PRODUCTION OF ZEOLITE AND CHITOSAN NANOPARTICLES FOR *IN VITRO* ADSORPTION OF FUNGI IN SELECTED LOCALLY PRODUCED FISH FEEDS IN MINNA

BY

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MTech/SLS/2018/9274

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FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA

AUGUST, 2023

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THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGER-STATE, NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF TECHNOLOGY IN BIOCHEMISTRY

AUGUST, 2023

DECLARATION

I hereby declare that this thesis titled: **"Production of Zeolite and Chitosan Nanoparticles for i*n Vitro* Adsorption of Fungi in Selected Locally Produced Fish Feeds in Minna"** is a collection of my original research work and it has not been presented for any other qualification anywhere. Information from other sources (published or unpublished) have been duly acknowledged.

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CERTIFICATION

This thesis titled: **"Production of Zeolite and Chitosan Nanoparticles for *in vitro* Adsorption of Fungi in Selected Locally Produced Fish Feeds in Minna"** by SALAWU, Samuel (MTech/SLS/2018/9274) meets the regulations governing the award of MTech of the Federal University of Technology, Minna, and it is approved for its contribution to scientific knowledge and literary presentation.

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ABSTRACT

The increase in consumption of fish has led to emergence of commercial fish farming. Majority of the farmers use local ingredients to produce the fish feeds which under favourable conditions support fungal growth and contamination. The use of nanotechnology is an effective approach to decreasing fungi contamination and mycotoxin toxicity in animals. The aim of this study was to produce zeolite from agricultural waste (rice husk and groundnut shell) with synthesized chitosan nanoparticles (CNPs) as additives for adsorption of fungi from selected locally produced fish feeds (A-E) in Minna. Proximate composition and morphological identification of fungi in five selected fish feeds were determined using standard procedure. Rice husk (RHS) and groundnut shell (GNS) were used as ash source for the production of zeolites and the zeolites were characterized with Xray diffraction (XRD). Chitosan nanoparticles were synthesized through ionic gelation method. The synthesized chitosan nanoparticles were characterized with UV-spectrophotometer and Zetasizer. The results of proximate composition showed high moisture content for Feed A and B with no significant difference in lipid, fibre and protein content in all the samples. Whereas feed E had the highest carbohydrate content and the lowest protein content. the fungal load of the five samples (A-E) were $172x10^5$, 85 $x10^{5,}$ 10 $x10^5$, 13 $x10^5$, 2 $x10^5$ ($x10^5$ CFU/g) respectively. The morphological identification of fungi in feeds confirm the presence of *Aspergillus fumigatus, Mucor pusillus, Trychphyton megnini, Candida tropicalis, Microsporum distortum* and *Aspergillus Niger.* The XRD patterns of GNS and RHS zeolite showed broad diffraction peaks at 2Ɵ angle range of 14-40° and 15-50° respectively. The UV-spectral showed that CNPs have maximum absorptions peak range of 260-340 nm with particle size range of 93.43-104.50 nm. The fungal load of the feeds with the inclusion of the RHS-nanoadditive and GNS-nanoadditive showed complete inhibition of the fungi growth with 50:50 chitosan nanoparticle/zeolite. The nanoadditive exhibited strong antifungal activities by inhibiting fungi growth in the feed samples as compared to the feeds without nanoadditives. In conclusion, the 50:50 chitosan nanoparticle/zeolite as nanoadditives can be suggested for an inclusion in local fish feed formulation.

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LIST OF ABBREVIATIONS

Food and Agricultural Organisation (FAO)

Butylated hydroxianisole (BHA)

Butylated hydroxytoluene (BHT)

Food and Drug Administration (FDA)

Aflatoxin B1 (AFB1)

Zerealenone (ZEN)

Deoxynivalenol (DON)

Fumonisins B (FB)

fusarium mycotoxins (FMs)

Nivalenol (NIV),

diacetoxyscirpenol (DAS)

European Union (EU)

Ochratoxin A (OTA)

Moniliformin (MON)

Red blood cells (RBCs)

Haemoglobin content (Hb)

Haematocrit value (Hct)

Mean corpuscular volume (MCV)

Mean corpuscular haemaglobin (MCH)

Mean corpuscular haemaglobin concentrate (MCHC)

Chronic Pulmonary Aspergillus (CPA)

Chronic Pulmonary Aspergillus (CPA)

World Health Organisation (WHO)

International Agency for Research on Cancer (IARC)

High-pressure liquid chromatography (HPLC)

Enzyme-linked immunoassay (ELISA)

Nanoparticles (NPs)

Titanium dioxide (TiO_2),

Chitosan (CS)

Sodium tripolyphosphates (TPP)

Chitosan nanoparticles (Ch NPs)

Polyelectrolyte complex (PEC)

Degree of deacetylation (DDA)

Rice husk (RHS)

Groundnut shell (GNS)

Association of Analytical Chemist (AOAC)

Potato Dextrose Agar (PDA)

Nutrient Agar (NA).

Colony forming units per gram (CFU/g)

National Agency for Food and Drug Administration and Control (NAFDAC)

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

The increase in consumption of fish has led to emergence of commercial fish farming (Adekambi *et al.,* 2020). There is a considerable potential to expand aquaculture in Africa in order to improve food security. Although potentials abound in the continent for the development of viable fish farming, one of the major hindrances to the development of

aquaculture industry in Africa is the lack of locally produced high-quality fish feed, which fishes require for growth and proper health condition (Marijani *et al*., 2019).

Fish is an important and good source of protein, providing a good balance of proteins, vitamins, minerals, and polyunsaturated fatty acids (Sunarto-Kadir, 2021). Fishes are often recommended as part of healthy diet by government food agencies. Fish feeds provides the balance nutrition in a stable and concentrated form, enabling the fish to feed efficiently and grow to their full potential. Commercially produced fish feeds can either be complete or supplemental. Complete diets supply all the nutrients necessary for the optimal growth and health of the fish. In contrast, supplemental (i.e., incomplete or partial) diets are intended only to help support the natural food normally available to fish in ponds or outdoor raceways. Supplemental diets do not contain a full complement of vitamins or minerals but are typically used to help fortify the naturally available diet with extra protein, carbohydrate, and lipids. While, most fish farmers use complete diets, local producers formulate feed using ingredients which includes maize, soya mill, fish, groundnut, wheat, cassava etc (Chakraborty *et al.*, 2019).

Fungi are groups of spore-producing organisms feeding on organic matter, including molds, yeast and mushrooms. Fungi are heterotrophs, they acquire their food by absorbing dissolved molecules, typically by secreting digestive enzymes into their environment. Growth is their means of mobility, except for spores (a few of which are flagellated), which may travel through air or water. Fungi are ubiquitous in the environment, being found in water and suitable organic nutrients when appropriate temperature conditions prevail. Fungi have been reported to occur in food and feed worldwide with some of them capable of producing a wide array of mycotoxins. (Gonçalves *et al.*, 2018).

Various methods have been used to reduce exposure to fungi and mycotoxins in contaminated cereals. The addition of binding agents to food and feed that are contaminated is one of the most effective approaches (Kolawole *et al.,* 2021). In this regard, the use of microporous aluminosilicate materials, which include zeolites as toxin binders can be beneficial due to their extraordinary chemical and physical features such as high surface charge, cation-exchange, and adsorption capacity.

Zeolites are minerals that contain mainly aluminum or silicon compounds. They are used as adsorbents in microbial contamination. They are also used as catalysts, detergents and air purifiers (Bacakova *et al.,* 2018). Zeolites are also marked as dietary supplements to treat cancer, diarrhea, autism, herpes and hang over. It is also used to balance pH and remove heavy metals in the body (Eroglu *et al*., 2017).

Chitosan is a linear polysaccharide of natural origin composed essentially of β-(1,4)-linked glucosamine units (2-amino-2-deoxy-β-D-glucopyranose) together with some proportion of N-acetylglucosamine units (2-acetamino-2-deoxy-β-D-glucopyranose) (Garg *et al.,* 2019). Chitosan is characterized by its chemical and physical properties such as anti-microbial activities, cationic character, biocompatibility, non-toxicity, adsorption power, small particle size and high surface area. Thus, the addition of chitosan nanoparticles as binding agents to feeds that are contaminated can also be used to prevent or minimize exposure to fungi and mycotoxins (Desai, 2016). It possesses antifungal activity wherein the mechanism of action involves morphogenesis of the cell wall, directly interfering with fungal growth. Low molecular weight chitosan passes through cell membrane and interact with DNA to interrupt their functions. Chitosan is widely used in the preservation of agricultural commodities in

the food industry and among many other industrial applications (Bautista-Banos, *et al.*, 2016; Alqahtani *et al.,* 2020).

1.2 Statement of the Research Problem

The increase in demand and consumption of fish as a source of protein is on the rise in Africa. Many fish farmers use locally made fish feed in order to reduce production costs and increase profit margins (Adekambi *et al.,* 2020). The locally made fish feeds are usually from locally available plant and animal wastes like rice, maize, wheat bran, blood meal, cottonseed cake sunflower seedcakes, soybeans and cassava. These ingredients are often subjected to contamination by molds during preharvest/or due to poor storage conditions (Marijani *et al*., 2019). Prolonged storage, high temperature and humidity conditions are some of the factors that facilitate fungal development and production of mycotoxins, compromising feed quality that can adversely affect the health of animals and humans (Almeida *et al.,* 2019). Contamination of fish feeds by mycotoxins and the possible transfer of these toxins into farmed fish and fish-derived products for human consumption remain a serious food safety concern (Almeida *et al*., 2019). However, in Nigeria there is a weak regulatory system that guides the formulation process (Puri *et al.,* 2019). Mycotoxin affect feed quality by reducing the nutritive value in contaminated feeds and also serves as vehicle for animal and human infection. Mycotoxin are known to cause a number of toxic effects in animal species and they have the ability to become part of animal products because they are largely lipophilic. Mycotoxin contamination has been implicated with a reduction in fish productivity, anemia, hemorrhaging, liver impairment, weight loss, increased vulnerability to secondary infectious diseases, reduced reproductive capacity, and even mortality resulting in serious economic losses (Mahfouz & Sherif 2015).

1.3 Justification for the Study

Fish is an importance source of both food and income to many people in developing countries. Fish requires high quality nutritional balanced diet for growth. Fish feeds are frequently contaminated by fungi and subsequently mycotoxins. Food and Agricultural Organisation (FAO) statistics estimates that 25% of world's food crops are lost to mycotoxin yearly and a substantial part of the wastage is in Africa (Eskola *et al*., 2019). Several fungi mitigation processes have been employed for treatment of contaminated food. However, an effective and popular approach to decreasing fungi contamination and mycotoxin toxicity in animals is the use of toxin binders as feed additives. These binders can reduce the contamination of feed by fungi and mycotoxins either by suppressing/reducing the absorption or promote the excretion of fungi and mycotoxins or modify their mode of action (Kolosova *et al.*, 2012). The advantage of nanoparticles in reducing fungi contamination in feeds is their high surface area to volume ratio, which enables binding of higher concentrations of toxigenic fungi (mycotoxins). Nanoparticles can pass through the cell membrane because of their size and cause disruption which can interfere with fungi growth (Gontero *et al.*, 2016). Therefore, considering the health hazards linked to the fungi presence in fish feed, there is need for development of a novel and low-cost material for the removal of fungi.

1.4. Aim and Objectives of the Study

1.4.1 Aim

The aim of this study was to produce zeolite from agricultural waste rice husk and groundnut shell doped with chitosan nanoparticles for *in vitro* adsorption of fungi from selected locally produced fish feeds in Minna.

1.4.2 Objectives

The objectives of the study were to;

i. Determine the nutritional composition of the selected locally produced fish feeds in Minna.

ii. Establish the fungal profile of the selected locally produced fish feeds in Minna.

iii. Produce and characterize silica and zeolite using rice husk and groundnut shell.

iv. Synthesize, optimize and characterize chitosan nanoparticles.

v. Formulate zeolite and chitosan nanoparticles as nanoadditives and determine their *in vitro* fungal adsorption capacity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aquaculture in Nigeria

Until recently, capture fisheries have dominated fish production rather than aquaculture in Nigeria. In 2007, about 460,000 tonnes of fish came from capture fisheries and less than 50,000 tonnes from aquaculture (Ayinla, 2019). However, by 2012, the aquaculture sector had grown five-fold to 250,000 tonnes (Food and Agricultural Organization-FAO, 2018). The need to close the gap between production and demand for fish is the major driver of

aquaculture growth in Nigeria. The domestic demand for fish is estimated as 1.5 million metric tonnes (Agboola *et al*., 2019). Catfish production takes place in all the geopolitical areas of the country but the South-South and South-West regions produce the largest shares (Agboola *et al*., 2019).

The global capture production of *Clarias gariepinus* has been rising steadily across the years. In the year 2004, 46,859 metric tonnes were captured as compared to the 2014 record of 55,417 metric tonnes (FAO, 2018). Aquaculture production of the species, has seen a significantly higher rise between the same period. Global production figures have risen about seven folds from 35,400 metric tonnes in the year 2004 to 237,124 metric tonnes in 2014 (FAO, 2018). This is a clear indication that the aquaculture production of *Clarias gariepinus* is gaining grounds globally and especially in Nigeria, West Africa.

Fish farming is fast becoming the bail out point of the protein need of Africans and Nigerians in particular. In this scenario, the production ecology has it that fish production from brackish water is about 0.60%, fresh water 72.3% and marine water 27.1%. Of all these production level, catfish (*Clarias spp*) ranks the highest of the cultured fish in Nigeria with about 33%, *Oreochromis species* 13%, *Channa species* 4%, *Heterotis species* 4%, *Synodontis species* 3%, *Cyprinids* 1% and others in the likes of *Gymnachus niloticus, Citherinus species, Hepsetus odeo* and *Papychranus afer* recording about 22%. As a result of this level of production, Nigeria ranks top in Africa in the use of aqua feed both of local and exotic sources (Puri *et al.*, 2019).

2.1.2 Classification and description of an African catfish

The African catfish (*Clarias gariepinus*) locally referred to as mudfish belongs to;

Phylum: Chordata;

Class: Osteichytes;

Order: Siluriformes;

Family: Clariidae and

Genus: *Clarias*.

In Africa, more than one hundred different species (100) have been identified for which about nine species feature prominently in African aquatic ecology. These are the *Clarias gariepinus, Clarias anguillaris, Clarias pachynema, Clarias macromystax, Clarias agbonyiensis, Clarias buthupogon, Clarias lazera, Clarias macracanthus and Clarias tsanensis* (Idodo-Umeh, 2018). Morphologically, the *Clarias gariepinus* is a scale less fish with smooth skin and soft ray fin, dorsal-ventrally flattened bony head and elongated body (Idodo-Umeh, 2018) (Figure 1). Its dorsal fin has about 61-80 rays and anal fin of 50 -65 rays. Its head is between rectangular and pointed dorsal outline with broad snout. It is often described as depressed and long body shaped fish. The eyes are positioned in the flat depressed head and are relatively small in size. The depth of its body is 6-8 times its standard length. Its teeth are vomerine, granular, fine, and are arranged in rows. These common species of *Clarias* possess characteristically elongated four pairs of barbells like the cat whiskers which are 20-50% as long as the head when the fish is longer than 30cm and 50-80% of the head when the length is smaller in size. The *Clarias gariepinus* has a number of gill rakers which are long, slender and closely fitted of about 24 -110 in numbers that increases as the fish grows. The fish has short dorsal fins that extend as far as to the caudal fin. The pectoral fin extends from the operculum to below the dorsal fin ray. Its lateral line is swap with white coloration extending from the posterior end to the middle of the caudal fin base. The fish has two characteristic colour of dark grey/greenish black colour at the

dorsal part while the ventral part is predominantly white. In some modification, some of them shows band of pigmentation on both side and irregular black sports (Shadyeva *et al.*, 2019).

Ecologically, Abdul-Kari *et al.* (2020) confirmed that the *Clariidae* can be found in stagnant water, lake, pool, and running water body. They are hardy such that they can survive wide range of extreme aquatic conditions. This distinct characteristic is traced to their accessory breathing lungs that complement their gills which enable them to live several hours outside water body. The fish is very active at night, a bottom feeder and omnivorous in its feeding pattern. However, they also exhibit predatory behavior mostly at night hence they are mostly referred to as nocturnal fish and do not reproduce in captivity. Naturally, they are involved in migratory breeding during the onset of rains where they move from deep water to shallow water especially with running water. In captivity pond, they often wriggle out of the body of water to carry out land excursion for a long distance.

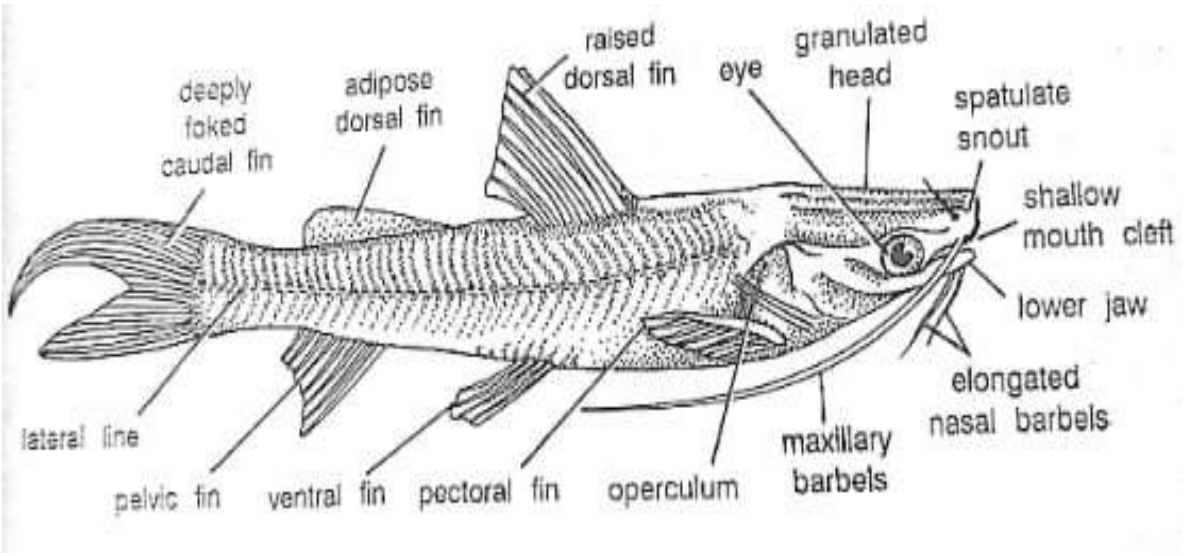


Figure 1: Pictorial View of the African Catfish (*Clarias gariepinus*)

(Idodo-Umeh, 2018).

2.1.3 Feeding behavior of catfish

Feeding is a complex behaviour of animals including fish. In fish, it involves several responses which includes modes of feeding and feeding habits, mechanism of feed detection, frequency of feeding and preferences of feed provided or found. Fish feeding behaviour ranges from plant and detritus feeders to predatory feeding. Some fishes based on their feeding behaviour have become dormant feeders by the fact that they remain in one spot and source of feeds. Others are sub-dormant feeders which generally could move away from spot or source of feed or aggressive areas and some are also sub- ordinate feeders in behaviour. These can stay out of feeding spot or source and still survive on whatever feed items that pass their way (Sunil, 2019).

In addition, Colgan (2020) classified some fish as “generalists” feeders, highly specialized feeders and opportunistic feeders. It also added that hunger is one great factor that stimulate fish feeding behaviour and that feeding initially occur at a faster rate in starved fish and degreases as feeding increases. This is thus in support of restriction feeding and skip a -day feeding practice. Sorum *et al.* (2018) suggested that aggression is one feeding behaviour that could be fixed owe to threat display and fight and every fish has its own life strategy. These different characteristics response to fish feeding behaviour is due to three factors that have been identified as factors that affect feeding behaviour of fish. These are the environmental factors, fish physiological factors and the feeds factors.

2.1.4 Feeding practices of catfish production in Nigeria

Clarias gariepinus has an advantage over other aquaculture species cultured in many parts of the world because of its ability to withstand adverse environmental conditions, utilize

atmospheric oxygen and effectively convert different feedstuff to flesh (Okomoda, 2019). This edge over other fish species comes with some challenges that hinge on its commercial propagation ranging from poor water quality to the practices of feeding (Tiamiyu *et al.,* 2018). Significant mortality can occur from poor feeding practices at different stages of development of the fish. A study by Tiamiyu *et al.* (2018) shows that the growth advantage and size homogeneity linked with ad libitum feeding of *C. gariepinus* is detrimental to the survival rate of the fish; especially if feeding is done too frequently. This is because aggressive swimming behaviour increases, consequent upon heightened anticipation of the fish for food, eliciting cannibalism and increased mortality as well as energy expenditure (Al-Khafaji *et al.,* 2017).

The study of Okomoda *et al.* (2019) shows that the response of fish to feeding and its utilization of feed can be greatly influenced by its stage of development as well as the timing of feeding. Feed optimization in terms of feeding rate and quantity has become a crucial area of study in the culture of many aquaculture species (Tiamiyu *et al.,* 2018). By identifying the optimum feeding practices, farmers can successfully optimize production time, maximize feed utilization, improve the rearing environment (water quality) and facilitates the production of even-sized fish. These practices may differ for different species, size/age, feed composition, and rearing environment (Okomoda *et al.,* 2019).

2.1.5 Fish nutritional requirements

Feeding fish can no doubt increase fish production. In feeding fish, greater challenges exist than in feeding terrestrial farm animals as the feeding behaviour of the farm animals can easily be monitored than fishes which dwell in water. In feeding fish, the nutritional requirement changes with the physiological changes that occur in the fish and in whichever

stage, the fish nutritional needs comprise protein, carbohydrates, fats, vitamins and minerals. These in their formulation should be balanced to meet up with their growth need and targets (Okomoda *et al.,* 2019). Similarly, Cho (2019) added that feeding standard which is the feeding practice employed to deliver nutritionally balanced and adequate amount of diet to the fish to enable them maintain normal growth and reproductive potentials together with efficient growth and or performance of work should be high. Until now feeding of fish has been based on instinct and folkloric practice and this has not met the nutritional requirement of the fish.

Quantitatively and qualitatively speaking, fish need protein of high value of essential amino acids as fishes are efficient in the use of protein for growth and reproduction. The dietary protein requirement of fish varies from species. Rainbow trout (*Salmon gairdneri*) needs 40-60% of protein, channel catfish 30- 36%, carp (*Cyprinus carpio*) 38%, African catfish 35-45%, etc. The protein requirement of fish is higher compared to other farm animals like poultry. This is because they are naturally more efficient in eliminating nitrogenous waste in the form of soluble ammonia compounds through the gills tissues directly into the water and use less energy as a result of less activity, thus the proteins are converted more to flesh (Edwin *et al.*, 2018). It should be added that the protein need of fish is influenced by various factors such as size of the fish, water temperature, feeding rate, availability of planktons in the water, energy level of the feed and the quality of protein in term of its amino acids composition. As important as this nutrient is, its deficiency has been noticed. This ranges from poor feed efficiency, poor growth rate to poor utilization of other feed nutrients (Al-Khafaji *et al.*, 2017).

On the energy need of fish, Cho (2019) reported that energy is not a nutrient rather it is an end product of absorbed macro nutrient when oxidized and metabolized. It is added that most carbohydrate sources such as starch from plant sources are not utilized as energy sources but as simple energy. Therefore, lipids and protein provide the energy need of the fish. Physiologically, lipids and protein help to form important structures of the fish but the need for energy can prevent their incorporation into the body tissue and may involve their catabolism as source of energy. Thus, the utilization of the nutrients depends on the level of intake and the make-up of the diet. The overriding important of food as an energy source means that the major factor regulating the food intake of the animal is its energy value in relation to the animal energy need (Yinka *et al.*, 2015).

In the same vein, Jonathan and Niall (2018) added that the carbohydrates consumed by fish are digested by several enzymes as in other higher terrestrial animals. The mechanisms of using carbohydrate vary between carnivorous and omnivorous species. Among the carnivorous species are the carp, catfish, eel, etc. which can ingest 80% starch as against carnivorous species like Rainbow trout which are poor in starchy feed stuff utilization due to inaction of several enzymes involved in digestion and catabolism of carbohydrate.

Lipids are other nutritional need of fish. These are also source of energy as 1g of lipid yields 8.5 kcal of energy. During digestion, lipids are broken down into fatty acids and glycerol components which are absorbed by the fish. Body fats are synthesized from excessive fatty acids and glycerol and are stored in the subcutaneous tissue, muscle, space between connective tissue and abdominal cavity. The deficiency of these nutrients has been linked to skin discolouration, fin erosion, fatty liver, poor growth among other. Lipids tend to deteriorate through oxidation producing substances that are toxic to fish and destroy vitamin

E. When fish feed is made with rancid fat (oxidized lipid), incidence of muscular atrophy, weight loss and neutrality can result (Okomoda *et al*., 2019).

Fish also need vitamins and minerals as nutrients for their physiological function, growth and maintenance of body metabolism. These are specially included in the feed as premix with varying composition (Jonathan and Niall, 2018).

Feed additives, including binders, antioxidants and antibiotics, are added to catfish feeds to improve their quality and performance. Binders are added to improve the quality of steamed pellets, increase their durability and improve their stability in water. Common pellet binders include the bentonites, which are clay compounds mined from deposits, and lignin sulfonates, which are by-products of the wood processing industry. However, extruded catfish feeds do not require additional pellet binder, because these feeds contain a high percentage of grain or grain by-products which improves feed gelatinization and expansion. The synthetic antioxidants used in channel catfish feeds include butylated hydroxianisole (BHA), butylated hydroxytoluene (BHT) and ethoxyquin. These compounds may be added to fats and sprayed on catfish feeds or added directly to feeds during mixing to avoid lipid peroxidation. United States Food and Drug Administration (FDA) permissible levels for BHA and BHT are 0.02 percent of dietary fat content; while for ethoxyquin, the permissible level is 150 ppm (Al-Khafaji *et al.,* 2017). Antibiotics are incorporated into feeds to be fed to catfish diagnosed with specific diseases. The main antibiotics added to catfish feeds are oxytetracycline (Terramycin) and a combination of sulfadimethoxine and ormetoprim (Romet).

2.2 Forms of Fish Feeds

The use of formulated feed in enhancing fish productivity in pond is very essential and these feeds must be in the right forms of preparations. The preparation of fish feeds is classified based on the moisture content of the feed, feed buoyancy, shape, appearance and the stocks they are made for (Figure 2). Michael *et al.* (2022) said that feed preparation based on moisture content of the feed are classified into dried, moist, wet feed and crumbs. That the dry feeds have about 10% moisture content, moist feed with 30-40% moisture contents and wet feed above 50% moisture contents. In feeding practice, dry and moist feeds are very common and have excellent result than the others. Local fish famers prefer dry feeds which are easily made in large scale, easier to store, transported and fed to the fish than the rest. Thus, they are more palatable, attractive and easily ingested by the fish than the wet and crumbs (Cruz *et al.*, 2020).

On feed preparation based on buoyancy, there are the floating and the sinking feeds. While the floating feed when fed float on the water, the sinking feeds go down the water base. In whichever forms, both gives adequate growth under normal condition. However, performances of floating feeds are high though expensive. It is also reported that a mixture of 15% floating feed and 8% sinking feed can be made (Zakes *et al.*, 2016). These feeds in

any moisture content and buoyancy are in various shapes of pellets, crumbles, granules, balls, cake etc. and are fed to the fish based on the fish size also (Figure 2).

Plate I: Sample Fish Feeds of Different Feed Buoyancy, Shape & Appearance

2.2.1 Aquafeed industry in Nigeria

The development of aquafeed in Nigeria has always been in correspondence with the growth of commercial fish farming. Before 2000, the contribution of aquafeed to total animal feed production was negligible. An estimated 35,570 tonnes of aquafeed was used in 2000, representing less than 1% of national feed production (Fagbenro and Adebayo, 2021). Poultry feed has always been the main product accounting for 90% of animal feed produced. In 2015, an estimated 5.3 million metric tonnes of feed were produced, of which aquafeed contributed about 12 %, second behind poultry feed (Udo and Umoren, 2017). The production is dominated by large-scale commercial feed industries. The commercial feeds are largely high-quality starter feeds. The farmers prefer using commercial starter feeds throughout the early phase (1-2 months) before switching to local feeds to reduce cost of production with optimum growth (Agboola *et al*., 2019).

It is also recorded that farm-made feeds accounted for 70% of total aquafeed produced in 2000. However, recent investments by feed companies like Skretting, Olam, and others establishing their factories in the country have contributed to the increase of more local production of commercial fish feeds. Nonetheless, some farmers still produce feeds on-farm to reduce the cost of feed. The quality of locally made feeds depends on the feed formulation, ingredient quality and the manufacturing processes (Agboola *et al*., 2019). A farm feed is mostly made from locally available raw materials.

Apart from the conventional feed ingredients, there are other cheaper resources, not suitable for human consumption, which have been characterized, explored and investigated as fish feed ingredients. Some of these non-conventional fish feed ingredients are mucuna seed meal, jack bean, pigeon peal, brewer's dry grain, insect meal, winged bean, sesbania, leucania, maggots, earthworms, toad meal and rumen epithelial scrapings (Agboola *et al.*, 2019). The use of these non-conventional feed ingredients is limited because they are not readily available in quantities for large-scale fish feed production. Non-conventional feed materials like insect meal are a viable alternative in fish feed in terms of cost, feed quality, and yield for several fish species such as rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis spp*.), and African catfish (*Clarias spp.*) (Mogaji and Ibiyo, 2016).

2.2.2 Commercial and locally made fish feed in Nigeria

The growth of fish is influenced by its feed utilization which in turn is a function of the nutrient composition and digestibility of the feed (Moshood *et al.*, 2019). A few studies have compared the growth performance of fish to commercial and locally made feeds (Moshood *et al.*, 2019) but different results have been reported due to different species of fish and various methods of formulating the local feeds from a variety of sources. The locally made feed has led to questionable formulations, nutrients composition and production techniques by most farmers who opt for cheaper feed without considering salient factors like proximate composition and processing technique. Some of these studies which investigated feed types on different parameters in fish include Chakraborty *et al.* (2019) who compared the effect of local and commercial feeds on the growth and survival of *C. gariepinus* juveniles in Nigeria, showed better performance with commercial Coppens feed in weight gain, specific growth

rate, protein efficiency ratio and food conversion ratio than the local feed. However, the growth performance reflected the proximate composition of the two diets with the crude protein of Coppens feed having 42 % and local feed with 11 %.

C. gariepinus does not utilize large amounts of carbohydrate for growth but needs protein as observed in fish fed with Coppens, which contained a high percentage of protein as reported by Moshood *et al.* (2019). This study also linked the slow growth performance in fish fed with the local feed with the high percentage composition of fibers in the feed and could be due to the inability of the fish digesting and utilizing the feed (Moshood *et al.,* 2019). A high level of fiber content in feed has been observed to slow the growth of *C. gariepinus* fingerlings (Agbabiaka *et al.,* 2018).

2.2.3 Effects of feed on fish flesh quality

The quality of fish flesh which is composed of muscle reflects a nutritional diet. The study of Zhang *et al*. 2016 reported that muscle lipid, calcium, phosphorus, moisture and protein contents, responded to iron levels in grass carp. It was also reported that the fish muscle tissue with high levels of PUFA is sensitive to oxidation. Another report surmised that oxidative damages are the main non-microbial cause of quality deterioration of fish flesh (Terevinto *et al*., 2020). Lipid oxidation is thought to induce changes in protein structures and thereby influence firmness and water-holding capacity of fish muscle (Lund *et al.,* 2021). Furthermore, another report has shown that nutrients like myo-inositol deficiency depressed antioxidative capacity of muscle leading to loss of flesh quality.

2.2.4 Feed care and storage

Bagged feed should be kept out of direct sunlight and as cool as possible. Vitamins, proteins, and lipids are especially heat-sensitive and can be readily denatured by high storage temperatures. High moisture stimulates mold growth and feed decomposition. Avoid unnecessary handling and damage to the feed bags that could break the pellets and create fines (powder) that will not be consumed by fish (Fagbenro and Adebayo, 2021).

Feed should not be stored longer than 90 to 100 days and should be inventoried regularly. Bags should not be stacked more than 10 high because the excessive weight from the upper bags will crush pellets in lower bags, creating excess fines (dust). Older feed should be used first, and all feed should be regularly inspected for mold prior to feeding. All moldy feed should be discarded immediately. Mice, rats, roaches, and other pests should be strictly controlled in the feed storage area because they consume and contaminate feed and transmit diseases (Fagbenro and Adebayo, 2021).

2.3 Mycotoxins in Fish Feeds

2.3.1 Mycotoxins

Mycotoxins are toxic secondary metabolites with a low molecular weight, which are mainly produced from filamentous fungi species under specific conditions (e.g., high humidity, damaged and contaminated crops, or poor agricultural practices (Horky *et al*., 2018; Agriopoulou *et al*., 2020). The mycotoxins in foods and feeds have highly toxic effects on both humans and animals and can lead to some adverse health impacts (e.g., central nervous system disorders, hepatotoxicity, cardiotoxicity, nephrotoxicity, gastrointestinal tract damage, carcinoma, and even death), which have been an enormous threat to the public health worldwide (Alshannaq and Yu, 2017; Horky *et al*., 2018; Sobral *et al*., 2018; Khaneghah *et*

al., 2019). For instance, severe aflatoxicosis outbreaks have occurred in Malaysia, India, and Kenya, leading to the death of hundreds of humans (Yang *et al*., 2018). Notably, it has been reported that Fumonisins exhibited the toxicity in animals, leading to the equine leukoencephalomalacia of horses and the porcine pulmonary edema syndrome, hydrothorax, and thorax swelling of pigs (Agriopoulou *et al*., 2020). In addition, the corn contaminated by mycotoxins has been a chronic economic and health concern in the United States (Yang *et al*., 2020). Mycotoxin producers mainly include *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps* (Cunha *et al*., 2018). By now, there are approximately 10,000 fungi identified and more than 500 species of mycotoxins have been reported. In addition, it is estimated that there are another 1,000 species undiscovered. Particularly, masked mycotoxins have brought a great challenge due to no routine strategy established for the detection of them (Haque *et al*., 2020). Among these reported mycotoxins, there are several frequently encountered mycotoxins (e.g., aflatoxins, zearalenone ochratoxins, fumonisins, patulin, and trichothecenes) that cause the most important concern to food systems and public health (Luo *et al*., 2018; Haque *et al*., 2020).

2.3.2 Mycotoxin contamination in fish feed and feed ingredients

Plant proteins such as oilseeds are excellent alternatives to animal proteins in fish feeds because they are less expensive and are more abundant in many parts of the world (Anater *et al*., 2019). Diets for Nile tilapia and warm water species such as carp and channel catfish are predominantly formulated using high amounts of grains and plant proteins, and as such, the feeds are at high risk to contamination by mycotoxins. Cereals are common ingredients used in fish feeds and like the oilseeds, they are the main point of entry for many mycotoxins in humans and fish dietary systems, particularly in Africa (Marijani *et al*., 2017). Bran, which

is also a common ingredient in fish feed, is usually derived from any cereal grains such as rice, maize, wheat, oats, barley, rye, and millet during the dry milling process. Unfortunately, this dry milling is not likely to destroy mycotoxins. Mycotoxins are generally concentrated in the bran and outer layers of grains but are less in the endosperm (Rai *et al.,* 2015). This suggests that bran or whole meal grains have the potential to contain higher concentrations of certain mycotoxins than those manufactured from flours or grits milled from the grain endosperm. The use of mycotoxin contaminated bran and other ingredients provide an avenue for the finished fish feed to contain similar mycotoxins posing a health hazard in fish (Rai *et al.*, 2015).

2.3.2.1 *Occurrence of aflatoxin*

The incidences of aflatoxin contamination in fish feed have been reported in many countries of the world especially in the tropical and subtropical regions. Therefore, this means fish feed in both tropical and subtropical regions are more prone to aflatoxin contamination compared to the temperate regions (Odoemelam and Osu, 2019). The recommended regulatory limit for aflatoxin in fish feeds is 20 $\mu g \cdot kg^{-1}$, but a majority of samples from tropical countries are above this limit (Marijani *et al*., 2017).

The incidence of aflatoxin in sorghum and millet from northern Nigeria was investigated by Daniel *et al*. (2021), and they found out that 28.6% sorghum (0.96–21.74 $\mu g \cdot kg^{-1}$) and millet grain (105–14.96 $\mu g \cdot kg^{-1}$) were contaminated with aflatoxin.

In the study of Marijani *et al*. (2017), levels of mycotoxins in fish feeds and feed ingredients from fish farms, imported fish feeds, and feeds made by local feed millers in East Africa were analyzed. Results obtained revealed that aflatoxin contamination was higher in feed processed at farm level in terms of incidence rate (64.3%), feed ingredients (50%), and local commercial feed mills (35.7%), but not in imported feed. Inclusion of antifungal agents in imported feeds to prevent fungal growth during prolonged and varied storage conditions in farms might be a possible reason for the absence of aflatoxin in imported feed samples (Marijani *et al*., 2017). In the same study, fish feed samples from Kenya were found to be highly contaminated with aflatoxin at concentrations ranging from <2− 806.9 $\mu g \cdot kg^{-1}$, followed by those from Tanzania (<2–377.9 $\mu g \cdot kg^{-1}$), Uganda (<2− 28.0 $\mu g \cdot kg^{-1}$), and Rwanda (<2–4.8 $\mu g \cdot kg^{-1}$). In another study from Egypt, around 42.86% of fish feed were contaminated with aflatoxins at a value higher than the permissible limit of 20 $\mu g \cdot kg^{-1}$ (Kholife *et al*., 2019).

Gonçalves *et al*. (2020) screened raw materials and finished fish feeds for aflatoxin contamination in Brazil. Aflatoxin B1 (AFB1) was detected in the mean level of 1.1 $\mu g \cdot kg^{-1}$, 7.4 $\mu g \cdot kg^{-1}$, and 3.8 $\mu g \cdot kg^{-1}$ in maize bran, other cereal products, and finished fish feed, respectively.

Aflatoxin contamination of cottonseed cake has been a major concern worldwide as extremely high contents ranging between 200 and 300 $mg \cdot kg^{-1}$ was reported in samples exported from the USA to the European markets (Hussaini *et al*., 2017). In a survey done by Rodriguez *et al*. (2021) on contamination of aflatoxins in feeds and their ingredients in the Middle East and Africa, it was found out that sunflower meal has the highest contamination level in the whole survey (556 $\mu g \cdot kg^{-1}$). A survey done in East Africa by Marijani *et al*.

(2017) found that cottonseed cake intended for fish feeds was contaminated by aflatoxin with a maximum concentration of 377.9 $\mu g \cdot kg^{-1}$, while soybeans were not contaminated. Sunflower seed cake was the only ingredient that contained the highest AFB1 concentration of 806.9 $\mu g \cdot kg^{-1}$ when compared to maize bran, soybeans, cottonseed cake, and rice bran (Marijani *et al*., 2017). In another survey from Tanzania conducted by Mmongoyo *et al*. (2017), sunflower seed cake was contaminated with aflatoxin with a maximum concentration of 662.7 $\mu g \cdot kg^{-1}$ recorded. Interventions to control aflatoxin contamination along the oilseed product value chain should be implemented to enhance feed safety in African countries.

From the report of the studies above, it can be confirmed that the occurrence of aflatoxin is very high from fish feeds from Africa. This is worrying as the high incidences of aflatoxicosis in human were also reported in Africa. During human aflatoxicosis outbreaks in 2005 and 2006, maize was heavily contaminated with aflatoxin with maximum levels of 48,000 and 24,000 $\mu g \cdot kg^{-1}$, respectively (Daniel *et al*., 2021). Maize intended for fish feeds was contaminated by AFB1 at a maximum concentration of 135 $\mu g \cdot kg^{-1}$ (Marijani *et al*., 2017). Similar results were also reported by Reddy and Salleh (2018), who found out that 22.5% of samples of maize had AFB1 contamination ranging from 20.6 to 135 $\mu g \cdot kg^{-1}$. The poorest quality maize is used for animal feeding, which makes animals more at risk to aflatoxicosis (Mutiga *et al*., 2022).

Rice is another important staple food in Africa and Asia and its bran is widely used for animal feeding. Rice bran intended for fish feed from East Africa were not contaminated by aflatoxin (Marijani *et al*., 2017). In another study from Iran, rice bran was contaminated by aflatoxin with the mean concentration of 18 $\mu g \cdot kg^{-1}$. Wheat does not grow well in tropical climates; however, its bran is widely used as a component of animal and fish feeds (Pinotti *et al*., 2019).

Out of 52 ingredients intended for fish feed from East Africa, sorghum and wheat bran were detected at a very low concentration of less than 3 $\mu g \cdot kg^{-1}$ (Marijani, 2019). Another study from Brazil reported that out of 140 sorghums collected, only 12.8% were contaminated by aflatoxin (Da Silva *et al*., 2020). Shetty and Bhat (2017) found out that 20% of normal sorghum and 89% of normal maize samples also contained aflatoxin B1. Bandyopadhyay *et al*. (2021) suggested that if the primary cereal is sorghum instead of maize, then the risk of aflatoxin-related problems is reduced by 4-fold. Low level of aflatoxin contamination in wheat bran, sorghum, and soya bean suggests that they are likely to be useful in the formulation of fish feeds with aflatoxin below levels that could elicit any adverse complications on fish health.

2.3.2.2 *Occurrence of fusarium*

There are several reports on the contamination of cereal grains and animal feed with *Fusarium* mycotoxins worldwide (Pinotti *et al*., 2019). The most important among them are the trichothecenes, Zerealenone (ZEN), and the fumonisins. The trichothecenes are subdivided into four basic groups, with types A and B being the most important. Type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol (DAS) (Streit *et al*., 2022). Type B trichothecenes include DON also known as vomitoxin, nivalenol (NIV), and fusarenon-X. Fumonisins, particularly Fumonisins B1 (FB1), are found in maize grain which is a major component of feeds for warm water fish (Santana-Mayor *et al*., 2020). Contamination of fumonisin in cereals is dependent on the geographical region, season, and conditions under which the particular cereal is grown, harvested, and stored (Mutiga *et al*., 2022). The prevalence of fumonisin has been reported to be 100% or close to it in all surveillance studies on maize from different parts of Africa. Several studies have shown that

maize bran which is mainly used for animal/fish feed has been contaminated with FBs (Pinotti *et al*., 2019). Fumonisin was detected at the concentration of 1 $mg \cdot kg^{-1}$ on the maize bran used for animal feed from Tanzania (Nyangi, 2019). The prevalence of *F. verticillioides* and production of FB1 in cereal grains and oilseeds in Zimbabwe was established by Gamanya and Sibanda (2021). While the authors did not find *Fusarium* and FB1 contamination in sunflower and soybean samples tested, high incidences were recorded for maize followed by wheat and sorghum. Maize bran and soybeans from East Africa used for fish feeds contained a maximum of up to 3970.1 $\mu g \cdot kg^{-1}$ and 1402.3 $\mu g \cdot kg^{-1}$ of FB1, respectively, while cottonseed cake, sunflower seed cake, and rice bran were not contaminated with FB1 (Marijani *et al*., 2017). This suggests that maize is more susceptible to FBs when compared to other feed ingredients. In the same study, they found out that fish feeds processed at the farm level contained a maximum FBs concentration of 2834.6 $\mu g \cdot kg^{-1}$ and samples tested were below the regulatory limits of 5000 $\mu g \cdot kg^{-1}$ recommended by EU (Mariana and Vitor, 2020).

Deoxynivalenol (DON) is the most often occurring trichothecene and is prevalent in crops used for food and feed production, generally found in various cereal crops such as wheat, barley, oats, rye, rice, and maize (Streit *et al*., 2022). Natural occurrence of DON in cereals is certainly prevalent, and surveys from South America, Canada, China, and many countries of Europe have shown contamination levels in excess of 50% in oats, barley, and wheat with mean concentrations as high as 9 mg·kg–1 in barley (Yazar and Omurtag, 2018). In a survey carried out between 2004 and 2007, DON was the predominant mycotoxin with highest levels detected in wheat bran. Few studies have been carried out on the contamination of DON in finished fish feeds. A survey done in Central Europe has shown that more than 80% of the

samples from commercial fish feed were contaminated with DON with a mean concentration of 289 $\mu g \cdot kg^{-1}$ recorded (Pietsch *et al*., 2018). Fish feed processed at farm level were contaminated with the mean DON concentration of 755 $\mu g \cdot kg^{-1}$ while among the ingredients, maize bran was highly contaminated with 984 $\mu g \cdot kg^{-1}$ (Marijani *et al*., 2017). Another study from Nigeria found out that fish feeds were contaminated with a mean DON concentration of 85.9 $\mu g \cdot kg^{-1}$ (Pietsch *et al*., 2020). All fish feeds from these studies were below the regulatory limits of 5000 $\mu g \cdot kg^{-1}$ recommended by European Union (EU) (Mariana and Vitor, 2020). Sixty-eight samples of shrimp and fish feed from Asia and Europe were contaminated with DON at a mean concentration of 162 $\mu g \cdot kg^{-1}$ and maximum level of 413 $\mu g \cdot kg^{-1}$ (Gonçalves *et al*., 2018).

Zearalenone, a toxic metabolite of *Fusarium* fungi is commonly found as contaminant in maize, and also it may occur in oats, barley, wheat, and sorghum. However, the production of ZEN is favored by high humidity and low temperature conditions. It may co-occur with DON in grains such as wheat, barley, oats, and maize and FBs in maize. ZEN was found in fish feed from Asia with average concentrations of 76.2 $\mu g \cdot kg^{-1}$ (Bai *et al*., 2017). Other studies from Europe reported that fish feeds were contaminated with ZEN with a maximum concentration of 511 $\mu g \cdot kg^{-1}$. However, the ZEN values found in these studies do not exceed the values (5000 $\mu g \cdot kg^{-1}$) currently recommended by the European Commission in animal feeds (Mariana and Vitor, 2020). DON and ZEN in unprocessed cereals and soybean were detected at the mean concentrations of 1,461 ± 2,265 $\mu g \cdot kg^{-1}$ and 656 ± 853 $\mu g \cdot kg^{-1}$, respectively, in samples collected in 2014, while in 2015 these means were 2,687 ± 2,731 $\mu g \cdot kg^{-1}$ and 1,140 ± 1,630 $\mu g \cdot kg^{-1}$, respectively (Pleadin *et al*., 2017).

The authors suggested that higher contamination determined during 2015 could be explained by high to extreme humidity evidenced in the period of cereals' growth and harvesting. The occurrence of DON and FBs in fish feeds, even at low levels, may be of concern, since it can cause growth retardation and immunotoxic effects in fish. These results suggest that ZEN contamination may pose little health risk (if any) to the consumers of the fish (Wo´zny *et al*., 2018).

2.3.2.3 *Other mycotoxins*

Other mycotoxins like OTA, NIV, DAS, T−2 toxin, alternariol (AOH), and ROQ-C have been reported to occur in fish feeds and ingredients intended for fish feed formulation. Cottonseed cake for fish feed formulation was the only ingredient contaminated by OTA with a maximum concentration of 24.2 $\mu g \cdot kg^{-1}$ (Marijani *et al*., 2017). Nivalenol was detected in fish feeds processed at farm level with a maximum concentration of 732.5 $\mu g \cdot kg^{-1}$, while no ingredients intended for fish feeding was contaminated by NIV. Other mycotoxins like DAS, T-2, and ROQ-C were detected in fish feeds and their ingredients but at very low concentrations. Also, an immunosuppressive mycotoxin, gliotoxin, was detected in oilseed cakes at levels up to 45 $\mu g \cdot kg^{-1}$, which was associated with the presence of toxigenic isolates of *A. fumigatus* (Lanier *et al*., 2019).

2.3.3 Effects of mycotoxin on fish health

The toxic effects of mycotoxins are not only depended on the dose in feeds but as well as on the duration of toxin exposure, species, as well as the sex and age of the animal (Pleadin *et al*., 2017). Among the mycotoxins, AFB1 is the most studied in fish, possibly because of its

natural occurrence being most widely found in tropical countries and that it is a known human carcinogen and most potent hepatotoxin (El-Sayed and Khalil, 2019).

The biological effects of AFB1 in aquatic species depend on the toxin's concentration in feed and species. Channel catfish, *Ictalurus punctatus*, appears to be one of the most resistant among fish species when exposed to AFB1, while rainbow trout is the most sensitive to AFB1, and exposing them at concentrations as low as 0.4 μg AFB1 kg^{-1} may cause a 14% chance of developing tumors (Nomura, 2021). Previously, there are no studies on hepatocellular carcinomas in channel fish caused by AFB1, but there are reports that dosing them with higher concentrations of AFB1 resulted in decreased growth rate and moderate internal lesions. European sea bass is also sensitive to AFB1; El-Sayed and Khalil (2019) found that exposing them for 4 days with median lethal concentration (LC_{50}) of 180 μg kg^{-1} AFB1 causes aflatoxicosis. Other studies on fish have shown reduced growth rates particularly on Nile tilapia and channel catfish-fed diets containing 1880 and 10000 μg AFB1 kg^{-1} feed, respectively. The mortality rate of 17% was reported in Nile tilapia fed diets containing 2000 μg AFB1 kg^{-1}. Aflatoxin is also known to affect eye opacity resulting in cataract and blindness, yellowing of the body surface, wounds on the body surface, fin and tail rot, abnormal swimming, feeble and stationary movements, and reduced appetite in tilapia fed aflatoxin-contaminated diet (El-Sayed and Khalil, 2019).

Aflatoxin has been reported to disrupt the reproductive system in both male and female animals; however, very few studies had been reported in aquatic animals (Huang *et al*., 2017). The few existing studies show a significant decrease in ovary weight, fecundity, and egg size of gibel carp fed on AFB1-treated ration (Huang *et al*., 2019). In Nile tilapia, a negative effect on gonadosomatic index, fecundity, sperm count, sperm activity, and serum estradiol-17β

concentrations was observed after feeding them with 1 and 3 mg·kg^{-1} AFB1 contaminated for 3 months. The contamination values of AFs found in fish feeds and their ingredients from Africa were high and, can negatively affect farmed fish, thus leading to economic loss to fish farmers (Diab, 2020).

Fusarium mycotoxins are able to induce both acute and chronic toxic effects. Previous studies have shown that these effects depend on the dose, duration of exposure, and fish species that are exposed. Rainbow trout are sensitive to DON when exposed at low dose, while channel catfish are much less responsive. Hooft *et al*. (2021) reported that feeding rainbow trout with low, graded levels of DON ranging from 3.0 ×10− 4 to 2.6 ×10− 3 mg·kg^{-1} from naturally contaminated maize resulted in highly significant decrease in growth, feed intake, feed efficiency, and protein and energy utilization, whereas channel catfish fed of diets containing up to 10 mg·kg^{-1} DON from either a purified source or naturally contaminated wheat had no effect on the feed consumption, growth, hematocrit values, or liver weight. Also, the rainbow trout liver is sensitive to FB1 because it induces changes in sphingolipid metabolism and is a cancer promoter in this species. Growth performance of Nile tilapia fingerlings was negatively affected when fed with both moniliformin (MON) and FB1 at 70 and 40 mg·kg^{-1}, respectively (Manning *et al*., 2018).

However, when compared to channel catfish, Nile tilapia appears to be more resistant to these two mycotoxins as no mortality and histopathological lesions have been reported (Tuan *et al*., 2022). Reduction in growth, feed efficiency, and feed intake in fish fed with DON-contaminated diet was reported by Hooft *et al*. (2021). However, D¨oll *et al*. (2021) reported that eye development and upward curvature of the body axis of were observed when zebrafish larvae were exposed to 500 μg·L^{-1} or higher of ZEN. By considering the few studies made

in aquaculture species, we would presume that ingestion of ZEN may affect growth performance, but it depends on species, dose, and duration of exposure, and it can result in complications in broodstocks of farmed species and monosex-cultured species. Scarce information is available on the toxicity of OTA in aquatic species. A significant reduction in weight gain, poorer feed conversion rate, lower survival, and hematocrit was observed in channel catfish fed with OTA-contaminated diets (Manning *et al*., 2018).

Furthermore, moderate-to-severe histopathological lesions of the liver and posterior kidney were observed (Manning *et al*., 2018). While a significant decrease in erythrocyte count (Red blood cells -RBCs), haemoglobin content (Hb), and haematocrit value (Hct) in Nile tilapia exposed to low OTA level (400 $\mu g \cdot kg^{-1}$) was seen, in Nile tilapia exposed to 600 $\mu g \cdot kg^{-1}$ diet mean corpuscular volume (MCV), mean corpuscular haemaglobin (MCH), and mean corpuscular haemaglobin concentrate (MCHC) blood indices significantly reduced (Manning *et al*., 2018). OTA has a negative impact on shrimp even after feeding them with 1000 $\mu g \cdot kg^{-1}$ contaminated diet for 8 weeks. However, increasing the dose and exposure duration might affect the shrimp negatively.

Other mycotoxins like sterigmatocystin, which is closely related to the aflatoxin as a precursor in aflatoxin biosynthesis and carcinogenic, have been studied in Nile tilapia (Diab, 2020). Stg has genotoxic and toxicopathological effects in Nile tilapia, (Diab, 2020). Stg also decreases body weight and increase in frequencies of micronucleated red blood cells (MN RBC) and chromosomal aberrations in the kidney of Nile tilapia. Studies on the effects of ROQ-C on animals and fish are limited; however, Chronic Pulmonary Aspergillus (CPA) has been reported to cause anorexia, diarrhea, pyrexia, dehydration, weight loss, ataxia, immobility, and extensor spasm at the time of death in several animals (Manning *et al*., 2018).

Different studies highlight that mycotoxins are a serious problem to farmed aquatic species. A majority of these experiments were conducted based on chronic character, contaminated feed as the route of exposure, and several different doses. It is important to maintain standards when performing the experiments in relation to sex, as studies show that male animals are more susceptible to mycotoxin, divide the toxicology tests on acute, subchronic, and chronics, and also consider species, age, rearing conditions, route of exposure, and dose according to each type of mycotoxins, mainly to detect toxic effects on fish.

2.3.4 Mycotoxin residues in fish and risks for public health

In order to protect consumer's safety, rules and safety limits for several mycotoxins in certain foodstuffs are contained in certain laws or regulations by governmental agencies of different countries. For most mycotoxins, a tolerable daily intake (TDI) has been established, which estimates the quantity of mycotoxin that someone can be exposed to daily over a lifetime without significant risk to health (FAO, 2018).

The main source of human exposure to aflatoxins is the ingestion of contaminated food, with the burden of dietary exposure being particularly high in developing countries. In a study reported by Ezekiel and Sombei (2014), the mean aflatoxin levels are estimated to be 1.18 µg/kg and <1 ng/g/body weight per day in Nigeria. While, the mean aflatoxin exposure is estimated to be less than 1.0 ng/kg of body weight per day in developed countries, whereas in sub-Saharan African countries it exceeds 100.0 ng/kg of bodyweight per day (World Health Organisation-WHO, 2018). Exposure can also be indirectly by consuming animal protein (e.g. fish) in which aflatoxin residues accumulated in the muscle after they were fed with feed contaminated by mycotoxins. Aflatoxin B1 has potent genotoxic and carcinogenic effects. It has been classified as a group-1 carcinogen by the International Agency for

Research on Cancer (IARC) of the World Health Organization, being particularly toxic to individuals who are infected by the hepatitis B virus (WHO, 2018). Chronic exposure to aflatoxins has been associated with 28.0% of hepatocellular carcinoma globally, but the percentage of cases attributable to these toxins ranges from 0.0% in Europe and North America to 40.0% in Africa (Liu and Wu, 2018). Acute exposure, on the other hand, results in severe damage to the liver and a high mortality rate. Acute aflatoxicosis has been described ever since the 1960s and the most recent episodes occurred in eastern Kenya (2004) and central Tanzania (2016) (Kamala *et al*., 2018). Together, these cases of acute aflatoxicosis affected 385 people, out of which 145 died. The signs of aflatoxicosis shown by these patients were jaundice, abdominal pain, vomiting, diarrhea and ascites. The apparent cause of both acute aflatoxicosis cases appears to be the ingestion of maize contaminated by aflatoxins.

Fumonisins on the other hand appear to only be dangerous to humans when they are chronically exposed to this toxin. Fumonisins B1 are a group-2B carcinogen and, as such, are cancer-promoting toxins. They have been associated with a higher incidence of esophageal and hepatic cancer in China and in Africa, in regions where contamination by fumonisins is highly frequent. Additionally, exposure to fumonisins during pregnancy appears to be related to a higher neural tube deformity risk in offspring (Missmer *et al*., 2016).

Studies regarding the effects of ochratoxin A on humans are scarce and thus they are widely unknown. However, it has been considered a group-2A carcinogen, i.e., a probable carcinogen (International Agency for Research on Cancer - IARC, 2020). It affects mainly the kidney, liver and blood, where it accumulates. Ochratoxin A has genotoxic effects which result in DNA damage which, in turn, is the first step to carcinogenesis. As such, exposure to this toxin may be involved in the development of hepatic cancer, urinary tract tumors and

testicular cancer, among other diseases which have been widely reviewed by Malir *et al*. (2016).

Ochratoxin A also seems to accumulate with high incidence, particularly in developing countries, in the breast milk of lactating women, which might lead to infant exposure. Deoxynivalenol produces its toxic effects by inhibiting protein synthesis. It does not pose a health threat to humans compared to other mycotoxins as its effects are generally gastrointestinal, i.e., short-term nausea and vomiting, diarrhea, abdominal pain, headache, dizziness and fever. In fact, trichothecenes in general, but particularly deoxynivalenol, have been associated with an outbreak of acute mycotoxicosis which occurred in India after consumption of bread made using mold-damaged wheat and led to severe gastrointestinal problems (Bai *et al.*, 2017).

As zearalenone resembles the chemical structure of naturally occurring estrogen, it can bind to its receptors on the human cell leading to hormonal imbalances. In turn, this can result in a number of pathologies of the reproductive system. For example, zearalenone has been detected in hyperplastic and neoplastic endometrium, possibly contributing to carcinogenesis. However, according to IARC, zearalenone is not classifiable regarding its carcinogenicity to humans, i.e., there is no evidence that it causes cancer in humans (IARC, 2020). Thus, more studies are needed in order to fully elucidate the effects of zearalenone on human health.

2.4 Nanotechnology and Food Safety

Nanotechnology is a multidisciplinary and promising technology that involves the advancement of organic and inorganic materials and the conversion and manipulation of

these materials on an atomic and molecular scale, which feature custom-made biological, chemical, and physical properties (Dera and Teseme, 2020). Specifically, when the structure of materials is changed, and the particle size is reduced below its outset to approximately 1–100 nm, the physical and chemical properties of the material will show significant differences compared to the original materials even though they both contain the same base materials. Due to their unique chemical and physical characteristics, nanoscale materials provide exceptional benefits to a wide variety of fields of sciences and technology, including engineering, materials science, chemistry, physics, biology, and medicine (Chaudhry *et al*., 2017).

Despite the advances in technology in the areas of food preservation, sanitation and regulations, food safety continues to be a great public concern both nationally and internationally (Garcia-Pinilla, 2019). Foodborne pathogens and toxins can cause foodborne illnesses and present serious risks to human health. Nowadays, nanotechnology applications have expanded into the food industry and play significant roles in all aspects of this sector (Garcia-Pinilla, 2019). With regard to food safety, nanotechnology offers various tools and techniques that can solve food safety issues, including microbial and toxin detection, shelf-life extension, and improvements in food packaging. Nanotechnology approaches in food safety are concentrated on the antimicrobial properties of nanoparticles and nanosensors for foodborne pathogens detection and other contaminants (Nasr, 2018).

2.4.1 Applications of nanomaterials to mycotoxin control

Nanomaterials are defined as the materials with a small size (the length below 100 nm) along at least one dimension, which are synthesized by inorganic and organic materials (Pavel *et al.,* 2018). Many nanomaterials have been successfully applied in the development of

effective sample pretreatment technology and employed as the heart of sensing methods in aspects of signal readout for the accurate mycotoxin detection, greatly improving detection performances including sensitivity, detection time and accuracy. (Eivazzadeh-Keihan *et al*., 2017; Goud *et al*., 2018; Jiang *et al*., 2018; Yang *et al*., 2018). For the mycotoxin elimination, there are some strategies (e.g., chemical, biological, and physical methods) successfully developed (Luo *et al*., 2018; Agriopoulou *et al*., 2020). Excitingly, some emerging nanomaterials recently have been widely applied in the mycotoxin production inhibition, adsorption, and detoxification, which have exhibited a great potential in the control of mycotoxin contamination (Horky *et al*., 2018; Gonzalez-Jartin *et al*., 2019). At present, advanced nanomaterials have been widely used in mycotoxin contamination control, which have attracted rising interests.

2.4.1.1 ***Nanoparticles for the detection of mycotoxin***

Regarding mycotoxins, many analytical methods have been developed for their reliable determination. Currently, the most commonly used approaches in practice are high-pressure liquid chromatography (HPLC) and mass detection and enzyme-linked immunoassay (ELISA) (Chauhan, *et al*., 2019). Since early detection is needed to protect health, current research has focused on improving the detection limit, time consumption, sample consumption, and ease of use. Practice distinguishes two different types of Nanoparticles (NPs) utilization in detection systems (Rhouati *et al*., 2017). On the receptor level, NPs directly react with the detected molecule. This system requires adequate specificity and reproducibility. NPs could be evolved as a transducer-enhancing signal to the detector. An overview of these technologies is given by Rai *et al*. (2015). This work summarizes the possibilities of the immobilization of biomolecules and states that mycotoxins warrant further

research regarding the construction of nanobiosensors with more stability and durability. In this case, the advantage of NPs is their high surface-area-to-volume ratio, which enables the binding of higher concentrations of mycotoxins (Rhouati *et al*., 2017). Over the past decade, research in nanomaterials has focused on carbon nanotubes, polymers, superparamagnetic NPs, quantum dots, and metal NPs. In addition, the use of nanoparticles allows various modifications with the specific ligands or surface decoration by functional groups such as CH_3, -OH, -COOH, $-NH_2$, or $-CONH_2$. Much of the current literature on mycotoxin detection pays particular attention to immunodetection of mycotoxins (Figure 2.1).

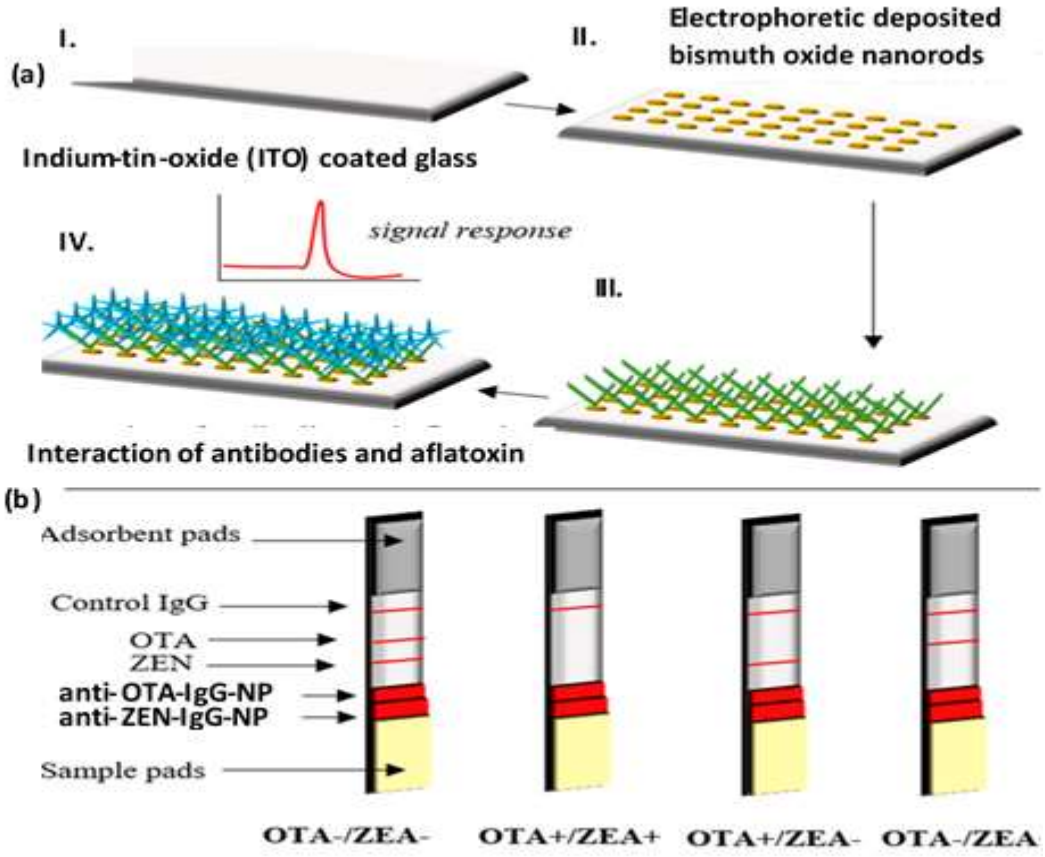


Figure 2.1: Different Immunodetection Arrangements of Antibodies, Nanoparticles and Mycotoxins.

(Solanki *et al*., 2017).

Lateral flow immunochromatographic assay is a rapidly developing technique which combines antibodies (for specificity) and NPs (for sensitivity). Taking advantage of gold NPs of quantum dots, the limit of detection for various mycotoxins is in the range of 0.1–10,000 ng/mL. Immunoelectrodes have been designed based on bismuth oxide nanorods for AFL B1 detection (Solanki *et al*., 2017) (Figure 2.1 a). The third generation of immunosensors is characterized by rapid response (15 s), high sensitivity (1.132 ng/dL), broad linear range (1–70 ng/dL), and low detection limit (8.715 ng/dL) and operates by direct electron transfer of analytes to electrodes. Immunochromatographic ready-to-use test strips have recently been proposed for the simultaneous detection of ZEA and T2 toxin with detection limits of 0.1 and 0.05 ng/mL. Perfect sensitivity and rapid testing have been achieved using antibody-labeled magnetic NPs for sample pretreatment (Petrakova *et al*., 2017). Multilayer NPs can perform several functions simultaneously. Luo *et al*. (2017) designed RuSi-Ru(bpy)3(2+) loaded with gold-functioned nanoporous CO/CO_3O_4 electro-chemiluminescence biosensors for sensitivity (5 ng/mL) detection of DON. Ag-Au Core-Shell NPs have been used for surface-enhanced Raman scattering aptasensors for the double detection of OTA A and AFL B1. Principally, Raman scattering aptasensors produce stable and quantitative signals that emerge from the plasmonic coupling at the junction of a silver core and a gold shell (Lara *et al*., 2015). The latest development has focused on one-step multiplex detection of various mycotoxins. Whether in the form of a strip or whatever form is needed for a device, this simplifies the whole analysis (Sun *et al*., 2016) (Figure 2.1 b). Kong *et al*. (2016) made semi quantitative and quantitative multi-immunochromatographic strips based on gold NPs as a label for the detection of 20 mycotoxins. An advantage of this is the ability to read results by the naked eye. The visual limits of detection for ZENs, DONs, T2s, AFs, and FMs were

estimated to be 0.1–0.5, 2.5–250, 0.5–1, 0.25–1, and 2.5–10 g/kg, respectively (Kong, *et al*., 2016).

The above mentioned findings demonstrate the great potential of NPs for mycotoxin detection. Therefore, detection requirements are greater than sensitivity and reliability. Mycotoxin derivatives are commonly undetectable by conventional analytical techniques due to their changed structure by plant enzymes (Solanki *et al*., 2017). Enzymatic or acid hydrolysis is often employed prior to mycotoxin determination as an effective pretreatment step of masked derivatives. Also, mass spectrometry could expose masked mycotoxins (Solanki *et al*., 2017). The design of a proof of concept for the identification of unknown modified masked mycotoxins is still a big challenge, which could be facilitated with NPs for both mycotoxin isolation or detection.

2.4.1.2 *Nanoparticles as antifungal for mold inhibition and mycotoxin production*

The past decade has seen the rapid development of antibacterial nanoparticles as a solution for antibiotic resistance of pathogenic bacteria. Their applicability against mycotoxin occurrence has been limited by differences between bacteria and fungi. Bacteria are single celled, whereas most fungi are multicellular; bacteria have three distinct shapes, while fungi have various shapes which lead to mycelium formation; bacteria reproduce sexually, whereas fungi are capable of reproducing both sexually or asexually. All these differences make fungi more durable and resistant against some antibiotics (Sureka *et al*., 2019). Until now, research has tended to focus on antibacterial nanoparticles rather than on nanoparticles against fungi. The latest findings in the field of antifungal nanoparticles have been summarized between 2016 and 2017 (Roque *et al*., 2017). In practice, the prevention of mycotoxin occurrence could be mediated via antifungal nanoparticles, which are easy to produce in large scale.

According to recent scientific articles, the antifungal strategy is oriented in two directions. Firstly, an antifungal compound is encapsulated into a polymeric nanocage. Perhaps the most serious disadvantage of this method is the instability in air, although nanopolymers allow cargo release under the appropriate conditions (e.g., presence of enzymes, higher temperature, pH change). Secondly, inhibition effect is reached by nanoparticles alone. This method mainly relies on metal nanoparticles which are stable, act immediately, and offer the possibility of green synthesis. Moreover, the advantage of green synthesis is the formation nanobiocomposites using plant, micro-organism, and animal sources which show less toxicity and improved their main features (Pavel *et al*., 2018)

The relationship between a NP's antifungal activity and mechanism of action has been investigated. He *et al*. (2021) studied the influence of ZnO nanoparticles on *Botrytis cinerea* and *Penicillium expansum*. ZnO NPs producing reactive oxide species (ROS) leads to the damage of the lipid bilayer cell membrane and the breakdown of the affected cell (He *et al*., 2021). Scanning electron microscopy (SEM) showed the formation of unusual bulges on the surface of fungal hyphae and deformation of fungal hyphae after treatment with 12 mmol/L ZnO NPs. More recent attention has been focused on silver nanoparticles. The findings indicate that Ag NPs inhibit fungal growth as well as morphological and metabolic changes (Lara *et al*., 2015). For instance, the application of 45 ppm Ag NPs caused a decrease of organic acid (oxalic, maleic, and citric acid) production, mycotoxin production (up to 80%), and changes in the enzymatic profile in Aspergillus niger and Penicillium chrysogenum (Lara *et al*., 2015). All the studies reviewed so far, however, suffer from the fact that the interaction of NPs with the individual components of the fungi cells has not been investigated yet.

2.4.1.3 *Nanoparticles for the adsorption of mycotoxins*

Due to their great surface area, superior affinity to organic compounds, and easily functionalized modification to improve the selectivity to specific target contaminants, emerging nanomaterials have exhibited a great potential in the adsorption of mycotoxins (Abd-Elsalam *et al*., 2017; Horky *et al*., 2018; Ramadan *et al*., 2020; Santana-Mayor *et al*., 2020). Mycotoxins possess structural diversities, which lead to various physical and chemical properties. Generally, mycotoxins are able to be divided into nonpolar and polar molecules, but several mycotoxins are reported to fall in between. ZEN is non polar and trichothecene is polar, while FMs and AFs are reported to be highly polar (Stroka and Maragos, 2016; Horky *et al*., 2018). Nanomaterials that can change their properties as different physicochemical conditions and serve as nonpolar or polar substances provide a great opportunity to response the diversities of mycotoxins. Currently, plenty of nanomaterials such as carbon nanomaterials, chitosan polymeric NPs, and magnetic Fe_3O_4 modifiers have been widely applied to mycotoxin adsorption (Horky *et al*., 2018; Gonzalez-Jartin *et al*., 2019; Ramadan *et al*., 2020). Carbon nanomaterials (e.g., nanodiamonds, CNTs, and magnetic graphene oxide (MGO) have been successfully used for the adsorption of mycotoxins due to their merits of superior adsorptive capability, large surface area per weight, high stability, inherent inertness, and colloidal stability under different pH (Horky *et al*., 2018).

Nanodiamonds possess the chemical structures (e.g., hydroxylation, carboxylation, and hydrogenation) that permit the surface functionalization, offering a binding affinity to different kinds of mycotoxins (Shoala, 2020). As reported by Pirouz *et al*. (2017) nanodiamond could be used for adsorbing mycotoxins (i.e., OTA and AFB1) through electrostatic interactions that rely on the distinct functional groups on the nanodiamond surface. In addition, Gibson *et al*. (2011) evaluated that the adsorption capacities of

nanodiamonds for OTA and AFB1 were approximately 15 μg/mg and around 10 μg/mg, respectively.

Due to their adsorption properties, single/multi-walled CNT have also been widely applied in the adsorption of mycotoxins, such as trichothecenes, ZEN, and AFTs (Horky *et al*., 2018; Shoala, 2020). MGO are prepared by using graphene oxide and iron oxide NPs, which have been used for the adsorption of mycotoxins. The oxygen functional groups of MGO surface can interact with fusarium mycotoxins (i.e., DON, HT-2, T-2, and ZEN). MGO is used to effectively reduce the levels of DON, HT-2, T-2, and ZEN in palm kernel cake, which can be achieved with 69.57%, 57.40%, 37.17%, and 67.28%, respectively (Pirouz *et al*., 2017). Chitosan (CS) NPs have been reported for simultaneous adsorption of diverse mycotoxins. Glutaraldehyde crosslinked CS exhibited an extremely promising adsorption property for FUM1, OTA, ZEN, and AFB1 with levels of 99%, 97%, 94%, and 73%, respectively.

However, glutaraldehyde cross-linked CS showed no obvious adsorption effects (<30%) for DON and T-2 (Pleadin *et al*., 2017). As Yue's group reported, a nontoxic CS-coated Fe_3O_4 NPs were successfully synthesized for the adsorption of PAT in a juice-pH simulation aqueous. The prepared CS coated Fe_3O_4 adsorbent possessed excellent adsorption properties, low toxicity, and good magnetic properties and exhibited an effective adsorption for PAT with a maximum adsorption ability of 6.67 mg/g (Luo *et al*., 2017). Notably, Turan and Sahin (2016) synthesized magnetic NPs (MIP MNPs) by modifying molecularly imprinted polymers onto the of MNP surface for the specific recognition of OTA (Figure 2.2). The prepared MIP MNPs exhibited high adsorption capacity, high selectivity, and rapid adsorption (Turan and Sahin, 2016). Recently, Sun *et al*. (2016) developed a new, highly effective, and magnetic molecularly imprinted adsorbent (i.e., Fe_3O_4-SiO_2-CS-GO-MIP) for the removal of PAT in apple juice (Figure 2.2 b). Moreover, the proposed Fe_3O_4-SiO_2-

CSGO- MIP showed a maximum adsorption capacity of 7.11 mg/g for PAT, and more than 90% of the total PAT could be removed within 24 hr (Sun *et al*., 2019).

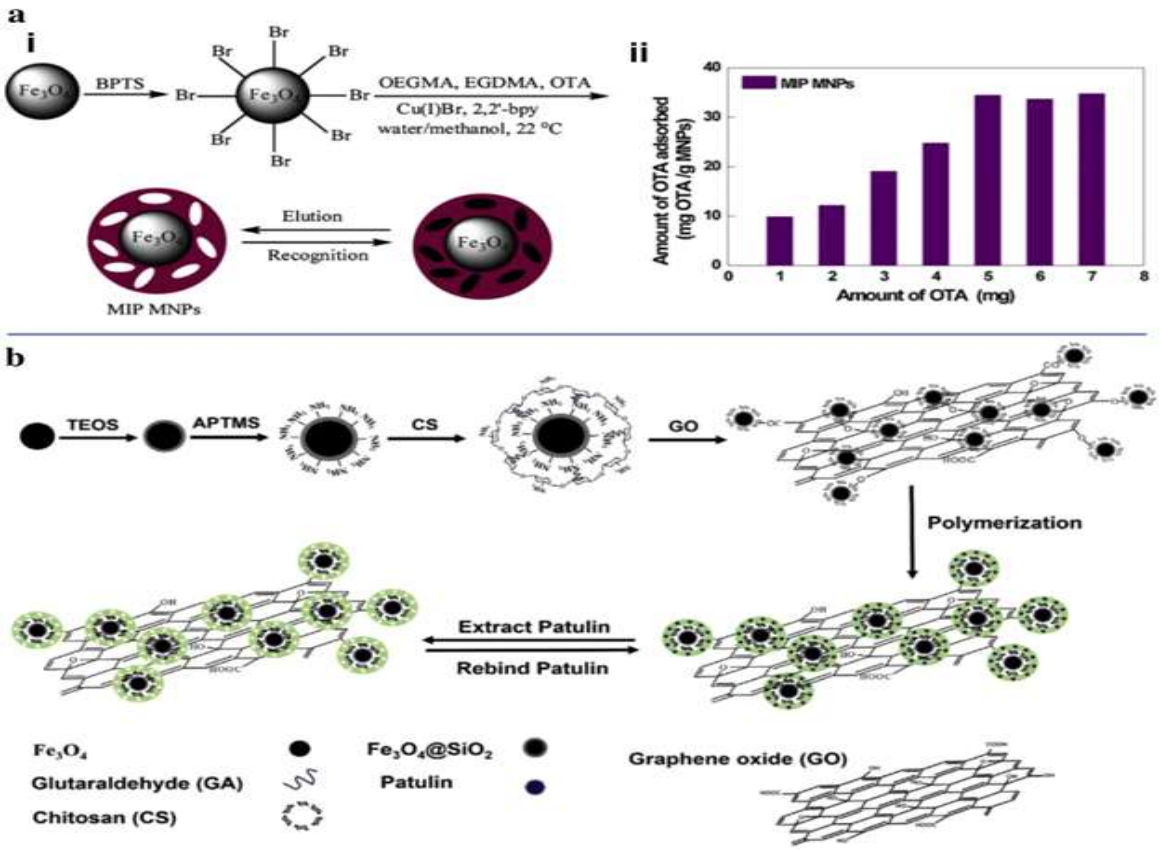


Figure 2.2: Synthesis magnetic NPs (MIP MNPs) by modifying molecularly imprinted polymers onto the of MNP surface for the specific recognition of OTA.

(Turan and Sahin, 2016)

2.4.1.4 *Nanomaterials for the detoxification of mycotoxins*

Detoxification of mycotoxins is considered as a continuous challenge in the food industry. Currently, a great many strategies have been proposed for the mycotoxin detoxification, such as chemical methods (e.g., ozonation, ammoniation, and chemical agents), physical methods (e.g., adsorption, cold plasma, thermal processes, ultraviolet light, microwave heating, sorting, and irradiation), and biological methods (such as enzyme and microorganism)

(Pankaj *et al.*, 2018; Sun *et al.*, 2019). However, these methods for the detoxification of mycotoxins still suffer from some limitations to some extent. For example, chemical approaches may cause residue problems, the adsorption of mycotoxins may lead to a secondary pollution, and biological strategies may suffer from the limits caused by harsh environmental requirements, long growth period, and high cost (Sun *et al*., 2019). Thus, a simple, highly efficient, and safe degradation technology is urgently required for the mycotoxin detoxification. In recent years, photocatalytic degradation as a progressive oxidation technology have exhibited an enormous potential in the detoxification of mycotoxins due to their merits of low cost, environmental-friendly, easy operation at only mild pressure and temperature conditions, and no secondary pollution (Bai *et al*., 2017; Jamil *et al*., 2017). Amazingly, the state-of-the-art nanomaterials have played a key role on the photocatalytic degradation of mycotoxins and have gradually been an attractive study hotspot in mycotoxin detoxification fields (Wu *et al*., 2020).

To date, plenty of nanomaterials such as graphene/ZnO hybrids, g-C_3N_4, WO_3/RGO/g-C_3N_4, Fe_2O_3, titanium dioxide (TiO_2), and UCNP-TiO_2 have been widely applied in the photocatalytic degradation of mycotoxins (Bai *et al*., 2017; Mao *et al*., 2018; Sun *et al*., 2019; Wu *et al*., 2020). For instance, Bai's group successfully synthesized a graphene/ZnO hybrid with a high photocatalytic activity through one-step hydrothermal strategy for the DON photo-degradation under the UV light irradiation. Based on the photocatalytic activity of the prepared graphene/ZnO hybrid under the UV light irradiation, the photocatalytic degradation of DON could be achieved about 99% within 30 min (Bai *et al*., 2017). Notably, Mao *et al*., (2018) developed a novel and highly efficient WO_3/RGO/g-C_3N_4 composite with an enhanced photocatalytic activity for the degradation of AFB1 under the irradiation of visible

light. The as-prepared WO_3/RGO/g- C_3N_4 composites containing g-C_3N_4 nanosheets, RGO, and dispersive WO_3 nanowires were employed as a solid electron mediator. The ·OH, $\cdot O_2^-$, and H^+ were found to be major active radicals in the process of the photodegradation of AFB1 using WO3/RGO/g-C_3N_4 composites.

Moreover, the photocatalytic degradation mechanism ofWO_3/RGO/g- C_3N_4 composites toward AFB1 was studied (Figure 2.3) (Mao *et al*., 2018). Soon after, their group also prepared a novel nanosized g-C_3N_4 sheets with the size range of 50–150 nm for the efficient photocatalytic degradation of AFB1 under a visible light (Figure 2.3). Compared with bulk g-C_3N_4, nanosized g-C_3N_4 sheets showed an enhanced photocatalytic activity toward the degradation of AFB1 under the visible-light irradiation, which was attributed to the larger surface area and better charge separation efficiency of the newly prepared nanosized g-C_3N_4 sheets. During nanosized g-C_3N_4 for the photocatalytic degradation of AFB1, O_2^- and holes served as major active radicals for degrading AFB1, and some ·OH groups also participated in this process (Mao *et al*., 2018). The double bond (C8=C9) in the terminal furan ring of AFB1 is considered as the key hypertoxic site, while the reaction mechanism between ROS and the hypertoxic site during nanomaterials for the detoxication of AFB1 has been rarely studied. To explore the photocatalytic inactivation mechanism of the hypertoxic site in AFB1, Mao *et al.* (2018) creatively prepared a class of CdS/WO_3 composites via the deposition of CdS onto the clew-likeWO_3 surface. The fabricated CdS/WO_3 composites not only could prolong the charge life time and promote the charge separation, but also could oxidize the OH^- to produce the ·OH radicals (the main active radicals for the photocatalytic inactivation) by the high oxidation ability of WO_3, remarkably reducing the toxicity of AFB1 under the irradiation of visible light. By using radical trapping test, high resolution mass

spectrum, and 18O isotope labeling studies, the reaction mechanism between the ·OH radicals and the C8=C9 could be systematically confirmed, and the inactivation of C8=C9 through the ·OH addition reaction was concluded to be the main pathway for the AFB1 detoxification (Figure 2.3 c). Moreover, based on DFT theoretical calculations, the ·OH radicals were verified to tend to react with C9 site instead of other sites (Mao *et al*., 2018). In another study, based on a facile hydrothermal strategy, their group successfully fabricated a new dendritic-like α-Fe_2O_3 with a superior activity for the photocatalytic degradation of DON under the irradiation of visible light. With the prepared dendritic-like α-Fe_2O_3 under visible-light irradiation (λ > 420 nm), 90.3% of DON could be degraded within 2 hr (Wang *et al*., 2019). In addition, TiO_2 and its composites have been employed as the catalyst for the photocatalytic degradation of mycotoxins (Wang *et al*., 2019). As reported by Sun *et al*., (2019) a kind of activated carbon supported TiO2 catalyst (AC/TiO_2) was synthesized by a simple hydrothermal method for the photocatalytic degradation of AFB1. Under the irradiation of UV—Vis light, the degradation efficiency of AC/TiO_2 composite toward AFB1 could reach 98%, which was higher than degradation efficiency of bare TiO_2 (76%) due to an enhanced visible-light intensity and higher surface area of AC/TiO_2 composites (Sun *et al*., 2019). Remarkably, Wang's group prepared a new photo-catalyst (i.e., NaYF4:Yb,Tm-TiO_2 composite (UCNP-TiO_2) for the photocatalytic degradation of DON under the irradiation of NIR light. Moreover, the *in vitro* toxicity of the degradation products in different degradation time was assessed via the influence on Hep G2 cells (including ROS levels, cell morphology, cell apoptosis, cell cycle, antioxidant capacity, and cell viability) (Figure 2.3d; Zhang *et al*., 2016). Recently, their group employed UCNP-TiO_2 for the photocatalytic degradation of DON under the irradiation of UV—Vis light and identified three degradation products of DON (Wu *et al*., 2020). Furthermore, TiO_2 NPs was also

applied in the degradation of PAT in apple juice under UV light intensity. With the TiO_2 NPs, the PAT in apple juice could be degraded to below 10 ng/L in 180 min (Wu *et al*., 2020). Although some nanomaterials have been developed for the detoxification of mycotoxins, much more nanomaterials with higher photocatalytic activity still remained to be further developed.

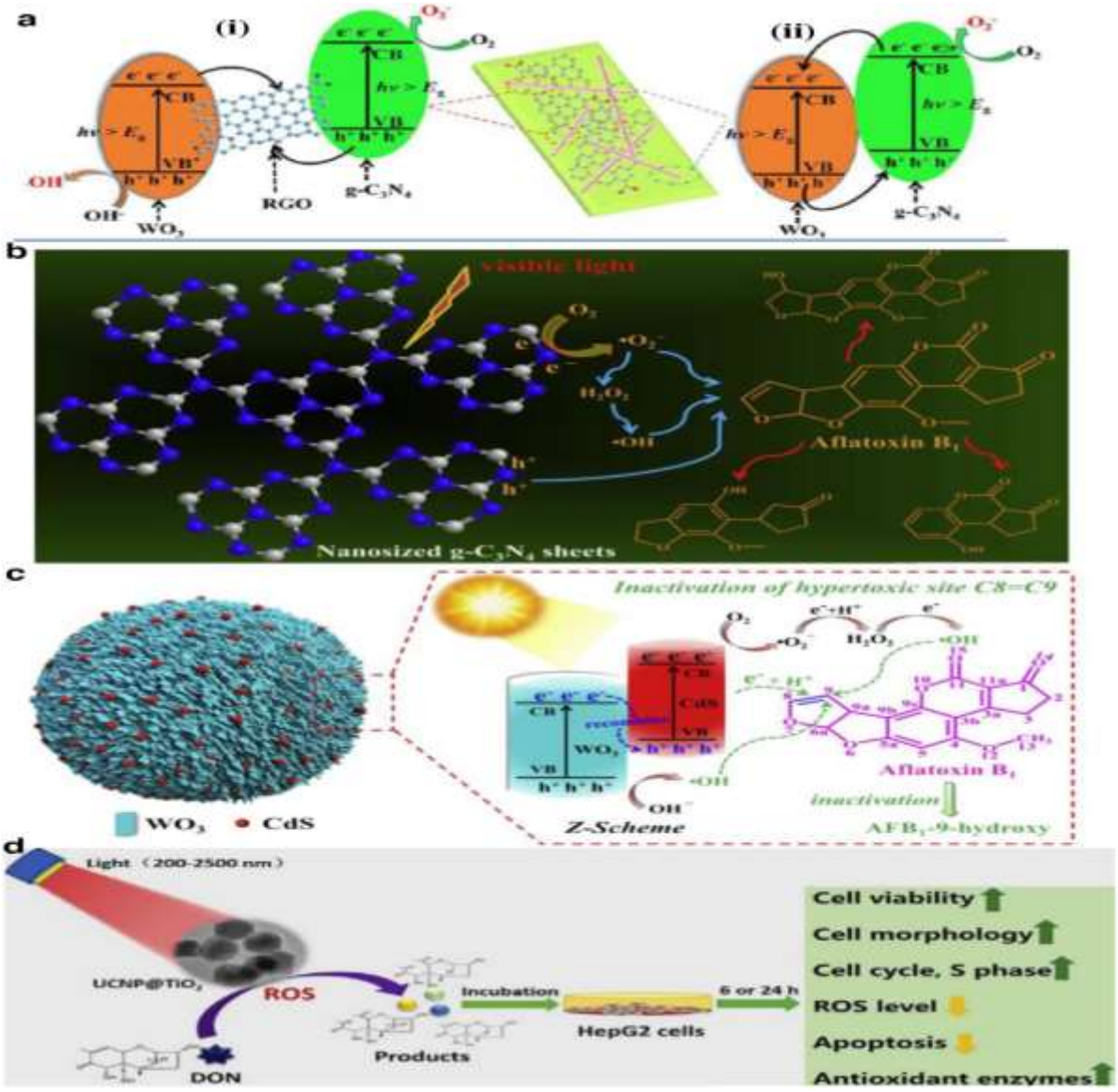


Figure 2.3: WO_3/RGO/g-C_3N_4 composite with an enhanced photocatalytic activity for the degradation of AFB1 under the irradiation of visible light.

(Mao *et al*., 2018).

2.5 Chitosan Polysaccharide

Chitosan is a linear polysaccharide of natural origin composed essentially of β-(1,4)-linked glucosamine units (2-amino-2-deoxy-β-D-glucopyranose) together with some proportion of N-acetylglucosamine units (2-acetamino-2-deoxy-β-D-glucopyranose) (Bautista-Baños, *et al.,* 2016). Chitosan (CS) is a natural cationic polysaccharide produced from chitin, which is the structural element found in the exoskeleton of crustaceans. In contrast to similar polysaccharide celluloses, CS contains hydroxyl groups, acetylamine, or free amino groups which has attracted attention in many fields of applications. CS is nontoxic, biodegradable, and possesses low immunogenicity. Therefore, CS has shown promising results for mycotoxin elimination from different raw materials. In 1990s, CS began to be considered as a suitable mycotoxin adsorbent with approximately 70% efficacy (Khajarern *et al*., 2018).
In addition, a CS solution in a mixture with the minerals rektorit and attapulgit has been patented for removing feed zearalenone and reducing diarrhea due to its antimicrobial properties. Although carbon nanostructures are the focus of much research nowadays, chitosan polymer and nanoparticles have been highlighted in the years 2010–2015. CS is easily subjected to nanoparticles via the gelatation process using aldehydes (e.g., glutaraldehyde) and acids (e.g., thioglycolic, acrylic, and oxalic acids) (Desai *et al*., 2016). Another way for nanoparticle formation is ionic cross links based on electrostatic interaction with phosphoric acid derivatives such as sodium tripolyphosphate (TPP) (Huang and Lapitsky, 2017).

CS's ability to quickly gel relies on the formation of inter- and intramolecular cross linkages between sodium tripolyphosphates (TPP) and CS amino groups. CS can be easily processed

in diverse forms, such as films, threads, tablets, membranes, and micro-particles/ nanoparticles. This allows to design a variety of medical and pharmacological devices adaptable to end purposes. The properties of prepared CS nanoparticles depend on physicochemical conditions such as pH, temperature, time, and functionalization or modification by specific ligands (Khan *et al*., 2019).

2.5.1 Chitosan as adsorbents for mycotoxins

Chitosan (CS) is a natural-based polyaminosaccharide and usually is obtained from waste biomass (shrimp, crabs and seashell) during seafood process (Crini, 2020). The biopolymer is non-toxic, biocompatible and biodegradable. It has been widely used as a promising biosorbent for the removal of various heavy metal ions and dyes. However, CS is very sensitive to pH and easily is dissolved in acid media (pH < 3), which limits its wide application in many fields. The chemical modification of CS with epichlorohydrin, tripolyphosphate and glutaraldehyde is regarded as an alternative to overcome the weakness (Yong *et al*., 2018). The cross-linked CS polymers not only enhance chitosan's resistance to acid media but also improve its adsorption capability (Yong *et al*., 2018).

Currently, only few studies are reported regarding the use of chitosan as adsorbent to sequester mycotoxin. For instance, chitosan bead could efficiently bind OTA in wine and improve wine safety. Chitosan with different molecular weights reduced the bio accessibility of beauvericin in the simulated gastrointestinal fluid (Meca *et al*., 2017). Besides, a commercial adsorbent consisting of chitin, chitosan and chitosan oligosaccharides could provide protection against the combined toxicity of aflatoxins (AFs) and ZEN in duck. The utilization of cross-linked chitosan polymers used for simultaneous removal of multiple mycotoxins in maize and wheat (Zachetti *et al.,* 2019).

2.5.2 Chitosan nanoparticles as antimicrobics and mycotoxin adsorbent

Chitosan nanoparticles (Ch NPs) are formed through ionic gelation, where the positively charged amino groups of chitosan electrostatically interact with the polyanions engaged as cross-linkers (Ahmed and Aljaeid, 2019) (Figure 2.4). For example, Tripathi *et al*. (2019), proposed chitosan nanoparticles based antimicrobial film consisting of chitosan and polyvinyl alcohol. The developed film showed the antibacterial activity against *E. coli*, *S. aureus*, and *B. subtilis* and it has also increased the shelf life of tomatoes. Another study conducted by Burdock (2017) suggested hydroxypropyl methylcellulose act as a potential material for edible packaging films. However, De-Moura (2019) noticed that the incorporation of chitosan-based nanocomposite in hydroxypropyl methylcellulose enhanced its mechanical and barrier characteristics. Moreover, antimicrobial activities of chitosan/gold and chitosan/silver nanocomposite against *E. coli*, *S. aureus*, *P. aeruginosa*, and *Aspergillus niger* have been evidenced in other studies (Youssef *et al*., 2014).

It is widely known that Ch NPs are able to encapsulate various compounds. Glutaraldehyde crosslinked chitosan showed promising adsorption ability for AFL B1 (73%), OTA (97%), ZEN (94%), and FUM 1 (99%) but no obvious adsorption for DON and T2 (<30%) in a buffer system simulating gastrointestinal conditions (Yang *et al*., 2018). Although having great binding capacity, glutaraldehyde is considered to be toxic. LD_{50} for rats has been determined to be 1.30 mL 50% /kg body wt. On the other hand, TPP is a nontoxic, anionic chelating agent forming stable Ch NPs. Using a water-in-oil microemulsion encapsulation method, magnetic Fe_3O_4 CS NPs were synthetized as patulin adsorbents with high magnetic properties, adsorption capabilities, and hypotoxicity. Patulin molecules were adsorbed completely after 5 h with an adsorbent concentration of 400 g in conditions mimicking the

pH of juice. In vitro cytotoxicity and acute toxicity tests showed negligible cytotoxicity, no toxic response, or histopathology in treated mice.

Ch NPs have been recently gaining a lot of popularity. Its bio nature, abundance, degradability, and special properties are its strengths. Ch NPs are reported to exhibit antitumor properties by improving the body's immune function (Yang *et al*., 2018). Ch NPs is useful in bandages to reduce bleeding and as an antibacterial agent and for drug delivery.

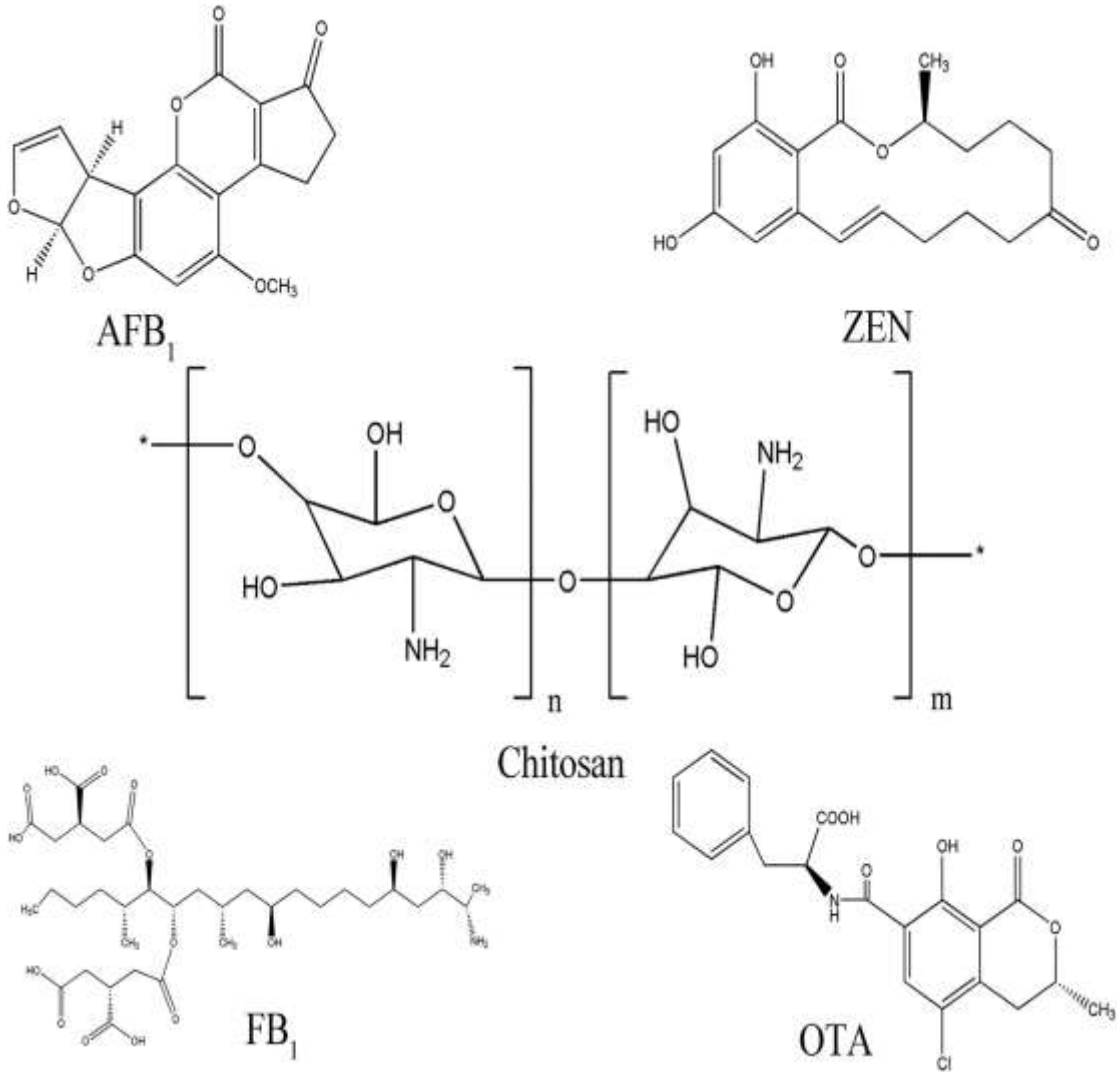


Figure 2.4: Chemical Structures of AFB1, ZEN, FB1, OTA and Chitosan.

2.5.3 Preparation of Ch NPs

Ch NPs have been reported to be prepared by emulsion droplet coalescence, a reverse micellar method, ionic gelation, precipitation, sieving, and spray drying (He *et al*., 2021). All of the above-described techniques follow a bottom-up approach. Bottom–up techniques arrange smaller components into complex assemblies and top-down approaches begin with large sized materials and break them into smaller ones. Routine conventional NPs synthesis usually follow bottom–up techniques. Chitosan micro- and nanoparticles have been prepared using varied techniques. The particle size, stability of the active constituent and the final product, residual toxicity present in the final product, and their drug release kinetics are what go into the selection of an appropriate preparation method (Agnihotri *et al*., 2018). It is confirmed that the size of the prepared particles depends on the molecular weight and chemical structure and degree of deacetylation (DDA) of chitosan, including the method used. The higher the molecular weight of chitosan, the larger the particle size (Anihitri *et al.*, 2021).

The most common methods for obtaining Ch NPs are: ionotropic gelation, microemulsion, emulsification solvent diffusion, and emulsion-based solvent evaporation. Each of these methods influence the particle size and surface charge of nanochitosan and impact the molecular weight and degree of acetylation. The coacervation method involves the separation of spherical particles by mixing electrostatically driven liquids (Zhong, 2019). In the polyelectrolyte complex (PEC) method, an anionic solution is added to the cationic polymer, under mechanical stirring, to obtain nanoparticles. The co-precipitation method involves the addition of a chitosan in low pH to a high pH solution, resulting in co-precipitation of highly monodisperses chitosan nanoparticles. In the microemulsion method, chitosan in acetic acid

solution and glutaraldehyde are added to a surfactant in an organic solvent such as hexane. NPs form overnight as the cross-linking process is completed, resulting in the formation of small-sized nanoparticles (Zhong, 2019). The Emulsification Solvent Diffusion Method is where an o/w emulsion is prepared with mechanical stirring and high-pressure homogenization to achieve 300–500 nm sized Ch NPs. Emulsion Based Solvent Evaporation Method is a slight modification of the above method but avoids high shear forces. In reverse micellar method, the surfactant is dissolved in an organic solvent, to which chitosan, and drug and crosslinking agents are added under constant overnight vortex mixing, leading to the formation of Ch NPs of fine sizes (Banerjee *et al*., 2022).

2.5.4 Properties of chitosan nanoparticles

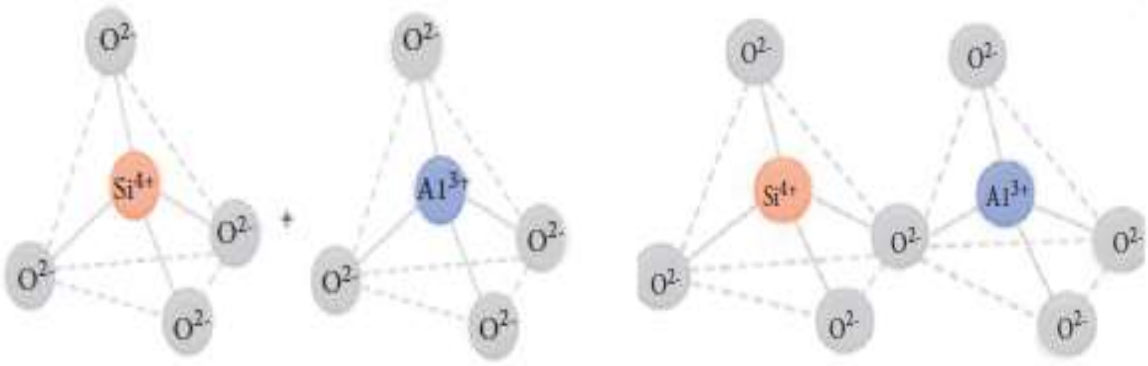
The physiochemical properties of chitosan nanoparticles (Ch NPs) refer to its thermal and morphological properties. The thermal properties of Ch NPs reduce its movement and increases its stability (Abdulhussien and Tamadhur, 2017). The physiochemical properties reveal high degree of deacetylation, stable positive zeta potential, low molecular weight and high water binding capacity. Ch Nps have antioxidant reducing power and scavenging ability against superoxide radicals. Moreover, the excellent antimicrobial ability proposes that the Ch Nps can be used to control, suppress, or inhibit the growth of bacterial and fungal organisms. Ch Nps exhibits low toxicity which is an indicator cytocompatibility and safety (Anusha and Fleming, 2015).

2.6 Zeolite

Zeolites are hydrated aluminosilicate that is made from tetrahedral alumina (AlO_4^{5-}) and silica (SiO_4^{4-}) through interlinkage of oxygen atoms (Odebunmi *et al*., 2018). The term

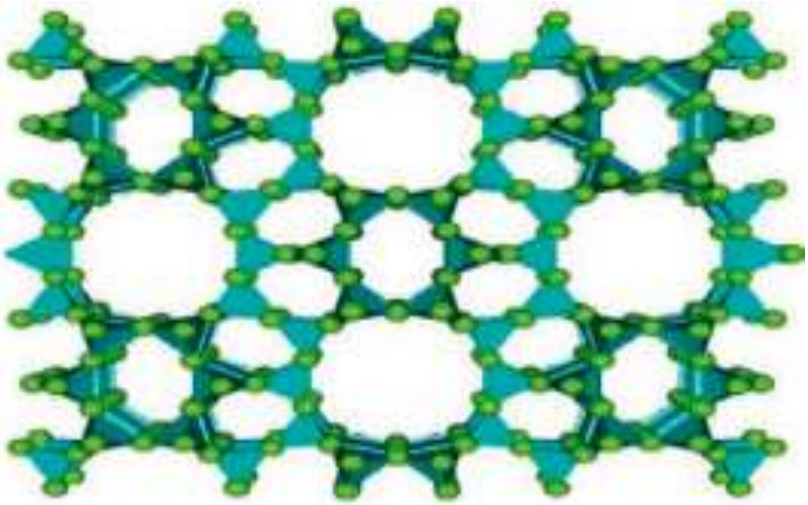
zeolite is derived from Greek words “Zeo” and Lithos” which means boil and stone, respectively; zeolites have high ability of absorbing water and releasing it when they get heated (Moshoeshoe *et al*., 2017). They have an open void with 3D crystal structure having aluminum, silicon, and oxygen coordination with active metals. Zeolites are composed of central atoms (Al, Si or P), and terminal oxygen atom in tetrahedral structure to form primary building blocks (as indicated in the left side of Figure 2.5). They can also form secondary building blocks by bridging through oxygen-oxygen atoms to form rings, prisms, and various sizes (as it is seen at the right side in Figure 2.5).

Zeolites are composed of an elementary structure of an aluminosilicate framework, which comprises a tetrahedral arrangement of silicon ion (Si4+) and aluminum ion (Al_3^+) that are surrounded by four oxygen anions (O_2^-) (Ramezani *et al*., 2019). Each oxygen ion within Si-O and Al-O bonds connects with two cations and which are shared between two tetrahedron structures (Bacakova *et al*., 2018). This results in the tetravalent electroneutral Si in $SiO_{4/2}$ and the trivalent negatively charged Al in $AlO_{4/2}$. The negative charge and the pores of zeolites can be occupied by group IA or IIA metal ions and water molecule (Bacakova *et al*., 2018).



Primary build unit (PBU) of Zeolites

Secondary building unit (SBU) of Zeolites



Thee PBU is combined by sharing oxygen atom with adjacent tetrahedral to form the SBUs. SBUs might be single rings, double rings, polyhedral or more complex

Figure 2.5: Primary and Secondary Building Units of Zeolites. (Moshoeshoe *et al*., 2017).

Zeolites are referred to as 'molecular sieves. They are low-density, crystalline aluminosilicate materials that possess regular micropores with a dimension range of 0.3-2 nm. 232 different zeolite framework types are currently known. Open zeolite structures offer high accessibility of their atoms, which is combined with unrivalled shape selectivity. However, a serious drawback in zeolite-catalysed reactions is that reactants and products may face severe diffusion limitations that often lead to pore blocking and catalyst deactivation by

coke formation. The efficiency of separation processes in zeolites is strongly influenced by intra-pore diffusion. A lot of work has been dedicated to the preparation of zeolites with enhanced accessibility to the micropore volume. A straightforward approach relies on the shortening of the diffusion path by decreasing the zeolite crystal size (Banerjee *et al*., 2022). The impact of diffusion limitation is substantially reduced when nanocrystals with a size of below 50 nm are obtained. In such a case up to 20% of tetrahedrally (T) coordinated atoms can be located on the external surfaces. There are several reviews addressing diverse aspects of nanosized zeolite preparation and their uses (Banerjee *et al*., 2022).

2.7 Efficiency Evaluation of Nanoparticles as a Solution for Eliminating the Risk of Mycotoxins

The issue of nanomaterials has received considerable critical attention regarding their toxicity on the living organisms. In addition, the massive increase of nanotechnology in many applications has caused the emergence of an opposing discipline (nanotoxicology). It could be assumed that newly synthetized nanoparticles are immediately examined for their toxicity and applicability. Nanotoxicity depends on particle solubility, surface area, number of particles per volume, surface charge, size, and tendency to agglomerate, which determine the elimination nanoparticles from the body (Suresh *et al*., 2018). The mentioned properties indicate the mechanism of action. Among those that have been described are the interaction with cell membrane, apoptosis induction, ROS production, inhibition of mitochondrial functions, lipid peroxidation, or autophagy (Suresh *et al*., 2018). Experimental studies have proven that nanoparticles could act as oxidative stress inductors, cytotoxically, as inflammatory agents, or could interact with nucleic acids and thereby contribute to the damage of both microorganisms and higher plants and humans (Suresh *et al*., 2018). Current toxicologic studies have found that the most organism-friendly are cerium oxide

nanoparticles, fullerenes, or polymeric nanoparticles (Polyethylenglycol or chitosan). On the other hand, many inorganic nanoparticles have been considered as hazardous (Chatterjee *et al*., 2017). An objective assessment of toxicity seems to be difficult. A number of publications have been created on this topic and consortia and research groups have emerged as well. So, the status of this issue is still evolving. Interestingly, particle toxicity often works differently on model stem cells and organs.

Furthermore, viability tests, such as the MTT assay, are sensitive to pH and medium, as found in Jo *et al*. (2019). The relationship between the dose and the response of the organism must be taken into account as well as the accumulation in the environment and the chronic exposure of the organism to low doses (Goyal and Basniwal, 2017). In terms of efficiency evaluation, the toxicity of anti-mycotoxin nanomaterials and the toxic effect of mycotoxins have to be carefully considered. A key problem with nanomaterial efficiency against mycotoxin evaluation is the uncomplicated studies that are unilaterally focused. In reviewing the literature, no relevant data was found on the association between nanomaterial effectivity, toxicity, and the received dose of mycotoxins from food and feed. There are predominantly studies that focus on nanoparticle synthesis. Large-scale production has been improved by the fact that the prices of nanoparticles are comparable to those of commonly used bentonite for the elimination mycotoxins from feed (2–8 USD per gram in the Czech Republic). This review outlines the relationship between the threat of concentration of mycotoxins taken from fish feeds by fish or as food from mycotoxin poisoned fish and the effectiveness of nanoparticles to eliminate them. Nanoparticle efficiency against mycotoxins vary depending on the nanomaterial used. The scientific literature shows the toxic effect of the most common mycotoxins to be in the range of 1–30 mg/kg of the feed dose (Alshannaq and Yu, 2017).

Theoretically, only 30 mg of nanoparticles per 1 kg of compound feed is sufficient to eliminate the toxic effect. In the case of the toxicity of selected nanoparticles, it has been established from literature that the safe level for mice is on average 0.3–16,000 mg/kg. Taken together, the results of Nano adsorbent efficiency against mycotoxins would have been far more persuasive if the authors had considered the actual practical feed dose of mycotoxins (Alshannaq and Yu, 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Petroleum ether, sulphuric acid, boric acid, ammonia, hydrochloric acid, $KMNO_4$, H_2SO_4, Na_2SO_4, CuSO4, selenium, NaOH, NH_3, NH_4OH, Potato Dextrose Agar, Nutrient Agar, chloramphenicol, lactophenol blue, sodium tripolyphosphate and Chitosan powder. All the reagents and chemicals were of GPR grade and were purchased by Sigma Aldrich Chemical Company Incorporation in Milwaukee, Wisconsin, USA, and British Drug House (BDH) Limited in England.

3.1.2 Collection of samples

Locally produced fish feeds were collected from local producers (millers) at Talba Farms, located in Bosso local government area in Niger State.

Rice husk (RH) and groundnut shell (GNS) were collected from a rice and groundnut milling factory in Gbeganu area of Minna, Niger state.

3.2 Methods

3.2.1 Proximate composition analysis

The proximate analysis of the feed samples for moisture, total ash, crude fibre and fat were carried out in triplicate using methods described by Onwuka (2005). The nitrogen was determined by micro Kjeldah method described by Onwuka (2005) and the nitrogen content

was converted to protein by multiplying by a factor of 6.25. Total carbohydrate content was estimated by 'difference'. All the proximate values were reported in percentage (%).

3.2.1.1 ***Determination of moisture***

Moisture was determined by oven drying method. About 2g of well-mixed samples was accurately weighed in clean, dried crucible (W_1). The crucible was allowed in an oven at 100-105 C for 6-12 h until a constant weight was obtained. Then the crucible was placed in the desiccator for 30min to cool. After cooling it was weighed again (W_2). The percentage moisture was calculated by following formula.

$$\% \text{ Moisture} = \frac{W_1 - W_2 \times 100}{\text{Weight of sample}}$$

Where,

W = Initial weight of crucible + Sample 1

W = Final weight of crucible + Sample 2

3.2.1.2 ***Determination of ash***

For the determination of ash, clean empty crucible was placed in a muffle furnace at 550^0C for an hour, cooled in desiccator and then weight of empty crucible was noted (W_1). Two grams of each of sample was taken in crucible (W_2) and was purchased over a burner, until it was charred. Then the crucible was placed in muffle furnace for ashing at 550^0C for 2-4 h. The appearance for gray white ash indicates complete oxidation of all organic matter in the sample. After ashing the crucible was cooled and weighed (W_3). Percentage ash was calculated by the following formula.

$$\% \text{ Ash} = \frac{\text{Difference in Weight of Ash} \times 100}{\text{Weight of Sample}}$$

Difference in weight of ash = $W_3 - W_1$

3.2.1.3 *Determination of crude protein*

Protein in the sample was determined by kjeldahl method 0.25g of dried samples was taken in digestion flask, with 6ml of concentrated H_2SO_4 and a speck of kjeldah1 catalyst (mixture of 10g Na_2SO_4+5g CuSO4+ 0.05g selenium). The flask was swirled in order to mix the contents thoroughly then digested on the digestion block till the mixtures become clear (colourless or greenish in color). The digest was cooled and transferred to 100ml volumetric flask and volume was made up to mark by the addition of distilled water. Distillation of the digest was performed in Markham Distillation Apparatus. Ten milliliters of digest were introduced in the distillation tube then 10 ml of 40% NaOH was gradually added through the same way. Distillation was continued for at least 10 min and NH_3 produced was collected as NH_4OH in conical flask containing 5ml of 4% boric acid solution with few drops of methyl red indicator. During distillation yellowish color appears due to NH_4OH. The distillate was then titrated against standard 0.1 N HCI solution till the appearance of pink color. A blank was also run through all steps as above. Percentage crude protein content of the sample was calculated by using the following formula;

$$\% \text{ Crude Protein} = 6.25^* \times \%N \quad (*. \text{ Correction factor})$$

$$\%N = \frac{(S-B) \times N \times 0.014 \times D \times 100}{\text{Weight of the sample} \times V}$$

Where,

S = Sample titration reading — B = Blank titration reading

N = Normality of HCI — D = Dilution of sample after digestion

V = Volume taken for distillation — 0.014 – Milli equivalent weight of Nitrogen

3.2.1.4 *Determination of crude fat*

Crude fat was determined by ether extract method using Soxhlet apparatus. Approximately 2g of moisture free sample was wrapped in filter paper, placed in fat free thimble and then introduced in the extraction tube. A weighed, cleaned and dried receiving flask was filled with petroleum ether and fitted into the apparatus. The Soxhlet apparatus was assembled and allow refluxing for 6hrs; extract was transferred into clean glass dish with either washing which was evaporated on water bath. Then the dish placed in an oven at 105^0C-110^0C for 1hr and cooled it in a desiccator. The percentage crude fat was determined by using the following formula:

$$\% \text{ Crude Fat} = \frac{\text{Weight of either x } 100}{\text{Weight of sample}}$$

3.2.1.5 *Determination of crude fiber*

For the determination of crude fiber, 2g of sample was defatted with per ether; boiled under reflux for 30min with 200ml a solution containing 1.25g of H_2SO_4 per 100ml of solution. The solution was filtered through linen or several layers of cheese cloth on fluted funnel, washed with boiling water until the washings are no longer acidic then the residue was transferred into a beaker and boiled for 30min with 200ml of solution containing 1.25g of carbonate free NaOH per 100ml, the final residue was filtered through a thin but close pad of washed and ignited asbestos in a Gooch crucible, then dried in an electric oven and weighed after which it was incinerated, cooled and reweighed. The loss in weight after incineration /2 x 100 is the percentage crude fiber.

3.2.1.6 *Carbohydrate content determination*

The nitrogen free method described by Association of Analytical Chemists-A.O.A.C (1990) was used to determine the carbohydrate content. The carbohydrate is calculated as weight by difference between 100 and the summation of other proximate parameter as Nitrogen Free Extract (NFE) percentage carbohydrate (NFE) = 100- (m+p+F+A+F_2).

Where, M=moisture, P=protein, F_1=Fat, A=ash, F_2=crude fiber

3.2.2 Fungal profile

The methods described by Nirmala (2016) was employed in establishing the fungal profile of five locally produced fish feeds in Minna.

3.2.2.1 *Preparation of the media*

The media used were Potato Dextrose Agar (PDA) and Nutrient Agar (NA). Nutrient Agar was prepared by adding 7g of agar into a 500ml conical flask and dissolving it with 250ml distilled water. The conical flask was plugged with cotton wool wrapped with aluminum foil and the mixture was warmed on a heating mantle to homogenize. Then the medium in the flask was sterilized in an autoclave at 121 ˚C for 15 mins.

Potato Dextrose Agar was prepared by weighing 9.75g of the agar into a 500ml conical flask and dissolving it with 250ml distilled water. Then, chloramphenicol was added aseptically to molten PDA at 45 ˚C in order to retard bacterial growth. The isolation of microorganisms was done by using the pour plate method.

3.2.2.2 *Serial dilution*

Serial dilution was done as follows. To prepare stock solutions, 1g of each sample was added into test tubes containing 10ml sterile water. Then, 1ml was removed from each of the

solution and added to another set of test tubes containing 9ml sterile water which made 10^{-1} dilution. The same procedure was repeated to make 10^{-4} dilution. Then, 0.5ml of the 10^{-4} dilution was added into sterile petridishes and sterile molten agar was poured into the plates. The inoculated plates were allowed to set and incubated. The plates were kept in an inverted position to avoid the condensation of water vapour on the plate cover from dropping on the culture. The NA plates (bacterial cultures) were incubated at 37 ˚C for 24hours while PDA plates (for fungal cultures) were incubated at 25 ˚C for 72 hrs. The number of colonies found on each media was counted.

The number of fungal colonies per gram of sample was calculated and expressed in colony forming units per gram of sample (CFU/g) as:

CFU/g= Number of colonies in plate × Reciprocal of dilution factor.

3.2.2.3 *Pure isolation*

Isolation of microorganisms was done. Sterile molten agar (NA and PDA) was poured into petridishes and allowed to solidify. Different colonies were taken from the mixed culture plates and streaked on plates separately. The streaked plates were incubated at 25 ˚C for 72 hours for fungal growth. Similar sub-culturing was done until pure cultures were obtained. The pure cultures were stored in Mc Cartney bottles. PDA and NA were prepared in Mc Cartney bottles and sterilized in an autoclave at 121˚C for 15 mins. Then, the media were allowed to set in an incline position to prepare agar slants. The pure fungal isolates were inoculated into PDA slants and incubated at 25 ˚C for 48 hours.

3.2.2.4 *Identification*

The fungal isolates were stained with lactophenol blue and will be mounted between microscopic slides and cover slides. Micro-morphological characteristics will be observed under an optical microscope.

3.2.3 Production of silica from rice husk and groundnut shell

The methods described by Yunusa *et al.* (2016) was employed in the production of silica and synthesis of zeolite.

3.2.3.1 *Leaching of rice husk and groundnut Shell*

Rice husk and groundnut shell was sieved to eliminate clay particle. The rice husk and groundnut shell were washed thoroughly with distilled water to remove any adhering impurities and dried in an oven at 100°C for 24hrs. Acid leaching of the rice husk and groundnut shell was carried out to remove soluble elemental impurities such as iron, magnesium, calcium etc. Leaching was carried out at 10 wt% solids in 10wt% Hydrochloric acid (HCl). The HCl solution was prepared from a standard HCl stock of mean concentration of 37wt% and density of 1.19 g/ml. The dried RH and GNS were soaked in the prepared solution of 10wt% HCl for 24hrs. The treated rice husk and groundnut shell were washed again thoroughly with distilled water until pH became 7 and then dried at 100^0C for 24hours.

3.2.3.2 *Pyrolysis of rice husk and groundnut shell*

The dried RH and GNS was pyrolyzed in an oxygen atmosphere at 500^0C, 450^0C and 400^0C respectively for 6 hours. The Rice Husk Ash (RHA) and Groundnut Shell Ash (GNSA) obtained were used in the synthesis of zeolite.

3.2.3.3 *Zeolite synthesis*

The methods described by Yunusa *et al.* (2016) was used for the synthesis of zeolite. The three major Steps involved in the synthesis of zeolite Y are preparation of Seeding gel, Feedstock gel and over all gel.

Preparation of Seed Gel: About 1.7g sodium hydroxide pellets were dissolved in 7.5ml deionized water and stirred until clear and homogenous solution appeared. 2 ml of the aqueous NaOH were added to 0.75g sodium aluminate and stirred with heating until a homogenous mixture was formed. 1.5g silica source (RHA, cab-o-sil and sodium metasilicate and GNSA, cab-o-sil and sodium metasilicate) were added separately to 5.5 ml sodium hydroxide aqueous and stirred while heating on the magnetic stirrer until homogenously mixed (Odebunmi *et al*., 2018). The two solutions were then mixed simultaneously in a 150 ml propylene bottle and stirred with heating for 30 minutes. The gel formed was then aged for 24 hrs.

Preparation of Feedstock Gel: About 7.8g NaOH pellets were dissolved in 142 ml distilled water and stirred until a clear solution formed. 42.5 ml of NaOH solution were added to 13.7711g sodium aluminate and stirred with heating gently on the hot plate until clear solution appeared then 28.1463g silica source (RHA, cab-o-sil and sodium metasilicate and GNSA, cab-o-sil and sodium metasilicate) were added to 100ml of NaOH solution in a polypropylene bottle. The mixture was then stirred and heated on the hot water bath. The aluminate and silicate solutions formed were then mixed together in a polypropylene bottle and stirred for 2hrs.

Preparation of Overall Gel: The seed gel and feed stock gel were then mixed. The seed gel was added slowly into the feed stock gel and the mixture was continuously stirred with magnetic stirrer for 2hrs at room temperature. The mixture was then transferred into 150 ml propylene bottle and was aged for 24hrs at room temperature. After ageing, the mixture was inserted into oven at 100^0C for 22hrs. The propylene bottle was removed after 22 hrs in the oven and the cap was quickly opened and left to cool to room temperature. After cooling the mixture was, filtered and washed with hot de-ionized water, then dried overnight in the oven at 100^0C.

3.2.4 Characterization of zeolite

The X-ray Diffraction Patterns of zeolite from rice husk (Z-RHS) and groundnut shell (Z-GNS) were carried out using the method described by Tariqul and Changsheng (2019). The zeolite samples and were evaluated by XRD spectrometry. It was scanned for 2h ranging from 5^o to 60^o with CuKα radiation and an acceleration voltage of 35.4 kV and current of 45 mA at a rate of 5^o/min. The percentage of crystallinity of each synthesized zeolite was calculated from the XRD patterns by using the equation below:

$$\text{Crystallinity \%} = ([I_{cx} - I_a] / [I_{c100} - I_a]) \times 100$$

where I_{c100} is the intensity for fully crystallized zeolite at 2Θ 27^o and I_a is the intensity when we have completely amorphous nature at 2Θ 26.65^o and I_{cx} is the intensity of each desired sample at 2Θ 27^o.

3.2.5 Synthesis of chitosan nanoparticles

The methods described by Vaezifar *et al.* (2013) was used for the preparation of chitosan nanoparticles.

Original chitosan was dissolved in acetic acid 1 (w/v) %. The pH of the solutions was raised to 4.6–4.8 by addition of appropriate amount of NaOH. Aqueous sodium tripolyphosphate solutions were prepared by dissolving in distilled water. Sodium tripolyphosphate serves as a cross linking agent, it increases stability and decrease mobility of chitosan nanoparticles. The tripolyphosphate solution was then added dropwise to a chitosan solution while stirred with magnet stirrer at room temperature to form chitosan nanoparticles spontaneously. The mixture was then stirred during the reaction time. The resulting suspension was subsequently centrifuged at 20,000 rpm for 10 min. The chitosan nanoparticles were extensively washed with distilled water to remove any impurity. Finally, the nanoparticles formed were precipitated and dried at 70 ^{0}C for 24 hours and characterized. The temperature of reaction and pH were kept constant for all experiments. Thus, the effects of chitosan concentration (CHT), tripolyphosphate concentration (TPP), and reaction time (t) on the particle size distribution were investigated.

3.2.5.1 *Optimization of chitosan nanoparticles*

The methods described by Vaezifar *et al.* (2013) was used to determine optimum conditions, the tests were designed by Design Expert software, using Taguchi method. The best conditions were determined based on three factors at three levels to determine the optimum conditions for preparing chitosan nanoparticles by ionic gelation. Ionotropic gelation is a mild method for preparing nanoparticles in an aqueous environment which does not need the introduction of chemical groups into the chitosan molecules. Design of Experiments Examinations were designed by Qualitek-4 software (Nutek, Inc.) using Taguchi method based on three factors in three levels. The optimized factors were CHT, TPP and t. Chitosan solutions were prepared at three levels of concentrations (1.0, 2.0, and 3.0 mg/ml). Aqueous

sodium tripolyphosphate solutions were also prepared at three levels of concentrations (0.5, 1, and 2 mg/ml). The three levels for reaction time were chosen as 30, 60, and 90 min. The experimental parameters based on three factors in three levels were designed and examined using Qualitek-4 software. The size of nanoparticles in this method is affected by the chitosan concentration, tripolyphosphate concentration and reaction time. To prevent flocculation, the nanoparticle concentration must be kept at low level.

3.2.5.2 *Characterization of chitosan nanoparticles*

The chitosan nanoparticles were analyzed with basic characterization techniques. UV- Vis Spectroscopy was used to study the formation of chitosan nanoparticles and nature of particle size. Particle size analysis was carried out to analyze the average particle size and size range (Rai *et al.*, 2015).

3.2.6 Doping of chitosan nanoparticles on the zeolite

The synthesized chitosan nanoparticles were added to the produced Z-GNS and Z-RHS. This was done to boost and increase the synergistic effect of the nanoadditives.

3.2.6.1 *Ratio formulation for the nanoadditive and feed inclusion*

S1= (100% Chitosan nanoparticles)
S2 = (50% Chitosan nanoparticles + 50% Zeolite-GNS/RHS)
S3 = (100% zeolite-GNS)
S4 = (100% zeolite- RHS)

3.2.7 Determination of *in vitro* adsorption capacity of the formulated nanoadditive

The methods described by Nirmala (2016) was employed in determining the in vitro adsorption capacity of five locally produced fish feeds with the inclusion of the formulated nanoadditives. The antifungal activity of the produced nanoadditives was carried out by

mixing various concentrations of the nanoadditives with the feed samples. Serial dilution of the fish feed was carried out and 1ml of the 10^{-5} dilution was aseptically transferred into sterile petri dishes. Molten Nutrient agar and Sabouraud dextrose agar medium were poured into respective petri dishes and allowed to solidify before incubating. The plates were screened for the presence of discrete colonies after incubation period and the actual numbers of organisms were estimated in colony forming unit per ml (cfu/ml).

3.3 Data Analysis

All experimentsnumeric data obtained were analyzed by one-way analysis of variance (ANOVA) using SPSS version 20.0. The results were expressed as the mean ± standard error of mean (SEM). The significance difference between the selected locally produced fish feeds were compared and a probability level of $P \leq 0.05$ was considered significant.

CHAPTER FOUR

4.0. RESULTS AND DISCUSSION

4.1 Results

4.1.1 Proximate composition of selected locally produced fish feeds in Minna

The percentage composition of five selected commercial locally produced fish feeds is presented in Table 4.1. The percentage of carbohydrate in all the samples range from 60.98±2.19 to 76.65±0.15^{c} % with feed E having highest of about 76.65±0.15^{c} % and feed B with lowest 60.98±2.19 %. Feed A and B has no significant different in percentage moisture content when compared to feed C, D and E. While feed E has the lowest percentage crude protein of 1.50±0.15^{a} as compared to other feeds. All the samples have a significant percentage of crude fibre with feed E having the highest of about 4.30±0.23^{c} and feed B with lowest 2.50±0.60^{a}. Feed A and E has no significant ash content when compared with feed B, C and D.

Table 4.1: Proximate composition of five selected locally produced fish feeds

	Proximate Parameters					
Feed sample	**Moisture content (%)**	**Crude Protein (%)**	**Crude Lipid (%)**	**Ash content (%)**	**Crude Fibre (%)**	**Carbohydrate (%)**
A	13.33±0.64^{d}	3.96±0.35^{d}	3.16±0.60^{a}	14.00±0.29^{b}	3.00±0.24^{b}	65.54±0.89^{b}
B	12.93±0.59^{d}	3.34±0.33^{c}	3.00±0.29^{a}	19.20±1.80^{d}	2.50±0.60^{a}	60.98±2.19^{a}
C	6.13±0.35^{b}	2.25±0.14^{b}	4.50±0.87^{b}	18.16±0.44^{c}	3.34±0.29^{b}	68.94±0.66^{c}
D	9.73±1.05^{c}	4.40±0.15^{d}	3.16±0.44^{a}	18.66±0.88^{c}	2.90±0.20^{a}	64.03±1.96^{b}
E	4.20±0.23^{a}	1.50±0.15^{a}	5.00±0.58^{c}	13.16±0.44^{a}	4.30±0.23^{c}	76.65±0.15^{d}

Values are in ± mean S.E. (S.E = Standard error of Mean)
Values between experimental treatments Within column bearing the same superscript are not significantly different at the 5% level (P<0.05).

4.1.2 Qualitative characterization of fungi isolated from selected locally produced fish feeds in Minna

The cultural characteristics and morphological features following staining and microscopy characterize the fungal species present in five selected locally produced fish feeds having varying genera with *Aspergillus spp.* appearing the most. Other species of fungi confirmed to be present are *Mucor spp.*, *Trycophyton spp.*, *Microsporum spp.*, *Trichoderma spp.*, and *Candida spp*. as shown in Table 4.2.

Table 4.2: Typical Colonial and Morphological Features of fungi isolates from locally produced fish feeds

Morphological characteristics	Microscopic features	Suspected organism	Feed sample with fungi isolate
Black colony and powdery, initially appears whitish and as it changes it turns to Black	Smooth conidiophores, no septate on hyphae, dark brown conidia contain in Conidiophores which are produced from vesicle surface.	*Aspergillus niger*	**A, B, D** and **E**
Gray-green colony and Fluffy	Long conidiophores, scattered green conidia (spore). Hyphea has no septate	*Aspergillus fumigatus*	**A, D** and **E**
Whitish wooly with numerous black dots on top	Contain Micro-conidia build up in Conidiophores	*Mucor pusillus*	**A** and **B**
Dark Brown	Numerous single celled micro-conidia which are formed laterally on smooth walled and are Spherical in Shape.	*Trichophyton megninii*	**B, C** and **D**
Velvety colony with a radial grooves with a raised center	Contains septate hyphae, macrocondida which are irregular and distorted	*Microsporum distortum*	**A**
White mycelia with concentric rings center	Forms white and transparent mycelia with little or no candida	*Trichoderma koningii*	**A** and **B**
Dull, dry semi-white with slightly cream colour mycelia border	Contains an oval blastospores sparsely along the hypae in small cluster	*Candida tropicalis*	**A**

4.1.3 Characterization of the synthesized Zeolite from Groundnut shell and Rice husk

Figure 4.1 and 4.2 represent the patterns of X ray diffraction pattern of zeolite synthesized from groundnut shell and rice husk respectively. According to the X ray diffraction of the synthesized zeolite, the spectra micro structure for groundnut shell have peaks and intense set located between 10-20° and 31-40° of 2θ which confirm combination of amorphous and crystalline material (fig. 4.1). While, rice husk peak and intense set was observed to be located between 2θ = 14-50° (fig. 4.2), typical confirming a crystalline material.

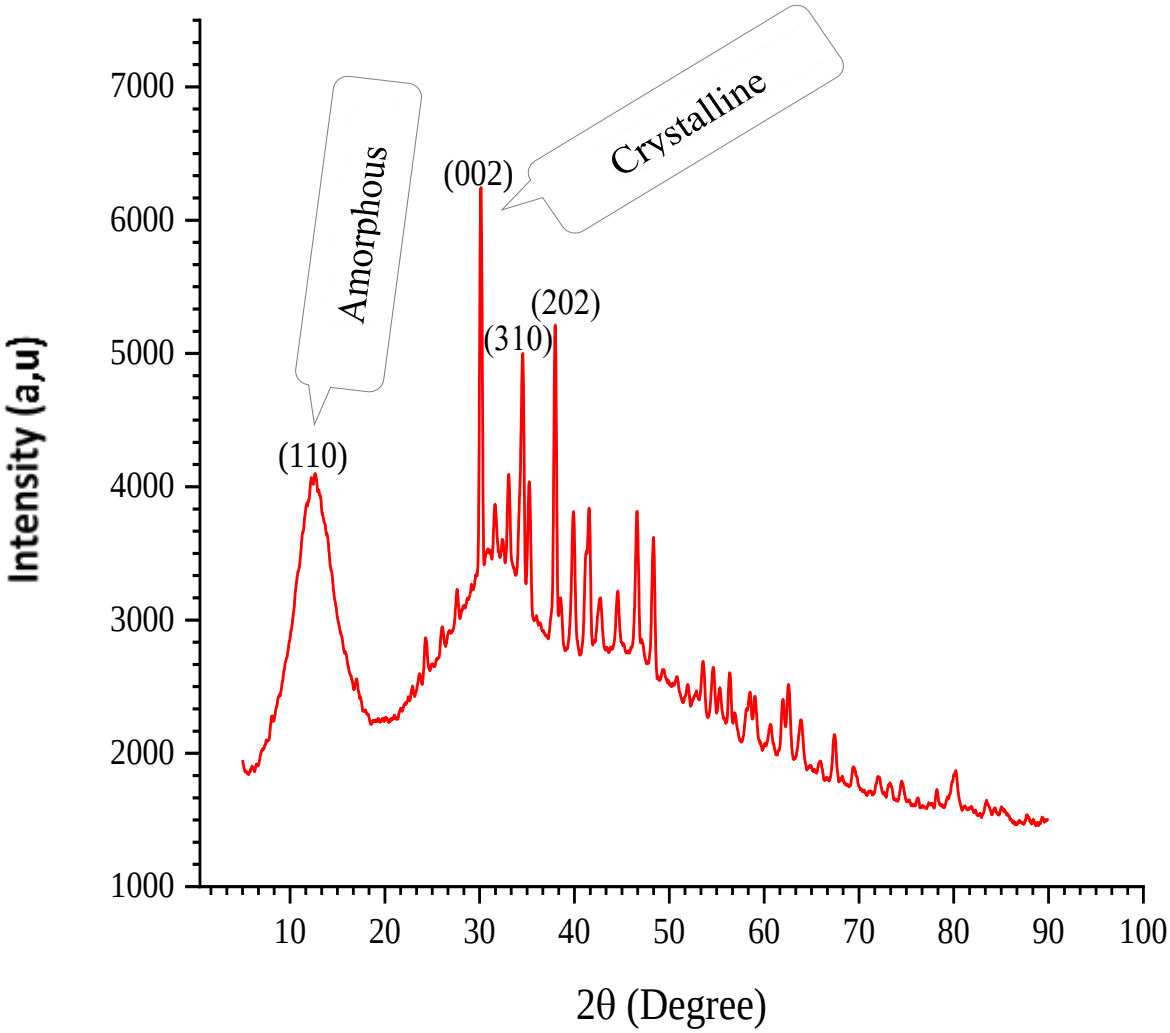


Figure 4.1: **X-ray Diffraction Patterns of Zeolite Synthesized from Groundnut Shell.**

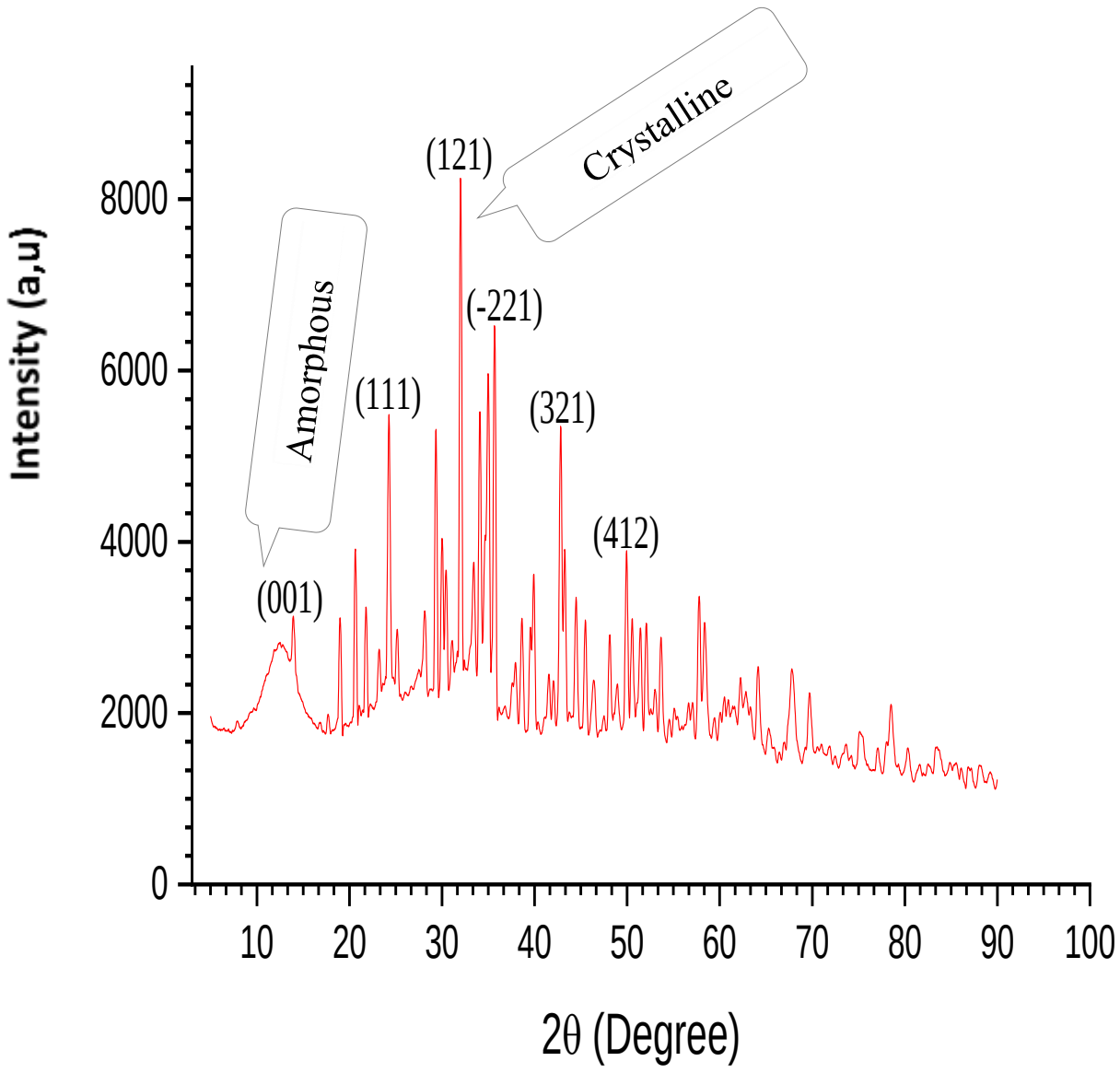
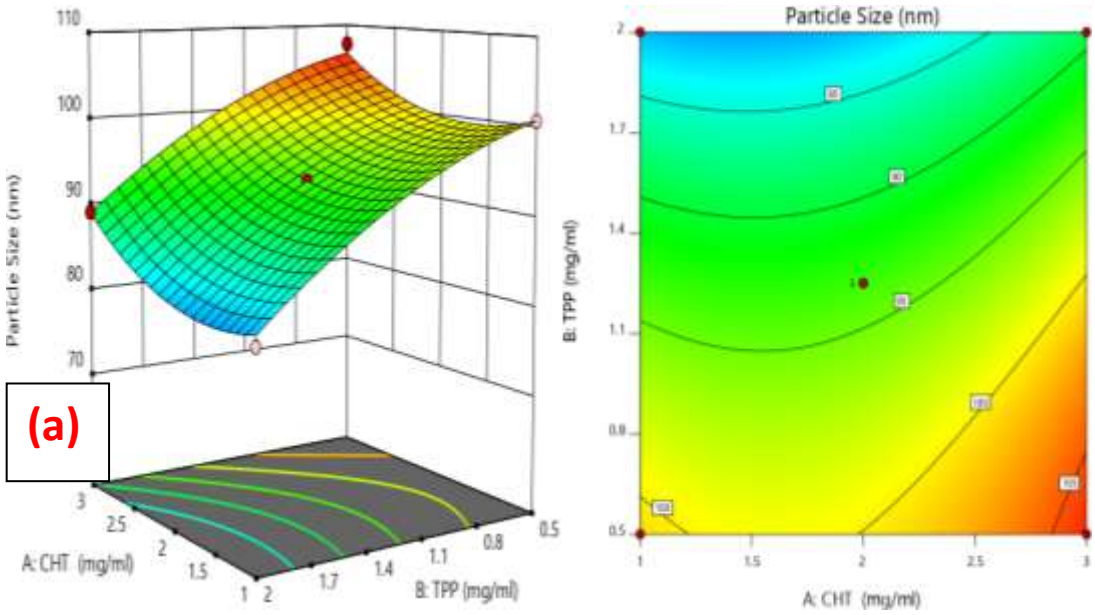


Figure 4.2: X-ray Diffraction Patterns of Zeolite Synthesized from Rice Husk.

4.1.4 Optimization of Chitosan Nanoparticles

4.1.4.1 *The central composite design (CCD) model using the 3d plot and response surface methodology (RSM) design model*

Figure 4.3 shows the relationship and effect of the process variables of chitosan concentration (CHT), tripolyphosphate concentration (TPP) and reaction time (t) on the response variables of particle size distribution using the 3D plot. Figure 4.3(a) illustrate the relationship between the CHT, TPP and particle size. It is shown that the particle size is significantly ($P \leq 0.05$) increased from ≤85 to 105 nm when the CHT increased from 1 to 3 (mg/ml). By increasing reaction time (t) from 30 to 90 Min, the size of the Ch NPs increases (Figure 4.3(b). Figure 4.3(c) demonstrate that the decrease in TPP, especially below 0.8 (mg/ml), results with the particle size increase up to 104nm.



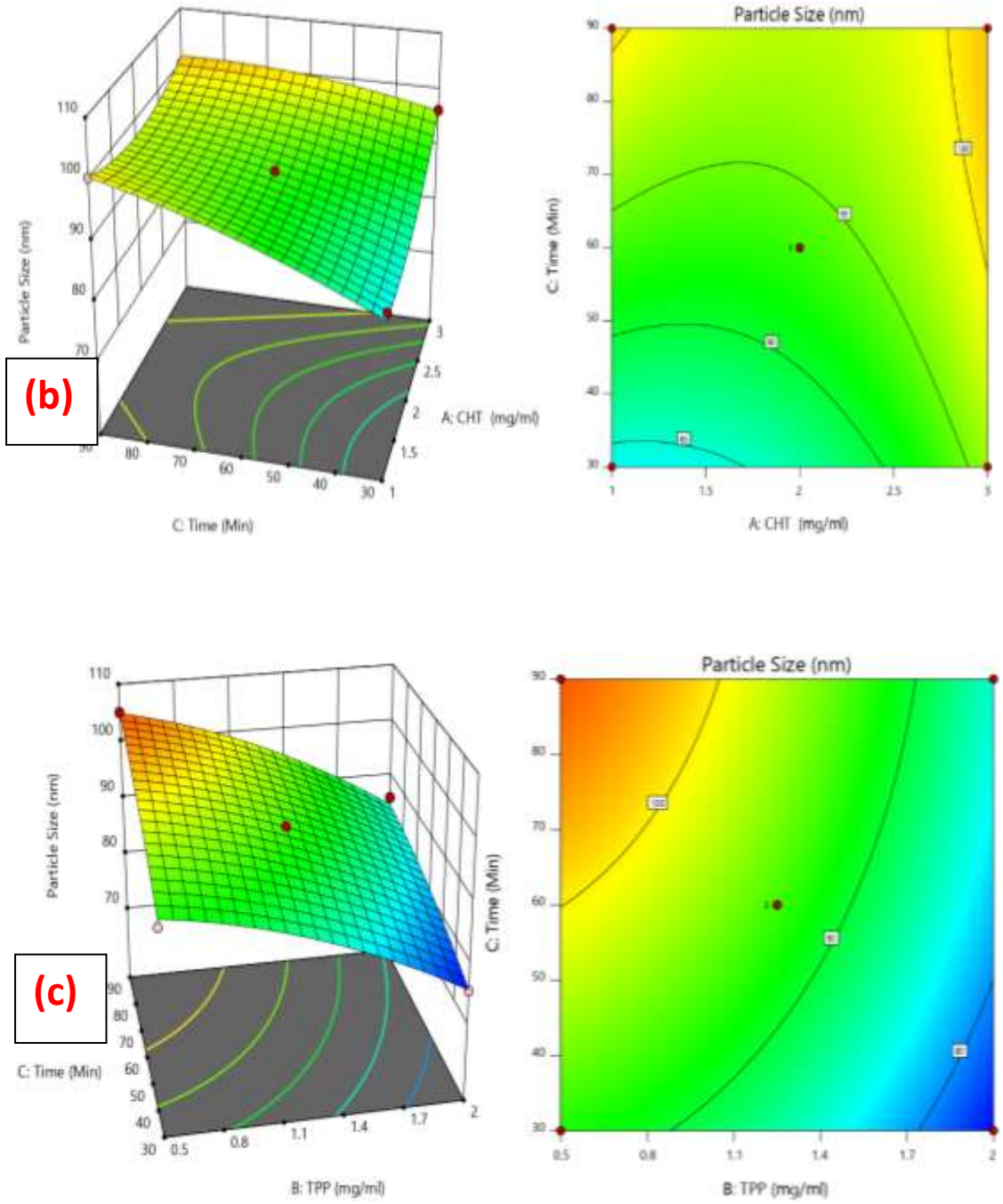


Figure 4.3: 3D Response surface (*left*) and contour plots (*right*) (a−c) for the mean particle size of Chitosan nanoparticles (Ch NPs).

4.1.4.2 *The ramp function*

The ramp function (Figure 4.4) shows the prediction ability for the particle size of chitosan nanoparticles. The ideal parameters (conditions) for the synthesis of chitosan nanoparticles of particle size (75.49nm) were chitosan concentration of 1.31 mg/ml, tripolyphosphate concentration 1.93 mg/ml and a reaction time of 35 h, as showed in the arrangement of ramps (Figure 4.4).

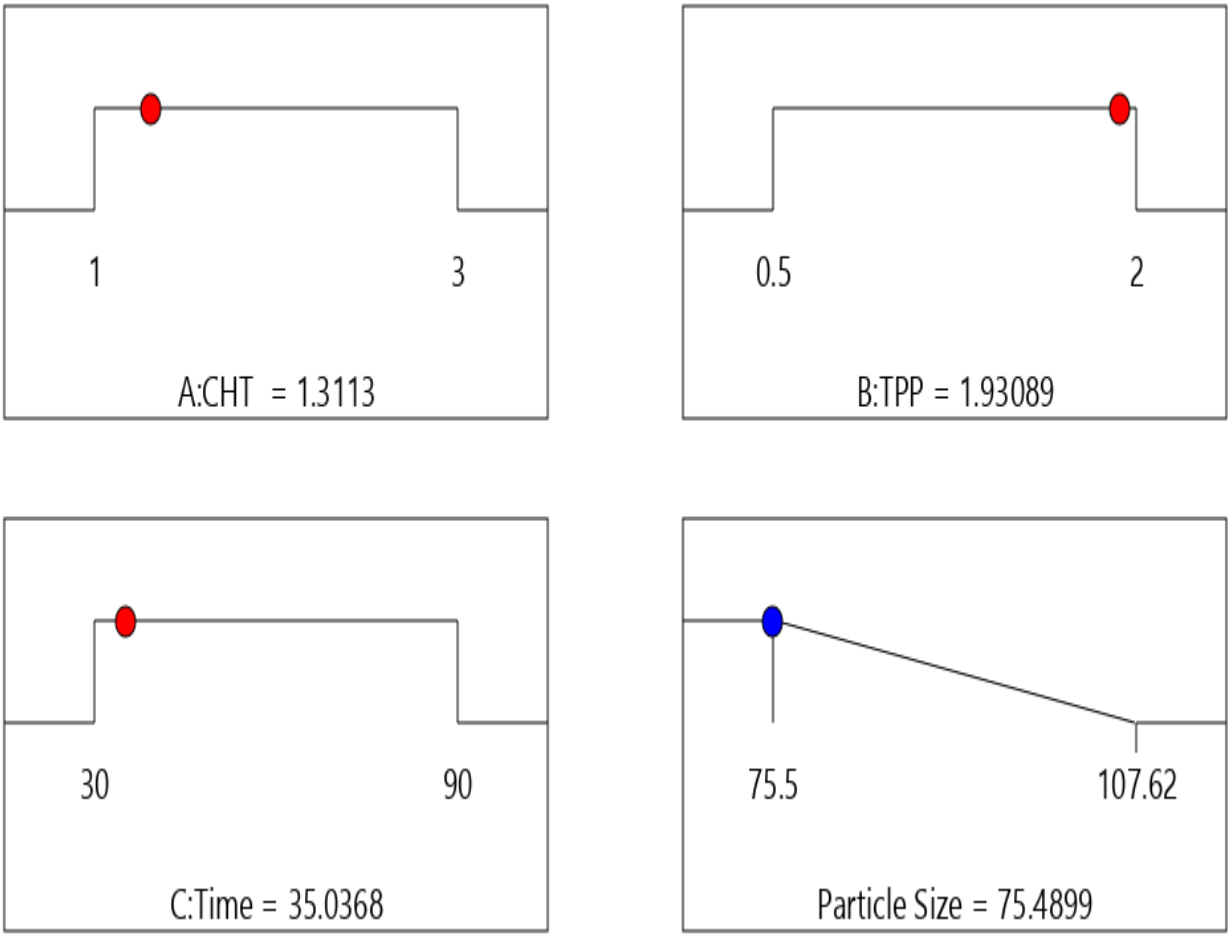


Figure 4.4: Ramp function for the predictive particle size of chitosan

4.1.4.3 *The optimization of the model using the analysis of variance (ANOVA)*

The results for the particle size distribution model are given in Table 4.3. The model establishes a relationship between Chitosan Concentration (CHT), Sodium Tripolyphosphate Concentration (TPP), Time (t) and particle size. The p-value of the model is <0.0001, proving that the model is significant while the R^2 calculate the error in fit, confirmed by coefficient R^2 (Table 4.4).

Table 4.3: Quadratic model for Particle Size of the synthesized chitosan nanoparticles

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	1127.31	9	125.26	64.69	0.0001
A-CHT	89.18	1	89.18	46.05	0.0011
B-TPP	675.28	1	675.28	348.73	< 0.0001
C-Time	240.57	1	240.57	124.24	0.0001
Residual	9.68	5	1.94		
Lack of Fit	9.68	3	3.23		0.2651
Pure Error	0.0000	2	0.0000		
Cor Total	**1136.99**	**14**			

Table 4.4: Fit statistics of the quadratic model.

Std. Dev.	1.39	**R^2**	0.9915
Mean	93.19	**Adjusted R^2**	0.9762
C.V. %	1.49	**Predicted R^2**	0.8638
		Adeq Precision	27.0973

4.1.5 Characterization of chitosan nanoparticles (CNPs)

4.1.5.1 *Ultraviolet-visible spectra*

The bio-reduction of chitosan by aqueous sodium tripolyphosphate assessed by UV-Vis spectrophotometer was presented in Figure 4.5. The UV-Vis spectra of the different concentrations of chitosan showed range absorption peaks of Ch NPs 1 at 217.50-340.50 nm, Ch NPs 2 at 223.50-260.00 nm, Ch NPs 3 at 216.00-340.50 nm, Ch NPs 4 at 219.00-338.50 nm and Ch NPs 5 at 219.50-340.50 nm (Figure 4.5).

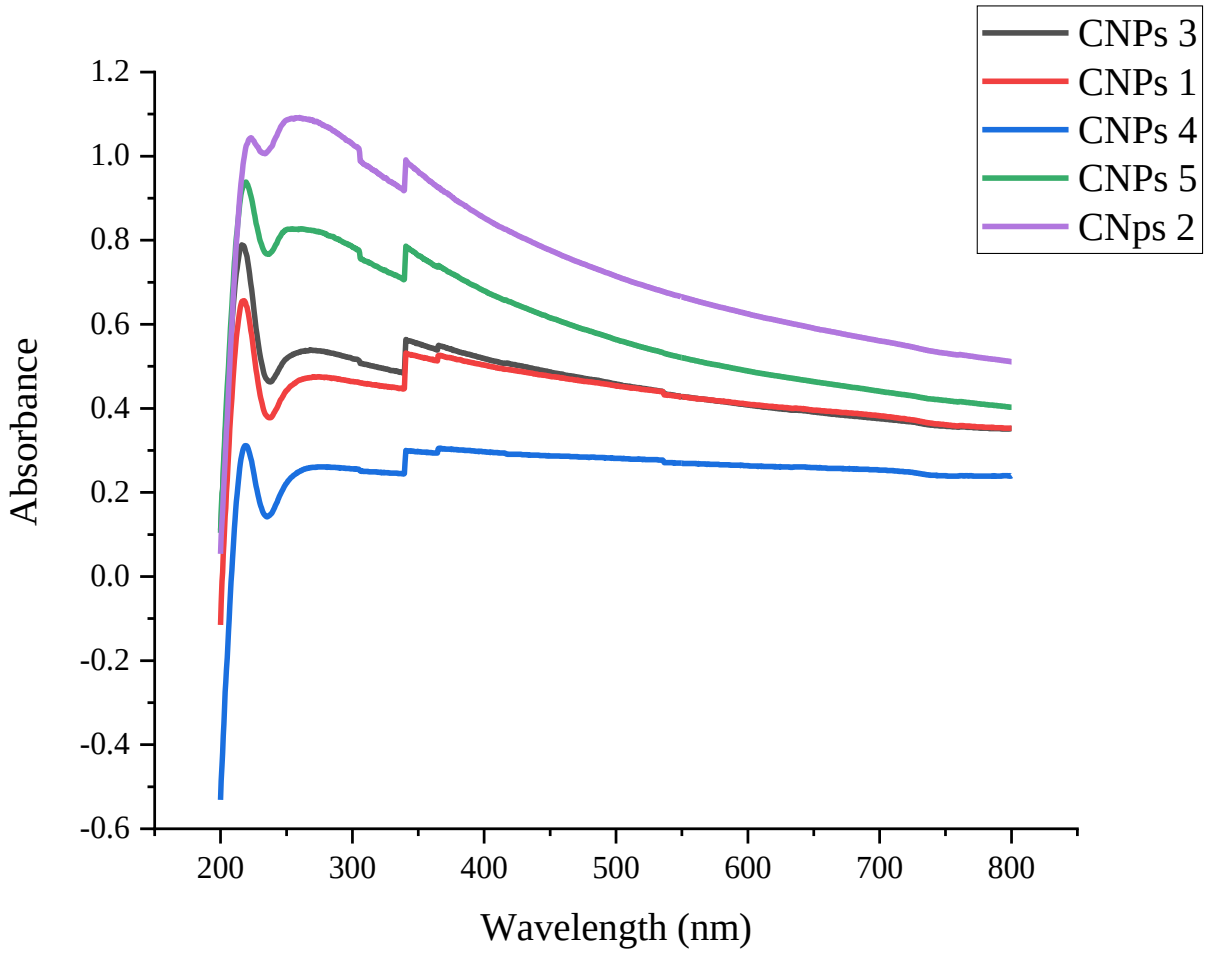


Figure 4.5: **Ultraviolet-Visible Spectra of Chitosan Nanoparticles**.

Key:
Ch NPs 1 - CHT 2mg/ml, TPP 1.25mg/ml, t 60 min; **Ch NPs 2** - CHT 1mg/ml, TPP 0.5mg/ml, t 60 min; **Ch NPs 3** - CHT 1mg/ml, TPP 1.25mg/ml, t 90 min; **Ch NPs 4** - CHT 2mg/ml, TPP 2mg/ml, t 30 min; **Ch NPs 5** - CHT 2mg/ml, TPP 0.5mg/ml, t 90 min.

4.1.5.2 Particle size of synthesized chitosan nanoparticles

The particle size of synthesized chitosan nanoparticles for all the conditions were Ch NPs 1 at 96.16nm, Ch NPs 2 at 93.43nm, Ch NPs 3 at 96.62nm, Ch NPs 4 at 104.5nm and Ch NPs 5 at 103.7nm (Fig 4.6) which are not significantly different from each other.

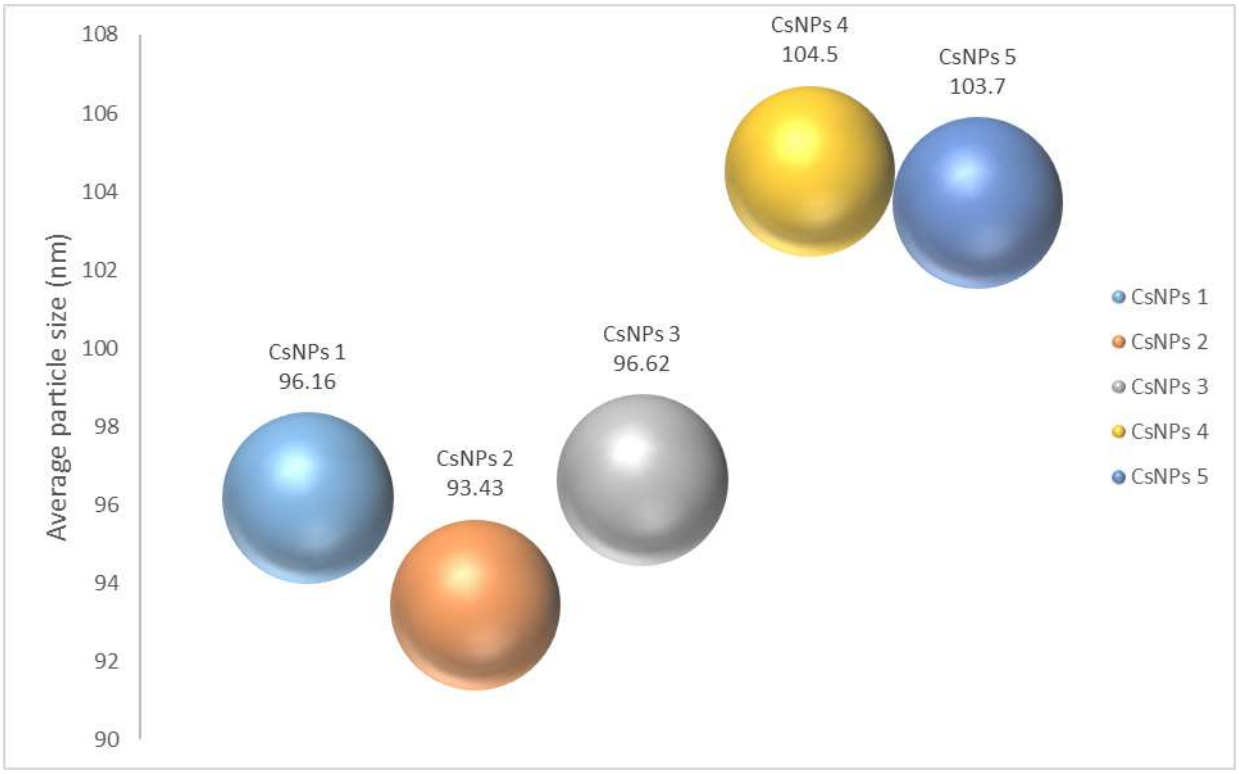


Figure 4.6: Average particle size distribution of synthesized chitosan nanoparticles with varied condition.

4.1.6 The *in vitro* antifungal activity of the formulated Nanoadditives in the selected locally produced fish feed samples in Minna

The mean fungal counts of the fish feeds (A-E) with nanoadditive inclusion are presented in Table 4.5. All nanoadditives inclusion feed causes a significant reduction in the mean total fungal count of the treated fish feed samples as compared to the control fish-feed sample without nanoadditives (A-E). Nanoadditive S2 shows a 100% antifungal activity with (0.00±0.00) recorded fungal load count in all the fish-feed samples, followed by nanoadditive

S1 with 75% antifungal activity, recording (1.03±0.02) fungal load count in fish-feed sample B as compared with the control. Whereas, nanoadditive S4 shows the least antifungal activity (25%) with (34.33±0.21) recorded as the highest fungal load count in fish feed sample A as compared with other nanoadditive. The mean total fungal count of the treated fish feed samples was statistically significantly different at ($P<0.05$).

Table 4.5: Fungal Load Of Selected Locally Produced Fish Feeds with GNS and RHS Nanoadditive Inclusion

Ratio of Nano-additives	A	B	C	D	E
	Mean Fungi Count (MTFC) $\times 10^5$ (mfc/g)				
Control	172.31±0.64^c	85.23±0.59^c	10.11±0.35^b	13.12±1.05^c	2.16±0.23^a
S1	0.00±0.00	1.03±0.02^a	0.00±0.00	0.00±0.00	0.00±0.00
S2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
S3	26.04±0.11^a	2.12±0.14^a	0.00±0.00	2.01±0.32^a	0.00±0.00
S4	34.33±0.21^b	13.21±0.47^b	3.04±0.01^a	4.31±0.02^b	0.00±0.00

Values are in ± mean SEM (SEM = Standard Error of Mean)
Values between experimental treatments Within column bearing the same superscript are not significantly different at the 5% level ($P<0.05$).

Key: S1= (100% Chitosan nanoparticles)

S2 = (Chitosan nanoparticles 50:50 Zeolite-GNS/RHS)

S3 = (100% zeolite-GNS)

S4 = (100% zeolite- RHA)

4.2 Discussion

4.2.1 Proximate composition of selected locally produced fish feeds

Fish are cold-blooded aquatic creatures and an important source of proteins for humans. It has been reported that properly formulated and balanced diet fish feed fulfill all nutritional requirement for optimal growth gain (Keremah and Esquire, 2014).

However, protein is the major growth promoting factor in feed and formed by linkages of individual amino acids. Although, more than 200 amino acids occur in nature, but of these, 10 are essential (indispensable) amino acids that cannot be synthesized by fish and must be supplied by fish diet (Steven *et al.,* 2021). It has been reported that the dietary protein and amino acid requirements of fish are influenced by several factors, including fish age and size. Protein requirements are generally higher for early life stage of fish. As fish grow larger, their protein requirements usually decrease (Steven *et al.,* 2021). However, this study confirmed the nutrient composition of the selected locally produced feeds protein contents of Feed A and D to be higher than other feed samples (Table 4.1). Thereby, suggesting fish feed A and D with higher protein content will improve/increase growth performance in the fingerling fishes sample as reported by Steven *et al.* (2021).

Lipids (fats) are high-energy nutrients that can be utilized to partially spare substitute for protein in aquaculture feeds. Lipids have about twice the energy density of proteins and carbohydrates (Yildirim and Beck, 2017). Lipids make up about 7-15 percent of fish diets, supply essential fatty acids, and serve as transporters for fat soluble vitamins (Steven *et al.,* 2021). The mean range of crude lipid recorded in this study was 3.0-5.0% (Table 4.1) which is within the range of work done by Okomoda *et al*. (2019). The fatty acid (FA) requirements of fish from recent studies indicate that catfish may require higher lipid levels for optimum

spawning performance. Also, Sink and Lochmann (2018) found that supplementation of catfish broodstock diets with 10 percent fish oil increased spawning success, fecundity, total egg volume, egg weight, total egg lipid concentration, hatching success and fry survival.

Carbohydrates (starches and sugars) are the least expensive sources of energy for fish diets. In fish, carbohydrates are stored as glycogen that can be mobilized to satisfy energy demands. Carbohydrates are included in aquaculture diets for the purpose of their binding activity and floating of feeds (Steven *et al.*, 2021). The carbohydrate content of the studied fish feeds ranged from 60.98±2.19 to 76.65±0.15% and this was within the acceptable range recommended for commercial fish (National Research Council - NRC 2017). The ash content of the fish feed has been reported to be a useful content to extrapolate the minerals concentrations provided by the feeds (Raghavendra, 2022). The ash content of the feeds studied range from 13.16±0.44 to 19.20±1.80 (Table 4.1) and was within the acceptable range recommended for fish feeds (NRC 2011). In fish, the minerals regulate osmotic balance and aid in bone formation as well as components in enzyme and hormone systems. However, dietary requirements for minerals in fish cannot be well elucidated because fish can also absorb many minerals directly from the water through their gills and skin, allowing them to compensate to some extent for mineral deficiencies in their diet (Steven *et al.*, 2021).

Moisture content of the fish feeds determined the environmental condition for storage. The NRC (2011) reported that feed stored in an area with high moisture and/or high temperatures will cause oxidation of the lipid and degradation of vitamins. However, this study confirmed the moisture content in feed A and B to be higher than C, D and E (Table 4.1) suggesting the vulnerability of feed A & B to fungi contamination and possibly mycotoxin production and therefore lowers feed quality and may pose a threat to fish and humans (Saad, 2016). Fibre refers to plant materials such as cellulose, hemicellulose, lignin, pentosums and other

complex carbohydrates. These are indigestible and do not play an important role in nutrition. Fibre adds bulk to feed but increases the amount of feca material produced. The goal in commercial aquaculture is to limit the fibre content in feeds (Krontveit *et al.,* 2014). A certain amount of fibre permits better binding and moderates the passage of feed through the alimentary canal. However, it is not desirable to have a fibre content exceeding 8% in diets for fish. When the fibre content is excessive, it results to lower digestibility of nutrients. The analysed crude fibre content of all the feeds were within the safe dietary limit for fish which is within the range of work done by Isah *et al*. (2021).

4.2.2 Fungal profile of selected locally produced fish feed samples in Minna

Fungi are ubiquitous in the environment, which have been reported to occur in food and feed worldwide with some of them capable of producing a wide array of mycotoxins (Bryden, 2017). The high fungal counts depict the level at which feed ingredients used in feed formulation is contaminated during storage and handling (Ubiebi, 2017). The total fungal counts of the selected fish feeds studied ranged from $2.16 \times 10^5 – 172.31 \times 10^5$ cfu/g which exceeds the accepted National Agency for Food and Drug Administration and Control (NAFDAC) standard for finished feed (1×10^5 cfu/g) (NAFDAC, 2000). Thereby, revealing the colonization by fungal species, as presented in Table 4.2.

Mycotoxin are secondary metabolic products from fungi belonging to the Aspergillus, Penicillium and Fusarium genera. However, one of the fungal identified *Aspergillus flavus* and *Aspergillus fumigatus* are major aflatoxin B1 producers that have been reported for causes of decreased growth rate and aflatoxicosis in fish with carcinogenesis in human thereby suggesting the health hazard of the studied fish feeds (El-Sayed and Khalil, 2019). Previous studies have reported that samples contaminated with *Trichophyton* and

Microsporum fungi had a detectable level of fumonisin (Victoria *et al*., 2020). Fumonisins are known to cause health effects especially in the liver and kidney, although effects of fumonisins in humans remain limited (Joint Food and Agriculture Organization-JECFA, 2018). *Mucor pusillis* have also been linked to cause lungs infection as a result of necrosis of infected tissues and pen neural invasion in the lungs ascribed to contamination in feeds (Al-Khafaji *et al.,* 2017). *Aspergillus* spp. observed in the fish-feed samples is one of the major fungi genera that produces aflatoxins B1.

4.2.3 Characterisation of the synthesized zeolite from groundnut shell and rice husk

Agricultural residues such as rice husk and groundnut shell are less costly, easily available and possess large percentage of Aluminium Silicate (SiO_2/Al_2O_3). Using waste materials for the purpose of zeolite synthesis helps to mitigate environmental pollution. Aluminium Silicate present in rice husk and groundnut shell are useful as an alternative source of zeolite synthesis (Gautam *et al*., 2021). Presently, synthetic zeolites are used commercially more often than natural zeolites due to the microporous and purity of crystalline products, biocompatibility, nontoxicity, large surface areas, high pore volumes and the uniformity of particle sizes (Zhu *et al*., 2019). Zeolite is of great industrial importance due to its molecular sieving, ion exchange and adsorption properties. The unique topology of the crystalline structure and subnano micropore channels endow zeolites with a molecular sieving ability and provide an intra-crystal diffusion path and reaction space for the substrates, while their framework heteroatoms determine the catalytic functions (Xu *et al*., 2020).

The phase composition of the synthesized zeolite analyzed using X-ray diffractometry (XRD) in this study suggest that the synthesized zeolite products contain both diffraction (crystalline) and non-diffraction (armorphous) peaks as seen in Fig. 4.1 and 4.2 by the

presence of a broad diffraction ‘hump’ in the region between 18 to 32 degrees 2θ confirming amorphous phase.

The synthesized products matched the characteristic peaks of zeolite at 2θ values as reported by Treacy and Higgins (2021). Also, Amir *et al*. (2019) reported the presence of inert binders, such as amorphous silica, which are not porous, usually leads to a slower uptake adsorbent as compared to the pure crystalline adsorbent. This has been observed for zeolites, that the presence of amorphous silica notably decreases the diffusivity and absorbability (Amir *et al*., 2019). Crystalline zeolites of a tetrahedra framework has been reported to compose different open structures or cavities ranging from 0.8-1 nm in diameter which are the order of molecular dimension (Uga *et al*., 2020).

The experimental results of the Rice Husk XRD data, (Fig. 4.1 and 4.2 show that great quantities of crystalline zeolite formed with chemical composition of inorganic cations such as Na, K, Li, and SiO_2/Al_2O_3 ratio that might be responsible for difference in the crystallinity of the synthesis mixture (Nicholas, 2019). More also, the rice husk has a higher SiO_2/Al_2O_3 ratio than the groundnut shell which in the synthesis gel places a constraint on the framework composition of the zeolite produced. Thereby, making rice husk producing better zeolite crystals and a promise substrate for zeolite synthesis.

4.2.4 System based optimization studies

The optimization of cultivation conditions is an important problem in the development of economically feasible bioprocesses. Central Composite Design (CCD) based Response Surface Methodology (RSM) are used to analyze the effects of the process parameters and this is applied in this study based on the following parameters such as chitosan concentration

(CHT), tripolyphosphate concentration (TPP), and reaction time (t) on the particle size distribution chitosan nanoparticles.

A significant interactive outcome of CHT and TPP on the synthesis of chitosan nanoparticles shows that the particle size is significantly ($P \leq 0.05$) increased when the CHT increased from 1 to 3 (mg/ml) (Fig. 4.3a) and the interaction of CHT and reaction time (t) was exposed as shown in Fig. 4.3b. An increase in the synthesized chitosan nanoparticle was also observed by an increase in reaction time. However, the effect of TPP with time (Fig. 4.3c) showed that there was a significant increase in the synthesized chitosan nanoparticle size as the TPP concentration decreases, especially below 0.8 mg/ml (Peng *et al*., 2020).

The ramp function details (Figure 4.4) were used to ensure the prediction ability for the particle size of chitosan nanoparticles. Similar adequacy tests were also suggested in the reported literature by Danish *et al*. (2020). However, the final optimum values of the parameter predicted by RSM to obtain a synthesized chitosan nanoparticle size of 75.5nm were chitosan concentration of 1.31 mg/ml, tripolyphosphate concentration 1.93 mg/ml and a reaction time of 35 h, as showed in the arrangement of ramps (Figure 4.4). The outcomes of these studies exhibit that multi-objective optimization via RSM are crucial for the design and operation of particle size equipment. Invariably, the ANOVA results (Table 4.3) show that the confirmed model is appropriate. This result demonstrated that the response should be considered significant as the p-value corresponding to the F-value is less than 0.05 (model significant). The regression coefficients and determination coefficient (R^2) for the fitted statistics of the established design (Table 4.4) confirmed the value of intended R^2 (0.9915) which implies that 98.85% variation in the optimization design is due to the independent variables in the synthesis of chitosan nanoparticles. The Predicted R^2 of 0.8638 is in reasonable agreement with the Adjusted R^2 of 0.9762; at the difference less than 0.2 and the

adequate precision measures signal to noise ratio with ratio greater than 4 desirable. The ratio of 27.097 indicates an adequate signal that can be used to navigate the design space.

4.2.5 Characterization of chitosan nanoparticles

Due to its several unique properties, such as biodegradability, biocompatibility, and low toxicity, chitosan nanoparticles has been extensively investigated for applications in many fields (Peng *et al*., 2020).

The synthesized chitosan nanoparticles in this study was confirmed by the UV–visible spectrophotometer with absorption peak within the range of 216 nm to 389 nm (Figure 4.5) that is in line with the characteristic surface absorption of chitosan nanoparticles reported by Schlachet *et al*. (2020). Similarly, Yasin *et al*. (2021) also reported absorption spectra of chitosan nanoparticles found to be in range from 250 to 400 nm. These absorption peaks are the points at which each material absorbs a specific range of light and shows the behavior accordingly (Peng *et al*., 2020).

The particle size of chitosan nanoparticles measured using ZETA nanosizer showed the average particle size range of 96.16nm to 104.5nm for all conditions. The size distribution profile of chitosan nanoparticles, as shown in Figure 4.6, represents a typical batch of nanoparticles having a narrow size distribution with the volume average hydrodynamic diameter. This result is in agreement with the report of Ganna *et al*. (2020) who described the characteristics of *Curcumin* loaded chitosan nanoparticles with particle size ranging from 1 to 100 nm.

4.2.6 The *in vitro* antifungal potentials and adsorption capacity of the formulated nanoadditives

Due to ignorance and profit maximization by farmers, contaminated grains are sold and use for animal feed production. However, protection against aflatoxins has been demonstrated with the use of a number of compounds that either increase animal's detoxification processes or prevent the production of fungi in feed (Wang *et al*., 2019). The control of fungi growth in selected fish feeds studied using nanoadditive adsorption process shows that every formulated nanoadditive decreased fungi growth. However, the absolute inhibition of the growth of fungi contaminant was recorded in nanoaddtive S2 = (Chitosan nanoparticle 50:50 Zeolite-GNS/RHA) as shown in Table 5. The experimental treatment of fish feeds with nanoadditive correlates with the results as recorded by Greene (2018). Results from this research show inhibitory capabilities of nanoadditive S2 in biological control of food pathogenic fungi contamination in locally produced fish feed.

There are three possible mechanisms of the antifungal activities of the nanoadditive. The first mechanism involves the interactions between the NH_3^+ sites of chitosan nanoparticles with the negatively charged phospholipids of the fungi cell membrane. This will increase the permeability of membrane and cause the leakage of cellular contents, which susbsequently leads to cell death (Garc´ıa-Rinc´on *et al.,* 2010). For the second mechanism, chitosan acts as chelating agent by binding to trace elements, causing the essential nutrients to be unavailable for normal growth of fungi (Roller and Covill, 2009). Thirdly, chitosan could penetrate cell wall of fungi and bind to its DNA. This will inhibit the synthesis of mRNA and affect the production of essential proteins and enzymes (Kong *et al.,* 2010). The alliance between a microporous (zeolite) and biocompatible (chitosan) material with the

accompaniment of negative and positive charges exhibit strong adsorption properties of fungi growth (Ahlafi *et al.,* 2013).

Nanoadditives are widely used as adsorbents because of its easy function, low level of energy consumption, simple preservation, high capacity of adsorption efficiency (Jain *et al*., 2015). Recently, due to availability, cost effectiveness and lack of toxicity, biopolymers have been used as adsorbent (Chuanqiang *et al*., 2016). The *in vitro* adsorption capacity of the formulated nanoaddtives can be deduced from quantitatively comparing the fungi profile results of the fish feed samples with the control (Table 4.5). The result shows that the *in vitro* fungi adsorption capacity of the formulated nanoadditives was S2> S1> S3> S4 respectively. This results shows that the synthesized chitosan nanoparticle doped with zeolite-RHS/GNS has a better adsorption capacity as compared to the pure chitosan nanoparticles as nanoadditives. Similarly, Yong *et al*. (2018) also discovered that the chemical modification/doping of chitosan with zeolite showed high adsorption capability of fungi.

CHAPTER FIVE

5.0 CONCLUSION, RECOMMENDATIONS AND CONTRIBUTION OF RESEARCH TO KNOWLEDGE

5.1 Conclusion

This study demonstrated that the locally produced fish feeds had a significant amount of nutrient composition with an indication of more room for improvement and regulation in the development of local formulation of the African catfish feed. Mycotoxigenic fungi of genera *Aspergillus, Trichophyton* and *Microsporum* were confirmed and isolated from locally produced fish feed samples with varying frequencies suggesting the degree of mycotoxigenic fungi in fish production and threat to consumers. Diffraction pattern of the zeolite material shows positions and intensities of peaks of both amorphous and crystalline components in Rice Husk. While, a CCD/RSM 3D plot revealed that increase in CHT, reaction time (t), and decrease in TPP increases the particle size of the synthesized chitosan nanoparticles. The optima particle size computed to be 75.5nm at corresponding values for the 1.13mg/ml chitosan concentration, 1.93mg/ml tripolyphosphate concentration and 35h reaction. Thereby, suggesting an effective model and optimize multi-objective parameters for chitosan nanoparticle. While the adsorption treatment of fish feeds with the nanoadditives especially S2 show inhibiting and degradability of fungi in all the feeds.

5.2 Recommendations

To reduce the exposure of fishes to fungi and mycotoxin, it is suggested that the appropriate precautions should be undertaken to protect fish feed from contamination and future studies can be carried out on the toxicity studies of the effect of these nanoadditves through *in vivo* studies.

5.3 Contribution of Research to Knowledge

This research work attempts to address multiple gaps and in doing so makes important contributions to scientific knowledge.

Firstly, the study extends the limited research on locally made fish feeds, their nutritional composition, exposure to fungi and mycotoxins and the impact on animal and human health.

The percentage composition of selected fish feeds (A-E) showed range of 60.98±2.19 to 76.65±0.15, 1.50±0.15 to 4.40±0.15, 4.20±0.23 to 13.33±0.64, 2.50±0.60 to 4.30±0.23, 13.16±0.44 to 19.20±1.80, 3.00±0.29 to 5.00±0.58 for carbohydrate, crude protein, moisture content, crude fibre, ash content and crude lipid respectively. This finding can contribute to the development of policies and guidelines for ensuring nutritional development and abolishing malnutrition of fishes due to poor economic conditions in Nigeria and underscores the need for more research and investment in this area.

The high fungal counts depict the level at which feed ingredients used in feed formulation is contaminated during storage and handling. The total fungal counts of the selected fish feeds studied ranged from 2.16×10^5 – 172.31×10^5 cfu/g which exceeds the accepted National Agency for Food and Drug Administration and Control (NAFDAC) standard for finished feed (1×10^5 cfu/g). Thereby, revealing the colonization by fungal species, *Aspergillus Niger*, *Aspergillus fumigatus*, *Mucor pusillus*, *Trichophyton megninii*, *Microsporum distortum*, *Trichoderma koningii*, *Candida tropicalis* were confirmed to be present.

Secondly, the study validates the need for the development of a novel and low-cost material for the removal of fungi in feeds.

Thirdly, the study assesses the mitigation processes which have been employed for decreasing fungi contamination and mycotoxin toxicity in animals through the use of toxin binders as feed additives. The inclusion of nanoadditives in the selected fish feeds causes a significant reduction in the mean fungal count of the treated fish feed samples as compared to the control fish feed samples without nanoadditives. Nanoadditive S2 (Chitosan nanoparticles 50:50 Zeolite-GNS/RHS) shows a 100% antifungal activity with (0.00±0.00) recorded fungal load count in all the fish feed samples.

Hence, zeolite with chitosan nanoparticles at optima concentration suggests an effective model for the adsorption of fungi in fish feeds.

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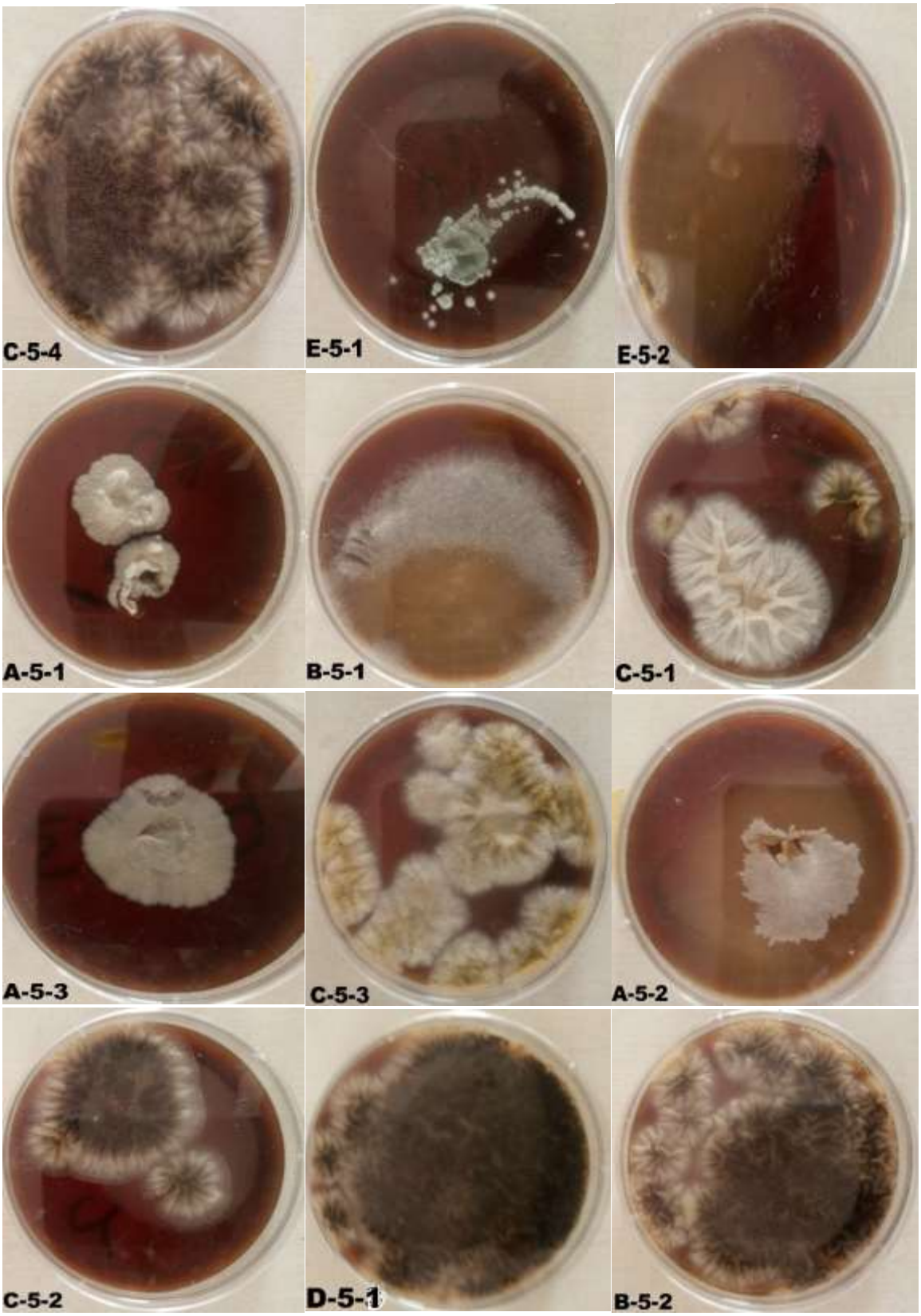
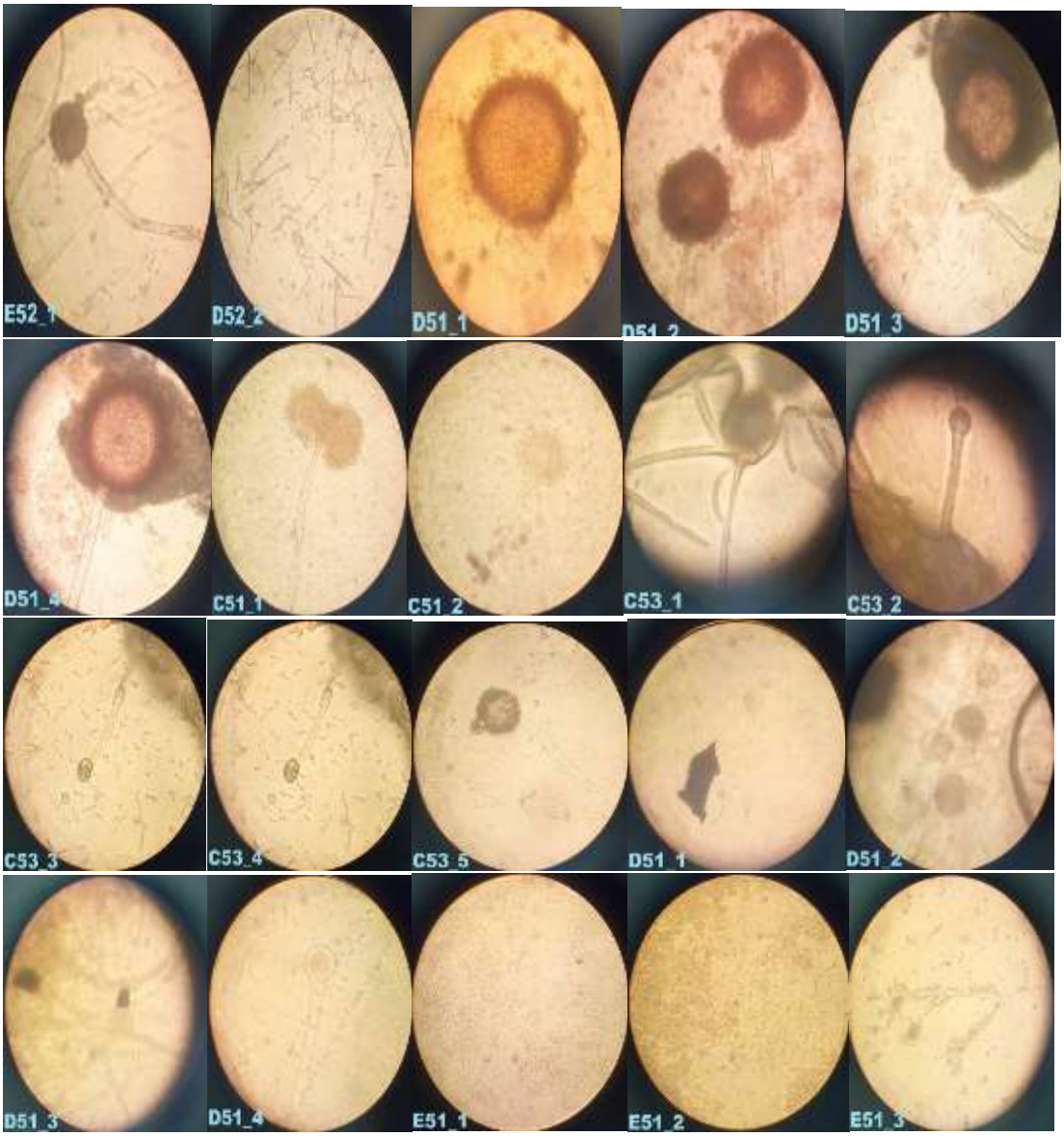
APPENDICES

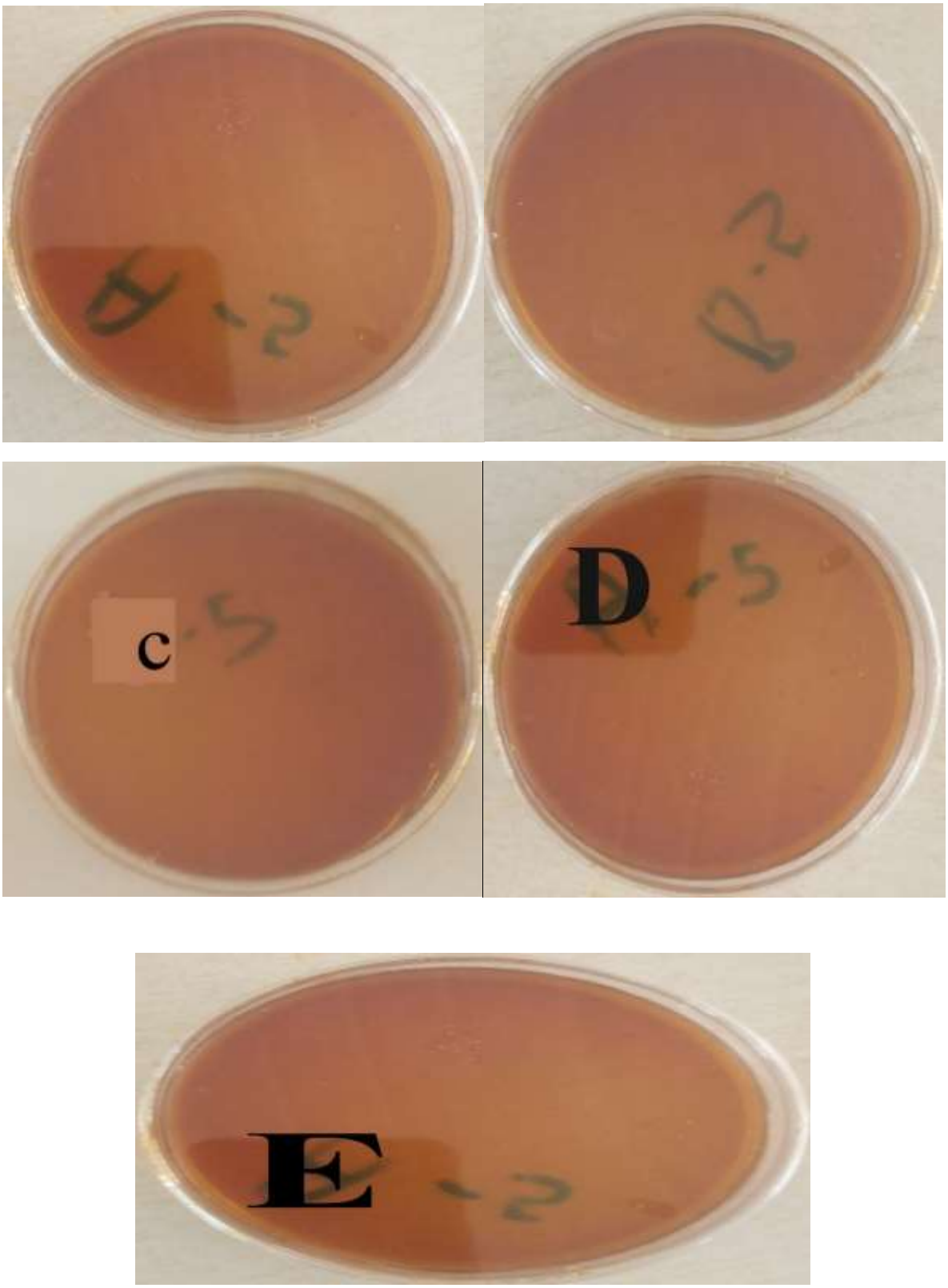
Appendix A: **Colonies of Different Species of *Aspergillus spp. Mucor spp., Trycophyton spp., Microsporum spp., Trichoderma spp.,* and *Candida spp*.**



Appendix B: Pure isolates of the various types of fungi.



Appendix C: Microscopic examination of the various types of fungi.



Appendix D: Plates with Nanoadditive S2 showed complete inhibition of fungi in the fish feeds.