# Proximate analysis of Azolla and effect of formulated fish diet supplemented with Azolla on the growth performance and feed utilization of juveniles of Asian sea bass (*Lates calcarifer*)

A Dissertation Submitted in partial fulfillment of the degree of Master of Science in Zoology

By

**VIBHUTI KASAR**

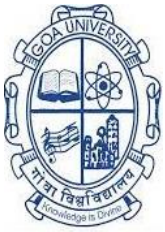
Roll no: 21P044018

Under the Supervision of

**MS. GANDHITA KUNDAIKAR**

School of Biological Sciences and Biotechnology

Discipline of Zoology




**GOA UNIVERSITY**

Date: April 2023

Examined by: Seal of the School

## DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled- **Proximate analysis of Azolla and effect of formulated fish diet supplemented with Azolla on the growth performance and feed utilization of juveniles of Asian sea bass (*Lates calcarifer*).** is based on the results of investigations carried out by me in the Discipline of Zoology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of **Ms. Gandhita Kundaikar** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

I hereby authorize the University authorities to upload this dissertation to the dissertation repository or anywhere else as the UGC regulations demand and make it available to anyone as needed.

Vibhuti Kasar

21P044018

Discipline of Zoology

School of Biological Sciences and

Biotechnology

Date:

Place: Goa University

# COMPLETION CERTIFICATE

This is to certify that the dissertation **"Proximate analysis of Azolla and effect of formulated fish diet supplemented with Azolla on the growth performance and feed utilization of juveniles of Asian sea bass (*Lates calcarifer*)"** is a bonafide work carried out by **Ms. Vibhuti Kasar** under my supervision in partial fulfillment of the requirements for the award of the degree of **Master of Science in Zoology** in the Discipline of Zoology at the School of Biological Sciences and Biotechnology, Goa University.

Ms. Gandhita Kundaikar

Discipline of Zoology

Date:

Dr. Savita Kerkar

Zoology Discipline

School of Biological Sciences and Biotechnology

Date:

Place: Goa University

# ACKNOWLEDGEMENT

First and foremost, I would like to express my utmost gratitude and appreciation to my guide, Ms. Gandhita Kundaikar for her supervision, guidance and assistance throughout my dissertation.

I express my gratitude to Dr. Nitin Sawant, Programme Director, Discipline of Zoology for providing me an opportunity to carry out the dissertation and providing me well-equipped laboratories needed for my dissertation.

I thank the Discipline of Zoology, Government of India for constant support by providing us with the required funding for the project.

I would also like to extend my deepest regards and express gratitude to other faculty members Dr. Shanti Dessai, Dr. Minal Shirodkar, Dr. Avelyno D'costa, Dr. Shamshad Shaikh without the support and encouragement of whom this journey would not have been complete.

I would like to extend my heartfelt thanks to my dear friend Ms. Abhigya Sharma for being a constant source of motivation and encouragement throughout my dissertation journey.

I would like to express my sincere appreciation to my friend Mr. Vianney Carvalho and Ms. Vrunda Gayak for making a significant contribution to the successful completion of this dissertation.

A special thanks to Mr. Stanley, Green Lake Farm, for providing the Fish seeds.

I would like to thank Dr. Elroy Pereira for the constant availability and insightful suggestions throughout the dissertation.

I express my heartfelt gratitude to the non-teaching staff of our department, for their help during the tenure of the project.

To my family, thank you for always believing in me. I am especially grateful to my parents who have always encouraged me in all my pursuits and inspired me to follow my dreams.

Last but not the least, I would like to show my appreciation to my friends and those who lent me a hand, and supported and guided me in the process of completing my dissertation.

# CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF PLATES

# 1. INTRODUCTION

## 1.1 Importance of fish feed

Fish is a very nutrient-dense food and a great source of protein from animals. In low-income, food-deficient nations, fish makes up 20% of animal protein. Fish account for over 17% of all protein consumed worldwide. Increased aquaculture production might reduce protein deficiency, especially in underdeveloped rural parts of the country. Reducing reliance on wild fish and strengthening fish farming is required to achieve sustainability in the aquaculture industry (Delgado et al., 2002).

According to the World Bank (2013), by 2030, aquaculture will generate two-thirds of the fish consumed worldwide. As a result, it is expected that the aquaculture business will expand tremendously.

It is essential to give fish artificial feed so that they can develop quickly and reach their maximum weight in the shortest period of time for the benefit of fisheries and to get the maximum output from freshwater resources. Fish meal is considered to be the best component among frequently used feed ingredients since it is compatible with the protein needs of fish (Alam et al., 1996).

Many fish farmers have greatly limited their activity as a result of the high expense of fish diets. Indeed, the prepared fish diet that is often offered incorporates expensive, variable-quality fish meals. As a result, it becomes crucial to replace the expensive protein source with cheaper ingredients to minimise cost and maximise benefit. (Olvera-Novoa et al., 1990)

Due to escalating costs and irregular supplies of fish meal, cheaper ingredients of plant origin must be utilized in place of fish meal in fish feed (Higgs et al. 1995).

The expense of fish meals can be reduced by using plant protein supplements in fish feed (Rumsey, 1993).

In animal production systems, a healthy, high-quality product can only be produced economically with good nutrition. Nutrition is important in aquaculture since feed generally accounts for around 50% of the variable production cost. The evolution of the aquaculture industry to meet the rising demand for fish and seafood products that are cost-effective, safe, and of high quality is aided by the development of improved species-specific diet formulations (Craig et al., 2017).

The diet has to be well proportioned and contain all the key nutrients. In many third-world nations, the meal used in fish farming is highly costly, irregular, and in limited supply. In both intensive and semi-intensive fish farming, fish feed accounts for a significant amount of the production costs. The nutritional and commercial performance of farmed fish heavily depends on the selection of feed components used in aquafeed. Fish meal is an increasingly costly by-product of industrial fish farming and a rich source of high-quality protein (Das et al., 2018).

It is crucial to adequately determine the protein requirements for each species and cultured life stage, considering that protein is the costliest component of fish feed. Methionine content is often low in fish diets made with plant proteins. Similarly, methionine and lysine are frequently insufficient in fish diets made from bacterial or yeast proteins. As a result, when these sources of protein are employed in place of fishmeal in diets, these amino acids must be supplied (Craig et al., 2017).

## 1.2 Formulation of fish feed

The formulation of a low-cost, balanced diet utilizing readily accessible agricultural by-products is necessary for the commercial culture of fish. Fish meal has now surpassed other protein ingredients as the costliest component in aquaculture diets. Several studies have shown significant effectiveness in partially substituting fishmeal in the diets of various fish species with soybean meal and other soybean derivatives (Boonyaratpalin and Tunpibal 1998; Quartararo *et al*. 1998; Hernadez *et al*. 2007).

The method of integrating feed components into a combination that will meet the particular production goals is known as "feed formulation and preparation." While preparing the feed, some aspects must be taken into consideration, including cost, palatability, availability of the products utilised, and anti-nutritional elements (Azevedo, 1998).

Fish feed formulation is based on the apparent digestibility of protein, energy, and specific amino acids, using data acquired for a variety of raw materials, such as plant by-products that are frequently utilised in the feed industry. Numerous studies on a variety of fish species have been conducted over the past few decades, and information on the majority of nutrients' digestibility has been gathered. (Tacon, 1994).

The prepared or synthetic feed can be consumed whole or as a supplement. Fish are given a complete meal that includes every nutrient required for their growth and well-being. The nutritional content of the feed will vary depending on the species of fish being reared and their developmental stage. When fish are housed in cages or high-density indoor systems and are unable to eat naturally, a full diet must be given to them. On the other hand, supplemental diets are specifically developed to supplement the natural food that fish frequently have access to in ponds or outdoor raceways. Supplemental diets are often used to significantly enhance the

naturally available food with more protein, carbs, or lipids rather than providing a complete complement of vitamins and minerals (Craig et al., 2017).

The typical percentage of protein in aquaculture feeds is 30 to 35 percent for shrimp, 28 to 32 percent for catfish, 35 to 40 percent for tilapia, 38 to 42 percent for hybrid striped bass, and 40 to 45 percent for trout and other marine finfish. In general, the protein needs of omnivorous (fish that eat both plants and animals) and herbivorous (fish that consume plants) fish are lower than those of carnivorous (fish that eat animal meat). When compared to low-density culture, fish raised in high-density systems (such as recirculating aquaculture) have greater protein needs, e.g., in ponds (Craig et al., 2017).

Fries and fingerlings at an earlier stage of development typically have higher protein needs. Protein requirements decrease for juvenile and adult fish. The habitat in which the fish are raised, the temperature and quality of the water, their genetic makeup, and their eating habits all influence their protein requirements. If the food contains sufficient amounts of carbs and lipids, protein can be utilised for fish development. If not, the more expensive protein can be used for supporting life and energy needs instead of development of fish (Craig et al., 2017).

- **Importance of Aquatic Plants in Feed**

Research on the use of aquatic plants for feeding livestock and aquaculture is becoming increasingly important since they are recognised to have a high feed value. They can provide a vital and affordable source of protein, particularly in aquaculture and cattle farming.

Since they contain the necessary nutrients, water weeds may be used as an alternative to traditional feed, but because they have previously been disregarded, using water weeds as feed may help create new livestock and fishing production methods.

By exploiting this peculiar plant material in the cattle and aquaculture industries, as well as for other medical uses, the problem of how to use these rapidly spreading aquatic plants may be resolved (Magouz et al., 2020).

A cheap, easily accessible, and environmentally benign substitute ingredient is needed since fish feed, the most expensive component of aquaculture, accounts for between 50 and 60 percent of the overall cost of production. Azolla enhances fish growth, development, nutrient digestion, and immunity. The inclusion of Azolla in the fish's diets can support immunological responses (lysozyme activity and phagocytosis) that promote pathogen resistance (Magouz et al., 2020).

Because fish feed is expensive in aquaculture, a low-cost, readily available, and ecologically friendly replacement ingredient is required (Sithara and Kamalaveni, 2008).

## 1.4 Importance of Azolla

Azolla is a small, fast-growing aquatic fern that is rich in protein, essential amino acids (EAA), and vitamins. Azolla is distinguished by its greater availability and nutritional value. Azolla is a high-yielding, easy-to-cultivate plant. It grows in conjunction with the nitrogen-fixing organism Anabaena azollae. In fish, Azolla improves growth, development, nutritional digestibility, and immunity. (Kumari et al., 2017).

The adventitious, determinate roots of the diploid Azolla plant's sporophyte generation, which produces spores, arise from the main rhizome, which can be horizontal or vertical. These roots are multi-branched, prostrate, floating stems with deeply lobed leaves.

Freshwater environments may be found with Azolla species all around the world. Azolla has a total of seven recognised species, namely *A. caroliniana, A. filiculoides, A. mexicana, A. microphylla, A. rubra, A. niilotica, A. pinnata. Among these species, A. pinnata is widespread in the country.*

Both macronutrients and micronutrients are necessary for the growth of Azolla. Such nutrients are obtained by them straight from the soil.

Azolla has a high nutritional value and is used as cattle feed because it contains essential amino acids, proteins, vitamins, and minerals. Additionally, it contains omega-3 and six polyunsaturated fatty acids. As a result, it is used to facilitate the digestion of livestock, poultry, and aquatic organisms (Chandewar et al., 2017).

Previous research has suggested that a 10–45 percent Azolla level can be integrated into the diet of tilapia species that require more than 40% protein content (Abou et al., 2007).

Incorporating azolla into a fish's diet can help sustain immune responses (lysozyme activity and phagocytosis) that aid pathogen resistance (Magouz et al., 2020).

Considering its nutritional value, due to its low protein digestibility and high fibre content, azole inclusion levels in tilapia diets are extremely limited in aquaculture (Leonard et al., 1998).

In terms of the environment, Azolla has several drawbacks. Azolla may have tremendously adverse consequences for wetlands that are irreversible because they may change the local flora and fauna. In addition, they could change the biological, chemical, and physical aspects of aquatic ecosystems, which might result in a decline in ecological quality. By threatening communities of native wildlife and endangered species, it was discovered to harm biological diversity (Sax et al., 2005; Vander Zanden and Olden, 2008).

Moreover, it damages the aquatic ecology by lowering nutrient and oxygen levels while raising water turbidity. Moreover, these invaders can survive and reproduce under a range of environmental conditions (Olenin et al., 2007).

Azolla is often used in rice as an azo-biofertilizer and has a variety of additional uses, such as the removal of pollutants and heavy metal contamination, animal feed, wastewater treatment, azo-biofuel, mosquito control, and weed control. Additionally, it is used in antibacterial drugs for fungi and bacteria. It may also be used for other things, including medical applications (Kumar et al., 2015).

### 1.5 Asian Sea Bass (*Lates calcarifer)*

Asian sea bass, sometimes also known as "barramundi," is a finfish that is primarily carnivorous and is found in several tropical and subtropical regions of countries in the western Pacific and Indian Ocean (Greenwood, 1976).

Its life cycle is biphasic in the wild, with an initial stage of 2–3 years of growth in inland freshwaters followed by a subsequent migration into the sea for sexual development and spawning. Asian sea bass are certainly a desirable species for cultivation in captivity due to their characteristics of being adaptable to a diverse range of salinity and turbidity (Boonyaratpalin et al., 2002).

Asian sea bass are fish with rapid growth. It often has a high market price and has white flesh, which is favoured by customers. It is a large-bodied marine teleost from the tropics. Its cultivation was initially introduced in Papua New Guinea, Australia, and Southeast Asia (Cheong, 1989).

It is one of the most widely consumed fish species as food. Presently, local family-owned and mid-sized farms produce the majority of the species' commercially viable stock, utilising brooders procured from either the sea or unidentified sources. Asian sea bass have a high reproductive rate and can endure high salinity (Orban et al., 2021; Lawley, 2010; Robinson *et al*., 2010).

In Thailand and other Asian nations, Asian sea bass, often known as giant sea bass, have a significant economic impact. Both earthen ponds and sea cages are used to cultivate the fish, with the majority of the sea cages generally located around river mouths or estuaries. Cage farming has become more prevalent over the past few years in various parts of the nation. Fishermen that cultivate sea bass frequently utilise trash fish as feed. There are increasing issues

with the availability of trash fish, both in terms of quantity and quality, as well as poor fish development when trash fish is used as feed. Formulated feeds will probably be required shortly, as expected. It has also been attempted to use plant protein sources in place of fish meals. (Boonyaratpalin et al., 1989).

*Lates calcarifer*, which grows rapidly, completely depends on fishmeal as its primary source of nutrition. Because of the growing competition for this conventional protein for use in aquaculture, human consumption, and animal feed, the price of this feed is now rising. The carnivorous fish L. calcarifer develops quickly and is entirely dependent on the use of fishmeal (FM) as the primary source of protein in its diet. The cost of this feed is currently rising due to increased competition for this traditional protein for industrial usage, aquaculture diets, human consumption, and animal diets, which drives up their costs and lowers the profitability of fish farming as well as its yield in terms of quantity and quality (Erlinda et al., 2014).

## 1.6 Review of literature

In the present study, a sun-dried sample of Azolla pinnata was utilized for chemical composition analysis. Standard methods were used to determine the proximate composition, fiber fractions of cell wall components, and mineral content. Standard techniques were used to estimate the approximate composition, cell wall constituents, and trace minerals (Cu, Zn, Mn, and Fe) of sun-dried Azolla. There were 90.00±0.77 of dried matter, 22.05±0.72 of crude protein, 81.05±0.44, 3.25±0.76 of organic matter, and 18.94±0.31 of total ash. The contents of lignin, hemicellulose, NDF, ADF, cellulose, and hemicellulose were respectively 48.25±0.48, 37.14±0.11, 11.11±0.29, 8.07±0.25, and 28.87±0.64. Zn, Cu, Mn, Mn, Fe, and Ca concentrations were 30.02±2.39, 26.29±1.41, 348.17±7.26, 533.12±96.56, and 0.33±0.03, respectively (Gupta et al., 2018).

In order to enhance feed, the nutritional value of Azolla pinnata was investigated. Under the shed, Azolla was grown, picked, and dried. The proximate principles of the dried Azolla sample were examined. The sun-dried Azolla meal's dried matter (DM) content was 91.78 percent. It was 74.50% organically composed, included 22.25% crude protein, 11.19% crude fibre, 2.45% ether extract, 25.50% total ash, 38.61% nitrogen-free extract (NFE), and 7.94% acid-insoluble ash. According to the chemical analysis, Azolla has a high crude protein content and may be used as a novel natural protein source in animal and poultry feeds (Kumar et al., 2018).

A feeding experiment was conducted to assess the effects of Moringa oleifera and Lam leaf meals on growth, feed conversion ratio, protein efficiency ratio, proximate body composition, and survival rates in Asian sea bass, Lates calcarifer. Four isonitrogenous test diets with a 40% crude protein content (CP) were developed. 15 groups of sea bass fingerlings with an initial weight of 2 g were randomly distributed in three replicates and fed three times a day for 75 days. Feed utilization efficiency and survival rates of sea bass given diets containing up to 30%

dietary inclusion level of MOLM were comparable to those of the diet containing 20% dietary inclusion. When compared to fish-fed diets containing 10, 20, and 30% MOLM, seabass-fed a control diet (0% MOLF) showed the highest percentage weight gain and protein efficiency ratio. The proximate body composition of Asian sea bass showed that crude protein and crude ash significantly reduced as the dietary inclusion level of MOLM was increased from 10 to 30%. When the fish meal was substituted with more MOLM, the body's crude lipid, on the other hand, displayed the contrary pattern. The capacity of L. calcarifer to grow and sustain its health was found to be unaffected by the use of MOLM, up to a maximum of 10%, as a plant protein alternative in diets based on fish meals (Erlinda et al., 2014).

Azolla meal was added to fish feed at three different levels (10%, 20%, and 30%) and given to GIFT (genetically enhanced farmed tilapia) for 90 days. The collected data revealed that in fish fed 30% Azolla, the ultimate body weight, weight gain, and specific growth rate all fell significantly ($P>0.05$). Azolla incorporation in tilapia diets did not affect the length of the villus in the foregut ($P>0.05$). The villus length in the midgut increased substantially ($P>0.05$). The tilapia foregut mucosal length increased considerably ($P>0.05$). The number of goblet cells considerably increased in the foregut and hindgut in fish fed Azolla at 3% compared to the control, with no changes between those fed at 10% and 20% ($P>0.05$), while the number of goblet cells dramatically increased in the midgut ($P>0.05$). The haematological and biochemical functions of tilapia-fed Azolla were normal, with no significant differences for the measured parameters except for the red blood cell count, which increased significantly ($P>0.05$) in fish-fed Azolla at 20% compared to the control, with no differences between those fed at 20% and 30% (Magouz et al., 2020).

Azolla filiculoides (water fern), Elodea sp., and Pistia stratiotes (water lettuce), three species of aquatic weeds, were fed to 60 young Oreochromis niloticus (Nile tilapia) to find out which one would prove to be more selectively ingested and favoured. Twenty Nile tilapia were fed compound feed as part of the control group. Azolla filiculoides, Elodea sp., and the roots of Pistia stratiotes were observed to be the three most frequently utilized food sources, indicating the selection of the weeds. The fish-fed compound feed showed the greatest growth response to the diets, followed by Azolla filiculoides and Elodea sp., whereas Pistia stratiotes showed declining solid growth. Hence, it is hypothesized that Azolla filiculoides and Pista sp. are suitable feeds for O. niloticus, particularly when utilized with other feeds (Agbede et al., 2004).

There has been a review of the use of Azolla meal as a plant-based source of protein for the culture of fish, with a particular emphasis on the tilapia species and the Cyprinidae family. The nutrition of aquaculture was researched in around 30 online journal articles from Research Gate and Google Scholar. Among the publications that were evaluated, it was discovered that dietary Azolla supplementation at a specific amount improves feed efficiency, protein conversion ratio, mobilization and usage of glycogenic amino acids, and growth performance. Except for T. zillii, which requires a diet with a protein content of at least 40%, this research thus proposes that a level of 10-45% Azolla inclusion can be included in the diet for Tilapia species (Mosha et al., 2018).

Dried and fresh Azolla pinnata were assessed as food sources for fingerling and adult Nile tilapia, Oreochromis niloticus (L.). To replace 25, 50, 75, and 100% of the fish meal (FM) protein in the control diet, dried Azolla was added to practical meals that had about 30% crude protein and 360–400 kcal/lOOg of GE. Fresh Azolla was also evaluated as a whole diet for these fish. For ten weeks, duplicate groups of fish were fed diets that had been prepared at rates of 5% and 3% of their body weight per day, respectively, for fingerlings (2-54g ± 0093) and adults (4033g ± 103) of fish. Fish fed the control diet showed significantly greater growth and feed utilization efficiency than fish fed diets supplemented with Azolla. The fish performance continued to deteriorate with increasing dietary Azolla levels in the diets. This reduction was especially severe when dried and fresh Azolla were given as complete diets. Furthermore, adult tilapia-fed fresh Azolla started losing weight from the 7th week. Fish-fed fresh Azolla had significantly higher moisture content than those fed formulated diets. While body ash content exhibited a positive association with Azolla levels in the meals, body protein, and lipid contents showed a negative correlation. Results of this study indicate that young Nile tilapia utilizes Azolla more efficiently than adults (Sayed et al., 1992).

In order to replace nearly 50% of the protein in soybean meal in the control diet (or about 30% of the diet's total protein), sun-dried Azolla was crushed and integrated into experimental diets at different amounts (10.6, 21.2, 31.8, and 42.2% of the diets). All experimental feeds contained 30% crude protein, 300 Kcal digestible energy/100g, and a protein/energy ratio of about 70 mg protein/Kcal. Nile tilapia (Oreochromis niloticus) fingerlings were given diets for 90 days at 3% of fish biomass daily with an initial mean body weight of 8.1g ± 0.3g. The obtained results showed that Azolla meal (in dried pellet form) is a good ingredient for Nile tilapia fingerling diets since growth performance in all experimental and control diets was nearly similar without significant variances. Also, increasing the percentage of Azolla meals in the diet seemed to have no impact on the hepatosomatic index or survival rate. While the amount of Azolla meal

in the diets steadily grew with an increase in the feed conversion ratio, there were no appreciable differences up to the inclusion level of 31.8% before the ratio reduced significantly. Increasing the amount of Azolla meal in the meals resulted in a considerable decline in body protein and lipid content. However, when the amount of Azolla meal in the meals increased, moisture and ash content also increased substantially. (Ebrahim., 2007).

In this study, the feeding frequency of Asian seabass, Lates calcarifer, was studied to find out its consequences on growth performance, feed consumption, chemical body composition survival rate, cannibalism, and morphological indices. For the trial, 140 sea bass with an average weight of 5.47± 0.11 g were housed at random in 4 concrete tanks, each measuring 914 cm x 183 cm x 122 cm and holding 18,399 L, for a total of 68 days. A pelleted meal yielding 46% crude protein was given to the fish in various feeding groups known as (T1, T2, T3, and T4). The meal was given to each group three times a day at rates of (T1) 3%, (T2) 4%, (T3) 6%, and (T4) 9% of fish biomass per day. After the trial, the physicochemical properties of water were within expansion limits for Asian sea bass. In comparison to the T1 and T2 group, the average daily weight increase (g), weight gain (g), and specific growth rate (%) were all considerably greater in the T3 and T4 group ($p < 0.05$). The T1 group, which generated 3% biomass per day, demonstrated the lowest feed conversion ratio. The cannibalism rate was substantially ($p < 0.05$) higher in T1 (3%) compared to T3 and T4 treatments (Hassan et al., 2021).

In the present study, the effects of ascorbic acid and the herb garlic (Allium sativum) on the haemato-biochemical parameters and growth performance of juvenile Asian sea bass were examined. 600 fish (body weight: 43.14± 0.23 g) were split into four groups. As a control group, fish in the first group were fed a basal diet. Fish in groups 2, 3, and 4 were given a baseline di*et al*ong with ascorbic acid (1.5 g/kg food), 40 g/kg of garlic, or a combination of 20 g/kg of garlic and 0.75 g/kg of ascorbic acid for 12 weeks. In comparison to the control and other treatment groups, fish fed just garlic showed significantly ($p < 0.05$) improved growth performance, feed consumption, and chemical body composition. When compared to the control during the experiment, none of the hematological indicators, biochemical parameters, or survival rates substantially varied ($p > 0.05$) in any of the groups. Fish fed just garlic exhibited a significant ($p < 0.05$) reduction in total cholesterol and feed conversion ratio when compared to the control and other treatment groups. In conclusion, dietary supplementation with garlic only (40 g/kg diet) was significantly more effective than dietary supplementation with ascorbic acid only (0.75 g/kg diet) in improving most of the measured parameters (Abdelwahab., 2020).

This study examined the effects of various dosages of two species of Bacillus (Bacillus licheniformis and Bacillus subtilis) on growth parameters, fish chemical composition, liver activity, and digestive enzymes in Asian sea bass. In addition to a control meal without supplementary microorganisms during eight weeks, juvenile Asian sea bass consumed diets supplemented with 1, 103, 106, and 109 CFU g-1 probiotics. At the end of the experiment, body composition (crude protein, crude lipid, ash, and dried matter), digestive enzymes (protease, lipase, and amylase), liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP)), immunologic indicators (lysozyme), and haematological parameters red blood cells (RBCs), white blood cells (WBCs), haemoglobin (Hb), and haematocrit (Hct) were analysed. Asian sea bass fed probiotic Bacillus (Bacillus licheniformis and Bacillus subtilis)-supplemented diets exhibited significantly greater

growth than those on the basal diet (control). When compared to the control group, fish given the probiotic-containing food showed greater levels of total protein, dried matter, and lipids while reducing the total levels of all three ($P < 0.05$). Fish given diets supplemented with 1× 106 CFU g1 probiotic Bacillus showed the greatest levels of digestive enzymes (protease, lipase, and amylase) and hematological parameters (RBC, WBC, and Hb). Also, fish fed a diet supplemented with 1 106 CFU g1 probiotic Bacillus showed lower levels of the liver enzymes AST, ALT, and ALP. Due to the fact that adding 1 ×106 CFU g1 of Bacillus to the diet is the dose that yields the greatest effects (Adorian et al., 2019).

Two experiments were used to perform this investigation. In Experiment 1, the effects of Azolla nilotica (AZN) and Spirulina platensis (SP) on the growth and resistance of Oreochromis niloticus to oxidative stress were examined. Seven fish groups (G1-G7) were given a basic diet (control), AZN 5%, AZN 10%, SP 0.5%, SP 1%, a mixture of AZN 5% and Spirulina platensis 1% (AZN 5%-SP 1% mix), and a mixture of AZN 10% and Spirulina platensis 1% (AZN 10% - SP 1% mix), respectively, for three months. In comparison to the control group, all supplemented groups showed significantly higher growth metrics (weight gain, specific growth rate, average length gain, and feed efficiency ratio) and lower feed conversion ratios. Intestinal-somatic index in G4 - G6 and in G5 - G6 groups, as well as the hepato-somatic index, were higher than the control in the G4 and G5 groups. Spleno-somatic index and the antioxidant enzymes GSH-px, SOD, and CAT were significantly higher in G5 and G6 than in the control group. White blood cells substantially increased in G2 and G4 ($p<0.05$). Experiment 2 showed the impact of a one-month SP 1% supplementation on both the male and female fertility of O. niloticus. When compared to the control, males administered SP 1% exhibited higher rates of liveability, spermatozoa motility, and density of sperm (Daim et al., 2021).

The objective of this research was to determine the fish Labeo rohita fingerlings' growth performance will be affected by Azolla supplementation. Weight gain, weight gain as a percentage, specific growth rate (SGR), food conversion ratio (FCR), and gross conversion efficiency (GCE) considerably exceeded controls in treatments. The highest weight increase (27.450±3.839g), percent weight gain (45.863±1.677%), SGR (1.024±0.051%), and GCE (0.257±0.010) were however observed in T2 (200g Azolla per kg basal diet). Compared to other treatments, the fish given T2 showed improved food utilization and a lower food conservation ratio (FCR), measuring 3.903±0.163. Hence, it can be said that a food supplemented with @200g/kg Azolla performed a significant part in enhancing the development of Labeo rohita. For L. rohita, it is therefore suggested to supplement its food with 200 g/kg of Azolla (Ojha et al., 2017).

The major objective of this study is to examine the effect of supplemental feeding, Azolla pinnata, and probiotics on the protein and amino acid content, as well as the quality and profile of lipidty acids in patin fish oil extracts. Patin fish were fed one of three distinct feeding regimens in this study: pellets alone (P1), A. pinnata plus pellets (P2), or pellets and probiotics (P3). These fish were evaluated based on their development pattern, the quality of their fish oil, and the amount of protein and amino acids they contained. With the highest body weight in comparison to other fish samples, P3 has the greatest development profile. Analysis of the lipidty acid composition using gas chromatography was combined with analysis of the acid value, saponification number, peroxide value, and iodine number to assess the quality of fish lipids. As a consequence of dried rendering, the data demonstrate that P3 produced the maximum oil production from patin fish meal. Moreover, P3 has the lowest peroxide value and saponification number. The main components of the unsaturated and saturated lipidty acids in all of these fish samples are oleic acid and palmitic acid, respectively. The Kjeldahl technique was used to evaluate P3's greatest crude protein concentration. In P1, P2, and P3, tryptophan

constitutes 13 amino acids, asparagine is 14 amino acids, and glutamic acids constitute 13 amino acids, making up the majority of the amino acid composition in each protein (Oktavianawati et al., 2016).

The objective of this study was to evaluate the impact of using fresh A. pinnata in replacement of commercial fish feed (CFF) for Thai silver barbs (Barbonymus gonionotus). Post fingerlings of B. gonionotus were raised in five treatments, marked T1 to T5, by replacing 0%, 25%, 50%, 75%, and 100% of the protein in CFF with A. pinnata protein (dried matter basis), respectively, for 56 days. The fish raised entirely on CFF and those with a 25% replacement of A. pinata did not vary greatly in their specific growth rate, net production rate, protein efficiency ratio, proximate composition, or general characteristics. However, in the case where A. pinnata was substituted for more than 25% of the CFF, there was a considerable decline in fish development and other parameters that were observed. In T5, fish fed just A. pinata displayed the lowest performance. As per the studies, it is feasible to substitute 25% of the CFF of Thai silver barb with fresh A. pinata without significantly affecting growth or product quality, which might increase profit margin (Das et al., 2018).

The potential of Azolla filiculoides Lam. as fish food was studied through pond cultivation. The amount of growth in the pond, (Komoike pond, Sakai), 1,200 kg/l00 m2, was smaller than that in a synthetic fertilizer solution and a dilute secondary treated effluent. The gradual decrease in growth in the pond water was mostly impacted by phosphorus deficiency. Azolla plants may be a suitable source of fish feed because of their considerable crude protein content and higher lysine concentration. Azolla cultivated for three days indicated a significantly higher amount of dehydro-ascorbic acid (1,909 mg kg-1 dried weight) than L-ascorbic acid (10 mg kg-1 dried weight). During this time, the ratio of linolenic acid in the lipidty acid composition of Azolla slowly increased. In a feeding experiment with Tilapia nilotica, a feed incorporating 20.7, 34.4, and 48.2% of the weight of dried Azolla, fed for three weeks, caused a decline in a

fish weight gain of 5.2, 16.8, and 17.1%, respectively, in comparison to the control (Shiomi et al., 2001).

The objective of the comparative study was to compare the growth rates, biomass yields, and proximate composition of six distinct Azolla species. Also, the essential amino acids (EAA) of several Azolla species during the linear growth period were studied. To test the effectiveness of dried Azolla mixture as a feed ingredient in the diet of Labeo rohita, a feeding trial was carried out in the cement tanks (8.0 m × 1.25 m × 1.0 m). Azolla mixture was included in the diet at levels of 15%, 25%, and 35%. The diet containing a 25% Azolla mixture and a specific growth rate of 0.7468% per day resulted in the highest weight gain. The experimental fish reported the value of exponent "b" in the range of 2.5155 to 2.7760. The condition factor "K" of each experimental fish was above 1.0 (1.2237-1.2326), showing that the fish were in good condition. Azolla was supplemented with fish diets, which lowered the amount of lipid in fish muscle (Datta et al., 2011).

The objective of this research was to evaluate the growth performance of Nile tilapia, Oreochromis niloticus, fed on fresh and dried Azolla pinnata as a partial or complete replacement of the control diet for six months in 16 outdoor concrete ponds (about 2.5 m water/ pond). For each treatment, growth, feed conversion ratio, protein intake, and body composition were all studied. Results indicate that when Azolla meal was supplemented with the control diet up to 50%, O. niloticus growth and feed utilization were not significantly affected ($P>0.05$). Fish fed just on fresh Azolla alone, however, showed exceedingly low development performance ($P<0.05$). In terms of body composition, the amount of protein and lipids declined as the amount of Azolla in the diet increased, achieving their lowest levels when Azolla was consumed fresh only. however, there was an inverse correlation with ash content (Tharwat et al., 1999).

During 70 days, Etroplus suratensis were fed diets with different percentages (10, 25, 50, 75, 90, and 95%) of dried Azolla powder. The percentage weight increases and food conversion ratios (FCRs) obtained for a fishmeal-based control diet were not considerably different (P>0.05) from those obtained at 10% and 25% dietary inclusion levels. Fish given diets with greater dietary inclusion levels (50, 75, 90, and 95%) of dried Azolla powder, on the other hand, showed considerably lower percentage weight gain and higher (P0.05) FCRs. A rise in dietary levels of Azolla above 25% resulted in an essentially linear decline in growth performance and feed utilization efficiency. There were no appreciable changes in the muscle composition of the fish given the control diet and diets containing lower levels of Azolla (10% and 25%) (P>0.05). Fish given diets with higher Azolla levels showed muscular tissue which had considerably higher moisture content and reduced protein and lipid contents (P<0.05). The results suggested that up to a level of 25%, dried Azolla powder may be included in the diet of E. suratensis (Joseph et al., 1994).

The effects of feeding Bacillus subtilis fermented Azolla (BSFA) on nonspecific immunity, antioxidative status, intestinal digesting enzymes and histomorphometry, and disease resistance in Nile tilapia were studied for 95 days. Five isonitrogenous and isocaloric experimental diets were prepared with BSFA at a concentration of 1%. (0 percent, 15 percent, 30 percent, 45 percent, 60 percent). The BSFA30 tilapia group showed a considerable rise in growth performance parameters (FBW, BWG, SGR, PER, and FCR) as compared to the control group, followed by BSFA45 (P 0.05). When compared to the other groups, the BSFA30 group had the highest non-specific immunity parameters (lysozyme activity, phagocytic index, and phagocytic activity) (P 0.05). Surprisingly, Nile tilapia fed with the BSFA15 diet had the highest level of protease activity (P 0.05), while those fed the BSFA30 diet had the highest level of amylase activity (P 0.05). Surprisingly, Nile tilapia fed the BSFA15 diet had the highest level of protease activity (P 0.05), while those fed the BSFA30 diet had the highest level of

amylase activity. The progressive increase in administered BSFA improved the histomorphology of the intestines significantly. BSFA significantly reduced cumulative mortality in tilapia disease resistance against Aeromonas septicemia as compared to the control group. To summarise, BSFA was more effective in boosting Nile tilapia development and immunity (Ismail et al., 2022).

The study improved the stocking density for ponds where carp are raised in polyculture using Azolla according to three different treatments, T1: 10,000 fish ha-1, T2: 11,500 fish ha-1, and T3: 12,500 fish ha-1. Moreover, six months were spent growing fish (Hypophthalmichthys molitrix, Catla catla, Labeo rohita, Cirrhinus cirrhosus, Cyprinus carpio, Ctenopharyngodon idella, and Barbonymus gonionotus). H. molitrix, C. catla, L. rohita, C. cirrhosus, C. carpio, C. idella, and B. gonionotus, with mean initial stocking weights of 60, 65, 58, 52, 61, 70, and 22 g. There were three replications of each treatment in this research. Liming (250 kg ha-1) and basal fertilization with TSP, urea, and cow dung were used in all of the treatments. Triple Super Phosphate (TSP) (2.5 kg ha-1 day-1 in all treatments) was used as a periodic fertilizer. Weight increase and Specific Growth Rate (SGR) of fish were assessed monthly, whereas water quality parameters (temperature, clarity, DO, pH, alkalinity, and free CO2) were measured every two weeks. Also, the economics of fish farming were evaluated in terms of the total cost, gross benefit, net profit margin, and cost-benefit ratio (CBR). Between the treatments, there were no notable differences in the mean values of the water quality measures. Except for survival rate, all growth metrics demonstrated a significant difference ($P<0.05$) from the treatments. The mean total yield for treatment T3 varied more substantially ($P< 0.05$), whereas treatment T1 (fish stocking density of 10,000 ha-1) was shown to be the best in terms of the total cost, gross benefit, net benefit, net profit margin, and CBR. The research showed that 10,000 fish per hectare of economical Azolla-based fish farming in Bangladesh can be a good option. (Asadujjaman et al., 2016).

The effects of using Azolla filiculoides as a substitute protein source on Nile tilapia, Oreochromis niloticus, growth rates, and productivity were assessed. Three identical ponds of male O. niloticus (starting to mean weight = 15.5 g) were fed three isonitrogenous (27 percent CP) and isocaloric (4019.14kcal/g) diets, A0, A10, and A20, containing three levels of Azolla 0, 10, and 20, accordingly. The control diet contained zero percent Azolla. After 90 days of the experiment, the growth performances and production are satisfactory in all treatments. Daily rates of growth (1.15-1.26 g/day), specific growth rates (2.27-2.35 percent/day), survival (74.78-77.43 percent), annual fish yield (31.01-32.75 kg/acre/year), and feed conversion ratio did not significantly differ across dietary treatments (P > 0.05) (1.06-1.13). Fish fed with A0 and those fed with A20 showed comparable growth and output results. Therefore, adding up to 20% of Azolla to the O. niloticus diet had no negative effects on either growth or production. With rising Azolla levels in the diet, the cost-benefit analysis showed a significant decrease in the production costs of one kilogram of fish, equivalent to US$0.09 between A0 and A10 (P > 0.05) and US$0.14 between A0 and A20 (P 0.05). These results indicate that using Azolla at up to 20% of a diet can perform as a viable substitute source of protein for O. niloticus in tropical wetlands on an isonitrogenous and isocaloric basis (Abou et al., 2007).

In the study, 25% and 30% of dried Azolla were used to make fish diets using wheat flour, fish meal, and groundnut oil cake. Fish feeding studies using Catla fries were undertaken in tanks using commercial feed (C), 25% Azolla substitution (T1), and 30% Azolla substitute (T2) diets. Fish reared on Azolla-replaced feed formulations exhibited faster development and a 2% increase in body weight compared to the control. Additionally, fish reared on dried Azolla-based diets displayed greater survival rates and nutritional characteristics, mainly carbohydrate and protein, than fish reared on commercial feed. This study confirms the use of dried Azolla as a low-cost nutritional component in fish meals (Rajan et al., 2021).

The main objective of the current feeding experiment was to determine the effects of switching from fishmeal to zooplankton biomass meal (ZBM) on grey mullet, Mugil Cephalus (initial weight of 0.10 ± 0.01 g)., growth performance, nutritional consumption, gut, and liver histological alterations. The control diet (Z0) and the other four diets (Z25, Z50, Z75, and Z100), in which 25%, 50%, 75%, and 100% of the fishmeal was substituted by ZBM, respectively, were isoproteic (35 percent crude protein) and isolipidic (8% crude lipid) diets. The grey mullet on the Z100 diet had greater final weights, weight gains, and daily growth indices than those on the control diet after 60 days of feeding ($p<0.05$). Additionally, fish given the Z100 diet showed improved feed conversion ratio, protein efficiency ratio, and lipid efficiency ratio values. ZBM insertion also substantially increased muscle thickness, crypt depth, and intestinal villus length ($p<0.05$). When compared to the control group, no histopathological alterations were found in the liver. Economically stated, replacing fishmeal in the diet with ZBM (Z100) lowers the price per kg of weight gain by around 40% and the expense of feed additives by 18%. Overall, the findings of this study indicated that replacing fishmeal with ZBM up to 100% might enhance the growth performance, feed consumption, gut health, and economic viability of raising juvenile M. cephalus (Taleb et al., 2021).

In this study, the advanced fingerlings of Labeo rohita were raised in aerated indoor plastic tanks, to assess the dried matter and nutrient digestibility of dried Azolla powder mixed with the control diet. The principal components of the Control diet, groundnut cake, and rice bran, were replaced at 10, 20, 30, and 40% levels with Azolla powder in the almost isocaloric diets for the fish. using acid-insoluble ash as the indicator, the digestibility of total dried matter and key nutrients were calculated. With increasing Azolla incorporation, there was an inclination for the digestibility (%) of dried matter, protein, lipid, and nitrogen-free extract (NFE) to decline. Only the protein and lipid digestibility of the 20% Azolla diet was similar to that of

the Control, with greater incorporation levels leading to a considerable decline in the digestibility values (Gangadhar et al., 2021).

## 1.7 Objectives

- To evaluate the proximate analysis of Azolla.
- Formulation of Fish Feed Supplemented with Azolla.
- Effect of Azolla – based formulated diet on the growth and well-being of Asian Sea Bass.
- The following parameters will be invested for the comparative studies after the feeding trials:

1. Food conservation ratio (FCR)
2. Growth of the fish
3. Conditioning factor (‘K’)
4. Muscle tissue biochemical composition

**1.8 Study plan**

Phase I - Azolla cultivation and drying (Shiomi et al., 2012)

Phase II- Proximate analysis of Azolla **(**AOAC, 2000)

Phase III – Formulation of fish feed (R. Shakti et al., 2021)

Phase IV: - Fish-feeding experiments (Mosha et al., 2020)

Phase V: - Growth performance and Feed utility in Sea Bass (Kumari et al., 2017)

Phase VI: - Data Collection and Statistical Analysis

## 2. MATERIALS AND METHODS

**Species of Azolla: Azolla pinnata**

**Phase I - Azolla cultivation and drying (Shiomi et al., 2012).**

Fresh Azolla was brought from a shallow pond near Goa University and cultivated in a small container using soil and water mixed with NPK fertilizer spread across the container. After two weeks, the container was completely covered with grown Azolla. It was then harvested, thoroughly washed with clean water, and kept in the sunlight for drying. It was then kept in the oven overnight to remove all moisture and used for the proximate analysis.

**Phase II- Proximate analysis of Azolla (AOAC, 2000)**

The proximate analysis of both fresh and sun-dried Azolla was done. Both samples were put through standardized procedures for various nutritional analyses.

Proximate principles including, dried matter, crude protein, ether extract, crude fibre, total ash, and moisture were done as per standards using, the Association of Analytical chemistry methods, (2000).

**Processing of Azolla sample:**

5g of fresh Azolla leaves were homogenized in 20 ml of ethanol. The solution was centrifuged and the supernatant was used for the analysis of protein carbohydrates.

**Protein (Lowry et al., 1951)**

The phenolic group of tyrosine and tryptophan residues (amino acids) in a protein will form a blue colour complex with maximal absorption in the area of 660 nm wavelength when using the folin-Ciocalteu reagent, which is composed of sodium tungstate molybdate, and phosphate. As a result, the presence of these aromatic amino acids affects the colour intensity, which varies for various proteins.

**Preparation of Reagents:**

1. Lowry's reagent:

- 4% sodium carbonate was prepared by dissolving 4g of sodium carbonate.
- 2% copper sulphate was prepared by dissolving 2g of copper sulphate
- 4% sodium potassium tartrate was prepared by dissolving 4g of sodium potassium tartrate in 100 mL of distilled water.
- All these solutions were mixed (a+b+c) in (98 ml + 1 ml + 1 ml) to prepare Lowry's reagent.

2. Folin's reagent was prepared by adding 10 mL of Folin-Ciocalteu reagent to 10 mL of distilled water (10:10).
3. BSA Standard: 5mg BSA was dissolved in 20 ml of NaOH (freshly prepared).

**Protocol:**

1. 1 ml of the Azolla sample was added to each of the 5 test tubes.
2. 5 ml of Lowry's reagent was added and incubated for 15 minutes at room temperature.
3. After 15 minutes, 0.5 ml of Folin's reagent was added to all 5 test tubes and incubated at room temperature for 10 minutes.
4. Absorbance was measured at 660 nm using a spectrophotometer.
5. BSA was used as the working standard.

**Carbohydrate (Hedge and Hofrreiter, 1962)**

Concentrated $H_2SO_4$ dehydrates carbohydrates, forming furfural in the process. The enol tautomer of Anthrone, which is the active form of the reagent, interacts by condensing with the carbohydrate furfural derived to produce a green color in diluted solutions and a blue color in concentrated solutions. The blue-green solution exhibits a peak in absorption at 620 nm.

**Reagents**:

1. Anthrone reagent: 2g of Anthrone was dissolved in 1 liter of concentrated $H_2SO_4$
2. Glucose stock solution: 200 g of glucose was dissolved per mL of distilled water.

**Protocol**:

1. 1 mL of the Azolla sample was added to each of the 5 test tubes, and then 5 mL of Anthrone was added.
2. The tubes were then kept in a boiling water bath for 15 minutes.
3. OD was taken at 620 nm using a spectrophotometer.
4. Finally, using the standard glucose graph, the quantification of all carbohydrates was calculated.

**Lipid (Bligh and Dye, 1959)**

- **Reagents**: Methanol, Chloroform

**Protocol**:

1. 1g of Azolla sample was weighed and homogenized with 20 ml of a 2:1 chloroform: methanol mixture in a mortar and pestle.

2. The sample was then covered with foil in a beaker.

3. Then the beaker was kept in the sonicator bath for 2 hours.

4. The aqueous solution was then transferred into the separating funnel overnight.

5. Two different aqueous phases were visible after homogenization; the upper layer was separated and measured using the measuring cylinder as the total lipid content in Azolla.

**Fibre (AOAC, 2000).**

Cellulose and lignin make up about 97% of crude fibre, along with small amounts of other mineral components. The amount of energy in the feed may be roughly estimated using the crude fibre content. The natural cellulose is significantly degraded by oxidative hydrolysis and the subsequent alkali digestion significantly deteriorates lignin. After final filtering, the residue is collected, which is then weighed, ignited, cooled, and weighed once again. The weight loss indicates the crude fibre content.

**Reagents**:

0.255 N sulphuric acid

0.255 N sodium hydroxide

10% potassium sulphate

**Protocol:**

1. 2g of defatted Azolla sample was in 200 ml of 0.255 N sulphuric acid for 20 minutes.
2. The extract was filtered through four to five folds of muslin cloth before it was washed in hot water.
3. The residue was again filtered through muslin cloth for 20 minutes of boiling in 200 mL of 0.255N sodium hydroxide solution.
4. After that, the residue was rinsed with cold water, 10% potassium sulfate, boiling water, and at last alcohol.

5. The extra material was dried for 1 hour in a hot air oven set to 110°C, cooled in a desiccator, and weighed.
6. The dried contents were heated for 20 minutes in a muffle furnace, then cooled and weighed. Crude fibre has been demonstrated by weight reduction.

**Calculation:**

W1: loss in weight.

W: weight of sample used

Crude fibre (%) = W1/W ×100

**Estimation of Moisture (AOAC, 2000).**

The sample was weighed at around 1 g in the crucible. It was subsequently heated to 105 + 1 degrees Celsius for 4 hours. The crucible was taken out, put in desiccators to cool, and then weighed. At 30-minute intervals, the drying, chilling, and weighing processes were repeated until there was less than 1 mg of difference between the two resulting weights. The lowest weight was noted. then the following formula was used to get the percentage of moisture content.

**Calculations:**

W1 (g)= weight, in g, of the dish with the material before drying

W2 (g) = weight of the crucible with the material after drying

W (g)= weight, in g, of the empty crucible

Moisture content (%) = 100 × (W1-W2)/ (W1-W)

**Estimation of Ash (AOAC, 2000).**

Ash content is an estimate of the sample mineral content. The sample was weighed at about 1g and heated in a hot air oven for about an hour to remove extra moisture and lipid. The crucible

was then ignited in the muffle furnace at 500 °C for 4 hours until ash was collected. The crucible was taken out, cooled in desiccators, and weighed. The following formula was used to determine the percentage of ash content.

**Calculations:**

W1 (g) = weight of the empty crucible

W2 (g)= weight of the empty crucible with ash

W (g) = weight of the test sample

Ash content (%) = 100 × (W2-W1)/W

**Total Carotenoid estimation (Singh et al., 2003)**

85% acetone was used as the solvent to extract the total carotenoids from the fern Azolla. 20 ml of acetone was added to the exact weight of 5g of fresh leaves in a 50 ml Falcon tube. To separate the two phases, the mixture was mashed in a mortar and pestle and centrifuged at 3000 rpm for 10 minutes. The pigment-containing supernatant was collected and kept at 4°C for further use. Acetone was used to extract the sample repeatedly until the supernatant was colourless. A final known volume was created by combining all the supernatant fractions. At 450 nm, optical density was measured using 85% acetone as a blank.

**Calculations:**

A= absorbance measured

V= total extract volume

W (g)= sample weight

2500= β-carotene absorption coefficient in acetone

Content of carotenoids (µg/g) = A×V(ml) ×104 /A1% ×W(g) (Oliverial *et al*., 2017)

**Phase III – Formulation and proximate analysis of fish feed (R. Shakti et al., 2021)**

**Sundried Azolla was used in the feed formulation since it has a higher protein content than fresh Azolla.**

| Diet | No. of juveniles | Azolla | CFF (Commercial Fish Feed) |
|---|---|---|---|
| **Control** | **8** | **-** | **100%** |
| **Experimental Diet 1** | **8** | **10%** | **90%** |
| **Experimental Diet 2** | **8** | **15%** | **85%** |
| **Experimental Diet 3** | **8** | **20%** | **80%** |

**Diet formulation:** The Control diet included commercial fish feed brought from Green Lake Farm, Majorda, Goa, partially replaced with dried Azolla in the three experimental diets that were cultured and sundried in the Animal House, Goa University. Fish weights were used to weigh out the ingredients, which were then manually mixed with water and 5% oil to form the dough. The dough was then cooked for 5–10 minutes in warm water. The extruder was used to make the noodles, which were then dried in the oven for three days before getting crushed to obtain small particles. Fish were fed 10% of their body weight twice daily at 9:00 am and 2:00 pm. Every fish in every tank was weighed separately twice every week. The feeding portions were then modified in consideration of the updated weights. After 45 days of the feeding study, all fish were euthanized in iced water, weighed, and tests were performed.

**Proximate analysis of the formulated fish feed was performed separately for commercial fish feed as a control and also for 10, 20, and 30 percent of the Azolla-incorporated experimental diets.**

**PROTEIN TEST (Lowry et al., 1951).**

The phenolic group of tyrosine and tryptophan residues (amino acids) in a protein will form a blue color complex with maximal absorption in the area of 660 nm wavelength when using the folin-Ciocalteu reagent, which is composed of sodium tungstate molybdate, and phosphate. As a result, the presence of these aromatic amino acids affects the colour intensity, which varies for various proteins.

**Preparation of Reagents:**

1. Lowry's reagent:

- 4% sodium carbonate was prepared by dissolving 4g of sodium carbonate.
- 2% copper sulphate was prepared by dissolving 2g of copper sulphate
- 4% sodium potassium tartrate was prepared by dissolving 4g of sodium potassium tartrate in 100 mL of distilled water.
- All these solutions were mixed (a+b+c) in (98 ml + 1 ml + 1 ml) to prepare Lowry's reagent.

2. Folin's reagent was prepared by adding 10 mL of Folin-Ciocalteu reagent to 10 mL of distilled water (10:10).
3. BSA Standard: 5mg BSA was dissolved in 20 ml of NaOH (freshly prepared).

**Protocol:**

1. 1 ml of supernatant of control, 10%, 15%, and 20% Azolla-incorporated experimental diets was added to the test tubes.
2. 5 ml of Lowry's reagent was added and incubated for 15 minutes at room temperature.
3. After 15 minutes, 0.5 ml of Folin's reagent was added to all 5 test tubes and incubated at room temperature for 10 minutes.
4. Absorbance was measured at 660 nm using a spectrophotometer.
5. BSA was used as the working standard.

**Carbohydrate (Hedge and Hofrreiter, 1962)**

Concentrated $H_2SO_4$ dehydrates carbohydrates, forming furfural in the process. The enol tautomer of Anthrone, which is the active form of the reagent, interacts by condensing with the carbohydrate furfural derived to produce a green colour in diluted solutions and a blue colour in concentrated solutions. The blue-green solution exhibits a peak in absorption at 620 nm.

**Reagents**:

Anthrone reagent: 2g of Anthrone was dissolved in 1 liltre concentrated $H_2SO_4$

Glucose stock solution: 200 g of glucose was dissolved per mL of distilled water.

**Protocol**:

1. 1 ml of supernatant of control, 10%, 15%, and 20% Azolla-incorporated experimental diets was added to the test tubes, and then 5 mL of Anthrone was added.
2. The tubes were then kept in a boiling water bath for 15 minutes.
3. OD was taken at 620 nm using a spectrophotometer.

4. Finally, using the standard glucose graph, the quantification of all carbohydrates was calculated.

**Lipid (Bligh and Dye, 1959)**

**Reagents**: Methanol, Chloroform

**Protocol**:

1. 1g of control, 10%, 15%, and 20% Azolla-incorporated experimental diets was weighed and homogenized with 20 ml of a 2:1 chloroform: methanol mixture in a mortar and pestle.
2. The sample was then covered with foil in a beaker.
3. Then the beaker was kept in the sonicator bath for 2 hours.
4. The aqueous solution was then transferred into the separating funnel overnight.
5. Two different aqueous phases were visible after homogenization; the upper layer was separated and measured using the measuring cylinder as the total lipid content in Azolla.

**Fibre (AOAC, 2000).**

Cellulose and lignin make up about 97% of crude fibre, along with small amounts of other mineral components. The amount of energy in the feed may be roughly estimated using the crude fibre content. The natural cellulose is significantly degraded by oxidative hydrolysis and the subsequent alkali digestion significantly deteriorates lignin. After final filtering, the residue is collected, which is then weighed, ignited, cooled, and weighed once again. The weight loss indicates the crude fibre content.

**Reagents**:

- 0.255 N sulphuric acid
- 0.255 N sodium hydroxide

- 10% potassium sulphate

**Protocol:**

1. 2g of control, 10%, 15%, and 20% Azolla-incorporated experimental diets were boiled in 200 ml of 0.255 N sulfuric acid for 20 minutes.
2. The extract was filtered through four to five folds of muslin cloth before it was washed in hot water.
3. The residue was again filtered through muslin cloth for 20 minutes of boiling in 200 mL of a 0.255 N sodium hydroxide solution.
4. After that, the residue was rinsed with cold water, 10% potassium sulphate, boiling water, and, at last, alcohol.
5. The extra material was dried for 1 hour in a hot air oven set to 110°C, cooled in a desiccator, and weighed.
6. The dried contents were heated for 20 minutes in a muffle furnace, then cooled and weighed.

**Calculation:**

Crude fibre (%) = W1/W ×100

W1= loss in weight.

W= weight of sample used

**Estimation of Moisture (AOAC, 2000).**

1g of each of the four different compositional diets (control, 10%, 15%, and 20%) was weighed. It was subsequently heated to 105 + 1 degrees Celsius for 4 hours. The crucible was taken out, put in desiccators to cool, and then weighed. At 30-minute intervals, the drying, chilling, and weighing processes were repeated until there was less than 1 mg of difference between the two

resulting weights. The lowest weight was noted. then the following formula was used to get the percentage of moisture content:

**Calculations:**

W1 (g)= weight, in g, of the dish with the material before drying.

W2 (g) = weight of the crucible with the material after drying.

W (g)= weight, in g, of the empty crucible.

Moisture (%) = 100 × (W1-W2)/ (W1-W)

**Estimation of Ash (AOAC, 2000).**

Ash content is an estimate of the mineral content of the sample. 1g of each of the four different compositional diets (control, 10%, 15%, and 20%) was weighed and heated in a hot air oven for about an hour to remove extra moisture and lipid. The crucibles were then ignited in the muffle furnace at 500 °C for 4 hours until ash was collected. The crucible was taken out, cooled in desiccators, and weighed. The following formula was used to determine the percentage of ash content:

**Calculations:**

W1 (g) = weight of the empty crucible

W2 (g)= weight of the empty crucible with ash

W (g) = weight of the test sample

Total ash (%) = 100 × (W2-W1)/W

**Total Carotenoid estimation (Singh et al., 2003)**

85% acetone was used as the solvent to extract the total carotenoids from the different compositions of Azolla-incorporated diets (control, 10%, 15%, 20%). 20 ml of acetone was added to the exact weight of 5g of fresh leaves in a 50 ml Falcon tube. To separate the two

phases, the mixture was mashed in a mortar and pestle and centrifuged at 3000 rpm for 10 minutes. The pigment-containing supernatant was collected and kept at 4°C for further use. Acetone was used to extract the sample repeatedly until the supernatant was colourless. A final known volume was created by combining all the supernatant fractions. At 450 nm, optical density was measured using 85% acetone as a blank.

**Calculations:**

A= absorbance measured

V= total extract volume

W (g)= sample weight

2500= β-carotene absorption coefficient in acetone

Carotenoid content (μg/g) = A×V(ml) ×104 /A1% ×W(g) (Oliverial *et al*., 2017)

**Phase IV: Fish feeding experiments (Mosha et al., 2020)**

Fish-feeding experiments were carried out in the Animal House of Goa University. 40 juvenile Asian sea bass were brought from Green Lake Farm, Majorda, Goa, and housed in the Aqua room of the Animal House. Eight juveniles were then placed in each of the four tanks after they had been well-cleaned and filled with 20 litres of tap water from the Animal House. Before experimenting, fish underwent a two-week acclimatization period. Water was exchanged at a rate of 20% of the total volume. The total experimental duration was 45 days. Fish were fed 10% of their average body weight, twice a day. Dried Azolla-based fish feed formulations T1–10% Azolla was replaced by T2-15% and T3-20%. Azolla was replaced with commercial feed, and the C-Control is only commercial feed. After 10 minutes, the leftover feed was removed from the water to maintain the quality of the water. After the feeding trial of 40 days, fishes were starved for 12 hours and then, all the fish were euthanized in ice water, weighed, and tests were conducted.

**Phase V: Growth Performance and Feed Utility in Asian Sea Bass (Kumari et al., 2017)**

After the experiment, the nutritional index characteristics of the tested feed, including the survival rate (SR), weight gain (WG), length gain (LG), feeding rate, specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER), were evaluated to study the effect of the Azolla-incorporated diet on the fish. (Tekinay and Davis, 2001).

1. **Food conservation ratio (FCR) (Kumari et al., 2017)**

FCR (g) = 100 × (weight gain/total feed intake)

2. **Growth parameters of the fish (Kumari et al., 2017)**

- **Weight Gain (g)** = Final weight (g) – Initial weight (g)/ Initial weight (g)
- **Length Gain (cm)** = Final Length (cm) – Initial Length (cm)/ Initial Length (cm)
- **Specific Growth Rate (SGR)** = Final weight (g) – Initial weight (g)/ time (duration in days) X 100
- **Daily Growth Index (%)** = Final body weight 1/3 – Initial body weight1/3/ time (duration in days) X 100
- **Survival Rate (%)** = Final number of fish/ Initial number of fish X 100

3. **Conditioning factor ('K') (Adorian et al., 2018)**

- **Condition Factor (%) =** (Final weight/ Final length3) x100

4. **Protein efficiency ratio (PER) (Erlinda et al., 2014)**

- **(PER) =** Weight gain (g)/Protein intake (g) x100

5. **Lipid efficiency ratio (LER)**

- **(LER) =** Weight gain (g)/Lipid intake (g) x100

6. **Muscle tissue biochemical composition**

**Sample preparation**

**Reagents:**

**Phosphate buffer**: 0.077g of Na2HPO4 and 0.029g of NaH2PO4 were dissolved in 50 ml of distilled water, adjusting the pH to 7.

All the fish were weighed and dissected to remove the muscle mass for further analysis. 5g of muscle tissue of each fish in the 4 consecutive experimental tanks, i.e., control, 10%, and 15%. 20% was homogenized individually in 10 ml of phosphate buffer and then centrifuged at 4000 rpm for 10 minutes. A supernatant was used for the analysis of proteins and carbohydrates, and the remaining muscle tissue was used to estimate the lipids as well as the total carotenoid, fibre, dried matter, ash, and moisture content.

**1. Protein (Lowry et al., 1951)**

The phenolic group of tyrosine and tryptophan residues (amino acids) in a protein will form a blue colour complex with maximal absorption in the area of 660 nm wavelength when using the folin-Ciocalteu reagent, which is composed of sodium tungstate molybdate, and phosphate. As a result, the presence of these aromatic amino acids affects the colour intensity, which varies for various proteins.

**Preparation of Reagents:**

1. **Lowry's reagent:**

- 4% sodium carbonate was prepared by dissolving 4g of sodium carbonate.
- 2% copper sulphate was prepared by dissolving 2g of copper sulphate
- 4% sodium potassium tartrate was prepared by dissolving 4g of sodium potassium tartrate in 100 mL of distilled water.
- All these solutions were mixed (a+b+c) in (98 ml + 1 ml + 1 ml) to prepare Lowry's reagent.

2. **Folin's reagent**

- was prepared by adding 10 mL of Folin-Ciocalteu reagent to 10 mL of distilled water (10:10).

**Protocol:**

1. 1 ml of the muscle tissue extract of 8 individual fish from each of the 4 experimental tanks was added separately to test tubes.
2. 5 ml of Lowry's reagent was added to each test tube and incubated for 15 minutes at room temperature.
3. After 15 minutes, 0.5 ml of Folin's reagent was added to all the test tubes and incubated at room temperature for 10 minutes.
4. Absorbance was measured at 660 nm using a spectrophotometer.
5. Finally, using the BSA standard graph, the quantification of total protein was computed.

2. **Carbohydrate (Hedge and Hofrreiter, 1962)**

Concentrated $H_2SO_4$ dehydrates carbohydrates, forming furfural in the process. The enol tautomer of Anthrone, which is the active form of the reagent, interacts by condensing with the

carbohydrate furfural derived to produce a green colour in diluted solutions and a blue colour in concentrated solutions. The blue-green solution exhibits a peak in absorption at 620 nm.

**Reagents**:

- Anthrone reagent: 2g of Anthrone was dissolved in 1 litre of concentrated H2SO4
- Glucose stock solution: 200 g of glucose was dissolved per mL of distilled water.

**Protocol**:

1. 1 ml of the muscle tissue extract of 8 individual fish from each of the 4 experimental tanks was added separately to test tubes, and 5 ml of Anthrone was added to it.
2. The tubes were then kept in a boiling water bath for 15 minutes.
3. OD was taken at 620 nm using a spectrophotometer.
4. The OD was put in the standard equation obtained from the glucose standard graph to compare the total carbohydrate content in the fish fed with different compositional diets (control, 10%, 15%, and 20%).

**3. Lipid (Bligh and Dye, 1959)**

**Reagents**:

- Methanol
- Chloroform

**Protocol**:

1. 1g of muscle tissue from 8 individual fish from all 4 experimental tanks was weighed and homogenized with 20 ml of a 2:1 chloroform: methanol mixture in a mortar and pestle.

2. The sample was then covered with foil in a beaker.
3. Then the beaker was kept in the sonicator bath for 2 hours.
4. The aqueous solution was then transferred into the separating funnel overnight.
5. Two different aqueous phases were visible after homogenization; the upper layer was separated and measured using the measuring cylinder to compare the total lipid content in the fish fed with different compositions (control, 10%, 15%, and 20%).

**4. Fibre (AOAC, 2000).**

Cellulose and lignin make up about 97% of crude fibre, along with small amounts of other mineral components. The amount of energy in the feed may be roughly estimated using the crude fibre content. The natural cellulose is significantly degraded by oxidative hydrolysis and the subsequent alkali digestion significantly deteriorates lignin. After final filtering, the residue is collected, which is then weighed, ignited, cooled, and weighed once again. The weight loss indicates the crude fibre content.

**Reagents**:

- 0.255 N sulphuric acid
- 0.255 N sodium hydroxide
- 10% potassium sulphate

**Protocol:**

1. 2g of *defatted* muscle tissue sample of fish from all 4 experimental tanks was boiled in 200 ml of 0.255 N sulphuric acid for 20 minutes.
2. The extract was filtered through four to five folds of muslin cloth before it was washed in hot water.

3. The residue was again filtered through muslin cloth for 20 minutes of boiling in 200 mL of 0.255N sodium hydroxide solution.
4. After that, the residue was rinsed with cold water, 10% potassium sulphate, boiling water, and at last alcohol.
5. The extra material was dried for 1 hour in a hot air oven set to 110°C, cooled in a desiccator, and weighed.
6. The dried contents were heated for 20 minutes in a muffle furnace, then cooled and weighed. Crude fiber has been demonstrated by weight reduction.

**Calculations:**

W1= loss in weight.

W= weight of sample used

Crude fibre (%) = W1/W ×100

**5. Estimation of Moisture (AOAC, 2000).**

1g of muscle tissue of fish from each tank was weighed in the crucible. It was subsequently heated to 105 + 1 degrees Celsius for 4 hours. The crucible was taken out, put in desiccators to cool, and then weighed. At 30-minute intervals, the drying, chilling, and weighing processes were repeated until there was less than 1 mg of difference between the two resulting weights. The lowest weight was noted. then the following formula was used to get the percentage of moisture content.

**Calculations:**

W1 (g)= weight, in g, of the dish with the material before drying

W2 (g) = weight of the crucible with the material after drying

W (g)= weight, in g, of the empty crucible

Moisture (%) = 100 × (W1-W2)/ (W1-W)

**6. Estimation of Ash (AOAC, 2000).**

Ash content is an estimate of the mineral content of the sample. 1g of fish muscle tissue from all 4 experimental tanks was weighed in the crucible and heated in a hot air oven for about an hour to remove extra moisture and lipid. The crucible was then ignited in the muffle furnace at 500 °C for 4 hours until ash was collected. The crucible was taken out, cooled in desiccators, and weighed. The following formula was used to determine the percentage of ash content.

**Calculations:**

W1 (g) = weight of the empty crucible

W2 (g)= weight of the empty crucible with ash

W (g) = weight of the test sample

Total ash (%) = 100 × (W2-W1)/W

**7. Total Carotenoid estimation (Singh *et al*., 2003) (Oliverial *et al*., 2017)**

Fish were gutted and their heads removed before the measurement. Then, 1 g of fish muscle tissue from each treatment was weighed and placed in the marked 10 ml glass bottles. 2.5 g of anhydrous sodium sulfide was added to the sample and mixed gently using a glass rod against the vial wall. The chloroform was then added, and it was kept overnight at 0° C in a refrigerator. Each vial contained 0.3 ml of chloroform overall, which was taken out and diluted with 100% ethanol to make 3 ml. Similar steps were used to prepare a blank for comparison. The optical density was measured using a spectrophotometer at wavelengths 380, 450, 470, and 500 nm.

**Calculations:**

A= absorbance measured

V= total extract volume

W (g)= sample weight

2500= β-carotene absorption coefficient in acetone

Content of carotenoids (μg/g) = A×V(ml) ×104 /A1% ×W(g)


**Plate 1: Azolla cultivation**

**Plate 2: Collection of Azolla**

**Plate 3: Drying of Azolla**

**Plate 4: Azolla powder**


**Plate 5: Proximate Analysis of Azolla**


**Plate 6: proximate analysis of Exp. diets**

**Plate 7: Juvenile Asian Sea Bass**

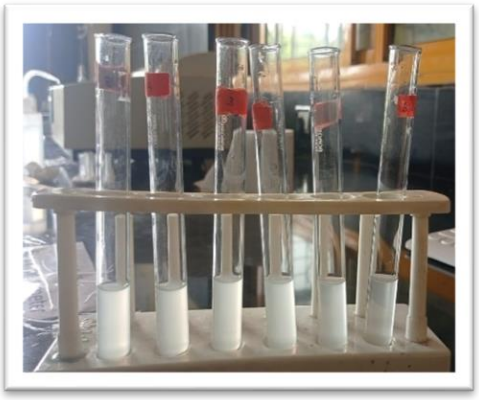


**Plate 8: Proximate analysis of fish carcass**


**Plate 9: Weighing of the fish**


**Plate 10: Length measurement of fish**

| Proximate principle | Dried Azolla | Fresh Azolla |
|---|---|---|
| **Crude Protein (%)** | 30 | 25.4 |
| **Carbohydrate (%)** | 23.2 | 18.1 |
| **Crude Lipid (ml/g)** | 6.5 | 5.8 |
| **Crude Fibre (%)** | 18.5 | 14.8 |
| **Moisture** (%) | 11 | 18 |
| **Ash** (%) | 30 | 33 |
| **Carotenoids (µg/g)** | 6.9 | 7.5 |

**Table 1: Proximate Analysis of Dried and Fresh Azolla**

| Composition | Control (CFF) (C) | Exp. Diet 1 (10%) | Exp. Diet 2 (15%) | Exp. Diet 3 (20%) |
|---|---|---|---|---|
| **Crude Protein (%)** | 30 | 29.3 | 29.8 | 29.7 |
| **Carbohydrate (%)** | 6.06 | 6.04 | 6.13 | 6.2 |
| **Total Lipid (ml/g)** | 5.4 | 5.1 | 4.9 | 5.2 |
| **Crude Fibre (%)** | 13.6 | 13.2 | 13.09 | 13.14 |
| **Total Ash (%)** | 23.2 | 23.5 | 30 | 30.4 |
| **Total Moisture (%)** | 12 | 14.5 | 12.98 | 13.03 |
| **Total Carotenoids (µg/g)** | 4.8 | 5.11 | 5.06 | 5.31 |

**Table 2: Proximate Analysis of Control and experimental diets**

**Note: Control**: commercial fish feed; **Experimental diet 1**: diet with 10% Azolla; **Experimental. diet 2**: diet with 15% Azolla; **Experimental diet 3**: diet with 20% Azolla.

| Growth parameters | Control | | Experimental Group 1 (10%) | | Experimental Group 2 (15%) | | Experimental Group 3 (20%) | |
|---|---|---|---|---|---|---|---|---|
| | Initial | Final | Initial | Final | Initial | Final | Initial | Final |
| Weight Gain (gm) | 0.189995± 0.0067173 | 0.182086± 0.064377 | 0.290308± 0.102639 | 0.17792 ± 0.06290 4 | 0.208048± 0.073556 | 0.152684± 0.053982 | 0.193907± 0.068557 | 0.170079± 0.060132 |
| P Value | <0.0001 | | <0.0001 | | <0.0001 | | <0.0001 | |
| Length Gain (cm) | 0.176777± 0.0625 | 0.148805± 0.05261 | 0.225198± 0.07962 | 0.119523± 0.042258 | 0.306769± 0.108459 | 0.225198± 0.07962 | 0.256348± 0.090633 | 0.213391± 0.075445 |
| P Value | <0.0001 | | <0.0001 | | <0.0003 | | <0.0001 | |
| DGR (%) | 0.005191± 0.001835 | | 0.004398±0.001555 | | 0.006071± 0.002146 | | 0.005128±0.001813 | |
| P Value | | | 0.0015 | | 0.0005 | | P<0.0001 | |
| SGR (%) | 1.656608±0.585699 | | 1.399563±0.49482 | | 1.66119±0.587319 | | 1.365074±0.482627 | |
| P Value | | | 0.0019 | | 0.0011 | | 0.0002 | |
| Condition factor (k) | 0.050766±0.017949 | | 0.041959±0.014835 | | 0.062978±0.022226 | | 0.066295±0.023439 | |
| P Value | | | 0.3112 | | 0.0056 | | 0.0738 | |
| FCR | 0.274155±0.096929 | | 0.298479±0.105528 | | 0.239143±0.08455 | | 0.334857±0.11839 | |
| P Value | | | 0.0007 | | 0.0007 | | 0.06 | |
| PER (%) | 0.07139±0.02524 | | 0.051539±0.018222 | | 0.072366±0.025585 | | 0.060917±0.021538 | |

| | | | | |
|---|---|---|---|---|
| **P Value** | | 0.0019 | 0.0005 | 0.0001 |
| **LER (%)** | 0.01285±0.004543 | 0.008971±0.003172 | 0.011899±0.004207 | 0.010666±0.003771 |
| **P Value** | | 0.0048 | 0.0002 | 0.0001 |
| **Survival (%)** | 100% | 100% | 100% | 100% |

**Table 3: Means (±standard deviation) of growth parameters, condition factor, and food conversion ratio, of juvenile Asian Sea Bass fed experimental diets**

| Proximate Composition | Control | Experimental Group 1 (10%) | Experimental Group 2 (15%) | Experimental Group 3 (20%) |
|---|---|---|---|---|
| **Crude Protein (µg/ml)** | 79.23387± 28.01341 | 137.2797± 48.53572 | 23.98954± 8.481584 | 6.213144± 2.196678 |
| **P Value** | | 0.0451 | 0.0013 | 0.0002 |
| **Total Carbohydrates (mg/ml)** | 0.002381± 0.000842 | 0.005287± 0.001869 | 0.008745± 0.003092 | 0.003594± 0.001271 |
| **P Value** | | 0.0517 | | 0.0167 |
| **Total Lipid (ml/g)** | 0.795362± 0.281203 | 0.757966± 0.267981 | 0.725608± 0.256541 | 0.74457± 0.263245 |
| **P Value** | | <0.0001 | <0.0001 | <0.0001 |
| **Crude Fibre (%)** | 1.199869± 0.424218 | 0.766024± 0.27083 | 1.085744± 0.383869 | 0.757712± 0.267891 |
| **P Value** | | 0.3565 | 0.06 | 0.0403 |
| **Total Moisture (%)** | 1.933169± 0.683478 | 2.418825± 0.855184 | 2.372118± 0.83867 | 2.054916± 0.726522 |
| **P Value** | | 0.7554 | 0.2417 | 0.0641 |
| **Total Ash (%)** | 1.022113± 0.361371 | 1.502379± 0.531171 | 1.102995± 0.389968 | 0.514636± 0.181951 |
| **P Value** | | 0.136 | 0.0116 | 0.0019 |
| **Total Carotenoids (%)** | 0.0465 | 0.0589 | 0.08601 | 0.0862 |

**Table 4: Proximate muscle tissue composition of juvenile Asian Sea Bass, Lates calcarifer, after the feeding trial of 40 days**

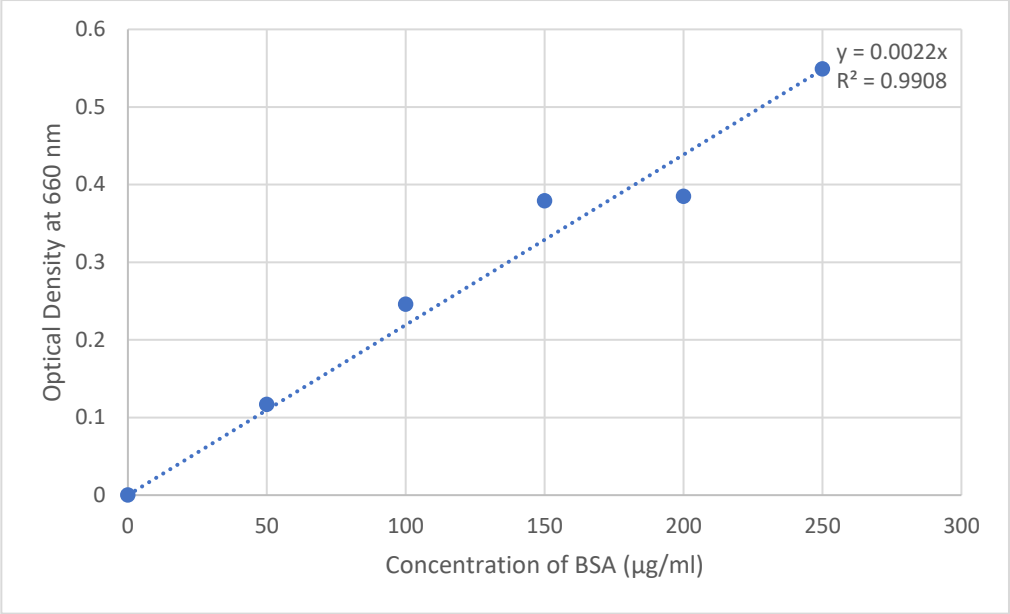



**Figure 1: Standard curve of protein (µg/ml)**

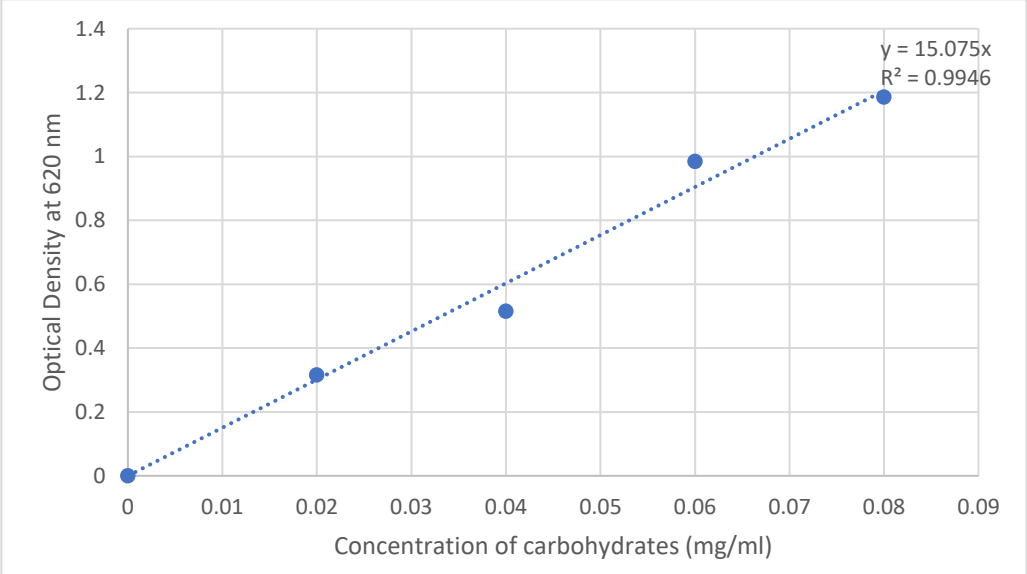



**Figure 2: Standard curve of carbohydrate (mg/ml)**

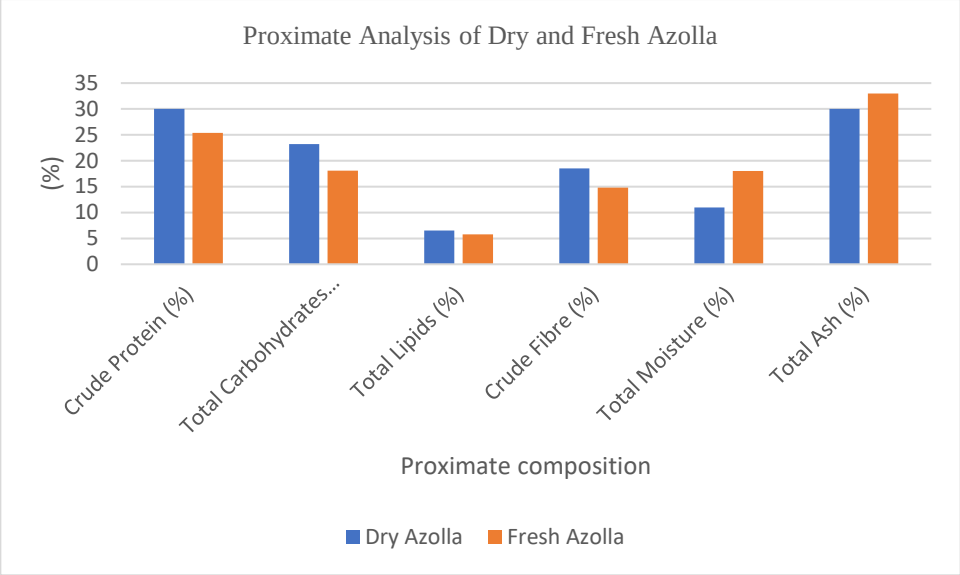



**Figure 3: Comparative study of Proximate Analysis of dried and fresh Azolla**

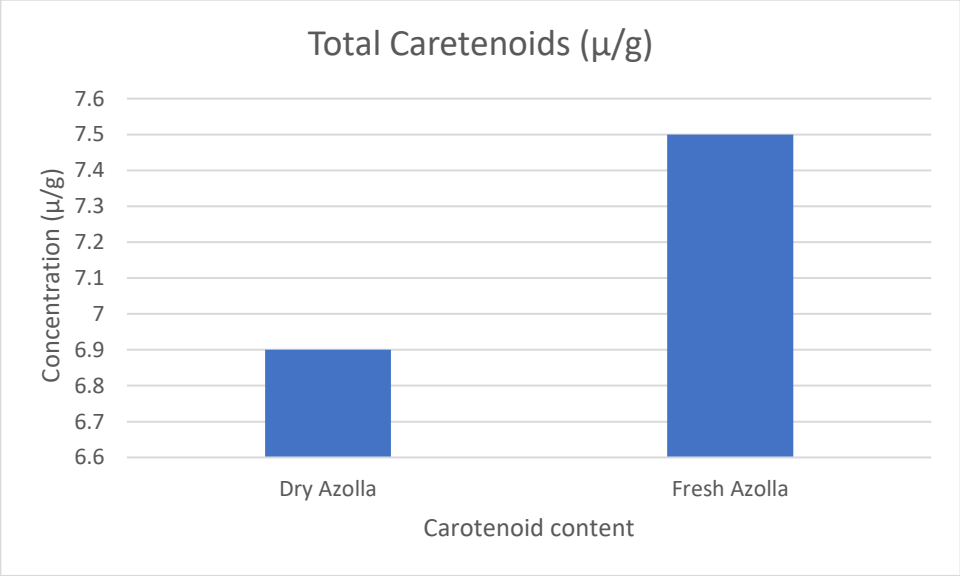



**Figure 4: Comparison of Carotenoid content in dried and fresh Azolla**

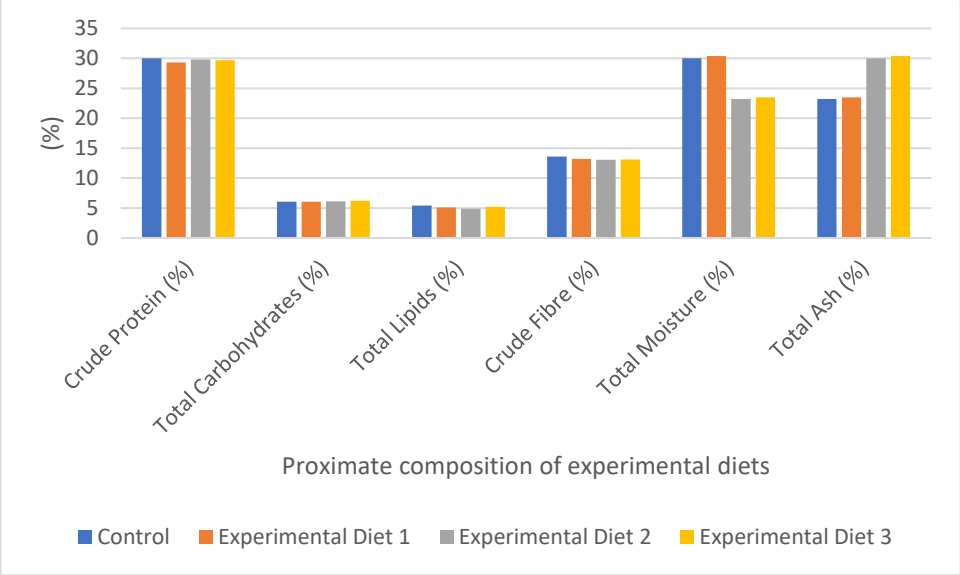



**Figure 5: Comparative study of Proximate Analysis of CFF and Experimental diet.**

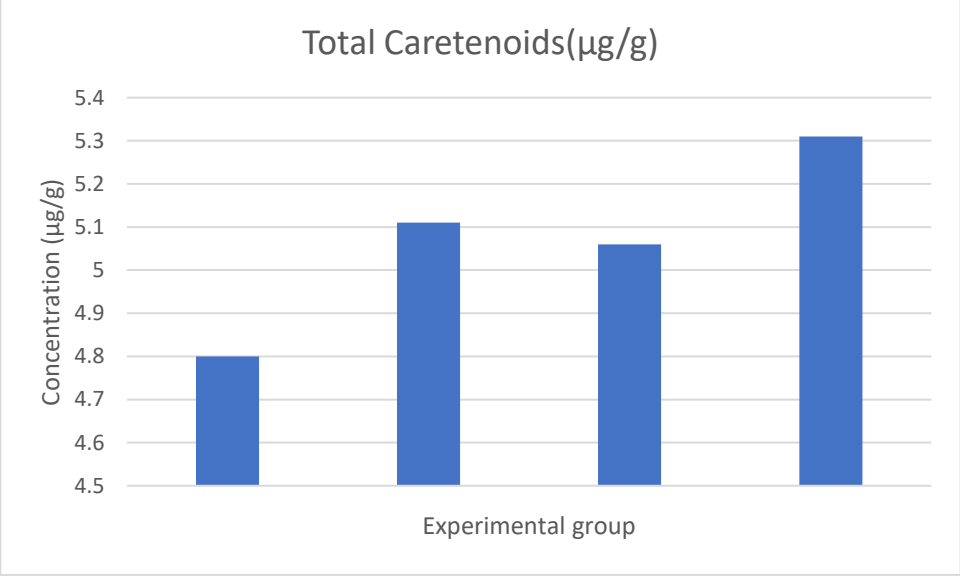



**Figure 6: Comparative study of Carotenoid content (µg/g) CFF and Experimental diet.**

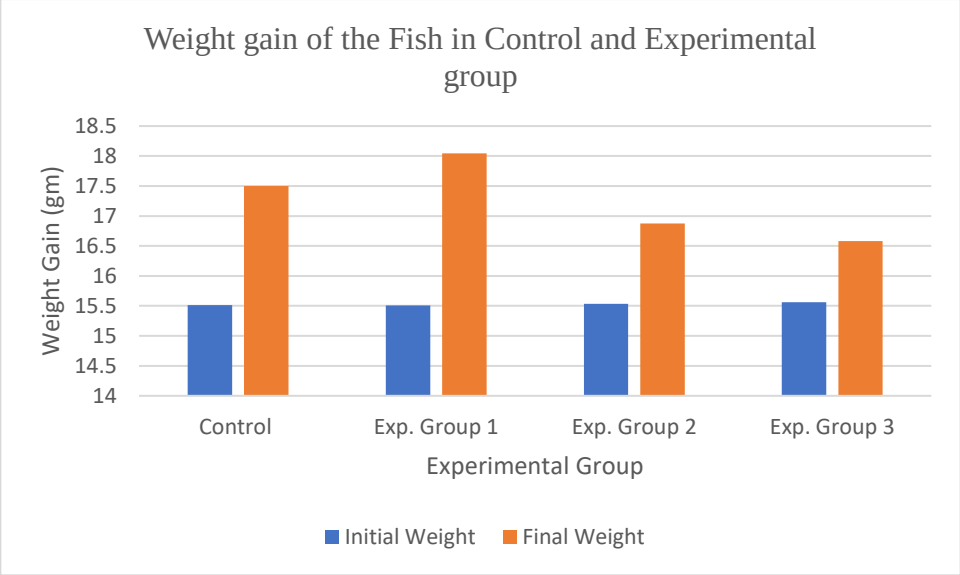



**Figure 7: Comparative study of weight gain (g) in fish fed experimental diets for 40 days**

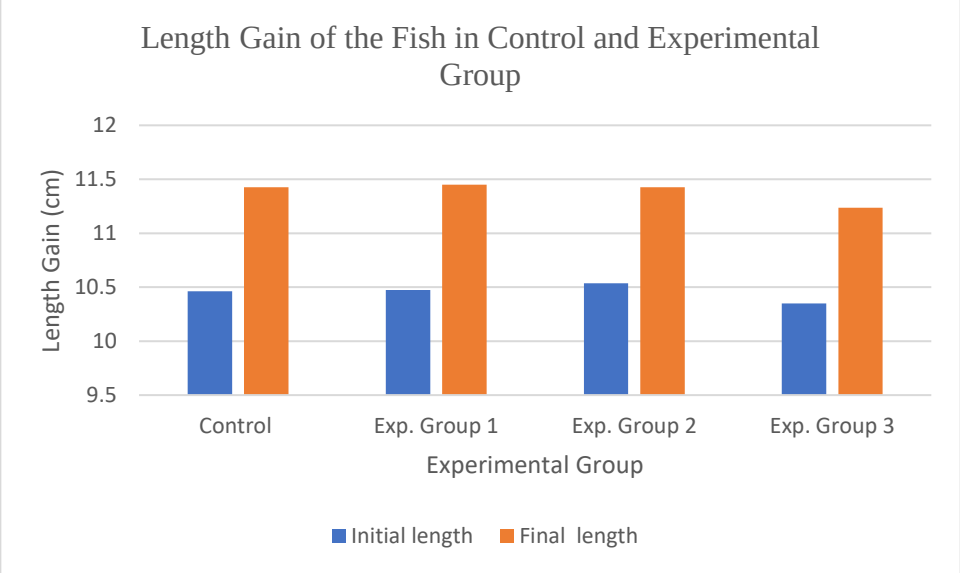



**Figure 8: Comparative study of length gain(cm) n fish fed experimental diets for 40 days**

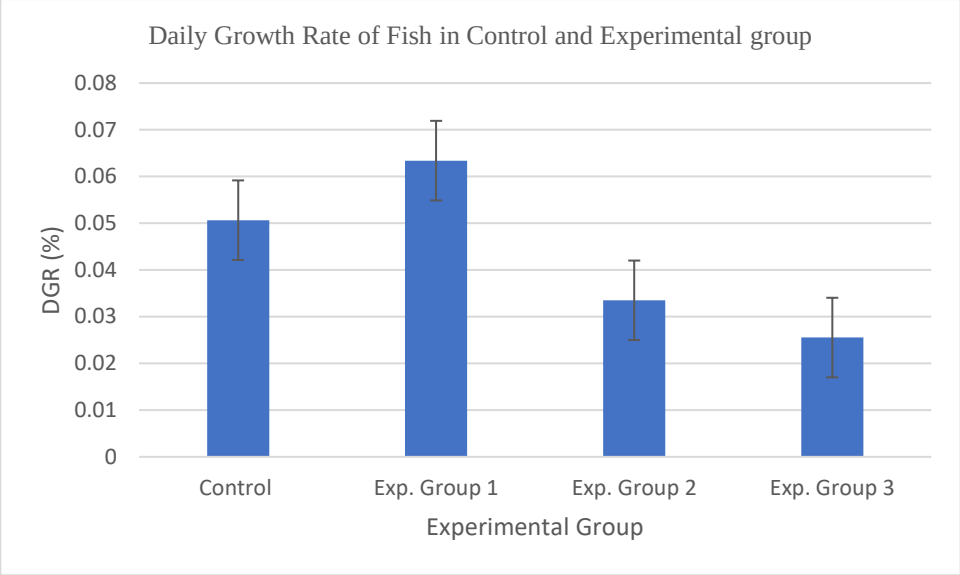



**Figure 9: Daily Growth Rate of the fish-fed experimental diets for 40 days**

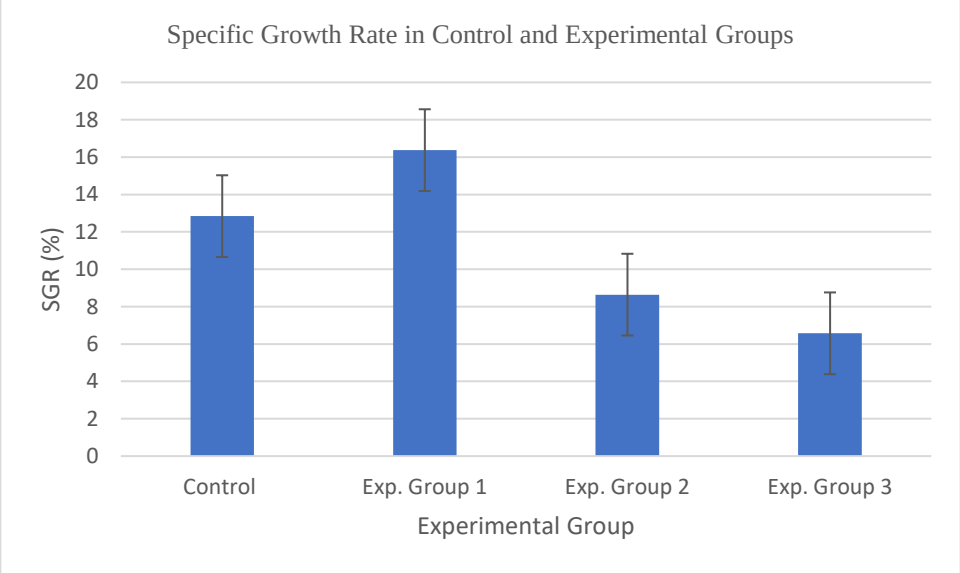



**Figure 10: Specific Growth Rate of the fish-fed experimental diets for 40 days**

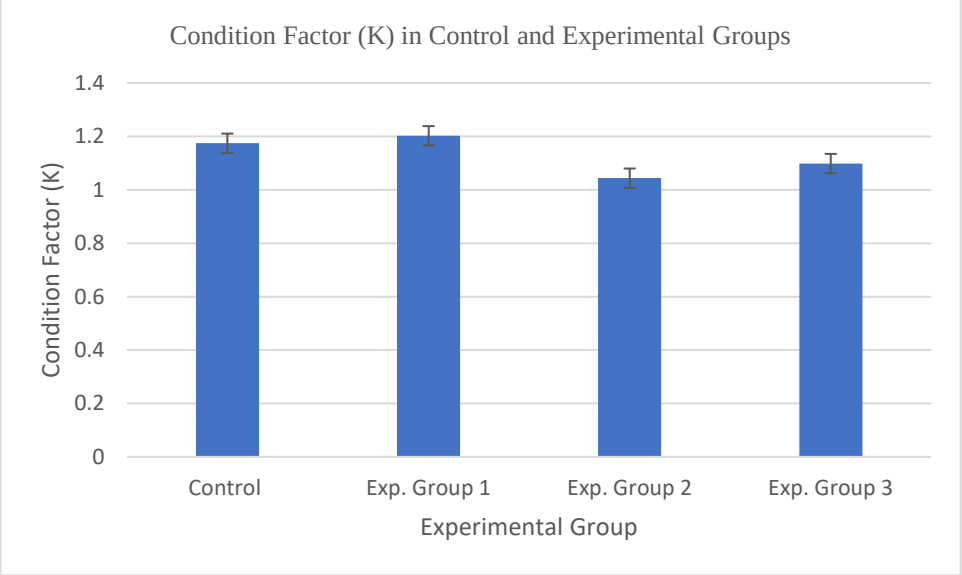



**Figure 11: Condition Factor (K) of the fish-fed experimental diets for 40 days**

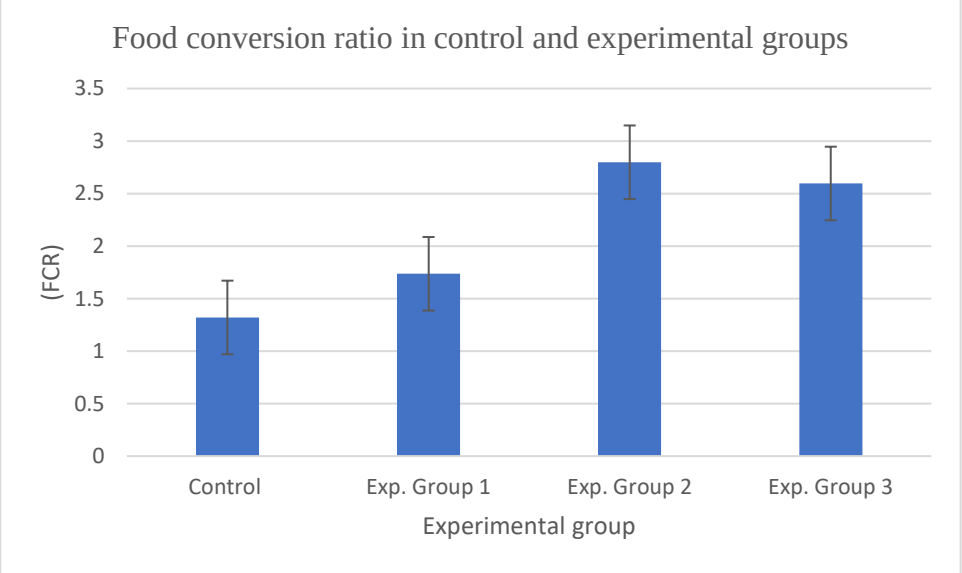



**Figure 12: Food conversion ratio of the fish-fed experimental diets for 40 days**

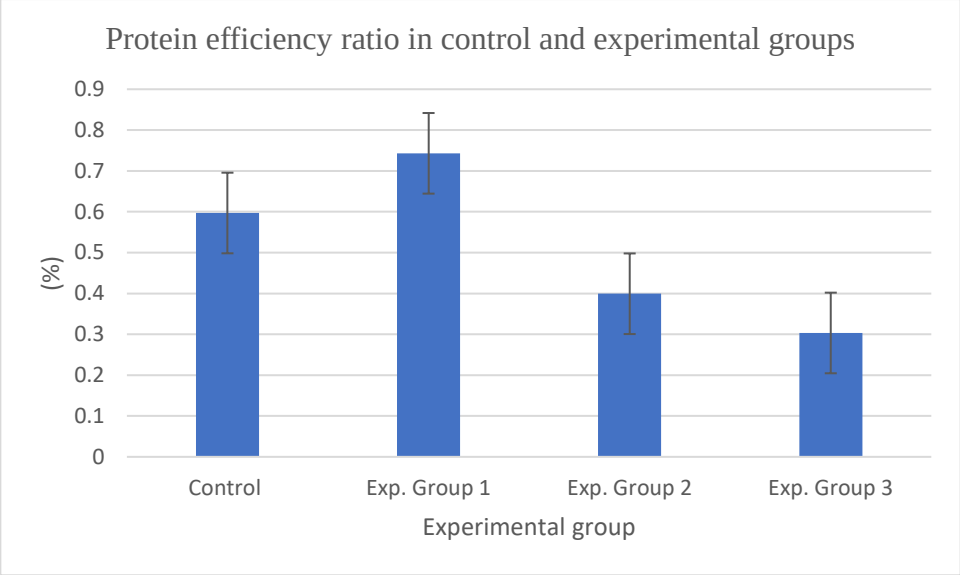



**Figure 13: Comparative study of protein efficiency ratio (%) of the fish-fed experimental diets for 40 days**

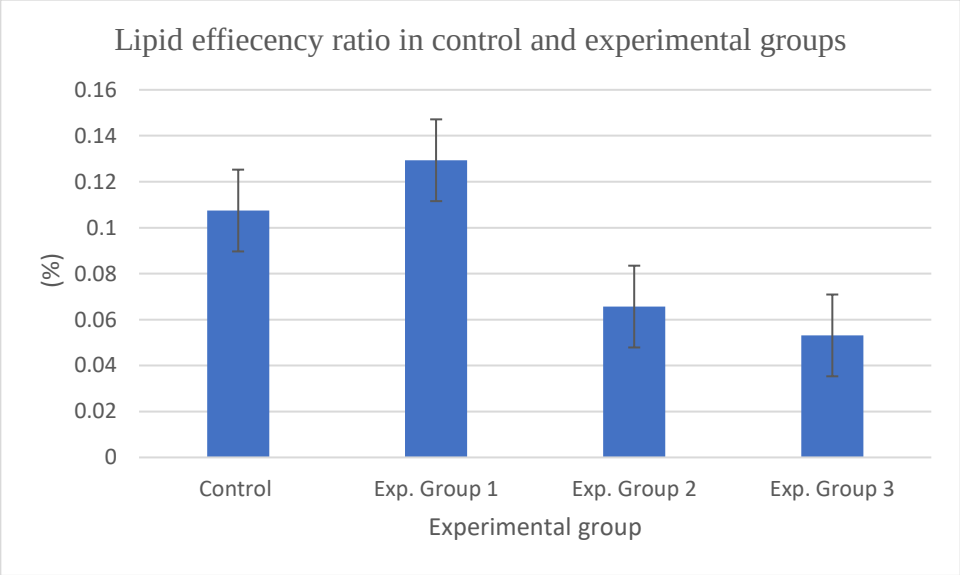



**Figure 14: Comparative study lipid efficiency ratio (%) of the fish-fed experimental diets for 40 days**

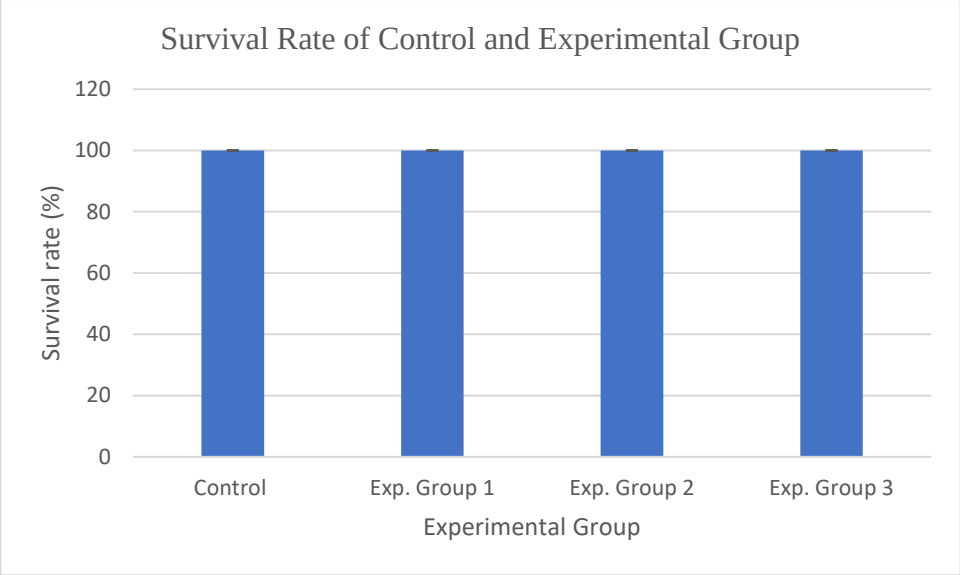



**Figure 15: Survival rate (%) of the fish-fed experimental diets for 40 days**

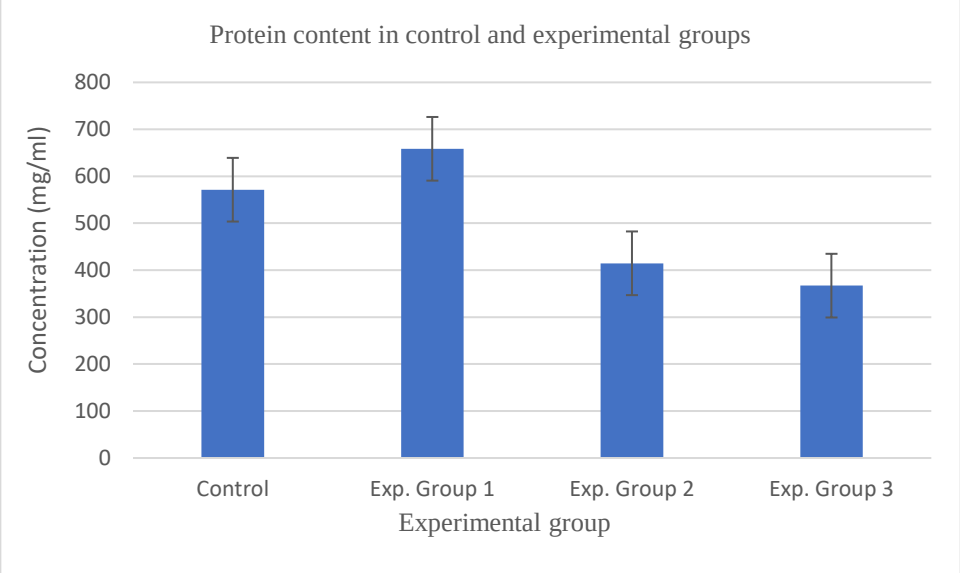



**Figure 16: Comparative study of protein in fish-fed experimental diets for 40 days**

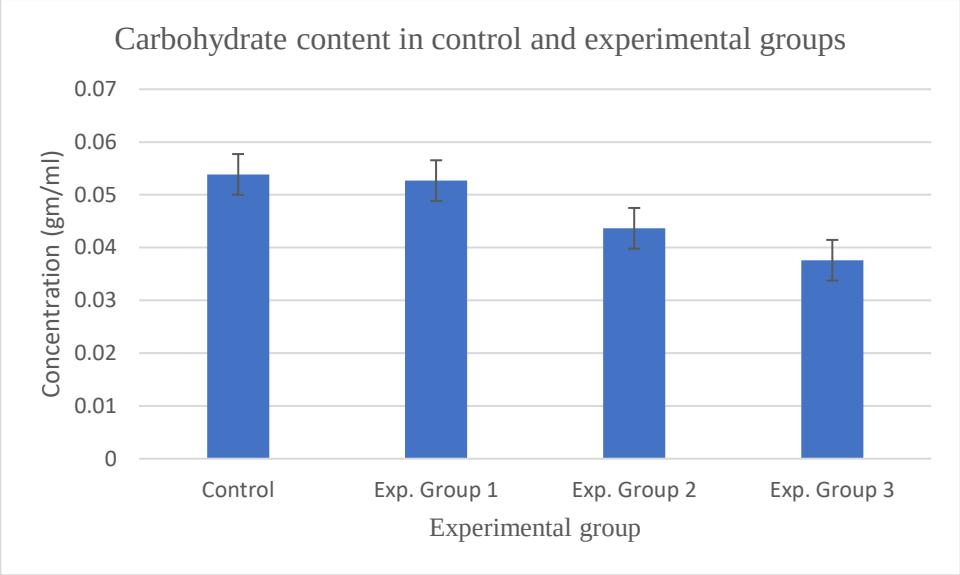



**Figure 17: Comparative study of carbohydrate in fish-fed experimental diets for 40 days**

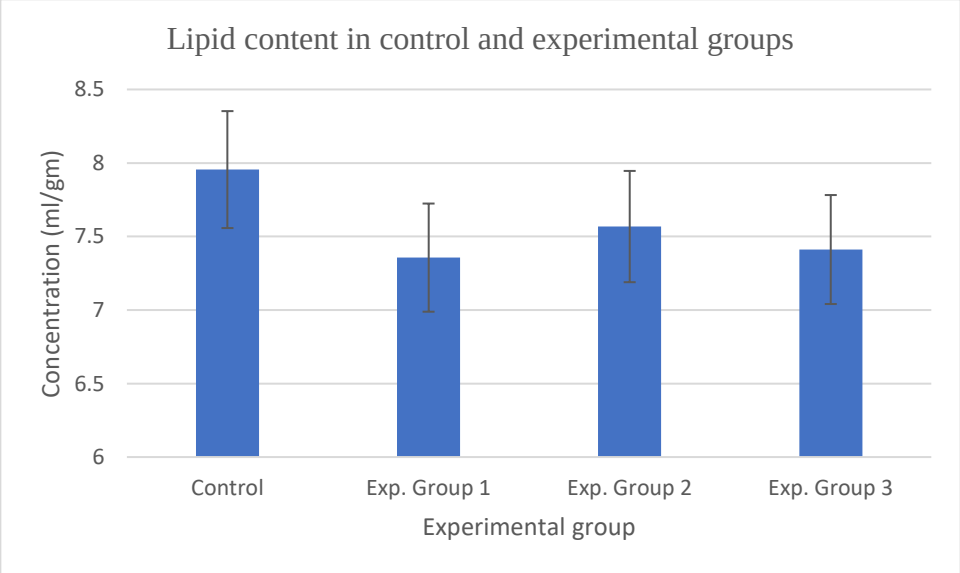



**Figure 18: Comparative study of lipid in fish-fed experimental diets for 40 days**

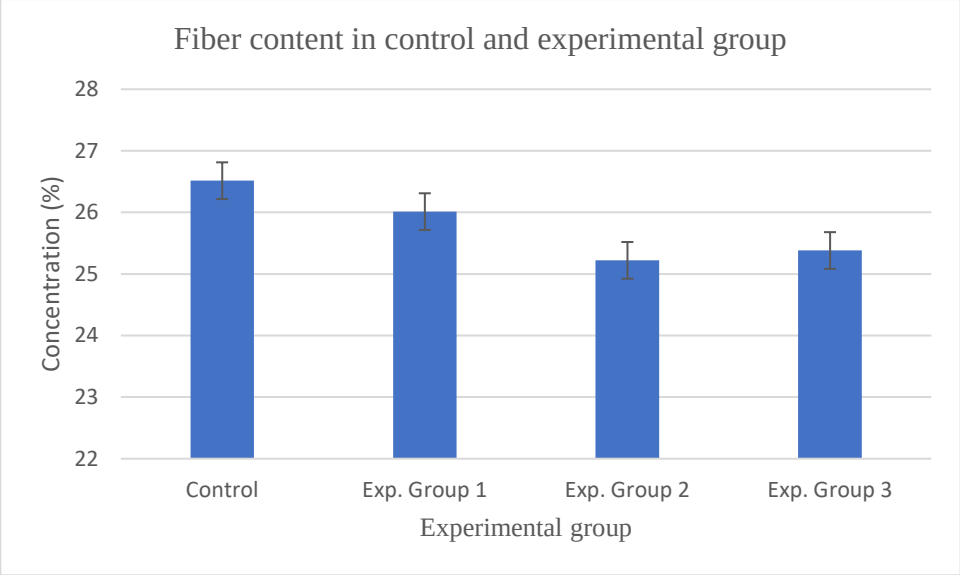



**Figure 19: Comparative study of fiber in fish-fed experimental diets for 40 days**

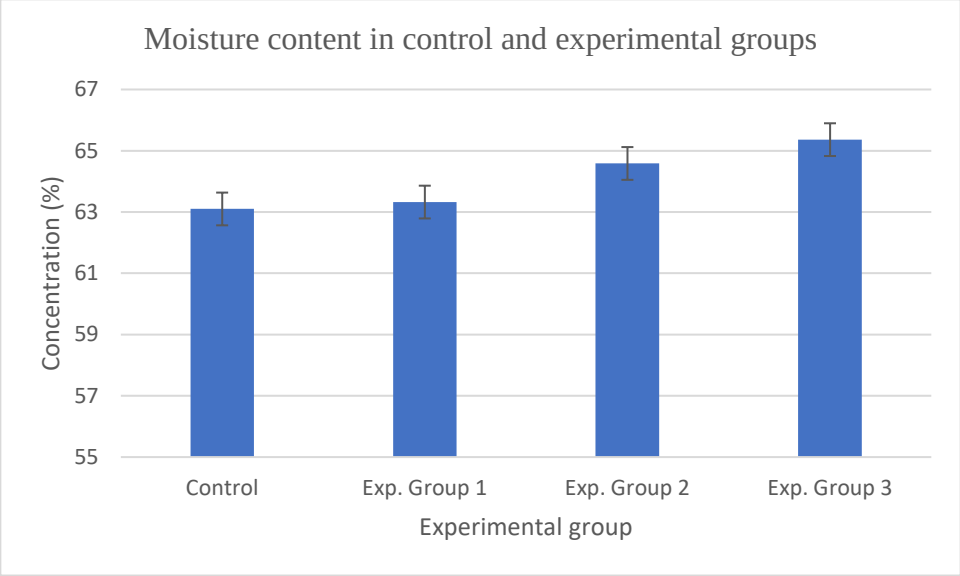



**Figure 20: Comparative study of moisture in fish-fed experimental diets for 40 days**

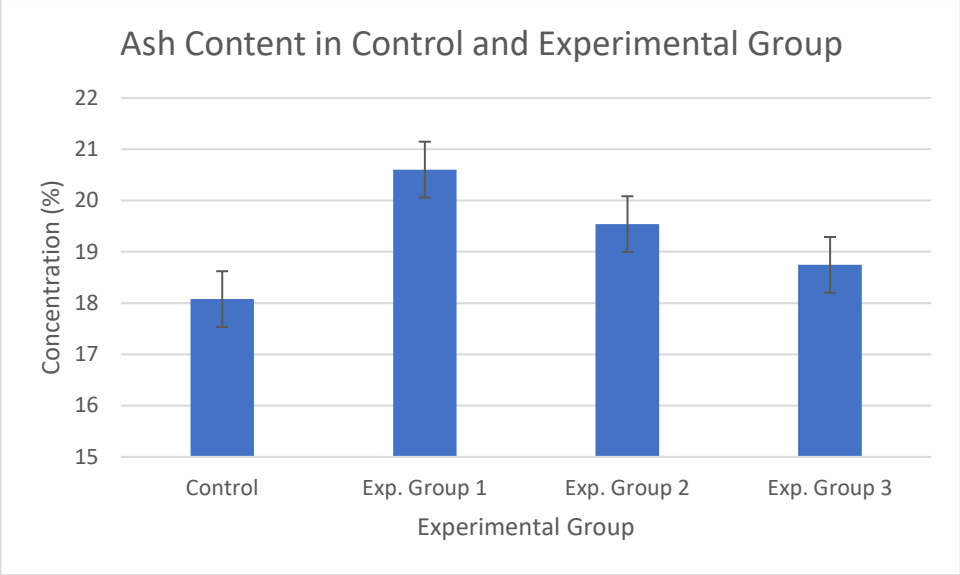



**Figure 21: Comparative study of ash in fish-fed experimental diets for 40 days**

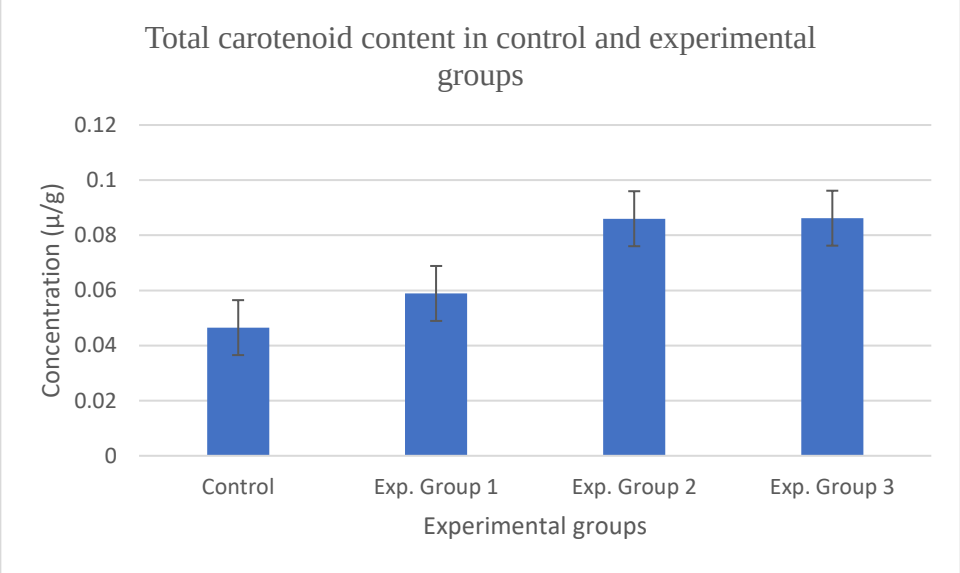



**Figure 22: Comparative study of Carotenoid in fish-fed experimental diets for 40 days**

# 3. Results

## 3.1 Proximate composition of Azolla

The proximate composition of dried and fresh azolla was determined separately to determine their crude protein, crude fibre, crude lipid, ash, moisture, carbohydrate and carotenoid content. As shown in Table 1, the protein content in dried azolla estimated in the present study was 30%, which is greater than the 25.4% estimated in fresh azolla.

The carbohydrate content in dried azolla was determined to be 23.2%, which is higher than that found in fresh azolla at 18.1%. The crude fat content in fresh azolla analysed in this study was 5.8%, lower than the 6.5% observed in dried azolla. The crude fibre content in dried azolla (18.5%) was higher than in fresh azolla (14.8%). The moisture content in dried azolla found in this study was 11% lower than the 18% found in fresh azolla. Ash content in fresh azolla recorded was 33%, compared to more than 30% in dried azolla.

The carotenoid content in dried azolla obtained was 6.9%, which was lower than the 7.5% obtained in fresh azolla.

## 3.2 Proximate analysis of formulated diets

In Table 2, the nutritional composition of the Azolla-based dry feed formulations (Exp. Group 1, Exp. Group 2, Exp. Group 3) and control feeds is compared. It was found that the protein and carbohydrate content of the test diets and the control diet were comparable. The lipid content was slightly lower at 4.9% in exp. Group 2, with 5.1% and 5.2% in exp. Group 2 and exp. Group 3, respectively. The moisture content of 14.5% in exp. Group 1 was determined to be higher than other exp. Groups and Control, as shown in Figure 2. The fibre content in the control and all three experimental groups was found to be similar. The high ash content was

observed in the control and exp. Group 3, with lower ash content in control and exp. Group 1. The carotenoid content was determined to be lowest in the control feed (CFF) and was observed to increase with increasing inclusion levels of azolla (10%, 15%, 20%).

### 3.3 Growth Parameters

The highest observed weight gain (%) was 16.35% in exp. Group 1 (10%) and 12.8% (CFF) in control, suggesting ($P < 0.001$) that there is a significant difference between control and exp. Group 1. The difference between exp. Group 2 (15%) and exp. Group 3 (20%) was significant for weight gain ($p<0.001$). Similarly, the highest increase in length (%) was in exp. Group 1. The ($P < 0.001$) indicates a highly significant difference between the control and exp. Group 1 compared to those in exp. Group 2 and exp. Group 3 ($p<0.0002$).

In the present study it was discovered that the daily growth rates in the control and experimental groups were greater (0.050% and 0.063%, respectively) than those of experimental groups 2 and 3 ($P < 0.001$). There is a substantial ($P < 0.001$) difference between the control group and exp. group 1.

The higher specific growth rate of 16.37% was discovered in Experiment Group 1, and the p-value of 0.0019 demonstrates that there is a significant difference in the specific growth rate between the control and Experiment Group 1. The p-value of 0.0011 shows that the difference between the control group and experimental group 2 is significant. There is a very significant difference between the control group and experiment group 3 for SGR, as shown by the p-value of 0.0002.

The highest condition factor (K) was observed in exp. Group 1, with a p-value of 0.3, shows that there is no significant difference between control and exp. Group 1. The p-value of 0.0056

suggests that there is a significant difference in control in exp. Group 2, and the condition factor for Experiment Group 3 of 1.09 with a p-value of 0.07 shows no significant difference. Exp. Group 2 and Exp. Group 3, fish fed 15% and 20% of the Azolla-supplemented diet, The p-value of 0.0007 indicates that there is a highly significant difference in control, exp. Group 2, and exp. Group 3, and the p-value of 0.18 shows that there is no significant difference between control and exp. Group 1.

The protein efficiency ratio and lipid efficiency ratio (%) was higher in the control and experimental group than in exp. groups 2 and 3. The survival rate was 100% in all experimental groups.

### 3.4 Proximate analysis of fish muscle

In the present study, proximate analysis of the fish muscle in different experimental groups was performed to study the effect of different inclusion levels of Azolla (10%, 15%, 20%) on Asian Sea Bass muscle tissue.

**Protein**: The protein content varied significantly in all experimental groups. The crude protein content of 658.568 µg/ml and 571.42 µg /ml was observed in control and exp. Group 1, as shown in Table 4. The p-value=0.0451 shows that there is no significant difference in the control and experimental group 1. The p-value = 0.001 indicates a highly significant difference between the control and exp. group 2, and the p-value = 0.0002 indicates a highly significant difference between the control and exp. group 3.

**Carbohydrate:** The carbohydrate level in the control was found to be 0.053 mg/ml, which is slightly higher than the 0.052 mg/ml found in Experiment Group 1 with a p-value of 0.0517, indicating that concentration is either equal or slightly different. The p-value = 0.01 shows that

there is a significant difference between control and exp. Group 1, and the lowest concentration observed was 0.037 mg/ml in exp. Group 3 with a p-value < 0.0001, indicating a highly significant difference between control and exp. Group 3.

**Lipid:** The highest lipid content of 7.955 ml/g was found in the control, and the lowest of 7.35 ml/g, 7.56 ml/g, and 7.41 ml/g were found in exp. group 1, exp. group 2, and exp. group 3, respectively. The p-value of 0.0001 suggests that there is a highly significant difference between the control and all other groups.

The significantly higher crude fibre content (%) of 26.51% and 26.01% in control and experiment group 1, respectively, indicates there is no statistically significant difference between the two groups, according to the p-value of 0.3565. The lowest crude fibre amounts were found in experiment groups 2 and 3, at 25.22% and 25.38%, respectively. A p-value of 0.06 shows that there is no statistically significant difference between experiment group 2 and the control group. The p-value of 0.04 shows a significant difference between the control group and experiment group 3.

**Moisture**: In this investigation, it appeared that there was no significant difference between the control and experimental groups for **moisture content** ($p = 0.06$). There was no significant difference between the moisture content in Experimental Groups 1 and 2, which were respectively 63.32% and 64.58% ($p=0.24$). Experiment Group 1 ($p = 0.001$), which had the highest estimated ash content (%), showed a significant difference. The reduced ash content was determined to be 19.54% and 18.74% in experimental Group 2 and 3, respectively. A significant difference between experimental Group 2 and control was shown by the p-value of

0.001, whereas no significant difference was shown by the p-value of 0.136 for experimental Group 3.

**Carotenoid**: It was observed that the carotenoid content (0.08 µg/g) in Experiment Group 3 (fish fed with a 20% Azolla-incorporated diet) was higher than in the control(0.04 µg/g).

## 4. Discussion

One of the food sectors with the most rapid growth is aquaculture. Fish makes up 17% of all protein consumed globally and 20% of animal protein. Protein deficiency may be reduced by increased aquaculture production, particularly in rural regions. However, this industry is limited by several factors, including an inadequate supply of cost-effective feed and poor feed management techniques (Kumari et al., 2017). Fish species, a nutrient in the feed, feed additives, and the habitat in which the fish are raised are just a few of the factors that influence the intricate method of fish growth (Sithara and Kamalaveni., 2008).

In the current study, the effectiveness of a fish meal formulation containing Azolla on the feed utilisation and growth performance of juvenile Asian Sea Bass (*Lates calcarifer*) was investigated. In the animal house at Goa University, azolla was grown (as shown in plate 1)and its proximate analysis was performed. The result of the proximate analysis of dried and fresh Azolla was compared as shown in Table 1.

The study found that the crude protein content of dried azolla was 30%, which is higher than the crude protein content of fresh azolla, which was found to be 25.4%. This is because dried azolla contains essential amino acids and has a higher crude protein content because the water content has been removed. Due to the reduction in water content, other nutrients such as protein, carbs, crude fibre, and minerals are present in larger amounts in Azolla. The high moisture content in fresh azolla makes up a substantial proportion of its weight. When the water is taken out of the plant during drying, the remaining plant matter, including the fibre content, becomes more concentrated. The estimated percentage of crude lipid in dried azolla was 6.5 ml/g, which is higher than that estimated in fresh azolla. This may be because the higher water content dilutes the fat content of the fresh azolla (Pullin and Almazan, 1983).

In this present study, the carotenoid content analysed in fresh azolla was 7.5%, which is higher than the 6.9% found in dried azolla, as shown in Figure 1. This may be due to its exposure to sunlight; as the carotenoids are sensitive to heat and light, some of the carotenoid content in the dried azolla might have degraded (Mohseni et al., 2016).

Dried azolla was powdered and partially used to replace some commercial fish feed. four treatments (control, experimental diet 1, experimental diet 2, and experimental diet 3) were formed to partially replace commercial fish feed at substitution rates of 0%, 10%, 15%, and 20% with Azolla, and the proximate analysis of each experimental diet was done separately.

Fish were starved for 12 hours, individually weighed using an electric balance, and then put to death in ice water after a 40-day feeding experiment. The growth parameters, such as weight increase, length gain, specific growth rate, daily growth rate, and survival rate, were then examined. Additionally, Table 3 shows the approximate composition of juvenile Asian sea bass fed experimental diets for 40 days, including their feed value and amounts of crude protein, crude fibre, ash, moisture, and crude lipid, as shown in Table 4. The lipid efficiency ratio and the protein efficiency ratio were also calculated.

Table 4 illustrates the findings of the proximate analysis of fish given an Azolla-incorporated diet in the control and experimental groups. In the current study, dried Azolla protein replacements in fish diets of 10%, 15%, and 20% were evaluated. The development characteristics and feed utilization of Asian sea bass demonstrated that the fish, particularly at low concentrations of 10%, appreciated the replacement of commercial feed with the addition of Azolla.

The current study indicated that 10% of Azolla showed higher weight gain (%) and length gain (%), but as the inclusion levels of Azolla increased, the weight and length gain were found to

be lowered. This may be because Asian Sea Bass is a highly carnivorous fish in nature and it might show difficulties with feeding on a plant-based diet after a certain level (Erlinda., 2014)

In the current study, the growth parameters were studied after the feeding trial of 40 days, and the highest weight gain (16.35%) and length gain (9.3%) were observed in exp. Group 1 (10%) with a higher specific growth rate (16.37± 0.49482) and daily growth rate (0.063± 0.001555) ($p < 0.001$) than that in control and exp. Group 2 (15%) and exp. Group 3. The lowest weight gain and length gain were observed in exp. Group 3, possibly because Asian Sea Bass is a highly carnivorous fish in nature and is not compatible to consume the plant-based diet after a certain level of inclusion or due to the better feed efficiency.

Abou et al., (2008) reported improved growth performance at an inclusion level of 25% for common carp fed with different Azolla inclusion levels (0, 10%, and 20%). The increased inclusion level of Azolla in the current investigation resulted in observable reductions in growth performance. Due to its high fibre content and antinutritional factors (ANFs), azolla may have an adverse effect on fish growth performance and feed efficiency. Depending on Azolla inclusion levels, intake periods, experimental strategies, fish species, and the size of the fish, the amount of Azolla needed to provide the maximum growth performance might vary (Sotolu et al., 2013). Similar studies were done to see the effects of the MOLM diet (Moringa olifera) on Asian Sea Bass which reported that the 10% of MOLM diet showed good results on the weight gain (%) and specific growth rate for Asian Sea Bass (Erlinda., 2014).

In the study, FCR was noted to be decreasing with the increase in the inclusion levels of Azolla. The lowest FCR was reported in control, and Exp. Group 1 (10%), 1.32, and 1.7, respectively, showed no significant difference ($p=0.1889$). The highest FCR of 2.79 and 2.59 was observed in exp. Group 2 (15%) and exp. Group 3 (20%) and showed a significant effect ($p<0.0002$). This may have been due to either inadequate protein quality or poor protein absorption of

supplemented A. pinnata by the fish. In a previous study, Das et al., (2018) reported no significant difference in FCR at 25% replacement of CFF with Azolla on *Barbonymus gonionotus.*

The condition factor (K) in the present study was found to be lower (1.04) in exp. Diet 2 (15%). The highest condition factor was reported in control and exp. Group 1, it was observed that the 10% of Azolla has shown good results on weight and length gain of the Asian Sea Bass

In this study, the highest protein efficiency ratio (PER) was observed in Experiment Group 1 (0.74) as compared to the control and other experimental groups, indicating the protein has been utilised by the fish in Experiment Group 1. This suggests that the PER has decreased with the increase in the Azolla incorporation level. Previous findings of Lee and Kim., (2001) for the salmonids PER in this research ranged from 0.30 to 0.74. This indicates that a high-energy diet from non-protein sources cannot provide a larger protein proportion for development. Similarly, the lipid efficiency ratio was reported to be lower (0.05) in Experiment Group 3 (20%), suggesting that LER decreases with the increase in the inclusion levels of Azolla.

In the current study, the proximate muscle tissue analysis of Asian sea bass was done in terms of crude fibre, ash, moisture, crude protein, and crude lipid; additionally, the carbohydrate and carotenoid content were determined.

In the present study, the crude protein content and carbohydrate content for control and exp. Group 1 showed a significant difference from those found in exp. Group 2 and exp. Group 3. The crude protein content was found to be higher (658.56 µg/ml) in Experiment Group 1 than that found in Control; this may be because Azolla is also regarded as a major source of nitrogen, and because of the excessive amounts of non-protein nitrogen compounds found in Azolla, a reduction in the crude protein content in fish muscle is observed. (Garrido et al., 2016).

The carbohydrate content in Experiment Group 3(0.043 mg/ml) and Experiment Group 4 (0.037 mg/ml) was found to be lower than that found in Control(0.053mg/ml) and Experiment Group 1(0.052 mg/ml). Nayak et al., (2014) reported that feeding grass carp with Azolla caused a decline in the total amount of carbohydrates in the fish, and this may have been caused by the limited availability of carbohydrates in the Azolla diet and not by a direct impact of Azolla on fish carbohydrate metabolism.

The crude fibre content was observed to be highest in control than exp. group 1 ($p=0.3$), and exp. group 3, with no significant difference, only the exp. group 2 showed a crude fibre content of 25.22%) with a significant difference. This may be because different Azolla inclusion levels have varying effects on the nutrient digestibility of fish. This might be a result of fish digestive enzymes not being able to break down fibre, which is an indigestible component of plants.

The crude lipid content was reported to be different in all experiments. According to the proximate study, the protein from A. pinnata increased as the composition of fish carcasses, particularly their lipids, disintegrated. When more than 25% of the CFF protein was replaced with plant protein, there was a noticeable reduction in the amount of fat present. (Das et al., 2018).

In the present study, the ash content (%) was observed to be higher (20.60%) in exp. group 1, followed by exp. group 2 (19.54%) and exp. group 3 (18.74), respectively. and that shows a significant difference. The ash content found to be lower in control may be because azolla is rich in minerals, and as the levels of azolla increased in the diets, the ash content was also observed to increase (Oluwaniyi et al., 2012).

The moisture content in this study was found to be higher (65.36%) in Experiment Group 3 (fish fed with 20% of Azolla) and the lowest (63.1%) in Control. This may be because the high-

water content of azolla itself can be correlated to a spike in moisture content in fish carcasses with increasing levels of azolla in the diet. Azolla has a high-water content, normally between 93% and 99% (Kumari et al., 2015). A large amount of water is also consumed by fish when they consume diets rich in azolla. The total moisture content of the fish carcass increases as a result of this. The high fibre content of azolla may also help fish retain more water in their guts, which could increase the moisture content of the fish carcass (Fagbenro and Eyo, 2006).

In this study, the carotenoid content (µg/g) of 0.0862 µg/g in exp. group 3 was found to be higher than that found in exp. group 2 (0.0860 µg/g). Carotenoid content was reported to be lowest in control (0.046 µg/g) and exp. group 3 (0.0589 µg/g). This is supported by the study wherein Mosha et al., (2020) used the total carotenoid content in the muscular tissues of GIFT strains as a substitute for consumer approval of fish products. They found that the total carotenoid content of the fish muscles increased along with the Azolla levels.

Based on the overall observations, 10% of Azolla inclusion level can be effective on the growth performance and food utilisation of Asian Sea Bass.

## 5. CONCLUSION

A study was conducted to study the effect of a formulated fish diet supplemented with Azolla on the growth parameters and feed utility of Asian Sea Bass *(Lates calcarifer).* The fish were fed different inclusion levels of Azolla (10%, 15%, 20%), and the result revealed that up to 10% of Azolla can be included in the diet of juvenile Asian Sea Bass without showing a negative impact on growth performance, feed utilization, or muscle tissue, with enhanced growth and optimum feed utilization and increased carotenoid content. therefore, maximum level of 10% could be optimal and acceptable as a plant protein replacement in fish feed for Asian Sea Bass.

It can be concluded that Azolla has the potential to be used as a good protein source in fish feed which is cost-effective and easily available. Further investigations can be done to study the nutritional requirements of Asian Sea Bass with respect to plant-based fish feed which can be a reliable and alternative source for cost-effective fish feed in aquaculture.

## 6. REFERENCES


1. Gupta, S. K., Chandra, R., Dey, D., Mondal, G., & Shinde, K. P. (2018). Study of chemical composition and mineral content of sun-dried Azolla pinnata. *Journal of Pharmacognosy and Phytochemistry*, *7*(6), 1214-1216.
2. Ganzon-Naret, E. S. (2014). Utilization of Moringa oleifera leaf meals as plant protein sources at different inclusion levels in fish meal-based diets fed to Lates calcarifer. *Animal Biology & Animal Husbandry*, *6*(2), 158-167.
3. Kumar, M., Dhuria, R. K., Jain, D., Sharma, T., Nehra, R., & Prajapat, U. K. A NUTRITIONAL EVALUATION OF AZOLLA (AZOLLA PINNATA) AS FEED.
4. Bhaskaran, S. K., & Kannapan, P. (2015). Nutritional composition of four different species of Azolla. *European Journal of Experimental Biology*, *5*(3), 6-12.
5. Agbede, S. A., Akinyemi, O., Falaye, A. E., Atsanda, N. N., Adeyemo, A. O., & Adesina, A. A. (2001). Selectivity of three aquatic weeds as diet for Nile tilapia (Oreochromis niloticus).
6. Mosha, S. S. (2018). A review on significance of Azolla meal as a protein plant source in finfish culture. *Journal of Aquaculture Research and Development*, *9*(544), 2.
7. El-Sayed, A. F. (1992). Effects of substituting fish meal with Azolla pinnata in practical diets for fingerling and adult Nile tilapia, Oreochromis niloticus (L.). *Aquaculture Research*, *23*(2), 167-173.
8. Ebrahim, M. S. M., M. M. Zeinhom, and R. A. Abou-Seif. "Response of Nile tilapia (Oreochromis niloticus) fingerlings to diets containing Azolla meal as a source of protein." *Journal of the Arabian Aquaculture Society* 2, no. 1 (2007): 54-69.
9. Hassan, H. U., Ali, Q. M., Ahmad, N., Masood, Z., Hossain, M. Y., Gabol, K., ... & Kamal, M. (2021). Assessment of growth characteristics, the survival rate and body composition of Asian Sea bass Lates calcarifer (Bloch, 1790) under different feeding

rates in closed aquaculture system. *Saudi Journal of Biological Sciences*, *28*(2), 1324-1330.

10. Abdelwahab, A. M., El-Bahr, S. M., & Al-Khamees, S. (2020). Influence of dietary garlic (Allium sativum) and/or ascorbic acid on performance, feed utilization, body composition and hemato-biochemical parameters of juvenile Asian Sea Bass (Lates calcarifer). *Animals*, *10*(12), 2396.
11. Adorian, T. J., Jamali, H., Farsani, H. G., Darvishi, P., Hasanpour, S., Bagheri, T., & Roozbehfar, R. (2019). Effects of probiotic bacteria Bacillus on growth performance, digestive enzyme activity, and hematological parameters of Asian sea bass, Lates calcarifer (Bloch). *Probiotics and antimicrobial proteins*, *11*, 248-255.
12. El-daim, A., El Asely, A., Kandiel, M., El-Gawad, A., Elabd, H., Shaheen, A., & Abbass, A. (2021). Effect of Spirulina platensis and Azolla nilotica as feed additives on growth performance, antioxidant enzymes and fecundity of Oreochromis niloticus. *Iranian Journal of Fisheries Sciences*, *20*(3), 846-862.
13. Kumari, R., Ojha, M. L., Saini, V. P., & Sharma, S. K. (2017). Effect of Azolla supplementation on growth of rohu (Labeo rohita) fingerlings. *Journal of entomology and zoology studies*, *5*, 1116-1119.
14. Oktavianawati, I., Andinata, D., Isnaeni, A. N., Hermiastuti, M., Rahmawati, N., Handayani, W., & Winata, I. N. A. (2016). Effects of feeding diets containing Azolla pinnata and probiotic on the growth and nutritional content of Patin fish (Pangasius djambal). *Agriculture and Agricultural Science Procedia*, *9*, 403-410.
15. Das, M., Rahim, F. I., & Hossain, M. A. (2018). Evaluation of fresh Azolla pinnata as a low-cost supplemental feed for Thai Silver Barb Barbonymus gonionotus. *Fishes*, *3*(1), 15.

16. Shiomi, N., & Kitoh, S. (2001). Culture of Azolla in a pond, nutrient composition, and use as fish feed. *Soil science and plant nutrition*, *47*(1), 27-34.
17. Datta, S. N. (2011). Culture of Azolla and its efficacy in diet of Labeo rohita. *Aquaculture*, *310*(3-4), 376-379.
18. Abdalbakee, T. T., & Mohammed, H. N. (2019). Effect of using different levels of Azolla as a substitute for soybean meal in the production performance of fish carp. *Plant Archives*, *19*(1), 573-577.
19. Abou, Y., Darchambeau, F., Fiogbé, E. D., & Micha, J. C. (2012). Ecology of feeding Nile tilapia under Azolla cover in earthen ponds: an assessment using structural equation modelling. *IJB*, *2*, 105-111.
20. Tharwat, A. A. (1999). Production evaluation of Nile tilapia Oreochromis niloticus utilized fresh and dried Azolla pinnata in semi-intensive fish culture. *Egyptian J. Anim. Prod*, *36*(1), 71-82.
21. Méndez-Martínez, Y., Chacón Matos, D., Pérez Tamames, Y., & Ramírez de la Ribera, J. L. (2018). Growth and survival of Clarias gariepinus fingerlings with the inclusion of Azolla in diet. *REDVET*, *19*(4).
22. Joseph, A., Sherief, P. M., & James, T. (1994). Effect of different dietary inclusion levels of Azolla pinnata on the growth, food conversion and muscle composition of Etroplus suratensis (Bloch). *Journal of Aquaculture in the Tropics*, *9*(1), 87-94.
23. Fasakin, A. E., & Balogun, A. M. (1998). Evaluation of dried water fern (Azolla pinnata) as a replacer for soybean dietary components for Clarias gariepinus fingerlings. *Journal of Aquaculture in the Tropics*.
24. Ismail, T., Hegazi, E., Nassef, E., Habotta, O. A., & Gewaily, M. S. (2022). The optimized inclusion level of Bacillus subtilis fermented Azolla pinnata in Nile tilapia (Oreochromis niloticus) diets: immunity, antioxidative status, intestinal digestive

enzymes and histomorphometry, and disease resistance. *Fish Physiology and Biochemistry*, *48*(3), 767-783.

25. Asadujjaman, M., & Hossain, M. A. (2016). Fish growth, yield and economics of conventional feed and weed based polyculture in ponds. *Journal of Fisheries*, *4*(1), 353-360.
26. Abo-Taleb, H. A., El-feky, M. M., Azab, A. M., Mabrouk, M. M., Elokaby, M. A., Ashour, M., ... & Sallam, A. E. (2021). Growth performance, feed utilization, gut integrity, and economic revenue of grey mullet, Mugil cephalus, fed an increasing level of dried zooplankton biomass meal as fishmeal substitutions. *Fishes*, *6*(3), 38.
27. Rajan, S. P., Subramanian, S., & Chellamuthu, M. (2021, December). Dried Azolla Based Feed Formulation for Fresh Water Carp. In *Proceedings of the First International Conference on Combinatorial and Optimization, ICCAP 2021, December 7-8 2021, Chennai, India*.
28. BESLIN, L. G., & Rajan, M. (2021). Azolla a Boon for the Growth of Gift Tilapia, Oreochromis Niloticus (Linnaeus, 1758).
29. Gangadhar, B., Umalatha, H., & Hemaprasanth, K. (2021). Apparent digestibility of dried azolla powder incorporated diets in rohu Labeo rohita (Hamilton, 1822). *Indian Journal of Fisheries*, *68*(4).
30. Mishra, S., Khune, V. N., Bara, S., & Banjara, S. (2013). Nutritional evaluation of Azolla pinnata. *Science and Technology*, *4*(3), 207-214.
31. Pullin, R. S. V. and G. Almazan, 1983. Azolla as a fish food. ICLARM News letter, Metro Manila, Philippines, 6: No L
32. Delgado, C. L., Rosegrant, M. W., Wada, N., Meijer, S., & Ahmed, M. (2002). Fish as food: projections to 2020 under different scenarios (No. 595-2016-39966).

33. Sotulu, A., Osagie, A. U., & Ikhajiagbe, B. (2013). Nutrient composition of Azolla pinnata and its potential as a supplement in livestock feed. African Journal of Agricultural Research, 8(9), 776-780.
34. Oluwaniyi, O. O., & Adewolu, M. A. (2012). Growth performance and carcass composition of Nile tilapia, Oreochromis niloticus fed Azolla meal-supplemented diets. Journal of Fisheries and Aquatic Science, 7(6), 421-429.
35. Fagbenro, O. A., & Eyo, A. A. (2006). Growth and nutrient utilization of Clarias gariepinus (Burchell, 1822) fingerlings fed diets containing graded levels of Azolla meal. Turkish Journal of Fisheries and Aquatic Sciences, 6(1), 1-5.
36. Mohseni, M., & Pourkazemi, M. (2016). Carotenoid content of muscle and liver of five fish species from the Persian Gulf. International Journal of Aquatic Biology, 4(6), 404-410.
37. Alam, S. M. (1996). Azolla: a natural aquatic plant for sustainable agriculture and environment. Journal of Crop Production, 1(1), 241-263.
38. Lee, K. J., & Kim, J. D. (2001). Replacement of fish meal by Azolla meal in diets for coho salmon, Oncorhynchus kisutch. Aquaculture Nutrition, 7(1), 47-51.
39. gariepinus (Burchell, 1822) fingerlings fed diets containing graded levels of Azolla meal. Turkish Journal of Fisheries and Aquatic Sciences, 6(1), 1-5.
40. Nayak, D., Singh, S.B., Mishra, B.K., and Pradhan, C. (2017). Azolla as a Feed Supplement for Livestock-A Review. Journal of Pharmacognosy and Phytochemistry, 6(5), 1130-1133.
41. Garrido, M. D., Auil, R. P., Palma, G. A., & Espinosa, M. M. (2016). Azolla filiculoides as a sustainable feedstock for biogas production. Environmental Technology, 37(4), 446-453.