

FISH MEAL REPLACEMENT WITH KRILL MEAL IN THE DIET OF MARINE FISH ON GROWTH AND METABOLIC RESPONSE

Submitted in partial fulfillment of the requirements for the award of Bachelor of
Technology Degree in

Biotechnology

By

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DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

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MARCH 2021



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BONAFIDE CERTIFICATE

This Project thesis is the bonafide work of **SHUNMUGA PRIYA.L (37230084)** who carried out the final year project entitled "**FISH MEAL REPLACEMENT WITH KRILL MEAL IN THE DIET OF MARINE FISH ON GROWTH AND METABOLIC RESPONSE**" from 15th December 2021 to 21st February 2021.

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This is to certify that Miss. SHUNMUGA PRIYA L. (Register Number: 37230084), B.Tech (Biotechnology), Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai has undergone in-plant training on Marine Biotechnological Applications and done a work on “Fishmeal replacement with krill meal in the diet of marine fish on growth and metabolic response” under my guidance at ICAR-Tuticorin Regional Station of CMFRI, Thoothukudi during December 2020-February 2021. This research work or any part of its results were not submitted elsewhere for any other degree.

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DECLARATION

I **SHUNMUGA PRIYA.L** hereby declare that the final year project thesis entitled **“FISH MEAL REPLACEMENT WITH KRILL MEAL IN THE DIET OF MARINE FISH ON GROWTH AND METABOLIC RESPONSE”** was done by me under the guidance of **Mr.D.Linga Prabu** at **ICAR-CMFRI, TUTICORIN REGIONAL STATION, THOOTHUKUDI** is submitted in partial fulfillment of the requirements for the award of **Bachelor of Technology** degree in **Biotechnology**.

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ABSTRACT

As the demand for the inclusion of fishmeal in aquafeed is very high, an alternative source should be identified and used. Krill meal is a source of quality protein comparable to fishmeal is an untouched animal source ingredient and available in huge quantity. It is also act as an effective attractant in the diet of marine fishes for the effective consumption of feed and to promote better growth and health in the fishes. With this background, the krill meal was used to replace the fish meal as a protein source ingredient in the diet of juveniles of *Epinephelus coioides* (orange spotted grouper) in an experiment that was conducted for 63 days. There were five treatments each with triplicates in this experiment and fed to experimental fishes by varying level of inclusion of krill meal in place of fishmeal at the rate of 0, 15, 30, 45 and 60% of protein content of fishmeal. After the completion of experimental duration, the fishes were sampled to access the growth response and feed utilization. The experimental fishes were anesthetized using clove oil to collect blood and tissue samples for enzyme analysis, histological analysis and functional gene expression profile. Among the five treatments, highest weight gain, specific growth rate, average daily growth and weight gain percentage was observed in the T5 group. The feed utilization in terms of feed intake, PER, FE, FCR was better in T5 group due to higher concentration of krill meal in the diet as it was better attracted and enhanced feed consumption by fishes. The VSI and HSI were found higher in the fishes of T5 group due to better accretion of nutrients. The condition factor was also found to increase with increasing level of krill meal in the diet which indicates good health of the fish. The serum total protein, albumin, cholesterol, triglycerides and glucose levels were better in the higher level of krill meal fed groups. The tissue samples were found to contain moderate amount of digestive enzymes such as amylase, protease and lipase. The survival rate was found higher in T4 group followed by T3 group. The histological result indicates that no noticeable changes among the treatments. The proximate analysis results did not show any clear trend among the treatments; however, the ether extract was increased with increasing level of krill meal in the diet. Hence, 60% inclusion of krill meal is considered as effective level for the replacement of fishmeal in the diet of orange spotted grouper.

Keywords: Fishmeal, Krill meal, Orange spotted grouper.

CONTENTS

S.NO	TITLE	PAGE NO
1	Introduction	1
2	Review of Literature	2
2.1	World Aquaculture Scenario	2
2.2	Indian Aquaculture Scenario	2
2.3	Indian Mariculture Scenario	3
2.4	Fish meal and its importance in aquaculture Industry	4
2.5	Alternate feed ingredients	5
2.6	Krill meal	5
2.7	Orange Spotted Grouper (<i>Epinephelus coioides</i>)	6
3	Aim and Objective	7
4	Materials and Methods	8
4.1	Materials	8
4.1.1	Chemicals	8
4.1.2	Instruments	9
4.1.3	Softwares	10
4.2	Methods	11
4.2.1	Experimental Setup	11
4.2.2	Monitoring the water parameters	11
4.2.2.1	Salinity	11
4.2.2.2	pH	12
4.2.2.3	Temperature	12
4.2.2.4	Water quality	12
4.2.2.4.1	Nitrate	12
4.2.2.4.2	Nitrite	13
4.2.2.4.3	Ammonia	13

4.2.3	Feed Preparation	14
4.2.4	Sampling in ethical way	16
4.2.5	Fish sampling	17
4.2.6	Blood sampling	18
4.2.7	Isolation of Serum	19
4.2.8	Enzyme analysis	20
4.2.8.1	Amylase	20
4.2.8.2	Protease	21
4.2.8.3	Lipase	23
4.2.9	Serum Analysis	23
4.2.9.1	Total protein	23
4.2.9.2	Albumin	23
4.2.9.3	Triglycerides	23
4.2.9.4	Cholestrol	24
4.2.9.5	Glucose	24
4.2.10	Proximate analysis	24
4.2.11	RNA Isolation	25
4.2.12	DNA Isolation	26
4.2.13	cDNA Synthesis	27
4.2.13.1	Removal of Genomic DNA	27
4.2.13.2	First strand cDNA Synthesis	27
4.2.14	Polymerase Chain Reaction	28
4.2.14.1	Primers	28
4.2.15	Agarose Gel Electrophoresis	29
5	Results	31
5.1	Water Parameters	31
5.1.1	Water quality	31
5.1.2	Salinity	31
5.1.3	Temperature	31
5.2	Feed Utilisation	32
5.3	Growth estimation	33

5.4	Specific growth rate	34
5.5	Fish sampling	35
5.6	Serum analysis	38
5.7	Amylase analysis	39
5.8	Protease analysis	41
5.9	Lipase analysis	44
5.10	Proximate analysis	46
5.11	RNA isolation	47
5.12	DNA isolation	48
5.13	Polymerase Chain Reaction	48
5.14	Histological Analysis	49
5.15	Discussion-Results	50
6	Summary and Conclusion	51
6.1	Discussion	51
6.2	Summary	51
7	References	52

LIST OF ABBREVIATIONS

RNA	Ribonucleic acid
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
FRP	Fibre-reinforced plastic
NaOH	Sodium Hydroxide
ICAR-CMFRI	Indian Council of Agricultural Research – Central Marine Fisheries Research Institute
ppt	Parts per trillion
L	Litre
TCA	Trichloro acetic acid
mg	Milligrams
ml	Millilitre
dl	Decilitre
G	Gram
μl	Microlitre
μg	Microgram
Ng	Nanogram
M	Molar
mM	Millimolar

LIST OF FIGURES

FIGURE.NO	TITLE	PAGE NO
2.1	Krill meal	5
2.2	Orange spotted grouper (<i>Epinephelus coioides</i>)	6
4.1	Grouper juveniles	11
4.2	Experimental Setup (side view)	11
4.3	Experimental Setup (Top view)	11
4.4	Nitrate test	14
4.5	Nitrate colour chart	14
4.6	Nitrite test	14
4.7	Nitrite colour chart	14
4.8	Ammonium test	14
4.9	Ammonium colour chart	14
4.10	Kneading of feed	16
4.11	Preparation of dough	16
4.12	Cooking in pressure cooker	17
4.13	Pelletization	17
4.14	Drying of pelletized feed	17
4.15	Grinding of feed	17
4.16	Seiving and size grading of feed	17
4.17	Feed pellets	17
4.18	Anaesthetising of fish	18
4.19	Fish sampling	18
4.20	Viscera	19
4.21	Liver	19
4.22	Intestine	19
4.23	Muscle	19
4.24	Blood sampling	20
4.25	Collection of blood	20
4.26	Clotting of blood	20
4.27	Isolated serum	20

4.28	Wet fish	26
4.29	Dried fish	26
4.30	Crushed and powdered fish sample	26
5.1	Salinity	32
5.2	Temperature	32
5.3	Standard graph – Amylase	40
5.4	Standard graph – Protease – Higher concentration	42
5.5	Standard graph – Protease – Lower concentration	43
5.6	RNA Isolation	48
5.7	DNA Isolation	49
5.8	Polymerase Chain Reaction	49

LIST OF TABLES

TABLE.NO	TITLE	PAGE NO
4.1	Fish feed ingredients for experimental feed	15
4.2	Primer Specification	29
4.3	Temperature Schedule for PCR Programme	30
5.1	Water quality readings	32
5.2	Feed utilisation	33
5.3	Growth estimation	34
5.4	Specific growth rate	35
5.5	Weight of fishes per tank	36
5.6	Length and weight of fishes	37
5.7	Weight of organs sampled	38
5.8	Absorbance readings of components in serum	39
5.9	Amount of components present in serum	39
5.10	Readings of Standard graph – Amylase	40
5.11	Amylase concentration – Liver samples	41
5.12	Amylase concentration – Intestine samples	41
5.13	Readings of standard graph – Protease – Higher Concentration	42
5.14	Readings of standard graph – Protease – Lower Concentration	43
5.15	Protease concentration – Liver samples	44
5.16	Protease concentration – Intestine samples	44
5.17	Titration readings – Lipase – Liver and Intestine samples	45
5.18	Lipase concentration – Liver and Intestine samples	46
5.19	Proximate analysis	47
5.20	Proximate composition analysis of feed and whole fish body samples	47
5.21	Histological analysis	50

CHAPTER 1

INTRODUCTION

Fish meal is a significant component present in the feed for aquaculture to grow fishes. Fish meal is the most scarce marine resource that generally occupies significant proportion of marine fish feed. As the demand for the inclusion of fishmeal in aquafeed is very high, an alternative source should be identified and used. Krill meal is a carnivores meal for fishes obtained from (Antarctic Krill) *Euphasia superba*. Antarctic krill is a small crustacean which is found in the southern oceans of Antarctica. The krill meal is an untouched animal source of fish meal and is abundantly available in the oceans. It is a highly effective attractant. It can be used as an additive in the fish feed. Because of the high protein content in the krill meal it can be used as a replacement for fish meal and is being fed to the fishes. The protein content in the krill meal is as same as that of the fish meal. Krill meal is highly nutritious and it can increase the intake of feed by the fishes. The orange spotted grouper (*Epinephelus coioides*), is a species of marine fish, which belongs to the subfamily Epinephalinae and the family Serranidae. It is found in the Indo-Pacific region of oceans. It is found in marine and brackish waters. The maximum publishes length is 120 centimeters and the maximum weight is 15 kilograms. It can be found in the coral reefs along the mainland coasts and large islands. They feed on shrimp, crab and benthic crustaceans. They are diandric protogynous hermaphrodites, in which some male juveniles continue to be male during and after the growth, while some functionally female juveniles turn to be male during growth and continue to be male after the growth. They are slow growing and lazy to prey. They also mature late and produce little eggs. They are very sensitive and is often harvested by the humans and exploited for food. The formulated experimental feed with the inclusion of various amounts of krill meal was fed to orange spotted grouper for a period of 63 days. Krill meal is expected to increase the growth and health of the fishes which forms the background of this work. The outcome of this work is to find the better replacement for fishmeal without causing any harm to the fishes.

CHAPTER 2

REVIEW OF LITERATURE

2.1 WORLD AQUACULTURE SCENARIO:

Aquaculture is the fastest growing food-producing sector in the world. It is developing, expanding and intensifying in almost all regions of the world. The global aquaculture has grown significantly in the present times. The aquaculture animal harvest has increased from 76.6 million tonnes as per the records in 2015. The aquaculture flora contributes to 27.7 percent of the total global aquaculture harvest. Achieving the long term goal of economic, social and environmental sustainability of the aquaculture sector, to ensure its continued contribution of nutritious food to keep the world healthy, will develop primarily on continued commitments by the governments. As the sector further expands and diversifies and intensifies, it should recognize the relevant environmental and social concerns and make conscious efforts to address them in a transparent manner, backed with scientific evidence (Subasinghe, R., 2017). It is increasingly realized that sustainable development and responsible production of aquaculture, in a long run, cannot be achieved without the full participation of the producers in the decision-making and regulation process, which has led to efforts to empower farmers and their associations and move toward increasing self-regulation. These factors are all contributing to the improvement in the management of the sector, typically through the promotion of 'better management' practices of producers.

2.2 INDIAN AQUACULTURE SCENARIO:

Fish play an important role in nutrition of people in India. Per capita availability of fish in the country is 9.5 kg with production at 5.8 million tonnes during 1999-2000. This production level makes India the second largest aquaculture producing country in the world. India cultures many different species including Indian major carps (catla, *Catla catla*; rohu, *Labeo rohita*; naini, *Cyprinus mrigala*; and calbasu, *Labeo calbasu*), marine shrimp, freshwater shrimp, oysters, green and brown mussels, and pearl oysters for domestic and

international markets. To meet the increased demand for fisheries products by 2010, India should increase its current level of production to 7-8 million tonnes per year. With marine fishery production capture steady at 3 million tonnes per year, inland aquaculture production must increase from its current level of 2.5 million tonnes to 5 million tonnes by 2010. India should intensify culture production methods for this increased production values to be achieved (Gopakumar K.,2003).

2.3 INDIAN MARICULTURE SCENARIO:

Mariculture is the specialized branch of aquaculture involving the cultivation of marine organisms for food and other products in an enclosed section of the sea (cages/pens), or in tanks, ponds or raceways which are filled with the saline water. Generally the mariculture production data focuses on the commercial data, which is the output of marine and brackish farming activities for profit whose final harvest is used for human consumption. It is the promising sector by which an additional marine fish requirement can be met in the future years. It is also the fastest growing sub-sector of aquaculture. At global level, mariculture produces many high value fishes, crustaceans, and molluscs such as oysters, mussels, clams, cockles and scallops (Gopakumar, G.,2015). With support of the government initiatives and policies, various innovations by scientific community, support from private industries and companies, fishermen, farmers, NGOs and self help groups, the sector have evolved from a modest, traditional and subsistence level to a sophisticated and modern enterprise. India now occupies, third position in total world production and second in aquaculture production. 'Fish for All forever' necessitates the sustained development of sector to cater nutritional needs of millions of people in future.

2.4 FISHMEAL AND ITS IMPORTANCE IN AQUAFEED INDUSTRY:

Fishmeal is recognized by nutritionists as a high-quality, digestible feed ingredient that is favoured for the addition to diet of most farm animals, especially fish and shrimp. Fishmeal carries large quantities of energy per unit

weight and is an excellent source of protein, lipids (oils), minerals, and vitamins; there is very little carbohydrate in fishmeal. Fishmeal is a generic term for a nutrient-rich feed ingredient used primarily in diets for domestic animals, sometimes used as a high-quality organic fertilizer. Fishmeal can be made from almost the type of seafood but it is generally manufactured from wild-caught, small marine fish that contain the high percentage of bones and oil, and usually deemed not suitable for direct human consumption. These fishes are considered 'industrial' since most of them are caught for the sole purpose of fishmeal production (Miles R D.,2006). Among various animal proteins, fish meal is rich in protein, readily available throughout the world, and competitively priced against other animal proteins (e.g. milk and blood). In addition, many researchers reported that fish meal is traditionally recognized as a high digestible protein with a high content of amino acids, vitamins and minerals for pigs. Supplementation of fatty acids by fish meal is one strategy to modify an animal immune response. As a result of fish meal effects, those supplementations can result in improved performance and immunity of young animals (Cho J H.,2011). Fish meal is the major dietary source of n-3 long chain poly unsaturated fatty acids (PUFA), docosahexaenoic acid (22:6n-3) and eicosapentaenoic acid (20:5n-3). These fatty acids affect heart disease, autoimmune disease and inflammatory diseases in humans and laboratory animals (Simopoulos et al., 1986). Korver and Klasing (1997) reported that diets with a relatively high ratio of the n-3 PUFA to n-6 PUFA offer protection against growth retardation following an immunological challenge. Fish meal carries large quantities of energy per unit weight and is the source of high-quality protein and highly digestible essential amino acids and fatty acids (Zinn et al., 2009). Numerous studies have shown that fish meal generally improves the growth and performance of starter and grower pigs (Kjeldsen et al., 1981; Newport and Keal, 1983; Pike et al., 1984).

2.5 ALTERNATE FEED INGREDIENTS:

Several records are available for various alternate feed ingredients for fish meal. Plant products such as soyabean meal (Carter C G.,2000), wheat

gluten, rapeseed meal, extruded peas (Sitjà-Bobadilla, A. et al., 2005) can be used as an alternative feed ingredient. Animal products such as meat meal, bone meal (Millamena, O. M., 2002), krill meal (Yoshitomi B et al., 2001), black soldier fly pre-pupae meal (Magalhães, R et al., 2017). The algae spirulina maxima can also be used as a fish meal replacement (Olvera-Novoa, M. A et al., 1998).

2.6 KRILL MEAL:

Krill is one of the most abundant species on Earth and, at current levels, is one of the world's underexploited marine stocks. Although krill is food for whales, penguins and many fishes, there is a scientific confidence that Antarctic krill is in no danger of over harvesting anytime in the near future. Krill has amino and fatty acid profiles that closely resemble the profiles of fish meals, making it a suited alternative source in the diets for farmed marine fish (van der Meeren et al., 2008). The total replacement of fish meal by krill meal was successful without any changes in the growth of the fish. The protein value of krill meal is same as that of the fish meal (Yoshitomi et al., 2006). It performed even better than fish meal due to the higher contents of phospholipid, vitamins, nucleotides, chitin and natural carotenoids (Everson, 2000; Hansen et al., 2011; Lu et al., 2014).



Figure 2.1 Krill Meal

2.6 ORANGE SPOTTED GROUPER (*Epinephelus coioides*):

Epinephelus coioides is a marine brackish water fish which is found to be

associated with the coral reefs in the maincoastlands and islands. This belongs to the family Serranidae and the subfamily Epinephelinae. (Romero P et al.,2002).

Juveniles are common in shallow waters of estuaries over sand, gravel, mud and among mangroves (Kailola P J et al.,1993). Females mature at 25 to 30 cm, sexual transition occurs at 55 to 75 cm (Heemstra P C et al.,1999). Eggs and early larvae are probably pelagic (Kailola P J et al., 1993). It has been tested in several countries as a potential species for mariculture (Golani D L et al., 2002).



Figure 2.2 Orange Spotted Grouper (*Epinephelus coioides*)

CHAPTER 3

AIM AND OBJECTIVE

AIM:

The aim of this study is to understand the growth and metabolic response of marine fish fed with krill meal in place of fishmeal in the diet to reduce the dependency on fishmeal.

OBJECTIVE:

- To study the effect of fishmeal replacement with krill meal on growth performance of fish.
- To assess the metabolic response of fishes fed with krill meal based diets.
- To study the expression profile of genes related to metabolism in the experimental fishes.

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS:

4.1.1 CHEMICALS:

The chemicals used for the experiment are listed in the table below.

S.NO	CHEMICALS USED	MANUFACTURER
1	EDTA	Himedia
2	Sucrose	Himedia
3	Neutral Buffer Formalin	Himedia
4	Clove Oil	Himedia
5	Sodium Dihydrogen Phosphate	Himedia
6	Disodium Hydrogen Phosphate	Himedia
7	Ethanol	Himedia
8	Olive Oil	Himedia
9	Phenolphthalein	Himedia
10	Sodium Hydroxide	Himedia
11	Gum acacia	Himedia
12	Sodium Benzoate	Himedia
13	Potassium Phosphate Dibasic Trihydrate	Himedia
14	Caesin	Himedia
15	Trichloro acetic acid	Himedia
16	Folin Ciocalteu's Phenol Reagent	Himedia
17	Sodium Carbonate Anhydrous	Himedia
18	Sodium Acetate Trihydrate	Himedia
19	Calcium Acetate	Himedia
20	L-Tyrosine	Himedia
21	Sodium Chloride	Himedia
22	Sodium Potassium Tartarate	Himedia
23	Starch	Himedia

24	Sodium Phosphate	Himedia
25	Dinitro salicylic acid	Himedia
26	Maltose	Himedia
27	EtBr	
28	Bromophenol blue	
29	Agarose	Himedia
30	Trizol	
31	Chloroform	
32	2-propanol	
33	Tris Base	
34	Acetic Acid	
35	RNAse free water	

4.1.2 INSTRUMENTS:

S.NO	INSTRUMENTS USED
1	Homogenizer
2	Magnetic Stirrer
3	Weighing Balance
4	Hand held Refractometer
5	Laboratory Thermometer
6	pH meter
7	Burette
8	Glass Pipettes
9	Micro Pipettes
10	Refrigerated Centrifuge
11	Refrigerator
12	Microwave oven
13	Pelletizer
14	Pressure Cooker
15	Autoclave
16	Hot Air Oven

17	UV Spectrophotometer
18	Gel Documentation System
19	Water bath
20	Induction Stove
21	PCR
22	Agarose Gel Electrophoresis Unit
23	Eppendorf tubes
24	Screw Cap Tubes
25	Glasswares
26	Test Seives
27	Deep Freezer (-20°C)
28	Microtips
29	Whatman Filter Papers

4.1.3 SOFTWARES:

S.NO	SOFTWARE USED
1	Microsoft Excel

4.2 METHODS:

4.2.1 EXPERIMENTAL SETUP:

The *Epinephelus coioides* (Orange spotted grouper) juveniles were stocked in the FRP tanks. Five treatments, each with a triplicate were kept in this experiment. The FRP tanks were filled with 300L of sea water. Fourteen juveniles per tank were stocked in the 15 tanks.



Figure 4.1 Grouper Juveniles
view)



Figure 4.2 Experimental Setup (Side



Figure 4.3 Experimental Setup (Top view)

4.2.2 MONIROTING THE WATER PARAMETERS:

4.2.2.1 SALINITY:

1. Salinity was monitored using the hand-held refractometer.
2. The sensor in the refractometer is rinsed with freshwater and ensured its salinity is set zero.

3. The sensor was then rinsed with the seawater at which the fishes are being reared.
4. The reading in the refractometer is viewed through the lens.
5. The salinity of the water is maintained at 26-32 ppt.

4.2.2.2 pH:

1. The digital pH meter is used to measure the pH of water.
2. The pH meter is rinsed with the freshwater and the pH was set zero.
3. The pH meter is then dipped in the seawater and waited for a stable reading.
4. The pH is maintained at 8.2.

4.2.2.3 TEMPERATURE:

1. The laboratory thermometer is used to measure the temperature.
2. The thermometer is dipped in the water.
3. The level of the mercury denotes the temperature of the water.
4. The temperature was maintained at 27-29°C.

4.2.2.4 WATER QUALITY:

The water testing kit was used to measure the amount of nitrate, nitrite and ammonia in the water.

NITRATE:

1. 10ml of water sample is taken in the test tube.
2. A pinch of nitrate reagent-1 was added and the solution was agitated for 5 minutes.
3. The supernatant solution was decanted to a fresh tube and allowed to stand for a few minutes.
4. drops of nitrate reagent-2 was added to the supernatant solution and mixed well.
5. After occasional shaking, the final colour that formed is compared with nitrate colour chart and the nitrate value was recorded.



Figure 4.4 Nitrate Test

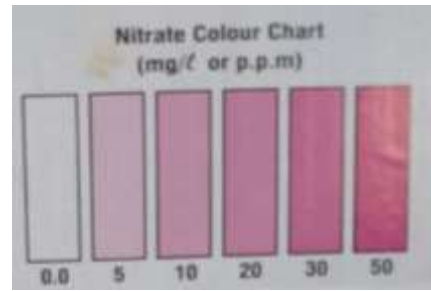


Figure 4.5 Nitrate colour chart

NITRITE:

1. 5 ml of water was taken in the test tube.
2. 3 drops of nitrite reagent-1 was added and mixed well. After occasional shaking, the final colour that formed was compared with the nitrite colour chart and the value was recorded.



Figure 4.6 Nitrite test

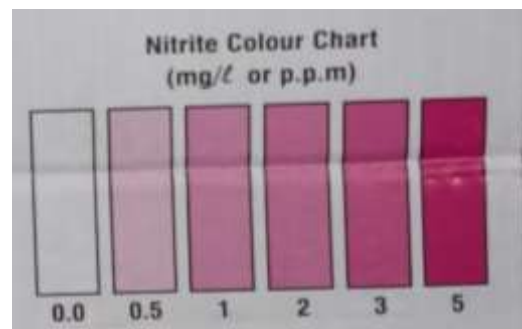


Figure 4.7 Nitrite colour chart

AMMONIUM:

1. 5 ml of water sample was taken in the test tube.
2. 5 drops of Ammonium reagent-1 was added and mixed well. The colour that formed is compared with ammonium colour chart immediately and the ammonium value was recorded.



Figure 4.8 Ammonium test

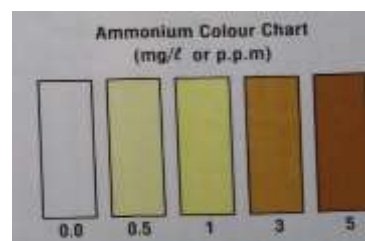


Figure 4.9 Ammonium colour chart

4.2.3 FEED PREPARATION:

1. The feed was prepared by adding the ingredients in the required amounts as given in the table below.
2. The feed pellets were prepared by following the steps.

Table 4.1 Fish Feed Ingredients for Experimental feed

INGREDIENTS	OKM	15KM	30KM	45KM	60KM
Fish meal	43.5	37	30.5	24	17.5
Wheat gluten	3	3	3.5	4	4.4
Soybean meal	14.15	14.6	14.5	13.6	13
Squid meal	5	5	5	5	5
Groundnut oil cake	5	5	5	5	5
Shrimp meal	5	5	5	5	5
Krill meal	0	7.2	14.4	21.6	28.8
Wheat powder	13.2	13	12.9	13.25	13.3
Sunflower oil	2	2	1.7	1.5	1.5
Fish oil	3.5	2.75	2.4	2	1.5
Vitamin	2	2	2	2	2
Mineral	2	2	2	2	2
BHT	0.1	0.1	0.1	0.1	0.1
SMB	0.1	0.1	0.1	0.1	0.1
Methionine	0.3	0.25	0.15	0.1	0.05
Lysine	0.4	0.25	0	0	0
Vitamin C	0.25	0.25	0.25	0.25	0.25
Immunostimulant	0.5	0.5	0.5	0.5	0.5



Figure 4.10 Kneading of feed



Figure 4.11 Preparation of dough



Figure 4.12 Cooking in pressure cooker



Figure 4.13 Pelletization



Figure 4.14 Drying of pelletized feed



Figure 4.15 Grinding of feed



Figure 4.16 Sieving and Size grading of feed



Figure 4.17 Feed Pellets

4.2.4 SAMPLING IN ETHICAL WAY:

1. The fish should be anesthetized with any one of the suitable anesthesia such as clove oil, MS222 and benzocaine.
2. In ICAR-CMFRI the anesthetization protocol was standardized using clove oil and MS222 for orange spotted grouper.
3. For collecting blood, catch the fish using a hand net and transfer carefully into the bucket with anesthetic agent.
4. The fish is allowed to swim until lose its equilibrium to get anesthetized absolutely. Once the fish completely lose its equilibrium, it should be taken out and kept in soft wet cloth and ensured that the fish should not show any movement.
5. Therefore, the fish won't feel pain while collecting blood. According to the size of the fish the needle and syringe should be selected.
6. For small size fish (<30 g), 26 G needle fixed in 1 mL tuberculin syringe will be ideal. The needle should be rinsed with anticoagulant (2.75% EDTA solution or heparin) to prevent blood coagulation during blood collection.
7. Insert the needle beneath the scales to reach caudal peduncle area of fish just below the lateral line, until the needle touches the vertebral column. This can be felt by hard impenetrable surface and thereafter, the syringe should be withdrawn slightly, so that the blood vessel beneath the vertebral column could be pierced and sampled.

4.2.5 FISH SAMPLING:

1. Fish sampling and anesthetization of fish was done based on the protocol of ICAR-CMFRI.
2. The fishes were kept for fasting overnight. So that the foecal matter present in the intestine will be released.
3. The fishes were caught and anesthetized by using clove oil. Clove oil was added 100 μ L per litre.
4. The length and weight of the fishes were measured.
5. A small slit was given at the anus using a scalpel blade.
6. The fish was cut vertically by inserting the scissors at the slit made.
7. A perpendicular cut was made at the muscle, the viscera was visible.
8. A cut was made at the oesophagus and the viscera was removed carefully and weighed.
9. The liver was separated from the viscera and weighed.
10. The intestine was uncoiled, separated and the anus part was cut. The intestine was weighed.
11. The muscle was removed from the skin part and weighed.
12. The isolated internal organs were stored in the deep freezer by adding required amount of 0.25M sucrose for the enzyme analysis.
13. The liver and intestine samples were stored in the neutral buffer formaline for histology analysis.
14. The liver samples were stored in 500 μ l of trizol for RNA isolation.
15. The sampled fishes were kept in the hot air oven for drying. The dried fishes were used for proximate analysis.



Figure 4.18 Anesthetizing of fish



Figure 4.19 Fish sampling



Figure 4.20 Viscera



Figure 4.21 Liver



Figure 4.22 Intestine



Figure 4.23 Muscle

4.2.6 BLOOD SAMPLING:

1. The fishes were caught and anesthetized by using clove oil. Clove oil was added 100 μ L per litre.
2. The anesthetized fish was taken for blood sampling.
3. The syringe was rinsed with 2.75% EDTA to prevent immediate clotting of blood.
4. The syringe was inserted and the dorsal vein was found.
5. The blood was collected from the fish.
6. The blood was allowed to clot in order to isolate the serum.
7. The serum was used for the biochemical analysis to evaluate the amount of total protein, albumin, globulin, triglycerides, cholesterol and glucose.

4.2.7 ISOLATION OF SERUM:

1. The blood should be released into the anticoagulant free container (red top) and kept in slanting position.
2. Allow the blood to clot by leaving it undisturbed at room temperature for 2 h for better separation of serum.
3. Remove the clot portion by centrifuging at 5000 rpm for 10 minutes. The resulting supernatant is serum and transfer into a clean tube.
4. The serum samples can be stored for 7-10 days at 2–8°C. If the serum is not analyzed immediately, it should be stored at –20 °C until analysis not exceeding 3 months.



Figure 4.24 Blood Sampling



Figure 4.25 Collection of Blood



Figure 4.26 Clotting of blood



Figure 4.27 Isolated serum

4.2.8 ENZYME ANALYSIS:

4.2.8.1 AMYLASE:

1. The amylase analysis was carried out for the liver and intestine samples.
2. The required reagents were prepared.
3. 20mM Sodium phosphate buffer was prepared. 6.7mM Sodium chloride solution was prepared.
4. Starch was dissolved by adding in the buffer. Starch is to be prepared freshly before the commencement of the analysis.
5. Sodium Potassium tartarate solution was prepared by dissolving 12g in 8ml of 2M NaOH.
6. 96mM Dinitro salicylic acid was prepared. The prepared sodium potassium tartarate solution and the DNS were mixed to prepare the colouring reagent.
7. 0.2% Maltose was prepared to plot the standard curve.
8. The reagents were added as given in the procedure.
9. 1ml of starch sample was added to all the tubes.
10. 1ml of enzyme sample was added to the test tubes. It was incubated for 3 minutes at 20°C.
11. 1ml of the prepared colouring reagent was added. The tubes were kept in water bath for 15 minutes.
12. The enzyme solution was now added to the blank tube.
13. 9ml of distilled water was added to all the tubes.
14. After the incubation of samples, the absorbance was recorded at 540nm using UV Spectrophotometer.
15. The standard graph was plot and the enzyme activity was calculated.

Standard curve:

$$A_{540\text{nm}} \text{ Standard} = A_{540\text{nm}} \text{ Standard} - A_{540\text{nm}} \text{ Standard Blank}$$

Plot $A_{540\text{nm}}$ of the standards Vs milligrams of maltose.

Sample Determination:

$$\Delta_{540\text{nm}} \text{ sample} = A_{540\text{nm}} \text{ Test} - A_{540\text{nm}} \text{ Blank}$$

Determine the milligrams of maltose liberated using the standard curve.

$$\text{Units/ml enzyme} = \frac{(\text{mg of maltose released} \times \text{dilution factor})}{1}$$

$$\text{Units/mg solid} = \frac{\frac{\text{unit}}{\text{ml enzyme}}}{\frac{\text{mg solid}}{\text{ml enzyme}}}$$

4.2.8.2 *PROTEASE:*

1. The protease analysis was done for liver and intestine samples.
2. The required reagents were prepared.
3. Potassium phosphate buffer was prepared.
4. 0.65% casein was prepared by dissolving in the buffer. Casein is to be freshly prepared before the commencement of the analysis.
5. 110mM TCA was prepared.
6. 0.5M Folin's Phenol reagent was prepared.
7. L-Tyrosine was prepared to plot the standard curve.
8. The reagents were added as given in the procedure.
9. 5ml of casein was added to all the tubes.
10. The tubes were kept in water bath at 37°C for 5 minutes.
11. 100µl of enzyme samples were added to the test tubes.
12. Incubate at 37°C for 10 minutes.
13. 5ml of TCA was added in all the tubes to stop the enzyme reaction.
14. The enzyme samples were added to the blank tube.
15. The tubes were incubated for 30 minutes.
16. The contents were filtered using Whatman filter paper to a fresh tube.
17. The 2ml of the filtrate was taken and 2.5ml of Sodium carbonate and 0.5ml of Folin's ciocalteau reagent was added.
18. The tubes were incubated at 37°C for 30 minutes.
19. The contents were filtered to a fresh tube using Whatman filter paper.
20. 2ml of the obtained filtrate was used for spectrophotometer analysis.
21. The absorbance was recorded at 660nm.
22. The formulae used are

$$\text{Units/ml enzyme} = \frac{\mu\text{mole tyrosine equivalents released} \times 11}{1 \times 10 \times 2}$$

$$\text{Units/mg solid} = \frac{\frac{\text{units}}{\text{ml enzyme}}}{\frac{\text{mg solid}}{\text{ml enzyme}}}$$

4.2.8.3 LIPASE:

1. The lipase analysis was done for the liver and intestine samples.
2. The required reagents were prepared.
3. Phosphate buffer was prepared by mixing 0.1M of Sodium dihydrogen phosphate and 0.1M of Disodium hydrogen phosphate in the ratio of 61:39.
4. Olive Oil Emulsion was prepared using Olive oil, Gum acacia and Sodium benzoate.
5. 95% Alcohol was prepared.
6. 0.05N NaOH was prepared.
7. Phenolphthalein was used as the indicator.
8. The reagents were added as given in the procedure.
9. 3ml of distilled water was added to all the tubes.
10. The enzyme sample was added to all the tubes.
11. The control tubes were boiled for 5 minutes at 100°C and cooled.
12. 0.5ml of prepared buffer was added to all the tubes.
13. 2ml of the prepared olive oil emulsion was added to all the tubes.
14. The tubes were shaken well and incubated at 27°C for 24 hours.
15. 3ml of 95% alcohol and 2 drops of phenolphthalein was added to all the tubes before titration.
16. The tubes were titrated against the 0.05N NaOH till the appearance of the end point, permanent pale pink colour.
17. The volume 0.05N NaOH required for each samples was recorded and the calculations were done to find the enzyme activity.
18. The formula used is

Units of intestinal lipase activity per g of tissue = $\frac{\text{Volume of NaOH solution required for experimental solution} - \text{Volume of NaOH solution required for the control tube}}{\text{Weight of tissue}}$

4.2.9 SERUM ANALYSIS:

Kit was used for the biochemical analysis of serum.

4.2.9.1 TOTAL PROTEIN:

1ml of biuret reagent was added to all the tubes.

20µl of distilled water was added to the blank tube.

20µl of BSA was added to the standard tube.

20µl of serum sample was added to the test tubes.

The absorbance was recorded using UV Spectrophotometer at 550nm.

The amount of total protein was calculated using the formula.

The formula used is

$$\text{Total protein in g/dl} = \frac{\text{Absorbance of Test solution}}{\text{Absorbance of Standard}} * 8$$

4.2.9.2 ALBUMIN AND GLOBULIN:

1ml of BCG reagent was added to all the tubes.

10µl of distilled water was added to the blank tube.

10µl of albumin standard was added to the standard tube.

10µl of serum sample was added to the test tubes.

The absorbance was recorded using UV spectrophotometer at 630nm.

The amount of albumin was calculated using the formula.

The formulae used are

$$\text{Albumin in g/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} * 4$$

$$\text{Globulin in g/dl} = \text{Total proteins in g/dl} - \text{Albumin in g/dl}$$

$$\text{Albumin/Globulin ratio} = \frac{\text{Albumin in g/dl}}{\text{Globulin in g/dl}}$$

4.2.9.3 TRIGLYCERIDES:

1ml of working reagent was added.

10µl of distilled water is added to the blank tube.

10µl of triglyceride standard was added to the standard tube.

10µl of serum samples were added to the test tubes.

The absorbance was recorded using UV spectrophotometer at 505nm.

The amount of triglyceride was calculated using the formula

The formula used is

$$\text{Triglycerides in mg/dl} = \frac{\text{Absorbance of Test solution}}{\text{Absorbance of Standard}} * 200$$

4.2.9.4 CHOLESTEROL:

1ml of working reagent was added.

10µl of distilled water is added to the blank tube.

10µl of cholesterol standard was added to the standard tube.

10µl of serum samples were added to the test tubes.

The absorbance was recorded using UV spectrophotometer at 505nm.

The amount of triglyceride was calculated using the formula

The formula used is

$$\text{Cholesterol in mg/dl} = \frac{\text{Absorbance of Test solution}}{\text{Absorbance of Standard}} * 200$$

4.2.9.5 GLUCOSE:

1ml of glucose reagent was added.

10µl of distilled water is added to the blank tube.

10µl of glucose standard was added to the standard tube.

10µl of serum samples were added to the test tubes.

The absorbance was recorded using UV spectrophotometer at 505nm.

The amount of triglyceride was calculated using the formula

The formula used is

$$\text{Total glucose in mg/dl} = \frac{\text{Absorbance of Test solution}}{\text{Absorbance of Standard}} * 100$$

4.2.10 PROXIMATE ANALYSIS:

The sampled fishes were then kept in the hot air oven for drying.

The dried fishes were then crushed and powdered for proximate analysis.

The wet weight and the dry weight of the fishes were noted.

4.2.10.1 PROXIMATE COMPOSITION ANALYSIS:

Proximate analysis of crude protein (Kjeldahl nitrogen × 6.25) was determined by

the Kjeldahl method after acid digestion using a Kjeldahl System (FOSS Kjeltac 2300), total fat /ether extraction was done using a Soxhlet System (FOSS Soxtec 2043), ash content was determined by incinerating the samples in muffle furnace at 600 °C for 4 h and moisture (weight loss after drying at 105 °C 4-5 h) was estimated using hot air oven by AOAC (1995) method.



Figure 4.28 Wet fish



Figure 4.29 Dried Fish



Figure 4.30 Crushed and Powdered fish sample

4.2.11 RNA ISOLATION:

RNA isolation was done by using trizol method.

Liver samples were used for RNA isolation.

20-30mg of the sample was taken.

The sample was homogenized by adding 500µl of trizol and incubated for 5 minutes at room temperature.

250µl of chloroform was added to the samples and shaken vigorously for 15 seconds.

The sample was incubated at room temperature for 5 minutes.

The sample tubes were centrifuged at 12,000rpm for 15 minutes at 4°C.

The aqueous phase thus obtained is collected in the fresh tube in which the RNA is present.

250µl of 2-propanol was added and mixed well.

The sample was incubated at room temperature for 5 to 10 minutes and centrifuged at 12,000rpm for 15 minutes at 4°C.

The RNA is deposited on the sides of the tube. The supernatant should be removed.

The pellet of washed with 75% ethanol.

The sample was then centrifuged at 7,500 rpm for 5 minutes.

The pellet was air dried and dissolved by adding appropriate volume of RNase free water.

Agarose Gel Electrophoresis was done to confirm the presence of RNA in the isolated sample.

4.2.12 DNA ISOLATION:

DNA isolation was done using a kit.

The liver samples were used for DNA isolation.

30mg of the liver sample was taken and homogenized.

200µl of buffer GA was added and vortexed for 15 seconds.

4µl of RNase A was added, vortexed and incubated for 5 minutes at room temperature.

20µl of proteinase K was added and mixed by vortex.

The tubes were incubated at 56°C for 0.5 to 2 hours.

200µl of buffer GB was added and mixed by vortex.

The tubes were incubated at 70°C for 10 minutes.

200µl of 96-100% ethanol was added and vortexed for 15 seconds.

The mixture was transferred to fresh tube and centrifuged at 13,400 rpm for 30 seconds.

The supernatant was discarded.

500µl of buffer GD (after adding ethanol) was added and centrifuged for 13,400 rpm for 30 seconds.

The supernatant was discarded.

600µl of buffer PW (after adding ethanol) was added and centrifuged at 13,400 rpm for 30 seconds.

The supernatant was discarded. This was repeated once.

The tubes were centrifuged again for 2 minutes at 13,400 rpm.

50-200µl of buffer TE was added and incubated at room temperature for 5 minutes.

The tubes were centrifuged for 2 minutes at 13,400 rpm twice.

The sample then obtained is stored at 4°C.

Agarose Gel Electrophoresis was done to confirm the presence of DNA in the isolated sample.

4.2.13cDNA SYNTHESIS:

4.2.13.1 REMOVAL OF GENOMIC DNA:

The genomic DNA contamination is to be removed in the sample by adding the following

RNA	- 1µg
10X Reaction buffer	- 1µl
DNase	- 1µl
Nuclease free water	- 7µl

The mixture was incubated at 37°C for 30 minutes.

1µl of 50mM EDTA was added and incubated at 65°C for 10 minutes.

4.2.13.2 FIRST STRAND cDNA PROTOCOL:

The following reagents were added for the development of first strand of cDNA.

Total RNA 0.1ng	- 5µg
Oligo dT 18 primer	- 1µl
Nuclease free water	- 6µl

The tubes were incubated at 65°C for 5 minutes.

After incubation, the following reagents were added.

5X Reaction buffer - 4µl

RNAse Inhibitor - 1µl

10mM dNTP mix - 2µl

Revert aid M-MuLV RT - 1µl

The mixture was mixed gently and centrifuged briefly.

The tubes were incubated at 42°C for 1 hour.

The reaction was terminated by heating up at 70°C for 5 minutes.

The samples were then kept in the PCR as per the instructions the samples were run in the PCR.

4.2.14 POLYMERASE CHAIN REACTION:

4.2.14.1 PRIMERS:

1. The primer for leptin gene was designed from the most conserved regions of selected species of grouper fish.
2. The organs such as muscle and liver were collected using sterile scissors and forceps and RNA was isolated from these tissues.
3. From RNA, cDNA was synthesized and used for PCR amplification using the following primers. The PCR amplification was carried out at the following cyclic conditions in the Thermal Cycler.

Table : 4.2 Primer Specification

S. No.	Primer name	Primer sequence from 5' to 3'
1.	Leptin Forward	ACTACACTCTGGCCCTGTTG
2.	Leptin Reverse	GTCAAACCTTGACCTGGGAGA

Table. 4.3 Temperature schedule of PCR programme

Step	Temperature	Time
1. Initial denaturation	94°C	3 min
2. Denaturation	94°C	40 sec
3. Primer annealing	55°C	45 sec
4. Extension	72°C	45 sec
Step 2 to 4 repeated for 32 cycles		
5. Final extension	72°C	7 min
6. Hold	4°C	-

Then the PCR product was load in the 1.5% agarose gel electrophoresis and the product was confirmed in the gel and eluted using gel elution kit. The eluted DNA was sent for sequencing. The sequence will be submitted in NCBI for getting accession number.

4.2.15 AGAROSE GEL ELECTROPHORESIS:

The required reagents were prepared.

The 10XTAE buffer was prepared by adding Tris HCl, EDTA and Acetic acid.

The Gel tray was set up by sealing the sides with cellophane tape and the comb was placed.

1% agarose was prepared by adding 0.5g of agarose to 50ml of the 1XTAE buffer.

The agarose was melted in the microwave oven.

EtBr was added once the agarose is cooled to the room temperature.

The melted agarose is then poured on the gel tray set up and kept aside for solidification.

Once the agarose gets solidified, the cellophane tape is removed and the tray is placed in the electrophoresis tank.

The electrophoresis tank was filled with 1XTAE buffer and the electrodes were connected.

The power supply was started and the gel was viewed in the Gel documentation system after 45 minutes of run.

CHAPTER 5

RESULTS

5.1 WATER PARAMETERS:

5.1.1 WATER QUALITY:

Table 5.1 Water Quality readings

COMPOUND IN mg/L	STORAGE WATER	UV TREATED WATER	OUTLET WATER
NITRATE	30	20	20
NITRITE	0	0	0
AMMONIA	0.25	0.25	0.25

5.1.2 SALINITY:



Figure 5.1 Salinity

5.1.3 TEMPERATURE:



Figure 5.2 Temperature

5.2 FEED UTILISATION:

Table 5.2 Feed Utilisation

TANK	WEIGHT GAIN	FEED CONSUMED	NO.OF.FISHES	FEED INTAKE/FISH	AVERAGE	FEED CONVERSION RATIO	AVERAGE	PROTEIN CONSUMED	PROTEIN EFFICIENCY RATIO	AVERAGE	FEED EFFICIENCY	AVERAGE
1A	1.87857143	85	11	7.727273	9.157576	4.113377	3.585742	3.709091	0.506478	0.600293	0.243109	0.28814
1B	4.45714286	122	10	12.2		2.737179		5.856	0.761124		0.36534	
1C	1.93142857	83	11	7.545455		3.90667		3.621818	0.533276		0.255972	
2A	8.14857143	165	9	18.33333	14.27525	2.249883	1.869724	8.8	0.925974	1.136049	0.444468	0.545303
2B	6.73142857	139	12	11.58333		1.720784		5.56	1.210689		0.581131	
2C	7.87857143	142	11	12.90909		1.638507		6.196364	1.271483		0.610312	
3A	10.5057143	166	11	15.09091	12.41492	1.436448	1.898841	7.243636	1.450337	1.281969	0.696162	0.615345
3B	2.85714286	113	13	8.692308		3.042308		4.172308	0.684787		0.328698	
3C	11.0542857	175	13	13.46154		1.217766		6.461538	1.710782		0.821176	
4A	11.4742857	159	13	12.23077	13.14744	1.065929	1.028551	5.870769	1.954477	2.027817	0.938149	0.973352
4B	12.6814286	162	13	12.46154		0.98266		5.981538	2.120095		1.017646	
4C	14.2228571	177	12	14.75		1.037063		7.08	2.008878		0.964262	
5A	16.0428571	186	10	18.6	14.8619	1.159394	1.115862	8.928	1.796915	1.872526	0.862519	0.898813
5B	14.4428571	167	10	16.7		1.156281		8.016	1.801754		0.864842	
5C	8.99857143	130	14	9.285714		1.03191		4.457143	2.01891		0.969077	

Based on the results obtained it is clear that the feed consumption and the growth rate is higher in T5 group and lower in T1 group.

5.3 GROWTH ESTIMATION:

Table 5.3 Growth Estimation

Tank	Initial weight	No of fishes	Average weight	Final weight	No of fishes	Average weight	Weight gain	Average	Weight gain percentage	Average	Survival	Average
1A	134	14	9.571429	126	11	11.45	1.87857142		19.6268656		78.57142	
1B	135	14	9.642857	141	10	14.1	4.45714285	2.7557142	46.2222222	28.77798	71.42857	76.19047
1C	132	14	9.428571	125	11	11.36	1.93142857		20.4848484		78.57142	
2A	141	14	10.07143	164	9	18.22	8.14857142		80.9078014		64.28571	
2B	139	14	9.928571	200	12	16.66	6.73142857	7.5861904	67.7985611	77.0066	85.71428	76.19047
2C	134	14	9.571429	192	11	17.45	7.87857142		82.3134328		78.57142	
3A	143	14	10.21429	228	11	20.72	10.5057142		102.853146		78.57142	
3B	142	14	10.14286	299	13	13	2.85714285	8.1390476	28.1690140	81.32855	92.85714	88.09523
3C	137	14	9.785714	271	13	20.84	11.0542857		112.963503		92.85714	
4A	130	14	9.285714	270	13	20.76	11.4742857		123.569230		92.85714	
4B	139	14	9.928571	294	13	22.61	12.6814285	12.792857	127.726618	131.8619	92.85714	90.47619
4C	138	14	9.857143	289	12	24.08	14.2228571		144.289855		85.71428	
5A	131	14	9.357143	254	10	25.4	16.0428571		171.450381		71.42857	
5B	138	14	9.857143	243	10	24.3	14.4428571	13.161428	146.521739	137.329	71.42857	80.95238
5C	134	14	9.571429	260	14	18.57	8.99857142		94.0149253		100	

The growth rate is higher in T5 group as the feed consumption was higher and lower in T1 group as the feed consumption was lower. The survival rate is higher in the T4 group and lower in the T1 group.

5.4 SPECIFIC GROWTH RATE:

Table 5.4 Specific growth rate

TANK	SPECIFIC GROWTH RATE					
	Initial weight	Final weight	Specific Growth Rate	Average	ADG	Average
1A	2.258782	2.437989	0.298678	0.414176	0.031309	0.045929
1B	2.266217	2.646174	0.633262		0.074285	
1C	2.243744	2.430098	0.310589		0.032190	
2A	2.309702	2.902519	0.988028	0.950538	0.135809	0.126437
2B	2.295416	2.813010	0.862656		0.112190	
2C	2.258782	2.859339	1.000928		0.131309	
3A	2.323787	3.031099	1.178853	0.950801	0.175095	0.135651
3B	2.316769	2.564949	0.413632		0.047619	
3C	2.280923	3.036874	1.259917		0.184238	
4A	2.228477	3.033028	1.340918	1.400395	0.191238	0.213214
4B	2.295416	3.118392	1.371626		0.211357	
4C	2.288196	3.181381	1.488642		0.237047	
5A	2.236139	3.234749	1.664348	1.424252	0.267380	0.219357
5B	2.288196	3.190476	1.503799		0.240714	
5C	2.258782	2.921547	1.104608		0.149976	

The specific growth rate is found to be higher in the T5 group and lower in T1 group.

5.5 FISH SAMPLING:

Table 5.5 Weight of fishes per tank

TANK	WEIGHT (g)	NO.OF.FISHES	AVERAGE WEIGHT (g)
1A	126	11	11.45
1B	141	10	14.1
1C	125	11	11.36
2A	164	9	18.22
2B	200	12	16.66
2C	192	11	17.45
3A	228	11	20.72
3B	299	13	13
3C	271	13	20.84
4A	270	13	20.76
4B	294	13	22.61
4C	289	12	24.08
5A	254	10	25.4
5B	243	10	24.3
5C	260	14	18.57

Table 5.6 Length and Weight of fishes

TANK	LENGTH (cm)	WEIGHT (g)	CONDITION FACTOR
1A	9.7	14.07	1.541
1B	10.4	18.47	1.641
1C	10.5	14.98	1.294
2A	11.6	21.07	1.349
2B	10.6	20.60	1.729
2C	10.6	19.24	1.615
3A	9.8	16.08	1.708
3B	10.8	18.87	1.497
3C	10.9	21.17	1.643
4A	9.7	16.00	1.753
4B	10.5	19.94	1.722
4C	12	29.89	1.729
5A	10	16.78	1.678
5B	10.6	18.53	1.555
5C	11	25.25	1.897

The condition factor is higher in T5 group which indicates healthy growth of fishes.

Table 5.7 Weight of Organs sampled

TANK	VISCERAL WEIGHT (g)	WEIGHT OF LIVER (g)	VISCERO SOMATIC INDEX	HEPATO SOMATIC INDEX
1A	0.564	0.107	4.008	0.760
1B	0.766	0.115	4.174	0.622
1C	0.861	0.118	5.747	0.787
2A	0.873	0.173	4.143	0.821
2B	0.956	0.203	4.640	0.985
2C	0.693	0.098	3.601	0.509
3A	0.740	0.075	4.601	0.466
3B	1.060	0.162	5.617	0.858
3C	1.007	0.097	4.756	0.458
4A	1.060	0.352	6.625	2.2
4B	0.968	0.160	4.854	0.802
4C	1.704	0.358	5.700	1.197
5A	1.205	0.426	7.181	2.538
5B	0.966	0.193	5.213	1.041
5C	2.093	0.621	8.28	2.459

5.6 SERUM BIOCHEMICAL PROFILE:

Table 5.8 Absorbance readings of components in serum

SAMPLES	TOTAL PROTEIN Absorbance at 550nm	ALBUMIN Absorbance at 630nm	CHOLESTROL Absorbance at 505nm	TRIGLYCERIDES Absorbance at 505nm	GLUCOSE Absorbance at 505nm
BLANK	0	0	0	0	0
STANDARD	0.443	1.025	0.481	0.297	0.320
TANK 1	0.036	0.022	0.015	0.065	0.242
TANK 2	0.128	0.118	0.124	0.320	0.102
TANK 3	0.153	0.041	0.054	0.084	0.082
TANK 4	0.142	0.077	0.084	0.257	0.055
TANK 5	0.140	0.020	0.071	0.193	0.045

Table 5.9 Amount of components present in serum

TANK	TOTAL PROTEIN (g/dl)	ALBUMIN (g/dl)	GLOBULIN (g/dl)	ALBUMIN GLOBULIN RATIO	CHOLESTROL (mg/dl)	TRIGLYCERIDES (mg/dl)	GLUCOSE (mg/dl)
1	0.650	0.085	0.564	0.152	6.237	43.77	75.62
2	2.311	0.460	1.851	0.248	51.55	215.4	31.87
3	2.76	0.160	2.6	0.061	22.45	56.56	25.62
4	2.56	0.300	2.26	0.348	34.92	173.06	17.18
5	2.52	0.078	2.442	0.031	29.52	129.96	14.06

The value of the components present in the serum indicates the healthy growth of fishes and the good development of components needed for the growth.

5.7 AMYLASE ANALYSIS:

Table 5.10 Readings for Standard graph - Amylase

ABSORBANCE	CONCENTRATION
0.006	7.14
0.069	21.43
0.139	35.71
0.207	50
0.291	64.29
0.338	71.43
0.42	100
0.502	125
0.598	150
0.801	200

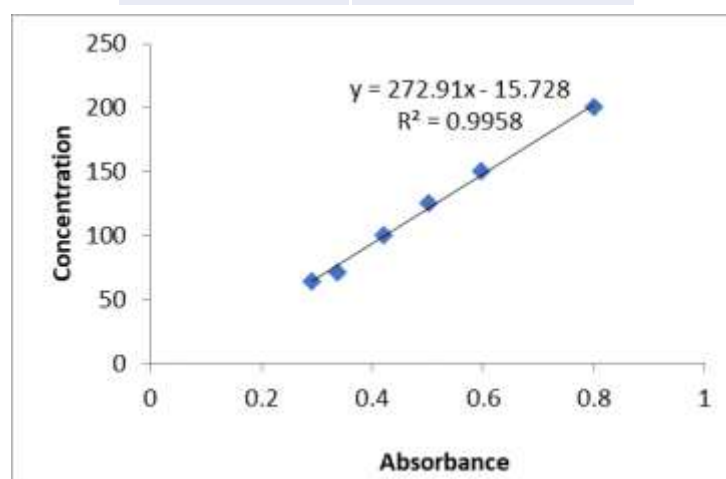


Figure 5.3 Standard Graph - Amylase

Table 5.11 Amylase concentration – Liver sample

SAMPLES (LIVER)	ABSORBANCE AT 540nm	DIFFERENCE	NANOMOL/Min/mgPROTEIN	AVERAGE
BLANK	0	-	-	-
1A	0.342	0.342	2.215119	1.822660032
1B	0.393	0.393	2.417688	
1C	0.155	0.155	0.835173	
2A	0.344	0.344	1.732698	1.970162667
2B	0.445	0.445	3.162642	
2C	0.158	0.158	1.015148	
3A	0.124	0.124	0.478413	2.349054814
3B	0.254	0.254	1.330748	
3C	0.676	0.676	5.238004	
4A	0.277	0.277	1.581324	1.877775419
4B	0.261	0.261	1.51448	
4C	0.477	0.477	2.537522	
5A	0.479	0.479	3.282108	2.015309756
5B	0.402	0.402	2.083672	
5C	0.158	0.158	0.680149	

The values indicates that good amount of amylase is secreted in T3 group which is responsible for digestion.

Table 5.12 Amylase concentration – Intestine samples

SAMPLES (INTESTINE)	ABSORBANCE AT 540nm	DIFFERENCE	NANOMOL/Min/mgPROTEIN	AVERAGE
BLANK	0	-	-	-
1A	0.647	0.647	3.273756	2.208398
1B	0.317	0.317	1.9529	
1C	0.264	0.264	1.398539	
2A	0.269	0.269	1.060971	1.209733
2B	0.289	0.289	1.254315	
2C	0.300	0.300	1.313912	
3A	0.220	0.220	1.018803	1.087631
3B	0.221	0.221	1.243833	
3C	0.189	0.189	1.000256	
4A	0.163	0.163	0.706852	1.284969
4B	0.336	0.336	1.497213	
4C	0.367	0.367	1.65084	
5A	0.211	0.211	0.74764	1.298481
5B	0.276	0.276	1.138262	
5C	0.455	0.455	2.00954	

The amount of amylase secreted is good in T1 group of fishes which is responsible for the digestion of feed.

5.8 PROTEASE ANALYSIS:

Table 5.13 Readings for standard graph – Protease – Higher Concentration

CONCENTRATION	ABSORBANCE
75	0.252
100	0.288
150	0.388
200	0.592
400	0.963
500	1.231
750	1.486

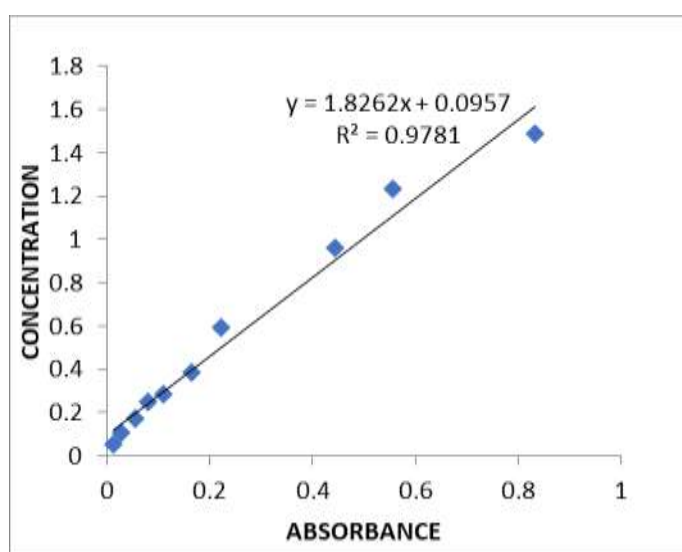


Figure 5.4 Standard graph – Protease – Higher Concentration

Table 5.14 Readings for standard graph - Protease

CONCENTRATION	ABSORBANCE
0.007	0.024
0.014	0.053
0.028	0.109
0.055	0.175

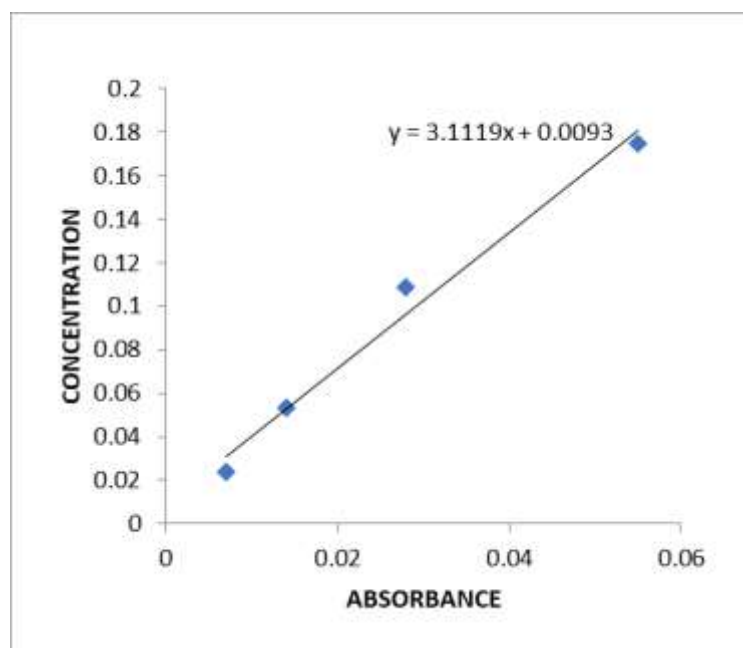


Figure 5.5 Standard graph – Protease - Lower Concentration

Table 5.15 Protease concentration – Liver sample

SAMPLES (LIVER)	ABSORBANCE AT 660 nm	DIFFERENCE	SAMPLE CONCENTRATION	MICROMOLES OF TYROSINE EQUIVALENTS RELEASED	UNIT/ml/min	MICROMOLES OF TYROSINE/ml/min
BLANK	0.020	-	-	-	-	-
1A	0.100	0.08	0.003932967	0.029146181	64.12159774	0.353891483
1B	0.093	0.073	0.000236713	0.026896751	59.1728526	0.32657902
1C	0.256	0.236	0.086306611	0.079276326	174.407918	0.962569226
2A	0.035	0.015	-0.030389385	0.00825862	18.1689643	0.100275756
2B	0.071	0.051	-0.011380083	0.019827115	43.61965359	0.240739851
2C	0.053	0.033	-0.020884734	0.014042868	30.89430894	0.170507804
3A	0.060	0.04	-0.017188481	0.016292297	35.84305408	0.197820266
3B	0.159	0.139	0.035087101	0.048105659	105.8324496	0.584096526
3C	0.109	0.089	0.008685292	0.032038305	70.48427006	0.389007506
4A	0.152	0.132	0.031390848	0.045856229	100.8837045	0.556784064
4B	0.156	0.136	0.033502993	0.047141618	103.7115589	0.572391185
4C	0.154	0.134	0.03244692	0.046498923	102.2976317	0.564587624
5A	0.106	0.086	0.007101184	0.003107	6.8354	0.03772504
5B	0.068	0.048	-0.012964191	0.01886	41.492	0.228997185
5C	0.042	0.022	-0.026693132	0.010508049	23.1177078	0.12758821

The table indicates good amount of protease secretion in T4 group fishes.

Table 5.16 Protease concentration – Intestine samples

SAMPLES (INTESTINE)	ABSORBANCE AT 660nm	DIFFERENCE	SAMPLE CONCENTRATION	MICROMOLES OF TYROSINE EQUIVALENTS RELEASED	UNIT/ml/min	MICROMOLES OF TYROSINE/ml/min
BLANK	0.091	-	-	-	-	-
1A	0.265	0.174	0.052432301	0.092706166	203.9535648	1.125633671
1B	0.140	0.049	0.012981105	0.024258022	53.36764867	0.294539702
1C	0.153	0.062	0.085571305	0.031376629	69.02858394	0.380973475
2A	0.370	0.279	0.085571305	0.150202607	330.4457343	1.823752604
2B	0.159	0.068	0.018977687	0.03466214	76.25670792	0.420865986
2C	0.098	0.007	-0.000274496	0.001259446	2.770780856	0.015292129
3A	0.204	0.113	0.033180118	0.059303472	130.4676377	0.720059814
3B	0.307	0.216	0.065687903	0.115704742	254.5504326	1.404881244
3C	0.101	0.01	0.000672333	0.002902201	6.384842843	0.035238384
4A	0.180	0.089	0.025605488	0.046161428	101.5551418	0.560489772
4B	0.175	0.084	0.02402744	0.043423502	95.53170518	0.527246013
4C	0.208	0.117	0.034442556	0.061493812	135.286387	0.746654821
5A	0.260	0.169	0.050854253	0.08996824	197.9301281	1.092389912
5B	0.280	0.189	0.057166444	0.100919943	222.0238747	1.225364947
5C	0.439	0.348	0.107348365	0.187985982	413.56916	2.282516474

The amount of protease secretion is good in T4 group fishes.

5.9 LIPASE ANALYSIS:

Table 5.17 Lipase - Titration readings – Liver and Intestine samples

TANK	LIVER		TANK	INTESTINE	
	CONTROL	TEST		CONTROL	TEST
1A	1.2	1.5	1A	1.1	1.3
1B	1.3	1.6	1B	0.7	0.9
1C	1.4	1.7	1C	0.7	1.0
2A	1.0	1.0	2A	0.9	1.0
2B	1.1	1.2	2B	0.9	1.0
2C	1.2	1.3	2C	0.9	0.9
3A	1.2	1.3	3A	0.8	0.8
3B	1.2	1.4	3B	0.8	1.0
3C	1.2	1.4	3C	0.8	1.0
4A	1.0	1.2	4A	0.8	0.9
4B	1.3	1.4	4B	0.8	0.9
4C	1.2	1.3	4C	1.0	1.1
5A	1.5	1.7	5A	0.9	0.9
5B	1.3	1.4	5B	1.1	1.2
5C	1.4	1.4	5C	1.1	1.3

Table 5.18 Lipase concentration – Liver and Intestine samples

TANK	UNITS OF LIPASE ACTIVITY PER GRAM OF TISSUE	
	LIVER	INTESTINE
1A	0.3	0.2
1B	0.3	0.2
1C	0.3	0.3
2A	0	0.1
2B	0.1	0.1
2C	0.1	0
3A	0.1	0
3B	0.2	0.2
3C	0.2	0.2
4A	0.2	0.1
4B	0.1	0.1
4C	0.1	0.1
5A	0.2	0
5B	0.1	0.1
5C	0.0	0.2

Lipase secretion is good in T1 group fishes.

5.10 PROXIMATE ANALYSIS:

Table 5.19 Proximate analysis

TANK	NO.OF.FISHES	WET WEIGHT		DRY WEIGHT		DRY MATTER (%)	MOISTURE (%)
		PETRI PLATE WEIGHT	FISH WEIGHT	PETRI PLATE WEIGHT	FISH WEIGHT	-	-
1	7	95.10	68.92	96.042	17.529	25.43	74.57
2	5	102.61	78.08	102.731	20.588	26.35	73.65
3	5	104.47	76.39	104.587	20.499	26.82	73.18
4	4	107.55	80.00	107.613	21.390	26.73	73.27
5	4	100.27	67.58	100.303	19.352	28.63	71.37

Dry matter (%) = (Dry weight/Wet weight)*100; Moisture(%)=100-Dry matter

The moisture content is higher in T1 group fishes.

Table 5.20 Proximate composition analysis of feed and fish whole body samples

Fish feed (in %)					
Parameter	K1	K2	K3	K4	K5
Moisture (%)	4.56	4.22	3.94	4.56	3.97
Crude protein (%)	47.99	48.01	46.71	48.11	47.94
Crude fibre (%)	1.23	1.39	1.59	2.02	2.69
Ether extract (%)	8.98	9.11	9.28	9.56	10.15
Total Ash (%)	18.98	18.78	18.81	17.35	15.61
Fish whole body (in %)					
Moisture (%)	3.91	6.04	5.49	6.37	7.66
Crude protein (%)	66.25	65.49	65.18	67.98	64.48
Crude fibre (%)	0.15	0.11	0.13	0.10	0.05
Ether extract (%)	10.45	13.47	15.21	14.53	14.47
Total Ash (%)	20.19	18.07	16.19	13.78	14.65

Based on the results obtained, the best results are obtained for T5 group.

5.11 RNA ISOLATION:

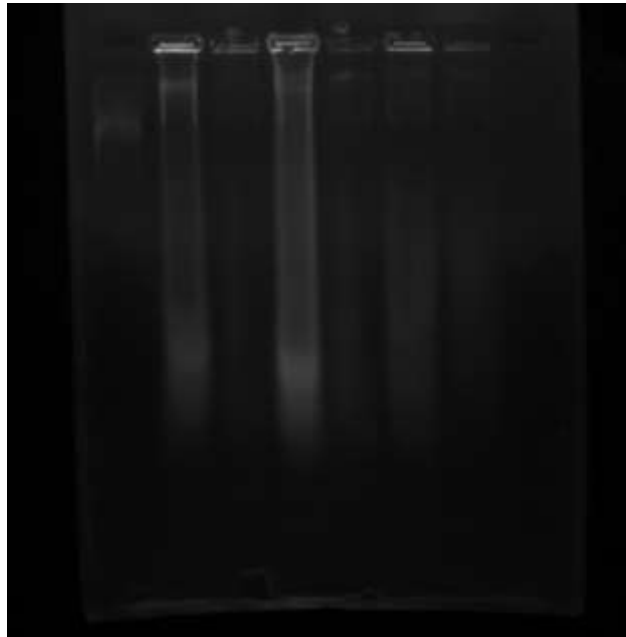


Figure 5.6 RNA Isolation

5.12 DNA ISOLATION:

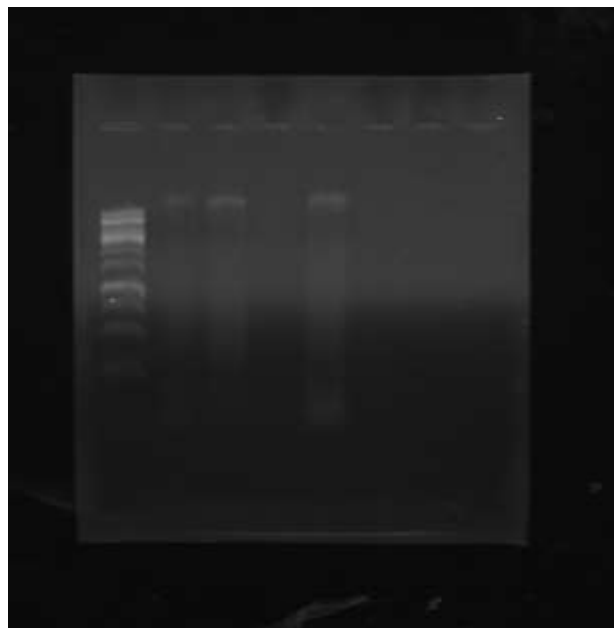


Figure 5.7 DNA Isolation

5.13 POLYMERASE CHAIN REACTION:

Semi-quantitative RT-PCR analysis and expression level of Leptin gene of orange spotted grouper

The cDNA derived from liver and muscle samples collected from orange spotted grouper was used to find the tissue level expression of leptin gene using appropriate primers. The expression level showed that no significant difference observed between the liver and muscle samples.

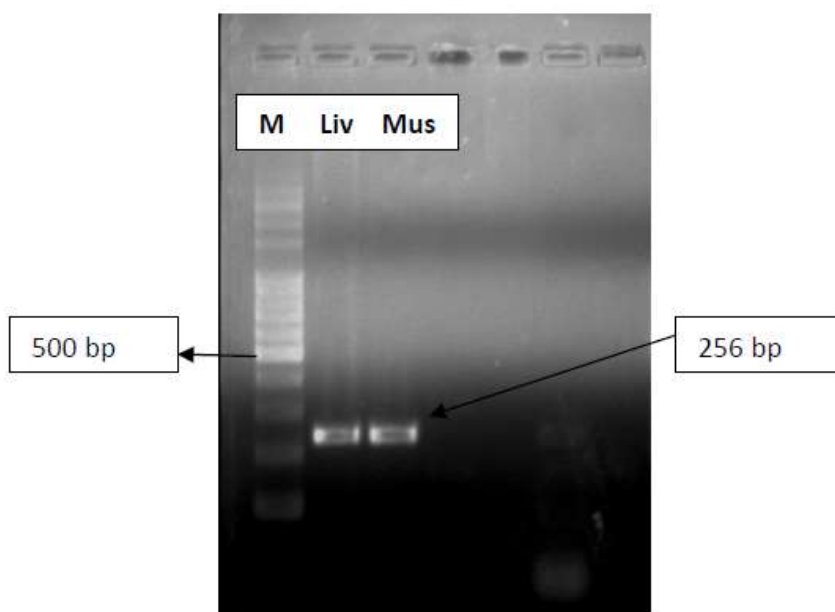
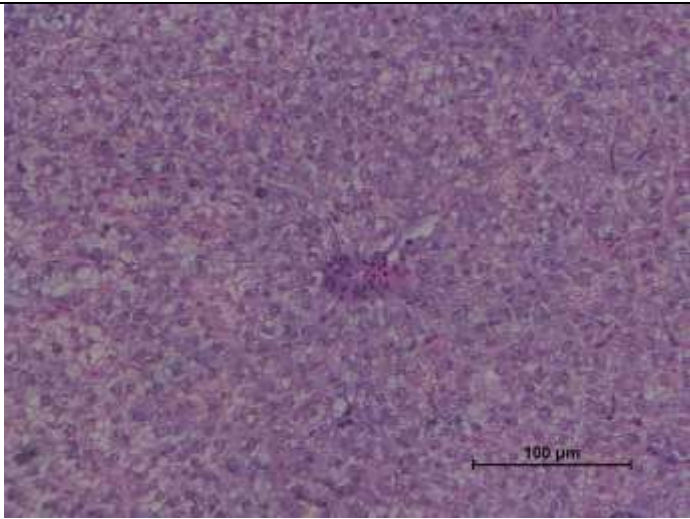
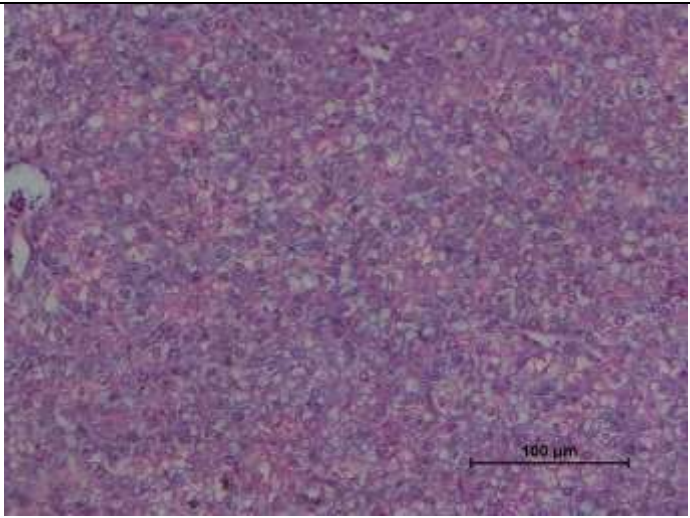
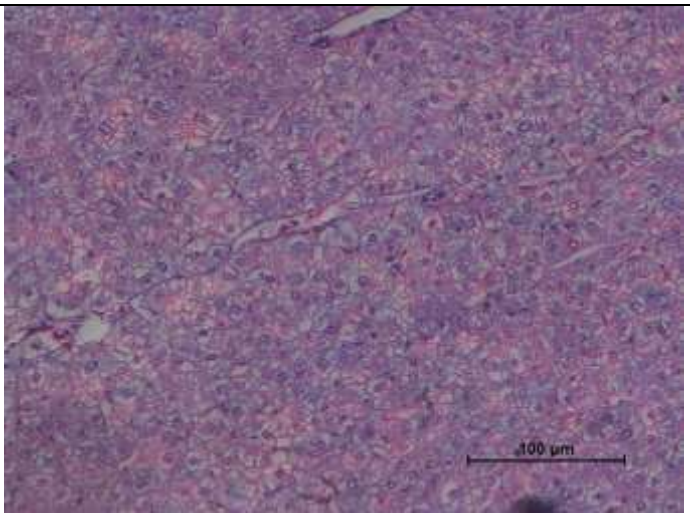
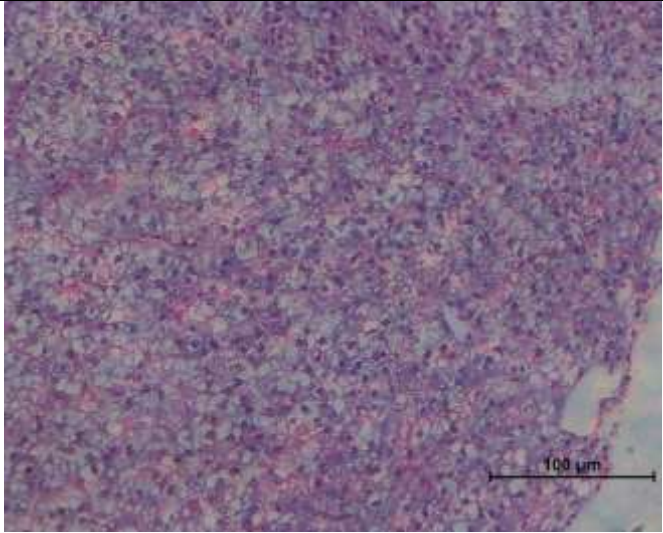
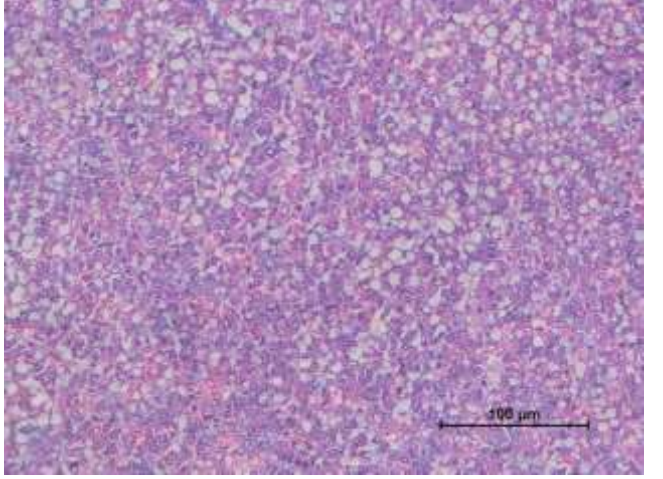


Figure 5.8 Polymerase Chain Reaction

5.14 HISTOLOGICAL ANALYSIS:

Table 5.21 Histological Analysis

	Liver section of orange spotted grouper fed control diet showing mild congestion & vacuolation of hepatocytes. Sinusoids with focal extravasations of red cells surrounding the central veins (stained with H&E; x20).
	Liver section of orange spotted grouper fed 15% krill meal diet showing fat vacuolation of hepatocytes. (stained with H&E; x20).
	Liver section of orange spotted grouper fed 30% krill meal diet showing fat vacuolation of hepatocytes. Sinusoids seen (stained with H&E; x20).

	Liver section of orange spotted grouper fed 45% krill meal diet showing cytoplasmic fat vacuolation of hepatocytes (stained with H&E; x20).
	Liver section of orange spotted grouper fed 45% krill meal diet showing Intra cytoplasmic round circumscribed fat vacuoles of hepatocytes (stained with H&E; x20).

5.15 DISCUSSION - RESULTS:

Among the five treatments, highest weight gain was observed in tank 5, which was fed with 60% of krill meal in the diet in place of fishmeal. The specific growth rate, average daily growth and weight gain percentage was higher in the T5 group. The feed utilization in terms of feed intake, PER, FE, FCR was better in T5 group due to higher concentration of krill meal in the diet as it was better attracted and enhanced feed consumption by fishes. The Viscero Somatic Index and Hepato Somatic Index was high in the fishes of T5 group due to better accretion of protein and lipids. Hence, 60% inclusion of krill meal is considered as effective level for the replacement of fishmeal in the diet of orange spotted grouper.

CHAPTER 6

SUMMARY AND CONCLUSION

6.1 DISCUSSION:

The results obtained in the present study is in agreement with Saleh et al.,2018, who observed that krill meal inclusion used in their experiment was 3%,6% and 9% and reported better growth response at 9% krill meal inclusion in feed.. The survival rate in the present study is higher at T4 (45% inclusion) that is in agreement with Tharaka et al.,2020, who reported better survival rate and growth at 6% krill meal that is the penultimate higher krill meal inclusion. Tharaka et al.,2020 reported that the feed consumption rate and specific growth rate were higher in 6% krill meal likewise the feed consumption rate and specific growth rate is high in 45% and 60% replacement of fishmeal by krill meal in the present experiment.

6.2 SUMMARY:

The results of the growth estimation, feed utilization, enzyme analysis and biochemical analysis of this study revealed that as the inclusion level of krill meal increased the growth of the experimental fishes. Better survival was recorded in T4 group followed by T3 group and least survival was recorded in T1 group which was due to lower feed consumption as it contains no krill meal in the diet. As the level of inclusion of krill meal increased in the diet the fat accumulation was also increased which was evident from the enhanced VSI and HSI in the experimental fishes. Based on the results obtained, it is concluded that the krill meal is an effective feed attractant and growth enhancer and served as a promising fishmeal replacing ingredient in the aquafeed.

CHAPTER 7

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