Sudan University of Science and Technology
Faculty of Graduate Studies

Preparation and Characterization of Chitosan/Silver Nano-composite and its Application on Nile Water as Antibacterial Materials

تحضير وتشخيص الشيتوزان/الفضه النانويه المركبه وتطبيقها كمضاد للبكتريا على مياه النيل

A Thesis Submitted to Sudan University of Science and Technology college of
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Master of Science in Chemical Engineering

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سورة النجم الايه(39-40)

Dedication

I dedicate this work,

To whom my life worth nothing without them; my father and mother.

To the great human being who stood up to her wonderful endowment, without her efforts this research wouldn't become possible; MissWala Elsayed

To myself.

Acknowledgment

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List of abbreviations

ChNP	Chitosan nanoparticles
AgNP	Silver nanoparticles
DD	Degree of Deacetylation
DM	Demineralization
DP	Deproteinization
DC	Decolorization
DA	Deacetylation
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
rpm	rotation per minute
HOAc	acetic acid

Abstract:

The prepared chitosan nanocomoposite in this study may demonstrate the potential in optimizing the minimum amount required to achieve complete inactivation of various bacteria in Nile water. The objective of this research is to evaluate the in vitro antibacterial activity of chitosan nanoparticles and silver-loaded nanoparticles against E. coli, S. choleraesuis, S. typhimurium, and S. aureus. Firstly, Chitin was isolated from Jinga shrimp shell waste by sequential chemical treatments and then chitosan was prepared by deacetylation and chemical hydrolysis, respectively. The physicochemical properties of both chitin and/or chitosan were determined (Viscosity, Ash, Protein, Degree of Deacetylation and Molecular Weight). On the other hand, Chitosan nanoparticles were prepared based on the ionic gelation of the prepared chitosan with tripolyphosphate anions. While silver nanoparticles were synthesized using the biosynthesis method in which the solution of aqueous solenostemma argel was used to reduce silver ions. Finally, the prepared silver nanoparticles were dispersed onto the chitosan nanoparticles to prepare chitosan / silver-loaded nanoparticles. The resulted chitosan nanocomposite was characterized by FTIR and SEM. The antibacterial activity of chitosan nanoparticles and silver-loaded nanoparticles were evaluated against the bacteria. Results show an improvement in the inhibition of the growth of various bacteria tested when silver nanoparticles were introduced. Consequently, chitosan silver-loaded nanoparticles could be recommended as an efficient antibacterial material for water disinfection.

المستخلص:

قد يُظهِر الشيتوزان المركب النانوي المعد في هذه الدراسه القدره على تحقيق الحد الأدنى من الكميه المطلوبه لتحقيق التعطيل الكامل لمختلف البكتيريا في مياه النيل. الهدف من هذا البحث هو تقييم النشاط المضاد للبكتيريا في المختبر من الجسيمات النانويه الشيتوزان والجسيمات النانويه المحمله بالفضه ضد E. coli و S. choleraesuis و S. typhimurium و S. aureus.

أولاً ، تم عزل الكيتين من نفايات قشور الروبيان الشحاميه عن طريق المعالجات الكيميائيه المتسلسله ثم تم تحضير الشيتوزان بواسطة نزع الأملاح والتحلل الكيميائي على التوالي. تم تحديد الخواص الفيزيائيه والكيميائيه لكل من الكيتين و/ أو الشيتوزان (اللزوجه، الرماد، البروتين والوزن الجزيئي).

من ناحيه أخرى ، تم تحضير جسيمات الشيتوزان النانويه بناءً على الجيل الأيوني للشيتوزان المحضر مع أنيونات ثلاثية الفوسفات. بينما تم تصنيع الجسيمات النانويه الفضيه باستخدام طريقة التخليق الحيوي التي أستخدم فيها محلول نبات الحرجل المائي لتقليل أيونات الفضه. وأخيراً ، تم توزيع الجسيمات النانويه الفضيه المعده على الجسيمات النانويه الشيتوزان لتحضيرالجسيمات النانويه المحمله بالفضه والشيتوزان. تم تشخيص الماده النانويه المركبه الناتجه عن الشيتوزان بـFTIR وSEM .

تم تقييم النشاط المضاد للبكتريا من الجسيمات النانويه الشيتوزان والجسيمات النانويه محمله بالفضه ضد البكتريا. أظهرت النتائج تحسنا في تثبيط نمو البكتيريا المختلفه التي تم أختبارها عند ادخال الجسيمات النانويه الفضيه. وبالتالي ،يمكن التوصيه بالجسيمات النانويه المحمله بالفضه من الشيتوزان باعتبارها ماده فعاله مضاده للجراثيم لتطهير المياه.

Chapter One

Introduction

1.1 Definition of Chitosan:

Chitosan is a natural modified carbohydrate biopolymer, nontoxic, a copolymer of glucosamine and N-acetylglucosamine. It is a white, hard, inelastic and nitrogenous polysaccharide. Prepared from chitin by a chemical process involving demineralization, deproteinization, decolorization, and deacetylation, which in turn, is a major component of the shells of crustaceans, such as crab, shrimp, and crawfish. Chitosan and its derivatives have attracted considerable interest and multifaceted applications due to their antimicrobial, biodegradability and antifungal activity. It is used in biomedical industries, agriculture, genetic engineering, food industry, environmental pollution control, water treatment, paper manufacture, photography and so on.

With regards to their chemical structure (Figure 1.1), chitin and chitosan have similar chemical structure(Ocloo et al., 2011).

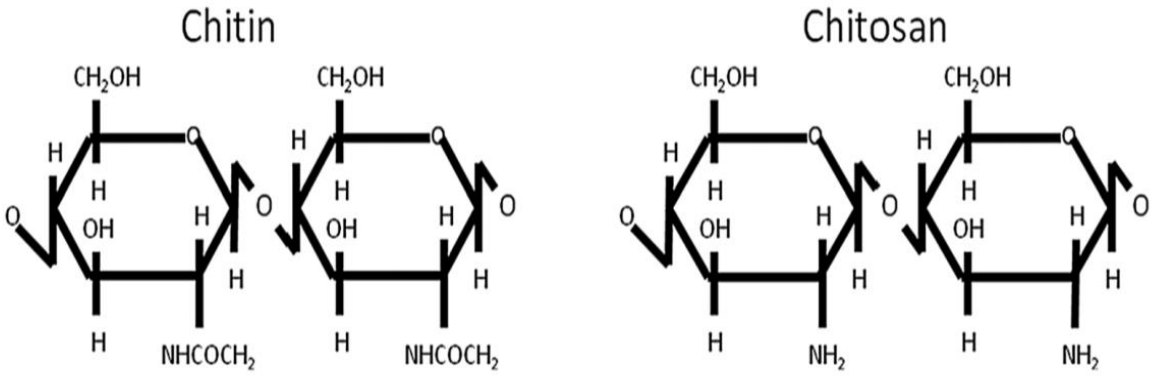


Figure (1.1) Structure of Chitin and Chitosan(Divya and Jisha, 2018).

1.2 Nanotechnology:

Nanoscience and nanotechnology are the study and application of extremely small things. It is a multidisciplinary science that across all the other science fields, such as chemistry, biology, physics, materials science, and engineering. Nanoparticles range in dimension from 1 to 100 nm. They have unique properties compared to their bulk equivalents due to the decrease in dimension to the atomic level. This is because bulk materials have relatively constant properties regardless of their size, but as the size decreases, the percentage of surface atoms compared to bulk material increases. This causes unexpected properties of nanoparticles. Nanoparticles are synthesized by size reduction using either top-down methods such as milling, high-pressure homogenization and sonication, example: Polymeric nanoparticles can be synthesized from natural and synthetic polymers. Or bottom-up processes like reactive precipitation and solvent displacement.

Polymeric nanoparticles are used owing to their stability and ease of surface modification. Biopolymeric nanoparticles have added advantages, like availability from marine (chitin and chitosan) or agricultural (cellulose, starch, pectin) resources, biodegradability, biocompatibility, and nontoxicity.

Chitosan, which considered a biopolymer, the biodegradable polymers is studied mainly as delivery systems for controlled release of active ingredients, stabilization of biological molecules like proteins, peptides or genetic material.

Nanomaterials are excellent adsorbents, catalysts, and sensors due to their large specific surface area and high reactivity. More recently, several natural and engineered nanomaterials have also been shown to have strong antimicrobial properties, including chitosan nanoparticles (ChNP), silver nanoparticles (AgNP) , photocatalytic TiO_2, fullerol, aqueous fullerene nanoparticles , and carbon nanotubes(Divya and Jisha, 2018).

1.2.1 Chitosan nanoparticles:

Chitosan nanoparticles (ChNP) are natural materials with excellent physicochemical, antimicrobial and biological properties, which make them a superior environmentally friendly material and they possess bioactivity that does not harm humans; Due to these unique properties. Chitosan nanoparticles have the characteristics of chitosan and the properties of nanoparticles such as surface and interface effect, small size and quantum size effects. Owing to the enormous potential of chitosan nanoparticles(Divya and Jisha, 2018).

1.2.2 Silver nanoparticles:

Silver nanoparticles (AgNP) have proved to be most effective because of its good antimicrobial efficacy against bacteria, viruses and other eukaryotic micro-organisms. They are undoubtedly the most widely used nanomaterials among all, there by being used as antimicrobial agents, in textile industries, for water treatment, sunscreen lotions etc. Studies have already reported the successful biosynthesis of silver nanoparticles by plants(Hasan, 2015).

1.3 Water treatment:

Water is the most essential substance for all life on earth a precious resource for human civilization. Clean water (i.e., water that is free of toxic chemicals and pathogens) is essential to human health. Clean water is also a critical feedstock in a variety of key industries including electronics, Pharmaceuticals and food. The world is facing formidable challenges in meeting rising demands of clean water as the available supplies of freshwater are decreasing due to (i) extended droughts, (ii) Population growth, (iii) more stringent health based regulations(Savage and Diallo, 2005).

The use of sand filtration and chlorine disinfection marked the end of waterborne epidemics in the developed world more than a century ago. Worldwide, waterborne diseases remain the leading cause of death in many

developing nations. The consequences are daunting: diarrhea kills about 2.2 million people every year, mostly children under the age of 5. The importance of water disinfection and microbial control cannot be overstated.

The rapid growth in nanotechnology has spurred significant interest in the environmental applications of nanomaterials. In particular, its potential to revolutionize century-old conventional water treatment processes has been enunciated recently.

ChNP-coated 4-micron membranes were tested for their drinking water purification ability in a flow through membrane filtration systems. The ChNP-coated membranes held good bacterial growth compared to noncoated membranes.
Also, the filtered water showed the maximum removal of coliforms using multiple tube fermentation(Li et al., 2008).

1.4 Characterization Methods:

1.4.1 Scanning Electron Microscope (SEM):

SEM is widely used to investigate the microstructure and chemistry of a range of materials. The main components of the SEM include a source of electrons, electromagnetic lenses to focus electrons, electron detectors, sample chambers, computers, and displays.

Electrons, produced at the top of the column, are accelerated downwards where they passed through a combination of lenses and apertures to produce a fine beam of electrons. The electron beam hits the surface of the sample mounted on a movable stage under vacuum. The sample surface is scanned by moving the electron beam coils. This beam scanning enables information about a defined area of the sample. The interaction of the electron beam with the sample generates a number of signals, which can then be detected by appropriate detectors.

The scanning electron microscope is able to observe objects with 3-D image.

Images have high magnification and high resolution, but they appear in black white. To make the detailed images, a specimen is coated gold particles. Electrons bounce off of the gold plating to produce the image. This type of microscope is useful in viewing very tiny objects in fine detail and images are saved to be viewed later. Common objects that are viewed are detailed images of bacteria, viruses and some cellular components(Ouellet et al., 2008).

1.4.2 Fourier-transform infrared spectroscopy (FTIR):

FTIR is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high-spectral-resolution data over a wide spectral range. This confers a significant advantage over a dispersive spectrometer, which measures intensity over a narrow range of wavelengths at a time(Ouellet et al., 2008).

1.5 Aim of study:

The aim of this study is:

1. Characterization of Chitosan/Silver Nano-composite using FTIR and SEM.
2. Use Chitosan/Silver Nano-composite as antibacterial on Nile water.

1.6 Objectives:

There are many objectives for this study:

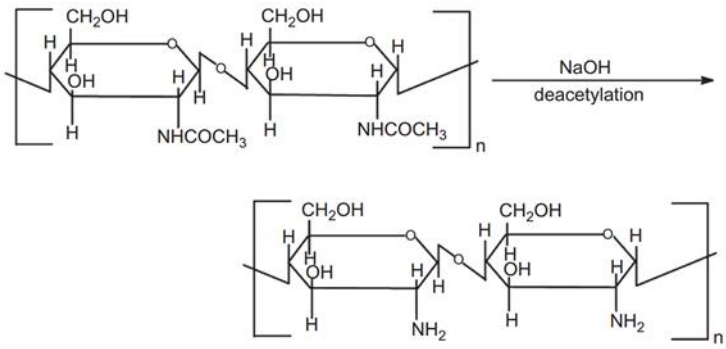
1. To produce chitosan from (Jinga) shrimp shell waste by a chemical processes.
2. To produce chitosan nanoparticles by convert chitosan yield.
3. To produce silver nanoparticles using silver nitrate.
4. To produce chitosan/silver Nano-composite by mixture of chitosan nanoparticles and silver nanoparticles.
5. To test to antibacterial activity by chitosan /silver Nano-composite on Nile water.

Chapter Two

Literature Review

2.1 Definition of Chitin and Chitosan:

Chitin is the second most abundant organic compound in nature after cellulose. Chitin is widely distributed in marine invertebrates (Figure 1), insects, fungi, and yeast. However, chitin is not present in higher plants and higher animals. Consists of 30-40% protein, 30-50% calcium carbonate and calcium phosphate, and 20-30% chitin. Chitin is widely available from a variety of source among which, the principal source is shellfish waste such as shrimps, crabs, and crawfish. It also exists naturally in a few species of fungi (No and Meyers, 1995).



Figure(2.1): N-deacetylation of chitin to produce chitosan(Kumar, 2000).

2.2 Characteristics and Composition of Chitosan:

Chitosan is a nontoxic, biodegradable polymer of high molecular weight, and is very much similar to cellulose, a plant fiber (Figure 2.2).

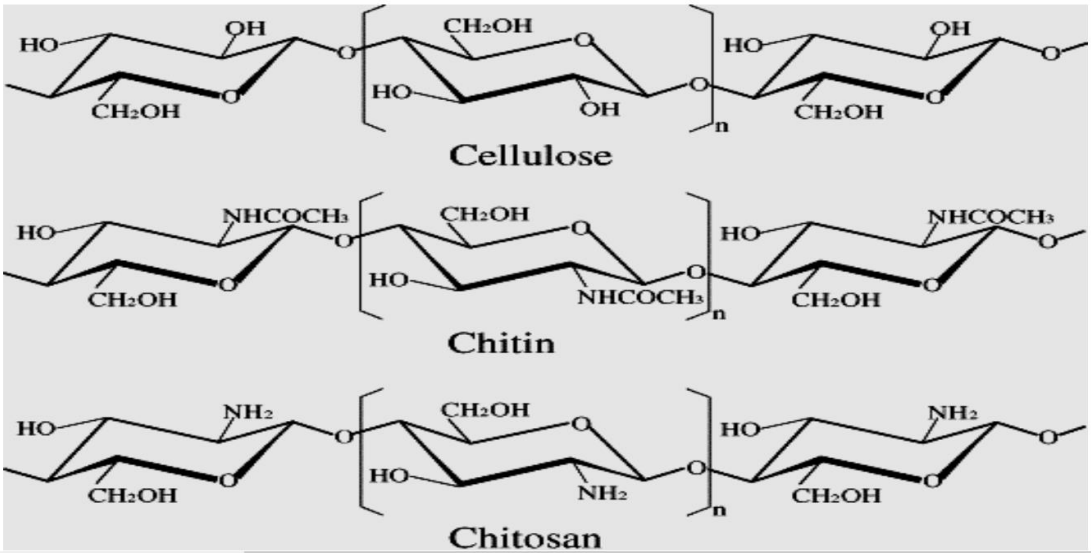


Figure (2.2) Structure of Cellulose, Chitin, and Chitosan(Fernandez-Kim, 2004).

As seen in Figure (2.2), the only difference between chitosan and cellulose is the amine (-NH_2) group in the position C-2 of chitosan instead of the hydroxyl (-OH) group found in cellulose. However, unlike plant fiber, chitosan possesses positive ionic charges, which give it the ability to chemically bind with negatively charged fats, lipids, cholesterol, metal ions, proteins, and macromolecules. In this respect, chitin and chitosan have attained increasing commercial interest as suitable resource materials due to their excellent properties including biocompatibility, biodegradability, adsorption, and ability to form films, and to chelate metal ions(Fernandez-Kim, 2004).

2.2.1 Degree of Deacetylation (DD):

The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a compound (chitosan) with a high degree chemical reactive amino group ($-NH_2$). This makes the degree of deacetylation (DD) an important property in chitosan production as it affects the physicochemical properties, hence determines its appropriate applications. Deacetylation also affects the biodegradability and immunological activity(Fernandez-Kim, 2004).

2.2.2 Viscosity:

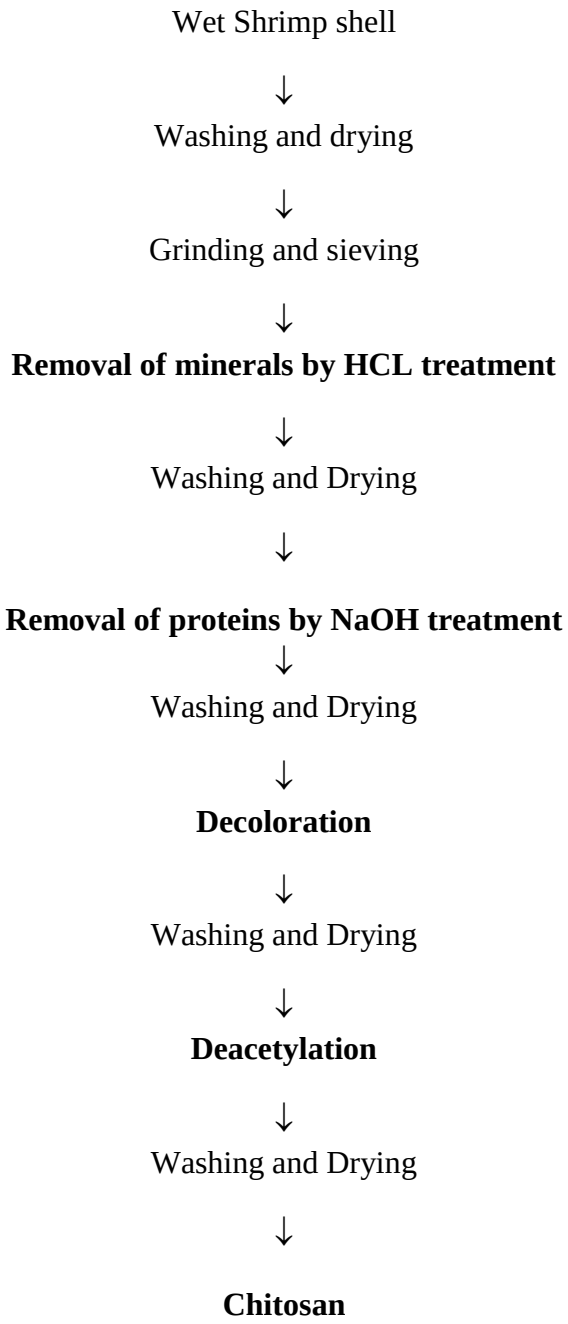
Viscosity is an important factor in the conventional determination of molecular weight of chitosan and in determining its commercial applications in complex biological environments such as in the food system. Higher molecular weight chitosan often render highly viscous solutions, which may not be desirable for industrial handling.

Some factors during processing such as the degree of deacetylation, molecular weight, concentration of solution, ionic strength, pH, and temperature affect the production of chitosan and its properties. For instance, chitosan viscosity decreases with an increased time of demineralization. Viscosity of chitosan in acetic acid tends to increase with decreasing pH but decrease with decreasing pH in HCl, giving rise to the definition of 'Intrinsic Viscosity' of chitosan which is a function of the degree of ionization as well as ion strength(Fernandez-Kim, 2004).

2.2.3 Molecular Weight:

Chitosan is a biopolymer of high molecular weight. Like its composition, the molecular weight of chitosan varies with the raw material sources and the method of preparation. Molecular weight of native chitin is usually larger than one million Daltons while commercial chitosan products have the molecular weight, depending on the process and grades of the product. In general, high temperature, dissolved oxygen, and shear stress can cause degradation of chitosan, molecular weight of chitosan can be determined by methods such as chromatography and viscometer(Fernandez-Kim, 2004).

2.3 Production of Chitosan:



Figure(2.3): Traditional Shrimp shell Chitosan Production Flow Scheme(Skaugrud and Onsoyen, 1990).

Isolation of chitosan from shrimp shell wastes involves four traditional steps (Figure 2.3): demineralization (DM), deproteinization (DP), decolorization (DC), and deacetylation (DA).

However, the isolation of chitin specifically consists of only two steps: demineralization (DM) and deproteinization (DP), which involves the dissolution of calcium carbonate with HCl and the removal of proteins with NaOH, respectively(Fernandez-Kim, 2004).

2.4 Applications of Chitosan:

The poor solubility of chitin is the major limiting factor in its utilization. Chitosan is considered as a potential polysaccharide because of its free amino groups that contribute polycationic, chelating, and dispersion forming properties along with ready solubility in dilute acetic acid. Chitosan possesses exceptional chemical and biological qualities that can be used in a wide variety of industrial and medical applications. Some of these are listed below Table (2.1)

Table (2.1) Applications of Chitosan:

Wastewater Treatment	Removal of metal ions, flocculant/coagulant, protein, dye, amino acids.
Food Industry	Removal of dye, suspended solids, preservative, color stabilization, food stabilizer, thickener and gelling agent, animal feed additive, etc.
Medical	Wound and bone healing, blood cholesterol control, skin burn, contact lens, surgical sutures, dental plaque inhibition, clotting agent, etc.
Agriculture	Seed coating, fertilizer, controlled agrochemical release.
Cosmetics	Moisturizer, face, hand, and body creams, bath lotion, etc.
Biotechnology	Enzyme immobilization, protein separation, cell recovery, chromatography.

2.4.1 Chitosan in the Wastewater Treatment:

The prime commercial applications for chitosan currently are in industrial wastewater treatment since chitosan carries a partial positive charge and binds to metal ions, thus makes the metal ions removal from waste streams

or contamination sites easier. In terms of utilization, crawfish chitosan as a coagulant for recovery of organic compounds in wastewater was demonstrated to be equivalent or superior to, the commercial chitosan from shrimp and crab waste shell and synthetic polyelectrolytes in turbidity reduction.

The wastewater released from food processing plants typically seafood, dairy or meat processing industries contain appreciable amounts of protein which can be recovered with the use of chitosan; this protein, after drying and sterilization, makes a great source of feed additives for farm animals.

The removal of dyes is difficult to achieve because of their high resistance to degradation by light, chemical, biological, and other exposures. However, chitin and chitosan have been found to have an extremely high affinity for dyes which may contribute to aquatic toxicity(Fernandez-Kim, 2004).

2.5 Chitosan nanoparticles production:

Chitosan has the ability to form a gel on contact with anions and form beads. This property enables its use in drug delivery. But still, the large size of these beads (1–2 mm) limits its application.

Chitosan nanoparticles are natural materials with excellent physicochemical, antimicrobial and biological properties, which make them a superior environmentally friendly material and they possess bioactivity that does not harm humans. Due to these unique properties, chitosan nanoparticles find a wide array of applications. Some of them are discussed below (Divya and Jisha, 2018).

They used ChNP prepared by emulsifying and crosslinking for intravenous delivery of anticancer drug 5-fluorouracil. Since then, many methods have been employed for the synthesis of ChNP. Five methods are presently available. They are ionotropic gelation, microemulsion, emulsification solvent diffusion, polyelectrolyte complex and reverse micellar method. Out of this, the most widely used methods are ionotropic gelation and polyelectrolyte complex. These methods are simple and do not apply high shear force or use organic solvents. The schematic representation of different methods of ChNP synthesis is depicted in Figure (2.4) (Divya and Jisha, 2018).

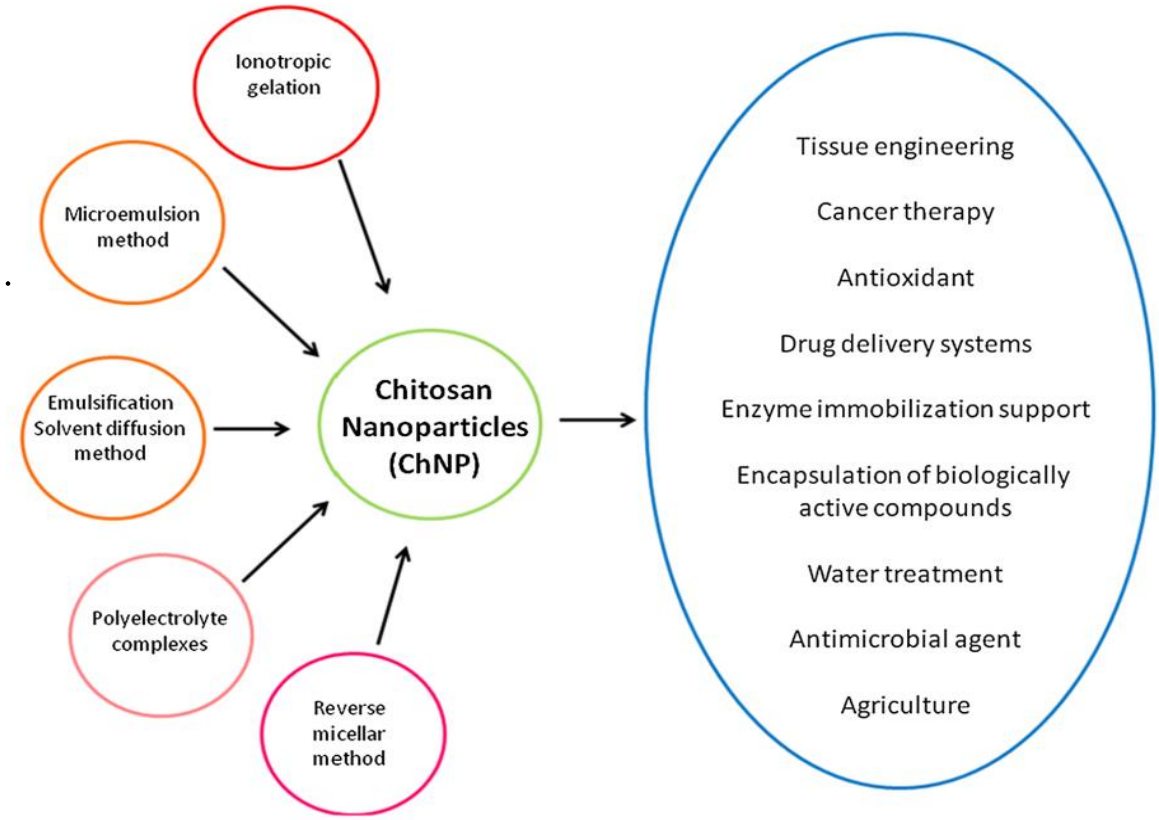


Figure (2.4): Schematic representation of different methods of ChNP synthesis and its various applications.

2.5.1. Methods of Chitosan nanoparticles synthesis:

2.5.1.1 Ionotropic gelation:

The method utilizes the electrostatic interaction between the amine group of chitosan and a negatively charged group of polyanion such as tripolyphosphate. Chitosan can be dissolved in acetic acid in the absence or presence of the stabilizing agents, such as poloxamer. Polyanion was then added, and nanoparticles were formed spontaneously under mechanical stirring at room temperature. The size and surface charge of particles can be modified by changing the ratio of chitosan to the stabilizer. A general increase in particle compactness and size was observed on increasing the chitosan concentration and on increasing the polymer to polyanion ratio. They also reported that nanoparticles dispersed in saline solution were more stable due to the smaller particle size found in the presence of sodium

chloride. This is because a monovalent salt like sodium chloride when added to the solvent screens out to the electrostatic repulsion between the positively charged amine groups on the chitosan backbone. This will increase the flexibility of the polymer chains in solution and thus increase its stability(Divya and Jisha, 2018).

2.6.1Applications of Chitosan nanoparticles:

2.6.1.1 Water treatment:

Water pollution has raised serious concerns lately mainly due to the inadequacy of conventional water treatment methods. Even though, activated carbon can be used for adsorbing impurities though effective is not cost or energy efficient.

The low-cost adsorbents like chitosan and cellulose are interesting options in this context. The functional groups of chitosan, hydroxyl and amino groups make it an excellent absorbent and enable to be used in water treatment for removal of functional matrices like pesticides and metal pollutants. Nanochitosan were tested effectively for adsorptive capacity of Pb(II), Cr (VI) , Cd(II) , arsenate, acid Green 27 (AG27) dye of anthraquinone type , etc. Chitosan nanofibers owing to their high porosity and higher surface area per unit mass are potential adsorbents. They were tested to remove Pb(II) and Cu (II) while retaining their inherent characteristics.

ChNP-coated 4-micron membranes were tested for their drinking water purification ability in a flow through membrane filtration systems. The ChNP-coated membranes held good bacterial growth compared to noncoated membranes.
Also, the filtered water showed the maximum removal of coliforms using multiple tube fermentation (MPN) test.

Magnetic chitosan possesses good dye adsorbing capacity and can also be easily recovered from the treated water using magnetic force thus exhibiting excellent reusability(Divya and Jisha, 2018).

2.7 Silver nanoparticles:

Silver has long been known to exhibit a strong toxicity to a wide range of micro-organisms; for this reason silver-based compounds have been used extensively in many bactericidal applications. Silver compounds have also been used in the medical field to treat burns and a variety of infections. Several salts of silver and their derivatives are commercially employed as antimicrobial agents. Commendable efforts have been made to explore this property using electron microscopy, which has revealed size dependent interaction of silver nanoparticles with bacteria. Nanoparticles of silver have thus been studied as a medium for antibiotic delivery, and to synthesize composites for use as disinfecting filters and coating materials. However, the bactericidal property of these nanoparticles depends on their stability in the growth medium, since this imparts greater retention time for bacterium–nanoparticle interaction. There lies a strong challenge in preparing nanoparticles of silver stable enough to significantly restrict bacterial growth(Wang et al., 2015).

Silver nanoparticles exhibit many unique electronic, catalytic, optical, and other physical and chemical properties that their bulk counterparts do not have. Silver nanoparticles have been applied in many fields including photonics, microelectronics, photocatalysis, lithography, and surface-enhanced Raman spectroscopy. Several types of chemical and physical technologies, such as chemical reduction, electrochemical reduction, photochemical reduction, and heat evaporation have been developed for the synthesis of silver nanoparticles. In recent years, bio- or green-synthesis of silver nanoparticles has become an emerging science. For example, using plant extracts for reducing silver nitrite to obtain nanoparticles with antimicrobial activities has emerged as a cost-effective and eco-friendly approach(Wang et al., 2015).

2.7.1Colloidal Silver:

Scientists have discovered that the body's most important fluids are colloidal in nature: suspended ultra-fine particles. Blood, for example, carries nutrition and oxygen to the body cells.

This led to studies with colloidal silver (electrical silver atoms). An electro-colloidal process, which is known to be the best method, is used for manufacturing the Colloidal silver. Colloidal silver appears to be a powerful, natural antibiotic and preventative against infections. Acting as a catalyst, it reportedly disables the enzyme that one-celled bacteria, viruses and fungi need for their oxygen metabolism. They suffocate without corresponding harm occurring to human enzymes or parts of the human body chemistry. The result is the destruction of disease-causing organisms in the body and in the food (Singh et al., 2008).

2.7.2 Synthesis of silver nanoparticles:

Nanometer sized silver particles were synthesized by inert gas condensation and condensation techniques. Both techniques are based on the evaporation of a metal into an inert atmosphere with the subsequent cooling for the nucleation and growth of the nanoparticles. The size and morphology of the nanoparticles were analyzed with a transmission electron microscope (TEM). The stability of nanoparticles was examined by exposing them to ambient conditions for one month. The antibacterial efficiency of the nanoparticles was investigated by introducing the particles into a media containing bacteria. The antibacterial investigations were performed in solution and on petri dishes. The silver nanoparticles were found to exhibit antibacterial effects at low concentrations. The antibacterial properties were related to the total surface area of the nanoparticles. Smaller particles with a larger surface to volume ratio provided a more efficient means for antibacterial activity. The nanoparticles were found to be completely cytotoxic to E. coli for surface concentrations as low as 8 µg of Ag/cm^2(Singh et al., 2008).

2.8 Chitosan Nano-composite in Water Treatment:

The applications of nanomaterials, including nano-rods, nano-pin, nano-sheet, nano-fiber, and nanoparticles opened up a whole new chapter in wastewater treatment researches. This material is known to work efficiently as they possessed greater Specific Surface Area (SSA) compared to their existence in bulk volume. SSA is the total surface area of a material per unit mass (m^2/kg or m^2/g), thus defined the property of the solids itself. The increased in SSA of the materials will increase the "working site" to remove any specific contaminants found in wastewater. Besides that, the alteration of nanomaterials' electronic properties due to the quantum size effect is another momentous aspect that should be highlighted. The reduction of particle size from macro to micro dimensions may not come into play in changing their electronic properties, but as the nanometer range is reached, this effect becomes prominent(Aizat and Aziz, 2019).

However, the application of nanoparticles solely may not be good enough. For example, TiO_2 is known to be a very good photocatalytic material and the degradation ability was proven by multiple studies. However, TiO_2 is only effective in the degradation of various organic pollutants, but not the nonbiodegradable metal ions, inorganic compounds, and heavy metal

constituents. It may also build up in concentrations in food chains to toxic levels as they have infinite lifetimes. On the other hand, the common usage of the photocatalytic particle suspension method while carrying out studies regarding the photocatalysts possessed few major drawbacks, such as the final recovery step. This step is necessary as the release of nanoparticles in the environment (commonly through streams and underground water) is very dangerous towards humans, animals, and nature. Other than that, the risk of poisoning and strenuous regeneration of photocatalysts may hinder the industrial application of this advanced oxidation process (AOP) if these problems are not overcome(Aizat and Aziz, 2019).

Due to this fact, the immobilization of nanoparticles such as TiO_2 has been carried out as soon as the 1980s and since that, multiple supports and binders that will actually be contributed to the photocatalytic process has been

investigated. The utilization of chitosan as the TiO_2 binder is one of the perfect approaches as the presence of NH_2 and OH group on the polymer will serve as the binding and reaction site for those materials via adsorption, thus providing extensive ways in treating various wastewater pollutants(Aizat and Aziz, 2019).

Chapter Three

Material and Methods

3.1 Materials:

Cooked undersized Jinga shrimp shell waste was obtained from a commercial Sea food (Red sea, Port Sudan). For Demineralization, Deproteinization, Decolouration, and Deacetylation we used hydrochloric acid, acetic acid, acetone (ALPHA CHEMIKA) and Sodium hydroxide, ethanol (LOBA Chemie). To convert chitosan to nanoparticles sodium tripolyphosphate (TTP) was used which was obtained from (LOBA Chemie). Silver nitrate (LOBA Chemie) was converted to nanoparticles by using leaves of Solenostemma argel which was obtained from the local market.

All chemicals were of laboratory grade and used without further purification.

3.2 Methods:

3.2.1 Shrimp Chitosan Production:

The shells of tail and the head were separated, and placed separately in to black polyethylene bags. These were stored at freezer until utilized. Washed under warm tap water to remove soluble organics, adherent proteins and other impurities, then put in vessel and boiled in water for 1 h to remove the tissue. Then dried in an oven at 160 °C for 2h (Mukherjee, 2001). To obtain a uniform size product, the dried shells were ground in a fine powder through a Manual Vibratory Disc Mill RS 200, speed 1500 rpm, range (100-200 mesh) as show in figure (3.1).

Figure (3.1) Manual Vibratory Disc Mill RS 200

Dried ground shell was placed in opaque plastic bottles and stored at refrigerator until further use.

3.2.2 Isolation of Chitosan:

To obtain chitosan you must do four processes:

3.2.2.1 Demineralization:

It is known that shells contain carbonate, so carbonate must be removed in this experiment. Dilute hydrochloric acid (HCL) was used to prevent hydrolysis of chitin. The hydrochloric acid concentration (1.5M). The ratio of dried shells to acid solution 1:10 (w:v). Then was stirred for 1 h at room temperature, filtering the mixture and washed by tap water then distilled water to remove HCL, using PH paper to make sure all HCL is removed. Finally, we dried shell powder by put in oven at 80 C° for 12 h.

3.2.2.2 Deproteinization:

The shells was deproteinized with sodium hydroxide (NaOH) concentration (2M). The ratio of dried shells to solution 1:20 (w: v). Then heat stirred with temperature 80°C for 2h, to broke protein. Then filtered the mixture and washed by tap water then distilled water to remove sodium hydroxide, used

PH paper to sure all sodium hydroxide remove. Finally, we dried shell powder by placing in oven at 80 °C for 12h(Aizat and Aziz, 2019).

3.2.2.3 Decolouration:

The dry shell powder was already became white after deproteinization, So doesn't need added Naocl to bleaching, But added mixture of Acetone and Ethanol (1:1) ratio, And stirred for 2h, then filtered and washed. Finally, we dried shell powder (Chitin) by placing in oven at 80°C for 12h.

3.2.2.4a Deacetylation:

To obtain chitosan must convert the chitin involved deacetylation. The sodium hydroxide concentration (2M), the ratio of dried shell to solution 1:50 ,then heat stirred with temperature 80 °C for 7h.Then filtered the mixture and washed by tap water and distilled water to remove sodium hydroxide, we dried shell powder in oven 80°C for 12h. Finally, washed the dried powder by ethanol and dry only.

3.2.2.4b Deacetylation:

A suspension of 1g of chitosan in 50 ml of aqueous sodium hydroxide, as deacetylation reagent (50% by weight), was mixed at fixed temperature 100°C, under constant stirring. After 5hr, the filtered, washed with distilled water and 70% ethanol until the filtrate was neutral. Then it was oven-dried at 80°C overnight(Aizat and Aziz, 2019).

3.3 Preparation of Chitosan nanoparticles:

Chitosan was dissolved at 0.5% (w/v) with 1% (v/v) acetic acid (HOAc) and then raised to pH 4.6-4.8 with 10M NaOH, put in stirring about 1 h, after that filtered by pump. Chitosan nanoparticles formed spontaneously upon addition of 1ml of an aqueous tripolyphosphate solution (25%, w/v) to 3ml of chitosan solution under magnetic stirring(Benhabiles et al., 2012).

3.4 Preparation of Silver nanoparticles:

About 3g of the leaves of (Solenostemma argel) was soaked in 90ml of boiled distilled water overnight. The extract was filtered, and the filtrate was immediately

used for preparation of the nanoparticles. About 0.84g silver nitrate ($AgNO_3$) was dissolved in 50ml of distilled water, the solution has been diluted, by taking 0.5ml from solution and adding 50ml distilled water. Then 5ml of (Solenostemma argel) extract was added to the solution after diluting. Then put in Autoclave at 121^0C, 0.2 MPa, for 15 min(Awad et al., 2018).

3.5 Preparation of Chitosan / Silver Nano-composite:

The solution of silver nanoparticles was mixed with the solution of chitosan nanoparticles by ultrasonic about 1 hr. To obtain final an aqueous solution containing both silver and chitosan nano-composite. The mixture of chitosan and silver nano-composite solutions were purified by centrifugation at 15^0C, 3600 rpm, for 30 min. Supernatants were discarded, and the mixture of chitosan and silver nano-composite were extensively rinsed with distilled water to remove any sodium hydroxide and then freeze-dried before further use and analysis.

3.6 Physicochemical Properties Measurements:

3.6.1 Ash:

Ash measurement is an indicator of the effectiveness of the demineralization step for removal of calcium carbonate.

Ash of the shrimp chitosan was calculated according to the standard method # 923.03 (AOAC, 1990), brought 3 crucibles (A,B,C) were weighed before burning (27.2773g, 30.2871g, 35.2490g). Then placed 2.0 g of shrimp shells in crucible(A), 2.0 g of chitin in crucible(B) and 2.0 g of chitosan in crucible(C). The samples were heated in a furnace perheated to 600 ºC for 6hr, and then the crucibles were cooled by placing in to desiccators for overnight and weighed (27.6513 g, 30.3009 g, 35.2665 g).

Calculation: $\frac{\text{Weight of residue, g}}{\text{Sample weight, g}} \times 100 = \%\ \text{Ash}$ (3.1)

3.6.2 protein:

Nitrogen is one of the five major elements found in organic materials such as protein. This fact was recognized by a Danish chemist, Johan Kjeldahl, who used it as a method of determining the amount of protein in samples taken from a wide variety of organisms. In 1883 Kjeldahl presented to the Danish Chemical Society a method (much revised since his day) for determining the amount of nitrogen in mixtures of substances containing ammonium salts, nitrate, or organic nitrogen compounds.

The central basis used in this procedure is the oxidation of the organic compound using strong sulfuric acid. As the organic material is oxidized the carbon it contains is converted to carbon dioxide and the hydrogen is converted into water.

The nitrogen, from the amine groups found in the peptide bonds of the polypeptide chains, is converted to ammonium ion, which dissolves in the oxidizing solution, and can later be converted to ammonia gas. The Kjeldahl method of nitrogen analysis is the worldwide standard for calculating the protein content in a wide variety of materials ranging from human and animal food, fertilizer, waste water and fossil fules(Blamire, 2003).

3.6.2.1 Kjeldahl method:

The Kjeldahl method consists of three steps, which have to be carefully carried out in sequence:

a. The sample is first digested in strong sulfuric acid in the presence of a catalyst, which helps in the conversion of the amine nitrogen to ammonium ions.
b. The ammonium ions are then converted into ammonia gas, heated and distilled. The ammonia gas is led into a trapping solution where it dissolves and becomes an ammonium ion once again.

c. Finally the amount of the ammonia that has been trapped is determined by titration with a standard solution, and a calculation made(Blamire, 2003).

3.6.3 Viscosity:

The Viscosity of chitosan was determined with Ostwald viscometer.

i. Weigh approximately 1mg of finely selected polymer and place it in a standard 100 ml dry beaker.
ii. Add 80 ml of solvent to the beaker until the polymer is completely dissolved, then complete the volume to the mark with the solvent. (If not fully melting polymer the beaker can be placed in a water bath at 25 ° C for the purpose of accelerating the dissolution process.
iii. Prepare the following solutions: (0.2, 0.4, 0.6, 0.8mg / 100ml) in standard flasks by diluting the solution from the original polymer. (The standard flasks shall be 25 mL or 50 mL as determined by the teacher).
iv. Measuring the viscosity of the solvent using a viscometer (Ostwald) provided that the device was washed with solvent before use. Apply a quantity of 10 ml of solvent is applied if the solvent flow time is measured (t_0 sec).
v. Prepare the previous solutions in 25 mL flasks and 20 mL of solvent if prepared in flasks 50 ml capacity.
vi. Wash the machine with a solvent and then with the desired solution of the polymer solution (using 10 or 20 ml of the solvent) the flow time of the solution (t) sec is measured.
vii. Calculate the time required for the flow of all remaining solutions taking into account that we start with the diluted solution and then the highest concentration with washing the device Twice solvent after each solution, the same volume is used in each measurement.

Figure (3.2) Ostwald viscometer

3.6.4 Molecular Weight:

For the determination of viscosity-average molecular weight (Mv) was obtained from viscosity equation using Marke-Houwink parameters according to the equation:

$$[\eta] = k.\,(Mv)^{\alpha} \qquad (3.2)$$

Where [η] is the intrinsic viscosity and k, α are constants. These parameters were determined for chitosan (k =0.078 $cm^3\ g^{-1}$ and α =0.76)(Mirzadeh et al., 2002).

3.6.5 Degree of deacetylation:

DD = 118.883-[40.1647 *(A1655 / A3450)] (3.3)

3.7 Characterization:

3.7.1. Fourier-transform infrared spectroscopy (FT-IR):

Chitin and Chitosan samples were loaded directly, introduced into the FT-IR and scanned at 4000-400 cm-1 at resolution intervals of 4.000 cm-1 using NICOLET (370 DTGS). A description of the spectrum is attached.

Figure (3.3) FTIR analysis

3.7.2. Scanning Electron Microscope (SEM):

SEM is widely used to investigate the microstructure and chemistry of a range of materials. The main components of the SEM include a source of electrons, electromagnetic lenses to focus electrons, electron detectors, sample chambers, computers, and displays.

Figure (3.4) Scanning Electron Microscope (SEM)

3.8 Antibacterial activity of chitosan/Silver Nano-composite on Nile water:

The membrane filter method gives a direct count of total coliforms present in a given sample of water. A measured volume of water is filtered, under vacuum, through a cellulose acetate membrane of uniform pore diameter, usually 0.45 mm. Bacteria are retained on the surface of the membrane which is placed on a suitable selective medium in a sterile container and incubated at on appropriate temperature. If coliforms are present in the water sample, characteristic colonies form that can be counted directly(Bartram and Ballance, 1996).

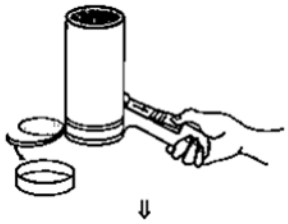
3.8.1 Preparation of Endo Medium:

We weighted 1.2g coliform medium, added 25ml distilled water, 0.5 Ethanol and shaking, then put the mixture in Autoclave about 15min.

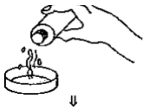
Figure (3.5) Endo Medium.

3.8.2 Steps in the membrane filter technique:

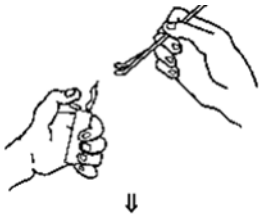
a. Add absorbant pad to Petri dish.



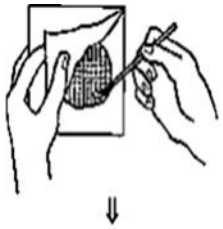
b. Soak pad in M-endo medium.



c. Disinfect tips of blunt-ended forceps and cool.



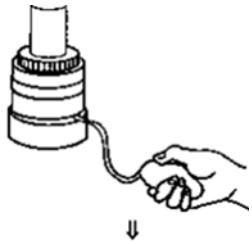
d. Remove membrane filter from sterile packet.



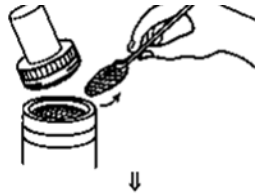
e. Place membrane filter in filtration apparatus.

f. Add sample to filtration apparatus.

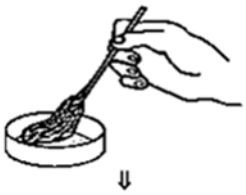
g. Apply vacuum to suction flask.



h. Remove filter with sterile forceps.



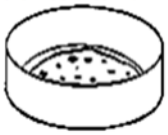
i. Place filter in prepared Petri dish.



j. Label Perti dish.

k. Leave to resuscitate and then incubate.

L. Count colonies after full incubation.



Chapter Four

Result and Discussion

The present work investigates the various physicochemical functional properties of chitosan.

4.1 Preparation of chitosan:

Figure (4.1): Shrimp shell after dry an oven160 ^{0}C.

Figure (4.2) Chitin after Decolouration

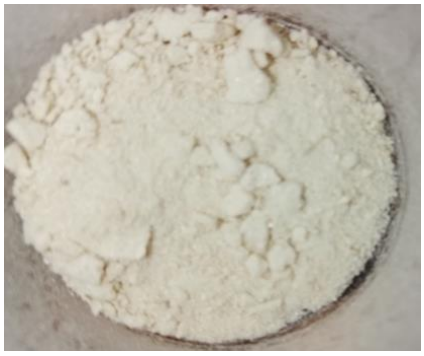


Figure (4.3): Chitosan yield.

These samples have been according to the method mentioned in chapter there.

4.2 Experimental results:

Some residual ash of chitosan may affect their solubility, consequently contributing to lower viscosity, or can affect other more important characteristics of the final product. A high quality grade of chitosan should have less than 1% of ash content, in this study obtained excellent result (0.68%) of ash content and protein content (1.7%).

The shells contained relatively high protein content (32.43%) and ash (18%) on a dry basis (Table 4.1).

The viscosity-average molecular weight, Mv, of chitosan obtained in this study was determined to be 30.6 with a degree of deacetylation (DD) of 67.83% and lower viscosity 1.05(Table 4.1).

Table (4.1): Characteristics of chitin and chitosan produced:

The Physicochemical Properties	**Jinga Shrimp**	**Chitin**	**Chitosan**
% of Ash (wt/wt)	18	0.87	0.68
% of Protein (wt/wt)	32.43	2.68	1.7
Intrinsic viscosity(100ml/mg)	–	–	1.05
Molecular weight	–	–	30.6
Degree of deacetylation(%)	–	70.3	67.83

4.2.1Viscosity result:

Table (4.2): Viscosity test data:

C(mg/100ml)	t_0(sec)	t(sec)	$\eta_r=\eta/\eta_0=t/t_0$	$[\eta/\eta_0-1]=\eta_{sp}$	(η_{sp}/C)	$In(\eta_r/C)$
0.2	79.8	81	1.01	0.01	1	3.92
0.4	79.8	125.4	1.57	0.57	14.25	3.67
0.6	79.8	133.8	1.68	0.68	11.33	3.33
0.8	79.8	145.2	1.82	0.82	10.25	3.12

***We calculated viscosity by using two points from C, $ln(\eta_r/C)$:**

$(x_1 - y_1)$ and $(x_2 - y_2)$

$\eta = y_2 - y_1 / x_2 - x_1 = 3.33 - 3.12 / 0.8 - 0.6$

$\eta = 0.21/0.2 = 1.05$

4.3 Preparation of silver nanoparticles:

A change in color of colloidal solution occurred, which confirmed the reduction of Ag+ ions the formation of silver nanoparticles.

Figure (4.4): Silver nitrate solution

Figure (4.5): Silver nanoparticle

4.4 Characteristics of Methods:

4.4.1 Fourier Transform Infrared Spectroscopy (FT-IR) analysis:

The spectrum of Chitosan was showed in Fig (4.6) where: A3450, A2870, A1655, A1420, A1320, are values of absorbance from baseline 1, 2, 3, 4, 5 to maximum, respectively. In Figure1, on the basis IR spectrum of chitosan, baseline settings and individual bands ascribed for characteristic groups in chitosan are presented.

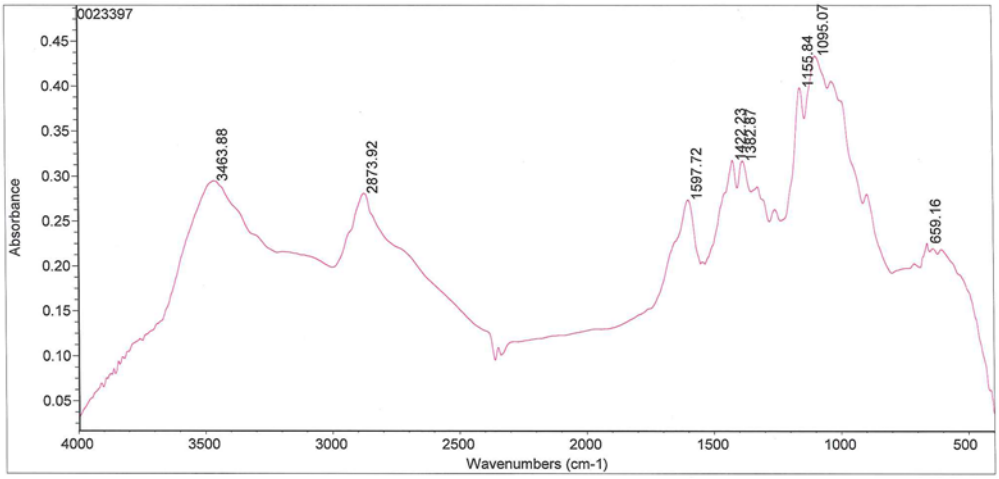


Figure (4.6): I.R. spectrum of chitosan showing the two baselines (‘a’ and ‘b’) for calculating the amide I band absorbance for the ratio A1655 /A3450.

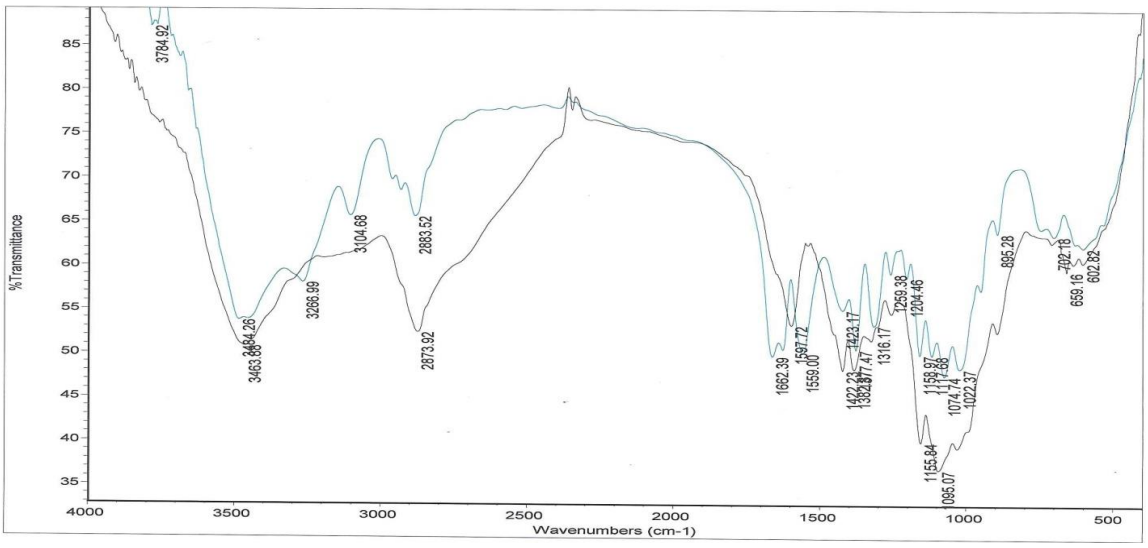


Figure (4.7): I.R. spectrum of chitin and chitosan, the green baseline is chitin and black baseline is chitosan.

4.4.2 Scanning Electron Microscope (SEM):

Scanning Electron Microscopy is a microscopic morphology observation method between transmission electron microscope and optical microscope. It can directly use the material properties of the sample surface material for microscopic image.

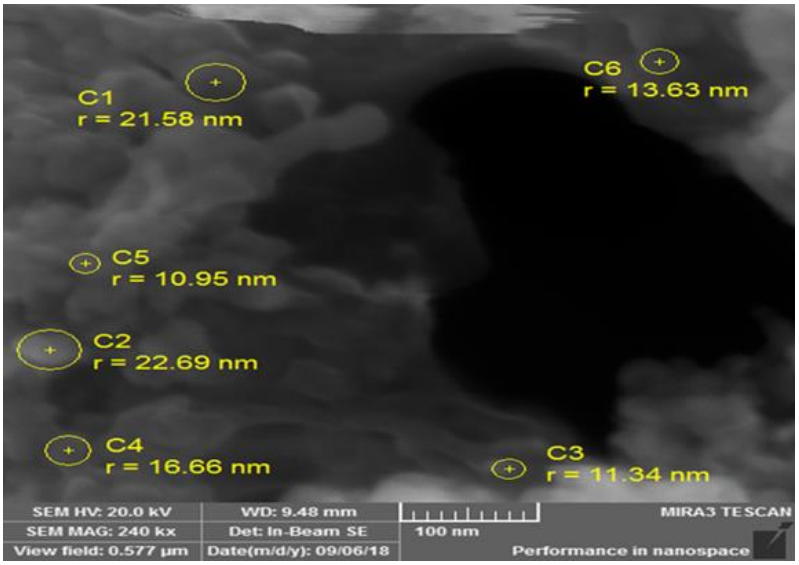
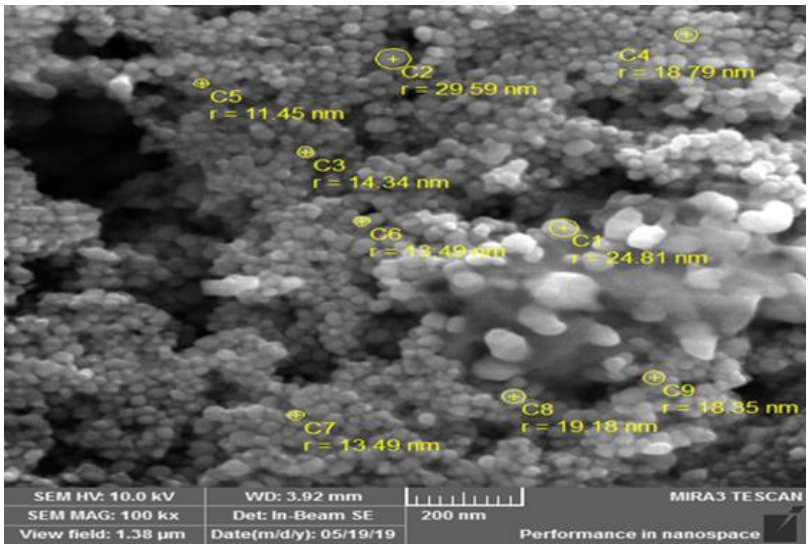


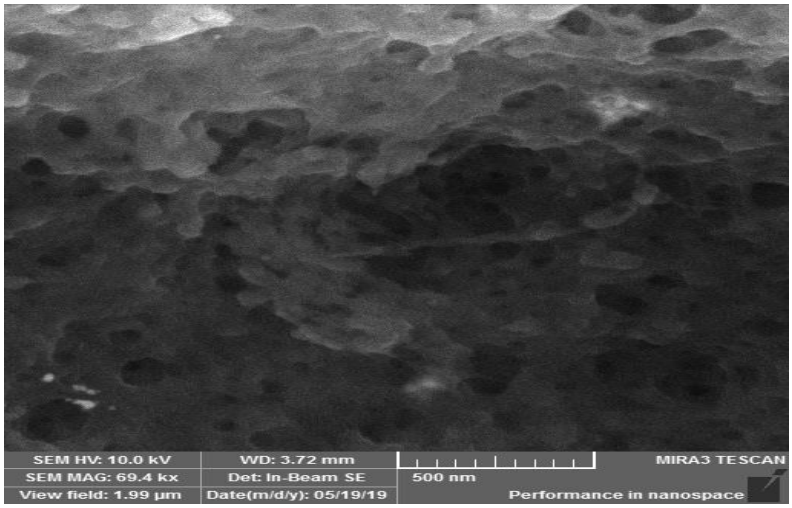
Figure (4.8) The SEM photographs of the synthesized chitosan nanoparticles.

SEM Analysis: The morphology of Silver nanoparticles was observed and the results were shown in (Fig.4.9) Silver nanoparticles revealed a very homogenous morphology and they are spherical in shape.



Figure(4.9): The SEM photographs of the synthesized silver nanoparticles.

SEM analysis: shows that the surface of Silver/ Chitosan nano-composite is not flat and has irregular scattered conglomerates of different sizes and shapes, typical for powder systems (Fig 4.10).



Figure(4.10): The SEM photographs of the synthesized silver/chitosan nano-composite.

4.5 Antibacterial assessment:

Chitosan /Silver nanocomposite exhibit higher antibacterial activity in this study than chitosan nanoparticles on account of the special character of the nanocomposite could be tightly adsorbed onto the surface of the bacteria cells so as to disrupt the membrane, which would lead to leakage of intracellular component, thus killing the bacteria cells. Chitosan/Silver nanocomposite showed in Table (4.3) a negative of bacterial/100ml Nile water and this result is excellent, compared to chlorine and chitosan nanoparticles.

4.5.1 Chitosan /Silver Nano-composite as antibacterial activity:

We added about 3mg Chitosan / Silver Nano-composite in 100ml of the treated Nile Water by chlorine, then shake the mixture and passed by the filter. Placed the filter on the plate agar. Finally incubated for 24 hours at 35°C.

4.5.2 Chitosan nanoparticles as antibacterial activity:

We added about 3mg Chitosan nanoparticles in 100ml of the treated Nile Water by chlorine, then shake the mixture and passed by the filter. Placed the filter on the plate agar. Finally incubated for 24 hours at 35°C.

Table (4.3): The result of antibacterial activity on treated Nile water by chlorine, chitosan nanoparticles and chitosan/silver nanocomposite.

Materials	Dose	Total Coliform Bactria / 100 ml Nile Water
Chlorine	10mg	38 Colony /100 ml
Chitosan nanoparticles	3mg	19 Colony / 100 ml
Chitosan/Silver nanocomposite	3mg	Negative.

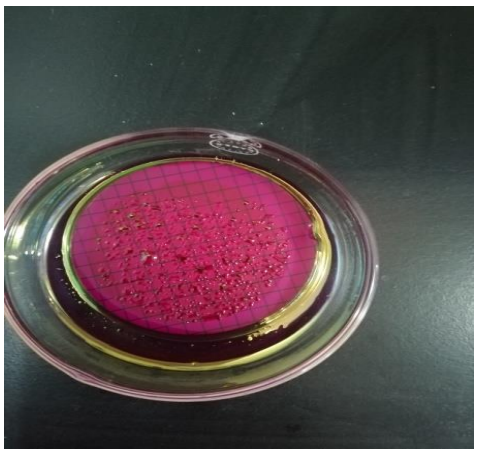


Figure (4.11)

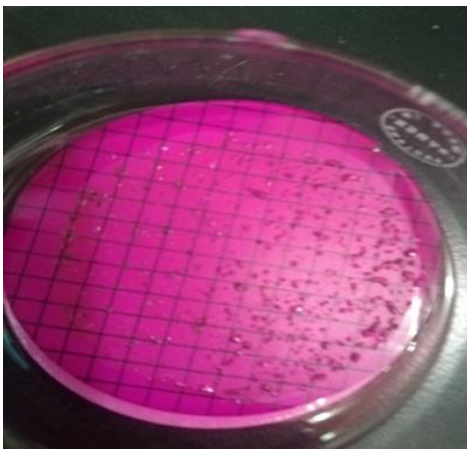
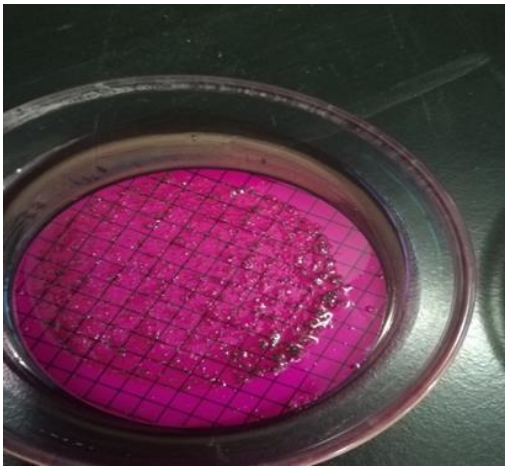


Figure (4.12)

Figure (4.11) Sample of treated Nile water by 10mg chlorine

The coliform bacteria not killed all and showed by green color**.**

Figure (4.12) Sample of treated Nile water by 3mgChitosan/Silver nano composite. All the coliform bacteria were killed.



Figure(4.13): Sample of treated Nile water by 3mg Chitosan nanoparticles

Not all coliform bacteria were kill.

Chapter Five

Conclusion and Recommendation

5.1 Summary and Conclusion:

In summary, chitosan /silver Nano-composite have been synthesized and characterized in the present study. Chitosan was a naturally occurring biopolymer originating from several shells of crustaceans, such as shrimp and lobster. Its excellent physical and chemical properties allow its use in various environmental applications, especially in water treatment. In particular, chitosan nanoparticles, such as those prepared in this study, as their unique character exhibit potential antibacterial activity. The purpose of this study was to evaluate the antibacterial activity of chitosan and silver nanocomposite against various microorganisms. Chitosan nanoparticles were prepared based on the ionic gelation of chitosan with tripolyphosphate anions. Silver nanoparticles were prepared by biosynthesis, providing a first aqueous solution of silver nitrate, providing a second solution of an aqueous solenostemma argel, and combining the first solution and the second solution to produce a solution including nanoparticles of silver. The solution of silver and chitosan nanoparticles were mixed and purified by centrifugation, Supernatants were discarded, and the mixture of chitosan and silver nanocomposite were extensively rinsed with distilled water and then freeze-dried before further use and analysis. The physicochemical properties of chitosan and/or chitin were determined (Viscosity for Chitosan 1.05%, Ash for (Chitosan: 0.68%, Chitin: 0.87%) ,Protein for (Chitosan: 0.35% , Chitin: 2.68%), Degree of deacetylation for (Chitosan: 67.83%, Chitin: 70.3%) and Molecular Weight. The prepared sample was characterized by Fourier transform infrared spectroscopy (FTIR) analysis, Scanning Electron Microscope (SEM), the minimum particle size of SEM photographs of the synthesized chitosan nanoparticles, r=10.95nm, the minimum particle size of the synthesized silver nanoparticles, r=11.45 nm. The antibacterial activity tested by chitosan/silver nanocomposite in Nile water; showed that excellent

results in concentrate 3mg/100ml could inhibit the growth of various bacteria tested.

5.2 Recommendation:

Chitosan silver-loaded nanoparticles could be recommended as an efficient antibacterial material for water disinfection.

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