MASS PRODUCTION OF AQUATIC MACROPHYTE *SPIRODELA POLYRHIZA*, ITS AMINO ACID AND FATTY ACID PROFILES STUDY AND ITS USE IN CARPS' FEED FORMULATION

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Dedicated
To My
Beloved Parents

CERTIFICATE

This is to certify that the Ph.D. thesis entitled "**Mass Production of Aquatic Macrophyte *Spirodela polyrhiza*, its Amino Acid and Fatty Acid Profiles Study and its use in Carps' Feed Formulation**" submitted to Delhi Technological University, Delhi, for the award of Doctor of Philosophy is based on the original research work carried out by me under the supervision of **Prof. JaiGopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi, India and **Prof. R. Chakrabarti**, Department of Zoology, University of Delhi, Delhi, India. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I, **Avanish Kumar Shrivastav,** certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of **Prof. JaiGopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi and **Prof. R. Chakrabarti**, Department of Zoology, University of Delhi, Delhi, India for a period of July 2017 to July 2022 at the Department of Biotechnology, Delhi Technological University, Delhi and Department of Zoology, University of Delhi, Delhi, India. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have devotedly acknowledged, given credit and refereed to the research workers wherever their work has been cited in the text and the body of thesis. I further certify that I have not wilfully lifted up some other's work, paragraph, text, data, results etc. reported in the journal, books, reports, dissertations, thesis etc., or available at websites and included them in Ph.D. thesis and cited as own my work.

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ABSTRACT

Aquaculture is one of the fastest growing food producing sectors in the world. It contributes immensely in food security, livelihood and employment generation. Fish and fishery products provide a healthy food for human consumption with high protein and low saturated fat. In commercial aquaculture, feed constitutes around 60-70% of total expenditure due to the use of fish meal and fish oil as a prime source of protein and lipid, respectively. The limited availability of fishmeal and its price are major concerns in the present scenario. Therefore, the rising price and shortage of diet components created a crucial call to find out an inexpensive, abundantly available and innovative alternative raw material to be used in place of fish meal for fish feed without compromising the growth and meat quality of fish. Freshwater duckweeds are a potential ingredient to replace the costly fishmeal in aquaculture. The present study aimed to evaluate the application of freshwater macrophytes as fish feed ingredient. In the present study twelve macrophytes were collected from different water bodies and were cultured in outdoor cemented tanks. Among twelve different cultured macrophytes, greater duckweed S*pirodela polyrhiza* was selected as a potential fish feed ingredient for its nutritional value. The culture technique of selected macrophyte *S. polyrhiza* was developed in outdoor cemented tanks using different manures. The organic manure was found suitable for the production of *S. polyrhiza*. The organic manure was used for the mass production of *S. polyrhiza* in ponds. The production rate of *S. polyrhiza* in ponds was 2020±150 tonnes/ha/year. Fish feeds were formulated by using Winfeed 2.8 software package (WinFeed UK Limited, Cambridge, United Kingdom). The incorporation of *S. polyrhiza* at various levels in the diets of *Labeo rohita* and *Cyprinus carpio* showed the beneficial effect on the fishes. It improved the digestive enzyme physiology of the fishes, thereby enhanced the growth rate. The biochemical composition study of fish showed that there was improvement in the compositions of fishes like, crude protein, crude lipid and amino acids contents. The omega-3 fatty acids eicosapentaenoic acid (EPA)

and docosahexaenoic acid (DHA) contents increased in *S. polyrhiza* supplemented diets fed common carp compared to other diets fed fish. Expression levels of delta-6-desaturase (*fads2d6*), elongation of very long-chain fatty acids protein 2 (*elovl2*), elongation of very long-chain fatty acids protein 5 (*elovl5*) and fatty acid synthase *(fas*) were up-regulated in common carp fed greater duckweed based diets compared to others. The knowledge generated from the present study is useful in the formulation of cost-effected diets for carps and thereby, the production of nutrient rich fishes for consumers.

CONTENTS

Chapter 1

Introduction

INTRODUCTION

The rapid growth of human population and increasing urbanization reduce the land resources for agricultural activities, and thereby, it poses a great threat to the food security in this millennium. In 2050, the human population is estimated to reach 9 billion that may impose an anthropogenic pressure on the food security (HLPE, 2017). The climate change is severely affecting the agricultural production. Over the last two decades, the fisheries and aquaculture sector recognized for their critical role in ensuring food security and nutrition globally (FAO, 2022). No other food-producing sector is parallel to the steep growth of aquaculture sector. Aquaculture supplies nutritious food at reasonable cost. It has been estimated that fish contributing nearly 16% of the animal protein consumed by the global population (Hua et al., 2019, FAO, 2020).

According to the recent reports, the total fisheries and aquaculture production in 2020 attained a record of 214 million tonnes, which comprises 178 million tonnes of aquatic animals and 36 million tonnes of algae, mainly due to the growth of aquaculture in Asian countries (FAO, 2022). The contribution of aquaculture sector is nearly 58% of the total fisheries and aquaculture production. The global aquaculture production in 2020 was 122.6 million tonnes, comprising 87.5 million tonnes of aquatic animals and 35.1 million tonnes of the algae. In terms of livelihoods, 58.5 million people are employed in the primary fisheries and aquaculture sector, whereas about livelihood of 600 millions depends on the products of fisheries and aquaculture sector (FAO, 2022).

The consumption of fish has reached a new record of 20.5 kg per capita and it will increase further in the upcoming decades (FAO, 2022). Out of 178 million tonnes of fish produced worldwide in 2020, 154.8 (87% of total production) million tonnes were used for human consumption, whereas the remaining 23.14 million tonnes (13% of total production) was used to make fishmeal and fish oil (FAO, 2022). Aquaculture sector converts their 65% of the captured fish into fishmeal (Jackson, 2009; Kaushik and Troell, 2010; Merino et al., 2012).

Fish is receiving all nutrients through food chain. Therefore, the supply of nutritious food is the primary requisite for the production of quality fish. Usually, fish oil and fish meal are used as prime source of lipid and protein, respectively in the fish diets (Shepherd and Jackson, 2013). Traditionally, fishmeal has been used as a prime protein source in aquafeed due to its high nutritional value, digestibility, high palatability and low carbohydrate content (NRC, 2011). Moreover, fishmeal is a rich source of essential amino acids and polyunsaturated fatty acids (n-3 and n-6), which are essential for fast growth and better performance of fishes (Lech and Reigh, 2012). In commercial aquaculture, feed constitutes around 60-70% of total expenditure (Akiyama et al., 1992; Singh et al., 2006; Iskander et al., 2019). Fish meal and fish oil production rose along with the expansion of aquaculture over the past two to three decades. The limited availability of fishmeal and its price are major concerns in the present scenario of growing demand of fishmeal (Tacon and Metian, 2015). Therefore, the rising price and shortage of feed ingredients created a crucial call to find out an inexpensive, abundantly available and innovative alternative raw material to be used in place of fish meal for feed

formulation without compromising the growth and meat quality of fish (Bureau and Meeker, 2011; Liland et al., 2012; Ghosh and Ray, 2017; Fiordelmondo et al., 2022). The nutrients like, protein, lipid, vitamins, minerals, amino acids and fatty acids should be present in ingredients. A good alternative ingredient should posses certain characteristics such as availability, sustainability, cost-effectiveness and relatively high protein with easy digestibility (Gatlin et al*.,* 2007). Keeping in mind the importance of a nutritionally balanced and affordable fish diet, there is a rising effort to investigate the nutritional value of various non-traditional resources of fish feed ingredients *viz.,* aquatic macrophytes and terrestrial plants (Mondal and Ray, 1999; Bairagi et al., 2002; Kalita et al., 2007).

Freshwater macrophytes, the fastest growing aquatic plants, are abundant in tropical and subtropical countries. They grow profusely in nutrient-rich water. They can grow rapidly and can be reproduced without competing with agricultural land (Bhanthumnavin and Mcgarry, 1971; Said et al., 1979). These macrophytes are broadly classified into four major groups based on their occurrence in the water body: the surface floating (*Azolla* spp.), submerged (*Hydrilla* spp.), emergent (*Potamogeton* spp.), and marginal (*Ipomoea* spp.). Some commonly occurring freshwater macrophytes are: mosquito fern *Azolla microphylla* (Azollaceae), water velvet *A. pinnata* (Azollaceae), buffalo spinach *Enhydra fluctuans* (Asteraceae), water thyme *Hydrilla verticillata* (Hydrocharitaceae), water spinach *Ipomoea aquatica* (Convolvulaceae), duckweeds *Lemna minor* (Lemnaceae), water clover *Marsilea quadrifolia* (Marsileaceae), water lettuce *Pistia stratiotes* (Araceae), giant salvinia *Salvinia*

molesta (Salviniaceae), floating fern *S. natans* (Salviniaceae), greater duckweed *Spirodela polyrhiza* (Lemnaceae), and Asian watermeal *Wolffia globosa* (Lemnaceae). These macrophytes are distributed throughout the temperate, sub-tropical and tropical regions of the world. Most of these macrophytes, except *H. verticillata* (submerged plant), *M. quadrifolia,* and *E. fluctuans* (marginal plants) are surface floating macrophytes. All these macrophytes propagate through vegetative reproduction.

The nutritional value of freshwater macrophytes has been recognized globally and their values in terms of proteins, lipids, ash etc. varies greatly (Hassan and Chakrabarti, 2009). Macrophytes are a rich source of protein, lipid, amino acids, fatty acids, and minerals (Hasan and Chakrabarti, 2009). Duckweeds, *Lemna minor, Spirodela polyrhiza* and *Wolffia globasa* are rich sources of α-linoleic acid which is the precursor of ecosapentenoic acid (EPA) and decahexanoic acid (DHA) (Appenroth et al., 2017). Dietary inclusions of these polyunsaturated fatty acids (PUFAs) have several health benefits for humans and other animals. Fish are unable to synthesize two essential fatty acids like n-6 (derived from linoleic acid, LA) and n-3 (derived from alpha-linolenic acid, ALA). So these fatty acids should be supplied in the diets of fishes (Horrobin and Manku, 1990). In duckweeds the protein content ranged from 20-35% (dry weight) with all essential amino acids required for the preparation of animal feeds (Appenroth et al., 2017; Herawati et al. 2020, Yahaya et al., 2022). The leaf protein extracted from freshwater macrophytes may be used for human or non-ruminant animals. The extracts of seven freshwater macrophytes show no cytotoxic and anti-proliferative effects on human cell

lines (Sree et al., 2019). Therefore, macrophytes should be considered as useful feed ingredients.

Around fifty species of macrophytes are used for herbivorous and omnivorous fish either directly or indirectly (Mandal et al., 2010; Akmal et al., 2014; Naseem et al., 2021). Several studies show the effect of freshwater macrophytes supplemented feeds for different fish species. Dietary supplementation of *Lemna minor* (20%) and *Azolla pinnata* show positive impacts on the growth performance and feed utilization of common carp *Cyprinus carpio* (Yılmaz et al., 2004; Gangadhar et al., 2017). Feeding of rohu *Labeo rohita* with 20 and 30% *L. minor* supplemented diets shows enhanced weight gain, specific growth rate (SGR), and low food conversion ratio (FCR) compared to the control diet fed fish (Bairagi et al., 2002; Mer et al., 2016). The supplementation of duckweed in the diet of common carp increased the antioxidant capacity as evidenced by enhanced activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and reduced levels of malondialdehyde (MDA) (Yang et al., 2019). Even in several countries like, Thailand, Laos, and Cambodia the duckweeds serve as human food. The *W. globosa* are easily available in food market of Thailand (Shree et al., 2019). In these countries it is also used to prepare several dishes like salad, omelets and vegetable curries (Saengthongpinit, 2017). Some of the macrophytes are like, *Enhydra fluctuans, Ipomea aquatica* (Kalmi sak) and *Marsilia quadrifolia*, are consumed as a vegetable by humans in Bangladesh and India (Dewanji et al., 1993; Islam et al., 2021).

Digestive enzymes are a collection of enzymes present in the digestive system of animals that break down macromolecules into their most basic forms to speed up the body absorption (Walting, 2015). Evaluation of the quantity and activity of digestive enzymes is employed as a relative indicator of feed acceptability, digestive capacity, and the survival rate (Suzer et al., 2008). Amylolytic, proteolytic, and lipolytic enzymes are associated with digestion of carbohydrates, proteins, and lipids, respectively in the fishes (Bairagi et al., 2002; Falcon- Hidalgo et al., 2011; Ray et al., 2012). The activity of digestive enzymes amylase, protease, and lipase in fish can be affected by the type of food, fish age, fish species as well as feeding habitat (Falcon-Hidalgo et al., 2011; Lopez-Vasquez et al., 2009). The digestibility of ingredients plays a very significant role in the overall bioavailability of the nutrients present in feed (Chakrabarti and Rathore, 2009). The *in vitro* digestibility study shows the suitability of duckweeds as a fish feed ingredient (Sharma et al., 2016).

Supply of sufficient amounts of the essential n-3 long chain PUFA (LC-PUFA), specifically eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), to support optimal human nutrition is a global problem that transcends geographical and political boundaries. While the n-3 LC-PUFA is completely absent in conventional plant meals and vegetable oils, their precursor ALA can be abundant in terrestrial and freshwater plants. The conversion of ALA and linoleic acid (LOA, 18:2n-6) to LC-PUFA requires a series of fatty acyl desaturase (*fads*) and elongation of very long chain fatty acid (*elovl*) enzymes such as *elovl5* and *elovl2* (Kuhajda et al., 1994;

Torstensen and Tocher, 2010; Castro et al., 2016; Monroig et al., 2016; Xie et al., 2021). The products of the Δ*6fads* and *elovl5* genes are key enzymes in the biosynthesis of EPA and DHA (Fonseca-Madrigal et al., 2005; Torstensen and Tocher, 2010). Importantly, many freshwater fishes including common carp have metabolic capacity to convert dietary ALA to the n-3 LC-PUFA, EPA, and DHA (Tocher et al., 2002; Glencross, 2009; Tocher, 2010; Tasbozan and Gökçe, 2017).

Indian major carp rohu *Labeo rohita* and exotic common carp *Cyprinus carpio* are extensively cultured freshwater species. Rohu is an herbivore, predominantly column feeder species and *Cyprinus carpio* is an omnivore, bottom feeder fish. Both are belonging to the family Cyprinidae. The common carp is the fourth most cultured freshwater fish in the world and contributed 7% of total aquaculture (fish) production in 2018 (FAO, 2020). In India, It is extensively used in composite fish culture (Rathore et al., 2005). There is an increasing demand for the formulation of cost-effective diets with adequate nutritional value and high digestibility for these cultivable species.

The selection of aquatic macrophytes as food source requires an investigation related to their nutritional composition. The fatty acid and minerals composition of some freshwater macrophytes, likes *Spirodela polyrhiza, Lemna punctata, Lemna minor, Lemna gibba, Wolffia hayalina* and *Wolffia microscopia* are documented (Appenroth et al., 2017). Amino acids and vitamins are essential nutrients required for optimum general health and physiological function development, growth, maintenance and reproduction

(Alagawany et al., 2021). Vitamins exert catalytic functions that facilitate nutrient synthesis. Thus, it plays an important role in the metabolism and influences the health of farmed animals.

The survey of literature shows that there is a scarcity of information related to biochemical composition (especially amino acids and vitamins) of different freshwater macrophytes. The culture techniques for large scale production of freshwater macrophyte are also not standardized. Macrophytes are usually collected from the natural resources for various studies. The evaluation of impact of the macrophytes supplemented pelleted feeds in fish is most essential for the production of cost-effective feed and sustainable aquaculture development.

The overall aim of the present investigation is to formulate pelleted diets using non-conventional ingredients and also the evaluation of the impact of the prepared diets on the physiology of fishes. The specific objectives are:

- Selection of macrophytes and evaluation of their nutritional value as non-conventional ingredients for fish feed formulation.
- To standardize the culture technique of selected macrophyte for large scale production.
- To formulate diets for carps rohu *Labeo rohita* and common carp *Cyprinus carpio* using *Spirodela polyrhiza* as a protein source.
- Evaluation of nutritional quality and physiological responses (*viz*. digestive enzyme profiles, expression of selected genes) of rohu *Labeo rohita* and common carp *Cyprinus carpio* fed with formulated diets.

Chapter 2

Review of Literature

REVIEW OF LITERATURE

The role of nutrition is crucial in aquaculture because it influences the physiology and growth of fish and thereby, the production costs. Rapid expansion of intensive aquaculture elevated the demand for specific formulated diets. The prime area of fish nutrition research is the formulation of cost-effective diet with effective feed conversion ratio. It is utmost important to know the nutritional requirement of the cultivable species for sustainable aquaculture (Hardy and Kaushik, 2021). In commercial aquaculture, feed represents 60-70% of the total costs (Iskandar et al., 2019). Therefore, the growth of aquaculture industry is determined by the fabrication and commercialization of quality diets (Daniel, 2018). Fish meal is the major source of protein and the most expensive component in fish diet. The low availability of fishmeal and augmentation in market price obligated the need of appropriate cost-effective alternatives to fish meal. The digestive physiology, metabolism and bioenergetics, assessment of the requirements of amino acids and essential fatty acids like ecosapentanoic acid (EPA) and docosahexaenoic acid (DHA) are important areas in fish nutrition research.

This chapter focuses the following topics as a comprehensive review for the assessment of the study completed within the areas of fish nutrition.

2.1. Nutritional requirements of fish

2.1.1. Protein and amino acids

2.1.2. Lipid and essential fatty acids

2.1.3. Vitamins

2.2. Replacement of fishmeal

2.2.1. Replacement of fishmeal with conventional ingredients

2.2.1.1. Soybean (*Glycine max* meal)

2.2.2. Replacement of fishmeal with non-conventional ingredients

2.2.2.1. Freshwater macrophytes

2.2.2.2. Duckweeds

2.2.2.3. The greater duckweed *Spirodela* spp.

2.3. Digestive enzymes and its importance

2.3.1. Proteases

2.3.2. Amylase

2.3.3. Lipase

2.4. Genes involved in fatty acids metabolism

2.4.1. Desaturases

2.4.2. Elongases

2.1. Macronutrients requirements of fish

2.1.1. Protein and amino acids

Protein is the most important and expensive factor in fish feed formulation that provides essential and non-essential amino acids responsible for both maintenance and growth of the fish (Hardy, 2010). Carnivorous fish requires

higher content of protein in their diet (40-55%). The requirement of protein in the diets of herbivorous and omnivorous fishes is less (20-35%) compared to the carnivore fishes (Lall and Tibbetts, 2009). Fishmeal is the prime source of protein for different fish species. However, it is now regarded both economically and environmentally unsustainable, and the aquaculture industry is under socio-economic pressure to create alternative proteins. The most widespread replacement for fishmeal in aquafeed is plant protein. Plants have been known to be the inexpensive and most plentiful source of protein (Fasuyi and Aletor, 2005). Insufficiency of any essential amino acids has a negative impact on growth even though other feed ingredients are available in adequate quantity (Dutta, 1994). Methionine and lysine are the chief limiting amino acids in aquafeed, which act as precursor to carnitine, essential for the transportation of long chain fatty acyl groups into the mitochondria for beta oxidation (Walton et al., 1982). It was reported that deficiency of methionine decrease the growth, activity of digestive enzyme and antioxidant ability in hepatopancreas and intestine of grass carp *Ctenopharyngodon idella* (Wu et al., 2017). They reported that the arginine requirement of fish ranged from 3.0-8.1% of dietary protein. The requirement of tryptophan in Nile tilapia *Oreochromis niloticus* fingerlings has been reported to be 3.4 g/kg of dietary protein (Zaminhan et al*.,* 2017). Amino acids are necessary for various vital functions in fish like stress responses, ammonia detoxification, immune function, ureagenesis and antioxidant defence (Hoseini et al., 2019). The requirement of amino acids of rohu *Labeo rohita,* common carp *Cyprinus carpio,* catla *Catla catla,* Nile tilapia *Oreochromis niloticus*, rainbow trout

Oncorhynchus mykiss and grass carp *Ctenopharyngodon idella* have been documented (Table 1)

Table 1: Essential amino acids requirement (g/100g diet) for *L. rohita, C. carpio, C. catla,* O*. niloticus*, *O. mykiss* and *C. idella* (NRC, 2011; FAO, 2013)

Amino acids	***L. rohita***	***C. carpio***	***C. catla***	***O. niloticus***	***O. mykiss***	***C. idella***
Histidine (His)	0.90	0.5	0.98	1.0	0.7	0.67
Isoleucine (Ile)	1.20	1.0	0.94	1.0	0.8	1.18
Leucine (Lue)	1.50	1.4	1.48	1.9	1.4	1.98
Lysine (Lys)	2.27	2.2	2.49	1.6	1.8	-
Methionine (Met)	1.42	0.7	1.42	0.7	1.0	-
Phenylalanine (Phe)	1.48	1.3	1.48	1.1	1.2	1.09
Threonine (Thr)	1.71	1.5	1.98	1.1	0.8	1.01
Tryptophan (Trp)	0.45	0.3	0.38	0.3	0.2	0.24
Valine (Val)	1.50	1.4	1.42	1.5	1.3	1.36
Arginine (Arg)	2.30	1.7	1.92	1.2	2.0	1.68

2.1.2. Lipid and essential fatty acids

Lipids are diverse class of compounds and sources of high dietary energy. Dietary lipids are the sources of fatty acids, steroids, phospholipids and fat soluble vitamins for appropriate functioning of metabolic activities (Ghanawei et al., 2011). Growth and development of gonads are two important processes in fish which requires large amount of fatty acids (Ghanawei et al., 2011). Fatty acids with single double bond known as monounsaturated fatty acids (MUFA) and fatty acids with two or more double bonds are known as polyunsaturated fatty acids (PUFA). The vital essential n-3 and n-6 fatty acids

are linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) (Chen, 2018). Essential fatty acid, LA can be metabolized into arachidonic acid (ARA, 20:4n-6) and ALA can be metabolized into eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6 n-3) and such conversion is limited in most vertebrates (Chen, 2018). Freshwater fish can desaturate and elongate ALA to EPA and DHA (Chen, 2018). However, fish and shellfish possess high EPA and DHA and fish oil is the most essential source of highly unsaturated fatty acid. The functionality of cell membrane mainly its fluidity is crucial and fluidity depends on the essential fatty acids (LA and ALA) and the ratio of n-3 and n-6 PUFA. Their ratio is determined by the dietary intake (Cartwright et al., 1985). Fish diet consists of about (7-15%) lipids and dietary lipids mainly phospholipids that embedded in membrane structure reflects the composition of PUFA in formulated diet. The shorter PUFA like ALA and LA are usually desaturated and elongated prior amalgamation into phospholipids (Olsen et al., 1991). The necessity of essential fatty acids and their optimum level of requirement to maintain health of fish and growth are varied from one species to other and it is dependent on its ability to synthesize fatty acid endogenously (Olsen et al., 1991).

Traditionally, fish oil is known to be major dietary lipid source because of its rich nutritional importance. However, increasing global aquaculture industry demands alternative lipid sources in recent time. Therefore, recent studies have aimed on the uses of economical lipid dietary supplements. Vegetable oils are rich in n-6 and n-9 PUFA (Yildiz et al., 2018). As a result, some fish species can biosynthesize long chain PUFA using vegetable oil as lipid

sources (Yildiz et al., 2018). It was observed that vegetable oils can improve growth performance in *C. idella* and *O. niloticus* (Du et al., 2008; Li et al., 2019). Dietary lipid and composition of fatty acid are vital factor that plays an important role in the reproductive function of the fish (Luo et al., 2017). Balanced dietary lipid are essential for better follicle maturity, composition of membrane, quality of oocyte and quality of embryo (Khalil et al., 2013; Ghaffarilaleh et al., 2014). It was reported that EPA and ARA deficiencies in diets affected sperm and egg quality resulted in negative impact on the development of gonads in fishes (Estefanell et al., 2015). Dietary n-3 Long chain-PUFA (LC-PUFA) are engaged in steroidogenesis by modifying estradiol-17β (E2) secretion and synthesis of prostaglandin and vitellogenin in fishes (Wathes et al., 2007; Peng et al., 2015).

Tilapia required of 5-12% lipid and 0.5-1.0%n-6 fatty acids in their diets for growth and development (Lim et al., 2011). It was studied that n-6 and n-3 PUFA had an effect on survival, growth and development of brood stock of Japanese flounder *Paralichthys olivaceus* (Furuita et al., 2003) and grouper, *Epinephelus malabaricus* (Wu et al., 2002). Modification of ARA and DHA contents in diets showed effect on the survival, growth and composition of essential fatty acids in tissue of white bass *Morone chrysops* (Harel et al., 2000). Better growth and survival was reported in Indian major carps with the inclusion of 4% dietary phospholipids (Paul et al., 1998). A 7-9% dietary lipid has been considered as optimum level for the Indian major carps (Mohanty, 2006). The supplementation of 1% n-3 PUFA in the diets of juvenile rohu elevated the immunity of fish against bacterial pathogen (Mishra et al., 2006).

2.1.3. Vitamins

Vitamins are groups of organic compounds which are indispensable micronutrients for the animals. Vitamins are important cofactors for various physiological and cellular processes, essential for immune system functions, operation of endocrine system, integrity of cell membrane and other metabolic functions (Halver, 2002; Gatlin 2002). The requirement of dietary vitamin level for various health benefit is much higher than that needed for normal growth and development (Sealey and Gatlin, 2001; Koshio, 2007). Various natural sources of vitamins are plants, animals and microorganisms. Vitamins are also supplemented in various crystalline or coated forms that vary in its activity (Gatlin, 2002). Vitamins are categorized as water soluble or fat soluble. Water soluble vitamins includes vitamin B-complex consists of 11 compound which includes 8 well organized members. The dietary requirement of the 8 members of vitamin B complex is little but its role for the growth and development is crucial. The requirement of vitamins are essential because of its vital role in growth performances, more definite biomarkers like activity of particular enzyme and gene markers and also vitamin deficiencies lead to the signs of various diseases (Halver, 2002). Fat soluble vitamins are A, D, E and K which play important role in various physiological functions. They are soluble in non-polar solvent, not in water and present in different chemical forms. Citrine, lipoic acid, p-aminobenzoic acid and astaxanthin are some vitamin like compounds with less known actions in fish (Sealey and Gatlin, 2001). The requirements of vitamins depend on the stage of development as well as it is species-specific (Sealey and Gatlin, 2001).

Growth and development of salmon *Salmo salar* also dependent on the availability of 11 water soluble vitamins (Halver, 1957). Various studies shows the qualitative requirements of water soluble vitamins for carp (Ogino, 1967), cobia (Mai et al., 2009), Japanese sea bass (Ai et al., 2004) and large yellow croaker (Ai et al., 2006). The early mortality syndrome in wild Atlantic salmon was reported due to the deficiency of thiamine (Harder et al., 2018). Low survival, reduced growth and feed conversion were observed in tiger shrimp while fed thiamine free diet (Chen et al., 1991). Riboflavin (vitamin B_2) supplementation to a level of its requirement in Jian carp elevated the activity of enzymes in intestine (Li et al., 2010). Elevated malondialdehyde, a marker for lipid peroxidation was observed in intestine and gills of riboflavin deficient diet fed grass carp (Chen et al., 2015). Niacin (vitamin B_3) deficient diet diminished the growth performances and feed efficiency in shrimp (Shiau and Suen, 1994). Traditionally, sufficient niacin provided by the animal and plant ingredient used in aquafeed to fulfill the requirement for growth and welfare of fish. Panthotenic acid (vitamin B_5) is the key compound involves in intermediate metabolism of protein, lipid and carbohydrate. Therefore, responsible for the normal physiology and metabolism of growing fish (NRC, 2011). Gill disorder due to low panthotenic acid has been observed in most fishes like carp, rainbow trout, eel, salmon, yellow tail, catfish, red sea beam and grouper (Halver and Hardy, 2002). Dietary vitamin B_6 (pyridoxine) plays a protective role in jian carp (Feng et al., 2011). The specific symptoms of vitamin B_6 deficiency in fish are gasping breathing (hyperventilation), nervous disorder like hyperirritability, epileptic and dark, green pigmentation in the

skin, primortem rigor and peritoneal edema (Feng et al., 2011). Blue slime patch is a symptom of vitamin B_7 (Biotin) deficiency in brook trout (Halver and Hardy, 2002). Folic acid (vitamin B_9) and vitamin B_{12} plays complementary roles in metabolism (Halver and Hardy, 2002). Deficiency of folic acid shows similar symptoms to the deficiency of vitamin B_{12} (NRC, 2011). Vitamin B_{12} (cobalamine) is crucial for cell division like erythropoiesis and energy metabolism of fatty acid and amino acid (NRC, 2011). Vitamin C also known as ascorbic acid helps in performing various physiological functions like antioxidant activity, collagen synthesis, improving ion absorption and utilization, immunomodulation, enzyme cosubstrate functions and stress resistance. Ascorbic acid increases antioxidant protection to sustain the structural integrity of cells in various organs of grass carp (Xu et al., 2016). Similarly, antioxidant protection function of vitamin C was observed in rainbow trout, prawn and pacific abalone (Dabrowski et al., 2004; Wu et al., 2014; Asaikkutti et al., 2016). Abscorbic acid helps in wound healing, hemorrhages prevention, increased collagen content of bone, increased bone health in channel catfish and rainbow trout (Lim and Lovell, 1978; Wahli et al., 2003). Ascorbic acid increases antibody production by enhancing the immune responses in Indian major carp (Nayak et al., 2007). The requirement of ascorbic acid at hatchling stage of common carp and Indian major carp ranged from 45-354 and 650-750 mg/kg diet, respectively (Mahajan and Agrawal, 1980; Gouillou-Coustans et al., 1998). Mostly, the water soluble vitamins are excreted in urine but excess fat soluble vitamins can be stored in the liver (hepatopancreas) of animals. In Nile tilapia, dietary lipid and vitamin

E levels affects some immune parameters like lysozyme or alternative complement activity (Lim et al., 2009).

2.2. Replacement of Fishmeal

In aquaculture industry, fishmeal is the chief source of protein (Chakrabarti et al., 2018). At present, 65% of captured fish converts to fishmeal in aquaculture industry (Jackson 2009; Kaushik and Troell 2010; Merino et al., 2012). Commercial aquaculture production of feed is calculated to be around 40 million tons globally (Alltech, 2018) which is assumed to be elevate above 85 million tons by 2025 (Tacon and Metian, 2015). Overharvesting of the pelagic fish includes capelin, herring, sardines, mackerel and anchoveta to manufacture fishmeal collected from the coastal zones of Chile, Peru and the Atlantic Ocean leads to limited production potential and also loss of sea food consumption by humans (Naylor et al., 2000; Worm et al., 2009; Merino et al., 2012; Ghosh et al., 2018).

The augmentation of price and limited feed components necessitated the requirement of cheap and plentiful alternatives (Ghosh and Ray, 2017). Balanced nutrition in supplementary feeding is crucial to promote the productivity of cultured fish (Baruah et al., 2018). Digestible and econonomically viable alternative feed with balanced nutrition are essential to substitute partially or fully the fishmeal. Alternate feeding stuffs not utilized by human will reduce the production cost of animal protein and enhances ecological footprint (Goldstein, 2015). The sustainability of the aquaculture industry sustainability is also dependent on the utilization of high grade and

low cost diets (Ghosh and Ray, 2017). Moreover, researchers suggested that plant and animal protein combination is more efficient to substitute than that of a single protein (Noor et al., 2000). Numerous researches have been reported on the partial substitution of fishmeal with regard to the nutritional and health aspects of fish (Kaushik et al., 1995; Bransden et al., 2001; Refstie et al., 2001; Torstensen et al., 2008; Draganovic et al., 2011; Zhang et al., 2018; Chen et al., 2019). There is an increasing effort to study the nutrition value of various non-conventional feed resources including terrestrial plants and aquatic macrophytes (Kalita et al., 2007).

2.2.1. Replacement of fishmeal with conventional ingredients

Oil cakes or oil meals are the by-products of edible industry after extraction of oil from oil seeds (Khan et al., 2003). Edible oil cakes are considered to be good quality protein source for fish diet and its composition might vary depending on its growth condition, variety and method of extractions (Mazurkiewicz, 2009). Oil seeds extraction by-products like cakes of mustard, groundnut, sunflower, almonds and soybean have been utilized as protein source in aquaculture industry (Khan et al., 2003). A diversity of oil cakes includes sesame, linseed, groundnut, soybean etc. have been investigated in carp as plant derived protein sources diets (Hossain and Jauncey, 1989; Hossain et al., 2001; Mazurkiewicz, 2009). The replacement of fishmeal with bio-processed groundnut oil cake showes no adverse effect on the survival, carcass composition, digestive enzyme activity, growth and feed conversion of rohu (Ghosh and Mandal, 2015). Enhanced growth has been observed in

sunflower oil-cake fed (30-50%) tilapia (Hossain et al., 2018). Groundnut oil cake (35%) has been used as an ingredient in the formulation of fish diets (ICAR, 2006). Groundnut oil cake and soybean oil cake are used in the diet of silver barb *Puntius gonionotus* as substitute of fishmeal (Mohanta et al., 2009).

2.2.1.1. Soybean (*Glycine max*) meal

Soybean meal is known to be the best vegetable source of protein (Gatlin et al., 2007). The protein content of soybean meal ranges from 40-49%. The amino acid composition makes it a good supplement of grain protein and fulfills the requirement of animals (Zhang et al., 2018). Soybean possess highly digestible protein (methionine and lysine), therefore, replacement of fishmeal by soybean meal has been reported to be effective for fish growth and health without any negative impact (Karalazos et al., 2007; Zhang et al., 2018; Ye et al., 2019). It is reported that soybean meal has anti-nutritional factors (ANFs) which affect negatively the digestion of food and the growth of the fish (Zhang et al., 2016; Ye et al., 2019). Therefore, attempts are made to improve the nutritional values of soybean meal with advance technologies (Li et al., 2015; Kissiger et al., 2016; Wang et al., 2017). Various products of soybean including toasted and extracted soybean meal, low oligosaccharides soybean meal, full-fat soybean meal and SPC (Refstie et al., 1998) are used in the fish feed formulation. The inclusion of soybean meal is limited for many carnivorous fish species due to ANF and high carbohydrate content (Fowler, 1980; NRC, 2011). ANF can be eliminated to a certain limit in soybean meal and several techniques have been applied for such deduction. During extrusion technique of feed, the generated heat lowers the level of lectines

and protease inhibitors (Barrows et al., 2007; Gatlin et al., 2007; Krogdahl et al., 2010). Alcohol extraction method reduces saponins, sterol and oligosaccharides (Krogdahl et al., 2010). Reduction in feed intake and suppression in growth are observed in rainbow trout, *O. mykiss* at an inclusion of level >20% (Oliva-Teles et al., 2012). In *Heteropneustes fossilis*, 15% fishmeal has been replaced with soybean meal without affecting growth, feed and protein efficiencies (Siddiqui et al., 2014). In the diets of juvenile, *Pseudobagrus ussuriensis,* fishmeal is replaced at a level of 40% by soybean meal without affecting its growth and feed efficiency but high level of soybean meal reduces the growth performance of fish (Wang, 2016). Fishmeal is replaced at a level of 25% by soybean meal in tilapia (Sharda, 2017). In the diet of rainbow trout, 80% of fishmeal has been effectively replaced by bio-processed soybean meal (Voorhees et al., 2019). It is reported that the proximate composition of muscle, parameters of plasma, hepatic and muscular metabolites and fatty acid profile in the *Senegalese sole* are not affected by replacing 30% fishmeal with plant protein sources like soybean meal, wheat gluten and soybean protein concentrate (Rodiles et al., 2015). Shimei and Li (2010) has reported that soybean meal protein can substitute fish meal at a level <75% without affecting the growth of tilapia. They have found that increasing level of soybean meal uplifted the trend in feed intake.

2.2.2. Replacement of fishmeal with non-conventional ingredients

Sustainability of fishery industry depends on the accessibility of high grade and reasonably priced diets (Ghosh and Ray, 2017). Partial or complete plant ingredient incorporating fish feed have been known to be more efficient and

economical (Ofojekwu and Ejike, 1984; Robinson et al., 1984). Several studies have been carried out for the substitution of expensive marine origin proteins with economical plant protein, like, *Glycine max*, *Psophocarpus tetragonolobus, Leucaena leucocephala, Cynodon dactylon* (Refstie et al., 1998; Fagbenro, 1999; Bairagi et al., 2004; Kaleeswaran et al., 2011). *Mucuna pruriens* seed meal, *Morus indica* leaf meal, leaf meal and tuber meal of *Manihot esculenta*, leaf protein concentrate and leaf meal of *Medicago sativa* (Olevera- Novoa et al., 1990; Siddhuraju and Becker, 2001; Mondal et al., 2012; Lukuyu et al., 2014;), some legumes such as *Vigna radiate* and *Vigna ungiculata* (Ghost et al., 2018). Plants with their different parts have been utilized as alternative sources of protein in aquaculture. Plant ingredients to be used in aquafeed are chiefly divided into seed meals and leaf meals. Leaves of various aquatic macrophytes, vegetables, grasses and terrestrial plants have been studied as potential feed ingredients in aquaculture.

2.2.2.1 Freshwater macrophytes

Herbivorous and carnivorous fishes are consuming around 50 species of macrophytes directely/indirectely (Akmal et al., 2014). Incorporation of 30% macrophytes in feed shows a better profile of amino acids (Mandal et al., 2010). In comparison to standard marketable plant ingredients, macrophytes possess a satisfactory crude protein content of 241-264 g/kg (Cruz et al., 2015). Several studies shows the rich nutritional value of macrophytes in terms of protein, lipid, carbohydrate, amino acids, fatty acids, vitamins and minerals (Appenroth et al., 2017, 2018) (Table 2).

Table 2: Nutritional value of macrophytes (% dry matter basis)

Macrophyte	Protein	Lipid	Carbohydrate	Ash	Reference
Azolla microphylla	20.2%	3.50%	-	16.30%	Datta, 2011
Azolla pinnata	21.67%	3.27%	-	19.33%	Kumari et al., 2018
Enhydra fluctuans	8%	1.10%	9.64%	15.15%	Datta et al., 2019
Ipomoea aquatica	26.45-29.46%	1.7 - 2.19%	10.51- 40.50%	7.28-16.37%	Adelakun et al. 2016; Ali and Kaviraj,2018
Lemna minor	24 – 28%	5%	42%	25%	Kalita et al., 2007; Appenroth, 2017; Pagliusoetal, 2022.
Pistia stratiotes	23.27% 11.80%	14% 2.90%	39.75% 60.70%	16.20% 17.00%	Adelakun et al. 2016; Mandal and Ghosh, 2018
Salvinia cuculata	11%	7.0%	50.80%	31.20%	Kalita et al., 2007
Spirodela polyrhiza	25.60–34.5%	4.5%	11.14-29.8% (Starch)	15.20%	Rusoff et al., 1980; Fasakin et al., 2001; Xu et al., 2011; Yu et al., 2011; Tang et al., 2015; Appenroth et al., 2017
Wolffia arrhiza	20.40%	4.63%	-	17.60%	Chareontesprasit and Jiwyam (2001)
Wolffia hyliana	35%	6.5%	-	-	Appenroth et al., 2017

Various aquatic macrophytes, such as waterthyme (*Hydrilla verticillata*), waterspinach (*Ipomoea aquatica*), duckweed (*Lemna* spp*.*, *Spirodela* spp. and *Wolffia* spp*.*), water cabbage (*Pistia stratiotes*), water fern (*Salvinia* spp.), mosquito fern (*Azolla* spp.), water clover (*Marsilea quadrifolia*) and water cress (*Enhydra fluctuans*) are used as suitable sources of nutrients in fish feed (Bairagi et al., 2002; Kalita et al., 2008; Gangadhar et al., 2015; Nisha and Geetha, 2017). These plant proteins are considered as rich sources of amino acids that increase the value of conventional diet as well as helpful for the growth of carps (Jackson et al., 1982; Mohapatra and Patra, 2013) (Table 3). Various study shows that macrophytes are sustainable alternative to

replace fishmeal in the diets of fishes, for instance, feeding of rainbow trout (*O. mkiss*) with *S. polyrhiza* (Stadtlander et al., 2019), Nile tilapia with *Azolla filiculoides (A. filiculoides)* (Abou et al., 2011, 2013)*, A. africana* (Fasakin et al., 2001)*, A. microphylla* (Fiogbe et al., 2004) and ducweed based diets (De Matos et al., 2014). Similarly, the feeding of rohu *L. rohita* fingerling with raw or fermented *Pistia* leaf (Mandal and Ghosh, 2019), *Salvinia cuculata* (Ray and Das, 1992), *Ipomea aquatica* (Ali and Kaviraj, 2018), *Azolla* species (Datta, 2011), fermented *Eichhornia* leaf meal (Ghosh et al., 2004) shows promising results.

Table 4: Amino acid composition of various freshwater macrophytes (g/100 g protein)

Amino acid	*Spirodela polyrhiza*[1]	*Lemna minor*[1]	*Azolla piliculoides*[2]	*Azolla pinnata*[3]	*Azolla microphylla*[4]	*Salvinia molesta*[5]
Essential amino acids						
Arginine	4.7	4.81	1.66	1.15	2.04	4.7
Histidine	1.6	1.5	0.24	-	3.3	1.7
Isoleucine	3.3	3.7	0.8	0.93	5.75	3.6
Leucine	6.8	7.3	1.51	1.65	10.6	7.5
Lysine	4.2	5.0	1.24	0.98	2.34	3.7
Methionine	1.6	1.6	0.22	0.34	0.46	1.4
Phenylalanine	3.97	4.4	1.32	1.01	5.1	5.0
Threonine	4.2	4.0	2.35	0.87	7.61	4.8
Tryptophan	-	-	-	0.39	1.0	1.1
Valine	4.4	4.6	1.10	1.18	7.67	5.2
Non-essential amino acids						
Alanine	5.4	5.1	1.42	-	7.86	5.9
Aspartic acid	7.8	8.2	2.05	-	11.58	9.2
Cystine	0.8	0.9	-	0.18	-	1.8
Glutamic acid	9.6	9.8	4.54	-	14.2	10.4
Glycine	4.3	4.6	1.6	1.0	8.59	5.8
Proline	3.5	3.8	0.6	-	1.62	4.5
Serine	4.1	4.1	1.96	0.9	9.36	4.8
Tyrosine	3.1	3.1	0.65	0.68	0.92	3.5

Source: [1]Appenroth et al., 2017; [2]Shiomi and Kitoh, 2001; [3]Alalade and Iyayi, 2006; [4]Bhaskaran and Kannapan, 2015; [5]Moozhiyil and Pallauf, 1986.

2.2.2.2 Duckweeds

Duckweeds, mainly *Spirodella polyrhiza*, *Lemna minor* and *Lemna gibba* are considered as an important source of protein. They occupy a key position as feed ingredients in the diets of fishes in aquaculture industry and also preferred as nutrient rich feed to many herbivores fishes (Singh et al., 1967; Gaigher et al., 1984; Azim and Wahab, 2003; Mandal et al., 2010). The protein and fibre contents of duckweeds are found 28-43 and 5%, respectively (Chaturvedi et al., 2003; Aslam et al., 2017). Anti-nutritional factors (ANF) are present in aquatic macrophytes. However, application of various processing technique have the potentiality of inactivating or eliminating the harmful properties of ANFs. Fasakin et al. (2001) reported that the replacement of 10% fishmeal in the diet of Nile tilapia. The cellulotytic and amylolytic bacteria are reported in the gut of duckweed fed mrigal *Cirrhinus mrigala* (Ghosh and Ray, 2014). Duckweeds are distinguished by better absorbtion and availability of amino acids like methionine, lysine, carotenoids and vitamins A, B and E (Chojnacka, 2006; Showqi et al., 2017). Earlier studies have reported the presence of essential amino acids (valine, phenylalanine, isoleucine, leucine, threonine) (Goopy and Murray, 2003) and non-essential amino acids (glycine, lysine, methionine, serine, tyrosine) in duckweeds . High lysine concentration in duckweed is beneficial for fish growth (Yilmaz et al., 2004).

2.2.2.3. The greater duckweed *Spirodela* spp.

Duckweeds are monocotyledons of the Lemnaceae family. Duckweed represents a small family with five genera that includes *Spirodela, Landoltias,*

Lemna, Wolffiela and *Wolffia* with 37 species reported worldwide (Landolt, 1986; Appenroth et al., 2017). Species of Lemnaceae family belongs to small floating aquatic plants with reduced morphology and fastest-growing ones among the species of higher plants. *Spirodela* is a fast growing, free-floating freshwater macrophyte with smallest genome among the duckweed family. It belongs to subfamily Lemniodeae and has two species such as, *Spirodela polyrhiza* (L.) Schleiden and *Spirodela intermedia* (W. Koch). It has fronds of large size (4-12 m). The importance and utilization of *Spirodela polyrhiza* has been recognized recently.

The culture of *Spirodela polyrhiza* requires specific environmental conditions. The water quality parameters influence the growth of duckweeds which includes temperature, pH, ammonia, phosphates etc. Temperature, the master abiotic factor plays a vital role in the growth of duckweed (Culley et al., 1981). Sonta et al. (2019) has suggested that 20-28°C temperature as optimum growth condition for the production of the greater duckweeds.

The optimum pH for the production of *S. polyrhiza* ranged from 6.5-8.5 (Kaul and Bakaya, 1976; Gopal and Chamanlal, 1991; Islam and Khondkar, 1991). The light intensity should be 4,200-6,700 lux with 14-16 h photoperiod for the optimum production of duckweed (Mkandawire and Dudel, 2007). The maximum production of *S. polyrhiza* was possible with optimum conductivity of 650-1000 µS/cm (Gopal and Chamanlal, 1991). The complete disappearance of *S. polyrhiza* was observed in the month of May because of low alkalinity and conductivity (Khondker et al., 1993). There was a production potential of

duckweed in freshwater bodies (Chakrabarti, 2017). The ammonia concentration should be maintained between 7-12 mg N/l to obtain the required content of protein in duckweed (Leng et al. 1995). Phosphorus level should be maintained 4-8 mg/l for the good growth of the duckweeds (Hassan and Chakrabarti, 2009). Within 2-4 days, the biomass of duckweeds gets doubled under optimal conditions of temperature, pH, nutrient availability and light.

Duckweeds are rich sources of protein (40%) when grows in the rich culture system (Robinette et al., 1980; Hassan and Edward, 1992; Hanczakowaski et al., 1995; Ahmad et al., 2003). Minerals and protein concentration in duckweed largely depends on the degree of nitrogen, potassium, phosphorous and other elements availability in the growing media. Duckweed as a replacement for soybean appeared to be cost-effective at 40% protein level (Bhatnagar et al., 2012; Bhatnagar and Raparia, 2014; Bhatnagar and Lamba, 2015; Bhatnagar and Dhillon, 2017).

Protein content in *S. polyrhiza* varied between 23.8-40.9% (Hillman and Culley, 1978; Hassan and Edwards, 1992). Appenroth et al. (2017) reported 25% protein content in *S. polyrhiza*. The crude protein content of leaf protein concentrate and residual pulp fibres of *S. polyrhiza* were 64.6% and 19.9%, respectively on dry matter basis (Fasakin, 1999). The nutritional value of *S. polyrhiza* was as follows: 180g/kg protein, 31 g/kg lipid and 218 g/kg ash on dry matter basis (Stadtlander et al., 2019). They found that essential amino acids, methionine and tryptophan contents were higher in *S. polyrhiza* cultured on diluted slurry as compared to soybean and lupine. The amino acid

profile of *S. polyrhiza* fulfilled the essential amino acids requirement of Nile tilapia and common carp (NRC, 1998, 2011). The total fatty acid content in duckweeds ranged from 1.05-1.62% (Tang et al., 2015). The fatty acids, linolenic acid, linoleic acid and palmitic acid accounted approximately 80% of total fatty acids and these were most dominant fatty acids (Tang et al., 2015). Carbohydrates in duckweed encompassed polysaccharides, starch and sugars (Shen et al., 2016; Turker and Baran, 2018).

2.3. Digestive enzymes and its importance

Digestive enzymes are the vital component of digestive system. The activities of digestive enzymes determined the digestive capacity of fish (Ali and Jauncey, 2004). Digestive enzyme plays an important function in the physiology of vertebrate in nutrient digestion and absorption of nutrients like protease, α-amylase and lipase (Johnson, 1994; Zhou et al., 2010). Intestinal digestion and absorption capacity to different diets by various fish species conveyed the status of fish health (Deng et al., 2010). Different factors such as age of species, environmental condition and sources of nutrients are responsible for the digestive capabilities of fish (Peres et al., 1998; Debnath et al., 2007; Kumar et al., 2018). The composition of diets also influences the activities of digestive enzymes (Peres et al., 1998; Perez- Jimenez et al., 2009). Secretion of digestive enzymes by the exocrine pancreas of fish in the anterior region of the intestine is more active than its posterior region concerning digestion and absorption of nutrients (Kuz'mina et al., 2008; Chikwati et al., 2013). High protease, α-amylase and lipase activity leads to more protein, carbohydrate and lipid digestion in fish. Amylolytic, cellulolytic,

proteolytic and lipolytic enzymes are responsible for the growth and development of cultured fish and are also involved in the digestion of carbohydrate, cellulose, proteins and lipids respectively (Bairagi et al., 2002). Digestive enzyme activities vary among fish species, in a similar way the activity of the same digestive enzyme differs within a fish in various regions of the intestine (Yang et al., 2018). The digestive mechanism is unique in common carp and being a stomachless fish, common carp changes its activity level of various digestive enzymes to become accustomed to various diets. In the foregut of common carp, digestion and absorption of protein occurs, while digestion and absorption of starch and cellulose occurs throughout the intestine (Zhao et al., 2020). Vegetable proteins are not easy to digest, therefore, protein has to be well utilized by herbivorous fish. Better understanding of nutritional physiology of fishes requires the detailed knowledge of the change associated with the development of digestive system, degradation of food and assimilation efficiency mechanism (Gisbert et al., 1998; Fernández et al., 2001). Numerous studies are reported on the digestive enzyme activities of different fish species (Fernández et al., 2001; Furne et al., 2005; Kumar et al., 2007; Zhang et al., 2018; Kumar et al., 2019).

2.3.1 Protease

The digestive enzyme protease is essential in digestion of food, energy storage and development of fish (Sainz et al., 2004; Kumar et al., 2007). Protease is responsible for the breakdown of proteins to peptides, hence, catalyze proteolysis. Pepsin, trypsin and chymotrypsin are the major digestive proteases. Earlier studies show highest protease activity in carnivorous fish followed by

omnivorous and then herbivorous fishes (Wu and Zhu, 1994). Trypsin belongs to serine proteinases family, it hydrolyzes protein and peptides at carboxyl side of amino acids lysine and arginine residues. Protein digestion, activation of zymogen of chymotrypsin and other enzymes are the important functions of trypsin (Cao et al., 2000; Klomklao et al., 2008). It also plays vital role in all cyprinids at their early development (Chakrabarti and Rathore, 2010).

2.3.2 Amylase

Amylase catalyzes the hydrolysis of carbohydrates or starch into simple sugars (Sundarram and Murthy, 2014). Amylase hydrolyses the internal α-1, 4- glycosidic links in polysaccharides to yield simple sugars such as glucose and maltose. Various studies have reported higher amylase activity in herbivorous fish compared to carnivorous fish (Kuz'mina, 1978; Hofer et al., 1982; Chan et al., 2004; Horn et al., 2006). Fish fed with plant based diets shows significantly enhanced amylase activity (Magalhaes et al., 2016; Ranjan et al., 2018). Activity of amylase is family specific and independent of diet (Hofer et al., 1982). Amylase activity has increased significantly in *L. rohita* fed solid state fermentation *Pistia* leaf (Mandal and Ghosh, 2019). Higher α-amylase activity has been found in the foregut, midgut and hindgut of duckweed fed common carp (Zhao et al. 2020).

2.3.3 Lipase

Lipases catalyzes the hydrolysis of triacylglycerols and breaks down the ester bonds of lipids and fats to convert into fatty acids, glycerols and other alcohols (Melani et al., 2020). Lipase is secreted from the hepatopancreas and is

stimulated by the presence of substrates such as triglycerides and phospholipids, vitamin esters and cholesteryl esters (Wong and Achotz, 2002). In marine fish, lipase activity was observed before starting exogenous feeding, while in freshwater fish, lipase activity was reported after 4 days of hatching (Chakrabarti and Rathore, 2010).

Lipase activity has been reported in *L. rohita* fed fermented *Ipomoea aquatica* and bio-processed *Pistia* leaf (Ali and Kaviraj, 2018; Mandal and Ghosh, 2019). Significantly lower lipase activity has been found in the in the liver of duckweed fed grass carp than those fed with chironomid larvae (He et al., 2013). Higher lipase activity has been found in earthworm fed common carp than those fish fed earthworms with duckweed and only duckweed (Zhao et al., 2020).

2.4. Genes involved in fatty acids metabolism

Accumulation of lipid results from the balance between the fatty acid biosynthesis called as lipogenesis and catabolism of fat by β-oxidation, commonly known as lipolysis. Many key enzymes and transcriptional factors are involved in these processes (Chen et al., 2015). The chief enzyme involved in the lipogenesis pathway is catalyzed by the multienzyme complex, fatty acid synthase (FAS). The lipogenic enzymes also includes acetyl-CoA carboxylase (ACC) and lipolytic enzymes are adipose triacylglyceride lipase (ATGL), hormone sensitive lipase (HSL) and carnitinepalmitoyltransferase I (CPT I) (Eliott and Elliot, 2009). The rate limiting step in fatty acid biosynthetic pathway is the irreversible conversion of acetyl-CoA to malonyl-CoA catalyzed by the enzyme ACC (Cheng et al., 2011). The *de novo* biosynthesis of long-

chain fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH catalyzed by the key enzyme FAS (Dong et al., 2014). Stearoyl-CoA desaturase (SCD) is responsible for synthesis of unsaturated fatty acids (Ardiyani et al., 2012). Fatty acid oleate (oleic acid) is essential for the biosynthesis of triglycerides and other lipids are synthesizes by SCD (Miyazaki *et al*., 2004).

Fish lipid are rich in n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), and it plays an important role in fish nutrition as well as human nutrition. The naturally occurring prime saturated fatty acids in animal fats, including fish lipids are palmitic acid (16:0) and stearic acid (18:0), whereas oleic acid (18:1n-9) and palmitoleic acid (16:1n-7) are the main monounsaturated fatty acids. The major PUFAs in fish are eicosapentaenoic acid, EPA (20:5n-3) and docosahexaenoic acid, DHA (22:6n-3) and their metabolic precursor is α-linoleic acid, LNA (18:3n-3), together with arachidonic acid, ARA (20:4n-6) and its metabolic precursor is 18:2n-6 (linoleic acid, LA) as the major n-6 PUFAs (Tocher, 2003). The unsaturated fatty acids 16:0 and 18:0 are *de novo* biosynthesizes by all known organisms, including fish and desaturate them to yield oleic acid (18:1n-9) and palmitoleic acid (16:1n-7), respectively (Sargent et al., 2002). Elongation reactions which add sequentially two carbon atoms to the carboxyl ends of fatty acyl CoA substrates to form longer fatty acids are catalyzed by elongase enzymes in eukaryotes (Sargent et al., 2002; Tocher, 2004). In aquaculture, the composition of fatty acid of farmed fish depends on the dietary fatty acid supply and the capacity of endogenous C:20 and C:22 LC-PUFAs biosynthesis.

The plant-derived C:18 PUFAs conversion to health essential C:20 and C:22 LC-PUFAs varies species to species and greatly dependent on the complement of their fatty acyl desaturases and elongases. Most animals and plants possess Δ9 desaturase and Δ12 and Δ15 desaturases are found in plants only. Therefore, linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3*n*-3) cannot be synthesized by most animals from oleic acid (18:1n-9), and so there is requirement in dietary supplement of LA and ALA (Sargent et al., 2002). The enzyme Δ6 desaturase catalyzes ALA (18:3n-3) to synthesizes EPA (20:5n-3) by desaturation at the Δ6 position, followed by a 2-carbon elongation and another desaturation at Δ5 position. ARA (20:4n-6) is synthesized from LA (18:2n-6) upon the same enzymatic pattern. Synthesis of DHA (22:6n-3) from EPA (20:5n-3) has been known to proceed through two consecutive chain elongations, another Δ6 desaturation and a peroxisomal β-oxidation chain-shortening reactions. It indicates that last Δ4 ethylene bond insertion does not occur in DHA (22:6n-3) through Δ4 desaturation of its immediate precursor EPA (20:5n-3) (Tocher, 2003). Direct Δ4 desaturation from EPA (20:5n-3) to synthesize DHA (22:6n-3) has been observed in some fishes like, *Siganus canaliculatus* and *Solea senegalensis* (Li et al., 2010; Morais *et al*., 2012). The mean end product of ALA (18:3n-3) is DHA (22:6n-3), while the mean end product of LA (18:2n-6) is ARA (20:4n-6) (Tocher, 2003).

2.4.1. Desaturase

Desaturases are iron-containing, oxygen dependent non-heme enzymes that introduces double bonds at specific positions in fatty acyl chains (Pelley,

2012). Desaturase enzyme are classified into two classes on the basis of its subcellular location, soluble and membrane-bound desaturases (Castro et al., 2016). Fatty acid desaturase enzymes are membrane bound in endoplasmic reticulum introduces double bonds in palmitate and stearate between carbons 9 and 10, producing palmitoleic acid (16:1n-7) and oleic acid (18:1n-9), respectively and this enzyme requires oxygen and nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate (NADPH) (Pelley, 2012). Fatty acid desaturase (*fads*) are a family of genes with three members; *fads1*, *fads2* and *fads3* in mammals. The genes *fads1* and *fads2* in mammals and cartilaginous fish encodes Δ5 and Δ6 desaturases, respectively (Guillou et al., 2010; Lee et al., 2016).

Recently, many fish desaturases have been isolated and characterized functionally. Hasting et al. (2001) reported zebrafish with both Δ5 and Δ6 desaturase bifunctional activity, while common carp, turbot, gilthead seabream and rainbow trout chiefly showed activity of Δ6 desaturase (Zheng et al., 2005). The first fish species to possess separate and distinct Δ6 and Δ5 desaturases genes is Atlantic salmon (Zheng et al., 2005). In Atlantic salmon, four genes encoding putative fatty acyl desaturases (*fad)* protein have been cloned and characterized, *Δ5fad, Δ6fad-a, Δ6fad-b* and *Δ6fad-c* (Hastings et al., 2004; Zheng et al., 2005, Monroig et al., 2010). The genes *Δ6fad-a* and *Δ6fad-b* were primarily expressed in brain, liver and intestine, with also high expression of *Δ6fad-b* in gill and predominant expression levels of *Δ6fad-c* in brain, with little expression in all other tissues. Fish fed diets of low levels of LC-PUFA, shows elevated expression of *Δ6fad-a* and *Δ6fad-b* in intestine and

liver respectively and with no response of *Δ6fad-c* (Monroig et al., 2010). Several studies in fish reported the conversion rates of fatty acid substrates by *Δ6*-desaturase (Seiliez et al., 2001; Zheng et al., 2009). Ren et al. (2012) have reported a high ALA and LA content diets up-regulates the expression level of *Δ6*-desaturase gene (Tocher et al., 2006; Li et al., 2008).

2.4.2. Elongases

Very long chain fatty acyl elongases (elovl) are membrane-bound proteins catalyzed the condensation of two-carbon units to the carboxyl end of a fatty acyl CoA, with malonyl-CoA in the fatty acid elongation pathway (Leonard et al., 2004). It is a gene family of seven members found in vertebrates; differ in specificity of fatty acid substrate and have temporal and spatial expression patterns (Jakobsson et al., 2006). Fatty acid elongation takes place on the cytoplasmic face of the endoplasmic reticulum as well as in the mitochondria (Cook and McMaster, 2002). The elongation of fatty acid substrate is synthesized endogenously from fatty acid supplemented diets (Cook and McMaster, 2002; Leonard et al., 2004; Guillou et al., 2010). There are four steps in each elongation cycle, including conduction, reduction and dehydration. The reactions of reduction are catalyzed by four enzymes resemblance to the de novo fatty acid synthetic pathway of palmitic acid. In vertebrates, *elovl2, elovl4* and *elovl5* of elovl family plays important roles in the biosynthesis of PUFA (Leonard et al*.,* 2004; Jakobsson et al., 2006).

In fish, the enzyme *elovl5* elongates C:18 and C:20 PUFA, with lower elongase capacity towards the substrate C:22 (Castro et al., 2016). The

enzyme, *elovl2* as preferred elongation substrates in the elongation of C:20 and C:22 in fish (Monroig et al., 2009; Oboh et al., 2016; Ferraz et al., 2018) but lost activity towards expansion of teleosts and no activity in recent emerged lineages of teleost (Castro et al., 2016). In teleost, *elovl4* plays critical role in biosynthesis of both saturated and polyunsaturated very long chain fatty acids (>C:24) (Carmona-Antonanzas et al., 2011), also able to convert EPA and DPA (docosapentaenoic acid; 22:5n-3) to 24:5n-3, intermediate substrate for the synthesis of DHA (Carmona-Antonanzas et al., 2011). It has been suggested in various studies that synthesis of DHA in rainbow trout, proceeds via intermediates of C:24 (Buzzi et al., 1997), involves indirect insertion of *Δ4* double bond. In Atlantic salmon, three elongases: *elovl5a, elovl5b* and an *elovl2*-like elongase have been cloned (Hastings et al., 2001, Morais et al., 2009). These are chiefly expressed in liver and intestine, the major sites of lipid distribution and synthesis (Morais et al., 2009). The genes *elovl5b and elovl5a* codes for a protein involved in the elongation of C:18 to C:20 PUFA and C:20 to C:22 (Hasting et al., 2001; Morais et al., 2009). Several studies have reported many *elovl5* elongase genes from fish species which preferred C18 and C20 PUFA, but not C22 PUFAs (Hastings et al., 2005; Zheng et al., 2009). The expression of gene *elovl5* has been elevated in liver when was fish oil has replaced by linseed oil (Zheng et al., 2004)

Chapter 3

Materials and Methods

MATERIALS AND METHODS

3.1. Culture of macrophytes

Freshwater macrophytes *viz*., mosquito fern *Azolla microphylla,* water velvet *Azolla pinnata,* buffalo spinach *Enhydra fluctuans,* water thyme *Hydrilla verticillata,* water spinach *Ipomea aquatica,* duckweed *Lemna minor,* water clover *Marsilea quadrifolia,* water lettuce *Pistia stratiotes,* giant salvinia *Salvinia molesta,* floating fern *Salvinia natans,* greater duckweed *Spirodela polyrhiza* and Asian watermeal *Wolffia globosa* were collected from different water bodies of Delhi, Uttar Pradesh and West Bengal and then identified on the basis of their morphological characteristics. The macrophytes were cultured in cemented tanks (1.2 × 0.35 m) and tanks were filled with clean tap water (30 cm water depth). The surface floating macrophytes *A. microphylla*, *A. pinnata*, *E. fluctuans*, *H. verticillata*, *I. aquatica*, *L. minor*, *M. quadrifolia*, *P. stratiotes*, *S. molesta*, *S. natans*, *S. polyrhiza* and *W. globosa* were cultured without soil (Fig. 1). The soil (10 cm layer) was applied for the culture of *E. fluctuans, I. aquatica* and *M. quadrifolia*. The combination of organic manures, *viz.* cattle manure, poultry droppings and mustard oil cake (1:1:1) was used at the rate of 1.052 kg/m^3 for the culture of macrophytes (Srivastav et al., 2006, DWRP, 1998; BFRI, 1997). The combination of manure was kept in 5 L plastic bucket with water to decompose for five days. After decomposition, the manure was applied in individual tank. One fourth of the initial dose of manure was applied at every tenth day for continuous nutrient supply in the culture tank. This schedule was followed throughout the culture period. The tanks

were monitored regularly and macrophytes were harvested when the whole surface of the tank was covered. The freshly harvested macrophytes were washed twice with tap water and then then with distilled water.

Azola microphylla ***Azolla pinnata*** ***Enhydra Fluctuans***

Hydrilla verticilleta ***Ipomea aquatica*** ***Lemna minor***

Marsilia quadrifolia ***Pistia stratiotes*** ***Salvinia molesta***

Salvinia natans ***Spirodela polyrhiza*** ***Wolffia golobosa***

Fig.1: Freshwater macrophytes grown in the outdoor facility

After washing, macrophytes were air dried and kept at 40°C for 3 h in an oven. Then ground, sieved, powders were kept in air tight containers and stored in a refrigerator at 4°C for proximate composition analysis.

3.2. Culture of selected macrophyte *Spirodela polyrhiza*

3.2 .1.Culture in outdoor tanks

The proximate composition, amino acid, vitamins composition and yield shows that the greater duckweed *Spirodela polyrhiza* is a nutrient rich plant ingredient for fish feed formulation. Therefore, the culture technique of the selected macrophytes was devolved using different organic manures, inorganic fertilizers and their combinations. The experiment was conducted in outdoor cemented tanks during December 2016 - February 2017 and the duration of the experiment was 120 days (Fig.2). Three different combinations of manures were used for the culture of *S. polyrhiza* culture. In manure 1 (organic manure, OM), cattle manure (local), poultry droppings (local) and mustard oil cake (Double Hiran Mustard Oil-cake, Malook Chand Food Pvt. Ltd., Aligarh, U.P. India) (1:1:1) were used at the rate of 1.052 kg/m^3 (Srivastava *et al.*, 2006). In manure 2 (Inorganic fertilizer, IF), urea (IFFCO, Indian Farmers Fertilizer Cooperative Limited, New Delhi, India), potash (Narmada, Gujarat Narmada Valley Fertilizers and Chemicals, Gujarat, India) and triple superphosphate (IPL, Indian Potash Limited, Chennai, India) were used at the rate of 15, 3 and 3 kg/ha/day, respectively based on the study of DWRP (1998). In manure 3 (combination of cattle manure and inorganic fertilizers, OM + IF**),** cattle manure, urea, potash and triple super phosphate

were used at the rate of 750, 7.5, 1.5, 1.5 kg/ha/day, respectively based on the study BFRI (1997).

In all these experiments, organic manures were applied at the rate of one fourth dose of initial dose of every 10 days interval. In inorganic fertilizer, similar dose (initial amount) of manures was applied at every 10 days interval. Manures for individual tank were mixed with tap water and allowed to decompose for 5 days before application. All manures, except cattle manure were applied in dry conditions. The moisture content of cattle manure was measured and the weight was adjusted.

Plants were harvested when the surface of the tanks were covered with culture plants. The half of the covered surface were harvested and rest half was left for further growth. At the end of experiment all plants were harvested, production and relative growth rate (RGR) were calculated at each harvest. Harvested plants were washed thrice with distilled water, air dried, powdered and stored at 4°C till further use.

Fig.2. Culture of *S. polyrhiza* in outdoor cemented tanks

3.2.2. Culture of *S. polyrhiza* in pond

Three cemented ponds at the Central Institute of Fisheries Education (Indian Council of Agricultural Research), located at Rohtak, Haryana were used for the production of *S. polyrhiza* during July - August 2017 (Fig.3). Each pond was 200 m^2 in size (20m×10 m) and a 50 cm water level was maintained in the pond. Based on the results of the outdoor culture, the combination of organic manures (cattle manure, poultry dropping and mustard oil cake, 1:1:1) was selected for the pond culture of *S. polyrhiza*. Manures were decomposed for 5 days and then *S. polyrhiza* were introduced in each pond at the rate of 1 kg/pond (fresh weight). Initially, these greater duckweeds covered a small area of the water body (Fig.4). In each pond, after the initial dose, one fourth dose of manure was applied at 10 days interval. Greater duckweeds were harvested thrice at 10 days interval during 30 days of culture period. The harvesting pattern was similar to tank production, i.e. duckweeds were harvested when the whole water surface was covered. In first and second harvest, 50% duckweeds were harvested and plants were totally collected during the third harvest (Fig.). The production rate of cultured *S. polyrhiza* was expressed as kg/ha/month (DW).

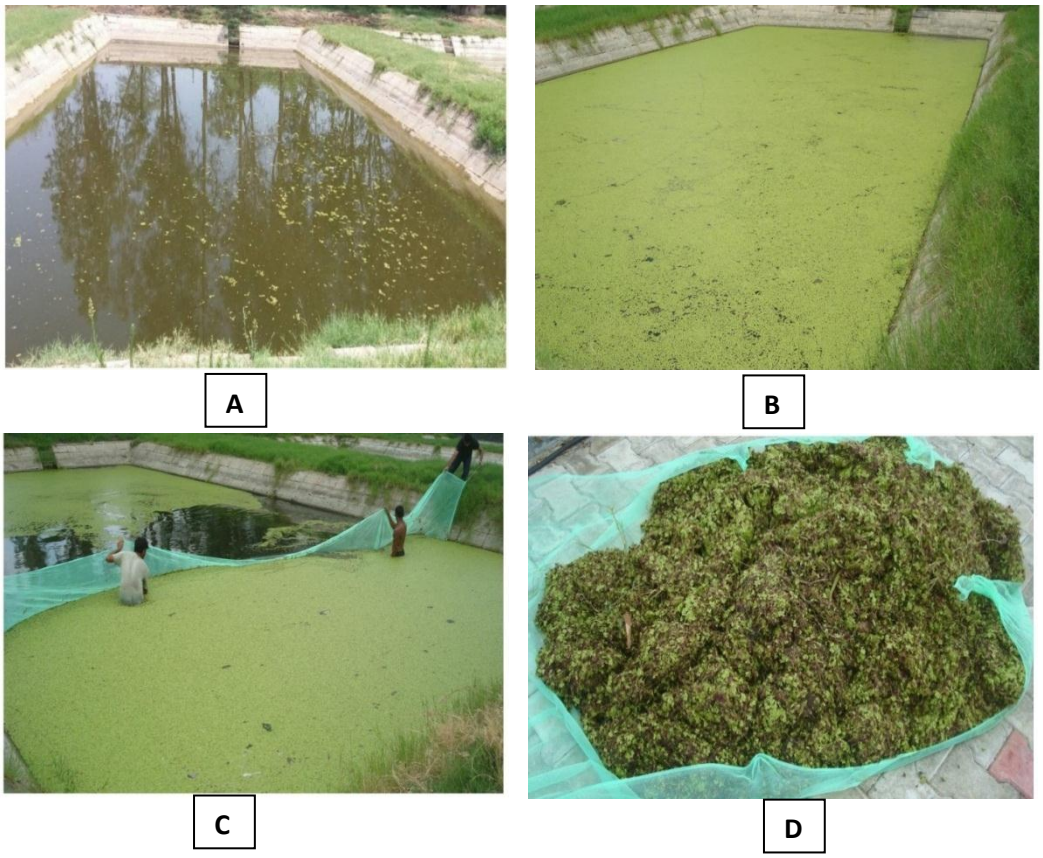

Fig.3: Introduction of *S. polyrhiza* in the pond of Central Institute of Fisheries Education, Rohtak, Haryana, India. **(B)** Production of *S. polyrhiza* in the pond after 10 days of culture. **(C)** The harvesting (50%) of *S. polyrhiza* in pond. (**D**) Freshly collected *S. polyrhiza.*

3.3. Water quality

Major water quality parameters *viz*. temperature, pH, dissolved oxygen, ammonia (NH_3) nitrate (NO_3^-) and conductivity were monitored regularly using digital multi-parameter (HQ 40d, Hach, USA). Light intensity was measured at the surface of the water with probe (PMA 2130) attached with a lux meter (Solar Light, PMA 2100, USA). Phosphate (4500-P D. Stannous Chloride Method) and nitrite (4500-NO_2^- B. Colorimetric Method) levels were measured following standard protocols (APHA, 2017).

3.4. Relative growth rate

Fresh *S. polyrhiza* was used for the estimation of relative growth rate (RGR). The RGR was calculated using the formula:

$RGR = \ln (W_t/W_0)/t$

Where, W_t and W_0 are the weight of *S. polyrhiza* at the time t and zero reference time, respectively; t is the time interval in days. RGR was expressed as g/g/day

3.5. Formulation of fish feed

Two feeding trial were conducted to evaluate the suitability of cultured greater duckweed *S. polyrhiza* as feed ingredient for rohu *Labeo rohita* and common carp *Cyprinus carpio*. The greater duckweed *S. polyrhiza* was cultured using organic manures *viz*., cattle manure mustard oil-cake and poultry dropping. The greater duckweed was collected, cleaned, dried, and ground. The meal was stored at 4°C till feed formulation. Other feed ingredients *viz*., soybean meal (Ruchi Soya Industries Limited, Mumbai, India), wheat flour (Aashirvaad Atta, ITC Limited, Bangalore, India), corn flour (Ahaar, Private Limited, New Delhi, India), sunflower oil (Fortune, Adani Wilmar Limited, Gujarat, India), amino acids (Himedia, Mumbai, India), vitamin and mineral premixes (Piramal Enterprises Limited, Mumbai) were purchased. Five isoproteic, isolipidic, and isoenergetic experimental feeds were prepared with graded inclusion of *S. polyrhiza* meal replacing soybean meal as the primary protein source (Table 1). Fish feeds were formulated using the Winfeed 2.8 software package

(WinFeed UK Limited, Cambridge, United Kingdom). The control feed (SP0) contained soybean meal as the only primary source of protein. In the four experimental feeds, greater duckweed was incorporated at the levels of 5, 10, 15, and 20% of total feed at the expense of soybean meal, wheat flour, corn meal, and sunflower oil (to maintain constant crude protein, crude lipid, and gross energy levels) to produce feeds SP5, SP10, SP15, and SP20 (Fig.3). The soybean meal was replaced in a graded manner, which resulted in changes in the proportions of amino acids in the feeds. Therefore, some specific amino acids such as histidine, methionine, lysine, and threonine were supplemented to the feeds based on the reported requirements of common carp and rohu (NRC, 2011). The inclusion levels of these four amino acids were determined using the Winfeed software to ensure the requirements of the fish were satisfied. All dry feed ingredients were blended for 10 min and mixed with the oil, then warm water was added slowly and everything was mixed thoroughly. The entire mixture was placed in the hopper of the Twin-Screw extruder (Basic Technology Private Limited, Kolkata, India) (Fig.4). The feed pellets were prepared and extrusion conditions were as follows: cutter 134 rpm; feeder 10 rpm; extrusion 190 rpm; extrusion torque 9.22; heater 1 temperature 65^0C; heater 2 temperature 70^0C; and final mass temperature 75^0C.

Table 1: Ingredient proportions and proximate composition of diets. Values are given as Means + SE (n = 3)

Ingredients	**SP0**	**SP5**	**SP10**	**SP15**	**SP20**
Soybean meal	500.0	475.0	450.0	425.0	400.0
S. polyrhiza	--	50.0	100.0	150.0	200.0
Wheat flour	245.0	224.0	204.0	183.0	162.0
Corn meal	147.0	144.0	147.0	138.0	135.0
Sunflower oil	62.0	60.0	57.0	55.0	52.0
Vitamin/minerals premix	5.0	5.0	5.0	5.0	5.0
Mono calcium phosphate	20.0	20.0	20.0	20.0	20.0
Choline chloride	1.0	1.0	1.0	1.0	1.0
Histidine	1.0	1.0	2.0	2.0	2.0
Methionine	12.0	12.0	12.0	12.0	12.0
Lysine	5.0	6.0	6.0	7.0	7.0
Threonine	2.0	3.0	3.0	4.0	2.0

Diets: SP0 = Control, Soybean; SP5 = Soybean + 5% *S. polyrhiza*; SP10 = Soybean + 10% *S. polyrhiza*; SP15 = Soybean + 15% *S. polyrhiza*; SP20 = Soybean + 20% *S. polyrhiza*.

The diameter of the produced pellets was 1 mm. All feeds were stored at 4^0C prior to use. A common difficulty in the use of feeds based on plant ingredients is their palatability to the fish (Rodriguez et al., 1996), but this can be mitigated by the extrusion process. Anti-nutritional factors such as trypsin inhibitor, phytic acid tannins, oxalates etc. are found in greater duckweed (Cruz et al., 2011). However, the preparation of the feed by the extrusion technique helped to mitigate the impact of anti-nutritional factors as high temperature and pressure inactivate many of these factors and control enzymatic rancidity of nutrients (Rokey, 2004; Stadtlander et al., 2019).

Fig.3: Experimental and test diets prepared for *Labeo rohita* and *Cyprinus carpio*

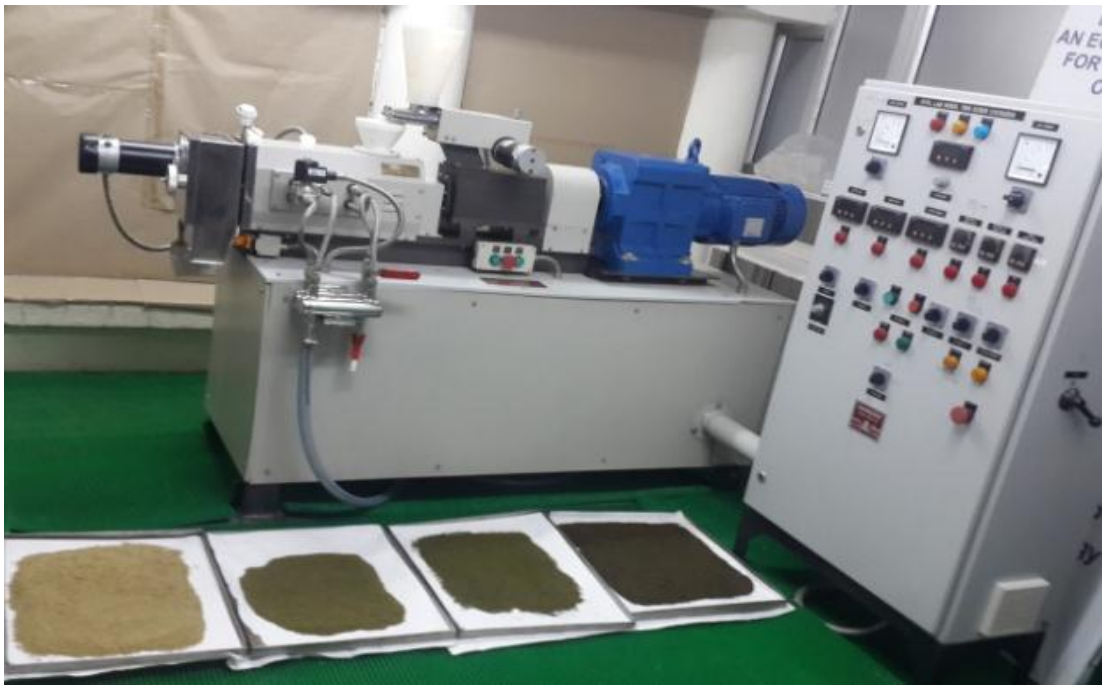

Fig.4: Twin-Screw Extruder (Basic Technology, Kolkata, India) used for the preparation of diets.

3.6. Culture of fish and sampling

The fishes were collected from a local fish farm and acclimated in the aquarium for 1 week. Each glass aquarium was connected to an external filtration unit (Sera fil bioactive 130, Germany). Water from the fish culture units was constantly filtered through the filtration unit to maintain ammonia levels in the fish culture unit. The dissolved oxygen level of water was maintained with the help of an aerator. Fishes were fed with soybean-based control feed during acclimation period. In the first experiment, rohu fingerlings (1.73±0.22 g) were placed randomly in the aquarium (Fig.5). The stocking density was 20 fingerlings/aquarium. In the second experiment common carp fry (0.473±0.006 g) were divided into 15 glass aquariums (50 L each) at random, with 30 fish in each aquarium. After 7 days of feeding experiment diet

started. The fish were fed one of the five different feeds SP0, SP5, SP10, SP15, and SP20 and three replicates were used for each treatment. The feeds were distributed *ad libitum* two times daily at 09:00 and 17:00 h and the weight of feed measured before distribution. Excess (uneaten) feed was collected after 1 h of feeding from each aquarium and oven dried.

Water quality parameters, namely, temperature, pH, dissolved oxygen, and conductivity were monitored in each aquarium using a probe connected to a portable meter (HQ40d Multiparameter, Hach, USA). The ammonia (NH_3) level was estimated using a probe, connected to Orion Versastar (Thermo Scientific, USA). The nitrite (NO_2^-), nitrate (NO_3^-), and phosphate (PO_4^{3-}) contents were analyzed regularly (APHA, 2017). After 60 days of culture, the feeding experiment was terminated, and fish were collected. Fish were starved for 24 h before harvesting, and then, all fish were euthanized with tricaine methane sulfonate (MS222, Sigma, USA), and the weight of individual fish was measured. Four fish from each tank were pooled (four fish/replicate) and stored at -80^{0}C for the assay of whole-body proximate composition, amino acid, and fatty acid profiles assays. Three replicates were used for each feeding regime (three replicates per diet, *n*=3). The digestive tracts of two individual fish per aquarium were collected (two fish/replicate, three replicates; 2×3=6 fish/diet) for the assay of digestive enzyme activities. The hepatopancreas from individual fish was collected (100 mg) and stored in 1 ml of TRIzol reagent (Ambion, Life Technologies, USA) for the gene expression analysis (four fish/treatment).

Fig.5: Culture of *Labeo rohita* under five different feeding regimes

3.7. Survival and growth parameters

Survival, final body weights (FBW), specific growth rate (SGR) and feed conversion ratio (FCR) were calculated as follows:

$$\text{Survival (\%)} = \frac{\text{Final number of fish in treatments at the time of harvesting}}{\text{Initial number of fish in treatments}} \times 100$$

$$\text{SGR (\%)} = \frac{\text{In Final weight of fish} - \text{In Initial weight of fish}}{\text{Duration of expriment}} \times 100$$

$$\text{FCR} = \frac{\text{Dry weight of feed consumed by individual fish during experiment}}{\text{Wet weight gain of individual fish}}$$

3.8. Biochemical assays

The proximate compositions of cultured macrophytes, feed samples and cultured fishes were determined following the standard protocol (AOAC, 2000). Three replicates were used for each assay.

3.8.1. Moisture content

Moisture content of the sample was estimated gravimetrically method. Briefly, 2 g of sample was taken in the Petri dish and spread uniformly. Then Petri dish containing sample was kept in an oven at 105°C for 3 h. After drying, it was kept in desiccators with partially covered lid. The final weight of the dried sample was taken for moisture content. The percentage of moisture was calculated using the following formula:

$$\text{Moisture (\%)} = \frac{\text{Initial weight of sample - Final weight of dried sample}}{\text{Initial weight of sample}} \times 100$$

3.8.2. Ash content

Ash content of the sample was determined using muffle furnace. Briefly, 2 g of sample was taken in crucible and then kept in oven at 105°C for 24 h. After drying, the sample was kept in a muffle furnace at 550°C for 8 h. Then the crucible was taken out from the muffle furnace and was kept in a desiccator for cooling. The percentage of ash was calculated using the following formula:

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

3.8.3. Crude protein

Crude protein content was determined using automated micro-Kjeldhal system (Pelican Instruments, Chennai, India). The Kjeldhal method was based on three successive steps: digestion, distillation and titration. The sample was digested with sulphuric acid in the presence of catalyst (potassium sulphate and cupric sulphate in 5:1) for complete hydrolysis. The

sample was converted into ammonium radicals by breaking entire nitrogen bond in the sample. During distillation, sodium hydroxide was added in the digested sample and then distillate was received in boric acid solution. Finally, the amount of ammonia-nitrogen in this solution was titrated with a 0.1N HCl. The percentage of nitrogen and protein were analyzed with the help of pre-installed programme software (TIAMO 2.2-81, Metrohm, Switzerland). The percentage of nitrogen was calculated:

$$N\ (\%) = \frac{14 \times \text{Normality of acid} \times \text{titrant value} \times 100}{\text{Weight of sample x } 1000}$$

Crude protein (%) was determined by multiplying nitrogen (%) with conversion factor 6.25.

3.8.4. Crude lipid

Crude lipid was determined following the chloroform: methanol extraction method (Folch et al., 1957). The sample (500 mg) was taken in a falcon tube and then chloroform: methanol (2:1, v/v) was added to the sample. Then it was vortexed for 1 min and centrifuged at 2057 x *g* for 15 min at 25°C (Sigma 3K30, Germany). The supernatant was collected in another tube and the process was repeated thrice. The pooled supernatant was passed through Whatman filter paper (no. 1) into a clean test tube and 2 ml milli-Q water was added for phase separation. Again it was centrifuged at 2057 x *g* for 5 min. The lower organic phase was collected carefully in a clean tube. The solution was evaporated under the stream of nitrogen gas in Nitrogen Evaporator (PCi

Analytic Private limited, Maharashtra, India) to remove the solvent. The percentage of crude lipid was calculated:

$$\text{Crude lipid (\%)} = \frac{\text{Final weight of tube - Initial weight of tube}}{\text{Weight of sample}} \times 100$$

3.8.5. Carbohydrate

Carbohydrate was estimated using the subtraction method (Aksnes and Opstvedt, 1998). The percentage of carbohydrate was calculated using the following equation:

Carbohydrate (%) = [100 – (Moisture + ash + crude protein + crude lipid %)]

3.8.6. Energy value

Energy value was measured following the standard method (Merrill and Watt, 1973). Energy (kcal/kg) = [(Crude protein/kg×4) + (Crude lipid/kg×9) + (Total carbohydrate/kg× 4)].

3.8.7. Amino acid analysis

The amino acid content of the cultured macrophytes, feed samples and cultured fishes were analyzed using Automatic amino acid analyzer, L-8900 (Hitachi Co. Ltd. Tokyo, Japan). The samples were digested with 6 N HCl in vacuumed hydrolysis tube at 110^0C for 22 h except, typtophan, methionine and cysteine (Fountoulakis and Lahm, 1998; Chakrabarti et al., 2018). The samples were digested with 4 N methanesulfonic acid and 3-(2-aminoethyl) for the estimation of tryptophan (Simpson et al., 1976). Methionine and cysteine were recovered by oxidizing the sample with performic acid and then

treated with 48% hydrobromic acid before acid hydrolysis. Treatment of performic acid converts cystine and cysteine to cystic acid and methionine to methionine sulfonate, both derivatives are stable to acid hydrolysis (Moore, 1963). Hydrolyzed sample was dried in nitrogen evaporator (PCi Analytic Private Limited, Maharashtra, India). Then 0.02 N HCl was added in the sample. The concentration of protein in the sample was 0.5 mg/ml. The sample was kept in the autosampler. Sample (20 µl) was injected by autosampler. Amino acids were separated by cation-exchange resin column (4.6 mm ID × 60 mm L) with 3 mm particle size using a gradient of lithium citrate buffers with increasing pH and temperature. The following analytical conditions were used for amino acid separation: column temperature 30-70^{0}C, reaction temperature 135^{0}C, and a ninhydrin flow rate of 0.35 ml/min. The reaction with α-amino group of primary amino acid with ninhydrin to forms Ruhemann's purple detectable at 570 nm. The reaction of ninhydrin with proline and hydroxyproline gives yellow colour compound detectable at 440 nm. The concentration of individual amino acid was compared with a standard solution (Wako Pure Chemical Industries Limited, USA) and expressed as g/100 g dry weight.

3.8.8. Fatty acid analysis

Fatty acid compositions of cultured macrophytes, feed samples and cultured fishes were analyzed with gas chromatography and flame ionization detection method (GC-FID) using a Clarus 580 (PerkinElmer, Waltham, USA). In brief, crude lipid was extracted from the samples following the protocol of Folch et

al. (1957) using chloroform/methanol (2:1, v/v). Three replicates were used for each treatment. Fatty acid methyl esters (FAME) were prepared from crude lipid extracts by acidic transesterification, treating the lipid with 1% sulfuric acid in methanol for 16 h at 50^0 C (Christie, 2003). After extraction and purification of FAME (Tocher and Harvie, 1988), a 1 ml aliquot was placed in a glass vial of the GC autosampler. Fatty acids were separated using a 60 m ZB-wax GC column, internal diameter of 0.32 mm, and film thickness of 0.25 mm (Phenomenex, Hyderabad, India). Data were collected using preinstalled programmed software (TotalChrom Workstation Ver6.3; PerkinElmer, USA). The FAME were identified and quantified with the help of standard (Supelco FAME 37 mix; Sigma-Aldrich, USA). The concentration of fatty acid was expressed as mg/100 g and area % of total fatty acids.

3.8.9. Water soluble vitamins

Vitamin contents of macrophytes were analyzed using UHPLC (Thermo Fisher Scientific, USA) with C18 column (3 µM, 150×4.6 mm). The water soluble vitamins thiamine (B_1), riboflavin (B_2), pyridoxine (B_6) and cobalamin (B_{12}) were assayed following the standard protocol (Sami et al., 2014). The dry sample (2 g) was placed in 0.1 N H_2SO_4 at 121^0C for 30 min. Then the samples were kept at room temperature and the pH 4.5 was adjusted with the help of 2.5 M sodium acetate. The enzyme takadiastase (50 mg) was added to the sample and incubated at 35^0C for 12 h. After incubation, the sample was filtered through Whatman filter paper (no. 4) and the filtrate was diluted with 50 ml distilled water. The diluted sample was again filtered with 0.45 μm

syringe filter, twenty microliters of the sample was injected by Autosampler in the injector of UHPLC system. The mobile phase (A/B 33/67; A: MeOH, B: 0.023 M H_3PO_4, pH 3.54) at a flow the rate of 0.5 ml/min used for chromatographic separation. Absorbance was recorded at 270 nm at 25^0C (Marzougui et al, 2009).

Vitamin C content of the macrophytes was analyzed (Sami et al., 2014; Babarinde and Fabunmi, 2009). Sample was blended and homogenized with the extracting solution (mixture of 0.3 M metaphosphoric and 1.4 M acetic acids). The sample was centrifuged at 10000 × *g* for 15 min and filtered through Whatman filter paper (no. 4). Filtered sample was again filtered with 0.45 μm syringe filter and twenty micro liter sample was injected in the UHPLC. The chromatographic separation was achieved on a RP-HPLC column through isocratic delivery of mobile phase (A/B 33/67; A: 0.1 M potassium acetate, pH = 4.9, B: acetonitrile: water [50:50]) at the flow rate of 1ml/min. The absorbance was recorded at 254 nm at 25°C.

3.9. Digestive enzyme assay

The digestive system was freeze-dried and homogenized in ice-cold Milli-Q R water (1:10) to maintain neutral pH of the extracts. Homogenate was centrifuged for 30 min at 10,000×*g* at 4^0C, and supernatants were collected for the assay of digestive enzyme activities using flurometer (Multimode reader, BioTek Synergy H1 Hybrid, USA). Three replicates were used for each treatment. The amylase activity was determined using an assay kit (E33651; Invitrogen, USA) and the fluorescence was measured at 485

(excitation) and 520 nm (emission). The enzyme activity was expressed as mU/mg protein/min. A protease kit (E6638; Invitrogen) was used to measure the total protease activity and the fluorescence was measured at 485 (excitation) and 530 nm (emission). The protease activity was expressed as fluorescence change/unit. The substrate *N*-benzoylL-arginine-methyl coumarinylamide (Sigma-Aldrich) was used for the estimation of serine proteases trypsin (Ueberschär, 1988). The activity was measured at 380 (excitation) and 440 nm (emission). The chymotrypsin activity was measured using succinyl-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (SigmaAldrich) as the substrate (Cao et al., 2000). The fluorescence was measured at 380 (excitation) and 450 nm (emission). The enzyme activities were expressed as mM 7-amino-4-methylcoumarin (AMC)/mg protein/min. The neutral lipase activity was determined following the method of Roberts (1985) using the substrate 4-methylumbelliferyl butyrate, 4-MU (Sigma-Aldrich, USA). The fluorescence was measured at 365 nm (excitation) and 450 nm (emission). The enzyme activity was expressed as mM 4-MU/mg protein/min. Protein content was estimated using bovine serum albumin (BSA, Sigma-Aldrich) as standard (Bradford, 1976).

3.10. Gene Expression

The levels of mRNA expression of delta-6 fatty acyl desaturases (*fads2d6*), elongation of very-long-chain fatty acids protein 2 (*elovl2*), elongation of very-long-chain fatty acids protein 5 (*elovl*5), and fatty acid synthase (*fas*) genes were determined in the hepatopancreas of common carp. Total RNA was

extracted using the TRIzol reagent (Ambion, Life Technologies, USA) following the protocol of the manufacturer. The absorbance of extracted RNA was examined at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific, USA) to determine the concentration and quality. The extracted RNA was treated with 1 U of DNase ISigma-Aldrich, USA) to avoid DNA contamination, and the quality of RNA treated with DNase was checked with 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed into cDNA by the reverse transcription reaction using high-capacity cDNA reverse transcription kit (Applied Biosystems, USA), using the protocol provided by the manufacturer. Quantification of gene expression was carried out with quantitative reverse transcription polymerase chain reaction (qRT PCR) using a Quant Studio 6 Flex system (Applied Biosystems) and PowerUp SYBRTM Green Master Mix (Applied Biosystems). Primers were designed using the online primer design tool of NCBI and *β-actin* was used as the reference (housekeeping) gene (Table 2). The efficiency of primers was evaluated using the melt curve and standard curve analysis with the QuantStudio 6 Flex Real-Time PCR system software v1 (Applied Biosystems). The reaction mixture (10 ml) used for the qRT-PCR was composed of 0.25 ml PCR forward primer (2.5 mM), 0.25 ml PCR reverse primer (2.5 mM), 1 ml of cDNA (1:3), 5 ml of 2 × PowerUpTM SYBRTM Green PCR Master Mix (Applied Biosystems), and nuclease-free water (3.5 ml). Samples were run in duplicate for each target gene with non-template control (NTC). The thermal cycling conditions were as follows: pre-denaturation of nucleic acid at 95^0C for 10 min followed by either 40 cycles of 15 s at 95^0C

and 1 min at 60^0C (primer T^M 60^0C) or 40 cycles of 15 s at 95^0C, 15 s at 55^0C, and 1 min at 72^0C for (primer T^M < 60^0C). The data of qRT-PCR was calculated using the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) method using ***β-actin*** as the internal control.

Table 2: Target genes and sequences of primers used for qPCR analysis in *Cyprinus carpio*.

Target gene	Primer	Primer sequence (5'-3')	Accession number	Primer efficiency (%)	Amplicon size (bp)
Cyprinus carpio					
Delta-6-desaturase (*fads2d6*)	*fads2d6* Fw	AGAAATCCGGAGAAATCTGGCT	AF309557	96.01	122
	fads2d6 Rv	ACTGGCGGTTTAGTTGATGTCT			
Elongation of very long chain fatty acids 2 (*elovl2*)	*elovl2* Fw	ATCAGTTTGGTCTGCCGGTT	KR706498	98.76	142
	elovl2 Rv	CAGCACAATGAAGATGGTGTCC			
Elongation of very long chain fatty acids 5 (*elovl5*)	*elovl5* Fw	GATTGACGACACTTCGTCCG	KF924199	97.51	122
	elovl5 Rv	GAAAGTGTGGCTGCAGTGTG			
Fatty acid synthase (*fas*)	*fas* Fw	AATGCTTGGCAGTCCAGAGT	KY378913	96.22	139
	fas Rv	AGACACCTGGAACAAGTCCTC			
β-actin	β-actin Fw	AGACATCAGGGTGTCATGGTTGGT	M24113.1	99.52	352
	β-actin Rv	CTCAAACATGATCTGTGTCAT			

3.11. Statistical Analysis

Data are presented as means with standard error (SEM) with n values as stated. The IBM SPSS 25.0 software (SPSS Inc., Michigan Avenue, Chicago, IL, USA) was used for statistical analysis. Data were analyzed using one way analysis of variance. Tukey's test was performed to compare the differences among experimental groups. The linear regression analysis was performed to check the effect of inclusion level of *S. polyrhiza* in the diets on growth performance, digestive enzyme activities, proximate composition, amino acid profile and fatty acid profile of fish.

Chapter 4

Results

RESULTS

4.1. Biochemical composition of macrophytes

Twelve freshwater macrophytes *viz*., mosquito fern *Azolla microphylla,* water velvet *Azolla pinnata,* buffalo spinach *Enhydra fluctuans,* water thyme *Hydrilla verticillata,* water spinach *Ipomea aquatica,* duckweed *Lemna minor,* water clover *Marsilea quadrifolia,* water lettuce *Pistia stratiotes,* giant salvinia *Salvinia molesta,* floating fern *Salvinia natans,* greater duckweed *S. polyrhiza* and Asian watermeal *Wolffia globosa* were collected from different water bodies, cultured and their biochemical compositions were assayed.

4.1.1. Proximate composition of collected macrophytes

The study showed variations in the compositions of the macrophytes (Table 1). The moisture content was significantly (P<0.05) higher in *E. fluctuans* compared to the other macrophytes. The moisture content was lowest in *A. pinnata*. Total protein contents was significantly (P<0.05) higher in *L. minor* and *S. polyrhiza* compared to the other macrophytes. There was no significant (P>0.05) difference in the protein content between *L. minor* and *S. polyrhiza*. Among the twelve macrophytes, protein content was >30% in three macrophytes *viz*., in *L. minor, S. polyrhiza* and *W. globasa*. In other macrophytes (except *P. stratiotes* and *M. quardifiollia*), protein contents were more than 20%. The protein content was minimum in *M. quadrifolia.* Significantly (P<0.05) higher lipid content was found in *L. minor* compared to other macrophytes. This plant was followed by *S. polyrhiza* and *I. aquatica*. Ash contents ranged from 11.46 (*P. stratiotes*) - 21.12% (*H. verticillata*).

Table 1: Proximate composition of freshwater macrophytes cultured with organic manures (%, dry weight).

Macrophytes	Moisture	Crude protein	Crude lipid	Ash	Carbohydrate	Energy (kcal/kg)
Azolla microphylla	7.67 ± 0.10^{ef}	24.39 ± 0.220^{e}	6.09 ± 0.017^{e}	14.48 ± 0.172^{e}	47.36 ± 0.482^{b}	3418.31 ± 8.72^{a}
Azolla pinnata	7.42 ± 0.175^{f}	29.45 ± 0.176^{c}	5.47 ± 0.043^{f}	17.47 ± 0.047^{d}	40.19 ± 0.084^{cd}	3278.11 ± 8.08^{c}
Enhydra fluctuans	11.87 ± 0.043^{a}	26.36 ± 0.08^{d}	4.53 ± 0.047^{j}	18.58 ± 0.192^{c}	38.65 ± 0.350^{d}	3008.50 ± 7.80^{f}
Hydrilla verticillata	9.90 ± 0.204^{c}	24.18 ± 0.274^{e}	4.99 ± 0.040^{hi}	21.12 ± 0.45^{a}	39.79 ± 0.282^{cd}	3008.63 ± 21.64^{f}
Ipomoea aquatica	10.73 ± 0.167^{b}	29.04 ± 0.371^{c}	7.12 ± 0.072^{c}	12.59 ± 0.035^{f}	40.50 ± 0.600^{c}	3423.33 ± 6.19^{a}
Lemna minor	8.17 ± 0.032^{e}	36.97 ± 0.091^{a}	8.10 ± 0.069^{a}	20.02 ± 0.061^{b}	26.73 ± 0.190^{g}	3277.55 ± 2.84^{c}
Marsilea quadrifolia	10.80 ± 0.099^{b}	16.78 ± 0.051^{g}	5.123 ± 0.018^{gh}	20.83 ± 0.092^{ab}	46.45 ± 0.195^{b}	2990.72 ± 7.67^{f}
Pistia stratiotes	10.93 ± 0.031^{b}	19.55 ± 0.315^{f}	5.99 ± 0.060^{e}	11.46 ± 0.119^{g}	52.05 ± 0.366^{a}	3403.43 ± 4.85^{a}
Salvinia molesta	7.17 ± 0.058^{f}	28.78 ± 0.383c	5.31 ± 0.069^{fg}	18.29 ± 0.185^{cd}	40.44 ± 0.237^{c}	3246.83 ± 5.24^{cd}
Salvinia natans	8.80 ± 0.107^{d}	28.04 ± 0.318^{c}	4.77 ± 0.044^{ij}	18.55 ± 0.108^{c}	39.81 ± 0.397^{cd}	3144.39 ± 3.52^{e}
Spirodela polyrhiza	7.564 ± 0.046^{f}	36.84 ± 0.241^{a}	7.72 ± 0.040^{b}	18.41 ± 0.014^{c}	29.45 ± 0.308^{f}	3346.89 ± 4.02^{b}
Wolffia globosa	6.33 ± 0.061^{g}	32.68 ± 0.122^{b}	6.48 ± 0.056^{d}	21.02 ± 0.051^{a}	33.48 ± 0.110^{e}	3229.77 ± 2.29^{d}
***P*-value**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
***F*- value**	274.566	439.408	516.141	361.526	467.620	359.840

Values having the means (n=3) in each row with different superscripts are significantly (P<0.05) different.

Carbohydrate content was significantly (P<0.05) higher in *P. stratiotes* compared to other macrophytes. The calorific value of macrophytes ranged from 3423.33 (*I. aquatica*) - 3008.50 kcal/kg (*E*. *fluctuans*). Significantly (P<0.05) higher and lower energy values were found in *I. aquatica* and *M. quadrifolia*, respectively compared to the other macrophytes.

4.1.2. Amino acid composition

4.1.2.1. Essential amino acids

The essential amino acid arginine content was significantly (P<0.05) higher in *I. aquatica* and *E. fluctuans* compared to other macrophytes (Table 2A). These macrophytes were followed by *W. globosa* and *L. minor*. Other essential amino acids histidine, valine and threonine contents were significantly (P<0.05) higher in *S. polyrhiza;* isoleucine, lysine and phenylalanine contents were significantly (P<0.05) higher in *L. minor* compared to others. There was no significant (P>0.05) difference between *L. minor* and *S. polyrhiza*. Leucine and methionine contents were significantly (P<0.05) higher in *L. minor* compared to others. This plant was followed by *S. polyrhiza.* Aromatic amino acid tryptophan was significantly (P<0.05) higher in *S. molesta* compared to other macrophytes. The lowest tryptophan content was found in *M. quadrifolia*.

4.1.2.2. Non-essential amino acids

The glutamate was the most abundant amino acid among the non-essential amino acids (Table 2B). Significantly (P<0.05) higher glutamate content was

found in *L. minor* and *S. polyrhiza* compared to others. Significantly (P<0.05) higher and lower alanine contents were found in *S. polyrhiza* and *P. stratiotes*, respectively compared to other macrophytes. There was no significant (P>0.05) difference in alanine content between two species of *Salvinia*. The aspartate content was significantly (P<0.05) higher in *S. polyrhiza* compared to other macrophytes and this plant was followed by *L. minor*. Sulfur containing amino acid cysteine was significantly (P<0.05) higher in *S. polyrhiza* compared to others. The glycine content was significantly (P<0.05) higher in *L. minor* and *S. polyrhiza* compared to others and these macrophytes were followed by *H. verticilleta, I. aquatica* and *S. molesta*. Proline content was significantly (P<0.05) higher in *W. globosa* compared to the other macrophytes. This plant was followed by *H. verticilleta* and *M. quadrifolia*. Serine and tyrosine contents were significantly (P<0.05) higher in *L. minor* compared to others.

The phosphoserine and taurine levels were significantly (P<0.05) higher in *L. minor* and *I. aquatica*, respectively compared to others. Significantly (P<0.05) higher and lower levels of phospho ethanol amine was observed in *S. polyrhiza* and *H. verticilleta*, respectively compared to others macrophytes. The phospho ethanol amine was absent in *A. microphylla, M. quadrifolia, P. stratiotes* and *W. globosa.* The α-amino adipic acid and β-amino isobutyric acid contents were significantly (P<0.05) higher *in L. minor* compared to others macrophytes. Significantly (P<0.05) higher levels of cystathionine and β-alanine were found in *L. minor* compared to others macrophytes. The γ-amino-n-butyric acid and 1 methyl histidine contents were significantly

(P<0.05) higher in *S. polyrhiza* and *I. aquatica*, respectively compared to other macrophytes. The 1 methyl histidine amino acid was absent in *W. globosa.* Significantly (P<0.05) higher hydroxyproline content was found in *S. polyrhiza* and *W. globosa* compared to others.

Table 2 (A): Essential amino acid composition of macrophytes cultured using organic manures (g/100 g).

Macrophytes	Essential amino acids										
	Arginine (Arg)	**Histidine (His)**	**Isoleucine (Ile)**	**Leucine (Leu)**	**Lysine (Lys)**	**Methionine (Met)**	**Phenylalanine (Phe)**	**Threonine (Thr)**	**Tyrptophan (Trp)**	**Valine (Val)**	**∑Essential**
A. microphylla	1.57 ± 0.001^{d}	0.46 ± 0.001^{ef}	1.24 ± 0.004^{c}	2.31 ± 0.002^{de}	1.48 ± 0.003^{cd}	0.33 ± 0.011^{d}	0.93 ± 0.001^{d}	1.31 ± 0.003^{cd}	0.19 ± 0.02^{ef}	1.58 ± 0.001^{c}	11.40 ± 0.01^{g}
A. pinnata	1.79 ± 0.01^{d}	0.62 ± 0.01^{bc}	1.42 ± 0.025^{b}	2.63 ± 0.028^{c}	1.81 ± 0.014^{b}	0.57 ± 0.002^{c}	1.70 ± 0.049^{b}	1.54 ± 0.009^{b}	0.28 ± 0.005^{d}	1.85 ± 0.007^{b}	14.22 ± 0.04^{cd}
E. fluctuans	4.16 ± 0.12^{a}	0.52 ± 0.003^{de}	0.97 ± 0.004^{d}	1.73 ± 0.005^{f}	1.31 ± 0.001^{de}	0.25 ± 0.014^{ef}	0.78 ± 0.001^{de}	1.03 ± 0.004^{e}	0.12 ± 0.02^{gh}	1.23 ± 0.003^{d}	12.11 ± 0.147^{fg}
H. verticillata	2.43 ± 0.01^{c}	0.36 ± 0.001^{g}	0.82 ± 0.005^{e}	1.67 ± 0.003^{f}	1.16 ± 0.002^{ef}	0.26 ± 0.011^{e}	0.76 ± 0.005^{de}	0.85 ± 0.002^{f}	0.11 ± 0.002^{gh}	1.04 ± 0.05^{e}	9.46 ± 0.023^{h}
I. aquatica	4.46 ± 0.01^{a}	0.56 ± 0.001^{cd}	1.27 ± 0.001^{c}	2.44 ± 0.001^{cd}	1.66 ± 0.001^{bc}	0.36 ± 0.001^{d}	0.98 ± 0.001^{d}	1.28 ± 0.001^{cd}	0.13 ± 0.001^{g}	1.59 ± 0.001^{c}	14.74 ± 0.001^{c}
L. minor	3.02 ± 0.02^{b}	0.78 ± 0.01^{a}	2.02 ± 0.01^{a}	3.95 ± 0.04^{a}	2.56 ± 0.04^{a}	0.85 ± 0.003^{a}	2.51 ± 0.01^{a}	1.78 ± 0.01^{a}	0.36 ± 0.003^{b}	2.43 ± 0.01^{a}	20.25 ± 0.03^{a}
M. quadrifolia	1.78 ± 0.042^{d}	0.36 ± 0.002^{g}	0.81 ± 0.012^{e}	1.56 ± 0.017^{fg}	0.96 ± 0.003^{fg}	0.17 ± 0.011^{g}	0.63 ± 0.033^{e}	0.81 ± 0.004^{f}	0.09 ± 0.004^{h}	1.03 ± 0.003^{ef}	8.20 ± 0.124^{i}
P. stratiotes	1.67 ± 0.01^{d}	0.27 ± 0.004^{h}	0.69 ± 0.011^{e}	1.29 ± 0.014^{g}	0.88 ± 0.009^{g}	0.19 ± 0.003^{fg}	0.93 ± 0.012^{d}	0.63 ± 0.008^{g}	0.21 ± 0.001^{e}	0.85 ± 0.008^{f}	7.61 ± 0.072^{i}
S. molesta	1.73 ± 0.015^{d}	0.67 ± 0.006^{b}	1.17 ± 0.01^{c}	2.063 ± 0.05^{e}	1.15 ± 0.007^{ef}	0.51 ± 0.007^{c}	1.35 ± 0.04^{c}	1.43 ± 0.01^{bc}	0.50 ± 0.01^{a}	1.68 ± 0.01^{bc}	12.24 ± 0.08^{fg}
S. natans	2.50 ± 0.21^{c}	0.44 ± 0.001^{ef}	1.27 ± 0.001^{c}	2.42 ± 0.002^{cd}	1.29 ± 0.001^{de}	0.33 ± 0.003^{d}	1.57 ± 0.015^{bc}	1.28 ± 0.008^{cd}	0.17 ± 0.001^{f}	1.61 ± 0.001^{c}	12.89 ± 0.21^{ef}
S. polyrhiza	2.52 ± 0.08^{c}	0.84 ± 0.051^{a}	1.93 ± 0.076^{a}	3.62 ± 0.160^{b}	2.46 ± 0.113^{a}	0.75 ± 0.023^{b}	2.36 ± 0.096^{a}	1.79 ± 0.100^{a}	0.28 ± 0.019^{d}	2.51 ± 0.112^{a}	19.06 ± 0.60^{b}
W. globosa	3.38 ± 0.004^{b}	0.43 ± 0.001^{fg}	1.22 ± 0.030^{c}	2.44 ± 0.040^{cd}	1.63 ± 0.001^{bc}	0.35 ± 0.021^{d}	0.88 ± 0.08^{d}	1.22 ± 0.004^{d}	0.33 ± 0.002^{c}	1.57 ± 0.005^{c}	13.46 ± 0.19^{de}
ANOVA ***P-value***	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ANOVA ***F-value***	160.774	124.518	258.568	232.646	234.699	354.809	225.337	146.499	362.162	243.537	368.181

Values having the means (n=3) in each row with different superscripts are significantly (P<0.05) different.

Table 2 (B): Non-essential amino acid compositions of macrophytes cultured using organic manures (g/100 g)

Macrophytes	Non- essential amino acids									
	Alanine (Ala)	**Aspartate (Asp)**	**Cysteine (Cys)**	**Glutamic acid (Glu)**	**Glycine (Gly)**	**Proline (Pro)**	**Serine (Ser)**	**Tyrosine (Tyr)**	**Phosphoserine (p-Ser)**	**Taurine (Tau)**	**Phospho ethanol amine (PEA)**
A. microphylla	1.58 ± 0.001^{c}	2.50 ± 0.001^{d}	0.19 ± 0.001^{f}	4.31 ± 0.005^{c}	1.44 ± 0.001^{c}	1.30 ± 0.006^{efg}	1.25 ± 0.001^{d}	1.48 ± 0.002^{bc}	0.08 ± 0.002^{e}	0.01 ± 0.002^{e}	-
A. pinnata	1.87 ± 0.010^{b}	3.01 ± 0.004^{c}	0.26 ± 0.005^{cd}	4.85 ± 0.51^{b}	1.75 ± 0.026^{b}	1.30 ± 0.044^{efg}	1.46 ± 0.034^{c}	1.31 ± 0.008^{d}	0.16 ± 0.007^{c}	0.06 ±0.004^{bc}	0.02 ± 0.002^{d}
E. fluctuans	1.17 ± 0.001^{d}	2.11 ± 0.007^{e}	0.25 ± 0.001^{de}	5.48 ± 0.007^{a}	1.13 ± 0.001^{d}	1.18 ± 0.008^{fgh}	0.90 ± 0.003^{f}	1.41 ± 0.004^{cd}	0.07 ± 0.001^{ef}	0.07 ± 0.002^{b}	0.02 ± 0.001^{e}
H. verticillata	1.11 ± 0.009^{d}	2.58 ± 0.002^{d}	0.22 ± 0.009^{e}	2.17 ± 0.021^{g}	1.65 ± 0.007^{bc}	3.80 ± 0.033^{b}	0.97 ± 0.005^{ef}	1.29 ± 0.005^{d}	0.05 ± 0.005^{fg}	0.02 ± 0.003^{de}	0.01 ± 0.001^{f}
I. aquatica	1.69 ± 0.001^{bc}	3.07 ± 0.001^{c}	0.02 ± 0.001^{de}	3.41 ± 0.001^{e}	1.59 ± 0.001^{bc}	1.44 ± 0.001^{e}	1.18 ± 0.001^{d}	1.60 ± 0.01^{ab}	--	0.09 ± 0.001^{a}	0.02 ± 0.001^{e}
L. minor	2.56 ± 0.05^{a}	3.47 ± 0.005^{b}	0.28 ± 0.004^{c}	5.43 ± 0.01^{a}	2.57 ± 0.01^{a}	1.08 ± 0.03^{h}	2.12 ± 0.01^{a}	1.61 ± 0.003^{ab}	0.46 ± 0.01^{a}	0.05 ± 0.005^{c}	0.020 ± 0.001^{e}
M. quadrifolia	1.02 ± 0.004^{de}	1.52 ± 0.003^{f}	0.11 ± 0.015^{g}	2.70 ± 0.016^{f}	0.96 ± 0.004^{de}	3.37 ± 0.028^{c}	0.68 ± 0.003^{g}	1.14 ± 0.024^{e}	0.06 ± 0.001^{ef}	0.02 ± 0.001^{de}	-
P. stratiotes	0.87 ± 0.007^{e}	1.38 ± 0.013^{f}	0.13 ± 0.003^{g}	1.83 ± 0.016^{g}	0.86 ± 0.007^{e}	2.87 ± 0.037^{d}	0.60 ± 0.016^{g}	0.46 ± 0.005^{g}	0.03 ± 0.001^{g}	0.02 ± 0.001^{de}	-
S. molesta	1.64 ± 0.025^{c}	3.01 ± 0.014^{c}	0.32 ± 0.004^{b}	4.06 ± 0.05^{cd}	1.55 ± 0.002^{bc}	1.14 ±0.001^{gh}	1.65 ± 0.04^{b}	1.31 ± 0.002^{d}	0.21 ± 0.002^{b}	0.02 ± 0.004^{e}	0.05 ± 0.001^{b}
S. natans	1.57 ± 0.001^{c}	2.48 ± 0.015^{d}	0.17 ± 0.001^{f}	3.95 ± 0.002^{cd}	1.51 ± 0.01^{c}	1.31 ± 0.021^{ef}	1.25 ± 0.008^{d}	0.89 ± 0.007^{f}	0.12 ± 0.001^{d}	-	0.04 ± 0.001^{c}
S. polyrhiza	2.58 ± 0.120^{a}	4.09 ± 0.212^{a}	0.44 ± 0.001^{a}	5.44 ± 0.304^{a}	2.50 ± 0.125^{a}	1.11 ± 0.056^{h}	1.79 ± 0.097^{b}	1.51 ± 0.036^{bc}	0.06 ± 0.003^{ef}	0.03 ± 0.002^{d}	0.07 ± 0.001^{a}
W. globosa	1.76 ± 0.006^{bc}	3.00 ± 0.003^{c}	0.17 ± 0.011^{f}	3.55 ± 0.058^{de}	1.48 ± 0.005^{c}	5.19 ± 0.001^{a}	1.10 ± 0.009^{de}	1.73 ± 0.075^{a}	0.22 ± 0.004^{b}	0.03 ± 0.004^{de}	-
ANOVA ***P* value**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ANOVA ***F* value**	203.457	156.402	336.661	185.476	197.474	2305.425	197.52	193.150	923.390	99.928	704.368

Table 2 (B): Non-essential amino acid compositions of macrophytes cultured using organic manures (g/100 g).

Macrophytes	Non- essential amino acids							
	α-amino adipic acid (α-AAA)	**Cystathionine (Cysthi)**	**β -Alanine (β -Ala)**	**β - amino isobutyric acid (b-AiBA)**	**γ -Amino-n-butyric acid (γ - ABA)**	**1 methyl histidine (1 Mehis)**	**Hydroxyproline (Hypro)**	**∑Non-Essential**
A. microphylla	0.01 ± 0.001^{cd}	0.08 ± 0.001^{cde}	0.05 ± 0.001^{e}	-	0.14 ± 0.001^{fg}	0.13 ± 0.002^{b}	0.12 ± 0.013^{bc}	14.67 ± 0.04^{ef}
A. pinnata	0.01 ± 0.001^{de}	0.15 ± 0.004^{ab}	0.25 ± 0.071^{b}	0.23 ± 0.007^{c}	0.27 ± 0.029^{cd}	0.05 ± 0.004^{e}	0.06 ± 0.003^{e}	17.08 ± 0.12^{c}
E. fluctuans	0.05 ± 0.001^{a}	0.05 ± 0.002^{e}	0.06 ± 0.001^{de}	-	0.18 ± 0.001^{ef}	0.13 ± 0.001^{bc}	0.07 ± 0.002^{de}	14.32 ± 0.03^{fg}
H. verticillata	0.01 ± 0.001^{cd}	0.07 ± 0.003^{de}	0.18 ± 0.001^{c}	0.10 ± 0.04^{d}	0.21 ± 0.010^{de}	0.03 ± 0.001^{v}	0.12 ± 0.002^{bc}	14.63 ± 0.037^{ef}
I. aquatica	-	0.07 ± 0.001^{de}	0.05 ± 0.001^{e}	-	0.31 ± 0.001^{c}	0.15 ± 0.001^{a}	0.12 ± 0.001^{bc}	15.04 ± 0.001^{e}
L. minor	0.039 ± 0.003^{a}	0.088 ± 0.003^{cde}	0.096 ± 0.005^{d}	0.565 ± 0.023^{a}	0.394 ± 0.003^{b}	0.083 ± 0.005^{d}	0.128 ± 0.006^{bc}	21.05 ± 0.08^{a}
M. quadrifolia	-	0.05 ± 0.007^{e}	0.05 ± 0.005^{e}	-	0.24 ± 0.001^{cde}	0.10 ± 0.002^{d}	0.05 ± 0.005^{e}	12.07 ± 0.06^{h}
P. stratiotes	0.01 ± 0.001^{de}	0.06 ± 0.003^{de}	0.06 ± 0.003^{de}	0.01 ± 0.001^{e}	0.10 ± 0.001^{g}	0.05 ± 0.002^{ef}	0.15 ± 0.008^{b}	9.50 ± 0.104^{i}
S. molesta	0.025 ± 0.04^{b}	0.172 ± 0.002^{a}	0.30 ± 0.01^{a}	0.35 ± 0.004^{b}	0.28 ± 0.01^{cd}	0.115 ± 0.005^{c}	0.125 ± 0.015^{bc}	16.33 ± 0.01^{d}
S. natans	0.01 ± 0.001^{de}	0.10 ± 0.003^{cd}	0.08 ± 0.004^{de}	-	0.08 ± 0.001^{g}	0.12 ± 0.003^{bc}	0.11 ± 0.008^{cd}	13.80 ± 0.03^{g}
S. polyrhiza	0.02 ± 0.001^{bc}	0.11 ± 0.001^{bc}	0.06 ± 0.01^{de}	0.35 ± 0.011^{b}	0.52 ± 0.02^{4a}	0.054 ± 0.003^{e}	0.20 ± 0.011^{a}	20.95 ± 0.34^{a}
W. globosa	-	0.09 ± 0.020^{cd}	0.25 ± 0.021^{b}	0.24 ± 0.015^{c}	0.25 ± 0.019^{cde}	-	0.22 ± 0.002^{a}	19.28 ± 0.18^{b}
ANOVA ***P* value**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ANOVA ***F* value**	**107.33**	**29.928**	**138.368**	**153.096**	**96.534**	**261.396**	**47.426**	**743.036**

4.1.3. Vitamins

Thiamine (vitamin B_1) content was significantly (P<0.05) higher in *S. polyrhiza* compared to other macrophytes. This plant was followed by *L. minor* (Table 3). The thiamine content was significantly (P<0.05) lower in *E. fluctuans* compared to others and it was absent in *A. pinnata*, *P. stratiotes* and *S. molesta*. The riboflavin (vitamin B_2) content was significantly (P<0.05) higher in *S. polyrhiza* compared to other macrophytes. This plant was followed by *I. aquatica*. Significantly (P<0.05) lower riboflavin content was observed in *E. fluctuans*. Pyridoxin (vitamin B_6) and cobalamin (vitamin B_{12}) contents were significantly (P<0.05) higher in *I. aquatica* compared to others. There was no significant (P>0.05) difference in cobalamin content between *A. microphylla* and *I. aquatica*. Vitamin C content was significantly (P<0.05) higher in *M. quadrifolia* compared to others macrophytes. There was no significant (P>0.05) difference in vitamin C content among *A. microphylla, A. pinnata* and *M. quadrifolia.*

Table 3: Vitamin contents of macrophytes cultured with organic manures (mg/100 g dry weight)

Macrophytes	Water soluble vitamins				
	Thiamine (B_1)	**Riboflavin (B_2)**	**Pyridoxine (B_6)**	**Cobalamin (B_{12})**	**Ascorbic acid (C)**
Azolla microphylla	7.79 ± 0.86^{e}	139.11 ± 3.65^{cd}	ND	41.14 ± 5.42^{a}	83.68 ± 0.99^{a}
Azolla pinnata	ND	ND	30.55 ± 0.28^{b}	ND	81.93 ± 4.83^{a}
Enhydra fluctuans	5.42 ± 0.04^{e}	56.85 ± 0.12^{e}	0.09 ± 0.001^{g}	ND	64.95 ± 0.74^{b}
Hydrilla verticillata	11.34 ± 0.64^{cd}	115.42 ± 7.78^{de}	ND	ND	ND
Ipomoea aquatica	14.17 ± 0.86^{c}	687.12 ± 30.88^{b}	58.35 ± 1.50^{a}	39.64 ± 0.181^{a}	38.10 ± 0.73^{c}
Lemna minor	17.56 ± 1.62^{b}	196.73 ± 8.55^{c}	6.26 ± 0.55^{d}	32.82 ± 0.27^{b}	ND
Marsilea quadrifolia	12.00 ± 1.30^{cd}	110.42 ± 1.18^{de}	ND	3.03 ± 0.09^{d}	90.99 ± 0.39^{a}
Pistia stratiotes	ND	102.98 ± 4.73^{de}	5.53 ± 0.26^{de}	$0.673 \pm .03^{e}$	36.48 ± 0.056^{c}
Salvinia molesta	ND	84.10 ± 11.92^{de}	ND	ND	ND
Salvinia natans	11.23 ± 0.006^{d}	ND	2.36 ± 0.006^{ef}	ND	ND
Spirodela polyrhiza	20.80 ± 1.61^{a}	1003.357 ± 6.93^{a}	10.49 ± 0.12^{c}	12.23 ± 0.07^{c}	28.20 ± 0.388^{d}
Wolffia globosa	10.96 ± 0.61^{d}	152.50 ± 10.11^{cd}	8.88 ± 1.07^{cd}	ND	ND
***P* value**	<0.001	<0.001	<0.001	<0.001	<0.001
***F* value**	64.061	480.057	799.87	208.73	183.90

Values having the means (n=3) in each row with different superscripts are significantly (P<0.05) different. (ND = Not detected)

4.2. Standardization of the culture technique of *Spirodela polyrhiza* in outdoor conditions

The proximate composition study showed that *S. polyrhiza* is one of the suitable macrophytes that may be used as a fish feed ingredient. The standardization of culture technique was essential for sustainable production of *S. polyrhiza* with required nutritional value. *S. polyrhiza* was cultured in cemented tanks using three different manures: manure 1 (organic manure, OM: cattle manure, poultry wastes and mustard oil cake), manure 2 (Inorganic fertilizer, IF: urea, potash and triple superphosphate) and manure 3 (combination of cattle manure and inorganic fertilizers, OM+ IF: cattle manure, urea, potash and triple super phosphate).

4.2.1. Water quality

Major water quality parameters were recorded in all treatments before the application of manures. There was no significant (P>0.05) difference in temperature, pH, dissolved oxygen, ammonia, nitrite, nitrate and phosphate levels among different treatments at the beginning of the study. A wide range of water temperature 9.4-26.7°C was recorded during the culture of duckweed during December 2016-March 2017 and this influenced the productivity of the duckweeds (Table 4). The whole culture period was broadly divided into three phases based on the temperature and light intensity. In phase I (December 2016-January 2017), water temperature and light intensity were 16.5°C and 26.0 µmol photons/m^2/s at the beginning and then gradually decreased. The lowest temperature and light intensity were recorded in January. In phase II

(February-March 2017) and phase III (March 2017), water temperature and light intensity showed increasing trends. There was no significant (P>0.05) difference in temperature and light intensity among the three different treatments during the culture period. Among these three different treatments, there was variation in pH in different phases.

Significantly (P<0.05) higher dissolved oxygen level was found in manure 2 (IF) compared to the other two treatments throughout the study period (Fig.1A). This group was followed by manure 3 (OM+IF) and lowest dissolved oxygen level (<1mg/L) was found in manure 1 (OM). Ammonia level was significantly (P<0.05) higher in manure 1 (OM) compared to the other two treatments throughout the study period (Fig.1B). In manure 1 (OM), ammonia levels ranged from 1.34-30.65, 7.52-18.57 and 15.25-17.85 mg/l in the first, second and third phases, respectively. In manure 2 (IF), ammonia levels ranged from 1.94-9.34, 0.03-7.71 and 1.44-3.33 mg/l in the first, second and third phases, respectively. In manure 3 (OM+IF), ammonia level ranged from 0.17-10.97, 0.27-4.08 and 0.2-0.41 mg/l in the first, second and third phases, respectively. The lowest ammonia level was found in the third phase regardless of manure.

Table 4: Environmental parameters observed in the culture tanks of *Spirodela polyrhiza*

Parameters	Manure 1		Manure 2		Manure 3	
	Range	**Mean ± SE**	**Range**	**Mean ± SE**	**Range**	**Mean ± SE**
			Phase I (December 2016 - January 2017)			
Temperature (^{0}C)	9.36 – 16.55	14.38 ± 0.34	9.36 – 16.55	14.38 ± 0.336	9.36 – 16.55	14.38 ± 0.34
Light intensity (µmol photon/m^2/ s)	14.56 – 49.43	27.29 ± 2.45	14.56 – 49.43	27.29 ± 2.45	14.56 – 49.43	27.29 ± 2.45
pH	7.20 – 7.91	-	7.04 – 7.86	-	6.98 – 7.85	-
Nitrite (mg/l)	0.007 – 0.26	0.116 ± 0.01	0.13 – 1.01	0.47 ± 0.05	0.06 – 1.04	0.49 ± 0.07
Nitrate (mg/l)	1.68 – 18.70	5.77 ± 1.13	6.58 – 43.66	30.76 ± 2.60	8.44 – 35.48	24.40 ± 2.01
			Phase II (February - March 2017)			
Temperature (^{0}C)	15.70 – 19.33	17.80 ± 0.43	15.70 – 19.33	17.80 ± 0.43	15.70 – 19.33	17.80 ± 0.43
Light intensity (µmol photon/m^2/s)	49.21 – 105.08	89.89 ± 3.25	49.21 – 105.08	89.89 ± 3.25	49.21 – 105.08	89.89 ± 3.25
pH	7.09 – 7.59	-	7.24 – 7.82	-	7.26 – 7.72	-
Nitrite (mg/l)	0.02 – 0.12	0.055 ± 0.015	0.11 – 0.84	0.44 ± 0.08	0.006 – 0.12	0.09 ± 0.02
Nitrate (mg/l)	5.95 – 44.73	25.77 ± 6.04	15.04 – 46.87	29.38 ± 4.58	16.15 – 34.94	24.42 ± 2.61
			Phase III (March 2017)			
Temperature (^{0}C)	23.26 – 26.70	24.98 ± 1.72	23.26 – 26.70	24.98 ± 1.72	23.26 – 26.70	24.98 ± 1.72
Light intensity (µmol photon/m^2/s)	137.41 – 151.16	143.79 ± 6.39	137.41 – 151.16	143.79 ± 6.39	137.41 – 151.16	143.79 ± 6.39
pH	7.27 – 7.56	-	7.18 – 7.43	-	7.28 – 7.39	-
Nitrite (mg/l)	0.015 – 0.02	0.016 ± 0.00	0.37 – 0.07	0.52 ± 0.16	0.082 – 0.12	0.10 ± 0.02
Nitrate (mg/l)	11.68 – 18.54	15.11 ± 3.44	33.51 – 36.95	35.23 ± 1.72	16.51- 34.94	24.41 ± 2.61

Nitrite level was significantly (P<0.05) higher in manure 2 (IF) and manure 3 (OM + IF) in the first phase compared to manure 1 (OM) (Table 4). There was no significant (P>0.05) difference between these two former groups. In the second and third phases, nitrite level was significantly (P<0.05) higher in manure 2 (IF) compared to the other two treatments. Nitrate level was significantly (P<0.05) higher in manure 2 (IF) compared to the other two treatments throughout the study period. Phosphate level was significantly (P<0.05) lower in manure 2 (IF) compared to the other two treatments throughout the study period (Fig.1C). Conductivity was significantly (P<0.05) higher in manure 1 (OM) compared to the other treatments throughout the study period (Fig.1D). In manure 1, the conductivity ranged from 516-1196 µS/cm.

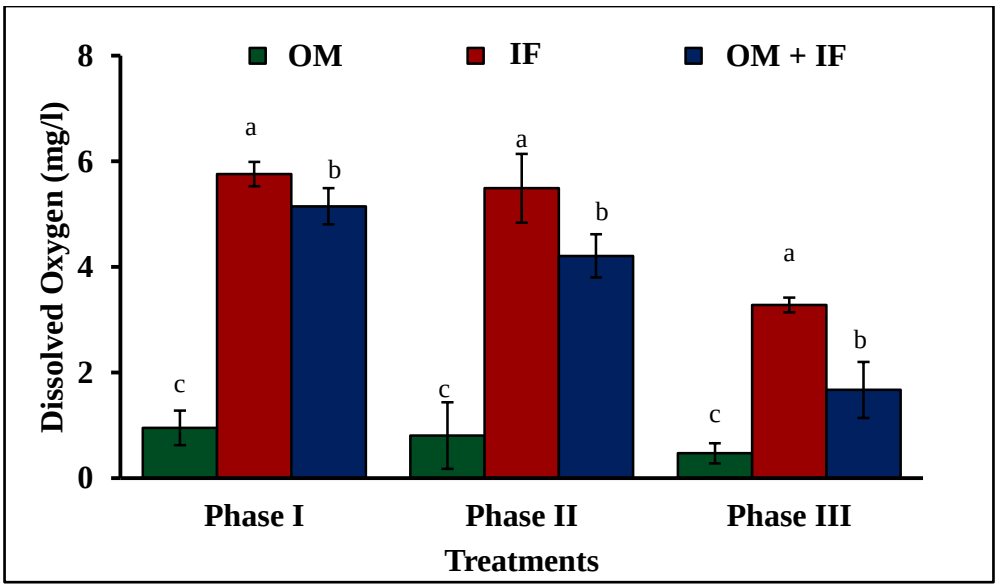

Fig.1A: Dissolved oxygen (mg/l) levels of water found in various treatments in different phases of culture of *S. polyrhiza*. Bars with different superscripts are significantly (P<0.05) different (n=3).

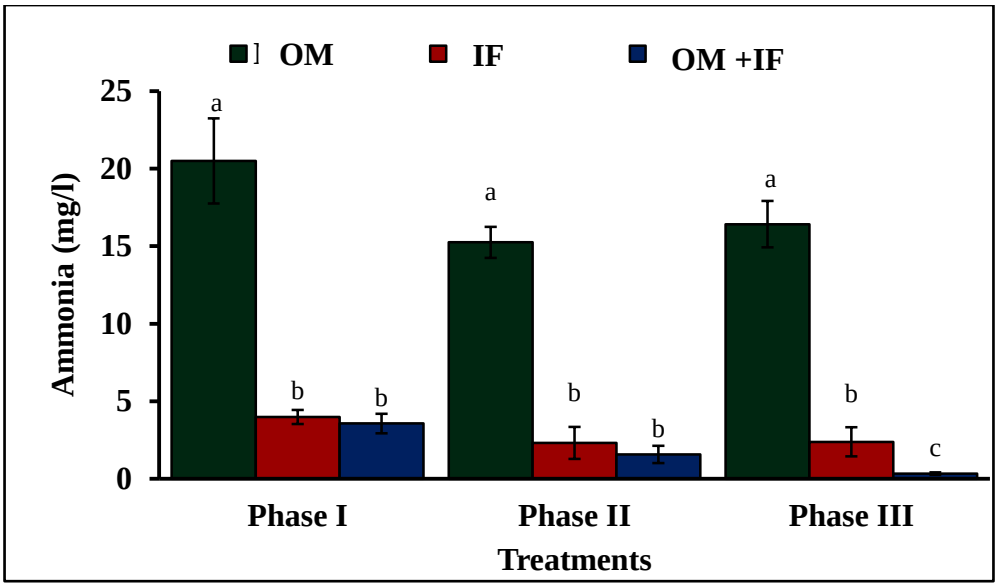

Fig.1B: Ammonia (mg/l) levels of water found in various treatments in different phases of culture of *S. polyrhiza*. Bars with different superscripts are significantly (P<0.05) different (n=3).

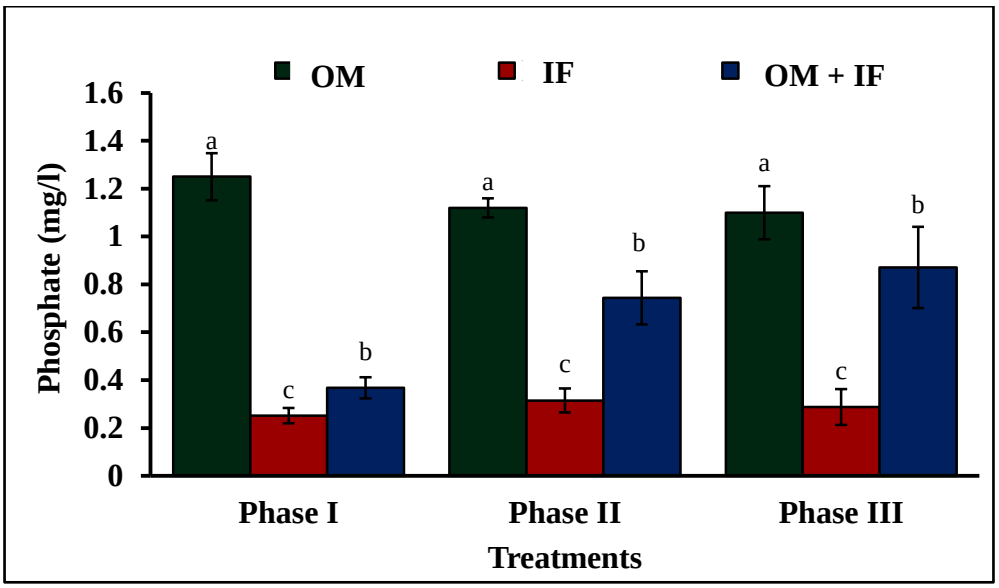

Fig.1C: Phosphate (mg/l) levels of water found in various treatments in different phases of culture of *S. polyrhiza*. Bars with different superscripts are significantly (P<0.05) different (n=3).

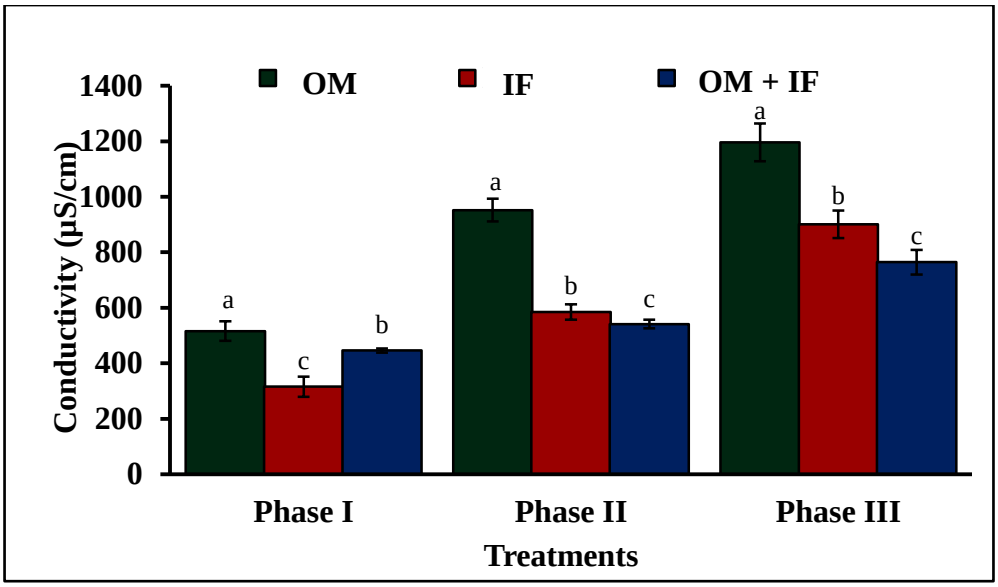

Fig.1D: Conductivity (µS/cm) of water found in various treatments in different phases of culture of *S. polyrhiza*. Bars with different superscripts are significantly (P<0.05) different (n=3).

4.2.2. Production and relative growth rate of *Spirodela polyrhiza*

The production of *S. polyrhiza* was affected by water temperature. The relative growth rate (RGR) of greater duckweeds was slow (0.02-0.04 g/g/day) at the beginning of the culture period due to low temperature regardless of treatments. Greater duckweeds were first harvested after 69 days of initial introduction in all three treatments. As water temperature increased, the growth rate also increased and duckweeds were harvested another four times; second and fourth harvests were performed after 10 days of the respective previous harvest and third and fifth harvests were after 12 days of the respective previous harvest. The RGR values ranged from 0.021-0.158, 0.007-0.12 and 0.024-0.129 g/g/day in manures 1 (OM), 2 (IF) and 3 (OM + IF), respectively throughout the study period. In manure 3 (OM + IF), the growth of plant was poor at fifth harvest compared to the previous one and

this resulted into negative RGR value. The average RGR values were 0.08±0.02, 0.06±0.03 and 0.07±0.03 g/g/day in manures 1 (OM), 2 (IF) and 3 (OM+IF), respectively. Total production rate of duckweeds was significantly (P<0.05) higher in manure 1 (OM) compared to the other manures (Fig.2). This group was followed by manure 3 (OM+IF) and minimum production was found in manure 2 (IF).

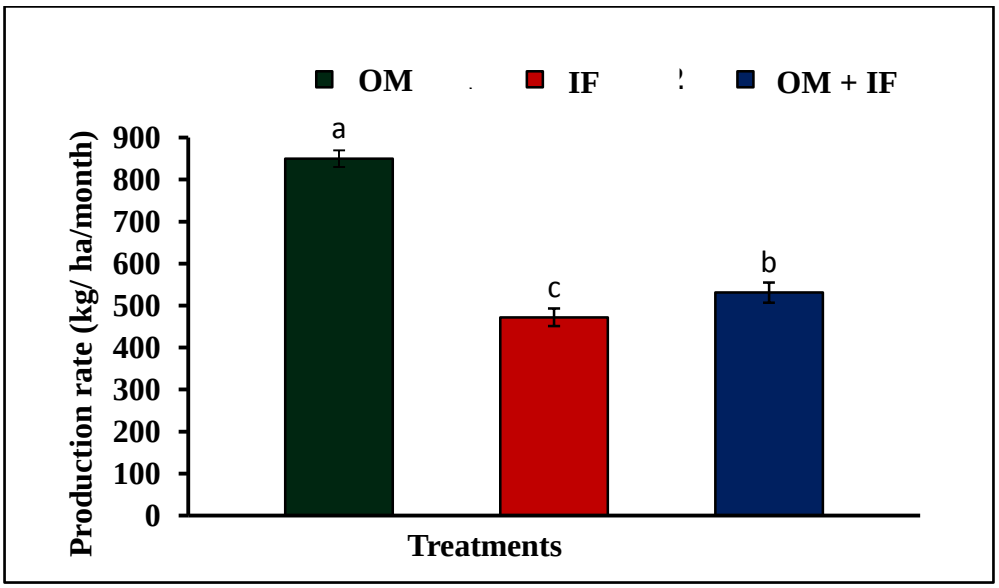

Fig.2: Total production rate *S. polyrhiza* in various treatments. Bars with different superscripts are significantly (P<0.05) different (n=3).

4.2.3. Biochemical composition

There was a difference in the proximate composition of greater duckweeds cultured with organic manures (OM) and inorganic fertilizers (IF) in tanks. Protein content was significantly (P<0.05) higher, and carbohydrate and ash contents were significantly (P<0.05) lower, in duckweed cultured in manure 1 (OM) compared to manure 2 (IF) (Table 5).

Table 5: Proximate composition of *S. polyrhiza* (% of dry weight)

Parameter	Manure 1 (Organic, OM)	Manure 2 (Inorganic, IF)
Protein	35.82 ± 0.14	30.50 ± 0.03*
Lipid	7.11 ± 0.11	7.19± 0.06
Ash	18.51 ± 0.02	20.64 ± 0.26*
Carbohydrate	38.38 ± 0.26	41.68± 0.17*

Data are presented as means ± SEM (*n*=3).

*Denotes significant difference (*P*<0.05) between the two manures as determined by Student's t-test

4.2.4. Amino acid composition

The amino acid profile of greater duckweeds cultured in organic manures showed the presence of essential (37.4%), non-essential (58.2%) and free amino acids (4.5%). Among essential amino acids, three branched chain amino acids, leucine, isoleucine and valine contributed 51.4%. Glutamic acid and glutamine consisted 28.3% of the total non-essential amino acids in the greater duckweed. The presence of taurine enhanced the nutritional value of greater duckweed (Table 6).

Table 4: Amino acid (g/100 g) profile of *Spirodela polyrhiza* cultured with organic manures

Amino acids	Concentration
Essential	
Histidine (His)	0.771 ± 0.053
Isoleucine (Ile)	1.703 ± 0.150
Leucine (Lue)	3.322 ± 0.207
Lysine (Lys)	2.280 ± 0.129
Methionine (Met)	0.694 ± 0.059
Phenylalanine (Phe)	2.159 ± 0.144
Threonine (Thr)	1.502 ± 0.386
Tryptophan (Trp)	0.282 ± 0.018
Valine (Val)	2.383 ± 0.139
Non-essential	
Alanine (Ala)	2.384 ± 0.130
Arginine (Arg)	2.386 ± 0.120
Asparatate (Asp)	4.094 ± 0.212
Cysteine (Cys)	0.369 ± 0.039
Glutamic acid (Glu)	5.103 ± 0.380
Glutamine ($GluNH_2$)	1.250 ± 0.300
Glycine (Gly)	2.369 ± 0.110
Proline (Pro)	1.001 ± 0.110
Serine (Ser)	1.904 ± 0.120
Tyrosine (Tyr)	1.558 ± 0.050
Phosphoserine (p-Ser)	0.060 ± 0.002
Taurine (Tau)	0.023 ± 0.006
Phospho ethanol amine (PEA)	0.072 ± 0.001
α Amino adipic acid (α -AAA)	0.020 ± 0.001
α Amino-n- butaric acid (α -ABA)	0.141 ± 0.014
Cystathionine (Cysthi)	0.115 ± 0.001
β -Alanine (β -Ala)	0.072 ± 0.011
β -Amino isobutyric acid (β -AiBA)	0.354 ± 0.015
Ethanol amine ($EOHNH_2$)	0.112 ± 0.004
Ornithine (Orn)	0.027 ± 0.002
1 Methylhistidine (1 Mehis)	0.048 ± 0.003
Hydroxy proline (Hypro)	0.197 ± 0.010
Ɣ- Amino isobutyric acid (Ɣ -AiBA)	0.478 ± 0.024

4.2.5. Fatty acid composition

The fatty acid composition of *S. polyrhiza* was dominated by polyunsaturated fatty acids (PUFA), which accounted for 47-53% of total fatty acids, primarily α-linolenic acid (ALA, 18:3n-3) at around 36–39% (Table 7). Total saturated fatty acids accounted for 32-39%, followed by linoleic acid (LA, 18:2n-6) at 11–14% and monoenes at 9–11%. As with proximate composition, fatty acid profile was affected by manures. *S. polyrhiza* grown in inorganic fertilizers (IF) showed a higher proportion of ALA, LA and total PUFA, and lower amount of saturated and monounsaturated fatty acids. Due to the slightly higher (although not statistically significant) lipid content of *S. polyrhiza* grown in manure 2 (IF), all fatty acids were in higher absolute amounts (mg/100 g dry mass) in macrophytes grown in inorganic fertilizers (IF).

Table 5: Fatty acid composition of *Spirodela polyrhiza* as percentage of total fatty acids (Percentage) or as mg fatty acids per 100 g dry weight (Absolute)

Fatty acid	Manure 1		Manure 2	
	Percentage	**Absolute**	**Percentage**	**Absolute**
14:0	1.01 ± 0.22	16.9 ± 1.86	1.10 ± 0.30	23.65 ± 7.42
15:0	0.60 ± 0.04	10.1 ±0.46	0.40 ± 0.01*	8.56 ± 0.55
16:0	31.22 ± 2.33	524.1 ± 18.32	25.50 ± 0.40	547.04 ± 33.88
18:0	2.33 ± 0.23	39.1 ± 0.35	2.02 ± 0.13	43.39 ± 4.69
20:0	0.40 ± 0.04	6.6± 0.10	0.33 ± 0.01	7.04 ± 0.55
22:0	0.77 ± 0.10	12.9 ± 0.32	0.85 ± 0.03	18.17 ± 0.24*
24:0	3.05 ± 0.15	51.3 ± 3.16	2.28 ± 0.05*	48.85 ± 1.15
Total saturated	39.38 ± 3.12	661.0 ± 20.21	32.48 ± 0.76	696.70 ± 48.49
16:1n-9	4.76 ± 2.23	86.4 ± 27.76	6.75 ± 0.14	144.61 ± 3.60
17:1 n	0.00 ± 0.00	0.0 ± 0.00	0.30 ± 0.02*	6.34 ± 0.22*
18:1n-9	2.09 ± 0.13	35.2 ± 1.68	3.01 ± 0.64	64.93 ± 16.74
18:1n-7	2.24 ± 0.23	37.6 ± 0.25	1.34 ± 0.06*	28.68 ± 2.59*
Total monoenes	9.09 ± 6.37	159.2 ± 124.19	11.39 ± 0.53	244.57 ± 22.72
18:2n-6	11.35 ± 0.76	190.7 ± 8.09	13.49 ± 0.23	289.08 ± 8.33*
20:4n-6	0.00 ± 0.00	0.0 ± 0.00	0.33 ± 0.01*	7.03 ± 0.08*
Total n-6 PUFA	11.35 ± 0.76	190.7 ± 8.09	13.82 ± 0.24	296.11 ± 8.41*
18:3n-3	35.75 ± 2.18	600.6 ± 29.28	38.95 ± 1.08	834.63 ± 15.44*
20:5n-3	0.38 ± 0.12	6.3 ± 1.37	0.60 ± 0.08	12.98 ± 2.30
Total n-3 PUFA	36.13 ± 0.30	606.9 ± 27.91	39.56 ± 1.00	847.61 ± 17.75*
Total DMA	4.04 ± 0.18	68.0 ± 4.35	2.76 ± 0.06*	59.09 ± 1.49
Total PUFA	47.48 ± 3.07	797.6 ± 35.99	53.37 ± 1.241	1143.72 ± 26.16*
Total Fatty acids		1685.8 ± 275.3		2144.1 ± 329.9

Data are presented as means±SEM (n=3). *Denotes significant difference (P<0.05) between the two manures as determined by Student's t-test. DMA, dimethyl acetals; PUFA, polyunsaturated fatty acids.

4.3. Culture of plant in pond

The application of organic manures resulted in the higher production rate of *S. polyrihiza* with required nutritional value. Therefore, this manure was selected for the mass production of *S. polyrhiza* in pond conditions.

4.3.1. Water quality

In three different ponds at the Rohtak centre, water temperature and pH ranged from 32.4-30.5°C and 7.76-8.30, respectively during the study period. Dissolved oxygen level ranged from 1.25-4.57 mg/l on various days of study. Ammonia, nitrite and nitrate levels of ponds ranged from 5.02-17.57, 0.003-0.12 and 0.23-2.44 mg/l, respectively. Phosphate level ranged from 1.1-2.0 mg/l during the study period (Table 6). Conductivity ranged from 1032-1251 µS/cm throughout the culture period.

Table 6: Water quality found in *Spirodela polyrhiza* culture ponds during the study period

Parameter	Range	Mean ± SE
Temperature (°C)	30.5 - 33.0	32.00 ± 1.0
pH	7.76 - 8.30	---
Dissolved oxygen (mg/l)	1.25 - 4.57	2.50 ± 0.25
Ammonia (mg/l)	5.02 - 17.57	15.25 ± 0.7
Nitrite (mg/l)	0.005 - 0.01	0.008 ± 0.002
Nitrate (mg/l)	0.05 - 2.05	0.921 ± 0.3
Phosphate (mg/l)	1.15 - 2.00	1.52 ± 0.07
Conductivity (µS/cm)	1032 - 1251	1150 ± 37.0

4.3.2. Production and relative growth rate (RGR)

S. polyrhiza was harvested three times from the ponds at 10 days intervals. After harvesting, duckweeds were cleaned thoroughly with tap water to remove organic material. The excess water was removed, air dried and then dried at 40°C. Dried duckweed were packed in airtight containers for further use. The RGR values were 0.48, 0.14 and 0.03 g/g/day in the first, second and third harvests, respectively. The average RGR value was 0.22±0.13 g/g/day. Total production was 2020±150 kg/ha/month on dry matter basis, equivalent to 24 tonnes/ha/yr (Fig. 3).

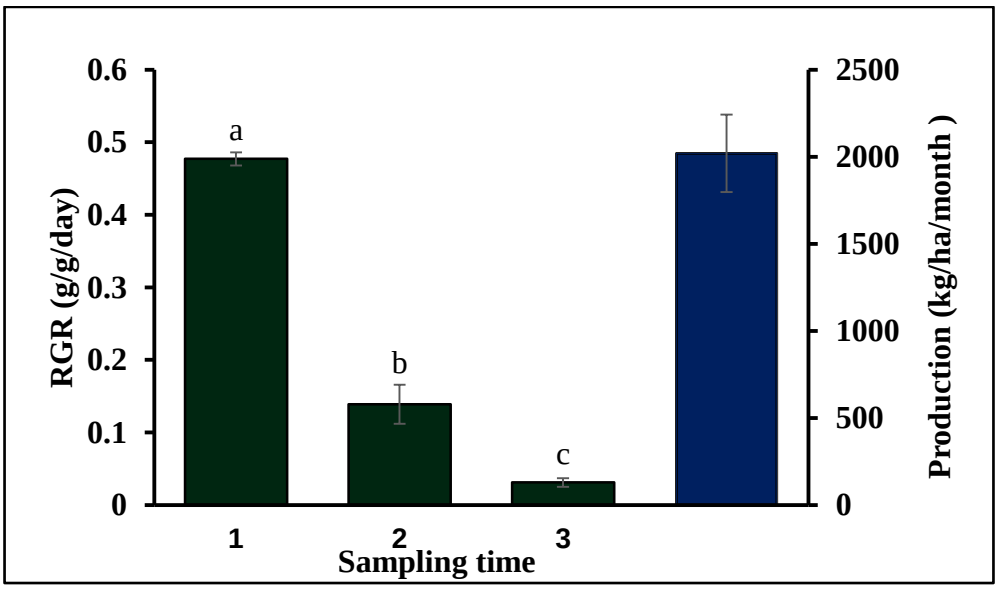

Fig.3: Relative growth rate (RGR) and total production of *S. polyrhiza* in ponds. RGR was measured thrice at 10 days interval. Bars with different superscripts are significantly (P<0.05) different (n=3).

4.4. Formulation of *Spirodela polyrhiza* supplemented feeds for fishes

The pelleted diets were formulated using *S. polyrhiza* for rohu *Labeo rohita* and common carp *Cyprinus carpio*. Five isonitogenous, isolipidic and isoenergetics fish feeds were formulated using the Winfeed 2.8 software

package (WinFeed UK Limited, Cambridge, United Kingdom). The soybean meal, wheat flour, corn flour, sunflower oil, amino acids, vitamin and mineral premixes were other ingredients used for feed formulation. In control feed (SP0), soybean meal was the primary source of protein, while in four experimental feeds, greater duckweed was incorporated at the levels of 5 (SP5), 10 (SP10), 15 (SP 15), and 20 (SP20)%.

4.4.1. Biochemical composition of prepared feed

There was no significant (P>0.05) difference in moisture, protein and lipid contents among five different feeds (Table. 10). Significantly (P<0.05) lower carbohydrate content was found in feed SP20 compared to others. A direct relationship was found between the increasing levels of *S. polyrhiza* and the ash contents of feeds. Significantly (P<0.05) higher ash content was found in the feed SP20 compared to others.

4.4.2. Amino acid composition of prepared feed

The amino acid compositions of the prepared feeds were influenced by the addition of *S. polyrhiza*. Significantly (P<0.05), higher contents of the essential amino acids, arginine, histidine, threonine, tryptophan and valine were found in *S. polyrhiza* supplemented feeds compared to the control one (Table 11).

Table 10: Proximate compositions of experimental feeds (g/kg of feed)

Parameters	Feeds				
	SP0	**SP5**	**SP10**	**SP15**	**SP20**
Moisture	63.3 ± 0.81	63.6 ± 0.20	63.2 ± 0.63	63.3 ± 0.84	62.5 ± 0.42
Protein	317.6 ± 0.50	318.4 ± 2.20	318.2 ± 3.10	322.2 ± 3.61	322.0 ± 0.70
Lipid	73.3 ± 0.81	73.4 ± 0.91	73.7 ± 0.25	73.2 ± 0.10	72.8 ± 0.17
Carbohydrate	474.6 ± 4.10	474.3 ± 1.72	472.0 ± 0.25	464.2 ± 2.21	460.6 ± 3.1
Ash	71.2 ± 0.28	70.3 ± 0.40	72.9 ± 0.60	77.1 ± 0.21	84.1 ± 1.1

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza.*

The non-essential amino acids, glutamic acid, proline and ethanol amine contents were significantly (P<0.05) higher in feed SP0 compared to the others. Other non-essential amino acids alanine, aspartate, cysteine, serine, tyrosine, cystathionine and γ- amino-n-isobutyric acid contents were significantly (P<0.05) higher in feed SP5 compared to others. The glycine and hydroxyproline contents were significantly (P<0.05) higher in feed SP20 compared to others; these two amino acids were minimum in feed SP0. The phosphoserine, taurine, phospho ethanol amine, β-alanine, β-amino-iso butyric acid and hydroxylysine contents were significantly (P<0.05) higher in feed SP15 compared to the others. The 1-methyl histidine and hydroxyproline contents were significantly (P<0.05) higher in feed SP20.

Table 11: The amino acid compositions of five different feeds of fishes (g/kg dry weight)

Essential	**SP0**	**SP5**	**SP10**	**SP15**	**SP20**
Arginine (Arg)	17.41 ± 0.2	19.92 ± 0.05	20.49 ± 0.06	19.74 ± 0.83	19.78 ± 0.08
Histidine (His)	6.98 ± 0.3	8.83 ± 1.02	7.73 ± 0.15	7.32 ± 0.07	7.80 ± 0.14
Isoleucine (Ile)	12.80 ± 0.2	13.11 ± 0.77	13.39 ± 0.47	12.47 ± 0.10	12.57 ± 0.22
Leucine (Lue)	22.21 ± 0.3	22.39 ± 0.25	22.41 ± 0.04	21.56 ± 0.13	22.15 ± 0.09
Lysine (Lys)	20.60 ± 0.1	20.93 ± 0.36	20.41 ± 0.04	19.03 ± 0.40	18.72 ± 0.29
Methionine (Met)	10.94 ± 0.2	12.39 ± 0.31	12.58 ± 0.1	12.02 ± 0.01	10.93 ± 0.48
Phenylalanine (Phe)	14.63 ± 0.03	15.27 ± 0.02	15.77 ± 0.13	14.57 ± 0.15	14.28 ± 0.38
Threonine (Thr)	11.23 ± 0.01	14.43 ± 0.24	14.33 ± 0.49	14.27 ± 0.06	14.50 ± 0.23
Tryptophan (Trp)	1.11 ± 0.01	1.47 ± 0.01	1.37 ± 0.2	1.59 ± 0.02	1.59 ± 0.2
Valine (Val)	13.71 ± 0.05	14.67 ± 0.15	14.17 ± 0.19	14.88 ± 0.06	14.04 ± 0.42
∑Essential	**131.62 ± 1.68**	**143.41 ± 2.20**	**142.69 ± 0.5**	**137.42 ± 2.3**	**136.25 ± 1.47**
Non-essential					
Alanine (Ala)	12.40 ± 0.01	13.21 ± 0.8	12.88 ± 0.01	12.58 ± 0.15	12.89 ± 0.10
Aspartate (Asp)	31.31 ± 0.07	31.87 ± 0.33	31.75 ± 0.21	29.63 ± 0.3	30.25 ± 0.06
Cysteine (Cys)	3.51 ± 0.04	3.80 ± 0.54	2.71 ± 0.16	2.36 ± 0.04	2.78 ± 0.07
Glutamic Acid (Glu)	68.41 ± 0.10	61.41 ± 0.81	61.55 ± 0.68	61.61 ± 0.32	62.27 ± 0.13
Glycine (Gly)	11.70 ± 0.2	12.54 ± 0.20	12.28 ± 0.01	11.72 ± 0.10	12.89 ± 0.10

Essential	**SP0**	**SP5**	**SP10**	**SP15**	**SP20**
Proline (Pro)	22.46 ± 0.01	15.69 ± 0.37	15.01 ± 0.05	14.88 ± 0.90	14.22 ± 0.06
Serine (Ser)	13.00 ± 0.11	14.00 ± 0.07	13.34 ± 0.24	13.50 ± 0.83	12.69 ± 0.06
Tyrosine (Tyr)	9.02 ± 0.21	9.44 ± 0.19	8.42 ± 1.36	8.84 ± 0.58	8.67 ± 0.06
Phosphoserine (p-Ser)	1.1 ± 0.3	1.65 ± 0.04	1.02 ± 0.17	1.83 ± 0.01	1.81 ± 0.28
Taurine (Tau)	0.35 ± 0.00	0.34 ± 0.01	0.37 ± 0.09	0.39 ± 0.01	0.33 ± 0.05
Phospho ethanol amine (PEA)		0.61 ± 0.12	0.17 ± 0.00	0.72 ± 0.00	
α Amino adipic acid (α-AAA)		0.05 ± 0.02	-	0.06 ± 0.00	0.23 ± 0.15
Cystathionine (Cysthi)	1.8 ± 0.7	1.57 ± 0.02	1.26 ± 0.02	1.46 ± 0.02	1.00 ± 0.4
β -Alanine (β -Ala)	0.2 ± 0.00	2.35 ± 0.36	1.79 ± 0.13	2.41 ± 0.02	2.01 ± 0.00
β Amino isobutyric acid (β-AiBA)	0.57 ± 0.5	-	1.51 ± 0.01	2.32 ± 0.1	1.49 ± 0.8
Ɣ-Amino-n-butyric acid (Ɣ-ABA)	0.31 ± 0.1	1.52 ± 0.40	1.10 ± 0.01	1.55 ± 0.2	1.16 ± 0.00
Ethanal amine (EOHNH2)	3.37 ± 0.12	1.30 ± 0.52	0.89 ± 0.09	1.30 ± 0.41	0.89 ± 0.00
Hydroxylysine (Hylys)	0.1 ± 0.04	0.93 ± 0.00	0.57 ± 0.00	1.33 ± 0.00	-
1 methyl histidine (1 Mehis)	0.58 ± 0.3	1.29 ± 0.00	0.89 ± 0.00	2.17 ± 0.66	2.55 ± 0.40
Hydroxy proline (Hypro)	0.65 ± 0.1	0.49 ± 0.03	0.57 ± 0.15	0.56 ± 0.22	0.74 ± 0.19
∑ Non-Essential	**180.84 ± 1.50**	**174.35 ± 1.00**	**168.67 ± 2.02**	**171.24 ± 7.81**	**168.71 ± 1.91**

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza*

4.4.3. Fatty acid composition prepared feed

Fatty acid composition of the feed was influenced by the addition of *S. polyrhiza* (Table 12). The saturated fatty acids, myristic acid (14:0) and lignoceric acid (24:0) contents were significantly (P<0.05) higher in feed SP0 compared to others. Other saturated fatty acids, palmitic acid (16:0), stearic acid (18:0) and arachidic acid (20:0) contents were significantly (P<0.05) higher in feed SP15 compared to others. Pentadecylic acid (15:0) and stearic acid (18:0) contents were significantly (P<0.05) higher in SP20 and SP10, respectively. Total saturated fatty acid content was significantly (P<0.05) higher in feed SP20 compared to others. This feed was followed by SP15, SP10, SP0 and SP5.

Among monounsaturated fatty acids, significantly (P<0.05) higher palmitoleic acid (16:1 n-9) content was found in feed SP20 compared to others. It was absent in SP0. Other mono-unsaturated fatty acids, oleic acid (18:1 n-9) and gondoic acid (20:1 n-9) contents were significantly (P<0.05) higher in feeds SP15 and SP0, respectively. The polyunsaturated fatty acids, linoleic acid (18:2 n-6) was the most abundant fatty acid in all feeds. It was significantly (P<0.05) higher in feeds SP10 and SP15 compared to others. The n-6 PUFA, eicosadienoic acid (20: n-6) and arachidonic acid (20: 4 n-6) contents were significantly (P<0.05) higher feed SP10 compared to others. The α-linolenic acid (ALA, 18:3 n-3) was the only n-3 PUFA and it was found in all feeds. The ALA content was significantly (P<0.05) higher in feed SP20 compared to others.

Table 12: Fatty acid compositions of five different feeds of fishes (mg/100 g dry weight)

Fatty acids	SP0	SP5	SP10	SP15	SP20
14:0	10.44 ± 0.07	5.91 ± 0.47	9.78 ± 0.86	9.40 ± 0.70	9.41 ± 0.25
15:0	5.20 ± 0.01	0.96 ± 0014	1.49 ± 0.10	1.83 ± 0.01	12.61 ± 0.58
16:0	581.01 ± 8.96	580.31 ± 5.78	600.05 ± 11.84	623.02 ± 7.69	619.99 ± 9.18
18:0	70.09 ± 0.99	38.84 ± 1.44	89.66 ± 3.84	88.69 ± 3.19	90.21 ± 1.98
20:0	3.26 ± 0.36	2.22 ± 0.04	5.71 ± 0.40	6.24 ± 0.21	5.65 ± 0.23
22:0	0.69 ± 0.01	-	-	-	-
24:0	15.72 ± 2.77	4.86 ± 0.82	11.86 ± 1.23	8.97 ± 0.46	8.46 ± 0.70
∑SFA	**686.41 ± 6.43**	**633.10 ± 6.12**	**718.55 ± 8.88**	**738.16 ± 9.52**	**746.33 ± 11.26**
16:1 n-9	-	0.02 ± 0.00	0.20 ± 0.01	0.84± 0.10	1.22 ± 0.07
18:1 n-9	1460.56 ± 3.81	1470.51 ± 10.68	1571.88 ± 8.28	1575.55 ± 1.72	1481.81 ± 2.88
20:1 n-9	7.86 ± 1.24	6.27 ± 0.02	6.97 ± 0.81	5.38 ± 0.19	3.57 ± 0.17
∑MUFA	**1468.42 ± 2.57**	**1476.80 ± 10.66**	**1579.06 ± 7.48**	**1581.77 ± 2.01**	**1486.6 ± 2.64**
18:2 n-6	3197.60 ± 22.9	3130.40 ± 7.24	3535.69 ± 3.74	3560.45 ± 8.26	3307.5 ± 8.34
20:2 n-6	1.09 ± 0.02	2.55 ± 0.56	1.19 ± 0.09	1.21 ± 0.10	0.30 ± 0.10
20:4 n-6	-	10.43 ± 0.27	0.38 ± 0.03	5.83 ± 0.61	3.58 ± 0.26
∑n-6 PUFA	**3198.7 ± 22.97**	**3143.39 ± 6.42**	**3537.26 ± 3.85**	**3567.49 ± 8.97**	**3311.46 ± 7.98**
18:3 n-3	10.98 ± 1.45	66.90 ± 1.90	83.00 ± 1.37	102.34 ± 0.59	121.24 ± 0.47
∑n-3 PUFA	**10.98 ± 1.45**	**66.90 ± 1.90**	**83.00 ± 1.37**	**102.34 ± 0.59**	**121.24 ± 0.47**

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza*

4.5. Evaluation of nutritional quality and physiological responses, *viz*. digestive enzyme profiles, expression of selected genes of cultured species fed with formulated diets

4.5.1. Performance of rohu *Labeo rohita*

Indian major carp rohu *Labeo rohita* were procured from Chatarji Brother's Fish Farm West Bengal, India and acclimated in the wet laboratory facility for 15 days. After the acclimation period, fish (1.74±0.02 g) were randomly divided in fifteen aquaria (50 l each). The stocking density was 20 fish/aquarium. Fish were fed with five different feeds: SP0, SP5, SP10, SP15 and SP20. Three replicates were used for each feeding scheme. Fish were harvested after 60 days of feeding. The performance of fish was evaluated.

4.5.1.1. Survival and growth of fish

After 60 days of feeding, the number of fish was recorded and the weight of individual fish was measured. There was no mortality of fish. The final weight was significantly (P<0.05) higher in the fish fed with diet SP5 compared to the other diets fed fish (Fig.6). There was no significant (P>0.05) difference in the final weight of SP10 and SP15 diets fed rohu. Significantly (P<0.05) lower final weight was recorded in rohu fed with diet SP20. Similarly, the specific growth rate was significantly (P<0.05) higher in SP5 diet fed rohu compared to others and this treatment was followed by SP10, SP15, SP0 and SP20 (Fig.7). The feed conversation ratio was significantly (P<0.05) lower in fish fed SP5 diet compared to others. This treatment was followed by SP10, SP15, SP0 and SP20 (Fig.8).

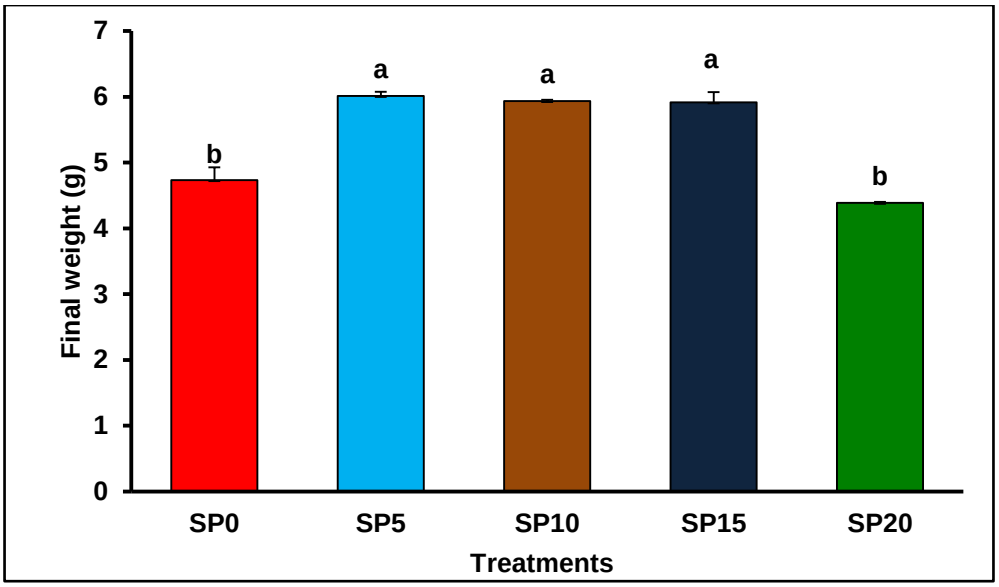

Fig.6: Final body weight of *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly (P< 0.05) different (n = 3).

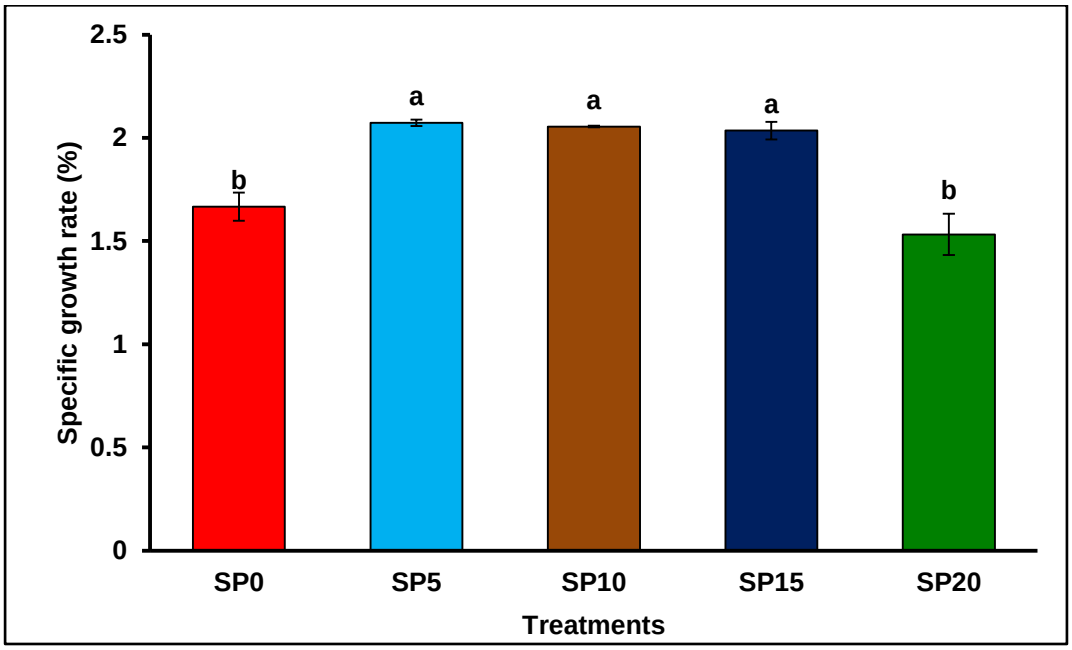

Fig.7: Specific growth rate of *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly (P< 0.05) different (n = 3).

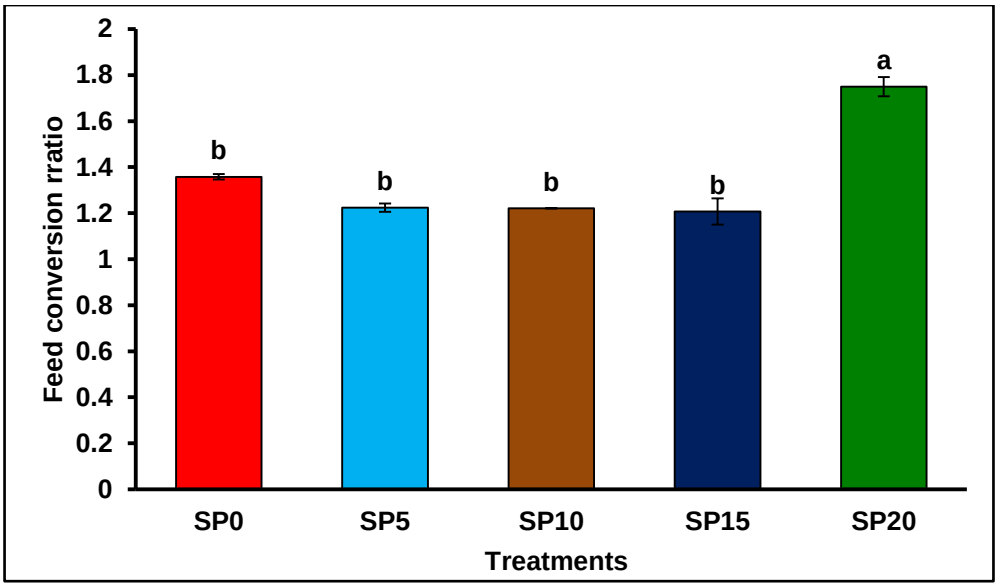

Fig.8: Feed conversion ratio of *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly (P< 0.05) different (n=3).

4.5.1.2. Digestive enzymes activities of fish

The type of diets influenced the digestive enzyme activities in rohu. The amylase activity was significantly (P<0.05) higher in SP5 diet fed fish (Fig.9) compared to other diets fed fish. The amylase activity was minimum in control diet (SP0) fed fish. Significantly (P<0.05) higher protease activity was found in fish fed diet SP5 compared to other diets fed fish (Fig.10). This treatment was followed by SP10, SP15, SP0 and SP20. The trypsin and chymotrypsin activities were significantly (P<0.05) higher in fish fed SP10 and SP5 diets, respectively compared to others (Figs.11 and 12). The lipase activity was significantly (P<0.05) higher in SP5 diet fed fish compared to others and this treatment was followed by SP15, SP10, SP0 and SP20 (Fig.13).

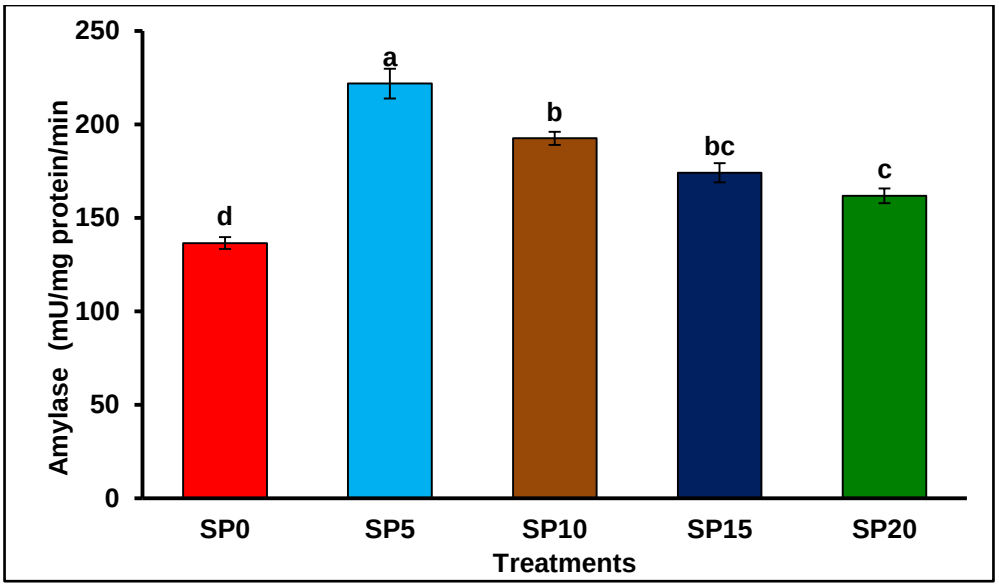

Fig.9: Amylase activity found in *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n = 3).

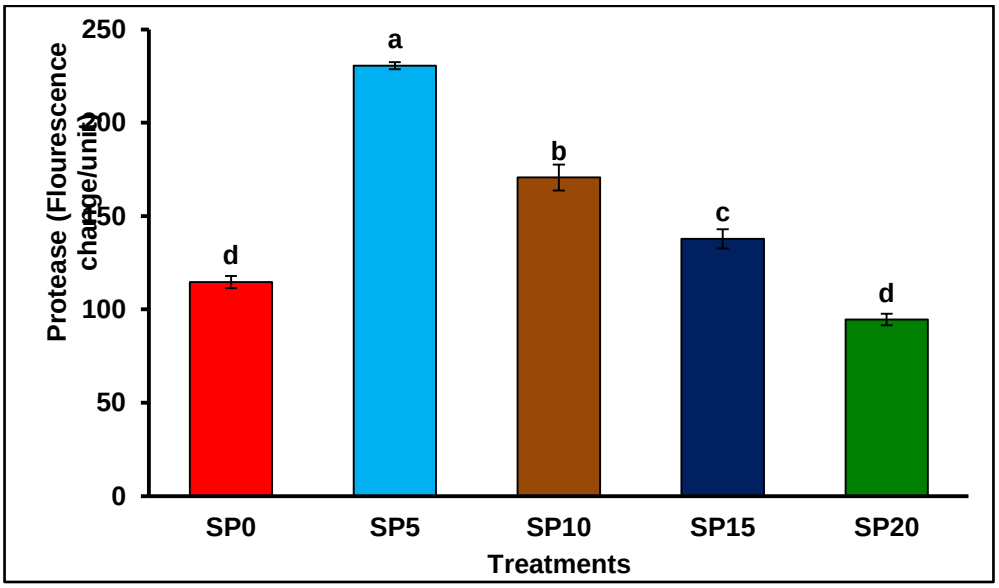

Fig.10: Protease activity found in *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n = 3).

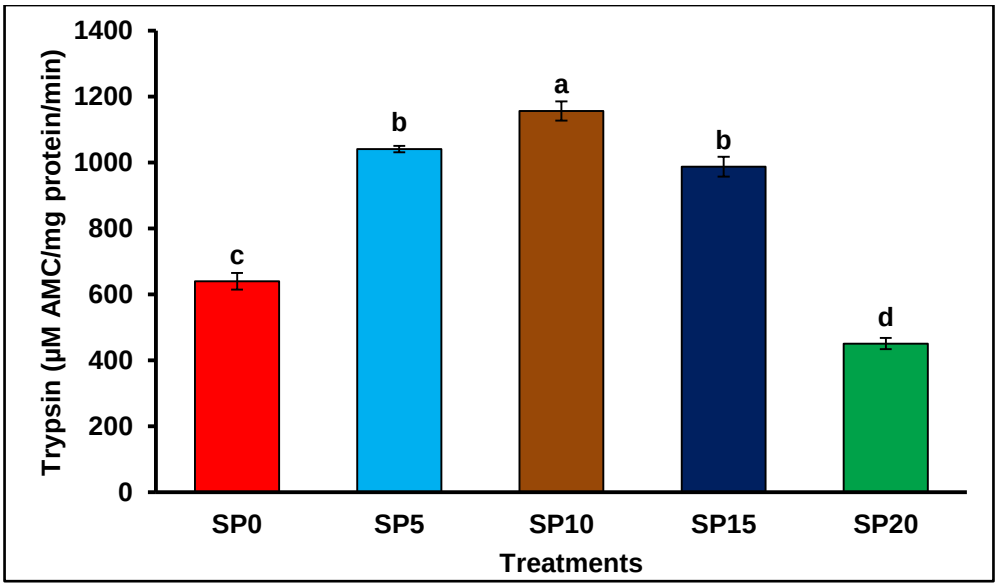

Fig.11: Trypsin activity found in *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n = 3).

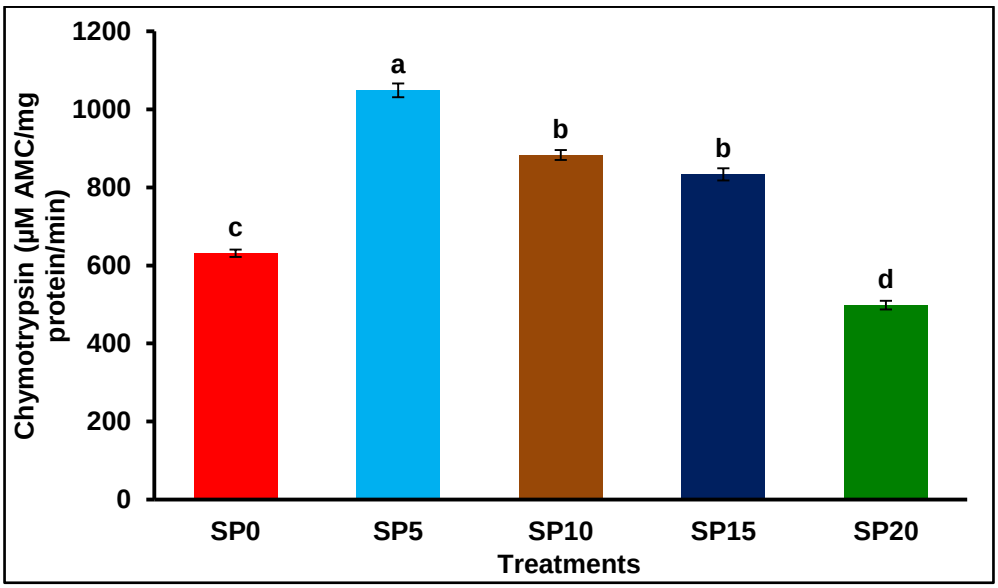

Fig.12: Chymotrypsin activity found in *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly (P < 0.05) different (n = 3).

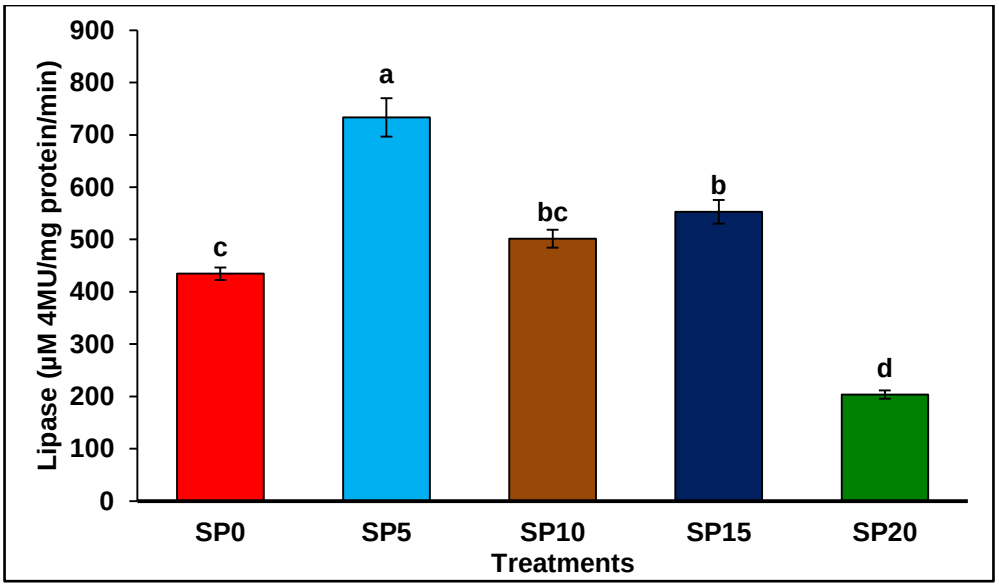

Fig.13: Lipase activity found in *Labeo rohita* cultured under five different feeding regimes. Bars with different superscripts are significantly ($P < 0.05$) different ($n = 3$).

4.5.1.3. Proximate composition of fish

There was no significant ($P>0.05$) difference in moisture content of the rohu fed with five different diets. The moisture content ranged from 77.6-78.4% in different diets fed rohu. There was no significant ($P>0.05$) difference in the protein and lipid contents of fish cultured under five different feeding regimes. The ash content was significantly ($P<0.05$) higher in SP20 diet fed fish compared to others. The ash content was minimum in SP0 diet fed fish.

4.5.1.4.

The supplementation of *S. polyrhiza* in the diets influenced the amino acids contents of rohu (Table 3). The essential amino acids, arginine, histidine and isoleucine contents were significantly ($P<0.05$) higher in SP15 diet fed fish compared to the other diets fed fish. There were no significant difference in arginine content among SP5, SP10, SP15 and SP20 diets fed rohu.

Table 13: Proximate compositions (g/kg, wet weight) of five different diets fed *Labeo rohita.* Values having the means (n=3) in each row with different superscripts are significantly (P<0.05) different.

Parameters	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear Regression R^2 Value
						P value	F value	
Moisture	779.13 ± 1.89^{a}	776.73 ± 1.56^{a}	784.03 ± 2.39^{a}	777.66 ± 0.95^{a}	782.46 ± 1.24^{a}	0.052	3.436	0.579
Protein	141.16 ± 1.04^{a}	145.96 ± 2.88^{a}	142.43 ± 1.10^{a}	143.66 ± 1.32^{a}	147.23 ± 2.56^{a}	0.143	2.193	0.467
Lipid	48.80 ± 1.33^{a}	50.20 ± 2.20^{a}	50.10 ± 0.65^{a}	52.20 ± 0.57^{a}	53.77 ± 0.99^{a}	0.103	2.567	0.507
Ash	23.43 ± 0.06^{c}	24.28 ± 0.14^{bc}	24.86 ± 0.29^{ab}	25.30 ± 0.29^{ab}	25.51 ± 0.30^{a}	0.831	12.296	0.831

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza*

Significantly (P<0.05) lower isoleucine content was found in fish fed diet SP0. The histidine and arginine contents were minimum in SP0 diet fed fish. The lysine was the most abundant amino acid and it was found in all fish. The leucine, lysine and valine contents were significantly (P<0.05) higher in diet SP10 fed rohu. The methionine and threonine contents were significantly (P<0.05) higher in SP20 and SP5 diets fed fish, respectively compared to others. Phenylalanine and tryptophan contents were significantly (P<0.05) higher in SP20 and SP5 diets fed rohu compared to others.

Among non-essential amino acids, alanine and aspartate contents were significantly (P<0.05) higher in fish fed SP20 and SP10 diets, respectively compared to the other diets fed fish. The cysteine and proline contents were maximum in SP5 diet fed rohu compared to others, whereas serine and alanine contents were significantly (P<0.05) higher in fish fed SP20 diet. Glutamate was the most abundant amino acid found in all fish. Highest glutamate content was found in SP10 diet fed fish. Significantly (P<0.05) higher serine and tyrosine contents were found in SP20 and SP10 diets fed fish, respectively compared to the other diets fed fish. The other non-essential amino acids *viz*. phosphoserine, β-amino isobutyric acid, hydroxylysine and hydroxyproline contents were significantly (P<0.05) higher in SP20 diet fed fish compared to others. Taurine, cystathionine and β-alanine content were significantly (P<0.05) higher in fish fed with diets SP10, SP20 and SP5, respectively. The 1-methyl histidine content was significantly (P<0.05) higher in SP0 diet fed fish compared to others and it was absent in SP15 and SP20 diets fed rohu.

Table 14: Amino acid compositions (g/kg, wet weight) of five different diets fed rohu. Values having the means (n=3) in each row with different superscripts are significantly (P<0.05) different

Amino acids	**SP0**	**SP5**	**SP10**	**SP15**	**SP20**	**ANOVA**		**Linear regression R^2 value**
Essential						**P value**	**F value**	
Arginine (Arg)	7.52 ± 0.04^{b}	8.38 ± 0.18^{a}	8.17 ± 0.11^{a}	8.52 ± 0.21^{a}	8.36 ± 0.03^{a}	0.004	7.964	0.761
Histidine (His)	3.18 ± 0.01^{d}	3.59 ± 0.08^{c}	4.15 ± 0.05^{b}	4.69 ± 0.09^{a}	4.31 ± 0.05^{b}	<0.01	80.142	0.970
Isoleucine (Ile)	5.62 ± 0.24^{bc}	5.44 ± 0.10^{c}	6.42 ± 0.01^{a}	6.64 ± 0.02^{a}	6.18 ± 0.01^{ab}	<0.01	18.205	0.879
Leucine (Leu)	8.71 ± 0.03^{c}	10.02 ± 0.18^{b}	11.16 ± 0.33^{a}	10.01 ± 0.11^{b}	10.46 ± 0.11^{ab}	<0.01	23.438	0.904
Lysine (Lys)	12.11 ± 0.07^{c}	11.14 ± 0.12^{d}	13.57 ± 0.01^{a}	12.15 ± 0.06^{c}	12.52 ± 0.03^{b}	<0.01	146.674	0.983
Methionine (Met)	2.79 ± 0.07^{b}	3.36 ± 0.12^{ab}	3.63 ± 0.24^{a}	3.95 ± 0.17^{a}	3.91 ± 0.02^{a}	0.001	10.450	0.807
Phenylalanine (Phe)	4.85 ± 0.06^{d}	5.44 ± 0.02^{c}	6.37 ± 0.03^{b}	5.64 ± 0.04^{c}	6.69 ± 0.11^{a}	<0.01	133.782	0.982
Threonine (Thr)	5.90 ± 0.01^{c}	6.51 ± 0.13^{b}	7.24 ± 0.05^{a}	7.25 ± 0.04^{a}	6.35 ± 0.04^{b}	<0.01	68.425	0.965
Tryptophan (Trp)	1.06 ± 0.005^{a}	1.59 ± 0.42^{a}	1.33 ± 0.001^{a}	1.22 ± 0.01^{a}	1.55 ± 0.002^{a}	0.301	1.403	0.360
Valine (Val)	6.38 ± 0.20^{bc}	6.12 ± 0.006^{c}	7.16 ± 0.03^{a}	6.68 ± 0.09^{ab}	6.21 ± 0.08^{bc}	<0.01	15.625	0.862
∑Essential	**58.16 ± 0.47^{d}**	**61.62 ± 0.87^{c}**	**69.24 ± 0.57^{a}**	**66.80 ± 0.22^{ab}**	**66.58 ± 0.47^{b}**	**<0.01**	**64.333**	**0.963**

Non-essential	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
						P value	F value	
Alanine (Ala)	7.99 ± 0.02^{d}	8.52 ± 0.01^{c}	9.02 ± 0.03^{b}	8.19 ± 0.13^{d}	9.52 ± 0.01^{a}	<0.01	106.536	0.977
Aspartate (Asp)	12.26 ± 0.02^{e}	13.01 ± 0.05^{d}	14.31 ± 0.12^{a}	13.42 ± 0.04^{c}	13.95 ± 0.04^{b}	<0.01	160.378	0.985
Cysteine (Cys)	2.13 ± 0.03^{a}	1.06 ± 0.01^{c}	1.08 ± 0.01^{c}	1.47 ± 0.01^{b}	1.41 ± 0.01^{b}	<0.01	952.545	0.997
Glutamic Acid (Glu)	19.46 ± 0.27^{b}	20.15 ± 0.37^{b}	22.39 ± 0.20^{a}	21.79 ± 0.33^{a}	22.86 ± 0.36^{a}	<0.01	22.071	0.898
Glycine (Gly)	8.50 ± 0.02^{d}	10.24 ± 0.11^{a}	8.78 ± 0.02^{cd}	9.04 ± 0.06^{c}	9.77 ± 0.05^{b}	<0.01	133.275	0.982
Proline (Pro)	10.27 ± 0.04^{a}	10.22 ± 0.13^{a}	8.45 ± 0.16^{b}	8.50 ± 0.30^{b}	8.32 ± 0.12^{b}	<0.01	34.027	0.932
Serine (Ser)	5.84 ± 0.08^{bc}	5.39 ± 0.07^{d}	6.11 ± 0.02^{ab}	5.56 ± 0.01^{cd}	6.43 ± 0.01^{a}	<0.01	37.993	0.938
Tyrosine (Tyr)	3.06 ± 0.17^{b}	2.41 ± 0.04^{c}	3.78 ± 0.03^{a}	2.16 ± 0.02^{c}	3.44 ± 0.25^{ab}	<0.01	24.463	0.907
Phosphoserine (p-Ser)	0.92 ± 0.01^{d}	1.60 ± 0.03^{b}	1.49 ± 0.00^{c}	2.18 ± 0.01^{a}	0.67 ± 0.03^{e}	<0.01	913.649	0.997
Taurine (Tau)	1.16 ± 0.04^{b}	1.29 ± 0.01^{ab}	1.50 ± 0.03^{a}	1.25 ± 0.03^{ab}	1.18 ± 0.01^{b}	<0.01	6.317	0.716
Cystathionine (Cysthi)	0.58 ± 0.02^{c}	0.54 ± 0.01^{c}	0.92 ± 0.01^{b}	0.33 ± 0.01^{d}	1.47 ± 0.03^{a}	<0.01	681.657	0.996
β -Alanine (β -Ala)	0.15 ± 0.01^{c}	0.99 ± 0.04^{a}	0.13 ± 0.01^{c}	0.88 ± 0.04^{b}	0.21 ± 0.09^{c}	0.026	4.420	0.639
β Amino isobutyric acid (β-AiBA)	0.05 ± 0.00^{b}	0.14 ± 0.04^{a}	0.15 ± .05^{a}	0.20 ± 0.01^{a}	0.07 ± 0.00^{b}	0.119	2.403	0.490
Hydroxylysine (Hylys)	0.08 ± 0.01^{b}	0.14 ± 0.04^{a}	0.15 ± 0.05^{a}	0.20 ± 0.01^{a}	0.18 ± 0.04^{a}	0.231	1.679	0.402
1 Methyl histidine (1 Mehis)	0.31 ± 0.05^{a}	0.20 ± 0.02^{b}	0.17 ± 0.01^{b}	-	-	<0.01	33.705	0.931
Hydroxy proline (Hypro)	2.79 ± 0.12^{a}	2.87 ± 0.05^{a}	1.64 ± 0.53^{b}	2.94 ± 0.03^{a}	1.35 ± 0.08^{b}	<0.01	9.536	0.792
∑Non- essential	**75.56 ± 0.18^{c}**	**78.77 ± 0.31^{ab}**	**80.04 ± 1.03^{ab}**	**78.06 ± 0.15^{bc}**	**80.96 ± 0.69^{a}**	**<0.01**	**12.767**	**0.836**

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza.*

4.5.2. Performance of common carp *Cyprinus carpio*

Common carp *Cyprinus carpio* juvenile were procured from Kaku Fish Farm, Rampur, Uttar Pradesh, India and acclimated in the wet laboratory facility for 15 days. After the acclimation, fish (0.473±0.006 g) were randomly divided in fifteen aquaria (50 l each). The stocking density was 30 fish/aquarium. Fish were fed with five different diets: SP0, SP5, SP10, SP15 and SP20. Three replicates were used for each feeding scheme. Fish were harvested after 60 days of feeding. The performance of fish was evaluated.

4.5.2.1. Survival and growth of fish

After 60 days of culture, the number of common carp in each aquarium was recorded. There was no mortality of fish; all fish survived. The broken-line regression analysis showed the impact of greater duckweed on the growth performance and specific (SGR) growth rate of common carp. The estimated break point for final weight and SGR were 10 and 13.4% of greater duckweed, respectively. The final weight of SP20 diet-fed common carp was significantly (P<0.05) higher compared with other diet-fed fish (Fig.14). Minimum weight was found in the SP0 treatment. Similar trend was also found with SGR (Fig.15). SGR was maximum in SP20 diet fed carp. FCR was minimum and maximum in SP20 and SP0 treatment, respectively (Fig.16).

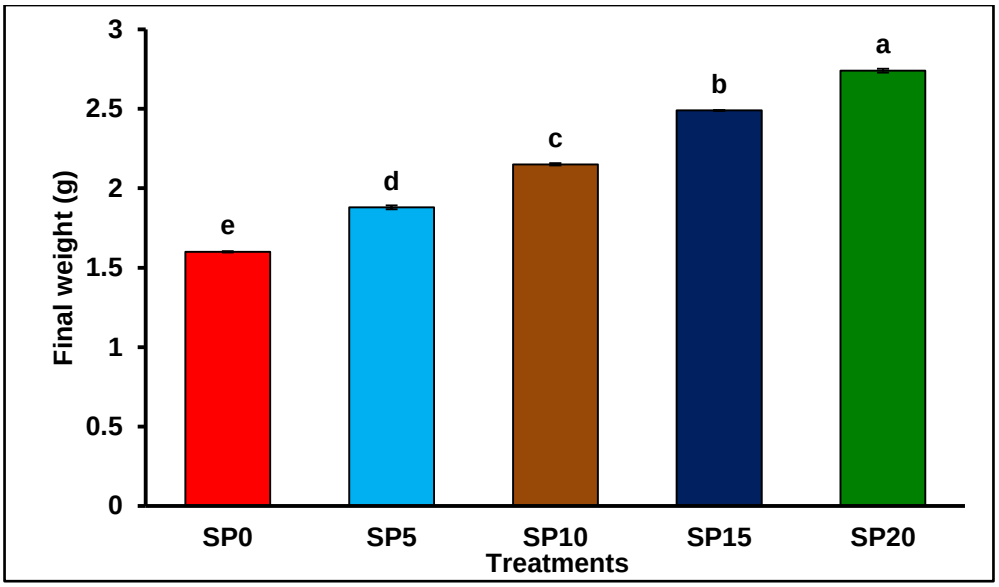

Fig.14: Final body weight of *Cyprinus carpio* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n=3).

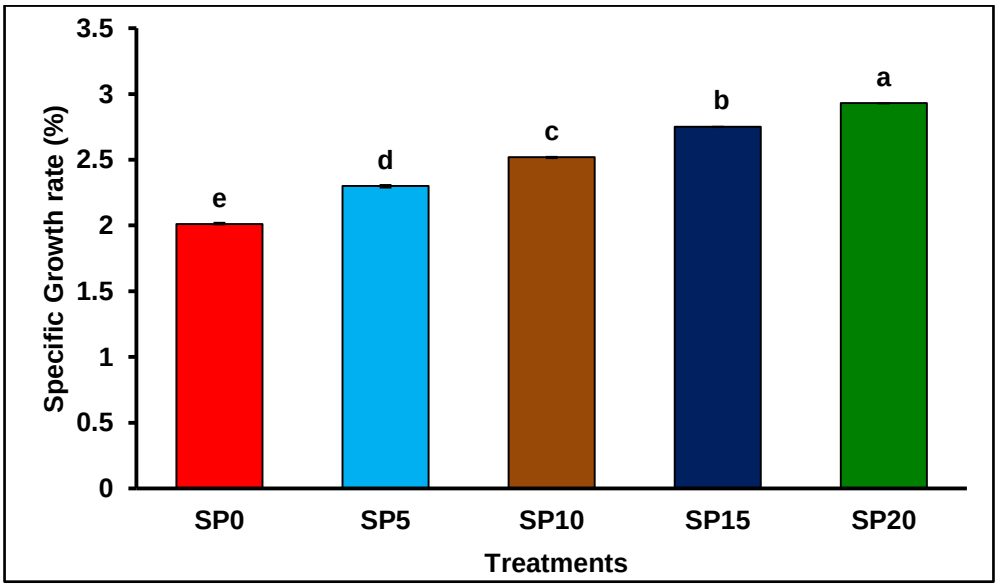

Fig.15: Specific growth rate of *Cyprinus carpio* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n = 3).

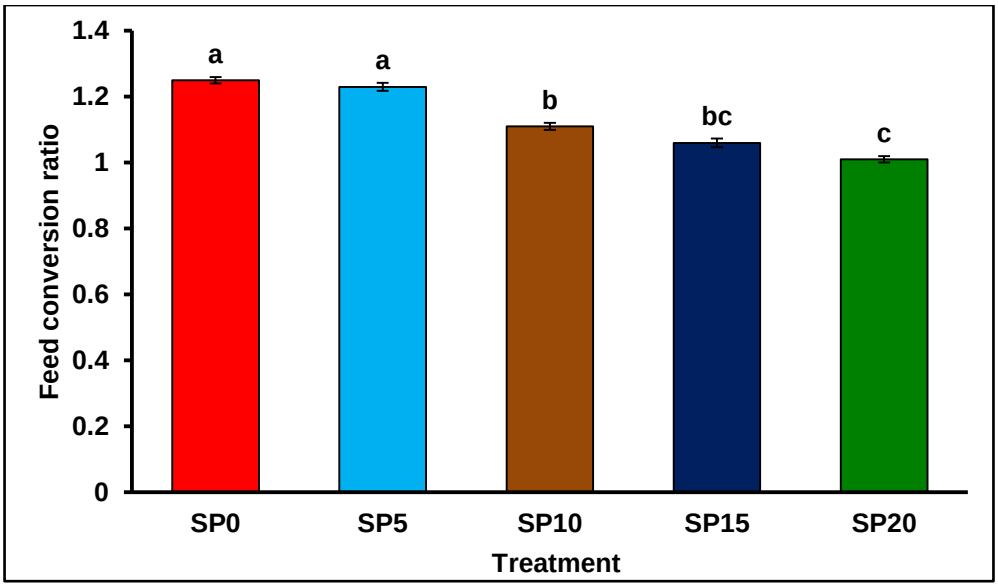

Fig.16: Feed conversion ratio of *Cyprinus carpio* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n = 3).

4.5.2.2. Digestive enzymes activities of fish

The amylase activity ranged from 98.91-126.48 mU/mg protein/min in five different diets fed common carp. The minimum amylase activity was found in SP5 diet-fed fish (Fig.17). The amylase activity was significantly (P<0.05) higher in SP0, SP15, and SP20 diets fed fish compared to others. The total protease activity was significantly (P<0.05) higher in SP10, SP15, and SP20 diets fed fish compared to other diets fed fish (Fig.18). Trypsin activity was significantly (P<0.05) higher in SP20 diet fed fish compared to other diets fed fish (Fig.19). Significantly (P<0.05) higher chymotrypsin and lipase activities were found in SP5 and SP15 diets fed common carp, respectively, compared to other treatments (Figs.20 and 21). The inclusion of greater duckweeds in the carp diet showed linear relationships (R^2=0.917-0.998) with amylase, trypsin, chymotrypsin, and lipase activities.

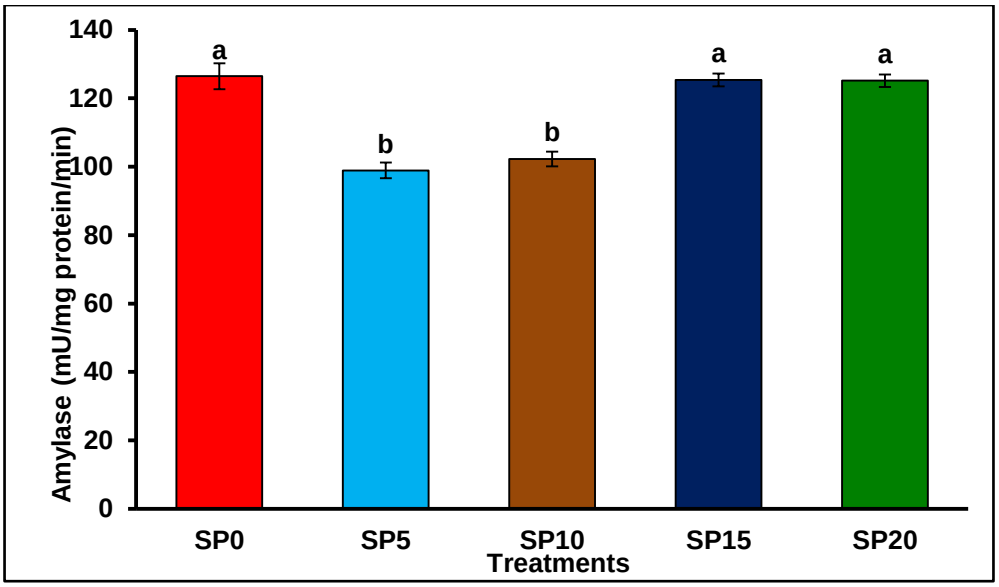

Fig.17: Amylase activity found in *Cyprinus carpio* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n=3).

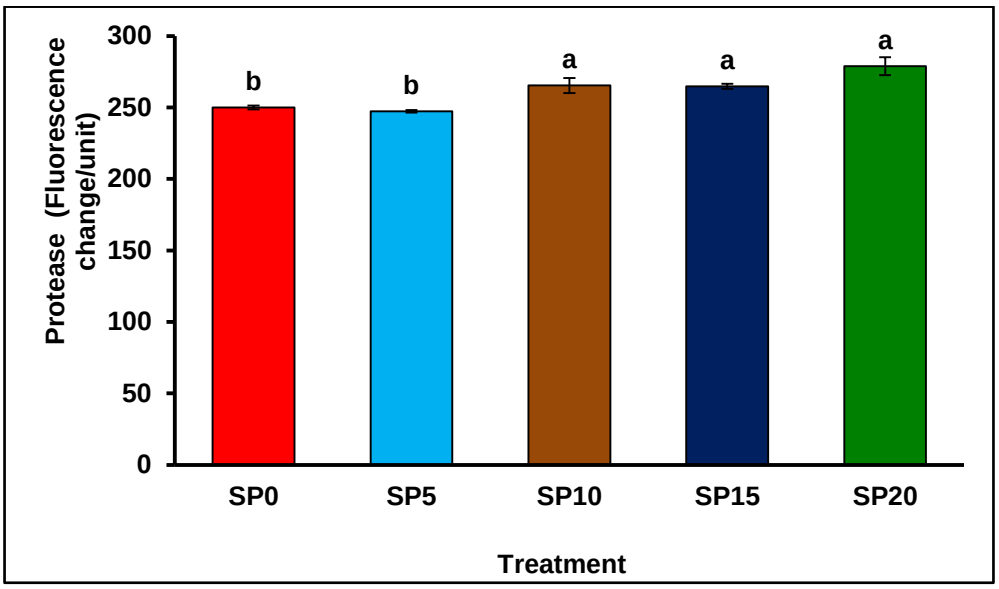

Fig.18: Protease activity found in *Cyprinus carpio* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n=3).

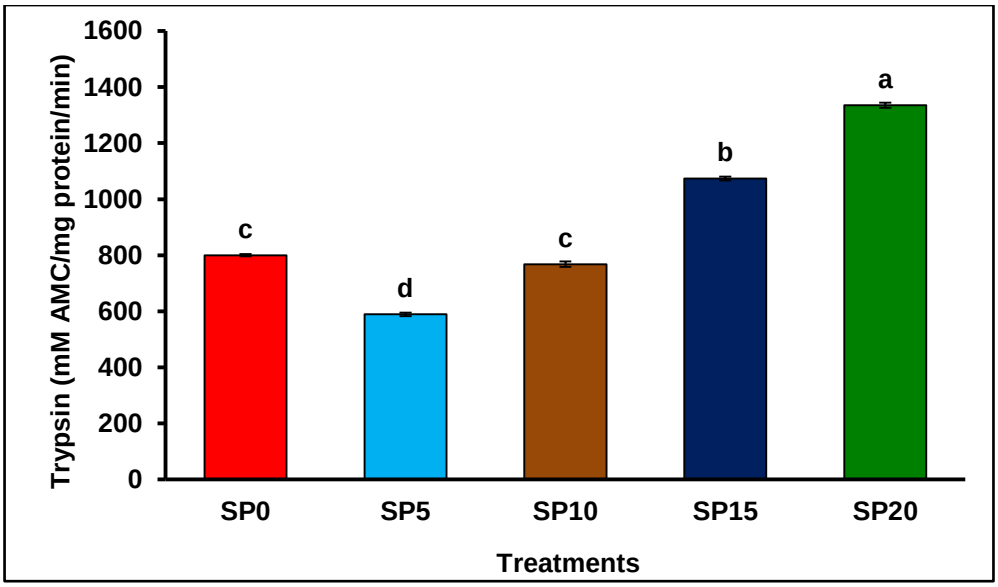

Fig.19: Trypsin activity found in *Cyprinus carpio* cultured under five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n=3).

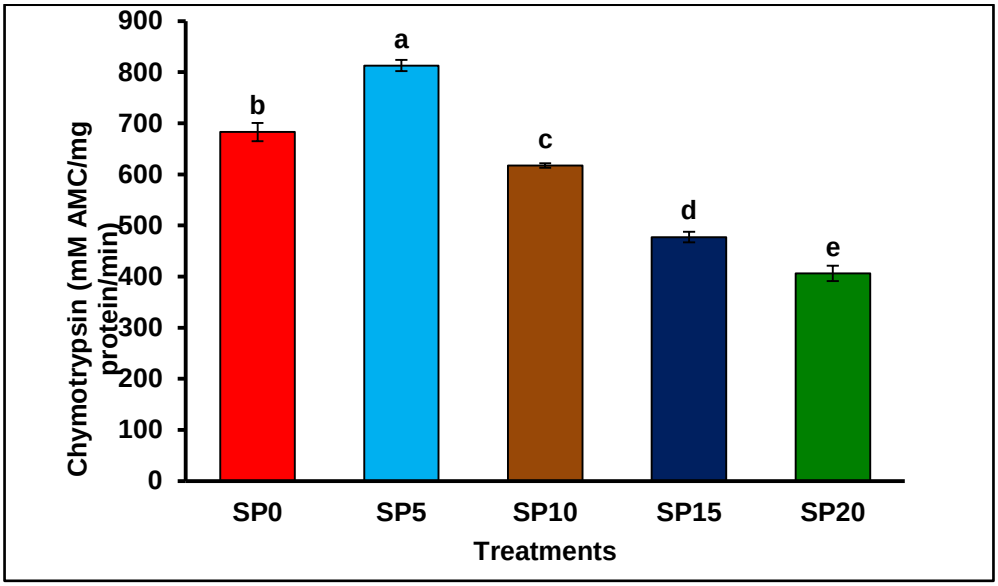

Fig.20: Chymotrypsin activity found in *Cyprinus carpio* cultured under five different feeding regimes. Bars with different superscripts are significantly (P < 0.05) different (n = 3).

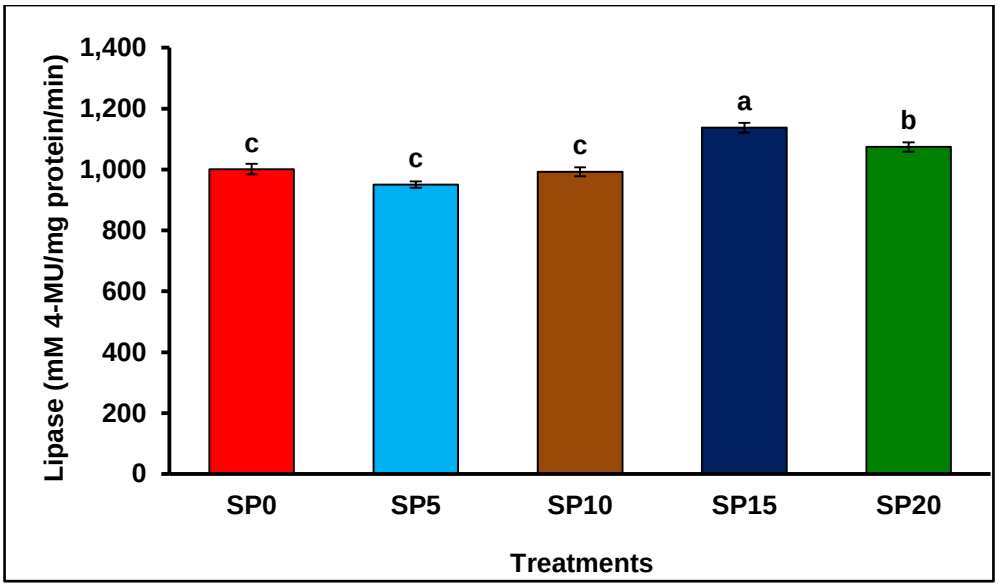

Fig.21: Lipase activity found in *Cyprinus carpio* cultured wunder five different feeding regimes. Bars with different superscripts are significantly (P<0.05) different (n=3).

4.5.2.3. Proximate Composition of fish

The moisture contents of fish varied from 747.6-756.4 g/kg in five different diets fed common carp (Table 15). The crude protein content was significantly (P<0.05) higher in SP10, SP15, and SP20 diets fed common carp compared with other diets fed fish. Significantly (P<0.05) higher crude lipid and ash contents were found in SP20 diet fed fish compared to others.

4.5.2.4. Amino acid composition of fish

The essential amino acids composition was similar in common carp cultured in five different feeding schemes (Table 16). The arginine level was 1.55-1.67 folds higher in 10-20% greater duckweeds supplemented diet fed common carp compared to SP0 and SP5 diets fed fish. Histidine (3.64-3.72 g/kg), isoleucine (5.13–5.39 g/kg), leucine (9.59-10.12 g/kg), and lysine (11.54-12.75 mg/kg) contents were significantly (P<0.05) higher in SP0 and SP5

treatments compared to other diets fed fish. Significantly (P<0.05) higher levels of methionine and valine were recorded in SP10 compared to others. The threonine and tryptophan levels were maximum in SP20 diet fed common carp. Among non-essential amino acids, alanine, aspartate, glycine, serine, taurine, and β-alanine contents were maximum in SP20 diet fed common carp. Highest levels of glutamic acid and tyrosine were observed in control diet (SP0) fed common carp. Aspartate contents were significantly (P<0.05) higher in SP10 and SP20 diets fed common carp compared to others. Highest levels of phosphoserine, cystathionine, and hydroxyproline were found in SP15 diet fed fish. The α-amino-n-butyric acid content was highest in SP15, and the citrulline content was maximum in SP15 and SP20 treatments. The α-amino-n-butyric acid and 3-methyl histidine were absent in SP0 and SP5 diets fed fish. The β -amino-isobutyric and γ-amino-butyric acids were absent in SP5, SP10, and SP15 diets fed common carp. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2 = 0.745–0.998) with different amino acids, except α-amino-*n*-butyric acid and β-alanine.

Table 15: Proximate compositions (g/kg, wet weight) of five different diets fed *Cyprinus carpio*. Values (*n*=3) with different letters in the same row are significantly different (*P*<0.05).

Parameters	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear Regression R^2 Value
						P value	F value	
Moisture	756.4 ± 3.6^{a}	752.8 ± 3.7^{a}	750.7 ± 1.02^{a}	750.5 ± 5.7^{a}	747.6 ± 1.2^{a}	0.524	0.852	0.254
Protein	150.3 ± 0.66^{c}	152.34 ± 0.25^{b}	156.73 ± 0.25^{a}	156.79 ± 0.12^{a}	157.06 ± 0.1^{a}	<0.01	81.09	0.970
Lipid	64.7 ± 0.1^{c}	64.4 ± 0.10^{C}	65.2 ± 0.1^{bc}	65.9 ± 0.2^{b}	68.1 ± 0.5^{a}	<0.01	33.949	0.931
Ash	19.6 ± 0.3^{c}	21.4 ± 0.1^{b}	21.8 ± 0.2^{b}	21.9 ± 0.3^{b}	22.5 ± 0.3^{a}	<0.01	28.401	0.919

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza*.

Table 16: Amino acid compositions (g/kg, wet weight) of five different diets fed *Cyprinus carpio*. Values (n=3) with different letters in the same row are significantly different (P<0.05).

Amino acids	**SP0**	**SP5**	**SP10**	**SP15**	**SP20**	**ANOVA**		**Linear regression R^2 value**
Essential						**P value**	**F value**	
Arginine (Arg)	6.77 ± 0.2^{c}	6.40 ± 0.1^{d}	10.7 ± 0.1^{a}	10.21 ± 0.03^{b}	9.94 ± 0.04^{b}	<0.01	905.511	0.997
Histidine (His)	3.72 ± 0.1^{a}	3.64 ± 0.2^{a}	2.85 ± 0.05^{b}	2.87 ± 0.06^{b}	2.73 ± 0.03^{b}	<0.01	156.009	0.984
Isoleucine (Ile)	5.13 ± 0.12^{ab}	5.39 ± 0.07^{a}	4.98 ± 0.01^{b}	4.25 ± 0.1^{c}	4.44 ± 0.13^{c}	<0.01	67.119	0.964
Leucine (Leu)	9.59 ± 0.19^{a}	10.12 ± 0.33^{a}	8.59 ± 0.04^{b}	8.28 ± 0.11^{bc}	8.01 ± 0.25^{c}	<0.01	53.555	0.955
Lysine (Lys)	11.54 ± 0.04^{b}	12.75 ± 0.08^{a}	10.1 ± 0.1^{c}	9.15 ± 0.05^{d}	9.47 ± 0.24^{d}	<0.01	323.243	0.992
Methionine (Met)	2.94 ± 0.04^{c}	3.37 ± 0.04^{ab}	3.63 ± 0.21^{a}	2.77 ± 0.09^{c}	3.01 ± 0.30^{c}	<0.01	12.611	0.835
Phenylalanine (Phe)	5.52 ± 0.12^{ab}	5.83 ± 0.1^{a}	5.16 ± 0.01^{bc}	4.91 ± 0.20^{c}	4.99 ± 0.14^{a}	<0.01	14.657	0.854
Threonine (Thr)	4.73 ± 0.01^{d}	5.16 ± 0.03^{c}	6.20 ± 0.1^{b}	6.12 ± 0.1^{b}	6.67 ± 0.08^{a}	<0.01	293.479	0.992
Tryptophan (Trp)	2.27 ± 0.03^{c}	1.60 ± 0.05^{d}	1.56 ± 0.003^{d}	2.32 ± 0.01^{b}	2.48 ± 0.002^{a}	<0.01	1705.8	0.998
Valine (Val)	5.98 ± 0.05^{d}	6.49 ± 0.05^{bc}	7.14 ± 0.06^{a}	6.25 ± 0.01^{c}	6.63 ± 0.17^{b}	<0.01	69.776	0.965
∑Essential	**58.2 ± 1.1^{b}**	**60.8 ± 0.1^{a}**	**61.1 ± 0.4^{a}**	**57.1 ± 0.7^{b}**	**58.4 ± 0.4^{b}**	<0.01	21.402	0.895

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza*.

Non-essential	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
						P value	F value	
Alanine (Ala)	8.03 ± 0.03^{b}	7.65 ± 0.03^{c}	8.03 ± 0.06^{b}	9.85 ± 0.07^{a}	9.94 ± 0.15^{a}	<0.01	363.836	0.993
Aspartate (Asp)	11.7 ± 0.03^{b}	11.4 ± 0.12^{b}	12.4 ± 0.36^{a}	11.3 ± 0.02^{b}	12.7 ± 0.12^{a}	<0.01	33.477	0.931
Cysteine (Cys)	1.14 ± 0.002^{c}	1.69 ± 0.07^{a}	1.12 ± 0.07^{c}	1.30 ± 0.17^{bc}	1.39 ± 0.02^{b}	<0.01	18.725	0.882
Glutamic Acid (Glu)	20.3 ± 0.08^{a}	18.8 ± 0.10^{c}	17.3 ± 0.01^{d}	17.3 ± 0.05^{d}	19.6 ± 0.17^{b}	<0.01	535.013	0.995
Glycine (Gly)	8.91 ± 0.09^{d}	10.0 ± 0.3^{c}	8.58 ± 0.01^{d}	10.80 ± 0.14^{b}	11.62 ± 0.03^{a}	<0.01	77.612	0.969
Proline (Pro)	27.8 ± 0.1^{c}	29.0 ± 0.1^{b}	31.37 ± 0.01^{a}	31.10 ± 0.20^{a}	25.1 ± 0.2^{d}	<0.01	387.422	0.991
Serine (Ser)	4.83 ± 0.02^{c}	4.81 ± 0.07^{c}	5.62 ± 0.01^{b}	5.48 ± 0.03^{b}	6.32 ± 0.08^{a}	<0.01	221.76	0.989
Tyrosine (Tyr)	4.10 ± 0.2^{a}	3.67 ± 0.34^{ab}	3.57 ± 0.02^{ab}	3.24 ± 0.16^{b}	3.34 ± 0.04^{b}	<0.01	7.311	0.745
Phosphoserine (p-Ser)	0.06 ± 0.00^{bc}	0.05 ± 0.00^{c}	0.10 ± 0.05^{bc}	0.28 ± 0.06^{a}	0.15 ± 0.01^{b}	<0.01	28.851	0.920
Taurine (Tau)	1.76 ± 0.03^{bc}	1.51 ± 0.15^{c}	1.74 ± 0.16^{bc}	1.96 ± 0.13^{b}	2.73 ± 0.04^{a}	<0.01	48.115	0.951
α Amino -n- butyric acid (α-ABA)	-	-	0.19 ± 0.02^{a}	0.20 ± 0.03^{a}	0.18 ± 0.00^{a}	0.221	1.961	0.395
Citrulline (Cit)	0.13 ± 0.01^{b}	0.15 ± 0.03^{b}	0.44 ± 0.00^{a}	0.54 ± 0.20^{a}	0.56 ± 0.00^{a}	<0.01	16.081	0.865
Cystathionine (Cysthi)	0.28 ± 0.05^{d}	0.11 ± 0.01^{d}	2.13 ± 0.14^{b}	2.60 ± 0.01^{a}	1.38 ± 0.04^{c}	<0.01	301.177	0.992
β -Alanine (β -Ala)	-	0.08 ± 0.02^{a}	0.06 ± 0.00^{ab}	0.11 ± 0.001^{a}	0.11 ± 0.05^{a}	0.185	2.051	0.435
β Amino isobutyric acid (β-AiBA)	0.04 ± 0.00^{b}	-	-	-	0.08 ± 0.00^{a}	-	-	-
Ɣ-Amino-n-butyric acid (Ɣ-ABA)	0.04 ± 0.00^{a}	-	-	-	-	-	-	-
Hydroxylysine (Hylys)	0.16 ± 0.05^{b}	-	-	0.24 ± 0.002^{a}	-	<0.01	518.664	0.992
3 Methyl histidine (3 Mehis)	-	-	0.07 ± 0.000^{a}	-	0.06 ± 0.000^{a}	0.02	13.914	0.777
Hydroxy proline (Hypro)	1.58 ± 0.09^{c}	2.28 ± 0.08^{b}	2.91 ± 0.22^{a}	3.13 ± 0.002^{a}	3.07 ± 0.16^{a}	<0.01	301.177	0.992
∑Non- essential	**90.95 ± 0.31^{c}**	**91.51 ± 0.21^{c}**	**95.76 ± 0.4^{b}**	**99.56 ± 0.50^{a}**	**98.16 ± 0.06^{a}**	**<0.01**	**136.54**	**0.982**

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza*.

4.5.2.5. Fatty acid composition of fish

The feeding of common carp with greater duckweed supplemented diets influenced the fatty acid composition of fish (Table 17). Among saturated fatty acids (SFA), palmitic acid (16:0) was the dominant one regardless of treatments. The myristic acid (14:0) was the second dominant SFA. Highest SFA was found in carp fed with SP5 diet. Mono-unsaturated fatty acids (MUFA) showed an inverse relationship with the inclusion level of greater duckweeds in the diet and the composition of the fish. Significantly (P<0.05) higher MUFA content was found in the control diet (SP0) fed common carp compared to others. Among MUFA, oleic acid (18:1n-9) was the dominant one in all treatments. Nervonic acid (24:1) was absent in fish fed diets SP15 and SP20. The greater duckweed supplemented diets enhanced the n-6 PUFA and linoleic acid (18:2 n-6, LA) content in fish. The highest level of LA was found in SP20 diet fed fish. The n-3 PUFA contents of fish showed an increasing trend with the increasing inclusion of greater duckweeds in the diet. The α-linolenic acid (18:3n-3, ALA), eicosapentaenoic acid (20:5n-3, EPA), docosapentanoic acid (22:5 n-3, DPA), and docosahexaenoic acid (22: 6n-3, DHA) contents were significantly (P<0.05) lower in SP0 diet fed common carp. The n-3 PUFA content was significantly (P<0.05) higher in SP20 diet fed common carp compared to other diet fed fish. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2=0.745-0.998) with different fatty acids, except stearic acid (18:0).

Table 8: Fatty acid compositions (mg/100 g, wet weight) of five different diets fed *Cyprinus carpio*. Values (n=3) with different letters in the same row are significantly different (P<0.05).

Fatty acids	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
						P value	F value	
14:0	22.92 ± 1.23^{b}	30.64 ± 1.00^{a}	22.80 ± 0.34^{b}	22.42 ± 0.11^{b}	15.78 ± 0.07^{c}	<0.01	156.593	0.984
15:0	4.21 ± 0.23^{c}	6.52 ± 0.25^{b}	4.89 ± 0.61^{c}	6.21 ± 0.34^{b}	7.29 ± 0.29^{a}	<0.01	22.074	0.898
16:0	374.00 ± 9.01^{d}	548.07 ± 20.3^{a}	507.88 ± 6.26^{b}	435.9 ± 1.41^{c}	369.36 ± 2.75^{d}	<0.01	176.633	0.986
18:0	1.90 ± 0.01^{a}	2.81 ± 0.68^{a}	3.00 ± 0.20^{a}	2.05 ± 0.29^{a}	1.24 ± 0.21^{a}	0.081	**2.856**	0.533
24:0	10.53 ± 0.01^{b}	14.68 ± 2.17^{a}	12.49 ± 0.04^{a}	7.21 ± 0.77^{c}	9.25 ± 0.11^{b}	<0.01	79.245	0.969
∑SFA	**413.58 ± 10.00^{d}**	**602.75 ± 22.66^{a}**	**551.09.1 ± 7.05^{b}**	**473.8 ± 3.20^{c}**	**403.20 ± 3.50^{d}**	**<0.01**	**165.49**	**0.985**
16:1 n-9	26.44 ± 0.02^{a}	14.10 ± 1.0^{b}	12.25 ± 2.35^{b}	24.44 ± 0.72^{a}	4.46 ± 0.73^{c}	<0.01	164.648	0.985
18:1 n-9	1013.68 ± 0.02^{a}	963.71 ± 46.81^{a}	889.60 ± 5.90^{b}	905.3 ± 1.80^{b}	699.36 ± 10.84^{c}	<0.01	91.965	0.974
24:1	2.51 ± 0.04^{b}	3.86 ± 0.44^{a}	2.33 ± 0.29^{b}	-	-	<0.01	22.290	0.881
∑MUFA	**1042.6 ± 1.75^{a}**	**981.688 ± 46.26^{b}**	**904.18 ± 3.83^{c}**	**929.79 ± 2.52^{bc}**	**703.82 ± 11.57^{d}**	**<0.01**	**107.854**	**0.977**
18:2 n-6	911.99 ± 8.58^{b}	989.75 ± 9.10^{a}	1030.2 ± 5.18^{a}	1016.81 ± 3.76^{a}	1053.6 ± 0.06^{a}	<0.01	11.145	0.817
20:2 n-6	32.67 ± 1.24^{b}	20.60 ± 0.03^{d}	32.55 ± 0.05^{b}	37.51 ± 1.82^{a}	24.48 ± 2.07^{c}	<0.01	72.707	0.967
20:3 n-6	13.44 ± 0.22^{b}	11.91 ± 0.78^{c}	10.77 ± 0.02^{d}	14.60 ± 0.30^{a}	10.85 ± 0.52^{d}	<0.01	55.671	0.957
20:4 n-6	26.89 ± 0.22^{b}	22.96 ± 0.32^{d}	28.17 ± 0.83^{a}	21.75 ± 0.15^{d}	25.12 ± 0.38^{c}	<0.01	102.122	0.976
22:5 n-6	71.09 ± 0.22^{ab}	66.27 ± 0.17^{c}	46.22 ± 0.002^{d}	69.43 ± 2.17^{b}	72.80 ± 0.144^{a}	<0.01	282.796	0.991
∑n-6 PUFA	**1056.1 ± 11.25^{b}**	**1111.5 ± 10^{ab}**	**1147.96 ± 62.51^{a}**	**1160.11 ± 8.01^{a}**	**1186.95 ± 2.2^{a}**	**<0.01**	**9.048**	**0.784**
18:3 n-3	15.10 ± 0.115^{d}	13.65 ± 0.18^{e}	18.09 ± 0.30^{c}	19.53 ± 0.40^{b}	32.63 ± 0.19^{a}	<0.01	2563.494	0.999
20:4 n-3	126.51 ± 0.07^{c}	137.58 ± 0.33^{b}	144.32 ± 0.56^{a}	125.9 ± 3.42^{c}	145.15 ± 0.80^{a}	<0.01	111.460	0.978
20:5 n-3	3.94 ± 0.06^{c}	5.01 ± 0.33^{bc}	4.09 ± 1.04^{c}	8.99 ± 0.01^{a}	6.00 ± 0.40^{b}	<0.01	46.860	0.949
22:5 n-3	5.39 ± 0.01^{c}	9.29 ± 0.311^{b}	11.32 ± 1.36^{a}	13.00 ± 0.58^{a}	8.06 ± 0.16^{b}	<0.01	51.941	0.954
22:6 n-3	48.03 ± 0.04^{d}	63.07 ± 0.240^{c}	65.64 ± 0.30^{c}	74.05 ± 0.41^{b}	81.92 ± 1.00^{a}	<0.01	315.64	0.992
∑n-3 PUFA	**198.9 7± 2.31^{d}**	**228.6 ± 0.54c**	**243.46 ± 3.52^{b}**	**241.47± 5.69^{b}**	**273.77 ± 0.45^{a}**	**<0.01**	**473.046**	**0.995**
EPA + DHA	**51.97 ± 2.19^{d}**	**68.08 ± 0.75c**	**69.73 ± 1.28^{c}**	**83.04 ± 1.29^{b}**	**87.93 ± 0.60^{a}**	**<0.01**	**598.073**	**0.996**

SP0 = Soybean meal only, SP5 = Soybean meal + 5%, *S. polyrhiza*, SP10 = Soybean meal + 10% *S. polyrhiza*, SP15 = Soybean meal + 15% *S. polyrhiza*, SP20 = Soybean meal + 20% *S. polyrhiza*.

4.5.2.6. Gene expression study

The expressions of various genes involved in the metabolism of fatty acid were recorded in the hepatopancreas of five different diets fed common carp. The expression of *fads2d6* was significantly (P<0.05) higher in fish fed diet SP5 compared to others (Fig.22). The expression levels of *elovl2* and *elovl5* were significantly (P<0.05) lower in SP20 diet-fed common carp compared to others (Figs.23 and 24). Significantly (P<0.05) higher expression of *fas* was observed in SP5 compared to other diet fed fish (Fig.25). The mRNA expressions showed polynomial 3 order relationships with different treatments.

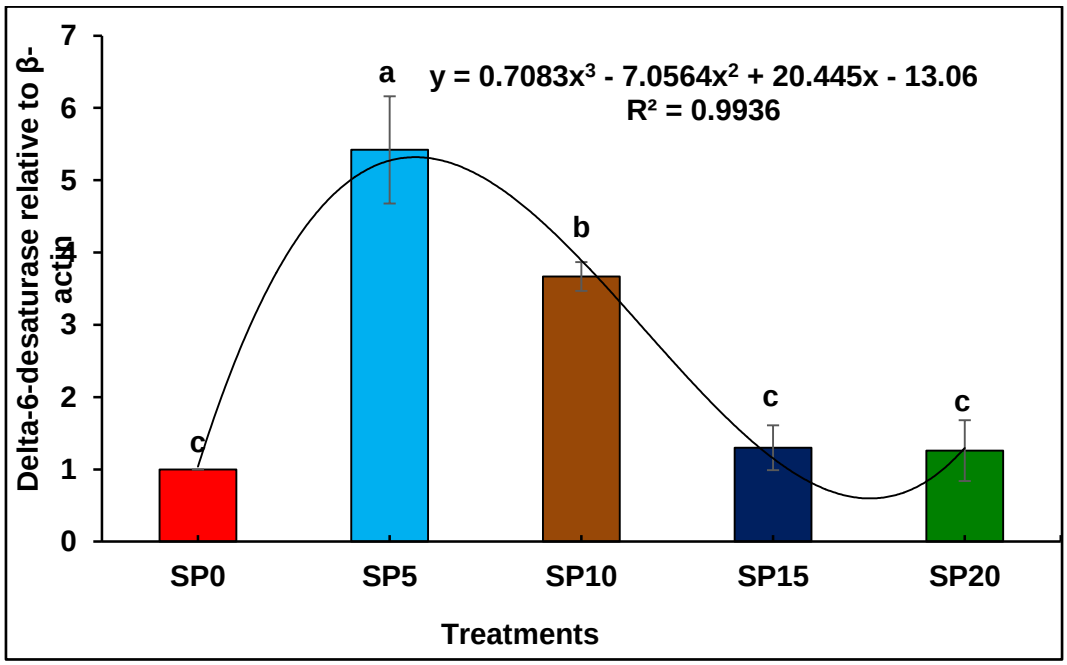

Fig.22: Expression of delta-6-desaturase (*fads2d6)* relative to *β-actin* in hepatopancreas of five different diets fed *Cyprinus carpio*. Bars with different superscripts are significantly different (n=3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2=0.994).

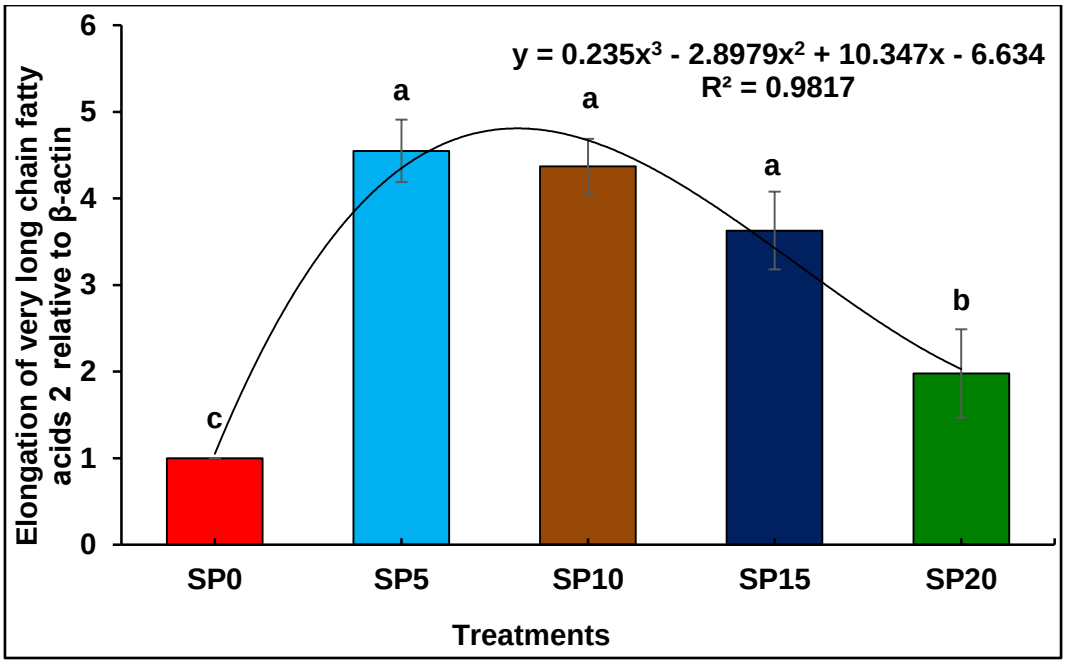

Fig.23: Expression of elongation of very long chain fatty acids protein 2 (*elovl2*) relative to *β-actin* in hepatopancreas of five different diets fed *Cyprinus carpio*. Bars with different superscripts are significantly different (*n*=3). A polynomial (order 3) relationship was found between the diet and expression of *elovl 2* (R^2=0.982).

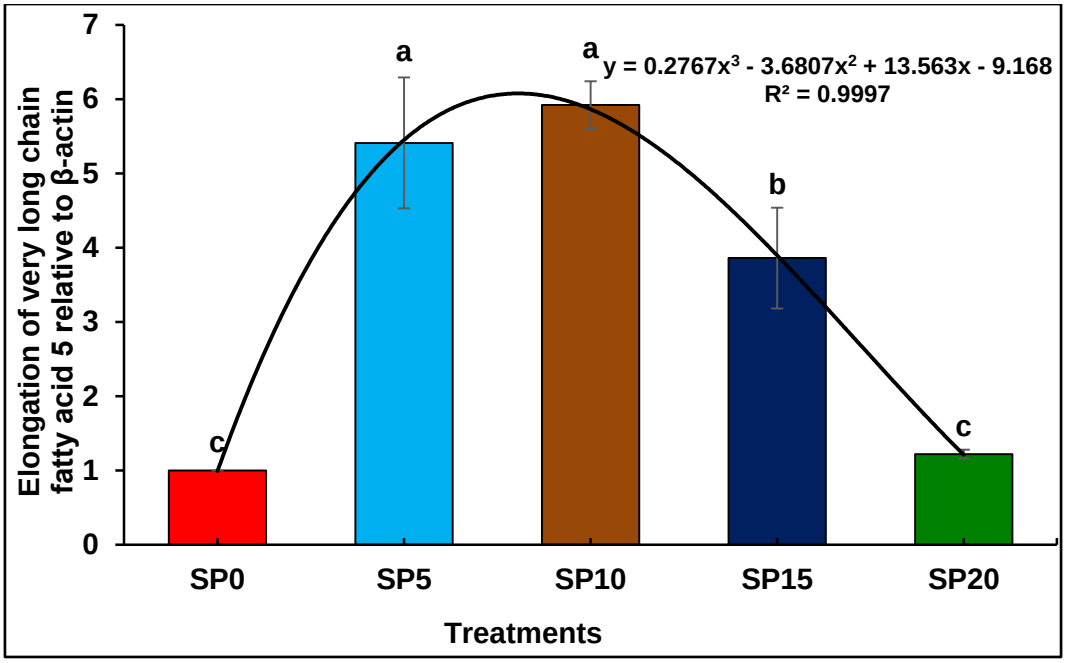

Fig.24: Expression of elongation of very long chain fatty acids protein 5 (*elovl5*) relative to *β-actin* in hepatopancreas of five different diets fed *Cyprinus carpio*. Bars with different superscripts are significantly different (*n*=3). A polynomial (order 3) relationship was found between the diet and expression of *elovl 5* (R^2 = 0.999).

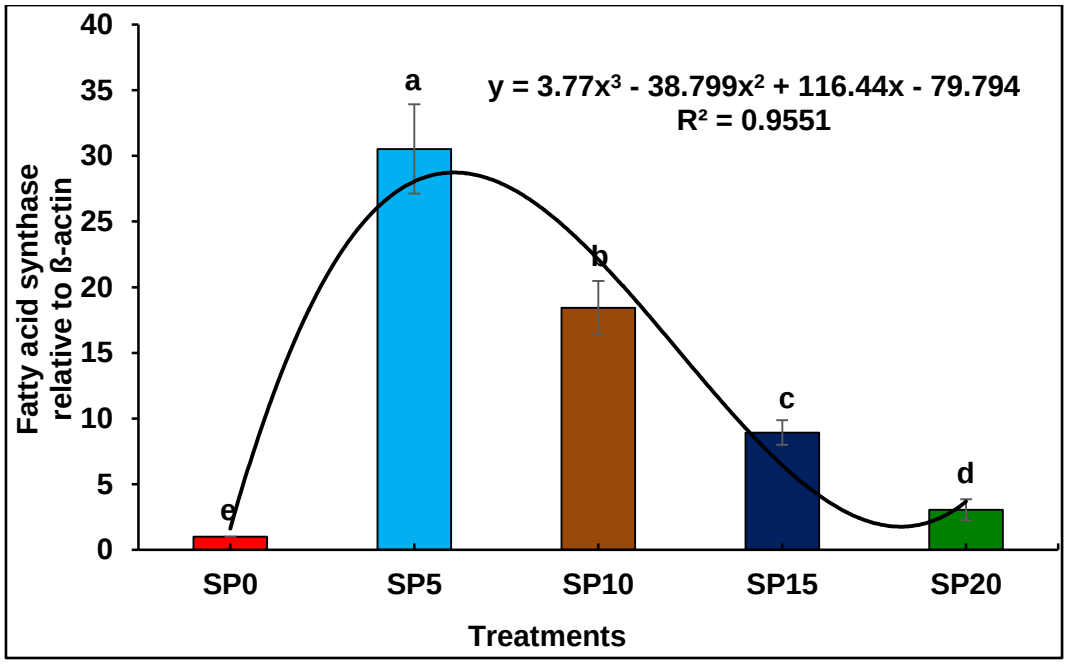

Fig.25: Expression of fatty acid synthase (*fas*) relative to *β-actin* in hepatopancreas of five different diets fed *Cyprinus carpio*. Bars with different superscripts are significantly different (*n*= 3). A polynomial (order 3) relationship was found between the diet and expression of *fas* (R^2=0.999).

Chapter 5

Discussion

DISCUSSION

5.1. The evaluation of nutritional value of freshwater macrophytes

5.1.1. Proximate composition

In the present investigation, the proximate composition study of twelve freshwater macrophytes showed significant differences in moisture, protein, lipid, ash, carbohydrate contents and calorific values. Proteins are the most important component of the nutrition and they are playing significant role in growth, physiological and immunological functions of fishes (Hoseini et al., 2019a). The protein content of three members of Lemnaceae family viz., *L. minor*, *S. polyrhiza* and *W. globosa* were more than 30% protein and the protein content of other cultured macrophytes (except *P. stratiotes* and *M. quardifiollia*) were more than 20%. The current study validates the previous findings that, aquatic macrophytes are rich source of the protein. In the present study, the protein content of the *L. minor*, *S. polyrhiza* and *W. globosa* were 36.97, 36.84 and 32.68%, respectively. These values were higher than the previous findings (Chareontesprasit and jiwyam, 2001; Fasakin et al., 2001; Bairagi et al., 2002; Kalita et al., 2007; Appenroth et al., 2017; Pagliuso et al., 2022). The protein content of *A. microphylla* and *A. pinnata* in the current study were 24.39 and 29.45%, respectively. Earlier study reported lower protein contents of these macrophytes (*A. microphylla*: 20.2%, *A. pinnata*: 21.67%) compared to the present observation (Dutta et al., 2011; Kumari et al., 2018). The protein content of the *I. aquatica* in the present study was 29.04%. This value was higher compared to the earlier findings of

Adelakun et al. (2016) and Ali and Kaviraj (2018). The protein content of the *S. molesta* and *S. natans* species in the present study were 27.78 and 28.04, respectively. Kalita et al. (2007) reported the protein content of *S. cuculata* as 11.0%. The protein content of *P. stratiotes* was 19.55% in the present study. Adelakun et al. (2016) reported the higher protein content (23.27%) of *P. stratiotes* compared to the present study. The protein content of the *E. fluctuans* in the present study was the 26.36% and it was higher than earlier study (Datta et al., 2019).

Significantly, higher lipid contents (>7%) were observed in *L. minor*, *S. polyrhiza* and *I. aquatica* in the present study. These values were higher compared to the findings (lipid content 5%) of Appenroth et al. (2017). The lipid content of *A. microphylla* and *A. pinnata* were 6.09 and 5.47%, respectively in the present study. Lower lipid contents were reported in *A. microphylla* (3.50%) and *A. pinnata* (3.27%) in earlier study (Dutta et al., 2011; Kumari et al., 2018). The lipid content of the *P. stratioetes* (5.99%) was higher in the current study compared to the earlier (2.90%) study (Mandal and Ghosh, 2018). The lipid contents of *S. molesta* (5.31%) and *S. natans* (4.77%) in the present study were lower than the earlier study of Kalita et al. (2007) with *Salvinia cuculata* (7.0%). The lipid content of the *E. fluctuans* (4.53%) in the current study was higher than the previous findings (1.10%) of Datta et al. (2019). The lipid content of the *W. globosa* (6.48%) in the current study was similar to the earlier finding (6.5%) of Appenroth et al. (2017) and it was higher compared to lipid content found in *Wolffia arrhiza* (4.63%) (Chareontesprasit and Jiwyam, 2001).

5.1.2. Amino acid composition

Amino acids are important component for living organisms. They are playing significant role in various physiological function, *viz*., protein synthesis, stimulating immune system, enhancing antioxidant responses etc. (Li et al., 2009). Balanced dietary amino acids content is very important in teleost. Each amino acid plays specific role in fish body (Wilson, 2002). In the present study, among twelve macrophytes, total essential amino acids were significantly higher in *L. minor* (20.25%) and *S. polyrhiza* (19.06%). Appenroth et al. (2017) reported that leucine and arginine were the most abundant amino acids among essential amino acids in duckweed *L. minor* and *S. polyrhiza*. Similar results were found in the present study. The lysine, methionine, cysteine, threonine, arginine, isoleucine, leucine, phenylalanine, tyrosine, glycine, serine and valine contents of *A. pinnata* were higher in the present study compared to the earlier finding (Alalade et al., 2006; Bhaskaran and Kannapan, 2015), whereas, the tryptophan content was lowered in present study. The non-essential amino acids glutamate and aspartate were most abundant amino acids and these two amino acids were found in all macrophytes in the present study. Similar results were reported in the earlier study (Shiomi and Kitoh, 2001; Appenroth et al., 2017). They reported that glutamate and aspartate were the most abundant amino acids found in macrophytes *A. filiculoides, S. polyrhiza, Landoltia punctata, L. minor, L. gibba, Wolffiella hyaline and W. microscopica.*

5.1.3. Vitamin contents

Thiamine (vitamin B_1) is essential for energy production, carbohydrate metabolism and nerve cell function (Dutta et al., 2019). The thiamine contents of the macrophytes in the present study ranged from 5.42 mg/100 g (*E. fluctuans*) - 20.80 mg/100 g (*S. polyrhiza*). The thiamine (vitamin B1), pyridoxine (vitamin B6) and ascorbic acid (vitamin C) contents of *I. aquatica* in present study were 14.17, 58.35 and 38.10 mg/100 g (dry weight) respectively. The vitamin contents of the macrophytes in the present study were higher compared to the earlier study (Misra and Misra, 2014; Datta et al., 2019). The vitamin B_1 and vitamin B_6 contents of the *E. fluctuans* in present study were 5.42, 64.95 mg/100 g (dry weight) and these values were higher than the earlier study (Datta et al., 2019). In the present study, vitamin C content of *Azolla* spp. ranged from 81.93-83.68 mg/100g (dry weight), whereas in other species *A. filiculoides,* the vitamin C content was 19.19 mg/100 g, dry weight (Shiome and Kitoh, 2012).

5.2. The culture of *Spirodela polyrhiza*

On the basis of proximate compositions of twelve macrophytes cultured in organic manure, the *S. polyrhiza* was selected as suitable fish feed ingredients. The culture technique of *S. polyrhiza* was standardized using various manures and the role of water quality parameters was recorded. Water temperature and sunlight are major environmental factors that influence the growth of duckweed compared to the nutrient concentrations in the water (Hasan and Chakrabarti, 2009). In outdoor cemented tank culture, *S.*

polyrhiza was first harvested after 69 days of culture. The water temperature was < 15°C during this period of culture. The lowest light intensity was also recorded during this period. Water temperature was >16°C at the second phase of culture and only then duckweed grew well and were harvested. Higher light intensity was also recorded at the second phase compared to the first one. In a comparative study, growth performance of *S. polyrhiza* was recorded at two temperature ranges of 10-12 and 26-28°C (Song et al., 2006). It was found that cell growth, the synthesis, and absorption ability of duckweed decreased at low temperature compared to duckweed cultured at higher temperature. There was no change in frond number for 15 days at low temperature.

In the present study, the relative growth rate (RGR) of greater duckweed was low during the first phase of outdoor cemented tank culture and then increased regardless of treatments. In manure 3 (OM+IF), RGR reduced in fifth harvest of phase three. Among the three manures, significantly higher production rate was found with manure 1 (OM) compared to the inorganic fertilizers, manure 2 (IF) and manure 3 (OM+IF). Therefore, organic manures (OM) were applied in pond culture of greater duckweed. In contrast to the cemented tank culture, RGR value was maximum at first harvest in pond culture of greater duckweed. The average RGR value was higher in pond compared to cemented tank production. Several studies showed similar the relative growth rate of duckweed, the RGR ranged from 0.03-0.3g/g/day in different culture conditions (Rejmankova, 1975; Porath et al., 1979; Oron 1994; Guy et al., 1990). The production rate of greater duckweed was

0.08±0.02 fronds/day in laboratory conditions (Lemon et al., 2001). Higher temperature also resulted in enhanced growth rate in ponds in the present study. In Bangladesh, highest growth of *S. polyrhiza* was found at 22.2-22.5°C in pond (Khondker et al., 1993). Though *S. polyrhiza* survived at low temperature, (10-12°C); it could not grow well at this temperature (Song et al., 2006). The duckweed exposed to oxidative damage at low temperature. Appenroth (2002) suggested that 15°C temperature (combined with 30 µM phosphate level) was the dominant turion formation inducing factor. In laboratory axenic culture, *S. polyrhiza* were exposed at 100 µmol/m^2/s white light (Appenroth et al., 2017). In the present study, good growth of *S. polyrhiza* was found at light intensity of 105-151µmol photons/m^2/s in outdoor conditions.

In Bangladesh and India, a pH ranged from 6.5-7.5 (Islam and Khondkar, 1991) and 6.8-8.5 (Kaul and Bakaya, 1976; Gopal and Chamanlal, 1991) was found to be optimum for the production of greater duckweed. In the present study, pH ranged from 6.98-7.86 and 7.76-8.30 in outdoor cemented tanks and pond culture systems, respectively. There was no direct effect of dissolved oxygen on the production of greater duckweed as highest production was recorded in manure 1 (OM) with minimum dissolved oxygen level in outdoor cemented tank culture. Leng et al. (1995) suggested that maintenance of low dissolved oxygen with 6-7 pH should be the strategy for duckweed pond management.

It was found that the root length was shorter in *S. polyrhiza* that grown at low temperature compared to the plants grown at a higher temperature. *S. polyrhiza* with shorter root length were inefficient in absorbing nitrogen, phosphorus and other nutrients from water (Reddy and DeBusk, 1985). In outdoor cemented tank culture, highest ammonia level was recorded in manure 1 (OM) at first phase and no production was recorded during this period. The ammonia level gradually reduced in the second and third phases and the growth of duckweed enhanced. Even with the same manure system manure 1 (OM), lower levels of ammonia were found in ponds compared to tanks. Absorption of nutrients helped in the higher production of duckweeds in ponds. The fluctuation of pH between 7.4 and 9.0 enhanced the ammonia toxicity in laboratory culture (Caicedo et al., 2000). In tank culture of duckweed, highest RGR was found in the second phase at 15.25 mg/l ammonia concentration in manure 1 (OM). It is also interesting to see that in tank culture, poor growth of duckweeds in manure (OM+IF) during fifth harvest might be related to the low ammonia level in the culture tank. Leng et al. (1995) suggested that 7-12 mg N/l was optimum to maintain a protein content of 40% in duckweed. A TKN content of 20-30 mg/l was required for optimum growth (Culley et al., 1981) and maintenance of high protein content. In the present study, the ammonia level in the pond water also helped in the proper growth of the duckweed. Nitrification rate was slower in manure 1 (OM) compared to the other two treatments in the tank culture of duckweed. In manure 1 (OM), nitrate level was significantly higher in the second phase compared to the other phases.

Phosphorus is a major limiting nutrient, although it is required in lesser amount. In the present study, the phosphate levels in manure 1 (OM) helped in the production of duckweed in both tanks and ponds. The optimum conductivity for maximum production of *S. polyrrhiza* was 650-1000 µS/cm (Gopal and Chamanlal, 1991). *S. polyrrhiza* completely disappeared in May due to reduced conductivity and alkalinity (Khondker et al., 1993). In the tank culture, the growth of greater duckweed was less in the first phase and the conductivity was minimum during this phase regardless of manures applied. Then conductivity increased with higher production of duckweed. In pond culture, the conductivity was always >1000 µS/cm.

In ponds, the production of greater duckweed was encouraging, 2020±150 kg ha/month (24 ton/ha/yr) on dry matter basis. Literature showed a wide variation in the production of duckweed, with various climatic conditions and nutrient availability mostly being responsible for this variation. Edwards et al. (1990) reported ~20 ton/ha/year (DM) production of *S. polyrhiza* during 1-3 months culture period; the yield decreased (~9 ton/ha/year) when the duration of culture period increased to 6 months. The yield of greater duckweeds in domestic wastewater (Reddy and DeBusk, 1985), sewage effluent (Sutton and Ornes, 1975) and nutrient non-limited water (Reddy and DeBusk, 1985) were 17-32, 14.6 and 11.3 ton/ha/yr, respectively. Based on the available data, an average harvest of 10-20 tons duckweed/ha/year could be expected under optimum environmental conditions (Hasan and Chakrabarti, 2009). In a similar study, *L. minor* was produced in ponds using organic manures. The initial amount of duckweed introduced for culture also influenced production. A

seeding rate of 60 kg/m^2 for *S. polyrhiza* was recommended (DWRP, 1998). In the pond culture, only 1 kg/pond (200 m2) *S. polyrhiza* was introduced in the present study.

5.3. Evaluation of physiological responses of cultured fishes

5.3.1. Performance of fishes

The effect of dietary inclusion of greater duckweed at four different levels (replacing soybean meal) on the performance of *Labeo rohita* and *Cyprinus carpio* was recorded in this study. The survival rate of fishes was not affected with the inclusion of greater duckweed in the diets. Fish were survived cent percent in both the experiments. The earlier study showed mixed results. The inclusion of greater duckweed more than 20% in diet resulted into mortality of *O. niloticus* (Fasakin et al., 1999, 2001), supplementation of *L. minor* (20%) in diets affected the survival rate of *C. carpio* (Yılmaz et al., 2004). *O. niloticus* (16.3 g) was fed with *A. filiculoides* supplemented feeds (10 - 50%) and 7-10% mortality was obtained (Abou et al*.,* 2013). In a 4 weeks of feeding trial, rainbow trout *Oncorhynchus mykiss* (0.28 g) were fed with *S. polyrhiza* supplemented diet at two levels of 6.25 and 12.5%. The mortality of *O. mykiss* increased with increasing dietary of duckweed level (Stadtlander et al*.,* 2019). *O. mykiss* and *O niloticus* have different feeding habits - carnivore and herbivore. Even the developmental stage (size) of the fish, their health status and temperature of culture system play significant role in the performance of fish. Like in various studies also showed a positive impact of duckweed on the survival of fishes *L. rohita* (Bairagi et al*.,* 2002; Pradhan et al*.,* 2019), *C.*

carpio (Yilmaz et al., 2004; Velichkova et al., 2020), *O. niloticus* (Fasakin et al*.,* 2001; Tavares et al., 2008). El-Shafai et al. (2004) reported that the inclusion of duckweed in the feed of *O. niloticus* improved the survival rate of fish. The incorporation canola meal at 50% level in the diet was not affecting the survival rate of *O. niloticus* (Iqbal et al., 2021).

The incorporation of greater duckweeds at different levels enhanced the final weight of rohu and common car*p*. The highest growth performance was observed in duckweed based diet fed fishes in the present study. Food was efficiently utilized in these treatments as minimum FCR was recorded. Similar trends were also observed in rohu fed 30% fermented *L. polyrhiza* (Bairagi et al*.,* 2002), 20% bio-processed *Pistia* leaves (Mandal and Ghosh, 2019) and *C carpio* fed up to 30% of *L. minuta* in their diets (Sirakov and Velichkova, 2018; Velichkova et al., 2020). Earlier study showed that the supplementation of 25% *A. microphylla* and *A. pinnata* mixture in diet enhanced the growth performance and specific growth rate of *L. rohita* (Datta, 2011). Feeding of raw *W. globosa* to *L. rohita* fry showed better growth compared to the formulated diet fed fish (Pradhan et al*.,* 2019). In *L. minor* supplemented diet fed *C. carpio* and *O. niloticus*, similar trends of growth performance, SGR, and FCR were found (El-Shafai et al., 2004; Yılmaz et al., 2004). The inclusion of *L. polyrhiza* in the diets of mrigal *Cirrhinus mrigala* and rohu improved the weight gain, SGR, and FCR (Bairagi et al., 2002; Ghosh and Ray, 2014). Inclusion of fermented *L. minor* at 2.5% level and canola meal at 50% level increased the growth of *O. niloticus* (Herawati et al., 2020; Iqbal et al., 2021). In this study, the SGR of common carp ranged from 2.01 to 2.93%.

Similar results were reported in earlier study like, in *L. minor* supplemented diet fed common carp, SGR ranged from 1.96-2.26% (Yılmaz et al., 2004), and in soy protein concentrate (SPC)-incorporated diet fed common carp, SGR ranged 2.01-2.93% (Zhu et al., 2020). The feeding of common carp with diets containing fishmeal and ultra-micro-ground mixed plant proteins (uPP)-based diets resulted in 540-560% growth of fish after 112 days of culture (Xie et al., 2021).

5.3.2. Digestive enzyme activities of fishes

The metabolism and digestion of meals are significantly influenced by the activities of digestive enzymes (Dabrowski and Glogowski, 1977; Perez-Jimenez et al., 2009). The activities of the appropriate digestive enzymes such as amylase, protease, and lipase are positively connected with the digestibility of nutrients like carbohydrate, protein and lipid in fish (Cho and Slinger, 1979; De et al., 2015). The study of digestive enzyme activities in five different diets fed fishes explained the reason of efficient utilization of consumed diet in plant based. Total protease and trypsin activities were maximum in *S. polyrhiza* supplemented diets fed fish; considerable amylase and lipase activities were also found in control diets fed fish. The digestive enzymes, namely, protease, lipase, and amylase played a significant role in digestion and absorption of nutrient (Zhou et al., 2010). An earlier study showed that, addition of 25% *Ipomoea aquatica* leaf meal (fermented with microorganisms) increased amylase activity of *L. rohita* (Ali and Kaviraj, 2018). In present study, the activities of digestive enzyme amylase and

protease increased in duckweed supplemented diets fed rohu and common carp. Similarly, inclusion of duckweed in the diets of grass carp *Ctenopharyngodon idella* and *O. niloticus* enhanced digestive enzyme activities like amylase, protease, trypsin, and chymotrypsin (Aslam et al., 2018; Zhao et al., 2020). The inclusion of the duckweed *L. minor* in the diets of mrigal *Cirrhinus cirrhosus* enhanced the population of amylolytic and cellulolytic bacteria in gastrointestinal tract (Ghosh and Ray, 2014). An *in vitro* digestibility study of duckweed showed the high degree of hydrolysis (DH%) of duckweed with the digestive juices of rohu and common carp (Sharma et al., 2016). Fish fed with different diets are able to adjust the activity of their digestive enzymes (Shiping and Zhao, 2005).

5.3.3. Biochemical composition of fish

The proximate composition study showed that the inclusion (10-20%) of greater duckweeds enhanced the crude protein contents of *L. rohita* and *C. carpio* in this study. The crude lipid and ash contents of fishes increased in a graded manner with the enhanced inclusion of greater duckweeds in the diet. An earlier study showed that supplementation of duckweeds improved the crude protein and crude lipid contents in fish (El-Shafai et al., 2004; Yılmaz et al., 2004; Fasakin, 2008; Abou et al., 2011). Aslam et al. (2021) reported significantly higher crude protein contents in *L. minor* incorporated diets fed grass carp *C. idella* and silver carp *Hypophthalmichthys molitrix* compared with the soybean-supplemented diet fed fishes. The inclusion of duckweeds increased the ash content in fish (Fasakin et al., 1999; El-Shafai et al., 2004;

Fasakin, 2008). This indicated that greater duckweed supplemented diets fulfilled the nutritional requirements of rohu and common carp. The amino acid profile of greater duckweed is comparable with soybean meal. Feeding with greater duckweed supplemented diets improved the essential and non-essential amino acid contents in *L. rohita* and *C. carpio*. The supplementation of fermented *L. minor* in the diet enhanced the lysine content in tilapia (Herawati et al., 2020).

In the present study, fatty acid composition of fish was influenced by the supplementation of greater duckweed. Highest and lowest SFA contents were found in SP5 and SP20 diets fed common carp, respectively. MUFA content showed an inverse relationship with the increased inclusion of greater duckweeds in diet of *C. carpio*. A direct relationship was found between the amount of greater duckweeds in the diet and n-6 PUFA and n-3 PUFA contents in common carp. Inclusion of greater duckweed in the diet enhanced the ALA, DHA, and EPA contents in common carp. The duckweeds are a rich source of fatty acids (Appenroth et al., 2017). The feeding of *A. filiculoides* enhanced the total n-3 PUFA (especially EPA and DHA) content (Abou et al., 2011), and fermented *L. minor* enhanced LA (Herawati et al., 2020) in *O. niloticus*. Similarly, the contents of EPA and DHA in *C. carpio* increased linearly with increasing greater duckweed level in the diet.

5.3.4. Expression of genes involved in the biosynthesis of fatty acids

In this study, the expression levels of key genes involved in the biosynthesis of fatty acids like *fads2d6*, *elovl2*, *elovl5,* and *fas* were evaluated in *C. carpio*.

Up-regulation of all these genes were found in fish fed greater duckweed supplemented diets compared with control diet fed fish. This might be due to the presence of LOA and ALA in the experimental diets. Earlier study showed that the higher contents of LA and ALA upregulated the expression of desaturases/elongases (Tocher et al., 2004; Turchini et al., 2006; Francis et al., 2007; Li et al., 2008). However, an excess of ALA in diet can inhibit the transcription of *fads2d6* gene (Bell et al., 1993). In this study, among the fish fed the experimental diets, the highest expression levels of genes were recorded in the SP5 treatment, and then the expression gradually decreased. The ALA content increased with increasing inclusion of greater duckweed in diet. EPA and DHA contents increased with decreasing expression of genes *elovl2, elovl5,* and *fads2d6*. Similar results were found in *C. carpio* and rainbow trout *O. mykiss* where the expressions of desaturases and elongases were higher in fish with lower contents of EPA and DHA (Ren et al., 2012; Lazzarotto et al., 2018).

Chapter 6

Summary and Conclusion

SUMMARY AND CONCLUSIONS

Aquaculture is the most diverse food producing sector in the world. It contributes immensely in human nutrition, food security and employment generation. Globally, around 600 million people are engaged in fisheries and aquaculture related activities and most of them are from Asian countries. Nutrition plays a central role in sustainable aquaculture. In intensive aquaculture, more than 60% of the production cost is due to fishmeal. It is used as a principle protein source. Currently, around 60% of the global supply of fishmeal is consumed by aquatic animals as feed. Therefore, it is essential to replace fishmeal with non-conventional alternative ingredients. Indian major carp rohu *Labeo rohita* and exotic common carp *Cyprinus carpio* are extensively cultured freshwater species in India.

In the present study twelve freshwater macrophytes *viz*., mosquito fern *Azolla microphylla,* water velvet *Azolla pinnata,* buffalo spinach *Enhydra fluctuans,* water thyme *Hydrilla verticillata,* water spinach *Ipomea aquatica,* duckweed *Lemna minor,* water clover *Marsilea quadrifolia,* water lettuce *Pistia stratiotes,* giant salvinia *Salvinia molesta,* floating fern *Salvinia natans,* greater duckweed *Spirodela polyrhiza* and Asian watermeal *Wolffia globosa* were collected from different water bodies. All macrophytes were cultured in outdoor cemented tanks (1.2 × 0.35 m) and tanks were filled with clean tap water (30 cm water depth). The combination of organic manures, *viz.* cattle manure, poultry droppings and mustard oil cake (1:1:1) was used at the rate of 1.052 kg/m^3 for the culture of macrophytes (Srivastav et al., 2006). After

culture, macrophytes were harvested, washed thrice with tap water and twice with distilled water, air dried, powdered and stored in -20^0 C till nutritional composition analysis.

The moisture content was significantly (P<0.05) higher in *E. fluctuans* compared to other macrophytes. The ash content was significantly (P<0.05) higher in *H. vertcillata* compared to the other macrophytes. The crude protein and crude lipid contents were significantly (P<0.05) higher in *L. minor* and *S. polyrhiza* compared to the other macrophytes. The carbohydrate content was significantly (P<0.05) higher in *P. stratiotes* compared to other macrophytes. All essential amino acids *viz.,* arginine, histidine, Isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine contents were significantly (P<0.05) higher in *S. polyrhiza* and *L. minor* compared to other macrophytes. Similarly, the non-essential amino acids alanine, aspartate, cysteine, glutamate, glycine, proline, serine, tyrosine, phosphoserine, taurine and phospho ethanol amine contents were significantly higher (P<0.05) in *S. polyrhiza* and *L. minor*. Amino acid phosphoserine was absent in *I. aquatica* whereas, taurine was absent in *S. natans*

The water soluble vitamins thiamine (B1) and riboflavin (B2) contents were significantly (P<0.05) higher in *S. polyrhiza* compared to other macrophytes. The pyridoxine (B6) and cobalamin (B12) content were significantly (P<0.05) higher in *I. aquatica* compared to the other macrophytes. The ascorbic acid (vitamin C) level was significantly (P<0.05) higher in *M. quadrifolia* compared

to other macrophytes. All the water soluble vitamins were present in *S. polyrhiza*.

On the basis of proximate and amino acid composition, vitamin content and yields, *S. polyrhiza* was selected as suitable fish feed ingredient for fish feed formulation. So, it was essential to standardize the culture technique of selected macrophytes. The culture technique of the *S. polyrhiza* was first standardized in outdoor tanks and then the technique was adopted in the ponds. Three different combinations of manure were used for the culture of the *S. polyrhiza* in outdoor cemented tanks. These manures were: manure 1 (organic manure, OM) containing cattle manure, poultry dropping and mustard oil cake in the ratio of (1:1:1) and the dose of manures were 1.052kg/m^3; manure 2 (Inorganic fertilizers, IF) containing urea, potash and triple super phosphate at the rate of 15, 3, 3 kg/ha/day, respectively and manure 3 (combination of cattle manure and inorganic fertilizers, OM+IF), containing cattle manure and inorganic fertilizers urea, potash and triple super phosphate at the rate of 750, 7.5, 1.5, 1.5 kg/ha/month.

Fresh duckweeds were introduced after 5 days of manure application in each culture units. Duckweeds were collected when the surface area of the culture units were covered with the plants. In each harvesting, the macrophytes were harvested from half of the tank and remaining plants were allowed to grow; plants were harvested completely at the time of final harvesting. One fourth of the initial dose of manure was applied in the duckweed culture unit at ten days interval. The relative growth rate (RGR), production and protein content were

significantly higher (P<0.05) in plants cultured in organic manure (OM) compared to other treatments. Therefore, organic manure (OM) was selected as suitable manure for mass production *of S. polyrhiza* in ponds. In the ponds, the average relative growth rate (RGR) of duckweed was 0.22±0.13 g/g/day. The production rate of duckweed in pond was 2020±150 kg/ha/month (dry weight).

The biochemical composition study showed that *S. polyrhiza* was a rich source of essential (37.40%) and non-essential (62.60%) amino acids. The leucine, isoleucine, and valine constituted 51.4% of the total essential amino acids. Glutamic acid content was 25.96% of total non-essential amino acids. The fatty acid composition study showed that *L. minor* was a rich source of polyunsaturated fatty acids (PUFA). The PUFA content was around 47-53% of total fatty acids, largely α-linolenic acid (ALA, 18:3*n*-3) around 38-39% and linoleic acid (LA, 18:2*n*-6) around 11-13%.

The pelleted diets were prepared using *S. polyrhiza* as a protein source. The diets were formulated with the help of Winfeed 2.8 software package (WinFeed UK Limited, Cambridge, United Kingdom). All diets were prepared with Twin-Screw Extruder. The extrusion technique helped to reduce the anti-nutritional factors present in the plant ingredients. It improved the digestibility of the prepared fish feed. Five experimental diets were formulated in which soybean meal simultaneously replaced by duckweed. The control feed (SP0) contained soybean meal as the only primary source of protein, while in the four experimental feeds, greater duckweed was incorporated at the levels of

5% (SP5), 10% (SP10), 15% (SP15), and 20% (SP20) of total feed. Incorporation of greater duckweed replaced the soybean meal at the level of 5%, 10%, 15% and 20% in the diets SP5, SP10, SP15 and SP20, respectively.

The proximate composition study of the prepared diets showed that, there was no significant (P>0.05) difference in moisture, protein, lipid, and carbohydrate contents of all prepared diets. Although, the ash content increased as the plant level increased in diets. The ash content was significantly (P<0.05) higher in diet SP20 compared to the other diets. The essential amino acid content was significantly (P<0.05) higher in diets containing *S. polyrhiza* compared to the soybean based diet SP0. There was no significant (P>0.05) difference in total saturated fatty acids, monounsaturated fatty acid and total n-6 polyunsaturated in all prepared diets. The n-3 polyunsaturated fatty acid ALA (α-linolinic acid) content increased with increasing level of plant in the diets. Significantly (P<0.05), higher ALA content was found in diet SP20.

Two experiments were conducted to evaluate the effect of plant-based diets on freshwater fishes. First experiment was performed with rohu *Labeo rohita,* whereas the second experiment was performed with common carp *Cyprinus carpio*. The duration of each feeding trail was 60 days and after feeding trail the performance of the fish was evaluated. Significantly (P<0.05) higher final bodyweight and specific growth rates were found in SP5, SP10 and SP15 diets fed rohu compared to fish fed other diets. The feed conversion ratio

showed the opposite trend. The amylase, protease, chymotrypsin and lipase activities were significantly (P<0.05) higher in rohu fed with diet SP5 compared to the other diets fed rohu. This treatment was followed by SP10 and SP15 diets fed rohu. The trypsin activity was significantly (P<0.05) higher in fish fed with diet SP10 compared to other diets fed fish. There was no significant (P>0.05) difference in moisture, protein and lipid contents of five different diets fed rohu. Whereas, the ash contents was significantly (P<0.05) higher in rohu fed with diet SP20 compared to other diets fed fish. The compositions of essential amino acids in fish flesh significantly (P<0.05) increased with the supplementation of *S. polyrhiza* in the diets of rohu.

The average final weight and specific growth rate of common carp increased with increasing *S. polyrhiza* content in the diets. Significantly (P<0.05) higher final weight and specific growth rate were observed in common carp fed with diet SP20 compared to other diets fed fish. Whereas, the lower FCR was observed in fish fed with diet SP20. Significantly (P<0.05), higher amylase, protease and trypsin activities were found in fish fed with diet SP20 compared to the other diets fed common carp. Significantly (P<0.05), higher chymotrypsin and lipase activities were observed in common carp fed with diet SP5 and SP15, respectively.

The moisture content was significantly (P<0.05) higher in common carp fed with control diet SP0 compared to the other diets fed fish. Whereas, the protein, lipid, ash content were significantly (P<0.05) higher in common carp fed with diet SP20 compared to the diets fed fish. The essential amino acid

composition of common carp was influenced by the addition of *S. polyrhiza* in the diets. The essential amino acid composition of common carp was enhanced by the incorporation of *S. polyrhiza* in the diets. The supplementation of *S. polyrhiza* in the diet of common carp influenced the fatty acid composition of fish. Total saturated fatty acid and total monounsaturated fatty acids contents were significantly (P<0.05) higher in common carp fed with diet SP5 and SP0, respectively. Total n-6 PUFA contents were increased with increasing level of plant in the diets. Significantly (P<0.05) higher content of n-6 PUFA was found in common carp fed with diet SP20. The total n-3 PUFA content was significantly (P<0.05) higher in common carp fed with diet SP20 compared to the other diets fed fish. The EPA and DHA contents were significantly (P<0.05) higher in common carp fed with diet SP15 and SP20, respectively compared to the other diets fed fish. The expression of gene delta 6 desaturase (*fads2d6*), elongation of very long chain fatty acid proteins type 2 (*elovl2*) and type 5 (*elovl5*) and fatty acid synthase (*fas*) were evaluated in fish. The expression of gene *fads2d6* and *fas* were significantly higher in common carp fed with diet SP5 compared to others. This treatment was followed by SP10, SP15 and SP20. The expression of gene *elovl2* and *elovl5* were significantly higher in common carp fed with diets SP5 and SP10.

The present study shows that duckweed *S. polyrhiza* is a potential ingredient to replace fishmeal and soybean meal in the diets of freshwater fishes. The application of organic manures helps in the sustainable production of quality duckweeds, rich in protein and unsaturated fatty acids. The extrusion process

improves the digestibility of the prepared diets and thereby, bioavailability of the nutrients. The inclusion of duckweed in the diets of freshwater fishes enhances the EPA and DHA levels of fishes. Therefore, the knowledge generated from the present study will help in the formulation of cost-effective and quality fish feed and thereby, production of nutrient rich fish for consumers.

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List of Publications

LIST OF PUBLICATION

Research Publications

1. Kumar, G., Sharma, J. G., Goswami, R. K., **Shrivastav, A. K.,** Tocher, D. R., Kumar, N., Chakrabarti, R. (2022). Freshwater macrophytes: Potential source of minerals and fatty acids for fish, poultry and livestock. ***Frontiers in Nutrition*** (*Frontiers Media S.A.*). 9:869425. (Impact factor: 6.590)

2. Goswami, R.K., Sharma, J., **Shrivastav, A.K.,** Kumar, G., Glencross, B.D., Tocher, D.R. and Chakrabarti, R. (2022). Effect of *Lemna minor* supplemented diets on growth, digestive physiology and expression of fatty acids biosynthesis genes of *Cyprinus carpio*. ***Scientific reports*** **(Nature Portfolio**), 12(1):1-13. (Impact factor: 4.996)

3. **Shrivastav, A.K.,** Kumar, G., Mittal, P., Tocher, D.R., Glencross, B.D., Chakrabarti, R. and Sharma, J. (2022). Effect of Greater Duckweed *Spirodela polyrhiza* Supplemented Feed on Growth Performance, Digestive Enzymes, Amino and Fatty Acid Profiles, and Expression of Genes Involved in Fatty Acid Biosynthesis of Juvenile Common Carp *Cyprinus carpio*. ***Frontiers in Marine Science*** **(Frontiers)**, p.10. (Impact factor: 5.247)

4. Kumar, N., Sharma, J. G., Kumar, G., **Shrivastav, A. K.,** Tiwari, N., Begam, A., Chakrabarti, R. (2021). Evaluation of nutritional value of prickly chaff flower (*Achyranthes aspera)* as fish feed ingredient. ***Indian Journal of Animal Sciences*** (*ICAR*) 91 (3):239-244. (Impact factor: 0.500)

5. Kumar, G., Sharma, J. G., Goswami, R. K., **Shrivastav, A. K.,** Kumar, N**.,** Chandra, S., Chakrabarti, R. (2021). The study of effect of vitamin C and *Achyranthes aspera* seeds enriched diets on the growth, biochemical composition, digestive enzyme activities and expressions of genes involved in the biosynthesis of fatty acids in Snow trout *Schizothorax richardsonii* (Gray, 1832). ***Journal of Applied Aquaculture*** (*Taylor & Francis Online*).

6. Goswami, R.K., **Shrivastav, A.K**., Sharma, J.G., Tocher, D.R., Chakrabarti, R. (2020). Growth and digestive enzyme activities of rohu *Labeo rohita* fed

diets containing macrophytes and almond oil-cake. ***Animal Feed Science and Technology*** **(Elsevier**), 263:114456. (Impact factor: 3.313)

7. Ahluwalia, S., Bidlan, R., **Shrivastav, A.K**., Goswami, R.K., Singh, P., Sharma, J.G. (2020). Optimization of protein extraction from detoxified Jatropha seed cake using response surface methodology and amino acid analysis. ***International Journal of Environmental Science and Technology*** **(Springer),** 17(2):1087-1100. (Impact factor: 3.519)

8. Sharma, J., Clark, W.D., **Shrivastav, A.K**., Goswami, R.K., Tocher, D.R. and Chakrabarti, R. (2019). Production potential of greater duckweed *Spirodela polyrhiza* (L. Schleiden) and its biochemical composition evaluation. ***Aquaculture*** **(Elsevier)**, 513:734419. (Impact factor: 5.135)

9. Chakrabarti, R., Clark, W.D., Sharma, J.G., Goswami, R.K., **Shrivastav, A.K**., Tocher, D.R. (2018). Mass production of *Lemna minor* and its amino acid and fatty acid profiles**.** ***Frontiers in chemistry*** **(Frontiers)**, 6:479. (Impact factor: 5.545)

Conferences Presentations

- **Shrivastav, A.K.,** Sharma, J.G., Chakrabarti, R. (2018). Effect of different organic and inorganic manures on growth, yield and nutritional composition of *Spirodela polyrhiza*. *8th International Science Congress.* Maharishi Markandeshwar University, Ambala, Haryana, India.

- **Shrivastav, A.K.,** Sharma J. G, Chakrabarti, R. (2021). Impact of *Spirodela polyrhiza* containing diets on growth, physiology and nutritional composition of common carp *Cyprinus carpio*. *International Colloquium on Regulatory Mechanisms Underlying Behaviour, Physiology and Development***.** Department of Zoology, University of Delhi, Delhi, India

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ORIGINAL RESEARCH
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Freshwater Macrophytes: A Potential Source of Minerals and Fatty Acids for Fish, Poultry, and Livestock

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The freshwater macrophytes are abundant in tropical and subtropical climates. These macrophytes may be used as feed ingredients for fish and other animals. The nutritional value of twelve freshwater-cultured macrophytes was evaluated in the present study. Significantly higher crude protein (36.94–36.65%) and lipid (8.13–7.62%) were found in *Lemna minor* and *Spirodela polyrhiza*; ash content was significantly higher in *Hydrilla verticillata*, *Wolffia globosa,* and *Pistia stratiotes* (20.69–21.00%) compared with others. The highest levels of sodium, magnesium, chromium, and iron levels were recorded in *P. stratiotes*. *H. verticillata* was a rich source of copper, manganese, cobalt, and zinc; the contents of calcium, magnesium, strontium, and nickel were highest in *S. polyrhiza*. Selenium and potassium contents were higher in *Salvinia natans* and *W. globosa*, respectively. The n-6 and n-3 polyunsaturated fatty acids (PUFAs) contents were significantly higher in *W. globosa* and *Ipomoea aquatica*, respectively compared with others. Linoleic and α-linolenic acids were dominant n-6 and n-3 PUFAs. The highest value (4.04) of n-3/n-6 was found in *I. aquatica.* The ratio ranged from 0.61 to 2.46 in other macrophytes. This study reveals that macrophytes are rich sources of minerals, n-6 and n-3 PUFAs.

Keywords: alpha-linolenic acid, linoleic acid, n-3 polyunsaturated fatty acids, sodium, iron, freshwater macrophytes

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INTRODUCTION

Freshwater macrophytes, the fastest growing aquatic plants, are abundant in tropical and subtropical countries. They grow profusely in nutrient-rich water. These macrophytes are broadly classified into four groups based on their occurrence in the water body: the surface floating (e. g. *Azolla* spp.), submerged (e. g. *Hydrilla* spp.), emergent (e. g. *Potamogeton* spp.), and marginal (e. g. *Ipomoea* spp.). The nutritional value of freshwater macrophytes has been recognized globally. The unchecked propagation of freshwater macrophytes creates problems in many water bodies. The judicial exploitation of these nutrient-rich plants may open a new avenue from a nutritional view point for humans and animals. The leaf protein extracted from freshwater macrophytes may be used for human or non-ruminant animals (1). Macrophytes are a rich source of protein, lipid, amino acids, fatty acids, and minerals (2). The amino acid and fatty acid profiles of duckweeds *Lemna minor* and *Spirodela polyrhiza* have been documented recently (3, 4). The mineral composition of macrophytes is different from the usual terrestrial vegetation. Calcium

(Ca), iron (Fe), and manganese (Mn) contents are higher in aquatic plants compared with the terrestrial ones (1). Minerals are important catalysts for various biochemical reactions. These are essential components for metabolism, growth, and development and help the animals to cope with the variable environmental conditions (5). There is an optimum dose for each mineral. Low/high concentrations may affect the physiology of the organisms. Toxic minerals like arsenic (As), mercury (Hg), antimony (Sb), cadmium (Cd) etc., are required by the body in little amounts, whereas excess levels of useful minerals like, sodium (Na), potassium (K), magnesium (Mg), Ca, Fe etc., may be harmful (5). Dietary inclusions of polyunsaturated fatty acids (PUFAs) have several health benefits for humans and other animals. The study of the profiles of fatty acids of feed ingredients ensures the quality of diets. Fish are unable to synthesize two essential fatty acids like n-6 (derived from linoleic acid, LA) and n-3 (derived from alpha-linolenic acid, ALA). So these fatty acids should be supplied to the diets of fishes (6).

The evaluation of minerals and fatty acids' compositions of aquatic macrophytes is essential for their selection as potential feed ingredients for fish and other animals. Some of the commonly occurring freshwater macrophytes are: *Azolla microphylla, A. pinnata*, *Enhydra fluctuans*, *Hydrilla verticillata*, *Ipomoea aquatica*, *Lemna minor*, *Marsilea quadrifolia*, *Pistia stratiotes*, *Salvinia molesta*, *S. natans*, *Spirodela polyrhiza,* and *Wolffia globosa*. These macrophytes are distributed throughout the temperate, sub-tropical, and tropical regions of the world. Some of these macrophytes like, *E. fluctuans*, *I. aquatica,* and *M. quadrifolia,* are consumed as vegetables by humans in India and Bangladesh (7), and *W. arrhiza* has been consumed in Thailand (8). Most of these macrophytes, except *H. verticillata* (submerged plant), *M. quadrifolia,* and *E. fluctuans* (marginal plants) are surface floating macrophytes. All these macrophytes propagate through vegetative reproduction. Mosquito fern *Azolla* spp. (Azollaceae) are heterosporous free-floating ferns. It lives symbiotically with nitrogen-fixing blue-green algae *Anabaena azollae*. Watercress *Enhydra fluctuans* (Asteraceae) is a hydrophytic plant and it grows in canals and marshy places. Waterthyme *Hydrilla verticillata* (Hydrocharitaceae) is a submerged, rooted aquatic plant. It can grow in water up to a depth of 6 m, and in transparent water it can survive up to a depth of 12 m. The water spinach *Ipomoea aquatica* (Convolvulaceae) with hollow roots floats in water easily. Three members of the family Lemnaceae, namely *Lemna* spp., *Spirodela* spp., and *Wolffia* spp. are known as duckweeds. The plant consists of a single leaf or frond with one or more roots. Water clover *Marsilea quadrifolia* (Marsileaceae) is a deciduous, aquatic fern. Each green and thin stalk rises from the rhizome to the water surface; it contains a single shamrock-like leaf with four leaflets. Water cabbage *Pistia stratiotes* (Araceae) is a perennial monocotyledon with thick, soft, and light green leaves that form a rosette. It floats on the surface of the water and roots are hanging beneath the leaves. The short stolon connects both the mother and daughter plants. Water fern *Salvinia* spp. (Salviniaceae) is a perennial free-floating macrophyte. During the period of high growth, leaf size decreases and both leaves and stems fold, doubling and layering to cover more of the water surface. The nutritional value of macrophytes in terms of proteins, lipids, ash etc. varies greatly (2). The culture medium influences the mineral contents of the macrophytes (8). The extracts of seven freshwater macrophytes show no cytotoxic and anti-proliferative effects on human cell lines (9). Therefore, macrophytes should be considered as useful feed ingredients. Production of macrophytes using a standard technique may help to maintain the nutritional value of the plant and also maximize the health benefits.

The aim of the present study is to evaluate the nutritional value, *viz.* proximate composition, minerals and fatty acids profiles of twelve cultured freshwater macrophytes. This study will help to evaluate the suitability of these macrophytes as feed ingredients for fish, poultry, and livestock.

MATERIALS AND METHODS

Culture of Macrophytes

Freshwater macrophytes were collected from water bodies of Delhi, Uttar Pradesh, and West Bengal and then identified. Macrophytes were cultured in outdoor cemented tanks (1.2 × 0.35 m) with clean dechlorinated tap water (3). A 10-cm layer of soil was used for the culture of *H. verticillata*, *M. quadrifolia,* and *E. fluctuans*. All other macrophytes were cultured without soil base. The depth of water was 30 cm in all culture tanks. A combination of organic manures *viz.* cattle manure, poultry droppings, and mustard oil cake was used (1:1:1) at the rate of 1.052 kg/m^3. All manures were decomposed for 5 days and then macrophytes were introduced individually in the outdoor cemented tanks. Three replicates were used for each macrophyte. For the steady supply of nutrients for the growth of macrophytes, the same combination of manures (at one-fourth dose of the initial one) was applied in the culture tanks. Manures were decomposed for 5 days in separate containers and then applied on day 6. This schedule was followed throughout the culture period. Culture tanks were monitored regularly and macrophytes were harvested when the whole surface of the tank was covered with plants. The freshly harvested macrophytes were washed twice with tap water and then with distilled water. After air drying, macrophytes were kept at 40°C for 3 h. Then the ground, sieved, and fine powders were kept in air-tight containers and stored in a refrigerator at 4°C for further assay.

Proximate Composition Analysis

The proximate composition of the macrophytes was analyzed (10). Three replicates were used for each assay. Moisture content was estimated after drying the sample at 105°C for 24 h. The dried samples were kept in a muffle furnace at 550°C for 8 h for the determination of ash contents. The crude protein contents were analyzed by measuring the nitrogen content (N x 6.25) with an automated micro-Kjeldhal apparatus (Pelican Instruments, Chennai, India). Crude lipid contents of the macrophytes were assayed gravimetrically (11). Carbohydrate contents were estimated by the subtraction method.

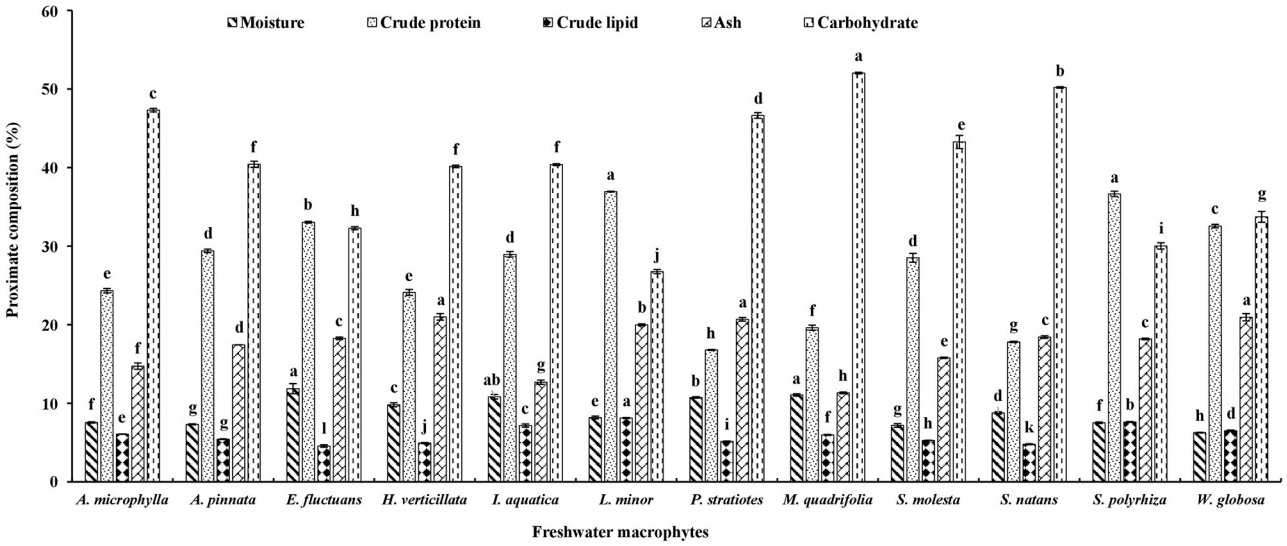

FIGURE 1 | Proximate composition of twelve freshwater macrophytes cultured with organic manures. Bars with different superscripts are significantly different ($n = 3$).

Mineral Assay

The mineral compositions of macrophytes were assayed using Inductively-Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7900, USA) following standard protocol at the Instrumentation Facility of Indian Institute of Technology, New Delhi. The powdered macrophyte sample (150 mg) was taken in a closed digestion vessel and 8 ml of suprapure 69% nitric acid (HNO_3, Merck, USA) was added to this. The sample was digested in Microwave digestion system (Multiwave PRO; Anton Paar, Austria). The digested sample was cooled at room temperature and transferred into a measuring cylinder; Milli-Q ultrapure water was added to make the volume 40 ml. Then the sample was filtered through a 0.2 μM syringe filter (Thermo Scientific, USA) and was collected in a glass vial. A 20 μL sample was injected through autosampler in the ICP-MS. The standard solution for each mineral was supplied with the equipment (Agilent Technologies, USA). It was diluted with Milli-Q ultrapure water containing 1% HNO_3 to make concentrations of 20, 40, 60, 80, 100, 250, 500, 1000 μg/l. The calibration (standard) curve was prepared. The blank was prepared with Milli-Q ultrapure water containing HNO_3 (1%). Minerals are divided into three major groups based on their concentrations in the mammal/human body *viz*. macro, trace, and ultra-trace minerals (5).

Fatty Acid Analysis

The fatty acid profiles of the macrophytes were analyzed using Gas Chromatograph (GC)–Flame Ionization Detector, Clarus 580 (Perkin Elmer, USA). The total lipid extracted from plants (11) was used to prepare fatty acid methyl esters (FAME) by transesterification using sulfuric acid in methanol at 50°C for 16 h (12). After extraction and purification of FAME (13), 1 ml sample was kept in a glass vial of autosampler of GC. The sample was separated and quantified in a GC column (60 m × 0.32 mm i.d. × 0.25 μm ZB-wax, Phenomenex, UK). The data were collected from pre-installed program software (TotalChrom Workstation Ver6.3, Perkin Elmer). The FAME was identified with the help of standards (Supelco FAME 37 mix, Sigma-Aldrich, USA).

Statistical Analysis

The compositions of twelve macrophytes are given as means ± standard error (SE). The differences in nutritional values of various macrophytes were tested using one-way analysis of variance (ANOVA) and Duncan's multiple range test (14). Statistical analyses were performed using the SPSS program (version 25.0). Statistical significance was accepted at $p < 0.05$.

RESULTS

Proximate Composition

The moisture content was highest (11.86%) and lowest (6.26%) in *E. fluctuans* and *W. globosa*, respectively (**Figure 1**). Significantly higher crude protein contents were found in two duckweeds, namely *L. minor* and *S. polyrhiza,* compared with others. The highest lipid content was also recorded in *L. minor*, followed by *S. polyrhiza*. The lipid content was minimum in *E. fluctuans*. Ash content was significantly higher in *H. verticillata*, *W. globose,* and *P. stratiotes* compared with other macrophytes. The ash content was minimum in *M. quadrifolia*. Carbohydrates levels were minimum and maximum in *L. minor* and *M. quadrifolia*, respectively.

Mineral Composition

Macrominerals

Among these twelve freshwater macrophytes, Na content was significantly higher in *P. stratiotes* compared with others (**Figure 2A**). This group was followed by *S. natans* and *E. fluctuans*. A significantly higher K level was found in *W. globosa* compared with others. This was followed by *L. minor*, *E.*

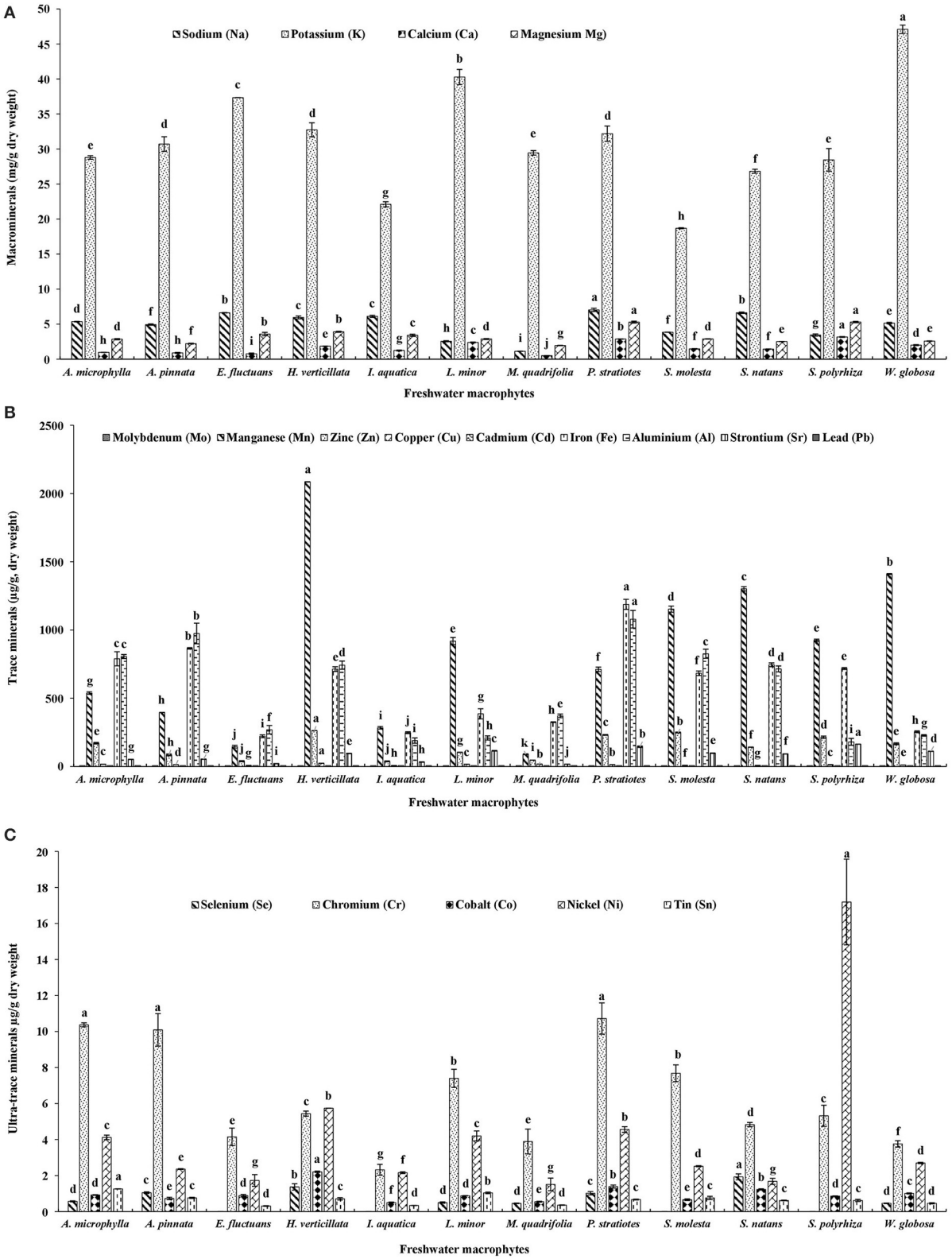

FIGURE 2 | (A) Macrominerals, **(B)** trace minerals, and **(C)** ultra-trace minerals contents of twelve freshwater macrophytes cultured with organic manures. Bars with different superscripts are significantly different ($n = 3$).

fluctuans, *H. verticillate,* and *P. stratiotes*. Ca content was highest in *S. polyrhiza*, followed by *P. stratiotes*. A significantly higher Mg level was found in *P. stratiotes* and *S. polyrhiza* compared with others. The Na, Ca, and Mg contents were minimum in *M. quadrifolia* compared with other macrophytes. It indicates the nutritional value of the macrophytes.

Trace Minerals

A total of nine trace minerals were found in these macrophytes (**Figure 2B**). Molybdenum (Mo) content was significantly higher in *A. microphylla, A. pinnata,* and *P. stratiotes* compared with others. Mn, zinc (Zn), copper (Cu), and Cd contents were significantly higher in *H. verticillata* compared with others. In *P. stratiotes*, significantly higher levels of Fe and aluminum (Al) were found compared with others. Among these macrophytes, *A. pinnata* ranked second for both Fe and Al. *A. microphylla* ranked third for Fe and fourth for Al contents. Maximum strontium (Sr) level was recorded in *S. polyrhiza* followed by *P. stratiotes*. In all these macrophytes lead (Pb) was found.

Ultra-Trace Minerals

Five ultra-trace minerals were found in these macrophytes (**Figure 2C**). A significantly higher level of selenium (Se) was found in *S. natans* compared with others. This plant was followed by *H. verticillata* and *P. stratiotes*. Se was absent in *S. molesta*, *E. fluctuans*, *I. aquatica,* and *S. polyrhiza*. Chromium (Cr) content was significantly higher in *P. stratiotes*, *A. microphylla,* and *A. pinnata* compared with others. Cobalt (Co) content was significantly higher in *H. verticillata* compared with others. This was followed by *P. stratiotes* and *S. natans*. Nickel (Ni) and tin (Sn) levels were significantly higher in *S. polyrhiza* and *A. microphylla*, respectively compared with others. Among these macrophytes, *P. stratiotes* ranked third for Ni content.

Fatty Acid Profile

The fatty acid profiles of twelve freshwater macrophytes were documented in the present study (**Tables 1–3**). The saturated fatty acids (SFAs) content was significantly higher in *W. globosa* compared with others. This was followed by *A. pinnata*, *L. minor,* and *I. aquatica*. SFA content was minimum in *P. stratiotes*. Among, SFA, palmitic acid (C16:0) was the dominant one in all these plants. Monounsaturated fatty acids (MUFA) content was significantly higher in *M. quadrifolia* compared with others. Among various MUFAs, oleic acid (C18:1n-9) was present in most of the plants and the amount was also higher compared with the others (**Supplementary Tables 1A–C**). MUFA content was also minimum in *P. stratiotes*. Though in small amounts two other monounsaturated fatty acids like, palmitoleic acid (C16:1n-9) and nervonic acid (C24:1), were present in all macrophytes, except *E. fluctuans* and *A. pinnata*. Another isomer of palmitoleic acid (C16:1n-7) was absent in two species of *Azolla* and *S. natans*.

The n-6 PUFA content was significantly higher in *W. globosa* compared with others. This was followed by *L. minor* and *A. pinnata*. The minimum level was found in *A. microphylla*. Among n-6 PUFA, LA (C18:2n-6) was the dominant one and was present in all macrophytes. Arachidonic acid (C20:4n-6) was the second dominant n-6 PUFA found in all macrophytes, except in *L. minor*. ALA (C18:3n-3) was the only member of n-3 PUFA present in all these macrophytes. ALA content was significantly higher in *I. aquatica* compared with others. This was followed by *L. minor* and *W. globosa*. The highest (4.04) n-3/n-6 was found in *I. aquatica* (**Supplementary Table 2**). The ratio ranged from 0.61 (*S. molesta*)−2.46 (*L. minor*) in other macrophytes.

DISCUSSION

A wide variation in the composition of freshwater macrophytes was recorded in the present study. The advantage of this study is that plants were cultured in the outdoor systems following a standard protocol (3). Therefore, almost the same quality of products is expected in a further study. There is scope for improvement in the nutritional value as the quality of the culture medium influences the composition of the plants.

In the present study, crude protein levels in three members of Lemnaceae family and *E. fluctuans* were above 30%, and protein contents of other macrophytes (except *P. stratiotes*, *S. natans,* and *M. quadrifolia*) were above 20%. The present study confirms the previous finding that macrophytes are rich sources of protein. The protein contents of *L. minor* and *S. polyrhiza* were 36.07 and 35.82%, respectively (3, 4). A previous study in Bangladesh reported that the protein contents of *E. fluctuans* and *I. aquatica* were 16.69 and 21.45%, respectively; macrophytes were collected from natural water bodies (15). In the present study, protein contents of *E. fluctuans* and *I. aquatica* were 16.35 and 7.51% higher compared with the same macrophytes studied in Bangladesh. Lipid contents of *I. aquatica*, *S. polyrhiza,* and *L. minor* ranged from 7.16 to 8.13% in the present study. The lipid contents of *E. fluctuans* and *I. aquatica* were 1.90 and 3.82% higher in the present study compared with the previous study (15). Ash contents of these two macrophytes were also higher in the present study compared with the previous one. Higher levels of ash contents of *H. verticillata*, *W. globosa,* and *P. stratiotes* compared with other macrophytes enhanced the nutritional value of these plants as feed ingredients for fish, poultry, and livestock. In the present study, lower levels of carbohydrates were observed in macrophytes compared with the plants harvested from the wild (15). Culture of macrophytes with organic manures enhanced the nutritional value of plants.

Among these macrophytes, highest levels of macrominerals, Na and Mg, were found in *P. stratiotes*. K and Ca were highest in *W. globosa* and *S. polyrhiza*, respectively. In the present study, among various macrophytes, *P. stratiotes* ranked second and fifth for Ca and K, respectively. A previous study reported the highest Ca level in *Hydrilla* sp., followed by *P. stratiotes* and *E. crassipes*. There was no variation in Mg level among these three macrophytes (16). Macromineral profile of leaves and roots of *P. stratiotes* collected from a natural water body of Nigeria was documented (17). This study showed that Na, K, Ca, and Mg contents were 3.73, 32.83, 2.30, and 3.70 g/kg of leaves, respectively. In the present study, Na, Ca, and Mg contents were 47, 20, and 30%, respectively, higher in *P. stratiotes* compared with the plants studied in Nigeria. K content was almost the same in the plants grown in two different conditions. The Na,

TABLE 1 | Saturated fatty acids (SFA) profiles of freshwater macrophytes cultured with organic manures (mg/100 g, dry weight).

Macrophytes /Fatty acids	C14:0	C15:0	C16:0	C18:0	C20:0	C22:0	C24:0	∑SFA
Azolla microphylla	6.55 ± 0.06^{fg}	-	541.84 ± 18.38^{de}	1.76 ± 0.89^{h}	1.39 ± 0.23^{h}	-	5.65 ± 0.23^{ef}	**557 ± 19.79^{fg}**
Azolla pinnata	16.99 ± 0.69^{e}	17.76 ± 8.72^{a}	815.78 ± 3.39^{b}	27.06 ± 0.36^{cd}	$4.08 \pm 0.42e^{f}$	17.58 ± 1.45^{a}	16.23 ± 1.09^{a}	**915.51 ± 8.08^{b}**
Enhydra fluctuans	9.46 ± 0.29^{f}	6.90 ± 2.29^{bc}	377.47 ± 0.07^{f}	29.34 ± 0.06^{c}	$2.01 \pm 0.17g^{h}$	0.07 ± 0.03^{d}	5.63 ± 1.02^{ef}	**431.01 ± 0.83^{h}**
Hydrilla verticillata	20.03 ± 1.90^{de}	4.40 ± 1.49^{cde}	377.33 ± 16.67^{f}	21.53 ± 1.68^{ef}	17.91 ± 0.17^{a}	0.89 ± 0.12^{cd}	8.03 ± 0.57^{de}	**450.14 ± 5.075^{h}**
Ipomoea aquatica	14.97 ± 0.73^{ef}	3.64 ± 0.89^{def}	622.79 ± 0.87^{c}	68.69 ± 0.80^{a}	4.54 ± 0.33^{de}	0.07 ± 0.01^{d}	14.47 ± 0.41^{ab}	**729.22 ± 1.90^{d}**
Lemna minor	40.11 ± 0.05^{a}	8.25 ± 0.13^{b}	753.13 ± 0.05^{b}	$17.69 \pm 0.06f^{g}$	5.65 ± 0.11^{d}	5.04 ± 0.10^{b}	12.81 ± 0.15^{bc}	**842.69 ± 0.55^{c}**
Marsilea quadrifolia	14.50 ± 0.46^{e}	1.92 ± 0.36^{efg}	542.58 ± 6.39^{de}	$16.81 \pm 0.47f^{g}$	1.38 ± 0.10^{h}	0.84 ± 0.02^{cd}	10.37 ± 0.81^{cd}	**588.42 ± 5.72^{ef}**
Pistia stratiotes	5.29 ± 0.40^{g}	3.85 ± 0.19^{cde}	268.75 ± 0.06^{g}	$14.07 \pm 1.16g$	8.11 ± 0.10^{c}	0.57 ± 0.04^{cd}	10.10 ± 0.45^{cd}	**310.77 ± 0.70^{i}**
Salvinia molesta	34.04 ± 5.04^{b}	6.14 ± 0.85^{bcd}	574.15 ± 19.84^{cd}	23.35 ± 0.72^{de}	2.99 ± 1.18^{fg}	5.13 ± 0.02^{b}	10.28 ± 0.48^{cd}	**656.11 ± 16.58^{e}**
Salvinia natans	4.04 ± 0.37^{g}	-	486.68 ± 2.72^{e}	3.26 ± 0.07^{h}	2.39 ± 0.18^{gh}	-	5.08 ± 0.41^{f}	**501.45 ± 4.02^{gh}**
Spirodela polyrhiza	24.26 ± 2.07^{cd}	0.81 ± 0.04^{fg}	509.94 ± 16.48^{e}	24.84 ± 1.58^{cde}	5.51 ± 0.62^{d}	1.42 ± 0.03^{cd}	7.85 ± 0.89^{de}	**574.66 ± 22.02^{fg}**
Wolffia globosa	28.24 ± 0.60^{c}	3.08 ± 0.29^{def}	1084.31 ± 63.38^{a}	54.25 ± 4.57^{b}	11.31 ± 0.90^{b}	2.07 ± 0.01^{c}	10.87 ± 0.46^{c}	**1194.14 ± 71.13^{a}**

Values having the means (n = 3) in each row with different superscript are significantly ($p < 0.05$) different.

TABLE 2 | Monounsaturated fatty acids (MUFA) profiles of freshwater macrophytes cultured with organic manures (mg/100 g, dry weight).

Macrophytes /Fatty acids	C16:1*n*-9	C16:1*n*-7	C17:1	C18:1*n*-9	C20:1*n*-9	C22:1*n*-9	C24:1	∑MUFA
Azolla microphylla	9.85 ± 0.70^{cde}	-	12.49 ± 0.75^{c}	77.22 ± 1.20^{e}	-	-	2.83 ± 0.74^{b}	**102.39 ± 1.91^{e}**
Azolla pinnata	9.39 ± 0.52^{cde}	-	91.52 ± 0.17^{b}	106.75 ± 0.34^{c}	-	-	-	**207.67 ± 0.35^{b}**
Enhydra fluctuans	-	25.29 ± 0.52^{c}	-	44.99 ± 0.21^{g}	0.28 ± 0.13^{cd}	-	0.18 ± 0.13^{c}	**70.75 ± 0.73^{f}**
Hydrilla verticillata	0.13 ± 0.02^{g}	8.81 ± 0.42^{d}	-	48.97 ± 1.60^{g}	14.15 ± 0.14^{a}	-	0.21 ± 0.03^{c}	**72.28 ± 3.67^{f}**
Ipomoea aquatica	0.33 ± 0.02^{f}	27.25 ± 0.29^{c}	-	24.16 ± 1.20^{h}	-	-	3.45 ± 0.62^{b}	**55.20 ± 1.73^{g}**
Lemna minor	51.60 ± 0.08^{a}	86.47 ± 0.15^{a}	-	9.67 ± 0.10^{i}	2.39 ± 0.04^{b}	-	7.01 ± 0.11^{a}	**157.17 ± 0.12^{c}**
Marsilea quadrifolia	7.86 ± 0.26^{de}	24.61 ± 0.42^{c}	168.24 ± 2.69^{a}	127.93 ± 2.59^{b}	0.54 ± 0.40^{cd}	7.58 ± 0.76^{b}	1.14 ± 0.74^{c}	**337.94 ± 7.36^{a}**
Pistia stratiotes	0.65 ± 0.08^{f}	10.59 ± 0.63^{d}	-	30.66 ± 0.03^{h}	0.25 ± 0.08^{cd}	5.39 ± 0.25^{c}	0.82 ± 0.03^{c}	**48.38 ± 0.14^{g}**
Salvinia molesta	6.51 ± 0.89^{e}	10.41 ± 2.38^{d}	9.53 ± 0.49^{d}	94.04 ± 0.02^{d}	0.39 ± 0.05^{cd}	3.63 ± 0.25^{d}	0.51 ± 0.01^{c}	**125.06 ± 4.06^{d}**
Salvinia natans	16.32 ± 0.74^{b}	-	4.71 ± 0.34^{e}	68.48 ± 1.18^{f}	-	-	0.65 ± 0.01^{c}	**90.16 ± 0.28^{e}**
Spirodela polyrhiza	13.81 ± 1.55^{bc}	38.93 ± 2.70^{b}	-	49.23 ± 5.09^{g}	0.13 ± 0.02^{cd}	3.30 ± 0.59^{d}	0.06 ± 0.01^{d}	**105.49 ± 5.59^{e}**
Wolffia globosa	12.00 ± 0.69^{bcd}	40.67 ± 0.54^{b}	-	137.22 ± 0.61^{a}	0.64 ± 0.03^{c}	17.65 ± 0.92^{a}	0.20 ± 0.02^{c}	**208.40 ± 2.60^{b}**

Values having the means (n = 3) in each row with different superscript are significantly ($p < 0.05$) different.

TABLE 3 | Polyunsaturated fatty acids (PUFA) profiles of freshwater macrophytes cultured with organic manures (mg/100 g, dry weight).

Macrophytes /Fatty acids	C18:2 *n*-6	C18:3 *n*-6	C20:2 *n*-6	C20:3 *n*-6	C20:4 *n*-6	∑*n*-6 PUFA	C18:3 *n*-3	∑ *n*-3 PUFA	*n*-3/n-6
Azolla microphylla	96.72 ± 4.71^{j}	-	-	-	16.71 ± 0.14^{d}	**113.43 ± 4.85^{i}**	149.05 ± 4.98^{i}	**149.05 ± 4.98^{i}**	1.31 ± 0.01^{f}
Azolla pinnata	443.29 ± 1.13^{c}	10.57 ± 0.31^{b}	-	3.17 ± 0.62^{de}	81.32 ± 0.42^{a}	**538.36 ± 2.49^{c}**	591.33 ± 0.66^{e}	**591.33 ± 0.66^{e}**	1.09 ± 0.01^{h}
Enhydra fluctuans	329.01 ± 3.35^{e}	-	1.11 ± 0.10^{bc}	-	5.24 ± 0.24^{e}	**335.38 ± 3.01^{e}**	608.37 ± 5.12^{e}	**608.37 ± 5.12^{e}**	1.81 ± 0.01^{d}
Hydrilla verticillata	168.35 ± 2.01^{g}	-	0.97 ± 0.08^{bc}	-	1.20 ± 0.17^{g}	**170.53 ± 1.89^{g}**	405.91 ± 5.50^{g}	**405.91 ± 5.50^{g}**	2.38 ± 0.06^{b}
Ipomoea aquatica	384.86 ± 1.85^{d}	-	0.95 ± 0.63^{bc}	-	2.87 ± 0.09^{f}	**388.69 ± 1.31^{d}**	1572.23 ± 2.87^{a}	**1572.23 ± 2.87^{a}**	4.04 ± 0.01^{a}
Lemna minor	601.47 ± 0.10^{b}	-	3.35 ± 0.10^{a}	5.33 ± 0.07^{c}	-	**610.16 ± 0.07^{b}**	1505.63 ± 10.10^{b}	**1505.63 ± 10.10^{b}**	2.46 ± 0.02^{b}
Marsilea quadrifolia	255.25 ± 1.33^{f}	-	0.91 ± 0.49^{bc}	55.44 ± 0.55^{a}	0.06 ± 0.01^{i}	**311.68 ± 2.31^{f}**	473.56 ± 1.79^{f}	**473.56 ± 1.79^{f}**	1.51 ± 0.01^{e}
Pistia stratiotes	132.20 ± 0.67^{h}	-	0.71 ± 0.04^{c}	1.03 ± 0.03^{f}	0.27 ± 0.01^{h}	**134.22 ± 2.11^{h}**	322.97 ± 0.32^{h}	**322.97 ± 0.32^{h}**	2.40 ± 0.03^{b}
Salvinia molesta	131.23 ± 1.29^{h}	-	2.39 ± 0.54^{ab}	23.88 ± 1.30^{b}	0.21 ± 0.01^{h}	**157.72 ± 0.56^{g}**	97.09 ± 1.18^{j}	**97.09 ± 1.18^{j}**	0.61 ± 0.01^{j}
Salvinia natans	118.57 ± 0.78^{i}	-	-	-	19.21 ± 0.22^{c}	**137.78 ± 1.00^{h}**	116.51 ± 0.89^{j}	**116.51 ± 0.89^{j}**	0.85 ± 0.01^{i}
Spirodela polyrhiza	368.28 ± 7.45^{d}	-	1.00 ± 0.01^{bc}	3.80 ± 0.34^{cd}	0.93 ± 0.02^{g}	**374.03 ± 6.06^{d}**	724.41 ± 12.66^{d}	**724.41 ± 12.66^{d}**	1.93 ± 0.02^{c}
Wolffia globosa	728.27 ± 17.19^{a}	16.54 ± 1.18^{a}	2.70 ± 0.05^{a}	1.65 ± 0.01^{ef}	22.44 ± 1.57^{b}	**771.63 ± 17.37^{a}**	909.28 ± 16.17^{c}	**909.28 ± 16.17^{c}**	1.17 ± 0.01^{g}

Values having the means (n = 3) in each row with different superscript are significantly ($p < 0.05$) different.

Ca, Zn, and Cu contents were higher in *I. aquatica* grown in Bangladesh compared with the macrophytes assayed in the present study (15). Although, Mg, K, and Fe contents were higher in the *I. aquatica* assayed in the present study compared with the plants studied in Bangladesh, Na, Mg, and K contents were higher in *E. fluctuans* evaluated in the present study compared with the previous study in Bangladesh. The Na, K, Mg, and Ca contents were higher in *A. filiculoides* and *S. molesta* grown in swine lagoons compared with the present study (18). In *A. filiculoides*, Na, K, Mg, and Ca contents were 2.77, 22.5, 5.04, and 9.3 g/kg (dry matter), respectively. In *S. molesta* Na, K, Mg, and Ca contents were 4.44, 34.7, 5.18 and 10.6 g/kg (dry matter), respectively.

In the present study, Na to K ratio ranged from 0.038 (*M. quadrifolia*) −0.276 (*I. aquatica*). The ratio was 0.063, 0.109, 0.121, 0.160, 0.177, 0.182, 0.185, 0.205, 0.218, and 0.238 in *L. minor*, *W. globosa*, *S. polyrhiza*, *A. pinnata*, *E. fluctuans*, *H. verticillata*, *A. microphylla, S. molesta*, *P. stratiotes,* and *S. natans*, respectively. In all these macrophytes, the ratio of Na to K is less than the WHO/FAO-recommended ratio for an adult human, i. e., <0.49 (19). Various studies showed the effect of culture medium on the mineral profile of macrophytes (8, 20, 21). In different species of duckweeds Na: K varied from 0.027–1.49 (K: Na = 0.67–37). In *Wolffia*, the ratio was 0.025 (K: Na = 40) and in another species, *W. microscopica* it was 0.003 (K: Na = 276).

In the present study, the Mg: Ca varied from 1.20 (*L. minor*)−4.65 in (*E. fluctuans*). Ca has been serving as the main structural mineral and helps in metabolism. It serves as a signal for vital physiological processes. Mg, the fourth most abundant cation in the body, is a co-factor for 350 cellular enzymes, most of which are involved in energy metabolism (22), hence, the ratio of Mg: Ca should be maintained. The Mg: Ca ratio was 0.4 in duckweed (21) and 0.5 in other species, *W. microscopica* (8). In the present study, the ratio was 1.28 for *W. globosa*.

The trace minerals analysis showed that among these macrophytes, *P. stratiotes* was a rich source for Mo, Fe, and Al. This macrophyte also has considerable amounts of Mn, Zn, and Sr. *A. microphylla* and *A. pinnata* were also rich sources of Fe and Mo. In a different strain of *W. arrhiza*, Fe contents ranged from 0.16–0.29 μg/g freeze-dried sample (23). The Fe content of *W. globosa* was 254.12 μg/g in the present study. Higher levels of Zn and Cu were found in *I. aquatica* grown in Bangladesh compared with the present study; Fe content was higher in the present study compared with the previous one (15). Fe content of *E. fluctuans* grown in two different environments was the same. Zn and Cu contents were lower in the plants assayed in the present study compared with the plants studied in Bangladesh. The Cu content of *S. molesta* grown in swine lagoons was 13 g/kg, dry weight (18). In the present study, Cu content of *S. molesta* was less compared with the previous study.

In the present study, the highest level of ultra-trace mineral Se was found in *S. natans*. This important mineral was also present in *H. verticillata* and *P. stratiotes*. It was interesting to record that Se was absent in *S. molesta*, *S. polyrhiza*, *E. fluctuans,* and *I. aquatica*. The Se content of freeze-dried *W. arrhiza* was <0.03 μg/g (23). In the present study, Se content of *W. globosa* was higher compared with the previous study. Significantly higher Cr levels were found in *P. stratiotes*, *A. microphylla,* and *A. pinnata* compared with other macrophytes. A significantly higher Co level was found in *H. verticillata* compared with the others. This macrophyte was followed by *P. stratiotes and S. natans*. Co content in all these macrophytes was >0.50 μg/g (dry weight). Among these macrophytes, the highest Ni content was found in *S. polyrhiza,* and this macrophyte was followed by *H. verticillata* and *P. stratiotes*. In the present study, the contents of heavy metals *viz*. Cd, Cu, Pb, and Sn of macrophytes were within the permissible limits (Cd: 0.2, Cu: 73.3, Pb: 0.3, Sn: 250 Zn: 99.40; mg/kg of wet weight) of WHO/FAO (24). In the present study, the mineral composition was evaluated in the dry sample. Therefore, the moisture (minimum 90%) contents of the samples should be considered at the time of comparison with the permissible limit of WHO/FAO for humans (where fresh plants were considered). In seaweeds, there is no regulation on the maximum heavy metals contents (25).

Various studies showed the dietary requirements of different macro, trace, and ultra-trace minerals for different animals (**Supplementary Tables 3A,B**). Na requirements of grass carp (*Ctenopharyngodon idella*), poultry, cattle, and humans are 2, 0.012–0.200, 0.96 g/kg diet, and 2.4 g/day, respectively. Among various fishes and prawns (*Pinneaus indicus*), Mg requirements vary from 0.4 to 0.946 g/kg of diets. K requirements recorded for common carp (*Cyprinus carpio*), grass carp, and Nile tilapia (*Orechromis niloticus*) are as follows: 0.9–12.4, 4.6, and 2.1–3.3 g/kg diets. K requirements for poultry, cattle, and humans are 0.3 and 2.4 g/kg diet and 3.5 g/day, respectively. Among different groups of fishes, rohu (*Labeo rohita*), common carp, grass carp, catla (*Catla catla*), and Nile tilapia require 1.9, 0.1, 2, 1.9 and 7 g Ca/kg diet, respectively. Ca requirements for poultry, cattle, and humans are 8 and 5.12 g/kg diet and 1.0 g/day, respectively. Among various fishes, Mn, Fe, Zn, and Co requirements vary from 12–25, 30–200, 15–79, and 0.01–0.5 mg/kg diet, respectively. Nile tilapia requires Se and Cr at the rate of 0.4 and 139.6 mg/kg diet, respectively. Fe, Zn, Cu, Se, Cr, and Co requirements are also evaluated for poultry, cattle, and humans. In channel catfish *Ictaluraus punctatus*, Fe, Cu, Mn, Zn, Se, and Co requirements were 30, 5, 25, 200, 0.1, and 0.05 mg/kg feed, respectively (26). In fish, Fe deficiency causes hypochromic microcytic anemia, Co and Mn deficiencies result in poor growth; Zn deficiency causes growth depression, cataract, and caudal fin and skin erosion; Se deficiency results in muscular dystrophy. In fish nutrition, Co plays a significant role. In common carp, the addition of cobalt chloride/cobalt nitrate enhanced the growth and hemoglobin formation (27). Therefore, supplementation of freshwater macrophytes may help to overcome the mineral deficiency in fish and other animals without showing any negative impact (9).

The fatty acid compositions of the two duckweeds *L. minor* and *S. polyrhiza* showed similarity with the previous study (3, 4). In the present study, palmitic acid and oleic acid were the dominant SFA and MUFA, respectively. Similar results were

also found in four duckweeds, *Landoltia*, *Lemna*, *Wolffiella,* and *Wolffia* (8). The fatty acid compositions of four aquatic plants *S. cuculata*, *Trapa natans*, *L. minor,* and *I. reptans* showed that cis-15 tetracosenoic acid and 9-hexadecenoic acid were the dominant fatty acids, and highly unsaturated fatty acids contents were higher compared with the saturated fatty acids (28). In the present study, LA was the major contributor for n-6 PUFA in all plants, and except in *L. minor*, arachidonic acid was also found in all macrophytes. ALA was the only member of n-3 PUFA present in these macrophytes. The presence of LA and ALA were recorded in duckweeds (8). The freshwater teleosts are capable of converting ALA to long-chain polyunsaturated fatty acids (LC-PUFA) like eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6n-3) (29–31). Therefore, the feeding of fish with freshwater macrophytes-based diets helps to fulfill the LC-PUFA requirements of cultured fish (32, 33). The n-6/n-3 PUFA was always <1; it ranged from 0.48–0.94 in different *Wolffia* species (23). A similar result was also found in the present study, except in two species of *Salvania*, where the ratio was >1.0.

CONCLUSION

Among these macrophytes, Na, Mg, Cr, and Fe contents were maximum in *P. stratiotes*; this macrophyte ranked second for Co, Sr, and Ca. *H. verticillata* was the richest source for Cu, Mn, Co, and Zn, and it ranked second for Se. Ca, Mg, Sr, and Ni contents were higher in *S. polyrhiza* compared with the others. *S. natans* and *W. globosa* were rich sources for Se and K, respectively. All these macrophytes were rich sources of n-6 and n-3 fatty acids. This study shows that macrophytes have an immense potential to be used as rich sources of minerals, as well as n-6 and n-3 PUFA for fish, poultry, and livestock.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JS, RC, and DRT designed the study. GK, RG, AS, NK, JS, and RC grown the macrophytes and analyzed samples. RC, DRT, GK, and JS wrote the manuscript. GK, AS, RG, NK, RC, DRT, and JS prepared tables. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.869425/full#supplementary-material

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Effect of *Lemna minor* supplemented diets on growth, digestive physiology and expression of fatty acids biosynthesis genes of *Cyprinus carpio*

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The potential nutritional value of duckweed *Lemna minor* (Lemnaceae) was evaluated for common carp *Cyprinus carpio* fry. Fish were fed diets containing five graded levels of duckweed: 0% (LM0, control), 5% (LM5), 10% (LM10), 15% (LM15) and 20% (LM20). The final weight and specific growth rate were significantly higher in LM15 and LM20 diets fed fish compared to others. Feed conversion ratio was minimum in fish fed diet LM20. Amylase activity was significantly higher in LM0 treatment. Total protease, trypsin and chymotrypsin activities showed linear relationships with the increased level of duckweed in the diet. Protein and essential amino acids contents were significantly higher in carp fed diets LM15 and LM20 compared to others. Lipid content was significantly higher in fish fed duckweed-based diets compared to control. A direct relationship was found between the inclusion level of duckweed in the diet and n-3 long-chain polyunsaturated fatty acid (LC-PUFA) content of carp. Contents of desaturated and elongated products of dietary linolenic acid (18:3n-3) including 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3 increased in a graded manner with increasing dietary duckweed. The monounsaturated fatty acids and n-6 PUFA contents reduced significantly in fish fed duckweed. Expression of *fads2d6*, *elovl2*, *elovl5* and *fas* were higher in carp fed diets LM10, LM15 and LM20 compared to control fish. The inclusion of *L. minor* in diet enhanced the nutritional value of carp by increasing protein, lipid, amino acids and n-3 PUFA contents.

Aquaculture is growing faster than other animal food-producing sectors and has raised expectations to bridge the gap between fish demand and supply[1]. The expansion of the aquaculture sector primarily depends on the supply of high-quality feed for cultivated fish. At present, soybean meal is one of the most commonly used alternative ingredients to replace fishmeal in aquafeed due to its high protein content and relatively well-balanced amino acid profile that can generally satisfy the requirements of many fish species[2–5]. Nevertheless, soybean meal is already in high demand in the human food chain both directly and indirectly in feeds for farmed terrestrial animals. This competition means soybean meal is an expensive ingredient and this may limit its use as an ingredient to meet future demands for fish feed. Therefore, there is a constant need to find other ingredients to replace both fishmeal and its main substitute, soybean meal, in feeds for farmed fish. Ideally, such ingredients should be non-conventional to avoid or minimize competition with other animal feed sectors.

The nutritional qualities of feed ingredients including amino acid and fatty acid compositions, micronutrient and fiber contents among others are major considerations when it comes to feed formulation[4–6]. The nutrients must be readily bioavailable to meet the energy and other physiological requirements of the fish and so the overall

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composition of the ingredient should be appropriate for the digestive enzyme (protease, trypsin, chymotrypsin, lipase etc.) profile of the species, which will help to maximize feed efficiency and production. However, in addition, the composition of feed itself can influence digestive enzyme activities[7]. Thus, feed intake and consumption rates as well as digestive enzyme profiles can be good indicators of health status of fish[8,9]. For plant ingredients, the presence of anti-nutritional factors is one of the potential limitations of their use in aquafeeds[10,11], although processing, such as fermentation and extrusion can reduce anti-nutritional effects[12–15]. In this overall context, the assessment of aquatic plants as potential ingredients for fish feed is an area of renewed interest and research[16,17].

Omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids are essential dietary nutrients for vertebrates including humans and fish[18,19]. They play important roles in many physiological processes like neural development, immune and inflammatory responses[18]. Primary production of the n-3 LC-PUFA, EPA and DHA, occurs largely in the marine environment and so dietary sources for fish feeds are limited largely to fish oil and fishmeal[20]. In contrast, plant meals and vegetable oils that are the main alternatives to the marine-derived ingredients are completely devoid of EPA and DHA[20]. However, many freshwater fishes are capable of converting the C_{18} PUFA found in plants, linoleic acid (LOA; 18:2n-6) and ALA, to the physiologically essential LC-PUFA arachidonic acid (ARA; 20:4n-6), EPA and DHA[21,22]. Consequently, dietary LOA and ALA can satisfy the essential fatty acid (EFA) requirements of fish species such as common carp and Nile tilapia *Oreochromis niloticus* that have been confirmed to have the metabolic capacity to convert dietary ALA to EPA and DHA[19,23,24]. The conversion of LOA and ALA to LC-PUFA requires a series of fatty acyl desaturase (*fads*) and elongation of very long-chain fatty acids (*elovl*) enzymes such as *elovl2* and *elovl5*[21,22,25,26]. Therefore, one potential option for increasing the amount of n-3 LC-PUFA available to human populations is to exploit the endogenous ability of freshwater fish species to produce EPA and DHA from ALA[20].

The nutritional value of freshwater duckweed *Lemna* spp. is well recognized, having relatively high protein, amino acid and fatty acid contents and low fiber, and so duckweeds can be used as dietary supplements for fish and other animals[16–27]. Specifically, lysine and methionine contents are higher in duckweeds compared to other plants and, therefore, more suitable as ingredients for animal feed[28,29]. *Lemna minor* is also a rich source of the omega-3 (n-3) polyunsaturated fatty acid (PUFA), α-linolenic acid (ALA, 18:3n-3)[16]. Several studies have investigated the use of duckweeds as an ingredient in feeds for carp species, such as *L. minor*[30] or *L. minuta*[31,32] in feeds for common carp *Cyprinus carpio*. Furthermore, *L. minor* has been supplemented in diets of rohu *Labeo rohita*[15], grass carp *Ctenopharyngodon idella* and silver carp *Hypophthalmichthys molitrix*[33].

Common carp is an omnivorous, bottom-feeding fish belonging to the family Cyprinidae and is one of the most cultured freshwater and economically valued species due to its fast growth and disease resistance[34]. This, combined with the inherent ability for the endogenous biosynthesis of n-3 LC-PUFA, makes common carp an appropriate candidate species to evaluate the utility of duckweed-based diets in freshwater aquaculture.

The overall aim of the present study is to evaluate the duckweed *Lemna minor* as a replacement for plant meals such as soybean meal in extruded pelleted diets for common carp *Cyprinus carpio*. The extrusion process helps to overcome the problems associated with the presence of anti-nutritional factors in *L. minor*. In the present study, the effect of *Lemna minor* supplemented diets on the survival, growth, digestive physiology, and biochemical composition of *Cyprinus carpio* are evaluated. The impact of dietary *L. minor* on the content of EPA and DHA and expression of *fads* and *elovl* genes in fish are also studied. This helps to understand the mechanism of endogenous biosynthesis of LC-PUFA from duckweed-derived ALA in freshwater fish like common carp. The n-3 PUFA content of control diet is very less and it helps to see the effect of *L. minor* supplemented diets on the composition of fish.

Results

Survival and growth of fish. After 60 days of culture, the number of fish in each aquarium was counted and no mortalities recorded in any dietary treatment. The final weight of carp fed diets LM15 (2.50 ± 0.012 g) and LM20 (2.66 ± 0.023 g) were significantly higher compared to the fish fed the other diets (Table 1). Final weight was lowest in control diet fed fish (LM0, 1.69 ± 0.005 g) with no inclusion of *L. minor*. The final weight and SGR of fish increased linearly ($p < 0.05$) with the increasing level of *L. minor* in the diet. There was no significant difference in feed intake of fish fed with five different diets. The feed intake of fish ranged from 2.36 to 2.46 g 100 g^{-1} BW day^{-1}. The FCR was lowest and highest in carp fed diets LM20 (1.02 ± 0.01) and LM0 (1.27 ± 0.06), respectively. The FCR decreased linearly with the increasing level of duckweeds in the diet of carp.

Digestive enzyme activities of fish. Amylase activity was significantly lower in carp fed the duckweed-based diets compared to fish fed control diet LM0 (Table 1). The total protease, trypsin and chymotrypsin activities increased linearly ($p < 0.05$) with the increased levels of duckweeds in the diet. Specially, the graded inclusion of dietary duckweed resulted in graded increased activities of protease and trypsin with highest activities recorded in fish fed diet LM20 followed by fish fed diet LM15. Chymotrypsin activity was also higher in fish fed diets LM10–LM20 compared to fish fed the control diet LM0. Lipase activity was significantly higher in fish fed diet LM5 compared to other treatments. The lowest lipase activity was observed in fish fed diet LM20.

Biochemical composition of fish. The protein and lipid contents of common carp showed linear relationships ($p < 0.05$) with the increased inclusions of duckweeds in the diet (Table 2). Protein content was significantly higher in carp fed diets LM15 (145.7 ± 0.52 g kg^{-1} of fresh weight) and LM20 (148.6 ± 0.57 g kg^{-1} of fresh weight) with the two highest inclusions of duckweed compared to fish fed the other diets. Significantly higher lipid content was observed in fish fed the duckweed-based diets (65.0–67.5 g kg^{-1} of fresh weight) compared to carp fed the control diet without duckweed. The moisture content was significantly higher in fish fed control diet

Parameters	LM0	LM5	LM10	LM15	LM20	Polynomial contrasts	
						Linear	Quadratic
Initial body weight (g)	0.48 ± 0.009^{a}	0.47 ± 0.008^{a}	0.48 ± 0.006^{a}	0.48 ± 0.007^{a}	0.47 ± 0.005^{a}	0.783	0.643
Final weight (g)	1.69 ± 0.005^{d}	1.94 ± 0.030^{c}	2.10 ± 0.009^{b}	2.50 ± 0.012^{a}	2.66 ± 0.023^{a}	< 0.001	0.425
Specific growth rate (g)	2.11 ± 0.03^{c}	2.35 ± 0.05^{b}	2.48 ± 0.02^{b}	2.76 ± 0.03^{a}	2.87 ± 0.01^{a}	< 0.001	0.474
Feed intake (g 100 g^{-1} BW day^{-1})	2.36 ± 0.10^{a}	2.46 ± 0.12^{a}	2.41 ± 0.04^{a}	2.44 ± 0.06^{a}	2.38 ± 0.02^{a}	0.925	0.479
Feed conversion ratio	1.27 ± 0.06^{a}	1.22 ± 0.07^{ab}	1.14 ± 0.02^{b}	1.08 ± 0.02^{c}	1.02 ± 0.01^{c}	0.001	0.969
Survival (%)	100	100	100	100	100		
Digestive enzymes							
Amylase (mU mg^{-1}protein min^{-1})	112.44 ± 1.67^{a}	75.16 ± 2.06^{d}	87.66 ± 0.78^{c}	87.22 ± 0.66^{c}	104.10 ± 1.14^{b}	0.264	< 0.001
Protease (Fluorescence change $unit^{-1}$)	104.31 ± 1.77^{d}	107.00 ± 0.53^{d}	114.70 ± 1.89^{c}	128.80 ± 4.40^{b}	151.70 ± 1.23^{a}	< 0.001	0.001
Trypsin (μM AMC mg^{-1} protein min^{-1})	822.95 ± 2.10^{d}	838.03 ± 4.65^{c}	847.41 ± 2.86^{c}	1146.73 ± 7.60^{b}	1320.30 ± 5.61^{a}	< 0.001	< 0.001
Chymotrypsin (μM AMC mg^{-1} protein min^{-1})	1772.69 ± 14.00^{d}	1782.77 ± 14.00^{d}	2075.77 ± 26.07^{c}	2341.07 ± 10.21^{a}	2128.35 ± 15.522^{b}	< 0.001	< 0.001
Lipase (μM 4-MU mg^{-1} protein min^{-1})	680.98 ± 4.73^{b}	731.04 ± 7.55^{a}	691.53 ± 6.96^{b}	642.67 ± 2.15^{c}	626.54 ± 11.84^{c}	< 0.001	< 0.001

Table 1. Initial and final weights, survival rate, specific growth rate, feed intake, feed conversion ratio and digestive enzyme activities of *Cyprinus carpio* fed with five different diets. Values (means ± SE, n = 3) in each row with different superscript are significantly different ($p < 0.05$). The polynomial orthogonal contrast was considered significant at $p < 0.05$ level. LM0 = Control, soybean; LM5 = 5% *L. minor*; LM10 = 10% *L. minor*; LM15 = 15% *L. minor*; LM20 = 20% *L. minor.*

Parameters	LM0	LM5	LM10	LM15	LM20	Polynomial contrasts	
						Linear	Quadratic
Moisture	771.8 ± 1.24^{a}	760.6 ± 0.58^{b}	759.6 ± 1.85^{b}	757.2 ± 3.10^{b}	752.1 ± 4.00^{b}	< 0.001	0.285
Crude protein	140.0 ± 0.92^{b}	140.8 ± 1.22^{b}	142.4 ± 0.85^{b}	145.7 ± 0.52^{a}	148.6 ± 0.57^{a}	< 0.001	0.099
Crude lipid	55.1 ± 1.00^{b}	65.0 ± 0.34^{a}	65.7 ± 0.62^{a}	67.1 ± 0.84^{a}	67.5 ± 0.44^{a}	< 0.001	< 0.001
Crude ash	20.0 ± 0.01^{a}	20.3 ± 0.02^{a}	20.9 ± 0.07^{a}	21.4 ± 0.03^{a}	21.7 ± 0.09^{a}	< 0.001	0.749

Table 2. Biochemical composition of *Cyprinus carpio* cultured under five different feeding regimes (g kg^{-1} of fresh weight). Values (means ± SE, n = 3) in each row with different superscript are significantly different ($p < 0.05$). The polynomial orthogonal contrast was considered significant at $p < 0.05$ level. LM0 = Control, soybean; LM5 = 5% *L. minor*; LM10 = 10% *L. minor*; LM15 = 15% *L. minor*; LM20 = 20% *L. minor.*

LM0 (771.8 ± 1.24 g kg^{-1} of fresh weight) compared to fish fed the duckweed-based diets. There was no significant difference in ash content among various diets fed carp.

Amino acid composition of fish. Total essential amino acid in fish increased linearly with the increased level of duckweed in the diet of carp (Table 3). Arginine, isoleucine and threonine contents were highest in carp fed diet LM20 while histidine, leucine and lysine levels were highest in fish fed the control diet LM0. Phenylalanine content was highest in LM15 diet fed common carp and LM20 followed this group. While there were no significant differences in total non-essential amino acid contents among fish fed the five diets, highest alanine, glycine and serine contents were found in carp fed diet LM20, whereas aspartate, cysteine and glutamic acid levels were highest in fish fed the LM0. Non-proteinogenic amino acids level was lower in fish fed the control LM0 diet compared to fish fed the diets including duckweed. Phosphoserine, taurine, cystathionine, ϒ-amino butyric acid and 1 methyl histidine levels were significantly higher in carp fed diet LM20, while β-alanine and hydroxyproline contents were significantly higher in fish fed diets LM15 and LM20, compared to fish fed the diets with lower duckweed inclusion. In carp fed diets LM0 and LM5, β-amino isobutyric acid was not detected. Like essential amino acids, total non-proteinogenic amino acids level in fish also showed a linear relationship with the increased level of duckweed in the diet.

Proteinogenic amino acids	LM0	LM5	LM10	LM15	LM20	Polynomial contrasts	
						Linear	Quadratic
Essential amino acids							
Arginine (Arg)	6.74 ± 0.13^{d}	7.99 ± 0.12^{c}	11.79 ± 0.10^{a}	10.81 ± 0.11^{b}	11.91 ± 0.03^{a}	< 0.001	< 0.001
Histidine (His)	3.83 ± 0.01^{a}	3.68 ± 0.10^{a}	3.29 ± 0.04^{b}	3.38 ± 0.03^{b}	3.27 ± 0.03^{b}	< 0.001	0.018
Isoleucine (Ile)	5.56 ± 0.07^{b}	5.46 ± 0.12^{b}	4.82 ± 0.07^{c}	5.57 ± 0.01^{b}	5.97 ± 0.02^{a}	0.002	< 0.001
Leucine (Leu)	10.10 ± 0.04^{a}	10.05 ± 0.07^{a}	8.43 ± 0.04^{d}	9.25 ± 0.03^{b}	8.89 ± 0.02^{c}	< 0.001	< 0.001
Lysine (Lys)	12.53 ± 0.02^{a}	11.76 ± 0.11^{b}	9.70 ± 0.01^{d}	10.24 ± 0.02^{c}	10.23 ± 0.03^{c}	< 0.001	< 0.001
Methionine (Met)	3.30 ± 0.05^{c}	3.37 ± 0.06^{c}	3.22 ± 0.06^{c}	5.32 ± 0.19^{a}	4.08 ± 0.02^{b}	< 0.001	0.303
Phenylalanine (Phe)	5.58 ± 0.03^{ab}	5.73 ± 0.12^{a}	5.42 ± 0.01^{b}	5.77 ± 0.02^{a}	5.75 ± 0.02^{a}	0.082	0.212
Threonine (Thr)	5.24 ± 0.03^{d}	5.40 ± 0.08^{c}	5.56 ± 0.01^{c}	6.19 ± 0.04^{b}	6.42 ± 0.08^{a}	< 0.001	0.005
Tryptophan (Trp)	1.61 ± 0.04^{d}	1.04 ± 0.05^{d}	3.55 ± 0.03^{a}	2.50 ± 0.08^{b}	2.30 ± 0.07^{c}	0.007	0.017
Valine (Val)	6.43 ± 0.14^{a}	6.45 ± 0.13^{a}	5.46 ± 0.70^{b}	5.66 ± 0.05^{b}	6.72 ± 0.04^{a}	0.517	< 0.001
Total	60.92 ± 0.50^{b}	60.93 ± 0.30^{b}	61.24 ± 0.24^{b}	64.69 ± 0.53^{a}	65.54 ± 0.49^{a}	< 0.001	0.096
Non-essential amino acids							
Alanine (Ala)	7.81 ± 0.06^{b}	7.11 ± 0.12^{d}	7.53 ± 0.06^{c}	7.94 ± 0.03^{b}	8.25 ± 0.01^{a}	< 0.001	< 0.001
Aspartate (Asp)	11.56 ± 0.01^{a}	11.39 ± 0.07^{a}	10.47 ± 0.07^{c}	11.26 ± 0.02^{b}	11.06 ± 0.08^{b}	< 0.001	< 0.001
Cysteine (Cys)	1.34 ± 0.04^{a}	1.48 ± 0.03^{a}	1.03 ± 0.03^{b}	1.36 ± 0.04^{a}	1.14 ± 0.03^{b}	0.001	0.718
Glutamic acid (Glu)	20.16 ± 0.04^{a}	19.57 ± 0.01^{b}	19.30 ± 0.06^{b}	18.33 ± 0.15^{c}	18.67 ± 0.08^{c}	0.001	0.003
Glycine (Gly)	8.29 ± 0.13^{d}	9.80 ± 0.08^{c}	10.52 ± 0.04^{b}	11.16 ± 0.00^{a}	11.33 ± 0.11^{a}	< 0.001	< 0.001
Proline (Pro)	18.48 ± 0.03^{b}	20.45 ± 0.13^{a}	18.05 ± 0.09^{c}	17.85 ± 0.08^{c}	16.73 ± 0.09^{d}	< 0.001	< 0.001
Serine (Ser)	4.06 ± 0.05^{c}	4.37 ± 0.08^{d}	4.66 ± 0.00^{c}	5.44 ± 0.03^{b}	5.74 ± 0.01^{a}	< 0.001	< 0.001
Tyrosine (Tyr)	3.57 ± 0.06^{c}	2.61 ± 0.04^{e}	4.92 ± 0.01^{a}	3.19 ± 0.01^{d}	3.85 ± 0.02^{b}	< 0.001	0.001
Total	75.27 ± 0.36^{a}	76.78 ± 0.20^{a}	76.48 ± 0.29^{a}	76.53 ± 0.32^{a}	76.77 ± 0.38^{a}	0.001	0.014
Non-proteinogenic amino acids							
Phosphoserine (p-Ser)	0.05 ± 0.00^{c}	0.12 ± 0.01^{b}	0.11 ± 0.02^{b}	0.08 ± 0.01^{bc}	0.20 ± 0.03^{a}	< 0.001	0.035
Taurine (Tau)	1.69 ± 0.08^{b}	1.78 ± 0.03^{b}	1.29 ± 0.02^{c}	1.80 ± 0.01^{b}	2.29 ± 0.02^{a}	< 0.001	< 0.001
Cystathionine (Cysthi)	0.42 ± 0.01^{b}	0.52 ± 0.02^{ab}	0.49 ± 0.04^{ab}	0.23 ± 0.02^{c}	0.65 ± 0.01^{a}	0.244	0.028
β Alanine (β-Ala)	0.05 ± 0.01^{b}	0.06 ± 0.01^{b}	0.08 ± 0.05^{b}	0.18 ± 0.02^{a}	0.19 ± 0.03^{a}	< 0.001	0.012
β Amino isobutyric acid (β -AiBA)	–	–	0.60 ± 0.01^{a}	0.37 ± 0.01^{b}	0.21 ± 0.02^{c}	< 0.001	0.001
ϒ Amino butyric acid (ϒ— ABA)	0.39 ± 0.06^{a}	0.09 ± 0.01^{b}	0.02 ± 0.00^{c}	–	0.42 ± 0.01^{a}	0.497	< 0.001
Hydroxylysine (Hylys)	0.47 ± 0.04^{a}	0.41 ± 0.01^{a}	0.17 ± 0.01^{b}	0.08 ± 0.01^{c}	0.03 ± 0.00^{d}	< 0.001	0.084
1 Methyl histidine (1 Mehis)	0.09 ± 0.02^{c}	0.09 ± 0.00^{c}	0.05 ± 0.01^{d}	0.19 ± 0.01^{b}	0.27 ± 0.01^{a}	< 0.001	< 0.001
Hydroxyproline (Hypro)	0.70 ± 0.10^{d}	1.23 ± 0.10^{c}	1.87 ± 0.07^{b}	2.12 ± 0.03^{a}	2.15 ± 0.03^{a}	< 0.001	< 0.001
Total	3.86 ± 0.16^{c}	4.30 ± 0.19^{b}	4.68 ± 0.22^{b}	4.43 ± 0.08^{b}	6.41 ± 0.14^{a}	< 0.001	< 0.001
Total amino acids	140.05 ± 0.08^{b}	142.01 ± 1.60^{b}	142.41 ± 0.21^{b}	145.65 ± 0.37^{a}	148.72 ± 0.25^{a}	< 0.001	0.129

Table 3. Amino acid composition of *Cyprinus carpio* cultured under five different feeding regimes (g kg^{-1} of fresh weight). Values (means ± SE, n = 3) in each row with different superscript are significantly different ($p < 0.05$). The polynomial orthogonal contrast was considered significant at $p < 0.05$ level. LM0 = Control, soybean; LM5 = 5% *L. minor*; LM10 = 10% *L. minor*; LM15 = 15% *L. minor*; LM20 = 20% *L. minor.*

Fatty acid composition of fish. Total n-3 PUFA content of fish increased with the increasing levels of duckweed in the diets (Table 4). The n-3 fatty acids found in the highest proportions in common carp were eicosatetraenoic acid (ETA 20:4n-3) and DHA, but it was particularly noteworthy that all n-3 PUFA increased in a generally graded manner with the graded increase in dietary duckweed inclusion. Thus, while the increased levels of ALA, the only n-3 PUFA present in the diets, could perhaps be expected, the more interesting result was that the levels of longer, more unsaturated n-3 PUFA including ETA, EPA, docosapentaenoic acid (DPA, 22:5n-3) and DHA also increased significantly with increasing dietary inclusion of *L. minor*. The levels of DPA, DHA and EPA + DHA were all two-fold higher in carp fed diet LM20 with highest duckweed inclusion compared to fish fed control diet LM0 with no duckweed (Table 4). In contrast, n-6 PUFA contents that were dominated by LOA were lower in carp fed the duckweed-based diets compared to fish fed the control diet LM0 and, consequently, the n-6 PUFA: n-3 PUFA ratio decreased from around 6 in carp fed the control diet to 3.8 in fish fed the highest inclusion of duckweed (LM20). However, monounsaturated fatty acids (MUFA), dominated by oleic acid (18:1n-9) regardless of diet, were even more reduced than n-6 PUFA by dietary inclusion of duckweed. Palmitic acid (16:0) was the dominant saturated fatty acid (SFA) in the carp and significantly higher 16:0 and SFA was found in fish fed diet LM5 compared to fish fed the other treatments. Both total SFA and total MUFA decreased linearly with the increased level of duckweed in the diet of carp.

Fatty acids	LM0	LM5	LM10	LM15	LM20	Polynomial contrasts	
						Linear	Quadratic
Saturated fatty acids (SFA)							
14:0	27.32 ± 0.89^{a}	23.61 ± 0.62^{b}	17.26 ± 0.41^{c}	16.39 ± 0.16^{c}	11.39 ± 1.26^{d}	< 0.001	0.111
15:0	4.45 ± 0.01^{c}	6.10 ± 0.48^{b}	5.48 ± 0.98^{c}	7.34 ± 0.08^{a}	6.39 ± 0.28^{b}	< 0.001	0.031
16:0	383.00 ± 0.01^{b}	434.12 ± 1.74^{a}	354.66 ± 6.64^{c}	364.53 ± 5.15^{c}	320.44 ± 5.60^{d}	< 0.001	< 0.001
18:0	1.90 ± 0.02^{c}	4.69 ± 0.20^{a}	3.31 ± 0.68^{b}	1.38 ± 0.39^{c}	3.15 ± 0.45^{b}	0.289	0.008
24:0	10.53 ± 0.07^{c}	8.49 ± 0.36^{d}	11.03 ± 0.09^{b}	9.79 ± 0.18^{c}	12.89 ± 0.65^{a}	< 0.001	< 0.001
Total SFA	427.21 ± 0.89^{b}	477.01 ± 1.31^{a}	391.74 ± 7.44^{c}	399.44 ± 4.78^{c}	354.26 ± 7.86^{d}	< 0.001	< 0.001
Monounsaturated fatty acids (MUFA)							
16:1n-7	26.44 ± 0.01^{a}	23.46 ± 0.93^{b}	8.21 ± 0.67^{c}	4.68 ± 0.41^{d}	2.29 ± 0.19^{e}	< 0.001	< 0.001
18:1n-9	1014.10 ± 0.40^{a}	959.98 ± 0.70^{b}	795.25 ± 5.50^{c}	745.45 ± 1.29^{d}	608.05 ± 6.44^{e}	< 0.001	< 0.001
24:1	2.52 ± 0.03^{c}	2.55 ± 0.37^{c}	3.23 ± 0.05^{b}	5.26 ± 0.37^{a}	2.78 ± 1.12^{bc}	0.010	0.012
Total MUFA	1043.06 ± 0.42^{a}	985.99 ± 0.13^{b}	806.69 ± 6.19^{c}	755.39 ± 1.32^{d}	613.12 ± 3.13^{e}	< 0.001	< 0.001
Polyunsaturated fatty acids (PUFA)							
18:2n-6 (LOA)	1087.98 ± 0.31^{a}	948.68 ± 2.69^{d}	982.46 ± 9.67^{c}	1033.81 ± 8.04^{b}	1099.49 ± 0.08^{a}	< 0.001	< 0.001
20:2n-6	32.73 ± 1.75^{a}	17.43 ± 1.14^{c}	24.44 ± 1.47^{b}	26.23 ± 3.15^{b}	30.66 ± 1.91^{ab}	0.230	< 0.001
20:3n-6	16.25 ± 0.92^{a}	11.28 ± 1.42^{c}	12.42 ± 0.42^{bc}	13.92 ± 0.25^{b}	10.61 ± 0.17^{c}	< 0.001	0.057
20:4n-6	27.41 ± 0.50^{a}	22.39 ± 1.12^{b}	22.80 ± 0.63^{b}	26.65 ± 1.67^{a}	21.51 ± 0.25^{b}	0.002	0.159
22:5n-6	66.97 ± 1.97^{a}	54.07 ± 0.34^{b}	49.53 ± 0.13^{c}	56.78 ± 0.99^{b}	46.22 ± 0.02^{d}	< 0.001	< 0.001
Total n-6 PUFA	1231.34 ± 1.52^{a}	1053.86 ± 8.75^{e}	1091.65 ± 8.85^{d}	1157.40 ± 7.20^{c}	1208.48 ± 2.42^{b}	0.002	< 0.001
18:3n-3 (ALA)	15.22 ± 0.01^{c}	14.92 ± 0.69^{c}	30.05 ± 0.33^{b}	38.35 ± 2.14^{a}	38.32 ± 0.78^{a}	< 0.001	0.021
20:4n-3	128.04 ± 1.36^{d}	166.65 ± 0.40^{a}	150.88 ± 4.39^{c}	161.30 ± 0.74^{ab}	156.96 ± 2.57^{bc}	< 0.001	< 0.001
20:5n-3 (EPA)	3.95 ± 0.02^{c}	5.01 ± 0.34^{b}	5.96 ± 0.05^{a}	6.00 ± 0.40^{a}	5.28 ± 0.12^{b}	< 0.001	< 0.001
22:5n-3	5.39 ± 0.01^{d}	9.09 ± 0.35^{c}	11.54 ± 0.14^{a}	11.10 ± 0.01^{ab}	10.42 ± 0.09^{b}	< 0.001	< 0.001
22:6n-3 (DHA)	53.60 ± 5.01^{e}	81.01 ± 1.77^{d}	86.01 ± 0.52^{c}	91.03 ± 1.61^{b}	107.50 ± 1.68^{a}	< 0.001	0.003
Total n-3 PUFA	206.20 ± 6.54^{d}	276.68 ± 2.81^{c}	284.44 ± 6.02^{c}	307.78 ± 1.67^{b}	318.48 ± 5.21^{a}	< 0.001	< 0.001
EPA + DHA	57.55 ± 5.01^{e}	86.02 ± 2.11^{d}	91.97 ± 0.58^{c}	97.04 ± 1.21^{b}	112.79 ± 1.57^{a}	< 0.001	0.001
n-6/n-3	5.97 ± 0.03^{a}	3.80 ± 0.01^{b}	3.84 ± 0.03^{b}	3.76 ± 0.02^{b}	3.79 ± 0.00^{b}	< 0.001	< 0.001

Table 4. Fatty acid composition of *Cyprinus carpio* cultured under five different feeding regimes (mg 100 g^{-1} of fresh weight). Values (means ± SE, n = 3) in each row with different superscript are significantly different ($p < 0.05$). The polynomial orthogonal contrast was considered significant at $p < 0.05$ level. LM0 = Control, soybean; LM5 = 5% *L. minor*; LM10 = 10% *L. minor*; LM15 = 15% *L. minor*; LM20 = 20% *L. minor*.

Gene expression. The expression levels of delta-6-desaturase (*fads2d6*) was significantly higher in common carp fed diets LM10, LM15 and LM20 compared to fish fed the control LM0 diet (Fig. 1a). The expression of elongation of very long-chain fatty acids protein 2 (*elovl2*) was significantly higher in LM20 diet fed common carp compared to others (Fig. 1b). There was up-regulation of elongation of very long chain fatty acids protein 5 (*elovl5*) in LM10, LM15 and LM20 diets fed fish compared to other group of fish (Fig. 1c). The fatty acid synthase (*fas)* expression was significantly higher in LM20 diet fed common carp compared to others (Fig. 1d). Expression levels of all the genes, except *fads2d6*, were lower in fish fed diet LM5 compared to carp fed the control diet. A polynomial relationship was found between the dietary inclusion levels of duckweed and expressions of various genes.

Discussion

The duckweed-based diets influenced the growth of common carp in the present study. Final weight and SGR of fish increased linearly as dietary level of duckweed inclusion increased. Similar results were observed in common carp fed diets supplemented with 30–50% *Lemna minuta*[31,32], grass carp *Ctenopharyngodon idella* fed water hyacinth *Eichhornia crassipes* leaf meal[35] and rohu *Labeo rohita* fed 20% processed *Pistia* leaves[36]. Feeding of Nile tilapia with fermented *L. minor* supplemented diet (2.5%) enhanced the growth and survival rate of fish[37]. Fresh *L. minor* was supplied with a commercial diet (protein 32%) of juvenile Nile tilapia[38]. Higher final body weight and SGR were found in the experimental diet fed fish compared to the control diet fed one. In the present study, the FCR of carp fed the duckweed-based diets was lower compared to fish fed the control diet suggesting that replacement of soybean meal with duckweed satisfied the nutritional requirements of common carp. Similar trends in FCR were also recorded previously in common carp fed diets including *L. minor* and *L. minuta*[30,32].

Digestive enzymes play a significant role in the utilization of diets[39] with the activities of enzymes affecting the efficiency of nutrient absorption, and so their characterization provides key information on the digestive ability of fish to hydrolyze protein, lipid, and carbohydrate in diets[40]. In the present study, amylase activity reduced in carp fed the duckweed-based diets compared to the fish fed the control diet. Similarly, lower amylase activity was observed in rohu fed diets formulated with raw *Pistia* leaves compared to fish fed a reference diet without *Pistia*[36].

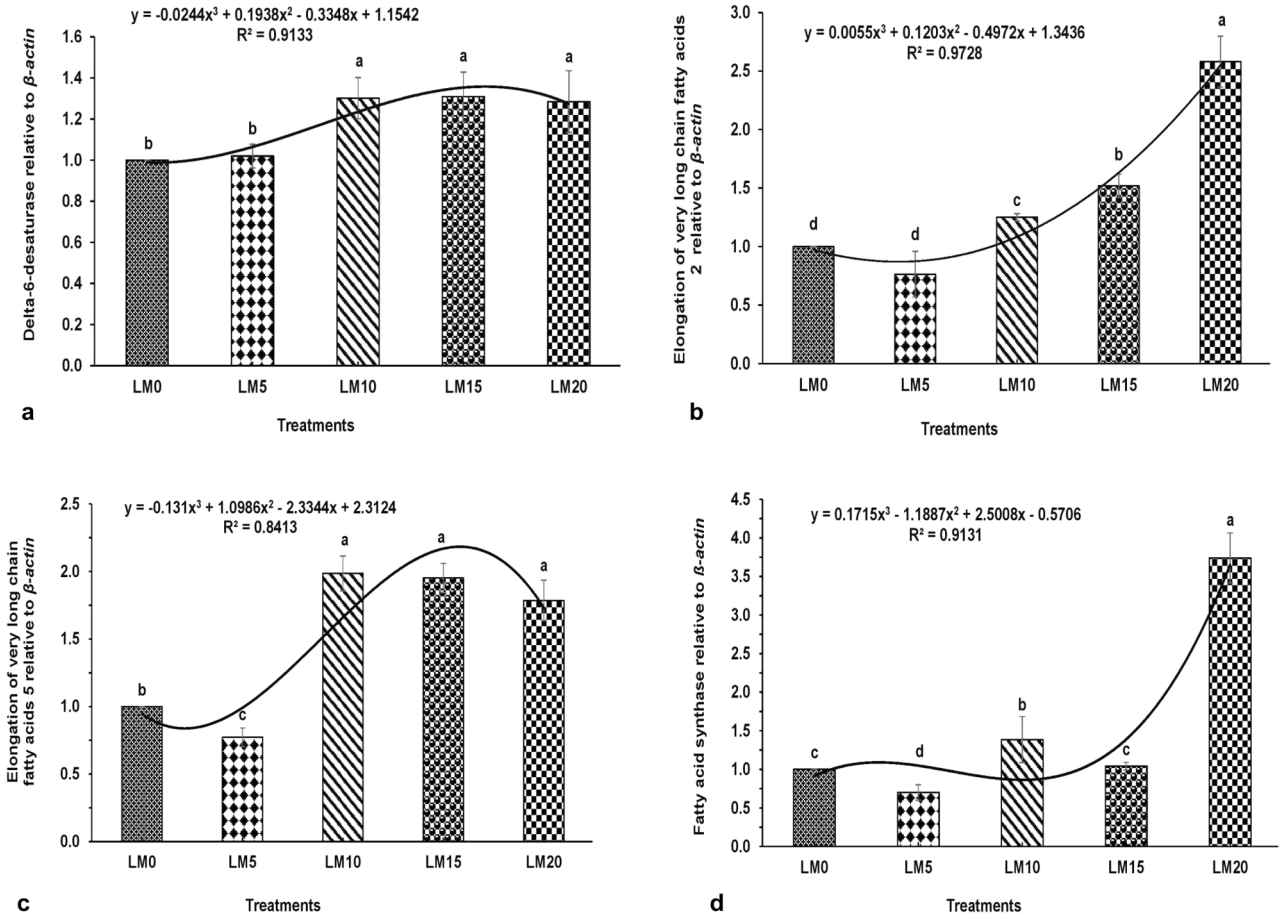

Figure 1. Expressions of (**a**) delta-6-desaturase (*fads2d6)*, (**b**) elongation of very long chain fatty acids protein 2 (*elovl2*), (**c**) elongation of very long chain fatty acids protein 5 (*elovl5*) and (**d**) fatty acid synthase (*fas*) relative to *β-actin* in hepatopancreas of common carp *Cyprinus carpio* cultured under five different feeding regimes. The polynomial (order 3) relationships were found between the diets and the expressions of *fads2d6* ($R^2 = 0.913$), *elovl2* ($R^2 = 0.973$), *elovl5* ($R^2 = 0.841$) and *fas* ($R^2 = 0.913$). Bars with different superscripts are significantly ($p < 0.05$; n = 3) different.

However, protein digestion in common carp was influenced by the duckweed-based diets with total protease, trypsin and chymotrypsin activities all significantly higher in fish fed the diets with the two highest levels of duckweed inclusion (LM20 and LM15) compared to fish fed the other diets. Similar results were reported in rohu fed a pelleted diet containing *L. minor*[15]. Higher activities of these enzymes suggested enhanced protein digestion in common carp fed the duckweed-based diets, which indicated that the consumed feed was used more efficiently, resulting in lower FCR and increased growth. Lower protease, trypsin, and chymotrypsin activities were recorded in carp fed the soybean-based diet in the present study. Lower enzyme activities were also observed in Atlantic salmon *Salmo salar*[41], Nile tilapia[42] and Japanese seabass *Lateolabrax japonicus*[43] fed soybean meal-based diets although, in these studies, the reference/control feeds were based on fishmeal. The presence of anti-nutritional factors including protease inhibitors may reduce digestive enzyme activities in these fishes[11,43,44].

The proximate composition of the carp showed that lipid contents were higher (and moisture lower) in fish fed the duckweed supplemented diets compared to fish fed the control diet. This was consistent with the findings of the previous studies[30,32] with common carp fed diets containing duckweeds. Higher lipid content was recorded in *I. aquatica* leaf meal and bio-processed *Pistia* leaves supplemented diets fed rohu[36,45]. These data may reflect higher lipid (fatty acid) biosynthesis, as evidenced by the increased expression of fatty acid synthase (*fas*), and tissue lipid deposition. Higher expression of *fas* was observed in tilapia fed palm oil-based diets[46].

Total amino acids contents (g kg^{-1}) would tend to increase in the present study with increasing inclusion of duckweed reflecting the increased protein content in fish fed the highest levels of *L. minor*. The amino acids content varied in different ingredients and thereby, their amount in five different diets. Finally, the composition of diets influenced the amino acid contents of common carp fed with five different diets. The composition of the diets influenced digestibility of the ingested feed. The in vitro digestibility study showed that the digestibility of *L. minor* (6.03 ± 1.78%) was significantly higher compared to soybean meal (3.35 ± 0.01%) in common carp[47]. Higher protein contents were reported in the muscles of *L. minor* supplemented diet fed grass carp and silver carp compared to the soybean supplemented diet fed fishes[33]. Duckweeds exhibit a well-balanced, highly bioavailable source of amino acids for fish[17,28,48], with essential amino acids such as arginine, histidine, isoleucine, leucine,

phenylalanine, threonine, valine, cysteine, and methionine present in relatively high contents in *L. minor*[15,16]. Therefore, duckweeds are regarded as a rich source of essential amino acids that can generally satisfy the amino acid requirements of common carp[5]. In this context, it was noteworthy that carp fed diets LM15 and LM 20 had higher total levels of essential amino acids with arginine, methionine, isoleucine, threonine, tryptophan and valine generally increasing with inclusion of duckweed. Thus, feeding of common carp with duckweed-based diets improved the amino acid composition of the fish.

Fish are an almost unique source of the health beneficial n-3 LC-PUFA, EPA and DHA, for humans[49,50]. Previously, higher levels of EPA, DHA and n-3 PUFA were recorded in Nile tilapia fed diets including *Azolla filiculoides*[51] and duckweeds are a good source of ALA, that is the precursor of n-3 LC-PUFA[16,17,52]. Therefore, in the present study, one focus was to determine if dietary duckweed can boost the levels of n-3 LC-PUFA in carp. The duckweed used in the present study was cultured with the addition of organic fertilizers that can boost lipid content to over 8% of dry weight and ALA content to over 40% of total fatty acids[16]. Furthermore, the inclusion of duckweed in the feeds was at the expense of soybean meal, wheat and corn flours, that are all derived from seeds where n-6 PUFA, specifically 18:2n-6, dominate the fatty acid content. Consequently, increasing inclusion of duckweed increased dietary ALA content by 14-fold while 18:2n-6 PUFA was reduced by around 20% with the n-6 PUFA: n-3 PUFA ratio in the feeds decreasing 17-fold from around 290 to 17. Thus, despite the relatively low lipid content that has precluded aquatic plants like duckweed from being regarded as dietary lipid sources, the inclusion of duckweed had a beneficial impact on the fatty acid composition of the carp feed[16].

While the increased dietary ALA was also reflected in increased ALA in carp in the present study, more importantly the levels of the n-3 LC-PUFA, ETA, EPA, DPA and DHA also all increased in the carp with the graded increased inclusion of duckweed in the diet. As the feeds were free of any marine ingredients and thus devoid of n-3 LC-PUFA, this result clearly indicated that there was active bioconversion of the dietary ALA, supplied by the duckweed, to DHA in carp resulting in accumulation of n-3 LC-PUFA. This confirmed the earlier findings that freshwater fishes, both herbivorous and omnivorous have the metabolic capacity to convert dietary ALA to EPA and DHA[24,53]. In the present study, the compositional data was supported by the gene expression studies. Several genes including delta-6-desaturase (*fads2d6*), elongation of very long-chain fatty acids protein 2 (*elovl2*) and elongation of very long chain fatty acids protein 5 (*elovl5*) play significant roles in the bioconversion of fatty acids[21,22,54]. Previous studies on the gene expression response of fish after replacing marine ingredients with plant-based diets/vegetable oils showed that the LC-PUFA biosynthesis pathway was stimulated in liver and intestine of various fish species[55–58]. In the present study, the *fads2d6*, *elovl2* and *elovl5* genes were all up-regulated in the hepatopancreas of common carp fed the duckweed diets compared to fish fed the control diet. This was consistent with previous studies that showed a higher level of dietary ALA increased expression levels of *fads2d6* and *elovl5* in common carp[59], and *fads2d6* and *elovl2* expression levels were up-regulated in juvenile and ongrowing rainbow trout fed plant-based diets[58]. Therefore, the higher levels of EPA + DHA in fish fed the duckweed-based diets confirmed the ability of common carp to synthesize LC-PUFA endogenously from dietary precursor, ALA through increased expression (and activity) of the biosynthetic desaturase and elongase enzymes.

In conclusion, the present study demonstrated that up to 20% duckweed *L. minor* can be included in feeds as a replacement for plant meals including soybean meal without affecting the survival or growth of common carp. The inclusion of duckweed in feeds also enhanced the nutritional value of the carp by increasing protein, lipid, and amino acids levels. Furthermore, duckweed also increased the ALA content of the feed and this enhanced the contents of n-3 LC-PUFA, including EPA and DHA of the carp.

Materials and methods

Ingredients and feed formulation. The duckweed *L. minor* was grown in outdoor cement tanks (150 L) fertilized with a mixture of organic manures including cattle manure, poultry wastes and mustard oil-cake as described in detail previously[16]. The duckweed was harvested, washed with clean water, and dried in an oven at 40 °C for 3 h. Other feed ingredients including soybean meal (Ruchi Soya Industries Limited, Mumbai, India), wheat flour (Aashirvaad Atta, ITC Limited, Bangalore, India), corn flour (Ahaar, Private Limited, New Delhi, India), sunflower oil (Fortune, Adani Wilmar Limited, Gujarat, India), amino acids (Himedia, Mumbai, India), vitamin and mineral premixes (Piramal Enterprises Limited, Mumbai) were purchased from the local market. After grinding, ingredients were sieved and stored at 4 °C prior to diet manufacture. The crude protein content of soybean meal, *L. minor*, wheat flour and corn flour were 50.0, 36.07, 12.0 and 12.0%, respectively. The lipid contents of soybean meal, *L. minor*, wheat flour and corn flour were 1.0, 8.45, 1.0 and 1.0%, respectively.

Five marine ingredient-free diets were formulated to contain 32% crude protein and 7% crude lipid using the Winfeed 2.8 software package (WinFeed (UK) Limited, Cambridge, UK) (Table 5). The control diet was based on plant meals with soybean meal as the major protein source (LM0). In four further experimental diets, duckweed was included at increasing levels of 5% (LM5), 10% (LM10), 15% (LM15) and 20% (LM20) of diet dry weight, largely at the expense of soybean meal, wheat flour and sunflower oil to maintain the diets as isonitrogenous, isolipidic and isoenergetic. Earlier study showed that in diet 20% inclusion of *L. minor* affected the survival rate of common carp[30]. Hence, in the present study the maximum inclusion of the duckweed was 20%. The amino acids viz. histidine, methionine, lysine and threonine were added in all diets[5]. All the dry ingredients were blended for 10 min and then mixed with the oil and warm water and feed pellets (1 mm diameter) produced using a BTPL Twin-screw-extruder (Basic Technology Private Limited, Kolkata, India). The pellets extrusion conditions were as follows: cutter rpm 134; feeder rpm 10; extrusion rpm 190; extrusion torque 9.22; heater 1 and heater 2 temperature 65 and 70 °C; final mass temperature 75 °C. The diets were dried at 40 °C before being stored at 4 °C prior to use. The proximate composition study showed that protein, lipid, carbohydrate and ash contents of *L. minor* were 360.70 ± 1.80, 84.50 ± 6.10, 340.70 ± 3.60 and 214.12 ± 2.00 g kg^{-1} (dry weight), respectively[16]. All essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine),

Ingredients	LM0	LM5	LM10	LM15	LM20		
Soybean meal	500.0	475.0	450.0	425.0	400.0		
Lemna minor	–	50.0	100.0	150.0	200.0		
Wheat flour	245.0	223.0	201.0	179.0	158.0		
Corn flour	147.0	147.0	146.0	146.0	146.0		
Sunflower oil	62.0	58.0	55.0	51.0	47.0		
Vitamin/minerals premix[1]	5.0	5.0	5.0	5.0	5.0		
Mono calcium phosphate	20.0	21.0	23.0	24.0	25.0		
Choline chloride	1.0	1.0	1.0	1.0	1.0		
Histidine	1.0	1.0	1.0	1.0	1.0		
Methionine	12.0	12.0	11.0	11.0	10.0		
Lysine	5.0	5.0	5.0	5.0	5.0		
Threonine	2.0	2.0	2.0	2.0	2.0		
						Polynomial contrasts	
						Linear	Quadratic
Proximate analysis (% dry matter basis)							
Moisture	6.34 ± 0.08[a]	6.38 ± 0.10[a]	6.20 ± 0.02[a]	6.27 ± 0.04[a]	6.21 ± 0.5[a]	0.089	0.902
Crude protein	31.61 ± 0.14[a]	32.05 ± 0.16[a]	31.42 ± 0.15[a]	31.68 ± 0.21[a]	31.63 ± 0.09[a]	0.285	0.796
Crude lipid	7.42 ± 0.06[a]	7.44 ± 0.12[a]	7.48 ± 0.13[a]	7.35 ± 0.04[a]	7.32 ± 0.05[a]	0.324	0.444
Carbohydrate	47.51 ± 0.08[a]	46.59 ± 0.10[a]	47.19 ± 0.09[a]	47.00 ± 0.09[ab]	46.02 ± 0.07[b]	0.003	0.257
Crude ash	7.22 ± 0.22[a]	7.54 ± 0.07[a]	7.72 ± 0.04[a]	7.70 ± 0.04[a]	7.82 ± 0.08[a]	0.001	0.077
Energy value (Kcal 100 g^{-1})	382.52 ± 0.36[a]	381.52 ± 0.44[a]	381.73 ± 0.58[a]	381.79 ± 0.60[a]	377.38 ± 0.31[a]	0.472	0.075

Table 5. Ingredients, dietary formulations (g kg^{-1}) and analysed proximate compositions of the control and experimental diets. LM0 = Control, soybean; LM5 = 5% *L. minor*; LM10 = 10% *L. minor*; LM15 = 15% *L. minor*; LM20 = 20% *L. minor.* [1]Supradyn multivitamin tablets (Piramal Enterprises Ltd., Mumbai, India) containing minerals and trace elements (as mg/kg in diets): = Vitamin A (as acetate) 12; Cholecalciferol 0.1; Thiamine mononitrate, 40; Riboflavine 40; Pyridoxine hydrochloride, 12; Cyanocobalamin, 0.06; Nicotinamide, 400; Calcium pantothenate 65.20; Ascorbic acid 600; α-Tocopheryl acetate,100; Biotin, 1.00. Minerals: Tribasic calcium phosphate, 516; Magnesium oxide, 240; Dried ferrous sulphate, 128.16; Manganse sulphate monohydrate 8.12; Total phosphorus, 103.20. Trace elements: Copper sulphate pentahydrate 13.56; Zinc sulphate, 8.80; Sodium molybdate dihydrate, 1.00; Sodium borate 3.52.

non-essential (alanine, arginine, aspartate, cysteine, glutamic acid, glycine, proline, serine and tyrosine) and non-proteinogenic (citrulline, hydroxiproline, taurine etc.) amino acids were found in *L. minor*. The fatty acid composition of *L. minor* was as follows: saturated fatty acids (SFA) 23–26%, monounsaturated fatty acids (MUFA) 11–12%, n-6 polyunsaturated fatty acids (PUFA), mostly linoleic acid (LOA) 17–18% and n-3 PUFA (α-linolenic acid) 41–47%. Based on this finding, the diets were prepared for the present study. The fatty acid compositions of the diets are presented in Table 6 and the amino acid compositions in Supplementary Table 1.

Fish culture and feeding. The Institutional Animal Ethics Committee (IAEC) of Delhi University approved the animal care and experimental procedure (DU/ZOOL/IAEC-R/2015/07). The following study was conducted in accordance with relevant guidelines and regulations. The study was carried out compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guideline.

Fry of common carp were collected from the local fish farm and fed the control diet for 1 week to acclimatize to the experimental conditions. At the initiation of the experiment a total of 450 fry (average weight, 0.47–0.48 g) were distributed randomly among 15 aquaria (30 fry 60 L $aquarium^{-1}$), Each aquarium was fed one of the five diets, LM0, LM5, LM10, LM15 and LM20, for 60 days, with each diet allocated randomly to three aquaria (n = 3). The fish were fed ad libitum to apparent satiation twice per day at 9.00 a.m. and 5.00 p.m. Any uneaten feed was collected after 1 h of feeding to provide an estimation of feed consumption rate. The dissolved oxygen level in the aquarium was maintained by an aerator, and an external filter (Eheim Classic 600, Germany) was employed to reduce ammonia level and maintain water quality of each aquarium. The water temperature (27.8–28.1 °C), pH (7.40–7.62), dissolved oxygen (5.5–5.82 mg L^{-1}), ammonia (0.02–0.05 mg L^{-1}), nitrate (0.85–1.01 mg L^{-1}) and conductivity (380.60–427.03 μS cm^{-1}) were regularly monitored in each aquarium using a digital water quality multi-parameter instrument (Hach HQ 40D; Loveland, Colorado, USA). The concentrations of nitrite (0.25–0.48 mg L^{-1}) and phosphate (0.08–0.12 mg L^{-1}) were measured regularly[60]. There were no significant differences in water quality parameters among the five dietary treatments throughout the study period.

Sampling. After 60 days of culture, the fish were starved for one day prior to the fish being harvested and euthanized with tricaine methane sulphonate (MS-222; Sigma-Aldrich, USA). The number of fish in each aquarium was counted and recorded to determine survival rate (SR), and the weight of individual fish was measured.

Fatty acids	LM0	LM5	LM10	LM15	LM20	Polynomial contrasts	
						Linear	Quadratic
Saturated fatty acids (SFA)							
14:0	10.44 ± 0.07^{a}	9.55 ± 0.06^{a}	8.82 ± 0.04^{a}	10.35 ± 0.20^{a}	10.88 ± 2.00^{a}	0.361	0.034
15:0	5.20 ± 0.01^{a}	4.59 ± 0.17^{b}	2.55 ± 0.01^{c}	0.81 ± 0.01^{d}	0.04 ± 0.00^{e}	< 0.001	0.981
16:0	581.01 ± 8.96^{a}	531.63 ± 8.38^{b}	531.73 ± 1.54^{b}	535.31 ± 4.55^{b}	538.91 ± 8.96^{b}	0.002	< 0.001
18:0	70.09 ± 0.99^{a}	64.13 ± 0.93^{b}	56.09 ± 0.27^{c}	66.21 ± 1.04^{b}	40.68 ± 0.51^{d}	< 0.001	< 0.001
20:0	3.26 ± 0.36^{a}	2.98 ± 0.33^{a}	2.44 ± 0.08^{ab}	3.12 ± 0.56^{a}	1.85 ± 0.25^{b}	0.005	0.414
22:0	0.69 ± 0.01^{b}	0.75 ± 0.01^{b}	0.14 ± 0.00^{d}	0.31 ± 0.07^{c}	0.90 ± 0.10^{a}	0.814	< 0.001
24:0	15.72 ± 2.77^{a}	16.77 ± 0.14^{a}	9.00 ± 0.46^{b}	8.29 ± 0.83^{b}	17.25 ± 2.76^{a}	0.250	0.001
Total SFA	686.41 ± 6.73^{a}	630.40 ± 8.97^{b}	610.77 ± 3.09^{b}	624.40 ± 3.65^{b}	610.51 ± 8.36^{b}	< 0.001	< 0.001
Monounsaturated fatty acids (MUFA)							
18:1n-9	1460.56 ± 3.81^{a}	1336.44 ± 3.95^{b}	1163.59 ± 6.70^{c}	1163.69 ± 2.02^{c}	1059.34 ± 2.20^{d}	< 0.001	< 0.001
20:1n-9	7.86 ± 1.24^{b}	8.19 ± 0.13^{a}	4.09 ± 0.28^{c}	3.46 ± 0.33^{c}	3.07 ± 0.26^{c}	< 0.001	0.369
Total MUFA	1468.42 ± 2.57^{a}	1344.63 ± 3.81^{b}	1167.68 ± 6.43^{c}	1167.15 ± 1.69^{c}	1062.41 ± 2.63^{d}	< 0.001	< 0.001
Polyunsaturated fatty acids (PUFA)							
18:2n-6 (LOA)	3197.60 ± 22.99^{a}	2925.76 ± 21.95^{b}	2862.71 ± 6.24^{b}	2769.39 ± 16.46^{c}	2600.36 ± 22.03^{d}	< 0.001	0.008
20:2n-6	1.09 ± 0.02^{cd}	1.92 ± 0.04^{b}	1.38 ± 0.10^{c}	0.92 ± 0.12^{d}	3.41 ± 0.21^{a}	< 0.001	< 0.001
18:3n-3 (ALA)	10.98 ± 1.45^{e}	51.10 ± 1.89^{d}	81.29 ± 1.35^{c}	115.44 ± 0.02^{b}	153.10 ± 0.26^{a}	< 0.001	0.741
Total PUFA	3209.67 ± 23.42^{a}	2978.78 ± 22.80^{b}	2945.38 ± 6.50^{bc}	2885.75 ± 16.46^{c}	2756.87 ± 21.82^{d}	< 0.001	0.006
n-6/n-3	291.32 ± 11.23^{a}	57.29 ± 2.55^{b}	35.23 ± 0.51^{c}	23.997 ± 0.21^{d}	17.01 ± 0.11^{e}	< 0.001	< 0.001

Table 6. Fatty acid composition of experimental and control diets (mg 100 g^{-1} of dry weight). LM0 = Control, soybean; LM5 = 5% *L. minor*; LM10 = 10% *L. minor*; LM15 = 15% *L. minor*; LM20 = 20% *L. minor.*

The specific growth rate (SGR), feed intake (FI) and feed conversion ratio (FCR) were calculated according to the following formulae.

$$\text{SR}\ (\%) = 100 \times \text{final number of fish/initial number of fish}$$

$$\text{SGR}\ (\%) = \left(\text{In final body mass} - \text{In initial body mass}\right) \times 100/\text{duration of culture}\ \left(\text{days}\right)$$

$$\text{FI}\ \left(\text{g}/100\ \text{g body weight/day}\right) = 100 \times \text{total feed fed}\ \left(\text{dry matter}\right)/[(\text{initial weight} + \text{final weight} + \text{dead fish weight})/2 \times \text{days}]$$

$$\text{FCR} = \text{feed}\ \left(\text{dry weight}\right)\ \text{consumed by fish individually during feeding trial/weight gain}\ \left(\text{wet weight}\right)\ \text{of individual fish}$$

For carcass composition, three fish from each aquarium were collected and pooled, this pooled sample served as one biological replicate (3 replicates per dietary treatment; n = 3). The entire digestive tracts (esophageal sphincter to anus) of another two fish per aquarium were dissected and pooled to provide 3 samples per diet (2 fish per aquarium, 6 fish per diet in 3 replicates; n = 3) for the study of digestive enzymes. The fish were kept in fasting condition for 24 h before sampling and the digestive tract was empty. The hepatopancreas of three individual fish per treatment (1 fish per aquarium, 3 fish per diet; n = 3) were dissected into TRIzol reagent (Ambion, Life Technologies, USA) for the gene expression study. Fish samples (number of fish for assays) were collected following the recommendation of IAEC. All samples were stored at − 80 °C prior to analyses.

Biochemical compositions of diets and fish. The biochemical compositions of diets and whole fish were determined following the procedures of the Association of Official Analytical Chemists (AOAC) International[61]. Diet samples were ground and the pooled fish samples (three fish) were blended to form a homogeneous paste prior to analyses. Moisture content was determined gravimetrically after drying samples in an oven at 105 °C for 3 h. Ash content was determined after incinerating the oven dried sample in a muffle furnace at 550 °C for 8 h. Crude protein content was determined by the Kjeldahl method (Nitrogen × 6.25; Pelican Instruments, Chennai, India). Crude lipid was determined following the chloroform/methanol extraction method[62]. The subtraction method was used for the determination of carbohydrate contents of feeds[63], and the energy value of each sample was calculated[64]. All samples were assayed in triplicate.

Amino acid analysis. Amino acid contents of diets and whole fish were analyzed using a dedicated Hitachi L-8900 High-Speed Amino Acid Analyzer (Hitachi Co. Ltd., Tokyo, Japan). Firstly, samples were hydrolyzed with 6 N HCl at 110 °C for 22 h under N_2 atmosphere other than tryptophan that was hydrolyzed in a separate sample using 4 N methanesulfonic acid and 3-(2-aminoethyl) indole. Methionine and cysteine were oxidized with performic acid before acid digestion. After digestion, all the samples were evaporated in a Nitrogen Evaporator

(PCi Analytic Private Limited, Maharashtra, India) before 0.02 N HCl was added to the dried samples to provide a protein concentration of 0.5 mg mL^{-1} in each sample and 1.5 mL was placed in a glass vial for the autosampler. The amino acids were separated using a cation-exchange resin column (4.6 mm ID × 60 mm L) with 3 µm particle size. The column temperature was 30–70 °C, reaction temperature was 135 °C with a ninhydrin flow rate of 0.35 mL min^{-1}. The amino acids were monitored at 570 nm other than proline and hydroxyproline that were monitored at 440 nm. The amino acids were compared with a standard amino acid solution (Wako Pure Chemical Industries Limited, USA). All samples were assayed in triplicate and concentrations expressed as g kg^{-1}.

Fatty acid analysis. The fatty acid compositions of diets and whole fish were measured by gas chromatography (GC). Total lipid was extracted from diet and fish paste samples after homogenization in chloroform: methanol (2:1, v/v) according[62]. Fatty acid methyl esters (FAME) were prepared from total lipid samples by transesterification using 1% sulphuric acid in methanol at 50 °C for 16 h[65]. FAME was separated on a Clarus 580 GC (PerkinElmer, USA) equipped with a ZB-wax column (60 m × 0.32 mm internal diameter × 0.25 µm; Phenomenex, UK). The data were collected from pre-installed programmed software (TotalChrom Workstation Ver6.3). The fatty acids present in diets and fish were identified by comparing the retention times of the sample peaks with a standard fatty acid mixture (Supelco FAME 37 mix, Sigma-Aldrich, USA) and published data[66]. All samples were assayed in triplicate and the concentration was expressed as mg 100 g^{-1}.

Digestive enzyme assays. The samples of whole digestive tract were freeze-dried and homogenized in chilled Milli-Q® water (1:10) to maintain neutral pH of the extracts. The homogenates were centrifuged at 10,000×*g* at 4 °C for 30 min and the tissue supernatants collected for assay of digestive enzyme activities by fluorometric methods (Multimode reader, BioTek Synergy H1 Hybrid, USA). Amylase activity was estimated using Ultra Amylase Assay kit (Invitrogen, USA), pH of the assay mixture was 6.9. The sample was incubated at 25 °C for 25 min. The fluorescence was recorded at 485 nm for excitation and 520 nm for emission. Total protease activity was estimated with EnzChek@ Protease Assay kit (Invitrogen) using phosphate buffer (pH 7.8) at 25 °C. The fluorescence was recorded at 485 nm for excitation and 530 nm for emission. Trypsin activity was estimated following the method[67] using Tris–HCl buffer (pH 8.0) and Na-benzoyl-L-arginin-methyl-coumarinylamide (Sigma-Aldrich, USA) as substrate. The sample was incubated at 30 °C for 10 min. Chymotrypsin activity was assayed using Tris–HCl buffer (pH, 7.5) and succinyl-Leu-Val-Tyr-4-methyl-coumaryl-7-aminde (Sigma-Aldrich) as substrate[68]. After 10 min of incubation at 37 °C, the fluorescence was recorded at 380 nm for excitation and 450 nm for emission. The substrate 4-methylumbelliferyl butyrate (4-MUB, Sigma-Aldrich) and Tris–HCl buffer (pH, 7.5) were used for the assay of neutral lipase activity[69]. Two sets of same samples were taken and one set was kept at 4 °C (ice bath) and the second one at 37 °C (water bath) for 10 min. The change in fluorescence was recorded at 365 nm for excitation and 450 nm for emission. The value obtained at 4 °C was subtracted from the value obtained at 37 °C. Protein content was estimated following the method using bovine serum albumin (Sigma-Aldrich) as standard[70]. All samples were assayed in triplicate.

Gene expression analysis. The mRNA expressions of *fads2d6*, *elovl2*, *elovl5* and *fas* genes were studied in the hepatopancreas of common carp. Total RNA was extracted in TRIzol reagent (Ambion, Life Technologies, USA) following the manufacturer's recommendations. The quality of extracted RNA was confirmed using a spectrophotometer (NanoDrop® ND-1000, Thermo Scientific, USA) and integrity of total RNA was determined on 1% agarose gel electrophoresis. The purified RNA (1 µg) was treated with DNase I (Amplification grade 1 kit, Sigma-Aldrich) to eliminate genomic DNA contamination. The DNase-treated RNA was reverse transcribed to cDNA using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). The cDNA sample for each tank was diluted and used for real-time qPCR to determine gene expression. Quantification of gene expression was performed by Quant Studio 6 Flex system (Applied Biosystems, USA) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA). Primers were designed using the online primer design tool of NCBI and *β-actin* was used as the reference gene (Supplementary Table 2). The reaction mixtures for qPCR (10 µL) were composed of 0.25 µL each of forward and reverse primers (2.5 µM), 1 µL of cDNA (1:3), 5 µL of 2X PowerUp™ SYBR™ Green PCR Master Mix and 3.5 µL of RNase-free water. All samples were run in duplicate with each reference gene and negative control (NTC; non template control, containing no cDNA). The cycling conditions of qPCR were as follows: initial activation step at 95 °C for 10 min followed by either 40 cycles of 95 °C for 15 s and 60 °C for 1 min (primer Tm at 60 °C) or 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 1 min (primer Tm < 60 °C). The data of qRT-PCR were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method with *β-actin* as the internal control[71].

Statistical analysis. Data were expressed as means ± standard error (SE) with n values as stated. The data were compared using one-way analysis of variance (ANOVA) followed where pertinent by Duncan's multiple range test, DMR[72]. The polynomial orthogonal contrasts were used to determine the linear and quadratic effect of increasing levels of dietary *L. minor*. The significance level was considered at ($p < 0.05$). All statistical analyses were performed using SPSS software (version 25.0, USA).

Ethical statement. The Institutional Animal Ethics Committee (IAEC) of Delhi University approved the animal care and experimental procedure (DU/ZOOL/IAEC-R/2015/07). The following study was conducted in accordance with relevant guidelines and regulations. The study was carried out compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guideline.

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R.C., D.R.T., J.G.S., B.D.G.: designed the study; R.K.G., R.C., J.G.S., A.K.S., G.K.: cultured the fish and analysed samples; R.C., D.R.T., B.D.G., J.G.S., R.K.G.: wrote the manuscript; R.K.G., R.C., D.R.T., J.G.S.: prepared graphs and tables.

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Effect of Greater Duckweed *Spirodela polyrhiza* Supplemented Feed on Growth Performance, Digestive Enzymes, Amino and Fatty Acid Profiles, and Expression of Genes Involved in Fatty Acid Biosynthesis of Juvenile Common Carp *Cyprinus carpio*

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The greater duckweed *Spirodela polyrhiza* (Lemnaceae) is a free-floating freshwater macrophyte. The effect of dietary inclusion of duckweed in the feed of common carp *Cyprinus carpio* fry was evaluated. The control feed (SP0) contained soybean meal as the primary protein source. In four experimental feeds, greater duckweed was incorporated at levels of 5% (SP5), 10% (SP10), 15% (SP15), and 20% (SP20) replacing soybean meal. Broken-line regression showed that incorporation of greater duckweed at 10 and 13.4% levels were the breakpoint for final weight and specific growth rate (SGR) of fish, respectively. The final weight and SGR of common carp fed diet SP20 were significantly higher compared with those of others. The feed conversion ratio was lowest in SP20 treatment. The inclusion of greater duckweed in the fish feeds showed linear relationships with amylase, trypsin, chymotrypsin, and lipase activities. The content of crude protein was significantly higher in SP10, SP15, and SP20 treatments compared with that of others. Significantly higher crude lipid and ash contents were found in SP20 diet-fed fish compared with other diet-fed fish. The essential amino acids composition was similar in five different diet-fed fish. The greater duckweed supplemented feeds influenced the fatty acid contents of fish. The monounsaturated fatty acids (MUFA) showed an inverse relationship with the inclusion level of greater duckweed in the feed. The highest MUFA content was found in fish fed SP0 diet. The highest level of linoleic acid was found in SP20 diet fed fish. The n-3 PUFA contents of fish showed an increasing trend with the increasing inclusion of greater duckweed, and a significantly higher level was found in SP20 compared with that of others. A significantly higher

expression of *fas* was found in SP5 and *fads2d6* in SP5 and SP10 compared with that of others. The expressions of *elovl2* and *elovl5* were significantly higher in SP5, SP10, and SP15 diet-fed fish compared with other diet-fed fish. The incorporation of greater duckweed in diets improved the growth performance and nutritional value of common carp.

Keywords: *Cyprinus carpio, Spirodela polyrhiza,* digestive enzymes, linoleic acid, eicosapentaenoic acid, docosahexaenoic acid, *fads2d6, elovl2*

INTRODUCTION

The application of freshwater macrophytes as fish feed ingredients is an emerging area of research. There is an increasing demand for quality ingredients that can replace fishmeal and fish oil without affecting the survival, growth performance, and quality of the farmed products. As an alternative to fish meal, plant protein is widely used in aquaculture as well as the poultry and swine feed industries (Hardy, 2010). The nutritional value (e.g., amino acid and fatty acid compositions, fiber content, and flavorings) of ingredients should be considered during fish feed formulation (Gatlin et al., 2007; Glencross et al., 2020). Meals and other products of soybean are the most commonly used plant-based ingredients in the aqua feed industry. However, soybean meal has great market demand as it is also used extensively by other animal feed industries. Therefore, there is a need to find other, non-conventional ingredients that have less or no use in other feed sectors, but that still have high-quality nutritional profiles with all the required amino acids and fatty acids. The greater duckweed *Spirodela polyrhiza* (family: Lemnaceae) is a free-floating freshwater macrophyte that has been considered as a suitable feed ingredient for both fish and livestock (FAO, 2001; Hasan and Chakrabarti, 2009; Cruz-Velásquez et al., 2014; Chakrabarti, 2017).

The study of the proximate composition showed that the crude protein content of soybean meal (460.7 g/kg) is higher compared with greater duckweed (366.5 g/kg), whereas crude lipid and ash levels are higher in greater duckweed (crude lipid: 76.2 g/kg, ash: 181.9 g/kg) compared with soybean meal (crude lipid: 11.0 g/kg, ash: 71.1 g/kg) (Lee et al., 2013). However, greater duckweed is a rich source of essential and non-essential amino acids (Sharma et al., 2019). The amino acid profile of greater duckweed fulfills all the recommended essential amino acid requirements of common carp *Cyprinus carpio* and Nile tilapia *Oreochromis niloticus* (NRC, 1998, 2011). Duckweeds are also known to be good sources of vitamins and fatty acids (Appenroth et al., 2017), with the fatty acid profile of greater duckweed being favorable in comparison with soybean meal. The n-3 PUFA content is 7.5-fold higher in greater duckweed than in soybean meal, with α-linolenic acid (ALA, 18:3n-3) being the predominant fatty acid in greater duckweed contributing 35.75% of total fatty acids (Sharma et al., 2019).

Supply of sufficient amounts of the essential n-3 long-chain PUFA (LC-PUFA), specifically eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), to support optimal human nutrition is a global problem that transcends geographical and political boundaries. While the n-3 LC-PUFA is completely absent in conventional plant meals and vegetable oils, their precursor ALA can be abundant in terrestrial and freshwater plants. The conversion of ALA (and linoleic acid, LOA, 18:2n-6) to LC-PUFA requires a series of fatty acyl desaturase (*fads*) and elongation of very long-chain fatty acid (*elovl*) enzymes such as *elovl5* and *elovl2* (Kuhajda et al., 1994; Torstensen and Tocher, 2010; Castro et al., 2016; Monroig et al., 2016; Xie et al., 2021). The products of the Δ*6fads* and *elovl5* genes are key enzymes in the biosynthesis of EPA and DHA (Fonseca-Madrigal et al., 2005; Torstensen and Tocher, 2010). Importantly, many freshwater fishes including common carp and Nile tilapia have the metabolic capacity to convert dietary ALA to the n-3 LC-PUFA, EPA, and DHA (Tocher et al., 2002; Glencross, 2009; Tocher, 2010; Taşbozan and Gökçe, 2017). Therefore, supplementation of the greater duckweed *S. polyrhiza* as a rich source of ALA in the feed of freshwater carp is a useful and cost-effective way to increase the n-3 LC-PUFA content of farmed fish for human consumption.

The omnivore common carp *C. carpio* (family: Cyprinidae) is the fourth most cultured freshwater fish and contributed 7% of total aquaculture (fish) production in 2018 (FAO, 2020) and is extensively used in composite fish culture in India (Rathore et al., 2005). The digestibility of ingredients plays a very significant role in the overall bioavailability of the nutrients present in feed (Chakrabarti and Rathore, 2009), and, recently, an *in vitro* digestibility study showed the potential suitability of greater duckweed as an ingredient in fish feed (Sharma et al., 2016). Rathore et al. (2005) have reported the variations in the activities of digestive enzyme in common carp during ontogenic development and observed significantly increased amylase activity in 30-day-old fish. This finding confirmed the capacity of common carp to digest plant-based feed. The presence of anti-nutritional factors is a major constraint to the application of plant-based ingredients in aquafeeds (Alarcón et al., 1998; Olsen et al., 2007; Hansen and Hemre, 2013).

Several studies have investigated the effects of freshwater macrophytes in feeds for different fish species. Dietary supplementation of *Lemna minor* (20%) and *Azolla pinnata* had no negative impacts on growth performance or feed utilization of common carp (Yılmaz et al., 2004; Gangadhar et al., 2017). Rohu *Labeo rohita*-fed diets containing 20 and 30% *L. minor* showed highest weight gain, SGR, and lowest FCR compared with the control diet without duckweed (Bairagi et al., 2002; Mer et al., 2016). The supplementation of duckweed in the diet of common carp increased the antioxidant capacity as evidenced by enhanced activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and reduced levels of malondialdehyde, MDA (Yang et al., 2019). The aim of the

present study was to investigate the effects of feeding diets containing greater duckweed *S. polyrhiza* on the survival, growth performance, digestive enzyme activities, and biochemical composition of common carp *C. carpio*. The greater duckweed was included in a graded manner at levels of 0%, 5%, 10%, 15%, and 20% of total diet in carp feeds. The expression of genes involved in the metabolic conversion of ALA was also studied to determine impacts on n-3 LC-PUFA biosynthesis.

MATERIALS AND METHODS

Feed Formulation

The greater duckweed *S. polyrhiza* was cultured using organic manures *viz*., cattle manure mustard oil-cake and poultry dropping (Sharma et al., 2019). These manures are cheap and easily available. The production cost of greater duckweed in this study was around Rs.14.0/kg. The greater duckweed was collected, cleaned, dried, and ground. The meal was stored at 4°C prior to further use. The moisture, crude protein, crude lipid, carbohydrate, and ash contents of the greater duckweed meal were 75.2, 366.5, 76.2, 300.2, and 181.9 g/kg, respectively. Five isoproteic, isolipidic, and isoenergetic experimental feeds were prepared with graded inclusion of *S. polyrhiza* meal replacing soybean meal as the primary protein source (**Table 1**). Fish feeds were formulated using the Winfeed 2.8 software package (WinFeed UK Limited, Cambridge, United Kingdom). The control feed (SP0) contained soybean meal as the only primary source of protein, while in the four experimental feeds, greater duckweed was incorporated at the levels of 5%, 10%, 15%, and 20% of total feed at the expense of soybean meal, wheat flour, corn meal, and sunflower oil (to maintain constant crude protein, crude lipid, and gross energy levels) to produce feeds SP5, SP10, SP15, and SP20. The soybean meal was replaced in a graded manner, which resulted in changes in the proportions of amino acids in the feeds. Therefore, some specific amino acids such as histidine, methionine, lysine, and threonine were supplemented to the feeds based on the reported requirements of common carp (NRC, 2011). The inclusion levels of these four amino acids were determined using the Winfeed software to ensure the requirements of the fish were satisfied. All dry feed ingredients were blended for 10 min and mixed with the oil before warm water was added slowly and everything mixed thoroughly. The entire mixture was placed in the hopper of the twin-screw-extruder (Basic Technology Private Limited, Kolkata, India), and feed pellets were formed with extrusion conditions as follows: cutter 134 rpm; feeder 10 rpm; extrusion 190 rpm; extrusion torque 9.22; heater 1 temperature 65°C; heater 2 temperature 70°C; and final mass temperature 75°C. The diameter of the produced pellets was 1 mm. All feeds were stored at 4°C prior to use. A common difficulty in the use of feeds based on plant ingredients is their palatability to the fish (Rodriguez et al., 1996), but this can be mitigated by the extrusion process. Antinutritional factors such as trypsin inhibitor, phytic acid tannins, oxalates etc., are found in greater duckweed (Cruz et al., 2011). However, the preparation of the feeds by the extrusion technique helped to mitigate the impact of antinutritional factors as high temperature and pressure inactivate many of these factors and control enzymatic rancidity of nutrients (Rokey, 2004; Stadtlander et al., 2019). In this study, the preparation of feed using the extrusion technology improved the digestibility of proteins and starches and destroyed the antinutritional factors present in the feed. The amino acid and fatty acid compositions of the feeds were measured and are presented in **Supplementary Tables 1**, **2**.

Culture of Fish and Sampling

Common carp were cultured and sampled following the guidelines of the University of Delhi Institutional Animal Ethics Committee (DU/ZOOL/IAEC-R/2015/07). The fish were collected from a local fish farm and acclimated in the aquarium at the University of Delhi for 1 week during which time the fry were fed the soybean-based control feed. The fry (0.473–0.479 g) were then distributed randomly into 15 glass aquaria (50 L each) with 30 fry per aquarium. The fry of common carp was selected to determine the influence of the plant-based diet on the digestive physiology of fish and to understand the suitability of these diets for early life stages. Each aquarium was connected to an external filtration unit (Sera fil bioactive 130, Germany). Water from the fish culture units was constantly filtered through the filtration unit to maintain ammonia levels of the units. The dissolved oxygen level of water was maintained with the help of an aerator. The carp were then fed one of the five different feeds SP0, SP5, SP10, SP15, and SP20 with three replicate aquaria per dietary treatment. The feeds were distributed *ad libitum* two times daily at 09:00 and 17:00 h and the weight of feed measured before distribution. Excess (uneaten) feed was collected from each aquarium 1 h after feeding, oven drying, and recording weight. Water quality parameters, namely, temperature, pH, dissolved oxygen, and conductivity were monitored in each aquarium using a probe connected to a portable meter (HQ40d Multiparameter, Hach, United States). The ammonia (NH_3) level was estimated using a probe, connected to Orion Versastar (Thermo Scientific, United States). The nitrite (NO_2^-), nitrate (NO_3^-), and phosphate ($PO4^{3-}$) contents were analyzed regularly (APHA, 2017). There was no significant difference in water quality parameters among the dietary treatments throughout the culture period. Temperature, pH, and dissolved oxygen ranged from 25.8 to 28.1°C, 6.66 to 7.56, and 6.45 to 7.48 mg/L, respectively, during the experimental period. The range of ammonia, nitrite, nitrate, and phosphate levels were 0.003–0.0530, 0.291–0.906, 0.956–3.84, and 0.013–0.097 mg/L, respectively, in different treatments and conductivity ranged from 636–811 μS/cm.

After 60 days of culture, the feeding experiment was terminated, and fish was sampled. Fish were starved for 24 h before harvesting, and then, all fish were euthanized with tricaine methane sulfonate (MS222, Sigma, United States), and the weight of individual fish was measured. Four fish from each tank were pooled (four fish/replicate) and stored at -80°C for the assay of whole-body proximate, amino acid, and fatty acid compositions. Three tank replicates were used for all assays (three replicates per diet, $n = 3$). The digestive tracts of two individual fish per aquarium were collected (two fish/replicate, three replicates; $2 \times 3 = 6$ fish/diet) for the assay of digestive

TABLE 1 | Formulations and measured proximate compositions of experimental diets.

Ingredients (g/Kg)	SP0	SP5	SP10	SP15	SP20
Soybean meal	500	475	450	425	400
Wheat flour	245	224	204	183	162
Corn flour	147	144	141	138	135
Sunflower oil	62	60	57	55	52
S. polyrhiza powder	0	50	100	150	200
Vitamin/minerals premix	5	5	5	5	5
Mono calcium phosphate	20	20	20	20	20
Choline chloride	1	1	1	1	1
Histidine	1	1	2	2	2
Methionine	12	12	12	12	12
Lysine	5	6	6	7	7
Threonine	2	3	3	4	4
Proximate composition (g/kg)					
Moisture	63.3 ± 0.81	63.6 ± 0.20	63.2 ± 0.63	63.3 ± 0.84	62.5 ± 0.42
Crude Protein	317.6 ± 0.50	318.4 ± 2.20	318.2 ± 3.10	322.2 ± 3.61	322.0 ± 0.70
Crude Lipid	73.3 ± 0.81	73.4 ± 0.91	73.7 ± 0.25	73.2 ± 0.10	72.8 ± 0.17
Carbohydrate	474.6 ± 4.10	474.3 ± 1.72	472.0 ± 0.25	464.2 ± 2.21	460.6 ± 3.1
Ash	71.2 ± 0.28	70.3 ± 0.40	72.9 ± 0.60	77.1 ± 0.21	84.1 ± 1.1

SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza.

TABLE 2 | Initial weight, survival rate, final weight, specific growth rate, feed conversion ratio, and digestive enzyme activities of *Cyprinus carpio* fed five different diets.

Parameters	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
						P value	*F* value	
Initial weight (g)	0.473 ± 0.001^a	0.479 ± 0.001^a	0.473 ± 0.003^a	0.473 ± 0.001^a	0.477 ± 0.002^a	0.093	2.692	0.518
Survival (%)	100	100	100	100	100	-	-	-
Final weight (g)	1.60 ± 0.004^e	1.88 ± 0.012^d	2.15 ± 0.007^c	2.49 ± 0.003^b	2.74 ± 0.013^a	< 0.01	7,507.44	0.999
Specific growth rate (SGR% BW/day)	2.01 ± 0.002^e	2.30 ± 0.011^d	2.52 ± 0.007^c	2.75 ± 0.001^b	2.93 ± 0.001^a	< 0.01	3,419.05	0.999
Feed conversion ratio (FCR)	1.25 ± 0.010^a	1.23 ± 0.012^a	1.11 ± 0.011^b	1.06 ± 0.013^{bc}	1.01 ± 0.010^c	< 0.01	74.394	0.967
Amylase (mU/mg protein/min)	126.48 ± 3.80^a	98.91 ± 2.30^b	102.28 ± 2.13^b	125.41 ± 1.87^a	125.14 ± 1.82^a	< 0.01	30.70	0.925
Protease (Fluorescence change/unit)	249.95 ± 1.40^b	247.31 ± 0.88^b	265.35 ± 5.30^a	264.78 ± 1.75^a	278.82 ± 6.28^a	0.091	2.715	0.521
Trypsin (μM AMC/mg protein/min)	799.93 ± 4.11^c	588.87 ± 6.18^d	768.03 ± 9.42^c	$1,073.91 \pm 6.72^b$	$1,335.45 \pm 9.52^a$	< 0.01	1,532.15	0.998
Chymotrypsin (μM AMC/mg protein/min)	683.17 ± 17.86^b	813.10 ± 10.84^a	617.5 ± 4.30^c	477.24 ± 10.24^d	406.38 ± 15.20^e	< 0.01	147.52	0.983
Lipase (μM 4-MU/mg protein/min)	$1,000.97 \pm 17.26^c$	950.40 ± 10.80^c	992.27 ± 14.82^c	$1,137.10 \pm 16.30^a$	$1,074.27 \pm 15.32^b$	< 0.01	27.50	0.917

Values (n = 3) with different letters in the same row are significantly different ($p < 0.05$).
SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza; BW, body weight.

enzyme activities. The hepatopancreas from individual fish was collected (100 mg) and stored in 1 ml of TRIzol reagent (Ambion, Life Technologies, United States) for the gene expression analysis (four fish/treatment; from two aquaria 1 + 1 fish and two fish from the third aquarium). Specific growth rate (SGR) and feed conversion ratio (FCR) of fish were calculated as follows.

SGR (% body weight/day) = (In final body mass - In initial body mass) × 100/duration of experiment (days).

FCR = feed (dry weight) consumed by individual fish during feeding trial/weight gain (wet weight) of individual fish.

Digestive Enzymes Activities

Intestinal samples were freeze-dried and homogenized in ice-cold Milli-Q® water (1:10) to maintain neutral pH of the extracts. Homogenates were centrifuged for 30 min at 10,000 × *g* at 4°C, and supernatants were collected for the assay of digestive enzyme activities using fluorimetry (Multimode reader, BioTek Synergy H1 Hybrid, United States) using three replicates per dietary treatment. The amylase activity was determined using an assay kit (E33651; Invitrogen, United States) with fluorescence measured at 485 (i.e., excitation) and 520 nm (i.e., emission). The enzyme activity was expressed as mU/mg protein/min. A protease kit (E6638; Invitrogen) was used to measure the total protease activity with fluorescence measured at 485 (i.e., excitation) and 530 nm (i.e., emission). The protease activity was expressed as fluorescence change/unit. The substrate *N*-benzoyl-L-arginine-methyl-coumarinylamide (Sigma-Aldrich) was used for the estimation of serine proteases trypsin (Ueberschär, 1988). The fluorescence was measured at 380 (i.e., excitation) and 440 nm (i.e., emission). The chymotrypsin was measured using succinyl-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Sigma-Aldrich) as the substrate (Cao et al., 2000). The fluorescence was measured at 380 (i.e., excitation) and 450 nm (i.e., emission). The enzyme activities were expressed as μM 7-amino-4-methylcoumarin (AMC)/mg protein/min. The neutral lipase activity was determined following the method of Roberts (1985) using the substrate 4-methylumbelliferyl butyrate, 4-MU (Sigma-Aldrich). The fluorescence was measured at 365 nm (excitation) and 450 nm (emission). The enzyme activity was expressed as μM 4-MU/mg protein/min. Protein content was estimated using bovine serum albumin (BSA, Sigma-Aldrich) as standard (Bradford, 1976).

Proximate Composition

Samples of feed were ground, and the fish samples (four fish pooled per replicate) were blended to form homogeneous paste prior to biochemical composition analyses. Proximate compositions of feeds and cultured fish were determined (three replicates for each assay) following the standard protocols of the Association of Official Analytical Chemists International (AOAC, 2000). The moisture level was calculated after drying samples at 110°C for 24 h, and ash content was measured after incineration of samples at 600°C for 16 h. The nitrogen content was first assayed using micro Kjeldahl method, and then, crude protein was calculated ($N \times 6.25$). The crude lipid content was measured gravimetrically following extraction of total lipid using chloroform/methanol (2:1, v/v) (Folch et al., 1957). The subtraction method was applied for the calculation of carbohydrate levels in the feeds (Aksnes and Opstvedt, 1998).

Amino Acid Analysis

Feed and whole fish samples were processed as described above and amino acid compositions assayed with an Automatic Amino Acid Analyzer L-8900 (Hitachi Co., Ltd., Tokyo, Japan) using three replicates for each sample. For all amino acids other than cysteine, methionine, and tryptophan, samples were hydrolyzed using 6 N HCl at 110°C for 24 h (Bassler and Buchholz, 1993; Chakrabarti et al., 2018). The sulfur-containing amino acids were analyzed after oxidizing the sample with performic acid prior to treating the sample with 6 N HCl. For the estimation of tryptophan, the sample was hydrolyzed using 4 M methanesulfonic acid with 0.2% 3-(2-aminoethyl). A nitrogen evaporator (PCi Analytic Private Limited, Maharashtra, India) was used to dry the digested samples, and 0.02 N HCl was added to obtain a protein concentration of 0.5 mg/ml in the sample, and 1.5 ml was placed in a glass vial of the auto sampler. A cation-exchange resin column (4.6 mm ID × 60 mm L) with 3 μm particle size was used for the separation of amino acids with the following analytical conditions: column temperature 30-70°C, reaction temperature 135°C, and a ninhydrin flow rate of 0.35 ml/min. All amino acids were monitored at 570 nm, except proline and hydroxyproline that were monitored at 440 nm. The concentration of individual amino acids was compared with a standard solution (Wako Pure Chemical Industries Limited, United States) and expressed as g/kg.

Fatty Acid Analysis

After processing feed and whole fish samples as described above, fatty acid compositions were analyzed by gas chromatography and flame ionization detection (GC-FID) using a Clarus 580 (PerkinElmer, Waltham, United States). In brief, crude lipid was extracted from the samples using chloroform/methanol (2:1, v/v) following the protocol of Folch et al. (1957) with three replicates per dietary treatment. Fatty acid methyl esters (FAME) were prepared from crude lipid extracts by acidic transesterification, treating the lipid with 1% sulfuric acid in methanol for 16 h at 50°C (Christie, 2003). After extraction and purification of FAME (Tocher and Harvie, 1988), a 1 ml aliquot was placed in a glass vial of the GC autosampler. Fatty acids were separated using a 60 m ZB-wax GC column, internal diameter of 0.32 mm, and film thickness of 0.25 μm (Phenomenex, Hyderabad, India). Data were collected using preinstalled programmed software (TotalChrom Workstation Ver6.3; PerkinElmer, United States). The FAME were identified and quantified by the comparison with standards (Supelco FAME 37 mix; Sigma-Aldrich, United States) and published data (Tocher and Harvie, 1988) and concentration expressed as mg/100 g.

Gene Expression

The levels of mRNA expression of delta-6 fatty acyl desaturase (*fads2d6*), elongation of very-long-chain fatty acids protein 2 (*elovl2*), elongation of very-long-chain fatty acids protein 5 (*elovl*5), and fatty acid synthase (*fas*) genes were determined in the hepatopancreas of common carp. Total RNA was extracted using the TRIzol reagent (Ambion, Life Technologies, United States) following the protocol of the manufacturer. The absorbance of extracted RNA was examined at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific, United States) to determine the concentration and quality. The extracted RNA was treated with 1 U of DNase I

TABLE 3 | Proximate compositions (g/kg, wet weight) of five different diets-fed *Cyprinus carpio*.

Parameters	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear Regression R^2 Value
						P value	*F* value	
Moisture	756.4 ± 3.6^{a}	752.8 ± 3.7^{a}	750.7 ± 1.02^{a}	750.5 ± 5.7^{a}	747.6 ± 1.2^{a}	0.524	0.852	0.254
Crude Protein	150.3 ± 0.66^{c}	152.34 ± 0.25^{b}	156.73 ± 0.25^{a}	156.79 ± 0.12^{a}	157.06 ± 0.1^{a}	<0.01	81.09	0.970
Crude Lipid	64.7 ± 0.1^{c}	64.4 ± 0.10^{c}	65.2 ± 0.1^{bc}	65.9 ± 0.2^{b}	68.1 ± 0.5^{a}	<0.01	33.949	0.931
Ash	19.6 ± 0.3^{c}	21.4 ± 0.1^{b}	21.8 ± 0.2^{b}	21.9 ± 0.3^{b}	22.5 ± 0.3^{a}	<0.01	28.401	0.919

Values (n = 3) with different letters in the same row are significantly different ($p < 0.05$).
SP0, soybean meal only; SP, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza.

(Sigma-Aldrich, United States) to avoid DNA contamination, and the quality of RNA treated with DNase was checked with 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed into cDNA by the reverse transcription reaction using high-capacity cDNA reverse transcription kit (Applied Biosystems, United States), using the protocol provided by the manufacturer.

Quantification of gene expression was carried out by using quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a Quant Studio 6 Flex system (Applied Biosystems) and PowerUp SYBR^{TM} Green Master Mix (Applied Biosystems). Primers were designed using the online primer design tool of NCBI with β−actin used as the reference (housekeeping) gene (**Supplementary Table 3**). The efficiency of primers was evaluated by using the melt curve and standard curve analysis using the QuantStudio 6 Flex Real-Time PCR system software v1 (Applied Biosystems). The 10 μl reaction mixture for qRT-PCR was composed of 0.25 μl PCR forward primer (2.5 μM), 0.25 μl PCR reverse primer (2.5 μM), 1 μl of cDNA (1:3), 5 μl of 2 × PowerUp^{TM} SYBR^{TM} Green PCR Master Mix (Applied Biosystems), and nuclease-free water (3.5 μl). Samples were run in duplicate for each target gene with non-template control (NTC). The thermal cycling conditions were as follows: predenaturation of nucleic acid at 95°C for 10 min followed by either 40 cycles of 15 s at 95°C and 1 min at 60°C (primer Tm 60°C) or 40 cycles of 15 s at 95°C, 15 s at 55°C, and 1 min at 72°C for (primer Tm < 60°C). The data of qRT−PCR were calculated using the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) method with β−actin as the internal control.

Statistical Analysis

Data are presented as means with standard error (SEM) with *n* values as stated. The IBM SPSS 25.0 software (SPSS Inc., Michigan Avenue, Chicago, IL, United States) was used for the statistical analysis. Data were analyzed using one-way analysis of variance, and the Tukey's test was performed to compare the differences among experimental groups. The linear regression analysis was performed to check the effect of inclusion level of *S. polyrhiza* in the diets on growth performance, digestive enzyme activities, proximate composition, and amino acid and fatty acid profiles of fish. The broken-line regression analysis was performed for average weight and SGR to determine the breakpoint, BP (Muggeo, 2008) with final BP estimated based on the least sum of squares of deviation (LS method) using R package. The significance was accepted at $p < 0.05$ level.

RESULTS

Performance of Fish

After 60 days of culture, the number of common carp in each aquarium was recorded. There was no mortality of fish; all fish survived (**Table 2**). The broken-line regression analysis showed the impact of greater duckweed on the growth performance and SGR of common carp. The estimated break point for final weight and SGR were 10 and 13.4% of greater duckweed, respectively. The final weight of SP20 diet-fed common carp was significantly higher compared with other diet-fed fish. Minimum weight was found in the SP0 treatment. Similar trend was also found with SGR. SGR was maximum in SP20 diet-fed carp. FCR was minimum and maximum in SP20 and SP0 treatment, respectively.

Digestive Enzymes

The amylase activity ranged from 98.91 to 126.48 mU/mg protein/min in five different diet-fed common carp with a minimum amylase activity in SP5 diet-fed fish (**Table 2**). The amylase activity was significantly higher in SP0, SP15, and SP20 diet-fed fish compared with other diet-fed fish. The total protease activity was significantly higher in SP10, SP15, and SP20 diet-fed fish compared with other diet-fed fish. Trypsin activity was significantly higher in SP20 diet-fed fish compared with other diet-fed fish. Significantly higher chymotrypsin and lipase activities were found in SP5 and SP15 diet-fed common carp, respectively, compared with other treatments. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2 = 0.917–0.998) with amylase, trypsin, chymotrypsin, and lipase activities.

Biochemical Composition of Fish

Proximate Composition of the Whole Body

The moisture contents of fish varied from 747.6 to 756.4 g/kg in five different diet-fed common carp (**Table 3**). The crude protein content was significantly higher in SP10, SP15, and SP20 diet-fed common carp compared with other diet-fed fish. Significantly

TABLE 4 | Amino acid compositions (g/kg, wet weight) of five different diets-fed *Cyprinus carpio*.

Amino acids	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
Essential						***P* value**	***F* value**	
Arginine (Arg)	6.77 ± 0.2^{c}	6.40 ± 0.1^{d}	10.7 ± 0.1^{a}	10.21 ± 0.03^{b}	9.94 ± 0.04^{b}	<0.01	905.511	0.997
Histidine (His)	3.72 ± 0.1^{a}	3.64 ± 0.2^{a}	2.85 ± 0.05^{b}	2.87 ± 0.06^{b}	2.73 ± 0.03^{b}	<0.01	156.009	0.984
Isoleucine (Ile)	5.13 ± 0.12^{ab}	5.39 ± 0.07^{a}	4.98 ± 0.01^{b}	4.25 ± 0.1^{c}	4.44 ± 0.13^{c}	<0.01	67.119	0.964
Leucine (Leu)	9.59 ± 0.19^{a}	10.12 ± 0.33^{a}	8.59 ± 0.04^{b}	8.28 ± 0.11^{bc}	8.01 ± 0.25^{c}	<0.01	53.555	0.955
Lysine (Lys)	11.54 ± 0.04^{b}	12.75 ± 0.08^{a}	10.1 ± 0.1^{c}	9.15 ± 0.05^{d}	9.47 ± 0.24^{d}	<0.01	323.243	0.992
Methionine (Met)	2.94 ± 0.04^{c}	3.37 ± 0.04^{ab}	3.63 ± 0.21^{a}	2.77 ± 0.09^{c}	3.01 ± 0.30^{c}	<0.01	12.611	0.835
Phenylalanine (Phe)	5.52 ± 0.12^{ab}	5.83 ± 0.1^{a}	5.16 ± 0.01^{bc}	4.91 ± 0.20^{c}	4.99 ± 0.14^{a}	<0.01	14.657	0.854
Threonine (Thr)	4.73 ± 0.01^{d}	5.16 ± 0.03^{c}	6.20 ± 0.1^{b}	6.12 ± 0.1^{b}	6.67 ± 0.08^{a}	<0.01	293.479	0.992
Tryptophan (Trp)	2.27 ± 0.03^{c}	1.60 ± 0.05^{d}	1.56 ± 0.003^{d}	2.32 ± 0.01^{b}	2.48 ± 0.002^{a}	<0.01	1,705.8	0.998
Valine (Val)	5.98 ± 0.05^{d}	6.49 ± 0.05^{bc}	7.14 ± 0.06^{a}	6.25 ± 0.01^{c}	6.63 ± 0.17^{b}	<0.01	69.776	0.965
Σ Essential	**58.2 ± 1.1**^{b}	**60.8 ± 0.1**^{a}	**61.1 ± 0.4**^{a}	**57.1 ± 0.7**^{b}	**58.4 ± 0.4**^{b}	<0.01	21.402	0.895
Non-essential	**SP0**	**SP5**	**SP10**	**SP15**	**SP20**	**ANOVA**		**Linear regression R^2 value**
						***P* value**	***F* value**	
Alanine (Ala)	8.03 ± 0.03^{b}	7.65 ± 0.03^{c}	8.03 ± 0.06^{b}	9.85 ± 0.07^{a}	9.94 ± 0.15^{a}	<0.01	363.836	0.993
Aspartate (Asp)	11.7 ± 0.03^{b}	11.4 ± 0.12^{b}	12.4 ± 0.36^{a}	11.3 ± 0.02^{b}	12.7 ± 0.12^{a}	<0.01	33.477	0.931
Cysteine (Cys)	1.14 ± 0.002^{c}	1.69 ± 0.07^{a}	1.12 ± 0.07^{c}	1.30 ± 0.17^{bc}	1.39 ± 0.02^{b}	<0.01	18.725	0.882
Glutamic Acid (Glu)	20.3 ± 0.08^{a}	18.8 ± 0.10^{c}	17.3 ± 0.01^{d}	17.3 ± 0.05^{d}	19.6 ± 0.17^{b}	<0.01	535.013	0.995
Glycine (Gly)	8.91 ± 0.09^{d}	10.0 ± 0.3^{c}	8.58 ± 0.01^{d}	10.80 ± 0.14^{b}	11.62 ± 0.03^{a}	<0.01	77.612	0.969
Proline (Pro)	27.8 ± 0.1^{c}	29.0 ± 0.1^{b}	31.37 ± 0.01^{a}	31.10 ± 0.20^{a}	25.1 ± 0.2^{d}	<0.01	387.422	0.991
Serine (Ser)	4.83 ± 0.02^{c}	4.81 ± 0.07^{c}	5.62 ± 0.01^{b}	5.48 ± 0.03^{b}	6.32 ± 0.08^{a}	<0.01	221.76	0.989
Tyrosine (Tyr)	4.10 ± 0.2^{a}	3.67 ± 0.34^{ab}	3.57 ± 0.02^{ab}	3.24 ± 0.16^{b}	3.34 ± 0.04^{b}	<0.01	7.311	0.745
Phosphoserine (p-Ser)	0.06 ± 0.00^{bc}	0.05 ± 0.00^{c}	0.10 ± 0.05^{bc}	0.28 ± 0.06^{a}	0.15 ± 0.01^{b}	<0.01	28.851	0.920
Taurine (Tau)	1.76 ± 0.03^{bc}	1.51 ± 0.15^{c}	1.74 ± 0.16^{bc}	1.96 ± 0.13^{b}	2.73 ± 0.04^{a}	<0.01	48.115	0.951
α Amino -n- butyric acid (α-ABA)	–	–	0.19 ± 0.02^{a}	0.20 ± 0.03^{a}	0.18 ± 0.00^{a}	0.221	1.961	0.395
Citrulline (Cit)	0.13 ± 0.01^{b}	0.15 ± 0.03^{b}	0.44 ± 0.00^{a}	0.54 ± 0.20^{a}	0.56 ± 0.00^{a}	<0.01	16.081	0.865
Cystathionine (Cysthi)	0.28 ± 0.05^{d}	0.11 ± 0.01^{d}	2.13 ± 0.14^{b}	2.60 ± 0.01^{a}	1.38 ± 0.04^{c}	<0.01	301.177	0.992
β -Alanine (β -Ala)	–	0.08 ± 0.02^{a}	0.06 ± 0.00^{ab}	0.11 ± 0.001^{a}	0.11 ± 0.05^{a}	0.185	2.051	0.435
β Amino isobutyric acid (β-AiBA)	0.04 ± 0.00^{b}	–	–	–	0.08 ± 0.00^{a}	-	-	–
γ-Amino-n-butyric acid (γ-ABA)	0.04 ± 0.00^{a}	–	–	–	–	-	-	–
Hydroxylysine (Hylys)	0.16 ± 0.05^{b}	–	–	0.24 ± 0.002^{a}	–	<0.01	518.664	0.992
3 Methyl histidine(3 Mehis)	–	–	0.07 ± 0.000^{a}	– –	0.06 ± 0.000^{a}	0.02	13.914	0.777
Hydroxy proline (Hypro)	1.58 ± 0.09^{c}	2.28 ± 0.08^{b}	2.91 ± 0.22^{a}	3.13 ± 0.002^{a}	3.07 ± 0.16^{a}	<0.01	301.177	0.992
Σ Non- essential	**90.95 ± 0.31**^{c}	**91.51 ± 0.21**^{c}	**95.76 ± 0.4**^{b}	**99.56 ± 0.50**^{a}	**98.16 ± 0.06**^{a}	**<0.01**	**136.54**	**0.982**

Values (n = 3) with different letters in the same row are significantly different ($p < 0.05$).
SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza. The bold values means summation/total value.

higher crude lipid and ash contents were found in SP20 diet-fed fish compared with other diet-fed fish.

Amino Acid Composition

The essential amino acids composition was similar in common carp cultured in five different feeding schemes (**Table 4**). The arginine level was 1.55- to 1.67-fold higher in 10–20% greater duckweed supplemented diet-fed common carp compared with SP0 and SP5 diet-fed fish. Histidine (3.64–3.72 g/kg), isoleucine (5.13–5.39 g/kg), leucine (9.59–10.12 g/kg), and lysine (11.54–12.75 mg/kg) contents were significantly higher in SP0 and SP5 treatments compared with other diet-fed fish. Significantly higher levels of methionine and valine were recorded in SP10; threonine and tryptophan levels were maximum in SP20 diet-fed common carp.

Among non-essential amino acids, alanine, aspartate, glycine, serine, taurine, and β-alanine contents were maximum in SP20 diet-fed common carp. Highest levels of glutamic acid and tyrosine were observed in control diet (SP0)-fed common carp. Aspartate contents were significantly higher in SP10 and SP20 diet-fed common carp. Highest levels of phosphoserine, cystathionine, and hydroxyproline were found in SP15 diet-fed fish. The α-amino-n-butyric acid content was highest in SP15, and the citrulline content was maximum in SP15 and SP20

TABLE 5 | Fatty acid compositions (mg/100 g, wet weight) of five different diets-fed *Cyprinus carpio*.

Fatty acids	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
						P value	*F* value	
14:0	22.92 ± 1.23^{b}	30.64 ± 1.00^{a}	22.80 ± 0.34^{b}	22.42 ± 0.11^{b}	15.78 ± 0.07^{c}	< 0.01	156.593	0.984
15:0	4.21 ± 0.23^{c}	6.52 ± 0.25^{b}	4.89 ± 0.61^{c}	6.21 ± 0.34^{b}	7.29 ± 0.29^{a}	< 0.01	22.074	0.898
16:0	374.00 ± 9.01^{d}	548.07 ± 20.3^{a}	507.88 ± 6.26^{b}	435.9 ± 1.41^{c}	369.36 ± 2.75^{d}	< 0.01	176.633	0.986
18:0	1.90 ± 0.01^{a}	2.81 ± 0.68^{a}	3.00 ± 0.20^{a}	2.05 ± 0.29^{a}	1.24 ± 0.21^{a}	0.081	**2.856**	0.533
24:0	10.53 ± 0.01^{b}	14.68 ± 2.17^{a}	12.49 ± 0.04^{a}	7.21 ± 0.77^{c}	9.25 ± 0.11^{b}	< 0.01	79.245	0.969
Σ SFA	**413.58 ± 10.00**^{d}	**602.75 ± 22.66**^{a}	**551.09.1 ± 7.05**^{b}	**473.8 ± 3.20**^{c}	**403.20 ± 3.50**^{d}	**< 0.01**	**165.49**	**0.985**
16:1 n-9	26.44 ± 0.02^{a}	14.10 ± 1.0^{b}	12.25 ± 2.35^{b}	24.44 ± 0.72^{a}	4.46 ± 0.73^{c}	< 0.01	164.648	0.985
18:1 n-9	1, 013.68 ± 0.02^{a}	963.71 ± 46.81^{a}	889.60 ± 5.90^{b}	905.3 ± 1.80^{b}	699.36 ± 10.84^{c}	< 0.01	91.965	0.974
24:1	2.51 ± 0.04^{b}	3.86 ± 0.44^{a}	2.33 ± 0.29^{b}	–	–	< 0.01	22.290	0.881
Σ MUFA	**1,042.6 ± 1.75**^{a}	**981.688 ± 46.26**^{b}	**904.18 ± 3.83**^{c}	**929.79 ± 2.52**^{bc}	**703.82 ± 11.57**^{d}	**< 0.01**	**107.854**	**0.977**
18:2 n-6	911.99 ± 8.58^{b}	989.75 ± 9.10^{a}	1, 030.2 ± 5.18^{a}	1, 016.81 ± 3.76^{a}	1, 053.6 ± 0.06^{a}	< 0.01	11.145	0.817
20:2 n-6	32.67 ± 1.24^{b}	20.60 ± 0.03^{d}	32.55 ± 0.05^{b}	37.51 ± 1.82^{a}	24.48 ± 2.07^{c}	< 0.01	72.707	0.967
20:3 n-6	13.44 ± 0.22^{b}	11.91 ± 0.78^{c}	10.77 ± 0.02^{d}	14.60 ± 0.30^{a}	10.85 ± 0.52^{d}	< 0.01	55.671	0.957
20:4 n-6	26.89 ± 0.22^{b}	22.96 ± 0.32^{d}	28.17 ± 0.83^{a}	21.75 ± 0.15^{d}	25.12 ± 0.38^{c}	< 0.01	102.122	0.976
22:5 n-6	71.09 ± 0.22^{ab}	66.27 ± 0.17^{c}	46.22 ± 0.002^{d}	69.43 ± 2.17^{b}	72.80 ± 0.144^{a}	< 0.01	282.796	0.991
Σ n-6 PUFA	**1,056.1 ± 11.25**^{b}	**1,111.5 ± 10**^{ab}	**1,147.96 ± 62.51**^{a}	**1,160.11 ± 8.01**^{a}	**1,186.95 ± 2.2**^{a}	**< 0.01**	**9.048**	**0.784**
18:3 n-3	15.10 ± 0.115^{d}	13.65 ± 0.18^{e}	18.09 ± 0.30^{c}	19.53 ± 0.40^{b}	32.63 ± 0.19^{a}	< 0.01	2,563.494	0.999
20:4 n-3	126.51 ± 0.07^{c}	137.58 ± 0.33^{b}	144.32 ± 0.56^{a}	125.9 ± 3.42^{c}	145.15 ± 0.80^{a}	< 0.01	111.460	0.978
20:5 n-3	3.94 ± 0.06^{c}	5.01 ± 0.33^{bc}	4.09 ± 1.04^{c}	8.99 ± 0.01^{a}	6.00 ± 0.40^{b}	< 0.01	46.860	0.949
22:5 n-3	5.39 ± 0.01^{c}	9.29 ± 0.311^{b}	11.32 ± 1.36^{a}	13.00 ± 0.58^{a}	8.06 ± 0.16^{b}	< 0.01	51.941	0.954
22:6 n-3	48.03 ± 0.04^{d}	63.07 ± 0.240^{c}	65.64 ± 0.30^{c}	74.05 ± 0.41^{b}	81.92 ± 1.00^{a}	< 0.01	315.64	0.992
Σ n-3 PUFA	**198.9 7 ± 2.31**^{d}	**228.6 ± 0.54c**	**243.46 ± 3.52**^{b}	**241.47 ± 5.69**^{b}	**273.77 ± 0.45**^{a}	**< 0.01**	**473.046**	**0.995**
EPA + DHA	**51.97 ± 2.19**^{d}	**68.08 ± 0.75c**	**69.73 ± 1.28**^{c}	**83.04 ± 1.29**^{b}	**87.93 ± 0.60**^{a}	**< 0.01**	**598.073**	**0.996**

Values (n = 3) with different letters in the same row are significantly different (p < 0.05).
SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza. The bold values means summation/total value.

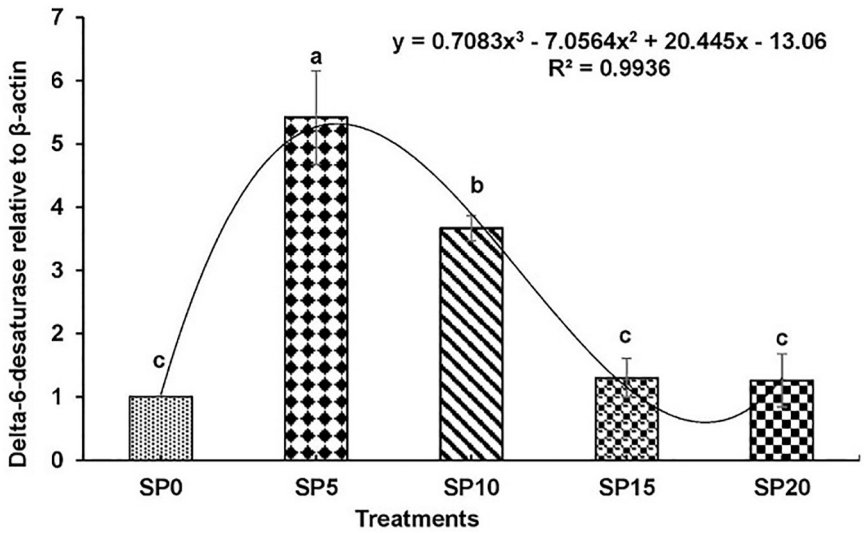

FIGURE 1 | Expression of delta-6-desaturase (*fads2d6)* relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (*n* = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.994).

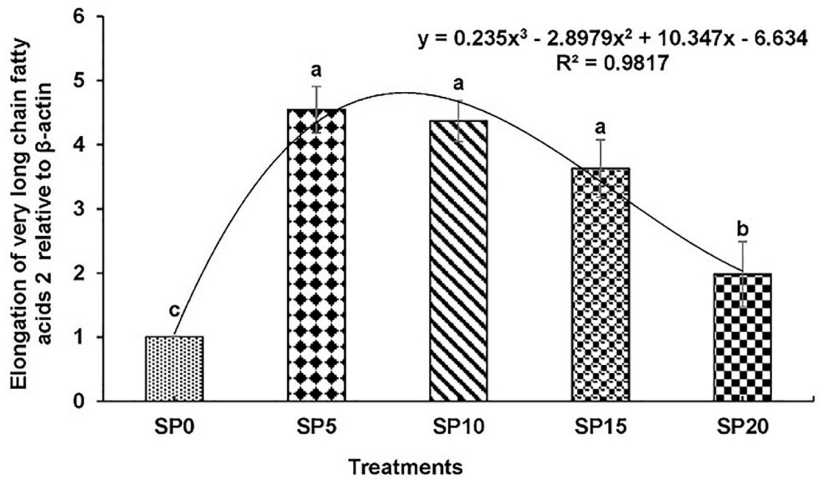

FIGURE 2 | Expression of elongation of very long chain fatty acids protein 2 (*elovl2*) relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (n = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.982).

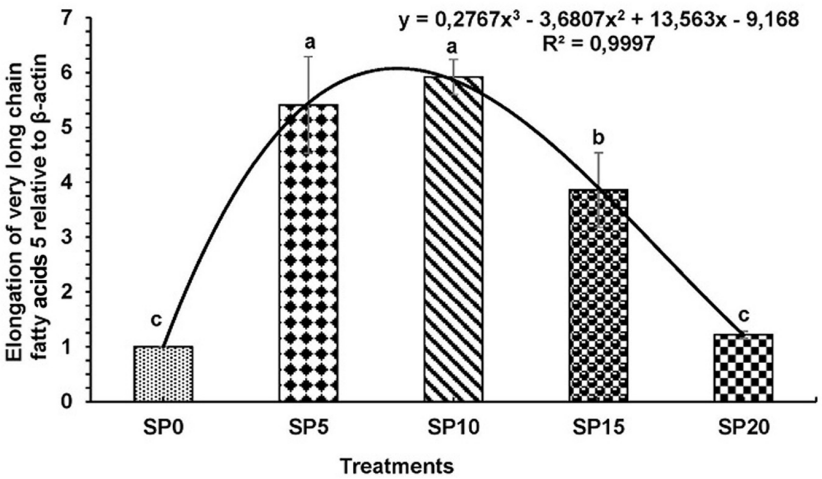

FIGURE 3 | Expression of elongation of very long chain fatty acids protein 5 (*elovl5*) relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (n = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.999).

treatments. The α-amino-n-butyric acid and 3-methyl histidine were absent in SP0 and SP5 diet-fed fish. The β-amino-isobutyric and γ-amino-butyric acids were absent in SP5, SP10, and SP15 diet-fed common carp. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2 = 0.745–0.998) with different amino acids, except α-amino-*n*-butyric acid and β -alanine.

Fatty Acid Composition

The feeding of common carp with greater duckweed supplemented diets influenced the fatty acid composition of fish (**Table 5**). Among saturated fatty acids (SFA), palmitic acid (16:0) was the dominant one regardless of treatments with myristic acid (14:0), the second most dominant SFA. Highest SFA was found in carp fed the SP5 diet. Monounsaturated fatty acids (MUFA) showed an inverse relationship with the inclusion level of greater duckweed in the diet. Significantly higher MUFA content was found in the control diet (SP0)-fed common carp compared with that of others. Among MUFA, oleic acid (18:1n-9) was the dominant one in all treatments. Nervonic acid (24:1) was absent in fish fed diets SP15 and SP20. The greater duckweed supplemented diets enhanced the n-6 PUFA and LOA (18:2 n-6) content in fish. The highest level of LOA was found in SP20 diet-fed fish. The n-3 PUFA contents of fish showed an increasing trend with the increasing inclusion of greater duckweeds in the diet. The ALA (18:3n-3), EPA (20:5n-3), docosapentanoic acid (22:5 n-3, DPA), and DHA (22: 6n-3) contents were significantly lower in SP0 diet fed common carp. The n-3 PUFA content was

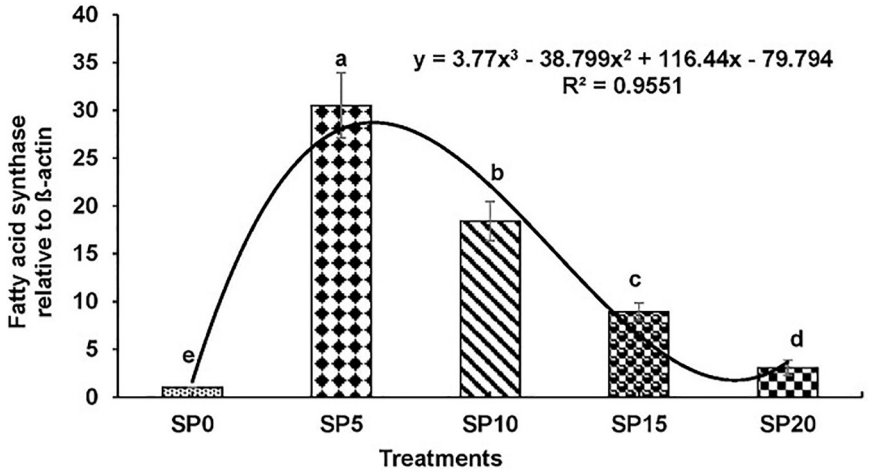

FIGURE 4 | Expression of fatty acid synthase (*fas*) relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (n = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.955).

significantly higher in SP20 diet-fed common carp compared with other diet-fed fish. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2 = 0.745–0.998) with different fatty acids, except stearic acid (18:0).

Gene Expression

Expressions of various genes involved in the metabolism of fatty acid were recorded in the hepatopancreas of common carp fed the five diets. The expression of *fads2d6 was* significantly higher in fish fed diet SP5 compared with others (**Figure 1**). The expression levels of *elovl2* and *elovl5* were significantly lower in SP20 diet-fed common carp compared with others (**Figures 2**, **3**). The significantly higher expression of *fas* was observed in SP5 compared with others (**Figure 4**). The mRNA expressions showed polynomial 3 order relationships with different treatments.

DISCUSSION

Performance of Common Carp

The effect of dietary inclusion of greater duckweed at four different levels (replacing soybean meal) on the performance of common carp was recorded in this experiment. The survival rate of fish was not affected with the inclusion of greater duckweed in the diets. The earlier study showed mixed results. The inclusion of greater duckweed more than 20% in diet resulted into mortality of tilapia (Fasakin et al., 1999, 2001), supplementation of *L. minor* (20%) in diets affected the survival rate of common carp (Yılmaz et al., 2004). El-Shafai et al. (2004) reported that the inclusion of duckweed in the feed of tilapia improved the survival rate of fish. The incorporation canola meal at 50% level in the diet was not affecting the survival rate of tilapia (Iqbal et al., 2021).

The broken-line regression showed that incorporation of greater duckweed at 10% level as a breakpoint for final weight of common carp, and it was 13.4% for SGR of fish. Highest growth performance was observed in SP20 diet-fed fish. Food was efficiently utilized in this treatment as minimum FCR was recorded. In *L. minor* supplemented diet-fed common carp and tilapia, similar trends of growth performance, SGR, and FCR were found (El-Shafai et al., 2004; Yılmaz et al., 2004). The inclusion of *L. polyrhiza* in the diets of mrigal *Cirrhinus mrigala* and rohu *Lebeo rohita* improved the weight gain, SGR, and FCR (Bairagi et al., 2002; Ghosh and Ray, 2014). Inclusion of fermented *L. minor* at 2.5% level and canola meal at 50% level increased the growth of tilapia (Herawati et al., 2020; Iqbal et al., 2021). In this study, the SGR of common carp (initial weight: 0.473–0.479 g) ranged from 2.01 to 2.93%. Similar results were reported in earlier study like, in *L. minor* supplemented diet-fed common carp (initial weight: 0.283–0.295 g), SGR ranged from 1.96 to 2.26% (Yılmaz et al., 2004), and in soy protein concentrate (SPC)-incorporated diet-fed common carp (initial weight: 2.43–2.47 g), SGR was 2.01–2.93% (Zhu et al., 2020). Xie et al. (2021) reported that feeding of common carp with diets containing fishmeal and ultra-micro-ground mixed plant proteins (uPP)-based diets resulted in 540–560% growth of fish after 112 days of culture. In this study with common carp, 238–474% weight gain of fish was recorded after 60 days of culture.

Digestive Enzyme Activities

The study of digestive enzyme activities in five different diet-fed common carp explained the reason of efficient utilization of consumed diet in SP20. Total protease and trypsin activities were maximum in SP20 diet-fed fish; considerable amylase and lipase activities were also found in SP20 diet-fed common carp. The digestive enzymes, namely, protease, lipase, and amylase played a significant role in digestion and absorption of nutrient (Zhou et al., 2010). Fish fed with different diets are able to adjust the activity of their digestive enzymes (Shiping and Zhao, 2005). The inclusion of duckweed in the diets of rohu and tilapia enhanced digestive enzyme activities like amylase, trypsin, and chymotrypsin (Goswami et al., 2020; Zhao et al., 2020).

Biochemical Composition of Fish

The proximate composition study showed that the inclusion (10–20%) of greater duckweed enhanced the crude protein content of common carp in this study. The crude lipid and ash contents of common carp increased in a graded manner with the enhanced inclusion of greater duckweed in the diet. An earlier study showed that supplementation of duckweed improved the crude protein and crude lipid contents in fish (El-Shafai et al., 2004; Yılmaz et al., 2004; Fasakin, 2008; Abou et al., 2011). Aslam et al. (2021) reported significantly higher crude protein contents in *L. minor*-incorporated diet-fed grass carp *Ctenopharyngodon idella* and silver carp *Hypophthalmichthys molitrix* compared with the *soybean*-supplemented diet-fed fishes. The inclusion of duckweed increased the ash content in fish (Fasakin et al., 1999; El-Shafai et al., 2004; Fasakin, 2008). This indicated that greater duckweed-supplemented diets fulfilled the nutritional requirements of common carp. The proximate composition study showed that the crude protein, crude lipid, and ash contents of greater duckweed were 36.65, 7.62, and 18.19 g/100 g (Sharma et al., 2019). The amino acid profile of greater duckweed is comparable with *soybean* meal. Feeding with greater duckweed-supplemented diets improved the non-essential amino acid contents in the common carp. The supplementation of fermented *L. minor* in the diet enhanced the lysine content in tilapia (Herawati et al., 2020).

In this study, fatty acid composition of fish was influenced by the supplementation of greater duckweed. Highest and lowest SFA contents were found in SP5 and SP20 diet-fed common carp, respectively. MUFA content showed an inverse relationship with the increased inclusion of greater duckweed in diet of common carp. A direct relationship was found between the amount of greater duckweed in the diet and n-6 PUFA and n-3 PUFA contents in common carp. Inclusion of greater duckweed in the diet enhanced the ALA, DHA, and EPA contents in common carp. The duckweeds are a rich source of fatty acids (Appenroth et al., 2017; Chakrabarti et al., 2018; Sharma et al., 2019). The feeding of *Azolla filiculoides* enhanced the total n-3 PUFA (especially EPA and DHA) content (Abou et al., 2011), and fermented *L. minor* enhanced LOA (Herawati et al., 2020) in Nile tilapia. Similarly, the contents of EPA and DHA in common carp increased linearly with increasing greater duckweed level in the diet.

Expression of Genes Involved in the Biosynthesis of Fatty Acids

In this study, the expression levels of key genes involved in the biosynthesis of fatty acids like *fads2d6*, *elovl2*, *elovl5,* and *fas* were evaluated in the common carp. Upregulation of all these genes was found in fish fed greater duckweed supplemented diets compared with control diet-fed fish. This might be due to the presence of LOA and ALA in the experimental diets. Earlier study showed that the higher contents of LOA and ALA upregulated the expression of desaturases/elongases (Tocher et al., 2004; Turchini et al., 2006; Francis et al., 2007; Li et al., 2008). However, an excess of ALA in diet can inhibit the transcription of *fads2d6* gene (Bell et al., 1993). In this study, among the fish fed the experimental diets, the highest expression levels of genes were recorded in the SP5 treatment, and then the expression gradually decreased. The ALA content increased with increasing inclusion of greater duckweed in diet. EPA and DHA contents increased with decreasing expression of genes *elovl2, elovl5,* and *fads2d6*. Similar results were found in common carp and rainbow trout *Oncorhynchus mykiss* where the expressions of desaturases and elongases were higher in fish with lower contents of EPA and DHA (Ren et al., 2012; Lazzarotto et al., 2018).

CONCLUSION

Greater duckweed (*S. polyrhiza*) may replace soybean meal up to 20% in the diet of *C. carpio* without affecting the digestive physiology and growth performance of fish even at an early life stage. Inclusion of greater duckweed enhanced growth performance and improved the quality of fish in terms of amino acids and n-3 PUFA, especially EPA and DHA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethics Committee, University of DelhiDU/ZOOL/IAEC-R/2015/07.

AUTHOR CONTRIBUTIONS

RC, DT, JS, and BG designed the study. AS, RC, JS, and GK cultured the fish and analyzed the samples. JS, RC, PM, DT, BG, and AS performed statistical analysis and wrote the manuscript. AS, RC, JS, and GK prepared graphs and tables. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.788455/full#supplementary-material

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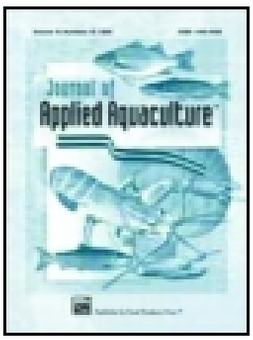

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The study of effect of vitamin C and *Achyranthes aspera* seeds enriched diets on the growth, biochemical composition, digestive enzyme activities and expressions of genes involved in the biosynthesis of fatty acids in Snow trout *Schizothorax richardsonii* (Gray, 1832)

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The study of effect of vitamin C and *Achyranthes aspera* seeds enriched diets on the growth, biochemical composition, digestive enzyme activities and expressions of genes involved in the biosynthesis of fatty acids in Snow trout *Schizothorax richardsonii* (Gray, 1832)

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ABSTRACT

Snow trout, *Schizothorax richardsonii* (Gray, 1832), is an economically important fish in the Himalayan region. Snow trout fry (118.53 ± 5.42 mg) were cultured in flow-through systems for 60 days. Fry were fed with four different diets containing: 0.08% vitamin C (D1), 0.5% *Achyranthes aspera* seeds (D2), both vitamin C and seeds (D3), and a control (D4). Average weight (271.82 ± 4.90 g) and specific growth rate (1.39 ± 0.03) were significantly higher in fish fed D3 compared to the others. Digestive enzyme activities were significantly higher in D3 compared to the others. EPA content was significantly higher in D2 (110.33 ± 0.94 mg 100 g^{-1}) and D3 (109.87 ± 1.87 mg 100 g^{-1}); DHA (342.70 ± 0.77 mg 100 g^{-1}) and *n*-3 PUFA (496.17 ± 1.82 mg 100 g^{-1}) contents were significantly higher in D3 compared to the others. The expression of *fads2d6* was significantly higher in D3 (6.70-fold) and *elovl5* in D2 and D3 (1.51–1.45-fold) compared to the others. Enriched diets improved fish flesh composition.

KEYWORDS

Schizothorax richardsonii; Vitamin C; *Achyranthes aspera*; DHA and EPA; *fads2d6* and *elovl5*

Introduction

Snow trout, *Schizothorax richardsonii* (Gray, 1832), belongs to the family Cyprinidae and is a commercially important fish of the Himalayan region. It contributes the maximum yield in the capture fisheries of hills and is a potential species for aquaculture (Kamalam et al. 2019; Petr 2002). Although artificial breeding of snow trout is successful (Joshi 2004), the proper culture technique has not been developed (Sarma et al. 2013). Moreover, the growth rate of snow trout is very slow, and the fish are susceptible to diseases

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Supplemental data for this article can be accessed on the publisher's website.

(Moses, Nilssen, and Chakrabarti 2017). Nutrition plays a significant role in the health status of the cultivable species (Lall and Olivier 1993). Supplementation of growth promoters and immunostimulants in the diet enhances the growth and improves the immunity of the fish (Kord et al. 2021). An earlier study shows that the enrichment of feeds with vitamin C and *Achyranthes aspera* seeds increases the growth rate of snow trout larvae. The enhanced levels of digestive enzymes, myeloperoxidase, nitric oxide synthase, etc., are found in fish fed enriched diets (Moses, Nilssen, and Chakrabarti 2017). The enzyme L-gulonolactone oxidase, which helps in the synthesis of ascorbic acid from L-gulonolacetone, is lacking in teleosts (Sato, Nishikimi, and Udenfriend 1976). Many studies show that supplementation of vitamin C in diets of fishes improves health conditions, e.g., in rohu *Labeo rohita* (Misra et al. 2007), Jian carp *Cyprinus carpio* (Liu et al. 2011), tilapia *Oreochromis karongae* (Nsonga et al. 2009), GIFT tilapia *O. niloticus* (Huang et al. 2016), and discus fish *Symphysodon haraldi* (Liu et al. 2018). Various factors like species, development stage, and environment etc., influence the requirement of vitamin C. The supplementation is needed more at the earlier stages compared to adults (Imanpoor, Imanpoor, and Roohi 2017; Kolkovski et al. 2000).

The herb *Achyranthes aspera* L. belongs to the family Amaranthaceae. It grows in tropical climatic conditions and has pharmaceutical applications. Many studies show that the seed of *A. aspera* enhances the immunity of different carps, and it protects the fishes against pathogens (Chakrabarti and Srivastava 2012; Chakrabarti et al. 2014; Kumar et al. 2019; Rao and Chakrabarti 2005; Rao et al. 2004; Sharma et al. 2019). The feeding of a seed-enriched diet protects the carps from harmful UV-B radiation (Singh, Sharma, and Chakrabarti 2013a, 2013b). The seed-supplemented diet also enhances the survival and growth rate of fishes. Chakrabarti et al. (2012) have observed the presence of saturated fatty acids (SFA), palmitic acid (23%), stearic acid (2%), and two long-chain polyunsaturated fatty acids (LC-PUFA)—oleic acid (33%) and linolenic acid (42%)—in the seeds of *A. aspera*.

Earlier studies show that freshwater teleosts are capable of converting linoleic acid (LA; 18:2 *n*-6) and α-linolenic acid (ALA; 18:3 *n*-3) to LC-PUFA—arachidonic acid (AA; 20: 4 *n*-6), eicosapentaenoic acid (EPA; 20:5 *n* − 3), and docosahexaenoic acid (DHA; 22:6 *n*-3) (Buzzi, Henderson, and Sargent 1997; Cook 1996; Glencross 2009). Therefore, the essential fatty acid requirements of freshwater teleosts can usually fulfill dietary LA and ALA (Tocher et al. 2001; Tocher, Dabrowski, and Hardy 2010). The conversion of LA and ALA to LC-PUFA requires a series of fatty acyl desaturase (*fad*) and elongation of very long-chain fatty acids (*elovl*) enzymes such as *elovl5* and *elovl2* (Torstensen and Tocher 2010). Fatty acid synthase (*fas*) is a key enzyme that regulates the de novo biosynthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH (Dong et al. 2014). So far,

no study has been conducted to evaluate the effect of seeds of *A. aspera* on the fatty acid composition and the expressions of genes involved in the biosynthesis of fatty acids in snow trout.

The present study aims to evaluate the effect of vitamin C and seeds of *Achyranthes aspera* supplemented diets on the growth performance, digestive enzyme activities, biochemical composition, and expressions of specific genes involved in the biosynthesis of fatty acids—*fads2d6, fas, elovl5*, and *elovl2*—in the fry of snow trout (*Schizothorax richardsonii*).

Materials and methods

Culture of fry

Snow trout were bred in the wet laboratory of DCFR, Champawat, Uttrakhand, India, in September 2017, and larvae were grown up to fry stage (118.53 ± 5.43 mg). The experiment was started October 8 and was continued up to December 6, 2017. Fry were cultured under four different feeding regimes and maintained in four separate flow-through systems. Each flow-through system consisted of four culture units (10 L each); these four units were considered replicates. The flow rate of the water was 1.5 L min^{-1}. The stocking density was 10 fry per $unit^{-1}$ (10 × 4 = 40 fry per treatment).

Formulation of diets and feeding

Four diets were formulated using fish meal as protein source (Table 1). The three experimental diets were: diet 1 (D1) was supplemented with vitamin C (0.08%), diet 2 (D2) was supplemented with *A. aspera* (0.5%) seeds, and diet 3 (D3) was enriched with vitamin C (0.08%) and seeds (0.5%); the control diet 4 (D4) had no vitamin C or seeds. The concentrations of vitamin C and seeds in the diets were selected based on a previous experiment by Moses, Nilssen, and Chakrabarti (2017). The amino acids and fatty acids compositions of the

Table 1. Formulation of diets and their composition.

Ingredient (g kg^{-1})	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal	450	450	450	450
Fish oil	10	10	10	10
Wheat flour	535.2	531	530.2	536
Vitamins & minerals	4	4	4	4
Vitamin C	0.8	–	0.8	–
A. aspera	–	5	5	–
Proximate composition of diets (% dry weight)				
Lipid	7.43 ± 0.03	6.86 ± 0.03	7.00 ± 0.05	7.09 ± 0.21
Protein	35.84 ± 1.12	35.92 ± 0.26	36.00 ± 0.23	36.15 ± 1.13
Moisture	7.37 ± 0.08	7.39 ± 0.12	7.00 ± 0.08	7.38 ± 0.03
Ash	9.36 ± 0.11	9.20 ± 0.03	8.84 ± 0.11	9.04 ± 0.05

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

formulated diets were evaluated (Tables S1 and S2 in the supplementary materials). Snow trout fry were fed at the rate of 5% of body weight every day. The whole amount of feed was divided into two parts and was given at 9:00 a.m. and 4:00 p.m. Satiation limit was assessed by feeding the fish with different amount of feeds; it was observed that feeding of fish at the rate of 2.5% of body weight was suitable. After 1 h of feeding, the uneaten feed was removed. The experiment was continued for 60 days.

Survival rate, growth, and feed conversion ratio

Survival rate (%) of snow trout was recorded. The weight of individual fish was recorded (Libror AEG-120, Shimadzu, Japan) after 60 days of culture. Specific growth rate (SGR) and feed conversion ratio (FCR) were calculated as follows:

$$Specific\ growth\ rate\ (SGR\ \%) = [ln\ (final\ weight) - ln\ (initial\ weight)] \times 100/study\ period$$

$$Feed\ conversion\ ratio\ (FCR) = feed\ intake/body\ weight\ gain$$

Digestive enzyme assays

The digestive tract was collected from individual snow trout. The digestive tracts of six fish were pooled to make one replicate, and four replicates were used per treatment. The sample was prepared in the following sequence: homogenization of pooled sample in ice-cold distilled water, following centrifugation (10,000 g for 30 min at 4°C) and collection of supernatant. The supernatant was used for the enzyme assay. All enzymes were assayed using a fluorometer (Biotek Synergy H1, USA). The EnzChek ® Ultra Amylase Assay Kit (E33651, Invitrogen, Oregon, USA) was used for the estimation of amylase activity. The excitation and emission were recorded at 485 nm and 520 nm respectively. The total protease activity was estimated based on a green fluorescence technique. The EnzChek® Protease Assay Kit (E6638, Molecular ProbesTM, Invitrogen) was used for the study. The change in fluorescence was estimated at excitation 485 nm and emission 530 nm. The trypsin activity was assayed using Na-benzoyl-L-arginin-methyl-coumarinylamide (Sigma, USA) as a substrate (Ueberschar 1988). The change in fluorescence was recorded at 380 nm (excitation) and 440 nm (emission). The chymotrypsin activity was assayed using succinyl-Leu-Val-Tyr-4-methyl-coumaryl -7-aminde (Sigma) as substrate (Cao et al. 2000). The excitation and emission were recorded at 380 nm and 450 nm respectively. Lipase was estimated using 4-methylumbelliferyl butyrate (4-MUB, Sigma) as substrate (Roberts 1985).

The excitation and emission were studied at 365 nm and 450 nm respectively. Total protein was estimated using bovine serum albumin as a standard (Bradford 1976).

Biochemical composition

The proximate composition—crude protein, crude lipid, moisture, and ash contents were assayed following standard methods (AOAC 2000). The crude protein was estimated following the Kjeldahl technique. In this assay, total nitrogen (%) was measured and was multiplied with 6.25 (N × 6.25) to obtain the protein (%) content. The crude lipid content was assayed gravimetrically (Folch, Lees, and Sloane-Stanley 1957). Moisture content was estimated by keeping the sample at 105°C for 3 h; for the estimation of ash, the sample was kept at 550°C for 8 h.

Fatty acids

The acid transesterification reaction was used for FAME (fatty acid methyl esters) estimation using a methylation reagent (1%). Crude lipid (1 mg) isolated from each sample was added with 2 mL of methylation reagent, 1 mL of toluene, and 1 mL of (C17:0) internal standard. After vortexing and proper mixing, the lipid sample was flushed with nitrogen. The sample was incubated at 50°C for 16 h (Christie 2003). The FAME (1 mg mL^{-1}) was isolated, purified, separated, and quantified using a Perkin Elmer Gas Chromatograph (Clarus 580 Autosampler, USA). Zebronics wax column 60 m (length) × 0.32 mm (internal diameter) × 0.25 μm (film thickness) was used for fatty acid analysis. FAMEs were compared with the known standards and the published data (Tocher and Harvie 1988). The fatty acid content was expressed as mg 100 g^{-1} of wet sample.

Amino acids

The amino acid composition of the sample was assayed using a Hitachi L-8900 Amino acid analyzer (Japan). All amino acids (except methionine, cysteine, and tryptophan) were hydrolyzed using HCl (6 N). In a digestion tube, a 30 mg sample was taken; 10 mL HCl was added to the sample, then flushed with nitrogen and sealed. The tube was kept for 22 h at 110°C for hydrolysis (Chakrabarti et al. 2018). Then the sample was kept in a nitrogen evaporator (PCi Analytics Pvt. Ltd., Maharastra, India) to evaporate acid. The dried samples were dissolved in HCl (0.02 N), and the final concentration of protein was 0.5 mg mL^{-1}. The sample (20 μL) was injected in the autosampler. The performic acid was first used to oxidize methionine and cysteine, and then hydrobromic acid (48%) was added in

the sample. The methanesulfonic acid (4 N) and 3-(2-aminoethyl) indole were used for the measurement of tryptophan. The amino acid concentrations were measured using standard solutions (Wako Pure Chemical Industries, Limited). The glutamine and tryptophan were estimated using freshly prepared standard (Sigma-Aldrich, USA). The amino acid content was expressed as g 100 g^{-1} of sample (wet weight).

Gene expression

Four genes—*fads2d6* (delta-6 desaturase), *fas* (fatty acid synthase), *elovl5* (elongase of very long chain fatty acids 5), and *elovl2* (elongase of very long chain fatty acids 2) were selected for the study; β-actin was used as reference gene for normalization. Primers were designed as per the sequences of genes available on the NCBI (Table 2). RNA was isolated using Trizol reagent following the instructions provided with the chemical. The purity of isolated RNA was estimated. The RNA was treated with DNase 1 to avoid DNA contamination (DNase kit, Sigma Aldrich). Purified RNA was converted to cDNA with an ABi kit (USA). The PCR amplification with β-actin confirmed cDNA, and bands were separated on agarose gel. SYBR green was used for the study of relative mRNA expressions (RT-PCR, Quant Studio 6 Flex, ABi, USA). The amplification efficiency of primers was estimated with their melt curves. Total volume of reaction mixture per well was 10 µL that was composed of 5 µL of SYBR green, 0.5 µL of forward primer, 0.5 µL of reverse primer, 3 µL of nucleic acid-free water, and cDNA 1 µL. The amplification cycle in the RT-PCR was as follows: 10 min for preliminary denaturation, number of cycles was 40 (like 15 s at 95°C and 1 min at 60°C). The plate was sealed with a 96-well optical adhesive cover. The optical plate was placed in RT-PCR and a program was run using QuantStudio 6 Flex software v1.1 on the computer. On the basis of CT value, the fold change of a particular mRNA was estimated (Livak and Schmittgen 2001).

Table 2. Primers used for gene expression analysis.

Gene	Accession No.	Direction	Primer Sequence
β-actin	KU715835	Forward	5′-GCCGTGACCTGACTGACTAC-3′
		Reverse	5′-CTGCTCGAAGTCAAGAGCCA-3′
fads2d6	KJ576791	Forward	5′-CACAATGCCTCGGCACAAT-3′
		Reverse	5′-CCAGCCAGAGTTCTCCAGATTT-3′
fas	MF289408	Forward	5′-CAATGCCCAGCAGCATAAGG-3′
		Reverse	5′-TGGGCTTGTTGAACCTCGG-3′
elovl5	KF924199	Forward	5′-GGGCTGGCTGTATTTCCAGAT-3′
		Reverse	5′-GATGCCACCCCATTAGTGTGA-3′
elovl2	KR706498	Forward	5′-ATCAGTTTGGTCTGCCGGTT-3′
		Reverse	5′-CAGCACAATGAAGATGGTGTCC-3′

Water quality

Water quality parameters were monitored regularly. The Hach multimeter (HQ40D, USA) was used for the estimation of temperature, pH, conductivity, dissolved oxygen, and ammonia levels of water. The nitrite and nitrate contents were measured following the APHA (2017) method. Temperature, pH, and conductivity ranged from 9.0 to 19.2°C, 7.15 to 7.88, and 241.3 to 251.6 μS cm^{-1} respectively in different culture systems throughout the study period. The experiment was started October 8 and completed December 6, 2017. At the start of the experiment the water temperature was 19.2°C; in November the water temperature gradually reduced. The minimum 9.0°C was recorded in the first week of December, and the experiment was completed. Snow trout is a coldwater fish and survives well in temperatures ranging from 5 to 20°C (Barat et al. 2019). Dissolved oxygen, ammonia, nitrite, and nitrate levels of fish culture units ranged from 7.36 to 9.38, 0.06 to 0.8, 0.10 to 0.18, and 0.21 to 0.28 mg L^{-1} respectively throughout the experiment.

Statistical analysis

The growth parameters, digestive enzyme activities, and biochemical composition of snow trout were expressed as means ± SEM. Data were analyzed using ANOVA, one-way analysis of variance, and Duncan's multiple range test (DMR) (Montgomery 1984). Statistical significance was accepted at the $P < 0.05$ level.

Results

Survival and growth

In snow trout fed the control diet (D4), the survival rate was 93%; all fish survived (100%) in the other treatments. A significantly higher final average weight was recorded in snow trout fed the D3 diet (271.82 ± 4.90 mg) compared to fish fed the other diets. The weight gain was 1.45–2.76-fold higher in fish fed the experimental diets compared to the control. Significantly higher SGR (1.39 ± 0.03%) and lower FCR (1.55 ± 0.07) were observed in snow trout fed the D3 diet compared to the fish cultured in the other three different feeding regimes (Table 3).

Table 3. Survival, average weight, specific growth rate (SGR), feed conversion ratio (FCR), and digestive enzyme activities found in *S. richardsonii* fed with four different diets. Means (n = 4) sharing different letters in the same row are significantly ($P < 0.05$) different.

Parameter	Diet 1	Diet 2	Diet 3	Diet 4
Survival (%)	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a	93 ± 2.50b
Average weight (mg)	198.72 ± 2.55b	201.22 ± 1.71b	271.82 ± 4.90a	173.74 ± 2.67 c
SGR (%)	0.86 ± 0.02b	0.87 ± 0.02b	1.39 ± 0.03a	0.64 ± 0.01 c
FCR	2.48 ± 0.03b	2.61 ± 0.16b	1.55 ± 0.07 c	3.26 ± 0.05a
Amylase (mU $\mu l^{-1}mg^{-1}$protein min^{-1})	19.93 ± 0.51b	21.46 ± 0.58b	33.28 ± 0.20a	12.09 ± 0.23 c
Total Protease (Fluorescence change $unit^{-1}$)	108.55 ± 4.07b	109.71 ± 1.83b	136.69 ± 3.0a	99.96 ± 2.54 c
Trypsin (μmol AMC mg^{-1}protein min^{-1})	141.66 ± 5.19b	150.87 ± 7.78b	254.63 ± 5.45a	72.86 ± 2.31 c
Chymotrypsin (μmol AMC mg_{-1}protein min^{-1})	74.80 ± 1.44b	80.34 ± 1.77b	162.35 ± 1.72a	59.83 ± 0.85 c
Lipase (μmol 4MU mg^{-1}protein min^{-1})	74.28 ± 2.06b	69.70 ± 1.66 c	157.68 ± 6.23a	78.68 ± 1.58b

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

Digestive enzyme activities

Significantly higher amylase, total protease, trypsin, chymotrypsin, and lipase activities were observed in snow trout fed the D3 diet compared to fish cultured in the other three different feeding regimes (Table 3). There was no significant difference in amylase, protease, trypsin, and chymotrypsin activities between snow trout fed the D1 and D2 diets. These enzyme activities were minimum in fish fed the control diet. Lipase activity was minimum in snow trout fed the D2 diet.

Proximate composition of muscle

The moisture contents ranged from 76.25% to 76.65% in different diets fed snow trout. The protein content was significantly higher in snow trout fed the D1 and D4 diets compared to the fish fed diets D2 and D3 (Table 4).

Table 4. Biochemical composition of muscle (wet weight) of *S. richardsonii* fed with four different diets. Means (n = 4) sharing different letters in the same row are significantly ($P < 0.05$) different.

Parameter (%)	Diet 1	Diet 2	Diet 3	Diet 4
Moisture	76.25 ± 0.36a	76.65 ± 0.54a	76.55 ± 0.17a	76.45 ± 0.35a
Protein	16.85 ± 0.10a	16.22 ± 0.27b	16.17 ± 0.16b	16.76 ± 0.20a
Lipid	3.20 ± 0.03b	3.32 ± 0.04b	3.54 ± 0.02a	3.11 ± 0.02 c
Ash	2.61 ± 0.05a	2.39 ± 0.02b	2.41 ± 0.01b	2.37 ± 0.02b

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

Table 5 (A) Amino acid profile (g 100 g^{-1} wet weight) of muscle of *S. richardsonii* fed with four different diets. Means ($n = 4$) sharing different letters in the same row are significantly ($P < 0.05$) different. Essential amino acids.

Amino acid	Diet 1	Diet 2	Diet 3	Diet 4
Histidine (His)	0.395 ± 0.001b	0.392 ± 0.001b	0.405 ± 0.003a	0.390 ± 0.001b
Isoleucine (Ile)	0.630 ± 0.002a	0.616 ± 0.002b	0.632 ± 0.001a	0.620 ± 0.001b
Leucine (Lue)	1.126 ± 0.004a	1.072 ± 0.005 c	1.101 ± 0.004b	1.109 ± 0.005b
Lysine (Lys)	1.247 ± 0.006a	1.210 ± 0.005 c	1.247 ± 0.002a	1.230 ± 0.002b
Methionine (Met)	0.396 ± 0.001a	0.289 ± 0.001 c	0.298 ± 0.003 c	0.388 ± 0.006b
Phenylalanine (Phe)	0.641 ± 0.010a	0.607 ± 0.003 c	0.624 ± 0.002b	0.628 ± 0.016b
Threonine (Thr)	0.725 ± 0.004a	0.686 ± 0.006 c	0.701 ± 0.001b	0.725 ± 0.012a
Tryptophan (Trp)	0.219 ± 0.002d	0.222 ± 0.002 c	0.228 ± 0.004a	0.226 ± 0.010b
Valine (Val)	0.731 ± 0.002a	0.715 ± 0.004 c	0.733 ± 0.006a	0.721 ± 0.004b
∑ Essential amino acids	6.109 ± 0.022a	5.808 ± 0.029d	5.970 ± 0.011 c	6.037 ± 0.046b

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

Table 5 (B) Nonessential and free amino acids.

Amino acid	Diet-1	Diet-2	Diet-3	Diet-4
Nonessential amino acids				
Alanine (Ala)	0.878 ± 0.002a	0.845 ± 0.005c	0.871 ± 0.002a	0.865 ± 0.003b
Arginine (Arg)	1.504 ± 0.009b	1.352 ± 0.211c	1.575 ± 0.046a	1.574 ± 0.002a
Asparatate (Asp)	1.500 ± 0.003a	1.435 ± 0.009d	1.465 ± 0.002c	1.485 ± 0.010b
Cysteine (Cys)	0.171 ± 0.001a	0.154 ± 0.003b	0.157 ± 0.001b	0.170 ± 0.001a
Glutamic Acid (Glu)	2.387 ± 0.009a	2.293 ± 0.006a	2.345 ± 0.002a	2.375 ± 0.016a
Glycine (Gly)	0.939 ± 0.004a	0.901 ± 0.008a	0.927 ± 0.001a	0.926 ± 0.003a
Proline (Pro)	0.526 ± 0.003b	0.527 ± 0.004b	0.541 ± 0.004a	0.518 ± 0.002 c
Serine (Ser)	0.697 ± 0.001a	0.658 ± 0.004c	0.671 ± 0.002b	0.695 ± 0.009a
Tyrosine (Tyr)	0.512 ± 0.002a	0.485 ± 0.001c	0.502 ± 0.002b	0.501 ± 0.004b
∑ Non-essential amino acids	9.113 ± 0.033a	8.650 ± 0.251b	9.054 ± 0.062a	9.110 ± 0.051a
Free amino acids				
Phosphoserine	0.034 ± 0.002a	0.023 ± 0.001b	0.025 ± 0.001b	0.022 ± 0.002b
Taurine	0.170 ± 0.001b	0.209 ± 0.001a	0.214 ± 0.004a	0.164 ± 0.001 c
Cystathionine	0.072 ± 0.002a	0.065 ± 0.001c	0.069 ± 0.001b	0.073 ± 0.001a
β-Amino isobutyric acid	0.266 ± 0.040a	0.204 ± 0.009b	0.207 ± 0.004b	0.259 ± 0.066a
γ-Amino-n-butyric acid	0.041 ± 0.016a	0.032 ± 0.002b	0.029 ± 0.001b	0.043 ± 0.002a
Ethanol amine	0.141 ± 0.007a	nd	nd	0.135 ± 0.013a
Hydroxylysine	0.144 ± 0.005a	0.142 ± 0.005a	0.147 ± 0.001a	0.154 ± 0.020a
Ornithine	0.034 ± 0.003a	0.035 ± 0.001a	0.039 ± 0.001a	0.036 ± 0.005a
3 Methylhistidine	0.013 ± 0.000a	0.016 ± 0.001a	0.017 ± 0.001a	0.012 ± 0.000a
Hydroxy proline	0.140 ± 0.006b	0.104 ± 0.007c	0.092 ± 0.005c	0.156 ± 0.004a
∑ Free amino acids	1.054 ± 0.154a	0.832 ± 0.030b	0.840 ± 0.015b	1.057 ± 0.213a

Note. Diet 1, vitamin C 0.08%; Diet 2, *A. aspera* seeds 0.5%; Diet 3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet 4, control.

A significantly higher lipid level was observed in snow trout fed diet D3 compared to the other treatments. The ash content was significantly higher in snow trout fed the D1 diet compared to the others.

Amino acid profile of muscle

Significantly higher histidine and tryptophan contents were observed in snow trout fed the D3 diet compared to fish fed the other diets (Table 5). Significantly higher isoleucine, lysine, and valine contents were found in snow trout fed diets D1 and D3 compared to the others. Significantly higher leucine, methionine, and phenylalanine levels were observed in snow trout fed the D1 diet compared to fish fed the other diets. Significantly higher threonine levels were recorded in snow trout fed the D1 and D4 diets compared to the fish fed diets D2 and D3. The total essential amino acids content was significantly higher in snow trout fed the vitamin C-supplemented diet (D1) compared to the fish cultured in the other three feeding regimes.

Nonessential amino acid alanine content was significantly higher in snow trout fed the D1 and D3 diets, and arginine content was significantly higher in snow trout fed the D3 and D4 diets compared to the others. Aspartate and tyrosine levels were significantly higher in snow trout fed the D1 diet compared to the fish cultured in the other three different feeding regimes. Cysteine and serine contents were significantly higher in snow trout fed the D1 and D4 diets compared to the D2 and D3 treatments. In snow trout fed different diets, glutamic acid and glycine contents were not significantly different. Proline content was significantly higher in snow trout fed the D3 diet compared to fish fed the other diets. The total nonessential amino acids content was significantly lower in snow trout fed the D2 diet compared to the others.

The free amino acids phosphoserine level was significantly higher in snow trout fed the D1 diet and taurine content in the D2 and D3 diets compared to the others. Cystathionine, β-amino isobutyric acid, and γ-amino-n-butyric acid contents were significantly higher in snow trout fed the D1 and D4 diets compared to the others. The hydroxyproline content was significantly higher in snow trout fed the D4 diet compared to the others. Significantly higher total free amino acids were recorded in snow trout fed the D1 and D4 diets compared to the D2 and D3 diets.

Fatty acid profile of muscle

In the muscle of snow trout fed the different diets, various saturated (SFA), monounsaturated (MUFA), *n*-6 polyunsaturated (*n*-6 PUFA), and *n*-3 polyunsaturated (*n*-3 PUFA) fatty acids were observed. Significantly higher myristic acid and pentadecanoic acid was observed in snow trout fed the D2 and D3 diets compared to the other two treatments (Table 6). Palmitic acid was significantly higher in snow trout fed the D2 diet compared to fish fed the

Table 6. Fatty acid profile (mg 100 g^{-1} wet weight) of muscle of *S. richardsonii* fed with four different diets. Means (n = 4) sharing different letters in the same row are significantly (P < .05) different.

Fatty acids	Diet-1			Diet-2			Diet-3			Diet-4		
Saturated fatty acids												
14:0	269.39	±	1.00b	271.31	±	1.34a	274.02	±	1.67a	264.34	±	2.09c
15:0	33.06	±	4.94b	38.63	±	0.84a	38.44	±	1.88a	33.48	±	3.56b
16:0	714.18	±	9.04b	728.72	±	1.14a	719.19	±	14.72b	705.27	±	10.99b
18:0	88.35	±	3.70ab	92.17	±	0.68a	85.03	±	4.16b	91.49	±	2.11a
22:0	5.37	±	0.31a	5.53	±	0.33a	4.41	±	0.55b	5.23	±	0.15a
24:0	9.78	±	0.82a	7.97	±	0.19b	7.96	±	0.41b	9.97	±	0.57a
∑SFA	1120.14	±	18.80a	1144.34	±	2.38a	1129.04	±	18.82a	1109.78	±	14.45a
Monounsaturated fatty acids												
16:1 *n*-9	669.40	±	1.49b	674.17	±	2.76a	679.20	±	7.07a	657.01	±	5.98c
18:1 *n*-9	829.03	±	3.05a	838.09	±	2.16a	838.70	±	7.41a	816.74	±	7.96b
20:1 *n*-9	100.17	±	0.64a	101.27	±	0.43a	100.55	±	0.43a	98.76	±	1.06a
22:1 *n*-9	37.70	±	0.31a	38.06	±	0.35a	37.00	±	0.91a	37.41	±	1.49a
24:1	62.98	±	0.83a	62.22	±	0.70a	63.81	±	0.93a	59.40	±	0.97b
∑ MUFA	1699.27	±	4.04b	1713.82	±	5.45a	1719.25	±	14.15a	1669.31	±	17.14c
n-6 Polyunsaturated fatty acids												
18:2 *n*-6	347.89	±	1.50b	351.11	±	0.54a	352.29	±	3.08a	343.82	±	2.90b
18:3 *n*-6	14.59	±	0.82b	15.91	±	0.20a	14.27	±	0.23b	14.59	±	0.36b
20:2 *n*-6	17.55	±	0.44a	17.32	±	0.49a	17.81	±	0.46a	18.55	±	0.65a
20:3 *n*-6	112.49	±	0.32a	113.18	±	0.35a	112.84	±	0.89a	111.48	±	0.70a
20:4 *n*-6	8.10	±	0.25a	7.94	±	0.31a	8.01	±	0.24a	7.63	±	0.41a
∑ *n*-6 PUFA	500.63	±	1.94a	505.46	±	0.64a	505.22	±	3.76a	496.07	±	4.23a
n-3 Polyunsaturated fatty acids												
18:3 *n*-3	33.89	±	0.15a	34.33	±	0.39a	34.44	±	0.25a	33.57	±	0.64a
18:4 *n*-3	8.79	±	0.26a	8.76	±	0.09a	9.16	±	0.19a	8.76	±	0.19a
20:5 *n*-3	107.81	±	0.78b	110.33	±	0.94a	109.87	±	1.88a	105.72	±	1.67b
22:6 *n*-3	339.73	±	2.67b	338.94	±	0.68b	342.70	±	0.77a	335.39	±	2.37c
∑ *n*-3 PUFA	490.22	±	2.37b	492.36	±	1.28b	496.17	±	1.82a	483.44	±	3.02c
n-3/*n*-6	0.98	±	0.00a	0.97	±	0.00a	0.98	±	0.00a	0.97	±	0.01a

Diet-1, vitamin C 0.08%; Diet-2, *A. aspera* seeds 0.5%; Diet-3, vitamin C 0.08% and *A. aspera* seeds 0.5%; Diet-4, control.

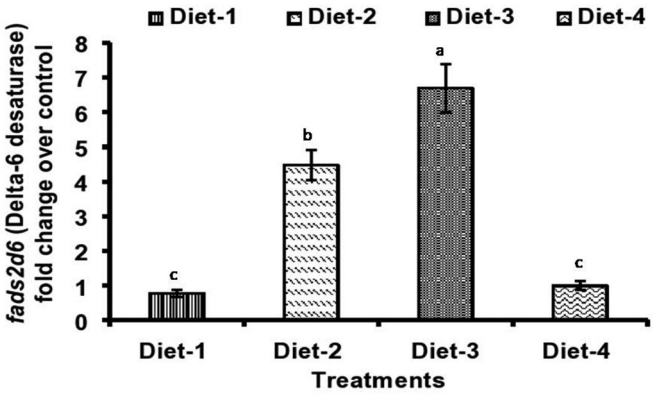

Figure 1. Relative mRNA expression of *fads2d6* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly (P < 0.05) different (*n* = 4).

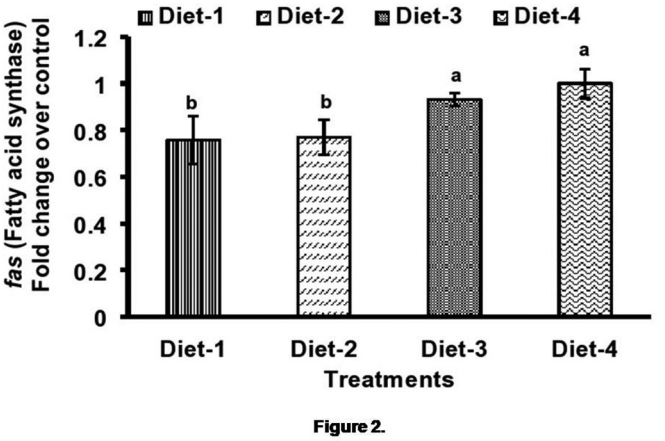

Figure 2. Relative mRNA expression of *fas* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly (P < 0.05) different (*n* = 4).

other diets. In snow trout fed the D3 diet, significantly lower stearic acid and behenic acid were found compared to others. SFA content was minimum in snow trout fed the control diet (D4).

Among MUFAs, palmitoleic and oleic acids contents were significantly higher in snow trout fed the D2 and D3 diets compared to the others. The gondoic acid and erusic acid levels were not significantly different among fish fed different diets. The nervonic acid content was significantly lower in snow trout fed the D4 diet compared to the others. The total monounsaturated fatty

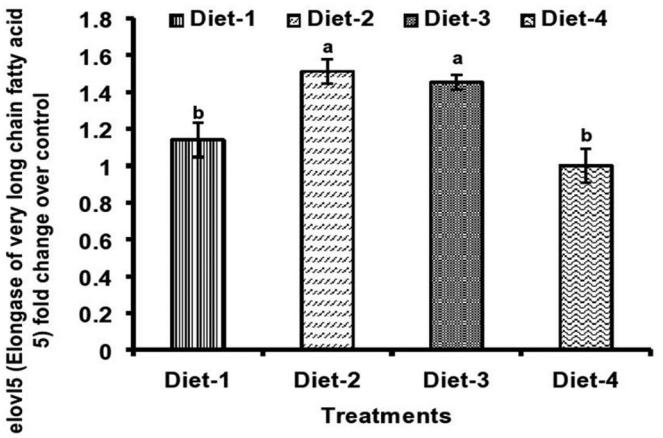

Figure 3. Relative mRNA expression of *elovl5* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly ($P < 0.05$) different ($n = 4$).

acid content was significantly higher in snow trout fed diets D2 and D3 compared to the others. In snow trout fed the D4 diet, the lowest MUFA content was observed.

Among *n*-6 PUFAs, the linoleic acid level was significantly higher in snow trout fed diets D2 and D3 and gamma linolenic acid in fish fed the D2 diet compared to the other diets. In snow trout fed the different diets, eicosadienoic, eicosatrienoic, and arachidonic acids contents were not significantly different.

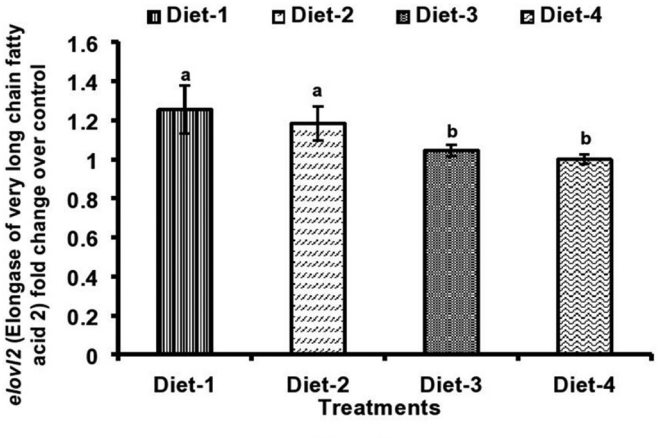

Figure 4. Relative mRNA expression of *elovl2* gene over control, with reference to *β-actin*. Bars with different superscripts are significantly ($P < 0.05$) different ($n = 4$).

In snow trout fed the different diets, α-linolenic acid and stearidonic acid contents were not significantly different. A significantly higher eicosapentaenoic (EPA) acid level was observed in snow trout fed the D2 and D3 diets compared to the others. The docosahexaenoic acid (DHA) content was significantly higher in snow trout fed the D3 diet compared to other diets. Total *n*-3 PUFA content was significantly higher in snow trout fed the D3 diet compared to fish cultured in the other feeding regimes. A minimum *n*-3 PUFA content was found in snow trout fed the control diet (D4). The *n*-3/*n*-6 ranged from 0.97 to 0.98 in fish fed different diets.

Gene expression analysis

There was upregulation of delta-6 desaturase (*fads2d6*) in snow trout fed the D3 diet, and this feeding regime was followed by fish fed the D2 diet (Figure 1). Fatty acid synthase (*fas*) mRNA expression was downregulated in snow trout fed the enriched diets compared to the control (Figure 2). There were upregulations of *elovl5* in snow trout fed all enriched diets (Figure 3) and *elovl2* in snow trout fed the D1 and D2 diets compared to the others (Figure 4).

Discussion

The diets of snow trout fry were enriched with vitamin C, seeds of the plant *A. aspera*, and a combination of vitamin C and seeds. The positive effect of enriched diets was reflected on the survival rate of snow trout. The survival rate of snow trout was 93% in the control; in other three feeding regimes all fish survived (100%). The enhanced average weight was observed in snow trout fed the enriched diets compared to fish fed the control diet. Previous studies showed that the addition of vitamin C in diets enhanced the survival and growth and reduced the FCR of cultivable fishes such as rohu (Misra et al. 2007) and snow trout (Moses, Nilssen, and Chakrabarti 2017). In discus fish, supplementation of vitamin C and vitamin E resulted in higher SGR and lower FCR compared to the fish fed the control diet (Liu et al. 2018). Zou et al. (2019) recommended 71.46–150.26 mg kg^{-1} vitamin C for optimum growth of Chu's croaker *Nibea coibor*. Significantly higher average weight and SGR were found in snow trout fed both vitamin C and seeds enriched diet, D3 compared to the fish fed diets enriched with vitamin C/ seeds/control. In rohu, incorporation of 0.5% seeds of *A. aspera* increased the average weight and SGR (Sharma et al. 2019). The presence of ecdysterone in seeds increased the growth of fish (Chakrabarti et al. 2012). Ecdysterone increased protein synthesis in skeletal muscle (Goerlich-Feldmann et al. 2008) and thereby enhanced the growth of fish. The enrichment of diet with both vitamin C and seeds gave better results compared to the diet enriched with individual ingredients (vitamin C or seeds).

The digestibility of the consumed feed depends on the digestive enzyme activities of fish. All digestive enzymes activities assayed were significantly higher in snow trout fed the D3 diet. This resulted in efficient digestion of consumed diet and thereby the lowest FCR compared to fish fed other diets. All digestive enzyme activities (except lipase) were higher in fish fed diets with vitamin C and seeds compared to fish fed the control diet. Therefore, the highest FCR was observed in snow trout fed the control diet. In rohu fed seed-enriched diets, a lower FCR was recorded (Sharma et al. 2019; Singh et al. 2019).

Snow trout fed the D1 and D4 diets showed significantly higher protein contents compared to fish fed the D2 and D3 diets. There was no variation in the amino acid composition in snow trout cultured in four different feeding regimes; the amount of a specific amino acid varied. There were higher values of seven essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine) in snow trout fed the D1 diet and five amino acids (histidine, isoleucine, lysine, tryptophan, and valine) in fish fed the D3 diet compared to the fish fed the control diet, D4. Among nonessential amino acids, a higher value of alanine was found in snow trout fed both D1 and D3 diets compared to the fish fed diet D4; aspartate and tyrosine contents were maximum in fish fed the D1 diet and proline content was higher in D3 diets compared to snow trout fed the D4 diet. The free amino acid taurine content was significantly higher in fish fed the D2 and D3 diets. The enrichment of the diet with vitamin C and seeds increased the specific amino acid level in the muscles of fish. Several amino acids were found in the seeds of *A. aspera* (Goyal, Goyal, and Mehta 2007). Thus, feeding snow trout a seed-supplemented diet improved the amino acids contents of muscle. The enrichment of vitamin C in the diet of snow trout enhanced the protein synthesis and produced a higher amino acid profile. Vitamin C is a hydrogen donor and is associated with many hydroxylation reactions—in collagen maturation, catecholamine synthesis, and carnitine synthesis. Vitamin C helps in the synthesis of phenylalanine and tyrosine (Guillaume et al. 2001).

A significantly higher lipid content was found in fish fed the D3 diet. Enrichment of diets with seeds or vitamin C and seeds improved the fatty acid contents of the muscle of snow trout. Vitamin C showed no influence on the lipid and fatty acid contents of muscle of pacu (*Piaractus mesopotamicus*) or rainbow trout (*Oncorhynchus mykiss*) (Trattner et al. 2007; Trenzado, Morales, and de la Higuera 2008). Supplementation of vitamin C in the diets of grass carp (*Ctenopharyngodon idella*) improved the fatty acids (palmitoleic, oleic, linoleic, and linolenic acids and *n*-3 PUFA) contents in muscle compared to fish fed the control diet (Han et al. 2019). In snow trout fed the seed-enriched diet (D2), seven SFAs (myristic, pentadecanoic, palmitic, stearic, behenic acids, etc.) were found in highest proportion

compared to fish fed the other diets. In the muscle of snow trout fed the D1, D3, and D4 diets, 2 (behenic and lignoceric acids), 2 (myristic and pentadecanoic), and 3 (stearic, behenic, and lignoceric acids) SFAs were maximum respectively. Three MUFAs (palmitoleic, oleic, and nervonic acids) contents were maximum in snow trout fed the D2 and D3 diets. Among *n*-6 PUFA, the highest amount of linoleic acid was found in snow trout fed the D2 and D3 diets; γ-linolenic acid was maximum in fish fed the D2 diet. Among *n*-3 PUFAs, EPA content was significantly higher in fish fed the D2 and D3 diets; DHA content was highest in fish fed the D3 diet. Minimum EPA and DHA contents were observed in snow trout fed the D4 diet.

The present study showed that the supplementation of seeds in diets enhanced the fatty acids, especially DHA and EPA levels in the snow trout muscle. In common carp, linseed oil and sunflower oil induced substantial LC-PUFA production (Nguyen et al. 2019). Enhanced levels of EPA and DHA were found in snow trout fed the D2 and D3 diets in the present study. The presence of palmitic, oleic, and linolenic acids in seeds influenced the fatty acid compositions in snow trout fed the seed-supplemented diets. Moreover, the higher levels of EPA and DHA in snow trout fed the vitamin C and *A. aspera* seed-supplemented diet showed that the fish has the capacity to synthesize long-chain polyunsaturated fatty acids endogenously.

A significantly higher expression of *fads2d6* was found in fish fed the vitamin C and *A. aspera* seed-supplemented diet (D3). The expression was also higher in fish fed the *A. aspera* seed-supplemented diet (D2) compared to the fish fed the vitamin C-supplemented (D1) and control diets (D4). This showed that the presence of fatty acids (Chakrabarti et al. 2012) in the seed influenced the *fads2d6* expression. A higher expression of *delta 6 desaturase* was found in rainbow trout (*Onchorynchus mykiss*) fed a plant-based diet (Ve´ron et al. 2016). The upregulation of *fads-6a* was found in common carp fed sunflower oil (Nguyen et al. 2019). There was downregulation of *fas* in fish fed all enriched diets compared to the control. The downregulation of *fas* was found in grass carp fed a vitamin C-supplemented diet (Han et al. 2019). The mRNA expression of *elovl5* was higher in fish fed the D2 and D3 diets. The *elovl2* was upregulated in snow trout fed the D1 and D2 diets. There was no effect of plant-based oil in the expression of *elovl5* in common carp (Nguyen et al. 2019).

Conclusions

The enrichment of diets with vitamin C and *A. aspera* seeds enhanced the survival rate and growth of *S. richardsonii*. The enrichment also improved the flesh composition. Vitamin C improved the amino acids composition; seeds

improved the fatty acid composition of fish. Seeds enhanced the EPA and DHA contents in fish.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contribution

RC, JGS, GK, and SC designed the experiment; GK and SC conducted the experiment; GK, RKG, NK, and AKS analyzed the samples; RC, JGS, and GK prepared the manuscript; GK and NK prepared the tables and figures.

Data availability statement

The data that support the finding of this study are available within the article.

Ethical clearance

The ethical clearance for conducting this experiment was approved by the animal ethics committee of the University of Delhi. Protocol No - DU/ZOOL/IAEC-R/02/2019.

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Short communication

Growth and digestive enzyme activities of rohu labeo rohita fed diets containing macrophytes and almond oil-cake

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ABSTRACT

The impact of plant-based diets on the digestive physiology of rohu *Labeo rohita* fingerlings (10.66 ± 0.53 g) was evaluated. A diet with all protein supplied by fishmeal was included as a control (F). Four test diets containing 300 g/kg protein were formulated using the following plant ingredients and fishmeal in a 1:1 blend: almond oil-cake *Terminalia catappa* (FTC), duckweed *Lemna minor* (FLM), water fern *Salvania molesta* (FSM) and combination of these three ingredients (FTCLMSM). The final body weight and specific growth rate were significantly higher in rohu fed diet FLM compared to the other treatments. Significantly lower feed conversion ratio in rohu fed diet FLM showed that diet was utilized efficiently in this feeding regime compared to the other diets. The composition of diets also influenced the digestive enzyme activities of the fish. Thus, amylase, trypsin and chymotrypsin activities were significantly higher in rohu fed diet FLM compared to the rohu fed the other diets. Protease activity was significantly higher in rohu fed diets FTC and F and lipase activity was significantly higher in rohu fed diet FTC compared to the rohu fed the other diets. The inclusion of raw duckweed in feed replaced 300 g/kg of dietary fishmeal without affecting growth.

1. Introduction

Sustainable aquaculture depends on the supply of quality feed to the farmed species. Protein plays significant role in fish nutrition and fishmeal has been traditionally used as a major protein source for the formulation of feed. The availability of quality fishmeal and its cost are two major constrains. Freshwater macrophytes are considered as potentially good sources of protein for formulation of feed for some fish species (Hasan and Chakrabarti, 2009; Chakrabarti, 2017). Several studies show the advantages of use of macrophytes as fish feed ingredients *viz.*, feeding of Nile tilapia *Oreochromis niloticus* with fresh *Lemna perpusilla* (Hasan and Edwards, 1992) or diets based on *Azolla africana*, *Spirodela polyrrhiza* (Fasakin et al., 2001) and *A. filiculoides* (Abou et al., 2011; Abou et al., 2013), common carp *Cyprinus carpio* with a diet based on L. *minor* (Yılmaz et al., 2004) and rohu *Labeo rohita* with raw/fermented L.

Abbreviations: ANOVA, Analysis of Variance; AOAC, Association of Official Analytic Chemists; APHA, American Public Health Association; BBSRC, Biotechnology and Biological Science Research Council; DBT, Department of Biotechnology; DF, Dry fish; DH, Degree of hydrolysis; F, Fishmeal; FAO, Food and Agriculture Organization; FBW, Final body weight; FCR, Feed conversion ratio; FI, Feed Intake; FLM, Fishmeal with *Lemna minor*; FSM, Fishmeal with *Salvinia molesta*; FTC, Fishmeal with *Terminalia catappa*; FTCLMSM, Fishmeal with *Terminalia catappa* Lemna minor, Salvinia molesta; IAEC, Institutional Animal Ethics Committee; IBW, Initial body weight; LM, *Lemna minor*; SGR, Specific growth rate; SM, *Salvinia molesta*; TC, *Terminalia catappa*; TCLMSM, *Terminalia catappa* Lemna minor, Salvinia molesta; WG, Weight gain

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Table 1
Chemical composition of feed ingredients used for the formulation of diets (g/kg as fed). Values are given as Means ± SE (n = 3).

Parameters	F[1]	TC[1]	LM[1]	SM[1]	TCLMSM[1]
Dry matter[2]	948.4 ± 0.78	942.2 ± 0.30	920.1 ± 1.80	928.8 ± 2.46	931.0 ± 0.49
Crude protein	689.0 ± 5.22	457.3 ± 3.03	364.7 ± 2.81	283.6 ± 3.61	351.2 ± 5.32
Crude lipid	82.0 ± 5.20	93.2 ± 2.32	73.9 ± 0.83	48.5 ± 1.20	69.8 ± 1.53
Total carbohydrate	15.4 ± 6.02	325.9 ± 0.10	263.9 ± 6.40	439.7 ± 0.91	362.8 ± 2.62
Crude ash	165.2 ± 3.34	65.8 ± 0.11	217.2 ± 0.24	157.0 ± 0.90	147.2 ± 1.14
Energy value (kcal/kg)[7]	3556.0 ± 32.0	3971.6 ± 23.4	3427.5 ± 24.31	3329.7 ± 28.88	3484.2 ± 25.43

[1]F, Fishmeal; TC, *T. catappa*; LM, *L. minor*; SM, *S. molesta*; TCLMSM, *T. catappa* + *L. minor* + *S. molesta*.
[2]Dry matter = Weight in g (1000 – Moisture) in 1 kg of feed.
[3]Energy (kcal/kg) = [(Crude protein g/kg × 4) + (Crude lipid g/kg × 9) + (Total Carbohydrate g/kg × 4)].

polyrhiza (Bairagi et al., 2002) or *Azolla microphylla* and *A. pinnata* (Datta, 2011) or *Ipomoea aquatica* supplemented diets (Ali and Kaviraj, 2018).

Labeo rohita rohu (family: Cyprinidae) is an economically important carp that is used extensively in composite fish culture. Rohu is an omnivore, column feeder fish and used in composite fish culture. Digestive tract analysis shows the presence of plant materials (Jhingran, 1991). The present study aims to evaluate the impact of diets supplemented with *Lemna minor* (LM), water fern *Salvania molesta* (SM) and oil-cake of almond *Terminalia catappa* (TC) on the growth and digestive enzyme activities of rohu.

2. Materials and methods

2.1. Ingredients and their composition

Locally available ingredients were used for the formulation of fish feed. The dry fish (DF), Bombay duck *Harpadon nehereus* was purchased from local fish market Ghazipur, New Delhi, India. The almond oil-cake *T. catappa* (TC) is a low-cost agricultural by-product. It was collected after the extraction of oil from a local oil extraction mill. The duckweed L. *minor* (LM) and water fern *S. molesta* (SM) were cultured in the outdoor cemented tanks using organic manures (Chakrabarti et al., 2018). Macrophytes were harvested, cleaned, air dried and kept in an oven at 40 °C. After drying, fishmeal, almond oil-cake and macrophytes were ground and sieved; fine powder were kept in air tight containers at 4 °C for further use.

Chemical composition of feed ingredients (Table 1 and Table 2) and diets (Table 3) were analyzed following the standard methods. Dry matter content was measured following the method 930.15 of the Association of Official Analytical Chemists (AOAC) (2000). The crude protein content was determined using the method 990.03 (Association of Official Analytical Chemists (AOAC), 2000) with an automated micro-Kjeldhal system (Pelican Instruments, Chennai, India). The nitrogen content was multiplied with 6.25 to calculate the amount of crude protein. The crude lipid content was measured (Folch et al., 1957) gravimetrically after extraction with chloroform/methanol (2:1, v/v). Carbohydrate content was then determined by the subtraction method. Ash contents of samples were determined following the method 942.05 of the Association of Official Analytical Chemists (AOAC) (2000). Energy value was determined following the standard method (Merrill and Watt, 1973).

The amino acids contents of ingredients were determined with Automatic Amino Acid Analyzer L-8900 (Hitachi Co. Ltd., Tokyo, Japan). Briefly, the sample was digested with 6 N HCl at 110 °C for 22 h except methionine, cysteine and tryptophan. Digested sample was dried in Nitrogen Concentrator (PCi Analytic Private limited, Maharashtra, India). Then 0.02 N HCl was added in the dried sample and made the concentration of protein 0.5 mg/mL. The sample (1.5 mL) was taken in glass vial and kept in the Auto sampler. In determination column, a 20 µL sample was injected with a flow rate of 0.35 mg/mL and the column temperature was 30 − 70 °C. In reaction column, reaction temperature was 135 °C with a ninhydrin flow rate of 0.35 mg/mL. The ninhydrin derivatives of proline and hydroxyproline were monitored at 440 nm, while other amino acids were monitored at 570 nm. The amino acids were compared with standards and quantified (Wako Pure Chemical Industries Limited, USA). All samples of ingredients and diets were analyzed in triplicates.

2.2. Formulation of diets and culture of fish

Five different diets were prepared. The control diet was prepared with only fishmeal (F). Four plant-based diets were formulated with the proportion of fishmeal and plant ingredient maintained at 1:1. In FTC diet, fishmeal was blend with TC; in FLM, fishmeal was blend with LM; in FSM, fishmeal was blend with SM and diet FTCLMSM was a blend of fishmeal and all three plant ingredients *viz.* TC, LM and SM (Table 3). All dried ingredients were collected in appropriate amount and mixed properly before addition of oil; then sinking pelleted diets (1 mm die) were prepared with a Twin-Screw-Extruder (Basic Technology, Kolkata, India). Diets were specially formulated to a fixed dietary protein content of 300 g/kg with equal amounts of plant material and fishmeal. Thus, the plant ingredients replaced 370, 300, 220 and 310 g/kg of fishmeal in FTC, FLM, FSM and FTCLMSM diets, respectively compared to the F diet.

Indian major carp rohu *Labeo rohita* were obtained from Chatterjee Brothers' Fish Farm, West Bengal. Fish (initial average weight: 10.66 ± 0.53 g) were randomly distributed in 15 glass aquaria (10 fish/50 L aquarium) in triplicate in laboratory facility of

Table 2
Amino acid composition of ingredients use in experimental diets as a protein source in (g/kg as fed). Values are given as Means ± SE (n = 3).

Amino acids	F[1]	TC[1]	LM[1]	SM[1]	TCLMSM[1]
Essential					
Arginine (Arg)	45.49 ± 0.021	56.58 ± 0.085	30.60 ± 0.452	17.53 ± 0.021	27.80 ± 0.202
Histidine (His)	18.63 ± 5.747	10.75 ± 0.653	8.94 ± 0.115	6.82 ± 1.727	7.43 ± 0.013
Isoleucine (Ile)	31.81 ± 2.067	18.84 ± 1.953	20.43 ± 0.646	11.90 ± 0.918	15.78 ± 0.426
Leucine (Leu)	55.83 ± 2.543	34.11 ± 2.399	41.32 ± 0.463	21.47 ± 2.065	28.17 ± 0.948
Lysine (Lys)	61.15 ± 1.265	13.51 ± 0.952	26.83 ± 1.614	15.64 ± 0.036	18.41 ± 1.389
Methionine (Met)	20.31 ± 0.016	3.63 ± 0.983	8.59 ± 0.142	5.86 ± 0.042	5.59 ± 0.474
Phenylalanine (Phe)	29.69 ± 0.288	26.54 ± 2.219	25.71 ± 0.344	14.38 ± 0.626	20.24 ± 0.844
Threonine (Thr)	33.41 ± 0.187	16.36 ± 0.492	19.24 ± 1.389	14.37 ± 0.001	15.02 ± 0.393
Tryptophan (Trp)	14.28 ± 0.001	3.86 ± 0.081	3.65 ± 0.107	5.54 ± 0.005	7.00 ± 0.024
Valine (Val)	37.07 ± 2.127	22.36 ± 2.060	26.64 ± 0.966	16.39 ± 1.241	20.41 ± 0.537
Non-essential					
Alanine (Ala)	46.54 ± 1.541	22.60 ± 1.152	28.82 ± 0.410	16.62 ± 1.436	19.72 ± 0.624
Aspartate (Asp)	68.83 ± 2.329	59.61 ± 0.103	37.14 ± 3.722	30.17 ± 0.104	39.98 ± 0.501
Cysteine (Cys)	6.40 ± 0.810	6.94 ± 0.683	3.81 ± 0.321	3.29 ± 0.200	4.22 ± 0.271
Glutamic acid (Glu)	129.48 ± 5.072	147.92 ± 14.353	64.27 ± 1.025	39.01 ± 0.436	78.70 ± 1.488
Glycine (Gly)	43.32 ± 0.610	31.08 ± 1.407	28.61 ± 0.312	15.30 ± 1.105	21.30 ± 0.620
Proline (Pro)	27.41 ± 1.220	20.11 ± 1.147	12.48 ± 0.353	11.59 ± 1.191	15.26 ± 0.611
Serine (Ser)	26.12 ± 0.295	20.61 ± 0.255	23.48 ± 3.209	13.90 ± 1.044	15.33 ± 0.317
Tyrosine (Tyr)	26.06 ± 2.342	16.18 ± 0.832	19.05 ± 1.250	11.36 ± 1.304	13.29 ± 2.411
Free amino acids					
Phosphoserine (p- Ser)	2.26 ± 0.064	3.11 ± 1.086	5.78 ± 0.001	2.19 ± 0.002	1.91 ± 0.337
Taurine (Tau)	2.44 ± 0.173	0.16 ± 0.011	0.41 ± 0.151	0.19 ± 0.031	0.15 ± 0.011
Phospho ethanol amine (PEA)	–	0.24 ± 0.014	0.23 ± 0.066	0.55 ± 0.141	0.41 ± 0.049
Sarcosine (Sar)	5.17 ± 1.399	–	0.97 ± 0.043	–	0.24 ± 0.012
α Amino-n-adipic acid (α - AAA)	2.73 ± 0.101	–	0.45 ± 0.136	0.28 ± 0.001	1.05 ± 0.735
α Amino-n- butaric acid (α - ABA)	–	–	1.50 ± 0.123	–	–
Cystathionine (Cysthi)	3.65 ± 0.107	2.39 ± 0.606	0.93 ± 0.198	1.75 ± 0.069	1.89 ± 0.197
β Alanine (β-Ala)	–	3.51 ± 1.010	1.11 ± 0.204	3.02 ± 0.540	1.00 ± 0.121
β Amino isobutyric acid (beta-AiBA)	17.49 ± 1.001	–	9.71 ± 2.711	3.90 ± 1.415	1.260 ± 0.230
ϒ Amino butyric acid (ϒ - ABA)	3.00 ± 1.016	2.67 ± 1.143	4.05 ± 0.149	2.80 ± 0.151	3.86 ± 0.102
Ethanol amine (EOHNH2)	2.86 ± 0.552	1.65 ± 0.019	1.46 ± 0.043	1.73 ± 0.452	1.60 ± 0.931
Hydroxylysine (Hylys)	4.48 ± 0.416	–	0.58 ± 0.070	3.78 ± 0.072	
Ornithine (Orn)	5.82 ± 1.664	0.86 ± 0.070	0.14 ± 0.017	0.69 ± 0.011	0.65 ± 0.050
1 Methyl histidine (1 Mehis)	0.99 ± 0.014	1.98 ± 0.021	0.87 ± 0.037	1.40 ± 0.448	1.09 ± 0.011
3 Methyl histidine (3 Mehis)	11.97 ± 1.101	–	1.17 ± 0.045	–	–
Carnosine (Car)		–	1.06 ± 0.019	–	–
Hydroxyproline (Hypro)	4.60 ± 0.183	1.29 ± 0.101	1.33 ± 0.157	1.48 ± 0.021	1.17 ± 0.044
Citruline (Cit)	2.03 ± 0.532	–	1.26 ± 0.024	–	–

[1] F, Fishmeal; TC, *T. catappa*; LM, *L. minor*; SM, *S. molesta*; TCLMSM, *T. catappa* + *L. minor* + *S. molesta*.

University of Delhi. Each aquarium was connected with an external, mechanical filter (Sera fil bioactive 130, Germany). Water from each fish culture unit came to the mechanical filter and after filtration, the water was back to the culture unit. Rohu were acclimated at 25 °C for 7 days to mitigate handling stress. Rohu were maintained on a 12 h light: 12 h dark regime throughout the study period. Fish were cultured under five different feeding regimes: F, FTP, FLM, FSM and FTCFLMFSM and feed was given at a rate of 3% of body weight every day. The amount of feed was adjusted as the weight of fish increased during the study period. The total amount of feed was divided in two parts and delivered at 9.00 a.m. and 5.00 p.m. Excess food was collected after 1 h of each feeding and it was used for the determination of actual feed consumption rate. All fish were harvested after 90 days of culture. Survival rate and final body weight of fish were recorded. The study was conducted following the guidelines of Animal Ethics Committee (IAEC), Department of Zoology, University of Delhi, Delhi, India (DU/ZOOL/IAEC-R/2015/07).

2.3. Water quality

Water samples were collected at weekly interval (4 samples/ month) from each treatment (3 replicates/treatment) and twelve samples were collected during 90 days culture period. There were 36 samples/ treatment (3 replicates x 12 samples). Water quality parameters including temperature, pH, conductivity and dissolved oxygen levels of aquaria were monitored regularly using a probe connected to a portable meter (IntelliCAL LDO101, Hach, USA). Similarly, ammonia was monitored using appropriate probe (HQ40d Multiparameter, Hach, USA). Nitrite (4500-NO_2^-) and nitrate (4500-NO_3^-) were measured following the methods of American Public Health Association (APHA) (2012).

Table 3
Composition of diets and their proximate analysis. Data are given as Means ± SE (n = 3).

Ingredients (g/kg diet)	Diets				
	F[1]	FTC[1]	FLM[1]	FSM[1]	FTCLMSM[1]
Fishmeal	316.4	198.6	221.2	245.7	219.5
Terminalia catappa oil-cake	———	198.6	———	———	73.2
Lemna minor	———	———	221.2	———	73.2
Salvinia molesta	———	———	———	245.7	73.2
Wheat flour	649.6	568.8	523.6	474.6	527.0
Cod liver oil	30.0	30.0	30.0	30.0	30.0
Vitamin-mineral premix[2]	4.0	4.0	4.0	4.0	4.0
Proximate analysis (g/kg)					
Dry matter[3]	928.6 ± 2.27	924.1 ± 2.15	939.2 ± 2.32	944.0 ± 1.17	925.3 ± 2.54
Crude protein	315.8 ± 1.50	313.0 ± 2.12	304.6 ± 1.55	303.5 ± 2.35	314.6 ± 1.25
Crude lipid	86.4 ± 2.55	87.3 ± 1.87	76.3 ± 1.24	79.8 ± 1.45	80.6 ± 1.25
Total carbohydrate[4]	454.5 ± 1.21	464.6 ± 2.25	468.9 ± 2.17	461.7 ± 1.2	453.5 ± 2.78
Crude ash	72.0 ± 2.12	59.2 ± 1.22	89.6 ± 1.17	99.2 ± 1.25	76.6 ± 2.15
Energy value (kcal/ kg)[5]	3858 ± 34.0	3896 ± 34.31	3780 ± 26.0	3778 ± 27.2	3798 ± 27.4

[1] F, Fishmeal; FTC, Fishmeal + *T. catappa*; FLM, Fishmeal + L. *minor*; FSM, Fishmeal + *S. molesta*; FTCLMSM, Fishmeal + *T. catappa* + *L. minor* + *S. molesta*.

[2] Supradyan multivitamin tablets with minerals and trace elements contains (as mg/kg in diets): = Vitamin A (as acetate) 12; Cholecalciferol 0.1; Thiamine mononitrate, 40; Riboflavine 40; Pyridoxine hydrochloride, 12; Cyanocobalamin, 0.06; Nicotinamide, 400; Calcium pantothenate 65.20; Ascorbic acid 600; α-Tocopheryl acetate,100; Biotin, 1.00. Minerals: Tribasic calcium phosphate, 516; Magnesium oxide, 240; Dried ferrous sulphate, 128.16; Manganse sulphate monohydrate 8.12; Total phosphorus, 103.20. Trace elements: Copper sulphate pentahydrate 13.56; Zinc sulphate, 8.80; Sodium molybdate dihydrate, 1.00; Sodium borate 3.52.

[3] Dry matter = Weight in g (1000 - Moisture) in 1 kg of feed.

[4] Carbohydrate = 1000 - ([Moisture + Protein + Lipid + Ash] contents of 1 kg feed).

[5] Energy (kcal/kg) = [(Crude protein g/kg × 4) + (Crude lipid g/kg × 9) + (Total Carbohydrate g/kg × 4)].

2.4. Sampling of fish

After 90 days of feeding trial, fish were fasted for 24 h. All fish were weighed and then anaesthetized with tricaine methanesulphonate MS-222; Sigma, USA. Fish were dissected on a glass plate maintained at 0 °C. The digestive tract of individual fish two fish per replicate; 2 × 3 replicates = 6 fish per treatment was collected, rinsed with chilled distilled water, blot dried and weighed. Then the entire digestive tract was homogenized in chilled distilled water 1:10 to maintain neutral pH of extract as this extract was used for various enzyme assays at different pHs. The homogenate was centrifuged at 10,000 x g for 15 min at 4 °C Sigma 3K30, Germany and the supernatant collected and used for enzyme activity study. Total soluble protein was measured following the method of Bradford (1976) using bovine serum albumin (Sigma, St Louis, USA) as a standard (1 mg/mL).

All enzymes were assayed using fluorometric methods (Fluoremeter, BioTek Synergy H1, USA). Amylase activity was measured with EnzChek@ Ultra Amylase Assay kit (E33651, Invitrogen, USA) with fluorescence measured at 485 nm for excitation and 520 nm for emission. Total protease activity was measured using EnzChek@ Protease Assay kit (E6638, Invitrogen, USA) with fluorescence measured at 485 nm (excitation) and 530 nm (emission). Trypsin activity was estimated using Na-benzoyl-L-arginin-methyl-coumarinylamide (Sigma, USA) as substrate (Ueberschär, 1988) with fluorescence measured at 380 nm (excitation) and 440 nm (emission). Chymotrypsin activity was measured following the method of Cao et al. (2000) using succinyl-Leu-Val-Tyr-4-methyl-coumaryl-7-aminde (Sigma, USA) as substrate and fluorescence measured at 380 nm (excitation) and 450 nm (emission). Neutral lipase activity was measured using 4-methylumbelliferyl butyrate (4-MUB, Sigma, USA) as substrate (Roberts, 1985) with fluorescence recorded at 365 nm for excitation and 450 nm for emission.

2.5. Specific growth rate, weight gain, feed intake and feed conversion ratio

The specific growth rate (SGR), weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) were calculated as follows:
SGR (%) = (In Final body weight - In Initial body weight) × 100/ Duration of expriment.
WG (%) = 100 [(Final body weight - Initial body weight)/ Initial body weight].
FI = 100 x Total feed fed (dry matter)/ [(Initial weight + Final weight + Dead fish weight)/ 2 x days].
FCR = Dry weight of feed consumed by individual fish during experiment/ Wet weight gain of individual fish

2.6. Statistical analysis

Chemical composition of feed ingredients, diets and water quality parameters were given as Means ± SE of three replicates and analyzed using one-way analysis of variance (ANOVA). Amino acids composition of ingredients and proximate composition of feeds were given as Means ± SE of three replicates. Performance parameters (IBW, FBW, WG, SGR, FI and FCR) and digestive enzyme

activities (amylase, protease, trypsin, chymotrypsin and lipase) were given as means with pooled standard error (pSEM), using the aquarium as the experimental unit, and analyzed using one-way ANOVA and Duncan's multiple range test (Montgomery, 1984). Statistical analyses were performed using the Statistics 22 program (SPSS, 2013). Statistical significance was accepted at $P < 0.05$ level.

3. Results

3.1. Composition of ingredients

Analyses of chemical composition of raw ingredients showed that there was variation in the composition (Table 1). Protein, lipid and ash contents were significantly higher in fishmeal, almond oil-cake and duckweeds, respectively compared to the other ingredients. The amino acid profiles showed that all essential and non-essential amino acids were present in almond oil-cake, duckweeds, water fern and fishmeal although there was variation in their amount in different ingredients (Table 2). The highest amount of essential amino acids was found in fishmeal followed by duckweed, other than histidine content, that was higher in almond oil-cake compared to duckweed. Similar to the essential amino acids, non-essential amino acids contents were highest in fishmeal compared to other ingredients, other than glutamic acid that was highest in almond oil-cake. Some free amino acids such as sarcosine, α-amino-n-butaric acid, 3-methyl histidine and citruline were absent in almond oil-cake and water fern, but were present in fishmeal and duckweed.

3.2. Water quality

There were no significant differences in temperature, pH, dissolved oxygen, ammonia, nitrite, nitrate and conductivity of water in five different treatments throughout the study period (Table 4). Water temperature and pH ranged from 25.0 ± 0.5–27.0 ± 1.0 °C and 7.85–8.48 in different treatments, respectively during the study period. Dissolved oxygen level was always above 5 mg/L regardless of feeding regimes. Ammonia, nitrite and nitrate levels ranged from 0.54 - 0.69, 0.21 - 0.25 and 2.28–2.32 mg/L, respectively in different treatments. Ammonia and nitrite levels were below 1.0 mg/L in all treatments throughout the study period. Conductivity ranged from 609.41 to 632.00 µS/cm in various treatments.

3.3. Survival and growth of fish

There was hundred percent survival of rohu cultured under five different feeding regimes. All fish survived. There was no significant difference in the body weight of fish at the beginning of the study. The final body weight was significantly higher in rohu fed diet FLM compared to the fish fed the other diets (Table 5). There were no significant difference between the final body weights of fish fed diets FTC and FTCLMSM. Final body weight was lowest in fish fed diet FSM. Consequently, the weight gain and specific growth rate of rohu showed the similar trend. Highest SGR was found in fish fed diet FLM compared to the fish fed the other diets. Feed intake and feed conversion ratio showed the opposite trend. FCR was significantly lower in rohu fed diets FLM compared to fish fed the other diets.

3.4. Enzyme activities

Amylase activity was significantly higher in rohu fed diet FLM compared to fish fed the other diets (Table 6). This group was followed by fish fed diets FSM, FTCLMSM and FTC with lowest amylase activity in fish fed diet F. Total protease activity was significantly higher in rohu fed diets FTC and F compared to fish fed the other diets. There was no significant difference in total protease activity between these two former treatments. Significantly higher trypsin activity was recorded in rohu fed the duckweed-based diet compared to fish fed other diets. A similar trend was found with chymotrypsin activity with highest activity observed in fish fed diet FLM. Lowest trypsin and chymotrypsin activities were recorded in rohu fed diet FTC. In contrast, it was interesting that lipase activity was significantly higher in rohu fed almond oil-cake-based diet compared to fish fed other diets. This group was

Table 4
Dissolved oxygen, ammonia, nitrite, nitrate and conductivity of water found in different treatments during 90 days of culture. Water quality was monitored at weekly interval in various treatments (4 samples/month/treatment; 12 samples/ treatment in 3 months; 3 replicates/treatment). Data are provided as Means ± SE (n = 3).

Parameters	F[1]	FTC[1]	FLM[1]	FSM[1]	FTCLMSM[1]
Dissolved oxygen (mg/L)	6.98 ± 0.103	6.98 ± 0.184	6.79 ± 0.053	7.12 ± 0.069	7.10 ± 0.025
Ammonia (mg/L)	0.67 ± 0.009	0.54 ± 0.028	0.68 ± 0.057	0.57 ± 0.009	0.69 ± 0.025
Nitrite (mg/L)	0.23 ± 0.005	0.22 ± 0.005	0.21 ± 0.002	0.23 ± 0.002	0.25 ± 0.002
Nitrate (mg/L)	2.28 ± 0.039	2.29 ± 0.029	2.32 ± 0.024	2.30 ± 0.028	2.31 ± 0.026
Conductivity (µS/cm)	613.91 ± 4.763	610.08 ± 2.068	632.00 ± 3.079	609.41 ± 1.683	611.16 ± 4.041

[1] F, Fishmeal; FTC, Fishmeal + *T. catappa*; FLM, Fishmeal + *L. minor*; FSM, Fishmeal + *S. molesta*; FTCLMSM, Fishmeal + *T. catappa* + *L. minor* + *S. molesta*.

Table 5
Growth performance and feed conversion ratio of *L. rohita* fingerlings fed with five different diets for 90 days. There were three replicates/treatment and 10 fish/replicate (10 × 3 = 30 fish/treatment). Means with different superscripts in the same row are significantly different.

Parameters	Diets					pSEM	P-value
	F[1]	FTC[1]	FLM[1]	FSM[1]	FTCLMSM[1]		
IBW (g)[1]	10.66 [a]	10.66 [a]	10.66 [a]	10.66 [a]	10.66 [a]	0.007	0.998
FBW (g)[1]	20.89[c]	21.47[b]	22.45[a]	20.48[c]	21.37[b]	0.132	< 0.001
WG (%)[2]	96.00[c]	101.41[b]	110.60[a]	92.12[c]	100.50[b]	0.181	< 0.001
SGR (%)[3]	0.75[c]	0.78[b]	0.83[a]	0.73[c]	0.77[b]	0.062	< 0.001
FI (g/100 g BW/day)[4]	1.69[b]	1.64[c]	1.61[d]	1.71[a]	1.64[c]	0.001	< 0.001
FCR[5]	2.34[b]	2.22[c]	2.03[d]	2.44[a]	2.24[c]	0.003	< 0.001

[1] F, Fishmeal; FTC, Fishmeal + *T. catappa*; FLM, Fishmeal + *L. minor*; FSM, Fishmeal + *S. molesta*; FTCLMSM, Fishmeal + *T. catappa* + *L. minor* + *S. molesta*. IBW = Initial body weight, FBW = Final body weight.
[2] WG = Weight gain (%) = 100 [(Final body weight - Initial body weight)/ Initial body weight].
[3] FI = Feed intake = 100 x Total feed fed (dry matter)/ [(Initial weight + Final weight + Dead fish weight)/ 2 x days].
[4] SGR = Specific growth rate (%) = (In Final body weight - In Initial body weight) × 100/ Duration of expriment.
[5] FCR = Food conversion ratio = Dry weight of feed consumed by individual fish during experiment/Wet weight gain of individual fish.

Table 6
Amylase, protease, trypsin, chymotrypsin and lipase activities found in *L. rohita* cultured in five different feeding regimes. There were three replicates/treatment and two fish/replicate (2 × 3 = 6 fish/treatment). Means with different superscripts in the same row are significantly different.

Parameters	Diets					pSEM	P-value
	F[1]	FTC[1]	FLM[1]	FSM[1]	FTCLMSM[1]		
Amylase (mU/mg protein/min)	23.30[d]	41.30[c]	64.92[a]	48.77[b]	44.70[bc]	0.757	< 0.001
Protease (Fluorescence change/unit)	57.87[a]	58.19[a]	53.95[c]	53.25[c]	55.20[b]	0.515	0.015
Trypsin (μM AMC/mg protein/min)	32.20[d]	23.00[e]	76.63[a]	55.59[b]	42.90[c]	0.770	< 0.001
Chymotrypsin (μM AMC/mg protein/min)	21.20[c]	15.10[d]	29.29[a]	15.46[d]	24.70[b]	0.360	< 0.001
Lipase (μM 4-MU/mg protein/min)	12.37[b]	19.09[a]	9.31[c]	7.90[d]	10.26[c]	0.277	< 0.001

[1] F, Fishmeal; FTC, Fishmeal + *T. catappa*; FLM, Fishmeal + *L. minor*; FSM, Fishmeal + *S. molesta*; FTCLMSM, Fishmeal + *T. catappa* + *L. minor* + *S. molesta*.

followed by rohu fed diet F, the fishmeal-based diet.

4. Discussion

In the present study, highest growth was found in *L. minor* supplemented diet fed rohu. Earlier study showed that the supplementation of 25 % *A. microphylla* and *A. pinnata* mixture in diet enhanced the growth and SGR of rohu (Datta, 2011). Feeding of raw *Wolffia globosa*, the smallest duckweed to rohu fry showed better growth compared to the fish fed with formulated diet (Pradhan et al., 2019). Whereas, Stadtlander et al. (2019) reported that incorporation of another duckweed *Spirodela polyrhiza* at two levels of 6.25 and 12.5 % in the feed of rainbow trout affected the growth after 4 weeks of feeding. In the present study, supplementation of fish meal along with duckweed met the nutritional requirements of rohu. A lower FCR value showed that diet FLM was also utilized more efficiently in rohu compared to the other diets.

The study of chemical and amino acid compositions of almond oil-cake, duckweed and water fern largely showed the nutritional values of these ingredients as fish feed. The present study confirmed the earlier findings (Ahrens et al., 2005; Sharma et al., 2016; Chakrabarti et al., 2018). The presence of essential, non-essential and free amino acids in duckweed might influence the growth of rohu, despite the fact that their amounts were less in duckweed compared to fishmeal. Certainly, based on published amino acid requirements for rohu, duckweed protein could satisfy almost all the requirements. The essential amino acids requirements of rohu are reported as follows: arginine 2.30, histidine 0.90, isoleucine 1.20, leucine 1.50, lysine 2.27, methionine 1.42, phenylalanine 1.48, threonine 1.71, tryptophan 0.45 and valine 1.50 % of diet (Food and Agriculture Organization (FAO), 2013).

Amylase, trypsin and chymotrypsin activities were significantly higher in rohu fed diet FLM compared to fish fed other diets. The efficient enzyme activities in FLM might result in better FCR compared to the other feeding regimes in the present study. Earlier study showed that supplementation of 25 % *I. aquatica* leaf meal (fermented with bacteria) enhanced the α-amylase activity in rohu (Ali and Kaviraj, 2018). An *in vitro* digestibility study of almond oil-cake, duckweed and water fern showed the high degree of hydrolysis (DH%) of these raw ingredients with the digestive juices of rohu and common carp (Sharma et al., 2016). The effect of diet composition on digestive enzyme activities was found in the present study. Among the different ingredients used for diet formulation, the highest amount of lipid was found in the almond oil-cake and highest lipase activity was found in rohu fed with diet FTC, followed by fish fed with the diet F. In catla *Catla catla* larvae, effect of different type of diets was recorded (Meetei et al., 2014). Baragi et al.

(2002) found that incorporation of raw and fermented (with *Bacillus* sp.) leaf meal of *L. polyrhiza* resulted in replacement of 10 and 30 % fishmeal, respectively in diet of rohu fingerlings. In the present study, in diet FLM, 300 g/kg (30 %) of fishmeal was replaced with raw duckweed compared to the fishmeal-based control diet. Application of extrusion technique for the preparation fish feed increased digestibility and nutrient utilization of the ingredients (Stadtlander et al., 2019).This resulted in better performances of rohu fed with diet supplemented with raw duckweed.

5. Conclusions

The present study demonstrated that duckweed *Lemna minor* is a nutrient rich and digestible feed ingredient for carp rohu. The prepared pelleted feed may replace fishmeal up to 300 g/kg of feed and, thereby, reduce the cost.

Author contributions

RC, DRT, JGS: designed the study; RKG, AKS, RC, JGS: cultured the plants, prepared diets and conducted experiment with fish; analyzed samples; RC, DRT, JGS: wrote the manuscript; RKG, AKS: prepared the tables.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anifeedsci.2020.114456.

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ORIGINAL PAPER

Optimization of protein extraction from detoxified Jatropha seed cake using response surface methodology and amino acid analysis

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Abstract

Jatropha seed cake is a by-product of biodiesel industry which is rich in proteins and carbohydrates and contains many bioactive compounds. Besides having a good protein quality, the essential amino acid content (except lysine) is also high in *Jatropha* seed cake, and therefore, *Jatropha* proteins can be exploited for many technical applications. The major hindrance for its use as a protein supplement is the presence of anti-nutritional factors and toxins, especially the phorbol esters. The detoxification of *Jatropha* seed cake for the removal of phorbol esters has been shown in our earlier studies. Proteins were extracted from the detoxified *Jatropha* seed cake and analyzed for amino acid content. Various parameters such as temperature, solubilization pH, precipitation pH and extraction time were studied for their effect on protein content, and the process was optimized using response surface methodology among 5 best samples selected based on protein content and protein yield from dry concentrates. The amount of dry matter, protein content and protein yield of 17.1%, 41.98% and 38.66%, respectively, was obtained under the optimized conditions of 60 °C temperature, solubilization pH 11.0, precipitation pH 4.41 and extraction time of 0.78 h. Amino acid contents revealed that the extracted samples contained a higher concentration of essential amino acids, especially leucine (4.40 ± 0.98 g/100 g). The results indicated that the protein extraction procedure from *Jatropha* seed cake affected the amino acid content, yet the values were close to FAO/WHO reference protein, and thus, the *Jatropha* proteins can be explored further for their use as feed supplement.

Keywords *Jatropha* proteins · Isoelectric precipitation · Amino acids · Response surface methodology · Optimization process

Introduction

Jatropha seed cake which is the by-product of biodiesel industry is rich in proteins and carbohydrates and contains many bioactive compounds and has found many economical and technical applications (Ahluwalia et al. 2018). The seed cake can be used as a substitute for other organic fertilizers because of good content of nitrogen, phosphorus and potassium [NPK (%) 4.44: 2.09: 1.68] (Manyuchi et al. 2018) which is much higher than the NPK values of organic fertilizers such as cow manure [NPK (%) 0.97: 0.69: 1.66], compost of municipal wastes [NPK (%) 1.25: 0.25: 0.65] and Karanj oil cake [NPK (%) 4.0: 1.0: 1.0] (Achten et al. 2010). Also, the biological decomposition of *Jatropha* seed cake helps in improving soil fertility which enhances crop production (Jakub et al. 2016). Many researchers have reported the increase in yields when *Jatropha* press cake was used as an organic fertilizer (Ghosh et al. 2012). Being nutritionally rich, the seed cake can be used as animal feed, but the presence of toxins like phorbol esters makes it unsuitable for animal consumption, and therefore, the seed cake needs to be detoxified (Ahluwalia et al. 2017). Despite of this, the use of *Jatropha* protein is advantageous as the protein quality and the essential amino acid content (except lysine) are high in *Jatropha* seed cake as compared to Food and Agricultural Organization (FAO) reference protein (Yinuo et al. 2018; Makkar and Becker 2009). Amino acids are very important chemical compounds and play a major role as structural

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and functional components of the body. Many studies have been conducted on the functions of amino acids (essential or nonessential), their composition in proteins, their effect on promoting muscle strength and function, etc. Various analytical methods for the determination of amino acids have been explored like high-performance liquid chromatography (HPLC) using diode array detector (Calull et al. 1991) which has many photodiode arrays for obtaining information over different wavelengths at one time and which makes it different from other methods. Fluorescence detector has a high sensitivity and has been used for the analysis of amino acids after reaction with fluorescence reagent (Tateda et al. 1998). Combined HPLC system with mass spectrometry is another method which is accurate and has high precision and has been used by many authors in their study (Krumpochova et al. 2015; Vilches et al. 2017). It involves the separation of analytes by ion pair chromatography, and the detection is done by multiple reaction monitoring positive ion mode. Nuclear magnetic resonance (NMR) technique has been used by many research groups for the determination of amino acids (Sugiki et al. 2017; Mati et al. 2015). This method involves simple procedures for sample preparation, and the spectra are obtained is few minutes unlike other methods (Lars et al. 2004). Jimenez et al. (2012) and Monia et al. (2017) have used gas chromatography (GC) method coupled with mass spectroscopy for amino acid analysis which requires derivatization of polar amino acids before GC analysis which makes them less reactive and more volatile. The method is preferred over HPLC if the availability of instrumentation and operational costs is considered; however, the major disadvantage of this method is its sensitivity to moisture which results in unstable derivatized analytes. The proteins extracted from raw *Jatropha* seed cake were successfully used as reactive component in polyketone-based wood adhesive formulations which not only improved the performance but also contributed in making the process economical (Hamarneh et al. 2010). Also, *Jatropha* proteins have good functional properties such as emulsifying properties, foaming properties, solubility, film-forming properties which widen up the possibilities of using this protein for various technical applications like emulsifier or paper adhesive (Lestari et al. 2011), coating material for food packaging applications (Gabriele et al. 2015), etc. Several studies have been conducted on *Jatropha* protein extraction which includes steam injection (Devappa and Swamylingappa 2008) by which a yield of 70–77% was obtained, counter current multistage extraction (Lestari et al. 2010) resulted in 82% protein recovery, by isoelectric precipitation method (Hamarneh et al. 2010; Makkar et al. 2008) 40–53% proteins were recovered, and enzyme-assisted extraction process (Gofferje et al. 2014) yielded 76% proteins.

Every geographic region carries significance as far as the toxins like phorbol esters and other anti-nutritional factors are concerned. This study focuses on the J*atropha* seed cake obtained from the Delhi region of North India after the extraction of oil for biodiesel production. In our earlier studies, we have already shown the presence of various phorbol esters in these samples and the bio-detoxification, removing significant amounts of phorbol esters using the strain *Pseudomonas aeruginosa* DS1. In the present study, this detoxified *Jatropha* seed cake was analyzed for the presence of essential and nonessential amino acids. This study is important as India is an agriculture-based country with huge number of cattle and other animals being reared that demand for healthy feed. The population of India is increasing at an alarming rate, thereby making it imperative to focus on the food and feed requirements. The emergence of biofuel sector has given a sigh of relief for the scientists in protecting the environment, especially the air quality. The by-products of biodiesel industry using *Jatropha* seeds would produce tons of oil press seed cake carrying various anti-nutritional factors and other toxins like phorbol esters. These seed cakes can get in to the environment and lead to various other unstudied complications. Unintentional intake of these materials by animals might have a severe blow on the backbone of Indian agriculture, economy and heritage. The aim of this study was to extract proteins from detoxified *Jatropha* seed cake by applying the principle of isoelectric precipitation. Optimization of the process was done using one-factor-at-a-time approach and RSM. The effect of various factors such as extraction temperature, time, solubilization pH, precipitation pH variations was also studied for obtaining a high protein yield with high protein and amino acid content. Therefore, the present study carries a lot of significance in terms of safeguarding India from future set back and is a small step toward this.

Materials and methods

Materials

Jatropha seed cake, after the extraction of the oil from whole seeds by screw press, was obtained from Biodiesel Centre, Delhi Technological University (DTU), Delhi, India. All the chemicals used in the work were purchased from Fischer Scientific and were of analytical grade. For the detoxification of *Jatropha* seed cake, submerged fermentation was carried out using *Pseudomonas aeruginosa* DS1 isolated in our laboratory. Complete degradation of phorbol esters resulted when the seed cake was fermented for 15 h at 37 °C, pH 7, 100 rpm (Ahluwalia et al. 2017).

Methods

Protein extraction

The extraction of protein from detoxified *Jatropha* seed cake at different conditions was done according to the method described by Makkar et al. (2008) which followed the principle of isoelectric precipitation. 10-g seed cake was suspended in 100 ml water, and the pH was adjusted using 1 mol L^{-1} NaOH. The mixture was stirred for 1 h at different temperatures (Table 1). The pH was checked after every 20 min and adjusted to desired pH (shown in Table 1) using 1 M NaOH. The contents were then centrifuged at 6700*g* for 15 min to collect the supernatant. The acidic pH of the supernatant was adjusted using 4 M HCl. The mixture was stirred for 10 min and centrifuged at 6700*g* for 15 min for the separation of protein precipitates. The pellet was air dried, and the weight (dry wt.) was noted. Each experiment was done in duplicates.

Protein analysis

Protein content of the detoxified *Jatropha* seed cake and protein precipitates was determined by Kjeldahl method (AACC 2000), which consists of three steps: digestion, distillation and titration. The sample is digested in boiling concentrated sulfuric acid in the presence of a catalyst (potassium sulfate and cupric sulfate in 5:1 ratio) for complete dissolution and oxidation. The nitrogen in the sample is converted to ammonium sulfate. In the distillation step, sodium hydroxide is added into the solution, which converts the ammonium ion to ammonia, which is distilled and received in a boric acid solution. The amount of ammonia released and thus the amount of nitrogen present in the sample are then determined by titration. The results are expressed in % N, % NH_3 or protein (%N × factor). Dry matter recovery and protein yield were calculated according to the formula:

$$\text{Dry matter (\%)} = \frac{\text{Dry matter weight of protein precipitate}}{\text{Initial dry weight of seed cake}} \times 100$$

$$\text{Protein yield (\%)} = \frac{\text{dry mass extract (g)} \times \text{Protein content extract (\%)}}{\text{initial weight of seed cake (g)} \times \text{protein content in seed cake (\%)}} \times 100$$

Pilot-scale experiments

One-factor-at-a-time approach was applied to study the effect of temperature, solubilization pH and precipitation pH on protein content and protein yield. For this study, the following extraction conditions were performed: (1) temperature 25, 40, 55, 70, 85 °C; (2) solubilization pH 7.5, 8.5, 9.5, 10.5, 11.5; (3) precipitation pH 2, 3, 4, 5, 6. After analyzing the data of the former parameter, its optimum value was fixed in the subsequent experiments of pilot-scale studies. The quadratic model and R^2 value for each factor were found out. Similar methodology was followed by Chen et al. (2012) in their studies for cultural optimization of biosurfactant produced by *Acinetobacter* genus.

Optimization of protein extraction using RSM

Four different parameters, i.e., temperature, solubilization pH, precipitation pH and time, were varied at five levels for studying the combined effect of these independent variables on three response variables, i.e., dry matter, protein content and protein yield. Response surface methodology (RSM) was used to study the process and optimization of different parameters for the extraction of proteins from *Jatropha* seed cake. The experimental plan consisted of four factors at five levels. The optimal concentrations of each factor from the quadratic models (from pilot-scale experiments) were adjusted slightly and were applied as central value. Different levels of variation and codes of the independent variables of temperature (*A*), solubilization pH (*B*), precipitation pH (*C*) and time (*D*) are presented in Table 1. As per Central Composite Rotatable Design (CCRD), thirty sets of experiments were performed with varying levels of parameters. The analysis of variance was performed to evaluate the effect of the independent variables on the response variables. Design Expert (DX-11) was used for the selecting

Table 1 Levels and codes of the independent variables

Variables	Codes	Levels (coded and real values)				
		−2	−1	0	1	2
Temperature (°C)	*A*	30	40	50	60	70
Solubilization pH	*B*	8	9	10	11	12
Precipitation pH	*C*	3.5	4	4.5	5	5.5
Time (h)	*D*	0.5	0.75	1	1.25	1.75

appropriate model, and multiple regression analysis was performed considering a full second-order polynomial model.

Elemental analysis

Five best samples of dried protein concentrates on the basis of protein yield and protein content were selected and analyzed for carbon, hydrogen, oxygen, nitrogen and sulfur contents in CHNSO analyzer (Vario micro tube, Germany).

Amino acid analysis

The amino acid content of the five protein concentrates was estimated by amino acid analyzer (Hitachi, Model L-8900) using the method described by You Shin et al. (2013). In brief, the protein sample is made to pass through a cation exchange column where it is separated into amino acid components which are detected at a wavelength of 570 nm. Quantification was done by comparing sample chromatogram peaks with the standards. Proline and hydroxyproline (imino acids) have no absorbance maxima within the visible light spectrum and are therefore detected at 440 nm. In the deamination column, 20 µL of sample was injected with a flow rate of 0.35 mL min^{-1} and the column temperature was 30–70 °C. In reaction column, reaction temperature was 135 °C with a Ninhydrin flow rate of 0.30 mL min^{-1}.

Results and discussion

Pilot-scale experiments

The quadratic model and R^2 value for each factor (temperature, solubilization pH, precipitation pH) are shown in Fig. 1. On the basis of these models, the optimal concentration for each factor was used as a central value for optimization by RSM.

No significant change in the protein yield was seen at temperatures 25, 40 and 55 °C (Fig. 1a); however, a slight increase in protein yield was observed. As the temperature was increased from 55 to 85 °C, a significant decrease in protein yield was observed from 45.5 to 17.6%. An increase

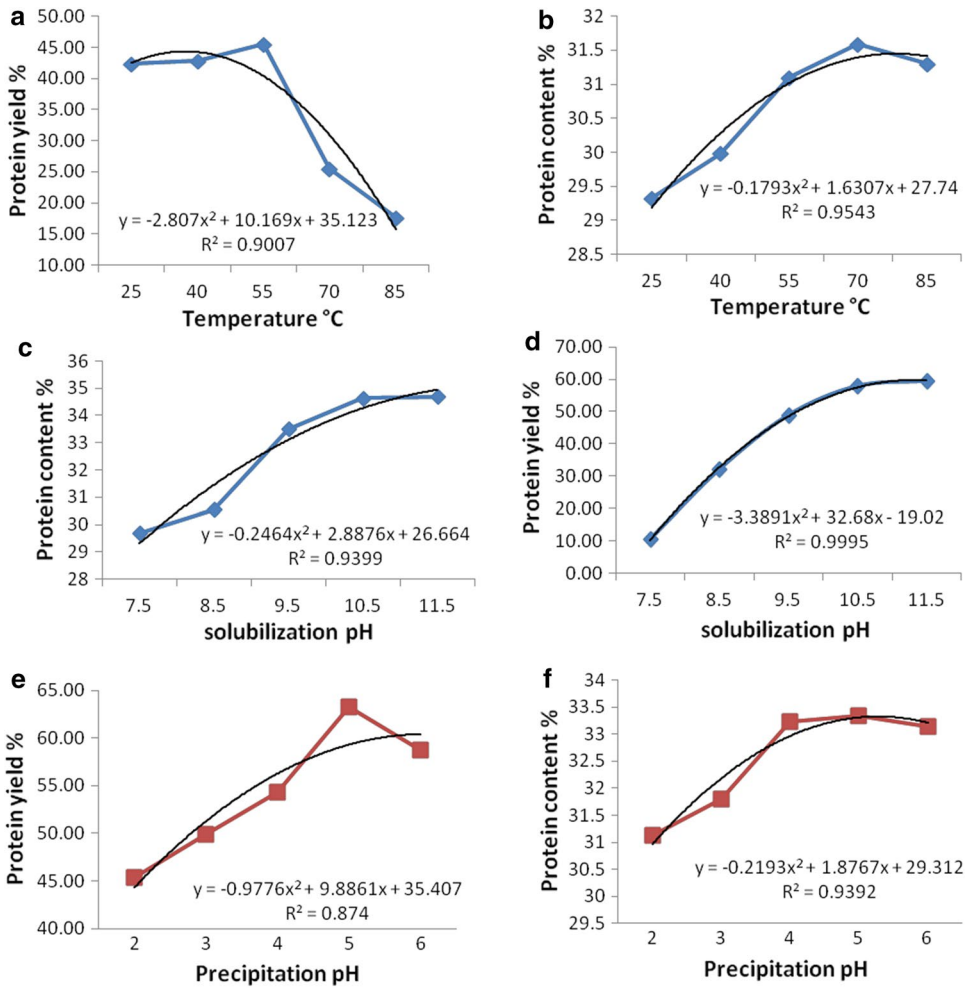

Fig. 1 Quadratic model and related equation for each factor after one-factor-at-a-time analysis

in protein content was observed with the increase in extraction temperature (Fig. 1b).

Figure 1c shows the increasing pH had a positive effect on the protein content of the concentrates. There was a gradual increase in the protein content when the pH was raised from 7.5 to 10.5; however, no significant change in protein content was observed when the pH reached 11.5. The highest protein content of 34.69% was observed in samples when they were solubilized at 11.5. The graphs in Fig. 1d clearly show that the alkaline conditions significantly affect the protein extraction yield and higher yields were obtained at pH 11.5 compared with pH 10.5.

There was a steady increase in protein yield when the precipitation pH was increased from 2 to 5. However, further increase in pH resulted in sharp decline in protein yield (Fig. 1e). This suggests that the isolectric point of *Jatropha* protein is between pH 4.5 and 5 which results in the maximum recovery of the solubilized proteins at this pH. The increase in protein content was observed when precipitation pH was raised from pH 2 to 4, and it was comparable at pH 4 and 5 while a decline in protein content was observed when the extraction was performed at pH6 (Fig. 1f).

Recoveries of protein concentrates

The data in Table 2 show the recoveries of dry matter, protein yield and protein content of protein concentrate (from detoxified *Jatropha* raw seed cake). The protein concentrate had protein content in the range from 29.47 to 45.62%, and the protein yield varied from 11.7 to 50.9%.

Optimization of protein extraction from detoxified *Jatropha* seed cake using RSM

Thirty experiments were performed according to CCRD to determine the effect of four parameters, i.e., temperature,

Table 2 Average values of the response parameters

S. no.	Temperature (°C)	Solubilization pH	Precipitation pH	Time (h)	Dry matter (%)	Protein content (%)	Protein yield (%)
1	40	9	4	0.75	6.1	31.72	11.7
2	60	9	4	0.75	10.8	30.07	18.4
3	40	11	4	0.75	15	31.11	26
4	60	11	4	0.75	17.5	37.02	36
5	40	9	5	0.75	8.2	30.12	13.7
6	60	9	5	0.75	8	33.58	15
7	40	11	5	0.75	15.6	31.72	27.5
8	60	11	5	0.75	15.2	40.87	34.5
9	40	9	4	1.25	12.5	32.98	23
10	60	9	4	1.25	18.3	33.72	34.2
11	40	11	4	1.25	11.7	33.17	21.6
12	60	11	4	1.25	20.1	42.18	47.1
13	40	9	5	1.25	15.3	31.72	31
14	60	9	5	1.25	17.5	35.18	34.2
15	40	11	5	1.25	16.7	31.94	29.6
16	60	11	5	1.25	23.1	42.75	50.9
17	30	10	4.5	1.00	11.2	34.54	21.5
18	70	10	4.5	1.00	16.7	45.62	42.3
19	50	8	4.5	1.00	9.1	29.47	14.9
20	50	12	4.5	1.00	19	39.56	41.8
21	50	10	3.5	1.00	16	30.61	27.2
22	50	10	5.5	1.00	17.1	33.35	31.7
23	50	10	4.5	0.50	11.8	33.12	21.7
24	50	10	4.5	1.50	18.4	36.31	37.1
25	50	10	4.5	1.00	16.8	34.88	32.6
26	50	10	4.5	1.00	17.5	35.33	34.3
27	50	10	4.5	1.00	16.1	34.01	30.4
28	50	10	4.5	1.00	17.3	35.01	32.6
29	50	10	4.5	1.00	16.4	34.98	32.9
30	50	10	4.5	1.00	15.9	35.97	31.4

solubilization pH and precipitation pH and time on dry matter, protein content and protein yield. All the experiments were performed in triplicates, and the average values of the responses were calculated and are given in Table 2.

Effect of parameters on dry matter

Experimental runs were performed, and the results were modeled according to a polynomial quadratic equation to identify the variables that affected significantly or non-significantly. The analysis of variance (ANOVA) was performed and is given in Table 3. Determination coefficient ($R^2 = 0.95$) illustrates that only 5% of the total variations are not described by the model. Adjusted determination coefficient (adjusted $R^2 = 0.92$) is also high, which indicates the model is highly significant. As shown in Table 3, the value of lack of fit (0.095) was nonsignificant which implies accuracy of the model. The response surface equation for dry matter obtained after eliminating the nonsignificant terms is:

$$\begin{aligned}\text{Dry Matter} = {} & 16.55 + 1.68A + 2.42B + 0.40C + 2.17D \\ & - 0.83AC + 1.01AD - 1.39BD + 0.77CD \\ & - 0.73A^2 - 0.71B^2 - 0.45D^2\end{aligned}$$

The probability value p is < 0.05 which indicates that the model is significant. The coefficients in the equation indicated that all the parameters significantly affect the dry matter content. The dry matter content was found to increase with the increase in temperature, pH and time. In a work done by Makkar et al. (2008), the increase in temperature increased the dry matter and protein recovery in seed cake when the extraction was carried out at pH 10. In our study as well, there was significant rise in dry matter content when the extractions were carried out at high temperatures. The response surface plots of dry matter content affected by temperature, solubilization pH, time and precipitation pH are shown in Fig. 2.

Effect of parameters on protein content

The protein content of the detoxified seed cake ranged from 30.07 to 45.62%. The second-order polynomial model had a high correlation coefficient ($R^2 = 0.95$), and it indicated that the model fitted suitably with the observed data. The analysis of variance (ANOVA) after omitting the nonsignificant terms is given in Table 4. The 3-D plots of protein content as a function of temperature, solubilization pH, precipitation pH and time are shown in Fig. 3. The response surface equation after removing the nonsignificant terms for protein content is:

$$\begin{aligned}\text{Protein Content} = {} & 34.50 + 2.63A + 2.16B + 0.47C \\ & + 0.99D + 1.80AB + 0.80AC \\ & - 1.17A^2 - 0.85C^2\end{aligned}$$

The coefficients of the first-order term in the equation showed that the protein content of the concentrate increased with the increase in temperature, solubilization pH, precipitation pH and time. An increase in protein content was observed with the increase in temperature which could possibly be due to the reason that high temperatures break the

Table 3 Pooled ANOVA showing the variables as a linear, quadratic and interaction terms on dry matter of *Jatropha* seed cake

Source	Sum of squares	*df*	Mean square	*F* value	*p* value
Model	421.46	11	38.31	34.06	< 0.0001
A—temp	68.01	1	68.01	60.46	< 0.0001
B—solubilization pH	140.17	1	140.17	124.62	< 0.0001
C—precipitation pH	4.00	1	4.00	3.56	0.0755
D—time	112.67	1	112.67	100.17	< 0.0001
AC	11.22	1	11.22	9.98	0.0054
AD	16.40	1	16.40	14.58	0.0013
BD	30.80	1	30.80	27.39	< 0.0001
CD	9.61	1	9.61	8.54	0.0091
A^2	15.23	1	15.23	13.54	0.0017
B^2	14.21	1	14.21	12.64	0.0023
D^2	5.67	1	5.67	5.04	0.0376
Residual	20.25	18	1.12		
Lack of fit	18.15	13	1.40	3.34	0.0957^{ns}
Pure error	2.09	5	0.4187		
Cor total	441.70	29			
				R^2	0.95
				Adj R^2	0.92

ns not significant terms and others are significant at 95% confidence

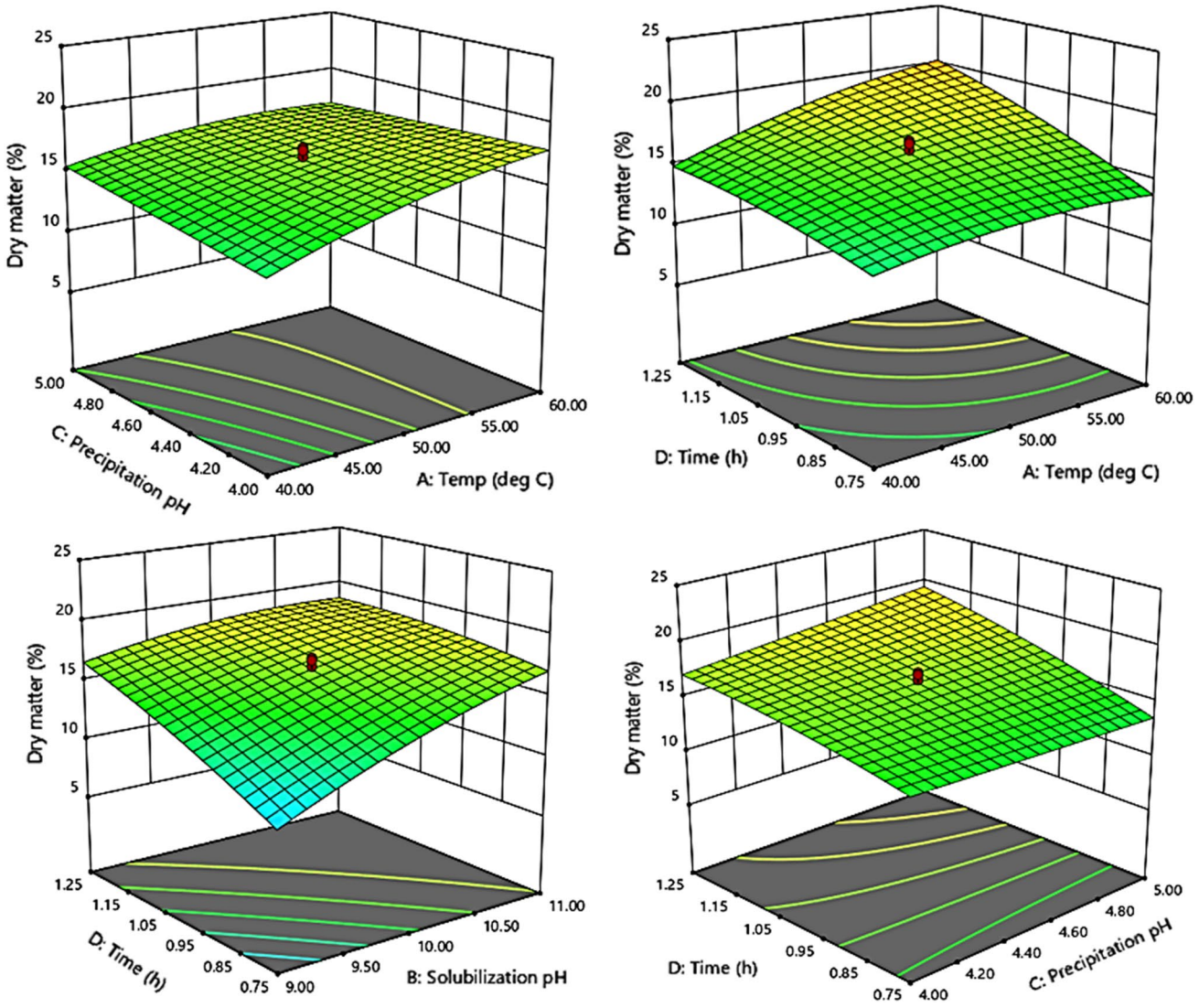

Fig. 2 Response surface plots of dry matter content affected by temperature, solubilization pH, time and precipitation pH

bonds in protein structure which increases the dissolution rate resulting in high protein content (Oliyaei et al. 2017; Liliana et al. 2013). However, temperature beyond a certain range may cause overheating and result in protein denaturation and the addition of impurities which may affect the protein content in the protein concentrate. In our study, temperature up to 70 °C was also found to be suitable for the extraction of proteins from detoxified *Jatropha* seed cake with good protein content.

Many researchers have observed increased protein content at higher pH because of the better solubility of proteins at alkaline pH which may attribute to good protein content in the protein concentrate (Rhee et al. 1972; Gisele et al. 2014). An increase in protein content was also observed with time as longer extraction time results in more solubility of proteins with a good amount of protein. According to Kain et al. (2009), the solubility of proteins is minimum at isoelectric pH because of balance between positive and negative charges which minimizes the repulsion forces among amino acids and thus reduces the solubility of proteins. Rhee et al. (1972) observed a decrease in protein content when the pH was increased beyond the isoelectric point. The positive coefficient of the interaction terms (AB and AC) indicated an increase in protein content with the addition of these variables.

Effect of parameters on protein yield

The analysis of variance (ANOVA) was performed to evaluate the effect of four factors on the protein yield. As shown in Table 5, the value of lack of fit (0.091) was non-significant which implies accuracy of the model. The R^2 and the adjusted R^2 value of the model was 0.96 and 0.94,

Table 4 ANOVA showing the variables as a linear, quadratic and interaction terms on protein content of *Jatropha* seed cake

Source	Sum of squares	*df*	Mean square	*F* value	*p* value
Model	435.99	8	54.50	52.00	< 0.0001
A—temp	165.64	1	165.64	158.03	< 0.0001
B—solubilization pH	112.02	1	112.02	106.87	< 0.0001
C—precipitation pH	5.41	1	5.41	5.16	0.0338
D—time	23.62	1	23.62	22.54	0.0001
AB	52.09	1	52.09	49.70	< 0.0001
AC	10.35	1	10.35	9.88	0.0049
A^2	38.86	1	38.86	37.08	< 0.0001
C^2	20.85	1	20.85	19.89	0.0002
Residual	22.01	21	1.05		
Lack of fit	19.97	16	1.25	3.06	0.1105^{ns}
Pure error	2.04	5	0.4079		
Cor total	458.00	29			
				R^2	0.95
				Adj R^2	0.93

ns not significant terms and others are significant at 95% confidence

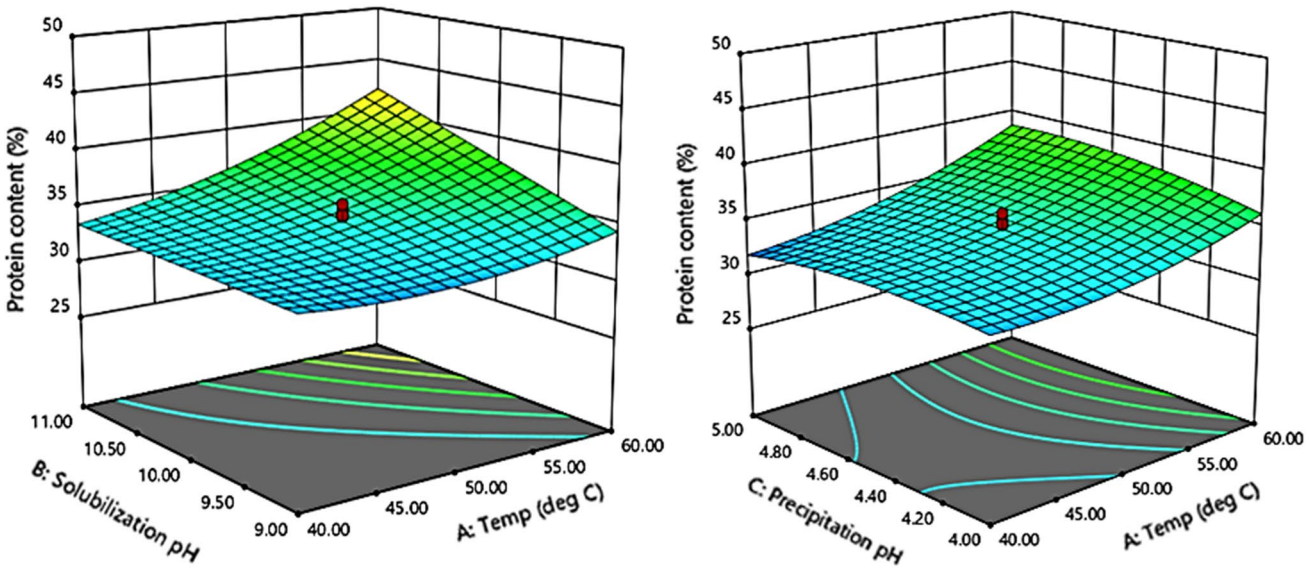

Fig. 3 Response surface plots of protein content affected by temperature, solubilization pH, time and precipitation pH and residual distribution plot

respectively, so it can be conferred that the obtained model is accurate and can be predicted for mentioned response. The independent variables having $p < 0.05$ indicate the significant model terms.

The response surface equation for protein yield after removing the nonsignificant terms in coded values is:

$$\begin{aligned}\text{Protein Yield} = {} & 31.97 + 5.32A + 6.08B + 1.14C + 4.98D \\ & + 2.59AB - 1.29AC + 2.26AD - 2.40BD \\ & + 1.33CD - 1.18B^2 - 0.90C^2 - 0.92D^2\end{aligned}$$

It can be observed from the response equation that all the four independent variables, i.e., temperature (*A*), solubilization pH (*B*), precipitation pH (*C*) and time (*D*), were showing significant effect on the protein yield. The interaction of temperature and solubilization pH had a positive effect on protein yield while all other interaction terms were negative.

As shown in Fig. 4, the increase in protein yield was observed with the increase in process temperature. In a work done by Selling et al. (2013), the highest protein yield (35%) was obtained when pennycress seeds were extracted with

Table 5 ANOVA showing the variables as a linear, quadratic and interaction terms on protein yield of *Jatropha* seed cake

Source	Sum of squares	*df*	Mean square	*F* value	*p* value
Model	2598.74	12	216.56	44.53	< 0.0001
A—temp	680.53	1	680.53	139.94	< 0.0001
B—solubilization pH	885.73	1	885.73	182.14	< 0.0001
C—precipitation pH	31.28	1	31.28	6.43	0.0213
D—time	596.01	1	596.01	122.56	< 0.0001
AB	107.12	1	107.12	22.03	0.0002
AC	26.52	1	26.52	5.45	0.0320
AD	81.90	1	81.90	16.84	0.0007
BD	92.16	1	92.16	18.95	0.0004
CD	28.09	1	28.09	5.78	0.0279
B^2	39.25	1	39.25	8.07	0.0113
C^2	23.13	1	23.13	4.76	0.0435
D^2	23.77	1	23.77	4.89	0.0410
Residual	82.67	17	4.86		
Lack of fit	73.74	12	6.14	3.44	0.0910^{ns}
Pure error	8.93	5	1.79		
Cor total	2681.41	29			
				R^2	0.96
				Adj R^2	0.94

ns not significant terms and others are significant at 95% confidence

water at 77 °C. In another work by Aleksandra et al. (2018), proteins were extracted from rape seed cake and primrose cake using a eutectic solvent and water at different processing temperatures and high yields of protein precipitates were obtained with increasing temperature. The rate of extraction varies linearly with temperature, and the increase in temperature causes molecules to move fast, thereby increasing the mass transfer rate between solid and liquid resulting in good solubility and increased extraction rate. But further increase in temperature resulted in decrease in protein yield. In a work done by Kain et al. (2009), the extraction of proteins from peanut at high temperatures (60 °C) resulted in decrease in protein yield. According to Chenyan et al., (2011), extractions at higher temperatures result in a decline in protein yield which could be due to thermal degradation or denaturation of proteins. In our study, temperature up to 60 °C resulted in a good protein content and protein yield, but as the temperature was further raised to 70 °C, protein yield declined, but the protein content was still higher. pH also plays a significant role on the extraction of proteins and protein yield. As the solubilization pH was increased an increase in protein yield was observed which could be due to increased extractability of proteins. In a study, the extraction of proteins from fish was done by the isoelectric precipitation method and high protein recovery was observed at pH 12 than pH 10 (Oliyaei et al. 2017). Similar results were obtained in various studies where a high protein yield was obtained at high alkaline pH (Martin et al. 2010; Saha et al. 2016; Shaviklo et al. 2017). Different studies have also shown an increase in solubility at precipitation pH. The graphs of precipitation pH indicated a good protein yield at pH 5. This suggests that the isolectric point of *Jatropha* protein is between pH 4.5 and 5 which results in the maximum recovery of the solubilized proteins at this pH. According to Kain et al. (2009), the solubility of proteins is minimum at isolectric pH because of balance between positive and negative charges which minimizes the repulsion forces among amino acids and thus reduces the solubility of proteins. Each protein has its own isoelectric pH depending upon its amino acid composition. Likewise, the method of extraction also affects the isoelectric pH of a particular protein. According to Yu et al. (2007), the maximum amount of peanut protein was extracted from supernatant when the extraction was carried out at pH 4. While in another work reported by Wu et al. (2009), pH 4.5 was found to be the isoelectric pH for the precipitation of peanut protein. The protein yield also increased with the increase in extraction time which is similar to the findings of Qiaoyun et al. 2017 who also observed a sharp increase in protein yield when the extractions were carried out for longer periods.

The interaction terms of solubilization pH and precipitation pH (*BC*) and precipitation pH and time (*CD*) were also found to be positive and significantly affecting the protein yield. It has been cited in many reports that both solubilization pH and precipitation pH influence the solubility properties of proteins in a solution and hence the protein yield. Since the interaction terms of solubilization pH and time

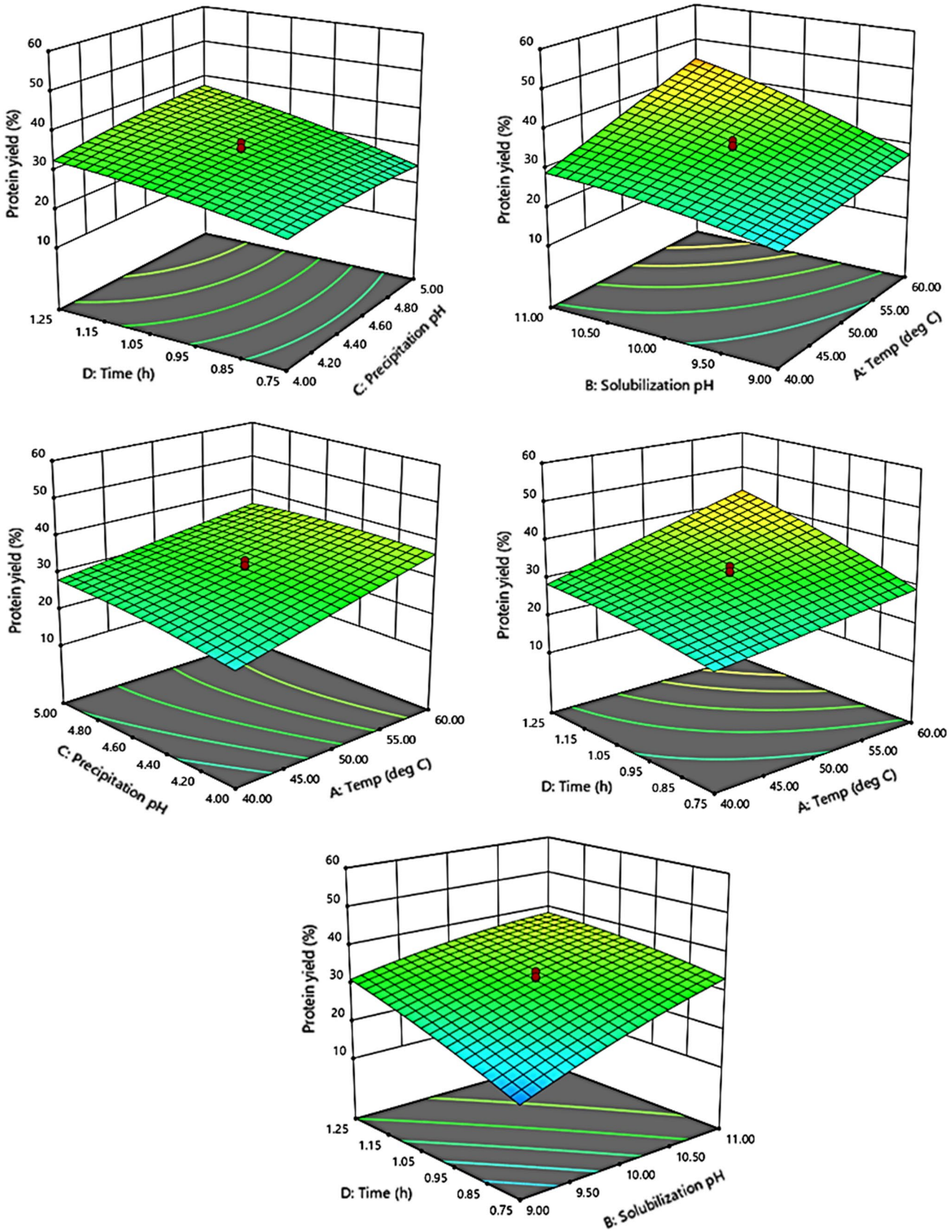

Fig. 4 Response surface plots of protein yield affected by temperature, solubilization pH, time and precipitation pH

were not significant, these factors are not dependant on each other in the chosen range.

Optimization

The optimum values as obtained by differentiation of the quadratic model, for achieving dry matter, protein content and protein yield, were temperature = 60 °C, solubilization pH = 11, time = 0.78 h and precipitation pH = 4.41. The predicted optimal dry matter, protein content and protein yield corresponding to these values were 17.4%, 41.12% and 39.86%, respectively. Additional experiments were performed in triplicates using these optimized levels to verify the accuracy of the model for predicting the three responses, i.e., dry matter, protein content and protein yield. The average of triplicate experiments yielded a dry matter, protein content and protein yield of 17.1%, 41.98% and 38.66%, respectively. In comparison, a good compliance between the experimental and predicted values was observed which confirms the reliability of model as well as the existence of optimal point.

Elemental analysis

Five best samples of dried protein concentrates were selected with the highest protein content and protein yield. These were analyzed for carbon, hydrogen, nitrogen and sulfur contents in CHNSO analyzer (Vario micro tube, Germany). The results are given in Table 6. The highest elemental percentage distribution recorded was 55.94% of carbon, and the lowest was of sulfur 0.19% in sample 2. The nitrogen content of protein concentrates varied from 5.81 to 7.30% while the C/N ratio ranged from 7.36 to 9.62. The lowest C/N ratio was obtained for sample 5 which is in suitable range for the application in soil amendments. Low C/N ratio allows fast mineralization which allows plants to take up nutrients easily but as the ratio increases, the decomposition and mineralization slow down.

Table 6 CHNS analysis of protein concentrates

	Samples				
	1	2	3	4	5
N (%)	6.33	5.81	5.90	6.75	7.30
C (%)	53.79	55.94	54.94	55.43	53.75
H (%)	7.21	8.41	8.32	8.43	8.05
S (%)	0.29	0.19	0.39	0.50	0.67
Protein (%)	39.56	36.31	36.87	42.18	45.62
N area	13,664	16,999	15,528	20,345	16,993
C area	71,450	102,715	90,143	106,530	78,773
H area	31,311	51,201	45,077	53,763	38,781
S area	23,970	23,639	29,308	35,801	36,130
C/*N* ratio	8.49	9.62	9.31	8.21	7.36
C/*H* ratio	7.46	6.64	6.59	6.57	6.68

Amino acid analysis

Amino acid composition of protein concentrates is an indicative of their nutritional value. The concentrations of amino acids (essential and nonessential) in all the samples (five best and optimized) varied considerably indicating the effect of extraction conditions on the structure and solubility of amino acids (Table 7). However, it was observed that *Jatropha* seed cake contained higher amounts of nonessential amino acids than essential amino acids. Leucine, valine, phenylalanine, isoleucine were the major essential amino acids found in all the samples of protein concentrates while the concentration of glutamic acid (10.92 g/100 g) and arginine (7.18 g/100 g) was higher in nonessential amino acids in *Jatropha* protein concentrates. Similar results were also observed by Makkar et al. (2008) and Ugbogu et al. (2014), who have reported higher percentages of these amino acids (leucine, valine, phenylalanine, isoleucine) in *Jatropha* seed cake which suggests that *Jatropha* seed cake can serve as a good source of these essential amino acids. Based on the results obtained for samples 1 and 2, it was observed that during the extraction of proteins, solubilization pH more toward alkaline side resulted in better recovery of amino acids from the protein concentrates. However, in case of tryptophan, acidic pH during extraction affected its content as lower pH leads to its degradation (Csapo et al. 2008). Sample 5 was found to have higher content of essential amino acids than sample 1 indicating the extraction of proteins at 60 °C didn't affect the amino acid concentration. According to Line et al. (2018), in the production of specialized protein products, heating at high temperatures resulted in oxidation, loss of cysteine, tryptophan and methionine which indicated that high temperatures lead to many undesirable physical and chemical changes. In a study by Beuk et al. (1948), there was complete retention of all amino acids (except cysteine) when the samples were autoclaved suggesting the thermo stability of amino acids except cysteine. Comparing the results obtained for sample 4 and sample 5, the amount of amino acids in sample 5 was higher than in sample 4 which suggested that the extraction of proteins at pH 5 (precipitation pH) is more efficient than at pH 4.

When the extraction pH was decreased slightly from 5 to 4.41, it was observed that the yield was comparable in 0.78 h (optimized sample, Table 7) to that of pH 5 in 1.25 h (sample 5, Table 7). This indicates that higher yield of amino acids can be obtained in less treatment time with slight variation in pH under same set of conditions. Further, these optimized conditions were validated with triplicate samples and found to be the best, yielding the maximum essential as well as nonessential amino acids, and the values were close to FAO/

Table 7 Amino acid content of five protein concentrate samples and optimized sample

S. no.	Amino acids	Concentration (g/100 g)					
		Sample 1 (50 °C, 12, 4.5, 1 h)	Sample 2 (50 °C, 10, 4.5, 1.5 h)	Sample 3 (60 °C, 11, 5, 0.75 h)	Sample 4 (60 °C, 11, 4, 1.25 h)	Sample 5 (60 °C, 11, 5, 1.25 h)	Optimized sample (60 °C, 11, 4.41, 0.78 h)
Essential							
1	Histidine (His)	1.21 ± 0.19	1.09 ± 0.46	1.19 ± 0.09	1.38 ± 0.69	1.45 ± 0.70	1.49 ± 0.51
2	Isoleucine (Ile)	2.32 ± 0.42	1.70 ± 0.71	2.28 ± 1.19	2.57 ± 2.19	2.63 ± 2.21	2.57 ± 0.30
3	Leucine (Leu)	3.91 ± 0.07	3.59 ± 2.17	3.94 ± 0.17	4.44 ± 2.28	4.63 ± 2.19	4.40 ± 0.98
4	Lysine (Lys)	1.40 ± 0.17	1.37 ± 0.72	1.43 ± 0.04	1.73 ± 0.75	1.82 ± 0.81	1.70 ± 0.06
5	Methionine (Met)	0.81 ± 0.01	0.64 ± 0.80	0.69 ± 0.57	0.73 ± 1.24	0.84 ± 0.12	0.75 ± 1.08
6	Phenylalanine (Phe)	2.51 ± 0.65	2.21 ± 1.35	1.44 ± 0.10	2.82 ± 1.26	2.91 ± 0.88	2.88 ± 0.75
7	Threonine (Thr)	1.77 ± 0.89	1.20 ± 0.00	–	2.17 ± 0.00	2.10 ± 2.60	2.15 ± 1.10
8	Tryptophan (Trp)	0.31 ± 0.00	0.29 ± 0.00	0.46 ± 0.00	0.51 ± 0.00	0.57 ± 0.00	0.51 ± 0.00
9	Valine (Val)	2.78 ± 0.41	2.23 ± 2.83	2.44 ± 1.43	2.87 ± 2.45	2.96 ± 2.75	2.84 ± 1.23
	Total EAA	17.58	14.36	13.89	19.26	19.94	19.32
Nonessential							
1	Alanine (Ala)	2.48 ± 0.27	2.20 ± 0.98	2.38 ± 0.04	2.72 ± 1.35	2.89 ± 1.33	2.78 ± 0.88
2	Arginine (Arg)	5.74 ± 0.87	5.38 ± 0.69	6.29 ± 0.67	6.66 ± 1.71	7.18 ± 2.07	6.70 ± 0.82
3	Aspartate (Asp)	4.61 ± 0.42	–	–	–	5.71 ± 0.00	5.53 ± 0.01
4	Cysteine (Cys)	0.59 ± 0.24	0.84 ± 0.10	0.94 ± 0.22	0.79 ± 0.59	0.67 ± 1.32	0.72 ± 0.72
5	Glutamic acid (Glu)	8.86 ± 0.32	8.37 ± 0.00	4.94 ± 0.00	9.95 ± 0.00	10.92 ± 3.95	10.04 ± 0.12
6	Glutamine (Gln)	–	–	3.99 ± 0.00	–	–	–
6	Glycine (Gly)	2.56 ± 0.45	2.32 ± 0.41	2.46 ± 0.33	2.84 ± 1.31	2.99 ± 1.21	2.82 ± 1.01
7	Proline (Pro)	1.33 ± 0.18	1.06 ± 0.46	1.11 ± 1.61	1.46 ± 0.40	1.46 ± 0.10	1.46 ± 0.75
8	Serine (Ser)	2.16 ± 1.00	1.55 ± 0.00	2.76 ± 0.00	3.02 ± 2.88	3.00 ± 0.66	3.03 ± 0.98
9	Tyrosine (Tyr)	1.48 ± 0.13	1.37 ± 0.37	1.62 ± 1.82	1.91 ± 0.60	1.99 ± 0.88	1.94 ± 0.65
10	Phosphoserine (p-Ser)	0.17 ± 0.08	0.12 ± 0.15	0.08 ± 0.00	–	0.24 ± 0.00	0.19 ± 0.02
11	Phospho ethanol amine (PEA)	0.01 ± 0.02	0.03 ± 0.00	0.04 ± 0.00	–	0.009 ± 0.00	–
12	α-Amino-n-adipic acid (a-AAA)	0.024 ± 0.00	–	–	–	–	–
13	α-Amino-*n*-butaric acid (a-ABA)	0.05 ± 0.02	0.04 ± 0.01	0.07 ± 0.16	0.07 ± 0.12	0.07 ± 0.12	0.07 ± 0.17
14	Cystathionine (Cysthi)	0.09 ± 0.00	8.04 ± 0.10	0.13 ± 0.53	0.12 ± 0.20	0.67 ± 1.32	0.44 ± 0.20
15	β-Alanine (b-Ala)	0.04 ± 0.03	0.05 ± 0.01	0.06 ± 0.05	0.03 ± 0.11	0.12 ± 0.00	0.04 ± 0.02
16	β-Amino isobutyric acid (b-AiBA)	0.03 ± 0.00	1.00 ± 0.00	0.11 ± 0.00	0.03 ± 0.00	0.05 ± 0.09	0.03 ± 0.05
17	ϒ-Amino butyric acid (g-AiBA)	0.01 ± 0.00	0.01 ± 0.01	0.009 ± 0..04	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
18	Ethanol amine ($EOHNH_2$)	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
19	Ornithine (Orn)	0.04 ± 0.00	0.03 ± 0.04	0.03 ± 0.03	0.03 ± 0.02	0.03 ± 0.04	0.03 ± 0.06
20	1 Methyl histidine (1 Mehis)	0.08 ± 0.00	0.08 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
21	Hydroxyproline (Hypro)	–	0.036 ± 0.00	–	0.04 ± 0.00	–	–
	Total	30.44	24.50	27.18	29.78	38.20	35.98

The range of detection of amino acids by the analyzer was 0.5–2 nM. The values presented here are within this range

WHO reference (1985; report 935), and thus, *Jatropha* proteins can therefore be used as feed supplement.

Conclusion

Various parameters such as temperature, solubilization pH, precipitation pH, and extraction time were tested for the extraction of proteins from the bio-detoxified *Jatropha* seed cake. The extraction was optimized using CCRD model of RSM. Four factors at five levels were allowed to interact in a set of 30 experiments. Multivariate ANOVA indicated that temperature and pH were the significant parameters affecting the protein content and protein yield. The optimum values of temperature, solubilization pH, precipitation pH and time for the extraction of protein from detoxified *Jatropha* seed cake were 60 °C, 11.0, 4.41 and 0.78 h, respectively, which yielded the maximum dry matter, protein content and protein yield of 17.1%, 41.98% and 38.66%, respectively. Elemental analysis showed the highest nitrogen and sulfur content of 7.3% and 0.67%, respectively, in sample 5. Amino acid contents were determined, and the results elaborate that though the extraction procedure affected the amino acid content, yet the values were close to FAO/WHO reference. The overall essential amino acid content was 19.32 g/100 g while the nonessential amino acids constituted 35.98 g/100 g under the optimized conditions of extraction. These *Jatropha* proteins can be used as food and feed supplement. Though this is a small step toward the essence of creating healthy environment and healthy livestock for future, further studies need to be carried out to ascertain the effect of these supplements on the overall health of animals. However, the present study indicates the high possibilities of supplementing the feed with essential amino acids, and the avenues for nutraceutical research are widely open for the development of suitable food and feed formulations.

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Production potential of greater duckweed *Spirodela polyrhiza* (L. Schleiden) and its biochemical composition evaluation

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ABSTRACT

The culture technique of greater duckweed *Spirodela polyrhiza* (L. Schleiden) was standardized in outdoor tanks using three different manures: manure 1 - cattle manure, poultry droppings and mustard oil cake, manure 2 - urea, potash and triple superphosphate and manure 3 - cattle manure, urea, potash and triple superphosphate. Significantly ($p < .05$) higher production was recorded in manure 1 compared to others. Manure 1 was subsequently selected for pond culture. In ponds, the production of duckweed was 2020 ± 150 kg ha^{-1} month^{-1} dry weight basis. Protein content was significantly higher ($p < .05$) in duckweed cultured in manure 1. The amino acid profile study showed the presence of essential (37.4%), non-essential (58.2%) and free (4.5%) amino acids. Leucine, isoleucine and valine contributed 51.4% of total essential amino acids. Duckweed contained 7% lipid and α-linolenic acid (36–37%) was the major fatty acid. The study showed the nutritional value of duckweed as an animal feed ingredient.

1. Introduction

The greater duckweed *Spirodela polyrhiza* (L. Schleiden) is a free-floating, fast growing aquatic plant, widely distributed in the still and slow-flowing water bodies globally. Morphologically, this monocotyledon plant is simple and lack specialised structures such as leaves or stems, but consist of flat ovoid leaf-like structures termed fronds with a rootlet for stabilisation. The bright green (upper part) and purple (lower side) colours of the fronds enhance its aesthetic value and make it suitable candidate for aquarium. Recent study shows the whole genome sequencing of *S. polyrhiza*, the most primitive member of Lemnaceae family (Michael et al., 2017; Hoang et al., 2018). It is a useful tool for further investigation with this duckweed. In accordance with other Lemnaceae, the usefulness and potential of *S. polyrhiza* has been recognized in recent days. It has utilisation for various purposes such as waste water remediation as it is able to remove nitrogen (particularly ammonia) with high efficiency (Culley and Epps, 1973; Sutton and Ornes, 1975, 1977), bio-fuel production (Jarvis et al., 1998; Zhao et al., 2012, 2014) and recombinant protein production (Khvatkov et al., 2018). It is also reported as a promising substrate for bio-hydrogen production, and recognized as an ideal plant in bioremediation and carbon cycle research (Kuehdorf et al., 2014; Olah et al., 2015; Tang et al., 2014; Wang et al., 2012, 2015; Xu and Deshusses, 2015; Xu et al., 2015). The copy number of the genes involved in the biosynthesis of two enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) are amplified in greater duckweed. GS and GOGAT are the major biochemical module for ammonium assimilation (Wang et al., 2014). In recent year, duckweeds are also considered as rich protein source for human consumption (Appenroth et al., 2018; de Beukelaar et al., 2019).

S. polyrhiza is also gathering interest as a feed material/ingredient for fish, poultry and pigs (Cruz-Velásquez et al., 2014; FAO, 2001; Hasan and Chakrabarti, 2009). Less fibre content of the plant makes it easily digestible. In grass carp *Ctenopharyngodon idella*, a 75% digestibility of *S. polyrhiza* has been observed (Wee, 1991). Similarly, analysis of the proximate composition showed that *S. polyrhiza* are a rich source of protein, although content varies from 23.8–40.9% (Hasan and Edwards, 1992; Hillman and Culley, 1978). Amado et al. (1980) reported the amino acid composition of 94 different strains of duckweeds. They suggested that all essential amino acids (except methionine) are present in sufficient amount in all strains of duckweeds. Recently, Appenroth et al. (2017) found around 25% protein level in *S. polyrhiza* cultured in nutrient medium. They also suggested that the levels of critical amino acids in duckweeds are within the recommended range of

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World Health Organization (WHO) for human. It is also a rich source of pigments, especially carotene and xanthophylls (Leng et al., 1995). Notably the nutritional and biochemical value of such macrophytes is highly variable and depends largely on water quality of the culture system (Boyd, 1971). Therefore, there is an urgent requirement to develop large-scale culture techniques for the production of nutrient-rich duckweeds. There is immense scope for large scale production of duckweeds in tropical climate (Chakrabarti, 2017).

In intensive management, supply of water and nutrient are essential for the continuous duckweed production of a predictable and useful biochemical composition (Hasan and Chakrabarti, 2009). Moreover, duckweeds are commonly cultured in wastewater which may contain unwanted components that are unsuitable for consumption by fish, other livestock, and ultimately human consumers. Inorganic and organic manures were successfully applied in Bangladesh for the production of duckweeds (BFRI, 1997; DWRP, 1998). The aim of the present study is to standardise the culture technique for the production of greater duckweed *Spirodela polyrhiza* in small tanks, and then large-scale production in ponds. The proximate, amino acid and fatty acid profiles of cultured *S. polyrhiza* are evaluated to establish its nutritional quality and suitability as an animal feed ingredient.

2. Materials and methods

2.1. Tank culture

S. polyrhiza were cultured in cemented outdoor tanks (1.2 m × 0.35 m × 0.30 m) using both organic manures and inorganic fertilizers between December 2016–March 2017. Three different combinations of manures used for the production of duckweeds were as follows. Manure 1: cattle manure, poultry droppings and mustard oil cake (1,1,1) were used at the rate of 1.052 kg m^{-3} (Srivastava et al., 2006). Manure 2: urea, potash and triple superphosphate were used at the rate of 20, 4 and 4 kg ha^{-1} day^{-1}, respectively (DWRP, 1998). Manure 3: cattle manure, urea, potash and triple superphosphate were used at the rate of 750, 7.5, 1.5 and 1.5 kg ha^{-1} day^{-1}, respectively (BFRI, 1997). There were three replicates for each treatment. *S. polyrhiza*, grown in the outdoor facility was introduced in the culture tanks (15 g $tank^{-1}$, fresh weight) after 5 days of manure application. All tanks were re-manured at 10 day intervals for sustainable duckweed production. In manure 1, organic manures were applied at a rate of one fourth dose of the initial dose. In manure 2 and manure 3, the amount of manure was equal to the initial dose. All manures were decomposed (5 days) before application. In each treatment, when the surface was fully covered, harvesting was initiated, except the fifth harvest in manure 3. At the time of fifth harvest, growth of duckweeds was poor in this treatment; duckweeds are totally harvested from all treatments. In all harvests (except the final), 50% of the total duckweeds were collected; all duckweeds were collected after 118 days of culture and the production was recorded as kg ha^{-1} $month^{-1}$ (dry weight, DW).

2.2. Pond culture

Three cemented ponds at the Central Institute of Fisheries Education (Indian Council of Agricultural Research), located at Rohtak, Haryana were used for the production of *S. polyrhiza* between July–August 2017. Each pond was 200 m^2 (20 m × 10 m) with water level maintained as 50 cm. Among the three manures used in the tank culture of greater duckweeds, highest production was obtained in manure 1, and so this treatment was selected for pond production. All the organic manures, cattle manure, poultry dropping and mustard oil cake (Srivastava et al., 2006) were decomposed for 5 days initially. *S. polyrhiza* cultures were produced in a clean environment (outdoor tanks of Department of Zoology, University of Delhi); then the plants were introduced in each pond at the rate of 1 kg $pond^{-1}$ (fresh weight). Initially, these greater duckweeds covered a small area of the water body (Fig. 1). In each pond, after the initial dose, one fourth dose of manure was applied at intervals of 10 days. Greater duckweeds were harvested thrice at 10 days intervals during 30 days of culture period. The harvesting pattern was similar to tank production, i.e. duckweeds were harvested when the whole water surface was covered. In first and second harvest, 50% duckweeds were harvested and plants were totally collected during the third harvest. The production was expressed as kg ha^{-1} $month^{-1}$ (DW).

2.3. Water quality

Major water quality parameters were recorded at weekly intervals in both tanks and ponds. A Solar Light lux meter (PMA 2100, USA) was used for the measurement of light intensity in the outdoor systems at fixed time (10.00 a.m.) and it was expressed as an average of replicates of individual treatment. A HACH Multi-meter (HQ 40d, USA) was used for the estimation of temperature, pH, conductivity, dissolved oxygen, ammonia and nitrate levels. Standard methods were followed for the estimation of phosphate and nitrite levels of water (APHA, 2012).

2.4. Relative growth rate (RGR)

The RGR of *S. polyrhiza* was estimated with the formula:

$$RGR = \ln (W_t/W_0)/t$$

where, W_t and W_0 were the weights of duckweeds at time t and zero reference time, respectively; t was the time interval in days. RGR was expressed as g g^{-1} day^{-1}.

2.5. Biochemical assays

The proximate composition of *S. polyrhiza* was assayed following standard methods (AOAC, 2000). Briefly, samples were dried for 24 h at 110 °C in an oven for the estimation of moisture contents. Ash content was determined after incineration at 600 °C for 16 h. Crude protein content was assayed by Kjeldahl distillation and nitrogen content (N x 6.25) was determined using a Tecator Kjeltec Auto 1030 analyser (Foss, Warrington, UK). Crude lipid level was determined gravimetrically using a Tecator Soxtec 2050 (Foss, Warrington, UK) after Soxhlet extraction by Hydrotec 8000 digester (Foss, Warrington, UK). Carbohydrate content of sample was subsequently determined by subtraction of protein, lipid and ash values.

The amino acid profile of greater duckweeds was estimated with an L-8900 Automatic Amino Acid Analyser (Hitachi Co. Ltd., Tokyo, Japan). The powdered duckweed sample was first hydrolysed using 6 N HCl for 22 h at 110 °C. Then hydrolysed sample was dried in a Nitrogen Evaporator (PCi Analytics, EV PLUS 08, Maharashtra, India). In the sample, 0.02 N HCl was added and the concentration of protein was 0.5 mg mL^{-1} of sample. The sample was kept in the Auto sampler and sample injection volume was 20 μL. As methionine, cysteine and tryptophan are destroyed during hydrolysis of sample with 6 N HCl, specific reagents are used for the estimations of these amino acids. Performic acid and hydrobromic acid (48%) were used for methionine and cysteine. For tryptophan, the sample was hydrolysed with 4 N methanesulfonic acid and 3-(2-aminoethyl) indole. The remaining methodology was identical for all amino acids. The ninhydrin derivative of proline and hydroxyproline was monitored at 440 nm, and other amino acids were monitored at 570 nm. The amino acids (peak areas) were quantified using the supplied Amino Acids Mixture Standard Solutions, Type B and Type AN-2 (Wako Pure Chemical Industries, Limited, Japan). Standard solutions for glutamine and tryptophan (Sigma-Aldrich, USA) were prepared before analysis.

Further *S. polyrhiza* samples were dried at 40 °C and ground prior to extraction of total lipid for fatty acid composition analysis. Total lipid was extracted from 1 g sample (DW) by homogenising in chloroform/methanol (2:1, *v*/v) using an Ultra-Turrax tissue disrupter (Fisher

Fig. 1.

Fig. 1. Introduction of *S. polyrhiza* (1 kg pond^{-1}) in Rohtak, Haryana.

Scientific, Loughborough, UK), and content determined gravimetrically (Folch et al., 1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 °C for 16 h (Christie, 2003), and FAME extracted and purified (Tocher and Harvie, 1988). The FAME were separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm (i.d.) × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector, and a flame ionisation detector. Data were collected and processed using Chromcard software for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME was identified by comparison to known standards and published data (Tocher and Harvie, 1988).

2.6. Statistical analysis

Data were presented as mean ± SE unless otherwise stated. One-way analysis of variance, ANOVA, Duncan's multiple range test, DMR (Montgomery, 1984). Student's *t*-test were used for the statistical analysis with significance accepted at $p < .05$ level.

3. Results

3.1. Culture in tanks

3.1.1. Water quality

Major water quality parameters were recorded in all treatments before the application of manures. There was no significant ($p > .05$) difference in temperature, pH, dissolved oxygen, ammonia, nitrite, nitrate and phosphate levels among treatments at the beginning of the study. A wide range of water temperature 9.4–26.7 °C was recorded during the culture of duckweed between December and March and this influenced the productivity (Table 1). The whole culture period was broadly divided into three phases based on the temperature and light intensity in the culture tanks. In phase I (December 2016–January 2017), water temperature and light intensity were 16.5 °C and 26.0 μmol photons m^{-2} s^{-1} at the beginning and then gradually decreased. The lowest temperature and light intensity were recorded in January. In phase II (February–March 2017) and phase III (March 2017), water temperature and light intensity showed increasing trends. There was no significant ($p > .05$) difference in temperature and light intensity among the three different treatments during the culture period. Among these three different treatments, there was variation in pH in different phases.

Significantly ($p < .05$) higher dissolved oxygen levels were found with manure 2 compared to the other two treatments throughout the study period (Fig. 2A). This group was followed by manure 3 and lowest dissolved oxygen level (< 1 mg L^{-1}) was found in manure 1. Ammonia levels were significantly ($p < .05$) higher in manure 1 compared to the other two treatments throughout the study period (Fig. 2B). In manure 1, ammonia levels ranged from 1.34–30.65, 7.52–18.57 and 15.25–17.85 mg L^{-1} in the first, second and third phases, respectively. In manure 2, ammonia levels ranged from 1.94–9.34, 0.03–7.71 and 1.44–3.33 mg L^{-1} in the first, second and third phases, respectively. In manure 3, ammonia level ranged from 0.17–10.97, 0.27–4.08 and 0.23–0.41 mg L^{-1} in the first, second and third phases, respectively. The lowest range of ammonia levels were found in the third phase regardless of manures.

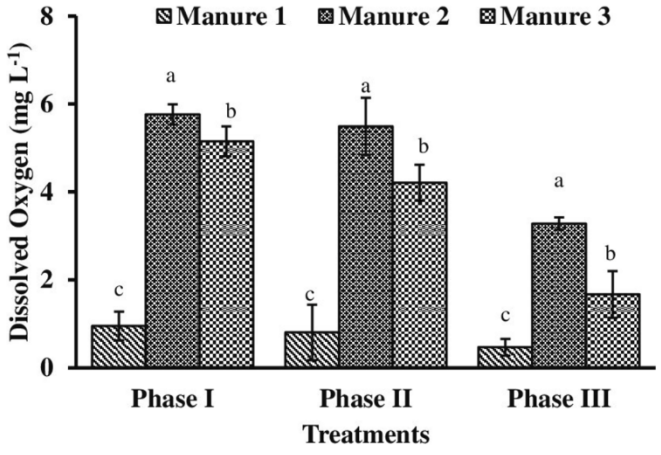
Nitrite level was significantly ($p < .05$) higher in manure 2 and manure 3 in the first phase compared to manure 1 (Table 1). There was no significant ($p > .05$) difference between these two former groups. In the second and third phases, nitrite levels were significantly ($p < .05$) higher in manure 2 compared to the other two treatments. Nitrate level was significantly ($p < .05$) higher in manure 2 compared to the other two treatments throughout the study period. Phosphate level was significantly ($p < .05$) lower in manure 2 compared to the other two treatments throughout the study period (Fig. 2C). Conductivity was significantly ($p < .05$) higher in manure 1 compared to the other treatments throughout the study period (Fig. 2D). In manure 1, conductivity ranged from 516 to 1196 μS cm^{-1}.

3.1.2. Production and relative growth rate (RGR)

The production of *S. polyrhiza* was affected by water temperature. The relative growth rate of greater duckweeds was slow (0.02–0.04 g g^{-1} day^{-1}) at the beginning of the culture period due to low temperature regardless of treatments. Greater duckweeds were first harvested after 69 days of initial introduction in all three treatments. As water temperature increased, the growth rate also increased and duckweeds were harvested another four times; second and fourth harvests were performed after 10 days of the respective previous harvest and third and fifth harvests were after 12 days of the respective

Table 1
Environmental parameters measured in tanks during the culture of *S. polyrhiza*.

Parameters	Manure 1		Manure 2		Manure 3	
	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Phase I (December 2016–January 2017)						
Temperature (°C)	9.36–16.55	14.38 ± 0.34	9.36–16.55	14.38 ± 0.336	9.36–16.55	14.38 ± 0.34
Light intensity (μmol photons $m^{-2}s^{-1}$)	14.56–49.43	27.29 ± 2.45	14.56–49.43	27.29 ± 2.45	14.56–49.43	27.29 ± 2.45
pH	7.20–7.91	–	7.04–7.86	–	6.98–7.85	–
Nitrite (mg L^{-1})	0.007–0.26	0.116 ± 0.01	0.13–1.01	0.47 ± 0.05	0.06–1.04	0.49 ± 0.07
Nitrate (mg L^{-1})	1.68–18.70	5.77 ± 1.13	6.58–43.66	30.76 ± 2.60	8.44–35.48	24.40 ± 2.01
Phase II (February–March 2017)						
Temperature (°C)	15.70–19.33	17.80 ± 0.43	15.70–19.33	17.80 ± 0.43	15.70–19.33	17.80 ± 0.43
Light intensity (μmol photons $m^{-2}s^{-1}$)	49.21–105.08	89.89 ± 3.25	49.21–105.08	89.89 ± 3.25	49.21–105.08	89.89 ± 3.25
pH	7.09–7.59	–	7.24–7.82	–	7.26–7.72	–
Nitrite (mg L^{-1})	0.02–0.12	0.055 ± 0.015	0.11–0.84	0.44 ± 0.08	0.006–0.12	0.09 ± 0.02
Nitrate (mg L^{-1})	5.95–44.73	25.77 ± 6.04	15.04–46.87	29.38 ± 4.58	16.15–34.94	24.42 ± 2.61
Phase III (March 2017)						
Temperature (°C)	23.26–26.70	24.98 ± 1.72	23.26–26.70	24.98 ± 1.72	23.26–26.70	24.98 ± 1.72
Light intensity (μmol photons $m^{-2}s^{-1}$)	137.41–151.16	143.79 ± 6.39	137.41–151.16	143.79 ± 6.39	137.41–151.16	143.79 ± 6.39
pH	7.27–7.56	–	7.18–7.43	–	7.28–7.39	–
Nitrite (mg L^{-1})	0.015–0.02	0.016 ± 0.00	0.37–0.07	0.52 ± 0.16	0.082–0.12	0.10 ± 0.02
Nitrate (mg L^{-1})	11.68–18.54	15.11 ± 3.44	33.51–36.95	35.23 ± 1.72	16.51–34.94	24.41 ± 2.61



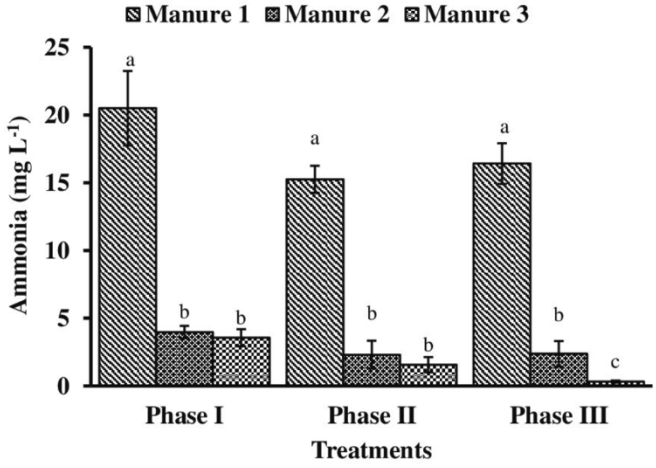


Fig. 2 (B)

Fig. 2. (*continued*)

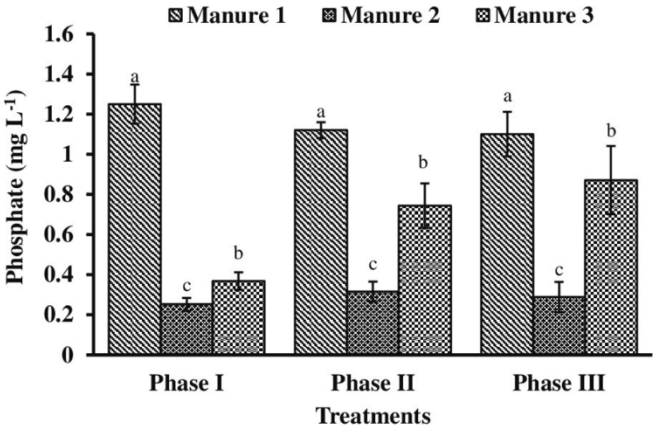


Fig. 2. (*continued*)

Fig. 2. Various water quality parameters (in parenthesis). (A) Dissolved oxygen, (B) ammonia, (C) phosphate and (D) conductivity of water found during three different phases of culture of *S. polyrhiza* in tanks. Phase I: December 2016–January 2017, Phase II: February–March 2017 & Phase III: March 2017. Bars with different superscripts are significantly ($p < .05$) different ($n = 3$).

previous harvest. The RGR values ranged from 0.021–0.158, 0.007–0.12 and − 0.024 - 0.129 g g^{-1} day^{-1} in manures 1, 2 and 3, respectively throughout the study period. In manure 3, poor growth of plant at fifth harvest compared to the previous one resulted into negative RGR value. The average RGR values were 0.08 ± 0.02, 0.06 ± 0.03 and 0.07 ± 0.03 g g^{-1} day^{-1} in manures 1, 2 and 3, respectively. Total production of duckweeds was significantly ($p < .05$) higher in manure 1 compared to the other manures (Fig. 3). This group was followed by manure 3 and minimum production was found in manure 2.

3.2. Culture in ponds

3.2.1. Water quality

In three different ponds at the Rohtak centre, water temperature and pH ranged from 32.4–30.5 °C and 7.76–8.30, respectively during the study period. Dissolved oxygen level ranged from 1.25–4.57 mg L^{-1} on various days of study. Ammonia, nitrite and nitrate levels of ponds ranged from 5.02–17.57, 0.003–0.12 and 0.23–2.44 mg L^{-1}, respectively. Phosphate level ranged 1.15–2.0 mg L^{-1} during the study period (Table 2). Conductivity ranged from 1032 to 1251 μS cm^{-1} throughout the culture period of greater duckweed.

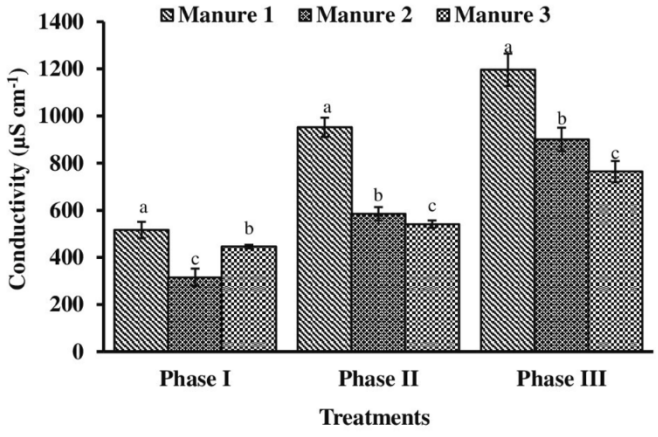


Fig. 2. (*continued*)

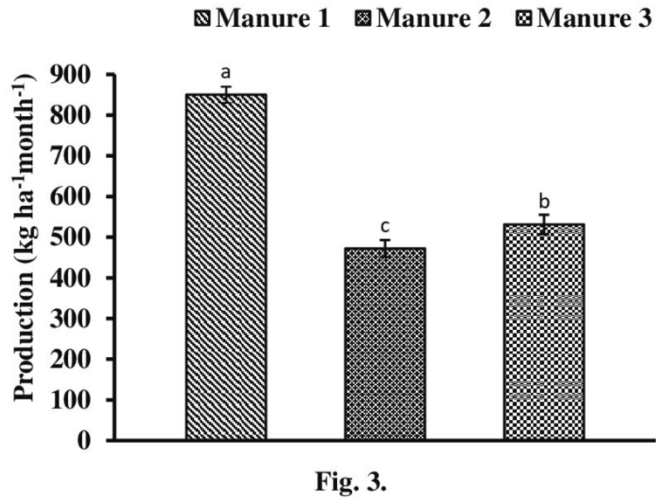


Fig. 3. Total production of *S. polyrhiza* cultured with three different organic manures and inorganic fertilizers in tanks. Bars with different superscripts are significantly ($p < .05$) different (n = 3).

Table 2
Environmental parameters measured in *S. polyrhiza* culture ponds during the study period.

Parameter	Range	Mean ± SE
Temperature (°C)	30.5–33.0	32.00 ± 1.0
pH	7.76–8.30	–
Dissolved oxygen (mg L^{-1})	1.25–4.57	2.50 ± 0.25
Ammonia (mg L^{-1})	5.02–17.57	15.25 ± 0.7
Nitrite (mg L^{-1})	0.005–0.01	0.008 ± 0.002
Nitrate (mg L^{-1})	0.05–2.05	0.921 ± 0.3
Phosphate (mg L^{-1})	1.15–2.00	1.52 ± 0.07
Conductivity (µS cm^{-1})	1032–1251	1150 ± 37.0

3.2.2. Production and relative growth rate (RGR)

S. polyrhiza was harvested three times from the ponds at 10 days intervals (Fig. 4A–B). Greater duckweeds were harvested from the ponds and were cleaned thoroughly with tap water to remove organic material, excess water was removed, air dried and then dried at 40 °C in an oven. Dried duckweed was packed in airtight containers for further use. The RGR values were 0.48, 0.14 and 0.03 g g^{-1} day^{-1} in the first, second and third harvests, respectively. The average RGR value was 0.22 ± 0.13 g g^{-1} day^{-1}. Total production was 2020 ± 150 kg ha^{-1} $month^{-1}$ on dry matter basis, equivalent to 24 t ha^{-1} yr^{-1} (Fig. 5).

3.3. Biochemical composition

There was a difference in the proximate composition of greater duckweed cultured with organic manures (manure 1) and inorganic fertilizers (manure 2) in tanks. Protein content was significantly ($p < .05$) higher, and carbohydrate and ash contents were significantly ($p < .05$) lower, in duckweed cultured in manure 1 compared to manure 2 (Table 3). The amino acid profile of greater duckweed cultured in organic manures showed the presence of essential (37.4%), non-essential (58.2%) and free amino acids (4.5%). Among essential amino acids, three branched chain amino acids, leucine, isoleucine and valine contributed 51.4%. Glutamic acid and glutamine consisted 28.3% of the total non-essential amino acids in the greater duckweed. The presence of taurine enhanced the nutritional value of greater duckweed (Table 4).

The fatty acid composition of *S. polyrhiza* was dominated by polyunsaturated fatty acids (PUFA), which accounted for 47–53% of total fatty acids, primarily α-linolenic acid (ALA, 18:3n-3) at around 36–39% (Table 5). Total saturated fatty acids accounted for 32–39%, followed by linoleic acid (LA, 18:2n-6) at 11–14% and monoenes at 9–11%. As with proximate composition, fatty acid profile was affected by manures. *S. polyrhiza* grown in inorganic fertilizers (manure 2) having a higher proportion of ALA, LA and total PUFA, and lower saturated and monounsaturated fatty acids. Due to the slightly higher (although not statistically significant) lipid content of *S. polyrhiza* grown in manure 2, all fatty acids were in higher absolute amounts (mg.100 g^{-1} dry mass) in macrophytes grown in inorganic fertilizers. *S. polyrhiza* lipid contained no long-chain PUFA such as docosahexaenoic acid (22:6n-3), although there was a trace level of eicosapentaenoic acid (20:5n-3), most likely due to minor microalgal contamination within the macrophyte biomass.

4. Discussion

Water temperature and sunlight are major environmental factors that influence the growth of duckweed compared to the nutrient concentrations in the water (Hasan and Chakrabarti, 2009). In tank culture, *S. polyrhiza* was first harvested after 69 days of culture. The water temperature was generally below 15 °C during this period of culture, and lowest light intensity was also recorded during this period. Water temperature increased above 16 °C at the second phase of culture and only then duckweed grew well and harvested. Higher light intensity was also recorded at the second phase compared to the first one. In a comparative study, growth performance of *S. polyrhiza* was recorded at two temperature ranges of 10–12 and 26–28 °C (Song et al., 2006). It was found that cell growth, the synthesis, and absorption ability of duckweed decreased at low temperature compared to duckweed cultured at higher temperature. There was no change in frond number for 15 days at low temperature range.

In the present study, the relative growth rate (RGR) of greater duckweed was low during the first phase of tank culture and then increased regardless of treatments. In manure 3, RGR reduced in fifth harvest of phase three. Among the three manures, significantly ($p < .05$) higher production was found with manure 1 compared to the inorganic fertilizers. Therefore, organic manures were applied in pond culture of greater duckweed. In contrast to the tank culture, RGR value was maximum at first harvest in pond culture of greater duckweed and the average RGR value was higher in pond compared to tank production. The production rate of greater duckweed was 0.08 ± 0.02 fronds day^{-1} in laboratory conditions (Lemon et al., 2001). Higher temperature also resulted in enhanced growth rate in ponds in the present study. In Bangladesh, highest growth of *S. polyrrhiza* was found at 22.2–22.5 °C in pond (Khondker et al., 1993), although *S. polyrhiza* survived at 10–12 °C, it could not grow well at a low temperature (Song et al., 2006). The duckweed exposed to oxidative damage at low temperature. Appenroth (2002) suggested that 15 °C temperature

Fig. 4(A)

Fig. 4. Production of *S. polyrhiza* (A) in ponds & (B) duckweeds after harvest.

(combined with 30 μM phosphate level) was the dominant turion formation inducing factor. In laboratory axenic culture, *S. polyrhiza* were exposed at 100 μmol $m^{-2} s^{-1}$ white light (Appenroth et al., 2017). In the present study, good growth of *S. polyrhiza* was found at light intensity between 105 and 151 μmol photons $m^{-2} s^{-1}$ in natural outdoor light.

In Bangladesh and India, a pH range from 6.5–7.5 (Islam and Khondkar, 1991) and 6.8–8.5 (Gopal and Chamanlal, 1991; Kaul and Bakaya, 1976) was found to be optimum for the production of greater duckweed. In the present study, pH ranged from 6.98–7.86 and 7.76–8.30 in tank and pond culture systems, respectively. There was no direct effect of dissolved oxygen on the production of greater duckweed as highest production was recorded in manure 1 with minimum dissolved oxygen level in tank culture. Leng et al. (1995) suggested that maintenance of low dissolved oxygen with 6–7 pH should be the strategy for duckweed pond management.

It was found that the root length was shorter in *S. polyrhiza* that grown at low temperature compared to the plants grown at a higher temperature. *S. polyrhiza* with shorter root length were inefficient in absorbing nitrogen, phosphorus and other nutrients from water (Reddy and DeBusk, 1985). In tank culture, highest ammonia level was recorded in manure 1 at first phase and no production was recorded during this period. The ammonia level gradually reduced in the second and third phases and the growth of duckweed enhanced. Even with the same manure system (manure 1), lower levels of ammonia were found in ponds compared to tanks. Absorption of nutrients helped in the higher production of duckweeds in ponds. The fluctuation of pH between 7.4 and 9.0 enhanced the ammonia toxicity in laboratory culture (Caicedo et al., 2000). In tank culture of duckweed, highest RGR was found in the second phase at 15.25 ± 1.0 mg L^{-1} ammonia concentration in manure 1. It is also interesting to see that in tank culture, poor growth of duckweeds in manure 3 during fifth harvest might be

Fig. 4(B)

Fig. 4. (*continued*)

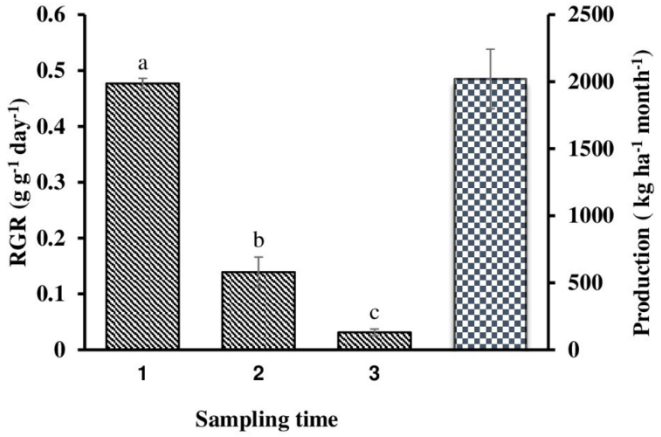


Fig. 5.

Fig. 5. Relative growth rate (RGR) and total production of *S. polyrhiza* in ponds. RGR was measured thrice at 10 days interval. Bars with different superscripts are significantly ($p < .05$) different (n = 3).

Table 3
Proximate composition of *S. polyrhiza* (% of dry weight).

Parameter	Manure 1 (Organic)	Manure 2 (Inorganic)
Protein	35.82 ± 0.14	30.50 ± 0.03*
Lipid	7.11 ± 0.11	7.19 ± 0.06
Ash	18.51 ± 0.02	20.64 ± 0.26*
Carbohydrate	38.38 ± 0.26	41.68 ± 0.17*

Data are presented as means ± SEM (n = 3).

* Denotes significant difference ($p < .05$) between the two manures as determined by Student's *t*-test.

related to the low ammonia level in the culture tank. Leng et al. (1995) suggested that 7–12 mg N L^{-1} was optimum to maintain a protein content of 40% in duckweed. A TKN content of 20–30 mg L^{-1} was required for optimum growth (Culley et al., 1981) and maintenance of high protein content. In the present study, the ammonia level in the pond water also helped in the proper growth of the duckweed. Nitrification rate was slower in manure 1 compared to the other two treatments in the tank culture of duckweed. In manure 1, nitrate level was significantly higher in the second phase compared to the other phases. Phosphorus is a major limiting nutrient, although it is required in lesser amount. In the present study, the phosphate levels in manure 1 helped in the production of duckweed in both tanks and ponds. The optimum conductivity for maximum production of *S. polyrrhiza* was 650–1000 μS cm^{-1} (Gopal and Chamanlal, 1991). *S. polyrrhiza* completely disappeared in May due to reduced conductivity and alkalinity (Khondker et al., 1993). In the tank culture, the growth of greater duckweed was less in the first phase and the conductivity was minimum during this phase regardless of manures applied. Then conductivity increased with higher production of duckweed. In pond culture, the conductivity was always > 1000 μS cm^{-1}.

In ponds, the production of greater duckweed was encouraging, 2020 ± 150 kg ha^{-1} $month^{-1}$ (24 t ha^{-1} yr^{-1}) on dry matter basis. Literature showed a wide variation in the production of duckweed, with various climatic conditions and nutrient availability mostly being responsible for this variation. Edwards et al. (1990) reported ~20 t ha^{-1} $year^{-1}$ (DM) production of *S. polyrhiza* during 1–3 months culture period; the yield decreased (~9 t ha^{-1} $year^{-1}$) when the duration of culture period increased to 6 months. The yield of greater duckweeds in domestic wastewater (Reddy and DeBusk, 1985), sewage effluent (Sutton and Ornes, 1975) and nutrient non-limited water (Reddy and DeBusk, 1985) were 17–32, 14.6 and 11.3 t ha^{-1} yr^{-1}, respectively. Based on the available data, an average harvest of 10–20 t duckweed ha^{-1} $year^{-1}$ could be expected under optimum environmental conditions (Hasan and Chakrabarti, 2009). In a similar study, *Lemna minor* was produced in ponds using organic manures. The production was lower (702.5 kg ha^{-1} $month^{-1}$, DW) compared to *S. polyrhiza* (Chakrabarti et al., 2018). The initial amount of duckweed introduced for culture also influenced production. A seeding rate of 60 kg m^{-2} for *S. polyrhiza* was recommended (DWRP, 1998). In the pond culture, only 1 kg pond $^{-1}$ (200 m^2) *S. polyrhiza* was introduced in the present study.

Table 4
Amino acid (g 100 g^{-1} of dry weight) profile of *S. polyrhiza* cultured with organic manures in tanks.

Amino acids	Concentration
Essential	
Histidine (His)	0.771 ± 0.053
Isoleucine (Ile)	1.703 ± 0.150
Leucine (Lue)	3.322 ± 0.207
Lysine (Lys)	2.280 ± 0.129
Methionine (Met)	0.694 ± 0.059
Phenylalanine (Phe)	2.159 ± 0.144
Threonine (Thr)	1.502 ± 0.386
Tryptophan (Trp)	0.282 ± 0.018
Valine (Val)	2.383 ± 0.139
Non-essential	
Alanine (Ala)	2.384 ± 0.130
Arginine (Arg)	2.386 ± 0.120
Asparatate (Asp)	4.094 ± 0.212
Cysteine (Cys)	0.369 ± 0.039
Glutamic acid (Glu)	5.103 ± 0.380
Glutamine ($GluNH_2$)	1.250 ± 0.300
Glycine (Gly)	2.369 ± 0.110
Proline (Pro)	1.001 ± 0.110
Serine (Ser)	1.904 ± 0.120
Tyrosine (Tyr)	1.558 ± 0.050
Free	
Phosphoserine (p-Ser)	0.060 ± 0.002
Taurine (Tau)	0.023 ± 0.006
Phospho ethanol amine (PEA)	0.072 ± 0.001
α Amino adipic acid (α-AAA)	0.020 ± 0.001
α Amino-n- butaric acid (α-ABA)	0.141 ± 0.014
Cystathionine (Cysthi)	0.115 ± 0.001
β -Alanine (β-Ala)	0.072 ± 0.011
β -Amino isobutyric acid (β-AiBA)	0.354 ± 0.015
Ethanol amine ($EOHNH_2$)	0.112 ± 0.004
Ornithine (Orn)	0.027 ± 0.002
1 Methylhistidine (1 Mehis)	0.048 ± 0.003
Hydroxy proline (Hypro)	0.197 ± 0.010
ϒ- Amino isobutyric acid (ϒ-AiBA)	0.478 ± 0.024

The proximate composition of greater duckweed varied with nutrient availability of the culture system. In the present study, the protein, lipid, ash and carbohydrate contents of greater duckweeds were influenced by the quality of the manures. The protein content of the duckweeds (30.5 ± 0.03–35.82 ± 0.14%) was higher in the present study compared to some previous studies. The duckweeds collected from Thailand showed 23.8 ± 0.8% protein content (Hasan and Edwards, 1992), whereas 25.6 ± 0.2% protein content was recorded in plants collected from a pond in Nigeria (Fasakin et al., 1999). In USA, 13.1% crude protein was found in greater duckweed collected from low-nutrient lagoon (Culley et al., 1981), whereas 40.9% crude protein was found in plants grown in a dairy cattle-waste lagoon (Hillman and Culley, 1978). In the present study, lipid contents of duckweeds ranged from 7.11–7.2%, whereas lipid contents of 2.5–6.7% were reported in the earlier studies (Hasan and Chakrabarti, 2009). Appenroth et al. (2017) found around 5% lipid content in duckweed. Similarly, the ash content of the duckweed in the present study (18.51 ± 0.02–20.64 ± 0.26%) was comparable with earlier studies, in which ash contents varied from 15.2 ± 0.4–18.3 ± 1.0% in greater duckweeds collected from different geographical areas (Hasan and

Table 5
Fatty acid composition of *S. polyrhiza* as percentage of total fatty acids (Percentage) or as mg fatty acids per 100 g dry weight (Absolute).

Fatty acid	Manure 1		Manure 2	
	Percentage	Absolute	Percentage	Absolute
14:0	1.01 ± 0.22	16.9 ± 1.86	1.10 ± 0.30	23.65 ± 7.42
15:0	0.60 ± 0.04	10.1 ± 0.46	0.40 ± 0.01*	8.56 ± 0.55
16:0	31.22 ± 2.33	524.1 ± 18.32	25.50 ± 0.40	547.04 ± 33.88
18:0	2.33 ± 0.23	39.1 ± 0.35	2.02 ± 0.13	43.39 ± 4.69
20:0	0.40 ± 0.04	6.6 ± 0.10	0.33 ± 0.01	7.04 ± 0.55
22:0	0.77 ± 0.10	12.9 ± 0.32	0.85 ± 0.03	18.17 ± 0.24*
24:0	3.05 ± 0.15	51.3 ± 3.16	2.28 ± 0.05*	48.85 ± 1.15
Total saturated	39.38 ± 3.12	661.0 ± 20.21	32.48 ± 0.76	696.70 ± 48.49
16:1n-9	4.76 ± 2.23	86.4 ± 27.76	6.75 ± 0.14	144.61 ± 3.60
17:1 n	0.00 ± 0.00	0.0 ± 0.00	0.30 ± 0.02*	6.34 ± 0.22*
18:1n-9	2.09 ± 0.13	35.2 ± 1.68	3.01 ± 0.64	64.93 ± 16.74
18:1n-7	2.24 ± 0.23	37.6 ± 0.25	1.34 ± 0.06*	28.68 ± 2.59*
Total monoenes	9.09 ± 6.37	159.2 ± 124.19	11.39 ± 0.53	244.57 ± 22.72
18:2n-6	11.35 ± 0.76	190.7 ± 8.09	13.49 ± 0.23	289.08 ± 8.33*
20:4n-6	0.00 ± 0.00	0.0 ± 0.00	0.33 ± 0.01*	7.03 ± 0.08*
Total n-6 PUFA	11.35 ± 0.76	190.7 ± 8.09	13.82 ± 0.24	296.11 ± 8.41*
18:3n-3	35.75 ± 2.18	600.6 ± 29.28	38.95 ± 1.08	834.63 ± 15.44*
20:5n-3	0.38 ± 0.12	6.3 ± 1.37	0.60 ± 0.08	12.98 ± 2.30
Total n-3 PUFA	36.13 ± 0.30	606.9 ± 27.91	39.56 ± 1.00	847.61 ± 17.75*
Total DMA	4.04 ± 0.18	68.0 ± 4.35	2.76 ± 0.06*	59.09 ± 1.49
Total PUFA	47.48 ± 3.07	797.6 ± 35.99	53.37 ± 1.241	1143.72 ± 26.16*
Total Fatty acids		1685.8 ± 275.3		2144.1 ± 329.9

Data are presented as means ± SEM (n = 3).
DMA, dimethyl acetals; PUFA, polyunsaturated fatty acids.
* Denotes significant difference ($p < .05$) between the two manures as determined by Student's *t*-test.

Edwards, 1992).

These data showed that culture of greater duckweed with a specific management strategy helped in the production of valuable animal feed ingredients. *S. polyrhiza* is a new generation sustainable crop (Hoang et al., 2018). Song et al. (2006) reported that temperature also influenced the soluble protein, chlorophyll α, chlorophyll β and carotenoid pigment of duckweeds. The present study confirmed the earlier study. The presence of essential amino acids viz. histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine were documented in greater duckweeds (Ismail, 1998). The present study showed that all the essential (including tryptophan) and non-essential amino acids were present in adequate quantity in cultured duckweed. The present study also showed the presence of taurine in the duckweeds. The presence of glutamic acid and glutamine confirmed the role of greater duckweed in reducing nitrogenous materials in the water. Similar amino acids composition was found in L. *minor* (Chakrabarti et al., 2018). The nutritional value of duckweed is comparable with alfalfa, being a rich source of lysine and arginine (Guha, 1997). The composition of essential amino acids in greater duckweed is comparable with soybean (NRC, 1998), the most commonly used ingredient in the diet formulation of fish (Table 6). The amino acid requirements of important cultivable species are documented (NRC, 2011). It is clear from the present study that the amino acid profile of greater duckweed meets the nutritional requirements of the cultivable species. The amino acid profiles of *Landoltia punctata* (= *S. oligorrhiza*) and different clones of *Wolffia arrhiza* were sufficient to fulfilled the requirements for human recommended by WHO ((Ismail, 1998; Appenroth et al., 2018).

In addition, *S. polyrhiza* demonstrated reasonable lipid content with ALA being the major fatty acid component in present study. Inorganic fertilizers resulted in slightly higher lipid content and relative percentage of ALA, which individually did not reach statistical significance, but together had a significant effect, increasing the absolute content of ALA. The PUFA content of *S. polyrhiza* grown in culture media was higher compared to the present study though the total lipid level was higher in the latter (Appenroth et al., 2017). It was interesting that in different species of *Wolffia* fat content was low, varied from 1 to 5%. PUFA levels were above 60% of total fat. The n-3 PUFA level was higher compared to n-6 PUFA (Appenroth et al., 2018). In the present study, the lipid and PUFA contents were higher in *S. polyrhiza* compared to *Wolffia* spp. In *L. minor*, 60–63% of total fatty acid was PUFA; around 41–43% α-linolenic acid and 17–18% linoleic acid (Chakrabarti et al., 2018).

Table 6
The essential amino acid profiles of soybean (*Glycine* max) meal and *S. polyrhiza* and their requirement for *Cyprinus carpio* and *Oreochromis niloticus* (NRC, 1998, 2011).

Amino acids	*Glycine max* meal (g 100 g^{-1})	*Spirodela polyrhiza* (g 100 g^{-1})	*Cyprinus carpio* (g 100 g^{-1} diet)	*Oreochromis niloticus* (g 100 g^{-1} diet)
Histidine (His)	1.17	0.77	0.5	1.0
Isoleucine (Ile)	1.99	1.70	1.0	1.0
Leucine (Lue)	3.42	3.32	1.4	1.9
Lysine (Lys)	2.83	2.28	2.2	1.6
Methionine (Met)	0.61	0.7	0.7	0.7
Phenylalanine (Phe)	2.18	2.15	1.3	1.1
Threonine (Thr)	1.73	1.50	1.5	1.1
Tryptophan (Trp)	0.61	0.28	0.3	0.3
Valine (Val)	2.06	2.38	1.4	1.5
Arginine (Arg)	3.23	2.38	1.7	1.2
Cysteine (Cys)	–	0.36	–	–
Tyrosine (Tyr)	–	1.55	–	–
Methionine + Cysteine	1.31	1.07	1.0	1.0
Phenylalanine + Tyrosine	–	3.7	2.0	1.6

5. Conclusions

The application of organic manures helped in the production of

greater duckweed *S. polyrhiza* in a sustainable manner. The temperature, light intensity, ammonia, phosphate and conductivity significantly influenced the productivity of the water bodies. Proximate composition, especially amino acid and fatty acid profiles confirmed the suitability of the greater duckweed as a potential ingredient for the development of diets for fish and other livestock.

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Mass Production of *Lemna minor* and Its Amino Acid and Fatty Acid Profiles

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The surface floating duckweed *Lemna minor* (Lemnaceae) is a potential ingredient to replace the application of fish-meal in the aqua-feed. The culture technique of the duckweed was standardized in outdoor tanks and then applied in the pond. Three consecutive experiments were conducted in tanks (1.2 × 0.35 × 0.3 m). In experiment 1, four different manures were used. In manure 1 (organic manure, OM) and manure 3 (2x OM), cattle manure, poultry droppings, and mustard oil cake (1:1:1) were used; in manure 2 (inorganic fertilizer, IF), urea, potash, triple superphosphate were used; manure 4 (2x OM+IF) was a combination of manure 2 and manure 3. In experiment 2, manure 1 (OM) and manure 2 (IF) were used, and manure 3 (OM+IF) was a combination of both manures. In experiment 3, OM and IF were selected. In pond (20 × 10 × 0.5 m), OM was applied. Fresh duckweed was seeded after 5 days of manure application. In experiments 1 and 3, total production was significantly ($P < 0.05$) higher in OM compared to other treatments. In experiment 2, there was no significant ($P > 0.05$) difference in production between OM and IF. In pond, relative growth rate (RGR) of duckweed ranged from 0.422 to 0.073 g/g/day and total production was 702.5 Kg/ha/month (dry weight). Protein, lipid, and ash contents were higher in duckweed cultured in OM compared to IF. The duckweed was a rich source of essential (39.20%), non-essential (53.64%), and non-proteinogenic (7.13%) amino acids. Among essential amino acids, leucine, isoleucine, and valine constituted 48.67%. Glutamic acid was 25.87% of total non-essential amino acids. Citrulline, hydroxiproline, taurine, etc. were found in the duckweed. The fatty acid composition was dominated by PUFA, 60–63% of total fatty acids, largely α-linolenic acid (LNA, 18:3n-3) at around 41 to 47% and linoleic acid (LA, 18:2n-6) at 17–18%. The nutritional value of duckweeds and their production potential in the pond conditions were evaluated. Duckweed biomass may thus be used to replace commercial fish-meal that is currently used in aquaculture.

Keywords: *Lemna minor*, organic manure, proximate composition, amino acids, fatty acids

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INTRODUCTION

The surface floating macrophyte duckweed *Lemna* is the largest genus of the family Lemnaceae. They are abundant in the tropical and subtropical countries; growing profusely in still, nutrient-rich small ponds, ditches, and swamps or in slowly moving water bodies. The entire plant body consists of metabolically active non-structural tissue (Wolverton and McDonald, 1980) and the low fiber content of the plant has a beneficial impact on digestibility when used in animal feed. Duckweed grows on water with relatively high levels of N, P, and K and concentrates the minerals and synthesizes protein. The reported presence of various essential (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, tyrosine) and non-essential amino acids (FAO, 2001), poly-unsaturated fatty acids (Yan et al., 2013), β-carotene, and xanthophylls has made *Lemna* spp. a potential feed source for livestock (Skillicorn et al., 1993; Leng et al., 1995). In Taiwan, duckweeds are used as food for pig and poultry (FAO, 2001). In fish feed, *Lemna* spp. are usually used in fresh state. In recent years, there is a growing interest in this free floating macrophyte in the aqua-feed industry (for production of pelleted diets) to replace the protein-rich and costly fish-meal. Chakrabarti (2017) reported the production potential of duckweeds in freshwater bodies. Duckweeds are also used for treatment of waste water (Culley and Epps, 1973; Sutton and Ornes, 1975, 1977) and production of bio-fuel (Jarvis et al., 1988; Zhao et al., 2012, 2014, 2015a,b).

Protein plays a significant role in fish nutrition. Fish-meal is one of the commonly used protein-rich ingredients in the aqua-feed industry. Non-availability of quality fish meal and competition for the same resources with the terrestrial live-stock industry has made aquaculturists search for economically viable alternative protein sources. The alternative protein source (to fish meal) should be available in the required amount, cost-effective and preferably non-conventional to avoid competition with other uses and industries. The amino acid profile of the ingredient should meet the nutritional requirement of the cultivable species and prepared feed should be palatable and digestible to the fish. Digestibility test of duckweeds in carps and tilapia showed promising results (Hassan and Chakrabarti, 2009). Sharma et al. (2016) reported that the protein content of *Lemna minor* was 39.75 ± 0.47% and that digestibility of this plant protein for rohu *Labeo rohita* and common carp *Cyprinus carpio* was high as determined by an *in vitro* digestibility study.

The application of *Lemna* spp. as potential aqua-feed ingredients requires continuous production. Sustainable production of this plant requires an understanding of its nutritional and environmental requirements. The nutritional value of a plant also depends on the culture medium. The growth rate of duckweed clones in different natural (Rejmankova, 1975) and laboratory (Landolt, 1957) conditions varied. Many studies showed the production of *Lemna* spp. in domestic waste water (Zirschky and Reed, 1988), septage-fed ponds (Edwards et al., 1990, 1992), and effluent water (Vroon and Weller, 1995). The production of *Lemna* spp. in clean water with a known manuring schedule is required for commercial aqua-feed production. Few studies have been conducted to find the best balance of nutrients that may provide maximum growth of duckweed (FAO, 1989), especially for *Lemna* spp. The requirement to fertilize duckweeds depends on the source of the water. Rainwater collected in ponds may need a balanced NPK application. In Bangladesh, inorganic fertilizers (IFs, urea, triple superphosphate, and potash) were used for the production of duckweeds (DWRP, 1998). Hassan and Chakrabarti (2009) suggested a wide range of organic waste materials *viz.* animal manure, kitchen wastes, wastes from a wide range of food processing plants, biogas effluents, etc. for the production of duckweeds. A periodic supply of nutrients helped to avoid nutrient deficiency in the culture systems (Sutton and Ornes, 1975; Said et al., 1979). A direct relationship was found between the crude protein content of duckweed and the nitrogen content of the culture system. Although many species survive at extreme temperature, warm and sunny conditions are preferable for faster growth of the plants (Skillicorn et al., 1993). The distribution of various members of duckweed has been influenced by the microclimatic factors such as light intensity, salinity, and regional temperature (Landolt, 1986). The growth of duckweed is largely a function of environmental temperature and light, nutrient status of the culture medium and the degree of crowding of the plants (Hassan and Chakrabarti, 2009).

The present investigation aimed to develop a suitable culture technique for the production of *L. minor* in a sustainable manner. In our earlier study, it was found that the application of organic manures (OM) viz. cattle manure, poultry wastes, and mustard oil-cake was very effective in the mass production of live food organisms (Srivastava et al., 2006). Application of these manures helped in the large scale production of zooplankton in the outdoor facility (Chakrabarti and Sharma, 2008). These manures are easily available. Therefore, in the present study, these OMs were selected along with the other IFs to evaluate their effect on the production of duckweed. The culture technique was first standardized in a small outdoor facility under controlled conditions using various organic and IFs. The best method was then adopted in pond conditions to evaluate the large scale production potential of the macrophyte. The nutritional value of the produced plant was determined to evaluate its suitability as a potential feed ingredient for the aqua-feed industry.

RESULTS

Culture of *L. minor* in Outdoor Tanks

Water Quality

Three consecutive experiments were conducted in outdoor tanks to generate the baseline data for the production of duckweed *L. minor* in the pond conditions. Four, three, and two different manures were used in experiments 1, 2, and 3, respectively. In the pond experiment, only OM were applied for the production of *L. minor*. In experiment 1, water temperature ranged from 28.0 ± 0.4 to 25.5 ± 0.3°C in various treatments during September–October 2016. The intensity of light was recorded as 7,353 ± 138 to 4,642 ± 114 lux during this period in different culture tanks. There was no significant ($P > 0.05$) difference in water temperature (**Figure 1A**) and light intensity (**Figure 2A**) among various culture tanks. Water temperature and light intensity were higher at the beginning of the study and

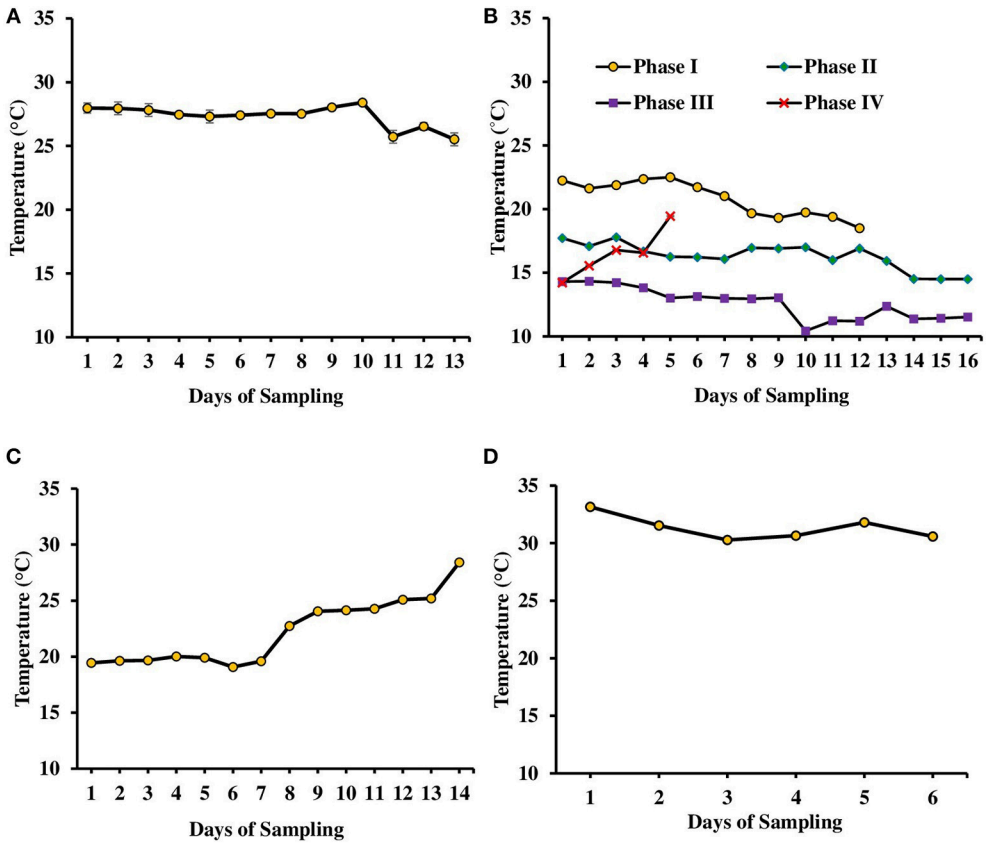

FIGURE 1 | Variation in water temperature found in tanks (experiments 1–3) and ponds during various days of culture of *L. minor*. **(A)** Experiment 1. **(B)** Experiment 2 has been divided in four phases depending on temperature—Phase I (October–November 2016), Phase II (November–December 2016), Phase III (December 2016–January 2017), and Phase IV (January–February 2017). The water temperature ranged from 22.5 to 18.5, 17.7 to 14.5, 14.3 to 11.2, and 14.2 to 19.4°C in first, second, third, and fourth phases, respectively. **(C)** Experiment 3 and **(D)** in ponds. There was no significant difference in temperature among various treatments of each experiment. Therefore, data for individual day of all treatments are presented as Mean ± SE. Data were collected at 9.00 a.m.

gradually reduced. The pH of water ranged from 7.10 to 7.41, 7.30 to 7.80, 6.96 to 7.35, and 6.99 to 7.52 in manures 1, 2, 3, and 4, respectively, throughout the study period. Dissolved oxygen level was significantly ($P < 0.05$) higher in the culture system fertilized with IFs compared to the other treatments throughout the study period. Dissolved oxygen levels in other treatments were always less than one (**Figure 3A**). Ammonia (NH_3) level was significantly ($P < 0.05$) higher in 2x OM+IF treatment compared to the others throughout the study period (**Figure 4A**). Among these four treatments, lowest ammonia level was found in IF. Ammonia level ranged from 0.585 to 4.65, 0.03 to 0.51, 8.4 to 22.6, and 15.65 to 42.97 mg/l in OM, IF, 2x OM, and 2x OM+IF, respectively, throughout the study period. Nitrite level was significantly ($P < 0.05$) higher in IF compared to the other treatments (**Figure 5A**). Highest level was recorded on day-1 of study in this treatment. Nitrite levels were 0.008 ± 0.002, 0.084 ± 0.024, 0.025 ± 0.008, and 0.033 ± 0.009 mg/l in OM, IF, 2x OM, and 2x OM+IF, respectively. Nitrate level was significantly ($P < 0.05$) higher in IF compared to the other treatments (**Figure 6A**). Nitrate levels were 1.30 ± 0.52, 15.30 ± 0.80, 5.23 ± 1.20, and 5.87 ± 1.22 mg/l in OM, IF, 2x OM, and 2x OM+IF, respectively. This showed the rate of nitrification among various treatments. Phosphate level was significantly ($P < 0.05$) higher in 2x OM+IF compared to the other treatments. This group was followed by 2x OM, OM, and minimum level was found in IF (**Figure 7A**). Highest level of phosphate was recorded on day-1 of study compared to the other days regardless of treatments. Conductivity was significantly ($P < 0.05$) higher in 2x OM+IF compared to the other treatments (**Figure 8A**). This group was followed by 2x OM, OM, and minimum level was found in IF.

The second experiment was conducted during October 2016–February 2017. In this experiment, seasonal effect on water quality and *L. minor* production was recorded (**Figure 1B**). Depending on the range of water temperature and light intensity the whole study period was divided into four phases. In the first phase (October–November) of the study, water temperature ranged from 22.5 to 18.5°C and then it reduced. Water temperature ranged from 17.7 to 14.5, 14.3 to 11.2, and 14.2 to 19.4°C in second (November–December), third (December–January), and fourth (January–February) phases, respectively. Light intensity also varied significantly in four different phases (**Figure 2B**). Light intensity ranged from 4,111 ± 232 to 2,322 ±

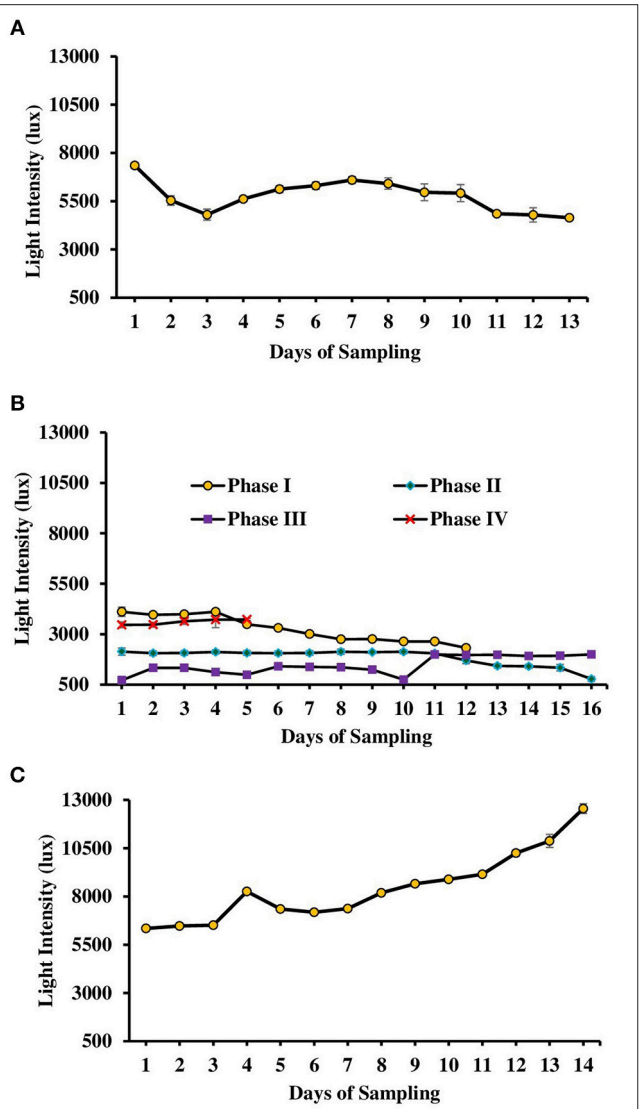

FIGURE 2 | Light intensity monitored during various days of culture of *L. minor* in tanks. **(A)** Experiment 1. **(B)** Experiment 2 has been divided in four phases depending on temperature—Phase I (October–November 2016), Phase II (November–December 2016), Phase III (December 2016–January 2017), and Phase IV (January–February 2017). **(C)** Experiment 3. There was no significant difference in light intensity among various treatments of each experiment. Therefore, data for individual day of all treatments were presented as Mean ± SE. Data were collected at 9.00 a.m. (Data for experiment 4 has not given.).

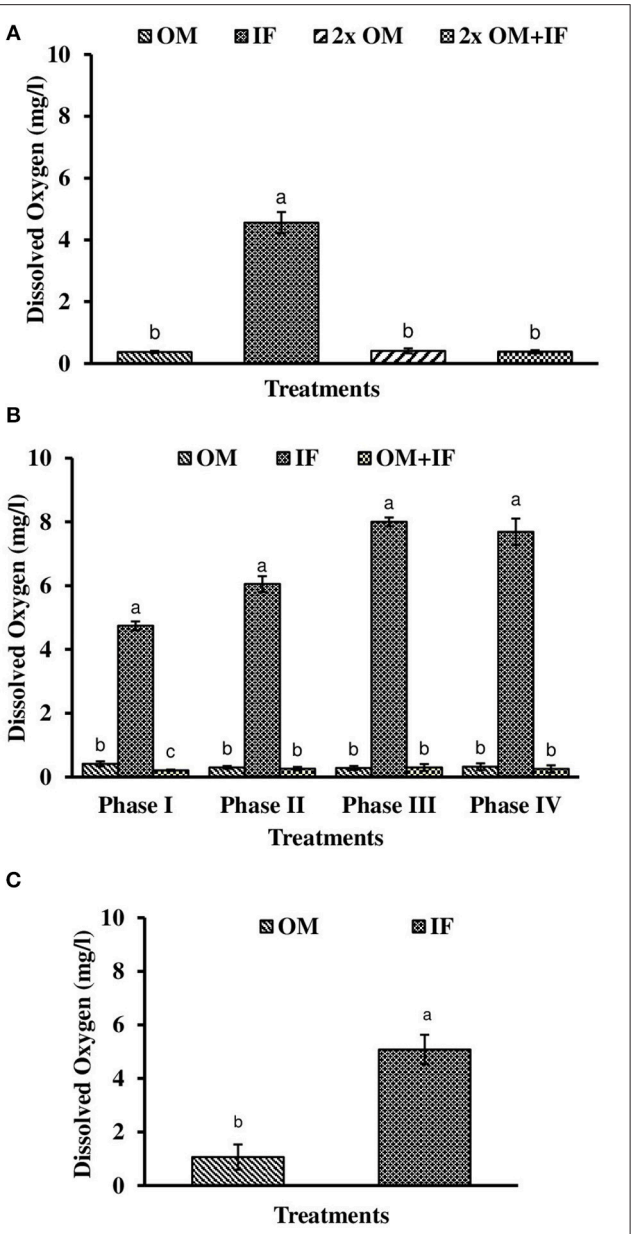

FIGURE 3 | Dissolved oxygen levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

130, 2,138 ± 178 to 781 ± 122, 718 ± 37 to 1,999 ± 34, and 3,463 ± 114 to 3,728 ± 57 lux in phase I, phase II, phase III, and phase IV, respectively. There was no significant ($P > 0.05$) difference in temperature and light intensity in various treatments throughout the study period. In OM, pH ranged from 7.19 to 7.60, 7.23 to 7.88, 7.52 to 7.91, and 7.66 to 8.13 in the first, second, third, and fourth phases, respectively. In IF, pH ranged from 7.50 to 7.86, 7.63 to 8.07, 7.68 to 8.05, and 7.60 to 8.09 in the first, second, third, and fourth phases, respectively. In OM+IF, pH ranged from 7.30 to 7.67, 7.32 to 7.86, 7.47 to 7.94, and 7.80 to 7.90 in the first, second, third, and fourth phases, respectively.

Dissolved oxygen level was significantly ($P < 0.05$) higher in IF compared to the other two treatments throughout the study period (**Figure 3B**). Dissolved oxygen level ranged from 4.7 to 8.7 mg/l in various days of study in IF. Dissolved oxygen level was <1 mg/l in most of the days of study in OM and OM+IF. Ammonia (NH_3) level was significantly ($P < 0.05$) higher in

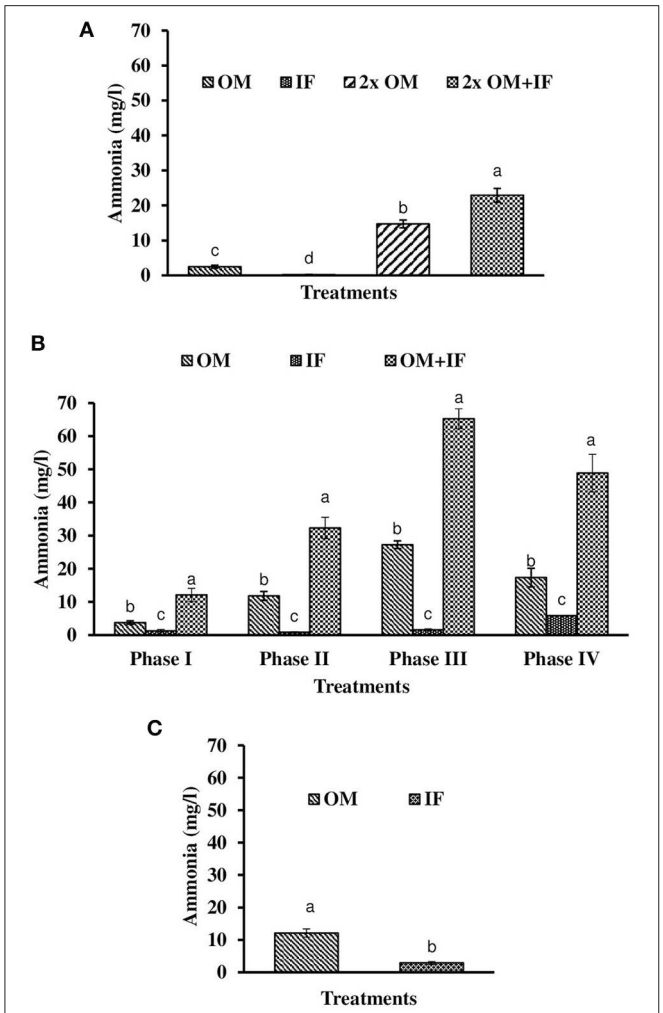

FIGURE 4 | Ammonia levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I−12, Phase II and III−16 each, and Phase IV−5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

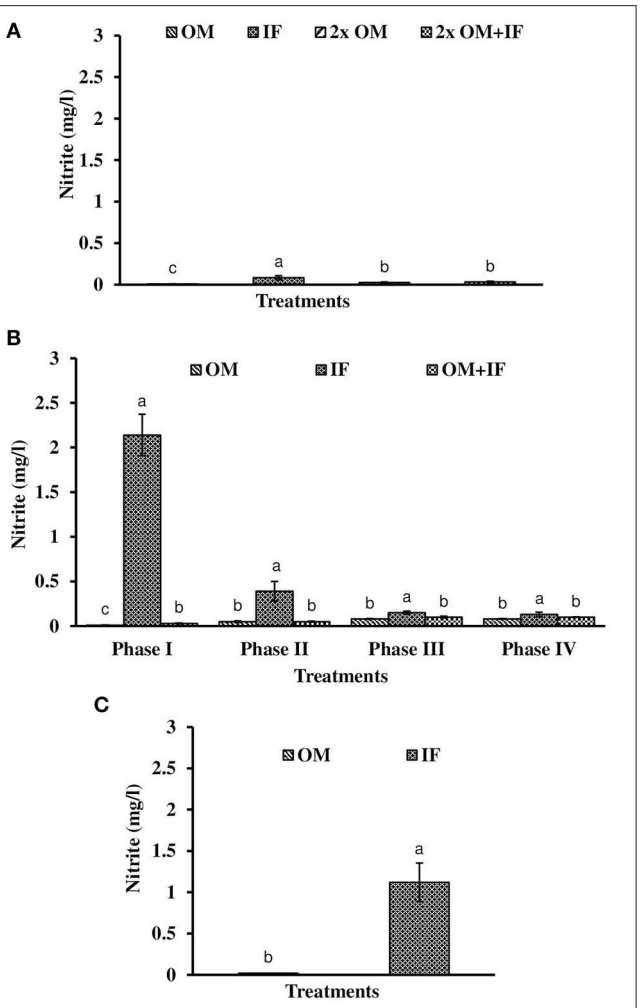

FIGURE 5 | Nitrite levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I−12, Phase II and III−16 each, and Phase IV−5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

OM+IF compared to the other two treatments in all four phases of the study (**Figure 4B**). In OM and OM+IF, highest ammonia level was found at phase III. In OM, ammonia level ranged 1.74 to 5.86, 4.61 to 21.06, 16.36 to 31.56, and 14.23 to 22.93 mg/l in the first, second, third, and fourth phases, respectively. In IF, ammonia level ranged from 0.20 to 2.88, 0.11 to 2.18, 0.57 to 5.37, and 5.80 to 5.92 mg/l in the first, second, third, and fourth phases, respectively. In OM+IF, ammonia level ranged from 5.42 to 19.96, 9.86 to 51.6, 47.70 to 88.5, and 42.96 to 60.16 mg/l in the first, second, third, and fourth phases, respectively. Nitrite level was significantly ($P < 0.05$) higher in IF compared to the other two treatments throughout the study period (**Figure 5B**). Nitrite levels were 0.01 to 0.08, 2.14 to 0.1, and 0.03 to 0.13 mg/l in OM, IF, and OM+IF, respectively, in all four phases. Nitrate level was significantly ($P < 0.05$) higher in IF compared to the other two treatments in first and second phases (**Figure 6B**). In OM+IF, significantly ($P < 0.05$) higher nitrate level was found in the third phase of the study. Nitrate levels were 0.16 to 1.95, 1.48 to 2.53, and 0.44 to 2.70 mg/l in OM, IF, and OM+IF, respectively, throughout the study period. Phosphate level was significantly ($P < 0.05$) lower in IF compared to the other two treatments throughout the study period (**Figure 7B**). In OM, phosphate level ranged 2.03 to 2.54, 1.22 to 2.22, 0.45 to 1.21, and 1.35 to 1.60 mg/l in the first, second, third, and fourth phases, respectively. In IF, phosphate level ranged from 0.15 to 0.42, 0.22 to 0.65, 0.30 to 0.55, and 0.28 to 0.45 mg/l in the first,

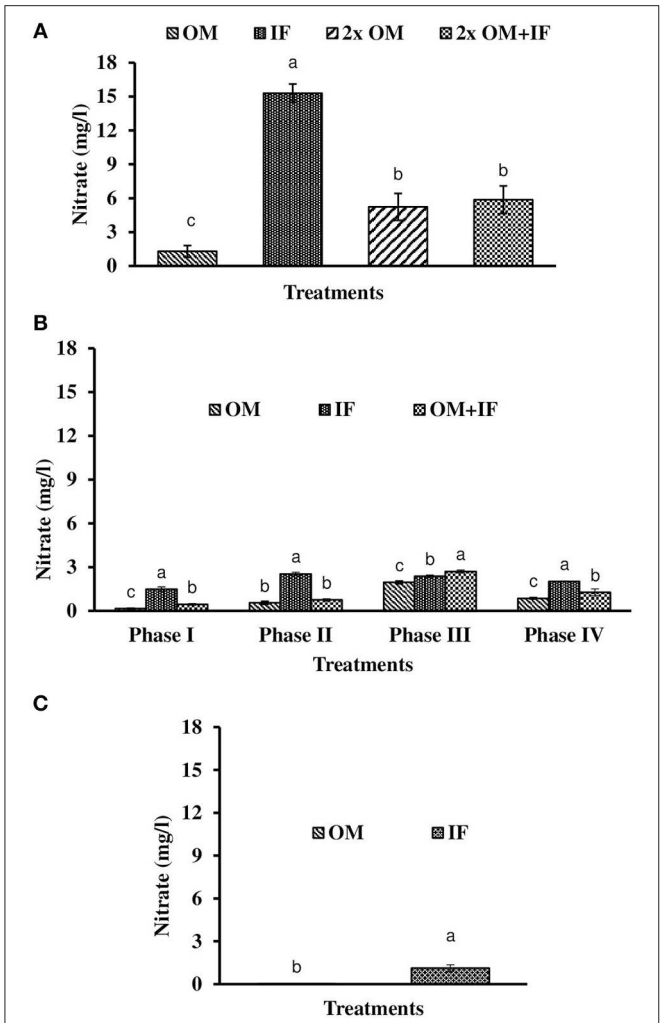

FIGURE 6 | Nitrate levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

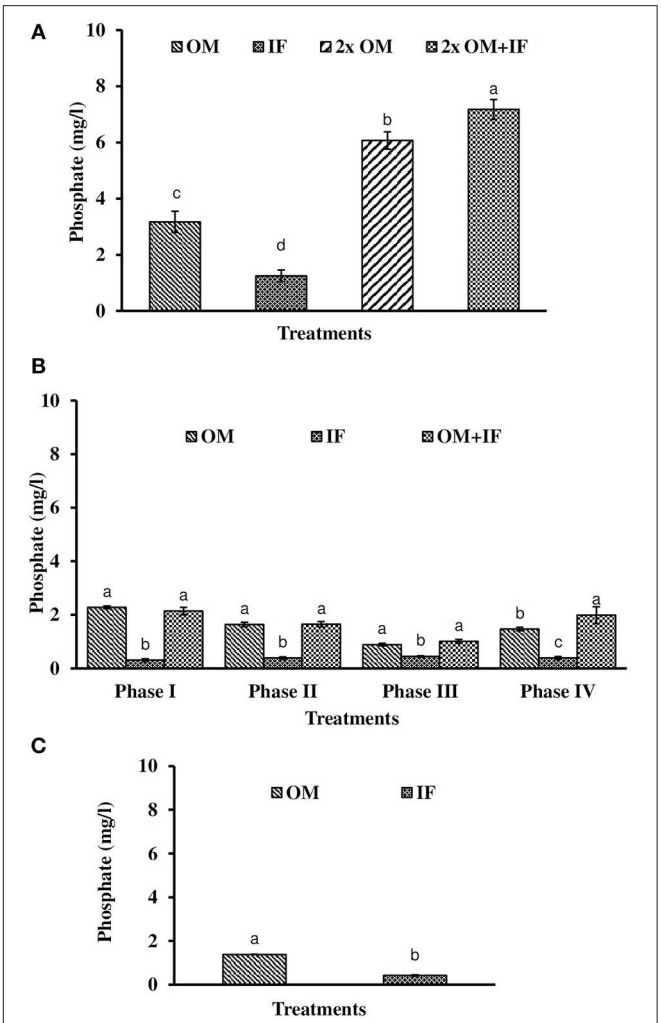

FIGURE 7 | Phosphate levels (in parenthesis) of water found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts were significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

second, third, and fourth phases, respectively. Conductivity was significantly ($P < 0.05$) higher in OM+IF compared to the other two treatments throughout the study period (**Figure 8B**). This group was followed by OM and IF.

The third experiment was conducted during February–April 2017. In this experiment, water temperature was minimum at the beginning and gradually increased ranging from 19.43 to 28.42°C. Light intensity also showed an increasing trend ranging from 6,341 ± 10 to 12,550 ± 283 lux throughout the study period. There was no significant ($P > 0.05$) difference in temperature (**Figure 1C**) and light intensity (**Figure 2C**) between the two treatments throughout the study period. The pH of the water ranged from 7.41 to 7.83 and 7.60 to 9.14 in OM and IF, respectively, during the study period. Dissolved oxygen level was significantly ($P < 0.05$) higher in IF compared to the OM throughout the study period. Dissolved oxygen level ranged from 1.27 to 0.12 mg/l in various days of study in OM. Dissolved oxygen level was always <1 mg/l in OM, except on the first day after manure application (**Figure 3C**).

Ammonia (NH_3) level was significantly ($P < 0.05$) higher in OM compared to IF throughout the study period (**Figure 4C**). Ammonia level ranged from 7.09 to 17.4 and 0.27 to 5.78 mg/l in OM and IF, respectively. Nitrite (**Figure 5C**) and nitrate (**Figure 5C**) levels were significantly ($P < 0.05$) higher in IF

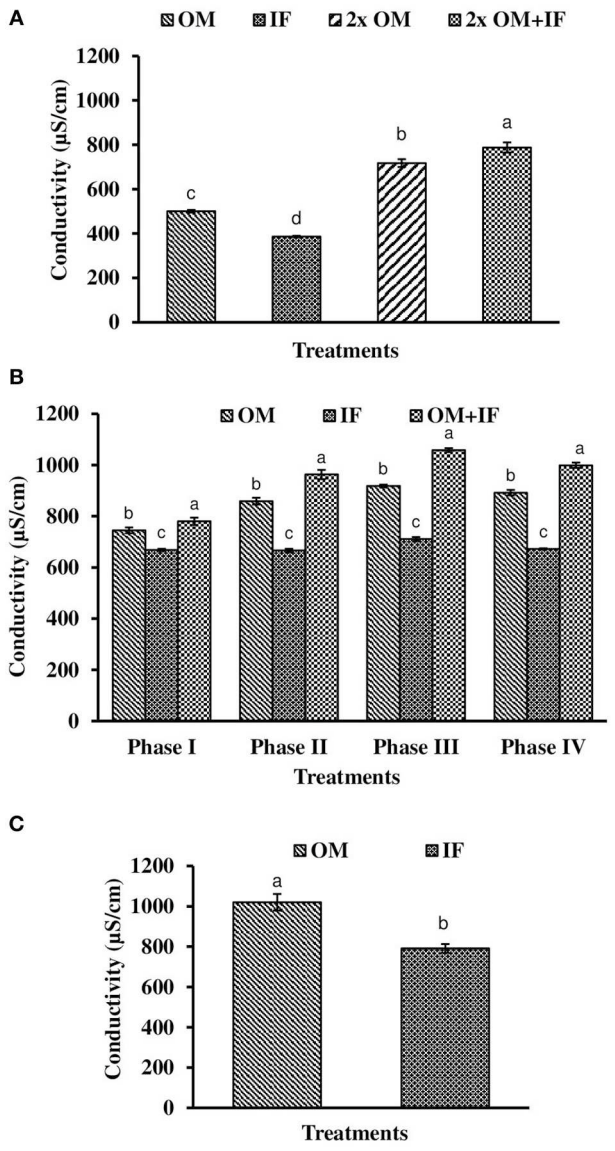

FIGURE 8 | Conductivity of water (in parenthesis) found in various treatments of tank experiments of *L. minor*. **(A)** Experiment 1, **(B)** experiment 2 (Phase I, Phase II, Phase III, and Phase IV), and **(C)** experiment 3. In each treatment (three replicates), average values of 13, 49 (Phase I–12, Phase II and III–16 each, and Phase IV–5 days), and 14 days of sampling for experiments 1, 2, and 3, respectively, were calculated. Data were presented as Mean ± SE. Bars with different superscripts are significantly ($P < 0.05$) different. Data were collected at 9.00 a.m. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

compared to the OM throughout the study period. Nitrite level ranged from 0.008 to 0.04 and 0.11 to 2.66 mg/l in OM and IF, respectively, during the study period. Nitrate level ranged from 0.05 to 1.61 and 1.13 to 4.32 mg/l in OM and IF (**Figure 6C**), respectively. Phosphate level was significantly ($P < 0.05$) higher in OM compared to IF throughout the culture period. Phosphate level ranged 1.29 to 1.65 and 0.24 to 0.50 mg/l in OM and IF, respectively, throughout the study period (**Figure 7C**). Conductivity was significantly ($P < 0.05$) higher in OM compared to IF (**Figure 8C**).

Relative Growth Rate (RGR) and Production

The relative growth rate (RGR) of *L. minor* varied among different treatments in all three experiments. In experiment 1, *L. minor* was harvested 6, 5, 4, and 3 times in OM, IF, 2x OM, and 2x OM+IF, respectively, during 30 days of culture period. The RGR was always highest at first harvest regardless of treatments (**Figures 9A–C**). In experiment 1, RGR ranged from 0.521 to 0.047, 0.463 to 0.083, 0.239 to 0.034, and 0.215 to 0.078 g/g/day in OM, IF, 2x OM and 2x OM+IF, respectively, in various days of sampling. In experiment 2, plants were harvested 5, 8, and 3 times from OM, IF, and OM+IF, respectively. The RGR ranged from 0.23 to 0.014, 0.196 to 0.021, and 0.169 to 0.056 g/g/day in OM, IF, and OM+IF, respectively, in various days of sampling. In experiment 3, plants were harvested 7 and 3 times from OM and IF, respectively. The RGR ranged from 0.30 to 0.035 and 0.140 to 0.023 g/g/day in OM and IF in various days of sampling.

Production of *L. minor* in Outdoor Tanks

In all these study, macrophytes were harvested when the surface area of the tanks were filled with macrophytes. There was difference in harvesting time in various treatments due to differences in the growth of plants. In all these treatments, 50% of the total biomass was harvested at each harvest, except the final one. In experiment 1, production of macrophytes was significantly ($P < 0.05$) higher in OM compared to the other treatments (**Figure 10A**). Lowest production was recorded in 2x OM+IF throughout the study period.

In experiment 2, *L. minor* production was significantly ($P < 0.05$) higher in OM compared to the other two treatments in first and second phases. In third phase, macrophyte was harvested thrice from IF. There was no harvesting from OM as the growth of duckweed was very less and it was not covering the whole surface area of the tank. Plants were harvested only when they covered the whole water body. The growth of macrophytes was very poor in OM+IF in the third phase. There was no survival of plants in this treatment. In fourth phase, *L. minor* production was significantly ($P < 0.05$) higher in OM compared to IF. Total production was significantly ($P < 0.05$) higher in OM and IF compared to OM+IF treatment. There was no significant ($P > 0.05$) difference in total production between OM and IF (**Figure 10B**).

In experiment 3, *L. minor* production was significantly ($P < 0.05$) higher in OM compared to the IF (**Figure 10C**). In OM, macrophyte was first harvested after 14 days of inoculation of plants when the tank was totally covered with macrophytes. In IF, tanks were filled with macrophytes after 28 days of inoculation and then plants were harvested. The type of manures influenced the growth of macrophytes.

Culture of *L. minor* in Ponds

Water Quality

This experiment was conducted in cemented ponds during July–August 2017. The variations in water quality parameters reflected the seasonal variations as well as effect of manure application.

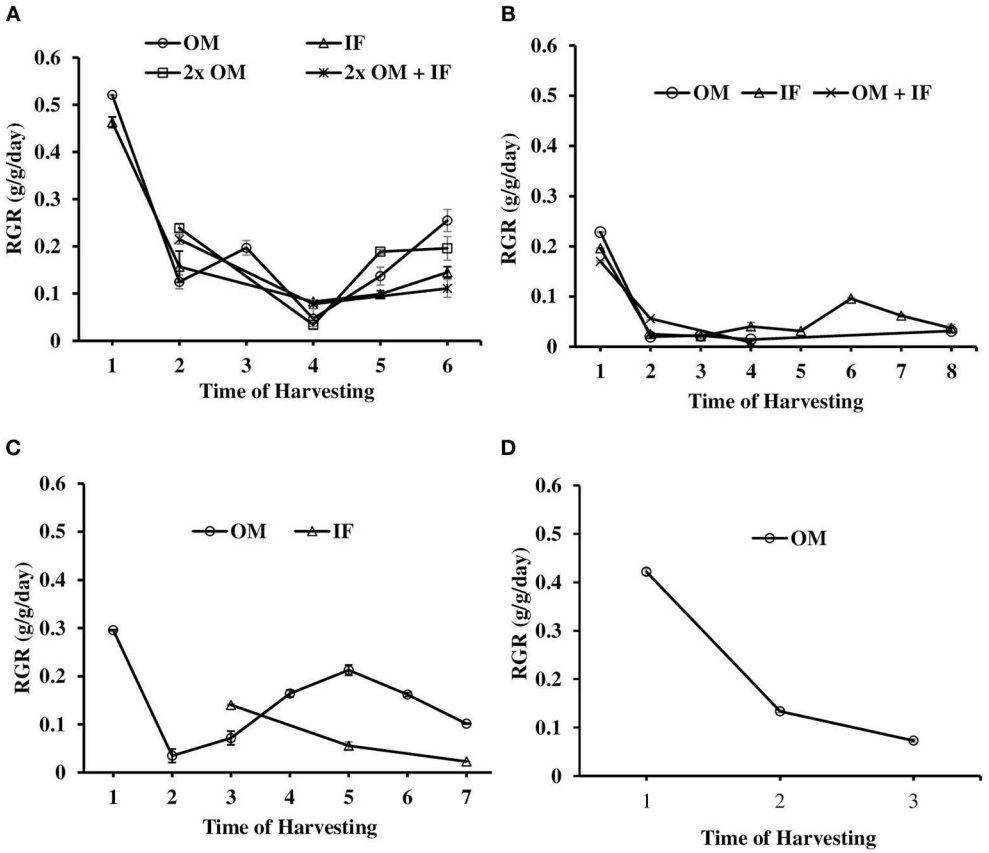

FIGURE 9 | Relative growth rate (RGR) of *L. minor* found in **(A)** experiment 1, **(B)** experiment 2, **(C)** experiment 3, and **(D)** ponds during various days of culture. In experiment 2, harvesting pattern was as follows: Phase I—first harvest, Phase II—second, third, and fourth harvests, Phase III—fifth, sixth, and seventh harvests, Phase IV—eighth harvest. In experiment 3, there was no harvesting in IF on 1, 2, 4, and 6 sampling. OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

Water temperature and pH ranged from 33.15 to 30.27°C and 7.32 to 8.04, respectively, throughout the study period. Water temperature was higher in July and gradually it decreased (**Figure 1D**). Dissolved oxygen level ranged from 1.04 to 3.57 mg/l on various days of study. Dissolved oxygen level was higher at the beginning of the experiment; the level decreased after the application of manures and with the growth of macrophytes as it covered the surface area of the ponds. Ammonia, nitrite and nitrate levels ranged from 5.02 to 10.57, 0.003 to 0.12, and 0.23 to 2.44 mg/l, respectively. Phosphate level ranged 1.15 to 1.70 mg/l during the study period (**Table 1**). Conductivity ranged from 1,022 to 1,351 μS/cm throughout the culture period of duckweed. Ammonia, nitrite, nitrate, and phosphate levels varied with the days of manure application.

Relative Growth Rate (RGR) and Production

The RGRs of *L. minor* were 0.422, 0.133, and 0.073 g/g/day in first, second, and third harvest times, respectively (**Figure 9D**). The duckweed was first harvested after 10 days of introduction. Macrophyte was harvested thrice during 1 month culture period. Fifty percent macrophyte was harvested at the time of first and second harvesting and all plants were collected at the time of third harvest. The production of *L. minor* was 1.17 ± 0.005 Kg/m^2/month (**Figure 10D**). Total production of duckweed in the pond was 702.5 Kg/ha/month (dry weight).

Composition of *L. minor*

Proximate composition analysis of duckweed showed that there was difference between the macrophytes cultured with OM and IF. Protein, lipid, and ash contents were significantly ($P < 0.05$) higher in macrophytes cultured in OM compared to IF. Balancing these higher levels, carbohydrate content was lower in macrophytes cultured in OM compared to IF (**Table 2**).

The amino acid profile of duckweed cultured in OM showed some interesting results (**Table 3**). The essential (39.20%), non-essential (53.64%), and non-proteinogenic (7.13%) amino acids were present in duckweed. All essential amino acids viz. histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine were found in adequate quantity. Leucine, isoleucine, and valine consisted 48.67% of the essential amino acids. Among non-essential amino acids, glutamic acid was 25.87%. Several non-proteinogenic amino acids viz. citrulline, hydroxiproline, taurine etc. were also present in the duckweed.

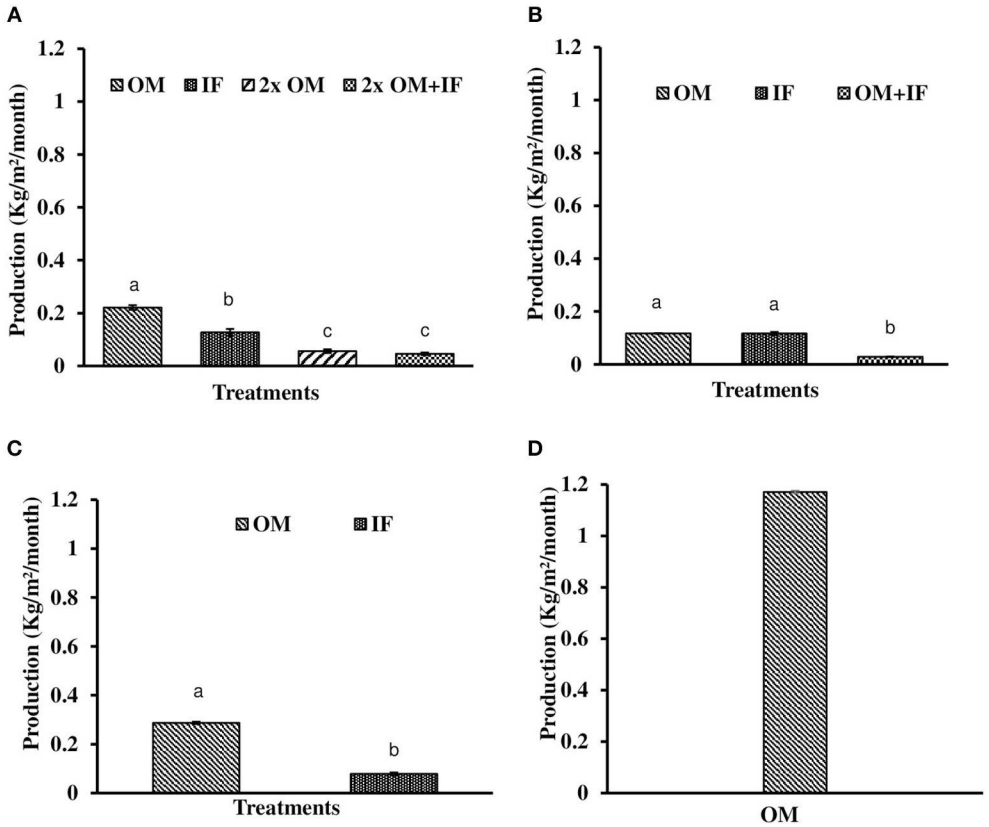

FIGURE 10 | Total production of *L. minor* found in **(A)** experiment 1, **(B)** experiment 2, **(C)** experiment 3, and **(D)** ponds. Bars with different superscripts are significantly ($P < 0.05$) different ($n = 3$). OM, organic manures; IF, inorganic fertilizers; 2x OM, double dose of organic manures.

TABLE 1 | Culture conditions of *L. minor* in ponds.

Parameter	Range	Mean ± SE
Temperature (°C)	30.27–33.15	31.32 ± 1.0
pH	7.32–8.04	–
Dissolved oxygen (mg/l)	1.04–3.57	2.25 ± 0.52
Ammonia (mg/l)	5.02–10.57	3.25 ± 0.7
Nitrite (mg/l)	0.003–0.12	0.045 ± 0.01
Nitrate (mg/l)	0.23–2.44	1.13 ± 0.01
Phosphate (mg/l)	1.15–1.70	1.52 ± 0.07
Conductivity (μS/cm)	1,022–1,351	1,161 ± 1.7

The average values (three replicates) of 6 days of sampling were presented as Mean ± SE.

TABLE 2 | Proximate composition of *L. minor* (% of dry weight) grown in the tanks.

	Organic manure (OM)	Inorganic fertilizer (IF)
Protein	36.07 ± 0.18	27.12 ± 0.40*
Lipid	8.45 ± 0.61	7.15 ± 0.06
Ash	21.41 ± 0.20	19.42 ± 0.30*
Carbohydrate	34.07 ± 0.36	46.31 ± 0.74*

**Denotes significant difference ($P < 0.05$).*

The amino acid profile of duckweed cultured in IF was not provided.

The fatty acid composition of *L. minor* was dominated by PUFA, which accounted for 60–63% of total fatty acids, largely α-linolenic acid (LNA, 18:3n-3) at around 41–47% and linoleic acid (LA, 18:2n-6) at 17–18%, followed by saturated fatty acids (∼23–26%) and monoenes (11–12%) (**Table 4**). As with proximate composition, fatty acid profile was also influenced by manures, with *L. minor* grown in OM having significantly higher proportions of LA, LNA, saturated, and monounsaturated fatty acids, and total PUFA when compared to the inorganic counterpart (IF). Due to the higher lipid content of *L. minor* grown in OM, all fatty acids were found in higher absolute amounts (mg/100 g dry mass) in macrophytes grown in OMs. Irrespective of manure, the *L. minor* lipid profile contained no long-chain PUFA (LC-PUFA) such as docosahexaenoic acid (DHA, 22:6n-3), although there was a trace of eicosapentaenoic acid (EPA, 20:5n-3).

DISCUSSION

In the present study, suitable manures and their dose for the production of *L. minor* was studied first in outdoor-tanks and

TABLE 3 | Amino acid profile of *L. minor* cultured with organic manures (OM).

Amino acids	Concentration (g/100 g)
ESSENTIAL	
Histidine (His)	0.894 ± 0.011
Isoleucine (Ile)	2.043 ± 0.064
Leucine (Lue)	4.132 ± 0.046
Lysine (Lys)	2.683 ± 0.161
Methionine (Met)	0.859 ± 0.014
Phenylalanine (Phe)	2.571 ± 0.034
Threonine (Thr)	1.924 ± 0.138
Tryptophan (Trp)	0.365 ± 0.010
Valine (Val)	2.664 ± 0.096
NON-ESSENTIAL	
Alanine (Ala)	2.882 ± 0.041
Arginine (Arg)	3.060 ± 0.045
Asparatate (Asp)	3.714 ± 0.372
Cysteine (Cys)	0.381 ± 0.032
Glutamic Acid (Glu)	6.427 ± 0.102
Glycine (Gly)	2.861 ± 0.031
Proline (Pro)	1.248 ± 0.035
Serine (Ser)	2.348 ± 0.333
Tyrosine (Tyr)	1.905 ± 0.125
NON-PROTEINOGENIC	
Phosphoserine (p-Ser)	0.578 ± 0.000
Taurine (Tau)	0.041 ± 0.015
Phospho ethanol amine (PEA)	0.023 ± 0.006
Sarcosine (Sar)	0.097 ± 0.004
α Amino adipic acid (a-AAA)	0.045 ± 0.013
α Amino-n-butaric acid (a-ABA)	0.150 ± 0.012
Cystathionine (Cysthi)	0.093 ± 0.019
β-Alanine (b-Ala)	0.111 ± 0.020
β-Amino isobutyric acid (b-AiBA)	0.971 ± 0.271
γ-Amino-n-butyric acid (g-ABA)	0.405 ± 0.014
Ethanol amine ($EOHNH_2$)	0.146 ± 0.004
Hydroxylysine (Hylys)	0.058 ± 0.007
Ornithine (Orn)	0.014 ± 0.001
1 Methylhistidine (1 Mehis)	0.087 ± 0.003
3 Methylhistidine (3 Mehis)	0.117 ± 0.004
Carnosine (Car)	0.106 ± 0.001
Hydroxy proline (Hypro)	0.133 ± 0.015
Citrulline (Cit)	0.126 ± 0.002

then the best condition was adopted in ponds. The results of three consecutive studies in outdoor systems showed that duckweed production was influenced by the quality of manures and doses, and environmental factors. Both organic and IFs were used separately and in combinations for the production of *L. minor*. In experiments 1 and 3, the average RGR of *L. minor* was higher in OM compared to IF. The average RGR-values of duckweeds in OM were same in experiment 1 in outdoor tanks and in the pond experiment (0.21 g/g/day). In experiment 1 and 3, total macrophytes production (**Figures 6A–C**) was significantly higher in OM compared to the other treatments; whereas in experiment 2, there was no significant difference in total production between OM and IF. The growth of duckweed was affected by low temperature in OM in the third phase (i.e., fifth, sixth, and seventh harvests) of experiment 2. Reduced growth of duckweed affected the total production. In contrast, in the culture system treated with IFs the growth was continued in the cold condition (**Figure 5B**). Production of macrophytes in 2x OM and 2x OM+IF of experiment 1, and OM+IF of experiment 2 were negligible. For this reason these manures were not adopted for the production of macrophytes and less discussed in the present study. Higher doses of manures resulted in significantly ($P < 0.05$) higher levels of ammonia that affected the growth and production of macrophytes in these treatments. RGR was always higher at first harvest regardless of treatments. In experiment 1, RGR in OM during second harvest was lower compared to 2x OM and 2x OM+IF as this was the first harvest for these two latter treatments. There was no production in all treatments, except OM at the time of third harvesting. Therefore, in experiment 1, the RGR was lower in OM during fourth harvest compared to the other treatments. Similarly, in experiment 3, the first harvest of *L. minor* from IF, was the third harvest for OM. The poor growth rate of duckweed resulted in slow production and delayed harvest. Availability of space and nutrient might influence the RGR in the first harvest compared to the successive harvests. The OMs are rich sources of nitrogen (N), phosphorous (P_2O_5), and potash (K_2O) and are usually applied in agricultural land in India (Gaur et al., 1990). The amount of nutrients of OM varied with season and geographical location. The mixture of these three manures fulfills the requirements of the plant. The decomposition of these manures enhances their availability to the plant. In the present study, mixture of manures was decomposed for 5 days before application in the water bodies.

Porath et al. (1979) reported the RGR of fresh *L. minor* cultured in both laboratory and field conditions. They obtained the highest value of 0.346 g/g/day in laboratory condition, whereas the value became 0.099 g/g/day in the field condition. The fresh yield of duckweed ranged from −0.026 to 0.66 Kg/m^2/week during 8 successive weeks of culture in the manured pond. Oron (1994) reported that the RGRs of duckweeds ranged from 0.10 to 0.35 g/g/day. RGR of *Lemma gibba* grown in desert ponds ranged from 0.081 to 0.191 g/g/day (Guy et al., 1990). Rejmankova (1975) observed 0.20 and 0.22 g/g/day RGR of *L. minor* and *L. gibba*, respectively, in field conditions. In the present study, RGR of duckweed was comparable with the earlier studies.

Hassan and Chakrabarti (2009) reported that variations in climatic conditions, nutritional status of the water body and differences in species resulted in the differences in the production of the macrophytes. Most of the data were generated from short-term studies in small-scale experimental systems. The data generated from longer duration study in commercial-sized systems are most wanted. In the present study, 702.5 Kg (dry mass)/ha/month (i.e., 8.43 tons/ha/year) *L. minor* were produced from pond with the application of OMs. In UASB effluent and nutrient non-limiting water, *L. minor* production were 10.7 and 16.1 tons (dry mass)/ha/year (Reddy and DeBusk, 1985; Vroon and Weller, 1995). In septage-fed pond, the production of *Lemna*

TABLE 4 | Fatty acid composition of *L. minor* as percentage of total fatty acids (%) or as mg fatty acids per 100 g dry weight (absolute).

	Organic manure (OM)		Inorganic fertilizer (IF)	
	Percentage	**Absolute**	**Percentage**	**Absolute**
14:0	1.10 ± 0.02	36.7 ± 3.3	1.14 ± 0.07	26.6 ± 1.0
15:0	0.31 ± 0.03	10.1 ± 0.4	0.36 ± 0.04	8.4 ± 0.22*
16:0	19.14 ± 0.03	634.8 ± 43.0	22.21 ± 4.32	516.7 ± 51.4
17:0	20.47 ± 0.11	79.5 ± 0.1	22.32 ± 2.49	77.7 ± 2.5
18:0	1.08 ± 0.01	35.7 ± 2.9	1.47 ± 0.57	33.9 ± 10.1
24:0	1.10 ± 0.00	36.4 ± 2.6	1.22 ± 0.17	28.4 ± 1.2
Total saturated	22.72 ± 0.03	753.7 ± 51.3	26.40 ± 5.17	613.9 ± 61.9
16:1n-9	5.60 ± 0.07	185.7 ± 10.6	5.36 ± 0.42	126.2 ± 21.8
16:1n-7	2.32 ± 0.02	77.0 ± 5.9	2.23 ± 0.12	52.4 ± 7.7
18:1n-9	2.15 ± 0.19	71.6 ± 11.3	2.88 ± 1.22	66.3 ± 22.3
18:1n-7	1.46 ± 0.03	48.4 ± 4.3	1.46 ± 0.04	34.3 ± 2.4
Total monoenes	11.53 ± 0.17	382.6 ± 32.2	11.93 ± 0.73	279.2 ± 9.7*
18:2n-6	16.88 ± 0.04	560.1 ± 40.1	18.07 ± 1.71	422.2 ± 0.6*
Total n-6 PUFA	16.88 ± 0.04	560.1 ± 40.1	18.07 ± 1.71	422.2 ± 0.6*
18:3n-3	46.35 ± 0.07	1,537.5 ± 108.9	41.24 ± 7.16	976.2 ± 260.9
20:5n-3	0.14 ± 0.02	4.5 ± 0.3	0.14 ± 0.02	3.6 ± 0.5
Total n-3 PUFA	46.49 ± 0.13	1,542.0 ± 102.6	41.38 ± 7.36	979.8 ± 265.9
Total DMA	2.37 ± 0.04	78.6 ± 4.1	2.23 ± 0.24	52.6 ± 10.7
Total PUFA	63.38 ± 0.10	2,102.1 ± 142.6	59.45 ± 5.66	1,402.0 ± 266.5
Total fatty acids		3,317.0 ± 230.3		2,347.6 ± 225.0

Results: mean ± SD. DMA, dimethyl acetals; PUFA, polyunsaturated fatty acids.
**Denotes significant difference between OM and IF ($P < 0.05$).*

perpusilla was 11.2 tons (dry mass)/ha/year (Edwards et al., 1990). It was suggested that in an aquatic environment with sufficient nutrients and optimum environmental conditions around 10–20 tons (dry mass)/ha/year duckweeds can be harvested (Hassan and Chakrabarti, 2009). Seeding of the plant also played important role in the production. DWRP (1998) recommended 40 Kg/100 m^2 for *L. minor* in order to obtain a dense cover in 3 days. But getting this amount of *L. minor* from control culture system for seeding was difficult. Therefore, only 1 Kg (wet weight) of plant was seeded in the 200 m^2 pond in the present study. The growth rate of *L. minor* was 3–6 folds higher at the time of first harvest compared to the second and third harvests. Optimum water quality parameters *viz*. temperature, ammonia, phosphate levels etc. influenced the growth of the macrophyte. In the month of July, the presence of moisture in the air also influenced the macrophytes production (compared to the dry season). Moreover, there was enough space at the initial phase; due to the growth of the plants, there was competition for space and nutrients at the later phase.

The impact of various water quality parameters on the production of *L. minor* was documented in the present study. Temperature is known to be the master abiotic factor. In an outdoor facility, *L. minor* were cultured under a wide range of temperature of 11.5–28.4°C during September 2016–April 2017. In the OM-based culture system, production was greatly reduced as the water temperature become <18.5°C from November onwards. An increasing trend in duckweed production was recorded from February onwards as temperature rose to 19.4°C. In pond the experiment, 31.5–30.3°C temperature was favorable for duckweed production. Temperature tolerance and optima are species-specific. Maximum growth for most of the species of *L. minor* was obtained between 17.5 and 30°C (Culley et al., 1981; Gaigher and Short, 1986). The growth rate declined at low temperature. Reduced growth rate was found in some duckweed at the temperature below 17°C (Culley et al., 1981). Most species seemed to die at 35°C water temperature. All these studies showed that 17–18°C was the critical temperature and 27–31°C was optimum temperature for the production of *L. minor*.

The intensity of light also played major role in the production of *L. minor*. In the experiment 2, as light intensity reduced, particularly in the second and third phases (718–2,138 lux), production reduced drastically; an increasing trend was recorded as light intensity increased (3,463–3,728 lux) in experiment 2. In the present study, maximum *L. minor* production was recorded at 7,353–10,878 lux light intensity. Mkandawire and Dudel (2007) suggested 4,200–6,700 lux light intensity at 14–16 h photoperiod for the optimum production of *L. gibba* and *L. minor*.

Duckweeds have wide range of pH tolerance. The biomass of duckweeds doubled in 2–4 days at pH 7–8 (Culley et al., 1981). Khondker et al. (1994) showed that pH 6.9–7.8 was suitable for *L. perpusilla* production. The optimum growth of *L. perpusilla* was found at pH 7.36 (Van der Does and Klink, 1991). In the present study, pH ranged from 7.32 to 8.04 in all outdoor and

pond culture systems in OM and IF. Application of higher dose of OMs resulted in pH <7.0 in 2x OM and 2x OM+IF in experiment 1. The pH of water decreased after the application of OMs in all experiments and then increased with the duration of study.

In the production of *L. minor*, a direct effect of dissolved oxygen was not recorded. In OM, dissolved oxygen level was generally <1.0 mg/l, whereas a higher concentration of dissolved oxygen was always recorded in IF. But the production was lower in IF compared to OM in most of the harvestings (except third phase of experiment 2). Application of OMs reduced the oxygen level in the culture system. Dissolved oxygen might influence the nitrification of ammonia as higher level of nitrite and nitrate levels were found in IF compared to OM.

Ammonia (NH_3) level was always lowest in IF regardless of experiment. Leng et al. (1995) suggested that ionized ammonia (NH_4^+) as preferable nitrogenous substrate for *L. minor* culture. The ammonium-ammonia balance shifted toward the un-ionized (NH_3) form at alkaline pH. This resulted in higher concentration of free ammonia in the culture system which affected the duckweed production. Leng et al. (1995) also suggested that the ammonia concentrations of cultured water should be 7–12 mg N/l for the maintenance of crude protein content of duckweed. In the present study, higher level of ammonia (18.8–32.0 mg/l) also affected the production of duckweed in culture systems fertilized with OMs, especially during winter. In experiment 2, significantly ($P < 0.05$) higher level of ammonia (combined with low temperature) affected the growth of macrophytes in OM+IF treatment. Porath and Pollock (1982) suggested that ammonia (NH_4^+) uptake is temperature-sensitive in duckweed. Nitrite and nitrate levels were lowest in OM. Higher levels of nitrite and nitrate in IF showed the better nitrification rate in this treatment compared to the OM. Duckweeds preferred ammonia as nitrogen source compared to nitrate and grew better in presence of the former nutrient (Lüönd, 1980).

Phosphorus is one of the limiting nutrients (after nitrogen) and is essential for rapid growth of the macrophyte. In OM, phosphate level was most of the time >1.0 mg/l, whereas phosphate level was <1.0 mg/l in IF. This showed the phosphate limitation of this treatment. In some species of duckweeds, decreased growth rate was obtained at *P*-values <0.017 mg/l (Lüönd, 1980). In *L. perpusilla*, a positive correlation was recorded between the concentrations of phosphate and silicate and the biomass (Khondker et al., 1994). Phosphorous (PO_4-P) should range between 4 and 8 mg/l for the optimum production of duckweeds (Hassan and Chakrabarti, 2009). The present study showed that the production of *L. minor* was dependent on nutrient availability in terms of nitrogen and phosphorous and environmental factors like temperature and light intensity of the culture system.

Many studies showed the proximate composition of several species of duckweeds from different geographical areas. Protein content ranged from 14.0 to 23.5, 25.3 to 29.3, 9.4 to 38.5, and 26.3 to 45.5% in *L. minor* (Majid et al., 1992; Zaher et al., 1995), *L. perpusilla* (Hassan and Edwards, 1992), *L. gibba* (Hillman and Culley, 1978; Culley et al., 1981), and *Lemma paucicostata* (Mbagwu and Adenji, 1988), respectively. In the present study, 36.07 ± 0.18 and 27.12 ± 0.4% protein contents were found in *L. minor* cultured in OMs and IFs. The nutritional status of the water body influenced the crude protein content of the duckweed; protein content ranged from 9 to 20% in nutrient-poor water or under sub-optimum nutrient conditions, whereas it ranged 24–41% in nutrient-rich water. The crude protein content of duckweed increased up to 40% at ammonia concentration of 7–12 mg N/l (Leng et al., 1995). The protein content of *L. minor* collected from a natural pond of northeast region of India was 28.0 ± 1.7 (Kalita et al., 2007). Appenroth et al. (2017) reported that in different species of duckweeds protein contents ranged from 20 to 35%.

Presence of high quality protein was reported in various studies (Porath et al., 1979; Rusoff et al., 1980). The essential amino acid profile of duckweed was better compared to the most of the plant proteins and more closely resembled to animal protein. Guha (1997) reported that the protein of duckweeds was rich in certain amino acids that were often low in plant proteins. The nutritional value of duckweeds is comparable with alfalfa in terms of two essential amino acids—lysine and arginine. These are required in animal feeds. High amount of leucine, threonine, valine, isoleucine, and phenylalanine and less amount of methionine and tyrosine are found in duckweeds. The amino acid content of the *L. paucicostata* was equivalent to that of blood, soybean, and cottonseed meals and considerably exceeded that of groundnut meal (Mbagwu and Adenji, 1988). Amino acids like, lysine (4.8%), methionine and cystine (2.7%), and phenylalanine and tyrosine (7.7%) were also present in the duckweeds (Appenroth et al., 2017). In the present study, the amino acid composition of *L. minor* confirmed its nutritional value as feed ingredient as it is a rich source for essential and non-essential amino acids. The presence of non-proteinogenic amino acids viz. taurine, citrulline, hydroxyproline, sarcosine etc. enhanced the nutritional value of duckweed.

Like protein, lipid, and ash contents of *L. minor* grown in OM were higher compared to the macrophytes grown in IF in the present study. Culley et al. (1981) reported 6.3% ether extracts in *L. gibba* from USA. In different species of duckweeds fat contents ranged from 4 to 7% (Appenroth et al., 2017). Lipid content of *L. minor* was higher (7.15–8.45%) in the present study, but slightly lower than the 10.6% reported by Yan et al. (2013). The duckweeds produced in nutrient poor water bodies showed lower lipid content (1.8–2.5%) compared to the plant grown (3–7% lipid) in water enriched with nutrient (Hassan and Chakrabarti, 2009). The lipid and ash contents of *L. minor* grown in natural pond were 5.0 ± 0.1 and 25.0 ± 1.6%, respectively (Kalita et al., 2007). Ash contents of various species of *L. minor* ranged from 11.1 to 17.6% (Hassan and Edwards, 1992; Zaher et al., 1995). The ash content of *L. minor* ranged from 19.42 to 21.41% in the present study. Duckweeds are known to accumulate large amounts of minerals in their tissues. Higher amount of ash and fiber and lower amount of protein were found in duckweed colonies with slow growth rate (Skillicorn et al., 1993). The ash content of duckweed was not influenced by the nutrient status of water (Leng et al., 1995). The fatty acid content of *L. minor* cultured in the present study was generally similar to that measured by Yan et al. (2013), who reported a composition of around 25% saturated fatty acids, 5% monoenes and 70% PUFA,

with a very similar level of LA (16%) but a higher proportion of LNA at ∼54% of total fatty acids. The trace level of EPA found in the present study most likely simply reflects the presence of a very small amount of freshwater microalgae, which can often contain some EPA but rarely DHA, in the *L. minor* harvest. Negesse et al. (2009) also reported the presence of short chain fatty acids (SCFA, 16.6%) in *L. minor* (C2 11%, C3 3.1%, C4 1.4%, and C5 0.4%) that could be useful for commercial utilization of duckweed as they can serve as preservatives, preventing bacterial growth. In recent study, Appenroth et al. (2017) reported that in different species of duckweeds, polyunsaturated fatty acids content ranged from 48 to 71%; the high level of n3 fatty acids resulted in a favorable n6/n3 ratio and enhanced the nutritional value of the duckweeds.

CONCLUSIONS

The present study showed that *L. minor* can be produced using cheap and easily available OMs and the produced macrophytes are rich source of protein, lipid, and minerals. Amino acid profile and fatty acid profile confirmed the suitability of the macrophytes in the production of aqua-feed.

Among water quality parameters temperature, light intensity, pH, ammonia, phosphate, and conductivity played major role. These factors should be maintained within reasonable limits for survival and growth of the macrophytes. The management strategies for duckweed culture should focus on the time of manure application and harvesting.

MATERIALS AND METHODS

Culture of *L. minor* in Outdoor Tanks

Lemna minor was collected from a pond located in the Department of Botany, University of Delhi and the plant was identified based on the morphological characteristics (oval shaped fronds, 2–5 fronds remained together, presence of three nerves in each frond and cylindrical root sheath with two lateral wings) with the help of scientist of Department of Botany. Since then the macrophyte was cultured in the Department of Zoology. Three consecutive experiments were conducted to generate the baseline data for the production of *L. minor*. Macrophytes were grown in cemented tanks (1.2 × 0.35 m) maintained in the outdoor facility of Department of Zoology, University of Delhi. The depth of water was 30 cm throughout the study period. Dechlorinated tap water, supplied by the Municipal Corporation of Delhi was used for all experiments. The first experiment was conducted during September–October 2016 and the duration of the experiment was 30 days. Four different manures were used. In manure 1 (OM) and manure 3 (OM 2x OM), cattle manure (local), poultry droppings (local), and mustard oil cake (Double Hiran Mustard Oil-cake, Malook Chand Food Pvt. Ltd., Aligarh, U.P., India) (1:1:1) were used at the rate of 1.052 and 2.104 Kg/m^3 (Srivastava et al., 2006); in manure 2 (IF), urea (IFFCO, Indian Farmers Fertilizer Cooperative Limited, New Delhi, India), potash (Narmada, Gujarat Narmada Valley Fertilizers & Chemicals, Gujarat, India), triple superphosphate (IPL, Indian Potash Limited, Chennai, India) were used at the rate of 15, 3, and 3 Kg/ha/day, respectively, based on the study of DWRP (1998); manure 4 (2x OM+IF) was a combination of manure 2 and manure 3. The amount of IFs was calculated for 10 days and applied in the culture tank. In all these experiments, OMs were applied at the rate of one fourth dose of initial dose at every 10 days interval. In IFs, similar dose (initial amount) of manures was applied at every 10 days interval. Manures for individual tank were mixed with tap water and allowed to decompose for 5 days before application. All manures, except cattle manure were applied in dry conditions. The moisture content of cattle manure was measured and the weight was adjusted.

The second experiment was conducted during October 2016–February 2017 and the duration of the experiment was 105 days. The three different manures used in this experiment were selected based on the results of the first experiment. The first two manures, manure 1 (OM) and manure 2 (IF) were similar to the earlier experiment, and manure 3 (OM+IF) was a combination of manure 1 and manure 2. Third experiment was conducted during February–April 2017. In this experiment, manure 1 (OM) and manure 2 (IF) were selected. This selection was based on the production potential of these manures compared to the others.

Fresh *L. minor* (15 g wet weight) was seeded after 5 days of manure application in each tank. Three replicates were used for each treatment. Harvesting of *L. minor* started when the plant covered the whole surface area of the tank; 50% of the total production was harvested during first and other consecutive harvests; all macrophytes were harvested at the end of the study. Production was expressed as Kg/m^2/month on wet weight basis.

Culture of *L. minor* in the Ponds

In CIFE, Rohtak Center (Indian Council of Agricultural Research), Haryana three cemented ponds (200 m^2, 20 m × 10 m) were prepared for the culture of *L. minor* in June 2017. The bottom was cleaned thoroughly and the pond was filled with ground water (50 cm). The OMs, like cattle manure, poultry dropping, and mustard oil cake (1:1:1) were applied at the rate of 1.052 Kg/m^3 (Srivastava et al., 2006). Organic manures applied for the production of duckweed were selected based on the results of outdoor cemented tanks. Three replicates were used for the study. The culture conditions of *L. minor* developed in the tank experiments were also applied in the pond. All manures were mixed properly and allowed to decompose for 5 days. Then fresh *L. minor* cultured in cemented tanks of Department of Zoology, University of Delhi was seeded (wet weight) at the rate of 1 Kg/pond. It covered a small area of the pond. The experiment was continued for 30 days. Like outdoor tank experiments, one fourth dose of initial dose of manure was applied at every 10 days interval. Harvesting started when the plant covered the whole surface area of the pond. Total pond area (200 m^2) was divided in four quadrates of 50 m^2 each. In first and second harvests, *L. minor* was collected from two quadrants for 50% harvesting (**Figures 11A,B**). In third harvest, all plants were collected and air dried (**Figure 11C**). Production was expressed as Kg/m^2/month on wet weight basis.

Water Quality

Various water quality parameters were monitored regularly in outdoor tanks and in ponds at 9.00 a.m. Water temperature and pH (PHC 10101), conductivity (CDC 40101), dissolved oxygen (LDO 10101), ammonia, NH_3 (ISENH318101), and nitrate (ISENO318101) were measured using HACH multimeter (HQ 40d, USA). Light intensity was measured with probe (PMA 2130) attached with a lux meter (SOLAR LIGHT, PMA 2100, USA) at the surface of the water. Phosphate (4500-P D. Stannous Chloride Method) and nitrite (4500-NO_2^- B. Colorimetric Method) were measured following the method of APHA (2012).

Relative Growth Rate

The RGR of *L. minor* was calculated using the formula: RGR = ln $(W_t/W_0)/t$.

Where, W_t and W_0 are the fresh weight of macrophytes at the time of harvest (t) and at the time of introduction of plant (zero reference time), respectively; t is the time interval in days.

Fresh *L. minor* was used for the study and RGR-value was expressed as g/g/day, i.e., production (g) of *L. minor* from 1 g of starter culture of *L. minor* per day.

Composition of *L. minor*

Biochemical (proximate) compositions of *L. minor* cultured in tanks (OM and IF) were determined by standard methods (AOAC, 2000). Moisture contents were recorded after drying at 110°C for 24 h, and the ash fraction obtained after incineration at 600°C for 16 h. The crude protein content was determined by measuring nitrogen content (N × 6.25) by Kjeldahl analysis (Tecator Kjeltec 1030 analyser, Foss, Warrington, UK). The crude lipid content was measured gravimetrically after extraction by Soxhlet (Tecator Soxtec 2050, Warrington, UK). Carbohydrate content was then determined by the subtraction method.

Amino acid composition was estimated using Automatic Amino Acid Analyzer L-8900 (Hitachi Co. Ltd., Tokyo, Japan). Dry and powdered plant sample was hydrolyzed with 6 N HCl at 110°C for 22 h. Hydrolyzed sample was dried in Nitrogen Evaporator (PCi Analytic Private Limited, Maharashtra, India). Then 0.02 N HCl was added in the sample and concentration of protein in the sample was 0.5 mg/ml. The sample was kept in the Auto sampler. Sample injection volume was 20 µl. Methionine, cysteine, and tryptophan are destroyed during hydrolysis with 6 N HCl; these amino acids are treated with specific reagents. Methionine and cysteine were oxidized with performic acid and then treated with 48% hydrobromic acid. Macrophyte sample was hydrolyzed with 4 N methanesulfonic acid and 3-(2-aminoethyl) indole for the estimation of tryptophan. Rest of the methods were same for all amino acids. The ninhydrin derivative of proline and hydroxyproline was monitored at 440 nm, and other amino acids were monitored at 570 nm. The contents of detected amino acids were quantified by comparing their peak areas with those of authentic standards provided with the equipment. Amino Acids Mixture Standard Solutions, Type B and Type AN-2 (Wako Pure Chemical Industries, Limited) were used. Standard solutions for glutamine and tryptophan (Sigma-Aldrich, USA) were prepared before analysis.

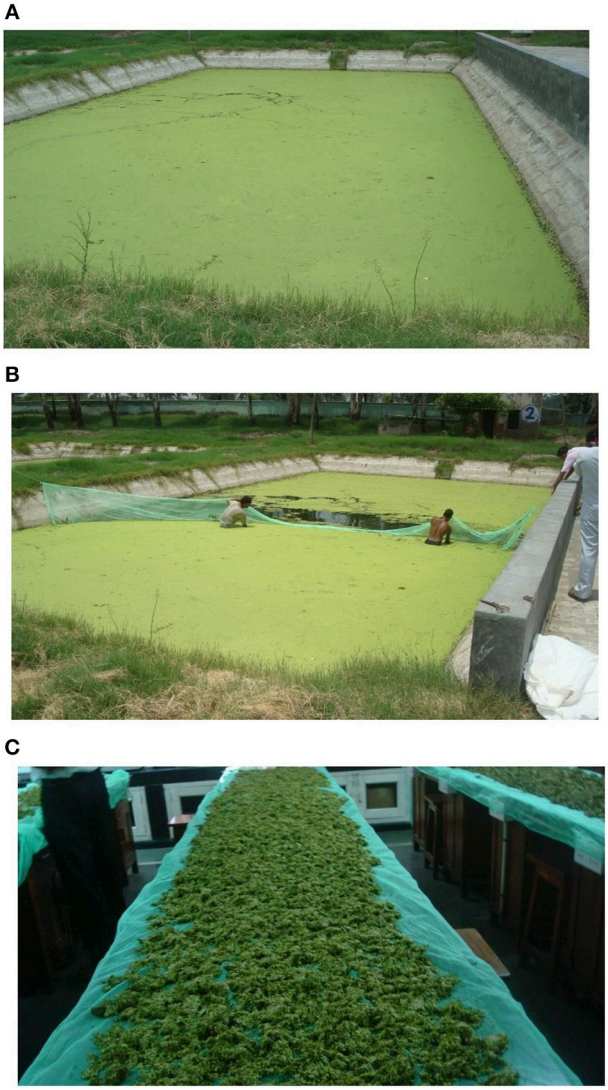

FIGURE 11 | (A) Production of *L. minor* after 10 days of culture in the pond of Central Institute of Fisheries Education, Rohtak, Haryana, India. **(B)** The harvesting (50%) of *L. minor* in pond. **(C)** Air drying of harvested duckweed at University of Delhi.

For fatty acid composition, *L. minor* samples were dried at 40°C and ground. The total lipid fraction was then extracted from 1 g of dried material by homogenization in chloroform/methanol (2:1, v/v) using a tissue disrupter (Ultra-Turrax, Fisher Scientific, Loughborough, UK), and lipid content determined by weighing (Folch et al., 1957). Fatty acid methyl esters (FAME) of total lipid were then prepared by acid-catalyzed transesterification for 16 h at 50°C (Christie, 2003). The FAME were extracted and purified as described in detail previously (Tocher and Harvie, 1988), and then separated and quantified by gas-liquid chromatography (Fisons GC-8160, Thermo Scientific, Milan, Italy) using a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), on-column injection

and ionization detection. Identification of FAMEs was by comparison to known standards and published data (Tocher and Harvie, 1988), and data collected and quantified using Chromcard for Windows (Thermoquest Italia S.p.A., Milan, Italy).

Statistical Analysis

Data were presented as mean ± SE unless otherwise stated. Data were analyzed using one-way analysis of variance (ANOVA), Duncan's multiple range test, DMR (Montgomery, 1984) and, where appropriate, by Student's *t*-test (SPSS software 19.0). Statistical significance was accepted at $P < 0.05$ level.

AUTHOR CONTRIBUTIONS

RC, DT, and JS designed the study; RG, AS, RC, and JS cultured the plant and analyzed samples; WC and DT analyzed samples; RC, DT, and JS wrote the manuscript; RG, WC, and AS prepared graphs and tables.

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