IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Coalescence of Chitosan Nanoparticles; Their Characterization and Efficacy against Fish Pathogen

Extraction and characterization of chitosan from shrimp waste and preparation and structural elucidation of chitosan nanoparticles for testing their efficacy against fish pathogen isolated in the study

[1]Anitha Abraham, [2]Achamma Thomas, [3]Laxmi M*, [4]Aleena Benny

[1]Assistant Professor of Zoology, [2]Chief Executive Officer, Trophic Biosystems Pvt Ltd, Cochin, [3]Scientist in charge*, Trophic biosystems Pvt Ltd, Cochin, [4]Research associate, Trophic Biosystems Pvt Ltd, Cochin

[1]Maharaja's College, Ernakulam, Kerala, 682011, India

***Abstract*:** Nanotechnology is a budding avenue utilized in disease prevention and treatment. Chitosan is an innate, biocompatible, biodegradable, nontoxic and feasible polymer that can be exploited for nanoparticle preparation. Chitosan nanoparticles (ChNP) are extensively used in pharmaceutical industries as an antimicrobial agent or as drug delivery vehicle. The present study was performed out to gauge the antimicrobial activity of CNP against various microorganisms isolated from diseased or healthy Nile tilapia, *Oreochromis niloticus*. The chitosan was extracted from shrimp shells and the ChNPs were synthesized by a novel method of ionic gelation with sodium tripolyphosphate (TPP) as a reducing agent. The physico-chemical characterization such as Chitosan yield (33%), average molecular weight (1,700,531.016 Da), degree of deacetylation (86.57%), pH (8) and ash value (0.39%) of prepared nanoparticles were found out to be similar with that of previous standard reports. The chitosan was morphologically characterized by Fourier transform infra red (FTIR) spectra and showed the characteristic presence of hydroxyl groups, bromoalkanes and primary alcohols. The antibacterial activity of CNPs against the isolated multidrug resistant (MDR) fish pathogen, *Aeromonas hydrophila* (MAR index-80%) was evaluated by well diffusion assay and compared with chitosan. CNP (24.5mm) exhibited superior antimicrobial activity against the MDR isolate on comparison with chitosan (14.2mm). Minimum inhibitory concentration analysis using resazurin assay revealed that, concentration required for pathogen inhibition was approximately the same for both antibiotic and CNP (62.5µg/mL). Accordingly, it can be anticipated that ChNPs can compete with and diminish the use of antibiotics in aquaculture.

Keywords: **Chitosan, Chitosan nanoparticles (ChNPs), Antimicrobial activity, FTIR, Multi Drug Resistant (MDR) fish pathogen, Resazurin assay, Antibiotics**

I. INTRODUCTION

Modest bioactive ingredients have gained plentiful attention in topical years from the scientific community, consumers and food manufacturers. Potential biologically active ingredients are vitamins, probiotics, peptides, antioxidants, etc. Scientific indications to support the concept of health-promoting ingredients are fattening unwaveringly. Innovative functional foods that convoy physiological benefits or lessen long-term risks of developing diseases are being developed by the scientific community. Nevertheless due to the instability of functional ingredients under conditions encountered during processing (temperature, oxygen, light) or in the gut (pH, enzymes, presence of other nutrients), it is strenuous to retain the health benefits of usable innards. Therefore, it is necessary to protect these molecules from harsh conditions by encapsulation or immobilization. Not long ago, various micro/nano carriers have become revolutionizing almost all realms in life sciences (Thiruchenduran *et al*., 2018).

For the time being, the exertion of nanomaterials is acquiring wider attention in pharmaceutical and biomedical research. Nanoparticles (NPs) of less than 100 nm in size present enhanced ability to get better patient compliance, enhanced biodistribution, and site-specific drug delivery. Several advanced nanomaterials are executed in the biomedical and pharmaceutical industries. These advanced functional nanomaterials include magnetic nanoparticles, silica-based nanomaterials, metal and metal-oxide nanomaterials,

and biological and carbon nanostructures, of name a few. Engineered nanomaterial-based biomedical device and biosensors can accomplish a new level of sensitivity, selectivity, effectiveness, and biological stability for biological application. Furthermore, nanomaterials are becoming an eco-friendly and cost-effective option for efficient biomedical applications in gene delivery and transfection, as well as drug delivery carriers and antibacterial agents, for wound healing, in nano systems against cancer and as therapeutic delivery systems (Reddy *et al*., 2012). Applications of biopolymers like starch, cellulose, silk fibroins, collagen, gelatin, albumin, and chitosan (Ch)-based nanomaterials endow the synthetic NPs with biocompatibility, biodegradability, and low toxicity. Biocompatible nanomaterials with raised specific surface area are desirable in a plethora of biological and biomedical applications, such as drug delivery, therapeutics, and gene delivery. In the latest years, several studies focused on the advances in this field, leading to substitute biocompatible nanomaterials considering the use of alternative resources, contemporory properties, and clampdowns.

Chitin is the excoriated material used for the production of chitosan. The primal materials used are the shells of crustaceans, mainly crabs and shrimps. The thin shrimp shells can be effortlessly be purified during the process. It can be used for biomedical purposes as well as for pharmaceutical purposes. If not, they can cause serious side effects. The end products should be exceedingly purified for better yield. Chitosan is a crucial linear polysaccharide cationic and hydrophilic polymer, obtained by alkaline hydrolysis of chitin; it is a non-toxic, biocompatible polymer consisting of randomly distributed β-(1, 4)-linked d-glucosamine (deacetylated) and N-acetyl-d-glucosamine (acetylated) units. Chitin is one of the most abundant natural amino polysaccharides obtained from the components of cell walls in fungi, and certain hard structures in invertebrates and fish. Chitosan nanoparticles (ChNPs) have an abundance of hydroxyl (–OH) and amine (–NH2) functional groups, which can be employed to react with cross-linking agents for in situ chemical cross-linking. CNPs are not only biocompatible and non-toxic; it is also biodegradable by certain enzymes into non-toxic oligosaccharides, making Chitosan appropriate for clinical use. In the biomedical application arena, they revealed great success as antimicrobial agents, as well as for membrane separation, as carriers for drug delivery, as sensing materials for biomolecule monitoring, and in tissue engineering. Additionally, Chitosan derivatives and Chitosan nanoparticles (ChNPs) depicted excellent performance in ophthalmology, dentistry, bio-imaging, bio-sensing, and diagnosis. Historically, Ch, derivatives and ChNPs are among the most extensively studied class of natural biopolymer materials for biomedical applications (Mohebbi *et al*., 2019).

Chitosan is considered as safe for human dietary use and in wound dressing applications. Nanoparticles can be obtained from chitosan by various routes of administration like carrier in drug delivery. Nanoparticles prepared from chitosan and chitosan derivatives possess a positive charge and mucoadhesive properties so that they can adhere to mucus membrane and carry out various processes. Chitosan based nanoparticles have different applications in the treatment of cancer, drug delivery, gastrointestinal diseases, pulmonary diseases and other infections. Because of its cationic functionality and solubility in the aqueous medium, chitosan become the most popular natural polymer and has wide varieties of applications in many fields, mainly in drug delivery. ChNPs, in the field of polymeric therapeutics, are availed for the treatment of pharmacological and medicinal release to the targeted site, especially due to their good biodistribution, as well as the sensitivity and specificity (Dash *et al*., 2011). For the large scale production of chitosan, chitin from shrimp and crab shells was basically used as raw material, which required high production cost and multiple chemical processes such as demineralization, deproteinization, and decolourization. Gladius of squid is another rich source of chitosan, which is a transparent material thrown as waste from seafood processing industries. Chitosan production is cost-effective and prevents the usage of excess acids and alkaline pollutants due to its low impurities and absence of coloured compounds. In addition, it shows better reactivity, solubility, and swelling than from other sources due to much weaker molecular hydrogen bonding. Most frequently, nanoparticles were synthesized according to bottom-up approach as a result of self-assembling or cross-linking process. Nanoparticles synthesized from chitosan have been widely studied as a bio-compatible alternative for metal nanoparticles in biological applications. Ch is made of non-toxic monomeric units, and their environmental degradation leads to non-toxic by-products. Consequently, research on Ch and its derivatives found its niche in the main area of clinical research and biomedical applications (Anusha & Fleming, 2016).

In the current study, the chitosan nanoaggregate had been extracted from the shrimp. The shrimp shells were subjected to deproteinization and demineralization to form *β*-chitin and further deacetylated to chitosan. The nanoaggregate of chitosan was then synthesized. The physical and chemical properties of as-prepared chitosan nanoaggregate were analyzed. Special attention was dedicated to the analysis of the antimicrobial, antioxidant, and cytotoxic activities in place to identify the bioactive potential of synthesized nanostructure.

II. MATERIALS AND METHODS

2.1. Sample collection

Local shrimp waste was collected, and the shells are washed thoroughly with water to remove impurities in a hot-air oven at 90 ◦C for 6 h. For chitin and chitosan productions, shells are homogenized in a blender into small sized pieces (<20 mesh). This material is kept frozen until used. The grounded material was transformed into chitosan via three successive steps (demineralization, Deproteinization, and Deacetylation).

2.2. Preparation of chitosan from chitin (Yuan *et al*., 2011)

2.2.1. Demineralization

Demineralization is carried out by adding 1 L of 1 M HCl to 100 g of shrimp shells. The reaction proceeded at room temperature under agitation at 250 rpm for predetermined times (0.5, 2, or 6 h). Afterwards, the demineralized shells are filtrated and washed with distilled water until neutral pH. They are bleached by immersing in ethanol for 10 min and dried in an oven at 70^0C.

2.2.2. Deproteinization

Deproteinization is performed by adding 1 M NaOH to the dried demineralized shells at a solid/liquid ratio of 1:10 (g/mL). Reaction is carried out under agitation at 80^0C for 3 h. The solid is filtrated and washed with distilled water until it achieved neutral pH. Then, it is immersed in ethanol for 10 min for further bleaching, and the resulting chitin is dried in an oven at 70^0C.

2.2.3. Deacetylation

Deacetylation of chitin is achieved by reacting chitin with 12.5 M NaOH at a solid/liquid ratio of 1:15 (g/mL).The reaction mixture is cooled down and kept frozen at −83 ◦C in an ultra-freezer for 24 h. Afterwards, the temperature of the mixture is raised to 115^0C,

and the reaction proceeded with agitation at 250 rpm for 4 or 6 h. The resulting chitosan is filtrated, washed with distilled water until neutral pH and dried in an oven at 70^0C.

2.2.4. Physico chemical characterization

2.2.4.1. pH

The pH measurements of the chitosan solutions will be carried out using a microprocessor pH meter (Fang *et al.,* 2010).

2.2.4.2. Ash value of chitosan

To determine the ash value of chitosan, 1.0 g of chitosan sample is placed and accurately weighed into clean, dry, pre-weighed porcelain crucible. The samples are heated in a muffle furnace preheated to 650°C for 4 h. The crucibles are allowed to cool in the furnace to less than 200°C and then placed into desiccators with a vented top. Percentage of ash value is calculated using the following (Qi and Xu, 2004):

Ash % = $(W_1\text{-}W_2) / W_1 \times 100$

where W1and W2 in grams are the weight of chitosan before and after ignition, respectively.

2.2.4.3. Determination of degree of Deacetylation (DA)

The degree of DA was measured by the acid-base titration method with modifications. In brief, chitosan (0.1 g) was dissolved in 30 ml HCl aqueous solution (0.1 mol/L) at room temperature with 2–3 drops of methyl orange added. The red chitosan solution was titrated with 0.1 mol/L NaOH solution until it turned orange. The DA was calculated by the formula:

DA % = $(C_1V_1 \times C_2V_2 / M \times 0.0994) \times 0.016$

where C1= concentration of standard HCl aqueous solution (mol/l), C2= standard NaOH solution (mol/l),V1= volume of the standard HCl aqueous solution used to dissolve chitosan (ml), V2=volume of standard NaOH solution consumed during titration (ml), and M= weight of chitosan (g). The number 0.016 (g) is the equivalent weight of NH_2 group in 1 ml of standard 1 mol/l HCl aqueous solution and 0.0994 is the proportion of NH_2group by weight of chitosan (Hossain and Iqbal, 2014).

2.2.4.4. Determination of molecular weight

Molecular weight was determined using viscosity measurement method; the viscosity was assessed using a Brookfield viscometer. In brief, 1% chitosan solution was prepared using 1% acetic acid. The measurement was made using a No. 5 spindle at 50 rpm at 25°C with values reported in centipoises (cps) units. Intrinsic viscosity was used to calculate the average molecular weight of the prepared samples using Mark-Houwink Sakurada equation: $\eta=KMa$ where K and 'a' are the constants for a given solute–solvent system is0.078 and 0.76, respectively (Alishahi *et al*., 2011).

2.3. Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by dissolving at 0.5 g of prepared chitosan in 100 mL of 1% acetic acid (v/v), and the pH of the solution was adjusted to be 4.6– 4.8 using 1N NaOH. Chitosan nanoparticles were formed spontaneously upon addition of 3 ml chitosan solution under vigorous magnetic stirring to 1 ml of an aqueous TPP (Sodium tripolyphosphate) solution (0.25% w/v), with a ratio of chitosan to TPP 3:1 at room temperature. Nanoparticles were separated by centrifugation at 9000×gfor 20 min. Chitosan nanoparticles were extensively rinsed with DI to remove any NaOH residues and dried at 40°C for 24 h before further use or analysis (Vaezifar *et al*., 2013).

2.4. Characterization of chitosan nanoparticles (Hu *et al.,* 2002)

2.4.1. UV-visible spectral analysis

UV-visible spectral analysis was measured in aqueous solution by using UV-VIS spectrometer with a resolution of 1 nm between 200 and 600 nm to confirm the formation of nanoparticles.

2.4.2. FTIR analyses of chitosan and chitosan TPP nanoparticles

Fourier transform infrared (FTIR) spectra were collected using a Perkin Elmer Spectrum One FTIR spectrophotometer (Perkin Elmer) using the KBr pellet method with a range of 4000–400 cm^{-1}. The surface morphology and atomic ratio of the NPs was determined by using FESEM-EDX (ZEISS supra 40VP, Oberkochen, Germany). The magnetic properties of the prepared Fe_3O_4 was revealed using a vibrating sample magnetometer (VSM, Lake Shore 7404, McCorkle Boulevard, Westerville, OH, USA) at room temperature 300 K. The magnetization measurements, M_s as a function of applied field (H) were measured under external magnetic fields up to ±14,000 Oe.

FTIR spectra of both chitosan and chitosan TPP nanoparticles were performed separately for comparison.

2.5. Isolation of pathogenic bacteria

2.5.1. Isolation of fish pathogen

The diseased fish was collected from fish farm and asceptically transprted to the laboratory. Pathogen was isolated from homogenized gut samples by spread plate - dilution technique into Thiosulfate-citrate-bile salts-sucrose agar (TCBS). The isolated colony was streaked on to nutrient agar plate and preserved on nutrient slant for further analysis (Madden, 2013).

2.6. Identification of fish pathogens

Colonies characteristics such as shape, size, margin and pigmentation were noted for the colonies obtained on Nutrient agar plates. Gram staining was done as differential staining and wet mount method was done to analyse the motility (Aneja, 2007). Genomic DNA was isolated and purified with the help of MagGenome XpressDNA Bacteria Kit (Mag genome, Cochin). The quality of the DNA isolated was checked using agarose gel electrophoresis. The gel was placed under UV transilluminator and DNA bands were visualized. Genomic DNA is further used for PCR amplification. PCR is mainly done using EMERALD KIT which consists of emerald mix. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Sequencing was performed by Agri Genome Kochi. The nucleotide sequence analysis (www.ncbi.nlm.nih.gov/BLAST) was done to to determine degree of similarity with the closest phylogenetic affiliate in the database. The sequence was compared with the reference sequences in the GenBank database by using the BLAST algorithm (Madden, 2013)

2.7. Antibiotic susceptibility testing and Evaluation of MAR indices

In vitro antibiotic sensitivity tests were done using disc diffusion test following the method described by Kirby-Bauer (Bauer *et al.,* 1966). Depending on the area of the zone diameters for individual antibiotic, the sensitivity was recorded as highly sensitive,

moderately sensitive, less sensitive and resistant as per manufacturer's instructions. Antibiotic sensitivity testing was done using ampicillin (AMP 10 mcg), cefuroxime (CXM 30 mcg), ciprofloxacin (CIP 5 mcg), gentamicin (GEN 10 mcg) and imipenem (IPM 10 mcg) as per CLSI guidelines. The multidrug resistant strain was identified by measuring the zone of inhibition in accordance with CLSI standard chart for *Enterobacteriaceae.* The isolates that showed resistance against three or more antibiotics were considered as MDR. Their multiple antibiotic resistance (MAR) Indices were calculated.

MAR index for each isolate is calculated as: - No. of antibiotics to which the isolate was resistant/ Total no. of antibiotics to which the isolate was subjected (Subramani & Vignesh, 2012).

2.8. Antibacterial activity of chitosan nanoparticles

Nanoparticles were dissolved in DMSO at a concentration of 10mg/mL. Antibacterial activity was determined by agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS). Well containing the same volume of DMSO served as negative control and antibiotic served as positive control. After incubation, the diameters of the growth inhibition zones were measured in mm (Saravanan *et al.,* 2010).

2.9. Assessment of Minimum Inhibitory Concentration (MIC)

MIC was determined by Resazurin method. The minimum concentration of the chitosan nanoparticle which permitted growth and beyond which there was no growth was considered as the minimum inhibitory concentration (MIC) against the strain tested. In order to confirm the results of the screening test, the screened strains were inoculated into nutrient broth, supplemented with varying concentration starting from 1mg/mL using Resazurin plate assay (Anoopkumar-Dukie *et al.,* 2005).

III. RESULTS AND DISCUSSION

3.1. Sample collection

Local shrimp waste was collected and was washed thoroughly with water to remove impurities. The cleaned shrimp shells were dried in a hot-air oven and were homogenized in a blender into small sized pieces (Fig. 1).

Figure 1: Sample collection and processing

3.2. Preparation of chitosan from homogenized shrimp shell

Chitosan preparation was carried out using a three-step process as per section 2.2 (Fig. 2, 3 and 4).

Figure 2: Demineralization of homogenized shrimp shell

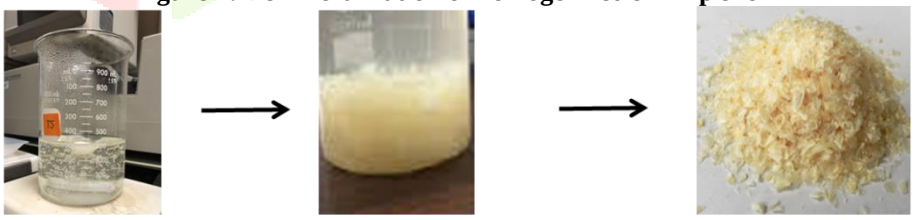


Figure 3: Deproteinization of demineralized shrimp shell

Figure 4: Deacetylation of chitin and preparation of chitosan

3.3. Physico – chemical characteristic of chitosan

The physic chemical analyses of chitosan are stated in table 1.

Table 1: Showing results of physic chemical characterization of chitosan

Characteristics	Chitosan
Chitosan yield	33%
Average molecular weight	1,700,531.016 Da
Degree of deacetylation	86.57%
pH	8
Ash value	0.39%

3.3. Preparation of chitosan nanoparticles

Chitosan nanoparticles prepared in the experiment were found in the form of white powder (Fig. 5).

Figure 5: Chitosan nanoparticle preparation

4.4. Characterization of chitosan nanoparticle

4.4.1. UV-Vis spectroscopy

The Chi -NPs composite exhibited peak at 425 nm is due to the excitation of surface plasmon resonance (SPR). The characteristic SPR band of Chi -NPs composites were an indicative of interaction between amino and hydroxyl groups of chitosan polymer with TPP (Fig. 6).

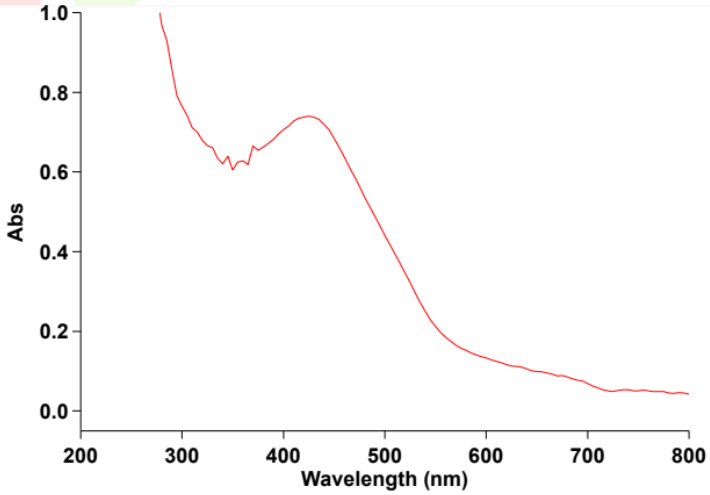


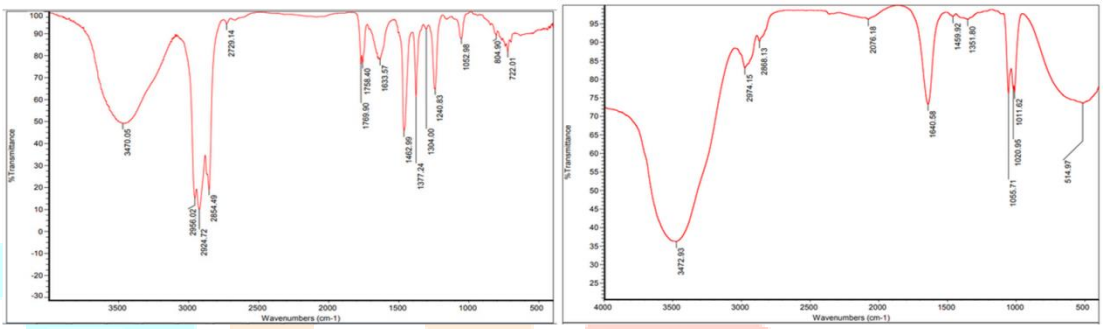
Figure 6: UV-Vis spectrum of chitosan nanoparticle.

3.4.2. FTIR spectra of chitosan and chitin

The FTIR spectrum of the prawn shell chitosan showed bands at 3470, 2924, 2854, 1769, 1633,1462, 1377,1240, 1052 and 722 cm^{-1}. The intense broad peak at 3470.05 cm^{-1} was the characteristic of the hydrogen-bonded OH stretch band. The intense broad band peak was characterized for the hydroxyl functional group in alcohol and phenol compounds. This indicated that chitosan is surrounded by

some proteins and secondary metabolites such as lignin sterols and alkaloids which have functional groups of hydroxyl, amines, alcohols, phenol, and carboxylic acids. The band at 2854.49 cm^{-1} showed CH stretch of CO stretching vibration corresponding to aldehydes compound. The band at 1633.57 cm^{-1} belonged to CO stretch amides compound. The major absorption band was observed between 1052 and 722 .01cm^{-1}, a major group present in chitosan absorption bands for the free amino group. FTIR spectroscopy measurements are carried out to identify the biomolecules that bound specifically on the given chitosan extract (Fig. 7 (a)).

FTIR spectroscopy was used to find out the chemical structure and bonding of CS NPs synthesized from shrimp shells. The FTIR spectrum of the CS NPs showed major absorption bands at 3472, 2974, 2076, 1640, 1459, 1351, 1055 and 514 cm^{-1} respectively. The observed intense and broad absorption band at 3472.93 cm^{-1} was due to the stretching vibration of O–H. The amount of water present in the CS NPs provided an indication of their hydrophilic nature, which was conformed through the –O–H bonding observed in the FTIR spectra of the CS NPs between 3200 cm^{-1} and 3550 cm^{-1}.The absorption band at 2974.15 cm^{-1} was corresponding to the asymmetric stretching vibration of methylene present in the CS NPs.The absorption band at 1640.58 cm^{-1} was due to the bending vibration of N–Hin chitosan/TPP nanoparticles after the addition of TPP.The bands centered at 1351.80 cm^{-1} could be assigned to the N–O stretching of aliphatic nitro compound. Regarding the C–O stretching, the absorption bands of 1633 .57cm^{-1} in pure chitosan have shifted to 1640.58 cm^{-1}, respectively, indicating the interaction between CS NPs. The peak at 1055.71 cm^{-1} for C–O stretching vibration of primary alcohol was observed. The absorption band at 514.97 cm^{-1} was indicated due to the bromoalkanes (Fig. 7(b).



FTIR spectrum for (a) chitosan (b) chitosan-TPP nanoparticle

Figure 7: FTIR spectra of chitosan and chitosan TPP nanoparticle

3.5. Isolation of fish pathogen

The diseased fish was collected from a nearby fish farm and aseptically transported to the laboratory (Fi. 8) Pathogen was isolated from homogenized gut samples by spread plate dilution technique into Thiosulfate-citrate-bile salts-sucrose agar (TCBS).The isolated colony was streaked on to nutrient agar plate and preserved on nutrient slant for further analysis (Fig. 9).

Figure 8: Infected fish

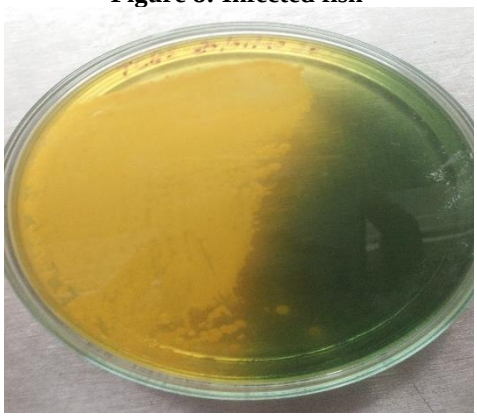


Figure 9: TCBS agar plate

3.6. Identification of fish pathogen

Gram staining showed that the isolate was a gram-negative bacillus which is motile. Genomic DNA was isolated. A portion of the 16S rDNA was amplified using a primer pair for16S rDNA (Fig. 10). The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm. The isolate was thus identified and confirmed to be *Aeromonas hydrophila* from NCBI BLAST analysis.

Figure 10: Agarose gel electrophoresis

3.7. Antibiotic susceptibility testing and Evaluation of MAR indices

The isolate was tested for antibiotic sensitivity, with 5 antibiotics (HiMedia, Mumbai) belonging to different classes, namely ampicillin (AMP 10 mcg), cefuroxime (CXM 30 mcg), ciprofloxacin (CIP 5 mcg), gentamicin (GEN 10 mcg) and imipenem (IPM 10 mcg). The results were interpreted as per the manufacturers' instructions and stated in table 2.

Table 2: Showing results of antibiotic susceptibility test

Sl. no	Antibiotic	Zone of inhibition	Activity
1	AMP 10 mcg	0.00mm	Resistant
2	CXM 30 mcg	9.00mm	Resistant
3	IPM 10 mcg	26.00 mm	Sensitive
4	CIP 5 mcg	0.00 mm	Resistant
5	GEN 10 mcg	0.00mm	Resistant

Multiple Antibiotic Resistance (MAR) Index was calculated. The MAR indices are listed in table 3.

Table 3: MAR indices

Organism	No. of antibiotics to which it is resistant (a)	Total No. of antibiotics used (b)	MAR Index (c) (a/b)	MAR Index (in %) (c X 100)
Aeromonas hydrophila	**4**	**5**	**0.8**	**80**

The isolates showed multiple antibiotic resistance against AMP 10 mcg, 30 mcg, CIP 5 mcg and GEN 10 mcg i.e. it was found to be resistant to four antibiotics out of the five tested. The results revealed that the isolate was sensitive only to the antibiotic Imipenem. MAR index was found to be 0.8 which is a high value and was found to be multi drug resistant.

3.8. Antibacterial activity of chitosan nanoparticles

Antibacterial activity was determined by agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS). Well containing the same volume of DMSO served as negative control and Imipenem served as positive control. The results are interpreted in table 4.

Table 4: Showing results of Antibacterial activity

Samples	Diameter of Zone of inhibition against *A. hydrophila (in mm)*
Chitosan nanoparticle	24.50
Chitosan	14.2
Imipenem (+ve control)	26.00
-ve control	0.00

3.9. Minimum inhibitory concentration of the most sensitive antibiotic and chitosan nanoparticle

The MIC is calculated by resazurin method. It showed significant degree of inhibition in all methanolic plant samples (Fig. 11). 62.5µg/mL was the concentration that was determined as the minimum inhibitory concentration for antibiotic as well as the chitosan nanoparticle against the pathogen.

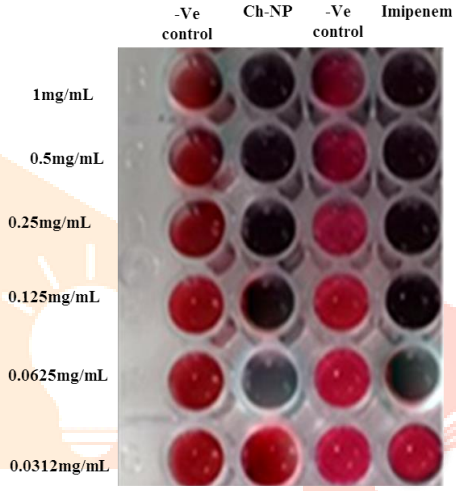


Figure 11: Minimum inhibitory concentration of antibiotic and nanoparticle

Shrimp is one of the major fisheries products worldwide. In the present study, shrimps shells were used as the source for extraction of chitin. Shrimp industries generate large amounts of shrimp bio-waste during processing, approximately 45-55% of the weight of raw shrimp (Lertsutthiwong *et al*., 2002). However, this bio-waste can be used to produce value-added products such as chitosan. Chitosan is a modified natural carbohydrate polymer derived from chitin which has been found in a wide range of natural sources such as crustaceans, fungi, insects and some algae. After cellulose, chitin is the second most abundant natural biopolymer found in nature (No and Meyers, 1989). 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. The yield obtained here found to be within the range reported by Lertsutthiwong *et al*., 2002, who showed that waste content varied approximately from 25 to 55% of the weight of raw shrimp. Yield of chitin extracted in this study from shrimp waste varied from was 33%. This is due to higher concentration of HCI which in-turn removes minerals from shrimp shell and subsequently increased the yield. Chitosan yielded in this study was higher than that reported by Brzeski (1982) (14% yield of chitosan from krill). The molecular weight of native chitin is usually larger than one million Daltons while commercial chitosan products fall between 100,000 to 1,200,000 Daltons. Several factors during production, including high temperature, concentration of alkali, reaction time, previous treatment of the chitin, particle size, chitin concentration, dissolved oxygen concentration and shear stress may influence the MW of chitosan (Li *et al*., 1992).The FTIR spectrum of the prawn shell chitosan showed similar results to that of standards (Puvvada *et al*., 2012). Tilapia is a fast growing, well-adapted fish species that has been commercially farmed all over 100 countries in the tropical and subtropical regions (El-Sayed, 2006). Like other aquaculture fish species, under stress conditions of intensive culture, tilapia is also susceptible to different bacterial infections such as hemorrhagic septicemia caused by *Aeromonas spp*. (Eissa *et al*., 2015). So in the present study also we focused on the isolation of fish pathogen from *O. niloticus* for studying the inhibitory effect of probiotic bacteria isolated from fish gut of fresh water fish. On morphological and molecular identification of the fish pathogen it is revealed that the isolate belongs to *Aeromonas hydrophila*. Usually, *A. hydrophila* is considered sensitive to antimicrobials, but during the last few years, the antimicrobial resistance has developed in many bacterial genera due to the unwarranted use of antimicrobials in agriculture and aquaculture structures (Adenipekun *et al*., 2016). In the present study also the isolate was found to be a multidrug resistant isolate. The results revealed that the isolate was sensitive only to the antibiotic imipenem among the tested five different groups of antibiotics. In the present work, it was demonstrated that chitosan nanoparticle has significant antimicrobial activity against the fish pathogen tested, viz., *A. hydrophila* and the MIC value obtained was also like that of the antibiotic used. The different physical composition and molecular weights of chitosan nanoparticles and its derivatives renders distinctive modes of antibacterial action. Low molecular weight water-soluble chitosan nanoparticles can penetrate the cell wall of bacteria and inhibit transcription of mRNA from DNA (Sudarshan *et al*. 1992). In this case, the oligomer molecules are assumed to be able to pass through the bacterial cell wall, composed of multilayers of cross-linked murine, and reach the plasma membrane. This is also supported by the work of Tsai and Su (1999) on E. coli, who investigated the antibacterial activity of shrimp chitosan nanoparticles and by Liu *et al*. (2006) who assessed the effect of molecular weight and concentration of chitosan

nanoparticles on antibacterial action. Chitosan nanoparticles, as a polyelectrolyte, can form electrostatic complexes under acidic conditions and its interactions depend directly on the charge density of chitosan nanoparticles (Rinaudo 2006). Several studies have indicated that the interactions between positively charged chitosan nanoparticles molecules and negatively charged residues on the cell surface play an important role in the inhibitory effect of chitosan nanoparticles on micro-organisms (Chung *et al*. 2004; Je and Kim 2006; Kong *et al*. 2010). Broadly, this suggests that the susceptibility of bacterial cells to chitosan nanoparticles may depend on surface electronegativity. For aquatic animal pathogens like those examined here, chitosan nanoparticles would have distinct advantages as novel antimicrobial agents due to their higher activity and increased solubility in water. Further investigation is warranted, particularly needed are those studies designed to extend the findings here in animal models to rigorously evaluate and characterize the efficacy and feasibility of CS-based strategies for disease prevention and control.

IV. CONCLUSION

Not only the antimicrobial property but also the characteristics such as nanoscale, low-toxicity, biodegradability, biocompatibility, derivatization, immunomodulatory effects, and easily affordable preparation conditions make chitosan a strong candidate for drug delivery into fish. Therefore, the use of chitosan in fish biotechnology has received growing attention in recent years. However, applications based on novel chitosan-based gene therapy methodologies to improve desirable traits in farmed fish have enormous potential for development. Most remarkable advances in the field addressed fish immunization, the control of reproduction for broodstock management and the modulation of gene expression to spare protein and overcome metabolic limitations of farmed fish. Further studies are needed for a better understanding of the extracellular and intracellular process, following chitosan-mediated gene delivery into fish. In addition, future trends in fish farming may greatly benefit from improved and more efficient chitosan formulations for enhancing gene delivery targeting and intracellular traffic in farmed fish and other aquacultural facilities.

V. ACKNOWLEDGEMENT

The work is fully supported and sponsored by Trophic Biosystems Pvt Ltd and PVT Merit Biolabs, Cochin, Kerala and the authors acknowledge the above mentioned institutions.

VI. REFERENCES

[1] Adenipekun, E. O., Jackson, C. R., Ramadan, H., Iwalokun, B. A., Oyedeji, K. S., Frye, J. G., ... & Oluwadun, A. (2016). Prevalence and multidrug resistance of Escherichia coli from community-acquired infections in Lagos, Nigeria. *The Journal of Infection in Developing Countries*, *10*(09), 920-931.

[2] Alishahi, A., Mirvaghefi, A., Tehrani, M. R., Farahmand, H., Koshio, S., Dorkoosh, F. A., & Elsabee, M. Z. (2011). Chitosan nanoparticle to carry vitamin C through the gastrointestinal tract and induce the non-specific immunity system of rainbow trout (Oncorhynchus mykiss). *Carbohydrate polymers*, *86*(1), 142-146.

[3] Anoopkumar-Dukie, S., Carey, J. B., Conere, T., O'sullivan, E., Van Pelt, F. N., & Allshire, A. (2005). Resazurin assay of radiation response in cultured cells. *The British journal of radiology*, *78*(934), 945-947.

[4] Anusha, J. R., & Fleming, A. T. (2016). Synthesis and characterization of chitosan nanoaggregates from gladius of Uroteuthis duvauceli. *International journal of biomaterials*, *2016*.

[5] Bauer AW, Kirby WM, Sherris JC. turck, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American journal of clinical pathology. 1966;45(4):493.

[6] Brzeski, M. M. (1982). Concept of chitin/chitosan isolation from Antarctic Krill (Euphausia superba) shells on a technique scale. In *Proceedings of the 2nd international conference on chitin and chitosan. The Japan Society of Chitin and Chitosan, Sapporo, Japan* (pp. 15-29).

[7] Chung, Y. C., Su, Y. P., Chen, C. C., Jia, G., Wang, H. L., Wu, J. G., & Lin, J. G. (2004). Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta pharmacologica sinica*, *25*(7), 932-936.

[8] Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in polymer science*, *36*(8), 981-1014.

[9] Eissa, S., Alshehri, N., Rahman, A. M. A., Dasouki, M., Abu-Salah, K. M., & Zourob, M. (2018). Electrochemical immunosensors for the detection of survival motor neuron (SMN) protein using different carbon nanomaterials-modified electrodes. *Biosensors and Bioelectronics*, *101*, 282-289.

[10] El-Sayed, A. F. M. (2006). Tilapia culture in salt water: environmental requirements, nutritional implications and economic potentials. *Avances en Nutricion Acuicola*.

[11] Fang, M., Long, J., Zhao, W., Wang, L., & Chen, G. (2010). pH-responsive chitosan-mediated graphene dispersions. *Langmuir*, *26*(22), 16771-16774.

[12] Hossain, M. S., & Iqbal, A. (2014). Production and characterization of chitosan from shrimp waste. *Journal of the Bangladesh Agricultural University*, *12*(1), 153-160.

[13] Hu, Y., Jiang, X., Ding, Y., Ge, H., Yuan, Y., & Yang, C. (2002). Synthesis and characterization of chitosan–poly (acrylic acid) nanoparticles. *Biomaterials*, *23*(15), 3193-3201.

[14] Je, J. Y., & Kim, S. K. (2006). Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. *Journal of agricultural and food chemistry*, *54*(18), 6629-6633.

[15] Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: a state of the art review. *International journal of food microbiology*, *144*(1), 51-63.

[16] Lertsutthiwong, P., How, N. C., Chandrkrachang, S., & Stevens, W. F. (2002). Effect of Chemical Treatment on the Characteristics of Shrimp Chitosan. *Journal of Metals, Materials and Minerals*, *12*(1), 11-18.

[17] Li, B., Wang, X., Chen, R., Huangfu, W., & Xie, G. (2008). Antibacterial activity of chitosan solution against Xanthomonas pathogenic bacteria isolated from Euphorbia pulcherrima. *Carbohydrate Polymers*, *72*(2), 287-292.

[18] Madden, T. (2013). The BLAST sequence analysis tool. In *The NCBI Handbook [Internet]. 2nd edition*. National Center for Biotechnology Information (US).

[19] Mohebbi, S., Nezhad, M. N., Zarrintaj, P., Jafari, S. H., Gholizadeh, S. S., Saeb, M. R., & Mozafari, M. (2019). Chitosan in biomedical engineering: a critical review. *Current stem cell research & therapy*, *14*(2), 93-116.

[20] No, H. K., Meyers, S. P., & Lee, K. S. (1989). Isolation and characterization of chitin from crawfish shell waste. *Journal of Agricultural and Food Chemistry*, *37*(3), 575-579.

[21] Puvvada, Y. S., Vankayalapati, S., & Sukhavasi, S. (2012). Extraction of chitin from chitosan from exoskeleton of shrimp for application in the pharmaceutical industry. *International Current Pharmaceutical Journal*, *1*(9), 258-263.

[22] Qi, L., Xu, Z., Jiang, X., Hu, C., & Zou, X. (2004). Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydrate research*, *339*(16), 2693-2700.

[23] Reddy, L. H., Arias, J. L., Nicolas, J., & Couvreur, P. (2012). Magnetic nanoparticles: design and characterization, toxicity and biocompatibility, pharmaceutical and biomedical applications. *Chemical reviews*, *112*(11), 5818-5878.

[24] Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in polymer science*, *31*(7), 603-632.

[25] Saravanan, P., Ramya, V., Sridhar, H., Balamurugan, V., & Umamaheswari, S. (2010). Antibacterial activity of Allium sativum L. on pathogenic bacterial strains. *Global veterinaria*, *4*(5), 519-522.

[26] Subramani, S., & Vignesh, S. (2012). MAR index study and MDR character analysis of a few golden staph isolates. *Asian Journal of Pharmacy and Life Science ISSN*, *2231*, 4423.

[27] Sudarshan, N. R., Hoover, D. G., & Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnology*, *6*(3), 257-272.

[28] Thiruchenduran S, Supraja N, Prasad T (2018) Synthesis, characterization, antimicrobial and antioxidant assay of costus igneus bio-active compounds loaded zinc nanoparticles for nano and bioactive. 2018 Oct 10; ABB. 000526.2018.

[29] Tsai, G. J., & Su, W. H. (1999). Antibacterial activity of shrimp chitosan against Escherichia coli. *Journal of food protection*, *62*(3), 239-243.

[30] Vaezifar, S., Razavi, S., Golozar, M. A., Karbasi, S., Morshed, M., & Kamali, M. (2013). Effects of some parameters on particle size distribution of chitosan nanoparticles prepared by ionic gelation method. *Journal of Cluster Science*, *24*(3), 891-903.

[31] Yuan, Y., Chesnutt, B. M., Haggard, W. O., & Bumgardner, J. D. (2011). Deacetylation of chitosan: material characterization and in vitro evaluation via albumin adsorption and pre-osteoblastic cell cultures. *Materials*, *4*(8), 1399-1416.