UTILIZATION OF ALLZYME® SSF AND CITRIC ACID IN THE FORMULATION OF DUCKWEED-INCLUDED DIETS FOR HYBRID GROUPER, *Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Hybrid grouper, tiger grouper (*Epinephelus fuscoguttatus*) x giant grouper (*Epinephelus lanceolatus*) (TGGG) has high market values in Asian region. In most commercial fish farms, groupers are fed mainly with fish meal-based diet. However, the expensive cost and uncertain availability of fish meal urged researchers to find alternative ingredients to curb this issues. The present study was conducted to find alternative ingredients and suitable feed additives to partially replace the fish meal protein for the hybrid grouper. In Experiment 1, graded levels of exogenous enzyme, Allzyme® SSF was used as feed additive to evaluate the growth and the suitable level of the enzyme for the fish. Six fish meal-based diets (50% protein, 16% lipid) were formulated with enzyme supplementation at 0 ppm (A0), 50 ppm (A50), 150 ppm (A150), 250 ppm (A250), 350 ppm (A350) and 450 ppm (A450). The feeding trial was conducted for 10 weeks. Results showed fish fed with diet A450 achieved the highest body weight gain (BWG), final body weight (FBW) and specific growth rate (SGR) without significant difference with others ($P>0.05$). Based on the feed utilization, all the fish utilized the feed well without significant differences among the groups. With enzyme supplementation, an increasing trend of protein efficiency ratio (PER), net protein utilization (NPU), trypsin-like, amylase and lipase in the stomach, apparent digestibility coefficient (ADC) of crude protein and crude lipid (CL) were observed. Thus, 450 ppm of Allzyme® SSF was selected for the following experiment. In Experiment 2, duckweed, *Lemna minor* (L) was used to replace 5% of fish meal protein in the diet of TGGG juvenile with 450 ppm of Allzyme® SSF and 3% citric acid supplementation. The experiment was carried out in a 10 weeks feeding trial to evaluate the effects of plant-based diet with feed additives on growth performance of TGGG. Four treatments were formulated as control diet without addition of duckweed and feed additives (CON), *L. minor* (DL) diet, *L. minor* with citric acid (DLC) and *L. minor* with enzyme (DLA) diets. At the end of experiment, the growth performance of DLC achieved significant higher BWG and SGR compared to CON ($P<0.05$) but not significantly different with other treatments ($P>0.05$). The food conversion ratio was not significantly different among treatments ($P>0.05$). However, the PER and NPU was highest in group fed citric acid-supplemented diet compared to other groups ($P>0.05$). The specific activities of trypsin-like enzyme were significantly higher in DLC and DLA groups compared to DL and CON groups ($P<0.05$). Highest amylase activities was observed in DLA than CON ($P<0.05$) while DL, DLC and DLA have significantly higher lipase activities in stomach than CON ($P<0.05$). The ADC of CP in fish fed DLC and DLA diets were enhanced compared to CON without significant difference ($P>0.05$). The histology of distal intestines and liver of TGGG showed no adverse effects that affected the performance of TGGG among the treatments. The present study showed that TGGG can utilize diet added with 5% of duckweed protein and the growth performance, feed utilization, digestive enzyme activities and apparent digestibility were improved with the addition of feed additives such as citric acid and Allzyme® SSF.

ABSTRAK

PENGGUNAAN ALLZYME® SSF DAN ASID SITRIK DALAM FORMULASI DIET KIAMBANG ITIK UNTUK KERAPU HIBRID, *Epinephelus fuscoguttatus x Epinephelus lanceolatus*

Kerapu hibrid, kerapu harimau (Epinephelus fuscoguttatus) x kerapu gergasi (Epinephelus lanceolatus) (TGGG) mempunyai nilai pasaran yang tinggi di negara Asia. Dalam kebanyakan ladang ikan komersial, kerapu diberi makanan yang diperbuat dari tepung ikan. Walau bagaimanapun, kos yang mahal dan ketersediaan yang tidak dijangka bagi tepung ikan menggesa penyelidik untuk mencari bahan-bahan alternatif untuk membendung isu-isu ini. Kajian semasa ini telah dijalankan untuk mencari bahan alternatif dan bahan aditif untuk menggantikan protein tepung ikan untuk kerapu hibrid secara separa. Dalam Eksperimen 1, tahap digred enzim eksogenus Allzyme® SSF telah digunakan sebagai bahan aditif untuk menilai pertumbuhan dan tahap enzim yang sesuai untuk ikan. Enam diet berasaskan tepung ikan (50% protein, 16% lipid) telah dirumuskan dengan penambahan enzim pada 0 ppm (A0), 50 ppm (A50), 150 ppm (A150), 250 ppm (A250), 350 ppm (A350) dan 450 ppm (A450). Percubaan makanan telah dijalankan selama 10 minggu. Keputusan menunjukkan ikan yang diberi makan dengan diet A450 mencapai pertambahan berat badan (BWG), berat akhir badan (FBW) dan kadar pertumbuhan tertentu (SGR) yang paling tinggi tanpa perbezaan yang signifikan dengan kumpulan lain (P>0.05). Berdasarkan penggunaan makanan, semua ikan menggunakan makanan dengan baik tanpa perbezaan yang signifikan antara kumpulan. Dengan suplemen enzim, corak yang meningkat dalam kadar kecekapan protein (PER), penggunaan protein bersih (NPU), tripsin, amilase dan lipase dalam perut, koefisyen penghadaman nyata (ADC) protein mentah (CP) dan lipid mentah (CL) dapat diperhatikan. Oleh itu, 450 ppm Allzyme® SSF telah dipilih untuk eksperimen yang berikut. Dalam Eksperimen 2, kiambang itik, Lemna minor (L) telah digunakan untuk menggantikan 5% daripada protein tepung ikan dalam diet juvana TGGG dengan penambahan 450 ppm Allzyme® SSF dan 3% asid sitrik. Eksperimen telah dijalankan dalam percubaan makanan selama 10 minggu untuk menilai kesan daripada diet berasaskan tumbuhan dengan bahan aditif terhadap prestasi tumbesaran TGGG. Empat jenis diet telah dirumuskan sebagai diet kawalan tanpa penambahan kiambang itik dan bahan aditif (CON), diet L. minor (DL), diet L. minor dengan asid sitrik (DLC) dan diet L. minor dengan enzim (DLA). Pada akhir eksperimen, prestasi pertumbuhan DLC mencapai BWG dan SGR yang ketara berbanding CON (P<0.05) tetapi tiada perbezaan ketara dengan kumpulan lain (P>0.05). Tiada perbezaan ketara dalam kadar penukaran makanan antara kumpulan (P>0.05). Walau bagaimanapun, PER dan NPU adalah paling tinggi dalam kumpulan yang diberi makan diet yang ditambah asid sitrik berbanding kumpulan lain (P>0.05). Aktiviti spesifik enzim tripsin adalah lebih tinggi dalam kumpulan DLC dan DLA berbanding kumpulan DL dan CON (P<0.05). Aktiviti amilase tertinggi diperhatikan dalam DLA berbanding CON (P<0.05) manakala DL, DLC dan DLA mempunyai aktiviti lipase dalam perut yang lebih tinggi daripada CON (P<0.05). ADC CP dalam ikan yang diberi makan diet DLC dan DLA telah dipertingkatkan berbanding CON tanpa perbezaan yang signifikan (P>0.05). Histologi usus distal dan hati TGGG tidak menunjukkan sebarang kesan buruk yang mempengaruhi prestasi TGGG antara kumpulan. Kajian semasa ini menunjukkan bahawa TGGG boleh menggunakan makanan yang ditambah dengan 5% protein kiambang itik dan prestasi pertumbuhan, penggunaan makanan, aktiviti enzim penghadaman dan

penghadaman nyata dapat dipertingkatkan dengan penambahan bahan aditif seperti asid sitrik dan Allzyme® SSF.

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LIST OF ABBREVIATIONS

α	-	Alpha
β	-	beta
µg	-	microgram
µL	-	microlitre
µmole	-	micromole
4-NPC	-	4-nitrophenyl caproate
ADC	-	Apparent digestibility coefficient
ANOVA	-	One –way Analysis Variance
AOAC	-	Association of Official Analytical Chemists
ATP	-	Adenosine triphosphate
BW	-	Body weight
BL	-	Body length
BWG	-	Body weight gain
CF	-	Condition factor
CL	-	Crude lipid
Cm	-	Centimetre
CMC	-	carboxymethylcellulose
CP	-	Crude protein
Cr_20_3	-	Chromium (III) oxide
$CuSO_4$	-	copper (II) sulphate
DM	-	Dry matter
DFI	-	Daily feed intake
EAAs	-	Essential amino acids
et al.	-	And others
FCR	-	Feed conversion ratio
FM	-	Fish meal
FTU/kg	-	Phytase activity unit per kilogram
g	-	gram
H&E	-	Haematoxylin and eosin
H_2O	-	Water
H_2SO_4	-	Sulphuric acid
ha	-	hectare
HSI	-	Hepatosomatic index
IPF	-	Intraperitoneal fat ratio
KOH	-	Potassium hydroxide
K_2SO_4	-	potassium sulphate
L	-	Litre
LM	-	*Lemna minor*
LP	-	Lamina propria
M	-	molar
$M^{-1}cm^{-1}$	-	Unit for molar attenuation coefficient
mg	-	milligram
mg/L	-	milligram per litre
mg/mL	-	Milligram per millilitre
ml	-	millilitre
mm	-	Millimetre
mM	-	Millimolar
MUFA	-	Monounsaturated fatty acid
N	-	Nitrogen

N	-	Normality
N/L	-	Nitrogen per litre
NaOH	-	Sodium hydroxide
NEAAS	-	Non-essential amino acids
NFE	-	Nitrogen-free extract
NH_4Cl	-	Ammonium chloride
NH_3	-	Ammonia
NIFES	-	National Institute of Nutrition and Seafood Research
NRC	-	National Research Council
nm	-	Nanometre
NPU	-	Net protein utilization
P	-	Phosphorus
PC	-	Pyloric caeca
PER	-	Protein efficiency ratio
ppm	-	Parts per million
ppt	-	Parts per trillion
PUFA	-	Polyunsaturated fatty acid
rpm	-	Revolutions per minute
S	-	stomach
SAFA	-	Saturated fatty acid
SBM	-	Soybean meal
SD	-	Standard deviation
SGR	-	specific growth rate
Spp.	-	species
SPSS	-	Statistical Package for Social Science
SSF	-	Solid state fermentation
TCA	-	Trichloroacetic acid
UV	-	Ultraviolet
V	-	vacuole
VSI	-	Viscerosomatic index

LIST OF SYMBOLS

®	-	Registered
^0C	-	degree Celsius
Σ	-	Sigma (sum)
%	-	percent
x g	-	Times gravity
III	-	three

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of study

With around three quarters of the world capture fisheries fully or overexploited (Huntington and Hasan, 2009) aquaculture is the main source for future fish production. Fish contributes to the nutritional security of poor households in developing countries in various ways (FAO, 2014). Capture fisheries and aquaculture supplied the world with 158 million tonnes fish in 2012 of which 136 million tonnes were used as human food (FAO, 2014). Further growth in the aquaculture production can therefore not depend on an expansion in the catch volume of wild fish but must rely on a further increase in the utilization of alternative feed resources (Bogevik, 2015).

Carnivorous fish farming was reported to place unnecessary pressure on world fish meal supplies (Naylor *et al.*, 2000). Carnivorous fish such as groupers, salmon and trout consume significantly more fish protein than they produced when observed through the discrepancies in the ratio of wild fish consumed to farmed fish produced (Huntington and Hasan, 2009). In order to ensure an optimal content of amino acids and other nutrients needed for fish growth and flesh quality, most fish feeds contain a minimum level of fish meal (FAO, 2014). However, the increasing price of fish meal in recent years has urged researchers to find alternatives for fish meal. Fish meal is used as a primary ingredient in high-protein feed production. Generally, fish feed constitute the major part of aquaculture production cost due to the reliance on fish meal as main protein source. A significant percentage of world fisheries production is still processed into fish meal and fish oil although with a declining trend (FAO, 2014). A total of 30.2 million tonnes for fish meal production was used up for aquaculture in 1994, dropped in 2010 due to reduced catches of

anchoveta, increased again in 2011 and then down to a declining trend in 2012 (FAO, 201). Currently, the usage of fish meal was expected to increase in order to cope with the increasing level of production and intensification of the aquaculture industry.

Due to the uncertain availability and peaked cost of fish meal, there has been a lot of efforts applied to find suitable sources of protein alternatives in order to replace the usage of fish meal. For grouper species, among the sources of protein investigated are animal protein such as poultry-by product meal in orange-spotted grouper, *E. coioides*, humpback grouper, *Cromileptes altivelis*, malabar grouper, *E. malabaricus* and also brown-marbled grouper, *E. fuscoguttatus* (Millamena and Golez, 2001; Millamena, 2002; Shapawi *et al.*, 2007; Wang *et al.*, 2008; Li *et al.*, 2009; Rachmansyah *et al.*, 2009; Gunben *et al.*, 2014). Plant protein alternatives has also been a great interest in aquaculture research for brown-marbled grouper, rainbow trout, *Oncorhynchus mykiss* and gilthead sea bream, *Sparus aurata* such as soybean meal products (Shapawi *et al.*, 2013a, b), lupin seed meal (Glencross *et al.*, 2004), pea seed meal (Pereira and Olivia-Teles, 2002), corn gluten meal (Pereira and Olivia-Teles, 2003), rapeseed meal and many others.

On the other hand, duckweed is the smallest angiosperms in the world, where it has a fast growth and is a simplest flowering plant (Patra, 2015). Moreover, due to its miniature sizes, capacity of rapid growth to form genetically uniform clones, easy management and high sensitivity to organic and inorganic substances, it is often used as model system for different types of experiment (Zhang *et al.*, 2010). In Malaysia, duckweed can be found almost anywhere such as ponds, lakes, and ditches. According to Goopy and Murray (2003), the members of Lemnaceae family are found almost worldwide but not present in polar region and deserts. Duckweed meal has been used for cattle, poultry, swine and fish which showed favourable results (Skillicorn *et al.*, 1993). In aquaculture, it has been used as partial replacement of fish meal in various fish species including Indian carp, *L. rohita* (Ahammad *et al.*, 2003; Gull *et al.*, 2005), common carp, *Cyprinus carpio* (Yilmaz *et al.*, 2004), and silver barb, *Barbodes gonionotus* (Noor *et al.*, 2000).

The problem of incorporation of plant protein sources in fish feed is the presence of various anti-nutritional factors which reduce the digestive enzyme activities and therefore decrease nutrient digestibility (Krogdahl *et al.*, 1994). Anti-nutritional factors also have limiting effect on the growth of fish and can cause pathomorphological changes in the intestines of fish (Krogdahl *et al.*, 2003). Studies on rainbow trout, *Oncorhynchus mykiss* and Atlantic salmon, *Salmo salar* revealed intestinal inflammatory developments promoted by anti-nutritional factors in soybean meal-based diets (Krogdahl *et al.*, 2003; Ostaszewska *et al.*, 2005). Soybean meal usage in formulated feed can also hamper growth due to the lack of amino acids especially lysine and/or methionine (Fagbenro and Davies, 2001). It is also validated that high dietary level of plant proteins which is over 40% of total protein for partial replacement of fish meal decreases feed efficiency as well as growth performance (Robaina *et al.*, 1995; Refstie *et al.*, 2000). Moreover, when plant-based ingredients increased, the intake of anti-nutritional factors and fibers will increase which will consequently interfere with nutrient digestibility in fish (Francis *et al.*, 2001).

Commonly, when plant source protein is added into the feed, supplementation of feed additives such as exogenous enzyme and organic acid has been reported to improve the utilization of the feed (Lin *et al.*, 2007; Pandey and Satoh, 2008; Yildirim and Turan, 2010). Poultry and pig industries have been using exogenous enzyme as supplementation in their feeds. The application of enzymes has shown that lower cost or cheaper ingredients can be used with equal and even better performance and subsequently increase the choice and flexibility of the feed manufacturer (Deguara *et al.*, 1999). In terrestrial animals, the beneficial effects of enzymes have been reported in a number of studies such as broiler chickens (Bedford and Classen, 1992; Walsh and Headon, 1994) and turkeys (Salmon *et al.*, 1986).

In aquaculture, exogenous enzyme has been used as feed additives in many species of fish to increase feed digestibility. There are still few studies evaluating enzyme supplementation in feed for fish and recommendations of dietary enzymes for aquatic organisms are built on results obtained for non-ruminants animals (Castillo and Gatlin, 2015). The practice of enzyme application for fish has yielded contrary results due to over-dependence on plant protein source types, enzymatic profile and addition level added to the diets, besides the fish species used as model