Impact of β-Mannanase from indigenous fungal isolates on growth, metabolism and immunity of omnivore fish

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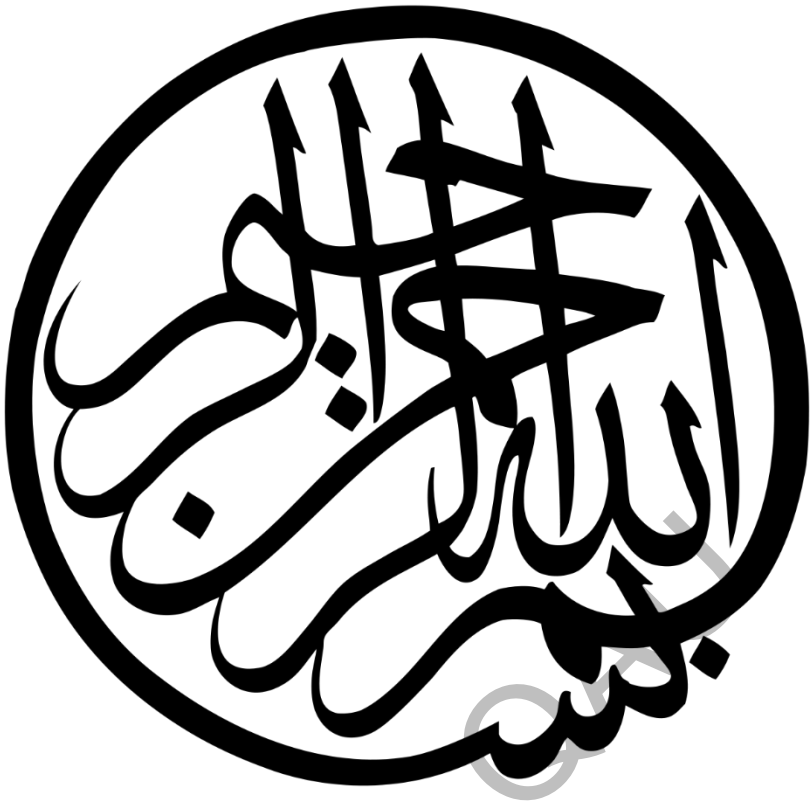

In the name of Allah, the Most Gracious, the Most Merciful

Dedicated to:

My beloved parents

Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviations	Full Name
µl	Micro litre
ALT	Alkaline amino transferase
AST	Aspartate aminotransferase
BSA	Bovine serum album
CMC	Carboxy methyl cellulose
Do	Dissolved oxygen
EDTA	Ethylenediamine tetraacetic acid
g/dL	Gram per decilitre
HDL	High density lipoprotein
LBG	Locust bean gum
LDL	Loe density lipoprotein
mg/L	Milligram per litre
min	Miuntes
M222	Tricaine methane sulphate
NSP	Non-starch polysacchrides
OD	Optical density
PBS	Phosphate buffer saline
Rpm	Rounds per minute
SDS	Sodium dodecyl sulfate
TG	Triglyceride

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Abstract

Nonstarch polysaccharides (NSP), in plant-based animal feed, are one of the major anti-nutritional factors which interfere with the digestion and absorption of the nutrients. Among NSP mannan occurs in the form of glucomannan and galactomannan and is widely present in plant-based feed sources like soybean meal, sesame meal, copra meal, palm kernel meal, guar gum meal, corn meal, canola meal and Corn distillers dried grain with solubles (DDGS) Thus mannan is a part of most feed formulations. As monogastric animals including fish lack the digestive enzymes needed to degrade mannan, this results in decreased nutrient digestibility and poor growth performance. Studies on poultry and swine have shown that β-mannanase can hydrolyze mannans in plant-based feed, thus increasing mineral bioavailability and nutrient digestibility while also providing health beneficial prebiotics in the form of mannan oligosaccharides (MOS). Most of the commercial β-mannanase enzyme preparations are well suited to function in gut of poultry and swine. But the gastrointestinal tract environment of omnivore fish is different from other monogastric animals like pigs and poultry. This creates a need for a suitable enzyme candidate that can used as a fish feed additive and can function well in the unique gastrointestinal tract environment of fish. In order to produce β-mannanase with the properties suitable for feed applications in aquaculture, new enzymes with desired characteristics need to be isolated from novel strains. Without supplementation of plant-based feed with suitable enzyme that degrade hemicellulose fraction in feed, the replacement of fishmeal with plant-based protein sources would not be feasible in aquaculture. Therefore, the present study's goal was to identify and characterize a mannan-degrading microbial enzyme from a novel fungal strain that may be employed as a feed supplement in plant-based feeds in aquaculture. The study was conducted in three phases.

In the first phase, isolation of various fungal strains from soil samples obtained from different parts of Punjab was carried out. Among many different strains tested for mannanase production, one fungal strain turned out to be an efficient producer of β-mannanase enzyme. Molecular and phenotypic identification of this strain revealed it to be *Aspergillus niger*. The DNA sequence was submitted in NCBI with accession number MN239884 and the strain was designated as *Aspergillus niger* AD-01. Different parameters for maximum production of β-mannanase from *Aspergillus niger* AD-01 were optimized. Optimum production of mannanase occurred at 30◦C, pH=5, substrate concentration=1% locust bean gum, nitrogen

source=yeast extract, days of incubation=5 days. When produced under optimized conditions, the specific activity of β-mannanase reached 12.49U/mg. After optimization of mannanase production its purification was carried out. Mannanase was purified in two steps. In the first step precipitation with ammonium sulphate was done and the enzyme was precipitated out. The precipitated enzyme was dialyzed against sodium phosphate buffer (PH=6) for overnight and then run through size exclusion Column containing sephadex gel G-75. The fractions containing highest activity of the enzyme were collected. The specific activity of purified enzyme reached 78.07 U/mg and it was purified by 6-fold with a percentage yield of 24%. Characterization of the enzyme revealed it to be a thermostable, low temperature active, weakly acidic and protease resistant enzyme. It's optimum temperature and pH were 40◦C and 6, respectively. The enzyme exhibited considerable stability when incubated in buffers with pH ranging from 4-8. Mannanase isolated from *Aspergillus niger* AD-01showed good thermostability. The enzyme was very stable at 37ºC and it retained half of its activity at 40◦C after 30 min but at 50ºC after 10 min incubation, its activity was reduced to mere 20%. The enzyme showed good resistance towards proteases and NaCl (1M and 2M) at 37◦C and pH=6.Mannanase enzyme did not lose its activity in presence of different metal except Ba^{2+} and Hg^{2+} where drastic reduction in activity was observed. Inhibition by Hg^{2+} suggests the presence of a crucial sulfhydryl group in the enzyme. This weakly acidic, protease resistant, low temperature active profile showed the potential of β-mannanase for use as a feed additive for agastric or omnivorous fish.

A 90-day feeding experiment was conducted in the second phase to examine the potential positive effects of β-mannanase supplementation in the plant-based feed on intestinal and biochemical enzymes activity, growth performance, digestibility of nutrients, immunological response and hematological indices of common carp. The experiment was carried out in triplicate, and 225 carp fingerlings (weighing a total of 13.17 ±0.12 g) were placed in 15 fiberglass tanks (15 fish in each tank). Five groups of fish were created with 45 fish in each group. The control group was provided with 35% basal crude protein diet and had 0 Ukg^{-1} diet of β-mannanase while Group A1 and A2 were supplemented with (500 and 1000 Ukg^{-1} diet of) commercial mannanase enzyme and group B1 and B2 with (500 and 1000 Ukg-1 diet of) isolated β-mannanase enzyme. Sampling was done at the end of the 90 day long feeding trial and it was observed that groups of fish that were supplemented with β-mannanase enzyme in the feed had enhanced growth performance, survival rate, immunological indices (respiratory burst activity, lysozyme activity, immunoglobulin activity

and phagocytic index) and hematological indices (Hb, MCHC, white blood cells and red blood cells). The activity of biochemical indices including AST and ALT decreased in the experimental groups but a reverse trend was observed with CHO and TG. The digestibility of different nutrients like fats, protein and carbohydrates also increased. Additionally, MyoD expression in muscles and TNF-α gene expression in muscles, intestine and liver was enhanced. The effect of mannanase supplementation on digestive enzymes was also investigated. The activity of all the digestive enzymes tested. i.e., cellulase, protease and amylase was increased significantly ($P < 0.05$) in treatment groups. Both sources of enzyme supplemented at two dosage levels had similar effect on different parameters of fish ($P > 0.05$).

In the third phase of the study the effect of β-mannanase supplementation on the intestinal health of common carp was investigated. For this the gene expression of crucial digestive and immune enzyme related genes and fish gut microbial diversity was studied. Again a 90-day feeding trial was run. The fish were divided into three groups: the control had 35% basal crude protein diet with 0 Ukg^{-1} diet of mannanase and the experimental groups (A1 and A2) were provided with 500 and 1000 Ukg^{-1} diet of isolated β-mannanase. Sampling was done at the end of the 90-day long feeding trial, and fish gut microbial diversity and the expression level of the selected immune and digestive enzyme related genes was studied. For investigation of the effects of β-mannanase supplementation on gut microbiota, the V4 region of bacterial 16S rRNA and ITS region of fungal 18sRNA was amplified and sequenced. Shannon and Simpson index showed that the experimental groups of fish had greater microbial diversity in the gut than control group. Enhanced microbial diversity in the experimental groups is an indication of the health of the fish and positive effects of β-mannanase supplementation in the diet. In the experimental groups *Lactococcus*, *Lactobacillus*, *Lachnospira* and *Geotrichum* increased and disease-causing opportunistic pathogens like *Vibrio* and *Aeromonas* were absent. Further evidence of beneficial effect of β-mannanase supplementation was provided by enhanced gene expression of important digestive enzyme (Amy, Lip, Trp, FAS, FASB) related and immune related (SOD, Lys, IL-β, Def, Nk-lys) genes.

Overall results indicated that the present study identified, isolated and characterized a stable, salt-tolerant, cold active extracellular endo-β-mannanase produced by *Aspergillus Niger*-AD-01, a new mannanase-producer. β-mannanase displayed good activity against

locust bean gum, guar gum, konjac powder and soybean meal. Furthermore, β-mannanase supplementation irrespective of microbial origin showed a beneficial effect on apparent digestibility coefficient of nutrients, the growth, body composition, intestinal enzyme activity, hemato-immunological indices, metabolic enzymes, and expression of genes related to growth and immunity in *C. carpio*. In addition, the supplementation of β-mannanase modulated the gut microbiota of *C.carpio,* while certain beneficial microorganisms were plentiful, opportunistic pathogens were absent. Mannanase also upregulated the expression of key digestive and immune-related genes in the intestine. Based on these findings, supplementation of β-mannanase may be recommended as a feed additive for low-cost production of common carp.

Introduction

Among different food producing sectors, aquaculture is the fastest growing one and has recently become global industry in many countries including Indonesia, US, and China (FAO, 2018) .It supports the food requirements of a large number of developing countries and reduces the burden on natural resources. Its contribution to world fish, mollusk and crustacean supply increased from 3.9% of total production in 1970 to 33% in 2005. This increase has averaged 9.2% every year since 1970. Aquaculture already contributes more than half of all seafood produced for human consumption worldwide, and this number is expected to climb (FAO, 2020).

World fish production rose to its highest level in 2016, when it reached 171 million tons, with aquaculture accounting for 47% of this figure. About 88% of the 171 million tons of overall fish production in 2016 has served direct human consumption, a proportion that has risen substantially over the last few decades(FAO, 2018). In 2016, the first combined sales volume of the aquacultural industry and the fishery was reported to be USD 362 billion of which USD 232 billion was from aquaculture production. Aquaculture has been responsible for the ongoing impressive growth in fish supply for human consumption with capture fish demand largely stagnant since the late 1980s. By 2025 we will be consuming the majority of seafood from aquaculture(FAO, 2018).

Fish meal, a commercial product primarily composed of fish, is currently used to feed farmed fish in the aquaculture industry. It has been the choice protein source for aqua feeds because of its high protein content, high nutrient digestibility and a general lack of anti-nutrients factors(Gatlin III et al., 2007). Whole fish such as menhaden, capelin or anchovy may be used in making fishmeal. Fishmeal can also be made from processing residue of salmon, herring, whiting or pollock. High in essential amino acids, fat content in fishmeal is 4-20% while ash content ranges from11-23% (Ween et al., 2017).

Recently the increasing demand of fishmeal, as aqua culture expands, is making its prices soar. As fishmeal is made from fish, trash fish such as menhaden anchoveta, and herring have been depleted due to overexploitation (Oliva-Teles et al., 2015).Thus the increasing use of fishmeal in aquaculture is becoming a serious cause of concern. Not just

overexploitation of fish to make fishmeal is a cause of concern but there are a few other reasons that necessitate for the reduction in use of fishmeal.

A prime reason is that it contains phosphorus more than a fish's nutritional need, which when released into water bodies causes eutrophication. This stimulates unbridled growth of some phytoplankton communities, especially cyanobacteria (Smith et al., 2006). Production of thick growth of cyanobacteria that release foul odor (sometimes described as septic or fishy) is one of the most harmful aspect of eutrophication because it deteriorate the quality of water and reduce its clarity (Dodds and Smith, 2016). Cyanobacterial blooms reduce penetration of light, thus affecting the efficiency of predators relying on light to chase and catch prey. In addition, these toxic overgrowth of cyanobacteria also limit growth of other plants or even cause them to die (Fitch and Kemker, 2014).

It has been reported that elevated rates of photosynthesis conducted by cyanobacteria consumes all of the dissolved inorganic carbon thus causing the pH of water to get very high (Lehtiniemi et al., 2005).By impairing chemosensory abilities of the aquatic animals, elevated pH will 'blind' species whose survival depends upon the detection of chemical cues dissolved in water (Turner and Chislock, 2010). The death of these huge blooms of cyanobacteria consumes all the oxygen in the water as microbes begin their decomposition of the dead matter. This creates areas within the water bodies where the concentration of oxygen is alarmingly low. These areas of deficient oxygen are called dead zones because a lot of animals cannot survive in such deficient amount of oxygen. These dead zones are present in many fresh water bodies including great lakes of the Laurentian (Willis et al., 2011). Hypoxic events have also been observed to stretch over 245,000 square kilometers in marine coastal ecosystems along vast waterways rich in nutrients (e.g., the Gulf of Mexico and the Mississippi River and; and the Susquehanna River and the Chesapeake Bay)(Diaz and Rosenberg, 2008). Eutrophication-induced anoxia and hypoxia appear to pose a threat to profitable industrial and recreational fisheries around the world. Unbridled growth of cyanobacteria is also harmful because they release toxic substances like microcystin and anatoxin-a(Bashir et al., 2020).

For the past few decades these toxic blooms of cyanobacteria have degraded quality of water, damaged valuable and lucrative fisheries, and caused public health hazards. Among

these toxic Cyanobacteria, *Cylindrospermopsis, Anabaena, Oscillatoria, Microcystis* and *Planktothrix* are the most prominent species (Lee et al., 2017; Bashir et al., 2020). They become very competitive when phosphorus to nitrogen ratio is high, light is less and nutrient concentrations are very high (Paerl and Paul, 2012). *Microcystis* seem to dominate nutrient-rich, freshwater environments. Domestic animals, birds, and even humans have been poisoned by harmful cyanobacteria blooms all over the world. First observation about the death of domestic livestock by harmful cyanobacteria was made as early as 1878 by Francis(Chorus and Welker, 2021).

Fish meal is also a major source of metals such as lead, cadmium, mercury, and arsenic in fish feed (Adamse et al., 2017). While numerous studies have been conducted on metal concentrations in water and its effect on fish, there are few studies on dietary exposure in fish (Briffa et al., 2020). To determine how metals get distributed in the target tissue in fish, the exposure pathway whether it is diet or water is critical. Dietary metals gather in the intestine and then circulate to the liver through the portal system before reaching other organs (Adamse et al., 2017). However, when compared to other organs, dietary metals are more likely to get deposited in the liver. The deposition rate in muscles is very low and a long-term exposure is required. Organic varieties of metals and metalloids, such as arsenobetaine and methylmercury are an exception, as they quickly penetrate the fish and readily settle in muscle tissue(Lie, 2008).

Ingestion rate and dietary metal bioavailability are prominent factors that decide the degree of tissue toxicity following intake of metal-contaminated feed (Schlekat et al., 2005). Metal consumption is greater in juvenile fish (% of body weight per day) than in adult fish. The efficiency of assimilation of a metal that is present in diet can determined in many different ways (Bosch et al., 2016) .The composition of food is most important factor in determining the bioavailability, digestion and the discharge of metal from food in the gastrointestinal tract (Lie, 2008).

There's considerable variation when it comes to determining the assimilation efficiency of a metal present in diet. The discharge of metal from feed components and its digestion in the intestinal tract, as well as bioavailability are highly affected by feed composition (Schlekat et al., 2005). The accumulation of a metal in the gut tract may be

influenced by organic ligands such as proteins, and/or amino acids phytates(Bosch et al., 2016). Interactions between different components of feed in commercial fish feeds that have vitamin and mineral supplementation for improving farmed fish quality and protection, can also affect metal absorption in the intestine(Lie, 2008). Once deposited in fish, metals like cadmium, mercury, lead and arsenic find their way from fish to humans as fish is an important source of protein and is consumed worldwide(Castro-González and Méndez-Armenta, 2008).

Trace metals are important for human health and help to deter illnesses and infections, but high concentrations of these elements can be dangerous to human health(Engwa et al., 2019). Lead (Pb), for example, has been found to be carcinogenic as it negatively affects both the digestive and respiratory processes as well as immune systems (Jan et al., 2015). Children are especially vulnerable to the toxic effects of increased concentration of this metal, which impairs their nervous systems and intellect. Cadmium (Cd) is toxic to gonads and bones and amasses easily in the circulatory system such as heart, lungs and kidney (especially the renal cortex)(Borges et al., 2003; Shabecoff and Shabecoff, 2010).The International Agency for Research on Cancer and the National Toxicology Program have also acknowledged these dangers, and Cd has been identified as a group-1 carcinogen (Mannan et al., 2018).

Due to the above-mentioned issues with fishmeal, the quest for sustainable options for aquaculture feeds has accelerated. Aside from fishmeal, animal by-products are also used as feed in aquaculture industry. Animal-byproducts are produced in poultry processing, meat-packaging and rendering industries(Gillund and Myhr, 2010). When these products are dried their protein content reaches to around 50-85%. It's required that the protein in animal by products meet a specific standard which is usually a minimum pepsin digestibility level. Whole egg protein is used as a standard to evaluate protein quality content. It's desired that animal by product meal should have essential amino acid compositions that is very close to whole egg protein. These products are rich in lysine, but poor in methionine and cysteine, usually regarded as restricting in dietary formulations(Miller, 2004). Following is a short explanation of different animal by-products.

Meat Meal and Meat and Bone Meal. This kind of feed is obtained from dried tissues of mammals excluding horn, hooves, hair, manure, hide trimmings, and stomach

contents(Lovell, 1989). Meat meal has protein content of 51% whereas the level of protein in meat and bone meal is approximately 50%. As far as lipid content in both of these products is concerned it is 9.1-9.7%. The major difference between the two items is the amount of phosphorus, which is less than 4.4% in meat meal. The amount of calcium in meat and bone meal and bone meal usually ranges from 8.8 to 12%.Both products i.e., the meat and meat and bone meal are comparatively high in ash, about 27 and 31% respectively(Hardy, 2002).

Blood meal is a dehydrated product that has been made from pure, fresh animal blood, free of contaminants and other materials (Lovell, 1989). Spray drying process is used to create the most common blood meal which involves vacuum evaporation at low temperature decreasing the moisture content to 70%. Flash-drying and traditional drying in a cooker are two other methods for drying blood. The protein content in blood meal is 85% while amount of lysine is 9–11 % with an availability of over 80 % (Hardy, 2002).

Feather meal is a product created from chicken feathers when they are hydrolyzed in the presence of calcium hydroxide and then dried(Guillaume et al., 2001). It contains 80–85 %protein, and the pepsin digestibility method requires that at least 75 % of the protein be digestible. However, because of it's low protein digestibility in fish, it is not commonly used in aquaculture (Hardy, 2002).

Poultry by-product meal is created by the waste resulting from the working of poultry processing plants, that is other than gizzard, feathers, and intestinal contents. The left-over product created after dressing the chickens is cleaned-up and then dried (Siddik et al., 2019). The ash content should not be more than 16 %, and the acid-insoluble ash must not be greater than 4%. The protein content in regular poultry by-product meal is about 58 % and the fat content 13%. Lower ash levels and higher protein are found in pet-food grade and low-ash poultry by-product meals than in regular poultry by-product meals(Hardy, 2002).

Bone meal, along with a variety of other animal by-product meals, especially meat meal, can cause the spread of mad cow disease also called bovine spongiform encephalopathy (Jedrejek et al., 2016). Another justification to restrict animal by-product use in aquaculture is the potential accumulation of pesticides, harmful heavy metals and drug residues. Due to these reasons animal byproducts are not considered very good options.

Plant protein sources are increasingly being identified as viable options for replacing fish meal partly or entirely in aqua feeds, thus satisfying the growing demands of aquaculture industry (Daniel, 2018). Moreover, several investigators have explored the possibilities of using plant proteins to supplement fish meal completely or partly, with the aim of not only assessing the nutritional importance of plant products but also understanding their long-term impact on fish health.(Nguyen et al., 2009; Thompson et al., 2012; Khalifa et al., 2018).

Blends of different plant meals like wheat, wheat gluten, soy concentrate, and corn gluten are used in commercial sector and they have far less levels of toxic metals (like mercury, arsenic, cadmium or lead) as compared to fishmeal (Hardy, 2010).In addition, they are cheaper and easily available. Thus, replacing fishmeal with plant-based products not only potentially reduce the amount of toxic metals in the feed but also prevents eutrophication. Soybean meal, canola meal, corn gluten meal and cereal concentrates like wheat and maize are most frequently used plant protein sources(Glencross et al., 2020).

The most essential plant-based protein supplements are oilseed meals prepared from cake left behind after oil is extracted from soybeans, canola, cottonseeds, coconuts, peanuts and sunflower seeds(Kaur and Saxena, 2005). Oils may be extracted physically or with the help of solvents. Soybean meal is North America's most preferred oilseed protein. Protein content is 48% in dehulled soybean meal, while it is 44% in defatted soybean. To prepare Full-fat soybean meal, oil is not removed from raw soybeans. It is often used to feed trout and salmon(Ruiz et al., 2020).

Corn gluten meal and Wheat gluten meal are plant derived proteins .These are high in protein content(60-75%) and are produced as a by-products during the production of starch (Loy and Lundy, 2019).

Wheat germ meal is mostly composed of wheat seed germ but also contains a little amount of bran and wheat middling. It is a byproduct that is produced during the milling of wheat (Brandolini and Hidalgo, 2012). The proximate composition of wheat germ meal changes according to milling techniques utilized. The protein content differs between 25% and 30%, whereas the fat percentage varies between 7 and 12 (Lim et al., 2008; Dapčević-Hadnađev et al., 2018).

Another prominent group of plant-based protein supplement are distiller and brewery's by-products. These items are made from the leftovers of liquor and beer productions(Lim et al., 2008). The fiber and protein content of these byproducts is relatively improved because much of the starch in the original substance is fermented to alcohol and extracted (Westendorf and Wohlt, 2002). As a result of yeast synthesis, distillers' products are fortified with nucleotides and vitamins.

The chief by-products of brewing are brewer's yeast and brewer's dried grains, which can also be used in the formulation of fish feed. Brewer's dried grains have a fiber content of 13% and protein content of 27%(Lim et al., 2008). The protein content of brewer's dried yeast is 44 % with just 3 % fiber. Brewer grains are not often used in diets of fish because they are bulky and it is difficult to grind them to a uniform particle size. In certain diet formulas the binder is made from brewer's yeast, but it is used rarely because it's very expensive (Westendorf and Wohlt, 2002; Lim et al., 2008; Bell et al., 2019).

Distillers' dried grains (a part left after removing the stillage), Distillers' dried soluble (a dried stillage fraction), and distillers' dried grains with solubles (a mixture of grain sediments and stillage), are the three major distilling by-products available(Lim et al., 2008). The concentration of protein in these products varies from 26 to 28 %, with dried grains having a crude fiber content (9-11%) higher than dried solubles (4–5 %)(Welker et al., 2014).

Pulses such as peas, lentils and beans are a fourth group of plant protein products(Lim et al., 2008). While these items have protein concentration of 20–30%, their methionine levels are less than desirable, restricting their use in the formulation of fish diets. Air classification can concentrate the protein fraction, resulting in protein concentrates of 50–60% protein (Daniel, 2018; Khazaei et al., 2019).

Plant proteins can be good substituents to fish meal because they are cheaper and available easily (Castillo and Gatlin III, 2015). The carbohydrate part of plant-based feed ingredients though can provide cheap energy, but it is not well utilized by many species of fish when compared to proteins and fats. Furthermore, the quality, palatability and the amount of protein in plant-based feed stuffs is low as compared to fishmeal (Sinha et al., 2011; Daniel, 2018). But the main issue with plant-based feed stuffs like soybean meal, corn

meal, canola meal, rapeseed meal is that they contain different antinational factors including protease inhibitors, tannins, allergens, saponins and non-starch polysaccharides (Samtiya et al., 2020).

Among different anti-nutritional factors, non-starch polysaccharides (NSPs) have a major role in fish digestion. They are present in the cell wall of the plant and are composed of a diverse set of molecules made up mostly interconnected monomers of pentoses and hexoses, such as arabinose, glucose, galactose, mannose and xylose(Sinha et al., 2011; Castillo and Gatlin III, 2015).

In monogastric animals, soluble NSPs may increase the viscosity of digesta and thus slow down its passage through the intestinal tract. On the other hand insoluble NSPs like hemicellulose and cellulose increase the viscosity of digesta (Johansen et al., 1996). Inclusion of soluble NSPs in fish diets slows stomach emptying, which can cause a delay in glucose absorption (Knudsen, 2001) and perhaps other nutrients. The addition of viscous cereal grains to the diet of African catfish lowered glucose and plasma cholesterol levels (Leenhouwers et al., 2007). Both plasma cholesterol levels and muscles were considerably reduced in common carp fed meals with sesbania endosperm (Hossain et al., 2001).

Various authors have also documented a considerable reduction in the total levels of cholesterol in the blood plasma of Atlantic salmon, yellow tail and rainbow trout when they were fed soybean diets high in NSP (Sinha et al., 2011). Reduced cholesterol levels in the blood are likely linked to increased viscosity of digesta because it entraps bile salts in the stomach, as has been seen in rats given galactomannans rich diets (Moundras et al., 1997).

In addition to increasing viscosity of the gut, the soluble NSPs also have an anti-nutritive impact through altering the different functions of gastrointestinal tract. Visceral secretion of electrolytes, water, lipids and proteins is hampered by these factors (Sinha et al., 2011). Bile acid secretion can be increased by non-starch polysaccharides, resulting in significant bile acid loss in the feces(Ikegami et al., 1990). In attempt to re-establish the balance,the liver may increase the production of bile acids from cholesterol, which can subsequently affect lipid and cholesterol absorption in the gut, resulting in reduced blood cholesterol levels(Hossain et al., 2001).

These effects might cause significant alterations in gut physiological dynamics, resulting in low nutrition absorption efficiency in the animal. Through their interactions with cholesterol, lipids and bile salts, non-starch polysaccharides may have an impact on lipid metabolism in the gut (Sinha et al., 2011). There have been no instances of NSPs directly inhibiting intestinal enzyme production, however binding to NSPs or limiting the access of enzyme to the substrates may lower the activity of most enzymes (Pettersson and Åman, 1989).Broiler chicks' digestive enzyme activity was affected by commercial NSP-supplemented meals (Iji et al., 2001a). Chicks fed diets supplemented with alginic acid (low viscosity) had the greatest jejunal maltase and sucrase activity, whereas chicks fed a gum xanthan-supplemented diet (high viscosity) had the lowest enzyme activities.

The consumption of a diet rich in NSP, changes the anatomy and physiology of the gut of monogastric animal, mainly by changing the digesta viscosities (Sinha et al., 2011). In the small intestine the active process of cell turnover is controlled mainly by, cell movement along the crypt—villus axis, crypt-cell proliferation, cell sloughing and cell expulsion from the villus top through apoptosis. When high viscosity digesta is present in intestinal lumen it may lead to deterioration of villus and increase the rate of cell loss from the villus (Montagne et al., 2003).

Because of the increased viscosity of digesta, it may take longer for it to travel through the digestive system, lowering the partial pressure of oxygen and thus favoring the proliferation of anaerobic bacteria (Choct, 1997). Albeit it is unclear if a quick change in the ecology of gut is harmful to nutrient utilization efficiency, the maintenance of health status of an animal is largely dependent on normal endogenous microbiota (Celi et al., 2017). The host's gut physiology benefits greatly from a healthy microbiome. The metabolism of organic substrates and nutrients are two of these advantages, in addition a healthy microbiome also contributes to the phenomena of colonization resistance(Sinha et al., 2011).

When the equilibrium of normal gut microbiota's is disturbed, disease causing microorganisms that have just arrived or were previously present but in small numbers grasp the opportunity to flourish and cause disease. Furthermore, the growth of some anaerobic organisms can result in the creation of toxins and the deconjugation of bile salts that are necessary to digest fats (Carre et al., 1995).

Presence of NSPs in the feed of monogastric animals, such as fish, have been shown to put off glucose absorption in the intestine. It was shown that feeding African catfish diets containing 400 grams of rye per kilogram of dry matter reduced glucose levels in plasma (Leenhouwers et al., 2007). When salmonid fish diet was supplemented with alginates and guar galactomannan (as NSP sources), glucose availability was decreased considerably as compared to diets that were NSP free (Sinha et al., 2011). Feeding Atlantic salmon a defatted soybean meal diet containing high (100g/kg diet) NSP level resulted in significantly decreased intestinal maltase activity(Kraugerud et al., 2007). Only a few studies have been carried out to understand how NSP affects carbohydrate metabolism and absorption in fish.

The addition of NSPs to a fish's diet has been shown to decrease the digestibility of amino acids. Increased Nitrogen secretion, either endogenously or via gut bacteria, is likely to be the cause of the reduction in nitrogen utilization efficiency following an NSP-rich meal(Sinha et al., 2011). According to Leenhouwers et al. (2006), adding soluble NSPs from guar gum to the African catfish diet (dry matter) at 40 and 80 g/kg significantly enhanced digesta viscosity in both distal and proximal gut.The apparent digestibility coefficient (ADC) of protein decreased in tandem with the rise in viscosity. Rainbow fish given 25–100 g of guar gum per kg diet have worse protein digestibilities(Storebakken, 1985).

It has been demonstrated that increasing the amount of NSP in a monogastric animal's diet lowers lipid use. Increased digesta viscosity generated by an NSP-rich diet has been found to have a detrimental impact on emulsification and lipolysis (Pasquier et al., 1996).Bile salts may get entrapped in NSP.This reduces their ability to solubilize lipids and, as a result,lipid absorption is affected (Sinha et al., 2011). In trout, NSPs rich feeds are thought to inhibit fat absorption by interfering with micelle production in the gastrointestinal system (Øverland et al., 2009). Likewise, when soybean meal that contained NSPs at 100 g/kg, was fed to Atlantic salmon (*Salmo solar*) a similar response was observed(Aslaksen et al., 2007).This is most likely due to cholesterol binding with bile salts in the intestine. Another probable cause for hypocholesterolaemia, according to Potter (1995), is an increase in excretion of bile acid, which results in the establishment of a situation whereby cholesterol is 'drawn' from the body. Metabolism of cholesterol in liver changes this condition to produce cholesterol for increased bile acid production(Sinha et al., 2011).

NSPs contain a number of components that negatively affect mineral absorption. The hydroxyl and carboxyl groups of phenolic substances, the carboxyl group in uronic acid, and the surface hydroxyl in cellulose are all polysaccharide and lignin components that interact with minerals (Sinha et al., 2011; Castillo and Gatlin III, 2015). Furthermore, mineral absorption has been demonstrated to be hampered by NSP-induced digesta viscosity (Van der Klis et al., 1995).

At present, the global production of cereal grains stands at 2790 million tonnes of legumes at 150 million tonnes, while the production of fibrous materials is approximately 115 million tons (FAO, 2020). If the NSPs in these plant materials are managed carefully, this large amount of plant resources might be used as cheaper fish feed components.

In legumes and cereal grains, NSPs mostly consist of arabinoxylan, cellulose and mannans. Mannan is often present in different fish feed ingredients like palm kernel meal, soy bean meal, copra meal, sesame meal and guar gum meal (Hsiao et al., 2006). As mannan is a part of soybean meal which is a widely used feed ingredient throughout the world, thus mannan is present in most feed formulations. In addition to soybean, Corn distillers dried grain with solubles (DDGS) and canola are also often added to monogastric diets thus adding to the quantity of mannan in the feed (Hsiao et al., 2006).

It has been reported in several studies that when mannan is present in animal feed, it can interfere with absorption of glucose in monogastric animals. The addition into salmon fish diets of guar galactomannan and alginates lead to a decreased supply of glucose in contrast to the control diet free from all these ingredients (Storebakken, 1985).Very few studies report the effect of mannan on glucose digestibility in fish, but significant amount of literature on mannan's effects on the digestion and metabolism of carbohydrates in swine is accessible (Sinha et al., 2011).

Rainbird and Low(1986) indicated that the absorption rate of glucose in jejunum was decreased by half when guar gum was included in the basal diet of growing pigs. Similarly, the addition of 40g/kg guar gum into the semi-purified diets of pigs reduced glucose absorption by 25 % (Sambrook and Rainbird 1985). In addition, Nune and malmlof (1992) showed that when guar gum (60g/kg) was included in swine diet, the glucose level dropped to

41%, insulin-like growth factor (IGF-1) to 58%, gastric inhibitory polypeptide to 55% and glucagon to 41%.

Feed ingredients containing mannan have been reported to show a detrimental effect on both growth performance and body composition. Hossein et al.(2001) observed that both feed utilization and growth were significantly reduced in common carp fed on galactomannan rich sesbania seed. Similarly, in an another study it was observed that when rainbow trout was fed a diet that contained 10% guar gum, it resulted a decrease in dry matter, growth and fat contents.Similar observations have been made from work on chickens. Nunes and Malmlof observed that elevated content of mannan in pig diet caused reduction in growth and interference with secretions of both IGF-I and insulin (Nunes and Malmlöf, 1992).

Mannan rich feed ingredients have been reported to increase viscosity of digesta (Sinha et al., 2011). This leads to ecological disturbance in the gut, reducing nutrient digestion and utilization. In a study carried out on rainbow trout fed guar gum supplemented diet, it was observed that digestibility of the nutrients was less compared to control. High viscosity of digesta was considered to be responsible for it(Storebakken, 1985).

Similar observations were made in common carp. Inclusion in the traditional carp diet of galactomannan-rich sesbania seeds (Sesbania aculeate) increased intestinal viscosity and negatively affected dietary absorption and utilization of nutrients(Hossain et al., 2001). High digesta viscosity has also been reported to adversely affect growth performance in poultry. The direct association between increasing mannan levels and ileal digesta viscosity was observed by Lee et al(2003b) . It was observed that as the guar gum concentration was increased in feed, the viscosity of digesta also increased. The broiler chicken showed poor growth which was attributed to high mannan content in the diet.

Mannan in the feed can degraded by mannanases (Malgas et al., 2015). Mannases though absent in monogastric animals are generally present in plants and microorganisms(Srivastava and Kapoor, 2017). Mannases are a kind of hemicellulase enzymes which randomly hydrolyze mannan chains into mannooligosachrides. These oligosaccharides can be further degraded by other hemicellulose degrading enzymes like α-D-galactosidase, β-D-mannosidase and β-D-glucosidase to produce galactose, mannose and glucose respectively(Dhawan and Kaur, 2007; Srivastava and Kapoor, 2017). The extent of

hydrolysis is determined by distribution of substituents and degree of substitution(Chauhan et al., 2012).

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Mannanases have been isolated from microorganisms, animals and plants(Kim et al., 2013; Wang et al., 2016; Jana et al., 2018). Most of the commercial β-mannanases have been produced from microbes due to their higher stability, production within limited time and space, cost effectiveness and ease of genetic manipulation. This increases their market value and makes them suitable candidates for applications in industry.

In the microbial world, numerous microbes possess the ability to degrade mannan efficiently. Among bacteria, most of the mannan degraders belong to the gram positive group such as *Bacillus* species(David et al., 2018). But there also some that belong to gram-negative group like *Klebsiella Oxytoca* that can efficiently hydrolyse mannan (Tuntrakool and Keawsompong, 2018). Competent mannan degraders among fungi are the members of genus *Aspergillus* while β-mannanases have also been isolated from *Trichoderma* sp. and *Penicillium* sp.(Agrawal et al., 2011; Blibech et al., 2011; Liu et al., 2020). Among actinomycetes group *Streptomyces* sp. and *Nocardiopsis* sp. have shown appreciable mannan degrading capability(Gohel and Singh, 2015; Pradeep et al., 2016).

Considering sequences of amino acids, mannanases are classified into glycoside hydrolase (GH) families 5 and 26.Bacterial mannanases may be classified in GH5 or 26 but fungal are classified in GH5(Chauhan et al., 2012).Mannanases metabolize cell wall mannans and thus promote maturation, ripening and plant growth. They also generate energy

equivalents by hydrolyzing substrates and thus support microbial metabolism(Chauhan et al., 2012; Srivastava and Kapoor, 2017).

Mannanases belonging to separate GH families differ in their primary structures, but have a similar spatial arrangement, a (β/α)8-barrel protein fold and are clustered together in clan GH-A. They often have modular architecture, which includes carbohydrate binding modules,additional functional domains and catalytic domains (Songsiriritthigul et al., 2011; Srivastava and Kapoor, 2017).

Site-directed mutagenesis and X-ray crystallographic studies in a broad variety of species have showed that β-mannanase needs a minimum of five substrate binding sites and a cleft shaped active site with nucleophile catalyst and a well conserved acid/base catalyst which are at a distance of 5.5 Å from each other for efficient hydrolysis of substrates(Tailford et al., 2009; Hekmat et al., 2010).

Mannanase when supplemented in a plant-based diet degrades mannan into mannooligosaccharides which have several health beneficial effects. Prebiotics are not digested by the host animal but metabolized by *lactobaccilus* and *Bifidobacterium* (Pandey et al., 2015b; Markowiak and Śliżewska, 2017). Such bacteria are considered to be beneficial for growth and health of the host because they reduce the risk of pathogen proliferation or produce metabolites associated with health (Singh et al., 2018).

For the past two decades, the frequent administration of antibiotics to livestock has been condemned due to destruction of microbial population in aquaculture ecosystem, the appearance of leftovers antibiotics in fish, and the impairment of immune response in aquatic animals(Ringø et al., 2010; Torrecillas et al., 2014).

Alternatively, prebiotics, which modify intestinal conditions in order to favor some beneficial microorganisms, are more feasible in aquatic organisms. This has a positive impact on fish development and reduces the vulnerability of the host's to disease (Dawood and Koshio, 2016). As several studies confirm that pathogenic microbes enter into body through gastrointestinal tract. The prebiotic ability of mannooligosaccharides and other fibers may be

beneficial to aquatic organisms in aquaculture industry, both for the promotion of robust intestinal health and for the suppression of potentially damaging bacteria. MOS is a complex of glucomannoproteins and its use is well documented in terrestrial animals(Ringø et al., 2010; Torrecillas et al., 2014). In several recent studies, the impact of MOS on marine animals was examined.

The mannose receptor (MR) is present on the surface of macrophages and it contains both microbial glycans and glycoproteins as natural ligands. It is also present in immature cultivated dendritic cells where it interacts withglycosylated antigens. Receptors like mammalian MRs are also present in Atlantic cod(Linehan et al., 2000). In addition, a C-type lectin with MR functionality in shrimp was discovered. Ligands containing mannose can also be linked to other receptors such as dectin-2 and DC-SIGN which can activate leucocyte(Gazi and Martinez-Pomares, 2009; Zhao et al., 2009). Because mannose containing molecules may increase production of proinflammatory cytokines through induction of intracellular signalling, MOS can be beneficial for both fish and shellfish. Thus enzymes like β-mannanase which degrades complex mannan fibers into mannan oligosaccharides holds increasing importance(Ringø et al., 2010; Dawood and Ma, 2020).

Addition of mannanase in the feed can be beneficial in a number of ways e.g., it degrades mannan present in the cell wall and thus releases trapped nutrients. It also enhances villus height in duodenum and jejunum thus surface for better adsorption of nutrients is increased and it produces mannooligosaccharides that favor the growth of probiotics(Castillo and Gatlin III, 2015; Dawood and Ma, 2020). Mannanases have also been reported to reduce digesta viscosity. Mannanase that are active in a wide range of pH and are resistant to action of proteases like pepsin and trypsin are desirable candidates in animal feed industry as biocatalysts(Dhawan and Kaur, 2007; Srivastava and Kapoor, 2017). Dawood et al.,(2019) have shown the potential of mannanses in animal feed by conducting experiments under simulated gastric conditions.Hemicell is widely used commercial mannanase enzyme produced by *Bacillus lentus* and marketed by chemgen in USA. It is a potent mannanase that can eand is often used in poultry and pig feed.

In the burgeoning aquaculture industry, which has to replace fishmeal (FM) with plant based proteins to maintain its sustainability, use of exogenous carbohydrase enzymes that can break the NSP in the plant cell walls, holds a lot of significance.

In the past few decades, β-mannanases have been isolated from different sources, characterized and their effectiveness as an animal feed enzyme in pigs and poultry has been demonstrated. β-mannanase supplementation in diet especially in high fiber diets or low energy diet has been reported to benefits the animals in many different ways.

β-Mannanase enhances growth and ileal digestible energy (IDE) and decreases digesta viscosity in broilers fed diets with varying levels of galactomannan (Latham et al., 2018). The observed beneficial effects of β-mannanase are considered to be contingent upon concentration of dietary galactomannan. In a study carried out on turkeys β-mannanase supplementation had a beneficial effect on morphology of jejunum mucosa (Ayoola et al., 2015). The villus tip width increased by 36%, villus height/crypt depth by 32%, villus surface area by 34%, and base width by 22.5%. Besides improving gut morphology Ayoola et al., (2015) observed that β-mannanase supplementation also causes thinning of ileal mucin layer. Increased mucin secretions have been linked to proliferation of intestinal pathogens. Thus, a reduction in mucin secretions may reduce the risk of pathogen proliferation in the gut and help establish symbiotic enteric ecosystem. β-mannanase supplemented with a cocktail of enzymes has also shown to be beneficial. Govil et al.,(2017) observes that β-mannanase supplementation in combination with other carbohydrase enzymes shows significant improvement in feed conversion efficiency, weight gain and performance index in broilers.

Copra meal is easily available in many parts of the world and can be a cheap alternative to commonly used feed ingredients like SBM and corn. But its reduced amino acid (AA) digestibility and low energy content can be a barrier to its frequent use. Kim et al.,(2017) demonstrate that copra meal if supplemented with β-mannanase (800 IU) can replace soybean meal and corn upto to 25% without negatively affecting the quality of pork and growth performance of pigs. El-Masry et al.,(2017) observes that addition of 5% guar meal (GM) in broiler diet has deleterious effect on growth performance. But with

supplemental β-mannanase GM can be used at 5% without adversely affecting blood biochemistry and growth.

Very few studies have been conducted on β-mannanase supplementation in aquaculture. (Dawood and Ma, 2020). The present need is not only supplementation of mannanase in fish feed and study of its effects but also the discovery of enzymes that can work specifically in the physiochemical conditions of the fish gastrointestinal tract and efficiently break down an antinutritive agent like mannan in soybean meal (Adeola and Cowieson, 2011; Dawood and Ma, 2020). Thus, helping to improve nutrient utilization of plant-based feed. Mannanases isolated from Fungi usually have acidic pH that lie in the range of 2.4-6.0(Srivastava and Kapoor, 2017).An enzyme with acidic pH has huge potential to be used in aquaculture industry. Some of the fungal species like *Trichoderma*, *Aspergillus* and *Penicillium* are ideal candidates for mannanase production because these microorganisms are nontoxic and produce high level of enzyme(Nigam, 2013).

Therefore, this study aimed to identify a potent β-mannanase producing strain that can produce β-mannanase with potential as a fish feed supplement. A local strain of *Aspergillus niger*AD-01 was isolated and identified. Experiments were conducted to select the most appropriate physicochemical conditions leading to highest β-mannanase production. Then we explored the efficient purification and characterization process of β-mannanase enzyme. We also tested its potential as a feed supplement in simulated intestinal digestion conditions. Then we carried out trials to test its potential in real application, using *Cyprinus carpio* as a model organism for omnivore fish.. In the first trial we assessed the effects of β-mannanase from two different microbial sources and its supplementation at two different levels, in pant-based fish feed to investigate the effect on growth and different hemato-immunological indices of the fish. The second trial was carried out to gain a deeper understanding of the effect of mannanase on intestinal health and thus create novel data concerning key digestive and immune genes and fish gut microbiota..

Materials and Methods

Chapter 1

Preparation of media

sterile Locust bean gum (LBG) agar was prepared by dissolving LBG=1g, $NaNO_3$=1g, agar=15g, K_2HPO_4=1g, yeast extract=1g, $MgSO_4.7H_2O$=0.5g in 1 liter distilled water.0.080g of streptomycin was also added to eliminate bacterial contamination.

Preparation of buffers

To prepare 50 mM of $Na_3\ PO_3$ buffer having pH=6.0, 0.6g of H_4NaO_5P and 0.18 g of $Na_2HPO_4 \cdot 7H_2O$ were dissolved in water.

Screening and Identification of Fungal strains

To isolate those fungal sterains that may produce β-mannanase enzyme, soil samples from different garden areas of Punjab, Pakistan were collected. The samples were serially diluted in distilled water and plated on sterile LBG agar(Locust bean gum=1g, $NaNO_3$=1g, agar=15g, K_2HPO_4=1g, yeast extract=1g, $MgSO_4.7H_2O$=0.5g in 1 liter distilled water.0.080g of streptomycin was also added to eliminate bacterial contamination)surface and incubated for 3-4 days at 37±2°C.The morphological study of the isolates was carried out by making microscopic observations.For further study of the isolates they were maintained on PDA (Potato dextrose agar)slants.

Secondary Screening

The microorganisms obtained from primary screening were then cultured in Erlenmeyer flasks containg liquid media composed of LBG=2%, K_2HPO_4=0.1% ,and $MgSO_4.7H_2O$=0.05% at pH=5.5.The flasks were then incubated on a rotary shaker at 30°C for 7 days. After seven days, the culture broth was centrifugated for 20 minutes at 12000 x g and the supernatant generated was used for enzyme assay. Out of six isolates tested,one isolate was observed to be a very effective producer of the β-mannanase. Following morphologic observation of this isolate it was designated as *Aspergillus niger* AD-01 . This Isolate was preserved in PDA slants for further production of β-mannanase enzyme.

Identification and Molecular characterization of selected β-mannanase producing fungal strain

- The fungal strain that was the most potent producer of β-mannanase enzyme was observed phenotypically and morphologically. For molecular identification of the strain, DNA was extracted by using QIAamp® DNA Mini Kit.
- Buffer sorbitol was prepared by mixing, 14 mM β-mercaptoethanol, 100 mM EDTA, 1M Sorbitol.
- Buffers AW1 and AW2= These are Wash solutions that remove contaminants from DNA attached in column membranes. Buffer AW1 and AW2 were supplied as a concentrate. An appropriate amount (as indicated on the bottle) of 70 % ethanol was added.
- Buffers ATL and AL: These are lysis solutions that break open cell membranes. These were stored at 25◦C and shaken thoroughly before use.

Procedure

1. yeast culture was grown in YPD (yeast extract peptone dextrose) medium to an OD600 of 10.

2. 3ml of culture was harvested and then centrifuged at 5000x g for ten minutes.

3.The pellet was resuspended in buffer sorbitol (600µl).Lyticase(200 U) was added and the solution was left to incubate for half an hour at 30◦C.

4.The spheroplasts were pelleted by centrifuging at 5000 x g for five minutes.

5.The spheroplasts were resuspended in 180 µl of buffer ATL(provided by QIAamp DNA Mini Kit).

6.Proteinsae K(20 µl) was added.To ensure efficient lysis ,the microcentrifuge tube solution was left for overnight.

7.The microcentrifuge tube(1.5 ml) was centrifuged briefly so as to remove drops present inside the lid.

8.Buffer AL(200µl) was added to the sample and then mixed by vortaxing briefly for efficient mixing of buffer AL and sample to create a homogenous solution. The sample was

left to incubate for 10 minutes at 70◦C.The white precipitate that was formed by mixing buffer AL was dissolved after incubation at 70◦C.The microcentrifuge tune was again centrifuged briefly so as to remove drops present inside the lid.

9.70% ethanol (200µl) was added to the sample and well mixed by vortexing for 15 seconds. Buffer AL,sample and ethanol were mixed well to create a homogenous solution. After well mixing, the microcentrifuge tube was centrifuged briefly to clean the inside of the lid from drops.

10. The mixture from step 9 (including the precipitate) was carefully transferred to the QIAamp Mini spin (QMS) column (in a 2 ml collection tube) while making sure it does not wet the edges. The tube was centrifuged for one minute at 6000 x g with the cover closed. After that, the QMS column was inserted in a clean collecting tube (2 ml) and the filtrate-containing tube was disposed off. After that, centrifugation at 6000 x g for one minute was conducted.

The QMS column was gently opened, and Buffer AW1 was carefully introduced, making sure it did not wet the rim. The QMS column was then put in a clean collection tube (2 ml), and the filtrate collection tube was disposed off.

11. The buffer AW2 was carefully put to the QMS column, making sure that it did not wet the sides. After closing the cover, the tube was centrifuged for three minutes at 20,000 x g.

12.The old tube holding the filtrate was removed and the QIAamp Mini spin column was put in a fresh collection tube (2 ml).After that, a one-minute centrifugation at 20,000 x g was done to eliminate the possibility of buffer AW2 carryover.

13.The QMS column was then put in a clean microcentrifuge tube (1.5 ml), and the filtrate collecting tube was disposed off. AE buffer (100µ l) was inserted to the QMS column after it was carefully opened. After incubating it for one minute, the material was centrifuged at 6000 x g for 1 minute. DNA was eluted and stored at -30°C.

PCR amplification and sequencing

PCR amplification and sequencing of 18s rRNA was carried out by Macrogen DNA

sequencing services USA. Mega 6 and NCBI-BLAST was used to examine the sequenced data. For phylogenetic analysis, comparison of the sequence was made with already available sequences in NCBI database.After molecular characterization DNA sequence was submitted in NCBI database.

Enzyme assay

For determination of β-mannanase activity 1%(W/V) locust bean gum (LBG) was used as substrate. An aliquot of 100µL of enzyme sample was mixed with 900µL of substrate solution in sodium phosphate buffer (pH6.0) for 20 minutes at 50°C.To stop the reaction 3,5-dinitrosalicylic acid was added and the reaction mixture was subsequently boiled at 90°C for 5 minutes in water bath. The reducing sugars released were measured at 540nm, using mannose as standard. Under standard assay conditions, the amount of enzyme that produced 1µmole reducing sugars per minute was expressed as one unit of β-mannanase activity.

Optimization of physicochemical conditions for β-mannanase production

Different physicochemical conditions were optimized to gain maximum β-mannanase output. The governing parameters were modified in a stepwise manner. First of all, the effect of carbon source (commercial mannans such as guar gum, LBG,Konjac powder and simple sugars, such as mannose, glucose, xylose and galactose) on enzyme production was investigated. Then other parameters such as the effect of temperature (20-60°C), pH(4.0-8.0),substrate concentration(%) and various nitrogen soucres was explored. After optimization of all these parameters the effect of incubation time for production of β-mannanase enzyme was studied.

Purification of β-Mannanase Ammonium sulfate [$(NH_4)_2SO_4$] Fractionation and Dialysis

500ml of crude β-mannanase was taken and calculated amount of solid ammonium sulphate was slowly added to achieve various saturation levels(40-80%) with thorough shaking on magnetic stirrer at 4°C.The mixture was left at 4°C overnight and followed by centrifugation at 13000xg for 15min.Collected precipitates were suspended in 50ml of 50mM Na_3PO_3(pH= 6.0) buffer. The solution was dialyzed for 15 hours at 4°C against the same buffer.The buffer was changed three times. After dialysis β-mannanase activity and protein

content of each fraction was determined.

Gel filtration or Size exclusion chromatography on Sephadex G-75

Precipitated protein was applied to Gel filtration column(2.5,45.0cm) that was packed with Sephadex G-75. 0.5 ml of concentrated enzyme sample was loaded on top of the column. The enzyme was eluted using 50mM Tris HCL buffer (pH=8) at flow rate of 30ml/h and fractions with maximum specific activity were collected for further analysis.

Estimation of molecular mass

For estimation of molecular mass of β-mannanase enzyme,SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) according to Laemmeli (1970) was used.

SDS-PAGE

Principle

The polyacrylamide gel-based separation method is a typical method for electrophoresis-based protein analysis. Sodium-Dodecyl-Sulfate-Polyacrylamide Gel Electrophoresis is another name for this technique (SDS-PAGE). Large molecules cannot migrate across polyacrylamide gels, whereas tiny (faster) molecules may. To employ this approach, the structure within the protein should first be deconstructed. Denaturation of the protein is caused by adding SDS and heating the sample. Irrespective of its isoelectric point, every protein gets a negative charge from SDS.

When an electric field is provided to the negatively charged proteins, they will flow through the gel to the anode. When the mass of the proteins exceeds the size of the pores, the proteins meet resistance from the gel and become trapped in it. Through staining, the proteins become visible. The molecular mass of the bands representing the proteins may be determined, and the bands can then be further analyzed to establish which band stands for which protein. Because proteins only migrate in one direction along the gel, protein samples are fed into wells produced in the gel side by side. Within each lane generated under the wells, proteins are sorted by mass into bands. One lane is set aside for a marker, which is a commercially

available combination of proteins with predetermined molecular weights that may be used to determine the size of proteins of interest.

Sample Preparation:

- Sample buffer was added to the samples and mixed by flipping the tube.
- The samples were heated for three minutes at 100◦C
- The samples were centrifuged for 1 minute at 1500rpm at 4◦C and the resultant supernatant was used for SDS-PAGE.
- Different solutions needed for SDS-PAGE were prepared according to table.1 and different buffers were prepared according to table.2

Protocol

- After adding APS and TEMED to the separation gel solution (table.3) the gel was poured into the SDS-PAGE apparatus quickly and carefully to avoid bubbles, leaving approximately 2 cm underneath the base of the comb for the stacking gel.

- Isopropanol was poured over the gel to remove air bubbles and keep the polymerized gel from drying out. In 30 minutes, the separation gel polymerized completely.
- After removing the isopropanol, any remaining residues were washed away with distilled water.
- The stacking gel (4%) was prepared and the ingredients were mixed according to the table 4.
- In the wells, combs were put. In 30 minutes, the stacking gel had completely polymerized. The spacer, binder clips and comb were removed from the gel assembly and the gel was positioned in the electrophoresis apparatus using binder clips. The electrophoresis buffer was loaded into the electrophoresis equipment' top and lower chambers. A syringe was used to remove air bubbles and tiny fragments of gel from the wells and beneath the gel.
- The wells were filled with samples and molecular weight indicators.
- The electrophoresis apparatus' gel assembly was removed.

- The power supply was turned on and the gel was run until the sample buffer dye (BPB) reached the bottom of the gel.
- Using a spatula, the gel was scraped from the glass plates and prepared for further investigation.

Staining & Destaining of the Gel

1. The gel was placed in a small dish.

2. A 20 mL staining solution was applied to the gel, which was then stained for 30 minutes while gently shaking it.

3. The staining solution was then poured off and saved.

4. A 5ml destaining solution was then applied, and the gel was gently shaken for one minute.

5. The destaining solution was poured off and disposed.

6.A destaining solution of 30 mL was added.

7. The gel was gently shaken to remove the stain until it was no longer noticeably discolored.

8. The destaining solution was poured off and thrown away once again.

9. Deionized water was used to rinse the gel. Approximately 30 ml of deionized water was poured and gently shaken for 5 minutes to rinse.

10. The gel was dried for 60 minutes at 60°C in a gel drier with a piece of Filter paper (whatman) underneath and a sheet of Saran wrap over it.

Characterization of Purified β-mannanase enzyme

Characterization and stability studies of purified β-mannanase were carried out to gain an insight into the nature and possible applications of the enzyme.

Determination of optimal pH and stability of β-mannanase

The effect of pH on mannanase activity was investigated by incubating purified β-mannanase enzyme in Na_3PO_3 buffers (pH ranging from=4.0-8.0) at 30°C.For investigation of pH stability, purified mannanase enzyme was preincubated in various Na_3PO_3 buffers(having

pH ranging from 4-8) at 30◦C for an hour.DNS procedure was immediately followed to determine residual acitivity.

Determination of Optimal temperature and thermostability of β-mannanase

The activity of purified mannanase enzyme was assayed at temperatures (ranging from 20-60 ◦C)and at pH=6.0 to investigate the effect of temperature on enzyme activity. For investigation of thermal stability purified β-mannanase was incubated for an hour without substrate at various temperatures(ranging from 20-60) and at pH=6.0.The residual activity was immediately determined by DNS method.

Effect of different metal ions and inhibitors on β-mannanase activity

For determination of the effect of different metal ions(Cu^{2+},Mn^{2+},Mg^{2+},Ca^{2+} ,Co^{2+} ,Zn^{2+},Ni^{2+},Hg^{2+},Ba^{2+}) and inhibtors (EDTA,N-bromosuccinimide, β-mercaptoethanol, 1, 10-phenanthroline) on β-mannanase activity, the purified enzyme was incubated at 30°C for 60 minutes in the presence of 1mM of each ion or inhibitor in the reaction mixture using 50mM sodium phosphate buffer pH6.0. Then the residual activity was promptly measured by DNS method.

Substrate specificity

For determination of substrate specificities of purified β-mannanase from *Aspergillus niger* AD-01, enzyme activities were assayed by DNS method towards soybean meal, coconut pulp, peanut shells, coffee beans, sugarcane bagasse, rice bran or 0.5%(w/v) of polysaccharides including locust bean gum(LBG), carboxy methyl cellulose(CMC) and oat spelt xylan.

Effect of Trypsin and Proteinase K

Resistance of β-mannanase to proteinases was determined by incubating β-mannanase enzyme for one hour at 37°C with trypsin (250 U mg−1, pH 7.0) or proteinase K (30 U mg−1, pH 7.0), at a ratio of 0.1:1 [mannanase to proteinase (w/w)]and the residual enzyme activity was measured in sodium phosphate buffer(pH6.0)at 50°C.

Potential assessment of β-mannanase enzyme as a feed additive for agastric fish

Simulated intestinal fluid containing 6.8mg/ml KH_2PO_4 and 10mg/ml trypsin at pH6.8, was prepared to determine the ability of β-mannanase to hydrolyze mannan in vitro. The reaction system (0.5U/ml) contained 2.5 U of β-mannanase enzyme,5.0 ml of SIF and 2.0%(W/V)wheat bran, soybean meal, rapeseed meal or sun flower meal. The mixture was left to incubate at 20°C for 4,8 and 12 hours at constant agitation. A similar experiment without the purified enzyme was done as a control experiment.

Statistical analysis

All experiments related to β -mannanase activity were performed in triplicate and expressed as mean ± SD (standard deviation). Graph pad prism software 5 was used to carry out the statistical analysis and to compare different treatment groups. One way analysis of variance (ANOVA) was used.

Table 1 : Preparation of Solutions for SDS-PAGE

Ingredient	Composition	Quantity
10%SDS	SDS	10g
	With dd H_2O the volume was brought to 100 ml	
10 % Ammonium Persulphate (APS)	Ammonium per Sulphate	1g
	With ddH_2O the final volume was brought to 10 ml	
Destaining solution	MeOH	1000ml
	ddH_2O	2700ml
	Acetic acid	300ml
Staining solution	ddH_2O	227ml
	Acetic acid	46ml
	Coomassie brilliant-blue-R250	1.3g
	MeOH	227ml

Table 2 : Preparation of Buffers for SDS-PAGE

Buffer	Composition	Quantity
5 M Tris-HCL.pH 8.8	Tris base	18.15g
	dd H_2O	50ml
	With cocn.Hcl pH was adjusted to 8.8.Final vol. was brought to 100 ml with dds H_2O and stored at 4C	
0.5M Tris-HCL,pH 6.8	dd H_2O	40ml
	Tris base	3g
	With conc.HCl pH was adjusted to 6.8.Final volume was brought to 50ml with distilled H_2O.	
4 X sample loading buffer	0.5MTris HcL,pH 6.8	5ml
	10 sds	0.4ml
	mercaptoethanol	18ml
	Bromophenol blue	5mg
	Glycerol	20ml
1X Electrophoresis buffer	Tris base	6g
	SDS	2g
	dd H_2O	2L
	Glycine	28.8g

Table 3 : Stacking Gel Preparation for SDS-PAGE

Ingredient	Quantity
H_2O	6.1 mL
10 % SDS	100 µL
Tris–HCl (0.5 M, pH 6.8)	2.5 mL
10% Ammonium persulfate (APS)	100 µL
TEMED	10 µL
Acrylamide/bis (30%, 37.5:1)	1.3 mL

Table 4 : Separation Gel preparation for SDS

Ingredient	Quantity
H_2O	3.8 mL
Tris–HCl (5 m, pH 8.8)	2.6 mL
TEMED	10 µL
10 % SDS	100 µL
Acrylamide/bis (30% 37.5:1;Merk)	3.4mL
10 % Ammonium persulfate (APS)	100 µL

Chapter.2

Fish collection and management

Healthy and active juvenile *C.carpio* were transferred from a local fish farm to Fisheries and Aquaculture Research Facility, Quaid-i-Azam University. Fish were transported by adopting the closed live hauling technique and after tempering, stocked in concrete raceways. Fish were acclimatized there for two weeks. During this period, carps were hand-fed twice a day 35% CP control diet (Table.5) to apparent satiation.

Preparation of diets

The commercial β-mannanase (BM_{Tr}) a propriety *Trichoderma reesei* fermentation product, having enzyme activity 20,000Uml^{-1} product at 37°C and pH 5.5 was supplied by YoutellBio, Bejing China while locally isolated β-mannanase (BM_{An}) having 78Uml^{-1} activity at 37°C and pH 6.0 was isolated from *Aspergillus niger* AD-01 in our lab. The detailed procedure of isolation and purification has been reported earlier(Dawood et al., 2019). Ingredients for feed were procured from a feed mill (Oryza Organics, Pvt Ltd).

A plant-based basal diet (35% CP) formulation containing soybean a major ingredient and its proximate composition are shown in Table 5. Five tested diets were prepared where β-mannanase was added in the basal diet at a dose of 0 units kg^{-1} diet (control), 500, and 1000 units kg^{-1} diet BM_{Tr} (A1 and A2 respectively) and BM_{An} (B1 and B2) respectively. Briefly, all dry ingredients except vitamins and minerals premix were finely ground with the help of electric grinder (GCG289, Geepas electronics, Dubai). Afterward, to obtain 35% CP, mixed along with vitamin-mineral premix in a fixed ratio and thoroughly mixed by using locally made mixer. Subsequently, oil and water were added in ingredients and made dough. The dough was passed through meat grinder and resultant noodles were cut into small pellets. The pellets were air-dried at low temperatures. For β-mannanase supplementation, the required amount of enzyme (both commercial and isolated) was dissolved in distilled water, and sprayed on experimental diets, while the control diet was sprayed with the same amount of water used for spraying on experimental diets. Each group of feed was dried once again. The pellets were transferred to a Ziploc bag and stored in a refrigerator. Fortnightly, a new batch of feed was prepared.

Experimental design

A bi-factorial feeding trial (3 β- mannanase dosage levels × 2 Enzyme sources) in a replicate of three was designed. Fish were weighed and 225 carps (*C. carpio*), mean body weight of 13.17±0.12g were selected at random and equally distributed into fifteen 250L circular fiberglass tanks (15 fish per tank with three replicates per treatment) at a stocking density of $1.5gL^{-1}$. Fish were acclimatized to laboratory conditions for three days. Afterward, tanks were divided at random into five groups and each group was provided their respective diet, i.e., control group fed β-mannanase free basal diet, A1 and A2 groups of fish received a basal diet supplemented with 500 and 1000 unit BM_{Tr} kg^{-1} diet respectively, while B1 and B2 groups of fish provided basal diet enriched with 500 and 1000 unit $BM_{An}kg^{-1}$ diet respectively. Fish were fed their respective diets twice a day (8:00 AM & 4:00 PM) for 12 weeks. Initially, they were fed at 4% body weight per day, and fortnightly, fish of each group were weighed by using an electronic balance (Shimadzu BL2200H) and the feeding rate was adjusted accordingly. During the trial, the temperature (°C), dissolved oxygen (DO) level (mg L^{-1}), and pH of water were checked daily by using the Hanna water testing instrument (HI-9828; Inc. Woonsocket, USA). However, total ammonia was checked weekly. During the experimental period, the DO level ranged from 5.5 to 6.1 mg L^{-1} while temperature increased from 21.5 to 23.5°C. However, no noticeable variations in other parameters were observed, i.e., pH (7.2 ±0.52) and total ammonia (<0.5ppm). To maintain optimum water quality and for calculation of FCR, daily uneaten feed and feces of each tank were collected after 2hrs and 6 hrs of feeding respectively with a partial change of water. Since all of the tanks were adjacent to one other and had a similar source of water, therefore the water quality parameters variation was negligible among all tanks. After 90 days, the feed was withheld for 24 hours before sampling.

Nutrients digestibility

For estimating the apparent digestibility coefficient ofnutrients, inert indicator method was used. Briefly, after 60 days of experiment, fish were fed with diets having 0.5% chromic oxide (Cr_2O_3).For apparent digestibility coefficient (ADC) of nutrients, daily uneaten feed

and feces of each tank were collected through siphoning after 2hrs and 6 hrs of feeding respectively with partial change of water. The collected fecal samples of each group were filtered, dried at 60°C in a preheated oven and stored at 20°C. Fecal collection continued for 30 days. For analysis, fecal samples of each group were pooled (10 days samples/pool thus 9 samples/experimental group) dried in oven and homogenized by using a pestle and motor. Chemical analysis of feed and faces samples was performed by adopting AOAC (2000) procedure. For *C.carpio*, apparent digestibility coefficients of crude protein, crude fat, and carbohydrates of the experimental diets were determined by using the following equation.

$$ADC\ (\%) = 100 - \left[\frac{\%\ Cr\ in\ feed}{\%\ Cr\ in\ feces} \times \frac{\%\ nutrient\ in\ feces}{\%\ nutrient\ in\ feed} \right] \times 100$$

Percentage survival and Growth measurements

On the day of sampling, fish of each tank were weighed collectively, counted their numbers and mean body weight of fish as well as the survival (%) (100 × Final number of fish / initial number of fish) of each group were determined. Based on observed initial and final weight of fish, the growth performance parameters like specific growth rate (SGR= 100 × ln (final body weight- Initial body weight)/ Total experimental days), percent weight gain (WG% = final body weight- initially body weight/ initial body weight × 100) and feed conversion ratio (FCR = dry weight of feed consumed / wet weight gain) of each group were calculated .

Sample collection

Thirty-six fish from each group (12 fish from each replicate) randomly captured and were anesthetized with buffered Tricaine methanesulfonate (MS-222, 0.10 gL-1). The blood of 18 fish (6 fish per replicate) was drawn from caudal vein by using 3 ml sterile syringe. For collecting enough serum, blood from two fish was pooled in the same tube (3 samples/tank or 9 samples /treatment group) and allowed to clot at room temperature. Subsequently, the clotted blood was centrifuged (Kokusan, Ogawa Seiki Co., LTD, Tokyo, Japan) at 3000 rpm for 5 min and blood serum was collected in a new tube and saved at 4ºC until further analysis. For hematological analysis, the blood of other 18 fish (6 fish/tank) was collected from caudal vein with 3 ml syringe previously rinsed with EDTA (2.7% solution) and

collected in EDTA tubes (Liuyang Sanli Medical Technology Development Co., LTD). Again,blood of two fish was pooled (3 blood samples from each replicate or 9 samples /treatment group).

After being bled,6 fish per tank were dissected at low temperature by following a standard aseptic procudre and their intestinal tracts were removed carefully.The gut of 6 fish/tank,pool of two(3 samples/tank or 9 samples/treatment group) were frozen quickly in liquid nitrogen and saved at-80°C for later determination of intestinal enzyme activities. Similarly, the muscles of each group of fish after taking blood were saved for proximate composition. Moreover,for study of gene expression, the liver, muscle, and intestinal tissues of 3 fish per tank were preserved in RNA later and stored at −80°C.

Chemical composition of muscle, Feed and Feces

For chemical analysis of fish muscle, experimental diets and feces, samples were sent to accredited laboratory of Poultry Research Institute (PRI), where they analyzed by adopting standard protocol AOAC (2000).

Briefly, for determination of ash content, incineration in a muffle furnace was carried at 600◦C for 12 hours. For estimation of crude proetin (N x6.25) and crude fat, microkjeldahl apparatus and petroleum ether extraction method by soxhlet apparatus (Bligh and Dyer, 1959)were used respectively.Oxygen bomb calorimeter was used for determination of Gross energy and chromic oxide content in feces and experimental diets was determined after oxidation with perchloric reagent by using acid digestion method as described by Divakaran et al., (2002) through UV-VIS 2001 spectrophotometer.

Intestinal enzyme activity

The collected intestines were homogenized in 5 volumes (w/v) of chilled phosphate buffered saline (pH, 7.5) using hand-held homogenizer. Subsequently, the homogenate was centrifuged at 3000 × g for 10 min at low temperature (4ºC) and supernatant was collected in new tube and saved at -80ºC for subsequent intestinal enzymes analysis.

Amylase activity

For amylase assay, DNS(3,5 dinitrosallicylic acid) method based on determination at

560nm of reducing sugars using maltose as standard was used(Bernfeld, 1955).
0.5ml enzyme solution was mixed with equal amount of starch solution (1%) and was left to incubate at room temperature for 3-5 min. Afterward, 1 ml DNS reagent (prepared by mixing DNS=1g, 2M sodium hydroxide=20mL and sodium potassium tartrate=30g and diluted to 100 ml with distilled water) was added and was left to incubate on a preheated water bath for at least 5 minutes. The mixture was cooled and mixed with 10 mL reagent graded water. UV-Visible spectrophotometer (Agilent, 8453, USA) was set at λ 450 nm and absorption of resultant solution was noted for estimating amylase activity. One amylase unit was defined as the amount of enzyme in 1.0 ml solution that released1µg reducing sugar per min.

Cellulase activity

The production of reducing sugars as a result of cellulolytic activity was measured following DSN method. For this, I ml each of enzyme solution and CMC solution (1%) was taken in 10 ml glass tube and mixed with 1 ml of 0.1M citrate buffer. The resultant solution was left to incubate at 50°C for half an hour. Afterward, 3.0 ml DNS reagent was added and again incubated at boiling point for 15 min. Subsequently, I ml of 40% sodium potassium tartarate was mixed and test tube having solution was cooled at room temperature. The UV-Visible spectrophotometer (Agilent, 8453, USA) was set at λ 540 nm and reducing sugar i.e., glucose was measured for estimating the cellulolytic activity.One cellulase unit was defined as the amount of enzyme per ml solution that released 1 mg glucose per minute.

Protease activity

The protease activity was analysed by using casein as a substrate and L-tyrosine for the preparation of standard curve(Cupp-Enyard, 2008).For protease assay,100µl of enzyme extract was mixed with 0.65% of casein solution(5ml) and 110mM tricholoroacetic acid(5ml).The resultant solution was left to incubate at 37◦C for half an hour and then cooled at room temperature.Afterwards,the solution was filtered by Whatman filter paper.Subsequently, 2.0 ml filtrate was taken in separate clean 10 ml glass test tube and mixed with 5 ml Sodium Carbonate solution(500mM) and 1ml folin-Ciocalteau reagent (0.5mM) and heated for 30 min at 37ºC and then cooled down at room temperature. The UV-Visible spectrophotometer (Agilent, 8453, USA) was set at λ 660 nm and absorption of

resultant solution was noted for calculating protease activity.One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µg of tyrosine per ml filtrate under standard assay conditions.

Hematological indices

Hematological indices like WBCs(white blood cells),RBCs (red blood cells), MCV (Mean corpuscular volume), MCH (mean corpuscular hemoglobin), Hct (hematocrit), MCHC (Mean corpuscular hemoglobin concentration) and Hb (hemoglobin) were determined by using a pre-calibrated Hematology Analyzer (DxH 500, Beckman coulter).

Immunological and blood metabolic indices

Total serum proteins, immunoglobulin (IgM), aspartate aminotransferase (AST), and lysozyme activity was measured following the protocols used by Ullah et al. (2018) while protocol reported by Younus et al. (2020) was followed for the assessment of respiratory burst and phagocytic activity, from fresh heparinized blood. Cholesterol (CHO) and triglyceride (TG) assays were performed by adopting standard procedures as reported by Panigrahi et al.(2010).

Total serum protein and IgM level

Total serum protein was analyzed by following Lowry's method (1951), using bovine serum albumin (BSA) as a standard for the preparation of calibration curve and determination of protein concentrations. Stock solution of standard protein, i.e. bovine serum albumin was prepared by dissolving 1 mg BSA in 1ml distilled water (1mg/1ml) in test tube. Standards were prepared by diluting the stock solution i.e. (BSA ranged from 0.05 to 1 mg/ ml) with distilled water such that every solution would reach to 2 ml in test tube. In each test tube 2000 µl alkaline copper sulfate reagent was added and mixed well. Then these solutions were incubated for 10 minutes at room temperature. After that 200 µl Folin Ciocalteau solution was added in each tube and again incubated for 30 minutes. Finally, absorbance was measured at 660 nm. The absorbance against protein concentration was plotted to get a standard calibration curve. The absorbance of unknown sample was noted and concentration

of the unknown sample using the standard curve plot was determined.

For the determination of serum IgM level, Anderson and Siwicki (1995) procedure with some modification was adopted. Briefly, 0.1 ml each of serum and 12 % polyethylene glycol were mixed and the solution was incubated at ambient temperature under constant shaking(shaking incubator, ISS Innova 43)for 2 hrs. Afterwards, the solution was centrifuged at 7000 rpm for 10 min and supernatant was separated. The protein concentration in the supernatant was determined by adopting Lowry's method(Lowry et al., 1951) and IgM level was calculated by subtracting the IgM value from total serum protein.

Lysozyme activity

To determine the lysozyme activity,Anderson and Siwicki (1995) method reported by Ullah et al.(2018) was followed. Briefly serum (100µl) was taken with the help of micropipette in fresh test tube and mixed with 900 µl of 750 µg ml^{-1} Micrococcus lysodeikticus (Sigma, USA) suspended in saline phosphate buffer (pH 6.2) solution. Bacteria were thoroughly mixed and the rate of absorbance change was observed by using spectrophotometer set at 450 nm. The reading was noted after 1 min intervals for 10 minutes. Lysozyme activity was measured by using lysozyme from hen egg white (Sigma-Aldrich) as a standard.

Phagocytic activity

To determine phagocytic activity, heparinized blood (100µl) was mixed killed *S.aureus*(1 × 107cells) suspended in saline phosphate buffer(pH=7.2)(Anderson and Siwicki, 1995).The mixture was left to incubate at room temperature for half an hour.Afterwards, a smear was prepared by transerfing the mixture(5µl) to a glass slide.The smear was air-dried,fixed in ethanol (95%) for five minutes and then air dried again and then dipped in Gimsa stain.From each smear a total of 100 phagocytic cells were read under the microscope and the number of phagocytic cells and phagocytosed bacteria were counted.Phagocytic activity(PA) and phagocytic index(PI) were measured by following formulas:

PA = No.of phagocytic cells with ingested bacteriaNo. Of phagocytes x100

PI = No.of engulfed bacteria/phagocytic cells

Alanine aminotransferase (ALT)

ALT activity was measured by using alanine aminotransferase Activity Assay KIT MAK-052(SIGMA-ALDRICH). Assay was performed by adopting standard procedure. Before use, all the reagents were left for a while at room temperature. Subsequently, Master reaction mix (1 ml) was prepared. It's constituents were fluorescent peroxidase substrate(20µl), ALT Assay buffer (860µl), ALT enzyme mix (20µl) and ALT substrate (100 µl). In the next step, serum sample(10µl) and ALT buffer (10µl

) were added to 96 well microplate. Then, Master reaction mix(100µl) was added in each of standard, positive control and sample wells. The plate was placed on a horizontal shaker(VEVOR Orbital Rotator) so that the mixture in each well is mixed properly. After that, a measurement was taken and absorbance was read at 570nm on a spectrophotometer (Agilent,8453,USA).Repeated measurements were taken after every 5 minutes for 4 times. The change in absorbance for initial and final reading for samples and positive control was measured. Activity of ALT was calculated and expressed as UL^{-1}.One unit of ALT is defined as amount of enzyme that generates 1.0 nmole of pyruvate per min.

AST Activity

AST activity was estimated by assaying the rate of NADH oxidation, which is proportional to decrease in absorbance at 340nm over time.AST/GOT kit (AMEDA Laborodiagnostik GmbH Graz Austria) was used for measurement of AST activity. Briefly, 100µl serum was taken in 2 mL Eppendorf tube and added 1000µl working reagents(910µl reagent A and 91µl reagent B).The mixture was mixed gently and incubated for 1 min at 30◦C.UV-Visible spectrophotometer (Agilent 8453,USA) was adjusted at λ 340nm and the absorbance was noted thrice after every 1 minute interval. The AST activity is denoted in UL^{-1}.

Total Cholesterol

Griner Diagnostic GmbH (Unter Gereuth, Bahlingen, Germany) was used for determination of total cholesterol. Briefly, serum sample (10 µl) was mixed with Reagent R

(1ml) and was allowed to incubate for 5 minutes. Then absorbance at 500nm was measured by using Merck laboratory analyzer(MICRO-LAB 200,Germany).Total Cholesterol (CHO) was calculated by mg/dl.

Triglycerides

AMP diagnostic kit(AMEDA labordiagnostik GmbH,Germany) was used to measure serum Triglycerides(TG) level. For this, a small amount of serum sample (1 µl) was mixed with Reagent R(1ml) and left to incubate for 15 min at room temperature. The absorbance was read by using at 500 nm by using Merck laboratory analyzer(MICRO-LAB 200,Germany).Triglyceride level was measured as mg/dl.

Respiratory burst activity

Briefly,heparinized blood(100µl) was placed in a microplate and mixed with 100µl of 0.2% of nitro blue tetrazolium(NBT) dye(Anderson and Siwicki, 1995).The mixture was left to incubate at room temperature for half an hour and then 0.05ml of this mixture was transfered to a glass tube containing 1ml of N-N di-ethyl methyl formamide solution and centrifuged for five minutes at 3000rpm.The supernatant's absorbance was read at 540nm.

Total RNA extraction and cDNA synthesis

From each sample, the total RNA was extracted using buffer RLT (Qiagen, Mississauga, Canada) and 0.12M β-mercaptoethanol (Sigma). The extracted RNA was quantified at 260 nmand 280 nm (A260/A280) by using NanoDrop® ND-1000Spectrophotomete, (Thermo Scientific, Wilmington, USA).The cDNA was synthesized via RevertAid First-strand cDNA synthesis kit (Thermo Fisher Scientific, Lithuania)by following manufacturer's protocol. Briefly, RNA (1 µg) along with Random Hexamer primers, was incubated for 5min at 70°C followed by cooling for 10 min at room temperature, thus the primers anneal appropriately to RNA. Afterward, RT-buffer, dNTPs, RNAs inhibitor and RT enzyme were added to the mixture, and incubated for 5min at 25°C, then for 60 min at 42°C, and finally at 95°C for 3 min in a thermal cycler (Master cycler, Gradient Eppendorf, USA). Immediately the resultant sample was stored at -20°C until further analysis.

QPCR-Conditions for Analysis of Gene Expression

Housekeeping gene, β-actin was used to analyze the relative expression of the selected genes, i.e., MyoD in muscle and TNF-α gene in the intestine, liver, and muscle tissue of each group of fish. The sequence of primers of reference gene and selected genes are shown in Table 6. The amplification of cDNA was performed on a LightCycler® 480 Instrument by using SYBR®Premix ExTaqTM kit (Takara Bio, Japan). The 20µl mixture was prepared by mixing 1.0µl of the forward and reverse primers (10 mM), 10µl of SYBR Premix Ex Taq™, 1.0 µl of cDNA with 8µl ultra-pure water. The thermal cycling conditions involved initial denaturation at 95°C for 10 min followed by 40 cycles at the same temperature, i.e., 95°C for 15s, then annealing at 56°C for 30s, and final extension at 72°C for 30s.

For each sample qPCR was run two times with three replicates. Equation, $\%E = 10(-1/\text{Slope}) - 1 \times 100$ was used to determine the efficiency and validity of qPCR primers (Radonićet et al. 2004).For relative gene expression, ΔΔCt method based on the expression of target gene relative to the expression of housekeeping gene (reference) was adopted by using IQ5 software (Bio-RAD).

Statistical analysis

All data have been expressed as mean± standard deviation (±SD). SPSS software (SPSS 18.0. Chicago, IL) was used to carry out all statistical procedures. Analysis of data was done by two-way ANOVA followed by Fisher's Post hoc LSD test to determine significant differences among experimental groups. Two-tailed T-test was used to compare the results of both enzymes at similar dosage level. The value $P<0.05$ was used as the criterion for statistical significance.

Table 5 : Formulation and proximate composition of 35% crude protein basal diet (% dry matter)

Ingredients	Quantity(g/100g)
Fishmeal	5
Soybean meal	60
Sunflower meal	10
Wheat bran	8
Rice bran	8.0
Fish Oil	1
Vitamin-mineral mixture[a]	3
DCP[b]	4
CMC[c]	1
Proximate Composition (%)	
Crude Protein	34.36
Crude Fat	12.90
NFE[d]	42.12
Chromium oxide	0.41
Crude fibre	5.32
Crude Ash	7.89
Gross energy (KJ g^{-1})	21.08

[a]Composition of vitamin-mineral mixture (quantity/kg): Vitamin A, 500, 000; Vitamin D3 820,000 IU; Vitamin K3, 800mg; Vitamin E, 6200mg ; Vitamin B2, 2500 mg; , Vitamin B_6 1000 mg; Vitamin B3, 15,100mg; L- lysine , 10,500mg 5; DL Methionine,50,500 mg; Cholin chloride, 125,500 mg; Manganese, 30,000mg; , Copper , 1000mg; Zinc, 17,555mg; Iodine, 30(mg; Cobalt, 50mg; Iron, 7500 mg.

[b]DCP:Dicalcium phosphate.[c]CMC:Carboxyl mythyl cellulose

Table 6 : Primers used for expression of MyoD in muscle and TNF- α in muscle, liver and intestine of C. carpio

Gene		Primer sequence (5′→ 3′)	Accession number	Reference
TNF-α	F	GGTGATGGTGTCGAGGAGGAA	XM_019088899.1	**(Hoseinifar et al., 2017a)**
	R	TGGAAAGACACCTGGCTGTA	XM_019088899.1	
Myo-D	F	TGCCTACTGTGGGCATGCAA	LN594833.1	**(Kobiyama et al., 1998)**
	R	ACTCACTTCTGCTGATCTGC	LN594833.1	
β-actin	F	AGAAGGACCACTTGCACTCA	KX622693.1	**(Duan et al., 2013)**
	R	GATGCCAAATACTGCTCAATGT	KX622693.1	

Chapter 3

Materials and Methods

Ethics statement

All experimental procedures were approved by the Quaid-I-Azam University's animal welfare committee in compliance with local legislation (BEC-FBS-67-QAU-2019).

Fish and Culture Conditions

Healthy Juvenile *C.carpio* with no sign of infection were bought from a commercial Fish farm and transferred to QAU Fisheries and Aquaculture research station. The fish were stocked in a concrete raceway and acclimatized for a week. Feeding was done twice a day to apparent satiation.

Preparation of diet

A plant-based 35% crude protein diet was formulated (Table.5). All ingredients used for the preparation of feed were purchased from a local feed mill (Oryza Organics Pvt, Ltd). The locally isolated β-mannanase having 78U/ml activity at 37◦C and pH 6.0 was isolated from *Aspergillus niger* AD-01 in our lab. The detailed procedure of isolation and purification has been reported earlier (Dawood et al.2019). To obtain a 35% crude protein diet, all feed ingredients were mixed in a fixed ratio (Table. 5). Dry ingredients were made into the dough by mixing them with water and oil and then passed through a meat grinder. The resulting noodles were cut and air-dried at low temperatures. The prepared feed was divided into three dietary treatment groups (A1, A2, and control). For β-mannanase supplementation, the proper amount of enzyme was dissolved in distilled water and sprayed on treatment groups at the rate of 1000, 500, and 0 units/kg respectively. The feed was dried once again. The pellets were transferred to a Ziploc bag and stored in the refrigerator.

Experimental Design and Feeding Trial

A completely randomized experiment was designed and conducted in replicates of four under controlled conditions. Fish having an initial weight of 12.32±0.11g were randomly selected and distributed into 12 circular fiberglass tanks (250L) at the rate of 1.5g/L (15 fish per tank). Each tank was well equipped with a heater and air stones connected to the air pump for maintaining the temperature at 22°C and DO level at 5.5 ± 0.5 mg/L, respectively. To prevent the fish from jumping out, the top of each tank was covered with a net. After acclimatization

to experimental conditions for three days, fish were divided into three groups (A1, A2, and control). Subsequently, the feeding trial was started and fish in each group were fed their respective diet at 4% of body weight twice (8:00 am and 4:00 pm) a day (Inayat and Salim, 2005). To maintain optimum water quality, one-third water of each tank was renewed daily. Furthermore, uneaten feed and feces were also removed. The feeding trial lasted for 12 weeks.

Sampling

At the end of the 90-day feeding experiment, six fish from each tank were randomly sampled and anesthetized with Tricaine methanesulfonate (MS-222) solution prepared in $NAHCO_3$ buffer (0.10 gL^{-1}).The fish were dissected at low temperature by following the standard aseptic method. The whole intestine of each fish was dissected out carefully with sterile scissors and forceps, snap-frozen in liquid nitrogen, and stored at −80◦C for the study of gene expression and composition of intestinal microbiota.

RNA extraction

RNeasy mini kit (Qiagen, Mississauga, Canada) was used for RNA extraction.

At room temperature, all of the RNA extraction processes were completed promptly.

All the steps of centrifugation were carried out at 20-25◦C in regular microcentrifuge tubes. It was ensured that centrifuge does not cool below 20◦C.

In all the steps the collection tubes used were of 2 ml except when mentioned otherwise.

Steps taken before starting out:

Before use, β-mercaptoethanol(β-ME) was mixed with buffer RLT in such a way that for every 10µl of β-ME,1000µl of buffer RLT was included.The mixing was dispensed in a fumehood after wearing protective clothing.

Buffer RPE was delivered in a concentrated form. Before use, to make a functioning solution, 4 volumes of ethanol (96–100 percent) were added as per instructions of the manufacturer.

Procedure:

1.The tissue sample was removed from storage. It was weighed and 30mg of tissue was selected for RNA extraction.

2. The tissue was weighed and immediately immersed in liquid nitrogen before being thoroughly pulverized with a blunt object. The liquid was promptly decanted into a centrifuge tube that had been chilled. The liquid nitrogen vaporized but the tissue did not thaw.600µl of buffer RLT was added. The lysate was transferred with the help of a pipette to QIAshredder spin column which was positioned beforehand in a collection tube . The tube was then centrifuged at maximum speed for a time period of 3 minutes.

4. using a pipette the supernatant was carefully shifted to a new microcentrifuge tube. Only this lysate was utilized in the next steps.

5. 700µl of this sample was shifted to an RNeasy spin (RNS) column that was put in a 2 ml collection tube. After careful closing of the tube, centrifugation at 10,000 rpm for a period of 15 Seconds was carried out. The resulting flow through was disposed off and the collection tube was used again in the next step.

6.700µl of RW1 buffer was put into the RNS column. After careful closing of the tube, centrifugation at 10,000 rpm for a period of 15 Seconds was carried out to wash up the spin column membrane. The resulting flow through was disposed off and the collection tube was used again in the next step.

7.500µl of RPE buffer was put into to RNS column. After careful closing of the tube, centrifugation at 10,000 rpm for a period of 15 Seconds was carried out to wash up the spin column membrane. The resulting flow through was disposed off and the collection tube was used again in the next step.

8. 500µl of buffer RPE was put into RNS column. After careful closing of the tube, centrifugation at 10,000 rpm for a period of 15 Seconds was carried out to wash up the spin column membrane. Thus the membrane of the spin column was dried out after extended centrifugation. This ensured that during the elution of RNA no ethanol was present. As a result of leftover ethanol, subsequent processes may be hampered.

9. RNS column was positioned in a fresh collection tube and the old one tube containing the flow through was thrown away. After closing the lid of the tube gently, centrifugation at max speed for a period of one minute was carried out to remove any likely remnants of RPE buffer, or if leftover flow through lingered on the rims of the RNS column after step 8.

10. The RNS column was shifted to into a fresh collection tube(1.5 ml). The spin column membrane was immediately injected with 30–50 µl of RNAse-free water. After closing the lid of the tube gently, centrifugation at 10,000 rpm for a period of one minute was carried out to elute the RNA.

RNA clean up

RNA was cleaned up by using RNeasy protect Mini kit (Qiagen inc., USA).

At room temperature, all of the RNA extraction processes were completed promptly.

All the steps of centrifugation were carried out at 20-25C in regular microcentrifuge tubes. It was ensured that centrifuge does not cool below 20C.

Buffer RPE was delivered in a concentrated form. Before use, to make a functioning solution, 4 volumes of ethanol (96–100 percent) were added as per instructions of the manufacturer.

In all the steps the collection tubes used were of 2 ml except when mentioned otherwise.

Procedure

1.Using RNAse-free water, the sample volume was adjusted to 100µl. A 350 µl volume of RLT buffer was added and thoroughly mixed.

2. 250 µl ethanol (96–100%) was pipetted into the diluted RNA and stirred well.

3. The sample having volume of 700 µl was shifted to an RNS column positioned in a collection tube. After careful closing of the tube, centrifugation at 10,000 rpm for a period of 15 Seconds was carried out . After carrying out centrifugation, the RNS column was cautiously taken out of the collection tube such that there was no contact between column and flow-through. The collection tube was emptied thoroughly. The flow through was

disposed off, RNS column was positioned in the tube and the collection tube was used again in the next step.

4. 500µl of buffer RPE was put in to RNS column. After closing the lid of the tube gently, centrifugation at 10,000 rpm for a period of 15 Sec was carried out to wash up the spin column membrane. After disposing off the flow through, the collection tube was used again in the next step.

5. RPE buffer having volume of 500 µl was put in RNS columnAfter careful closing of the tube, centrifugation at 10,000 rpm for a period of 15 Seconds was carried out to wash up the spin column membrane. Thus the membrane of the spin column was dried out after extended centrifugation. This ensured that during the elution of RNA no ethanol was present.As a result of leftover ethanol, subsequent processes may be hampered.

6. RNS column was positioned in a fresh collection tube and the old one with the flow through was thrown away. After closing the lid of the tube gently, centrifugation at max speed for a period of one minute was carried out to remove any likely remnants of RPE buffer, or if leftover flow-through lingers on the rims of RNS column after step 5.

7.The RNS column was shifted to a fresh collection tube(1.5 ml). The spin column membrane was immediately injected with 30–50 µl of RNAse-free water. After closing the lid of the tube gently, centrifugation at 10,000 rpm for a period of one minute was carried out to elute the RNA.

Gene Expression analysis

After RNA extraction and purification,the quality of RNA was checked by using 1.0% agarose gel electrophoresis and RNA samples were quantified by spectrophotometry at 260nm by using Nanodrop- 1000 spectrophotometer (Thermo Scientific, USA). For the synthesis of first-strand cDNA, total RNA (8.0 µg) was reverse transcribed by using Invitrogen SuperScript^{TM} IV Reverse Transcriptase kit (Thermo Fisher Scientific, USA), following the manufacturer's instructions. Real-time RT-qPCR was run using an SYBR Premix ExTag^{TM} II kit (TaKaRa, Japan) with a LightCyler 480 system. Specific primer sequences (listed in Table.7) of target genes were designed using the primer blast tool and

RT-qPCR was run by following the method reported by Duan et al. (2017) for investigation of the expression of digestion and immune-related genes including, Amy, Tryp, Lip, FAS, FASB, SOD, Lys, Def, NK-lys, and IL-β. For the calibration of the cDNA template and confirmation of successful reverse transcription, the β-actin gene of common carp was chosen as an internal control. 2─ΔΔCT comparative CT method was used to calculate relative gene expression, and shown as a fold change in expression, in comparison to the control group.

Fish gut microbiome Analysis

Intestinal DNA was extracted from 3 fish per tank, using DNeasy PowerSoil®Kit (Qiagen Inc., USA) according to manufacturers' instructions. The extracted DNA samples were qualitatively analyzed in 1.0% agarose gel electrophoresis and then quantity was determined at 260nm by using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). To generate a DNA library, the extracted DNA samples were subjected to PCR. The v4 region of bacterial 16S rRNA gene and internal transcribed spacer (ITS) regions of fungal 18S rRNA gene were amplified by using 515F (GTGCCAGCMGCCGCGGTAA) /806R (GGACTACHVGGGTWTCTAAT) and ITS2 primer pairs (ITS-3F, 5′-GCATCGATGAAGAACGCAGC-3′; ITS-4R, 5′-TCCTCCGCTTATTGATATGC-3′) respectively (Moncada et al., 2013; Walters et al., 2016). HotStarTaq Plus Master Mix Kit (QIAGEN, USA) was used to perform the PCR program. The PCR reaction conditions were initial denaturation at 95◦C for 5 min, followed by 27 cycles of denaturation at 95◦ C for 30s, 55◦C for 30s, and 72◦C for 45s. The final step was carried out at 72◦C and lasted for 10 min-. For the determination of length differences, the PCR fragments were subjected to 2.0% agarose gel electrophoresis. A PCR purification kit (QIAquick PCR Purification Kit) was used to purify the target bands. The purified PCR product was pooled and subjected to sequencing by Macrogen, USA using Illumina Miseq platform according to the manufacturer's instructions. The resulting sequences were processed and analyzed using the Bioinformatics package QIIME, version 2.0. For this, the raw data was imported into QIIME and the sequences for each sample were demultiplexed using the q2-cutadapt plugin. All the primer sequences and barcodes were removed. The sequences were also denoised and merged following the recommended protocol (Hall and Beiko, 2018). The resulting reads were clustered into operational taxonomic units (OTUs), defined at different similarity cut-off

levels. The taxonomic classification of the final OTUs was done by using BLAST against a curated database derived from RDPI, RDPII, and NCBI.

Statistical analysis

GraphPad prism5 software was used for statistical analysis. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple range test was used to carry out a comparison among treatments. Microbial diversity was assessed among samples using OTUs, Simpson, and Shannon indices. Significance was set at $P<0.05$

Table 7 : Primer sequences used in this study[1]

Primer Name	Sequence (5'–3')	Accession number
Lys-F	ACGCTGTGATGTTGTCCGTA	XM019104788
Lys-R	GTAGGCCGTGCACACATAGT	
SOD-F	ACAGTTGTGAATCGGGCCAA	XM019064760
SOD-R	GCAACGCCAGCAATATCGAG	
FAS-F	ATTGCTCCAGCCCTTCTGTC	XM019099327
FAS-R	ACGTGCTGATCCAACGTGAT	
FASB-F	TATTCTCTGGCAGACGGCAC	XM019107043
FASB-R	CTTTCCGTTGTCCTTGCGTG	
Amy-F	CAACCCCGACTCTACCTGTG	XM019081800
Amy-R	CGGAAGGCCACCATGTTTTT	
Lip-F	GAAGGTCCACTTCTTCGGCA	XM019064376
Lip-R	TTCCTTCTCGCCGTGGATTC	
Tryp-F	ACCACTTTTGTGGTGGCTCT	XM019110818
Tryp-R	ATGTTGTGCTCACCCAGACG	
Def-F	TGTGGATATCGAGGGCTGTG	NM001081555
Def-R	ACAGCACCTGTATCTTCGAG	
NK-lys-F	GACCATCCGAATCCTGTGAGG	XM019114515
NK-lys-R	TCATCTGTTTGTTCTCCGTTGC	
IL1-βF	ACCAGCTGGATTTGTCAGAAG	AB010701
IL1-βR	ACATACTGAATTGAACTTTG	

[1]Lys, lysozyme; SOD, superoxide dismutase; FAS, fatty acid synthase; Def, Defensin; FABP, fatty acid binding protein; Amy, amylase; Lip, lipase; Tryp, trypsin; Nk-lys, NK-lysin; IL1-β, interlukein1-β

Results

Chapter 1

Results

Screening of the High β -mannanase Activity Producing Strain

Six fungal strains that were active producers of β-mannanase and created colonies on mannan agar plate were selected for secondary screening. For this, these strains were grown in liquid media containing LBG as the only carbon source. Out of six isolates, one fungal strain proved to be a potent producer of β-mannanase enzyme. This strain was selected for further study and it was designated as *Aspergillus niger* AD-01.

Identification and molecular characterization of selected β-mannanase producing fungal strain

The selected strain was sub-cultured on LBG agar medium. At first the colonies were white, covered with feathery white aerial mycelia but as the culture matured, the colonies turned black and powdery. The back of the mature colony was buff colored (Fig. 1). Analysis of 18S rRNA sequence of the fungal isolate showed a homology of 98% with *Aspergillus niger* strains in NCBI database. The nucleotide sequence of the strain AD-01 was submitted in NCBI under accession number MN239884.

Optimization of culture conditions for enhanced β-mannanase production

Different parameters were optimized for maximum β-mannanase enzyme production: carbon source; pH; temperature; nitrogen source and time of incubation.

Among different carbon sources investigated, highest production of β-mannanase enzyme was achieved when locust bean gum (LBG) was used as the only carbon source (Table.8). Simple sugars like glucose, mannose, xylose and galactose did not induce β-mannanase production.In addition, how different nitrogen sources influence the production of β-mannanase enzyme by *Aspergillus niger* AD-01 was also investigated. β-mannanase enzyme production increased considerably when organic sources of nitrogen were used, instead of inorganic ones (Table 9).

In pH optimization studies, the effect of initial pH of the culture media on β-mannanase enzyme production was studied in the range of 3-8.In the present study, maximum production of β-mannanase enzyme was reached when the pH of the production medium was 5(Fig.2). To study the effect of temperature on β-mannanase enzyme production, different fermentation temperatures ranging from 20-60°C were tested. Maximum β-mannanase enzyme production

was achieved when the temperature of the production medium was 30°C(Fig3). For substrate concentration,maximum production of β-mannanase was achieved when substrate concentration was 1% (Fig4). After optimizing all the other parameters, the effect of time of incubation on β-mannanase enzyme production was investigated. For this, β-mannanase enzyme production was carried out under optimized conditions for 9 days. Maximum production of the enzyme was achieved at day 5(Fig 5).The enzyme production increased from 4.160 U/mg of protein in the nonoptimized media to 12.49U/mg of protein in the optimized medium. So there was 3 fold increase in the production of β-mannanase enzyme by optimizing the media composition.

Purification of β -mannanase

Crude β-mannanase enzyme produced on optimized culture parameters showed a specific activity of 12.49U/mg of total protein. The enzyme was purified by subjecting 500ml of fermentation liquid to ammonium sulphate precipitation and gel filtration chromatography. Different steps of purification like % yield, specific activity and purification fold are summarized in table (10).After multiple purification steps, β-mannanase enzyme was purified by 6 fold with 24% yield and the specific activity of β-mannanase, using LBG as sole carbon source reached 78.07U/mg. The purified β-mannanase was shown to be homogenous as examined by SDS-PAGE.

Apparent Molecular mass of β -mannanase

The apparent molecular mass of β-mannanase enzyme was found to be ~45kDa (Fig.6)

Characterization of β-mannanase

Biochemical characterization and stability studies were carried out in order to have a brief insight into the nature and possible applications of the isolated β-mannanase enzyme.The effect of temperature, pH, metal ions, solvents, proteinases and NaCl on the residual activity of β-mannanase enzyme was investigated.

Optimal temperature and thermostability of β-mannanase

The purified β -mannanase was maximally active at 40^{o}C (Fig .7) and showed >50% of the maximum activity at temperature 20^{o}C and at 10^{o}C the activity of ~ 20%. Figure. 8 shows the thermostability profile of β- mannanase enzyme. It is clear from Figure.8 that the enzyme is very stable at 37^{o}C after 60 min incubation. Approximately 50% of activity was retained at 40^{o}C for 30min. The half-life of the enzyme was 30min at 40^{o}C and only 10% of activity was retained after 10 minutes incubation at 50^{o}C.

Optimal pH and stability of β-mannanase

β-mannanase enzyme purified from *Aspergillus niger* AD-01 has been found to be maximally active at pH 6.0 and retains more than 25% of its maximum activity between pH 5.0 and 8.0 (Fig.9). The purified β-mannanase was found to have pH stability. It retained ~60% of its activity after incubating it in different buffers with pH ranging from 4-8 at 37°C for one hour(Fig.10)

The Effect of Inhibitors and Metal Ions on β -mannanase Stability

Table 11. Shows the effects of different inhibtors and metal ions on β-mannanase activity.It was found that the activity increased considerably in presence of β-mercaptoethanol and decreased drastically in presence of SDS.EDTA which envelops metal ions extensively, however did not inhibit β-mannanase activity . Among the different metal ions investigated β-mannanase activity was partially inhibited by Zn^{2+} and Co^{2+}(retaining80-85%) and strongly inhibited by Ba^{2+}, Pb^{2+} and Hg^{2+} (retaining <30% activity).

Substrate Specificity of β-mannanase enzyme

The purified β-mannanse enzyme activity was investigated against different substrates like LBG, CMC and oat spelt xylan with a concentration of 0.5%(w/v) and natural substrates like soybean meal, coffee shells, peanut shells, sugarcane bagasse and rice bran with a concentration of 2%(w/v).The results are presented in Table 12.It was observed that among the different tested substrates β-mannanase enzyme showed highest activity towards LBG while no activity towards oat spelt xylan or carboxy methyl cellulose. Among the natural

substrates tested the enzyme showed highest activity towards coconut pulp followed by soy bean meal.

Salt Tolerance

β-mannanase enzyme showed superior salt tolerance. It retained more than 80% of the activity in the presence of 0-4M NaCl (Fig.11).

The purified β-mannanase enzyme when incubated with 1M or 2M NaCl at 37^{o}C for one hour showed higher than 100% of its initial activity (Fig.12).

Proteinase resistance

Purified β-mannanase enzyme was resistant to proteases. There was no loss of activity when incubated for 60 min at 37^{o}C with trypsin and proteinase K.

Potential assessment of β-Mannanase enzyme as a feed additive for agastric fish

During the in vitro simulated intestinal digestion phase, 16.0-19.6,10.6-18.0,20.3-28.0 and 28.3-31.6 µmol/ml reducing sugars were amassed in 4-12 hours using 2.0%(w/v) Sun flower meal, wheat bran, rape seed meal and Soybean meal as simulated feeds respectively (Fig.13). The use of β-mannanase prevents viscosity of digesta because Mannan in the simulated feeds was degraded which is indicated by the release of reducing sugars.

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Table 8 : Effect of different carbon sources on production of β-mannanase enzyme by Aspergillus niger-AD01

Carbon Source (2% w/v)	Maximum β-Mannanase activity (U/ml)
Locust bean gum(LBG)	3.245±0.46
Guar gum	2.234±0.62
Konjac Powder	1.831±0.15
Glucose	0.003±0.05
Xylose	0.004±0.00
Mannose	0.001±0.00
Galactose	±0.002±0.02

Table 9 : Effect of different nitrogen sources on production of β-mannanase enzyme using 1% locust bean gum

Nitrogen Source (0.5% in the medium)	Maximum β-Mannanase activity (U/ml)
Ammonium Sulfate	4.243±0.87
Ammonium nitrate	3.165±1.30
Urea	2.851±0.99
Yeast extract	5.628±1.03
Sodium nitrate	3.678±1.45
Potassium nitrate	4.031±0.87
Peptone	4.819±1.40

Table 10 : Different steps of purification and percentage yield of β-mannanase enzyme from Aspergillus niger AD-01

Purification Stage	β-Mannanase activity(U/ml)	Total Protein(mg/ml)	Specific Activity(U/mg)	Purification fold	% yield
Crude enzyme extract	8.12	0.65	12.49	1	100
$(NH4)_3$ SO_3 precipitates	19.97	0.54	36.98	2.96	83.07
Purified enzyme	12.18	0.156	78.07	6.25	24

Table 11 : Effect of different metal ions (10mM) and chemical reagents on the activity of purified enzyme

Reagent	Relative Activity (%)
None	100±6.5
EDTA	118±12.5
SDS	0.0
Zn^{2+}	81.2±4.5
Co^{2+}	85.3±6.7
Ba^{2+}	13.5±5.4
Pb^{2+}	21.8±8.3
Mg^{2+}	140.6±9.8
Mn^{2+}	35.6±1.6
Cu^{2+}	110.5±1.8
Ca^{2+}	107.4±3.4
K^{2+}	92.6±2.5
β-Mercaptoethanol	160.5±1.5
Ni^{2+}	154.6±2.5
Hg^{2+}	15.7±3.5

Table 12 : . Effect of different substrates on β-mannanase activity

Substrate	Concentration	Relative β-Mannanase activity(%)
Locustbean Gum(LBG)	0.5	100±0.4
CMC	0.5	0
Oat Spelt Xylan	0.5	0
Soy bean meal	2	35.8±0.8
Coffee Shells	2	21.3±3.2
Coconut pulp	2	38.2±2.7
Peanut shells	2	13.6±2.3
Sugar cane bagasse	2	19.8±3.2
Rice Bran	2	26.6±2.6

Figure 1: selected β-mannanase producing fungal strain Aspergillus niger AD-01

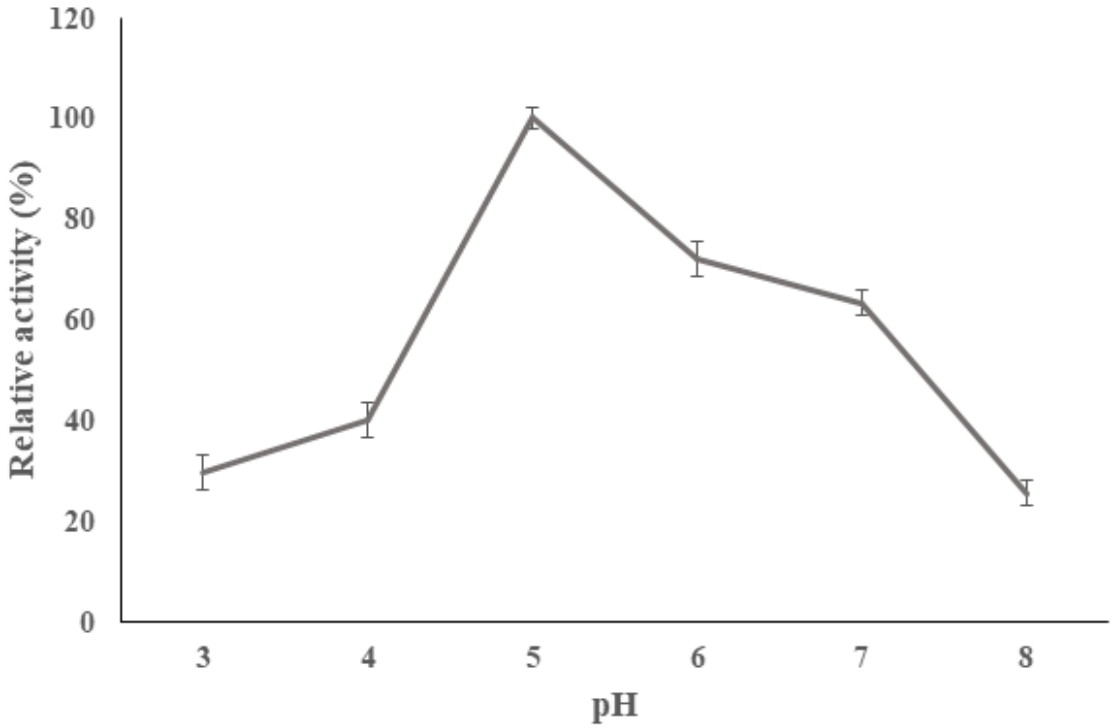

Figure 2: Effect of pH on production of β-mannanase

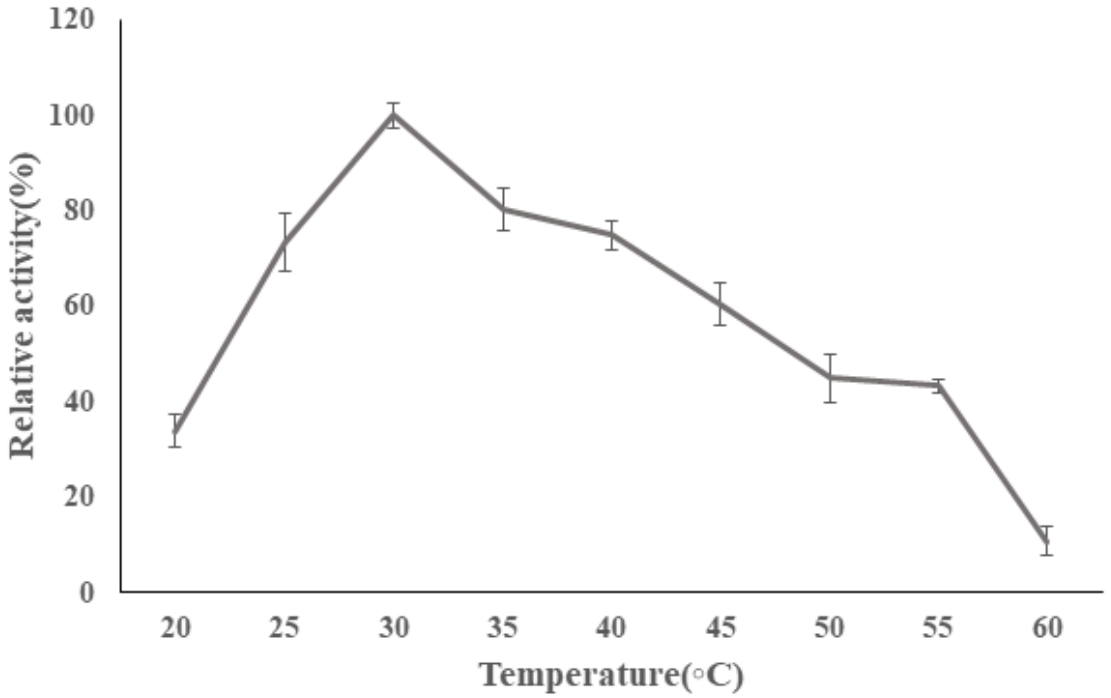

Figure 3: Effect of temperature on production of β-mannanase

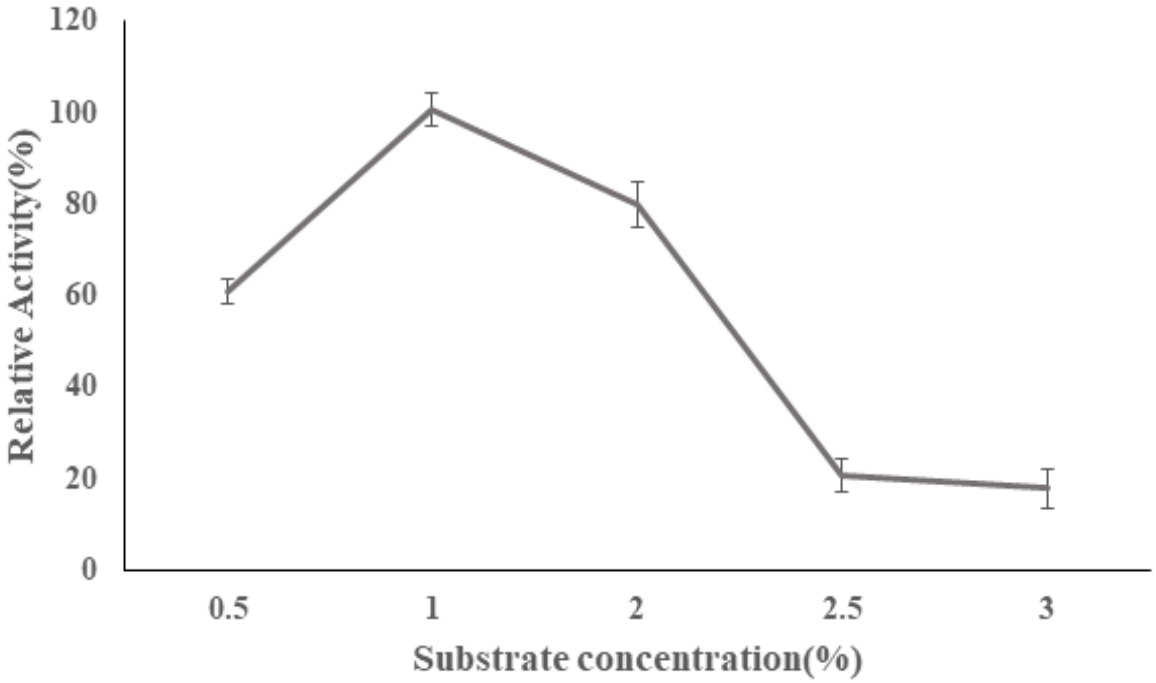

Figure 4 : Effect of substrate concentration on production of β-mannanase

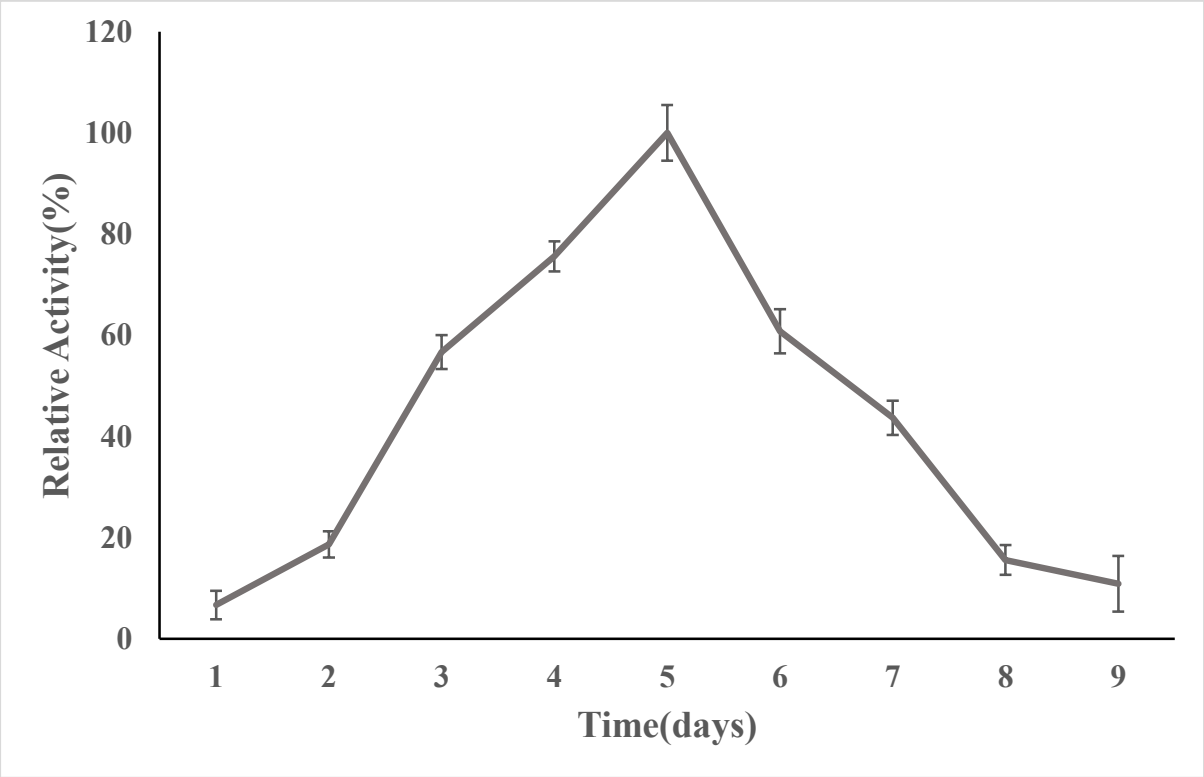

Figure 5 : Effect of time on production of β-mannanase

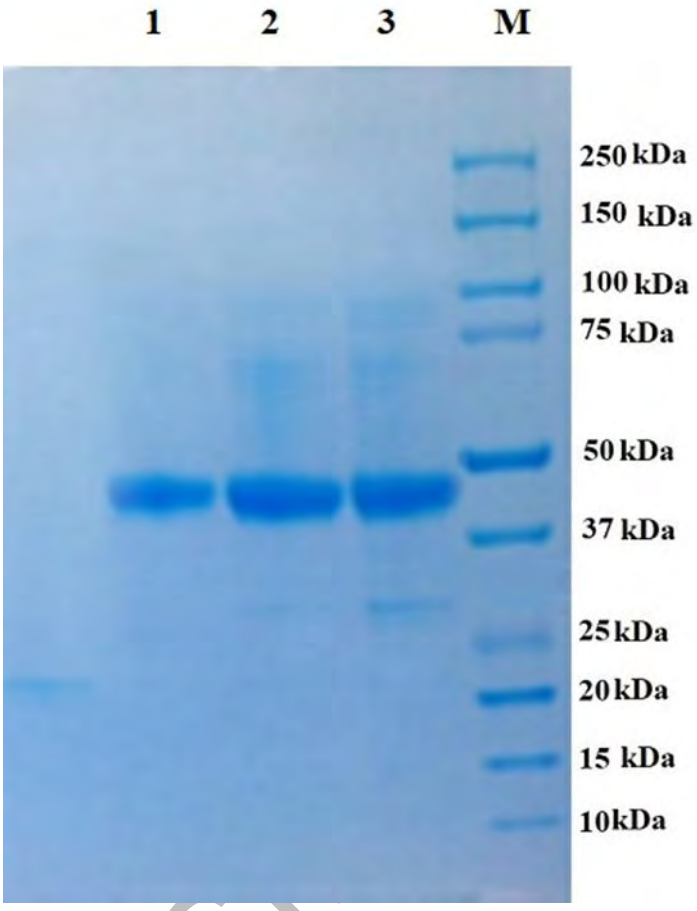

Figure 6 : SDS-PAGE analysis of purified β-mannanase enzyme. Lane M: protein molecular weight standard; Lane 1,2,3: purified β -mannanase

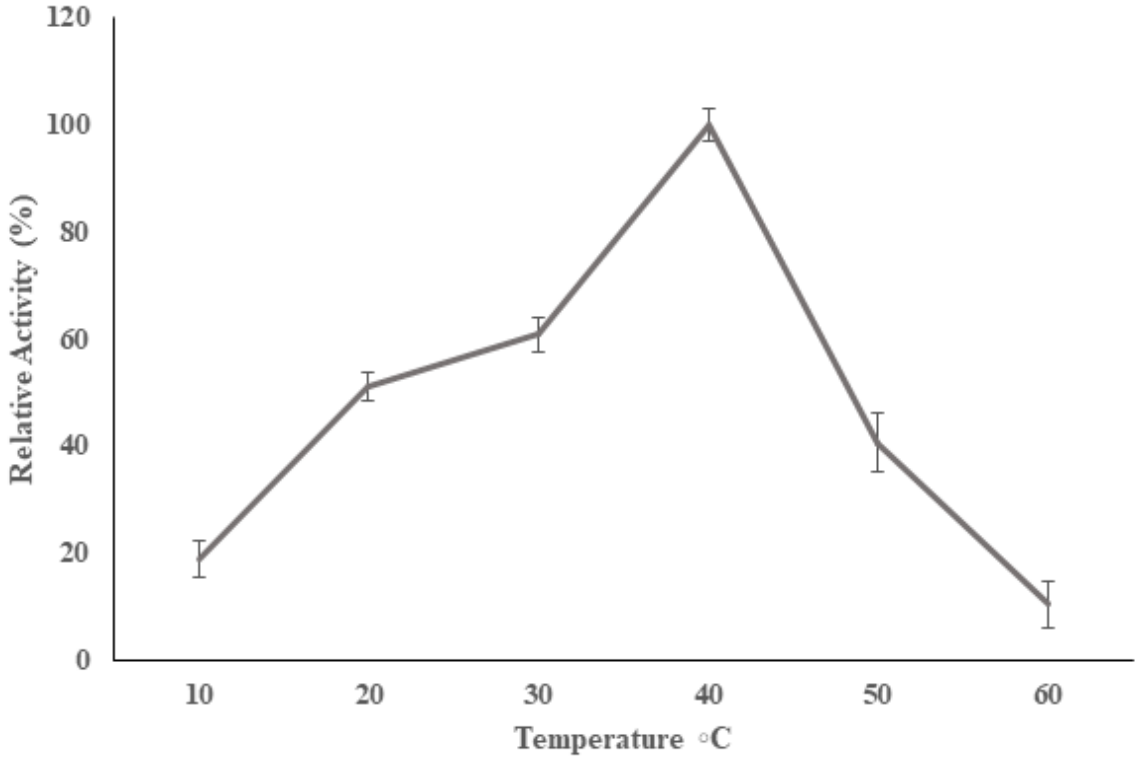

Figure 7 : Effect of temperature on activity of β-mannanase

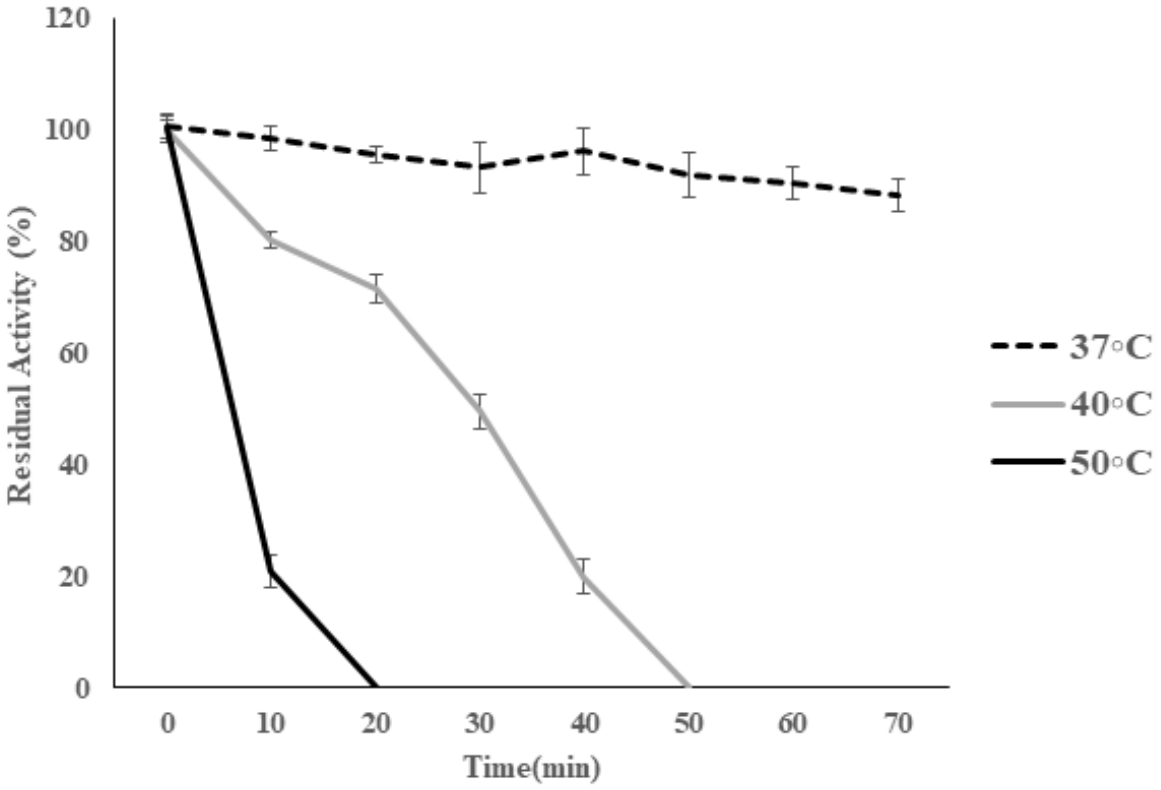

Figure 8 : Effect of temperature on stability of mannanase

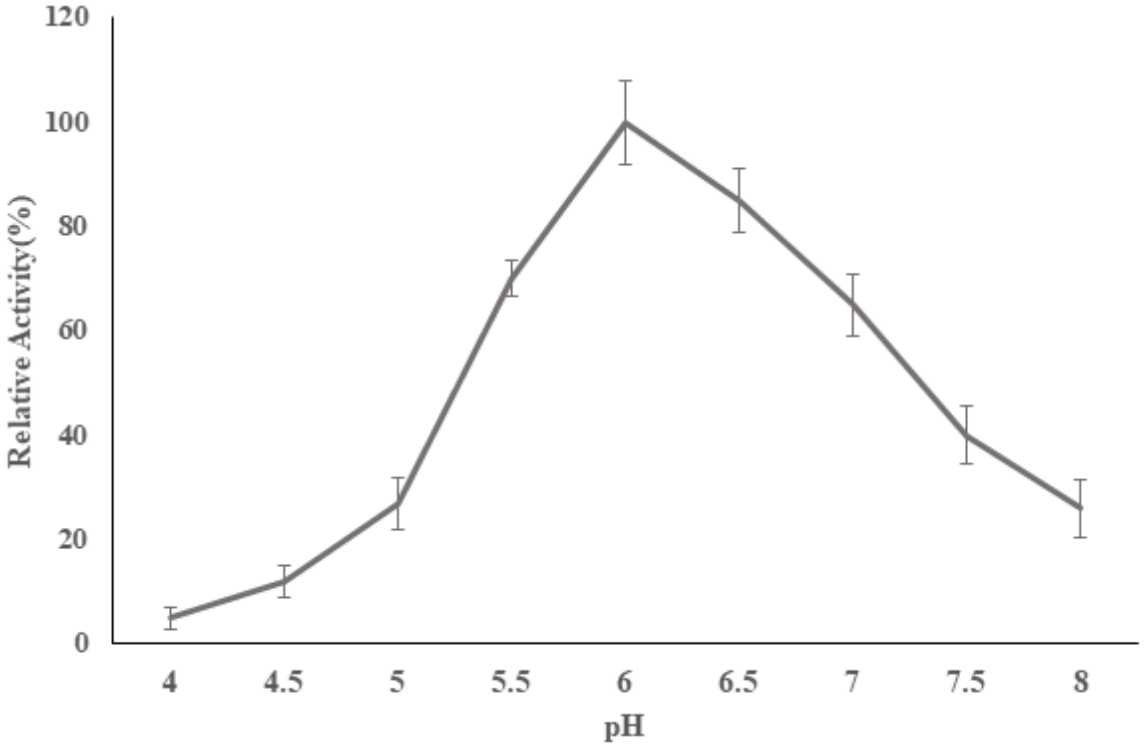

Figure 9 : Effect of pH on activity of β-mannanase

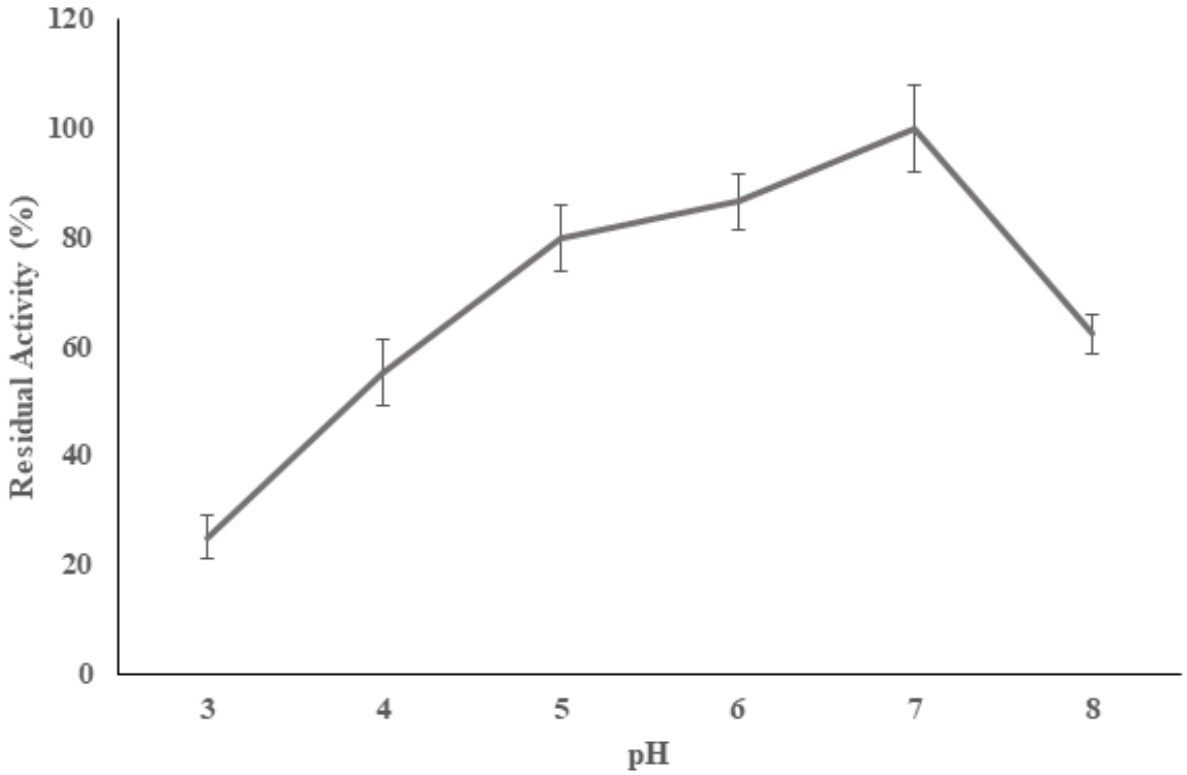

Figure 10 : Effect of pH on stability of β-mannanase

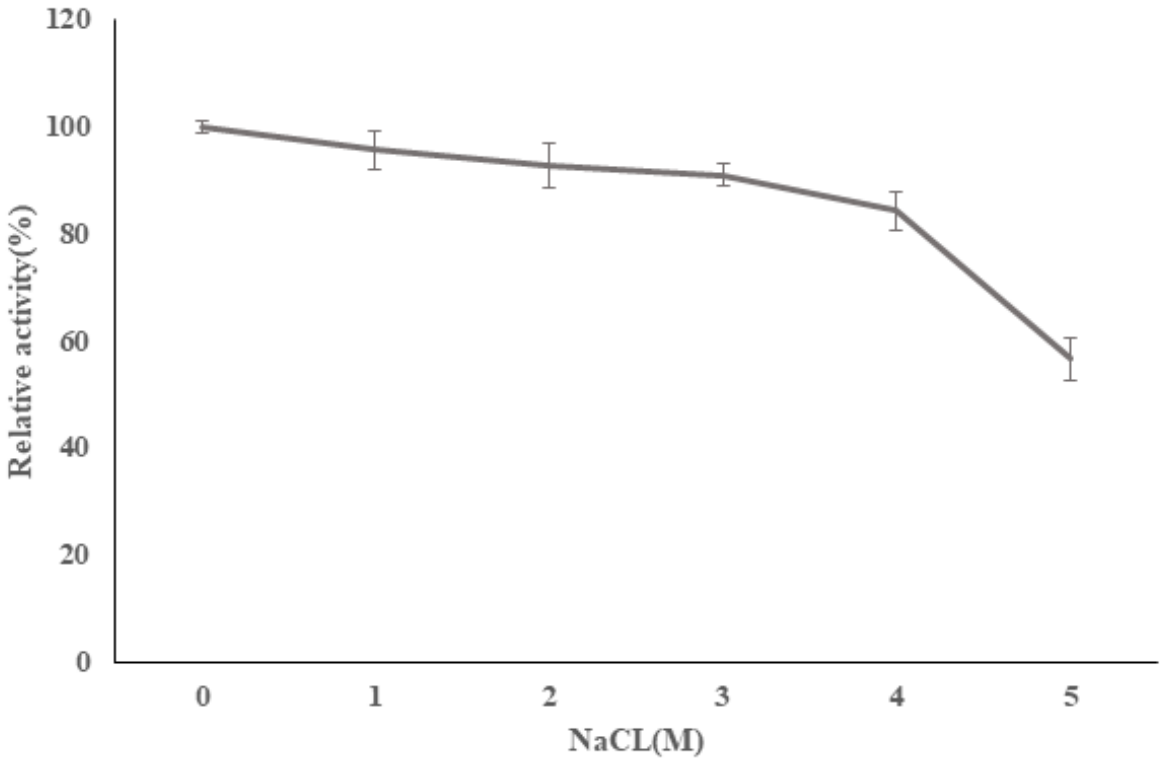

Figure 11 : Effect of NaCl on activity of β-mannanase

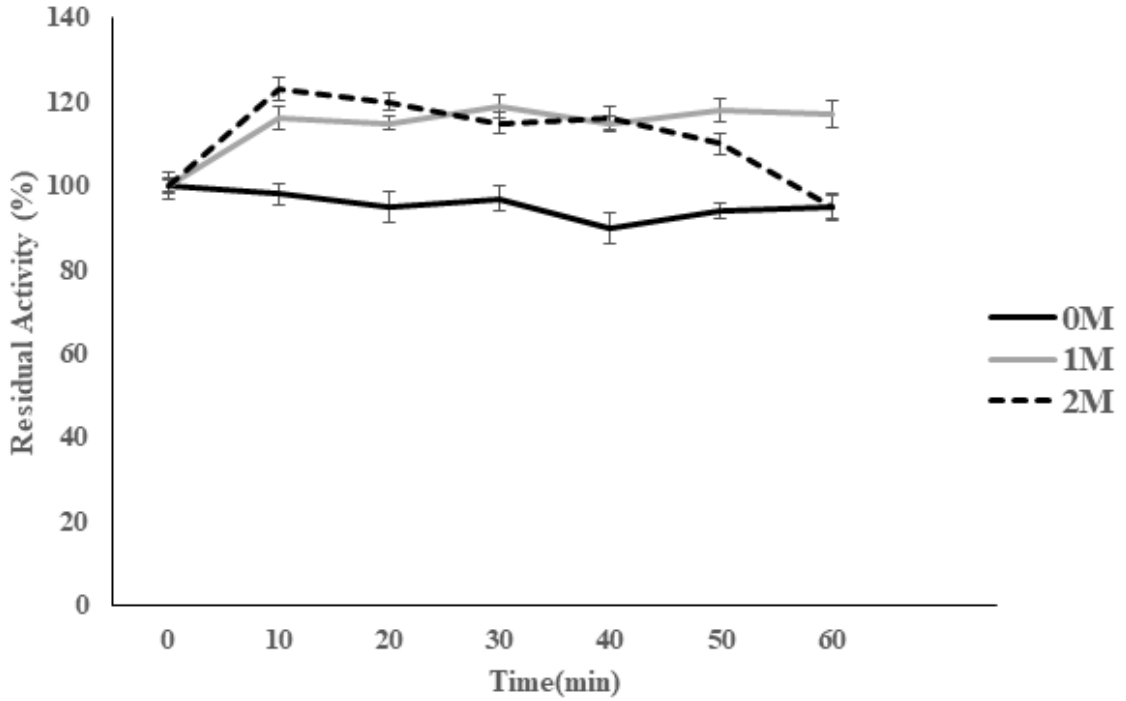

Figure 12 : Effect of NaCl on stability of β-mannanase

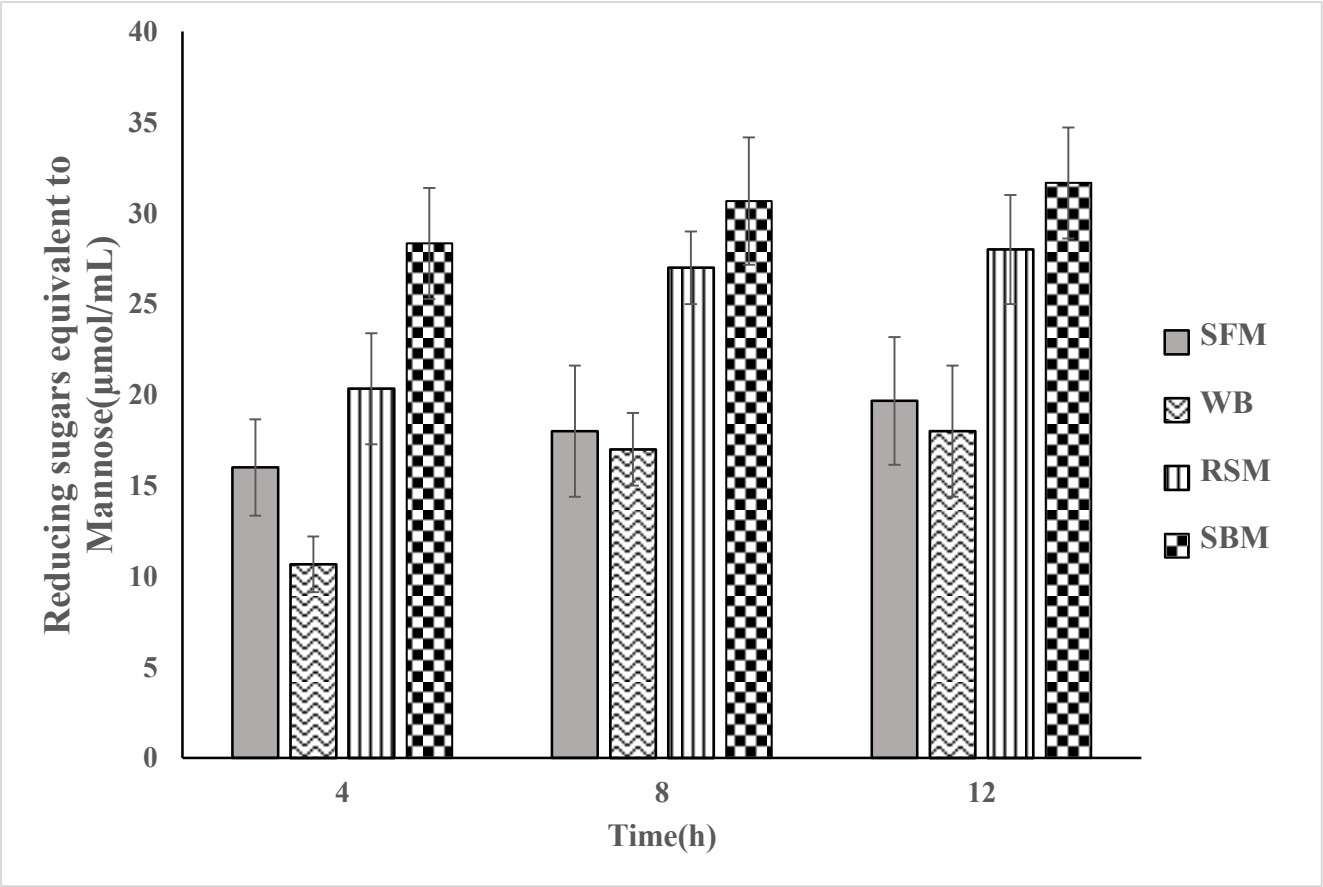

Figure 13 : Hydrolysis of 2.0%(w/v) substrates by the purified β-mannanase enzyme in simulated intestinal fluid.The error bars represent the means±SD(n=3)

Chapter.2

Apparent digestibility of nutrients and digestible energy

Exogenous enzyme β-mannanase addition showed significant effect on the apparent nutrient digestibility coefficients (ADC %) and digestible energy (Table 13). After 90 days experimental period, all groups of fish fed β-mannanase supplemented diet (A1, A2, B1, and B2) showed statistically similar (P> 0.05) but significantly higher (P<0.05) apparent nutrient digestibility coefficients of crude protein, crude fat and carbohydrates as compared to the control group of fish.Two-way ANOVA indicated significant effect of dosage level of β-mannanase (P<0.05) while non-significant effect of enzyme source (P>0.05) on the digestibility of crude protein, fats, carbohydrates and digestible energy.

Growth performance

The initial body weights of carp in control group C and experimental groups A1, A2, B1, and B2 were considerably similar ((P>0.05). However, at the end of the feeding trial, fish in groups A1, A2, B1, and B2 gained statistically similar (P> 0.05) but significantly more weight (P<0.05) than fish in group C. Similarly, the specific growth rate (%) was also significantly higher in groups of fish reared on β-mannanase supplementation than in control group C (Table 14). FCR value of all β-mannanase supplemented groups was also similar and lower than the control group.

Two-way ANOVA of all studied growth-related parameters, FCR and survival indicated significant difference (P< 0.05) among dosage levels, while non-significant difference (P> 0.05) among enzyme source, and the interaction between two variables (enzyme dosage × enzyme sources). However, all pairwise comparisons indicated statistically similar positive effects of both enzymes at both supplementation levels.

Muscle proximate composition

β-Mannanase supplemented diet showed a beneficial effect on the muscle composition of common carp (Table 15). We observed a significant increase in the protein and fat contents and a decrease in moisture (%) in the β-mannanase supplemented groups compared to a control group of fish. However, no significant difference was found in the ash content of the control and treatment groups. Again, two-way ANOVA showed significant

difference (P< 0.05) among dosage levels, a non-significant difference (P> 0.05) among sources of β-mannanase, and interaction between dosage levels and enzyme source (enzyme dosage × enzyme sources). However, all pairwise comparisons indicated statistically similar values of all proximate composition indices at both supplementation levels of both enzymes.

Intestinal enzyme activity

The activity of intestinal enzymes is an indication of digestion and absorption of the nutrients in the gastrointestinal tract. Here, supplementation of β-mannanase enhanced the intestinal enzyme activity of common carp (Table 16). After 90 days experimental period, all groups of fish fed β-mannanase supplemented diet (A1, A2, B1, and B2) showed statistically similar (P> 0.05) but significantly higher (P<0.05) amylase activity compared to the control group of fish. In comparison to amylase, the activity of protease, and cellulase increased with an increase in the dosage level of β-mannanase. Again, two-way ANOVA indicated an insignificant effect (P> 0.05) of enzyme source and interaction between dosage levels and enzyme source (enzyme dosage × enzyme sources) on intestinal enzyme activities.

Effect of β-mannanase supplementation on hematological indices of carp

β-mannanase also showed a significant effect on different hematological indices of carp. We found significantly higher (P<0.05) levels of WBC, RBC, Hb, HCT (%), and MCHC in the mannanase-fed groups than a control group (Table 17). However, MCH and MCV showed a significantly decreased level (P<0.05) in β-mannanase fed groups compared to a control group. Two-way ANOVA indicated an insignificant effect (P> 0.05) of enzyme source and interaction between dosage levels and enzyme source (enzyme dosage × enzyme sources) on all hematological indices of common carp. However, all pairwise comparisons indicated an almost similar positive effect of both enzymes on all hematological indices of common carp.

Effect of β-mannanase supplementation on immunological indices

Immunological indices like lysozyme activity, respiratory burst activity, immunoglobulin, and phagocytic activity also showed a significant increase in response to β-mannanase supplemented diets**.** Except for phagocytic activity and phagocytic index, all studied immunological indices showed similar levels in response to both sources of β-mannanase and at both dosage levels. However**,** phagocytic activity and phagocytic index showed a dose-dependent increasing trend ($P<0.05$). Similarly, significant interaction ($P<0.05$) between source and dosage effect was also found only on phagocytic activity (Table 18).

Effect of β -mannanase on metabolic enzymes and cholesterol level

β-mannanase supplemented diet also showed a significant effect on metabolic enzymes (Table 19). All groups of fish (A1, A2, B1, and B2) fed β -mannanase enriched diet showed a significant (($P<0.05$) dose-dependent decrease in serum AST and ALT activity while increase in cholesterol (CHO) and TG levels compared to the control group.

Two-way ANOVA indicated an insignificant effect ($P> 0.05$) of enzyme source and interaction between dosage levels and enzyme source (enzyme dosage × enzyme sources) on all hematological indices of common carp. However, all pairwise comparisons indicated an almost similar positive effect of both enzymes on both metabolic enzymes (AST and ALT activity) and serum cholesterol and triglyceride levels.

Gene expression

The β-mannanase supplemented diet showed a significant positive effect on MyoD and TNF-α gene expression in different tissues of *C. carpio* fingerlings. Result indicated significantly higher expression of the MyoD gene in the muscle of *C. carpio* fed diets supplemented with the enzyme (Fig. 14). Two tail tests indicated significantly higher MyoD expression in the muscle of B1 group (fed BM_{An}at the rate of500 units kg-1 diet), however at higher dosage level i.e., 1000 units kg-1 diet, both enzymes showed a statistically similar effect. Similarly, TNF-α gene in the muscle, intestine and liver of groups of fish fed β-mannanase supplemented diet also showed significantly higher expression (Fig. 15,16,17)

compared to a control group of fish. The pairwise comparison indicated that at higher dosage level (1000 units kg-1 diet) both enzymes showed statistically similar effect, i.e., higher expression of TNF-α gene in the muscle, liver, and intestine of *C. carpio* however, at lower dosage level (500 units kg^{-1} diet), both enzymes (BM_{An} and B_{MTr}) showed a variable effect in different tissues.

Table 13 : Effect of β-mannanase supplemented diets on apparent digestibility of major nutrients and digestible energy of common carp

Treatment groups	Crude protein	Crude fats	Carbohydrates	Energy	Digestible Energy (KJ g^{-1})
Control	70.76^{c}	73.6^{c}	64.01^{c}	68.16^{c}	14.08^{c}
A1	73.95^{a}	77.9^{ab}	67.58^{ab}	72.98^{ab}	15.66^{a}
A2	72.98^{ab}	76.78^{ab}	66.1^{b}	71.07^{b}	14.83^{b}
B1	71.8^{b}	76.1^{b}	67.36^{ab}	71.98^{ab}	15.2^{ab}
B2	73.50^{ab}	78.54^{a}	68.45^{a}	73.06^{a}	15.31^{ab}
Two-way ANOVA P-values					
Enzyme source	0.345	0.972	0.200	0.416	0.876
Dosage level	0.031	0.001	0.001	0.001	0.001
Interaction	0.118	0.052	0.119	0.357	0.265

Values are mean ±SD from triplicate groups of fish (n=6) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated β-mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

Table 14 : Effect of β-mannanase supplemented diets on growth performance of *C. carpio*

Treatment groups	Initial Body Weight (g)	Final Body Weight (g)	%Weight Gain	Specific Growth Rate (%/day)	Net Weight Gain	FCR	Survival (%)
Control	13.22±0.28^{a}	40.7	207.87±5.36^{b}	1.25±0.03^{c}	26.85±1.12^{c}	2.35±03^{a}	96.3±2.08^{b}
A1	13.19±0.36^{a}	46.62±1.9^{b}	253.49±14.72^{a}	1.39±0.04^{b}	33.43±1.88^{b}	1.9±0.19^{b}	100±0.0^{a}
A2	13.62±0.15^{a}	49.08±2.6^{a}	260.16±15.97^{a}	1.42±0.04^{a}	35.45±2.50^{b}	1.88±0.11^{b}	99.1±1.16^{a}
B1	13.35±0.28^{a}	51.44±2.9^{a}	285.68±29.86^{aa}	1.49±0.08^{a}	38.09±3.24^{a}	1.86±0.11^{b}	99.2±1.5^{a}
B2	13.05±0.20^{a}	48.1±1.57^{a}	268.53±10.53^{a}	1.44±0.02^{a}	35.05±1.47^{b}	1.83±0.12^{b}	98.6±1.65^{a}
Two-way ANOVA P-values							
Source		0.205	0.104	0.111	0.167	0.737	0.408
Dosage		0.001	0.649	0.001	0.000	0.000	0.004
Source × Dosage		0.062	0.313	0.223	0.097	0.969	0.817

Values are mean ±SD from triplicate groups of fish (n=9) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated β-mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

Table 15 : Effect of β-mannanase supplemented diet on the proximate composition of muscle of *C. carpio*

Treatment groups	Moisture (%)	Protein content (%)	Fat contents (%)	Ash (%)
Control	77.18±0.87[a]	13.70±0.74[b]	4.07±1.12[b]	2.76±0.25[a]
A1	72.08±1.79[b]	16.24±0.91[a]	5.95±0.54[a]	2.43±0.46[a]
A2	73.15±1.45[b]	16.95±0.73[a]	6.85±0.82[a]	2.32±0.50[a]
B1	72.81±1.35[b]	15.98±1.49[a]	6.10±1.27[a]	2.36±0.70[a]
B2	73.70±1.41[b]	16.55±1.2[a]	6.07±0.80[a]	2.57±0.64[a]
Two-way ANOVA P-values				
Source	0.245	0.470	0.374	0.677
Dosage	0.000	0.000	0.000	0.087
Source × Dosage	0.695	0.862	0.242	0.619

Values are mean ±SD from triplicate groups of fish (n=9) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated β-mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

Table 16 : Effect of β-mannanase on intestinal enzyme activity in *C. carpio*

Treatment Groups	Cellulase	Protease	Amylase
Control	0.426±0.16^{c}	0.34 ±0.09^{c}	0.47±0.17^{b}
A1	1.63±0.24^{b}	1.15± 0.13^{b}	1.37±0.27^{a}
A2	2.23±0.20^{a}	1.29± 0.14^{a}	1.45±0.23^{a}
B1	1.83±0.16^{b}	1.22±0.11^{b}	1.32±0.21^{a}
B2	2.33±0.27^{a}	1.43±0.20^{a}	1.53±0.21^{a}
Two-way ANOVA-P values			
Source	0.230	0.114	0.905
Dosage	0.0001	0.0001	0.0001
Source × Dosage	0.302	0.443	0.924

* Values are mean ±SD from triplicate groups of fish (n=9) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated β-mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

Table 17 : Effect of β-mannanase on hematological indices of *C. carpio*

Treatment Groups	RBC (10^6µL)	WBC(10^3µL)	Hb(g/dL)	HCT(%)	MCV(10^{-15}L)	MCH	MCHC
Control	1.56±0.12^{c}	142.2±1.21^{c}	5.14±0.12^{c}	19.3±1.42^{c}	201.66±7.09^{a}	33.74±1.98^{a}	14.72±0.85^{c}
A1	1.94±0.06^{b}	184.06±3.8^{a}	8.07±0.22^{a}	25.59±2.58^{b}	145.32±9.51^{c}	26.18±1.85^{bc}	18.79±1.96^{b}
A2	2.08±0.13^{a}	181.9±2.95^{a}	7.71±0.33^{b}	27.02±2.3^{b}	176.15±4.47^{b}	25.7±2.25^{c}	21.26±1.4^{a}
B1	1.92±0.10^{b}	178.2±4.45^{b}	7.65±0.24^{b}	29.59±2.15^{a}	174.6±10.01^{b}	25.93±2.65^{c}	18.46±0.58^{b}
B2	1.88±0.10^{b}	185.1±4.6^{a}	8.23±0.66^{a}	24.94±2.77^{b}	180.12±6.44^{b}	27.36±2.85^{b}	19.86±1.15^{b}
Two-way ANOVA P-values							
Source	0.270	0.265	0.844	0.239	0.006	0.413	0.104
Dosage	0.001	0.001	0.001	0.001	0.0244	0.001	0.001
Source× dosage	0.396	0.001	0.094	0.001	0.0042	0.336	0.238

* Values are mean ±SD from triplicate groups of fish (n=9) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

Table 18 : Effect of β-mannanase supplementation on immunological indices of *C. carpio*

Treatment Groups	Total serum Protein g/dl)	Globulin ($mgml^{-1}$)	Lysozyme Activity (µg/ml)	Respiratory burst Activity	Phagocytic activity (%)	Phagocytic index
Control	2.91 ± 0.24^{b}	17.82 ± 0.46^{b}	1.93 ± 0.25^{b}	0.28 ± 0.04^{b}	23.45 ± 1.0^{d}	1.38 ± 0.25^{c}
A1	3.71 ± 0.21^{a}	26.6 ± 2.57^{a}	3.29 ± 0.20^{a}	0.72 ± 0.03^{a}	57.82 ± 1.93^{c}	2.19 ± 0.187^{b}
A2	3.90 ± 0.22^{a}	25.3 ± 2.17^{a}	3.22 ± 0.37^{a}	0.81 ± 0.03^{a}	64.53 ± 1.36^{a}	2.45 ± 0.32^{a}
B1	3.76 ± 0.188^{a}	24.72 ± 2.70^{a}	3.13 ± 0.33^{a}	0.78 ± 0.07^{a}	61.87 ± 1.33^{b}	2.28 ± 0.414^{b}
B2	3.62 ± 0.36^{a}	25.53 ± 2.41^{a}	3.63 ± 0.27^{a}	0.74 ± 0.08^{a}	63.71 ± 0.97^{a}	2.35 ± 0.37^{a}
Two-way ANOVA p-values						
Source	0.304	.307	0.103	0.972	0.006	0.934
Dosage	0.001	0.001	0.001	0.001	0.001	0.001
Source × Dosage	0.230	0.373	0.410	0.021	0.001	0.645

* Values are mean ±SD from triplicate groups of fish (n=9) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated β-mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

Table 19 : Effect of β-mannanase on blood metabolic indices of *C. carpio*

Treatment	ALT(UL^{-1})	AST(UL^{-1})	CHO(mgdL^{-1})	TG(mgdL^{-1})
Control	25.73±0.	63.26±1.8	10.9±0.8	16.02±1.2
A1	19.04±0.	48.21±0.9	11.27±1.	16.9±0.98
A2	17.82±1.	46.29±1.1	12.75±0.	17.8±1.06
B1		48.66±0.9	11.53±0.	17.4±0.74
B2	18.86±0.	47.60±1.9	13.04±0.	18.2±0.95
Two-way ANOVA P-value				
Enzyme	0.877	0.175	0.574	0.297
Enzyme	0.001	0.001	0.001	0.001
Source ×	0.02	0.446	0.922	0.750

* Values are mean ±SD from triplicate groups of fish (n=9) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated β-mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

Table 20 : Proximate composition of faeces (% dry matter)

Treatment groups	Crude protein	Crude fat	NFE	Chromium oxide	Crude fibre	Crude Ash	Gross energy (KJ g^{-1})
Control	29.3	9.0	44.34	0.60	8.85	9.11	18.95
A1	27.5	8.15	42.62	0.64	9.5	9.91	18.28
A2	28.1	8.3	43.4	0.68	9.3	9.89	18.08
B1	27.9	8.29	42.73	0.68	9.21	9.78	18.12
B2	28.2	8.06	42.05	0.69	9.7	9.98	18.37

Values are mean ±SD from triplicate groups of fish (n=9) where the values within a column without a common superscript differ ($p<0.05$). A1 and A2 designate 500 and 1000 units kg^{-1} diet of commercial β-mannanase while B1 and B2 designate 500 and 1000 units kg^{-1} diet of isolated β-mannanase enzyme respectively. Control group contains 0 units kg^{-1} diet of β-mannanase.

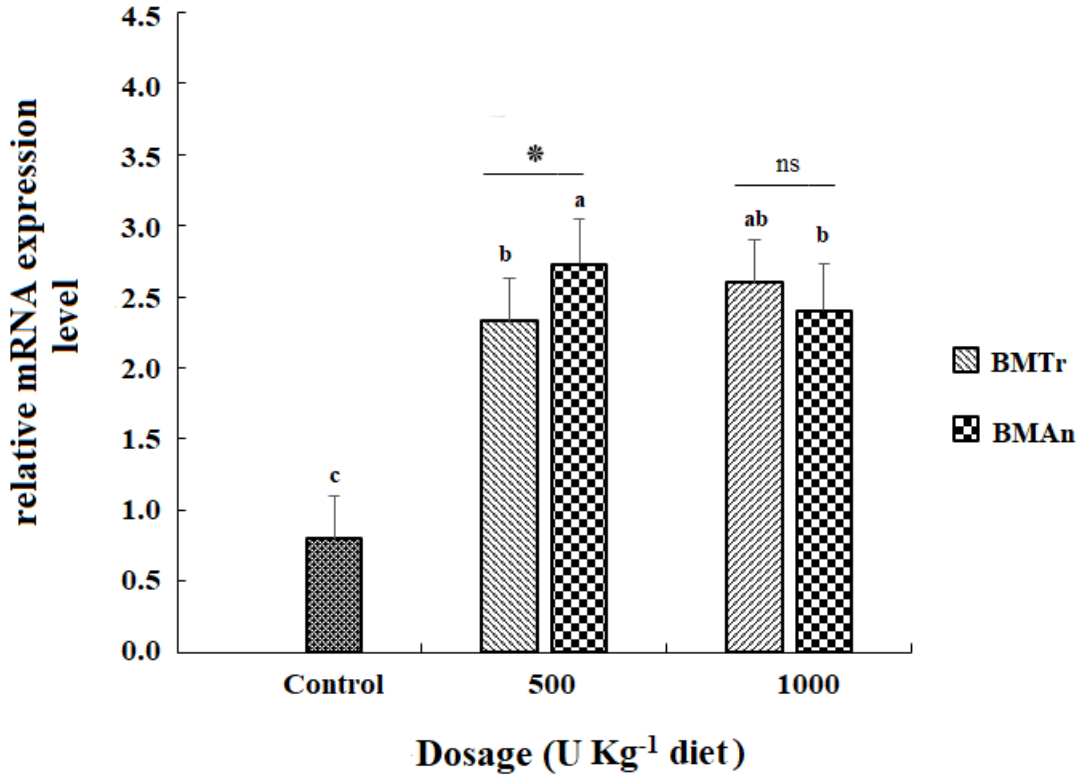

Figure 14 : MyoD gene expression in the muscle of *C.carpio* fingerlings after 90 days of feeding β-mannanase supplemented diet. The bar shows the values as average ± SD, n =9. ANOVA followed by LSD post hoc test represent comparisons between groups, while T-test compares the results of both enzymes (BM_{Tr} and BM_{An}) at similar dosage level. ns = non significant , * = P< 0.05. BM_{Tr} = fermentation product of *Trichoderma reesei* . BM_{An} = fermentation product of *Aspergillus niger*

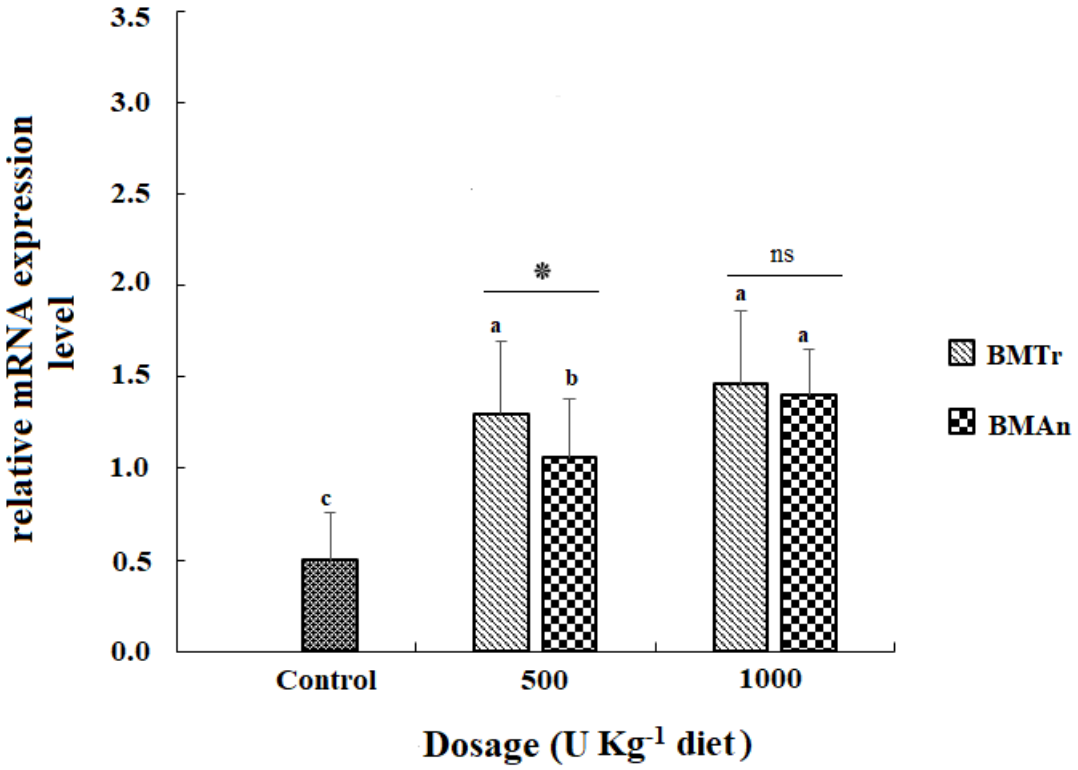

Figure 15 : TNF-α gene expression in the muscle of *C.carpio* fingerlings after 90 days of feeding β-mannanase supplemented diet. The bar shows the values as average ± SD, n =9. ANOVA followed by LSD post hoc test represent comparisons between groups, while T-test compares the results of both enzymes (BM_{Tr} and BM_{An}) at similar dosage level. ns = non significant , * = P< 0.05. BM_{Tr} = fermentation product of *Trichoderma reesei* . BM_{An} = fermentation product of *Aspergillus niger*

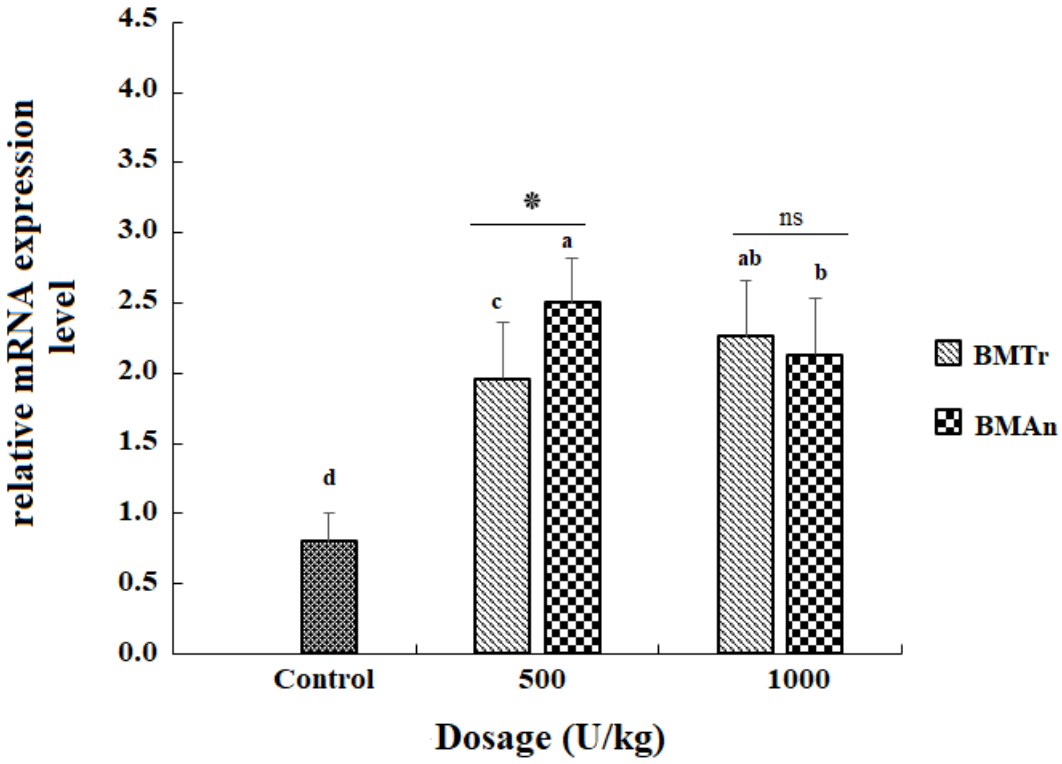

Figure 16 : TNF-α gene expression in the intestine of *C.carpio* fingerlings after 90 days of feeding β-mannanase supplemented diet. The bar shows the values as average ± SD, n =9. ANOVA followed by LSD post hoc test represent comparisons between groups, while T-test compares the results of both enzymes (BM_{Tr} and BM_{An}) at similar dosage level. ns = non significant , * = P< 0.05. BM_{Tr} = fermentation product of *Trichoderma reesei* . BM_{An} = fermentation product of *Aspergillus niger*

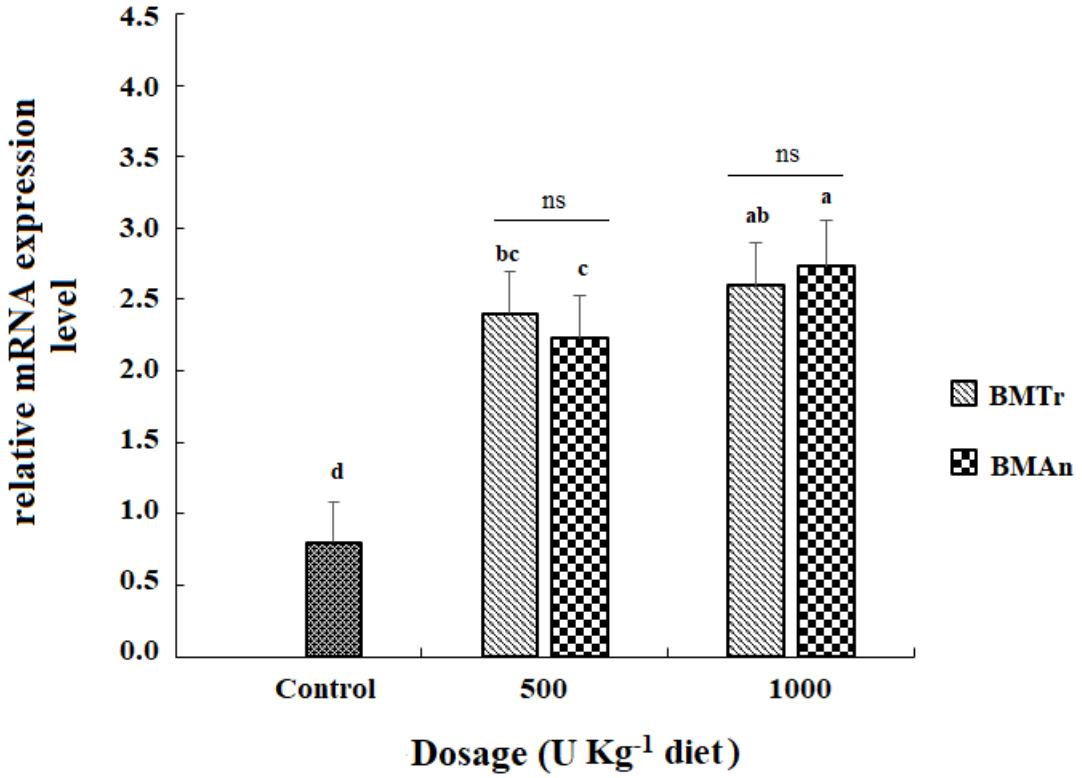

Figure 17*:* TNF-α gene expression in the liver of *C.carpio* fingerlings after 90 days feeding of β-mannanase supplemented diet. The bar shows the values as average ± SD, n =9. ANOVA followed by LSD post hoc test represent comparisons between groups,

, while T-test compares the results of both enzymes (BM_{Tr} and BM_{An}) at similar dosage level. ns = non significant, M_{Tr} = fermentation product of *Trichoderma reesei* . BM_{An} = fermentation product of *Aspergillus niger*

Chapter.3

Results

Expression levels of intestine immunity-related genes

The relative expression of immune-related genes, including NK-lys, Def, IL1-β, Lys and SOD increased in both treatment groups A1 and A2 compared to the control group ($P<0.05$) (Fig.1). However, there was no significant difference between antioxidant-related genes (SOD, Lys) between treatment groupA1 and groupA2 ($P > 0.05$). However, the expression of the mucosal immunity-related gene (IL1-β) and antimicrobial genes (Def, NK-lys) increased in a dose-dependent manner and thus was highest in the A2 group ($P<0.05$).

Expression levels of intestine digestion-related genes

The relative expression level of all the five genes related to digestion of nutrients, including Amy, Lip Tryp, FAS, and FABP was increased in the treatment groups (A1 and A2) as compared to control ($P<0.05$) (Fig.2). The highest expression level of all the five genes was detected in group A2. There was a significant difference ($P< 0.05$) between the expression level of the genes Amy and FAS in group A2 as compared to group A1. But no significant differences were observed in the expression level of Tryp, Lip, and FABP genes between groups A1 and A2 ($P > 0.05$).

Fish gut microbial analysis

Richness and diversity

DNA was extracted from 3 fish per tank (12 per treatment group). However, a few samples showed poor PCR amplification and thus were discarded, leaving 10 samples per treatment group for Illumina Miseq sequencing. After optimization and filtration of the raw data, a total of 2,524,849 high-quality sequencing reads were obtained from 60 samples with an average of 42,080 per sample. The total number of observed OTUs ranged from 81 to 169. Diversity analysis in terms of Simpson (alpha diversity) and Shannon (beta diversity) indices was increased in the treatment groups (A1 and A2) compared to control (Table 21).

Genera and phyla performances

Six phyla were detected in the control group: Proteobacteria (81.05%), Actinobacteria (12.6%), Bacteroidetes (4.21%), Firmicutes (1.03%), Planctomycetes (0.68%), and Chlamydia (0.0034%). Dominating bacterial genera were *Pseudomonas (74.27*%),

Streptomyces (9.08%), *Desulfobulbus* (1.78%) and *Haliobacteriovorax* (0.8%) (Fig.20). The control group showed five fungal phyla: Ascomycota (3.02%), Basidiomycota (96.28%) Glomeromycota (0.0085%), Neocallimastogomycota (0.0064%) and Cryptomycota (0.002%). Dominating fungal genera were *Debaryomyces* (80.23%) *Cryptococcus* (5.25%) and *Malassezia* (3.98%)(Fig.21)

Metagenomic analysis revealed that A1 group had 7 phyla Proteobacteria (91.7%), Firmicutes (3.06%), Actinobacteria (2.88%) Cyanobacteria (0.95%), Planctomyces (0.78%) Bacteroidetes (0.0456%) and Spirochete (0.0031%). At the genus level, dominating bacteria were *Achromobacter* (60.03%) *Klebsiella* (28.2%) and *Lactococcus* (3.02%) (Fig.22). Dominating fungal phyla in group A1 were Ascomycota (80.06%), Basidiomycota (19.02%) and Cryptomycota (0.0056%)(Fig.3b). Dominating fungal genera were *Galactomyces* (42.75%) *Geotrichum* (26.31%) and *Cryptococus* (16.78%). However, group A2 was represented by six phyla Proteobacteria (96.08%), Firmicutes (3.29%), Fusobacteria (0.0078%), Actinobacteria (0.0051%), Planctomycetes (0.0044%) and Bacteroidetes (0.0034%). Dominating bacterial genera were *Pseudomonas* (81.56%), *Serratia* (11.23%) *Lactobacillus (1.50%), Lachnospira* (1.34%) and *Bacillus* (0.45%) (Fig.4a). Among fungal phyla three phyla were dominant Ascomycota (97.05%) Basidiomycota (2.08%) and Glomeromycota (0.08%). Dominating fungal genera were *Galactomyces*(76.78%)*Geotrichum* (9.97%) *Knufia* (2.76%) and *Debaryomyces* (1.73%) (Fig.23)

Table 21 : Diversity measure of intestine microbial used in the study

Treatment Groups	No. of OTUs		Shannon index		Simpson index	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
A1	141.67±10.69[a]	81.26±10.71[b]	1.14[a]	1.24[a]	0.54[a]	0.60[a]
A2	169.16±28.30[a]	98.33±22.47[b]	1.37[a]	1.49[a]	0.65[a]	0.72[a]
Control	150.81±13.79[a]	160.48±12.02[a]	0.66[b]	0.53[b]	0.21[b]	0.54[a]

Vertical bars represent the mean± SE (N = 3). Data marked with different superscripts have significant differences ($P < 0.05$) among treatments.

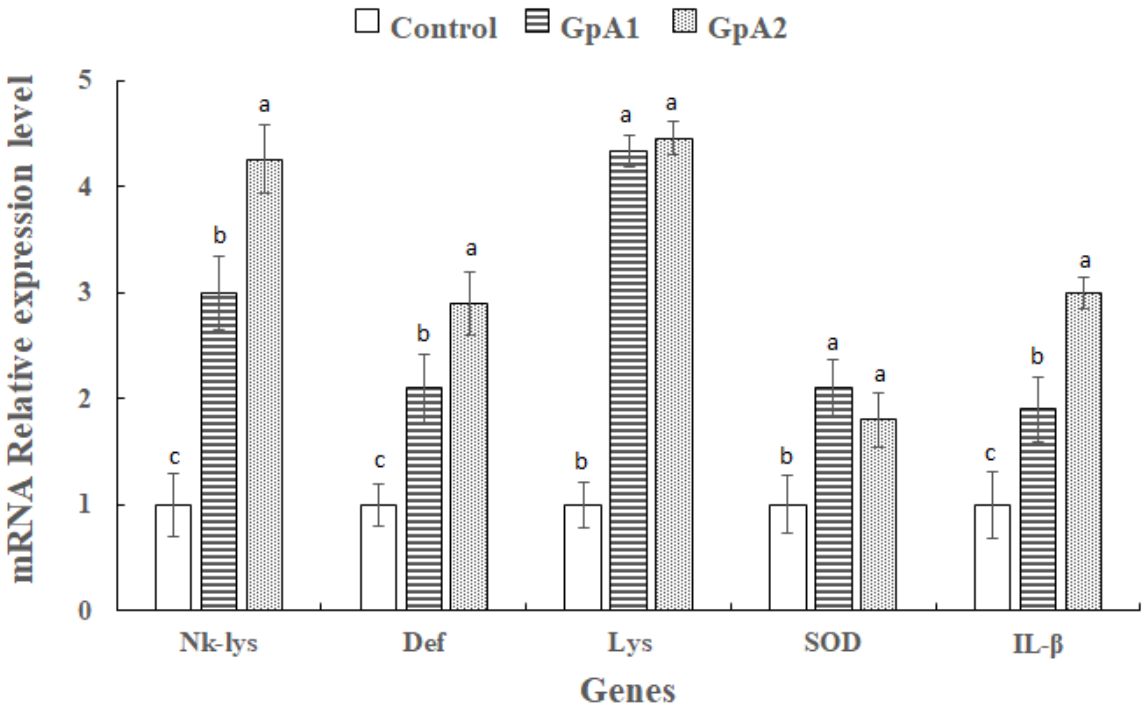

Figure 18 : Expression level of immunity-related genes in the intestine of common carp fed control and two different doses (500 &1000 units/kg^{-1} diet) of β-mannanase supplemented diet for 90 days. The reference gene is β-actin. Each bar represent data as mean± SE (N = 3). Bars marked with different letters have significant differences ($P < 0.05$).

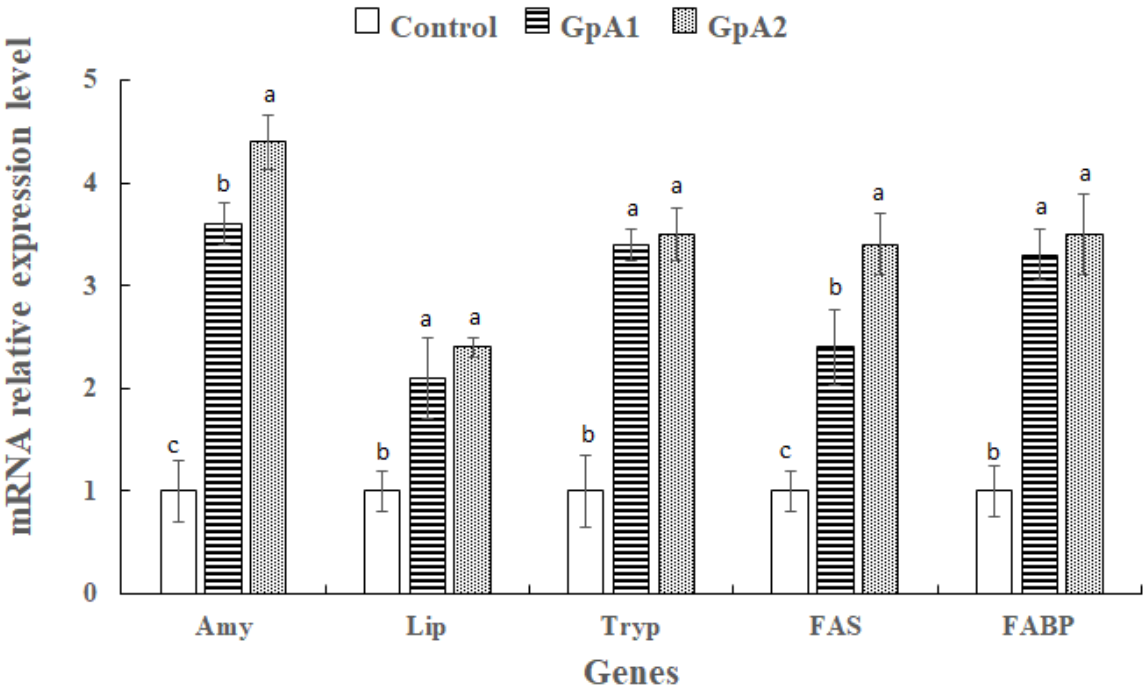

Figure 19 : Expression level of digestion related genes in the intestine of common carp fed control and two different doses (500 &1000 units kg^{-1} diet) of β-mannanase for 90 days. The reference gene is β-actin. Vertical bars represent the mean± SE (N = 3). Bars marked with different letters have significant differences ($P < 0.05$) among groups.

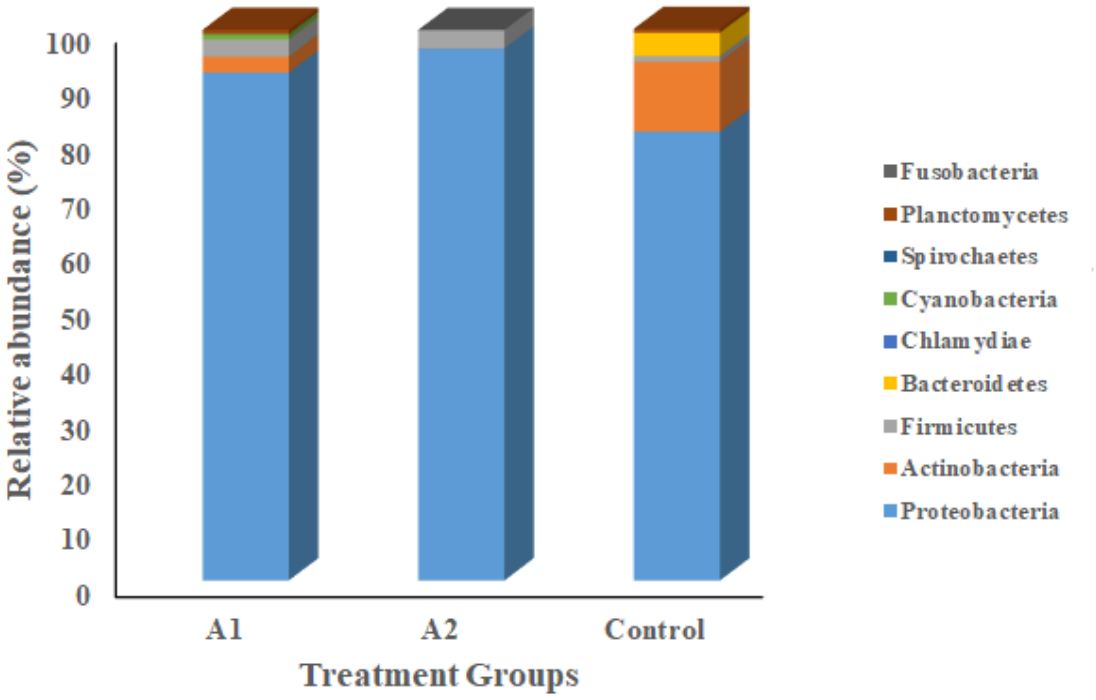

Figure 20 : Taxonomic composition of bacterial communities at phylum level

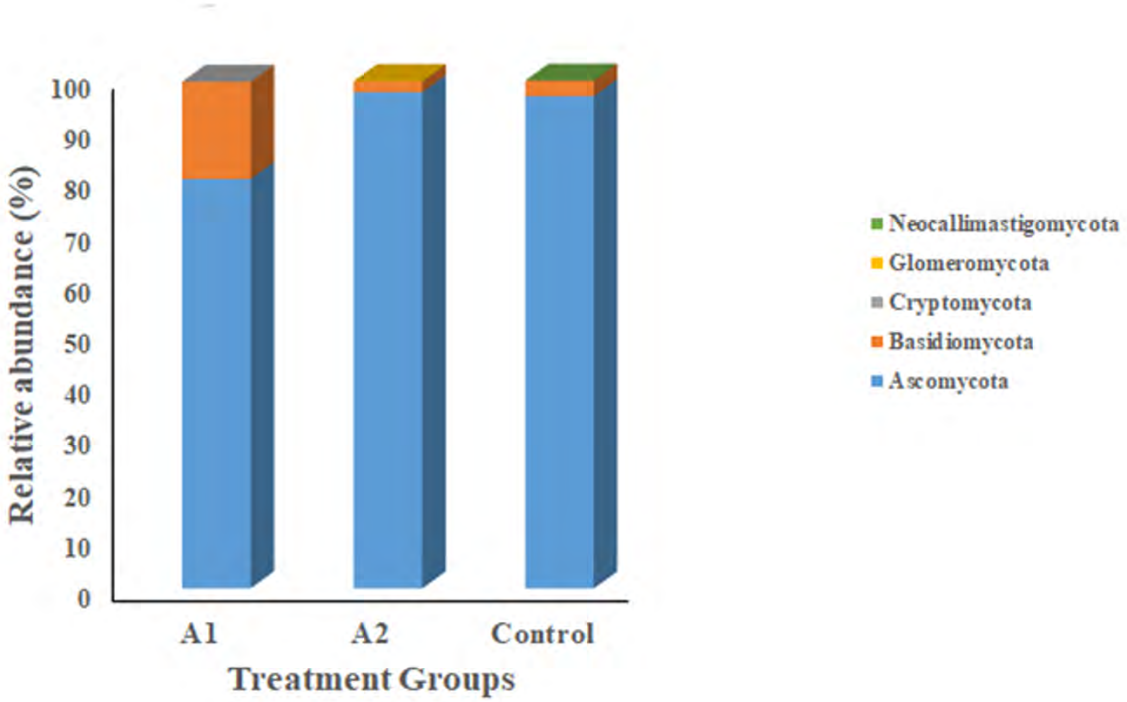

Figure 21 : Taxonomic composition of fungal communities at phylum level

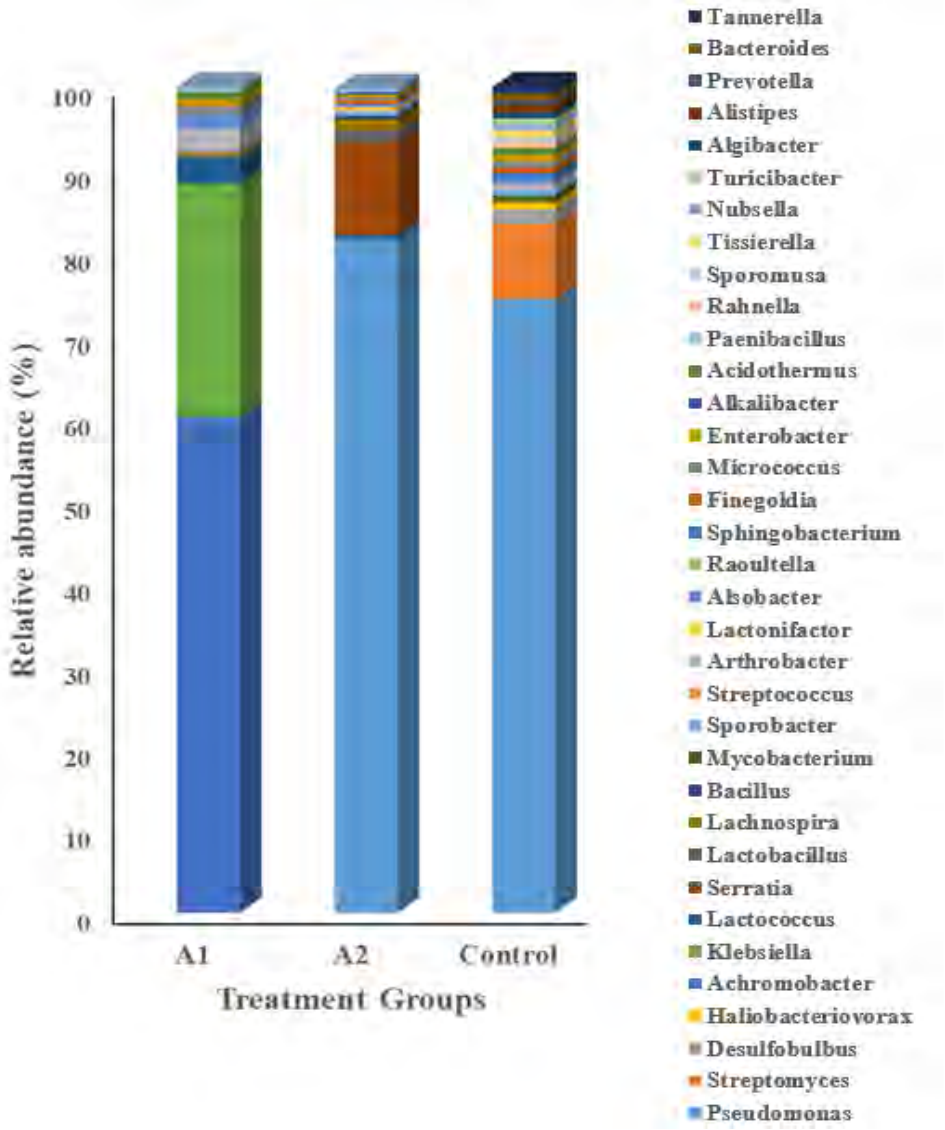

Figure 22 : Taxonomic composition of bacterial communities at genus level

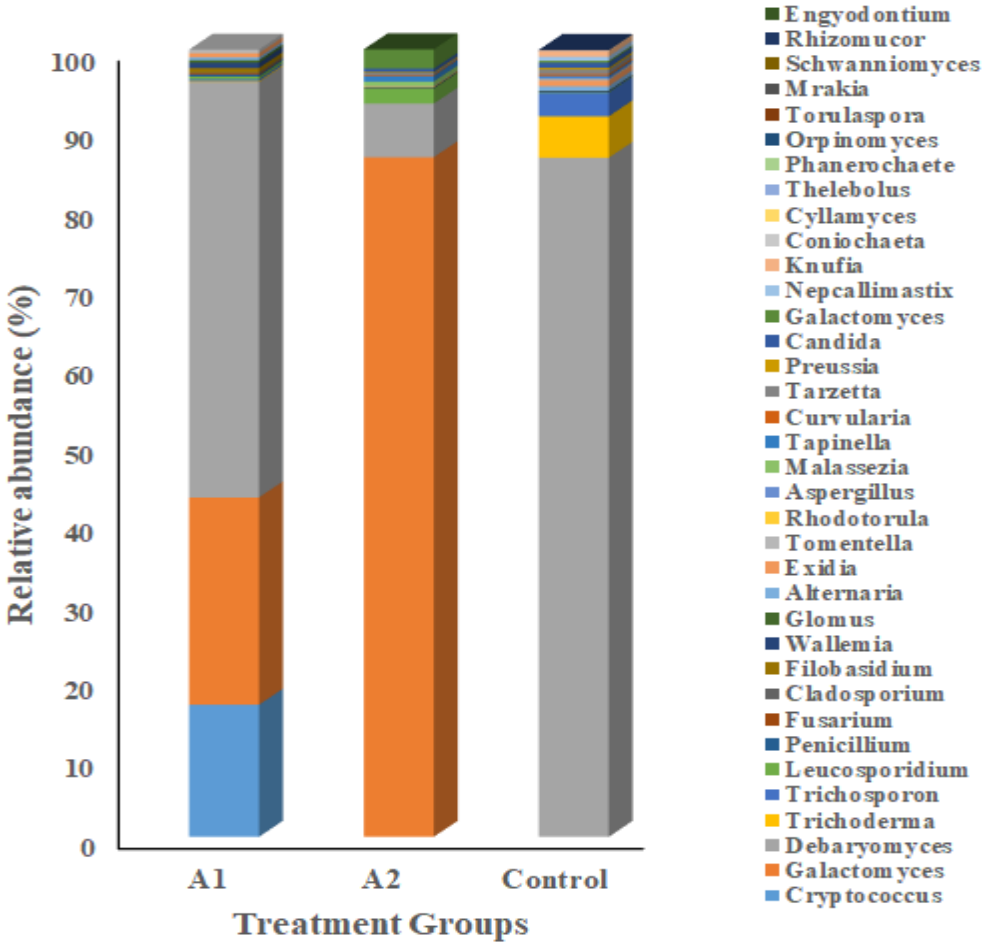

Figure 23: Taxonomic composition of fungal communities at genus level

Discussion

In recent years, one of the most important areas of investigation in aquaculture has been the substitution of fishmeal with less expensive alternatives in order to increase the expansion of the global aquaculture sector and assure its sustainability. Feedstuffs rich in plant proteins can be affordable replacements for fishmeal (Castillo and Gatlin III, 2015).But most plant-based feedstuffs contain anti-nutritional components like non-starch polysaccharides (NSP), phytin and protease inhibitors.These can adversely affect the intake of nutrients and fish health (Popova and Mihaylova, 2019).

Hydrothermal treatments, such as extrusion have been used to improve plant protein digestibility, and fractionation of crops have been carried out to reduce antinutritional components and enhance the concentration of protein. While these practices have shown to enhance the digestibility of energy and protein in a range of plant-based protein products, many constituents still have poor digestibility due to a shortage of enzymes required to break open the rigid cell wall surrounding these nutrients (Sinha et al., 2011; Glencross et al., 2012).In cereals and legumes, the cell wall consists mainly of cellulose, arabinoxylans, hemicellulose and minor quantities of β-glucan elements. When supplemented in the feed, NSP-degrading enzymes bring about hydrolysis of NSP. This breaks down the cell wall, enabling water hydration and accessibility to digestive enzymes like amylases and proteases. This improves nutrient uptake and the digestibility of substrates that are contained within the confines of cellwall (Castillo and Gatlin III, 2015).

Exogenous enzymes are currently utilized frequently worldwide after being thoroughly researched in swine and poultry, to increase the digestion of nutrients in plant-rich feed products. These enzymes are used to suppress the negative effects of phytic acid and nonstarch polysacchrides while enhancing phosphorus and carbohydrate bioavailability (Adeola and Cowieson, 2011). Utilizing phytase to increase the absorption of phosphorus from plant-rich feed ingredients has become relatively widespread in aquaculture (Lemos and Tacon, 2017).

However, carbohydrase enzymes have not been widely used in aquaculture diets,

necessitating more research on carbohydrases better suited to the aquaculture industry (Castillo and Gatlin III, 2015). Animal feed enzyme expertise is a vibrant field of study and development, and it's reasonable to believe new enzymes more suited to the environment of an animal's GI tract will be developed. These advancements will help to increase the efficiency and efficacy of enzyme supplementation in real-life settings (Adeola and Cowieson, 2011).

Carbohydrases are the enzymes that break down high molecular weight polymeric carbohydrates (Castillo and Gatlin III, 2015).A notable carbohydrase enzyme is β-mannanase which can breakdown mannan, a kind of NSP found in a variety of plant-based feed ingredients. If supplimented in feed, this enzyme breaks down high molecular weight mannan and may enhance nutritional digestibility in fish (Sinha et al., 2011).

Here, In the present study research was focused on isolation and characterization of a mannanase enzyme from a novel fungal strain, that could be used in aquaculture industry. Experimental trials were also carried out to test its potential as a feed additive in real applications.

For isolation of β-mannanase, soil samples were collected from different areas of Punjab and fungal strains were screened for mannanase activity. Among different strains isolated and screened *Aspergillus niger* proved to be a potent producer of mannanase (Kote et al.,2009; Youssef et al., 2006). The strain was molecularly characterized and its gene sequence submitted in NCBI and named as *Aspergillus niger* AD-01(Fig.1).

Fungal strain *A.niger* AD-01 produced 4.160 U/mg titer of β-mannanase under non-optimized conditions in submerged fermentation. Optimization of various parameters, viz. carbon source; pH; temperature; nitrogen source and time of incubation was carried out. Among different carbon sources tested, maximum production of β-mannanase enzyme was achieved when locust bean gum (LBG) was used as sole carbon source (Table.8). This proves that LBG is the best inducer of β-mannanase production from *Aspergillus niger* AD-01. 1% LBG produced maximum production of enzyme (3.245U/ml) followed by guar gum

(2.234U/ml). similar results were reported by other investigators while using commercial mannans for production of β-mannanase (McCutchen et al., 1996; Ademark et al., 1998; Odetallah et al., 2002).

The increased production of β-mannanase from LBG could be due to increased concentration of mannan present in LBG as compared to other sources. Konjac glucomannan has a 1:1.6 of mannose to glucose ratio (Kato and Matsuda, 1969) guar gum has a 1:2 galactose to mannose ratio, and locust bean gum has a 1:4 galactose to mannose ratio (Lawrence, 1973). This shows that LBG has the highest mannan content which might be the reason that maximum production of β-mannanase was achieved when LBG was used. Simple sugars like glucose, mannose, xylose and galactose did not induce β-mannanase production which might be due to repression of catabolite as seen for *Aspergillus sp.* (Haltrich et al., 1996; de Vries and Visser, 2001). Consequently, no evidence of enzyme synthesis was observed.

The impact of various nitrogen sources on *Aspergillus niger* AD-01's production of β -mannanase enzyme was also examined (Table.9). When organic nitrogen sources were employed instead of inorganic nitrogen sources, the synthesis of β-mannanase enzyme was significantly increased. This may be due to presence of carbohydrates and vitamins in addition to amino acids present in organic sources that the enhanced production of mannanase enzyme occurred. When yeast extract was employed as a nitrogen source, the highest output of β-mannanase enzyme was produced, followed by peptone. This is consistent with the findings of other investigators (Kote et al., 2009; Chantorn et al., 2013). In pH optimization studies, the effect of initial pH of the culture media on the production of β-mannanase enzyme was studied in the range of 3-8 (Fig.2). Highest mannanase production was observed when the pH of the production medium was 5. Similarly, Kote & Patil (2009) obtained optimum β-mannanase enzyme production from *Aspergillus niger* gr when the pH of the fermentation medium was 5. Mohammad *et al*. (2011) obtained maximum production of β-mannanase enzyme at pH 5.5.

To investigate how temperature influences β-mannanase production, different fermentation temperatures (ranging from 20-60°C) were tested (Fig.3). Highest β-mannanase

enzyme production was achieved when the temperature of the production medium was 30°C.Other investigators also observed maximum production of β-mannanase enzyme at 30°C (Youssef et al., 2006; Chantorn et al., 2013). Kote *et al*. (2009) however observed maximum β-mannanase enzyme production at 37°C.Rashid *et al*.(2011) obtained maximum β-mannanase enzyme production by *Aspergillus terreus* at 31°C.

Substrate concentration was also optimized. Highest production of β-mannanase was observed when the substrate concentration was 1% (Fig.4). After optimizing all the other parameters, the effect of time of incubation on β-mannanase enzyme production was investigated. For this, the production of β-mannanase enzyme was carried out under optimized conditions for 9 days (Fig.5). Maximum production of the enzyme was achieved at day 5.This is similar to the results reported in other studies (Kote et al., 2009). After day 5 the production of β-mannanase enzyme was greatly reduced which could be due to depletion of nutrients in the fermentation medium. The enzyme production increased from 4.160 U/mg of protein in the non-optimized media to 12.49U/mg of protein in the optimized medium (Table.10). So there was 3 fold increase in the production of β-mannanase enzyme by optimizing the media composition.

The enzyme was then purified by subjecting the fermentation liquid to ammonium sulphate precipitation and gel filtration chromatography. After multiple purification steps, β-mannanase enzyme was purified by 6 fold with 24% yield and the specific activity of β-mannanase, using LBG as sole carbon source reached 78.07U/mg (Table.10). After column chromatography, the enzyme yield rose dramatically, possibly due to the elimination of an inhibitor from the enzyme sample.

The main issue at large scale production of mannanase is the low stability and activity of mannanase, resulting in increased cost for enzyme production. There are some mannanases produced from bacterial genera of bacillus like *streptomyces* and *Cellulosimicrobium* that show appreciable specific activity as high as 2000U/mg against LBG. But most mannanases produced from wild bacteria have shown low activity. *Flavobacterium* sp. has specific activity of 2U/mg (Zakaria et al., 1998). *Paenibacillus cookie* has specific activity of 10U/mg (Yin et al., 2012). *W. viridescens* LB37 has been reported to have the specific activity of

63.53U/mg (Adiguzel et al., 2016).In *Bacillus* sp. the specific activity reached 32U/mg (Akino T,1988).This shows that β-mannanase isolated from *A.niger* AD-01 has higher specific activity (78U/mg) as compared to mannanases isolated from other wild strains of microorganisms.

In this study, characterization of β-mannanase reveals that the enzyme is a low temperature active, low molecular mass, weekly acidic, NaCl, and protease-tolerant β-mannanase.

The apparent molecular mass of β-mannanase enzyme was found to be ~45kDa (Fig. 6), which is less than most of the reported molecular masses of β-mannanase enzyme from *Aspergillus* species. The molecular mass of β-mannanase isolated from *Aspergillus oryzae* has been reported to be 110 kDa,from *Aspergillus niger* gr 66 kDa, from *Aspergillus terreus* FBCC1369 to be 49 kDa, and from *Aspergillus sulphureus* 48 kDa (Regalado et al., 2000; Chen et al., 2007; Naganagouda et al., 2009; Soni et al., 2016). Due to low molecular mass of β-mannanase enzyme isolated from *Aspergillus niger* AD-01, it has more ability to depolymerize mannan because it can penetrate the lignocellulosic systems more efficiently.

The majority of fungal β-mannanases that have been studied so far show optimum activity in the acidic (4.0-5.0) pH range (Van Zyl et al., 2010; Srivastava and Kapoor, 2017).β-mannanase enzyme purified from *Aspergillus niger* AD-01 has been found to be maximally active at pH 6.0 (Fig.9). From the literature it is evident that most of the β-mannanases from *Aspergillus* species have optimum pH that lies in the acidic range. β-Mannanase enzyme purified from *A.oryzae*, *A.awamori*, *A.niger*, *A.tamarii*, *A.fumigatus*, *A.aculeatus*, *A.terrus* and *A. sulphureus* had an optimum pH of 6.0, 3.0, 3.0, 4.5, 4.5, 5.0, 7.5, 2.4 respectively (Civas et al., 1984; Christgau et al., 1994; Regalado et al., 2000; Puchart et al., 2004; Chen et al., 2007; Hung et al., 2007). Therefore, the purified enzyme can be considered as a weekly acidic or neutral enzyme, making it suitable to be used a feed supplement for agastric fish which has a digestive system with a pH value of 6.8–7.3.

The optimal temperature for β-mannanase in the present study was 40 °C (Fig.7), comparable to that reported for *Aspergillus oryzae* (40 °C) β-mannanase but lower than most other Aspergilli viz. *A. awamori* (80◦C), *A. tamari* (60°C), *A. niger* (50°C), *A. sulphureus*

(50°C), *A. fumigatus*(60°C), *A. terrus* (55°C), and *A. aculeatus* (70°C) (Civas et al., 1984; Kofod et al., 1994; Ademark et al., 1998; Regalado et al., 2000; Chen et al., 2007; Huang et al., 2007). Compared with the optimal temperatures obtained for β-mannanase purified from different *Aspergillus* species mentioned above, β-mannanase of AD-01 showed a pronounced activity at lower temperatures. In the present study, the isolated β-mannanase enzyme had half-life of 30 min at 40°C and only 10% of activity was retained after 10 minutes incubation at 50°C (Fig.8). Similarly the thermotolerant *Aspergillus fumigatus* β-mannanases (MANI and MANII) were inactivated quickly when the temperature rose above 60°C(Puchart et al., 2004).

The gene encoding cold-active β-mannanase most likely originated in one of three ways. First, it is likely that the cold-active nature of enzymes is more prevalent in mesophilic organisms than previously theorized. Other than mannanase AD-01, there are several instances of enzymes that are cold-active in nature but have been procured from mesophilic species. During the evaluation of the catalytic characteristics of an enzyme from mesophilic species, a heightened activity at relatively low temperature is usually surprising. Similar was the case with a lipase enzyme from *Candida albicans* that has a low sequence similarity to reported lipases from psychrophilic species yet had a temperature optimum of 15°C (Lan et al., 2011). Another example is *Staphylococcus epidermidis* lipase, which was isolated from a vehicle servicing area and has an optimum temperature of 25°C.Monroe et al., (2014) described another fascinating situation in which the cold-active features of *Arabidopsis* β-amylase-3 were surmised from the fact that this enzyme showed more activity at night than β-amylase-1, which had the reverse behavior. Both amylase-3 and amylase-1 were overexpressed and extracted from *E. coli*, revealing that they have distinct temperature adaptations. When compared to β-amylase-1, β-amylase-3 had reduced optimal temperature, and thermal stability but higher residual activity at low temperatures.

Second, the gene might have come from a mannolytic psychrophilic species by lateral transfer; nevertheless, it appears that such a transfer would be unusual. Third, mannanase-AD-01 might have a marine origin, with the mannanase-AD-01 gene representing a vestigial gene that has not fully evolved, or it may have been lost from most *Aspergillus* species due to

its low activity in a purely mesophilic environment, It is solely found in the *A.niger*-AD-01 specie as was previously shown.

Enzymes should be active in the presence of additional additives or compounds that are required for the reactions to take place. Organic solvents are frequently used, whether pure or in combination with other solvents. To gain some understanding of the nature of cold-active enzymes, their residual enzymatic activity is evaluated in the presence of different inhibitors and additives (Santiago et al., 2016).

In the present study, β-mannanase was strongly inhibited by $Ba^{2+}$$Pb^{2+}$and Hg^{2+}(retaining <30% activity) (Table.11). Inhibition of purified enzyme by Hg^{2+}suggests that β-mannanase from A.*niger* AD-01 contains an essential sulfhydryl group. Enzyme activity is usually inhibited by ions such as Hg^{2+} because they react with sulfhydryl groups (Krajewska, 2008; Du et al., 2012). Similar to our results, β-mannanase from *Bacillus* sp. was strongly inhibited by Pb^{2+}(Cheng et al., 2016) and β-mannanase from *Biospora* sp. was strongly inhibited by Hg^{2+} (Luo et al., 2009).This suggests that *β*-mannanase from *Aspergillus niger* AD-01 should not be contaminated by $Pb^{2+}$and Hg^{2+}.β-mannanase activity was found to be considerably enhanced by presence of β-mercaptoethanol and EDTA but strongly inhibited by SDS. As is the case in the present study, β-mercaptoethanol has recently been reported to enhance enzyme activity for certain enzymes (Sharma and Satyanarayana, 2013) .

The findings of substrate specificity experiment showed that *A. niger* AD-01 β-mannanase exhibits highest activity toward locust bean gum (defined as 100 %) followed by coconut pulp (38.2%) and oat spelt xylan (35.8%) (Table. 12). In comparison, the β-mannanase from *Reinkea* sp. KIT-Y010 showed no activity towards guar gum or LBG but could hydrolyze linear mannans (konjac gum) effectively (Hakamada et al., 2014). Mannanase from *Aspergillus niger* AD-01 showed no activity towards oat spelt xylan or carboxy methyl cellulose (CMC) (Table.12). This indicated that β-mannanase from *Aspergillus niger*AD-01 was free from cellulase activity (towards CMC) and xylanase activity (towards oat spelt xylan). Similar results were obtained when activity of β-mannanase isolated from *philophora sp*. p13 and *Bispora sp*. MEY-1 was tested against LBG, CMC and oat spelt xylan (Luo et al., 2009; Zhao et al., 2010). β-mannanase isolated

from *Penicillium Pinophilum* C1 also showed no activity towards CMC-Na, birchwood xylan or p-nitrophenyl-β-D-mannopyranoside(Cai et al., 2011).Among the natural substrates tested the enzyme showed highest activity towards coconut pulp followed by soy bean meal. Similar results have been reported in another study with an *A.niger* strain that produced maximum production of the β-mannanase in the medium containing 2% (w/v) of coconut pulp among tested carbon sources (coconut pulp, soybean meal, date seeds, sucrose, mannose, fructose, glucose, carob pods, Rabbit feed)(Youssef et al., 2006).

Mannan is often present in different plant-based feed ingredients like soybean meal, sesame meal, copra meal, palm kernel meal, guar gum meal and corn meal. As mannan is a part of soybean meal which is a widely used feed ingredient throughout the world, thus mannan is present in most feed formulations(Van Zyl et al., 2010). In addition to soybean, Corn distillers dried grain with solubles (DDGS) and canola are also often added to monogastric diets thus adding to the quantity of mannan in the feed (Noblet et al., 2012). Studies on monogastric nutrition have found that mannan present in plant-based feed negatively affects animal growth performance and act as a strong antinutritive agent. Mannan present in the feed has been reported to put off glucose absorption in the diets of monogastric animals. Supplemenation of salmonid fish diet with guar galactomannan and alginates led to a reduced availability of glucose, as compared to control diet which was free of both of these ingredients (Storebakken, 1985). Very few investigators have assessed the effect of mannan on glucose digestibility in fish, but considerable literature is available about the effect of mannan on carbohydrate digestion and metabolism in swine. Rainbird and Low (1986) reported that when guar gum was included in the basal diet of growing swine, the rate of glucose absorption in the jejunum dropped to half. Similarly, 40g/kg guar gum inclusion to the semi purified diets of pigs resulted in a 25% reduction in glucose absorption (Sambrook and Rainbird, 1985). According to Nunes and Malmlof (1992) when guar gum was added at the rate of 60 g/kg to the diets of pigs, it caused the decrease in stomach inhibitory polypeptide by 55%, insulin production dropped by 30%, the insulin-like growth factor-1 (IGF-1) was reduced by 58% and glucagon by 41%.

Mannan-containing feed ingredients have been reported in several studies to negatively affect body composition and growth. Supplemenation of feed with sesbania

endosperm that is a mannan rich feed ingredient, at different rates (7.2%, 10.8% and 14.4%) significantly decreased feed utilization and growth in *C.carpio* (Hossain et al., 2001). In another study conducted on rainbow trout, feeding 10% guar gum diet significantly reduced growth, fat content and dry matter in the tissues (Storebakken, 1985). This is also known from work on chickens (Latham et al., 2018).Nunes and Malmlof (1992) reported that the high level of mannan in swine diet negatively affected growth performance and interfered with IGF-I and insulin secretion.

Presence of mannan in the diet has been reported to increase the viscosity of digesta (Lee et al., 2003b; Latham et al., 2018). High viscosity not only increases the residence time of digesta within the gut, but volatile fatty acid (VFA) production is also increased. Thus, the gut ecosystem is altered and the nutrient digestion and animal performance is reduced (Choct et al., 1996). In a study conducted on rainbow trout, poor nutrient digestibility with addition of guar gum was attributed to high viscosity of digesta (Storebakken, 1985). Similar observations have been made in common carp. Inclusion of galactomannan-rich sesbania seeds in the feed of common carp has been reported to increase intestinal viscosity, thereby negatively affecting nutrient absorption and utilization (Hossain et al., 2001). Highly viscous digesta also negatively affects the performance of poultry. Lee et al.,(2003b) observed a direct relationship between concentration of guar gum and digesta viscosity. As the guar germ concentration increased, the digesta viscosity also increased. For the control diet containing 0% guar germ, the observed ileal viscosity was 0.92 ± 0.21 cP. For 5.0% inclusion of guar germ, viscosity increased to 2.08 ± 0.67 cP, and for 7.0% guar germ concentration, viscosity reached 5.05 ± 1.41 cP. High viscosity caused by mannan content in guar germ was thought to be responsible for the poor performance of broiler chicken observed in the study.

The use of β-mannanase prevents viscosity of digesta and depression in growth because mannanase degrades mannan into mannooligosaccharides (Lee et al., 2003a; von Freiesleben et al., 2016). In the present study, mannan in the simulated feeds was degraded which is indicated by the release of reducing sugars during the simulated intestinal digestion phase in vitro (Fig.13).

Mannooligosaccharides (MOS) are a kind of prebiotics. Prebiotics are non-digestible carbohydrates that certain microorganisms, including *Bifidobacterium, lachnospira, Lactococcus* utilize as food source. These beneficial microorganisms or probiotics limit the spread of pathogens and enhance the production of beneficial compounds, which in turn are utilized by host for its own development and well-being (Dawood and Koshio, 2010). Some of the beneficial compounds produced by these bacteria are referred to as short chain fatty acids (SCFAs). SCFAs have frequently been reported as beneficial for colon health (McLoughlin et al., 2017). Prebiotics are also defined as carbohydrates entities that consist of monosaccharides, oligosaccharides, and polysaccharides depending on their molecular size or degree (the number of monosaccharide units) of polymerization (Ringo et al.,2010).

Dietary fiber is the sum of all lignin and polysaccharides which are left undigested by the GI tract's endogenous digestive enzymes. They can be divided into three categories: mixed (e.g. bran), insoluble (like cellulose), and water soluble (like inulin and oligofructose). The composition and activity of the GI tract's native microbiota are thought to be positively impacted by fermentable carbohydrates, which are regarded as being the most promising in this regard (Ringo etal.,2010). However, compared to the advancements made in the creation of prebiotics for land animals, study and implementation of orally delivered prebiotics in fish and shellfish production is still in its infancy.

For the past two decades farmers have been routinely using antibiotics to increase growth performance of fish and protect it from diseases. This use of antibiotics has become a source of concern and has been highly criticized because utilization of antibiotics involves a lot of danger (Cabello, 2004). The major concerns are that the routine use of antibiotics will produce antibiotics resistant bacteria, and the antibiotics fed to fish may leave residues in the digestive system of fish which may find its way to humans who consume the fish (Defoirdt et al.,2007). Because antibiotics are usually targeted against not only pathogenic microorganisms but all kinds of microorganisms, this may in turn lead to suppression of immune system and destruction of most of the microbial population in the gut. Thus, instead

of feeding antibiotics, probiotics have been preferred and have received a lot of attention from aquaculture industry (Merrifield and Carnevali, 2014)

Regarding the utilization of probiotics in the aquatic environment, numerous reports have been published (Zheng et al.,2017; wang et al.,2010; Ullah et al.,2018). However, the widespread use of probiotics in aquaculture industry has been restricted because probiotics are costly, difficult to administer in extruded diets, can potentially harm the environment, the labels on the bottles may not actually contain the desired microorganisms, may not be safe as feed supplement as some pathogenic bacteria may enter the body and the regulatory issues can be very hard to overcome (Ringo et al.,2010).

Instead, it seems more practical to alter the gut microbiota in aquatic animals by using prebiotics that change the gastrointestinal environment to favor the beneficial species of microorganisms over other. This may lead to better growth performance of fish increased, resistance to pathogens and illness because beneficial gut microbiota boost the immune system. However, knowledge of the value of prebiotics in the fish gut has only developed in the last two decades. This is evidenced by the fact that in 1995 the first study about effects of prebiotics was published (Markowiak and Śliżewska, 2017). Ringo et al., (2010) summarized many different kinds of prebiotics in their paper. These include: mannooligosaccharides, fructoologosacchride, xylooligosaccharides, inulin, galactooligosaccharide, arabinoxylan oligosaccharides. Among these many different types of prebiotics a lot of research has been done on mannooligosaccharides and they have proven to be beneficial for the health of many monogastric animals (Torrecillas et al.,2012; Yilmaz et al.,2007; Zhang et al.,2012). This is why the enzyme that is suitable to gastrointestinal environment of fish and degrades mannan into mannooligosaccharides in the fish gut holds a lot of significance. Invitro test with simulated intestinal fluid (Fig.13) shows that β-mannanase isolated in the present study is capable of degrading mannan and thus creation of mannanoligosacchrides that can act as prebiotics in fish gut and favor the growth of beneficial microorganisms. This skips the step of oral administration of probiotics.

β-mannanase AD-01 isolated and characterized in the present study is a cold active enzyme. This conveys several benefits to the enzyme. Due to flexibility of structure and

alterations in the active site for better binding of substrate, cold-active enzymes exhibit greater catalytic activity at low temperatures than mesophilic enzymes (Santiago et al., 2016).When compared to thermophilic and mesophilic rivals, cold-active enzymes, according to research exhibit various distinct properties. Reduced hydrophobicity of core, greater hydrophobicity of surface and glycine residue content, fewer disulphide bridges, electrostatic interactions and proline residues and enhanced loop structure development are among these (Feller and Gerday, 2003; Margesin et al., 2008; Santiago et al., 2016). Because of these enzymes their catalytic efficiency is higher at low or ambient temperatures than thermophilic and mesophilic rivals, they can save money by reducing energy consumption (Bakermans et al., 2011). Another benefit of cold active enzymes is that they avoid bacterial contamination and undesired chemical side reactions that might occur at elevated temperatures (Margesin et al., 2008; Kuddus and Ramteke, 2012; De Gobba et al., 2014). Due to these reasons, cold-active enzymes find applications in many industrial fields.

Cold-active enzymes are especially desirable for food processing because of their improved catalytic activity at temperatures that minimize degradation and changes in taste and nutritional content. Cold-active proteases and lipases can reduce production expenditure by replacing rennet and speeding up the maturation of cheese that ripens very slowly and requires special low moisture and temperature conditions (Yu et al., 2011). Cold-active proteases can also be employed to tenderize and enhance the flavor of frozen meat products, as well as to remove unwanted tissues from shellfish, such as the intestine and descaling and removal of fish skin, (Margesin, 2009; Joshi and Satyanarayana, 2013). In vegetable and fruit processing industries breakdown of pectin molecules in fruit and vegetables can be accomplished at low temperature via pectin-degrading enzymes such as pectate lyases and polygalactouronases, as well as the debranching activities of different hemicellulases(Margesin, 2009).

Cold-active galactosidases working at a neutral pH can help lactose-intolerant consumers digest dairy products more easily while simultaneously boosting sweetness at low temperatures. Cold-active galactosidases with acidic pH can decrease waste and enhance the practical usefulness of whey by generating sugary syrups which can be used as sweetening

agents in many different products of food and feed industry (Cieśliński et al., 2016; Mangiagalli and Lotti, 2021). Transglycosylation activities of cold-active galactosidases have been discovered, including lactose breakdown and the simultaneous production of oligosaccharides. Galactooligosaccharides can be used as supplement in prebiotic foods (as an ingredient in dairy product or directly in milk) to increase the growth of probiotics. People struggling with weight issues or diabetics can also use them as a low-calorie substitute for sugar, because they are resistant to digestion in the human gut (Mangiagalli and Lotti, 2021).

In baking, during dough preparation and processing, low temperature active biocatalysts including lipases, xylanases and amylases can bring about hydrolysis of the gluten, hemicellulose, starch, and free sulfhydryl groups at temperatures that are much lower than 35°C. The combined action of these biocatalysts can make the dough more elastic and manageable, which increases volume and improves structure of the loaf (Kuddus, 2018; Raveendran et al., 2018; Al-Maqtari et al., 2019). Recently yet another advantage of cold-active enzymes was discovered when a family 8 xylanase was shown to be hugely successful in baking and delivered a greater loaf volume when compared to a well-known commercial mesophilic enzyme preparation (Collins et al., 2006).

Cold-active enzymes, which have strong catalytic activity at ambient and low temperatures, are also beneficial to the drug industry. The growing demand for medications that are enantiomerically pure has spurred the usage of biocatalysts in organic synthesis (Margesin, 2009; Truppo, 2017). Because low moisture settings promote biosynthesis of hydrolase and transesterification reactions, as well as many substrates' solubility which are participating in biocatalytic conversions, enzymes used in several organic synthesis operations are required to be able to function effectively in both nonaqueous and aqueous/organic solvents (Schoemaker et al., 2003; Kaul and Asano, 2012) .

Cold-active enzymes' flexible design may be particularly useful when they are utilized in solvents that make thermophilic and mesophilic enzymes inoperative, such solvents seems to interfere with activation energy by increasing it many fold but reducing the conformational maneuverability that is needed for catalysis (Margesin, 2009; Santiago et al., 2016; Al-

Maqtari et al., 2019). A *Candida antarctica* heat-labile lipase has already been used for alteration of polysaccharide desymetrization of complicated pharmacological intermediates, and alcohol and amine resolution, among other things(Suen et al., 2004).

In the cosmetics industry, cold-active enzymes play a crucial role. They can increase the biotransformation of flavoring and scented ingredients (Trytek and Fiedurek, 2005; Margesin, 2009). Proteinases that work at room temperature could be beneficial. Topical gels are used to treat skin scarring, wound healing and infection (Barrett et al., 2012).

Although the standard ethanol manufacturing process is greatly refined, it is expensive in terms of cost and energy because high levels of heat and a special equipment is required. This reduces the output of biorefineries (Galbe and Zacchi, 2002; Hossain et al., 2017). According to some studies, the energy required for traditional cooking is 10–20 percent of the fuel value of ethanol produced. To overcome this issue, enzyme businesses have been partnering with researchers to create a low energy ethanol manufacturing technology that utilizes hydrolysis of starch which is raw. This is commonly referred to as cold hydrolysis. This approach efficiently bypasses the stage that requires costly equipment and energy demanding cooking step (Das et al., 2021; Karimi et al., 2021).

The STARGEN^{TM} series of fungal enzyme products from Genencor include different carbohydrases that can hydrolyze raw starch at 32°C in a concurrent fermentation and saccharification step. The combined action of these biocatalysts quickens the pace of the constant release of fermentable sugars from pulverized starch, which yeast consumes directly, resulting to more economical ethanol generation process (Margesin, 2009).Broin Companies has teamed up with Novozymes, another industrial enzyme company, to develop Broin's BPXTM technology. This is a cold hydrolysis process which uses microbial enzymes to effectively convert starch to simple sugars without the use of heat (Margesin, 2009; Singh et al., 2016).

Besides considerable energy savings, incorporating the raw starch hydrolysis process into production of ethanol has a number of other advantages, including higher ethanol yields,

fewer undesirable byproducts, lower plant emissions and waste, and lower capital costs (Bušić et al., 2018; Formann et al., 2020).

Furthermore, by continuously removing glucose and maltose during the fermentation process, SSF procedures result in higher enzyme efficiencies, which would otherwise cause end-product inhibition. To compensate for the lower specificity and activity associated with the cold hydrolysis stage, large enzyme concentrations are now required. This leads to skyrocketing of cost of production. Given their high activity at low temperatures which is ideal for processing, cold-active carbohydrases may allow for a decrease in loading of enzymes and thus demonstrate to be a practical and cheaper substitute to the present usage of mesophilic biocatalysts (Lin and Tanaka 2006). Cold-active glucoamylases and amylases may allow for a reduction in biocatalyst loading and thus prove to be a cheaper but efficient alternative to the current use of mesophilic enzymes (Santiago et al., 2016).

In the aquaculture industry, cold active enzymes hold considerable promise (Joseph et al., 2008; Boyd et al., 2020). Because of the pH of the digestive system of some aquatic organisms like grass carp and common carp and temperature of the water (Kapoor et al., 1976; Smith, 1980) . It is required that the enzyme used as fish feed additive should have neautral pH adaptivity and low temperature activity. Because secretory proteases are found in the gastrointestinal tract of monogastric animals, the enzyme needs to have good resistance against them (Sriket, 2014). Thus for an enzyme to be a good candidate as a feed additive for carp it should be resistant to proteases, be active at low temperature and nuetral pH conditions. Additionally, because of the typical sea salinity (3.5 percent, w/v), the use of biocatalysts in marine product aquaculture requires that the enzymes should have good salt tolerance.

Though cold active enzymes hold a lot of promise in aquaculture industry their potential has not been exploited like the other industries previously mentioned. In the present study, β-mannanase enzyme exhibited good salt tolerance and was resistant to proteinase. After treatment with trypsin and proteinase K for 60 minutes at 37°C, it exhibited no loss of activity. These properties point to mannanase's potential as a low-temperature-active β-

mannanase in marine and freshwater aquaculture applications as well as low-temperature operations. Furthermore, production from *A.niger* makes it even more suitable candidate in animal feed.

Experts from WHO, EFSA and FAO have repeatedly assessed and approved *A. niger* enzyme preparations, as well as the organism itself (Schuster et al., 2002; EFSA Panel on Food Contact Materials et al., 2019; EFSA Panel on Food Contact Materials et al., 2021). According to the FDA opinion letters from 1960s, cellulase, amylase, amyloglucosidase, glucose oxidase, catalase , pectinase and lipase from *A. niger* can be 'generally regarded as safe' (GRAS) if good manufacturing procedures are followed throughout the process (Singh et al., 2016). Godfrey and Reichelt (1983) claimed GRAS certification for protease and galactosidase from *A. niger* in addition to these enzymes. The FDA has also authorized cellulase and carbohydrase from *A. niger* as a secondary direct food additive for use in clam and shrimp processing (Subpart et al.).

Thus, the present study isolated and characterized an extracellular endo-β-mannanase enzyme generated by *Aspergillus niger*-AD01, a new mannanase-producer. This enzyme has good thermostability, is active at neutral pH and low temperature and resistant to salt and proteases. In addition, this enzyme displayed good activity against LBG, guar gum and konjac powder. The enzyme's broad working temperature and pH range, high activity particularly at low temperatures, salt tolerance, and resistance to proteases imply that it may have potential for use as a feed supplement in plant-based diets for omnivorous fish in aquaculture.

Many cold active enzymes have been reported in the last decade, with the majority claiming industrial use. Only a few, on the other hand, conducted a brief trial in a practical setting. These are 1). A thermostable β-galactosidase enzyme expressed both as inclusion bodies and soluble protein. The active inclusion bodies were then directly used for conversion of lactose.2) A galactosidase from *Lactococcus lactis* with appreciable activity at relatively low temperatures and a bioconversion rate of 98% for hydrolysis of lactose. 3)A cold-active and acidic pectin methylesterase enzyme PE8F46, isolated from *Penicillium chrysogenum* and effectively expressed in *Pichia pastoris.* In comparison to a commercial enzyme

complex, this enzyme considerably improved the hardness of pineapple juice when combined with calcium lactate. 4) Polygalacturonase, Endo-PG I, has been proven to lower papaya juice viscosity by 17.6% while increasing its transmittance by 59 %.5) a glycogen splitting enzyme (RmGBE) isolated from *Rhizomucor miehei*, a thermophilic fungus, has been shown to have superior cold adapted features. When added to wheat bread it caused an appreciable rise (28%) in specific volume and reduction (38%) in firmness of crumb.6) A galactosidase enzyme that showed remarkable ability to change RBC of blood type B into O (Balabanova et al., 2010; Tu et al., 2013; Dong et al., 2014; Pan et al., 2014; Wu et al., 2014).

In the present study the isolated β-mannanase enzyme was tested for its potential for use as a feed additive, not only in vitro but also in vivo. Conducting trials may help in rapid development of a feed additive or biocatalyst. In addition, it sheds light on the actual potential of enzyme to act as a biocatalyst in industrial applications.

For in vivo testing, β-mannanase (to minimize the NSP especially a mannan-associated problem) was supplemented in a plant-based diet to common carp.After 90 day feeding trial, a significant positive effect of the exogenous enzyme on the growth, immunity, and muscle proximate composition of fish was observed. Additionally, the comparative efficiency of β-mannanase isolated from two different fungal species was also evaulated and it was found that the efficiency of β-mannanase locally isolated from *Aspergillus niger* AD-01 (BM_{An}) was comparable to the commercial β-mannanase (BM_{Tr}) a propriety *Trichoderma reesei* fermentation product.

Although extensive literature reveals the beneficial effects of β-mannanase supplemented diets on different organisms, e.g., pigs (Pettey et al., 2002; Lv et al., 2013) , poultry (Jackson et al., 2004b; Li et al., 2010; Zangeneh and Torki, 2011a; Cho and Kim, 2013), yet scarce literature on fish (Yiğit et al., 2014; Chen et al., 2016b) is available to compare results of the present study. A significant and comparable increase in weight gain (%) and specific growth rate of *C. carpio* at both dosage levels (500 and 1000 units kg-1 diet) of β-mannanase compared to a control group (Table.14) was also observed. The up-regulation of Myo-D gene in the muscle (Fig.14) also indicated the positive effect of β-mannanase supplementation on the growth performance of carp. Like the results of the

present study, Chen et al. (2016b) also observed similar beneficial effects on weight gain and specific growth rate of tilapia. The findings of the current sudy are also in agreement with many studies on organisms other than fish. Many investigators reported a positive effect of mannanase supplementation on growth of broilers, pigs, toms and fish. Lee at al.,(2003b) reported that dietary supplementation of mannanase to broiler chicken removed growth depression, improved FCR and reduced high ileal viscosity that resulted from feeding different fractions (2.5 and 5.0%) of guar hull. This indicated that growth and digesta viscosity were inversely related. Latham et al.,(2018) also observed that increasing the concentration of guar gum in the diet of broilers increased intestinal viscosity and depressed body weight during the starter and grower phase. The negative effects were however reversed by supplementation with mannanase.Similarly, in another study the inclusion of different levels (0.5,1.0 and 2.0%) of guar gum negatively affected broiler body weight (BW)(Daskiran et al., 2004). The reduction in BW was especially severe at 2% inclusion of guar gum. The BW of all chicks was restored to control values with supplemental mannanase except for chicks fed 2% guar gum. Supplementation with β-mannanase improved weight gain even in chicks fed 2% guar gum, but the depression in growth was so great that the negative effects were not completely reversed with mannanase addition in the diet. This indicates that the amount of fiber in the diet should be monitored along with the dosage of enzyme.

In a dose response study, Jackson et al., (2004a) observed a significant increase in feed efficiency (3.5 to 3.8%) and weight gain (3.9 to 4.8%) in broilers fed corn-SBM-based diets supplemented with β-mannanase at 80 or 110MU/ton. However, inclusion of mannanase at the level of 55000U/kg in the feed caused no significant improvement in growth performance of broilers.

Conversely, some workers did not find the effect of corn-SBM-based β- mannanase supplemented diet on body weight gain and feed conversion of broiler chicken (Kong et al., 2011). Similarly, Yigit et al. (2014) also did not find a significant increase in the specific growth rate of trout in a 12-week trial in response to a soybean meal (44%) based diet supplemented with β-mannanase and other carbohydrases. The discrepancy in the results may

be related to many factors including temperature, age, species, mannan contents in feed ingredients, and dosage levels of β-mannanase (Sinha et al., 2011). It is well documented that the efficiency of a dietary enzyme decreases as the water temperature falls and increases with an increase in temperature towards optimum range (Forster et al., 1999; Vandenberg et al., 2012).

In addition to growth rate, a positive effect of β- mannanase on the FCR of *C. carpio* (Table.14) was also observed. The result of the present study is in agreement with literature that revealed improved FCR of tilapia (Chen et al., 2016b) and broilers (Daskiran et al., 2004; Jackson et al., 2004a) in response to β-mannanase supplemented feed. The improved growth performance and FCR observed here and reported by others may be anticipated with an improvement in energy metabolism with the digestion of mannan or via degradation of mannan in feed to mannan oligosaccharides (MOS) and the utilization of these oligosaccharides as prebiotic by gut microbiota (Chen et al., 2016b; Gainza and Romero, 2020).Similarly, the role of MOS in the colonization of beneficial intestinal microbiota and its effects on gut morphology, growth, FCR, innate immunity parameters and stress tolerance have been reported by many investigators.

Torrecillas et al. (2007) evaluated the effects of MOS included at two different levels (2 and 4%) in the diet of European sea bass (*Dicentrarchus labrax*) growth, feed consumption, immunological state, and infection susceptibility to *Vibrio alginolyticus*. Growth was greatly enhanced in fish fed MOS-supplemented diets at both tested concentrations. The researchers speculated that the better absorption of amino acids could be the reason for improved growth as was reported previously in chicken (Iji et al., 2001b). The researchers also discovered that at 4% inclusion level, dietary MOS greatly increased the phagocytic activity of kidney macrophages but this impact was dramatically diminished at 2%. Dietary MOS supplementation considerably reduced the number of fish afflicted by *Vibrio alginolyticus* during a 21-day challenge test.

Yilmaz et al., (2007) assessed the effects of MOS supplementation on rainbow trout's body composition, growth, foregut histology, and liver. MOS was included in the fish diet at four levels: 0 (control) to 4.5 g kg^{-1} with 1.5 g kg^{-1} increments. Overall, fish fed a diet

augmented with 1.5 g kg^{-1} had better growth results. The intestinal villous folds of fish that were given either 1.5 or 3.0 g kg^{-1} of mannanoligosacchrides were greater in length than those of fish that were fed 4.5 g kg^{-1} or control. At any dose, MOS exhibited no adverse effects on the morphology of intestine.

Dimitroglou et al., (2008) discovered greater surface area of absorption, as well as larger microvilli density and length, in rainbow trout that were fed 0.2% MOS for 56 days. Rodrigues-Estrada et al., (2008) studied the effects of MOS (4g Kg^{-1}) on rainbow trout development, immunological indices, and *Vibrio anguillarum* susceptibility. MOS boosted hemolytic activity, growth, phagocytic activity, survival and mucosa weight of rainbow trout when challenged with *Vibrio anguillarum*. Ghasemian and Jahanian (2016) reported several beneficial effects of MOS supplementation in laying hens. In comparison to control, MOS supplementation at 1–1.5 g kg^{-1} increased egg mass in the experimental groups. MOS supplementation also boosted antibody titers against bronchitis disease viruses and infectious Newcastle virus. In addition, they found that inclusion of MOS in the diet enhanced the crude protein and dry matter digestibility coefficients. In comparison to experimental groups, the ileal population of Salmonella was higher in control birds. Supplemental MOS at a dose of 1 g kg^{-1} reduced the number of *E.coli* and total bacteria found in the ileum. The authors concluded that, dietary MOS supplementation, especially at doses of 1 and 1.5 g kg^{-1}, might improve feed conversion efficiency and production performance in lying hens.

Fish lack endogenous enzymes required for the hydrolysis of NSP (Sinha et al., 2011), therefore indigestible dietary NSPs could not be used as the source of energy. It is well established that the viscous nature of most of the NSPs including mannan in feedstuff increases the viscosity of digesta, thus negatively affecting the residence time of digesta in the digestive tract, the interaction of digestive enzymes with the substrate, and absorption of nutrients (Amirkolaie et al., 2005; Sinha et al., 2011). Here, significantly improved digestibility of crude protein and crude fat and energy utilization (Table.13) may indicate that mannanase can relieve the negative effect of NSP by hydrolyzing the mannan. Several scientists studied the effect of exogenous enzymes supplementation on different organisms and suggested that dietary carbohydrase take off the nutrient encapsulating effect,

consequently enhancing the availability of nutrients to the endogenous enzymes and improving the digestibility of nutrients and environment for the intestinal microbes (Cowieson and Ravindran, 2008; Cozannet et al., 2017).Other researchers also investigated the effects of β-mannanase on digestibility of nutrients. Their findings support the results of the present study. Lv et al.(2013) reported an enhanced digestibility of CP, DM, NDF, calcium, and phosphorus by supplementing mannanase in growing pigs. Sundu and Kumar (2006) observed that dietary β-mannanase increased the lipid and protein digestibility in broilers fed different levels of copra meal. This increase in nutrient digestibility with supplementation of mannanase was attributed to the breakage of the cell wall, allowing the digestive enzymes to better access their substrate. Knudson et al. (1997) found that protein and lipids are enclosed within the endosperm cell wall and this prevents their digestion and absorption. Studies have shown that supplementation of mannanase not only results in improved digestibility of protein, lipid, or DM but also calcium and phosphorus (Lv et al., 2013). As phytate and minerals are bound to the cell wall, breakage of the cell wall releases them and leads to better bioavailability of minerals (Adeola & Cowieson, 2011).The digestibility of phosphorus and calcium was however not investigated in this study.

In the present study, the observed significant increase in protein and fat contents of muscle in mannanase supplemented groups may indicate the beneficial effect of the exogenous enzyme on the digestibility, absorption, and availability of nutrients (Table.15). The higher moisture % and lower contents of lipid and protein in the control group of fish may be because of the binding of mannan to water in the gut and formation of viscous fluid, which prevent the contact of enzymes with substrate and the formation of micelles, required for the absorption of lipid (Sinha et al., 2011; Castillo and Gatlin III, 2015) thus affecting the digestibility and availability of nutrients for synthesis and deposition rate in the muscles. No available study shows the effect of mannanase on muscle composition, however, Hossain et al.(2001) reported a decrease in whole-body fat and gross energy contents and an increase in whole-body moisture of tilapia with an increase in *sesbania endosperm* (having high contents of storage mannan and other NSP) in feed. However, some workers did not observe the effect of mannanase supplementation on carcass composition and whole-body composition of rainbow trout (Farhangi and Carter, 2007; Yiğit et al., 2014), which may be due to low

temperature. It is well established that enzyme activity is enhanced at higher water temperatures rather than low water temperatures (Forster et al., 1999; Vandenberg et al., 2012).

In the current study, the increased levels of AST and ALT and decreased levels of CHO and TG in a control group, while opposing trend in the mannanase supplemented groups (Table.19) also showed the negative effect of a plant-based diet and ameliorating effect of exogenous enzyme supplement. Like the results of the present study, Chen et al.(2016a) also reported a lower level of serum AST and ALT in tilapia fed β-mannanase supplemented diet, however they did not observe any significant effect on blood cholesterol, glucose, and HDL contents. Literature reveals reduced lipid absorption and hypocholesterolemic effect of NSP containing plant-based diet and the variable response of β-mannanase supplementation in different vertebrates. For instance broiler chicken fed hemicell enzyme (containing a higher amount of β-mannanase) supplemented corn/soybean-based diets, and laying hens fed β-mannanase supplemented plant-based diet (Zangeneh and Torki, 2011a) had not shown any significant effect on CHO, TG, and very low density lipoprotein (VLDL) of fish. However, decreased serum LDL-cholesterol level in response to β-mannanase in broiler was reported by Cho and Kim (2013). In the present study, dose-dependent increased in serum CHO and TG levels may indicate the increase in absorption of fat and CHO due to a decrease in the viscosity of digesta. Inconsistency in results could be related to the ratio of supplementation level of β-mannanase to the amount of mannan in feed (Azarfar, 2013).

Hematological indices are a key tool in assessing the changes in physiology and health of fish (Table.17). In the current study, the improved blood indices like HB, Hct, RBCs, and MCHC in response to exogenous enzymes may also indicate the better health status of fish. There's no comparable study on the effect of mannanase on the hematology of fish except Zamini et al. (2014) who observed no significant effect on hematological indices of Caspian salmon. However, Andrew et al. (2009b) reported the modulation of hematological indices in response to dietary MOS. The variation in the results can be attributed to the dosage level of mannanase, water temperature, or fish species.

The ability of a fish to digest food is influenced by the activity of its digestive enzymes, thus digestive enzyme activity determines the nutritional value of the feed. In the present study, a significant increase in intestinal enzymes (cellulase, protease, and amylase) activities of fish supplied with mannanase enzyme, as compared to a control (Table.16) was observed. Numerous studies have demonstrated that adding exogenous enzymes to feed can boost the release of endogenous enzymes (Lin et al., 2007; Zhou et al., 2013). Like our results, higher activity of amylase was reported in tilapia fed plant-based mannanase supplemented diet (Chen et al., 2016b) and in *L. rohita* when fed non-gelatinized diet supplemented with exogenous carbohydrase enzyme (Kumar et al., 2006). Moreover, other investigators reported improved activity of amylase, trypsin, and lipase in chicken(Almirall and Esteve-Garcia, 1995) and pigs(Jensen-Waern et al., 1998)fed barley-based diets. Although up till now, no study indicated the direct inhibitory effect of NSP including mannan on intestinal enzyme synthesis but suggested the restricted interaction of enzymes to substrates (Sinha et al., 2011). Thus, the increased activities of intestinal enzymes observed here and reported by others could be due to the release of the inhibitory effects of NSP on digestive enzymes by hydrolysis of NSP or may also be due to the release of enzymes from gut microbiota that flourishes in the presence of MOS (end product of mannan degradation).

In the present study, a positive effect of mannanase supplementation on the non-specific immunity of fish i.e., a significant increase in IgM level, total serum protein, lysozyme activity, respiratory burst activity, and phagocytic activity of fish (Table.18) was also observed. Similar enhancement of the immune response has been reported by many investigators in tilapia(Chen et al., 2016b) and broilers (Zou et al., 2006; Li et al., 2010), and suggested the indirect stimulation of immune response from the hydrolytic product of mannan i.e., MOS. Generally, MOS act as a prebiotic, block the colonization of pathogen in the GI tract by binding with mannose-specific type-I fimbriae, encourage the growth of beneficial microorganisms like *Bifidobacterium,lactococcus* and *Lactobacillus* species and trigger the immune system by activation of pattern recognition receptors and protein(Torrecillas et al., 2014). Many researchers reported the positive effects of MOS administration on the immune-related indices in different finfish species e.g., rainbow trout (Rodriguez et al., 2009), *L. rohita* (Andrews et al., 2009a), *P. olivaceus* (Ye et al., 2011) red

drum (Buentello et al., 2010), *Dicentrarchus labrax* (Torrecillas et al., 2012), Japanese flounder (Ye et al., 2011), shellfish e.g., crustaceans(Zhang et al., 2012), sea cucumber (Gu et al., 2011) and other vertebrates, e.g., broilers (Rehman et al., 2020) and pigs (Halas and Nochta, 2012).

Here, an enhanced expression of TNF-α gene in the intestine, liver and muscle of all the groups of *C. carpio* fed mannanase supplemented diet (Fig.15-17) was also observed. In the present study, TNF-α was elected because it is a pleiotropic cytokine produced predominantly by macrophages and plays a crucial role in the innate immune response, regulation of homeostasis, hematopoiesis and survival of lymphocytes (Sakai et al., 2020). There is no comparable study that indicated the regulation of TNF- α gene in fish following mannanase supplementation. However, many investigators reported a significant increase in the mRNA level of this gene in Nile tilapia (Pirarat et al., 2006; Al-Deriny et al., 2020) and common carp (Hoseinifar et al., 2017b) fed probiotics (*Spirulina platensis, Bacillus amyloliquefaciens* and *Lactobacillus rhamnosus*) and prebiotics (galacto-, fructooligosaccharide and inulin) supplemented diets respectively. The observed higher expression of TNF-α gene in present study may indicate the resistance of fish to infection(Idriss and Naismith, 2000).

There seems a direct link between enhanced level of TNF-α gene expression in the present study and increased respiratory burst activity and phagocytosis. Several studies have reported that Fish leucocytes' phagocytic activity is enhanced by the TNF-α protein (Zou et al., 2003; Garcia-Castillo et al., 2004; Grayfer et al., 2008). It has been demonstrated that TNF-α boosts survival of macrophages and restricts the proliferation of bacteria in diseased macrophages of *M. marinum* infected zebrafish (Clay et al., 2008). The researchers arrived at the conclusion that an increase in the formation of reactive oxygen species (ROS) seemed to be linked to bactericidal action . (Roca and Ramakrishnan, 2013; Lu et al., 2015).Another research study found that endothelial cells supernatants that were treated with TNF-α can enhance migration of leucocytes and respiratory burst activity in carp(Forlenza et al., 2009).

Normally circulating leukocytes have a restricted NADPH oxidase response to chemoattractant stimulation and phagocytosis. A number of pro-inflammatory stimuli,

including 1) tumor necrosis factor-α 2) granulocyte-macrophage colony-stimulating factor , and 3) bacterial lipopolysaccharide (LPS) enhance the phagocytes' respiratory burst activity (Ward et al., 2000; El- Benna et al., 2016; Nguyen et al., 2017). This enhancement of respiratory burst activity response is called priming. Priming increases the ability of leukocytes to destroy microbes by increasing their respiratory burst activity. The processes that prime leukocytes aren't completely understood (Ward et al., 2000)

Thus, in short β-mannanase supplementation irrespective of microbial origin showed a beneficial effect on apparent digestibility coefficient of nutrients , the growth, body composition, intestinal enzyme activity, hemato-immunological indices, metabolic enzymes, and expression of genes related to immunity and growth in *C. carpio* (Table.13-19). However, to gain even deeper understanding of the effect of mannanase on common carp, another trial (extending 90 days) was conducted to study the effect of mannanase on intestinal health.

For a long time, the digestive tract especially large intestine has been considered to be a microbial hotspot. A good interaction between microbiota and the host animal has beneficial effect on GIT (Míguez et al., 2016; Yang and Xu, 2018).Gut contains the highest number of microorganisms, with many parts that have diverse profiles of microbes and environmental conditions. The microbial community in the gut takes part in many different processes, which include vitamin and nutrient synthesis, fermentation of complex long chain carbohydrates, immune system homeostasis, defense from pathogens and physiological metabolism in distal tissues or organs(Han et al., 2018; Li et al., 2020).

In order to survive in the gut, the gut microbiota need to adapt to the environmental constraints present in the gut. Among these are the metabolic pathways that they have to utilize, such as those enforced by anaerobicity. In the gastrointestinal environment, sulfate reduction and fermentation of dietary carbohydrates can provide energy (Thursby and Juge, 2017).The microbial contact with intestinal mucosa, as well as indirect "cross-talk" between the microbial metabolites and host, are all important in the gut's growth, development, and physiologic equilibrium(Pan et al., 2019; Azad et al., 2020).

Recent research has found that gut microbiota are strongly affected by nutrition, and it is now considered a viable strategy for regulating any imbalance in microbial ecosystem (Donaldson et al., 2016; Lallès, 2016). According to several studies, the ability of gut microbiota to digest simple carbohydrates determines the profile of the microbiota in the gastrointestinal tract, demonstrating that the microbiota has adapted to the nutrients available in the small intestine (Zoetendal et al., 2012). Furthermore, many microorganisms present in the gut produce the enzymes and metabolic pathways required for the digestion of nondigestible and complex proteins and carbohydrates.

In addition, microorganisms are required by host to produce key vitamins such as vitamin K. Microbes are also needed for successful intake of several minerals such as iron, magnesium and calcium. Several studies have reported that microorganisms play important role in biotransformation of bile acid. After the fermentation cycle, short-chain fatty acids are produced, which supply energy to colonocytes. SCFA can also enhance intestinal epithelial cell proliferation and differentiation in vivo, as well as antimicrobial peptide synthesis and mucin secretions (Chang and Lin, 2016; Wang et al., 2017). Because of this, altering the gut microbiota has gained popularity as a method for improving the host's health, warding off illness and infection, and creating much needed energy and vitamins, the former of which might be crucial in physiological signaling pathways (Azad et al., 2020). In addition, studying how a feed additive affects the gut microbiota profile provides valuable information regarding the health of the host organism. Furthermore, studying the effect of a feed additive on different growth, immune and metabolic parameters without investigating its effect on microbiota of the animal is insufficient information. Thus, in the present study, mannanase enzyme was supplemented at two different doses to common carp and its effect on key digestive and immune genes as well as its gut microbiota was studied. Supplementation of mannanase enzyme in plant-based fish feed influenced the gut microbiota, immune and digestion-related genes in *C.carpio*. Illumina Miseq sequencing of gut microbiota of carp fed different doses of mannanase enzyme showed that supplementation with the enzyme altered its intestinal microbiota composition.

The metagenomic analysis showed Protobacteria and Firmicutes as dominant bacteria

in the gut of *C.carpio* of all groups (Fig.20). These bacteria make up the dominant phyla in the gut of most fish. It has been reported that Firmicute bacteria provide a good measure of intestinal health status. For example, *Lactococcus*, *Bacillus,* and *Lactobacillus* can not only provide protection against pathogen-induced intestinal function disruption but can also prevent inflammatory cytokines from being produced (Zheng et al., 2017), while Lachnospiraceae has been reported to take part in the fermentation of carbohydrates and production of short-chain fatty acids (SCFAs) and gasses (H2 and CO2) (Duncan et al., 2007). In the present study, dietary mannanase supplementation increased the growth of beneficial bacteria of phylum Firmicutes like *Bacillus, Lactobacillus,* and *Lachnospira* in both treatment groups (Fig.22).

Beneficial microorganisms, sometimes referred to as probiotics, are defined as living microorganisms that benefit the host's health when given in the adequate quantity. (Pandey et al., 2015a). Fish have been given a variety of microorganisms as possible probiotics including yeasts,bacteria,bacteriophages and microalgae (Egerton et al., 2018). The genera *Bacillus*, *Lactococcus*, *Shewanella*, are among the most commonly studied probiotics (Hagi et al., 2004; Merrifield and Carnevali, 2014; Egerton et al., 2018).

Carnevali et al., (2017)compiled a list of 61 papers that looked at the delivery of probiotics to teleosts in a recent review. Many investigators have reported enhanced growth rates and immunological status regulation in connection with gut microbial composition alteration (Bagheri et al., 2008; Huang et al., 2014; Tapia-Paniagua et al., 2014; Cordero et al., 2015). Most research to date has been done on larvae and juveniles, and it has been shown to have positive effects on the cells in mucus layer of the intestine and to stimulate the innate immune response. (Cerezuela et al., 2011; Abid et al., 2013).

In the present study, some opportunistic pathogens like *Aeromonas*, *Desulfobulbus* and *Desulfovibrio* were not observed in the mannanase supplemented groups (Fig.22). *Desulfobulbus* and *Desulfovibrio* are the chief sulfate-reducing bacteria and are ubiquitously present in both animals and humans (Ichiishi et al., 2010). The H_2S gas produced by these sulfate-reducing bacteria can damage the intestine epithelial cells. Thus, their absence in the treatment groups may indicate a reduced risk of opportunistic pathogen invasion in *C.carpio*.

Bacterial diseases like Vibriosis are a major constraint affecting the sustainability and production of the aquaculture industry(Abdelaziz et al., 2017; Haenen, 2017). Vibriosis is a practically lethal bacterial disease that is caused by the Vibrio bacterium (Chen et al., 2000). Vibrio bacteria belong to gram-negative group of bacteria found in marine and estuarine environments (Austin et al., 2007). They can be found floating freely in the water column, in combination with a host or a biofilm (Thompson et al., 2004). The number of Vibrio species discovered is rapidly increasing, and eleven of them have been connected to human ailments due to their potential to cause skin infections and gastrointestinal issues(Andrews, 2004; Novriadi, 2016).

Vibriosis has received considerable attention in the aquaculture industry, as it has become a major limiting factor and cause of death in aquafarming systems throughout the world. According to a reliable source, vibriosis has wiped out over 48 distinct species of marine fish in more than 14 countries (Austin et al., 2007). Vibriosis, for example, has resulted in a 30% drop in eel populations in Denmark.Vibriosis has been linked to major financial losses and other consequences in the aquaculture industry (Novriadi, 2016).

Vibrio anguillarum was the sole Vibrio species connected to fish disease until 1980 (Egidius, 1987). In recent years, several novel fish infections caused by this genus have been discovered, including the Characterization of different vibrio strains isolated from shrimp in a Rio Grande do Norte hatchery (Natal-Area, Brazil), where significant mortalities were observed at the time(Vanmaele et al., 2015). Severe necrosis of hepatopancreatic tissue and mass fatalities in farmed penaeid shrimp have also been related to the 69 kb plasmid pVPA3-1 identified in *Vibrio parahaemolyticus* (Han et al., 2015). This disease condition, also known as early mortality syndrome, has wreaked havoc on the shrimp aquaculture industry in both Central America and asia, resulting in losses of more than $1 billion(Zorriehzahra and Banaederakhshan, 2015).

In order to manage the devasting effects of vibriosis in aquaculture, antibiotic-medicated food is fed to diseased fish, and antibiotics are administered orally to all the fish that share cages or tanks (Defoirdt et al., 2007; Pridgeon and Klesius, 2012). However, due to

the development of antibiotic resistance in pathogens as a result of the regular use of antimicrobial chemicals, these treatments may be ineffectual. Another issue caused by unfettered antibiotic usage is the presence of leftover antibiotics in commercial products of aquaculture, which has resulted in human toxicity and allergy(Cabello, 2004). Because of the presence of antibiotic residues in farmed fish products, nations that want to import fish may reject them (Novriadi, 2016).

Data recently gathered by US,EU, Japanese and Austrailian authorities revealed that 2,400 export consignments of Vietnamese fishery products were turned down by US authorities (from 2002 to 2010), 422 shipments were refused by EU authorities, and 464 and 206 Vietnamese shipments were refused market entry by Japanese and Australian authorities, respectively (from 2003 to 2010) (Novriadi, 2016). Thus antibiotic usage resulted in a significant drop in output as well as a loss of competitiveness in the export market.

Comabtibg vibrosis without using antibiotics is the current challenge of aquaculture industry .In the present study, *Vibrio* spp. was not found in both the treatment groups which might be due to supplementation of feed with a carbohydrase enzyme-like β-mannanase that reduces the residence time of digesta in the gut of fish. An increase in residence time of digesta has been associated with the decrease in oxygen tension which may favor the growth of opportunistic pathogens like *Vibrio* and *Aeromonas* (Sinha et al., 2011). However, supplementation with carbohydrase enzyme reverses these effects and may prevent opportunistic pathogens to invade. Interestingly, *Vibrio* spp. was not found in the control group. The absence of *Vibrio* might be due to the presence of *Halobacteriovorax* that might suppress its growth(Williams et al., 2016). This phenomenon needs to be investigated further.

Metagenomic analysis of 18S rRNA of fish gut microbiota showed an abundance of *Geotrichum* spp. in both the treatment groups (Fig.23). *Geotrichum* is a part of normal human flora and infections from *Geotrichum* in animals are rare (Carter and Cole Jr, 2012). It has been reported that *Geotrichum* has broad-spectrum antibacterial activity against pathogenic microorganisms such as *E. coli*, *S. aureus, and Micrococcus* spp.(Mefteh et al., 2017). *Geotrichum* has been used as a probiotic in fish feed and its beneficial effects on growth,

immunity, and resistance against disease have been demonstrated in different studies (Amir et al., 2018; Ghori et al., 2018).

An enhanced expression level of genes related to key immune enzymes including, Lys, SOD, NK-lys, Def, and IL1-β in the present study also indicated the positive effect of dietary β- mannanase supplementation (Fig.18). The beneficial effects of dietary carbohydrase enzymes including xylanase and mannanase on the immunity of fish have been reported in other studies (Chen et al., 2016a; Saputra et al., 2016). However, no study has reported mannanase effect on the expression of immune-related genes. β-mannanase enzyme when supplied in the feed hydrolyses the mannan into manno-oligosachrides (MOS), which act as prebiotics (Ringø and Olsen, 1999; Huang et al., 2003). Many investigators have reported modulation of gut microbiota by MOS i.e., favoring the growth of certain bacteria while inhibiting proliferation of others (Wang et al., 2018; Zheng et al., 2018). The increased expression level of immunity-related genes in the present study might be due to changes in gut microbiota composition of *C.carpio*. Several studies have reported the causal link between gut microbiota and immune gene expression(Rupnik et al., 2009; Nie et al., 2017). Good evidence of this causal relationship has been provided by a study conducted on a mouse infected with *Clostridium difficile* (Rupnik et al.2009). It seems that some factors caused by specific gut microorganisms might influence the immune gene expression of the host, which in turn plays an important role in resistance towards disease in an animal (Nie et al., 2017).

According to some investigators, SCFAs generated by beneficial bacteria such as *Bacillus*, *Lactobacillus* and *Lachnospira* can enter a bacterial pathogen through its cell wall, expel its protons, impair the metabolism of cells, and ultimately cause death of the organism (De Schryver et al., 2010; Simonyte Sjodin et al., 2016; McLoughlin et al., 2017).These mechanisms reveal that mannanase may have played a role in not only improving the host immunity by a selective proliferation of beneficial microorganisms in the treatment groups but also contributed towards restricting the growth of the pathogenic microbes by increasing the SCFAs concentration in the intestines of *C.carpio.*The results of the present study are supported by other studies. Upadhaya et al.,(2016) observed that mannanase supplementation decreased coliform population and also tended to reduce ammonia emissions. Bortoluzzi and

Fernandes (2019)observed that β-mannanase supplementation altered the diversity and composition of gut microbiota of broilers either subjected or not subjected to coccidiosis challenge. Regardless of the presence of *Eimeria* spp. in broiler chicken, dietary mannanase modulated the gut microbiota and led to an increase in the beneficial groups such as Akkermansia, Ruminococcaceae and reduction of Bacteroidetes, which is related to poor feed efficiency in broiler chickens.

β-Mannanase supplementation in the present study, also led to an enhanced expression level of genes related to digestive enzymes including Lip, Amy, Tryp, FAS and FABP in both the treatment groups compared to control (Fig.19). Similar results have been reported by other investigators supplementing other carbohydrase enzymes to different fish species.Zhou et al., (2013) report that supplementation of cellulase enzyme in the duckweed-based diet increased the digestive enzyme activities of grass carp (*Ctenopharyngodon idella*). An increase in the intestinal enzyme activities following supplementation of feed with xylanase in Jian carp (*Cyprinus carpio* var. Jian) has also been reported (Jiang et al., 2014). This shows that exogenous enzyme supplementation can promote the production of endogenous enzymes. The consumption of a diet rich in NSP, changes the anatomy and physiology of the gut of the monogastric animal, mainly by changing the digesta viscosities. This leads to an increased residence time of digesta which may alter the pH of the intestine and disturb the microbial ecology within the gut(Sinha et al., 2011). Supplementation with β-mannanase may reverse these negative effects. In addition, the prebiotics (MOS) produced as a result of mannanase action promote selective growth of beneficial microorganisms(Markowiak and Śliżewska, 2017; Zhang et al., 2021). These beneficial microorganisms or probiotics have been linked to enhanced digestive enzyme activity. In a study reported by Yang and Chen(2005) lipase, Isomaltase and sucrase activities in rats fed with a high dose of synbiotic were observed to be increased. The reasons for the enhanced enzyme activity was attributed to the healthy gastro-intestinal microbial ecology or to modified bacterial enzyme secretions.

For Arbor Acres broilers fed with NJ0516 *Bacillus coagulans*, Wang and Gu(2010) observed that there was a significant increase in activity of amylase enzyme as compared to

control group. Similarly, Hu et al.,(2018) reported that activities of amylase in the small and large intestines of piglets administered by probiotic *bacillus* were substantially higher than those of the piglets fed diets supplemented with antibiotics.

Increase in enzyme activities of maltase, sucrase and lactase were also observed in jejuna mucosa of pigs when pigs were fed with diet supplemented with *Bacillus*(Hu et al., 2018). Similar observations were made when probiotic *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were fed to rats, an increase both in lactase and sucrase activity was observed in the intestinal mucosa (Southcott et al., 2008).

The probiotic *Lactobacillus Rhamnosus* GG was found to greatly enhance the lactase and sucrase activites in BALB/C mice (Goyal et al., 2013). Sankar et al.,(2017) report increased alkaline protease activity in *O. Mossambicus* when administered with *bacillus* alone or with another probiotic *micrococcus* ($P<0.005$).It was observed that whether the probiotic was administered for 14 days or 28 days, it had significant effect on enzyme activity. Thus, the enhanced gene expression of digestion-related genes Amy, Lip, Tryp in the present study might be due to these beneficial bacteria.

Probiotics also help to boost SCFAs synthesis. The preservation of cellular function and homeostasis depends on the synthesis of fatty acids (De Carvalho and Caramujo, 2018). Fatty acids whether produced by host or gut microbiota are kept as a reserve of energy and consumed when needed (Laplante and Sabatini, 2009). By promoting the growth of beneficial gut microbiota, dietary mannanase may enhance the intestinal SCFAs content of *C.carpio*. The significant increase in treatment groups of expression levels of the genes FAS and FABP might be linked to enhanced production of SCFAs by beneficial microorganisms. This shows that mannanase may have a role in *C.carpio* fatty acid production, specifically SCFAs.

In broilers, supplementation with xylanases and to some extent cellulases have shown good results, while in a soybean-based diet, pectinases and mannanases have been effective (Sinha et al.2011). The positive effects of dietary mannanase supplementation on growth performance of broilers (Zangeneh and Torki, 2011b; Cho and Kim, 2013) swine(Yoon et al.,

2010) and turkeys (Jackson et al., 2008) have been reported in several studies.Chen et al.,(2016) reported that mannanase addition in fish feed is beneficial for the growth of tilapia. It seems that the significant increase in growth performance observed in the present study was due to improved digestion and availability of nutrients, which is supported by our results related to enhanced digestion-related gene expression. However higher dietary mannanase concentrations, on the other hand, did not provide improved results. Microbial mannanase can be an excellent feed additive in terms of economic feasibility, and its optimal dosage for *C.carpio* health and performance regulation under the current study is 500U/kg.

Conclusion

In modern aquaculture, alternatives are required to substitute fishmeal with other easily available cheap raw materials since it is the present need of aquaculture industry to be sustainable in regards to energy usage, feed cost, and environmental effect. Enzyme usage in the feed industry is based on its effectiveness, safety, and cost, which is in line with the demands of a production system that uses less resources. The addition of carbohydrase enzymes to fish feed can increase the growth performance of farmed fish by degrading the hemicullolose fraction in the plant cellwall and thus enhancing the absorption and digestion of nutrients in plant-based feeds.

Over the past few decades, the use of β-mannanases in the anmal feed sector has progressively increased.The expansion in aquaculture industry and need for cheap plant-based feedstuffs has necessitated the search for mannanase enzyme that can work in the unique gastrointestinal environment of fish. The present study identified, isolated and characterized an enzyme that showed good thermostability, salt tolerance, resistance to proteases, appreciable activity at low temperature and neutral pH. This enzyme is the extracellular endo-β-mannanase produced by *Aspergillus Niger*-AD01, a new mannanase-producer.In addition the enzyme displayed good activity against mannan rich substrates including locust bean gum, guar gum, konjac powder and soybean meal. The enzyme's broad working temperature range, high activity particularly at low temperatures, tolerance towards salt and proteases, and broad range of working pH make it a suitable candidate for use as a feed supplement in plant-based diets for agastric fish in aquaculture. The enzyme showed its potential as a feed additive during simulated intestinal digestion phase. Feeding trials were run to test its potential in real applications. It was observed that β-mannanase supplementation irrespective of microbial origin showed a beneficial effect on apparent digestibility coefficient of nutrients, the growth, body composition, intestinal enzyme activity, hemato-immunological indices, metabolic enzymes, and expression of genes related to immunity and growth of *C. Carpio*. In addition, the supplementation of β-mannanase modulated the gut microbiota of *C.carpio*, while certain beneficial microorganisms were plentiful, opportunistic pathogens were absent. Mannanase also enhanced the expression of

key genes related immune and digetion enzymes in the gut of fish. Based on these findings, mannanase supplementation may be proposed for improving carp production on inexpensive feed, i.e., plant-based diet which can act as a substitute for fishmeal and its optimal dosage for *C.carpio* health and growth performance regulation under the current study is 500U/kg.

Future Prospects

Carp dietary enzyme usage has shown results that are comparable to those of probiotics and prebiotics in aquaculture. In aquaculture, all three of them function as alternatives to chemicals and antibiotics, providing significant advantages in terms of greater health, growth, and survival rates as well as the production of seafood that is safe for consumers. Modern biological agents may improve the two crucial aspects of farming of carp; resistance to disease and growth performance while utilizing cheap plant-based feeds. This will satisfy the needs of sustainable aquaculture development. Without the use of functional feed additives, intensive and large-scale carp aquaculture would fail.

The fundamental causes of the growth-promoting benefits of dietary enzyme supplementation are still under investigation in carp aquaculture and require additional studies.

The mechanisms underlying the immunity of gut mucosa enhanced by dietary supplementation of enzyme needs be thoroughly studied (e.g., employing immunohistochemistry, gene expression, and proteomics). In order to comprehend the mechanisms of action better, future research on dietary enzyme supplementation should utilize advanced biotechnological tools like Quorum sensing, various staining techniques, transmission electron microscopy, scanning electron microscopy, and high throughput genome technologies.

Even though dietary carbohydrase enzymes may have positive impacts on different growth parameters and immunity of fish in carp aquaculture, little is known about how these feed additives affect carp at different phases of its life. Future studies must conduct in-depth investigation to ascertain the impact of various enzyme supplementation at different phases of carp growth.

The present study uses culture-independent molecular approaches to examine the nutritional impact of a carbohydrase enzyme and the resultant production of prebiotics in carp gut microbiome. Further such studies are required to create more data about this important aspect of feed supplementation.

The preparation of the feed is crucial in creating an environment where dietary enzyme can work effectively on the hosts. The application of enzymes can be done as feed supplements or water additives. Therefore, it is essential to comprehend the proper circumstances (temperature and amount of time) for storage and preparation of diet.

New strategies have strived to enhance and optimize the enzyme activity and thus the aggressiveness of the enzymes toward the desired substrate has been increased. At the moment, enzymes added to commercial feeds are unable to endure the harshness of the feed processing methods, including pelleting or processing at temperatures above 95 °C. To prevent the damage caused to delicate protein structure of the enzyme from high processing temperature, exogenous feed enzymes can be mixed with a suitable oil and then sprayed on to feeds. Some feed enzymes have been given a protective covering that is mostly made of lipids in order to protect it during feed processing. Improving coating agents is one method that can now a days be utilized to enhance thermostability of enzymes. A desirable coating should have both ease of disintegration in the gastrointestinal tract during digestion and stability and durability throughout the process of feed production and preparation. Other strategies include discovering enzymes that are naturally thermostable, for instance from thermophilic bacteria, or engineering the enzyme to make it more thermostable.

Advances in protein engineering has brought forth new opportunities to introduce predesigned changes to create customized carbohydrases having the desirable properties. However, in engineering of feed enzymes a few challenges lie ahead: (1) It must be considered that enhancing the thermostability of an enzyme often decreases its overall flexibility, therefore the produced enzyme will likely have lower catalytic efficiency; (2) Before the engineered enzymes can be used in food and feed sector, there's need to investigate how these designed proteins interact with biological systems; (3) The paucity of general rules in prioritization of enzymatic properties must be improved while selection of appropriate methods is also a difficult issue in bioengineering of enzymes. Collection of successful studies in feed enzyme engineering should provide suitable guidelines; (4) The quest for an ideal carbohydrase enzyme that can be used as a feed additive is ongoing even after many research efforts, while the de novo design of highly optimized industrial feed remains still elusive. In order to develop an advance method to engineer an enzyme

containing highly desirable characteristics there's need to enhance the understanding of the relationship existing between function and structure of the enzyme. Once these advanced methods are developed, designed enzymes will be easier to manufacture and industrialize.

Exogenous dietary enzymes will also function differently in various fish species due to the variation in their gastrointestinal system. Fish without stomachs have neutral or slightly alkaline digestive environments, such as those in the cyprinidae family. The pH of the digestive system is lower in gastric fishes, even reaching 1.4 in *Tilapia nilotica*, as a result of gastric acid production. Thus, an exogenous enzyme's acid-base tolerance affects how it will be digested and consumed by the fish. There's need for the production of enzymes with altered amino acid structures that are more intrinsically acid-base tolerant or the exploration of such enzymes in natural environments. Additionally, specificity of an enzyme is the capacity to select a precise substrate among a collection of compounds that are identical. The complementarity of the conformational and structural properties of the enzyme and substrate serves as the basis for the specificity, which is basically a molecular recognition process. Different levels of substrate selectivity are displayed by enzymes for example., lactase, which can only bring about hydrolysis of the beta-glycosidic bond present in lactose sugar to yield glucose and galactose units, and maltase, which is limited to acting only on the beta-glycosidic bond situated between two molecules of glucose in maltose sugar. Trypsin, is a serine proteolytic enzyme and is usually present in the gastrointestinal tract, has the ability to bring about hydrolysis of peptide bonds in which basic amino acids like histidine, lysine, or serine provide an amino group. Thus, the effectiveness of exogenous enzyme employed in aquaculture feeds should be evaluated based on the specificity of the enzyme to its substrate present in feed.

The digestive tract contains inhibitors for exogenous enzymes, which may severely limit their potential actions. For instance, the protease released by fish may hydrolyze foreign enzymes or feed additives. To solve this issue and increase the effectiveness of exogenous enzymes, a reliable delivery mechanism must be created. Enzymes, medicines, and other substances are delivered more effectively in the medical area with the help of a variety of

polymeric substances and lipids. A few researchers have created a successful controlled delivery method based on chitosan nanoencapsulation for improving the effectiveness of exogenous dietary enzymes in fish digestive tract. But with carbohydrases such efforts are few and far in between and there is a need for more research in this area.

The ingredients that make up feed include crude lipid, crude carbohydrates, crude protein, some inorganic salts, and other constituents. Therefore, it has been hypothesized that utilizing a combined mixture of exogenous enzymes rather than a single enzyme might be more advantageous. Research has shown that, this assumption has not proven to be accurate. In several instances, adding a mixture of enzymes to feed had no appreciable impact on fish growth metrics, FCR, or nutrient digestibility. While in other instances, feeding fish a variety of carbohydrases resulted in significantly increased growth performance and immunity as compared to what can be achieved with a single enzyme. Therefore, to maximize the combined efficiency of enzymes, it is crucial to understand how enzymes cooperate to hydrolyze their unique substrates. This suggests that the creation of a mixture of enzymes that work together and degrade the target substrates will benefit from a better grasp of the substrates present in aquafeeds.

Finally, a principal obstacle in the marketing of enzymatic processes is the cheap, large scale production of dietary enzymes. To realize this goal, strategies need to be discovered which help facilitate the cheap production of bulk feed enzymes. It is hoped that in the near future, newer methods for simple and affordable production of carbohydrases, which can effectively meet the requirements of different industries will be discovered.

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Plant-based β-mannanase supplemented diet modulates the gut microbiota and up-regulates the expression of immunity and digestion-related genes in *Cyprinus carpio*

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ABSTRACT

Microbiota is an integral component of the intestinal tract and plays a significant role in health status of fish. However, diet and feeding habits are the key factors affecting the intestinal microbiota. Here, a 90-day feeding trial was conducted to investigate the effects of a plant-based β-mannanase supplemented diet on the growth, gut microbiome, and mRNA level of key digestion and immunity-related genes of common carp (*Cyprinus carpio*). Fish were evenly distributed in 3 groups: control, A1 and A2, and fed 35% CP supplemented with β-mannanase at the rate of 0, 500, and 1000 units/kg diet respectively. At the end of the feeding trial, the intestinal microbiota was profiled by sequencing the v4 region of bacterial 16S rRNA and internal transcribed spacer (ITS) regions of fungal 18S rRNA. Results indicated improved growth performance, changes in richness and diversity of intestinal microbiota and up-regulation of intestinal digestion (Amy, Lip, Tryp, FAS, FABP), and immunity-related (SOD, NK-lys, Def, Lys, IL1-β) genes of A1 and A2 groups of fish as compared to control. This study first time demonstrates that β-mannanase in a plant-based diet could improve the intestinal health of *C. carpio* via modulation of gut microbiota and up-regulation of host digestion and immunity-related genes.

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1. Introduction

In aquaculture feeds, fishmeal is the most prevalent and best source of protein. It has a high content of crude protein (65–72%), as well as all the ten necessary amino acids, essential fatty acids, some vitamins, and minerals (Tacon et al. 2009; Samaddar et al. 2015). However, for the past decade due to growing demand for fish meal in both livestock and aquaculture industry as well as a drop in its worldwide production and supply has made fish meal the most costly source of protein for fish feeds (El Sayed 1998). Furthermore, fishmeal contains significantly more phosphorus than is required for optimal fish development, resulting in the discharge of excess phosphorus into the environment causing eutrophication (Kalhoro et al. 2018). Because of these issues, replacing fishmeal with alternative sources of protein that function as well as or nearly as well as fishmeal is the current priority in the aquaculture industry.

At present, among different alternatives to fish meal, plant-based feedstuffs appear to be readily available and least expensive. Currently, worldwide cereal grain production is at 2790 million tonnes, legume production is at 150 million tonnes, and fibrous material production is about 115 million tons (FAO 2020). Although the carbohydrate content of grains and legumes provides a cheap source of energy for fish, most fish species do not use it as efficiently as protein and lipids (Turchini et al. 2019). Furthermore, plant-based feeds such as rapeseed meal, soybean meal, corn glutton meal, root tuber meal, sunflower meal, and legume seeds have some anti-nutritional factors such as protease inhibitors, tannins, saponins, phytates, lectins, and non-starch polysaccharide (NSP), which negatively affect digestion and fish growth (Francis et al. 2001).

Non-starch polysaccharides (NSP) are one of the major anti-nutritional compounds in plant-based feed, which are present in the cell wall as complex carbohydrates. They are responsible for increasing digesta viscosity, changes in morphology and physiology of the intestine, and alteration of the intestinal microbiota (Chen et al. 2016). However, it has not been reported that NSP directly inhibits digestive enzymes, but their activities might be affected due to increased digesta viscosity caused by NSP in the feed, thus restricting the enzyme to access the substrate (Sinha et al. 2011).

Among NSP mannan appear in the form of Glucomannan and Galactomannan. Mannan present in soybean meal, cotton-seed meal, rapeseed meal, and corn gluten meal has shown to be a strong anti-nutritive factor in various monogastric animals and fish species (Wu et al. 2005). Therefore, there is an increasing interest in the exploration of feed additives that could neutralize the anti-nutritional effects of mannan in the feed. Recent studies have shown that β-Mannanase can hydrolyze mannan present in the feed to manno-oligosaccharides (MOS), which act as prebiotics (Huang et al. 2003; Wu et al. 2005). For instance, the positive effects of dietary β-mannanase supplementation on growth performance and biological functioning of turkeys (Odetallah et al. 2002) laying hens (Jackson et al. 1999) broilers (Mussini et al. 2011), and swine (Pettey et al.

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