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Research Article

ANTIBACTERIAL EFFICACY OF CHITOSAN AND ITS MEDIATED SILVER NANOPARTICLES FROM SHELL WASTES OF *PORTUNUS PELAGICUS*

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ABSTRACT

At present, silver nanoparticles have found a distinctive place in the field of biomedicine due to their unique antibacterial property. In the present study, chitosan a non toxic bioploymer extracted from *Portunus pelagicus* crab shell wastes was used as a stabilizer and a reducing agent in the biosynthesis of silver nanoparticles. Chitosan mediated silver nanoparticles was synthesized and the antibacterial efficacy of both has been examined. Crab shell wastes were demineralized and deproteinized to yield chitin and it was partially deacetylated to yield chitosan. Antibacterial activity was conducted against two gram negative *E.coli*, *Pseudomonas aeruginosa* and two gram positive *Bacillus subtilis, Staphylococcus aureus* bacterial strains using disc diffusion assay. Streptomycin was used as positive control and acetic acid was used as negative control. Antibacterial activity of chitosan mediated silver nanoparticles showed pronounced activity compared to chitosan. Thus it can widely be used as good antimicrobial agent in biomedical and pharmaceutical industries.

INTRODUCTION

Nanotechnology has become an apparent field in modern material science fabricating nanoparticles (1-100 nm) with wider applications. Recently the attention has been focussed around the metallic nanoparticles such as silver (Sharma *et al*., 2009; Atta *et al*., 2014), gold (Yeh *et al*., 2012) and zinc oxide nanoparticles (Kavitha *et al*., 2017) etc. For many centuries, silver is the metal of choice by humans and has been used an effective antibiotic agent. Silver nanoparticles (AgNPs) have emerged as an arch product owing to broad antibacterial activity and low toxicity towards mammalian cells (Hien *et al*., 2015). To avoid the adverse effects of silver nanoparticles in medical field, researchers have turned towards green synthesis protocols using microorganisms, yeast and plant extracts (Mallikarjuna *et al*., 2011; Shah *et al*., 2015). Synthesis of AgNPs using plant extracts (Kuppusamy *et al*., 2016) have popped up as a novel approach but their stability and dispersion are very critical (Wang *et al*. 2015). Nowadays, stabilizers like natural polymers are gaining much interest among the scientific community than the synthetic polymers derived from petroleum oils as they are non-toxic and biodegradable materials.

Chitin is a linear, un-branched biopolymer consists of N-acetyl D-glucosamine units (Schiffman and Shauer, 2009) linked by β (1,4) glycosidic bonds. This polysaccharide is abundantly found in the exoskeleton of crabs, shrimps, prawns (Mohanasrinivasan *et al*., 2013), in cuticle of insects (Oduor-Odote *et al*., 2005) and in cell wall of fungi (Dhillon *et al*., 2013). There are 3 forms namely α,β,γ chitin (Prabhu and Natarajan, 2012). Applications of chitin has been restricted due to its compact structure it is insoluble in most of the solvents like water, acetone, dilute acids or dilute alkalines (Subhapradha *et al*., 2013). In order to make it applicable, chitin is partial deacetylated with 40% NaoH which yields chitosan. Chitosan is a straight chain polymer consists of both D-glcosamine and N-acetyl D- glcosamine (Lopez Mata *et al*., 2015). It consists of more amine group and readily soluble in acetic acid or formic acid. Thus it became highly biocompatible with unique physical, chemical and biological properties (Cheung *et al*., 2015). Molecular weight, degree of deacetylation and source of raw material used are the main factors in determining the physicochemical properties of chitosan (Chaandumpai *et al*., 2004). Being a polymer derived from natural resource, it possesses antimicrobial, antioxidant, antidiabetic, antitumor, hypocholesterolemic, haemostatic and wound healing properties (Si Trung and Bao, 2015). In the present study, antibacterial activity of chitosan and chitosan mediated silver nanoparticles (AgNPs) were evaluated against four human pathogenic bacterial strains namely *E.coli*, *Pseudomonas aeruginosa* (gram negative) and *Bacillus subtilis, Staphylococcus aureus* (gram positive) bacterial strains.

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MATERIAL AND METHODS

Collection of raw material

Crab shell wastes were collected from a seafood company, Palakkudi, Mimisal, Tamil Nadu and were brought to the laboratory. The shells were washed thrice until the sand and mud were removed. They were placed in Ziploc bags and brought to the laboratory. Approximately, 1500 grams of shell wastes were weighed and shade dried for 5 days. Then they were crushed into smaller pieces. The samples were oven-dried at 65°C until a constant weight was obtained. The dry weight of the samples were determined and used for further analysis.

Extraction of chitin and chitosan from crab shell wastes
Demineralization

20gm of sample powder was demineralised with 300ml of 2N HCl for 24 hours with constant stirring and then filtered. The filtrate was again washed with distilled water and filtered till the liquid showed neutral pH. The filtrate was then dried in hot air oven and weighed following the method of Takiguchi (1991a).

Deproteinization

The sample (filterate obtained after demineralization) was then deprotenized with 300ml of 1N NaOH at 80 °C for 24 h with constant stirring. After 24 h the sample was filtered. The sample filtrate was washed as before and dried. The weight was noted (Takiguchi. 1991a).

Deacetylation (Conversion of Chitin into Chitosan)

Chitosan was extracted from crab chitin through deacetylation process following the method of Takiguchi, 1991b. Briefly, chitin was deacetylated with 40% NaOH, heated for 6 h at 110°C in constant stirring then 10% acetic acid was added to the sample and stored for 12 h at room temperature with constant stirring. The dissolved sample was reprecipitated by adding 40% NaOH to pH 10. The sample was then dialyzed by deionized water to a pH of 6.5 and centrifuged at 10,000 rpm for 10 min and freeze dried.

Synthesis of Chitosan mediated silver nanoparticles

One step green synthesis of chitosan mediated AgNPs was done following the method of Wei *et al*., 2009 with slight modifications. 10 ml of 1% chitosan was mixed 4 ml of 52 mM $AgNO_3$ and kept at 95^0 C in a water bath and allowed to stand for 6 to 12 hours.

Antibacterial Activity

Bacterial strains

Two bacterial strains were tested for both chitosan and chitosan mediated AgNPs antibacterial efficacy. Gram positive bacteria *Staphylocccus aureus* and Gram negative bacteria *Pseudomonas aeruginosa* were purchased from CORX Life Sciences and Pharmaceutical Private Limited, Trichy.

Inoculum preparation

Nutrient broth was prepared and sterilized in an autoclave at 15 pounds pressure for 15 minutes. All the bacterial strains were individually inoculated in the sterilized nutrient broth and incubated at 37°C for 24 hours.

Evaluation of antimicrobial activity

Antibacterial activity of the chitosan (extracted) and chitosan mediated AgNPs was analyzed following the method of Bauer *et al*., 1996. 1% chitosan in 1% acetic acid was used as stock solution from which the desired working concentration was prepared. Chitosan and chitosan mediated AgNPs samples (100µg/ml) were tested for antibacterial activity using agar disc diffusion assay. Media were prepared using nutrient Agar (Himedia), poured on petridishes and inoculated with the test organisms from the seeded broth using cotton swabs. Sterile discs of six millimeter width had been impregnated with different concentrations of samples and introduced onto the upper layer of the seeded agar plate. The plates were incubated overnight at 37^0 C. Antibacterial activity was assessed by measuring the inhibition zone formed around the discs in millimeters. Streptomycin was used as positive control and acetic acid was used as negative control.

Statistical analysis

Results were expressed as mean ± S.D for triplicates. The data were subjected to analysis of variance (one way ANOVA) and significant differences (if $P < 0.05$) between the means were compared with Turkey's post hoc test using PAST 3.09 version.

RESULTS

Synthesis of chitosan and chitosan mediated AgNPs

In the present study, chitin (Figure. 1A) was successfully synthesized from crab shell wastes by demineralization and deproteinization process. Chitin was partially deacetylated with 40% NaOH to obtain chitosan (Figure. 1B). Chitosan mediated AgNPs were produced by green synthesis using $AgNO_3$. In this process, chitosan has acted as both a stabilizer and as a reducing agent. The colourless solution changed into yellow and finally yellowish brown which indicated the formation of AgNPs (Figure 2A and B). This approach appeared to be cost effective and an alternative to conventional methods of assembling silver nanoparticles.

Antibacterial activity of chitosan and chitosan mediated AgNPs

Antibacterial activity of was tested chitosan and chitosan mediated AgNPs against four bacterial strains two gram negative *E.coli* (Fig. 3), *Pseudomonas aeruginosa* (Fig. 4) and two gram positive *Bacillus subtilus* (Fig. 5) and *Staphylococcus aureus* (Fig. 6) on nutrient agar plates. It is concluded that chitosan mediated AgNPs showed higher activity than chitosan against all the four bacterial strains using disc diffusion method. The zone of inhibition is given in Table 1. Negative control acetic acid and positive control Streptomycin is also maintained and zone of inhibition was represented in Table.1.

Chitosan revealed highest antibacterial activity against *E.coli* (15.23 mm) followed by *Staphylococcus aureus* (14.9 mm) *Pseudomonas aeruginosa* (14.06 mm). The lowest antibacterial activity was against *Bacillus subtilus* (13.7 mm). Chitosan mediated AgNPs revealed highest antibacterial activity against *Staphylococcus aureus* (36.1 mm) followed by *Pseudomonas aeruginosa* (34.33 mm) and *E.coli* (33.83 mm). The lowest antibacterial activity was against *Bacillus subtilus* (33.66 mm).

Over all, Chitosan mediated AgNPs revealed highest antibacterial activity when compared with chitosan with all the four strains of bacteria.

Figure 1 A

Figure 1 B

Figure 2.A 2.B

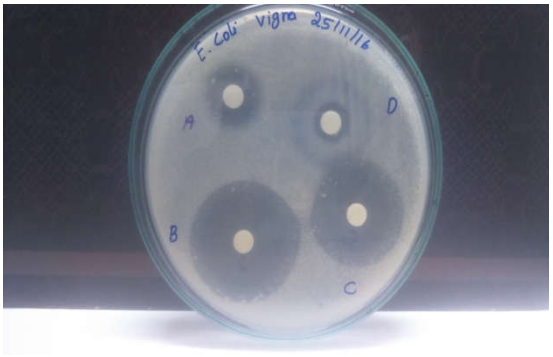

Figure 3

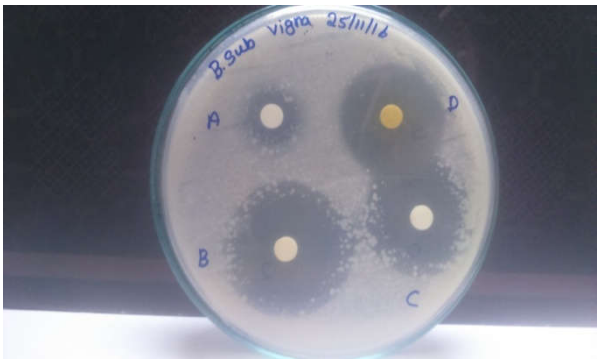

Figure 4

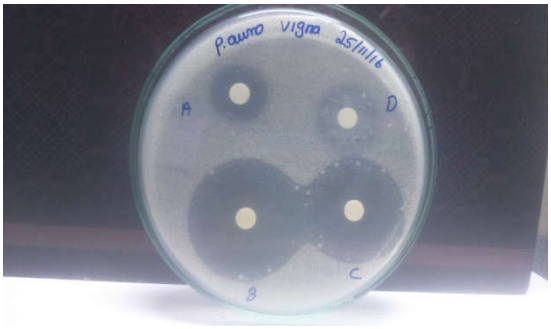

Figure 5

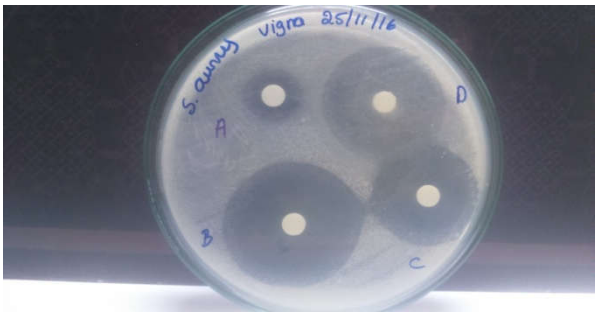

Figure. 6

A-Chitosan (100 µg/ml); **B**- Chitosan AgNPs (100 µg/ml); **C**- Negative Control (100 µg/ml); **D**-Streptomycin (100 µg/ml).

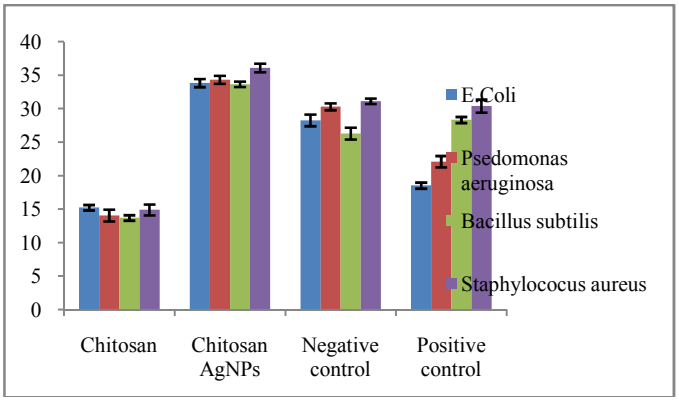

Figure 7 Antibacterial activity of chitosan against four bacterial strains

Table 1 Antibacterial activity of chitosan and chitosan mediated AgNPs

Bacterial strains	Zone of Inhibition (mm)			
	Chitosan	Chitosan Agnps	Negative control	Positive control
Gram-negative				
E.coli	15.23 ± 0.4	33.83 ± 0.61	28.26 ± 0.87	18.53 ± 0.45
Pseudomonas aeruginosa	14.06 ± 0.4	34.33 ± 0.41	30.3 ± 0.88	22.1 ± 0.45
Gram-positive				
Bacillus subtilis	13.7 ± 0.88	33.66 ± 0.61	26.3 ± 0.51	28.33 ± 0.85
Staphylococcus aureus	14.9 ± 0.81	36.1 ± 0.65	31.13 ± 0.41	30.4 ± 0.96

DISCUSSION

Disposal of enormous amount of seafood processing wastes in the coastal areas has led to a serious environmental problem (Blanco *et al*. 2007). All over the world, the amount of valuable chitin biomass production per year by the exoskeleton of crustaceans, molluscs, insects and fungi is estimated to be 100 billion tonnes (Bolat *et al*., 2010). The present study deals with the synthesis of chitosan from the shell wastes of *portunus pelagicus* collected from K.P.P seafood processing small scale industry. Our studies on extraction of chitin and chitosan from crab shell wastes are in accordance with Thirunavukkarasu and Shanmugam *et al*., 2009; Das and Ganesh, 2010; Webster *et al*., 2014.

Many reporters showed the antimicrobial activity of chitosan against several strains of bacteria (Cruz Romero *et al*., 2013; Mohanasrinivasan *et al*., 2011; No *et al*., 2003 and Liu *et al*., 2006). Ganan *et al*., 2009 showed chitosan with a molecular weight (120 kDa) was most active and highly sensitive against campylobacter species and it showed loss in membrane integrity of that species. Hafsa *et al*., 2014 stated that chitosan showed better antibacterial activity than chitosan ascorbate. The mechanism behind antibacterial activity involves interactions of positively charged chitosan molecules with negatively charged constituents of microbial cell walls thus results in interrupting normal cell metabolism of microorganisms (Monarul Islam *et al*., 2011). Antibacterial activity of chitosan is due to its polycationic nature (Raafat and Sahl, 2009) and thus used as a natural food preservative in food industries.

In a comparative study of antibacterial activity between chitosan and chitosan AgNPs, chitosan AgNPs showed higher activity than raw chitosan (Wei *et al*., 2009; Shameli *et al*., 2010). According to Hien *et al*., (2015) the antibacterial efficiency of chitosan AgNPs in cotton fabrics tested against *S. aureus* showed highly antibacterial efficiency. Venkatesham *et al*., (2014) also proved that the increased antibacterial efficacy of chitosan AgNPs against *E.coli* and *Micrococcus luteus* species. According to Akmaz *et al*., (2013), antibacterial activity of chitosan AgNPs increased with the increasing concentration of $AgNO_3$ used during the synthesis process. AgNPs penetrated into the cell membrane of bacteria and brought about cell death (Nyugen *et al*., 2014). It was evident from the present study, chitosan mediated AgNPs showed higher antibacterial activity when compared to chitosan as growth inhibitors of bacterial strains.

CONCLUSION

In the present study, simple, easy, eco-friendly procedure has been carried out to synthesis silver nanoparticles using chitosan, a natural polymer. In this method, chitosan has reduced and stabilized the dispersion of nanoparticles without any chemicals and requirement of high temperature or pressure. Being a natural compound and non toxic, it can be recommended to be widely used in food, pharmaceutical and biomedical field.

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