2. Synthesis and Characterization of Chitosan Nanoparticles

2.1. Introduction

The seafood processing industry has a key role in the Kerala economy by contributing US$5.5 billion in export alone (Ancy and Raju 2016). During the processing of seafood, the meat is only taken, while the head and shells are discarded as waste. This results in the generation of a large amount of shell waste globally. The shellfish industry which is prominent in all coastal countries generates about 60,000 to 80,000 tons of waste annually (Muzzarelli et al. 1986). Even though the wastes are biodegradable, the large quantities make degradation process slow resulting in accumulation of waste overtime which is a major environmental concern.

A quick and effective solution to this is a recycling of shell wastes and extraction of commercially viable substances like chitin from them. Chitin is the second most abundant polymer in nature after cellulose. Chitin is found in nature as a structural component of the exoskeleton of arthropods and fungal cell walls as uniform crystalline microfibrils (Jayakumar et al. 2010). Chitosan is a linear amino polysaccharide of (1→4) linked N-acetyl glucosamine and glucosamine units. It is a white, hard, non-elastic and nitrogenous polysaccharide (Badawy and Rabea 2011). Chitosan finds a variety of applications due to its high biodegradability, non-toxicity and antimicrobial properties. It is used in biomedical industries, agriculture, genetic engineering, food industry, environmental sector, pollution control, water treatment, paper manufacture, photography and so on (Cheba 2011).
The amino group present in the C2 position of chitosan makes it soluble in dilute acids (pH6). The protonated amino group of chitosan forms ionic complexes with anionic compounds like DNA, lipids, proteins etc. In fact, chitosan is the only natural positively charged polysaccharide available (Ibrahim and El-Zairy 2015). It could be understood that the physical properties of chitosan are dependent on the degree of deacetylation. For this reason, the most important parameter used for characterisation of chitosan is a degree of deacetylation and molecular weight (Younes and Rinaudo 2015).

Chitosan nanoparticles (ChNP) have an increased applicability compared to its bulk counterpart chitosan due to the increased surface to volume ratio and smaller size (Grenha 2012). There are many methods used for the synthesis of ChNP like coacervation, reverse micellar formation, emulsion droplet coalescence, self-assembly chemical modification etc (Tiyaboonchai 2003; Zhao et al. 2011). Ionic gelation of chitosan is but the widely accepted method owing to its simplicity and non-toxicity. The positively charged amino group of chitosan is crosslinked with negatively charged dialdehyde or multivalent anions (Calvo et al. 1997). The dialdehydes like glutaraldehyde, ethyl glycol etc are however not used due to its toxic nature. Sodium salts of tripolyphosphate (TPP) are the most common used multivalent anion because of its non-toxic nature and quick gelling property (Patil and Manisha 2014).

ChNP has increased activity than chitosan and this makes it an ideal compound for a variety of applications like agricultural, biomedical, industrial etc. Regardless of its potential applications, care has to be taken in selecting nanoparticles for agricultural use since nanomaterials might have toxic effects not found in its bulk counterpart due to their increased surface contact area (Tarafdar 2013). The transitional zone of
nanoparticle between an atom and the parent material is farther from one another, altering the original characteristics of the compound (Lee et al. 2010).

The effect of ChNP on the environment especially on human health and plant ecosystem has to be studied in detail before its agricultural application (Hu et al. 2011). There are various points for nanoparticles to enter into an ecosystem such as direct entry, through waste accumulation, by transformation, via food chain etc (Maurer-Jones et al. 2013). The difficulty in estimation of the environmental concentration of nanoparticles at any given time once it is released makes toxicity determination an important parameter.

The REACH regulations make mandatory for manufacturers and importers to produce the physicochemical properties and toxicity information of their nanoparticle products. The US EPA has declared silver nanoparticles (AgNP) as a pesticide and has issued a compulsory screening of all agricultural products for AgNP (Lee et al. 2010).

The seed priming of wheat and barley with ChNP and silicon dioxide nanoparticles (SiO$_2$NP) was studied by Behboudi et. al. (Behboudi et al. 2017) and it was reported that ChNP was far less toxic than SiO$_2$ NP. The 30ppm concentration of NP was found to be effective in increasing seed germination and growth while 90ppm and above was found to have toxic effects.

The cytotoxicity and genotoxicity of lecithin/ChNP were studied. The IC50 value of lecithin/ChNP was estimated using neutral red uptake cytotoxicity assay and was found to be 1.9%. Comet assay was done to analyse the DNA damage caused by lecithin/ChNP on human lymphocytes and it was found that high concentration of 4% was found
to be toxic but not as toxic as the positive control (50 μM H₂O₂) (Taner et al. 2014).

The cytotoxic effects of ChNP on human liver cancer cell model (HepG2) using sulforhodamine B colourimetric assays and flow cytometric analysis has been reported. The IC50 was estimated to be 239μg/ml and continued exposure of cells with ChNP for a period of 48h was found to have a genotoxic effect (Loutfy et al. 2016).

In this chapter, the extraction and subsequent characterization of chitosan from *Penaeus monodon* shells were done. The nanoparticle synthesis was carried using ionic gelation method and ChNP production was optimised and characterised. The cytotoxicity, seed toxicity and soil toxicity of ChNP were analysed in detail.

2.2. Materials and Methods

2.2.1. Sample Preparation: Shells of *Penaeus monodon* or Giant Tiger Prawn were obtained from the shellfish processing industries in Cherthala, Kerala. The shells were washed, air dried and refrigerated overnight. This was then oven dried for four consecutive days at 65°C.

2.2.2. Extraction of Chitosan: Chitosan was prepared using a combination of three procedures (Annadurai 2012; Puvvada et al. 2012; Biswas and Gargi 2013). Five gram of shrimp shell waste was treated with 4% NaOH at room temperature for 24h. The alkali was drained from the shells and washed with distilled water repeatedly till pH dropped to neutral. This process caused deproteinisation of shells. The deproteinised shells were treated with 4% HCl at room temperature for 12h for demineralization to yield chitin. The acid was drained off from chitin, washed with distilled water and finally dried at
room temperature. The process was repeated with 2% NaOH and 1% HCl. The chitin obtained still had a slight pink hue. Further decolourisation was achieved by soaking chitin in 1% potassium permanganate for 30min followed by 1% oxalic acid for 30min to 2h. The decolourised chitin was deacetylated to form chitosan by treating with 65% NaOH for 3 days at room temperature. Alkali was drained off and washed repeatedly with distilled water till pH was lowered. Chitosan was further dried at room temperature and stored. The weight of chitosan produced was measured and yield calculated using equation 2.1.

\[
\text{Yield(\%) = } \left(\frac{\text{Weight of Product}}{\text{Shrimp Shell Weight}}\right) \times 100
\]

2.2.3. Characterisation of Chitosan

2.2.3.1. Composition Analysis: Moisture content and residue on ignition or ash content were analysed based on methods by Association of Official Analytical Chemists (Kenneth Helrich 1990). For determining the moisture content, the 10g samples were dried at 60°C for 24h and percentage weight loss was calculated. The percent weight loss was equal to moisture content. For ash content determination, the samples were transferred to muffle furnace at 550°C until it turned white in colour. The samples were cooled to room temperature in a desiccator and reweighed.

2.2.3.2. pH: The pH measurement of chitosan solutions were carried out using EUTECH pH 700.

2.2.3.3. Viscosity: Viscosity of chitosan was determined at room temperature using a Brookfield digital viscometer (Mirzadeh et al. 2002).
2.2.3.4. **Degree of Deacetylation**: Chitosan homogenous solution was prepared using dilute HCl containing 0.01 mol/L and titrated against 0.1M NaOH. The end point was detected by the inflexions of pH values. Two inflexions were mainly noted. First, one corresponds to HCl neutralization and second to neutralization of ammonium ions of chitosan. The difference between two points gives the number of amino groups in chitosan chain also called the degree of deacetylation DD % = 100 - DA% (Zhang et al. 2011).

2.2.3.5. **Solubility of Chitosan**: Chitosan dissolves completely in 1% acetic acid. Weigh a few grams of chitosan and add 35ml 1% acetic acid. It was kept in a magnetic stirrer for 30mins. The sample was taken out and insoluble chitosan was removed by filtration through Whatman No.1 filter paper and weighed (Biswa and Gargi 2013).

2.2.3.6. **X-ray Diffraction spectrometer**: XRD analysis of chitosan was used to detect its crystallinity. A Bruker AXS D8 Advance diffractometer was used for the purpose.

2.2.3.7. **Scanning Electron Micrograph**: The structure of chitosan was examined using scanning electron microscope (SEM/EDAX JEOL (Japan), Model JSM 6390. The dried samples were mounted on specimen stubs with double adhesive tape and coated with platinum in a sputter coater and examined.

2.2.3.8. **FT-IR Studies of Chitosan**: The chitosan and chitin samples were characterized from 4000 to 400 cm⁻¹ using infrared spectrophotometer (Shimadzu IR Prestige 21 FT-IR – ATR attached).

2.2.4. **Synthesis of Chitosan Nanoparticles**

Chitosan nanoparticles were prepared using ionic gelation method (Calvo et al. 1997). Different concentrations of chitosan
(1mg-5mg) was added to 1% acetic acid (v/v) and mixed well using magnetic stirrer. The ChNP were formed by adding 1% TPP (w/v) drop by drop under magnetic stirring. Then the solution was centrifuged at 10,000 rpm for 10 minutes to remove residual TPP and the particles were freeze-dried. The practical yield was calculated from the following equation 2.2 (Nesalin and Smith 2012).

\[
PY(\%) = \left( \frac{\text{Weight of Nanoparticle}}{\text{Theoretical mass (Polymer+TPP)}} \right) \times 100
\]

2.2.5. Optimisation of Synthesis of Chitosan nanoparticles

Various parameters were analysed to optimise the nanoparticle synthesis to obtain the lowest nanoparticle size. XRD analysis of ChNP was done to detect its crystallinity and particle size was calculated. A Bruker AXS D8 advance diffractometer was used for the purpose. The size of nanoparticles was calculated using Debye Scherrer’s equation 2.3.

\[
d = \left[ \frac{k\lambda}{\beta \cos\theta} \right]
\]

Where \(d\) is mean grain size, \(\beta\) is Full Width at Half Maximum of diffraction peak (FWHM), \(\lambda\) is X-ray wavelength, \(\theta\) is Bragg’s diffraction angle and \(k\) is Scherrer’s constant with a value of 0.9.

2.2.5.1. Concentration of Chitosan

Different concentrations of chitosan were dissolved in 1% acetic acid and 1% TPP added drop by drop. 1,2,3,4,5mg/ml chitosan was used for the study (Mohammadpour et al. 2012).

2.2.5.2. Reaction Time

The effect of the different time period for the magnetic stirring of the Chitosan-TPP solution in 1% acetic acid was also studied. The reaction was carried out for 45, 60, 90, 120 and 150mins (Patil and Manisha 2014).
2.2.5.3. The rotation speed of magnetic stirrer
The number of rotations per minute of the magnetic stirrer was also optimised. For this, the magnetic stirrer was set to 500, 600, 700, 800 and 900 rpm (Gupta and Karar 2011).

2.2.6. Characterisation of Chitosan nanoparticles

2.2.6.1. Solubility of ChNP: The solubility of chitosan nanoparticles in distilled water and aqueous acetic acid solutions (1%, 0.5%, 0.1% v/v) was evaluated. 10mg/ml concentration of ChNP, chitosan and chitin were suspended in each solvent and allowed to stand for 24h at room temperature and solubility was checked after the incubation period (Badawy et al. 2014).

2.2.6.2. FT-IR analysis: Chitosan nanoparticles were characterized from 4000 to 400cm\(^{-1}\) using infrared spectrophotometer (Shimadzu IR Prestige 21 FT-IR – ATR attached).

2.2.6.3. Scanning electron micrograph: The structure of chitosan nanoparticle compounds was examined using scanning electron microscope. The dried samples were mounted on specimen stubs with double adhesive tape and coated with platinum in a sputter coater and examined under JEOL 6390 SEM JSM at 10 kV.

2.2.6.4. Transmission electron microscopy: The size and morphology of nanoparticles were observed using High-Resolution Transmission Electron Microscope (JEM 2100). The sample for analysis was prepared by placing a drop of the chitosan nanoparticles on a carbon coated copper grid (400 mesh size). This was air dried and observed.

2.2.6.5. Atomic Force Microscopy: AFM imaging was performed using Si3N4 probes with a spring constant of 0.06N/m (APER-A-100SPM). ChNP was dissolved in distilled water and a drop of ChNP solution was placed on clean dried glass slide. The drop was air dried and analysed.
2.2.7. Toxicity study of Chitosan nanoparticles

The cytotoxicity, phytotoxicity and soil toxicity of ChNP was studied to ensure the safety of nanoparticles for subsequent plant application.

2.2.7.1. In vitro Cytotoxicity studies

L929 (fibroblast) cells were obtained from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecos modified Eagles medium (DMEM) (Gibco, Invitrogen). The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100μl cell suspension (5×10^4 cells/well) was seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO₂ incubator. ChNP (50, 100, 150, 200, 300 mg/ml) was added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO₂ incubator. A control without ChNP was also kept. After 24 hours of incubation, the entire plate was observed in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera). Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

After 24 hours of the incubation, the sample content in wells was removed and 30μl of MTT solution reconstituted in PBS was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% CO₂ incubator for 4 hours. After the incubation, the supernatant was removed and 100μl of MTT solubilization solution was added and the wells were mixed gently. The absorbance values were measured at 540 nm (Talarico et al. 2004).
The percentage of cell viability and percent cytotoxicity were calculated using the formulas below.

\[
\% \text{ cell viability} = \frac{\text{Mean OD samples}}{\text{Mean OD control}} \times 100
\]

\[
\% \text{ Cytotoxicity} = 1 - \frac{\text{Mean OD samples}}{\text{Mean OD control}} \times 100
\]

The LD\(_{50}\) is the lethal dose at which 50% of cell viability was lost. The LD\(_{50}\) value was calculated using ED\(_{50}\)plus V1.0 software.

### 2.2.7.2. Seed Toxicity Study

Prior to application of ChNP as a growth elicitor, the toxicity of ChNP was studied according to standard protocols (U. S. Environmental Protection Agency 2012).

#### 2.2.7.2.1. Seed collection

Rice (*Oryza sativa* L. var. Jyothi) seeds were obtained from National Seeds Corporation Ltd, Palakkad Area Office. Uniform seeds were used for the study to reduce variation.

#### 2.2.7.2.2. Experimental Set up

Rice seeds were pre-soaked in water for 24hr. The pre-soaked seeds were then soaked in different concentrations of ChNP. Control seeds were soaked in water only. Each treatment had 20 seeds and was experiments were done in three replicates. The seeds were planted in an inert substrate (blotting paper soaked in sterile distilled water) in a sterile Petri dish giving it ample space for growth. The plates were incubated in dark. The test lasted till 50% of control plants have germinated. The number of germinated seeds was counted and the percent growth promotion was calculated using the equation 2.6. The
seeds were considered germinated when the radical was at least 2mm in length (Behboudi et al. 2017).

\[
\text{Percent Growth Promotion} = \frac{(c-t) \times 100}{c}
\]

2.6

Where \( c \) = number of germinated seeds in control; and \( t \) = number of germinated seeds in treatment.

2.2.7.3. Range Finding Test

The seeds were treated with a wide range of concentrations like 0.1, 0.5, 1, 5, 10, 15, 20 mg/ml ChNP. Treatment without ChNP was kept under control. The number of germinated seeds was counted and percent growth promotion was calculated for each treatment using equation 2.6.

2.2.7.4. Definitive Test

The definitive test helps in determining the measure of effect, concentration-response curve, and \( EC_{50} \) value with 95% confidence interval and standard error. The seeds were treated with five concentration of ChNP 0.5, 1.0, 1.5, 2.0, 2.5 mg/ml. Control without ChNP was kept. The number of germinated seeds was counted and percent growth promotion was calculated for each treatment using equation 2.5. The \( EC_{50} \) value was calculated using Probit Analysis Programme (Version 1.5).

2.2.7.5. Soil Toxicity Study

The soil toxicity study was carried out based on the United States Environmental protection Agency protocol for soil microbial community toxicity test (EPA 1996).
2.2.7.5.1. Experimental Set up

The surface soil was collected from Pallithode village of Alleppey District in Kerala, India. The soil characteristics were analysed and found to be of sandy loam texture, with pH 7.25; electrical conductivity, 0.24dS/m; organic carbon, 0.9%; available nitrogen, 54 Kg/ha; available phosphorus, 6.86 Kg/ha; available potassium 57.12 kg/ha and calcium 50ppm. The soil was sieved through a 2mm sieve. A total of three treatments including ChNP (1mg/ml), chitosan (1mg/ml) and control using sterile distilled water were maintained with five replicates per treatment. The 15ml of test solution was thoroughly mixed with soil and potted. Each pot received 500g treated soil. The pots were incubated in darkness at 22°C for a period of 30 days. The soil moisture content was maintained at 60% and sterilised water was added on weight loss basis.

2.2.7.5.2. Total Microbial Count

1g air-dried soil sample was taken. It was sieved and added to 10ml sterile distilled water. This was serially diluted six-fold by adding 1ml of soil solution to 9ml sterile distilled water and repeated five times. Aliquots from $10^{-5}$ dilution were spread on nutrient agar plates. The number of colonies forming units was counted after 24-48h incubation at 30°C and expressed as log CFU/ml (Stephen et al. 2015).

2.2.7.5.3. Soil dehydrogenase activity

In a test tube, 10g air-dried soil was mixed with 6ml freshly boiled and cooled water, 2ml 1% glucose solution and 2ml 0.5% tetrazolium chloride (TTC). A reaction mixture without soil sample served as control. The test tubes were sealed with rubber stoppers and incubated for 24h at 30°C. After incubation, 40ml acetone was added and mixed
well by shaking. This was incubated at room temperature for 2h in
dark with intermittent shaking. The soil suspension was filtered and
absorbance measured at 546nm (Shabanamol 2015).

2.2.7.5.4. Soil extraction

The soil from each treatment was sampled on 5th and 28th days of
experiment and analysed for nitrate and ammonia concentrations. First,
the soil samples were extracted using potassium chloride extraction
method (Smith and Li 1993). The 2g dry soil sample was transferred to
an extraction bottle containing KCl (2M/l) in the ratio 1:5. Cap the
extraction bottle and mixture was incubated in a shaker for 1h at room
temperature. After incubation, the solution was filtered using a
nitrogen-free filter paper. The filtrate was collected in a volumetric
flask and volume made up using KCl solution.

2.2.7.5.5. Soil nitrate level

0.25ml of soil extract was pipetted into a 50ml Erlenmeyer flask and
mixed with 0.8 ml 5% (w/v) salicylic acid in H₂SO₄. The solution was
incubated for 20 min at room temperature and 19ml 2N NaOH was
added. This was cooled to room temperature and absorbance measured
at 410nm (Singh 2017).

2.2.7.5.6. Soil ammonia level

One ml extract was pipetted out of a test tube and 5.5ml buffer solution
containing 0.1M/l Na₂HPO₄, 0.177 M/l Sodium potassium tartrate and
2.7M/l NaOH was added. This was mixed using a vortex and 4ml
salicylate/nitroprusside reagent was added followed by 2ml
hypochlorite solution. The solution was incubated for 45min at 25°C
and absorbance measured at 610nm.(Kempers and Zweers 1986).
2.2.8. Statistical analysis
The results are expressed as the mean ± Standard deviation (S.D). Experimental data were subjected to one-way analysis of variance (ANOVA) using Origin 8 SR4 to ascertain the significant difference between activity and treatment. A p-value < 0.05 was considered as statistically significant. The significance of soil toxicity assay was done with two way ANOVA using WASP 2.0 statistical package.

2.3. Results
2.3.1. Extraction and characterisation of chitosan from shrimp shells
Extraction of chitosan from shrimp shell requires harsh chemical treatments. The shrimp shell even though contains the majority of chitin, also has proteins and minerals. Proteins are removed by deproteinization and carbon and other salts are removed by demineralization. The current method of chitosan extraction is superior to other available methods based on the higher yield of pure quality chitosan (Plate 2.1). Deproteinization and demineralization steps were repeated twice. This aided in a higher yield of chitin from the shells. The final deacetylation of chitin at room temperature for 3 days gave a longer reaction time which resulted in higher yield of chitosan. The chitosan yield was found to be 46%. The physiochemical parameters are tabulated in table 2.1. The ash content of 2% is due to the presence of calcium carbonate which is found in large amount in shrimp shells. The moisture content was 5%. The chitosan obtained had low viscosity. The degree of deacetylation was found to be 85%. The solubility of chitosan in acetic acid is a mark of its purity. The concentration of chitosan in acetic acid is 7.7g/L. This indicates that the obtained chitosan was 77% pure.
Plate 2.1. Extraction process of chitosan from shrimp shells

Table 2.1: Characteristics of chitosan extracted from *P. monodon*.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Chitosan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan yield</td>
<td>46%</td>
</tr>
<tr>
<td>Moisture content</td>
<td>5%</td>
</tr>
<tr>
<td>pH</td>
<td>8</td>
</tr>
<tr>
<td>Residue on ignition</td>
<td>2%</td>
</tr>
<tr>
<td>Viscosity</td>
<td>80cps</td>
</tr>
<tr>
<td>Degree of Deacetylation</td>
<td>85%</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in 1% acetic acid</td>
</tr>
</tbody>
</table>
The XRD pattern of shrimp chitosan (Fig. 2.1) showed characteristic peaks at 2θ= 9.28° and 20.18°. The sharper peaks are an evidence of denser crystalline structure. The shrimp sample showed peaks at 1375 cm⁻¹, 1552 cm⁻¹, 1618 cm⁻¹, 1654 cm⁻¹. The bands can be seen in the Fig. 2.2. Chitosan spectra show a peak at 1029 cm⁻¹ and 1375 cm⁻¹. The bands are seen in Fig. 2.3. SEM analysis showed that chitosan had a long thin crystal structure on a smooth surface (Fig 2.4).

**Fig. 2.1:** XRD pattern of chitosan extracted from *P. monodon.*

**Fig. 2.2:** FT-IR spectra of chitin extracted from *P. monodon.*
Fig. 2.3: FT-IR spectra of chitosan extracted from *P. monodon*.

Fig. 2.4: SEM micrograph of chitosan extracted from *P. monodon*.

2.3.2. Synthesis and characterisation of chitosan nanoparticles

The nanoparticles were formed spontaneously through the formation of intra and intermolecular cross-linkages under a constant stirring at ambient temperature. The ChNP obtained were freeze-dried and stored for further use. The practical yield of ChNP compounds was calculated to be 76%.

2.3.3. Optimisation of chitosan nanoparticle synthesis

The X-Ray powder diffraction pattern of ChNP compounds for each parameter was recorded. The peak at $\theta = 18^\circ$ found in XRD pattern of ChNP is due to the presence of chitosan in the nanoparticle. Table 2.2 shows the size of nanoparticles calculated using Debye Scherrer’s equation. The concentration of chitosan
was found to have a linear relation with the size of nanoparticle such that, lower the concentration of chitosan, smaller the size of ChNP (Fig. 2.5). At 1mg/ml concentration, the nanoparticle size was 19nm. However since ChNP dissolved in water showed sedimentation when kept for few minutes, the 2mg/ml concentration that yielded nanoparticles of 21nm size was selected for further studies. The nanoparticle size increased with reaction time and was found to be lowest at 60min reaction time hence was chosen for further studies (Fig. 2.6). The speed of rotation did not show any marked relation with the nanoparticle size (Fig. 2.7). But the smallest nanoparticle size was obtained at 700rpm and hence was selected for further studies.

In accordance with the size of nanoparticles, the optimum conditions for nanoparticle synthesis that yield the smallest nanoparticles are 2mg/ml of chitosan dissolved in 1% acetic acid. To this 1%, TPP was added drop by drop and kept under magnetic stirrer for 60 min at 700rpm. The optimized response was 19nm size ChNP.
### Table 2.2. Determination of crystalline size of chitosan nanoparticles using Debye Scherrer’s equation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variables</th>
<th>ChNP size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. of Chitosan (mg/ml)</td>
<td>1</td>
<td>19 ± 2.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>21 ± 2.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>120 ± 4.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>153 ± 4.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>165 ± 5.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>C.D. (0.05)</td>
<td>77.75</td>
</tr>
<tr>
<td>Reaction time (min)</td>
<td>45</td>
<td>70 ± 4.54&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>21 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>41 ± 2.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>53 ± 3.87&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>65 ± 3.36&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>C.D. (0.05)</td>
<td>1.81</td>
</tr>
<tr>
<td>Rotation speed of magnetic stirrer (rpm)</td>
<td>500</td>
<td>92 ± 2.95&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>85 ± 1.65&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>700</td>
<td>19 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>60 ± 0.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>72 ± 2.59&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>C.D. (0.05)</td>
<td>1.81</td>
</tr>
</tbody>
</table>

*Data are mean ± S.D. of three replicates. Mean values are significantly different at p<0.05. Means with same letters are not significantly different.*
Fig. 2.5. XRD patterns of ChNP synthesized using (a) 1mg/ml, (b) 2mg/ml, (c) 3mg/ml, (d) 4 mg/ml and (e) 5 mg/ml of chitosan.
Fig. 2.6. XRD patterns of ChNP synthesized with reaction time (a) 45min, (b) 60min, (c) 90min, (d) 120min and (e) 150min.
Fig. 2.7. XRD patterns of ChNP synthesized with (a) 500rpm, (b) 600rpm, (c) 700rpm, (d) 800rpm and (e) 900rpm rotations of magnetic stirrer.

2.3.4. Characterization of chitosan nanoparticles

The solubility of ChNP in distilled water and aqueous solutions of acetic acid was studied and compared with chitosan and chitin (Plate 2.2). ChNP was found to be soluble in distilled water and all concentrations of acetic acid. Chitosan was insoluble in distilled water but formed a gelatinous mass of acetic acid solutions. Chitin was insoluble in all solvents used.
Plate 2.2. Solubility of (a) Chitin, (b) Chitosan and (c) ChNP in 1% acetic acid, 0.5% acetic acid, 0.1% acetic acid and Distilled water.

FTIR studies of ChNP were performed to analyze the chemical structure of nanoparticle. The FTIR spectrum is shown in Fig 2.8. A broadband of 3300-3200 cm\(^{-1}\) is observed. There are also distinct peaks observed at 2880 cm\(^{-1}\), 1643 cm\(^{-1}\) and 1419 cm\(^{-1}\).

Fig 2.8. FT-IR spectrum of Chitosan nanoparticles
Scanning electron micrograph of ChNP (Fig 2.9) shows spherical shaped chitosan nanoparticles. There was no agglomeration observed in the nanoparticles. TEM images (Fig 2.10) shows nanoparticles having nearly spherical shape with a smooth surface and size range of about 20-70 nm. Atomic force micrograph (Fig 2.11) shows spherical shaped nanoparticles which are in agreement with the SEM image.

Fig. 2.9. SEM image of Chitosan Nanoparticles.

Fig. 2.10. TEM image of Chitosan nanoparticles
Fig. 2.11. AFM image of Chitosan Nanoparticles.

2.3.5. Toxicity study of Chitosan nanoparticles

2.3.5.1. Cytotoxicity of Chitosan nanoparticles

The *in vitro* dye reduction test and microscopic observation using inverted phase contrast microscopy was used to analyse the cytotoxicity of ChNP in fibroblast cells. The inverted phase contrast microscopic image of cytotoxicity of ChNP is shown in Plate 2.3. Table 2.3 shows the cytotoxicity activity of ChNP in terms of percentage of viable cells and percent cytotoxicity after respective treatments. 300 mg/ml ChNP killed 74.16% cell after 24h incubation.
The LD<sub>50</sub> value was obtained as 64.21mg/ml. The high LD<sub>50</sub> value makes ChNP a potential antifungal, antioxidant as well as coating agent since optimal ChNP concentration for all above activities is well below the LD<sub>50</sub> value. The inhibition rate of ChNP increased with NP concentration indicating the inhibition of cell viability is dose-dependent.

Plate 2.3. Inverted phase contrast microscopic image of the morphology of ChNP (50, 100, 150, 200 and 300 mg/ml) treated L929 fibroblast cells and untreated L929 (control).
Table 2.3. Cytotoxic effect of different concentrations of ChNP on the growth of L929 fibroblast cell line measured using MTT assay.

<table>
<thead>
<tr>
<th>Conc. of ChNP (mg/ml)</th>
<th>% cell viability*</th>
<th>% cytotoxicity *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>85.6 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.33 ± 1.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>55.87 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>64.14 ± 3.72&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>150</td>
<td>38.52 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71.47 ± 0.84&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>200</td>
<td>27.74 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>82.25 ± 0.78&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>300</td>
<td>25.84 ± 0.004&lt;sup&gt;d&lt;/sup&gt;</td>
<td>84.15 ± 0.48&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C.D. (0.05)</td>
<td>9.36</td>
<td>9.61</td>
</tr>
</tbody>
</table>

* Data are mean ±S.D. of three replicates. Mean values are significantly different at p<0.05. Means with same letters are not significantly different.

2.3.5.2. Seed Toxicity of Chitosan nanoparticles

The seed toxicity study results showed that ChNP not only is non-toxic to rice seed germination but also happens to be a good elicitor of the growth (Plate 2.4). The number of germinated seeds was higher than control for all ChNP treatments. The results are shown in Fig 2.12. In the range-finding test, a maximum number of germination was found for seed treated with ChNP concentration 0.1mg/ml to 5mg/ml (Table 2.4). This range was hence chosen for the definitive test. In the definitive test, 0.5mg/ml ChNP concentration was found to have a positive effect on seed germination (Table 2.5). The IC50 value was calculated using Probit Analysis Programme (Version 1.5) and is found to be 4.91mg/ml ChNP.
Plate 2.4. Seed toxicity test of chitosan nanoparticles on rice seed germination (a) Range finding test (1) Control (2) 0.1mg/ml (3) 0.5mg/ml (4) 1mg/ml (5) 5mg/ml (6) 10 mg/ml (7) 15mg/ml (8)20mg/ml of ChNP (b) Definitive test (1) Control (2) 0.5mg/ml (3) 1mg/ml (4) 1.5mg/ml (5) 2mg/ml (6) 2.5mg/ml of ChNP.

Fig.2.12. Seed toxicity study of ChNP on rice seed germination (a) range finding test (b) definitive test.

Data are mean ±S.D. of three replicates. Mean values are significantly different at $p<0.05$. Means with same letters are not significantly different.
Table 2.4. Range finding test for determining seed toxicity study of ChNP on rice seed germination

<table>
<thead>
<tr>
<th>Concentration of ChNP (mg/ml)</th>
<th>Germination percent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55 ± 4.08</td>
</tr>
<tr>
<td>0.1</td>
<td>91.67 ± 2.37&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5</td>
<td>98.33 ± 2.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>95 ± 4.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>93.33 ± 2.37&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>86.67 ± 2.36&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>80 ±4.08&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>76.67 ± 4.71&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C.D. (0.05)</td>
<td>7.28</td>
</tr>
</tbody>
</table>

*Data are mean ±S.D. of three replicates. Mean values are significantly different at <i>p</i>&lt;0.05. Means with same letters are not significantly different.

Table 2.5. Definitive test for determining seed toxicity study of ChNP on rice seed germination

<table>
<thead>
<tr>
<th>Concentration of ChNP (mg/ml)</th>
<th>Germination percent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>56.67 ± 2.36&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5</td>
<td>98.33 ± 2.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>91.67 ± 2.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5</td>
<td>86.67 ± 2.36&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>83.33 ± 2.36&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td>76.67 ± 2.36&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C.D. (0.05)</td>
<td>5.14</td>
</tr>
</tbody>
</table>

*Data are mean ±S.D. of three replicates. Mean values are significantly different at <i>p</i>&lt;0.05. Means with same letters are not significantly different.
2.3.5.3. Soil Toxicity of Chitosan nanoparticles

In the present study application of both ChNP as well as chitosan has proved to have positive effects on soil environment and was enriching to the soil rather than toxic to it. The microbial population was found to be more than in control (table 2.6). The microbial population showed the normal life cycle of the microorganism with a log phase that lasted till day 15 and a gradual decline after that. ChNP treated soil had a microbial population of 2.18 log CFU/ml at day 15 compared to 1.96 log CFU/ml on day 15 (Fig. 2.13).

Table 2.6. Total microbial population (Log CFU/ml) of soil treated with ChNP and chitosan compared with control

<table>
<thead>
<tr>
<th>No of days</th>
<th>Total microbial count* (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ChNP</td>
</tr>
<tr>
<td>Day1</td>
<td>1.57</td>
</tr>
<tr>
<td>Day2</td>
<td>1.62</td>
</tr>
<tr>
<td>Day10</td>
<td>2.15</td>
</tr>
<tr>
<td>Day15</td>
<td>2.19</td>
</tr>
<tr>
<td>Day20</td>
<td>2.16</td>
</tr>
<tr>
<td>Day25</td>
<td>2.03</td>
</tr>
<tr>
<td>Day30</td>
<td>2.01</td>
</tr>
<tr>
<td>Mean</td>
<td>1.96</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>S.E. (m) ±</th>
<th>CD (0.05)</th>
<th>CD (0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>3.29</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Days</td>
<td>1.86</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Interaction</td>
<td>3.37</td>
<td>0.04</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Fig. 2.13. Total microbial population (Log CFU/ml) of soil treated with ChNP and chitosan compared with control.

Data are mean ±S.D. of three replicates. Mean values are significantly different at $p<0.05$. Means with same letters are not significantly different.

The soil dehydrogenase activity is a measure of the microbial population of soil. In the present study, the soil dehydrogenase activity was found to be the highest on day 15 like the total microbial count. The dehydrogenase activity of ChNP treated soil was found to be 3.64 $\mu$g TPF/g/soil/day while for control it was 3.11 $\mu$g TPF/g/soil/day (Fig. 2.14).
Table 2.7. Dehydrogenase activity (TPF/g/soil/day) of soil treated with ChNP and chitosan compared with control

<table>
<thead>
<tr>
<th>No of days</th>
<th>Soil dehydrogenase activity* (TPF/g/soil/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ChNP</td>
</tr>
<tr>
<td>Day1</td>
<td>2.89</td>
</tr>
<tr>
<td>Day5</td>
<td>3.32</td>
</tr>
<tr>
<td>Day10</td>
<td>3.41</td>
</tr>
<tr>
<td>Day15</td>
<td>3.63</td>
</tr>
<tr>
<td>Day20</td>
<td>3.12</td>
</tr>
<tr>
<td>Day25</td>
<td>2.89</td>
</tr>
<tr>
<td>Day30</td>
<td>2.77</td>
</tr>
<tr>
<td>Mean</td>
<td>3.14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>S.E. (m)±</th>
<th>CD (0.05)</th>
<th>CD (0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>2.67</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Days</td>
<td>8.95</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>Interaction</td>
<td>9.45</td>
<td>0.11</td>
<td>0.14</td>
</tr>
</tbody>
</table>
Fig. 2.14. Dehydrogenase activity (TPF/g/soil/day) of soil treated with ChNP and chitosan compared with control.

Data are mean ±S.D. of three replicates. Mean values are significantly different at $p<0.05$. Means with same letters are not significantly different.

The soil nitrogen levels can be understood by measuring the nitrate and ammonia levels. In the present study, the nitrate and ammonia levels were increased by ChNP and chitosan treatment. This implies the mechanism of action of soil enrichment is by increasing the soil nutrient level thereby improving the soil fertility. The application of ChNP was found to improve nutrient level causing it to double compared to the control. The nitrate level was 18.16ppm on day 5 and increased to 22.58ppm on day 28 while for control it was only 9.18ppm and 12.97ppm on day 5 and 28 respectively (Fig 2.15). The two way ANOVA showed that the results were statistically significant (Table 2.8).
Table 2.8. Soil nitrate level (ppm) of soil treated with ChNP and chitosan compared with control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Day 5</th>
<th>Day 28</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate level (ppm)*</td>
<td>ChNP</td>
<td>18.17</td>
<td>22.59</td>
<td>20.38</td>
</tr>
<tr>
<td></td>
<td>Chitosan</td>
<td>18.14</td>
<td>19.43</td>
<td>18.79</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9.19</td>
<td>12.98</td>
<td>11.09</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>15.17</td>
<td>18.33</td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td>S.E. (m)±</td>
<td>CD</td>
<td>CD</td>
<td></td>
</tr>
<tr>
<td>Treatments</td>
<td></td>
<td>(0.05)</td>
<td>(0.01)</td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td></td>
<td>0.11</td>
<td>2.35</td>
<td>3.20</td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td>0.02</td>
<td>1.92</td>
<td>2.61</td>
</tr>
</tbody>
</table>

Fig.2.15. Nitrate level (ppm) of soil treated with ChNP and chitosan compared with control.

Data are mean ±S.D. of three replicates. Mean values are significantly different at p<0.05. Means with same letters are not significantly different.
The ammonia level for ChNP treatment was 4.72ppm and 5.64ppm on day 5 and 28 whereas for control it was 2.29ppm on day 5 and 2.83ppm on day 28 (Fig 2.16). The ChNP treatment was found to be statistically superior to other treatments using two way ANOVA (Table 2.9).

**Table 2.9. Soil ammonia level (ppm) of soil treated with ChNP and chitosan compared with control.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Day 5</th>
<th>Day 28</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia level (ppm)*</td>
<td>ChNP</td>
<td>4.73</td>
<td>5.65</td>
<td>5.19</td>
</tr>
<tr>
<td></td>
<td>Chitosan</td>
<td>4.69</td>
<td>4.96</td>
<td>4.83</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.29</td>
<td>2.84</td>
<td>2.57</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>3.90</td>
<td>4.48</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>S.E. (m)±</th>
<th>CD (0.05)</th>
<th>CD (0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td>0.02</td>
<td>0.60</td>
<td>0.82</td>
</tr>
<tr>
<td>Days</td>
<td>0.11</td>
<td>0.495</td>
<td>0.87</td>
</tr>
<tr>
<td>Interaction</td>
<td>4.02</td>
<td>0.86</td>
<td>1.18</td>
</tr>
</tbody>
</table>
Fig. 2.16. Ammonia level (ppm) of soil treated with ChNP and chitosan compared with control.

Data are mean ±S.D. of three replicates. Mean values are significantly different at $p<0.05$. Means with same letters are not significantly different.

2.4. Discussion

2.4.1. Extraction and characterisation of chitosan from shrimp shells

Chitin is a linear polymer of 2-acetamido-2-deoxy-β-D-glucopyranose (Glc NAac). This is deacetylated to form chitosan. It is a linear binary polymer of Glc NAac and 2-amino-2-deoxy-β-D-glucopyranose (Glc N) (Badawy and Rabea 2011). The presence of protein and minerals makes the extraction process difficult. Hence obtaining a good yield is vital in the extraction process. In the present work, a yield of 46% chitosan was obtained. The yield reported by (Puvvada et al. 2012) was
34%. The difference in yield is due to reaction time which has a positive effect on the yield (Yen et al. 2009).

The biophysical characteristics of chitin and chitosan have a very important role in its properties and applications. Hence a detailed account of the various chemical and physical characteristics of chitin and chitosan is essential (Aranaz et al. 2009). The ash content of chitosan is an indication of the effectiveness of the method employed for removing inorganic materials. It is lowest in squid pen chitosan, about 0.17% (Shepherd et al. 1997). The moisture content may vary depending on the season, relative humidity and intensity of sunlight. Even though previous studies reported much less moisture content, permitted level is below 10% (Islam et al. 2011).

The viscosity of chitosan can be used to determine molecular weight. High molecular weight chitosan yields a high viscous solution. Hence low viscosity chitosan is more preferred (Badawy and Rabea 2011). In the present study, a low viscous chitosan is obtained.

Chitosan is fully or partially N-deacetylated derivative of chitin (Ravi Kumar et al. 2013). The higher DD value is due to high amount of protein (Puvvada et al. 2012). Chitosan, unlike chitin, has a high content of highly protonated free amino group that attracts ionic compounds. This is the reason for its solubility in inorganic acid (Mohammed et al. 2013).

The characteristic peaks of chitosan are reported in the range of 2θ= 9.9- 10.7 and 19.8- 20.7 (Prashanth et al. 2002). The SEM images of the present study showing long thin crystal chitosan structures were in accordance with previous data (Hwang 2013). Non-homogenous and non-smooth surface structure of chitosan was also reported by (Mohanasrinivasan et al. 2014). The FT-IR image obtained shows
characteristic peaks of chitin and chitosan. Chitin peak at 1375 cm\(^{-1}\) corresponds to symmetrical deformation to CH\(_3\) group. The peak at 1552 cm\(^{-1}\) corresponds to N-H deformation of amide II. 1618 cm\(^{-1}\) corresponds to vibration of amide I band and 1654 cm\(^{-1}\) corresponds to amide I stretching of C=O (Limam et al. 2013). The peak of chitosan FT-IR image at 1029 cm\(^{-1}\) corresponding to free amino group at the C2 position of glucosamine. It is a major peak of chitosan. The peak at 1375 cm\(^{-1}\) corresponds to C-O stretching of the primary alcoholic group (Rafeeq et al. 2010).

### 2.4.2. Synthesis and characterisation of chitosan nanoparticles

Chitosan nanoparticles were formed by ionic interaction between negatively charged polyanion, tripolyphosphate and positively charged amino group (–NH\(_3\)) of chitosan (Tarafdar and Biswas 2013). TPP forms five ionic cross-linkages with the cationic groups of chitosan (Karimi et al. 2013). The stability of nanoparticles formed can be attributed to the ionic cross-linkage as well as the mechanically robust nature of chitosan (Koukaras et al. 2012).

XRD pattern of ChNP done for optimisation purpose depicts diffractograms with a peak at 2\(\theta\)= 18°- 20° and diffractograms without peaks. The XRD patterns with no peak are a characteristic feature of amorphous structure (Sathiyanabam and Parthasarathy 2016). The shift between the amorphous and crystalline structure of ChNP is due to the semi-crystalline nature of chitosan (Jayakumar et al. 2010).

The broadband peak in FT-IR spectrum indicates the presence of –OH and –NH\(_3\) groups (Kaur et al. 2013). The peak at 2880 cm\(^{-1}\) corresponds to CH\(_2\)groups. The peak at 1643 cm\(^{-1}\) corresponds to cross-linkage of ammonium groups within phosphoric groups of TPP molecules (Qi et al. 2004). The peak at 1419 cm\(^{-1}\) corresponds to C-O
stretching (Saharan et al. 2016). HRTEM and SEM images of chitosan nanoparticles have been reported as smooth spherical structures with a size range between 10-150nm size (Sathiyabama and Parthasarathy 2016).

2.4.3. Toxicity study of Chitosan nanoparticles

Nanoparticles have increased activity compared to its bulk counterpart owing to its small size and a large surface to volume ratio (Heller et al. 2013). This increased activity could also mean that toxic effects not found in the parent compound might be present in the nanoform (Taraifar 2013). The agricultural application of ChNP leads to soil accumulation of nanoparticles that will lead to accumulation in the ecosystem through the food chain.

The cytotoxic effect of ChNP against HepG2 human liver cancer cell line was reported by Loutfy et al (Loutfy et al. 2016) and their results are in accordance with the present study. They reported that 100μg/ml of ChNP inhibited 12% of cells and had an IC₅₀ value of 230μg/ml.

Seed germination is a widely used phytotoxicity test owing to its sensitivity, low cost and simplicity (Wang et al. 2005). There have been reports of the beneficial effects of ChNP on plant growth and germination. This can be attributed to the absorption and utilization of nanoparticles by the embryo (Suriyaprabha et al. 2012). However, these effects seemed to diminish on exposure to higher concentrations (Behboudi et al. 2017). The low activity of nanoparticles at high concentration may be due to the fact that the nanoparticles were not able to penetrate endosperm and seed coat (Duke and Kakefuda 1981).

Soil application of a toxic substance will result in a reduction of the microbial population as well as adversely affects the soil enzyme and nutrient levels (Yuan et al. 2011). In this context, the analysis of soil toxicity of ChNP is important prior to its agricultural application (Yuan
et al. 2011). In the present study, ChNP and chitosan application were found to have soil enriching effect rather than toxic effect. The impact of the introduction of fullerene as either C_{60} or n C_{60} on soil environment was studied and found to have a very little effect on the structure and function of soil microflora (Tong et al. 2007). Whereas Silver nanoparticles and zinc oxide nanoparticles were reported to have a negative effect on the plant growth as well as on the soil environment (Lee et al. 2013).

2.4. Conclusion

Chitosan is acclaimed for its nontoxic, biodegradability, polycationic nature. Chitosan was obtained using the modified process of previous studies. The characteristics of produced chitosan were in accordance with the commercial standard. The obtained chitosan had low viscosity, high DD and a denser crystalline structure. Chitosan with such properties has many commercial applications and greater scope of applications. Chitosan nanoparticle was prepared by the incorporation of polyanion like tripolyphosphate (TPP) in chitosan solution under continuous stirring. The nanoparticle characterisation revealed spherical shaped nanoparticles of 19nm size. The low size and high surface to volume ratio of ChNP make it a more eligible for agricultural and other industrial applications. ChNP like its bulk counterpart was found to be non-toxic in nature and hence can be used in application studies to understand the versatile applications of chitosan with the main focus on agricultural applications.
2.6. References


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Guidel


