapidly to new environments, showing a wide range of biological responses to different environmental conditions both in culture and in nature (Jiang et al., 2016). As a result, this plasticity (or of the high degree of physiological tolerance found in the Nile Tilapia), the species has been able to successfully establish itself in extreme environmental conditions such as temperate winter conditions, hot springs and saline waters (Romano & Kumar, 2019).

According to Ishikawa et al. (2013), *Oreochromis niloticus* is one of the cultured tilapia species which appear to be a mouthbrooder. Based on the observation by Stickney & Perschbacher (2017), the mouthbrooding behaviour of the species involves the male digging a nest, after which the male courts a female that deposits her eggs into the nest while they are simultaneously being fertilized by the male. The female picks up the eggs and incubates them in her mouth. After several days, the eggs hatch and remain in the mouth of the female during egg yolk absorption. Once the larvae become free-swimming, they will leave the mouth of the female but will seek refuge there if startled for at least several more days.

2.5 Present Status of *Oreochromis niloticus*

The fish, *Oreochromis niloticus*, is an important food fish (Granada et al., 2015) for many countries trade. It is an affordable priced fish due to its sustainable stocks in the fresh market. *Oreochromis niloticus* is the most widely distributed and economically important member of the genus. It attains a length of 30 to 35 cm; common size 20 to 25 cm. It was

reported that the tilapia species was also known as *Tilapia nilotica* and *Sarotherodon niloticus* in the previous as stated by Chen et al. (2018). Until the identification as “*O. niloticus*” is verified, its reputation as the most widely cultured was referred as Nile tilapia.

Oreochromis niloticus is one of the most valuable fish in Asian people; the fish is mostly common staple food fishes in Thailand, Indochina and Malaysia (Stickney & Perschbacher, 2017), probable due to its firm, white and almost boneless tasty flesh and also easy to operate making commercially viable to culture (Wang & Lu, 2016). *O. niloticus* have the ability to adapt in any cultured environment make them distributed widely in many countries in the world. Due to this attribute, the fish can be afforded at a low price because the fish stock meet the demand and considered as a sustainable source of protein that can be obtained easily in the fresh market.

Oreochromis niloticus is presently referred as the “aquatic chicken” for the term in aquaculture (Perschbacher, 2014). This attribute due to low maintenance cultivation, and most importantly its widespread acceptance as a sustainable food. The fish also have an excellent tolerance to different environmental conditions, high survival rate, resistance to diseases and parasitic infections made them as the mainstay of many small-scale (Ndiwa et al., 2014). Furthermore, *O. niloticus* have a high growth rate and as an omnivorous fish in can depend on low protein diet for feeding. Besides, the fish also have high rate breeding success in captivity and easy to handle in cultured farm. Various report suggested that the suitable stocking density for tilapia for grow out in tanks can be increased to more than 30 m³ when food is not limited (Paulino et al., 2016). Despite of the high adaptation characteristics, optimal environmental is important to be maintained as the condition can help the fish gaining more weight and the greater size disparity.

Presently, the fish *Oreochromis niloticus* is used to control the outbreak of blue-green algal blooms in eutrophic lakes (Lu et al., 2006). Blue-green algal blooms are one of the consequences of eutrophication in freshwaters caused by increasing anthropogenic inputs of nutrients. Bloom formation damages water resources, alters the functioning of natural ecosystems and may lead to significant economic losses. Blue-green algal toxins also may pose health problems to human beings (Jochimsen et al., 1998). Therefore, in order to control the blue-green algal outbreak the aquaculture researchers has recommended to use omnivorous *Oreochromis niloticus* as it is a filter-feeding fish (Stickney & Perschbacher, 2017) helps to be the biological control which also more environmentally friendly. During the harvesting, these omnivorous tilapia not only acted as biological control of blue-green algal but also contributed to economic gain since it had high market value.

2.6 Economic Importance of *Oreochromis niloticus*

2.6.1 Native Region

Tilapia is a group of freshwater omnivorous cichlids that are native to Africa and subsequently have been introduced, either deliberately or accidentally, throughout the world (Dizhi et al., 2017). In Africa, tilapia is one of the most exploited inland water fish species in tropical to sub-tropical ecosystems which supporting livelihoods and providing vital food security. This fish is still largely available from traditional freshwater capture resources such as rivers, dams, lakes and much more than it is farmed. Over 350,000 metric tons of tilapia were captured annually during the peak production period of 2016 - 2018 (FAO, 2019).

However, FAO (2019) reported that the catches fell below 300,000 tons gradually. The fishermen reported that it is a drastic decline in catches compared to decades ago, largely due to overfishing fuelled by increase in populations and greater demand. In some cases,

decline in catches is attributed to climate change dynamics. Due to the supply gap, population rise, and increased knowledge of the “goodness” of tilapia products have caused huge demand for farmed tilapia in Africa. Governments in the region, in recognition of the importance of the aquaculture sector in national economies, have begun prioritising its development through several measures, including investments in farming. In fact, aquaculture production of tilapia has grown tremendously, at an annual average rate of 20% over the last decade and it is presently the top fastest growing aquaculture sub-sector. About 150,000 metric tons of tilapia were produced through aquaculture in the region (FAO, 2019).

Tilapia is a traditional and favourite dish in Africa. Some even call it a “democratic fish” in the sense that the fish is consumed as an affordable source of protein in poor rural communities, to be a premium product for the affluent in urban centres. The average annual per capita fish consumption in Africa is approximately 8.9 kg. It is difficult to quantify demand trends specifically for tilapia due to scanty data; however nearly all tilapia produced in Africa is locally consumed, with very limited exports to overseas markets. Notable countries with a strong demand for tilapia include Ghana, Malawi, Nigeria, Uganda and Zambia (FAO, 2019).

2.6.2 Introduced Region

Nowadays, all commercially important tilapia outside of Africa belong to the genus *Oreochromis*, and more than 90% of these farmed fish are Nile tilapia (*Oreochromis niloticus*). They are one of the most productive and internationally traded freshwater food fish. Farming of tilapia has increased in the last three decades as they are easy to grow and market. More than one hundred countries now farm tilapia (FAO, 2019), and 98% of them are grown outside their original habitat (introduced) (Chen et al., 2020). Currently, behind

carps, tilapia is the second most farmed fish in the world, with 3.96 million metric tons of fish produced, comprising 6.3% of global aquaculture production (FAO, 2018). China, Egypt, Indonesia, Philippines and Thailand produce the most tilapia (FAO, 2019). Africa and Asia consume them as a traditional food, and they are now eaten in non-traditional countries and regions such as USA, Canada, Europe, Central and South America as well (Ndiwa et al., 2014).

By far, the vast majority of aquaculture production of tilapia occurs in China at over 1.2 million metric tons in 2018, most of which is Nile tilapia (*O. niloticus*), with Indonesia being the second highest producer at over 250,000 metric tons in 2018 culturing both Nile tilapia and other tilapia species (FAO, 2019). While the USA contributed only 124,784 metric tons of farmed tilapia (< 0.5% of worldwide production) worth USD 439.4 million, their imports make up 32.8% of global exports, although there are indications that Europe will increasingly adopt tilapia as an alternative to traditional fish species. Indeed, the worldwide trade of tilapia has risen from USD 1.7 billion in 2010 to USD 5.0 billion in 2018 and this trend is likely to continue (FAO, 2019).

In Malaysia, aquaculture fisheries grown steadily, growing 36% from 2010 to 2015 and then growing 10% more in the past five years (DOF, 2019). Three major species farmed are tilapias, catfishes and carps. According to the FAO (2019), Nile tilapia (*Oreochromis niloticus*) accounts for 44.7% of the total freshwater aquaculture production in Malaysia. According to the Fisheries Department (2019), about 35,400 metric tons of tilapia worth USD 60.93 million in production value were recorded in Malaysia last year. Malaysian Fisheries Department has set a tilapia production goal of 130,000 metric tons by 2025 to meet the nationwide demand (DOF, 2019).

2.7 Feeding Mechanism and Nutrients Requirement of *Oreochromis niloticus*

2.7.1 Physiology of Digestive System

Oreochromis niloticus is a widely known tilapia species for its importance in aquaculture (Ndiwa et al., 2016). It is generally regarded as a warm-water omnivorous fish and member of the large and diverse family of Cichlidae (Qiang et al., 2014; Dizhi et al., 2017). Generally, herbivorous or omnivorous fish have a higher ability to utilize dietary carbohydrate than carnivorous fish (Krogdahl et al., 2005; Enes et al., 2009; Polakof et al., 2012; Azaza et al., 2015). The main reason may be due to the higher amylase activity in the digestive tracts and metabolic enzyme activity of herbivorous or omnivorous fish than carnivorous fish and the higher affinity of insulin receptors (Zhou et al., 2016; Yang et al., 2017).

Oreochromis niloticus can utilize a wide variety of foods including phytoplankton, periphyton and detritus (Boonanuntanasarn et al., 2018) and has a digestive system that differ from both of carnivores and any herbivorous fish (Chen et al., 2020). *Oreochromis niloticus* have teeth on their jaws as well as on the pharyngeal bones. The teeth are used to crush feed increasing the surface area which in turn facilitates enzyme-substrate interaction in the stomach. Digestion is involved two steps process which are gastric and intestinal components (Jafari et al., 2018). The stomach has an extremely acidic environment, often with very low pH which enables lysis of plant cells exposing them to digestive enzymes in the intestine (Rodrigues et al., 2012; Gominho-Rosa et al., 2015). The intestine is very long (about 7 to 13 times total body length of an adult fish), coiled and has a high pH due to secretion of bile salts (Kamalam et al., 2017). This facilitates action of enzymes like trypsin, chymotrypsin and amylase (Xie et al., 2017). Therefore, considering all these digestive

characteristics it is possible that *Oreochromis niloticus* have the abilities for digesting starch-rich feedstuffs.

2.7.2 Nutrient Requirements

There is widely literature about the nutrition of *O. niloticus* found (Azaza et al., 2015). As it is a freshwater omnivorous fish, the source of feed should be more variable and extensively available for feeding. Therefore, the aquaculture farmers fed with trash fish and cattle blood mixed with wheat flour or spent grains and rice bran (Zhou et al., 2011). Kumar et al. (2018) stated that during the first month of *O. niloticus* larvae, the best food should be *Artemia* and *Moina* compared to formulated diet; because the formulated diet during this time is much less acceptable and can be responsible to be low survival rate and growth rate. Qin and Fast in 1997 explained the feeding habitat in a well-organized where the author stated that for growing up the habituated on formulated diets, *O. niloticus* could be trained using two methods. Method 1: feed the larvae with *Artemia* nauplii supplemented with formulated feed for 30 days and slowly stop giving live food over a 7 to 10 days period. Method 2: feed the larvae with *Artemia* nauplii only for 30 days and feed *Artemia* and formulated feed for following 7 to 10 days. After that they can be adapted to consume formulated feed.

Numerous literatures stated that the artificial feed should contain the right protein or lipid/protein ratio in order to gain good growth (Couto et al., 2017). Generally, tilapia fry and spawning females have a higher protein requirement (30 to 40%) than the fingerlings and the grow-out stages (20 to 30%) for optimal growth performance (Abdel-Tawwab et al., 2010; Azaza et al., 2015). Besides, appropriate dietary lipid levels are also important to be considered since deficiencies or excesses can reduce growth or produce an excessively fatty

fish, respectively. The minimum requirement and optimal level has been shown to be 5% and 12% dietary lipid, respectively, in the feeds for hybrid tilapia (Maas et al., 2019). Moreover, at various tested protein levels of 6 to 30%, increasing dietary lipid inclusions from 6% to 18% linearly increased protein sparing in all diets, while a lipid level of 24% decreased the protein sparing (Haidar et al., 2016). The major nutrient requirements of cultured tilapia are reasonably well established and are summarized in Figure 2.3 and Figure 2.4 (FAO, 2019).

Life stage	Weight (g)	Requirement (%)
First feeding larvae		45-50
Fry	0.02-1.0	40
Fingerlings	1.0-10.0	35-40
Juveniles	10.0-25.0	30-35
Adults	25-200	30-32
	>200	28-30
Broodstock		40-45

Figure 2.3: Protein level requirements for tilapia.

Crude lipid, % min	10 - 15
Essential fatty acids, % min	
18:2n-6	0.5 - 1.0
20:4n-6	1.0
18:3n-3	
20:5n-3	
22:6n-3	
Carbohydrate, % max	40
Crude fibre, % max	8-10
Protein to energy ratio	110
(mg/kcal)	120

Figure 2.4: Essential crude lipid, fatty acid, carbohydrate, crude fibre and energy composition in the diet of tilapia.

Carbohydrates are categorized as monosaccharides, disaccharides, oligosaccharides or polysaccharides. Unlike protein and lipids, fish have no specific requirement for these nutrients (Hamid et al., 2011). Nevertheless, carbohydrates provide nutritive benefits including being an inexpensive alternative source of energy to protein (i.e. protein sparing) (Shiau & Peng, 1993), improving growth (Moura et al., 2019) as well as improving the stability of pelleted feeds (Ng & Romano, 2013). In practical terms for fish, carbohydrates

can be broadly categorized as starches (simple and complex) and non-starch polysaccharides (NSP). Starches include dextrin and glucose which are relatively well digested, and in contrast, non-starch polysaccharides are utilized much more poorly or not at all (Francis et al., 2001; Sinha et al., 2011). Based on the findings reported by Azaza et al. (2015), the essential level of dietary carbohydrate for tilapia were varied from 35% to 40% in order to gain the maximal growth performance. Gatesoupe et al. (2014) stated that the fish can utilize carbohydrate better than lipids, however more than 42% of carbohydrate tended to lower the specific growth rate.

Furthermore, the essential micronutrients (vitamins and minerals) also need to be considered in fish feed formulation because its involved in a wide variety of physiological processes necessary for the overall health of fish (Ng & Romano, 2013). Vitamins are organic nutrients that are essential for a wide variety of processes, and include aiding nutrient utilization, improving immunological responses, providing antioxidant protection and maintenance of other basic physiological processes (Krossøy et al., 2011; Liu et al., 2015). Minerals are inorganic elements that play vital roles in enzyme function, including many of those responsible for digestion, gas exchange, acid/base balance and osmoregulation (Shiau & Tseng, 2007; Cnaani et al., 2010; Da Silva et al., 2014). Commercially prepared vitamin and mineral premixes are often added to the formulation diets of fish. However, the composition of these premixes may be specific to one fish species and may not fulfil the optimal requirements of other species. Moreover, the mineral and vitamin requirements may change depending on the culture conditions or dietary composition.

2.8 Dietary Carbohydrate Sources

Starch is found in almost all tissues (leaves, roots, tubers, seeds, stems and flowers) in green plants. There are plenty number of plants which are commercially grown for starch including wheat, corn, and rice (cereal), potato (tuber), tapioca (root), and sago (stem) crops. All starches occur as minute granules in nature but the size, shape, and nature of molecular arrangement inside the granule depends on the species and sometimes on crop cultivar or variety. The processing methods employed for the extraction of different starch types were mainly depends on the nature and composition of raw material. This section reviews on the dietary carbohydrate sources from some major types of commercial starch crops.

2.8.1 Corn Starch

Corn, *Zea mays* L. is a member of Poaceae family. It has been developed as a staple food in several region of the globe (Scott & Emery, 2015). The botanical features of corn are well explained by Palanisamy et al. (2020). It is a heavy, vertical, solid stem and large, thin leaves form a tall annual grass. The female inflorescences (pistillate) that develop to be the torn ears are spikes with a consolidated hub, bearing matched spikelets in longitudinal columns; each line of combined spikelets as a rule makes two lines of grain. Corn is extensively cultivated all over the world, and a large quantity of corn is cultivated every year with total production about 1.04 billion metric tons. United States is the main producer of corn with total amount estimated around 366.3 million tons follows by China produced 257.3 million metric tons and Brazil produced 94.5 million metric tons (Palanisamy et al., 2020).

Zhao et al. (2009) stated that corns are rich in dietary fiber, nutritional supplements (vitamins A, B, E, and K), minerals (magnesium, potassium, and phosphorus), phenolic acids and flavonoids, plant sterols, and various phytochemicals (lignins and bound

phytochemicals). Around 80% of the world starch production is corn starch, which was extracted from corn pieces (64 to 80%) through the wet-processing process (Marichelvam et al., 2019). Corn starch is used in a broad variety of foodstuffs and applications. Basic corn starches have a small amount of protein (0.35%), lipid (0.8%), ash, and > 98% of two polysaccharides, namely amylose and amylopectin. Starch comes from plant sources that are insoluble in water, and at room temperature, it is in the form of granules (Srichuwong et al., 2017). Usually, corn and waxy maize starch granules differ in their size from 2 to 30 μm ; most fall in the range of 12 to 15 μm . They also differ in shape, appearing as cross-sections of polygons (Qin et al., 2016).

Corn starch is a recommended practical ingredient in aquaculture industry to reduce the production cost of fish feed (Hamid et al., 2011). The incorporation of corn starch in the formulation of aquaculture feeds are well documented. Gominho-Rosa et al. (2015) reported that Nile tilapia utilized corn starch more efficient and consequent increased feed absorption might be to the amylopectin fine structural characteristics of the starch. Li et al. (2019) stated that amylase-specific activity significantly higher in fish feed with corn starch shows that the amylase susceptibility of Type A starch cereal is higher than that of Type C. It is generally accepted in starch studies that the amylopectin unit chain length distribution, which is the basis for classification of starches, is one of the parameters that affects amylase activity (Marlida et al., 2000). Type A starch has “weaker points” that are more susceptible to amylase activity. These “weaker points” are believed to be derived from the branch linkage in the crystalline region and short double helices from short A-chains.

2.8.2 Potato Starch

Potato is a highly valuable vegetable crop with a wide variety of uses as fresh or processed foods. Total annual world production of all potato starches was approximately 85 million tons (Dupuis & Liu, 2019). Starch is the major carbohydrate in potatoes and is an agriculturally important commodity with many food and non-food uses (Yang et al., 2013). In food nutrition, starch plays a major role by supplying the metabolic energy that enables the body to perform its functions; it is the basic source of energy for a large portion of the world's population. Besides as a source of carbohydrates, potatoes also contain significant amounts of protein, ascorbic acid and other vitamins, phenolic substances, and minerals such as phosphorus, potassium, and calcium (Zhao et al., 2018). The chemical composition and structure of potato components, such as starch, non-starch polysaccharides, sugars and other carbohydrates, organic and inorganic compounds, and proteins are often influence the quality of potatoes and potato products.

Potato starch is a mixture of two polysaccharides, amylose and amylopectin. Amylose is a relatively long, linear α -glucan with few branches, containing approximately 99% α -(1,4) linkages and up to 1% α -(1,6) linkages, whereas amylopectin is a heavily branched structure, containing approximately 95% α -(1,4) and 5% α -(1,6) linkages in a hierarchical structure (Hong et al., 2018). The carbohydrates content of potatoes is 13.5 to 15% on a fresh weight basis, but on a dry weight basis it is approximately 75 to 80% (Kalita et al., 2018). Potato starch from various cultivars typically contains 20 to 33% amylose on a total starch basis, with the balance being amylopectin (Pycia et al., 2012; Wang et al., 2015). Potato starch is found in nature in discrete starch granules that have distinct refractive indices. This is due to differences in underlying structures, alternating crystalline and

amorphous layers. Potato starch granules are approximately 40 to 50 μm in diameter on average with a range of 10 to 110 μm (Waterschoot et al., 2015).

The applications of potato starch were potentially used for paper, textile, chemical, fermentation, pharmaceutical and petrochemical industries and in the production of industrial alcohol (Dupuis & Liu, 2019). Starch is indispensably used in paper, glue and corrugated paperboard industries. In the pulp industry, it is employed as a flocculating agent to increase the rate of dewatering of pulp. Starch remained in the finished paper act as an internal sizing agent and increase paper strength. It is also used for coating on paper sheets to increase smoothness and improve printability and writing properties by filling up the pores. In aquaculture, the used of potato starch in feed formulation have less favourable compared to corn and wheat starch (Type A starches). Type B starches, found mainly in tubers such as potatoes (Srichuwong et al., 2005), consist of amylopectin chains of 30 to 44 glucose molecules (Hamid et al., 2011). Its complexity and high number of amylose and amylopectin structure could be the main reason that lead to low utilization in fish digestibility.

2.8.3 Tapioca Starch

Tapioca starches are extracted from cassava (*Manihot esculenta* Crantz) which is a woody shrub grown in tropical and subtropical regions of the world (Hsieh et al., 2018). Due to the plant's drought tolerance, adaptability to marginal soil, and the rich store of starch in its tuberous root cassava has long been a staple food (Sanchez et al., 2010). Africa lead the cassava production estimated 57% (equivalent to 157 million metric tons) of the world output, followed by 32% for Asia, and 10% for South America (FAO, 2019). Nigeria (20.6% of total cassava production), Thailand (11.2%), Brazil (7.6%), and Indonesia (7.5%) are the

four countries contributing to the majority of global production. Although cassava is primarily consumed to provide food energy in Africa, most cassava in Asia and South America is processed into starch for industrial uses. In particular, the value of cassava starch exported from Thailand and Vietnam reached from USD 1180 to 2778 million in 2019, respectively (OEC, 2021), which amounted to 95% of global cassava starch export value. For years, cassava starch has been known to be a heavily traded starch, comparable to maize, potato, and wheat starches (OEC, 2021).

The name cassava usually refers to the tuber of the plant in North America and Europe, whereas tapioca refers to the starch or other processed products from the plant (Diaz et al., 2017). Tuber is the main source of starch meanwhile leaf is the rich source of protein, vitamins and minerals. Nutritionally, cassava contains potassium, iron, calcium, vitamin A, folic acid, sodium, vitamin C, vitamin B-6, and protein (Montagnac et al., 2009). According to Fakir et al. (2012), tapioca starches contain mainly carbohydrates (84.5%) with proximate composition of crude protein (6.86%), lipid (1.88%), fibre (0.60%) and moisture (15.20%). The starch primarily consists of two types of α -glucan distinct in molecular structure which is amylose that essentially as a linear linked glucan while amylopectin is the branched glucan as the structure backbone (Zhu, 2015). Tapioca starch granules have a round, truncated shape with an average granule size between 12.1 to 16.3 μm (Morante et al., 2016).

Recently, interests in development of biopolymer based edible/biodegradable films have increased because of the demand for natural food ingredients and their potential to increase product shelf life and decrease environment pollution (Shevkani & Singh, 2015). Tapioca starch can be an attractive choice for the preparation of edible and/or biodegradable films due to low cost, high availability, renewability and biodegradability. Tapioca starch

was evaluated for film-forming properties and it was concluded that tapioca starch-based films could be used in the food systems where edible films should disintegrate during cooking or mastication (Basiak et al., 2015). Based on the view of aquaculture, the application of tapioca starch in fish formulated diet shows much less attention compared to corn and wheat starch. This due to its high waxy level and might not compatible to incorporate with other ingredients during diet formulation which resulted into low digestibility and utilization (Romano & Kumar, 2019).

2.8.4 Rice Starch

Rice (*Oryza sativa* L.) is a major cereal crop and the staple food source for half of the world population. Starch is the major component of rice and accounts for more than 80% of the total constituents. The global rice (paddy) production for the year 2020 reached 115 million metric tons (FAO, 2020). Increase in rice production and demand for polished rice has significantly increased the amount of broken rice. Moreover, new rice cultivars are continuously being released and the total number has exceeded more than 2000 cultivars around the world (Deepa et al., 2008). Rice starch is reported as unique, with bland taste, creamy, spreadable, and smooth in texture. These characteristics include hypoallergenicity, digestibility, consumer acceptance, bland flavour, small granules, white colour, increased freeze-thaw stability of pastes, greater acid resistance, and a wide range of amylose: amylopectin ratios (Lawal et al., 2011). These unique properties have increased rice starch demand in the food and pharmaceutical industries.

The proximate composition of rice starch are approximately 12.49% (Moisture), 1.37% (ash), 1.73% (fat), 8.11% (protein) with 75% to 80% carbohydrate content. Starch occurs naturally as discrete particles, called granules (Mitchell, 2009). Rice starch granules

are the smallest known to exist in cereal grains, with the size reported in the range of 2 to 7 μm (Wang et al., 2012). These granules have smooth surface but angular and polygonal shapes. Starch properties depend on the physical and chemical characteristics such as mean granule size, granule size distribution, amylose/amylopectin ratio, and mineral content (Sandhu et al., 2010). Amylose is the major factor influencing the physicochemical properties of rice starch. Upon heating in aqueous solutions, starch swells irreversibly and its crystalline structure collapses, a phenomenon known as gelatinization. Starch swelling is a property of amylopectin, whereas amylose has been known to restrict it (Wani et al., 2012). Further, gelatinization converts starch into a physical form that is desirable in many food systems.

Rice starch is well known for its nutritive value and important carbohydrates that provide essential energy for living sustainability. Numerous study is recorded on the application of rice starch in the formulation of aquaculture feeds. It show that the inclusion of 200 g/kg or 300 g/kg rice starch in the diets, as a carbohydrate ingredient, did not cause any adverse effects on the growth and body chemical composition or the digestive enzymatic activities of fish (Loureiro et al., 2019). Hou et al. (2008) stated that rice starch is a commercially available ingredient widely used in the feed industry for terrestrial and aquatic animals. Furthermore, rice starch is also potentially be an alternative way to spare the expensive protein component in aquaculture feed production.

2.8.5 Wheat Starch

Wheat is one of the most grown, consumed and traded food grains of the world with a worldwide diversity of thousands of cultivars/varieties. It constitutes a major portion of diet of the world because of its agronomic adaptability, ease of storage, nutritional goodness

and the ability of its flour to prepare a variety of palatable and satisfying foods (Zhang et al., 2017). More than 713 million metric tons of wheat worth USD 85.94 billion is grown throughout the world on approximately 218 million hectares of land (FAO, 2020). It is the fourth most important food commodity in terms of both production quantity (after sugar cane, maize and paddy) and value (after meat, milk and paddy). China, India, USA, Russia, France, Canada, Germany, Pakistan, Australia and Ukraine are the top ten wheat producing countries in the world (FAO, 2020). Wheat is mainly utilized as food (67%); however, a significant portion is also used for feed (20%), seed (7%) and industrial purposes (6%), respectively (Sobota et al., 2015). *Triticum aestivum* is the most commonly grown species of wheat, accounting for 90 to 95% of total production. This wheat is most preferred because its primary used for making bread products, hence, referred as bread wheat (Gelinas & McKinnon, 2016).

Wheat starch is obtained by wet milling of white flour, preferably that from softer grade wheats. The “Martin Process” and the “Batter Process” are the processes applied to produce the wheat starch (Liu & Ng, 2015). Wheat starch is unique because of the properties of two principal macromolecular components, protein (gluten) and starch. Wheat gluten is responsible for the dough-forming ability of wheat flour to make bread, which is unmatched by any other proteins of plant, animal, or microbial origin (Lafiandra et al., 2014). It have a various granule sizes approximately range from 2 μm to 50 μm (Zhang et al., 2017). Wheat starch contain two granules called A-granules and B-granules. The ratios of A-granules to B-granules and amylose to amylopectin plus granular, structural, and molecular characteristics of wheat starch dictate the overall properties and behaviour of wheat starch in foods (Singh et al., 2015).

More than 60% of the total starch extracted is used for food and rest for non-food purposes. Wheat starch has an edge over other starches owing to brighter appearance and blander flavour (Yu et al., 2015). It is often used as an ingredient in several formulated foods to improve texture and mouth feel. It functions as tenderizer in cakes, doughnuts, cookies and cheese analogues. It is also incorporated to batters, ice creams, soups, gravies, etc., to provide adhesion, structure, moisture control and/or thickening. Besides, it is also used for the production of hydrolysis products such as syrups and sweeteners, which are widely used in beverages and confectionery industry (Shevkani et al., 2016). In aquaculture, wheat starch is a very common practical ingredient used in fish feed production. Jiang et al. (2019) stated that the inclusion of dietary wheat starches had no adverse effect on the growth of juvenile yellow perch. Besides, wheat starch was also reported to be efficiently utilized and shows better growth by gilthead seabream, *Sparus aurata* (Couto et al., 2016). This suggested that wheat starch potentially reduce the aquaculture feed cost by spar the expensive protein component in diet formulation.

2.8.6 Sago Starch

Sago (*Metroxylon sagu*) is a palm species which is indigenous to Southeast Asia region, specifically Malaysia, Indonesia, Papua New Guinea, and the Philippines (Jong, 2018). It is known for its tolerance to wet growing condition and has a main advantage of the ability to thrive in harsh swampy peat environment (Naim et al., 2016). In regard to sago production, currently Malaysia is the third largest sago producer in the world after Indonesia and Papua New Guinea which combined produce approximately 94.6% (Ahmad, 2014) of the world production. In Malaysia, the main production of sago is come from Mukah, Sarawak (67, 957 hectares) with yield about 42,000 to 50,000 metric tons procuring incomes between USD 3.4 million to USD 10.8 million (MAM, 2019). Based on the report stated by

Karim et al. (2008), the sago crop industry in Sarawak is well established and has become one of the important industries contributing to export revenue.

Sago starch is obtained from the trunk of sago palm (*Metroxylon sagu*) through the process of extraction and purification (Othman et al., 2015). According to Ishizuka et al. (1995), sago palm contains a large amount of starch in its trunk and its productivity was calculated to be 4 times that of paddy rice. Starch accumulation in the trunk of the sago palm can reach up to 250 kg dry weight per plant (Singhal et al., 2008). The crude starch usually referred to as 'sago flour' is essentially a raw material, which is refined, purified, or slightly bleached by oxidation with small quantities of hypochlorite (0.25 to 6% chlorine on dry starch basis) using modern processing machinery for sieving, dewatering and drying (Karim et al., 2008). Abd-Aziz (2002) reported that the starch obtained from sago palm is well known for its important in agriculture, ranking fifth highest in terms of agricultural revenue after pepper, palm oil, cocoa and rubber.

Sago starch comprises of about 27% amylose and 73% amylopectin (Ito et al., 1979). The sago starch behaves more like waxy corn, even though it is an amylose-containing starch (Singhal et al., 2008). Ahmad et al. (1999) concluded that the proximate composition and other physicochemical properties of sago starches from different origins did not vary significantly, except for the molecular weight in the amylose fraction and also the amylose content. Proximate composition studies showed that the moisture content in the sago starch varied between 10.6% to 20.0%, ash between 0.06% to 0.43%, crude fat between 0.10% to 0.13%, fiber between 0.26% to 0.32%, and crude protein between 0.19% to 0.25%. Sago starch has favourable carbohydrate profile (89.79%) and low priced approximately USD 450

per tons (Jalil et al., 2015). This has encouraged the sparing of fishmeal protein with sago starch as carbohydrates sources which can reduce the production cost of aquaculture feeds.

Sago (*Metroxylon sagu*) starch is regarded as a nutritive and important plant carbohydrates that has been considered for an alternative approach used to spare protein in the fish feed (Umar et al., 2013). Numerous study is reported on the application of sago starch as protein sparing in the formulation of aquaculture feeds. Hamid et al. (2011) reported that raw or cooked sago starch as carbohydrate source have no influence on the growth performance response and tolerable postprandial plasma glucose levels of *M. nemurus*. Umar et al. (2013) stated that the application of sago starch in aquaculture feed is very recommended due to its excellent moisture content in mixture feed to produce high quality of floating pellets. The level of carbohydrate included in the diet is an important consideration for formulating healthy metabolically efficient diets (Azaza et al., 2015).

2.9 Fish Haematology

Haematology plasma is a light yellow component that comprised of 55% of the total blood volume which contained water, salts, enzymes, antibodies and other proteins (Huyben et al., 2017). The main functions of haematology plasma are to maintain blood pressure and volume; transport nutrients, hormones, and proteins in the body; carry electrolytes such as sodium and potassium to muscles and to balance proper pH level in the body which supports cell function (Xu et al., 2019). Haematology plasma is extracted by centrifuging the blood sample in a tube contained of anticoagulant until a yellowish liquid formed on the top of the tube. Zhou et al. (2016) mentioned that glucose, triglyceride, and total protein contents in haematology plasma were related to animal health, and lower or higher plasma tissue will

reflect abnormal physical and chemical changes occurring in organisms, and it indicates general metabolism and physiological status.

Kamalam et al. (2017) stated that the optimum glucose level in blood plasma is very important to maintain the glucose homeostasis in the body. The glucose homeostasis is essential for vertebrates including fish because glucose is an important fuel for the functioning of specific tissues including the brain, gills, erythrocytes and gonads (He et al., 2015). To maintain the glucose homeostasis, cellular glucose transport and glucose utilization will be induced after feeding in insulin sensitive tissue such as liver, muscle and adipose tissue, whereas hepatic glucose production (HGP) will be provoked during the pre-prandial period (Chen et al., 2017). In mammals, cellular glucose utilization is always linked to increased glycogenesis, glycolysis and pentose phosphate pathway, while HGP is linked to enhanced glycogenolysis and gluconeogenesis from lactate, glycerol and some amino acids (NRC, 2011). Although lipogenesis is not directly linked to glucose metabolism, this pathway plays an important role in glucose homeostasis due to the conversion of glucose in excess into fatty acids (Liu et al., 2019). It is generally accepted that fish have a lower ability to utilize dietary carbohydrate than mammals because of their ectothermic lifestyle and evolutionary history (Polakof et al., 2012; Kamalam et al., 2017). To our knowledge, existing literature regarding the regulatory mechanism of glucose homeostasis mainly focus on omnivorous fish, especially in Nile tilapia, *Oreochromis niloticus*.

The triglyceride is well known for its water insolubility characteristic. It is transported in plasma with the combination of polar lipids (phospholipids) and proteins, as well as with cholesterol and cholesterol esters, in complex macromolecules known as lipoproteins (Lemos et al., 2018). Martins (2017) stated that the structure of the lipoproteins

are not depending on covalent linkages between the various components but determined by the combination of polar and non-polar interactions between them. The non-polar triglyceride (and cholesterol ester) is attached directly to the lipoprotein while the polar protein and phospholipid components are on the surface and these interactions help to stabilize the whole structure in the aqueous plasma environment (Wang et al., 2016). About 30 to 40% of the feed intake is normally in the form of fatty acids which consist of triglycerides. The digestion in the intestinal lumen breaks down the triglyceride into monoglycerides and absorbed by the intestinal cells which is released into the lymphatics in lipoproteins called chylomicrons (Sun et al., 2016b). It is important to maintain the concentration of lipoprotein to keep the balanced between the rate of entry and removal of triglyceride process in the plasma (Li et al., 2018). The imbalance toward the triglyceride concentration indeed may results into the excess fat production in the body as mentioned by Jia et al. (2019). Therefore, the appropriate level lipid/carbohydrate in formulated diet need to be considered and revised properly before the production of aquaculture feeds.

The presence of total protein in plasma is important for the indicators to determine the health, stress, and well-being status of terrestrial and aquatic organisms (Adorian et al., 2017). Kim et al. (2016) stated that the optimal growth and feed utilization in fish and other animals depend on the balanced total protein concentration in the plasma and muscle tissue as well as an optimum and appropriate source of the nonprotein energy. The utilization of dietary protein and amino acids depend on the processes of digestion and absorption. The digestive enzymes increased the efficiency of digestion process while protein plasma transport the digested protein and amino acids for the absorption process by the body (Ma et al., 2019). It has been obtained in fish that total protein plasma changes with the variation of dietary protein level (Huang et al., 2016; Sagada et al., 2017). Jiang et al. (2016) reported

that the amino acids absorption and transportation in the plasma is regulated by the transporters expressed on enterocytes. The Peptide Transporter 1 (Pep T1) is a low-affinity, high-capacity transporter that is fully responsible for the transport of di- and tripeptides from the intestinal lumen into the enterocytes (Deng et al., 2014). It has been shown in fish that different level of dietary protein, peptides and free amino acids affect Pep T1 expression (Bakke et al., 2010; Song et al., 2017) in the plasma. Therefore, the availability of total protein in the plasma lead to efficient protein absorption and consequently high growth and feed utilization.

Taking into account, the different source or type of feed intake is one of the factors which can affect the blood plasma compositions in the terrestrial animal and fish. Different fish species (carnivorous, herbivorous and omnivorous) may have differ ability to utilize their feed which associated with the absorption efficiency of nutrients in the plasma (Bonvini et al., 2018). Therefore, the feed formulation according to the respective species is considerable to improve cultivation efficiency and reduce feed cost production. Based on the fish physiological research, blood plasma biochemical and physiological parameters may change greatly depending on the sampling conditions (Luo et al., 2014). Thus, it is important to minimize the effects of handling stress, anaesthesia, and starvation, which can affect the physiological parameters in the blood of fish (Shi et al., 2010). Nordlie (2009) reported that the regulation of blood plasma components in fishes also influenced by the environmental factors in which freshwater fish have a balance blood plasma composition compare from fish from the saline environments.

2.10 Nutrient Digestibility and Digestive Enzymes Activities

2.10.1 Protein Digestibility

Debnath et al. (2017) defined the nutrient digestibility of a feed which determines the amount of nutrients that is actually absorbed by an animal and therefore the availability of nutrients for growth, reproduction etc. Usually the nutrient digestibility is determined by measuring the protein digestibility which involves a quantitative measurement of total protein in the diet ingested by the fish species which is not recovered in the faeces. Excellent digestibility cannot depend on the high content of protein and balanced amino acids profile especially if these protein and high amino acids are not well digested and absorbed by the fish. The efficiency of fish feed depends on the availability of the nutrients which are absorbed by the fish body (Sandrea et al., 2017) does not depend on the nutrient contents of the feedstuffs. Protein is noted as the most expensive nutrients in the aquaculture feed industries (Boonanuntanasarn et al., 2018). Because the cost of protein is estimated around 50% of the total cost production. In fact, the digestive protein plays a vital role in growth and health performance of cultured fish species including the *Oreochromis niloticus* which is considered as one of the best commercial important fish species (Sultana et al., 2010).

De Silva and Anderson (1995) stated that the protein digestibility depend on several factors such as the types and physical characteristics of the feedstuffs or diets, the experimental fish species and the physiological development stage of fishes which are relevant to the growth performance. There are four methods of measuring the protein digestibility described by De Silva and Anderson in 1995, which are based on (1) the use of markers; (2) the use of faecal indices; (3) *in vitro* analysis of consumed feed; (4) *In vivo* analysis of consumed feed. Of these, only the first three are relevant to the diagnostic phase of livestock systems research. The *in vivo* method is more applicable to on-station research

and involves intensive laboratory work and careful supervision. However, the present study was used the *in vitro* method for determining the protein digestibility. Therefore, the study has reviewed *in vitro* protein digestibility method only.

It is noted that information regarding the ability of intestinal proteases to hydrolyze the protein source and their final assimilation by the fish is crucial. This could be determined among all through nutrient digestibility studies using rapid *in vitro* method where use the crude enzymes which extract from the fish gut (stomach and intestine). Rahmah et al. (2016) stated that this methods are easy to analyze on different types of feed within an hour with high level of sensitivity. Many studies in aquaculture use the extracted crude enzymes from the fish gut to determine the *in vitro* protein digestibility of feedstuffs for cultured species (Sealey et al., 2011; Ngo et al., 2015; Debnath et al., 2017; Lewis et al., 2019). For determining *in vitro* protein digestibility method, various methods are assayed; among all, pH drop method is widely used (Rahmah et al., 2016; Lewis et al., 2019) because it is comparatively rapid, reproducible, cost and labour effective compared to other methods.

2.10.2 Carbohydrate Digestibility

Dietary carbohydrates, namely sugars, starch, and non-starch polysaccharides (NSPs), are major energy sources in the diet that support body metabolism. Food of any plant origin, such as fruits, vegetables, edible seeds, grains, legumes, and wholegrains, are reliable sources of dietary carbohydrates for any living creatures (Tian et al., 2016). However, the nutritional quality of carbohydrates strongly depends on the types, nature or source of the carbohydrate, interaction with other food constituents, its structures, processing methods that affect their physical and chemical characteristics, particle size of plant foods and their digestibility properties in the normal digestion system of living things (Wang et al.,

2015). This section provides the review of dietary carbohydrates based on its digestibility which can vary from simple and easily digestible carbohydrates to non-digestible carbohydrates.

The digestibility of some carbohydrates can be limited by the fact that the macromolecules are physically protected in specific compartments of the grains, seeds, or plant tubers that are inaccessible to the digestive enzymes because the carbohydrates naturally exist in a structural form that prevents the digestive enzymes from breaking them down or being easily digested (Hong et al., 2018). The digestibility of these carbohydrates can be substantially improved when appropriate food-processing techniques, such as milling are applied to the plant food. They ease the liberation of carbohydrates from the food matrix during ingestion. Moreover, the transformation of certain carbohydrates, such as starch, through conventional thermal or emerging nonthermal food-processing technologies into a structural form that affects their digestion rate has been evidenced (Juansang et al., 2012).

Starch is considered as the most important dietary carbohydrate that can provide an abundant amount of glucose upon human digestion because starch is a polysaccharide that is made up from polymers of glucose units bound together via glycosidic linkages (Lovegrove et al., 2017). Indeed, the living things may secrete a range of digestive enzymes that can help to degrade starch polymers into glucose molecules which are then absorbed into blood circulation (Xie et al., 2013). Being an energy storage carbohydrate in plants, starch exists in various botanical origins such as tubers, grains, and pulses. Hence, they are always consumed as staple foods (potatoes, wheat, maize, rice, cassava) by many populations around the world (Han et al., 2012). On average, starchy foods account for up to 60% of a human diet on a daily basis (Zeng et al., 2016). However, excess dietary

carbohydrates in diet may rapid the absorption of glucose in the body which might affect the blood glucose response and insulin demand that lead to metabolic disorders particularly a diabetic.

Compared to simple and easily digestible carbohydrates, non-digestible carbohydrates are those that cannot be hydrolysed by the digestive enzymes and absorbed in the upper gastrointestinal (GI) tract (Dharmaraj & Malleshi, 2012). Non-digestible carbohydrates in the diet include NSPs, β -linked disaccharides and oligosaccharides, and resistant starch. NSPs are plant components that make up the cell wall and all NSPs could be categorized as dietary fiber (Lovegrove et al., 2017). Most non-digestible carbohydrates are polymers of different monosaccharide units joined by β -glycosidic bonds. Enzymes in the digestive system are not able to cleave this kind of linkage and hence indigestible carbohydrates pass into the large intestine where they are fermented by the colonic bacteria to provide health benefit. The most common non-digestible carbohydrates is β -linked oligosaccharides (galacto- and fructo-oligosaccharides) which have the prebiotic properties that can promote the growth of beneficial lactic acid bacteria (probiotics) in the gut (Yang et al., 2017).

2.11 Protein Sparing of Carbohydrate on *Oreochromis niloticus*

It is well known that the non-nitrogenous foodstuffs may spare the body protein. The carbohydrates are unquestionably more efficient as protein sparer than are fats. Many studies have been performed with tilapia (*O. niloticus*), comparing sparing effect across a wide range of plant ingredients (carbohydrate source) used as substitutes for dietary protein (fishmeal). Carbohydrates are the cheapest energy source for human, fish and other animals. However, its requirements have not been extensively investigated compared to lipid and protein

requirements (Haidar et al., 2016). Carbohydrates is commonly accepted that appropriate levels of carbohydrates should be incorporated in fish diets to improve the energy availability, albeit sparingly for carnivorous fish like trout and salmon which are less well adapted to digest complex carbohydrates (Maas et al., 2020). Lack of carbohydrates in fish diets will increase the catabolism of protein and lipids (Schrama et al., 2012) while sufficient levels of easily digestible carbohydrates allow for protein sparing effects (Castillo & Gatlin, 2015).

The optimal level of dietary carbohydrates differs widely among fish species. In general, warm-water and freshwater fish are known to utilise higher levels of carbohydrates than cold-water or marine species (Kamalam et al., 2017). Omnivorous fish can handle higher levels of carbohydrates than carnivorous fish and show much higher rates of protein sparing (Hemre et al., 2002; Krogh et al., 2005). A review by Stone (2003) stated that a dietary digestible carbohydrate level of 40% for tilapia while the optimum for marine carnivorous and cold-water fish (i.e. Atlantic salmon, rainbow trout and plaice) is below 20%. Besides differences in optimal dietary carbohydrate levels, the way fish utilise digestible carbohydrates differs between species. The energy utilisation efficiency of digestible carbohydrates in common carp and Nile tilapia is found to be constant whereas for barramundi and rainbow trout, the utilisation efficiency decreases with increasing levels of digestible carbohydrate intake (Schrama et al., 2018; Phan et al., 2019). This suggests that some fish have a limited capacity to process carbohydrates.

Starches (corn, wheat, etc.) are often included to limit the amount of protein utilised for energy (Deng et al., 2016). Wang et al. (2005) showed an improvement in the protein efficiency ratio in Nile tilapia when including 22 to 46% of starch in the diet, compared to

levels of 6% or 14%. The total carbohydrate digestibility in the diet is largely dependent on the carbohydrate composition. The total carbohydrate digestibility depends on the starch level, the molecular complexity of the carbohydrate source and total carbohydrate level in the diet (Stone, 2003). The starch fraction of the diet is considered highly digestible with apparent digestibility coefficients (ADC) of 90%, up to 99% in Nile tilapia (Haidar et al., 2016). The ADC of the total carbohydrate fraction has been found to be as low as 30 to 60% and as high as 80 to 90% for Nile tilapia (Tran-Ngoc et al., 2016). The large variation in the digestibility of the total carbohydrate fraction is mainly caused by differences in the total dietary fibre fraction.

The dietary fibre fraction consists of remnants of plant cells, which are non-starch polysaccharides (NSP), oligosaccharides, lignin and gums (Dhingra et al., 2012). The NSP forms the bulk of the total dietary fibre fraction. There is a wide range of NSP, differing in both characteristics and properties (i.e. solubility), while the amount and type of NSP differ among plant ingredients. NSP is generally classified into cellulose, non-cellulosic polymers (hemicellulose) and pectic polysaccharides. It is often considered as indigestible and to be of little or no nutritional value (Sinha et al., 2011). However, a study by Leenhouwers et al. (2007) found a digestibility of up to 24% in Nile tilapia for the NSP fraction when using barley as the main NSP source. Amirkolaie et al. (2005) also showed digestibility for purified cellulose and guar gum in Nile tilapia, with an ADC of 2.8% and 20.8%, respectively. A more recent study using Nile tilapia by Haidar et al. (2016) showed a digestibility between 41% and 73%, depending on the feeding level, for a diet enriched with dried distillers grains with soluble from wheat (DDGS).

2.12 Digestive Enzymes

Diets are composed of protein, fat and carbohydrates. Digestive enzymes break down protein, carbohydrates and fats into progressively smaller components. Enzyme supplements normally contain amylase, protease and lipase. Cellulase, lactase, maltase, invertase (sucrase), glucoamylase, bromelain and papain may also be included in digestive products. These enzymes increase the level of digestion in the stomach, increasing the amount of food broken down (Soleimani et al., 2012). Therefore, less undigested food is passed into the colon, and food nutrients are more available for absorption (Jiang et al., 2015). Fish can utilize the nutrients of the diets if the digestive enzymes are appropriate. The gastrointestinal tract (GI) have several digestive enzymes subunits; however, the presence and quantity of digestive enzymes and its activities varies between species (Yang et al., 2012; Liu et al., 2015).

According to the feeding habitat fish are categorized into mainly three which are carnivorous, omnivorous and herbivorous, and it is generally believed that this is reflected in digestive enzyme activities (Falcon-Hidalgo et al., 2011; Nazemroaya et al., 2015). The relationships among feeding habits and proteolytic and α -amylase activities are still unclear (Leung & Woo, 2012). It has been shown that carnivorous fish have α -amylase activity although their natural diet has no starch (Ren et al., 2011). While most species have been reported to have appreciable activities of proteolytic digestive enzyme, the presence and activities of digestive carbohydrates is quite variable (Li et al., 2013). The predominant carbohydrates digestive enzymes are α -amylase, disaccharidase, and α -glucosidase and in combination these enzymes specifically hydrolyse the carbohydrates to yield glucose (Zeng et al., 2015). Therefore, the diet ingredients should be selected based on the digestive

enzymes activities of fishes, thus, the preparing formulated diet would be more effective for the digestive enzymes activities.

The growth capacity of fish can be controlled by several environmental and physiological factors (Wang et al., 2017). The enzymes activities in the gastrointestinal tract is one of the physiological factors that may restrict the growth of fish. Hansen and Storebakken (2007) found the higher amount of amylase, maltase and protease activities in fast-growing rainbow trout (*Oncorhynchus mykiss*) fish. The present study is conducted on omnivorous fish, *Oreochromis niloticus*. Various literature revealed that omnivorous fish fed on carbohydrate sources diets, therefore this fish has higher amylase activities (Chai et al., 2013). In fish, amylase activity has been reported to be highest in the anterior section of the intestinal tract, where the majority of the carbohydrate absorption takes place (Hamid et al., 2011). However, it is difficult to make a comparison of digestive enzyme activity between species because the activity levels may differ depend on whether the fish are carnivorous, omnivorous or herbivorous, respectively.

2.13 Carbohydrate Metabolism

Carbohydrates are organic molecules composed of carbon, hydrogen, and oxygen atoms and categorized as monosaccharides, disaccharides, oligosaccharides or polysaccharides (Ng & Romano, 2013). In practical terms for fish, carbohydrates can be broadly categorized as starches (simple and complex) and non-starch polysaccharides (NSP). Starches include dextrin and glucose which are relatively well digested, and in contrast, non-starch polysaccharides are utilized much more poorly or not at all (Francis et al., 2001; Sinha et al., 2011). Dietary carbohydrates are digested into glucose monosaccharides by hydrolysis off the bonds linking the monosaccharides before absorption occurs. Some hydrolysis occurs

in the mouth and stomach, but most occurs in the upper part of the small intestine, where the pH allows high activity of the specific enzymes secreted into the intestinal lumen. Just like the terrestrial animal, carbohydrate metabolism in fish also involved the process of glycolysis, the Krebs Cycle and oxidative phosphorylation.

The process of breakdown the dietary carbohydrates into glucose monosaccharides is referred as glycolysis (Cai et al., 2018). The energy produced from the catabolism of glucose during the glycolysis is conserved in the form of adenosine triphosphate (ATP), which can be used by the cell to power other metabolic processes (Polakof et al., 2012; Azaza et al., 2015). A specific transporter protein (GLUT 4) carries glucose across the cell membrane, where a phosphate group is transferred from ATP to form glucose-6-phosphate (Zhou et al., 2016). This irreversible reaction is catalysed by hexokinase which prevents the loss of nutrient from the cell because phosphorylated sugars cannot cross cell membranes. This also ensures a concentration gradient for glucose transport across the cell membrane. In the liver, the phosphatase enzyme catalyses the reverse reaction which allowed the free glucose to leave the cell, however, this enzyme is absent from muscle.

Further, the glucose-6-phosphate is converted to fructose-6-phosphate and a second phosphorylation step followed by converting fructose-6-phosphate to fructose-1,6-diphosphate (Ren et al., 2015; Huang et al., 2018). This reaction also required a phosphate group from ATP and is catalysed by phosphofructokinase (PFK). The activity of this complex enzyme is affected by many intracellular factors and it plays an important role in controlling flux through the pathway. The PFK reaction is the first opportunity for regulation at a point that affects the metabolism of both glucose and glycogen (Zhou et al., 2016; Zhang et al., 2019). In any sequence of metabolic reactions, there can only be one reaction that

determines the overall rate at which the sequence can proceed; it can go no faster than the slowest, or rate-limiting, reaction, and PFK is normally the point in glycolysis that determines the rate of the overall reaction (Maughan, 2013; Pérez-Jiménez et al., 2017).

The fructose-1,6-diphosphate then split into two three-carbon molecules which are glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Zeng et al., 2014; Wang et al., 2017). These two molecules are interconvertible, and further metabolism occurs only through glyceraldehyde-3-phosphate. Each of the succeeding steps in glycolysis thus occurs in duplicate. Glyceraldehyde-3-phosphate is converted to 1,3-diphosphoglyceric acid in a reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase and involving the simultaneous conversion of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) to its reduced form, NADH (Li et al., 2016). At physiological pH, 1,3-diphosphoglyceric acid exists as the ionized form, 1,3-diphosphoglycerate (1,3- DPG), so a hydrogen ion is released (Polakof et al., 2012). As well as accepting a hydrogen ion, NAD^+ also accepts two electrons. The additional phosphate group incorporated into 1,3-DPG is derived from inorganic phosphate, so there is no further input of ATP.

The reactions of glycolysis occur in the cytoplasm and the pyruvate formed is not phosphorylated which allowed movement to leave the cell (Storebakken et al., 2015; Song et al., 2018). Some pyruvate escapes from tissues such as muscle when the rate of glycolysis is high, but most is further metabolized. The fate of the pyruvate produced by glycolysis depends on a number of factors, including the metabolic capacity of the tissue. In the next step, the phosphate group is transferred from 1,3-DPG to adenosine diphosphate (ADP), with ATP being formed and the 1,3-DPG converted to 3-phosphoglycerate (Zhou et al., 2014; Chen et al., 2018). Internal reorganization of the 3-phosphoglycerate molecule shifted the

phosphate group into the 2 carbon position, to form 2-phosphoglycerate. A dehydration reaction then occurs, catalysed by enolase, which results in the formation of phosphoenolpyruvate. The last step in glycolysis results in the transfer of the phosphate group from phosphoenolpyruvate to ADP, with the formation of pyruvate and another ATP (Li et al., 2016).

Alternatively, the pyruvate undergo oxidative metabolism to CO₂ and water within the mitochondrion. The step involved the conversion by oxidative decarboxylation of the three-carbon pyruvate into two-carbon acetate group that is linked to coenzyme A to form acetyl-CoA (Maughan, 2013; Feng et al., 2019). This reaction converted NAD⁺ to NADH and catalysed by the pyruvate dehydrogenase enzyme complex. Acetyl-CoA is oxidized to CO₂ and water in the tricarboxylic acid (TCA) cycle which is also known as the Krebs' cycle or the citric acid cycle as citrate is one of the key reaction intermediates (Nie et al., 2015; Cheng et al., 2017). The reactions involve combination of acetyl-CoA with oxaloacetate to form citrate consist of six-carbon TCA. A series of reactions leads to the sequential loss of hydrogen ions and CO₂, resulting in the regeneration of oxaloacetate. Acetyl-CoA is also an intermediate of fatty acid oxidation, and the final steps of oxidative degradation which responsible for both fat and carbohydrate synthesis (Li et al., 2016; Huang et al., 2018).

The reactions of oxidative phosphorylation take place after the Krebs' cycle which also occurred within the mitochondria, whereas glycolysis is a cytosolic process, and the inner mitochondrial membrane is impermeable to NADH and NAD⁺ (Xia et al., 2015; Cai et al., 2018). A number of substrate shuttles act to transfer reducing equivalents into the mitochondrion. Some of the pyruvate formed may converted into amino acid alanine which can be released from the muscle and transport amino groups to other tissues such as liver

and kidney (Maughan, 2013). This shuttle involved both of three-carbon units and amino nitrogen groups between the tissues. Some pyruvates may also be converted to the four-carbon compound oxaloacetate by the incorporation of CO₂ in a reaction catalysed by pyruvate carboxylase. This conversion to oxaloacetate further undergo the resynthesis of glucose by the process of gluconeogenesis (NRC, 2011; Chen et al., 2017).

2.14 Application of Carbohydrates in Fish Diet

Carbohydrates are the most economical source of energy in formulated aquaculture diets (Zhou et al., 2016; Tran-Ngoc et al., 2019). Although no real carbohydrate requirements have been demonstrated in fish, an adequate level of carbohydrates in the diet is still necessary to reduce the catabolism of protein and lipids for energy and to provide metabolic intermediates for the synthesis of other biologically important compounds, such as ribose-5-phosphate for nucleotides and nucleic acid synthesis (Feng et al., 2019). Carbohydrate, if well utilized by fish, would be more economic compared with lipid because of its cheaper cost and better availability, although lipid constitutes an important source of non-protein energy for fish (Cheng et al., 2017). Besides, replacing the dietary protein by carbohydrate or lipid also not only reduces production cost but also important for nitrogen effluent from the culture system (Koch et al., 2016). Numerous studies have evaluated the maximum levels of carbohydrates that fish can tolerate without physiological disorders and growth impairment, rather than the optimal level for growth (Ma et al., 2014; Zhang et al., 2016). The provision of an appropriate amount of digestible carbohydrates in diets formulated for farmed aquatic species is important to spare the use of lipids and protein as sources of energy (Haridas et al., 2017).

Generally, fish utilize less carbohydrate than terrestrial animal while instead that carnivorous fish species utilize less than herbivorous and omnivorous fish (Hamid et al., 2011; Marandel et al., 2015). The main reason may be due to higher amylase activity in the digestive tracts and metabolic enzyme activity of herbivorous and omnivorous fish species and the higher affinity of insulin receptors than those for carnivorous fish (Liu et al., 2019). However, some carnivorous fish showed improved growth when fed the diet with an appropriate starch level compared with a non-starch diet (Ren et al., 2011; Luo et al., 2014). Inadequate carbohydrate in diet may lead to the degradation of protein and lipid, and adequate carbohydrate can spare the consumption of protein and fat for energy (Skov et al., 2017). Certainly, excessive carbohydrate in the diet of carnivorous fish depressed the growth performance and impaired some physiological functions (García-Meilán et al., 2020). Compared to lipid, carbohydrate can be well utilized by most fish species, however, adequate level of lipid in formulated feed is also essential as source of fatty acids, phospholipid and cholesterol which are required for normal growth, development and maintaining health (Kamalam et al., 2017). Goncalves et al. (2020) stated that high levels of dietary lipid could increase susceptibility to autoxidation and tissue lipid peroxidation. It is necessary to determine the optimum dietary CHO:L ratio to obtain better growth performance, physiological condition and health status for fish (Wang et al., 2014; Zhou et al., 2016).

The ability of dietary carbohydrate utilization by fish varies among fish species and can be affected by dietary carbohydrate level and source. Carbohydrate utilization in fish has been reviewed by Wilson (1994), and more recently by Chen et al. (2020) and García-Meilán et al. (2020) and come out with the conclusions that the ability of carbohydrate utilization by fish varies among species and warm water herbivorous or omnivorous fish species would utilize carbohydrate better than cold water marine carnivorous fish species.

This attributes might be due to their natural habitat, longer digestive tract and higher carbohydrates activities in digestive tracts and livers (Li et al., 2019). The complexity and utilization of the carbohydrate in fish is dependent on the source and the practical ingredients such as corn or a mixture of corn and wheat are common carbohydrate sources used in feed formulations (Wang et al., 2017). *Oreochromis* sp. is able to digest 57.9 g/kg of carbohydrates in corn and 71.7 g/kg in wheat and teleost is generally considered to be glucose-intolerant (Azaza et al., 2015).

Furthermore, different types of carbohydrates may not be equally utilized by fish. yellow catfish do not utilize simple sugars efficiently (Wang et al., 2014). Tilapia species utilize complex carbohydrates such as dextrin or starch for growth more readily than simple sugars such as glucose (Boonanuntanasarn et al., 2018). However, white sturgeon was found to have greater ability for glucose utilization than for starch utilization (Gong et al., 2015). The maximum dietary dextrin levels that optimal for cultivation and growth were 10% for yellowtail, 20% for red sea bream and 30% for common carp (Zhou et al., 2016).). The inclusion of dietary carbohydrates in aquafeed may lead to a reduction in the overall feeding costs of production. Based on the findings of many researchers, the recommended dietary inclusion of digestible carbohydrates is up to 20% for many carnivorous species, including salmonids and marine fish, and up to 40% from warm-water omnivorous species (Chen et al., 2020).

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview

The present research aimed to evaluate the effect of sago (*Metroxylon sagu*) starch-based carbohydrate on Nile tilapia, *Oreochromis niloticus* juveniles. The analyzed parameters in this study were growth performance, body indices, blood plasma compositions, whole body proximate compositions, nutrient digestibility and digestive enzymes activities. This chapter describes the complete research design with common methodologies that were adopted to perform the present study.

3.2 Research Design

The research was designed based on the objectives of the study. Figure 3.1 illustrated the research design of the comprehensive feeding trial on *O. niloticus* juveniles. A 12-week feeding trial was conducted where the fish were fed six formulated experimental diets and the control diet to observe the protein sparing effect based on different level of dietary protein and carbohydrate source (sago starch). In this research, the analysis were performed on growth performance, body proximate compositions, blood plasma compositions, nutrients digestibility, digestive enzyme activities and carbohydrate metabolic enzymes.

3.3 Feeding Trial

The feeding trial was carried out at the Aquaculture Laboratory of Universiti Malaysia Sarawak (UNIMAS), Kota Samarahan, Malaysia. A comprehensive feeding trial was designed for 12 weeks durations to conduct the present research. Briefly, the *O. niloticus* juveniles were rear using six experimental diets and a control to observe the effect

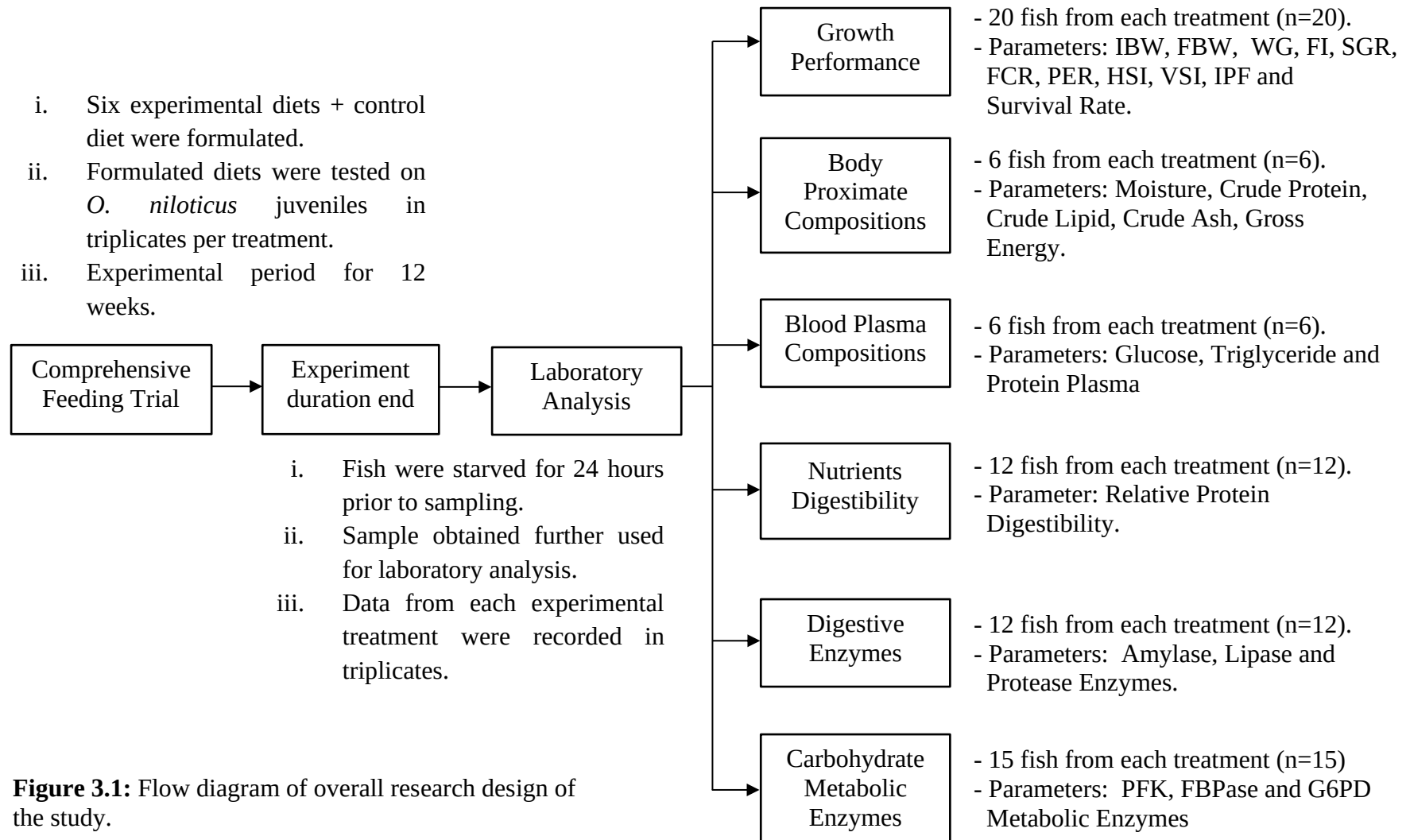


Figure 3.1: Flow diagram of overall research design of the study.

of sago (*Metroxylon sagu*) starch-based carbohydrate. During the whole period, fish were fed experimental diets twice daily at 09:00 and 17:00 hours to apparent satiation. The uneaten feeds were siphoned out and dried to determine feed intake measured by subtracting with the total feed fed.

3.4 Experimental Fish and Husbandry Conditions

Juveniles *O. niloticus* (mean weight of approximately 4.60 g) were purchased from the PM Aquaculture Sdn. Bhd. Sarawak, Malaysia and transported to the experimental facilities in aerated polyethylene bags. Fish were acclimated to laboratory conditions for 2 weeks in a 3000 L capacity polyester tank fitted to a flow-through system and fed a commercially fish feed (Dindings Soya & Multifeeds Sdn. Bhd., Kuala Lumpur, Malaysia), containing 320 g/kg crude protein. After this duration, fish were randomly introduced into 21 rectangular, experimental fish tanks (45×30×30 cm) of 40.5 L capacity supplied with de-chlorinated tap water and aerated continuously in a closed system. All fish were weighed individually at the beginning and in bulk fortnightly to monitor growth performance. Fish were reared under a natural photoperiod of an approximately 12/12 hours light/dark schedule. Trial conditions included 20 fish per tank, with each diet being experimentally tested in triplicate.

3.5 Experimental Diet Formulation and Preparation

The experimental feeding diets were prepared using sago starch as a carbohydrate source. Six isoenergetic (20.25 kJ/g) semi-purified experimental diets were prepared with three levels of protein, P(22%, 26% and 30%) and two levels of carbohydrates, C(38% and 44%) and the diets were designated as D1, D2, D3, D4, D5 and D6, respectively. Fishmeal was used as the protein source and lipid from fish oil were maintained at 90 g/kg in all the

diets. The control diet (D0) contained corn starch as the sole carbohydrate source (C40%). In the test diets, corn starch was completely substituted with either 38% or 44% of the sago starch. Ingredients were mixed in a feed mixer (Stand mixer, Model MK-GB1, Panasonic Co. Ltd., Taiwan) and passed through a feed extruder (Model TS102, Kimhill Ltd., Taiwan) to make 3 mm diameter pellets (Figure 3.2). The pellets were dried in a drying oven (Smith, Model A3018, United Kingdom Ltd., USA) maintained at 55 °C (Figure 3.3). Dried feed pellets were kept in separate, tightly capped bottles, labelled and stored in a deep freezer at -20 °C until used for the feeding trial. The formulation and proximate analysis of the experimental diets were given in Table 3.1.



Figure 3.2: Ingredients were passed through the mincer to form fish pellets.



Figure 3.3: Fish pellet were placed on a clean aluminium container for drying.

Table 3.1: Ingredients used and proximate composition of the experimental diets (g/kg).

Ingredients	Treatments						
	C40%	C38%			C44%		
	P30%	P22%	P26%	P30%	P22%	P26%	P30%
	D0	D1	D2	D3	D4	D5	D6
Danish fish meal ¹	300	220	260	300	220	260	300
Fish oil	90	90	90	90	90	90	90
Corn starch	400	-	-	-	-	-	-
Sago starch	-	380	380	380	440	440	440
Cellulose	150	250	210	170	190	150	110
CMC ²	20	20	20	20	20	20	20
Vitamin Mix ³	20	20	20	20	20	20	20
Mineral Mix ⁴	20	20	20	20	20	20	20
Proximate composition, g/kg							
Moisture	73.4	73.3	74.1	75.3	73.9	74.3	73.1
Crude protein	302.1	224.8	262.7	304.5	223.9	261.4	302.6
Crude lipid	122.5	107.9	114.7	124.3	104.8	112.6	121.1
Crude ash	47.6	47.8	48.1	48.7	46.5	46.9	47.4
Crude fibre	51.7	72.3	66.8	65.1	69.4	62.7	51.1
NFE ⁵	402.7	383.9	382.3	381.1	443.5	442.1	440.1
GE (kJ/g) ⁶	21.63	18.47	19.56	21.47	18.84	19.87	21.74

¹ Danish Fish Meal per kg = Crude protein 765.9 and crude lipid 97.6

² Carboxy methylcellulose

³ Vitamin mix per kg = Vit_A 50 million IU, Vit_{D3} 10 million IU, Vit_E 130 g, Vit_{K3} 10 g, Vit_{B1} 10 g, Vit_{B2} 25 g, Vit_{B6} 16 g, Vit_{B12} 100 mg, Niacin 200 g, Pantothenic acid 56 g, Folic acid 8 g, Biotin 500 mg and Anticake 20 g.

⁴ Mineral mix per kg = Copper 10 g, Iron 100 g, Manganese 100 g, Zinc 75 g, Cobalt 1 g, Iodine 1 g, Selenium 0.12 g and Anticake 10 g.

⁵ NFE = Nitrogen free extract was calculated as 1000 – (Moisture + Protein + Lipid + Ash + Fiber) g/kg.

⁶ GE = Gross energy was measured using bomb calorimeter, Parr 6400 bomb calorimeter.

3.6 Water Quality

Water quality parameters were determined throughout the feeding trial (Table 3.2). During the experiment, one fourth of the total of volume of water was removed daily to flush out excreta and unfed diet and replaced by the same volume of water. Water temperature and pH were determined using portable pH meter (Hanna, Model HI-98128, Hanna Instruments Company Ltd., USA, Appendix A) and dissolved oxygen were measured using portable DO meter (Extech, Model SDL150, FLIR Systems Company Ltd., USA, Appendix A). These three basic water quality parameters were measured twice daily (8:00 and 16:00) before the water was replaced accordingly. Additional water quality parameters (ammonium, nitrate, nitrite, and phosphorus) were measured every two weeks by using the portable spectrophotometer (Hach, Model DR 2800, Hach Company Ltd., USA, Appendix A). The methodology provided by the HACH kit was used to determine the additional water quality parameters and the detail methodology is given in Appendix A. Briefly, mineral stabilizer, polyvinyl alcohol and Nessler's reagent were used for the determination of ammonia. NitriVer®3 nitrite reagent powder pillow was used to measure the nitrate and nitrite while PhosVer®3 phosphate powder pillow was used to determine the presence of phosphorus in the rearing water.

3.7 Proximate Analysis

The feed ingredients, diets and fish muscle were analyzed using the standard protocols of the Association of Official Analytical Chemists (AOAC, 1997). The detail procedure is described in Appendix B. Briefly, the Precise HA60 infrared moisture determination balance was used to determine the moisture content of feed ingredients, prepared diets and fish muscle. After removing the moisture, the dried samples were used to

Table 3.2: Water quality parameters in the tanks used for the experiment of *O. niloticus* juveniles for 12 weeks.

Parameters	Treatments						
	C40%	C38%			C44%		
	<u>P30%</u> D0	<u>P22%</u> D1	<u>P26%</u> D2	<u>P30%</u> D3	<u>P22%</u> D4	<u>P26%</u> D5	<u>P30%</u> D6
Temp. (°C)	26.3±0.3	27.1±0.1	27.5±0.5	26.8±0.2	28.7±0.6	27.2±0.8	26.9±0.2
pH	6.7±0.4	7.1±0.7	5.8±0.1	6.9±0.3	7.3±0.8	6.7±0.5	6.5±0.2
DO (mg/L)	6.3±0.8	5.7±0.4	6.1±0.5	5.4±0.8	5.6±0.6	6.7±0.7	5.9±0.2
NH ₃ (mg/L)	0.05±0.02	0.04±0.01	0.03±0.01	0.02±0.01	0.04±0.02	0.06±0.03	0.03±0.01
NO ₃ ⁻ (mg/L)	0.13±0.04	0.11±0.03	0.06±0.02	0.09±0.01	0.12±0.02	0.14±0.05	0.11±0.03
NO ₂ ⁻ (mg/L)	0.06±0.02	0.02±0.01	0.04±0.03	0.03±0.01	0.05±0.02	0.07±0.04	0.04±0.02
PO ₄ ³⁻ (mg/L)	0.11±0.02	0.10±0.04	0.13±0.06	0.09±0.03	0.14±0.07	0.15±0.05	0.12±0.03

Temp.= Water temperature; DO= Dissolve oxygen; NH₃= Ammonia; NO₃⁻ = Nitrate; NO₂⁻=Nitrite; PO₄³⁻= Phosphate

°C= Degree Celsius as unit; mg/L= Milligram per Liter as unit

determine the content of the crude protein, lipid, fiber and ash (Appendix B). The Kjeldahl method using concentrated sulphuric acid (H_2SO_4) for digestion, 40% sodium hydroxide (NaOH) for distillation and 0.1N hydrochloric acid (HCl) was used as titration to determine the crude protein content in the feed ingredients, prepared diets and fish muscle. The automated protein quantification system (VAPODEST 50S, Gerhardt, Wiesbaden, Germany) was used during the distillation and titration activities. The estimated crude protein was determined using the VAPODEST-Manager software. A constant factor 6.25 was used to convert nitrogen to protein.

Crude lipid of the feed ingredient, prepared diets and fish muscle was determined by lipid extraction from the samples by homogenizing (Folch et al., 1957) in 20 volume of chloroform and methanol mixture in a ratio of 2:1 (v/v) and followed by the evaporation the solvent by heating in the oven at 60 °C. The ignition of dried lipid free residues after digestion with 5% H_2SO_4 and 40% NaOH was used to determine the crude fiber, whereas crude ash of feed ingredients, prepared diets and fish muscle was determined by incinerating the samples in a porcelain cup in a muffle furnace at 550 °C for 5 hours. Nitrogen free extract (NFE) was determined by deducting 100 from the sum of total crude protein, crude lipid, fiber and ash content values. Gross energy was estimated using bomb calorimeter (Model Parr 6400 calorimeter, Parr Instrument Company, Moline, IL, USA).

3.8 Data Sampling and Laboratory Analysis

At the end of the feeding trial after 12 weeks, fish were starved for 24 hours prior to sampling and randomly grouped within each treatment and used to determine growth performance, body indices, body proximate composition, blood plasma analyses, nutrient digestibility digestive enzymes and carbohydrate metabolic enzymes.

3.8.1 Growth Performance and Body Indices

The fish weight was taken fortnightly during the 12 weeks of feeding trial. The growth increment was measured by deducting of final weight from the initial weight done during stocking as described in section 3.3. During each sampling, a group of twenty fish were collected to measure weight from each triplicate tank. The studied parameters were on weight gain (WG%), the specific growth rate (SGR), survival rate (SR%) and for feed utilization, the protein efficiency rate (PER), food conversion ratio (FCR) was measured using the following formula:

Feed intake (FI) = Total feed intake (g)/ Number of fish (g)

Weight gain (WG%) = (Final weight (g) - Initial weight (g) / Initial weight (g)) x 100

Specific growth rate (SGR%) = (lnfinal weight (g) – lninitial weight (g) / No. of days) x 100

Protein efficiency rate (PER) = Wet weight gain (g) / Total protein intake

Feed conversion ratio (FCR) = Total feed intake (g) / Total weight gain (g)

Survival rate (SR%) = (Number of fish survived / Initial number of fish) x 100

The hepatosomatic index (HSI), visceral somatic index (VSI) and intraperitoneal fat (IPF) were done by sacrificing nine fishes per replicate tank making three groups of equal number of fishes at the end of experiment using the following formula:

Hepatosomatic Index (HSI %) = (Liver weight / Body weight) x 100

Viscerosomatic Index (VSI %) = (Viscera weight / Body weight) x 100

Intraperitoneal fat (IPF %) = (Intraperitoneal fat weight / Body weight) x100

3.8.2 Body Proximate Composition Analysis

The detail methodology for determining the proximate composition of prepared experimental diets is described in Section 3.6. Similarly, the methodology was followed to perform the proximate composition analysis of body fish muscle. Accordingly, out of the total fish from Section 3.8.1, about six fish were randomly collected from each triplicate tank and sacrificed for collecting the fish muscle. The fish muscles were kept in the small universal bottles and covered with aluminium foil sheet. The aluminium foils cover were pricked using a sharp needle and kept into freeze dryer (-70 °C to -75 °C) for 24 hours (non-stop). The freeze-dried muscles were crushed into powder using food blender (Mille, Model MEB-115) and kept into 50 mL autoclaved collection tubes for further analyzed of determining the proximate composition analysis following the AOAC (1997) guideline described in Appendix C.

3.8.3 Blood Plasma Composition Analysis

Blood samples were obtained randomly from six fish from each triplicate treatment and starved for 24 hours to determine blood plasma composition analysis (Stone et al., 2003). Accordingly, the fish were individually lightly anesthetized by using chilled water (4 °C) to reduce the stress during blood collection. The blood was extracted from the mid-ventral line behind the anal fin of each fish using a 1 mL syringe with 27-gauge hypodermic needle (Figure 3.3). Upon collection, the blood was transferred immediately into 2 mL blood tubes containing 5 mg/mL anticoagulant EDTA. Blood sample was centrifuged at 3500 x g for 5 minutes, plasma was separated and stored at -80 °C. The level of plasma glucose, triglyceride and total protein were analysed by using Haematology Analyzer (Abbott, Model CELL-DYN Emerald 18, Abbott Park, Illinois, USA).



Figure 3.4: Blood was extracted from the mid-ventral line of fish.

3.8.3.1 Plasma Glucose

The GOD-POD method as described by Ambade et al. (1998) was applied for the determination plasma glucose. The plasma content in the blood was tested using the glucose assay kit (Catalog No: E-BC-K234-S, Elabscience Biotechnology Inc., USA). The reagent was prepared by mixed thoroughly the Phenol solution and Enzyme solution at a ratio of 1:1 (v/v) respectively. Approximately, 20 μ L of the blood plasma extracted from fish fed with the six experimental diets including control respectively were pipetted into 5 mL test tube. Accordingly, 2.0 mL of the prepared reagent solution into were added each tube and fully mixed with a vortex mixer. The mixtures were incubated for 10 minutes at room temperature. Triplicates samples were taken from each feeding treatment. The blank (distilled water) was prepared using same procedure without the plasma sample. The absorbance were measured at 505 nm. The standard curve is given in Appendix C. The plasma glucose was expressed as mmol/L.

3.8.3.2 Plasma Triglyceride

The plasma triglyceride was determined by GPO-PAP method (Sullivan et al., 1985) using the triglyceride assay kit (Catalog No: E-BC-K238, Elabscience Biotechnology Inc.,

USA). Accordingly, 2.5 μ L of the blood plasma extracted from fish fed with the six experimental diets including control were pipetted into 96-well microtiter plate. Then, 250 μ L of Reagent enzyme solution were added into the assigned wells of the same microtiter plate. Both mixtures were thoroughly mixed and incubated at room temperature for 10 minutes. Triplicates samples were taken from each feeding treatment. After incubation, the mixtures were determined at 510 nm absorbance. The distilled water was used as blank using same procedure without the serum sample. The Glycerinum was used as a standard (Appendix D). The plasma triglyceride was expressed as mmol/L.

3.8.3.3 Plasma Protein

The application of Biuret and Lowry procedure modified by Ohnishi and Barr (1978) to measure the total plasma protein. The protein assay kit (Catalog No: E-BC-K165-S, Elabscience Biotechnology Inc., USA) was used to determine the protein content in the blood plasma. Both solutions of Copper reagent and Akali reagent were homogenize mixed at a ratio of 1:2 (v/v) to make the Biuret reagent. Standard protein concentration was prepared using Bovine Serum Albumin (BSA) with concentration of 0 to 30.0 g/L protein with 5.0 g/L interval. Approximately, 50 μ L of the blood plasma extracted from fish fed with the six experimental diets including control respectively were pipetted into 5 mL test tube. Added 2.5 mL of biuret reagent solution into each tube and mixed fully with a vortex mixer. The mixtures were incubated at room temperature for 10 minutes, and then cooled down. Triplicates samples were taken from each feeding treatment. After incubation, the absorbance of the mixtures were measured at 540 nm. The blank was prepared using distilled water with same procedure without the plasma sample. The standard curve was prepared (Appendix E) using the absorbance of different concentration of BSA. The total plasma protein was expressed as g/L.

3.8.4 Nutrients Digestibility Analysis

Relative Protein Digestibility (RPD) was determined with an in vitro technique using pH drop method (Section 3.7.4.3) prior to the test for intestinal enzymes (Section 3.7.4.1) using protein suspension mixture (Section 3.7.4.2).

3.8.4.1 Extraction of Crude Intestinal Enzyme

The modified method by Chisty (2005) was applied to extract the fish crude intestinal enzyme. Approximately, the enzyme were collected from 12 fish of each triplicate tank after 4 hours of feeding. The fish were sacrificed, and the intestines were collected to extract the crude enzyme. The fish were dissected, intestine removed, weighted and cleaned with the autoclave cold distilled water. At a ratio of 1:10 (w/v) (Wang, 2007), the rinsed intestines were cut into smaller and mixed with cold phosphate buffer solution, PBS (pH 7.4). The mixtures were homogenized using homogenizer (Model Ultra Turrax T25, IKA-Werke GmbH & Co. KG, Germany) and collected into 2 mL clean autoclaved eppendorf tubes, then centrifuged (Model CF15RX, Hitachi, Japan) at 10,000 x g for 15 minutes at 4 °C. The supernatant formed which also the extracted crude intestinal enzymes was collected into separate vials and kept at -70 °C prior for further analysis. During all the processes, ice blocks were used for maintaining cold environment to prevent the enzymes from denatured (Figure 3.4). Bradford (1976) method (Appendix F) was applied to determine the extracted crude intestinal enzyme protein concentration in which Bovine Serum Albumin (BSA) was used as standard (Appendix G).



Figure 3.5: Extracted crude enzymes were keep in box with ice blocks.

3.8.4.2 Preparation of Protein Suspension

Danish fishmeal with protein percentage $74.66\% \pm 0.09$ as determined by the procedure of AOAC (1997) was the only protein sources in the prepared diets. Briefly, the prepared six experimental diets included control diet were finely ground and sieved through a $250\ \mu\text{m}$ meshed sieve. The protein suspension was prepared with an equivalent amount of each diet that contained 312.5 mg of crude protein and mixed thoroughly with 50 mL of distilled water to produce $6.25\ \text{mg crude protein ml}^{-1}$ (Saterlee et al., 1979). The mixture was incubated for 1 hour at room temperature with stirring occasionally, were then vortex for mixing properly. The mixture was centrifuged at $10,000 \times g$ to obtain the supernatant and the protein concentration was measured by using Bradford (1976) with Bovine Serum Albumin (BSA) as a standard.

3.8.4.3 pH Drop Method

The pH drop method (Lazo et al., 1998) by *in vitro* method was used to determine the relative protein digestibility (RPD). The extracted crude enzymes of fish intestine and the protein suspension mixtures, PSM ($6.25\ \text{mg/mL}$) were used to determine the RPD (Saterlee et al., 1979; Rahmah et al., 2016). Accordingly, the PSM (30 mL) was mixed with

3 ml of extracted intestinal crude enzyme into a 50 mL autoclaved test tube and kept at 37 °C water bath. The pH was adjusted to 8.0±0.05 by using 0.01 mol HCl or 0.01 mol NaOH. After that the pH drop was measured and recorded at 1 minute intervals for 10 minutes. Triplicate samples per tank were tested. Casein was used as standard. RPD was calculated as follows:

$$\text{Relative Protein Digestibility (RPD)} = \{(-\Delta\text{pH feedstuff}) / (-\Delta\text{pH casein})\} \times 100$$

3.8.5 Digestive Enzymes Analysis

Protease, amylase and lipase enzyme activities were determined in digestive enzymes assays. The crude enzymes extracted from the fish intestine during determination of the relative protein digestibility were used (Section 3.7.4.1).

3.8.5.1 Amylase Assay

Worthington (1993) method as described by Akter et al. (2015) was followed to determine the amylase activities of *O. niloticus* juveniles. Briefly, 500 µL of extracted intestinal enzyme (Section 3.7.4.1) was taken into a clean test tube, concurrently another test tube was filled with 500 µL ddH₂O (autoclaved) as blank. These were incubated at 25 °C for 4 minutes. Then added 500 µL starch solution to each tube and again were incubated for 3 minutes at 25 °C. 1 mL dinitro salicylic acid colour reagent was then added to each tube and was incubated in the water bath at boiling temperature for 5 minutes. All tubes were cooled at room temperature, and 10 mL ddH₂O was added to each tube followed by thorough mixing. Absorbance was measured by using spectrophotometer (Model SP-880, Metertek Inc., Taiwan) at 540 nm. Triplicate biological sample per tank were assayed. Amylase activity in the extracted intestinal enzyme was determined using maltose as a standard

(Appendix H). One unit of amylase activity was defined as the micromoles of maltose released per minute per milligram protein.

3.8.5.2 Protease Assay

The modified method using casein digestion by Walter (1984) as described by Chong et al. (2002) was applied to determine the specific protease activities of *O. niloticus* juveniles. For the enzymatic reaction, the mixture was prepared with 0.25 mL casein (1% w/v in ddH₂O), 0.25 mL Tris-HCL buffer (0.1M, pH 8.5), and 100 µL of extracted intestinal crude enzymes (paragraph 4.2.9.1). The mixture was put into water bath for incubation for 1 hour at 37 °C. After 1 hour, 0.6 mL of 8% (w/v) trichloroacetic acid (TCL) was added in order to stop the reaction and left for 1 hour into normal freeze (4°C). After 1 hour incubation at 4 °C, the sample was centrifuged at 1800 x g for 10 min in order to collect the supernatant. It was measured at 280 nm wave lengths by using UV-Vis spectrophotometer (Agilent, Model Cary 60 UV, United State Ltd., USA). Blank sample was prepared followed by same above procedure except 100 µL extracted intestinal enzyme was added at the end of the incubation just after stopping the reaction with TCL. Triplicate samples per tanks were tested. The protease activity for *O. niloticus* juveniles was determined using L-Tyrosine as the standard (Appendix I). One unit of specific protease activity was defined as the amount of enzyme needed to release one micromole tyrosine per minute per milligram protein of the enzyme extract.

3.8.5.3 Lipase Assay

Bier (1955) method modified by Natalia et al. (2004) as described by Akter et al. (2015) was performed to determine the specific lipase enzyme. Accordingly, 1% solution of 1 mL polyvinyl alcohol (PVA) in ddH₂O was used to make the emulsion. 5 mL of 0.1 N HCl was added with the PVA solution and heated for 1 hour at 80 °C for 1 hour. The solution

was cooled down at room temperature, and then filtered. The filtered solution was adjusted the pH to 8.0 using 0.1 N NaOH. Then, 50 mL of this solution was taken and approximately 500 μ L of commercial virgin olive oil (extracted from the olive fruit as declared by the manufacturer) was added as substrate and homogenized for 5 minutes to make an emulsion, that was used for lipase determination. The mixture was composed of 1 ml of PVA solution-emulsified substrate, 500 μ L Mcllvaine buffer (0.1 M citric acid + 0.2 M disodium phosphate, pH 8), and 500 μ L extracted intestinal crude enzyme and then incubated with shaking for 4 hours at 37 °C. After the incubation, 3 mL of 1:1 ethanol-acetone solution was added to stop the reaction as well as to disrupt the emulsion. This was titrated with 0.01 M NaOH; before titration 2 to 3 drops of 1% phenolphthalein in ethanol were added. A blank was treated in the same way except the extracted enzymes were added after 4 hours incubation. Lipase activity was defined as the volume of 0.01 M NaOH required neutralizing fatty acid release during the 4 hours incubation period with the substrate and after correction by the appropriate blank.

3.8.6 Carbohydrate Metabolic Enzymes

The activities of 6-phosphofructokinase (PFK, EC 2.7.1.11), fructose 1, 6-bisphosphatase (FBPase, EC 3.1.3.11) and glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) were determined in the carbohydrate metabolic enzymes. The crude enzymes extracted from the fish liver were used for the carbohydrate enzyme assays determination.

3.8.6.1 Extraction of Liver Crude Enzyme

The crude enzyme of fish liver was extracted according to the method as described by Azaza et al. (2015). The liver were collected from 15 fish of each triplicate tank for the crude enzyme extraction. Accordingly, the fish were dissected, liver removed, weighted and cleaned with the distilled water. At a ratio of 1:5 (w/v), the rinsed liver was cut into smaller

and mixed with cold phosphate buffer solution, PBS (pH 7.4). The mixture was homogenized using homogenizer (Model Ultra Turrax T25, IKA-Werke GmbH & Co. KG, Germany) and collected into 2 mL clean autoclaved eppendorf tubes, then centrifuged (Model CF15RX, Hitachi, Japan) at 30,000 x g for 25 minutes at 4 °C. The supernatant formed which also the extracted liver crude enzymes was collected into separate vials and kept at -70 °C prior for further analysis. During all the processes, ice blocks were used for maintaining cold environment to prevent the enzymes from denatured.

3.8.6.2 Carbohydrate Enzyme Assays

The carbohydrate enzyme assays of 6-phosphofructokinase (PFK), fructose 1, 6-bisphosphatase (FBPase) and glucose 6-phosphate dehydrogenase (G6PD) were determined according to (Metón et al.,1999; 2003). The PFK enzyme assay were prepared by pipette approximately, 4 µL of the enzymes extracted from fish liver into the assigned wells of a 96-wall microtiter plate. Added 200 µL of ice-cold buffer containing 100 mM Tris-HCl, pH 8.25, 5 mM MgCl₂, 50 mM KCl, 0.15 mM ammonium sulphate, 4 mM 2-mercaptoethanol, 10 mM fructose-6-phosphate, 30 mM glucose-6-phosphate, 0.675 Uml⁻¹ aldolase, 5 Um⁻¹ triose phosphate isomerase, 2 Uml⁻¹ glycerol 3-phosphate dehydrogenase in all walls containing the extracted enzymes. PFK reaction was measured after addition of 1 mM ATP. Accordingly, the FBPase assay determination were assessed using 4 µL of fish liver crude enzyme extract pipette into 96-wall microtiter plate. The microtiter plate contained with extracted enzyme were added 200 µL of ice-cold buffer (85 mM imidazole-HCl, pH 7.7, 5 mM MgCl₂, 0.5 mM NADP, 12 mM 2-mercaptoethanol, 0.05 mM fructose-1,6-bisphosphate, 2.5 Uml⁻¹ phosphate glucose isomerase, 0.48 Uml⁻¹ G6P-DH) for enzyme reaction assayed. The assay of G6PD enzyme were measured with 4 µL of liver enzyme extract on the 96-wall microtiter plate by using the pipette. The final volume of 200 µL ice-

cold buffer contained with 78 mM imidazole-HCl, pH 7.7, 5 mM MgCl₂, 1 mM NADP and 1 mM glucose-6-phosphate were added to all wells of microtiter plate containing the extracted enzymes. The blank of each respective carbohydrate enzyme assays were prepared by using distilled water with the same procedure without the added of extracted enzyme. The prepared mixtures of all the specific enzyme assayed were incubated at room temperature for 10 minutes. After the incubation, the enzyme activity of PFK, FBPase and G6PD were measured at absorbance of 340 nm using spectrophotometer. Triplicates samples were taken from each feeding treatment. The protein concentration of extracted crude liver enzyme was determined at 30 °C using bovine serum albumin as a standard based on the method of Bradford (1976). One unit of enzyme activity was defined as the amount of NADH or NADPH generated by per mg protein per minute at 30 °C.

3.9 Statistical Analysis

All the data were collected in triplicate and presented as the mean $\pm$ S.D and analysed using one-way and two-way analysis of variance (ANOVA) for determined the effects of dietary protein and carbohydrate. *p*-values of < 0.05 were considered significant when compared by Duncan's multiple range test. All statistical analyses were compared using the Statistical Package for the Social Sciences (SPSS) program for Windows (Version 25.0. Armonk, NY: IBM Corp.).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Overview

Chapter 4 explains how the dietary sago starch as carbohydrate source incorporated with various protein level affect the growth performance, body indices, blood compositions, nutrient digestibility, digestive enzymes and carbohydrate metabolic enzymes in the diet for *O. niloticus* juveniles. The results obtained from this study were further discussed in the subsection of this chapter.

4.2 Results

4.2.1 Growth Performance, Nutrient Utilization and Body Indices

The results on final body weight (FBW), growth performances (WG% and SGR%), feed utilizations (FCR and PER) and body indices (HSI, VSI and IPF) of *O. niloticus* observed after 12 weeks were presented in Table 4.1. The two-way ANOVA result discovered that the interaction between different level of both protein and carbohydrate have significant ($p < 0.05$) effects to the dietary diets which were fed on *O. niloticus* juveniles. Different level of protein and dietary sago starch were found to have significantly ($p < 0.05$) influenced on the FBW and growth performance in *O. niloticus* juveniles. All diet treatments differed significantly ($p < 0.05$), with fish fed 38% sago starch diets (D1: 29.53 ± 2.50 g, D2: 31.87 ± 2.37 g and D3: 32.32 ± 2.25 g) having the highest FBW compared to all fish on 44% sago starch and the control diet, albeit D1 was not significantly different to D6 and control diet (D0). The same trend was observed in the weight gain (WG%) results. The WG% of fish fed on D3 had the significantly ($p < 0.05$) highest ($601.90 \pm 39.15\%$) with specific growth

rate, SGR (2.18%) compared to all test diets. Based on WG%, the second order polynomial regression analysis ($y = -4.6956x^2 + 14.23x + 535.73$, $r^2 = 0.2297$) was resulted into $X = 2.72$, which suggested that the appropriate level of protein and carbohydrates for protein-sparing effect to provide maximal growth of *O. niloticus* juveniles were between diets D2 (P26%, C38%) and D3 (P30%, C38%), respectively (Figure 4.1). However, all fish fed on 38% group diets (D1, D2 and D3) were performed better than those fed on diets with 44% sago starch level (Figure 4.2). During the experimental period, all the diets were well accepted by fish with no observed of rejection and all fish fed the diets actively. Feed intakes (FI) were ranged from 39.47 ± 1.88 to 44.16 ± 1.52 g and there was not significantly ($p > 0.05$) different was observed among all treatments. Feed conversion ratio (FCR) tended to increase with increased sago level in the test diets. FCR was recorded higher ($p < 0.05$) in all fish fed on 44 % sago starch group diets and become lower with decreased sago starch at 38% level different among all test diets, ranged between 1.55 ± 0.07 to 2.12 ± 0.23 , respectively. Protein efficiency ratio (PER) was dependent on incorporated between the level of protein and dietary sago starch in test diets, fish fed on optimum level (38%) performed more effectively ($p < 0.05$). Fish fed diet on 38% sago starch group diets exhibited higher PER compare with those fed on high sago level (44%), ranged from 2.12 ± 0.10 to 2.75 ± 0.14 , respectively. Based on the body indices (Figure 4.3) monitored, both HSI (2.04 ± 0.13 to $2.53 \pm 0.25\%$) and VSI (5.60 ± 0.49 to $6.41 \pm 0.73\%$) values were found to have high significantly ($p < 0.05$) different in fish fed on 44% sago starch level (D2 and D3) from those fed on 38% group diets (D4 and D5). Intraperitoneal fat (IPF%) showed significantly ($p < 0.05$) different among all dietary treatments, varied from 1.14 ± 0.20 to $1.89 \pm 0.32\%$, respectively. Overall, no fish died and as such fish survival rate was 100% throughout the feeding trial.

Table 4.1: Growth performance, feed efficiency, body indices and survival of *O. niloticus* juveniles fed on diets containing various levels of protein and carbohydrates for 12 weeks (n=20).

Parameters	Treatments										
	C40%	C38%			C44%						
	<u>P30%</u> D0	<u>P22%</u> D1	<u>P26%</u> D2	<u>P30%</u> D3	<u>P22%</u> D4	<u>P26%</u> D5	<u>P30%</u> D6				
IBW ¹ (g)	4.62±0.04	4.61±0.05	4.59±0.08	4.60±0.07	4.61±0.09	4.58±0.08	4.62±0.06				
FBW ² (g)	28.16±2.49 ^c	29.53±2.50 ^{cd}	31.87±2.37 ^{de}	32.32±2.25 ^e	23.29±2.19 ^a	25.65±2.46 ^b	27.71±2.57 ^c				
WG (%)	504.17±49.17 ^c	551.60±48.47 ^{cd}	599.78±44.19 ^{de}	601.90±39.15 ^e	404.13±42.37 ^a	459.25±46.34 ^b	500.81±47.76 ^c				
FI (g)	42.03±1.39	42.45±2.33	43.32±1.17	44.16±1.52	39.47±1.88	40.39±2.80	41.28±2.26				
SGR (%)	1.97±0.10 ^c	2.05±0.09 ^d	2.16±0.08 ^e	2.18±0.06 ^e	1.71±0.10 ^a	1.87±0.10 ^b	1.95±0.09 ^c				
FCR	1.82±0.14 ^{bc}	1.76±0.09 ^b	1.64±0.10 ^a	1.55±0.07 ^a	2.12±0.23 ^e	1.97±0.12 ^d	1.89±0.10 ^{cd}				
PER	2.20±0.11 ^a	2.75±0.14 ^e	2.54±0.15 ^d	2.32±0.10 ^b	2.61±0.17 ^d	2.43±0.13 ^c	2.12±0.10 ^a				
HSI (%)	2.25±0.18 ^c	2.16±0.16 ^b	2.04±0.13 ^a	2.06±0.19 ^a	2.53±0.25 ^e	2.50±0.21 ^e	2.36±0.15 ^d				
VSI (%)	5.86±0.54 ^c	5.75±0.52 ^b	5.60±0.49 ^a	5.64±0.57 ^a	6.41±0.73 ^e	6.39±0.68 ^e	5.97±0.55 ^d				
IPF (%)	1.53±0.23 ^{bc}	1.42±0.24 ^b	1.14±0.20 ^a	1.26±0.27 ^a	1.89±0.32 ^e	1.75±0.29 ^d	1.64±0.26 ^{cd}				
Survival (%)	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00				
<u>Two-Way ANOVA</u>											
<i>p</i> -value											
Interaction	IBW (g)	FBW (g)	WG (%)	FI	SGR (%)	FCR	PER	HIS (%)	VSI (%)	IPF (%)	Survival (%)
Protein	-	0.030	0.039	0.022	0.008	0.035	0.000	0.019	0.028	0.013	-
Carbohydrate	-	0.001	0.010	0.018	0.000	0.011	0.021	0.003	0.017	0.005	-
P x C	-	0.008	0.014	0.029	0.031	0.040	0.034	0.042	0.037	0.043	-

Mean values in same row with different superscript letters are significantly different ($p < 0.05$).

Result are mean ± SD of triplicate samples.

¹IBW, initial mean body weight.

²FBW, final mean body weight.

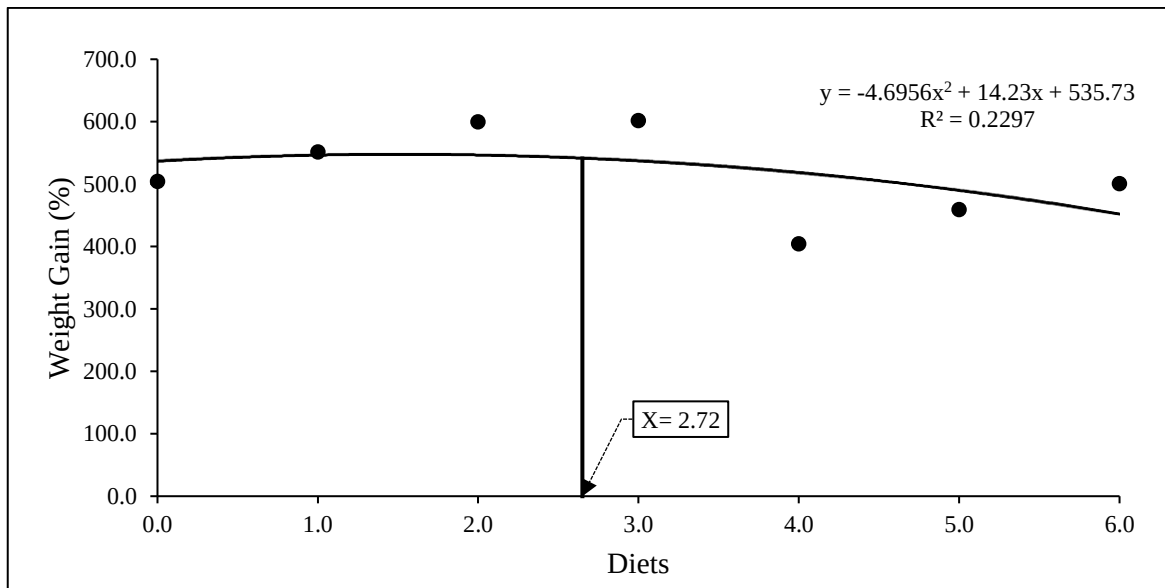


Figure 4.1: Relationship between dietary diets containing various levels of protein and carbohydrates and weight gain (%) of *O. niloticus* juveniles.

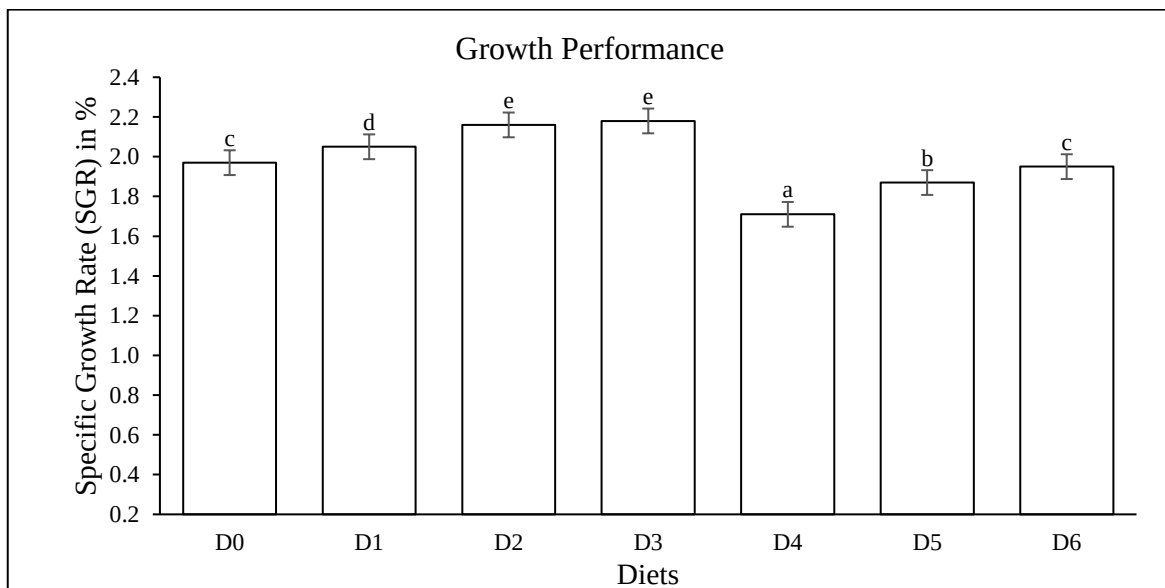


Figure 4.2: Effect between various levels of protein and carbohydrates on growth performance of *O. niloticus* juveniles. Result are mean $\pm$ SD of triplicate sample. Values assigned as different superscript letters are significant different ($p < 0.05$).

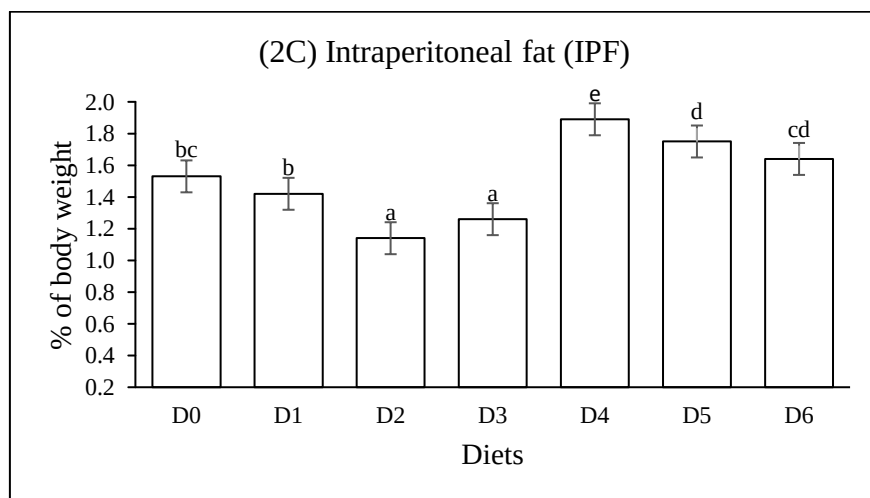
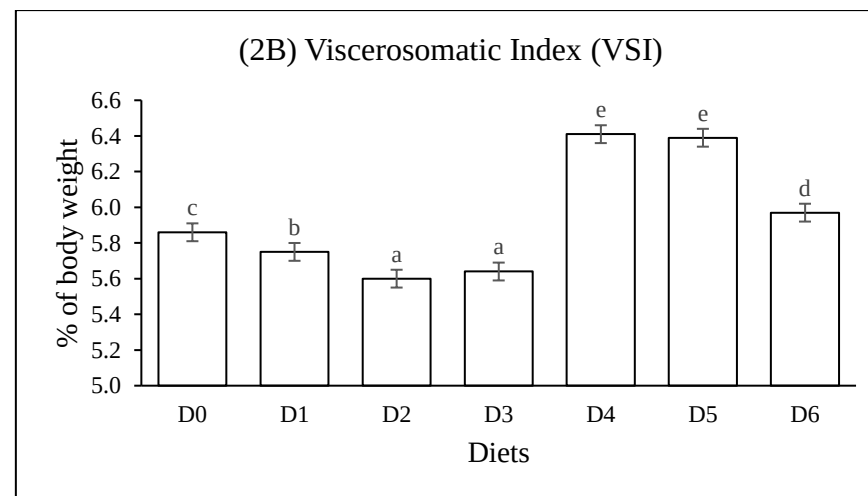
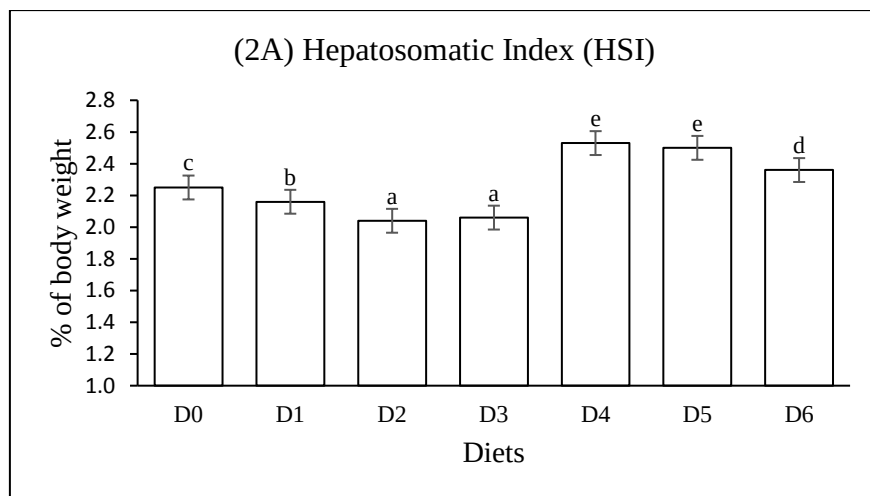


Figure 4.3: Effect between various levels of protein and carbohydrates on body indices (2A) Hepatosomatic Index; (2B) Viscerosomatic Index; (2C) Intraperitoneal fat of *O. niloticus* juveniles. Result are mean $\pm$ SD of triplicate sample. Values assigned as different superscript letters are significant different ($p < 0.05$).

4.2.2 Proximate Composition of Final Whole Body

The proximate of initial and final body composition of *O. niloticus* juveniles are shown in Table 4.2. Moisture content level in whole-body of the initial fish and the final treatment groups were varied from 74.88 ± 0.77 to $78.13 \pm 0.58\%$. The final moisture content was found to have high significantly ($p < 0.05$) different in fish fed the 38% sago starch group (D2 and D3) than those fed on D0 and 44% group test diets, however, have lower value compare with the initial body moisture contents. Whole-body protein was significantly higher ($p < 0.05$) in the final dietary treatment groups than those of the initial group. The whole-body protein content was maximum in the 38% group diets (D2 and D3) and decreased at 44% sago starch level among the final treatment groups. The whole-body lipid content was recorded lower in initial fish from those in final treatment groups, increased significantly ($p < 0.05$) as the dietary starch levels increased from 38 to 44% group diets, respectively. The whole-body ash content was significantly ($p < 0.05$) influenced by the sago starch level. Significant different ($p < 0.05$) of body ash content was higher in final treatment groups compare with in initial fish, increase with increased sago starch level in diets. The whole-body gross energy level for final treatment groups was significantly higher ($p < 0.05$) than the initial fish and it increase with sago starch level from 38% to 44% in diet groups. Overall, the two-way ANOVA result showed that different level of both protein and carbohydrate on the feeding diets were significantly ($p < 0.05$) influenced whole-body composition of *O. niloticus* juveniles.

Table 4.2: Whole-body proximate compositions (%) of *O. niloticus* juveniles fed on diets containing various levels of protein and carbohydrates for 12 weeks (n=6).

Proximate analysis	Treatments							
	Initial	C40 %	C38 %		C44 %		P30 %	P30 %
		P30 % D0	P22 % D1	P26 % D2	P30 % D3	P22 % D4	P26 % D5	P30 % D6
Moisture	78.13± 0.58 ^e	75.82± 0.63 ^{bc}	76.27± 0.81 ^c	76.53± 0.96 ^d	76.56± 0.87 ^d	74.88± 0.77 ^a	75.26± 0.65 ^a	75.69± 1.13 ^b
Crude protein	11.68± 0.21 ^a	14.57± 0.33 ^d	15.34± 0.42 ^d	15.65± 0.28 ^e	15.69± 0.45 ^e	13.16± 0.51 ^b	13.78± 0.36 ^c	14.32± 0.19 ^c
Crude lipid	3.64± 0.19 ^a	5.26± 0.24 ^d	4.92± 0.33 ^c	4.75± 0.21 ^{bc}	4.63± 0.51 ^b	5.89± 0.17 ^e	5.81± 0.39 ^e	5.42± 0.48 ^d
Crude ash	2.49± 0.53 ^a	2.83± 0.4 ^{cd}	2.72± 0.64 ^c	2.60± 0.72 ^b	2.64± 0.48 ^b	3.15± 0.36 ^e	2.97± 0.69 ^{de}	2.95± 0.29 ^d
GE (kJ/g) ¹	23.51± 0.18 ^a	25.86± 0.23 ^{cd}	25.57± 0.31 ^c	25.33± 0.38 ^b	25.21± 0.26 ^b	26.89± 0.45 ^e	26.85± 0.32 ^e	26.38± 0.40 ^d
Two-Way ANOVA								
<i>p</i> -value								
Interaction	Moisture		Crude protein		Crude lipid		Crude ash	
Protein	<0.043		<0.012		<0.024		<0.031	
Carbohydrate	<0.012		<0.000		<0.000		<0.026	
P x C	<0.038		<0.007		<0.011		<0.034	

Mean values in same row with different superscript letters are significantly different ($p < 0.05$).

Result are mean ± SD of triplicate samples.

¹GE = Gross energy was measured using bomb calorimeter, Parr 6400 bomb calorimeter.

4.2.3 Blood Plasma Compositions

Blood plasma composition of *O. niloticus* juveniles fed with different level of protein and sago starch for 12 weeks was presented in Table 4.3. The result based on two-way ANOVA revealed that different level of protein and carbohydrate have significant ($p < 0.05$) interaction on blood plasma compositions of *O. niloticus* juveniles. The effect of various levels of protein and carbohydrates on blood plasma composition for *O. niloticus* juveniles were statistically showed in Figure 4.4. Blood plasma glucose concentration was significantly affected by the sago starch level, decrease as the dietary starch levels increased. Statistically significant ($p < 0.05$) different with higher value was recorded in fish fed on 38% sago starch group (D2: 13.64 ± 0.43 mmol/L and D3: 13.68 ± 0.66 mmol/L) among the group diets. High significant difference ($p < 0.05$) of plasma triglyceride was recorded in fish fed on the 44% sago starch group diets ranged from 2.47 ± 0.38 to 6.52 ± 0.61 mmol/L, meanwhile, plasma protein concentration was showed significant difference ($p < 0.05$) varied from 4.18 ± 2.26 to 22.11 ± 2.19 g/L between the dietary treatments.

Table 4.3: Blood plasma composition analysis of *O. niloticus* juveniles fed on diets containing various levels of protein and sago starch for 12 weeks (n=6).

Blood Plasma	Treatments						
	C40%	C38%			C45%		
	<u>P30%</u> D0	<u>P22%</u> D1	<u>P26%</u> D2	<u>P30%</u> D3	<u>P22%</u> D4	<u>P26%</u> D5	<u>P30%</u> D6
Glucose (mmol/L)	12.86±0.26 ^c	13.25±0.37 ^d	13.64±0.43 ^e	13.68±0.66 ^e	12.19±0.18 ^a	12.42±0.59 ^b	12.71±0.71 ^c
Triglyceride (mmol/L)	4.18±0.86 ^b	3.72±0.75 ^b	2.84±0.55 ^a	2.47±0.38 ^a	6.52±0.61 ^e	5.63±0.29 ^d	4.75±0.42 ^c
Protein (g/L)	17.32±2.12 ^d	10.46±2.31 ^b	19.25±1.46 ^d	22.11±2.19 ^e	4.18±2.26 ^a	13.27±1.41 ^c	15.34±1.38 ^c
Two-Way ANOVA <i>p</i> -value							
Interaction	Glucose (mmol/L)		Triglyceride (mmol/L)		Protein (g/L)		
Protein	<0.022		<0.006		<0.031		
Carbohydrate	<0.001		<0.012		<0.008		
P x C	<0.015		<0.009		<0.014		

Mean values in same row with different superscript letters are significantly different ($p < 0.05$).

Result are mean ± SD of triplicate samples.

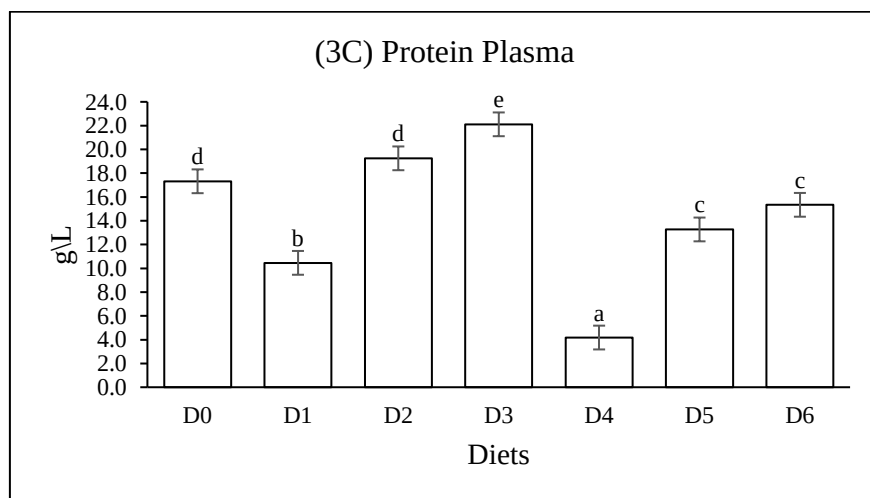
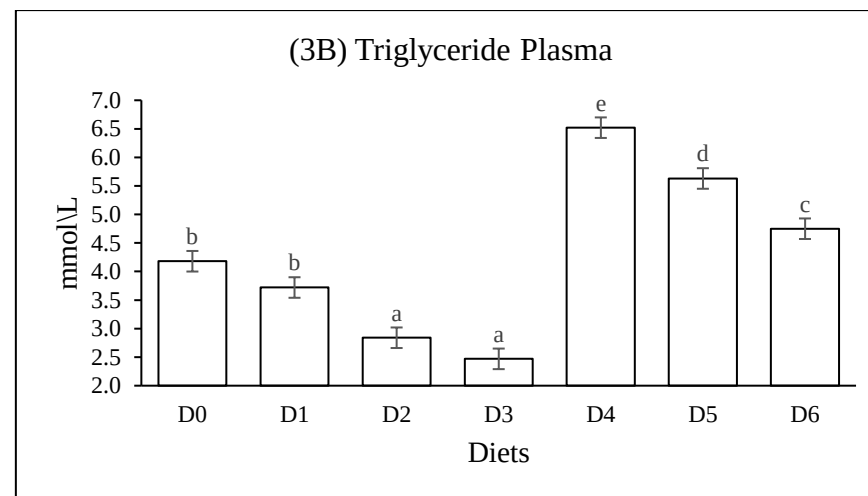
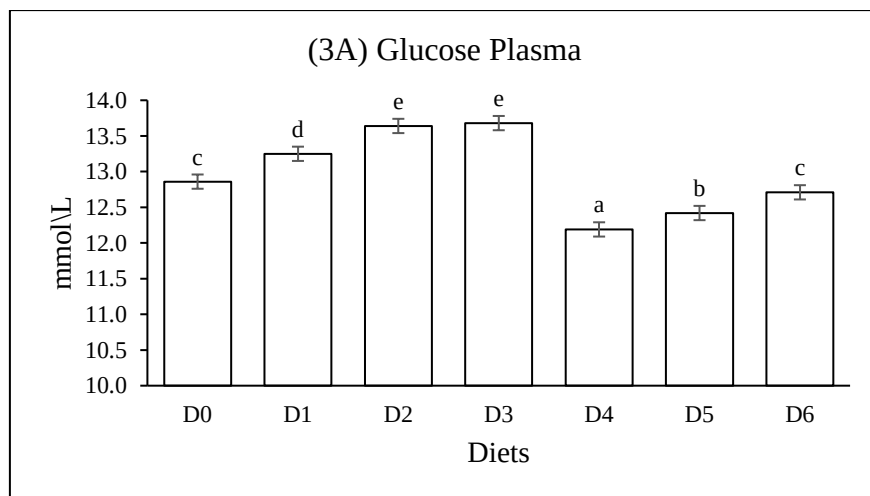


Figure 4.4: Effect between various levels of protein and carbohydrates on blood plasma (3A) Glucose plasma; (3B) Triglyceride plasma; (3C) Protein plasma of *O. niloticus* juveniles. Result are mean $\pm$ SD of triplicate sample. Values assigned as different superscript letters are significant different ($p < 0.05$).

4.2.4 Nutrients Digestibility

The results on nutrients digestibility of *O. niloticus* juveniles observed after 12 weeks were presented in Table 4.4. The two-way ANOVA result confirmed that the interaction between different level of both protein and carbohydrate have significant ($p < 0.05$) effects on nutrients digestibility of *O. niloticus* juveniles. High significant difference ($p < 0.05$) of relative protein digestibility (RPD) was recorded in fish fed on D2 ($77.54 \pm 3.56\%$) and D3 ($80.12 \pm 2.69\%$) between the group diets. Based on RPD (%), the polynomial regression analysis ($y = -0.2964x^5 + 4.7873x^4 - 26.194x^3 + 53.666x^2 - 29.665x + 70.2$, $r^2 = 0.8235$) was resulted into $X = 2.00$, which suggested that the minimum incorporation level of protein and carbohydrates to provide optimal nutrient digestibility of *O. niloticus* juveniles was diet D2 (P26%, C38%) (Figure 4.5). Overall, the formulated diets have positive effect on the relative protein digestibility (RPD) of *O. niloticus* juveniles where fish fed the 38% sago starch diets showed high statistic data compare to those from 44% group diets. This indicates that the formulated diets were well accepted and utilized by the fish at optimum level of 38% sago starch.

Table 4.4: Nutrients digestibility of *O. niloticus* juveniles fed on diets containing various levels of protein and carbohydrates for 12 weeks (n=12).

Nutrients digestibility	Treatments						
	C40%	C38%			C44%		
	<u>P30%</u>	<u>P22%</u>	<u>P26%</u>	<u>P30%</u>	<u>P22%</u>	<u>P26%</u>	<u>P30%</u>
	D0	D1	D2	D3	D4	D5	D6
Relative protein digestibility (%)	69.83±2.52 ^{cd}	74.72±1.71 ^d	77.54±3.56 ^{de}	80.12±2.69 ^e	50.29±2.38 ^a	57.40±1.47 ^b	65.67±3.63 ^c
<u>Two-Way ANOVA</u> (<i>p</i> < value)							
Interaction	Relative protein digestibility (%)						
Protein	<0.000						
Carbohydrates	<0.000						
P x C	<0.020						

Mean values in same row with different superscript letters are significantly different (*p* < 0.05).

Result are mean ± SD of triplicate samples.

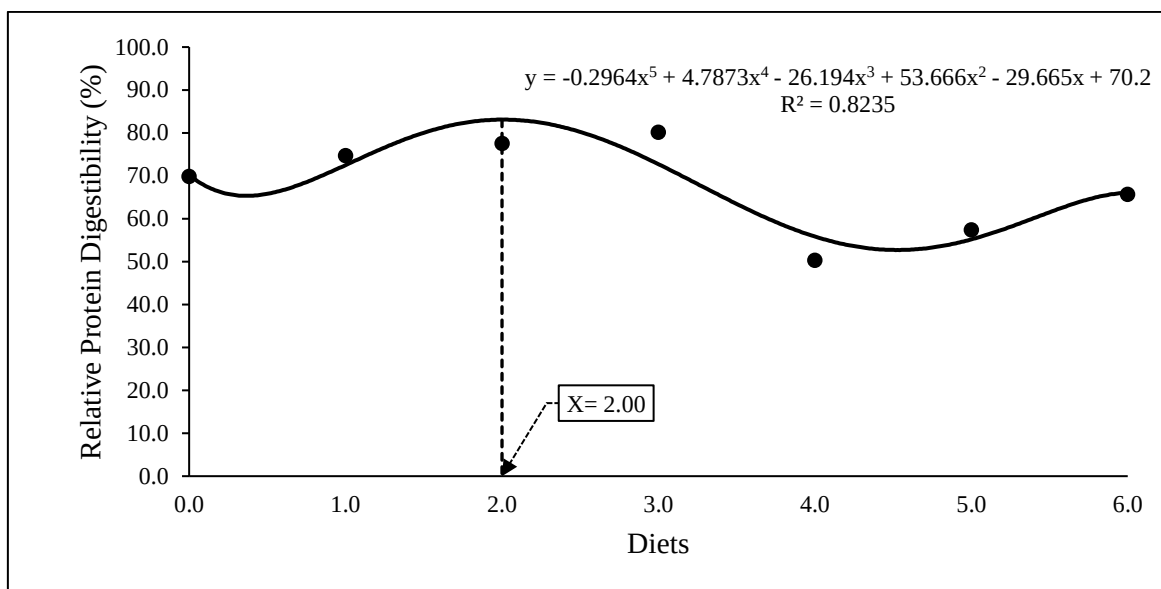


Figure 4.5: Relationship between dietary diets containing various levels of protein and carbohydrates and relative protein digestibility (%) of *O. niloticus* juveniles.

4.2.5 Digestive Enzymes

The digestive enzymes (amylase, lipase and protease) activities of *O. niloticus* juveniles are shown in Table 4.5. Amylase, lipase and protease specific activities were significantly ($p < 0.05$) affected by 38% and 44% of dietary sago starch among all diets. The amylase specific activity was found to be significantly high ($p < 0.05$) in fish fed the 38% sago starch diets (D2: $6.23 \pm 0.24 \text{ Umg}^{-1}$ and D3: $6.54 \pm 0.43 \text{ Umg}^{-1}$) followed by D1 than those fed on D0 and 44% group test diets. The similar trend was observed for specific activities of lipase enzyme ranged between $3.02 \pm 0.52 \text{ Umg}^{-1}$ to $5.68 \pm 0.75 \text{ Umg}^{-1}$ while the protease enzyme was recorded approximately from $0.38 \pm 0.07 \text{ Umg}^{-1}$ to $0.77 \pm 0.16 \text{ Umg}^{-1}$, respectively. The result based on two-way ANOVA revealed that different level of protein and carbohydrate have significant ($p < 0.05$) influenced on digestive enzymes activities of *O. niloticus* juveniles. Among all the enzyme activities, the amylase specific activity was found highest compared with both lipase and protease enzyme activities (Figure 4.6).

Table 4.5: Digestive enzymes activities of *O. niloticus* juveniles fed on diets containing various levels of protein and carbohydrates for 12 weeks (n=12).

Digestive enzymes	Treatments						
	C40%	C38%			C44 %		
	P30%	P22%	P26%	P30%	P22%	P26%	P30%
	D0	D1	D2	D3	D4	D5	D6
Amylase (Umg ⁻¹)	5.31±0.28 ^c	6.09±0.37 ^d	6.23±0.24 ^d	6.54±0.43 ^e	4.11±0.19 ^a	4.32±0.21 ^b	5.16±0.32 ^c
Lipase (Umg ⁻¹)	4.25±0.71 ^d	5.33±0.80 ^e	5.46±0.66 ^e	5.68±0.75 ^e	3.02±0.52 ^a	3.16±0.63 ^b	4.10±0.55 ^c
Protease (Umg ⁻¹)	0.58±0.12 ^{cd}	0.61±0.09 ^d	0.69±0.11 ^{de}	0.77±0.16 ^e	0.38±0.07 ^a	0.43±0.10 ^b	0.55±0.14 ^c
<u>Two-Way ANOVA</u>							
<i>p</i> -value							
Interaction	Amylase (Umg ⁻¹)		Lipase (Umg ⁻¹)		Protease (Umg ⁻¹)		
Protein	<0.023		<0.009		<0.014		
Carbohydrate	<0.000		<0.000		<0.002		
P x C	<0.016		<0.004		<0.011		

Mean values in same row with different superscript letters are significantly different ($p < 0.05$).

Result are mean ± SD of triplicate samples.

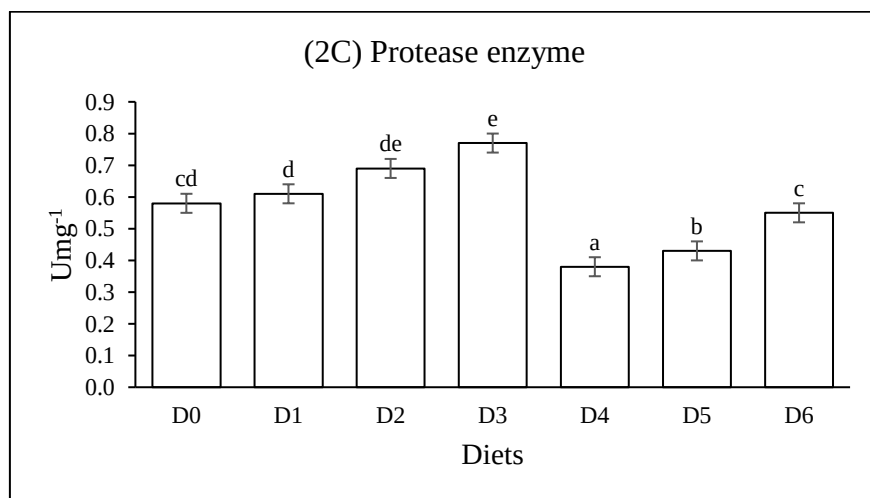
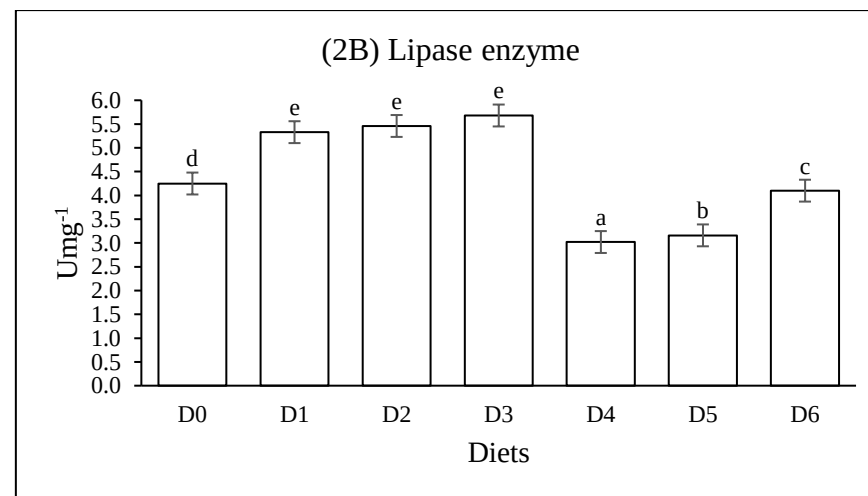
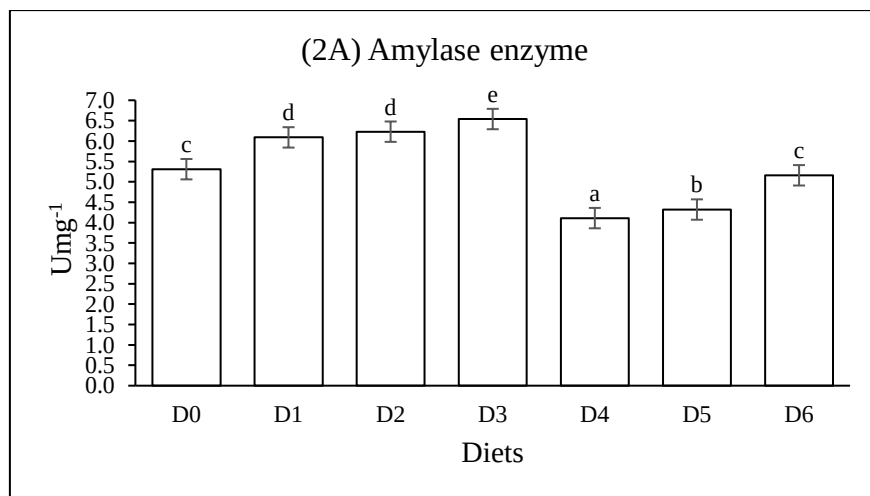


Figure 4.6: Effect of various levels of protein and carbohydrates on digestive enzymes (2A) Amylase enzyme; (2B) Lipase enzyme; (2C) Protease enzyme of *O. niloticus* juveniles. Result are mean±SD of triplicate sample. Values assigned as different superscript letters are significant different ($p < 0.05$).

4.2.6 Carbohydrate Metabolic Enzymes

The result on carbohydrate metabolic enzymes for PFK, FBPase and G6PD activities of *O. niloticus* juveniles were presented in Table 4.6. The analyzed data on two-way ANOVA indicated that various incorporation level of protein and carbohydrate have significant ($p < 0.05$) response on carbohydrate metabolic enzymes of *O. niloticus* juveniles. The PFK metabolic enzyme activity exhibited high significant affect ($p < 0.05$) in fish fed the 38% sago starch diets (D2: $0.94 \pm 0.02 \text{ Umg}^{-1}$ and D3: $0.97 \pm 0.01 \text{ Umg}^{-1}$) followed by D1 than those fed on D0 and 44% group test diets. The same trend was observed for metabolic activities of FBPase enzyme recorded approximately from 0.73 ± 0.04 to $0.96 \pm 0.02 \text{ Umg}^{-1}$. Meanwhile, the G6PD metabolic enzyme showed a promotive effect in fish fed on sago starch from 38 to 44% diet treatments which were ranged between 0.78 ± 0.07 to $0.95 \pm 0.02 \text{ Umg}^{-1}$, respectively. Overall, the PFK, FBPase and G6PD specific activities were significantly ($p < 0.05$) influenced by 38% and 44% of dietary sago starch among all diet treatments (Figure 4.7).

Table 4.6: Carbohydrate metabolic activities of *O. niloticus* juveniles fed diets containing various levels of protein and carbohydrates for 12 weeks (n=15).

Carbohydrate metabolic enzymes	Treatments						
	C40%	C38%			C44%		
	<u>P30%</u> D0	<u>P22%</u> D1	<u>P26%</u> D2	<u>P30%</u> D3	<u>P22%</u> D4	<u>P26%</u> D5	<u>P30%</u> D6
PFK ¹ (Umg ⁻¹)	0.87±0.05 ^c	0.91±0.04 ^d	0.94±0.02 ^{de}	0.97±0.01 ^e	0.76±0.02 ^a	0.79±0.03 ^b	0.85±0.04 ^c
FBPase ² (Umg ⁻¹)	0.83±0.02 ^b	0.87±0.03 ^c	0.92±0.05 ^d	0.96±0.02 ^e	0.73±0.04 ^a	0.75±0.02 ^a	0.81±0.03 ^b
G6PD ³ (Umg ⁻¹)	0.88±0.04 ^c	0.84±0.05 ^b	0.80±0.06 ^a	0.78±0.07 ^a	0.95±0.02 ^e	0.93±0.03 ^d	0.89±0.05 ^c
Two-Way ANOVA							
<i>p</i> -value							
Interaction	PFK ¹ (Umg ⁻¹)		FBPase ² (Umg ⁻¹)		G6PD ³ (Umg ⁻¹)		
Protein	<0.034		<0.011		<0.024		
Carbohydrate	<0.003		<0.001		<0.004		
P x C	<0.021		<0.008		<0.013		

Mean values in same row with different superscript letters are significantly different ($p < 0.05$).

Results are mean ± SD of triplicate samples.

¹PFK= 6-phosphofructokinase

²FBPase= Fructose 1, 6-bisphosphatase

³G6PD= Glucose 6-phosphate dehydrogenase

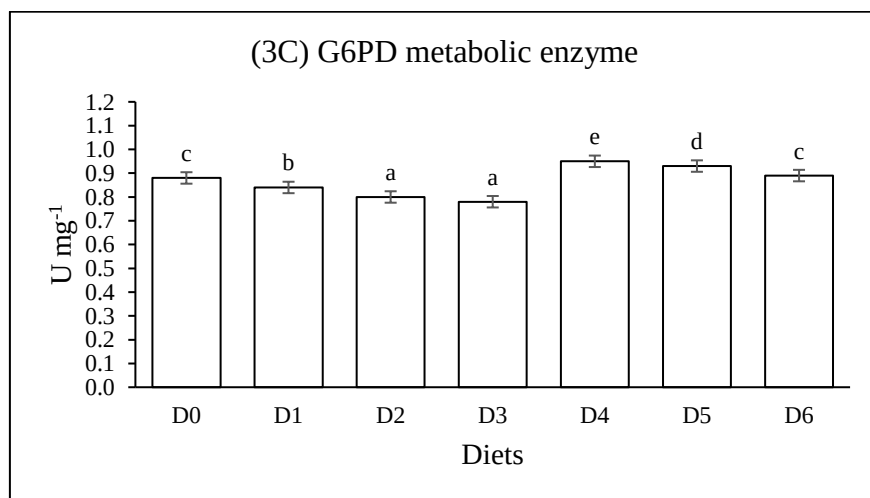
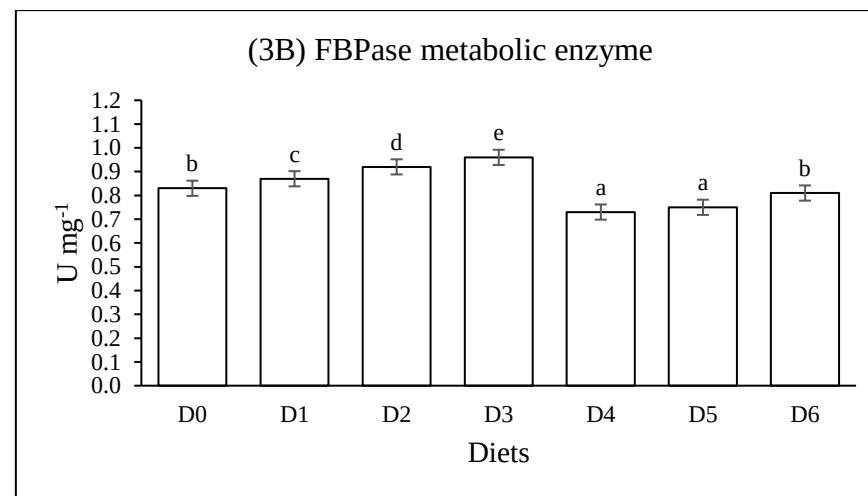
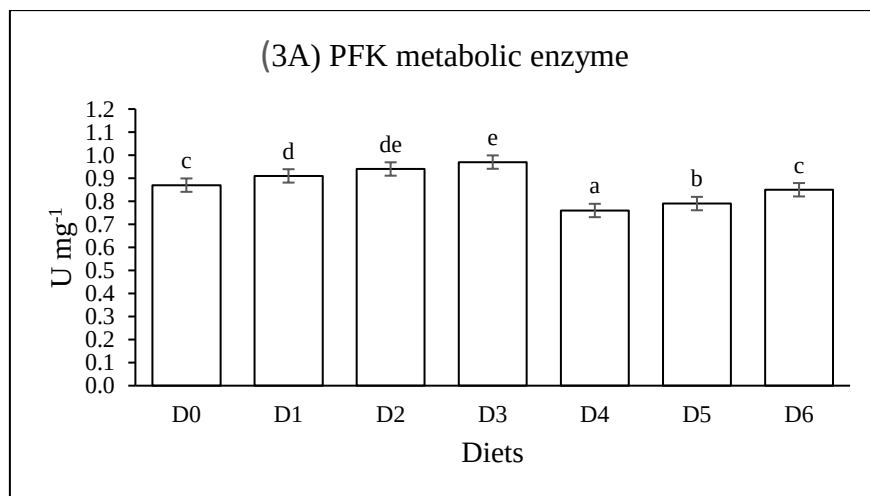


Figure 4.7: Effect of various levels of protein and carbohydrates on metabolic enzymes (3A) PFK (6-phosphofructokinase) enzyme; (3B) FBPase (fructose 1, 6-bisphosphatase) enzyme; (3C) G6PD (glucose 6-phosphate dehydrogenase) enzyme of *O. niloticus* juveniles. Result are mean $\pm$ SD of triplicate sample. Values assigned as different superscript letters are significant different ($p < 0.05$).

4.3 Discussion

The incorporation of proper carbohydrate levels in the diet has been reported to improve growth performance in different fish species (Stone et al., 2003b; Enes et al., 2008; Zhou et al., 2016). This study revealed that the optimum sago starch level required for maximum growth performance (WG% and SGR%) of *O. niloticus* juvenile was at 38% sago starch level (D1, D2 and D3) which was in agreement with the suggested requirement levels of dietary carbohydrate (starch), varied from 350 to 400 g/kg for tilapia (Azaza et al., 2015). Decrease in dietary protein level from 30 to 26% incorporated with 38% sago starch was observed not much influenced FBW in the fish, indicating that the level can spare some protein when the dietary protein is low (Mohanta et al., 2007). However, the growth performance of fish fed on 44% sago starch group was in contrast with those fed on 38% group diets. Azaza et al. (2015) stated that excess levels of starch exceed 401.3 g/kg intake by fish significantly reduce the growth rate and were accompanied by poor feed utilization. Besides, fish fed on high carbohydrate (starch) diet can reduce growth might depend on the nutrient digestibility efficiency as mentioned by Ren et al. (2011).

Survival rate at 100% was recorded in all the treatments after 12 weeks represent that tilapia fed on starch-based carbohydrates have high survival rates as reported by Wang et al. (2005). From the result, feed utilization (FCR and PER) were significantly affected by the sago starch levels. The FCR was observed higher in fish fed on 44% sago starch group diet (D4) with low protein (22%), showed that high carbohydrate incorporated with low protein level to produce high FCR value as recorded by Rahman et al. (2017). Our observation also suggested that fish fed on 44% group diets need to consume more in order to gain adequate amino acids levels for a better growth performance and it was similar with previous finding for juvenile Nile tilapia (Azaza et al., 2015). The fish fed on 38% sago starch group diets

have the highest PER and the result was in agreement with Xia et al. (2015) specified that protein efficiency ratio increased when dietary carbohydrate (starch) levels ranged from 25.61 to 37.55% and decreased in higher carbohydrate approximately at 45.31% of starch level. Singh et al. (2006) stated that feed utilization increased when the carbohydrate increases (optimum level at 30 to 40%) with low protein level, indicating that the protein was showed the sparing effect. The data obtained in the present investigation showed that higher growth in *O. niloticus* juveniles could be obtained even at a low dietary protein with optimum levels of carbohydrates in the diet.

In the present study, HSI and VSI with high significant values were observed on fish fed with 44% group diets of sago starch. The increases in the HSI with dietary starch have been observed by different authors (Moreira et al., 2008; Tian et al., 2012; Wang et al., 2016). This may occur due to high dietary starch level that lead to the increased of glycogen and lipid deposition on the liver (Xia et al., 2015). The liver was function as the preeminent site of lipogenesis in fish (Segner & Böhm, 1994) meaning that a portion of the absorbed carbohydrate, which is not used for energy, are converted and stored in the liver as both lipid and glycogen (Azaza et al., 2015). The VSI increased with starch level intake have been review in other previous studies (Peres & Oliva-Teles, 2002; Moreira et al., 2008). In fact, in juveniles of Nile tilapia, viscera represented about 6 to 8% of whole-body mass and significantly increased in excess dietary starch. This agreeable with other findings that many fish species can deposit significant amounts of fat in visceral depots (Rawles et al., 2008; Enes et al., 2010) when fed on high dietary starch level. Based on the result, intraperitoneal fat (IPF) was also significantly affected by the sago starch level. The IPF were found to have high values on fish fed with 44% group diets (higher) of sago starch compared to those fed on 38% group diets (lower). Wang et al. (2005) who reported high IPF ratio appeared in fish

fed on diets with high dietary starch level probably caused by high level of dietary starch may transfer to lipid and accumulated in the intraperitoneal cavity of the fish. Our results also in agreement with the findings in other fish species, showed that excess of dietary starch-based carbohydrate intake in diet increased the synthesis of fat in fish (Enes et al., 2009).

Body composition results showed considerable responses to dietary sago starch levels. In the present study, the whole-body moisture content tended to be higher at low dietary starch level (38% group diets). Such effect had been observed for *O. niloticus* x *O. aureus* (Wang et al., 2005), *Cirrhinus mrigala* (Singh et al., 2006), *Puntius gonionotus* (Mohanta et al., 2007), European sea bass (Moreira et al., 2008), and Yellow catfish (Ye et al., 2009). Crude protein content was recorded have high significantly influenced on fish fed with 38% sago starch group diets among all dietary treatments. The current result was agreeable with Mohanta et al. (2009) who mentioned that crude protein increased significantly at optimal starch level ranged between 220 g/kg to 380 g/kg, respectively. Both whole-body lipid and ash were positively related to starch concentration level. High value in body lipid content with increased dietary starch level could be explained by de novo synthesis of lipid from carbohydrate (Fernández et al., 2007). In our study although body ash content was low, it increases with increased dietary carbohydrate at each level of dietary protein and similar with the results for silver barb, *Puntius gonionotus* (Mohanta et al., 2007). The whole-body energy was observed to be higher at 44% sago starch group among all test diets in fact for omnivorous fish, high-carbohydrate diets stimulate lipogenic enzyme activities (Rahman et al., 2017). HSI was considered as a sensitive indicator for the available energy in fish because excess availability of digestible energy leads to deposition of either glycogen or lipid, resulting in higher HSI (Mohanta et al., 2009). We presume that the fat deposition in the liver Nile tilapia might have arisen from de novo synthesis of fat

carbohydrates when excess sago starch was fed on the fish, resulting in high energy due to the increased HSI value in the fish fed on 44% sago starch level.

It was well known that glucose, triglyceride and total protein contents in blood were correlated to animal health, general metabolism and physiological status (NRC, 2011). The amount of total protein in plasma were found significantly affected by different sago starch level in this experiment. The plasma protein values were varied among all dietary treatments, might due to the different protein content level incorporated in the experimental diets (Boonanuntanasarn et al., 2018). Plasma triglyceride and glucose were significantly influenced by the dietary sago starch. The present study showed that plasma triglycerides were increased with excess of dietary sago starch (44%) than those fish fed on the 38% group diets, respectively. This indicated that more active endogenous lipid transport into the blood, in response to fish fed on higher dietary starch level (Wang et al., 2014). The plasma glucose level was significantly increased at 38% starch group diets and this may due to the absorption of the glucose during efficient utilization by fish lead to increase of blood glucose level in blood plasma (Ren et al., 2011). The intensity of the blood glucose peak increases also deals with the level of digestible carbohydrate (starch) on diets (Stone et al., 2003a). Normal ranges for plasma glucose were not fully defined for many aquaculture fish species (Cui et al., 2010). In our study, plasma glucose was ranged from 12.19 to 13.68 mmol/L and the trend was similar with the result as recorded by Hemre et al. (2002) approximately above 11 mmol/L. The wide variation observed in the plasma glucose among fish also depend on the different species for feeding habit (such as carnivorous, herbivorous and omnivorous), different life stages or certain feeding regimes (Hemre et al., 2002). Additionally, we presume that the plasma glucose was significantly influenced may due to whether fish were fed or unfed the diet when sampling was carried out.

Indeed, the use of starch for carbohydrate-rich diets have the economic advantage as fish would utilize the inexpensive carbohydrate as the energy source instead of proteins, thus the absorbed protein will be spared for growth (Webb et al., 2010; Zhao et al., 2011). However excessive dietary starch more than 40 % in fish diet may lead to poor growth rate and low feed efficiency (Li et al., 2016) and it was in agreement with the present study in which 44% sago starch shows low growth performance compared to 38% group diets. Ren et al. (2011) also stated that increased carbohydrate starch in the fish diet depressed the growth performance and will be impaired with some physiological conditions matter. Furthermore, inadequate and excess level of starch may influence its incorporation between protein and lipid in diet formulation which result into low physical quality of feed and poor protein utilization (Zhou et al., 2016). Hence, the optimum supplementation of carbohydrate level in the fish diet is very important.

Starch based-carbohydrate utilization by fish were varies among species and it also depends on their ability to use the carbohydrates as an energy source, both for digestion and metabolism respective to their feeding habits (Boonanuntanasarn et al., 2018). Tilapia sp. are warm-water omnivorous fish that can utilize approximately up to 40% of digestible carbohydrate (starch) from their diets (Qiang et al., 2014) and the statement was support the current result in which fish fed with 38% sago starch show positive impact and perform more better. Nevertheless, too much dietary starch carbohydrate content and beyond optimal levels as observed in 44% group diets may generate problems in fish health through metabolic disturbances and clinical signs such as decreased growth, prolonged hyperglycaemia, increased glycogen deposition, liver hypertrophy, histopathological and in some cases increased glycosylated haemoglobin, retinopathy, hepatic steatosis and insulin resistance (Prisingkorn et al., 2017). Therefore, appropriate starch level is crucial in fish diet

as it also helps in reduce the amount of nitrogen from farms effluents and improved in the pellet binding, stability and buoyancy (Guerrero-Zárate et al., 2019).

The application of in vitro protein digestibility is one of the recommended approaches in order to determine the quality of potential feedstuffs which to be included in the fish diets (Chauranda & Soriab, 2019). The present study revealed high protein digestibility of *O. niloticus* juveniles fed on 38% compared to 44% sago starch group, especially for the diets with 26% and 30% protein. This pattern is comparable with the previous study conducted by Sandra et al. (2017). The incorporation of protein with proper dietary carbohydrate (starch) levels approximately between 35% to 40% in the diet has been reported to show a great impact in the digestibility of fish for better growth rate (Wang et al., 2016). This statement proved the current study that 38% sago starch level is at optimal range for the digestibility of *O. niloticus* juveniles which contribute to the better growth performance and feed utilization. However, the result of fish fed on 44% sago starch level was in contrast compare with those from 38% group diets. Excess dietary starch in diets indeed increased fat deposition on body indices (HIS, VSI and IPF) (Polakof et al., 2012), which is also proved by the low protein digestibility and digestive enzyme activities in the present study. The possible reason might be due to high dietary starch in diets increased viscosity in intestine which hinder the digestive enzyme activity in the digestive tracts of fish (Haidar et al., 2016). Furthermore, the variability of protein digestibility may also be related to formulate pelleting conditions and differences in feed intake by the fish (Saravanan et al., 2013).

Protein digestibility data reflect the percentage of a feed sample which is absorbed from the intestinal tract of an animal (Sales, 2010; Deng et al., 2016). Digestibility of protein and carbohydrates in formulated diets were well described by Hoseinifar et al. (2017). They

stated that appropriate level of both feedstuffs may have a positive effect on the feed utilization of fish which promote into better growth performance. To take into account, balanced level of protein combined with carbohydrates also had a great influence on digestive enzymes efficiency in intestinal tracts of fish. This may due to the positive response on the adaptation of intestinal microbial in the digestive tracts to produce more enzymes to assist in the utilization of feed diets (Maas et al., 2019). Fawole et al. (2018) mentioned that feed utilization efficiency and growth in fish also might be depends on the area of absorption of intestines in which wide area absorb more nutrients form the diets. Moreover, one must consider the environmental condition factors as it also can influence the nutrient digestibility and alter intestinal morphology of fish (Tran-Ngoc et al., 2018). Furthermore, the nutrient digestibility may depend on variability of energy and protein balance in the diets. Tran-Ngoc et al. (2019) reported that changes in the dietary macronutrient (protein) composition and nutrient type (starch) showed an effect on energy and protein utilization in fish.

Zhou et al. (2016) stated that amylase activity in both herbivorous and omnivorous are much greater than carnivorous and appropriate dietary carbohydrate (starch) levels can improve amylase activity in digestive enzyme. Nile tilapia, *O. niloticus* is an omnivorous fish that can utilize up to 40% starch level in its diet for growth (Azaza et al., 2015). These statements support the current study in which *O. niloticus* juveniles fed on 38% sago starch level have higher amylase activity in intestines if compared to those from the 44% group diets. Li et al. (2014) reported that amylase activity could be induced by appropriate dietary carbohydrate level; however, it was not found further increased but decreased when dietary carbohydrate level was higher than 44.31%, which indicated that excess carbohydrate level could hinder amylase activity and it is in an agreement with the present study. Amylase enzyme utilized dietary carbohydrates (starch) into glucose and stored as glycogen through

glycogenesis which catalysed by glycogen synthase (NRC, 2011). Xia et al. (2015) reported that high starch level in diet may cause insufficient amylase enzyme production in the intestine to breakdown the excess carbohydrate molecules during glycolysis which can lead to low amylase activity in the digestive tracts. This may result into the accumulation of glycogen and formed excess lipid which affect growth improvement in fish (Ren et al., 2011). Moreover, some researchers thought that the amylase activity may not only affected by different dietary carbohydrate level in diets but also determined by genome and development of the fish species (Li et al., 2014).

In this study, *O. niloticus* juveniles fed with 38% starch level appear to have a positive response on protease enzyme activity in the intestine than the 44% group diets. This is in agreement with Cai et al. (2018) stated that suitable starch level can improve feed utilization and promote protease enzyme secretions in the digestive tracts. Boonanuntanasarn et al. (2018) reported that fish fed diets containing 21.8% to 30.4% protein and 16.4% to 39.2% starch are ideal for constant rate of protease enzyme response in the intestine to digest the diets and the statement is corresponding with the formulated diets incorporation between protein and carbohydrates in our study. High dietary starch level indeed will reduce the efficiency of protein in formulated diets which resulted into poor activity of protease enzyme to breakdown the amino acid molecules during the diets digestion that is fundamental for growth performance (Wang et al., 2017). Furthermore, there are some evidence stated that excess starch in diets may increase acidic fluid within the digestive tracts that lead to negative effect on protease enzyme activity (Gominho-Rosa et al., 2015).

The similar trends were observed on lipase enzyme activity in the intestines of *O. niloticus* juveniles and our current finding is confirmed by Lu et al. (2018) stated that different level of starch in diets have a significance influence on digestive lipase enzyme. At

average starch level, lipase enzyme helps in breakdown the lipid formed from the digestible starch which further converted into energy that may essential for routine metabolism in fish (Long et al., 2015). Excessive absorption of dietary carbohydrates (starch) in diet could resulted into the body and liver lipid or glycogen depositions might due to low capability of digestive lipase enzyme (Wu et al., 2007). This indicates that more of the absorbed starch was used to synthesize lipid and not glycogen which required for body metabolism energy. As has been reported in previous studies (Peres & Oliva-Teles, 2002), liver lipid contents increase with the increase in available carbohydrate. Gao et al. (2011) reported that lipid accumulation in the liver potentially for serious health issues for cultured fish with the possibility of decreased resistance to disease and might lead to higher levels of oxidative stress.

Indeed, the intestinal digestive tract enzyme profile is an indicator which corresponding with nutrient digestibility and utilization. The digestibility in fish might depends on the presence of appropriate enzymes in proper locations in the wall and along the lumen of the intestinal tract (Deng et al., 2010). Zhang et al. (2010) described that digestive enzyme activities were depends on some factors such as food gene and nutritional habits. Taking into account, the feeding strategy of fishes and the enzymatic ability to digest the various kinds of food must be considered, respectively (Gao et al., 2011). Generally, the fish digestive structure was usually reflected their consumption habits which were obtained from their wild natural resource. Besides, the distribution of homogeneous enzyme in the fish intestine was frequently considered as their adaptation to the diversity of diet compositions from the natural habitat. Therefore, it is more sensible to be concerned about as it is a consequence of the fishes' history and their position in the evolution course.

The current study also measured on some carbohydrate metabolic enzymes which involved the 6-phosphofructokinase (PFK), fructose 1, 6-bisphosphatase (FBPase) and glucose 6-phosphate dehydrogenase (G6PD) to assess the response of the *O. niloticus* juveniles to the dietary starch levels. Kamalam et al. (2017) stated that the optimal dietary starch level varies among fish species. Liver is the main site that helps in regulating intermediary metabolism in response to the nutritional status, especially in key metabolic enzymes for carbohydrate (Enes et al., 2010). Generally, under aerobic conditions glucose is completely catabolized by glycolysis, the Krebs cycle and the respiratory chain for ATP production or through the pentose phosphate pathway leading to the production of nicotinamide adenine dinucleotide phosphate (NADP) for biosynthesis (Polakof & Panserat, 2012). Excess glucose may be stored as glycogen (glycogenesis) or converted into lipids (lipogenesis). Adaptation of hepatic enzymes to dietary carbohydrate levels have been reported consistently for fish species (Ekman et al., 2013; Zhou et al., 2015).

Based on the result, *O. niloticus* juveniles fed on 38% sago starch (carbohydrate) showed the higher PFK activity compared to those from 44% group diet and in agreement with the outcome reported by Azaza et al. (2015) stated that optimal carbohydrates level (< 40%) in formulated diet promotes the PFK activity in fish while beyond this level the enzyme may be associated with low activity. PFK metabolic enzyme is the key role for the glycolysis pathway (Ren et al., 2011). Higher activity of the glycolysis in *O. niloticus* juveniles fed on 38% group diet may suggest this level of digestible sago starch is the optimal tolerance threshold for metabolic utilization of glucose by the fish juveniles. Xia et al. (2015) stated that excess carbohydrate in feed intake decelerated the glycolysis of glucose metabolism which reduced the efficiency rate of PFK metabolic enzymes in fish to utilize the dietary carbohydrate, respectively. However, some studies suggested that the induction of PFK

activity by dietary carbohydrate levels might depend on the fish species and its categorized such as carnivorous, herbivorous or omnivorous (NRC, 2011).

The similar trend were observed on FBPase activity in which the *O. niloticus* juveniles fed with 38% sago starch level showed a positive response than those from 44% group diets. Ma et al. (2019) mentioned that high dietary carbohydrate level in diet could depressed and down-regulate FBPase enzyme activity in the fish liver and this statement supported the present finding. FBPase was known as one of the key metabolic enzyme that regulate the gluconeogenesis pathway (Enes et al., 2009; Cui et al., 2010). Pei et al. (2012) stated that the FBPase enzyme is important for promoting gluconeogenesis and also lipid degradation. Higher activity of the gluconeogenesis enzymes on the recent study may indicate that *O. niloticus* juveniles have metabolic ability to adapt to high-carbohydrate level was at 38% of the dietary sago starch. On the other hand, optimal level of carbohydrate in diets may induce insulin secretion, which stimulate hepatic glucose uptake, thus enhancing hepatic gluconeogenesis enzyme activities and leading to an increase in liver glycogen content to maintain glucose homeostasis (Maas et al., 2019).

The G6PD metabolic enzyme activities of *O. niloticus* juveniles showed a promotive effect with dietary carbohydrate level increased in the present study. The fish fed starch-rich diets had higher G6PD activities indicated increased capacities of NADPH generation which is necessary for the conversion of carbohydrate to fat (Kamalam et al., 2017). This statement confirmed the current result in which the G6PD activities were spotted increases in fish liver fed on the diet from 38% to 44% of the dietary sago starch. Glucose 6-phosphate dehydrogenase (G6PD) is one of the key metabolic enzyme that responsible for the lipogenesis pathway (Zhou et al., 2016). Azaza et al. (2015) reported that the increased in enzymes activities of lipogenesis is accompanied by increased of VSI, HSI and IPF values

in the fish and this statement support the current result presented on Table 4.1. Meanwhile, Boonanuntanasarn et al. (2018) stated that excess dietary carbohydrate in diet could increases the synthesis of lipid which further stored and accumulated as fat deposition on liver and stomach of the fish.

Taking into account, glucose metabolism and homeostasis are highly dependent on the feeding diet. Feeding fish with enriched carbohydrate diets have been primarily studied in cultured species (Polakof et al., 2012) even though it is generally accepted that fish do not have a specific requirement for dietary carbohydrate (Haidar et al., 2016). The incorporation level of dietary carbohydrates are varying in different fish species which associated with a persistent postprandial hyperglycaemia. Starch is the dietary carbohydrate that can be digested effectively by many fish species after extrusion and gelatinisation especially for herbivorous/omnivorous (Li et al., 2013). However, the potential mechanisms by which the starch level was induced are still unknown. Further investigation is needed to study the metabolism of carbohydrate in future studies for this species.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The present research was conducted through a comprehensive feeding trial aimed to determine the effect of sago (*Metroxylon sagu*) starch-based carbohydrate on the diet of Nile tilapia, *Oreochromis niloticus* juveniles. Briefly, different incorporation level between dietary protein and carbohydrate were formulated for feeding trials to evaluate the ability of the fish to spare the protein with dietary carbohydrate in the diet, respectively.

The results of the current study confirmed that sago starch as the source of carbohydrate in the formulated diet showed a protein sparing influenced on the *O. niloticus* juveniles. The results revealed that a positive relationship to the *O. niloticus* juveniles on growth performance, body indices, blood compositions, nutrient digestibility, digestive enzymes and carbohydrate metabolic enzymes. The results also proved that 38% of sago starch formulated in the diet tend to have a better effect than 44% level and the efficiency of all feeding diets tested in this study were significant. The present study showed that the protein level ranged from 26% to 30% spared with 38% of sago starch is best incorporation for feed formulation of fish. Therefore, this study concludes that the growth and feed utilization of fish can be maximized and improved by using the optimal level between protein and carbohydrate in fish feed production.

The application of sago starch as carbohydrate source can be considered as a suitable candidate to spare the protein in aquaculture feed industry. Besides, the low price, sago starch availability also wide and sustainable which can fully fulfil its demand in feed

industry. This is a great advantage for aquaculture industry as its help in reducing the production cost of the fish feed. To take into account, continuous study and research on dietary carbohydrate as energy source in fish is important to discover more about its advantages and benefits in aquaculture feed industry.

5.2 Recommendations

The current research demonstrates that the application sago starch as carbohydrate source is positively have the protein sparing effect on *O. niloticus* juveniles. As a follow up to this present research, further studies are recommended on:

- Conducting long-term growth trials using the sago starch practical diets in fertilised ponds under farmer managed conditions to ascertain their potential in increasing current yield levels among small scale fish farmers.
- Exploring other simple and yet efficient methods of improving the nutritional profile of ingredients such as removal of antinutritional factors as well as reducing fibre content to improve digestibility.
- Identifying and evaluating other locally available materials from both plant and animal sources which have a potential to serve as novel ingredients in formulating cost effective diets.

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APPENDICES

Appendix A: Water Quality Parameters Measurement Device and Methodology

A.1 Devices used for determining the water temperature, pH and DO



Portable pH meter by Hanna (HI-98128)



Portable Extech DO meter (SDL150)

A.2 Ammonia Nitrogen, $\text{NH}_3\text{-N}$ (Nessler Method)

Step 1: For ammonia nitrogen ($\text{NH}_3\text{-N}$), started the program 380N, Ammonia, Ness by pressing the dial. The display was instructed to fix the wavelength dial as 425 nm.

Step 2: The small display was fixed at 425 nm wavelength.

Step 3: The cylinder was filled with the 25 ml of prepared sample.

Step 4: The blank sample was prepared by using deionized water added in another cylinder.

Step 5: Added 3 drops of mineral stabilized to each cylinder and mixed properly.

Step 6: After mixing properly, added 3 drops of polyvinyl alcohol dispersing agent to each cylinder and mixed properly.

Step 7: Added 1ml Nessler reagent into each cylinder by using pipette and mixed properly.

Step 8: The mixed samples was kept for 1 minute. Time was measured by pressed SHIFT TIMES in the analyzing spectrophotometer.

Step 9: After 1 minute, each solution was poured into a sample cell.

Step 10: First the blank sample was placed for zeroing and then placed with the prepared sample. The light shield should be closed before pressing the READ button. The display showed the ammonia as mg/L.

A.3 Nitrate, $\text{NO}_3\text{—N}$ (Cadmium Reduction Method)

Step 1: For nitrate, $\text{NO}_3\text{—N}$, started the program 351 N, Nitrate, LR by pressing the dial. The display was instructed to fix the wavelength dial as 507 nm.

Step 2: The small display was fixed at 507 nm wavelength.

Step 3: The cylinder was filled with the 15 ml of prepared sample.

Step 4: Added a NitraVer 6 reagent powder pillow into the cylinder and kept for 5 minutes.

Step 5: After the timer expired, poured 10 ml of sample into a sample cell.

Step 6: Added a NitriVer 3 Reagent Powder Pillow to the prepared sample cell and kept for 15 minutes.

Step 7: The blank sample was prepared with the same process added 10 ml of deionized water into the sample cell.

Step 8: First the blank sample was placed for zeroing and then placed with the prepared sample. The light shield should be closed before pressing the READ button. The display showed the nitrate as mg/L.

A.4 Nitrite, NO₂—N (Diazotization method)

Step 1: For nitrite, NO₂—N, started the program 371 N, Nitrite, LR PP by pressing the dial. The display was instructed to fix the wavelength dial as 507 nm.

Step 2: The small display was fixed at 507 nm wavelength.

Step 3: The sample cell was filled with the 10 ml of prepared sample.

Step 4: Added a NitriVer 3 reagent powder pillow into the sample cell and kept for 20 minutes.

Step 5: The blank sample was prepared with the same process added 10 ml of deionized water into the sample cell.

Step 6: First the blank sample was placed for zeroing and then placed with the prepared sample. The light shield should be closed before pressing the READ button. The display showed the nitrite as mg/L.

A.5 Phosphorus, PO₄ 3– (PhosVer 3 method)

Step 1: For phosphorus, PO₄ 3–, started the program 490 P, React. PP by pressing the dial. The display was instructed to fix the wavelength dial as 880 nm.

Step 2: The small display was fixed at 880 nm wavelength.

Step 3: The sample cell was filled with the 10 ml of prepared sample.

Step 4: Added a PhosVer 3 phosphate reagent powder pillow into the sample cell and kept for 2 minutes.

Step 5: The blank sample was prepared with the same process added 10 ml of deionized water into the sample cell.

Step 6: First the blank sample was placed for zeroing and then placed with the prepared sample. The light shield should be closed before pressing the READ button. The display showed the phosphorus as mg/L.

Appendix B: Proximate Analysis (AOAC, 1997)

B.1 Determination of Moisture Content

Step 1: Heated up the infrared moisture determination balance (AND 4712) for about 20 minutes at 80°C.

Step 2: Labelled the aluminium pan; placed it on the balance and pressed “TARA” button for making it zero.

Step 3: An amount of approximately 4-5 g sample into the pan, spread evenly and recorded the sample. Triplicate samples were taken.

Step 4: Pressed the “START” button for making the balance start drying and waited until the reading remains unchanged.

Step 5: The reading was recorded in % dry after pressing the “STOP” button.

Step 6: Moisture content of the sample (%) = 100-% dry.

Step 7: Analysis was done in triplicates.

B.2 Crude protein measurement using Kjeldahl method

Step 1: 0.1 g moisture free sample was taken into ash free paper. Triplicates samples were taken.

Step 2: Weighted samples placed in different Kjeldahl flasks and numbered

Step 3: Added 1 teaspoon catalysts (containing $K_2SO_4 + CuSO_4$) and 25ml concentrated sulphuric acid

Step 4: Placed the flasks on Kjeldahl digestion unit for digesting

Step 5: The flasks were heated at 250°C for 20 minutes and shaken properly; the temperature increased up to the range of 350°C to 380°C for 1 to 2 hours depending on the colour which turned into light green; and then digested for another 45 minutes

Step 6: The heater was then switched off and the flasks were cooled down into room temperature for 10-15 minutes.

Step 7: Slowly added 300 ml distilled water in each flask

Step 8: Carefully added 100 ml of 40% NaOH for the distillation process and the flasks were vertically placed into the Kjeldahl Distillation Unit

Step 9: Prepared 25 ml boric acid and 2-3 drops of indicator (methylene red+bromocresol blue) mixture in a Erlenmeyer flasks, connected to the end of duct of the apparatus. It must be immersed into the solution during running the distillation which was conducted until as much as 75 ml boric acid was collected

Step 10: Titrated with 0.1N HCl until the colour of the solution turn greyish blue. The reading was recorded as ml of acid titrates.

Step 11: The crude protein was measured using following formula:

$$\% \text{ Nitrogen in sample} = 100 [(A \times B) \times 14.007] / C$$

Where, A=Volume of acid used in the titration

B= Normality of standard acid

C= Weight of sample in mg, while molecular mass (g) of nitrogen

$$\% \text{ Crude protein in the sample} = \% \text{ Nitrogen in the sample} \times 6.25$$

B.3 Determination of Crude Lipid

Step 1: 0.1 g moisture free sample was taken into a big clean glass tube. Triplicates samples were taken.

Step 2: Added 60 ml mixture of chloroform and methanol and homogenized for 2-5 minutes.

Step 3: Homogenized sample was filtered through funnel with filter paper (Whatman qualitative no. 1 or 2).

Step 4: Filter paper was stored for dry to measure the fiber.

Step 5: The filtered sample was washed with 40 ml mixture of chloroform and methanol and transferred to separating funnel.

Step 6: Added 20 ml 30% NaCl solution and mixed properly by shaking for 1 minute.

Step 7: The funnel was vertically left to separate into two phases. The upper phase was a mixture of NaCl solution and methanol; and the lower phase was a mixture of lipid extraction and chloroform. Lower phase was collected in a beaker after 2-3 hours; and weighted. After that, the beaker was put into oven at 80°C for 4 hours to evaporate the chloroform.

Step 8: After 4 hours, the beaker with dried lipid was taken out and kept into the desiccator for cooling.

Step 9: After cooling, the beaker with dried lipid was weighted and the crude lipid was determined using the following formula:

$$\% \text{ crude lipid} = \frac{\text{Beaker weight with lipid} - \text{weight of empty beaker} \times 100}{\text{Sample initial weight}}$$

B.4 Crude fiber determination

Step 1: 2 g fatless dried sample was taken from the filter paper used during crude lipid extraction and putted into 800 ml clean glass beaker. Triplicate samples were taken.

Step 2: Added 50 ml 5% sulphuric acid and 150 ml distilled water into the beaker

Step 3: The beaker was placed on the condenser and refluxed for 30 minutes; then left to cool at room temperature.

Step 4: Added 10 ml 40% NaOH and 3 drops phenolphthalein indicator and reflexed again for 30 minutes and then left to cool down at room temperature.

Step 5: The cool mixture was filtered through a funnel using 541 Whatman filter paper.

Before using the filter paper, it was numbered by sample.

Step 7: Rinsed the residue (from the beaker) with the boiling water, 1% HCl and again with boiling water to remove the HCl.

Step 8: Rinsed the funnel with methylated spirit and transferred the filter paper together with the sample to the porcelain crucible; then placed in the oven at 105°C until drying.

Step 9: After drying, the crucible was weighted, recorded and placed into the muffle at 550°C for about 5 hours; then transferred to the desiccators and weighted after cooled down and the crude fiber was calculated using following formula

Dried sample weight= Weight of fat free sample

100 - % lipid

% Fiber= (Crucible weight with ash – Weight of empty crucible) x 100

Dried sample weight

B.5 Crude ash determination

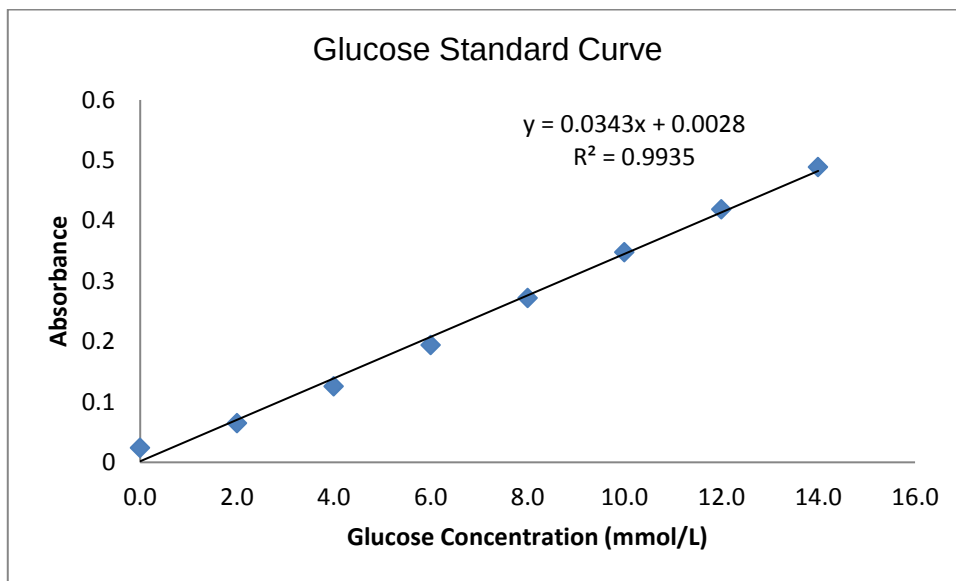
Step 1: The porcelain cup was taken and weighted after drying at 100°C for 1 hour. The weight was recorded

Step 2: 2 g sample was taken into the cup and the crucible cup was placed into the muffle at 550°C for about 5 hours; then transferred to the desiccators and weighted after cooled down and the crude ash was calculated using following formula:

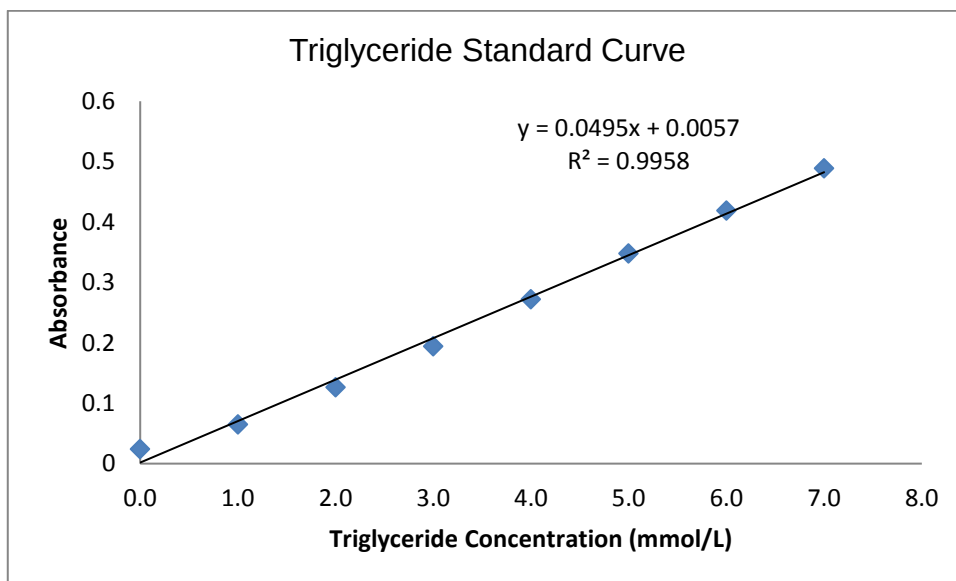
% crude ash= (Crucible weight with ash – Weight of empty crucible) x 100

Sample initial weight

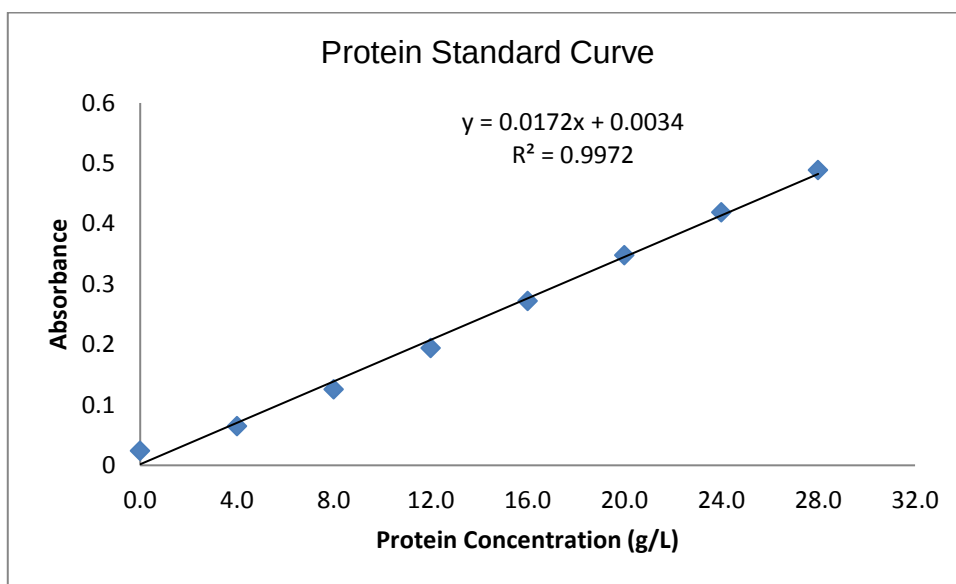
Appendix C: Standard curve of Glucose Plasma



Appendix D: Standard curve of Triglyceride Plasma



Appendix E: Standard curve of Protein Plasma



Appendix F: Bradford Protein Assay

Step 1: Bradford Reagent was prepared using Biorad Dye Reagent concentrate (#500-0006) which was diluted with distilled water 1:4 (v/v).

Step 2: Standard protein concentration was prepared using Biorad Assay Standard II Lyophilized Bovine Serum Albumin (BSA) (#500-0007) with concentration of 0 (distilled water) to 1.2 mg/ml with 0.1 mg/ml intervals.

Step 3: 10 ul of each concentration of BSA were pipette into assigned well of a 96-well micro titer plate in triplicates.

Step 4: 200 ul of prepared Biorad Reagent was added into each well containing the BSA.

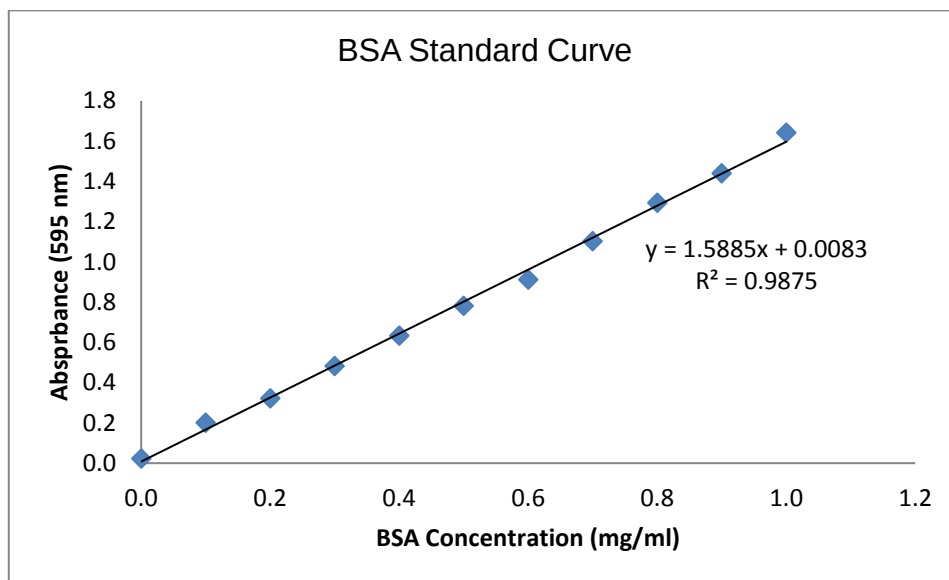
Step 5: The mixtures were incubated for 15 minutes at room temperature.

Step 6: The absorbance of the mixtures was determined using Biorad Microplate Reader Model 680 at 595 nm.

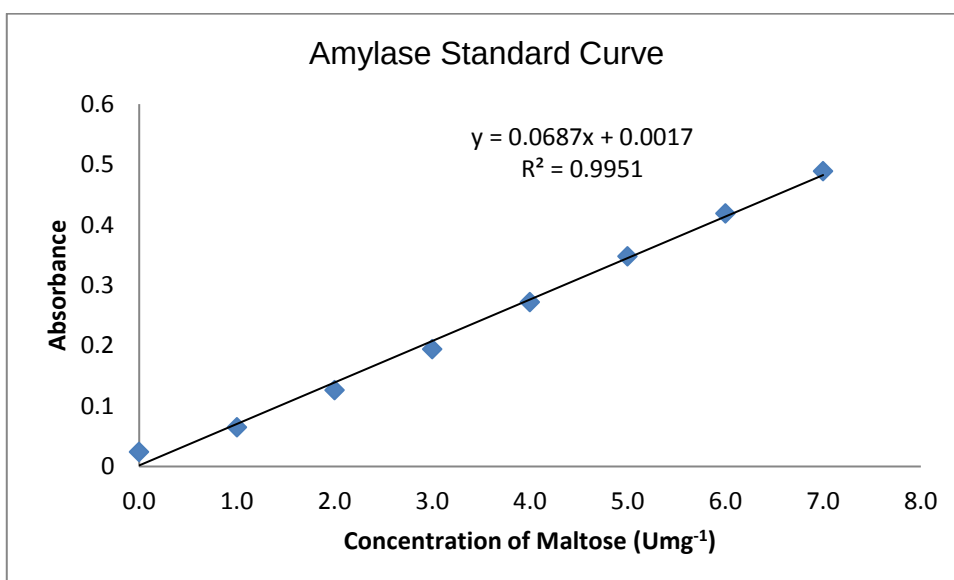
Step 7: Bovine Serum Albumin Standard Curve was plotted as absorbance against protein concentration to calculate the protein concentration in samples.

Step 8: Protein concentration of samples were determined using steps 3-6 except that BSA was substituted with samples.

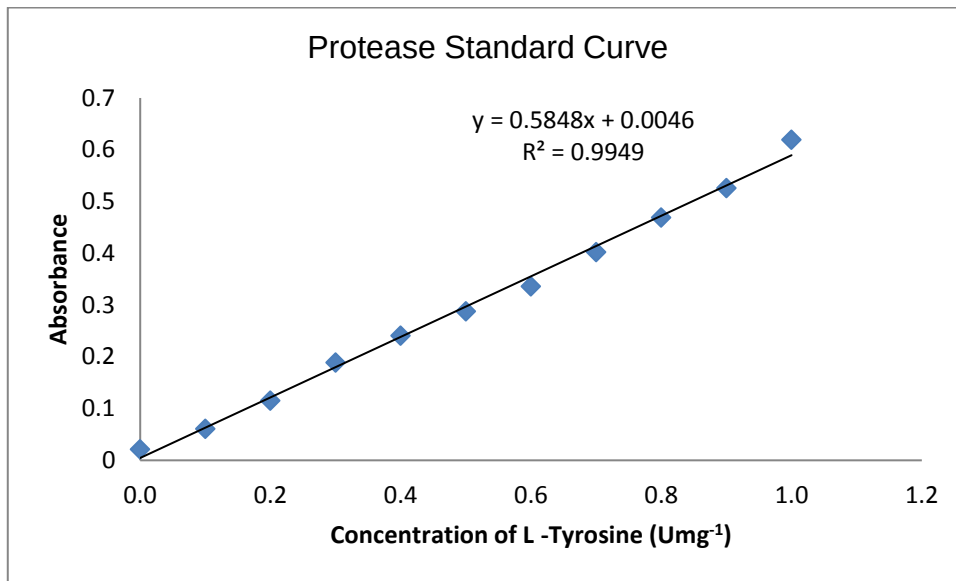
Appendix G: Standard curve of Bovine Serum Albumin (BSA)



Appendix H: Standard curve of Amylase Enzyme



Appendix I: Standard curve of Protease Enzyme



Publication

Senawi, B. L., Asdari, R., & Munir, M. B. (2020). Effects of Sago-Based (*Metroxylon sagu*) Carbohydrate on Growth Performance and Blood Plasma Compositions of Nile Tilapia, *Oreochromis Niloticus* (Linnaeus, 1758) Juveniles. *Journal of Sustainability Science and Management*, 15(7), 56–73.