"CHARACTERISATION AND APPLICATION OF SYNTHESIZED CHITOSAN NANOPARTICLES AGAINST *ESCHERICHIA COLI* BACTERIA"

DISSERTATION SUBMITTED TO MAHATMA GANDHI UNIVERSITY, KOTTAYAM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

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THRIKKAKARA

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CERTIFICATE

This is to certify that the project entitled **"CHARACTERISATION AND APPLICATION OF SYNTHESIZED CHITOSAN NANOPARTICLES *AGAINST ESCHERICHIA COLI* BACTERIA"** is a bonafide work done by **ANITTA JOSE** with **Register No: 180021043795** under the supervision of **Dr. K. S Rishad,** Unibiosys Biotech Research Lab, kalamassery ,Cochin during 2018-21 in partial fulfilment of the requirement for the award of the **Bachelor of Science in Zoology** of Mahatma Gandhi University, Kottayam.

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CERTIFICATE

This is to certify that the dissertation entitled **"Characterisation and application of synthesized chitosan nanoparticles against *Escherichia coli*"** is a bonafide record of the original research work done by **Ms. Anitta Jose, Bharata Mata College, Thrikkakara** partial fulfillment of the requirement for the award of **'Degree of Bachelor of Science in Zoology'** during the period of her study in the Department of Advanced Zoology and Biotechnology, under my direct supervision & guidance.

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DECLARATION

I do hereby declare that the work embodied in the dissertation entitled **"CHARACTERISATION AND APPLICATION OF SYNTHESIZED CHITOSAN NANOPARTICLES AGAINST *ESCHERICHIA COLI* BACTERIA"** submitted to the Mahatma Gandhi University, Kottayam in partial fulfilment for the award of Bachelor of Science in Zoology is record of bonafide dissertation done by me under the supervision of **Dr. K.S Rishad**, Unibiosys Biotech Research Lab, kalamassery , Cochin and that no part of this has been submitted for award of any other Degree/Diploma /Associate ship/Fellowship or any other similar title by any candidate of any university.

Place: THRIKKAKARA **ANITTA JOSE**

Date: **180021043795**

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ANITTA JOSE

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SYNOPSIS

Chitin is a potential energy source as well as gene and drug delivery carrier, and in the emerging field of nanobiotechnology. Chitosan, the product of chitin deacetylation, is known for its various uses in different fields. Due its increasing advantages, the chitosan nanoparticles has still importance in research purposes.

Chitosan is a natural, biocompatible, biodegradable, nontoxic and easily available polymer that can be used to prepare nanoparticles. Chitosan nanoparticles can be widely used in pharmaceutical industries as an antimicrobial agent. The present study focuses on the synthesis of chitosan nanoparticles its Characterisation and application. Chitosan nanoparticles are synthesised into desired size and surface charge using sodium Tripolyphosphate (STPP) initiated ionic gelation process. These chitosan nanoparticles were characterised by UV- VIS spectroscopy to know the kinetic behaviour that ranges between 200 to 700 nm. The antimicrobial properties of chitosan nanoparticles were studied as its application and it was done in *Escherichia coli* bacteria. The organism were identified by Gram's staining and biochemical test . Their activity was tested using well diffusion method and zone of inhibition was noted. The zone of inhibition obtained against *Escherichia coli* were 2.4cm for 10mg/ml, 2.7cm for 20mg/ml and 3.0cm for 30mg/ml.The study is thus a good demonstration of the applicability of chitosan nanoparticles as an effective antimicrobial agent.

INTRODUCTION

Chitin is the most abundant amino polysaccharide polymer occurring in nature, and is the building material that gives strength to the exoskeletons of crustaceans, insects, and the cell walls of fungi. Through enzymatic or chemical deacetylation, chitin can be converted to its most well-known derivative, chitosan. The main natural sources of chitin are shrimp and crab shells, which are an abundant by product of the food-processing industry, which provides large quantities of this biopolymer to be used in biomedical applications. In living chitin-synthesizing organisms, the synthesis and degradation of chitin require strict enzymatic control to maintain homeostasis. Chitin synthase, the pivotal enzyme in the chitin synthesis pathway, uses UDP-N-acetylglucosamine (UDPGlcNAc), produce the chitin polymer, whereas, chitinase enzymes degrade chitin. Bacteria are considered as the major mediators of chitin degradation in nature. Chitin and chitosan, owing to their unique biochemical properties such as biocompatibility, biodegradability, non-toxicity, ability to form films, etc., have found many promising biomedical applications. Nanotechnology has also increasingly applied chitin and chitosan-based materials in its most recent achievements.

Chitosan is an attractive natural biopolymer with the presence of reactive amino and hydroxyl functional groups. It has shown favorable biocompatibility characteristics as well as the ability to increase membrane permeability. Moreover, it is one of the most promising immobilization matrices due to an excellent membrane-forming ability, good adhesion, low cost, nontoxicity, high mechanical strength, and hydrophilicity as well as the improvement of stability. These properties have prompted extensive applications of chitosan as a matrix for enzyme immobilization. All of these excellent properties lead chitosan to be a proper material for enzyme immobilization, especially within biosensor applications that are mostly concerned with working of enzymes for detection mechanisms. Moreover, this excellent material, chitosan, can be prepared in nanoparticle form, which could enhance the benefit of improving characteristics in biosensor works. Many research papers mainly reported the preparation of chitosan in order to immobilize many enzymes for usage in biosensor, especially electrochemical detection.

Nanotechnology is enabling technology that deals with nano-meter sized objects. Nanotechnology is developed at several levels: materials, devices and systems. The nano materials level is the most advanced at present, both in scientific knowledge and in commercial applications. A decade ago, nanoparticles were studied because of their size dependent physical

and chemical properties. Now they have entered a commercial exploration period (Paull R *et al*., 2003). Living organisms are built of cells that are typically 10 μm across. However, the cell parts are much smaller and are in the sub-micron size domain. Even smaller are the proteins with a typical size of just 5 nm, which is comparable with the dimensions of smallest manmade nanoparticles. This simple size comparison gives an idea of using nanoparticles as very small probes that would allow us to spy at the cellular machinery without introducing too much interference. Understanding of biological processes on the nanoscale level is a strong driving force behind development of nanotechnology.

Nanoparticles are solid colloidal particles with diameters ranging from 1-1000 nm. They consist of macromolecular materials and can be used therapeutically as adjuvant in vaccines or drug carriers in which the active ingredient is dissolved, entrapped, encapsulated, adsorbed or chemically attached. Polymers used to form nanoparticles can be both synthetic and natural polymers. There are two types of nanoparticles depending on the preparation process: nanospheres and nanocapsules (Allemann E *et al*., 1993). Nanospheres have a monolithic-type structure (matrix) in which drugs are dispersed or adsorbed onto their surfaces; and nanocapsules exhibit a membrane-wall structure and drugs are entrapped in the core or adsorbed onto their exterior .The term “nanoparticles” is adopted because it is often very difficult to unambiguously establish whether these particles are of a matrix or a membrane type.

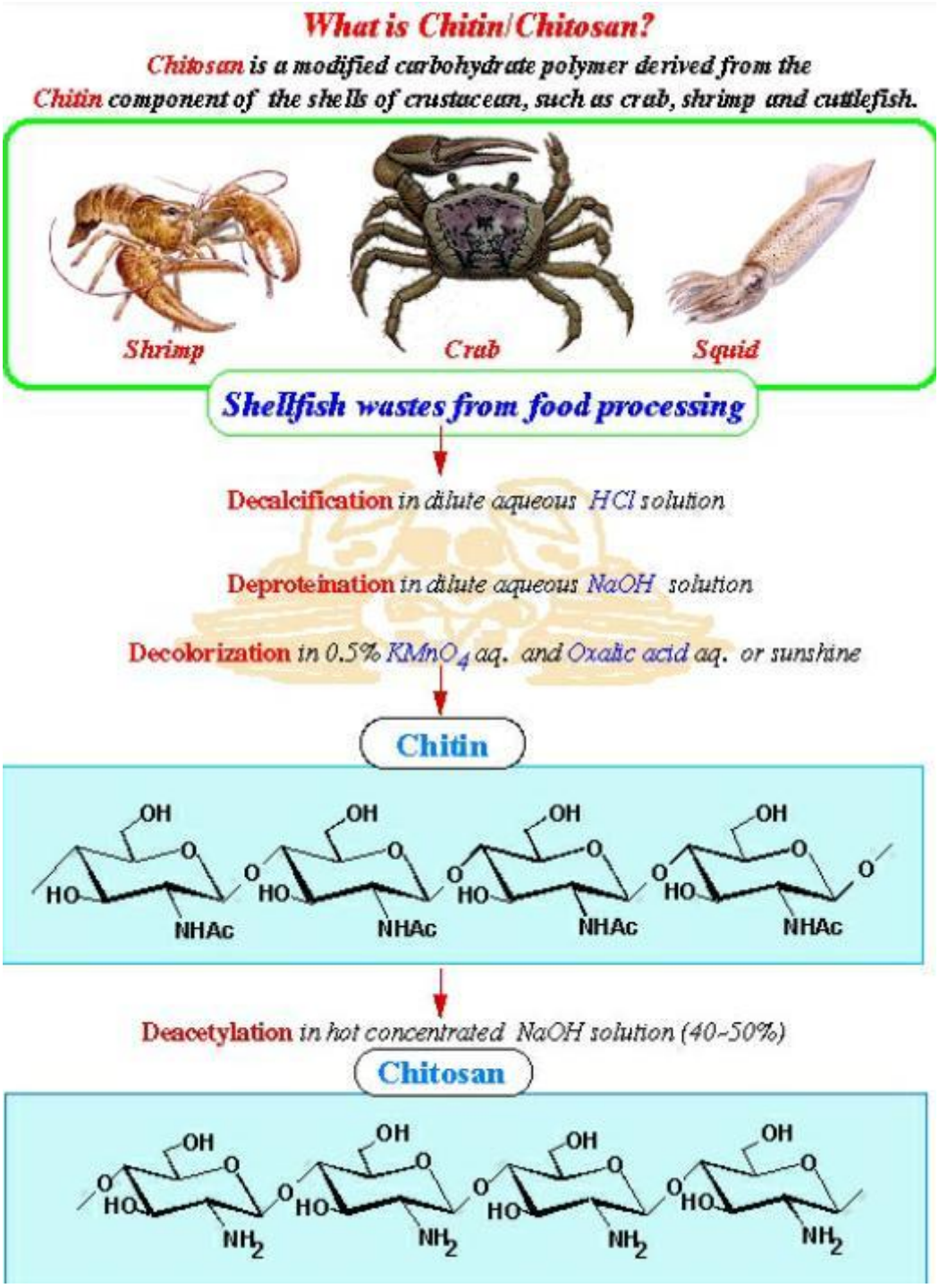

Figure 1: Preparation of Chitin and Chitosan

Escherichia coli is a Gram negative, facultatively anaerobic, rod-shaped bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms

(endotherms). They are about 2.0 μm long and 0.25–1.0 μm in diameter, with a cell volume of 0.6–0.7 μm3.Most *E. Coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2, and preventing colonization of the intestine with pathogenic bacteria. *E. Coli* and other facultative anaerobes constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination.

Most *E. Coli* strains do not cause disease, naturally living in the gut, but virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis, and Crohn's disease. Common signs and symptoms include severe abdominal cramps, diarrhea, hemorrhagic colitis, vomiting, and sometimes fever. Optimum growth of *E. Coli* occurs at 37 °C (98.6 °F), but some laboratory strains can multiply at temperatures up to 49 °C (120 °F).*E. Coli* grows in a variety of defined laboratory media, such as lysogeny broth, or any medium that contains glucose, ammonium phosphate monobasic, sodium chloride, magnesium sulfate, potassium phosphate dibasic, and water.

Figure 2 :*Escherichia coli*

E. Coli encompasses an enormous population of bacteria that exhibit a very high degree of both genetic and phenotypic diversity. Due to the low cost and speed with which it can be grown and modified in laboratory settings, *E. Coli* is a popular expression platform for the production of recombinant proteins used in therapeutics. Certain *E.* Coli strains and their derivatives are used by the biotechnology industry and also used as probiotic agent in medicine. *E. Coli* remains as one of the most diverse bacterial species: only 20% of the genes in a typical *E.Coli* genome is shared among all strains.

AIM AND OBJECTIVES

AIM

The project entitled " Characterisation and Application of Synthesised Chitosan Nanoparticles against *Escherichia coli* bacteria" mainly aim to synthesise, characterise chitosan nanoparticles and to check the antimicrobial activity of given *E.coli* bacteria as its application.

OBJECTIVES

The project undertaken has the following objectives,

- Synthesis of chitosan nanoparticles.
- Characterisation of chitosan nanoparticles.
- Identification of *E.coli* bacteria .
- Gram's staining of *E.coli* bacteria .
- Assess antimicrobial role of chitosan nanoparticles.

REVIEW OF LITERATURE

Chitin is one of the most abundant organic materials, beings second only to cellulose in the amount produced annually by biosynthesis. It occurs in animals, particularly in crustacea, molluscs and insects, where it is a major constituent of the exoskeleton, and in certain fungi, where it is the principal fibrillar polymer in the cell wall. Chitin has a crystalline structure and it constitutes a network of organized fibres, this structure confers rigidity and resistance to organisms that contain it (Roberts G, 1992). Chitin is poly [β-(1→4)-2- acetamido-2-deoxy-D-glucopyranose].

Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. Chitosan is also found in some microorganisms, yeast and fungi (Illum L, 1998). The primary unit in the chitin polymer is 2-deoxy-2-(acetyl amino) glucose. These units combined by β-(1,4) glycosidic linkages, forming a long chain linear polymer. Chitosan is soluble in most organic acidic solutions at pH less than 6.5 including formic, acetic, tartaric, and citric acid (LeHoux J and Grondin F, 1993).

It is insoluble in phosphoric and sulfuric acid. Chitosan is available in a wide range of molecular weight and degree of deacetylation. Molecular weight and degree of deacetylation are the main factors affecting the particle size, particles formation and aggregation. Chitosan possesses some ideal properties of polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic, and inexpensive. These properties render chitosan a very attractive material as a drug delivery carrier. In the last two decades, chitosan nanoparticles have been extensively developed and explored for pharmaceutical applications (Roberts G, 1992).

Nanotechnology is enabling technology that deals with nano-meter sized objects. Nanotechnology is developed at several levels: materials, devices and systems. The nanomaterials level is the most advanced at present, both in scientific knowledge and in commercial applications. A decade ago, nanoparticles were studied because of their sizedependent physical and chemical properties (Murray C *et al.*, 2000). Now they have entered a commercial exploration period (Paull R *et al.*, 2003). Living organisms are built of cells that are typically 10 μm across. However, the cell parts are much smaller and are in the sub-micron size domain. Even smaller are the proteins with a typical size of just 5 nm, which is comparable with the dimensions of smallest manmade nanoparticles. This simple size comparison gives an

idea of using nanoparticles as very small probes that would allow us to spy at the cellular machinery without introducing too much interference (Taton T, 2002).

Understanding of biological processes on the nanoscale level is a strong driving force behind development of nanotechnology (Whitesides G, 2003). Out of plethora of size dependant physical properties available to someone who is interested in the practical side of nanomaterials, optical and magnetic effects are the most used for biological applications (Parak W *et al*., 2003).

Nanoparticles are solid colloidal particles with diameters ranging from 1-1000 nm. They consist of macromolecular materials and can be used therapeutically as adjuvant in vaccines or drug carriers in which the active ingredient is dissolved, entrapped, encapsulated, adsorbed or chemically attached. Polymers used to form nanoparticles can be both synthetic and natural polymers. There are two types of nanoparticles depending on the preparation process: nanospheres and nanocapsules (Allemann E *et al*., 1993).

Silver nanoparticles (Ag NPs) were incorporated into biodegradable polymers that are chitosan, gelatin and both polymers via chemical reduction method in solvent in order to produce Ag BNCs. Silver nitrate and sodium borohydride were employed as a metal precursor and reducing agent respectively. On the other hand, chitosan and gelatin were added as a polymeric matrix and stabilizer. The antibacterial activity of different sizes of silver nanoparticles was investigated against Gram-positive and Gram-negative bacteria by the disk diffusion method using Mueller-Hinton Agar (Ahmad *et al.*,2012).

Chromium oxide nanoparticles (NPs) were rapidly synthesized by reduction of potassium dichromate solution with Arachis hypogaea leaf extract containing reducing sugars which act as reducing agent. The results indicated that the aldehyde groups present in the plant extract played an important role in the formation of Cr2O3 nanoparticles. The purification process of the Cr2O3 product does not require expensive methods, since a solid product is obtained from a reaction in liquid phase. The antibacterial effect of Cr2O3 nanoparticles against Escherichia coli was investigated as a model for Gram-negative bacteria. Bacteriological tests were performed in Potato Dextrose Agar (PDA) medium on solid agar plates and in liquid systems supplemented with different concentrations of nanosized Cr2O3 particles. These particles were shown to be an effective bactericide. The resulting Cr2O3 nanoparticles were characterized by X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM), UV-VIS absorption and Fourier transform infrared (FTIR) spectroscopy (Ramesh *et al*., 2012).

The internalization of chromium oxide nanoparticles in Escherichia coli cells was evaluated by flow cytometry using light scattering method. (Khatoon *et al*.,2011) The antibacterial activity of Cr (VI) and Cr (III) complexes against *P. aeruginosa* bacteria. (El-ajaily *et al*., 2007) Viability of an environmentally relevant bacterium, E. Coli exposed to varying concentrations of Chromium oxide nanoparticles was evaluated PMA (Propidium mono-azide) assisted Q-PCR. (Singh *et al*.,2011) The antibacterial effects of chromium (III) complexes using single representative strains of *E. Coli* and *Bacillus subtilis*. Although only a few studies have reported the antibacterial properties of chromium (III) complexes have a significant promise as bactericidal agent. (Vadde Ravinder *et al.,* 2009).

The physicochemical nature of chitin and chitosan, which influences the biomedical activity of these compounds, is strongly related to the source of chitin and the conditions of the chitin/chitosan production process. Apart from widely described key factors such as weight-averaged molecular weight (MW) and degree of N-acetylation (DA), other physicochemical parameters like polydispersity (MW/MN), crystallinity or the pattern of acetylation (PA) have to be taken into consideration. From the biological point of view, these parameters affect a very important factor—the solubility of chitin and chitosan in water and organic solvents. The physicochemical properties of chitosan solutions can be controlled by manipulating solution conditions (temperature, pH, ionic strength, concentration, solvent). The degree of substitution of the hydroxyl and the amino groups or the degree of quaternization of the amino groups also influence the mechanical and biological properties of chitosan samples. Finally, a considerable research effort has been directed towards developing safe and efficient chitin/chitosan-based products because many factors, like the size of nanoparticles, can determine the biomedical characteristics of medicinal products (Kumirska *et al*.,2011).

Several studies have shown chitosan to exhibit higher antibacterial activity against gram positive bacteria than gram negative bacteria, while some other studies have shown that gram negative bacteria as more susceptible than gram positive . Still many works have demonstrated that there is no significant difference between the antibacterial activity and bacterial species (K.Divya *et al*.,2016).

On addition of STPP to chitosan solution, an opalescent colour was observed, which indicates the formation of CSNPs. Different concentrations of STPP (0.5%, 1%, 1.5%, and 2%) were used for nanoparticle preparation and 1.0% STPP was found to be most suitable with sharpest

peak shown by UV spectroscopy, indicating the most CSNPs made.(Dolly Kain and Suresh Kumar,2020)

Chitosan nanoparticles was consider for carrying out antimicrobial study. (Rhoades and Roller 2000; Sebti *et al.,* 2005) Moreover in 2007 USFDA recognized the chitosan nanoparticles are admitted as generally as safe (GRAS). Worldwide (Japan, Korea, England, Italy, Portugal and United States) these nanoparticles are marketed as food additive(Novack *et al*., 2003). These nano-particles can be synthesized by deacetylation of crustacean shells, chitin contains β-1, 4-linked 2-amino-2-deoxy-β-D-glucose. Helander *et al.,* 2001 reported that chitosan nanoparticles effectively acted against some Gram-negative bacteria such as *E. Coli*, *Pseudomonas aeruginosa* and *S. typhimurium*. (Shahidi *et al*., 1999; No *et al*., 2007)studied the properties and applications of chitosan nano-particles and their derivatives in food storage. (Abdullah A. Alarfaj, 2019)

Chitosan is the second most abundant polymer obtained from the by product of seafood. Chitosan and its derivatives and chitosan loaded drugs are the recent area of interest against microbial pathogenesis. The cationic chitosan nanoparticles (ChNPs) interact with the anionic surfaces of the microbial cell membrane, which promotes antimicrobial activity. Although, ChNPs are potential against pathogenic microbes, selection of adaptable, suitable and cost effective synthesis method is much important. In the present study, ChNPs were synthesized adopting ionic gelation using sodium Tripolyphosphate as a cross linking agent and characterized by FTIR, DLS, SEM and TEM analysis. ChNPs were investigated for antimicrobial activity against bacterial (*Escherichia coli* and *Staphylococcus aureus*) and fungal (*Candida albicans*) pathogens. ChNPs showed bactericidal activity at the lower minimum inhibitory concentration of about 40–80 μg mL−1.(D.MubarakAli,2018).

The antimicrobial activities of chitosan modified compounds were assayed by agar wells diffusion technique. Agar plates were prepared using nutrient agar for bacteria while potato dextrose agar was applied for fungi. The plates were inoculated with 0.1 mL containing 106 cfu/mL of fresh bacterial cultures and spore suspensions of pathogenic strains. Wells of 7 mm in diameter were dug on the inoculated agar plates using a sterile cork borer in solidified agar. The tested compounds prepared in DMSO (60 μL) were added to the wells. DMSO as dissolving agent was tested as control. Plates were left for two hours at 4°C (in the refrigerator) to allow the diffusion and then incubated for 24 h at 37°C for bacteria, while fungi plates were inoculated at 28°C for 72 h. The antimicrobial activities of tested compounds were determined

by measuring the three replicates of the inhibition zones around the well.(Ahmed M. Khalil *et al.*,2017)

MATERIALS AND METHOD

GLASSWARES

All glass wares were used of Borosil glass. These were first washed with detergent, then with tap water and finally with distilled water. They were dried properly in hot air oven for future use.

CHEMICALS

All the chemicals used in the analytical method and media preparation were analytical grade with maximum available purity supplied by Hi-media.

INSTRUMENTS

- Magnetic stirrer
- Microwave Oven
- Laminar air flow
- Weighing balance
- UV VIS spectrophotometer
- Incubator
- Microscope
- Autoclave

MEDIA AND REAGENTS

- Nutrient Agar
- Nutrient Broth
- Mueller Hinton Agar
- Peptone Water
- Citrate Agar
- Kovac's Reagent
- Barritt's reagent A & B
- Methyl Red
- Crystal Violet
- Safranin
- Grams Iodine

METHODOLOGY

1.CHITOSAN NANOPARTICLES SYNTHESIS

Chitosan/Tripolyphosphate nanoparticles were prepared by ionic gelation process. 8ml of 0.4%STPP was added to 20ml of chitosan solution (0.1g/1%acetic acid) drop wise under constant stirring in magnetic stirrer. And the solution is stored at refrigerator for further studies.

Figure 3: Chitosan nanoparticle synthesis

2.CHARACTERISATION OF NANOPARTICLES

UV-VIS Spectroscopy: Nanoparticles were characterized in UV-VIS spectrophotometer (Systronics, Delhi) to know the kinetic behaviour of Chitosan nanoparticles. The scanning range for the samples was 200-540nm. The UV Vis absorption spectra of all the samples were recorded and numerical data were plotted.

3.ISOLATION OF BACTERIA

QUADRANT STREAK METHOD

It is the method used for the isolation of bacteria in pure culture. In this method, sterilized loop is dipped into detected suspension of organism which is placed on the surface of solidified agar plate to make a series of parallel non overlapping streaks.

PROCEDURE

Hold the inoculation loop in your right hand. Sterilize the inoculation loop in the bunsen burner by putting the loop into the flame until it is red hot . Allow it to cool. Lift the test tube containing the bacteria with your left hand. Remove the cotton plug of the test tube with the little finger of your right hand. Flame the mouth of the test tube. Dip the sterilized loop in the test tube and get a loop ful of bacteria. Replace the cotton plug of the test tube and place it in the rack. Lift the lid of the petridish . Place the loop ful of bacteria on the agar medium and spread it in the first quadrant. Flame the loop and allow it to cool. Turn the petridish and streak from the edge of the culture spread on the first quadrant. Flame the loop and allow it to cool. Streak again, from the edge of culture streaked on the second quadrant. Repeat this process till the fourth quadrant. Don not let the loop touch any of the previously streaked areas. . The flaming of loop after each quadrant is to effect the dilution of the culture so that fewer organisms are streaked in each area resulting in the final desired separation. Remove the loop , close the petridish and label it. Incubate the plates for 24 hours. Flame the loop before putting it aside.

4.IDENTIFICATION OF BACTERIA

A. GRAM'S STAINING

Gram staining was discovered by Hans Christian Gram in 1884.To differentiate between Gram positive and Gram negative bacteria based on their cell wall composition. Primary stain used is crystal violet which imparts colour to all cells. A decolourising agent ethyl alcohol acts as a lipid solvent and as a protein dehydration agent. Gram's iodine serves as the mordant substance that forms an insoluble substance binding to the primary stain. Safranin acts as a counter stain which imparts colour to Gram negative bacteria.

PROCEDURE

Isolated bacteria with maximum biofilm forming activity were taken and smears were prepared on clean grease free slide. Smear is heat fixed by passing over a flame 2-3 times. The slides were flooded with crystal violet solution for 1minute, washed with water and flooded with grams iodine for one minute. The slide was washed with water and decolourized with 95% ethyl alcohol .The slides were washed with water and counter stained with safranin stain for about 30 seconds and washed with water. The slides were air dried and examined under a microscope using 100X objective.

B.BIOCHEMICAL IDENTIFICATION

1. INDOLE PRODUCTION TEST

Indole test is performed to determine the ability of the organism to split tryptophan molecule into indole. Indole is one of the metabolic degradation product of the amino acid tryptophan Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and deaminating tryptophan with the production of Indole, pyruvic acid and ammonia. This test is performed to differentiate species of the family Enterobacteriaceae.

PROCEDURE

Peptone broth was inoculated with sample culture. It was then kept for incubation at 37ºC for 24 hours. After incubation add 1 ml of kovac's reagent to the tube including control. Shake the tubes gently for 2-5 minutes. Then the tubes were observed and results were noted.

2. METHYL RED TEST

All enteric organism utilize glucose for energy, as a result they produce acidic end products. In this test, pH indicator methyl red detects the presence of large concentrations of acid end products. The methyl red indicator in the pH range 4 will turn red which is indicative of positive. At a pH 6, still indicating the presence of acid ,due to the lowest ion concentration the indicator turns yellow and is a negative test.

PROCEDURE

Glucose phosphate peptone broth was inoculated with sample. It was then kept for incubation at 37ºC for 24 hours, and the tubes were added with methyl red indicator after incubation. The tubes were then observed for red colour formation.

3. VOGES –PROSKAUER TEST

To determine the ability of the organisms to produce neutral end product acetyl methyl carbinol (acetoin) from glucose fermentation the reagent used in this test consists of a mixture of alpha naphthol and 40% KOH solution. Detection of acetyl methyl carbinol requires that –this end product be oxidized to a diacetyl compound. This reaction will occur in the presence of alpha naphthol catalase and guanidine group that is present in the glucose phosphate peptone broth.

PROCEDURE

Inoculate pure culture of the test organism into broth and incubate for 24 hours at 37°C. A few drops of Barritt's reagent A & B were added to test tubes.The tubes are shaken gently to expose the medium to atmospheric oxygen and allowed the tube to remain undisturbed for 10 to 15 minutes and the colour change was observed.

4. CITRATE UTILIZATION TEST

The test is based on the ability of an organism to utilize citrate as its sole source of carbon and ammonia as its only source of nitrogen. The test organism was inoculated into a medium containing sodium citrate, an ammonium salt and the indicator bromothymol blue. Growth in the medium is shown by a change in colour of the indicator from light green to blue, due to alkaline reaction following citrate utilization.

PROCEDURE

The test organisms were inoculated into Simmon's citrate agar slant and incubated for 24-48 hours at 37ºC and the colour change was observed.

5.ANTIMICROBIAL ACTIVITY OF CHITOSAN NANOPARTICLES

Antimicrobial study was performed using well diffusion method against *Escherichia coli* sample. Varying concentration of chitosan nanoparticles (0.001%, 0.002%, 0.003%) were used for the study of MIC. 1% glacial acetic acid was used as a control.

RESULT

1. A clear solution with chitosan nanoparticle was obtained after magnetic stirring.

Figure 4: Chitosan synthesised from shrimp shell

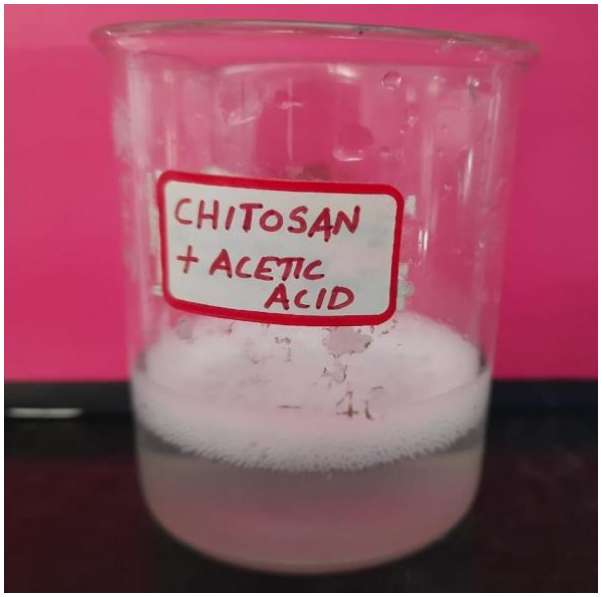

Figure 5: Clear solution with chitosan nanoparticle

2. A uv – visible spectrum of chitosan nanoparticles obtained a sharp intensity because of formation of nanoparticles. The absorption peak of chitosan nanoparticles was obtained at 200 nm in the uv region.

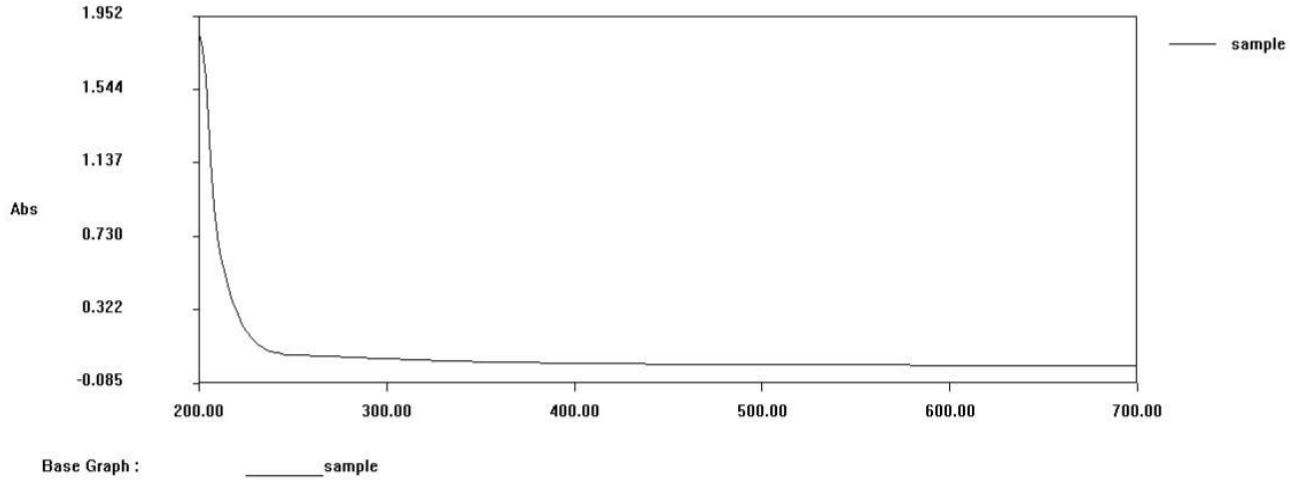

Figure 6: UV- Spectroscopy analysis of Chitosan nanoparticles.

3. On Nutrient Agar, after an incubation of 48 hours at 37°C, well isolated discrete colonies of bacteria were obtained.

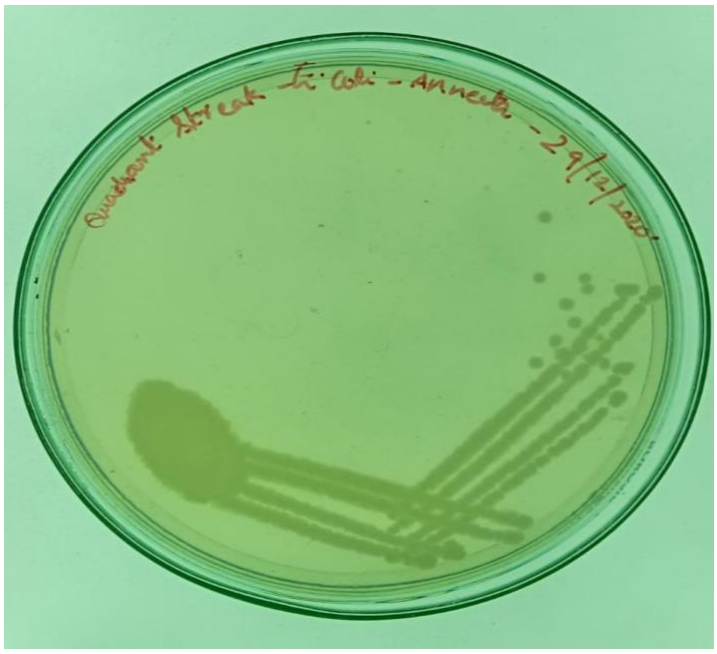

Figure 7: Isolation of bacteria on nutrient agar plate

4. Red colour Gram negative rod shaped bacteria were observed.

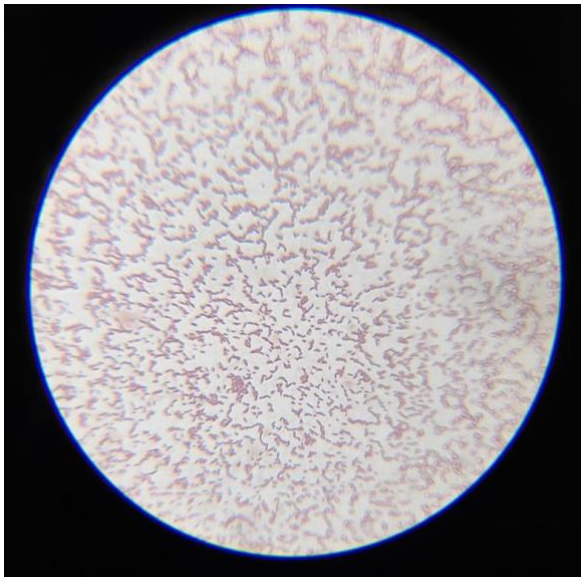

Figure 8: Gram staining

BIOCHEMICAL TESTS

Table 1:Biochmical tests performed for identification of the given *E. coli* bacteria.

BIOCHEMICAL TEST	OBSERVATION	INFERENCE
INDOLE TEST	Red colour ring is not formed.	Negative
CITRATE TEST	Blue colour is formed.	Positive

VOGES – PROSKAUER TEST	No Red colour is formed.	Negative
METHYL RED TEST	No Red colour is formed.	Negative

The given organism were identified on the basis of gram staining and biochemical analysis. Organism were identified as *Escherichia* species .

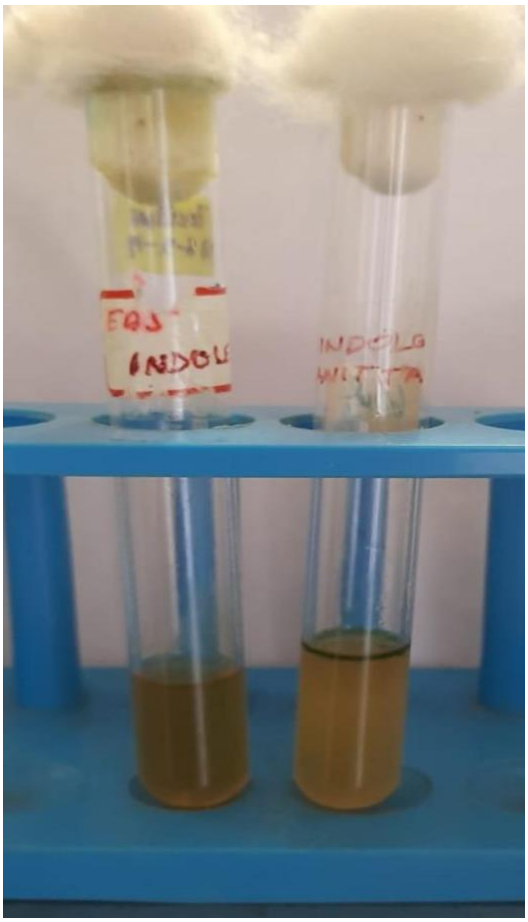

Figure 9: Indole test

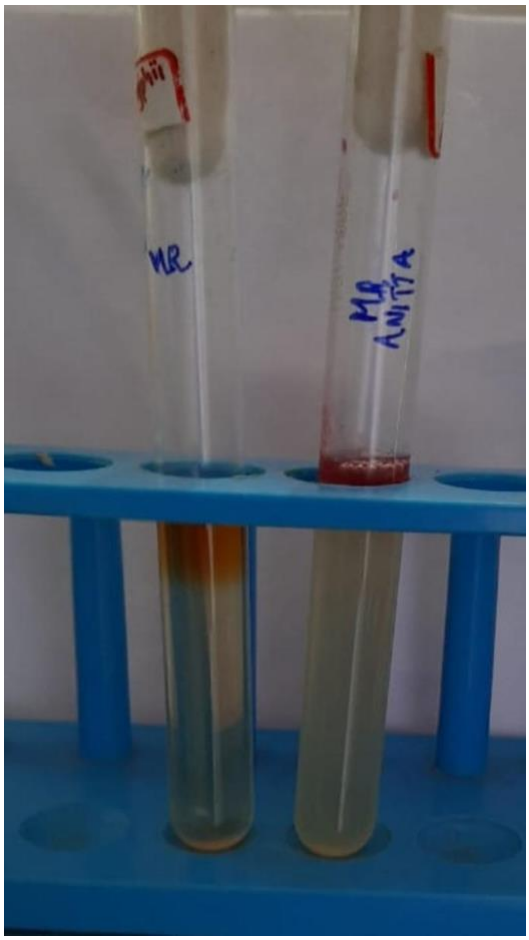

Figure10: Methyl Red test

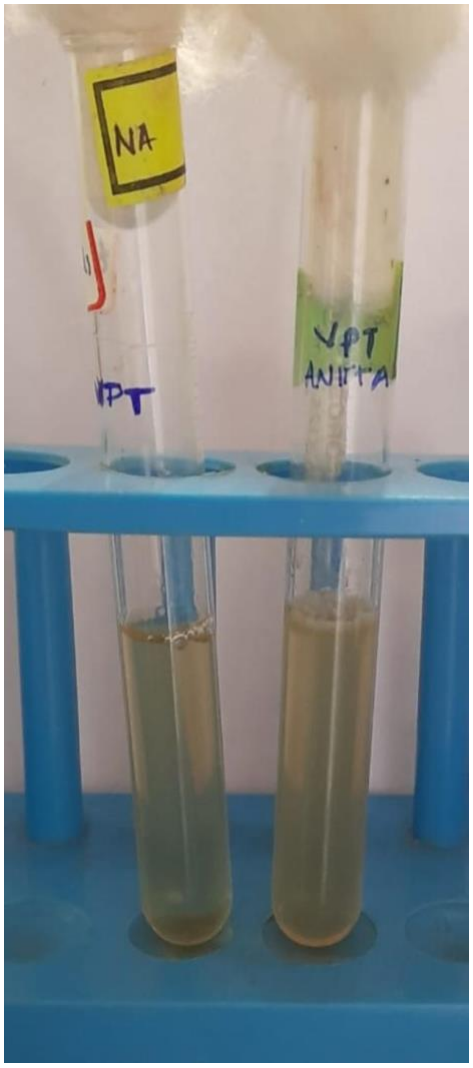

Figure 11: Voges-Proskauer test

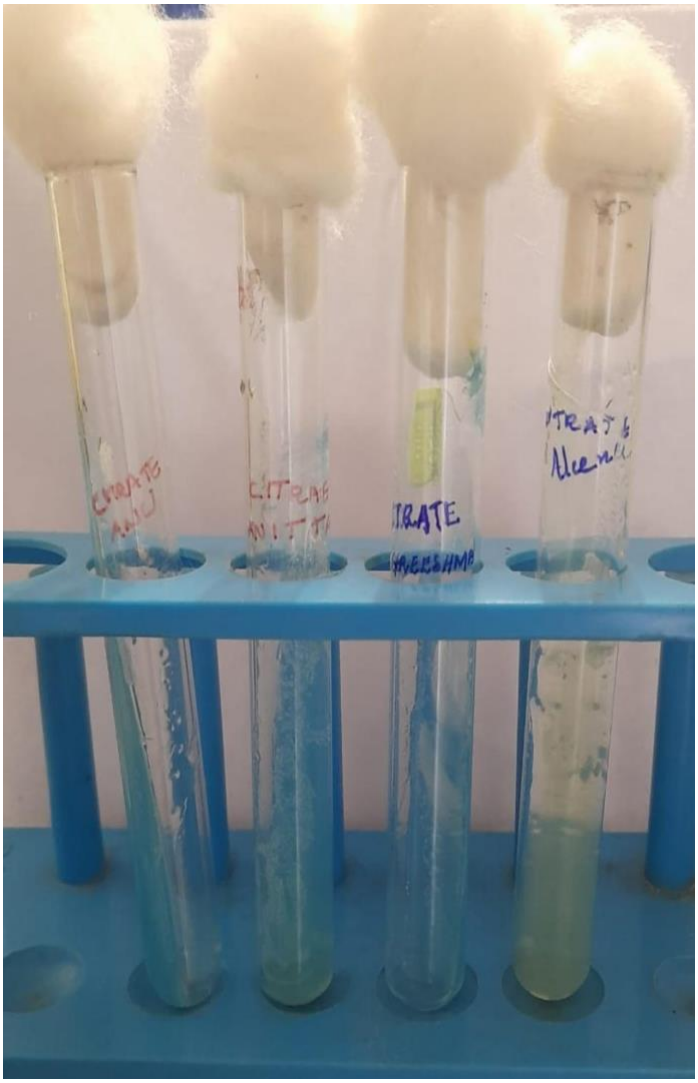

Figure 12: Citrate test

5. Chitosan nanoparticles produce a zone of inhibition of 2.4cm, 2.7cm and 3.0cm against *Escherichia coli* bacteria.

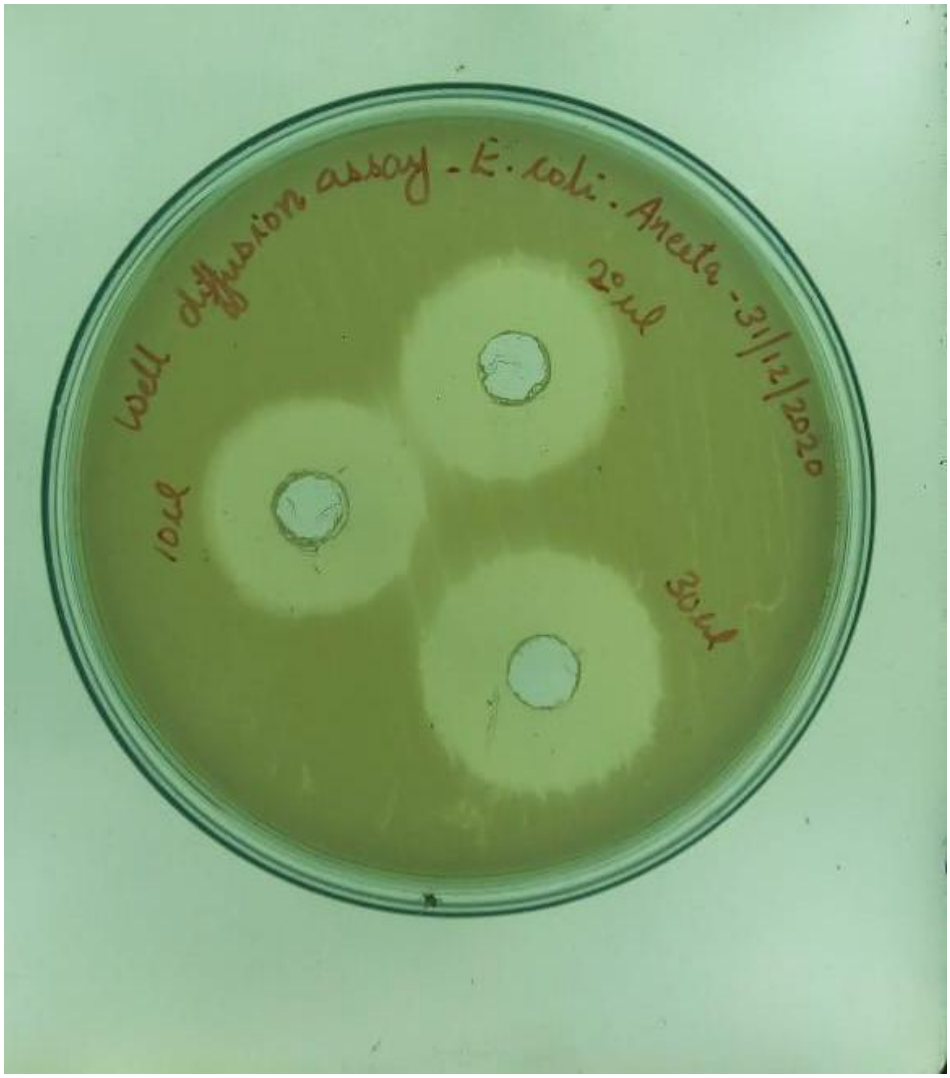

Figure 13: Antimicrobial activity by well diffusion method.

DISCUSSION

Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. Chitosan is also found in some microorganisms, yeast and fungi (Illum L, 1998).Molecular weight and degree of deacetylation are the main factors affecting the particle size, particles formation and aggregation. Chitosan possesses some ideal properties of polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic, and inexpensive. These properties render chitosan a very attractive material as a drug delivery carrier. In the last two decades, chitosan nanoparticles have been extensively developed and explored for pharmaceutical applications (Roberts G, 1992).

Nanoparticles are solid colloidal particles with diameters ranging from 1-1000 nm. They consist of macromolecular materials and can be used therapeutically as adjuvant in vaccines or drug carriers in which the active ingredient is dissolved, entrapped, encapsulated, adsorbed or chemically attached. Polymers used to form nanoparticles can be both synthetic and natural polymers. (Allemann E *et al*., 1993).

The present study focuses on the synthesis of chitosan nanoparticles its Characterisation and application. Chitosan nanoparticles are synthesised into desired size and surface charge using sodium Tripolyphosphate (STPP) initiated ionic gelation process. These chitosan nanoparticles were characterised by UV- VIS spectroscopy to know the kinetic behaviour that ranges between 200 to 700 nm and obtained an absorption peak at 200nm in the UV-region. Chitosan nanoparticles exhibit potential antimicrobial activity as their unique character . The antimicrobial properties of chitosan nanoparticles were studied as its application and it was done in *Escherichia coli* bacteria .This was performed using well diffusion method against *Escherichia coli* sample and a zone of inhibition of 2.4cm, 2.7cm and 3.0cm was produced by the synthesised chitosan nanoparticle in micro titre plate along with chitosan solution alone.

Several studies have shown chitosan to exhibit higher antibacterial activity against gram positive bacteria than gram negative bacteria, while some other studies have shown that gram negative bacteria as more susceptible than gram positive . Still many works have demonstrated that there is no significant difference between the antibacterial activity and bacterial species (K.Divya *et al*.,2016).

Chitosan nanoparticles was prepared by Ionic Gelation technique. This technique was first reported in 1997 .In this method chitosan is dissolved in acetic acid of appropriate concentration. As cross-linking agent used Sodium Tripolyphosphate(TPP) is most of research. The interaction of chitosan and sodium Tripolyphosphate (TPP) because of oppositely charged particles can be controlled by pH of the solution. Sudha. P. N. *et al* prepared nanochitosan by this technique;1gm of chitosan dissolved in 200ml. Of 2% acetic acid solution was stirred for 15 min. To get a homogeneous viscous gel. Then add .8 gm of TPP dissolved in 170 ml. Of conductivity water was added drop wise. Milky solution obtained ,this solution allow to rest and settled as suspension by adding conductivity water in excess for 24 hr. Chitosan nanoparticles were obtained . A mentioned above one technique but used different process for finding chitosan nanoparticles. The author's experience in this field and other works available in the literature, have concluded that the properties of chitosan nanoparticles obtained through interaction between chitosan and TPP are dependent on many parameters inherent to the preparation method. When increases the addition of cross-linking agent (TPP) increases the adsorption capacity of chitosan.

Surendra Nimesh described in his paper the reason of interaction, Due to the charge neutralization, polyelectrolyte complex are self-assembled and it leads to the rise in hydrophilicity. So nano formulated can be sizes from 50 to 700 nm. Many cationic polymers (i.e. gelatin, chitosan, polyethylenimine) also give this property. One author explains this method with the chitosan. The nanoparticles were spontaneously formed after addition of alginate solution into chitosan which was priorly dissolved in acetic acid solution, under mechanical stirring at room temperature. The complexes size range from 50 nm to 700 nm. Another study was done where author Nam *et al*. Used low molecular weight water soluble chitosan (LMWSC) nano carriers were developed by the similar methods for insulin delivery . The above method reported size of nanoparticles approximately 200 nm.

A study conducted by Lifeng Qi *et al* (2004) reported that chitosan is only soluble in acidic media, the precipitations of chitosan solution in acetic acid occurred upon addition to bacterial suspension, while chitosan nanoparticles could be well distributed in bacterial suspension after a slight shock for a nice dispersion. Bacteria can adhere to the surface of chitosan and chitosan nanoparticles significantly in short time of just 30min; thus chitosan and chitosan nanoparticles exhibit antimicrobial activity. According to the literature , chitosan possess antimicrobial activity against a number of Gram-negative and Gram-positive bacteria. The antibacterial activity of chitosan nanoparticles and copper loaded nanoparticles were compared with that of

chitosan in distilled water or 0.25% acetic acid, respectively. According to the data, the antibacterial activity of chitosan nanoparticles and copper-loaded nanoparticles are significantly higher than that of chitosan and doxycycline. Furthermore, the test samples exhibit lower antimicrobial activity in water compared with that of samples in 0.25% acetic acid. Moreover, the MIC and MBC values of copper-loaded nanoparticles against some bacteria are lower than those of chitosan nanoparticles, which indicate higher antimicrobial activity.

CONCLUSION

The current study entitled " Characterisation and Application of Synthesised Chitosan nanoparticles against *Escherichia coli* bacteria", indicates that the Chitosan nanoparticles synthesized from chitin using sodium Tripolyphosphate (STPP) showed it's applicability as an effective Antimicrobial agent. It was observed that preparing chitosan nanoparticles is a technology that requires a suitable technique among the various methods. Chitosan nanoparticles offer many advantages. Chitosan is a safe natural polymer material because of its two main properties: biocompatibility and biodegradable. Chitosan nanoparticles are suitable broad area of drugs including labile drugs and macromolecules.

Chitosan nanoparticles was prepared by Ionic Gelation technique. The prepared chitosan nanoparticles were characterized by UV- Vis spectroscopy showed the absorption peak of 200 nm. Chitosan nanoparticles exhibit potential antimicrobial activity as their unique character. It showed promising antimicrobial activity against *Escherichia coli* bacteria. This was studied by reading the zone of inhibition on the growth of *Escherichia coli* by the synthesised chitosan nanoparticle in micro titre plate along with chitosan solution alone. These studies show that chitosan nanoparticles could inhibit the growth of various microorganisms markedly and exhibit higher antimicrobial activity. It is anticipated that chitosan nanoparticles could be applied broadly as antimicrobial agents in medicine for their high antibacterial activity and acceptable biocompatibilities The current study gives an overview about the application of nano chitosan and also describes the method for preparing nano chitosan..

REFERENCE

1. Ahmed M. Khalil, Reham A. Abdel-Monem, Osama M. Darwesh, Ahmed I. Hashim, Afaf A. Nada, Samira T. Rabie, (2017)"Synthesis, Characterization, and Evaluation of Antimicrobial Activities of Chitosan and Carboxymethyl Chitosan Schiff-Base/Silver Nanoparticles", *Journal of Chemistry*, vol. 2017, Article ID 1434320, 11 pages, 2017.
2. A. K. Singla and M.(2001).Chawla. Chitosan: some pharmaceutical and biological aspects. Journal of Pharmacy and Pharmacology , 53: 1047–1067.
3. Alarfaj, A. A. (2019). Antibacterial Effect of Chitosan Nanoparticles against Food Spoilage Bacteria. *J Pure Appl Microbiol*, *13*(2), 1273-1278.
4. D. MubarakAli, F. LewisOscar, V. Gopinath, Naify S. Alharbi, Sulaiman Ali Alharbi, N. Thajuddin.(2018),An inhibitory action of chitosan nanoparticles against pathogenic bacteria and fungi and their potential applications as biocompatible antioxidants, Microbial Pathogenesis, Volume 114,2018,Pages 323-327.
5. E. I. Rabea, M. E. T. Badawy, C. V. Stevens, G. Smagghe, and W. Steurbaut.(2003). "Chitosan as antimicrobial agent: applications and mode of action," Biomacromolecules, vol. 4, no. 6, pp.1457–1465.
6. Gilles Dupuis and Jean-Guy LeHoux.(2007). Recovery of chitosan from aqueous acidic solutions by salting-out. Part 2: Use of salts of organic acids. Carbohydrate Polymers 68, 287–294.
7. H. K. No and S. P. Meyers.(1997) ."Preparation of chitin and chitosan," in Chitin Handbook, R. A. A. Muzzarelli and M. G. Peter, Eds., pp. 475–489, European Chitin Society, Grottammare, Italy.
8. Hyeon,T.(2003).Chemical synthesis of magnetic nanoparticles. Chemical Communications, (8), 927-934.
9. Huh, A.J. and Kwon, Y.J. (2011) "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. J. Control. Release 156, 128–145.
10. Islem Younes and Marguerite Rinaudo.(2015).Chitin and Chitosan Preparation from Marine Sources: Structure, Properties and Applications.Mar. Drugs, 13, 1133-1174.
11. Jaswal, V. S., Arora, A. K., Kinger, M., Gupta, V. D., & Singh, J. (2014). Synthesis and characterization of chromium oxide nanoparticles. Oriental Journal of Chemistry, 30(2), 559-566.

12. K. Divya, Smitha Vijayan, Tijith K. George, and M. S. Jisha.(2017). Antimicrobial Properties of Chitosan Nanoparticles: Mode of Action and Factors Affecting Activity.Fibers and Polymers , Vol.18, No.2, 221-230.
13. Kain, D., & Kumar, S. (2020). Synthesis and characterization of chitosan nanoparticles of Achillea millefolium L. and their activities. *F1000Research*, *9*(1297), 1297.
14. Kumirska, J., Weinhold, M. X., Thöming, J., & Stepnowski, P. (2011). Biomedical activity of chitin/chitosan based materials—influence of physicochemical properties apart from molecular weight and degree of N-acetylation. Polymers, 3(4), 1875-1901.
15. Kurita, K. (2006). Chitin and chitosan: functional biopolymers from marine crustaceans. Marine Biotechnology, 8, 203–226.
16. LeHoux, J. G., & Dupuis, G. (2007). Recovery of chitosan from aqueous acidic solutions by salting-out. Part 1. Use of inorganic salts. Carbohydrate Polymers, 68, 295–304.
17. Mohamed E. I. Badawy and Entsar I. Rabea.(2011).A Biopolymer Chitosan and Its Derivatives as Promising Antimicrobial Agents against Plant Pathogens and Their Applications in Crop Protection. International Journal of Carbohydrate Chemistry Volume, Article ID 460381.
18. M. Raffi, S. Mehrwan, T. M. Bhatti, J. I. Akhter, A.Hameed, W. Yawar, and M. M. Ul Hasan.(2010) .Ann. Microbiol.,60, 75 .
19. Phoenix, C., & Drexler, E. (2004). Safe exponential manufacturing. Nanotechnology, 15(8), 869.
20. Ravi Kumar, M.N.V.; Muzzarelli, R.A.A.; Muzzarelli, C.; Sashiwa, H.A.; Domb, J.(2004).Chitosan chemistry and pharmaceutical perspectives. Chem. Rev. 104, 6017–6084.
21. Ramesh, C., Mohan Kumar, K., Latha, N., & Ragunathan, V. (2012). Green synthesis of Cr2O3 nanoparticles using Tridax procumbens leaf extract and its antibacterial activity on *Escherichia coli.* Current Nanoscience, 8(4), 603-607.
22. R. A. A. Muzzarelli.(1983).“Chitin and its derivatives: new trends of applied research,” Carbohydrate Polymers, vol. 3, no. 1, pp.53–75.
23. R. C. Goy, D. De Britto, and O. B. G. Assis.(2009). “A review of the antimicrobial activity of chitosan,” Polimeros, vol. 19, no. 3, pp. 241–247.
24. Rinaudo, M.(2006). Chitin and chitosan: Properties and applications. Prog. Polym. Sci, 31, 603–632.

25. Russell JB, Jarvis GN (April 2001). “Practical mechanisms for interrupting the oral-fecal lifecycle of *Escherichia coli*”. Journal of Molecular Microbiology and Biotechnology. 3(2): 265–72.
26. S. Islam M. A., Rahman Bhuiyan and M. N. Islam.(2017). Chitin and Chitosan: Structure, Properties and Applications in Biomedical Engineering. J Polym Environ, 25:854–866.
27. Salata, O.(2004). Applications of nanoparticles in biology and medicine. J Nanobiotechnol 2, 3.
28. Singh, G., Vajpayee, P., Khatoon, I., Jyoti, A., Dhawan, A., Gupta, K. C., & Shanker, R. (2011). Chromium oxide nano-particles induce stress in bacteria: probing cell viability. Journal of biomedical nanotechnology, 7(1), 166-167.
29. Tanima Banerjee, Susmita Mitra, Ajay Kumar Singh, Rakesh Kumar Sharma Amarnath Maitra.(2002). Preparation, characterization and biodistribution of ultrafine chitosan nanoparticles. International Journal of Pharmaceutics,Volume 243,Pages 93-105.
30. Tankeshwar Acharya (July 16, 2016). Streak plate method; principle, purpose, procedure and results. Microbeonline.com.
31. Tenaillon O, Skurnik D, Picard B, Denamur E (March 2010). “The population genetics of commensal *Escherichia coli*”. Nature Reviews. Microbiology. 8 (3): 207–17.
32. Vlab.amrita.edu.,(2011) . Streak plate method. Retrieved 17 March 2021.
33. Whitesides, G. M. (2003). The’right’size in nanobiotechnology. Nature biotechnology, 21(10), 1161-1165.
34. Whitesides, G.M. (2005) Nanoscience, nanotechnology, and chemistry. Small 1, 172–179.
35. Xia, Y. (2008).Nanomaterials at work in biomedical research. Nat. Mater. 7, 758–760.
36. Yan Wu, Wuli Yang, Changchun Wang, Jianhua Hu and Shoukuan Fu.(2005).Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate.International Journal of Pharmaceutics,Volume 295,Pages 235-24.
37. Yeul VS, Rayalu SS (2013) J Polym Environ 21(2):606–614.
38. Zhong, Z.; Chen, R.; Xing, R.; Chen, X.; Liu, S.; Guo, Z.; Ji, X.; Wang, L.; Li, P.(2007). Synthesis and antifungal properties of sulfanilamide derivatives of chitosan. Carbohydr. Res, 342, 2390–2395.

APPENDIX

CULTURE MEDIAS

1. Nutrient Agar

Peptone - 5gm

Beef Extract – 3gm

NaCl -5gm

AAgar-15g

Distilled Water -1000ml

2.MR –VP Broth

Peptone – 7gm

Potassium phosphate – 5gm

Dextrose -5gm

Distilled water – 1000ml

3.Peptone water

Peptone – 1gm

NaCl – 0.5gm

Distilled water-100ml

4. Simmons citrate agar (pH -6.9)

Ammonium dihydrogen phosphate -1 gm

Dipotassium hydrogen phosphate -1gm

NaCl -5gm

Sodium citrate-2gm

Magnesium sulphate-0.2gm

Bromothymol blue-0.08gm

Agar -15gm

Distilled water-1000ml