

CHITOSAN NANOPARTICLE ENRICHED FISH FEED DIET

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Technology degree in **BIOTECHNOLOGY**

by

DIVYA K (REG NO - 39230014)

SAMYUKTHA E (REG NO - 39230060)



DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

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JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI- 600 119

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **SAMYUKTHA E (39230060)** and **DIVYA K (39230014)** who carried out the project entitled "**CHITOSAN NANOPARTICLE ENRICHED FISH FEED DIET**" under our supervision from **SEPTEMBER-2022 to APRIL-2023**.

Internal Guide

Dr. V RAMESH KUMAR

External Guide

Dr. S KUMARAN

Head of the Department

Dr. V RAMESH KUMAR

Submitted for Viva voce Examination held on 10/05/2023

10/5/23
Internal Examiner
External Examiner

DECLARATION

We, **SAMYUKTHA E (39230060)** and **DIVYA K (39230014)**, hereby declare that the Project Phase-2 Report entitled "**CHITOSAN NANOPARTICLE ENRICHED FISH FEED DIET**" done by us under the guidance of **Dr. V. RAMESHKUMAR, Ph.D.**, (Internal) and **Dr. S. KUMARAN, Ph.D.**, (External) at **COL.DR. JEPPIAAR RESEARCH PARK, CENTRE FOR DRUG DISCOVERY AND DEVELOPMENT** is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in **BIOTECHNOLOGY**.

1. E. S. Y. R.
2. D. J. A. K.

DATE: 10/05/2023

PLACE: CHENNAI

SIGNATURE OF THE CANDIDATE

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ABSTRACT

The danger to the coastal area and huge difficulty of disposing of shellfishery waste on a global scale. The usage of secure supplements to increase fish output and health is crucial for future aquaculture development. Some fish species employ chitosan (CS) as a feed ingredient to boost immunity and production. The objective of this research was to determine how chitosan nanoparticles (CSNPs) affected *Oreochromis niloticus* productive capacity, mortality rate, and protein conversion efficiency. This is the report on how *O. niloticus*'s growth performance and non-specific immunity were improved by CSNPs. Scanning and transmission electron microscopy, FT-IR, X-ray diffraction, and other methods were used to synthesize and analyze CSNPs. Zeta sizer and particle size distribution are also performed. Diets supplemented with CSNPs 1.0g/kg feed loaded were fed to fish (16.20 $\pm$ 0.08 g) for 45 days. In comparison to the control group, the tilapia's specific rate of growth, body weight, mortality rate, respiratory burst, and lysozyme activities were improved by the diet containing 1.0 g/kg CSNPs. The CSNPs are biologically active and powerful antimicrobial agents against a number of selected bacterial pathogen, according to this work. In conclusion, the results showed that the Nile tilapia's innate immunity qualities, growth performance, and resistance to disease were all greatly improved by the food supplement containing CSNPs.

Keywords: Chitosan, Chitosan nanoparticle (CSNPs), *Oreochromis niloticus*, Diet supplement, growth performance.

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LIST OF ABBREVIATIONS

ACRONYM	DESCRIPTIONS
CS	CHITOSAN
CSNPs	CHITOSAN NANOPARTICLES
O.niloticus	<i>Oreochromis niloticus</i>
FE-SEM	FIELD EMISSION SCANNING ELECTRON MICROSCOPE
HR-TEM	HIGH RESOLUTION-TRANSMISSION ELECTRON MICROSCOPE
XRD	X-RAY DIFFRACTION
TPP	TRIPOLYPHOSPHATE
GP	GROWTH PERFORMANCE
NAOH	SODIUM HYDROXIDE
FT-IR	FOURIER TRANSFORM INFRARED RAYS
DLS	DYNAMIC LIGHT SCATTERING
WG	WEIGHT GAIN
SGR	SPECIFIC GROWTH RATE
FI	FEED INTAKE
PER	PROTEIN EFFICIENCY RATIO
FCR	FEED CONVERSION RATIO
RBC	RED BLOOD CELL
WBC	WHITE BLOOD CELL
NBT	NITROBLUE TETRAZOLIUM
PDI	POLYDISPERSITY INDEX

CHAPTER-1

INTRODUCTION

1.1 GLOBAL STATUS AND DEVELOPMENTS IN AQUACULTURE

Over the last 50 years, aquaculture on a global scale has risen exponentially, reaching 52.5 million tonnes. Asia accounts for 89% of the volume and 79% of the value of this industry, with China being by far the highest producer. Many variables, such as earlier aquaculture techniques, population and economic expansion, a permissive regulatory environment, and growing export potential, have contributed to the region's rapid rise. In Aquaculture, which includes fishes, crabs, and shellfish but excludes primates, turtles, and plants, provided 43% of aquatic mammal food consumed by humans in 2007 and is predicted to rise in the future to satisfy demand (John Bostock et al., 2010). The fast growth of shellfish and fish farming, has now been fueled by the worldwide decrease of ocean fishery stocks. Around a quarter of all fish eaten by humans actually came from marine farming activities. The demand for fish farming as a significant protein source will grow as the global population continues to surpass 6 billion. Around four metric tons of marine fisheries were produced globally in 2018 with a 6 yearly growth rate estimated due to a sharp increase in the number of marine fisheries India has risen to the place of 4th largest manufacturer from 2017 the yield of shellfish in India 99 (*penaeus vanname*) increased to over 700000 tonnes.

1.1.1 TRASH GENERATION BY SHELLFISHERIES

For the long-term viability of oceanic fishery, though, its growth in aquaculture industry is a mixed blessing. A few mariculture practices, like prawns and salmonids, do have potential to harm ocean as well as coastal zones by destroying their habitats, disposing of waste, introducing exotic species and pathogens, and requiring a lot of fish meal and fish oil. This could further deplete wild fisheries stocks. Both aquaculture's reliance and influence upon marine fishery were expected to rise once productivity of an industry

grows and intensifies (Rosamond Naylor et al., 2000). Upcoming mariculture techniques will impact how produced and farmed fish were distributed, as well as the number of fish is obtainable overall for human utilization. The world's economy is rising, trash generation is growing, and then a vast quantity of the byproducts of modern foods are being wasted even though they can contain valuable substances. (Yadav et al., 2019; Subramanian Kumaran et al., 2021). The majority of solid trash is made up of food waste, and its volume is rising quickly globally (Marousek et al., 2020a; Kumaran et al., 2020).

Particularly, as soaring shellfish industry sectors directly correlate to tonnes of shrimp trash, which makes up 58% of chitin. Moreover, chitin, a polymer with remarkable qualities such biodegradability, biocompatibility, antibacterial, anticancer, and antioxidant capabilities, is present in significant proportions in the shrimp waste (Shanmuganathan et al., 2019; Kumaran et al., 2020).

1.2 AQUATIC NANOTECHNOLOGY

Nanotechnology offers a broad array of uses in fish farming, which include pond sanitation, water purification, aquatic disease detection and control, efficient nutrition delivery and medications (including hormones and vaccinations), and improving fish's ability to absorb these compounds (Bhattacharyya et al., 2015; Huang et al., 2015). Many nanotechnology-based approaches are being employed today to ramp up production, efficiency, and sustainability. The domains of health care are currently the focus of attention, enhancing the development of fish and shellfish through diets supplemented with nutraceuticals, when compared to other technologies, the transport of compounds by precision drug delivery and controlled vaccine release, applications of nanotechnology may be more efficient at preventing and treating illnesses, reducing dangers to human health and the environment limiting the usage of drugs and considering mental aspects. Nanoparticles for target delivery could lead to novel, less invasive, and more efficient ways to administer medications. The effectiveness of disease treatment will also be improved, and the costs will be greatly reduced, with

treatments that (theragnostics) also diagnosing them, therefore preventing sickness. (Chena & Yadab, 2011; Morris, 2009).

1.2.1 CONTEMPORARY NANOTECHNOLOGY AQUACULTURE

a. VACCINE DELIVERY

Vaccines have been used largely utilized in aquaculture as a pathogen defense mechanism to shield the host from illnesses brought on by these infections. In fisheries, oral administration or injection are the most dependable and efficient immunization methods. Nano-delivery systems have recently been proposed as an alternative method for administering vaccines to fish that is not only safer but also has the potential to increase effectiveness (Bakht Ramin Shah et al., 2019).

b. NUTRIENT DELIVERY

Nutraceuticals are undeniably important in increasing development and immunological indices in fish. Their execution, however, calls for greater costs as opposed to more reasonable standards. The use of nanomaterials in fishery for the efficient delivery of nutritional supplements and nutraceuticals is supported by a large body of evidence. These systems are principally created to boost nutrient bioavailability, accessibility, and consequently efficacy by enhancing nutrient solubility and safeguarding against the hostile environment of the gut. In this situation, it was discovered that selenium (Se), zinc (Zn), and manganese (Mn) nanoparticles helped gilthead seabream (*Sparus aurata*) develop a greater ability to withstand stress and develop bone mineralization. early weaning diets. (Izquierdo et al., 2017).

c. TOXICITY

The environment and human health are adversely affected by nanotechnology-based materials and technologies, in addition to their principal application in the growth and sustainability of aquaculture. Because of their greater sensitivity, aquatic organisms are

particularly vulnerable to the potential toxicity of these chemicals. As a result, it is critical to examine the negative and harmful impacts of nanomaterials on aquatic creatures (Wang et al., 2008). Nano-Se and selenite toxicity in selenium-deficient Medaka fish. Nano-Se had approximately sixfold higher liver accumulation and fivefold stronger toxicity (in terms of LC50) than selenite. The aggressive-accumulation of nano-Se was thought to be the cause of enhanced oxidative stress responses.

Thus, Aquaculture development and sustainability unquestionably benefit from the use of nanotechnology. So far, many nano-based solutions have been used to improve the critical pillars of aquaculture and fishing. However, there is rising worry regarding the toxicity of NPs, along with the misuse of antibiotics along with other synthetic drugs in those industries. It is therefore impossible to avoid using safe and ecologically beneficial practices. (Bakht Ramin Shah et al., 2019).

1.3 *Oreochromis niloticus* (TILAPIA)

The term "tilapia" is used to describe a wide variety of cichlid fish species that may be found in freshwater streams, ponds, rivers, lakes, and, less frequently, brackish water. Tilapias are growing more prominent in aquaculture despite once being considered an invasive species. As a result of its suitability for aquaculture, the second most widely cultivated fish in the world, tilapia production has grown fourfold in the last ten years. One of the most crucial fish species in tropical and subtropical fish farming is the Nile tilapia, *Oreochromis niloticus* (FAO, 2012). It provides significant sources of revenue and animal protein all throughout the world (Sosa et al., 2005). Tilapia can develop and reproduce in a wide range of circumstances and can withstand handling-induced stress (Siddik et al., 2014).

Tilapia, popularly referred as "aquatic chicken," emerged from today's aquaculture industry's brightest star, and its consumption has grown globally (Fitzsimmons, 2005).

It is now referred to as the "Food Fish of the 21st Century" (Costa-Pierce and Rakocy, 2000; Ramnarine, 2005; Prabu et al., 2017).

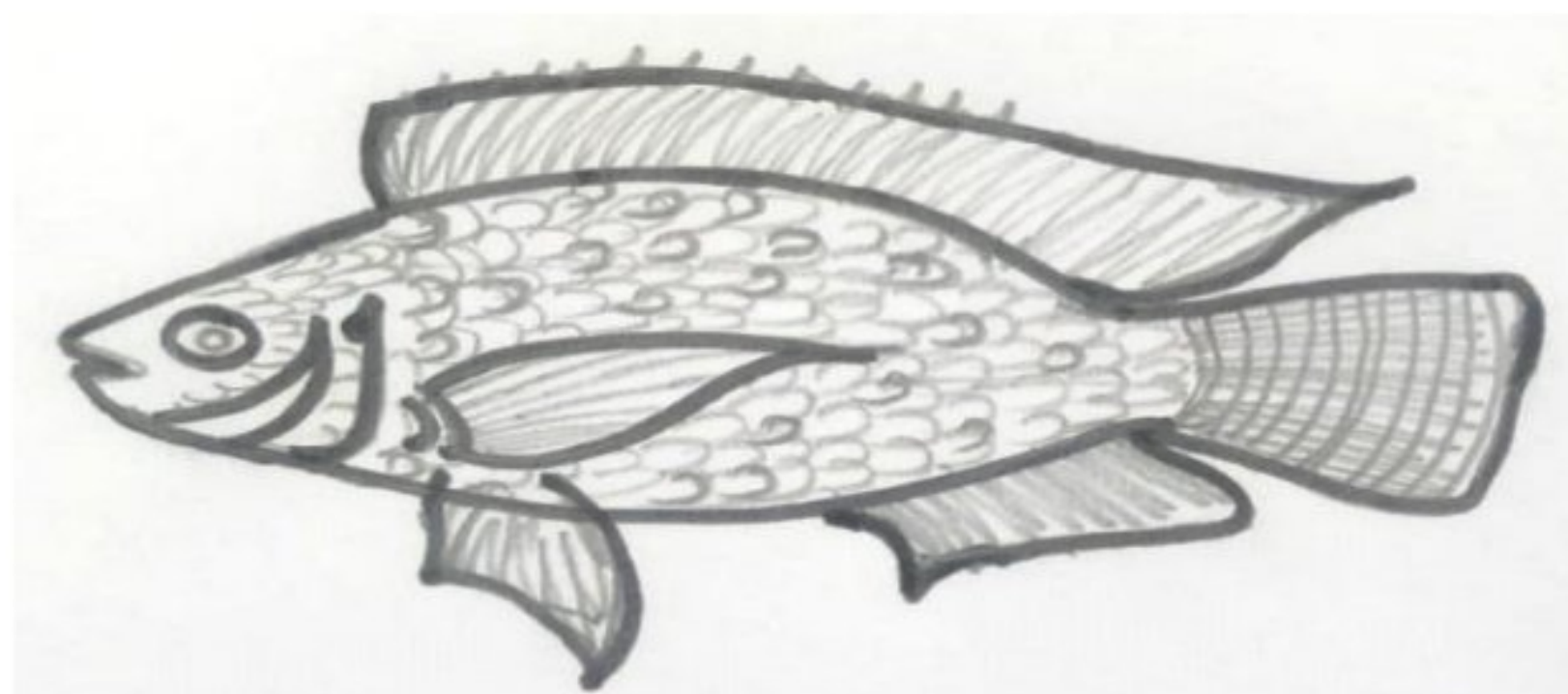


Fig 1.1 *Oreochromis niloticus*

1.3.1 HISTOPATHOLOGICAL STUDIES OF *Oreochromis niloticus*

Fish experience stress when they are confined, which can lead to tissue damage and production losses. Fish pathological and normal conditions can be determined through histopathological analysis. The ability to reduce sickness and stress in fish farming is a reflection of the wellbeing and health of the fish. Diverse methods for assessing aquatic species' health state may have an influence on fish welfare and greatly boost production. As tools for disease analysis and prevention, parasitological and histopathological diagnoses are among them (Sampaio et al., 2013). In response, histopathological analyses are highlighted because, in the face of unfavorable circumstances, they are described as confirmatory and conclusive diagnoses (McGavin and Zachary, 2013; Santos et al., 2017). Histopathological modifications come from a range of biological and physiological alterations in the living thing, which help to integrate the impacts of abiotic and biotic variables on organ function and fish health. (Van et al., 2009). The growth and survival of fish may be hampered by tissue changes brought on by environmental stressors (Brum et al., 2014) or pathogens (Guerra et al., 2012; Santos et al., 2017). Consequently, histopathological analysis is a lesion's relevance depends on how it affects organ function and the fish's capacity to survive.

A useful predictor of fish health condition is the severity of the lesions (Lilian Dordete Steckee et al., 2018).

1.3.2 GUT OF *Oreochromis niloticus*

Many biologists have wondered whether the tilapia's small, sac-like stomach can genuinely perform as a stomach. But considering that its contents have a low pH and that the pH maxima of its proteases are the same as those of the stomach's contents, it seems to have a gastric role (Kathiresan Purushothaman et al., 2016). Some of the researchers also stated that it is divided from the intestine by a pyloric sphincter.

O. niloticus's stomach was discovered to be separated into a large caecal zone and a pyloric section by (Morrison and Wright, 2005) reported three regions (initial or cardiac, middle or fundic and terminal or pyloric).

The structure of fishes' digestive systems has only occasionally been studied using the scanning electron microscope. A more accurate visualization of the structure-function connection in this organ system is made possible by combining SEM and light microscopy. The stomach mucosa's structure is ideal for both storing and digesting food.. It forms a narrow after being thrown up into relatively large longitudinal folds. Converting the empty stomach's lumen into a narrow stellate. The animal can handle wide changes in meal size thanks to this mechanism, which allows for significant distension in the presence of surplus food. In teleosts, the distension acts as a potent stimulant for stomach digesting secretions. Moreover, this folding pattern may separate the swallowed bolus into smaller pieces for more effective mixing with the digestive juices, slowing the flow of food through the stomach (Sensoy, 2021).

1.3.3 THE IMPACTS OF AMMONIA ON *Oreochromis niloticus*

Fish excrete between 60 and 80 percent of their nitrogenous waste as ammonia, a byproduct of protein catabolism (Ip YK and Chew, 2010). It can also enter aquatic environments through a variety of channels, including wastewater discharges, industrial effluent, fertilizer runoff, and the breakdown of biological wastes (Randall and Tsui, 2002). Total ammonia nitrogen (TA-N), which includes both ionized (NH_4) and unionized (NH_3) forms of ammonia, is the unit used to measure ammonia (Jackes Rodrigues da Silva et al., 2013). The ammonia that is produced during ionization (NH_4) is a bigger liquid form with charged particles that are difficult for the gill's hydrophobic micropores to readily pass through, whereas the ammonia that is produced during ionization (NH_3) is lipid-soluble and has no charge, allowing it to permeate over the gill membranes. Fish farming suffers from a serious ammonia problem, especially in recirculation networks, aquariums, and fish ponds. In intensive fish farming, where fish are housed in large numbers, an ammonia buildup may gradually take place, particularly when water circulation is limited (Person Le Ruyet et al., 2003). There have been several studies on the acute and long-term toxicity of ammonia to different fish species. (Dosdat et al., 2003; Lemarie et al., 2004). The majority of these research investigated the effects of ammonia on survival and growth. Investigations investigating the effects of ammonia exposure on Tilapia species are rather rare (Benli and Koksall, 2005). Excessive holding numbers and consumptive rates in intense fish breeding also raise the possibility of the animals being exposed to more waste products containing nitrogen, notably ammonia and nitrite (Abd El-Hack et al., 2022). At the generally accepted maximum safe levels of exposure to sublethal ammonia concentrations at or greater than $2 \text{ mg }^{-1} \text{ TA-N}$ for six weeks. Gills, liver, and kidney tissue all showed obvious histological alterations. To ascertain, further research is necessary to precise ammonia levels for Nile tilapia (Aysel Çag˘lan Karasu Benli et al., 2008)

1.4 CHITOSAN

Aquaculture requires the creation of novel, diets for fish that are not standard which can also support the physical needs and biological processes of cultivated fish in order to ensure continued growth. Therefore, in order to advance aquaculture, scientists must look for green, eco-friendly, renewable, valuable, and ecologically safe materials (Abdel-Ghany et al., 2020; Marwa El-Naggar et al., 2022).

Fish immune system growth and function are supported by chitosan (CS) and chitosan nanoparticles (CSNP), which are regarded as harmless, organic cationic polymers that degrade quickly [El-Naggar et al., 2020; Marwa El-Naggar et al., 2022).

Through the process of deacetylation, chitin, a crucial component of the exoskeletons of terrestrial animals and certain microorganisms' cell walls as well as those of aquatic crustaceans like shrimp, crabs, and crayfish, is transformed into CS (Xu et al., 2008). The prosperity of the nanotechnology sector is largely dependent on nanoparticles, which can range in size from 1 to 100 nm in at least one dimension. The physico-chemical properties of the nanoparticles are unique from those of their bulk constituents. They differ due to their increased proportion of surface to volume, which results in enhanced reactivity and distinguishing characteristics (Khosravi-Katuli et al., 2017).

1.4.1 CHITOSAN AND ITS APPLICATIONS

CS and CSNP have a variety of applications, including the treatment of water, crops, and cancer. Additionally, in aquaculture, they function as a nontoxic, and bio - based polymer, Due to its distinct biological features, the polymer chitosan (CS) is frequently employed in medical and biomedical formulations. It is a safe, versatile, and compostable polymer (Al-nemrawi et al., 2018). CS (Poliglusam) [-(1-4)-N-acetyl-D-glucosamine], is solvable in an aqueous solution of acidity. CS is unique in that it is non-toxic, recyclable, safe, and has enhanced solubility (Shard et al., 2014). These qualities make it appropriate to be utilized in a variety of industries, including aquaculture, cosmetics, cancer therapy, and drug delivery.

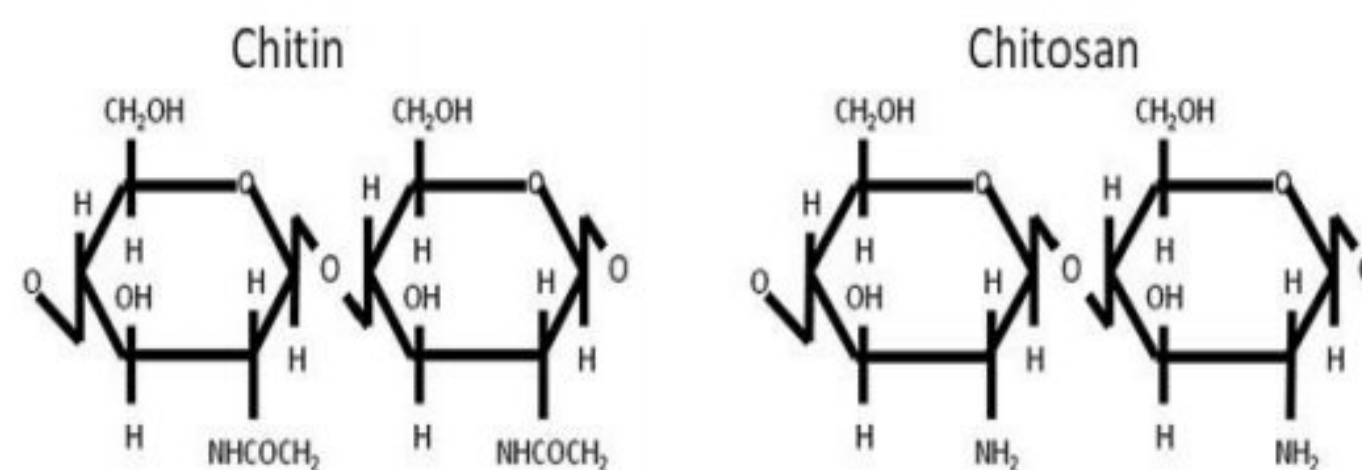


Fig 1.2 Structure of chitin and chitosan (kumar et al.,2000)

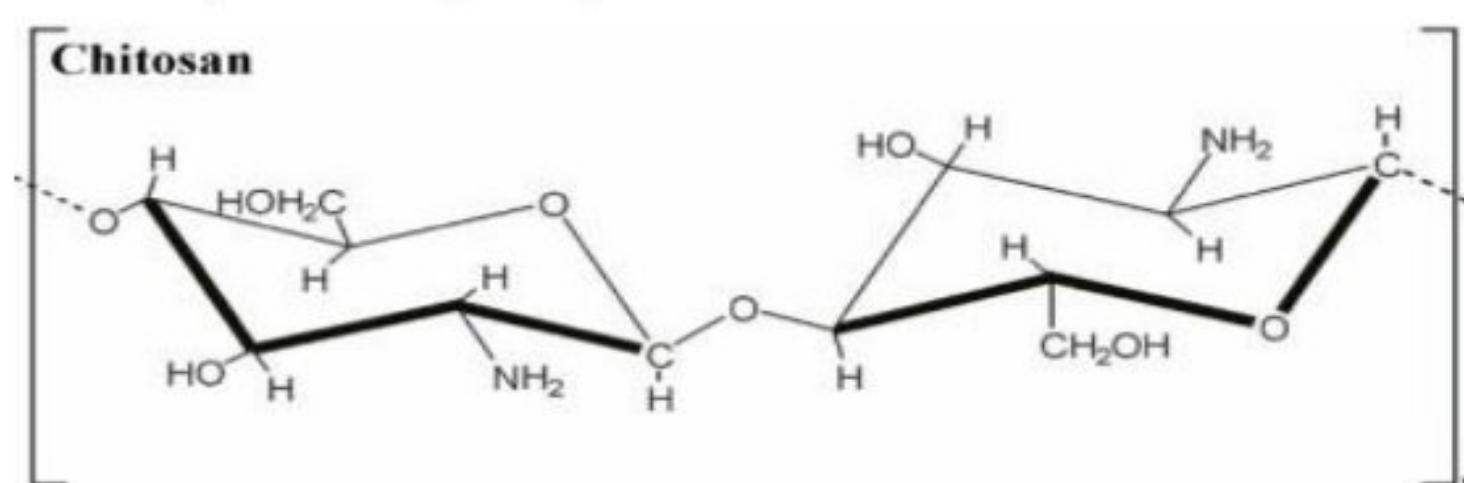


Fig 1.3 Chitin deacetylation (Saifuddin & Palanisamy, 2005)

1.4.1 CHITOSAN WITH TRIPOLYPHOSPHATE (TPP)

Chitosan nanoparticles (CSNPs) have been produced using a variety of techniques, ionotropic gelation, template polymerization, reverse micellar technique, oleyelectrolyte complex, precipitation, and crosslinking of emulsions are a few examples. The relationship between amino groups that are positively charged in chitosan and the negatively charged particles in a polyanion is the basis of the ionic gelation process. Most people refer to this polyanion as a crosslinker. Numerous crosslinkers have been employed, sodium tripolyphosphate (TPP) being one of them. TPP is the crosslinker that is most frequently used to create CSNPs due to its safety and multivalent characteristics. TPP and other polyphosphates are hydrolyzed into nutrient-rich, less complex phosphates. Because of this, polyphosphates are thought to be safe and have very low toxicity (AL-Nemrawi et al., 2018).

1.4.2 CHITOSAN'S IMPACT ON THE PERFORMANCE OF GROWTH

According to (Niu et al., 2011), chitosan, an active growth promoter, is crucial for the development of aquatic animals. According to (Zaki et al., 2015), The primary result of chitosan may be an improvement in the small intestine's morphological structure, which may enhance nutrient uptake and growth efficiency. Chitosan consumption at concentrations of 1 and 2 g kg⁻¹ Intestinal villi height, feed consumption metrics, and growth outcomes for sea bass were all improved by diet (*Dicentrarchus labrax*) (Zaki et al., 2015). (Shi et al., 2005) found that at low concentrations of dietary chitosan, nitrogen utilization and amino acid digestibility increased, which lends support to this conclusion. However, it is clear from these studies that the ideal dietary chitosan level varies by species. Additionally, the ideal chitosan concentration for the best fish growth performance may depend on the fish's size, age, and weight.

1.4.3 CHITOSAN AND CHITOSAN NANOPARTICLE PROPERTIES IN FISH AQUACULTURE

Considering the low harmful effects and fish growth capacity (GP) enhancement potential, anti-inflammatory characteristics, and immune reaction, CS and CSNP have been utilized extensively in a variety of applications.

Characteristics and training for the CSNP, in the 21st century has seen the rise of nanotechnology as a major industry. Due to its increasing surface area, which enables the use of nanoparticles in a wide range of fields, it is a new technology that affects the division of materials at a nano size of 1-100 nm. This can change the material properties and raise their potential to be used (Rathore & Mahesh, 2021). CSNP are biodegradable, biocompatible, and non-toxic materials that can be used for therapeutics, gene transport, and agriculture as biopesticides, as well as being efficient fish feed additives (Felt et al., 1998 and Augustine et al., 2019).

Furthermore, Due to its small size, which increases the surface area available to interact with physiological support, CSNP are favored. Unexpectedly, CSNP enhances the enables effective cell absorption, increases the bioavailability of necessary substances, and thoroughly reaches the desired locations (Alishahi et al., 2011).

1.5 FISH FEED FORMULATION

The intensive culture of many freshwater fish has increased, which has increased need for effective diets. Food output is negatively impacted by nutritionally deficient feed, and the accumulation of discarded feed has a significant negative influence on the environment. As a result, it is necessary to provide low-cost diets with the best possible nutritional composition. Since for production systems to generate a high-quality, healthy product, correct nutrition is crucial. Inexpensively, the quality of the feed components is the first element in the formulation of feed. One of the most expensive ingredients in prepared fish diets is fish meal. Fish nutritionists have experimented with substituting fish meal with partially or entirely with less expensive sources of plant protein. Soybean meal, which is utilized as the primary protein source in many fish diets, is thought to be the most nutrient-dense of all the plant protein feeds (Bhosale et al., 2010).

1.5.1 FISH FEED STABILITY

The majority of fish feed pellets on the market have poor water reliability and quickly swell when placed in water. As a result, the soluble vitamins and minerals in the pellet will be easily eroded out. They will cause nutrient deficiencies and environmental issues in ponds or fish tanks. In order to reduce swelling and mineral leaking while increasing floating time, a study was carried out. Many fish feed formulas were created and tested for these purposes. The recipe was based on readily available ingredients such as maize flour, soy flour, and tapioca flour. Palm oil stearin was added as a principal topic ingredient to the water to achieve water stability, resulting in better floating time and lower leachability (Saalah et al., 2010).

1.5.2 CHITOSAN AS AN IMMUNOSTIMULANT

Chitosan has been studied for its potential as an immunostimulant in Nile tilapia (*Oreochromis niloticus*), a fish that is regularly raised for food. Studies have shown that chitosan can enhance the immune response of Nile tilapia by stimulating the production and activity of immune cells. In one study, Nile tilapia were fed a diet supplemented with chitosan, and their immune response was evaluated. The researchers found that chitosan supplementation significantly increased the activity of lysozyme, an enzyme that plays a key factor in the innate immune reaction. Chitosan also increased the production of antibodies and enhanced the phagocytic activity of immune cells. Overall, these studies suggest that chitosan has potential as an immunostimulant in Nile tilapia, and could be used to enhance the fish's immune response and disease resistance. However, in order to establish the ideal dose, additional study is required. and application methods for chitosan supplementation in Nile tilapia diets.

1.5.3 CHITOSAN NANOPARTICLES AS FEED ADDITIVES

Feed additives are edible compounds that are sparingly added to livestock feed to improve feed quality, hence enhancing fish growth and lowering mortality risk (Dada et al., 2015). Due to the increased expense of fish meal, (ElNaggar et al., 2020) recommends looking for alternate protein sources, particularly plant-based alternatives. Chitosan can act as a prebiotic, promoting the growth of beneficial gut bacteria and improving digestive function in fish and shrimp. However, as with any feed additive, the optimal dosage and application methods of chitosan may vary depending on the species, feed composition, and other factors. To enhance *Oreochromis niloticus*'s immune system's capacity for development, it has been suggested that adding chitosan and chitosan nanoparticles to plant forward-based diets. Chitosan and Chitosan nanoparticles were advocated for use as immunostimulants and growth promoters in several studies (Gopalakannan & Arul, 2006; Wang & Li 2011; Alishahi et al., 2014; Ranjan et al., 2014; Abd El-Naby et al., 2019; Abdel-Tawwab et al., 2019 and Abdel-Ghany & Salem, 2020).

CHAPTER-2

LITERATURE SURVEY

Heba M. Abdel-Ghany and his team., (2019), stated that the negative polysaccharide is chitosan. obtained by varying degrees of exhaustive alkaline from chitin, a naturally occurring polymer of N-acetylglucosamine that is typically found in the cell walls of fungi, insects, and crustaceans. Is Chitosan has been discovered to possess a broad range of advantageous biological characteristics, including biodegradability, biocompatibility, and biosafety. It is an excellent material for a number of purposes because of these benefits. Chitosan is a substance found in land animals and is frequently added to feed since it has minimal negative effects. Intestinal microbial infections are suppressed, immune system function is improved, and cholesterol levels are decreased. It has more recently been utilized as an ingredient in fish feed. Chitosan is also gaining popularity as an active component. treatment of water for aquaculture. On the usefulness of chitosan in aquaculture, much study has been conducted. Therefore, in these reviews, we looked at the status of the world today of information on how dietary chitosan works Aquatic farm animals that have antibacterial, growth-enhancer, antioxidant, and immunostimulatory properties, with a focus on the best chitosan dose. This review also shown the potential for treating aquaculture water with chitosan. The research covered in this review indicate that Chitosan is a substance that has a lot of promise for use in aquaculture.

Fatma S. Abd El-Naby ang her team., (2018), stated that utilization of safe additives to enhance fish health and production is essential for aquaculture's continued development. To discover the outcomes of the analysis of chitosan nanoparticle (CSNP) supplementation on Nile tilapia (*Oreochromis niloticus*) growth, total body weight, gut number of bacteria and histomorphology, enzymes for digestion, hematological, immunological response, and liver status. The goal of this study was to track how CSNP affected the health and productivity of tilapia.

Mohsen Abdel-Tawwab and his team., (2019), reported that the fish feed supplements are typically used to combat any potential illnesses or to sustain ecologically friendly fish farming and to combat stresses. Chitosan, one of these feed supplements, stimulates the immune systems and growth of many aquatic organisms. It is anticipated that fish may respond more strongly to nanoscale materials than to materials of a more conventional size. In order to determine the impact of dietary chitosan nanoparticles (CSNP) on Nile tilapia, *Oreochromis niloticus* (L.) growth performance, antioxidant activity, the current study was carried out.

Alireza Alishahi and her team., (2012), In this review, they've stated that the chitosan is highlighted in relation to the numerous potential applications that could make use of its many qualities, including its antibacterial and antioxidant properties, capacity to form edible films and coatings, ability to treat wastewater from the seafood industry, improved gelling properties, capacity as a micro- and nanocarrier for bioactive compounds, functional foods, and drug compounds derived from aquaculture and seafood, are just a few.

Subashni Bhoopathy and her team., (2021), were to examined the antioxidant and immune-suppressive properties of curcumin-loaded chitosan nanoparticles (Cur-CSNPs) in *Lito Penaeus Vanname* and to show how Cur-CSNPs might boost the resistance of *Lito Penaeus Vanname* that had been exposed to *Vibrio harveyi*.

Udo and her team., (2017), has reported that the african catfish (*Clarias gariepinus*) culture is hindered by high feed costs, and efforts to reduce them have been focused on utilizing fishmeal alternatives that are currently in competition with both human and livestock consumption. More and more, it is necessary to look for waste materials that can help this situation. In order to better understand how chitosan and chitosan nanoparticles affect *C. gariepinus*' growth, feed utilization, survival, and body composition.

Marwa El-Naggar and her team., (2022), stated that the requirement for shock protection and growth of fish and disease, aquaculture seeks to provide steady environmental conditions. Well-balanced diets should be taken into account to ensure a consistent supply of fish species. The most significant feed additives for this purpose

are chitosan and chitosan nanoparticles, which have attracted the attention of researchers involved in the aquaculture field. The information about the antioxidant activity, growth stimulation, and immunostimulatory effects of chitosan and chitosan nanoparticles on fish was fully covered in the current review. Potentially useful materials include chitosan and chitosan nanoparticles. with a wide range of applications in fish aquaculture, according to the studies discussed in the current review.

Marwa El-Naggar and her team., (2021), reported that the chitosan is receiving much attention and is beginning to contribute significantly to aquaculture's sustainability. It satisfies the environmental requirements because it is an environmentally friendly compound that promotes the effective use of reagents and the minimization of potential waste. The aim of the current work was to determine the impact of feed supplements containing chitosan (CS) and chitosan nanoparticles (CSNP) at a level of 0.5% on two different diets on the innate immune system and general health of Nile tilapia. In order to maintain good health and resist infection, improvements, as well as a rise in the nutritional value of plant-based proteins, the current study suggests supplementing the Nile tilapia diet with CSNP.

Dina Nath Pandit and his team., (2020), has figured that the chitosan added to the bagrid catfish's diet raised survival rates and enhanced growth capabilities in fish. The result serves as a precedent for future research on locally available feed fixings like chitosan, particularly with *Mystus seenghala*.

Ali Khani Oushani and his team., (2020), reported that the growth and immune responses of rainbow trout (*Onchorhynchus mykiss*), weighing 27.75 ± 0.34 g, were assessed after 70 days of oral exposure to eight experimental diets, each of which contained varying amounts of chitosan and nano-chitosan loaded in clinoptilolite. The results to date imply that rainbow trout could benefit from diets supplemented with nchit + clin, particularly at 0.05 g kg⁻¹ nano-chitosan inclusion, in terms of growth performance and immune parameters.

SubramanianKumaran and his team., (2021), stated that the risk to the coastal area and major challenge of disposing of shellfishery waste on a global scale. The use of secure additives to increase the health and output of fish is crucial for future aquaculture

development. Several fish species use chitosan (CS) as a feed additive to boost immunity and production. The goal of the current work was to determine how NAG loaded (CSNPs) affected *Oreochromis niloticus* (L.) production rate, survival rates, and protein conversion efficiency. This is the first report on how *O. niloticus*'s growth enhancement and non-specific immunity were improved by CSNPs and CSNPs loaded with NAG. The results showed that the NAG-loaded CSNPs in the dietary supplement significantly improved growth performance, immune-modulatory properties, and other outcomes,

Yanbo wang and his team., (2011), stated that fishes were fed with both of these substances to assess the effects of feeding chitosan and chitosan nanoparticles to tilapia (*Oreochromis nilotica*) for 60 days at a dose of 5.0 g kg⁻¹ of food on the fishes' survival rate, growth capabilities, and meat quality. Fish were fed a meal containing chitosan (T-1) or the basal diet control, and the addition of chitosan nanoparticles (T-2) significantly increased ($P < 0.05$) final weight, daily weight gain (DWG), and feed conversion ratio (FCR) in contrast to fish fed the control diet (T-1). These results showed that dietary chitosan nanoparticle supplementation improved the meat quality status and growth performance of tilapia. The current study aims to examine the effects of chitosan and chitosan nanoparticles added to feed on Tilapia survival rate, growth potential, and meat quality.

Juliana M Araujo and her team., (2021), has clarified the results of adding selenium-loaded chitosan nanoparticles to feed on the Nile tilapia's (*Oreochromis niloticus*) antioxidant and growth responses. First, the physicochemical characteristics, monitored release, and antioxidant activity characteristics of selenium (Se)-containing chitosan-based nanoparticles were studied. This was done using the ionotropic gelation method. The effects on Nile tilapia growth, glutathione peroxidase activity, antioxidant activity (by radical scavenging activity), and body composition were then assessed. Overall, Se-loaded nanoparticles showed great promise as a reliable method of feeding dietary Se to Nile tilapia, positively influencing the species' growth, performance, feed effectiveness, resistance to oxidative stress, and antioxidant activity.

CHAPTER-3

AIM AND SCOPE OF THE PRESENT INVESTIGATION

3.1 AIM

To develop an economically cost-effective fish feed supplements, from Chitosan nanoparticles and observing the feed consumption, growth rate, weight gain of the fish *Oreochromis niloticus*.

3.2 SCOPE

The use of CSNPs with better immunostimulant properties in place of current conventional medications may be a potential strategy for increasing fish production in the aquaculture industry. To promote fish development, health, immunity, and productivity, it is essential to improve the diet quality utilizing organic diet supplements. Therefore, it is expected that the use of CSNPs will improve fish efficiency, diet quality, and immunological response. The present study's objective was to ascertain how CSNPs affected Nile tilapia growth, survival, and innate immune reaction. It also aimed to create an inexpensive diet composition that would increase fish immunological prophylaxis and disease resistance. thereby producing fish feed that's inexpensive. Hence the current work marks the beginning of a fresh era in the aquaculture industry.

CHAPTER-4

MATERIALS & METHODS

4.1 SYNTHESIS OF CHITOSAN NANOPARTICLES

CSNPs were created using the ionotropic gelation method, with minor modifications depending on the Chitosan and Tripolyphosphate concentrations. The Chitosan solution was prepared by; 3g of chitosan powder has been weighed and kept aside. At first, 1ml of acetic acid with 800ml of distilled water was stirred in the magnetic stirrer for 20 minutes. Then chitosan powder (3g) was added to the stirring solution. They were kept in the magnetic stirrer at 450rpm. Simultaneously, pH of the solution was maintained between 4-5, by adding Sodium hydroxide solution, that is 2 pellets of NaOH was diluted in purified water and then added to the chitosan solution. Kept for stirring for 12 hours. Meanwhile, sodium Tripolyphosphate solution was also prepared; 0.40g of STPP was added with 100ml of distilled water, kept in Motor stirrer for 30mins to completely dissolve the STPP with distilled water. After 30minutes, the subsequent steps involved adding little-by-little of STPP solution to the CS using instantaneous magnetic stirring for 40 minutes to create a 0.4% (w/v) STPP solution. Then, the created Chitosan-TPP solution was centrifuged at 8000 rpm, 4 °C for 45 min, effluent was completely removed. Subsequently, the pellets were thoroughly cleaned with distilled deionized water. Then the obtained CSNP's were kept in the hot air oven for drying. Dried pellets were further used for characterization studies and feed preparations (Kumaran subramanian et al., 2021).

4.2 CHITOSAN NANOPARTICLES CHARACTERIZATION

4.2.1 Ultraviolet visible Spectroscopy

The UV–visible spectra of Chitosan nanoparticles were measured using an 1800 UV visible spectrophotometer (Shimadzu, Japan). The wavelength measured was in the range of 200-500nm. In order to affirmed the production of CSNPs and determine the presence of impurities (Vaezifar et al., 2013).

4.2.2 Fourier-transform infrared (FT-IR) analysis

Using a Fourier transform infrared spectrometer (Bruker, France), it was possible to examine the functional groups tying chitosan, chitosan nanoparticles, and the drug tying to the nanoparticles. The Chitosan nanoparticles were measured with the frequency vary from $4000\text{--}400\text{cm}^{-1}$ through FT-IR spectra (Bruker, France). KBr processed the specimen to produce pellets for the CSNPs. At a frequency of 4cm^{-1} , sixteen measurements for each spectra were taken. (Ibrahim et al., 2011).

4.2.3 Field emission scanning electron microscopic (FE-SEM) analysis

A scanning electron microscope was used to investigate the shape, assess the size of surface pores and dispersion of nanoparticles (SEM). Utilizing a SUPRA 55 from Carl Zeiss NTS GMBH in Germany, FE-SEM analysis was carried out. A small number of CSNPs specimens were placed on a copper filter with a carbon coating to create a film of uniform thickness. After removing surplus material using filter paper, the film was dried on a SEM grid for 5 minutes using a mercury lamp with an output of 100–600 W. The film was then magnified with changing resolution intensity to interpret nanoparticles. (Shanmugasundaram et al., 2017 Kumaran Subramanian et al., 2021).

4.2.4 High – resolution transmission electron microscope (HR-TEM) analysis

Using an HR-TEM, the morphological characteristics of CSNPs were examined. The samples were laid out on a (Cu) grid with carbon coating (3.06 mm in diameter, 200 mesh, 125 m pitch, and 97 m hole W), and any moisture that remained was captured by filter paper on the copper grid. Finally, before analysis, the material was air-dried (Vaezifar et al., 2013).

4.2.5 X-ray diffraction (XRD) analysis

It is an essential tool to measure the crystal nature of nanoparticles in an ambient temperature. By, using an X-ray diffractometer, the crystallinity of the produced nanoparticles was measured. A Siemens D5000 X-ray diffractometer was used to measure the scattering patterns of CSNPs from a threshold angle of 5 to 50 at a transition frequency of 0.04/min (Hosseini et al., 2015).

4.2.6 Dynamic light scattering (DLS) and zeta potential analysis

DLS (Malvern, United Kingdom) assessed the particle dimension and index of dispersion of CSNP at 25 °C. The zeta sizer (Malvern, United Kingdom) was employed to look at the level vary between +100 mV to 100 mV using CSNPs produced at pH 4.6 and pH 7.4. Before analysis, samples were repeatedly inverted at 25 °C to check the integrity of the formed nanoparticles (Shanmugasundaram et al., 2017).

4.3 DIET PREPARATION AND FISH CULTURE

The *O. niloticus* (L.), was supplied to us as fingerlings from nursery ponds. Fish were housed in a spun glass aquarium that was aerated indoors for 2 weeks to help the fish get used to the lab setting. A light source was used to keep the light-dark cycle at 12 hours each day. A total of 30 Fish (1.49 ± 7.00 g) were dispersed at random among 10 fish per 3-L aquarium tanks in quadruplicates. They were separated in to three experimental groups. Such as Experiment 1 fed with commercially available feed, Experiment 2 as control, Experiment 3 were fed with chitosan loaded feed. Each aquarium was supplied with compressed air pump for aquariums is used. Fish were given food on one of the test diets for 45 days, twice a day at 9:00 and 14:00, until they appeared satisfied. Every day, settled fish excrement and half of the aquarium's water were evacuated and refilled with fresh, oxygenated tap water from a holding tank. Daily records of fish mortality were kept, and dead specimens were removed. (Kumaran Subramanian et al., 2021).

CHITOSAN loaded nanoparticles (g/kg diet)

COMPONENTS	0.0 (CONTROL)	FORMULATED FEED	CSNPs FORMULATED FEED
Chicken meal	15	15	15
Fish meal	10.4	10.4	10.4
Soya bean meal	33	33	33
Groundnut cake	20.3	20.3	20.3
Wheat bran	10.5	10.5	10.5
Cron oil	1.5	1.5	1.5
Codfish oil	2.3	2.3	2.3
Vitamins	1.0	1.0	1.0
Minerals	2.0	2.0	2.0
Starch	4.0	4.0	4.0
TOTAL	100	100	100

Elemental analysis (%)

Solid matter	94.6	93.7	93.8	94.1	93.5
Protein content	33.18	33.1	34.15	35.19	34.17
Crude fat	7.3	7.4	7.4	7.3	7.3
Ash content	5.3	4.2	5.4	5.8	5.4
Insoluble fiber	4.6	4.8	4.9	5.7	5.9
Nitrogen free extract	48.91	48.92	48.96	48.97	48.94
Total energy (Kcal/100g)	446.5	447.9	454.1	459.4	453.1

Table 4.1 Chemical components of feed additives with varying quantities of chitosan-loaded nanoparticles (CSNPs) (% dry weight).

4.4 CHITOSAN NANOPARTICLE LOADED WITH FISH FEED

The Chitosan solution was prepared by; 3g of chitosan powder has been weighed and kept aside. At first, 1ml of acetic acid with 800ml of distilled water was stirred in the magnetic stirrer for 20 minutes. Then chitosan powder (3g) was added to the stirring solution. They were kept in the magnetic stirrer at 450rpm. Simultaneously, pH of the solution was maintained between 4-5, by adding Sodium hydroxide solution, that is 2 pellets of NaOH was dissolved in distilled water and then added to the chitosan solution. Kept for stirring for 12 hours. Meanwhile, Tripolyphosphate solution was also prepared. 0.40g of TPP was added with 100ml of distilled water, kept in Motor stirrer for 30 mins to completely dissolve the TPP with distilled water. After 30 minutes, the successive steps involve adding the little-by-little STPP solution to the CS while rapidly magnetic stirring for 40 minutes to create a 0.4% (w/v) TPP solution. Then, the created Chitosan-TPP solution was centrifuged at 8000 rpm, 4 °C for 45 min, effluent was completely removed. Then, the pellets were thoroughly cleaned with distilled deionized water.

Formed Chitosan nanoparticle pellets was mixed up with distilled water and made in to a solution. The solution was taken in the 20ml syringe and sprayed in the prepared feed. Chitosan solution were uniformly mixed in the Fish feed and kept in the hot air oven for drying at 100°C. After drying, the chitosan loaded feeds were given to fishes of Experiment tank -3 for 1 week. The fish were weighed and their lengths were measured after a week of observation. Before weight of the fishes and after weight of the fishes were compared. Similarly, length of the fishes initially and finally was noted.

4.5 FEED CONSUMPTION AND GROWTH MEASUREMENT

Each tank's fish that had been fed chitosan-loaded CSNPs/kg of diet were collected and quantified, and documented after 45 days of feeding experiment analysis. The number of fish in each tank was counted, and this allowed us to calculate the fishes' survival rate. Based on the equations (2) through (8) suggested by (Abdel-Tawwab et al. 2019), the effectiveness of fish production and dietary intake metrics including

weight gain (WG), specific growth rate (SGR), feed consumption (FI), feed conversion ratio (FCR), and protein efficiency ratio (PER) was calculated. (Kumaransubramanian et al.,2021)

$$\text{Weight gain (WG) (g)} = W_2 - W_1$$

$$\text{Weight gain (\%)} = \frac{(W_2 - W_1) * 100}{W_2}$$

$$\text{Specific growth rate (SGR)} = \frac{[\ln W_2(g) - \ln W_1(g)]}{T}$$

$$\text{Feed consumed (FI) (g feed/ fish)} = \frac{\text{Total amount of food consumed by the fish across all of the experiments}}{\text{Fish Number}}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed consumption (g)}}{\text{Weight gain (g)}}$$

$$\text{Fish mortality (\%)} = \frac{(\text{Fish numbers at final}) * 100}{\text{Fish number at initial}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Total wet weight gain (g)}}{\text{Crude protein fed (g)}}$$

where, W2 – ending weight of the fish (g), W1 – beginning weight of the fish (g), and T-total experiment time (Kumaran Subramanian et al., 2021).

4.6 NON-SPECIFIC IMMUNE RESPONSE OF *Oreochromis niloticus*

For evaluation purposes the non-specific immunological parameters, Every week, fish blood samples (100 IU/mL) were aseptically collected from the caudal arteries and mixed with sodium heparin to prevent clotting.

4.6.1 THE COUNT OF WHITE AND RED BLOOD CELLS

Erythrocyte counts were originally commonplace in human medicine, but because to their huge inherent inaccuracies, they have been generally superseded by calculation of hemoglobin and hematocrit levels. Counts are mostly done selectively, and computerized procedures have essentially replaced optical counts. Electronic techniques of differential counting are not viable due to the combination of nucleated erythrocytes and leukocytes, fish blood. The visual counting of cells in a Neubauer hemocytometer is employed. The inability to specifically coagulate erythrocytes or leukocytes causes problems. The avian diluting fluid was used which contains two essential staining dyes that allow separation between RBC and WBC. The main drawback of this stain is unstable. For the evaluation of hematological parameters, Neubauer counting chambers (hemocytometers) were used to measure RBC and WBC counts.

4.6.2 LYSOZYME ACTIVITY AND NBT ASSAY

Fish blood components leucocytes were collected., and its respiratory burst activity was assessed using the NBT assay. The turbidometry approach was used to assess the lysozyme activity in plasma samples (Abu-Elal et al., 2013).

4.6.3 STATISTICAL ANALYSIS

The outcomes were shown as the average minus the standard error for each of the three analyses. One-way ANOVA analysis was carried out with SPSS software on all the data that has been collected (version 17.0, SPSS Inc., USA). ANOVA was performed to pinpoint group distinctions, and Dun-can's multiple range test was employed to identify any differences in means (Kumaran Subramanian et al., 2021).

CHAPTER-5

RESULT AND DISCUSSION

5.1 SYNTHESIS OF CHITOSAN NANOPARTICLES (CSNPs)

There are several methods for making CSNPs, including ionic cross-linking, complex-coacervation, and ionotropic gelation. The ionic gelation approach was used in the current study to create CS nanosuspension. The above-mentioned procedures are advantageous for CSNP synthesis since they are quick and gentle, requiring no organic solvents or high temperatures. It hinges on The ionic connection between the positively and negatively loaded amino groups in the acidic CS solution and the aqueous STPP solution. The molecules with opposing charges can form inter - molecular crosslinks due to the ongoing magnetic churning. TPP was gradually included into the CS solution to produce the ionotropic gelation of CSNPs. The synthesized CSNPs exhibited monodispersed nanoparticle sizes of around 100 nm and a low polydispersity index (PDI). The produced CSNPs had monodispersed nanoparticles of around 100 nm in size and consistently low polydispersity index (PDI) values. The stability and structural uniformity of nanoparticles were assessed using the PDI. The PDI value reflects the range of nanoparticle size; samples with a larger range of particle sizes have substantially higher PDI values, but samples comprised of particles of the same size have PDI values that are relatively low. In this latest study, low PDI values denote higher levels of monodisperse CSNPs and higher levels of particle equilibrium, whereas higher values suggest lower levels of particle equilibrium and CSNP accumulation. Opacity in the creation of CSNPs was verified by the presence of opalescence, which was evaluated for physio - chemical traits like crystalline structure, thermal potential, ultrastructural morphology, and the dimension of particles. The figure 5.1 provided below were required materials to synthesis CSNPs.



Fig 5.1 Materials for chitosan nanoparticle preparation:

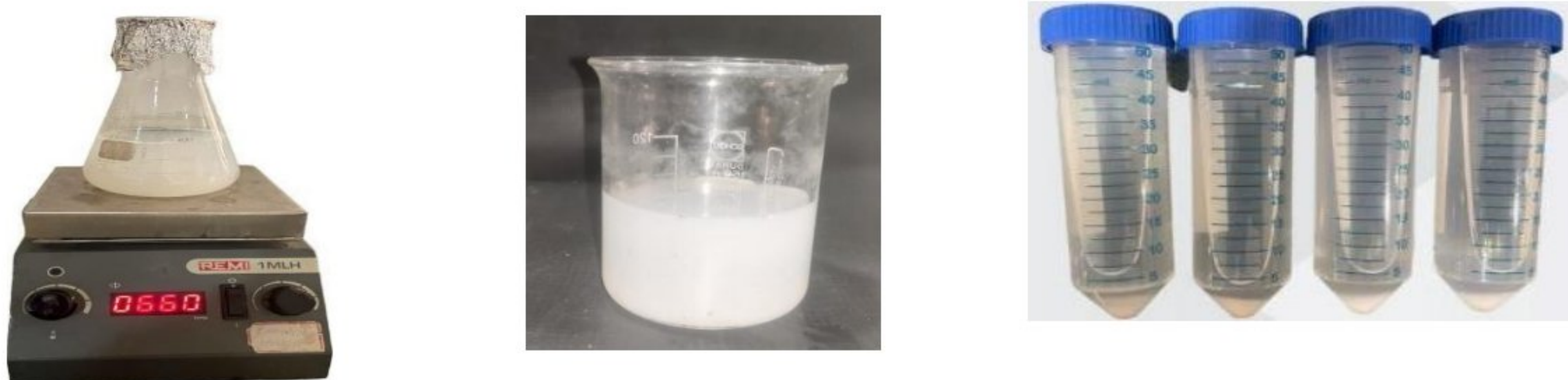


Fig 5.2 Magnetic stirring and centrifuging the chitosan- STPP solution

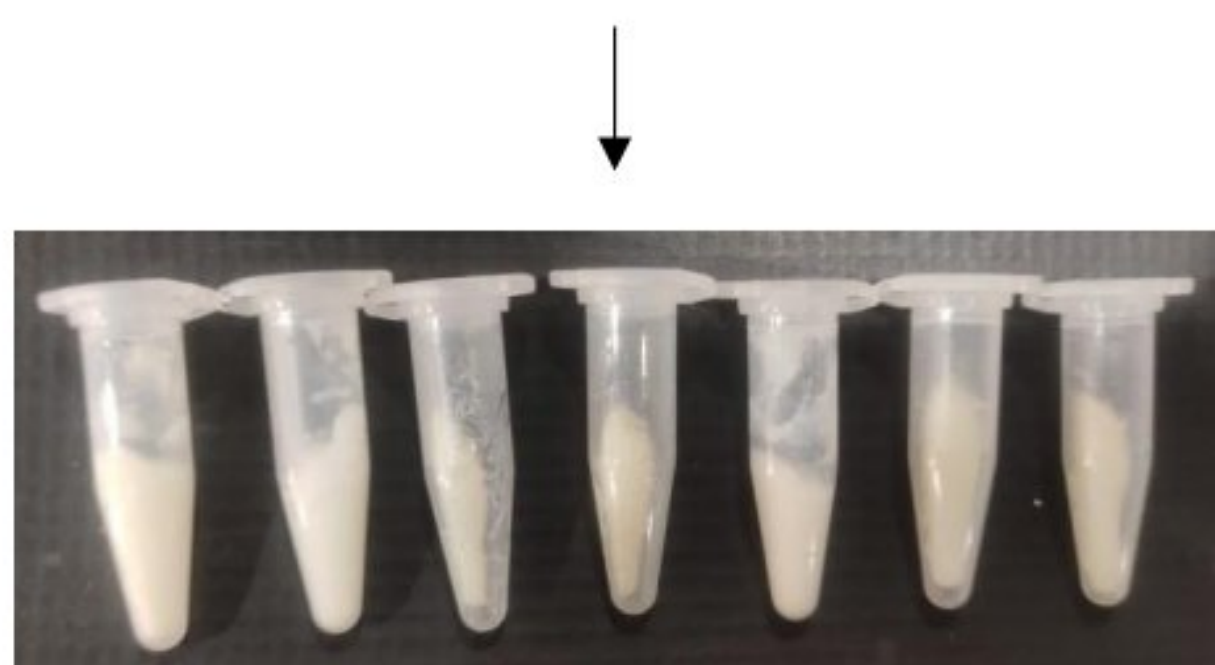


Fig 5.3 Chitosan Nanoparticle (CSNPs) used for further process.

3.2 CHARACTERIZATION OF CSNPs

1. UV-VIS ABSORPTION SPECTRUM

The optical properties of CSNPs were studied using the wavelengths in the UV-visible absorption, which range vary from 200 to 400. to 400 nm. Sharp absorption band strength was detected at 210 nm in a UV-visible CSNPs spectra. The formation of CSNPs was verified by UV-visible spectroscopy. After analyzing the UV-vis absorption spectra, it was discovered that the usual The loaded CSNPs' peak of absorption is at 210 nm. The absorption spectra of chitosan nanoparticle, on the other hand, were noticeably higher. The absence of a peak in the visible spectrum for the CSNPs solution between 250 and 400 nm further supports the creation of chitin nanoparticles.

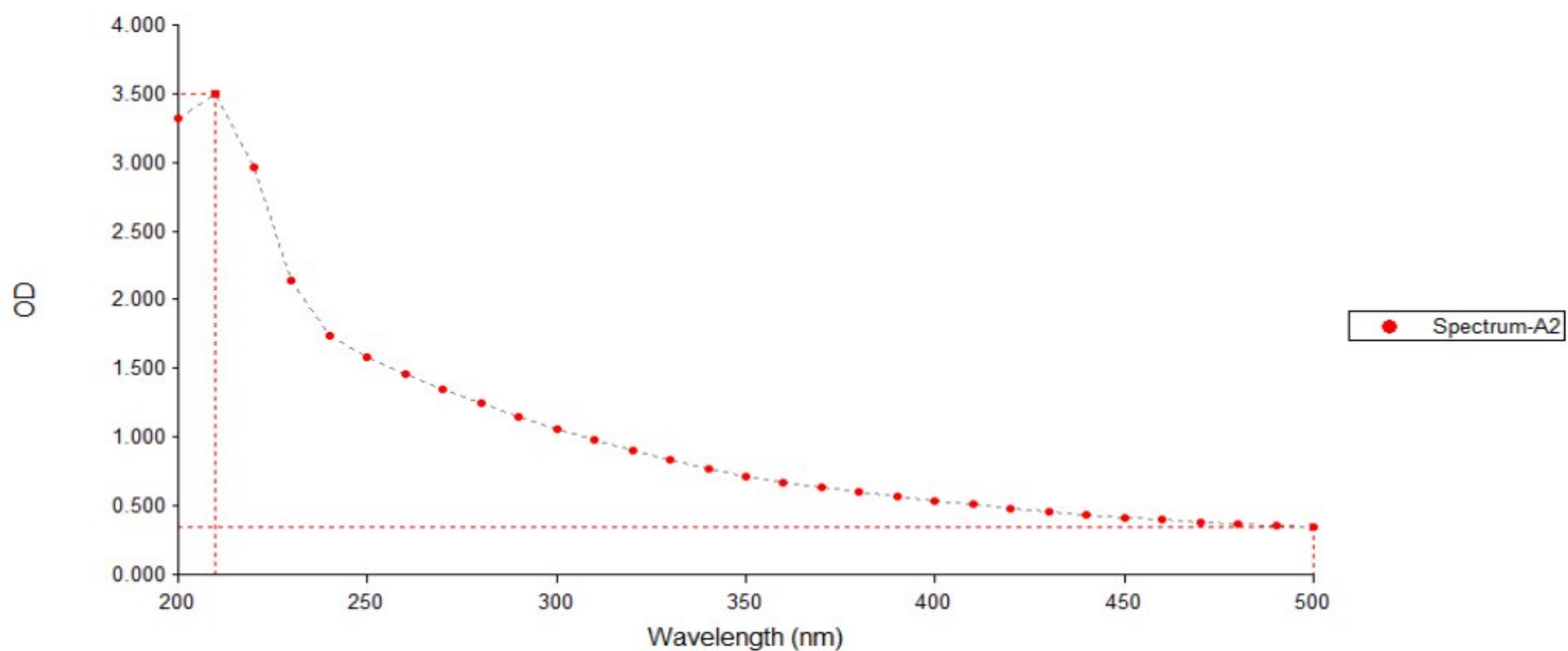


Fig 5.4 UV spectroscopy graph showing peak for CSNP

2. FT-IR SPECTRA ANALYSIS

The molecular structures of CSNPs were evaluated using the presented FT-IR studies. In this study, CSNP was 3252 cm^{-1} (-OH and -NH₂ stretching), 2621 cm^{-1} (OH stretching), 2063 cm^{-1} (C=C asymmetric stretching), 1715 cm^{-1} (C=O asymmetric stretch), 1640 cm^{-1} (amide I band), 1388 cm^{-1} (CH₃ stretch), 1276 cm^{-1} (C-O stretch), 1050–1075 cm^{-1} (C-O vibration), and 886 cm^{-1} (-CH elongation) are some examples of the lengths that certain molecules may be stretched to. The OH stretching band with hydrogen bonding characteristic was emphasized by a prominent broad peak at 3252 cm^{-1} . This asserts that certain amino acids and metabolic products, notably fatty acids and alkaloids are surrounded by functional groups such as CS and hydroxyls, amines, alcohols, phenols and carboxylic acids. Also, the band at 1638 cm^{-1} was greatly reduced and a new vibrational band appeared at 1021 cm^{-1} (C-O-C stretching). The strong peaks are due to symmetrical CH₃ oscillatory bending at 1380 cm^{-1} and 1419 cm^{-1} .

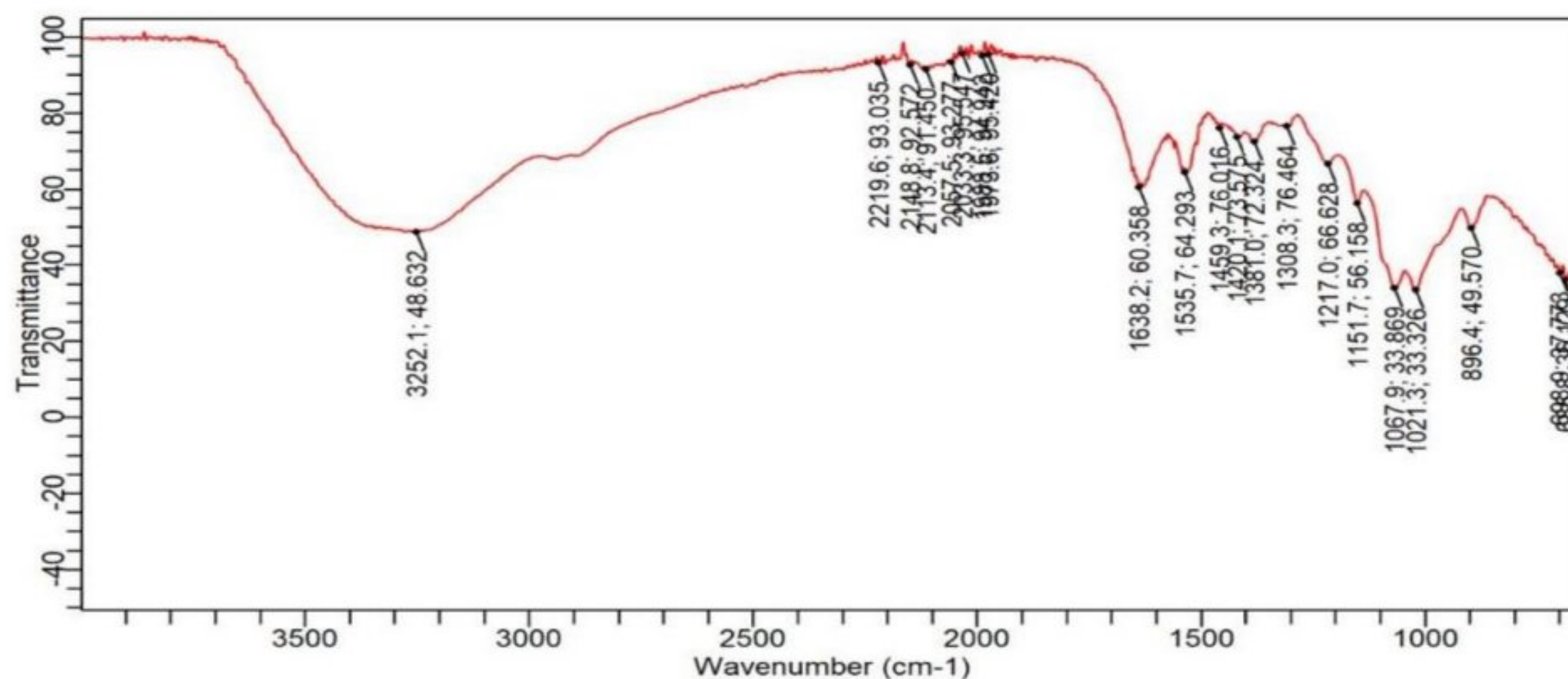


Fig 5.5 FT-IR spectra of Chitosan nanoparticle

3. FE-SEM ANALYSIS

SEM analysis was used to examine the particle microstructures of synthesized CSNPs, including their size, shape, and dispersion patterns. The CSNP SEM micrographs revealed that the particles had a thick morphology, with dense spherical bodies and a homogeneous microstructure. The spherical particles were well-distributed and tiny, suggesting that the coupling of CS and TPP molecules led to the formation of nanoscale CSNPs. Previous research has found CSNPs with similar appearances, suggesting that the CSNP synthesis process was effective in producing consistent particle morphology. The varied surface properties and spherical shape of CSNPs suggested that they had a porous surface structure due to their aggregation features. This porosity was reflected in the SEM micrographs, which showed a particularly dense surface shape for the CSNPs. Overall, SEM analysis was an effective tool for characterizing the microstructures of CSNPs and provided valuable insights into their physical properties. These insights can be used to guide further research into the potential applications of CSNPs in a number of disciplines, including tissue engineering, biomedical imaging, and medication delivery.

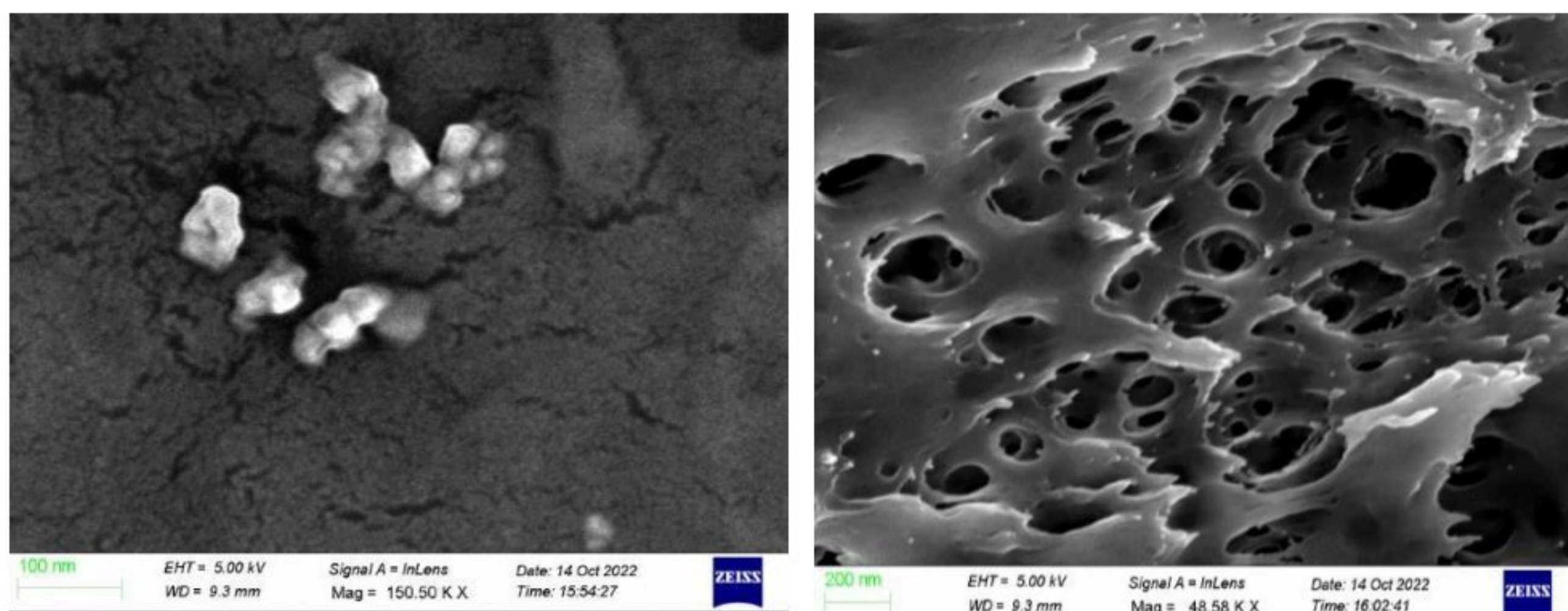


Fig 5.6 SEM images of CSNPs with dense spherical bodies and homogenous microstructure.

4. HR-TEM ANALYSIS

HR-TEM measurements affirmed the correct grain size and microscopic structure of CSNPs. Fine structure of CSNP synthesized under optimal conditions. The shape of the CSNPs was spherical, and the distribution of the particles' sizes ranged from 60 to 70 nm. TEM images showed that the lattice fringes of the samples indicated a high crystallinity level. The positively charged amino groups of chitosan and the negatively charged TPP ions of CS and TPP interacted to create CSNP. An exact dry size of CSNP is provided by HR-TEM. Because of the interaction of hydrogen bonds with CSNPs, which intensify throughout the drying process, CS/TPP NPs might aggregate. It can be observed that the NPs have a darker appearance in the center and on the surface, reflecting much greater scattering of the electron density. In spite of the fact that TPP has a greater electron density and includes elemental phosphorus than CS, it can be said that CS interacts with TPP often via hydrogen bonds in this situation.

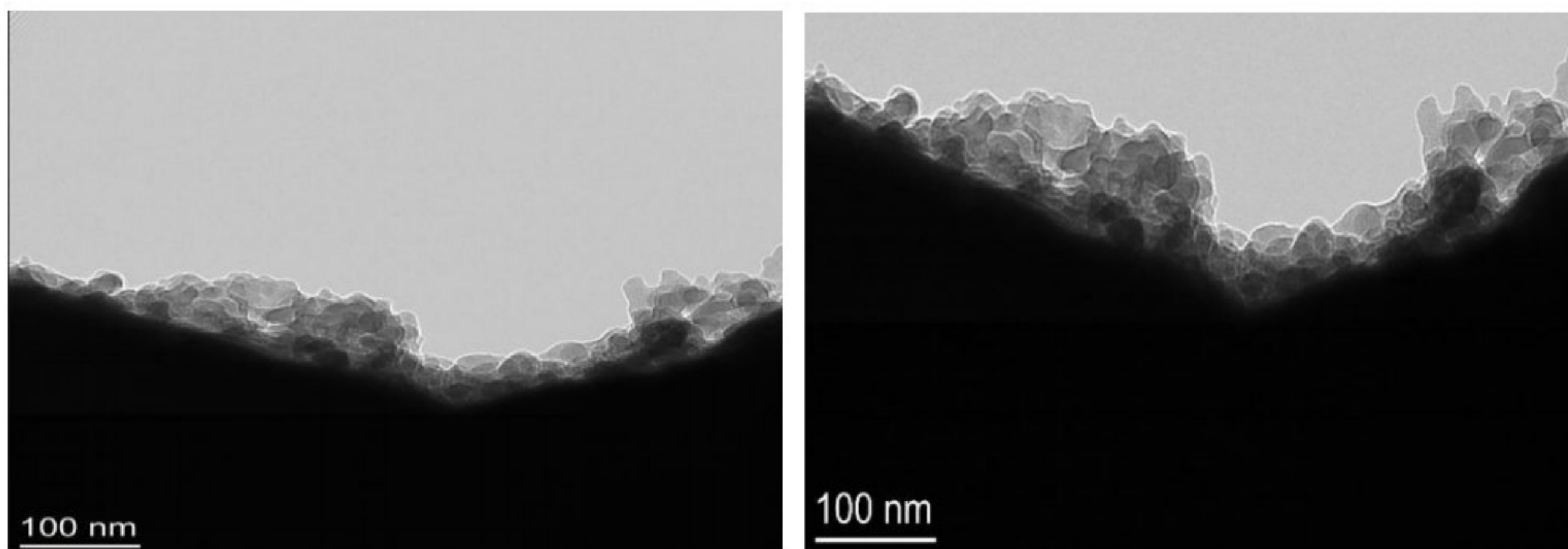


Fig 5.7 TEM microstructure of CSNPs shows smooth, spherical particles with a uniform size distribution.

5. XRD MEASUREMENT

Using XRD, the CSNPs' morphological conditions and dimension of crystallite were assessed. The CSNPs' XRD patterns are displayed. The good crystalline quality of the CSNPs was shown by a diffraction peak at 2θ range with a 2θ in CSNPs. The larger diffraction angle was also slightly displaced. The prominent diffraction peak (110) of both CSNPs spectra was determined to be a broad peak at 2θ . CS only had one large diffraction peak, which was centered at $2\theta = 20.20$. At $2\theta = 30.1, 44.6, 64.6$, and 77.5 , the CSNPs face the center cubic (fcc) lattice, accordingly. Also, it has been demonstrated that the scattering pattern of CSNPs would affect the hydrogen bonding contact between CS and TPP, causing the CS crystal structure to be disturbed. Additionally, freeze-drying has diminished the crystalline structure of CSNPs, making it clear that the crystal's size had an impact on the band's diameter. The larger band also shows an imperfect crystal structure.

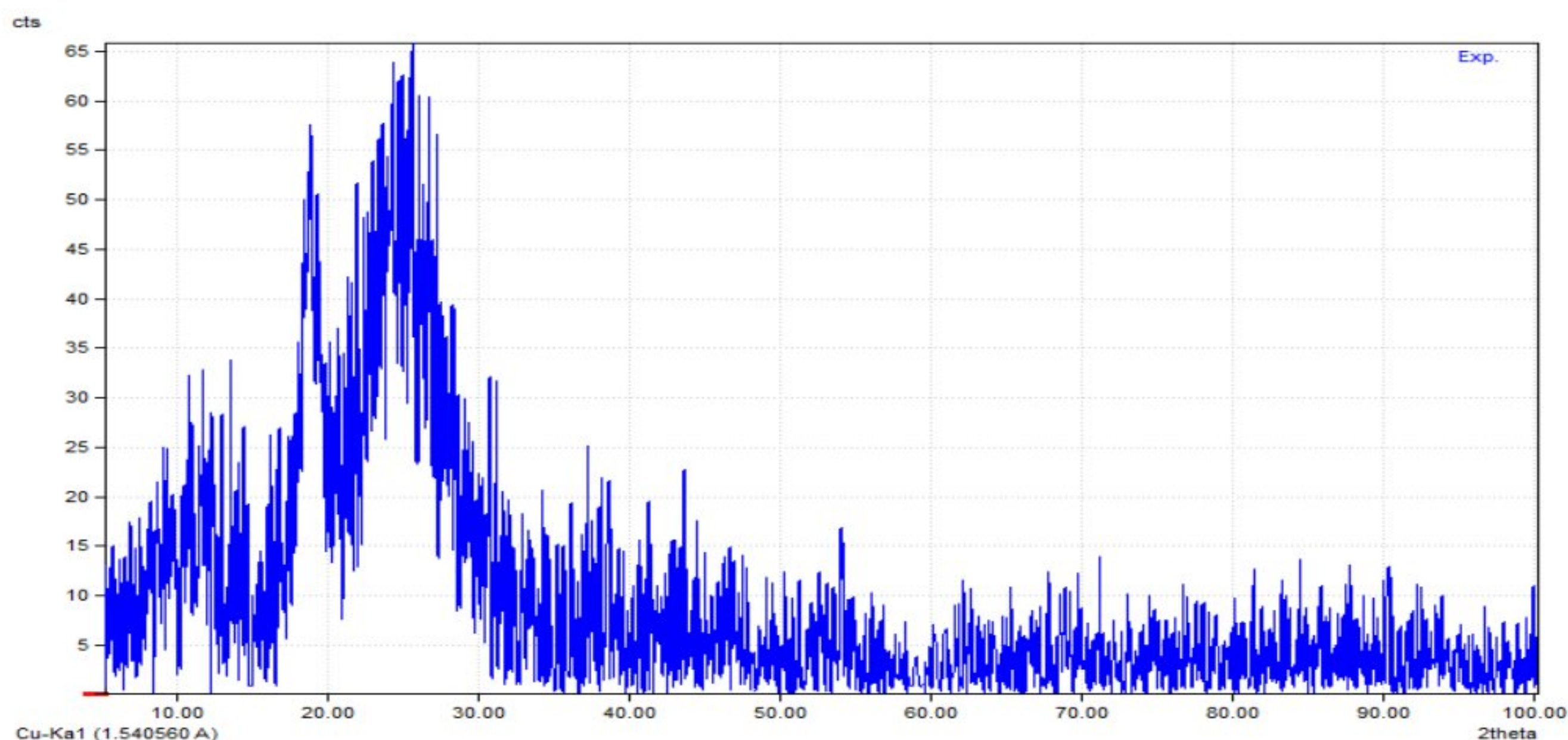


Fig 5.8 XRD pattern of CSNPs

6. PARTICLE SIZE DISTRIBUTION BY DLS AND ZETA POTENTIAL

This method is frequently utilized to verify the NPs' average particle size dispersion. The CSNP dispersion pattern is depicted in the figure below, with 341 nm, respectively, for the mean particle dimensions. The surface features of CSNPs created from CS and TPP molecules were disclosed by DLS data because nano-sized CSNPs were produced by cross-linking between CS and TPP molecules at a certain volume ratio differed substantially.

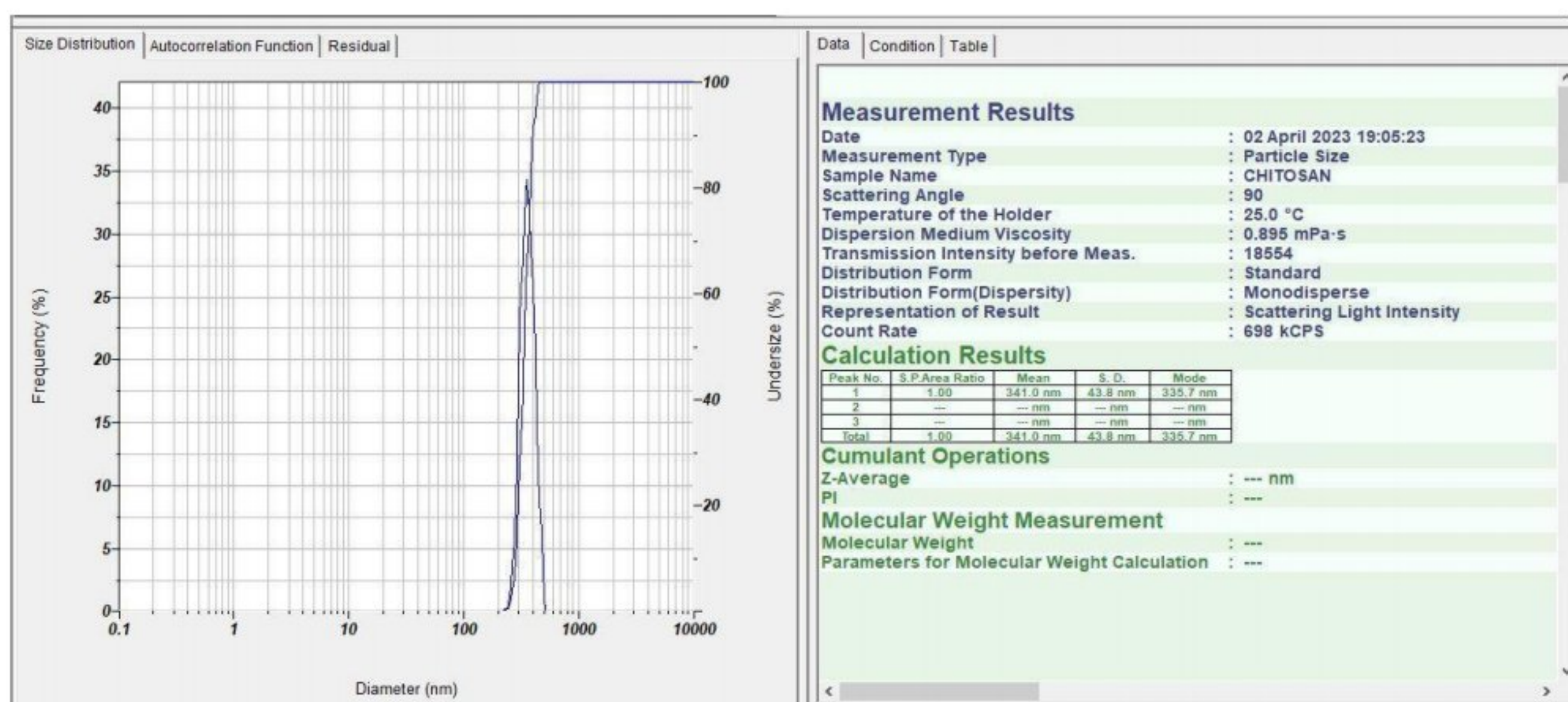


Fig 5.9 Graph showing particle size of CSNP

ZETA POTENTIAL

A key factor in determining the surface charges determine the degree of reliability and conductance of particle ensembles to biological layers. As a result, zeta potential research is essential to the characterization of nanoparticles. Zeta potential was used to assess the CSNPs and compare them to the precursor CS. Depending on the surface charge, CSNPs showed a positive zeta potential value in this investigation. Combining CS with TPP resulted in the fast generation of compact structures with significantly positive surface charges, which were later verified by measuring zeta

potential values. The zeta potential values for the CSNPs made at pH 4.6 and pH 7.4 were +5.37 and +11.3 mV, respectively. According to the current research, synthesized CSNPs had a higher zeta potential and were more reliable. That zeta potential is positive. With earlier findings, this positive zeta potential charge is stable. In this work, the low value of zeta potential showed that the aggregation of nanoparticles caused the creation of the enlarged second peak. The excellent stability of CSNPs is shown by the positive zeta potential value. The enhanced quantity of the electrical double layer and apparent decrease in clumping are likely caused by the extended peptide groups' apparent effects on the biosorption of anion. There have already been instances of similar zeta potential ranges.

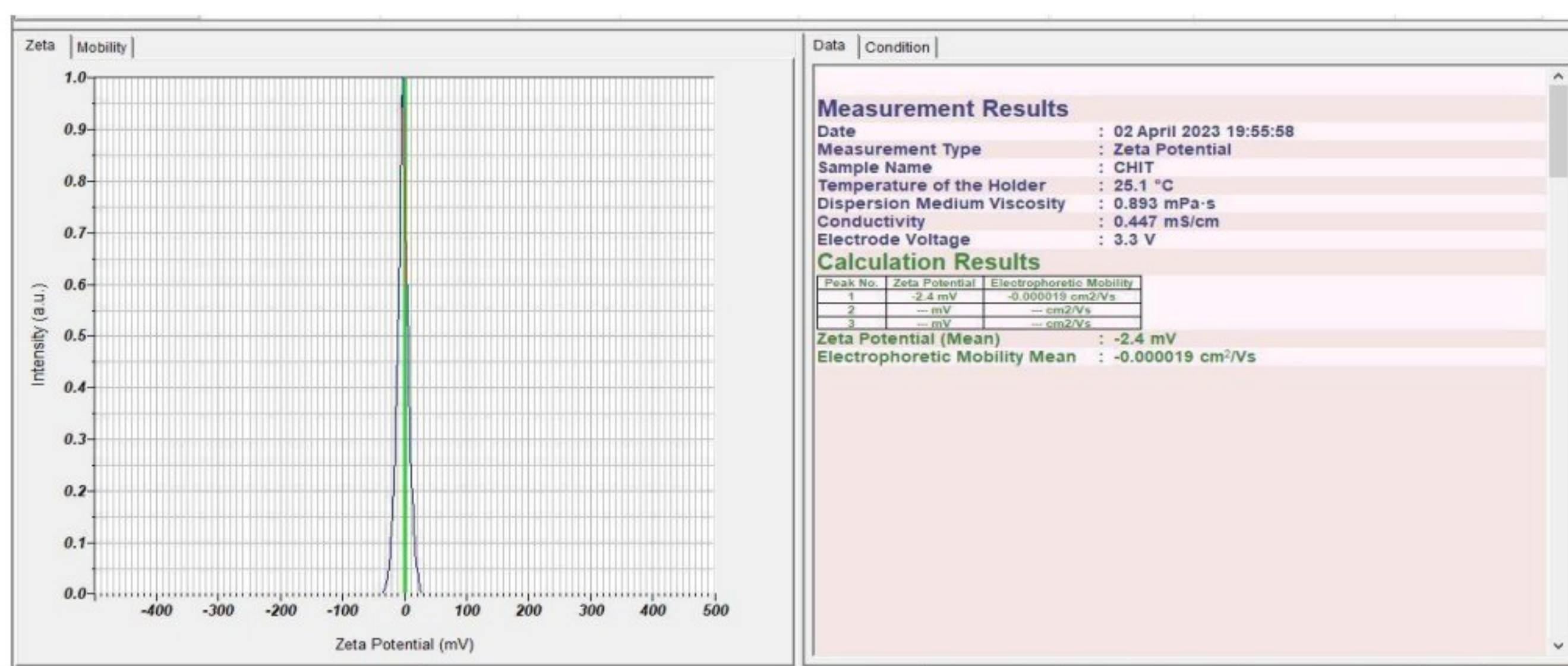


Fig 5.10 Zeta potential graph showing CSNP

5.3 *Oreochromis niloticus* (L.).

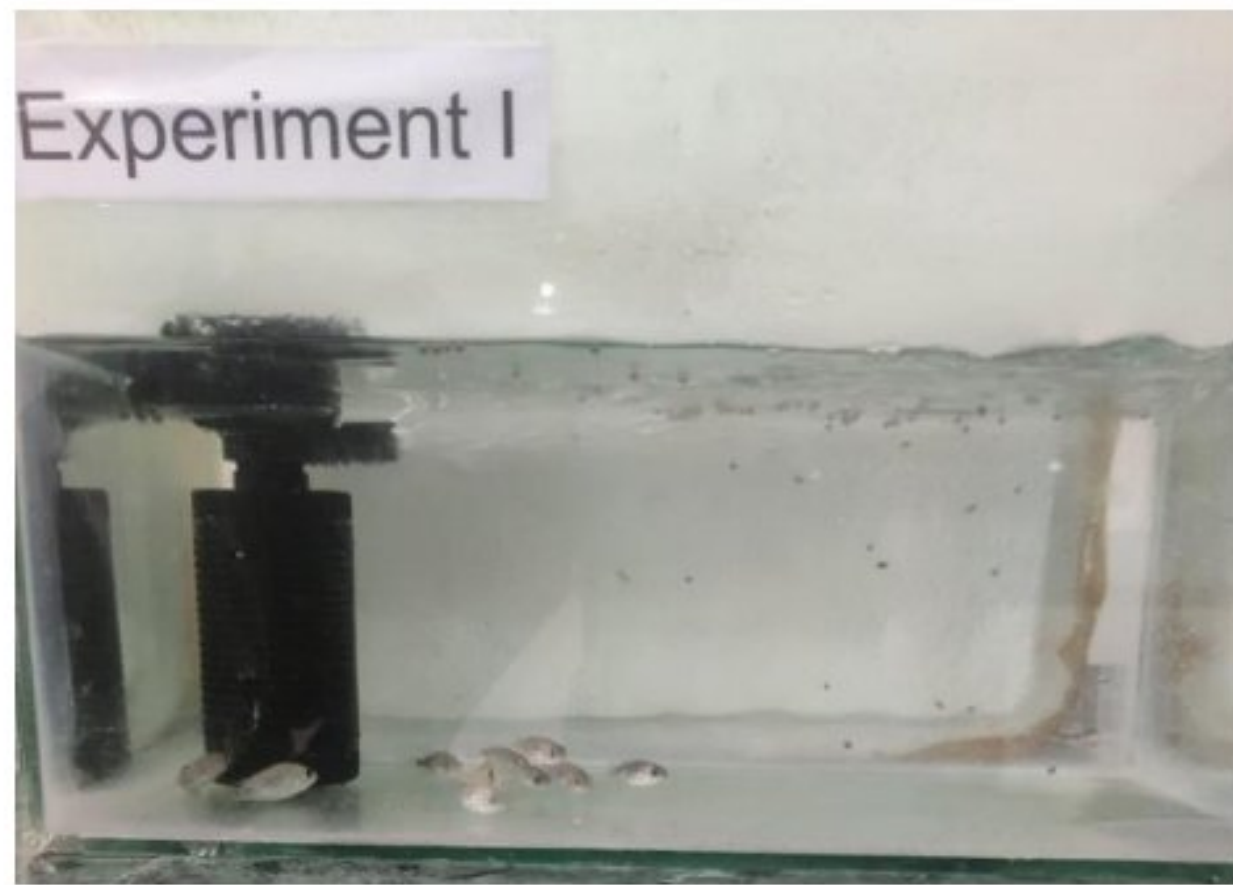
Oreochromis niloticus (L.). also known as *Nile tilapia*, has been brought and acclimatized for two weeks. After acclimatization period, all the fishes were subordinated to testing. Each fishes were noted accurately for their length and growth.



Fig 5.11 Niletilapia

5.4 EXPERIMENTAL SET UP

The *O. niloticus* (L.), was supplied to us as fingerlings from nursery ponds. Fish were housed in a spun glass aquarium that was aerated indoors for 2 weeks to help the fish get used to the lab setting. The periodicity of dawn and dusk was kept intact at the rate of twelve hours each day using a light source. Ten fish were randomly placed within each 3-L aquarium tank in quadruplicates. They were divided into three groups for the experiments. Such as Experiment 1 fed with commercially available feed, Experiment 2 as control and Experiment 3 was fed with chitosan nanoparticle loaded feed pellets. Using the aquarium's air pump, compressed air was provided to each tank. For 45 days, fishes were given one of the test diets twice daily at 9.00 and 14.00 until they were satisfied. By replacing them every 4-5 days, each aquarium is kept with clean water. Evidently, dead fish were removed.



***Fig 5.12 FED WITH
COMMERCIAL FEED***



***Fig 5.13 CONTROL- FED WITH
PREPARED FEED WITHOUT
CHITOSAN***



***Fig 5.14 FED WITH CHITOSAN NANOPARTICLE LOADED
FISH FEED***



Fig 5.15 EXPERIMENTAL SETUP OF THE FISH TANKS

5.5 COMMERCIAL FEED

This is the commercial feed which were given to the Experiment 1- tank fishes for 45 days and noted their feed consumption, growth and weight gain.



Fig 5.16 Commercial feed

5.6 PREPARATION OF FISH FEED

The below given components (fig 4.16) was measured approximately. Then all the components were added in to the tray for mixing and made in to dough. By the help of extruder, feed pellets were made and incubated for drying. After, drying prepared feed pellets, were broken into the size of 1mm in diameter and stored at room temperature.

The prepared feed pellets were given to Experiment 2 – tank fishes and noted their feed consumption, growth efficiency and weight gain. It was fed twice a day. The diet were given to the fishes properly and left over feed pellets were taken out. As because it contaminants the water.



Fig 5.17 Materials for fish diet preparation



Fig 5.18 Diet meal weighed for making feed pellets



Fig 5.19 Fish diet made in to dough, Syringe used as an extruder to form feed pellets



Fig 5.20 Feed pellets were formed by using extruder



Fig 5.21 Prepared Fish feed pellets

5.7 CHITOSAN NANOPARTICLES LOADING IN PREPARED FEED

Chitosan - Sodium tripolyphosphate solution was prepared with above given measurements and centrifuged, supernatants, were removed completely. CSNPs pellets were sprayed into the fish feed using a syringe along with a few amount of purified water. After chitosan nanoparticle loading, the loaded feeds were kept in hot air oven for two days to completely dry the formulated feed. Then, CSNPs loaded fish feed were given to test the Experiment 3- tank fishes. The diet fed for twice a day and the water quality checked and replaced apparently to maintain pH and ammonia level.

Remaining or left over feeds were taken out because to protect the fishes from contamination. Noted their feed consumption, growth efficiency and weight gain.



Fig 5.22 Chitosan solution



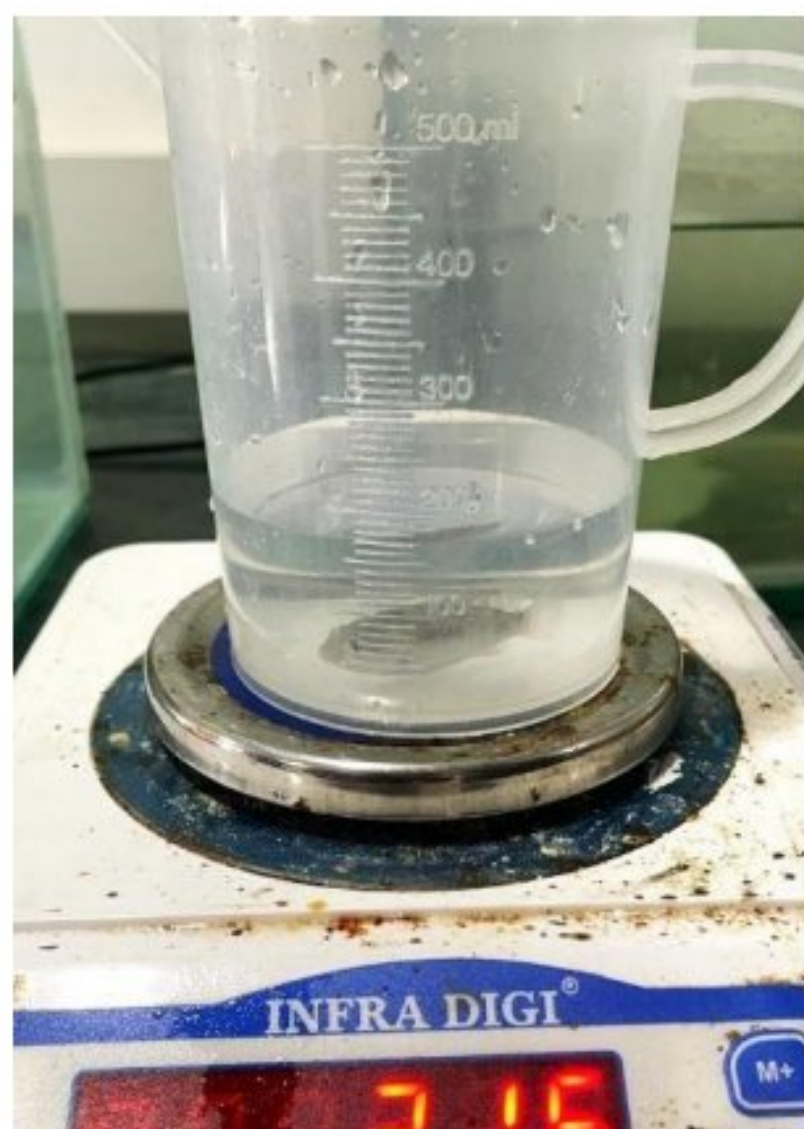
Fig 5.23 chitosan nanoparticle loading



Fig 5.24 Chitosan nanoparticle loaded feed

5.8 INITIAL WEIGHT OF THE FISHES

Fishes were weighed initially to determine their initial weight i.e., before giving feed using a weighing machine.



***Fig 5.25 Fish tank 1-
Commercial feed***



Fig 5.26 Fish tank 2- control



***Fig 5.27 Fish tank 3- CSNP's
loaded feed***

5.9 FINAL WEIGHT OF THE FISHES

Fishes were weighed finally at the 45th day to determine their final weight i.e. after giving the corresponding feeds to each tank. Fishes were weighed using a weighing machine and the observation of weight gain was noted, results were tabulated.

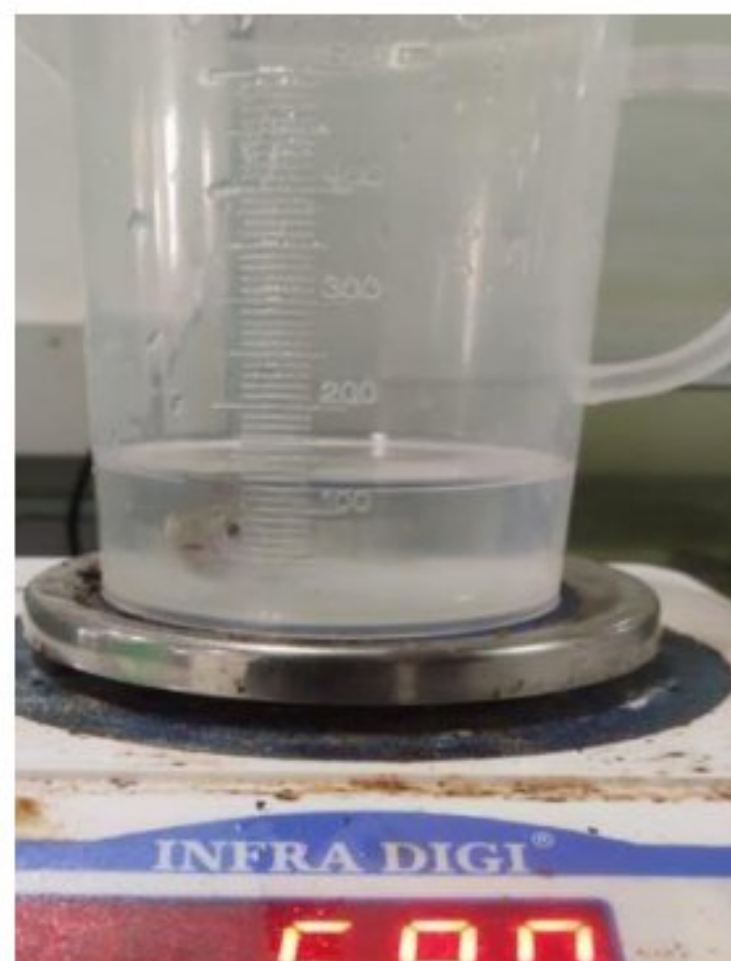


Fig 5.28 Fish tank 1- Commercial feed

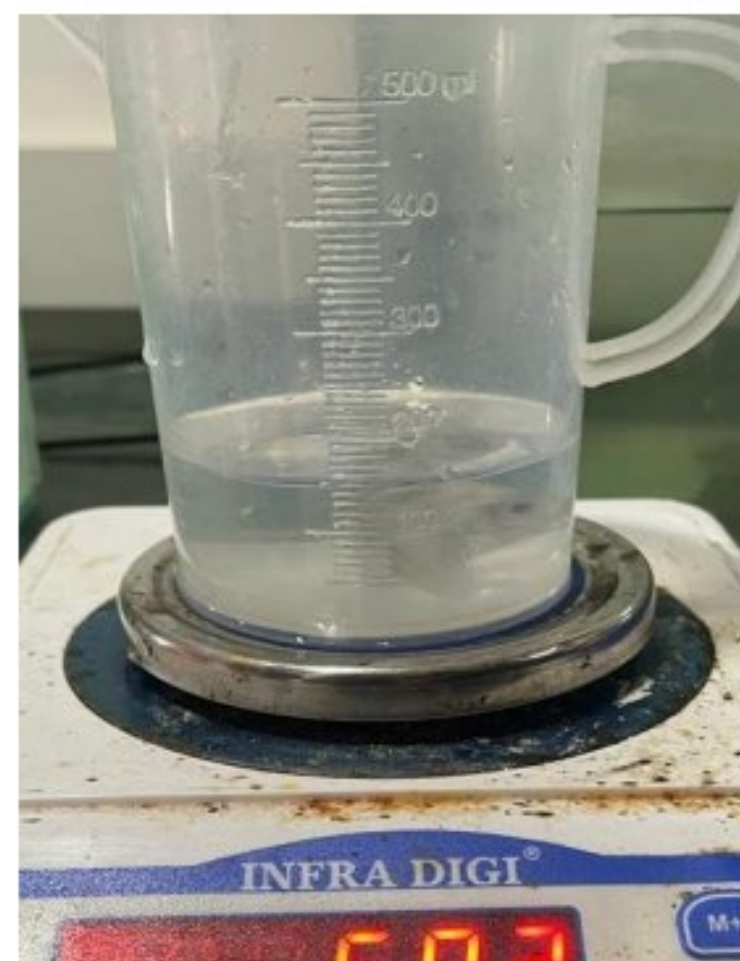


Fig 5.29 Fish tank 2- Control feed



Fig 5.30 Fish tank 3- CSNP's loaded feed

5.10 INITIAL LENGTH OF THE FISHES

Fish's length was initially measured to determine their growth rate i.e., before giving prepared feed and chitosan loaded feed. Below figures shows the initial length of fishes.



***Fig 5.31 Initial length of Fish tank 2-
Control feed***



***Fig 5.32 Initial length of Fish tank 3-
CSNPs loaded feed***

5.11 FINAL LENGTH OF THE FISHES

Fish's length was finally measured to determine their growth rate and feed consumption efficiency i.e., after giving formulated feed and chitosan loaded feed. Below figures shows the final length of fishes. The observations of length were noted and the results were compared.



***Fig 5.33 Final length of Fish
tank 2- Control feed***



***Fig 5.34 Final length of Fish
tank 3- CSNPs loaded feed***

5.12 OBSERVATION OF GROWTH AND FEED EFFICIENCY

PARAMETERS	CHITOSAN LOADED CSNPs (g/kg diet)			
	0.0 (CONTROL)	COMMERCIAL FEED	FORMULATED FEED	CHITOSAN FORMULATED FEED
INITIAL WEIGHT (g)	16.21 ± 0.24	16.62 ± 0.15	16.57 ± 0.09	16.84 ± 0.18
FINAL WEIGHT (g)	28.33 ± 0.43	30.73 ± 0.44	36.80 ± 0.20	39.3 ± 0.56
WEIGHT GAIN (g)	12.12 ± 0.43	14.11 ± 0.44	20.23 ± 0.20	22.46 ± 0.56
WEIGHT GAIN (%)	42.78 ± 4.23	45.91 ± 2.22	54.97 ± 1.90	57.15 ± 4.32
SPECIFIC GROWTH RATE (g)	26.93 ± 0.017	31.35 ± 0.02	44.95 ± 0.13	49.91 ± 0.16
FEED CONVERSION RATIO (g)	0.25 ± 0.28	0.22 ± 0.12	0.16 ± 0.02	0.15 ± 0.04
TOTAL FEED INTAKE (g)	100	100	100	100
PROTEIN CONVERSION RATIO (g)	0.36 ± 0.08	0.42 ± 0.05	0.59 ± 0.10	0.65 ± 0.22
SURVIVAL RATE	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00

Table 5.1 *Oreochromis niloticus*'s growth and feed consumption efficacy when supplemented with CSNPs

5.13 HAEMATOLOGICAL DATA

The RBC and WBC count in the control group considerably rose from the first to the third week as a result of the CSNPs diet augmented with fish. In a control group that did not receive any CSNP-enriched feed supplements, the total number of red blood cells fell. Fish RBC counts drastically rose after being fed an enriched diet starting in the first week, and the RBC level increased the blood system as a result of the loaded CSNPs diet. The control group gradually improved, but the fish treated with the CSNPs/kg diet considerably boosted the RBC and WBC levels. In contrast to other treatment groups, fish provided dietary supplements with CSNPs/kg diet showed higher RBC and WBC counts. Fish fed CSNP-containing diets showed an improvement in their blood parameters.



Fig 5.35 Graph depicting *O. niloticus*'s RBC and WBC counts after receiving various meal supplements

5.14 LYSOZYME ACTIVITY

Lysozyme activity in the testing and control groups considerably increased from the first to the third week as a result of the CSNPs diet supplemented with fish. Comparatively, fish fed on diets with CSNPs/kg of supplements had higher levels of lysozyme activity during their third week than other groups being treated.

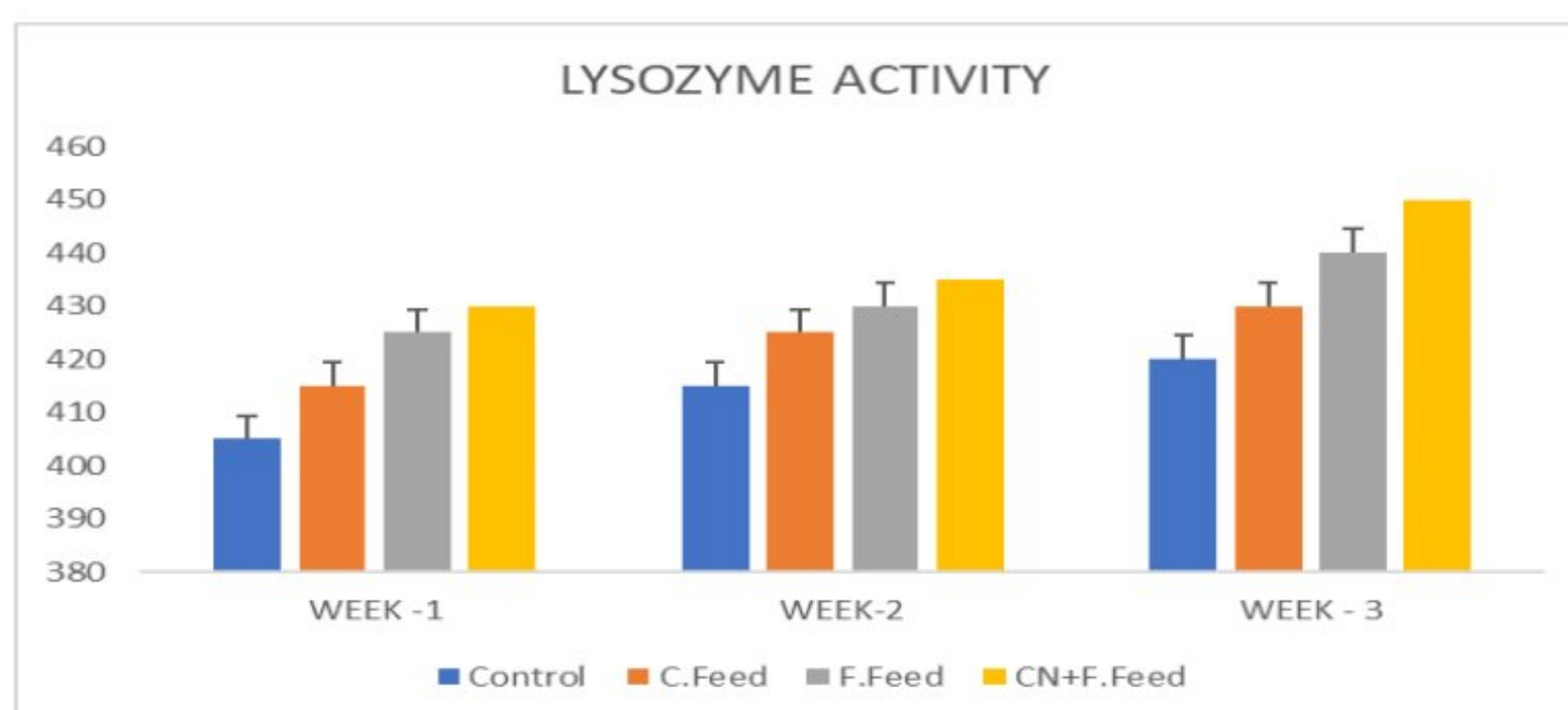


Fig 5.36 Graph depicting *O.niloticus*'s Lysozyme activity

5.15 NBT ASSAY

Findings on leukocyte activity revealed that CSNPs dietary supplements enhanced leukocyte respiratory burst (RB) behaviors as indicated by the higher NBT decrease. In contrast, fish fed dietary supplements have CSNPs.

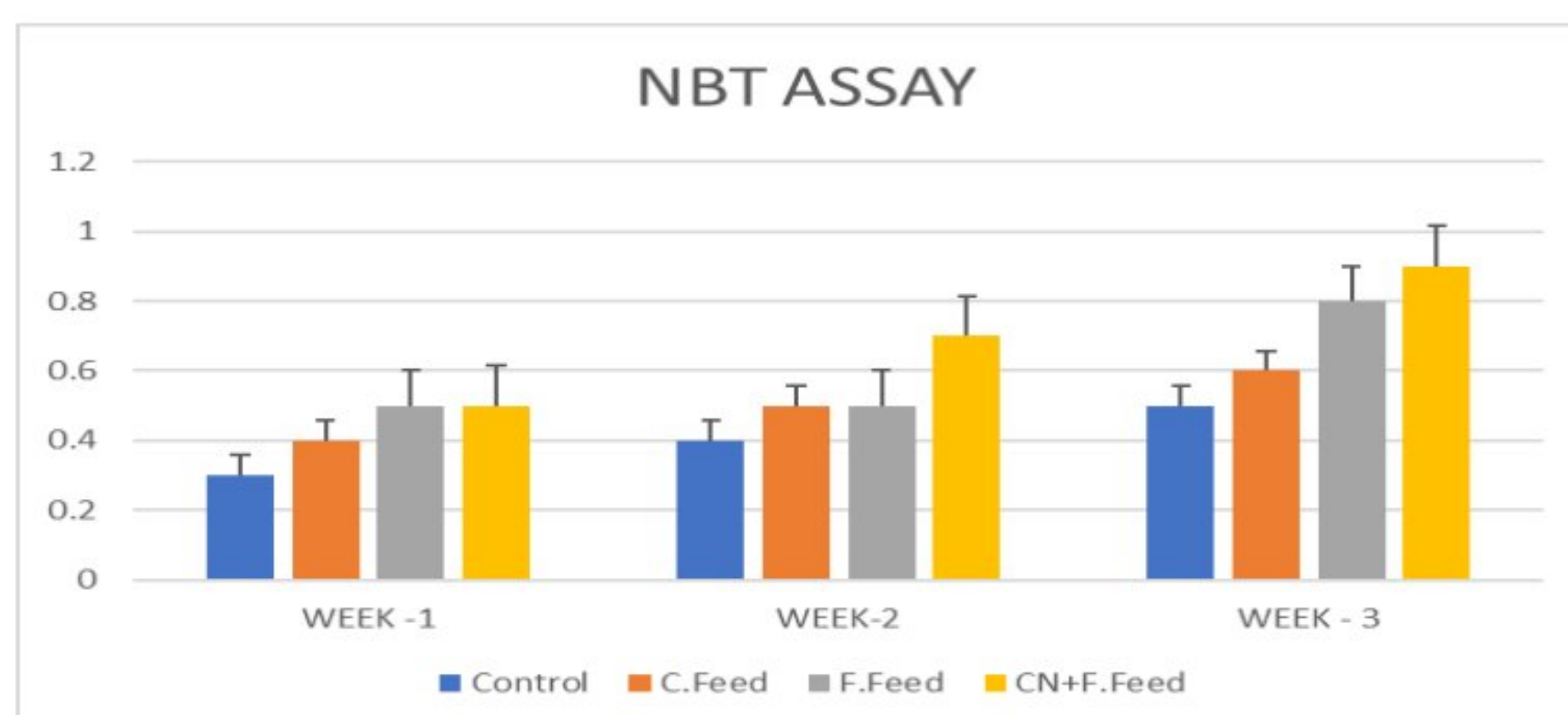


Fig 5.37 Graph depicting *O.niloticus*'s NBT assay

6. SUMMARY AND CONCLUSION

In conclusion, *Oreochromis niloticus* administered 1% CSNPs-loaded meals as therapeutic or preventative regimens significantly increased immunological response. To create an effective, affordable, and resistant to disease diet composition for CSNP-based Nile tilapia aquaculture production. Statistics show that for a single pond, modern growers of fish must spend approximately lakhs (INR) on imported feed. There is no doubt that aqua farmers will use this affordable feed product, and it will dominate both the worldwide and Indian hydroponics feed ventures. The goal of the current study is to develop a low-cost, immunostimulatory marine garbage diet for Nile tilapia for commercial usage that contains chitin derivatives products coated by nanomaterials to boost the fish's immune system and help them fend off infections. Through a coagulation and adsorption process, CS has proven efficient removal of several contaminants from aquaculture effluent. In addition, CSNPs provide wastewater treatment with an environmentally sound resource. Even though CS offers a practical method for treating wastewater in aquaculture, it still has to be further investigated if it can be used to actual fish farming. But in the long run, it may represent a large supply of items with added value generated from shrimp shell waste. According to the current research, feeding economically important fish *Oreochromis niloticus* CSNPs made from chitosan has a positive impact. Moderate doses of CSNPs (1.0 g/kg) significantly improved the performance of growth, intake of food, survivability rate, innate immune reaction, and the amount of feed converted. Results from both in the laboratory and in live testing unequivocally support the use of CSNPs made from shrimp discharge garbage as a cost-effective, nutritional supplement with added value for the aquaculture industry. This offers up new research directions for potent CSNP fish feed combinations that will enhance immune system response and proliferation in the growth of commercial aquaculture.

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