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Extraction And Characterization Of Chitin And Chitosan From *Aspergillus Niger*, Synthesis Of Silver-Chitosan Nanocomposites And Evaluation Of Their Antimicrobial Potential

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Abstract:

The fungal species such as *Aspergillus niger* are the rich source of antimicrobial compounds. The chitin which is the cell wall component are being isolated and used as potential source of antimicrobial along with its derivates. In the present study, the chitin and chitosan were purified from the mycelial mat of the *A. niger*. By using the chitosan isolated from the fungi, the chitosan-silver nanocomposite was synthesized under lab conditions. The comparative antibacterial potential of chitin, chitosan and nanocomposites were screened against *Bacillus subtilis* and *Bacillus cereus*, which are infecting mulberry silkworm (Bombyx mori) and humans respectively. The *in vitro* inhibition study results revealed that, Nanocomposites showed more inhibition of pathogens in comparison with other treatments along with positive control. Further, the highest antibacterial activity of chitosan-silver nanocomposites may be due to presence of two potential antimicrobial compounds in combination. Based on the results of the present study, chitosan based nanocomposites can be used for the management of the bacterial diseases of silkworm. The nanocomposite derived in the present work can be used for management of medically important pathogens infecting humans.

Key words:

Aspergillus Niger, Chitosan, Nanocomposites, Silkworm, Bacterial pathogens.

Running Title: Antibacterial Chitosan-silver nanocomposites from *Aspergillus niger*

Introduction

Aspergillus niger is a ubiquitous fungus which is present in all moist environment. The fungal cell is one of the dynamic structure which helps in the protection of the cell from the environmental stress. The structure of the fungal cell wall is unique and there by an excellent target for the development of antifungal drugs. Based on the studies in number of fungi, the cell wall is composed of chitin, glucans, mannans and glycoproteins.Chitin is one such product which contributes to strengthen the cell and necessary to maintain the cell wall integrity (1). Chitin and Chitosan are the naturally occurring biopolymers while Chitosan is the second most abundant polymer used after cellulose. The present trend of utilization of chitin and chitosan towards producing high value products in cosmetics, drugs carriers, feed additives, semi permeable membranes and pharmaceutics is gaining importance(1).

Chitin is insoluble linear beta,1-4 linked polymer of N-acetyl glucosamine. Chitosan is the cationic amino polysaccharide derived from the deacetylation of chitin. Few fungal organisms such as *Aspergilus niger, Aspergillus terreus*, *Aspergillus fumigatus* and *Penicillium species* give good yield of chitin and Chitosan showing the antimicrobial potential over the pathogenic organisms. Usually extraction of chitin and Chitosan from fungal organisms depends on species to species.

Synthesis and utilization of Chitosan silver nanocomposites is been studied by different researchers in various fields such as, induction of protective immune responses (2). Silver nanocomposite materials were synthesized by simple chemical method. These silver nanocomposites have therapeutic potential and exhibit good antimicrobial activity. They also exhibit high performance even at low temperature. Most importantly silver Chitosan nanocomposite is one of the rare composite materials which have good antimicrobial potential as well as biosensing activity. One more advantage is that Chitosan-Silver nanocomposite is biodegradable. Scanning Electron microscope (SEM), X-ray diffraction (XRD) and Dynamic Light Scattering (DLS) were used for the characterization of silver Chitosan nanocomposite.

In the existing scenario, researchers are paying more attention to find out new sources of organisms which are abundantly available and which can be easily cultured for the isolation of Chitin and Chitosan. The present work also aims to isolate, purify chitin and chitosan from *A. niger*. The study also concentrates on synthesis and characterization of antimicrobial chitosan-silver nanocomposite from the chitosan isolated from the fungus.

Materials and Methods

Isolation of fungi

The bread procured from the local shop was moistened by pouring little quantity of water. The moistened bread was left for the growth of the fungal organism for 5 to 7 days. After few days, the mycelia growth of the fungus was noticed regularly. After the attainment of its maximum growth, the single spore was inoculated to culture plate having Potato Dextrose Agar (PDA) medium (Fig. 1). The inoculated plates were incubated for 5 to 7 days. From the pure culture, the maximum production of fungi was carried out by inoculating fungi to Potato Dextrose Broth. After 7 days of inoculation, the fungal mat grown on PDB was used for the extraction of chitin.

Extraction of chitin and chitosan

The mycelia mat of *A. niger* was grinded into a powdery form using liquid nitrogen in a pre-chilled pestle and mortar. Extraction involved two steps viz., Deproteinization and Deacetylation. Deproteination of extract was done by using sodium hydroxide (2% W/V) at ratio 30:1 V/W at the temperature of 90^0C for 2 hours. After deproteination, the filtrate was centrifuged at 4000 rpm for 15 minutes. Chitin was collected separating the insoluble fractions. Chitin was deacetylated with 10% v/v Acetic acid (40:1v/w) and incubated for 6 hours at 60^0C and centrifuged at 6000rpm for 15minutes.After deacetylation, Chitosan was extracted as final product. Precipitation of Chitosan was done by maintaining pH at 9.0 by adding Sodium Hydroxide (4M). After precipitation, the filtrate was centrifuged at 4000 rpm for 15 minutes. The collected Chitosan was further used for the synthesis of nanocomposites. Based on the biomass production, the recovery % of both chitin and Chitosan was 8.46% and 9.83% respectively.

Synthesis of chitosan-silver nanocomposites

For the extraction of nanocomposites, purified Chitosan (0.1g) was taken in a microcentrifuge tube and dissolved with 100πl acetic acid. The mixture was dispensed in the 50ml distilled water. The solution was heated upto 95^0C or more such that the Chitosan gets completely dissolved. Under the boiling condition, silver nitrate (1ml; 30mM) and Sodium hydroxide (1ml; 0.3M) was added slowly with constant stirring. The solution was allowed for cooling at room temperature. The precipitated nanocomposites were centrifuged and pellet was collected.

Subculture of pathogens

The bacterial cultures of medically important *Bacillus cereus* and *Bacillus subtilis* (pathogenic to mulberry silkworm) which were pre-maintained in the Silkworm Pathology section of Central Sericultural Research and Training Institute, Mysore, Karnataka, INDIA were sub-cultured on the Nutrient Agar (NA) media by using standard inoculation method.

In vitro inhibition of bacterial pathogens by using Chitin, Chitosan and Chitosan silver nanocomposites

Each Pathogenic bacterial suspension was prepared in sterilized distilled water and the initial concentration of bacteria was adjusted to approximately 10^8 colony-forming units (CFU)/ml. Suspension of microbial cultures was inoculated on the entire surface of the Nutrient agar media in a Petri plate using sterile swab sticks. The sterile discs of diameter 6 mm were impregnated with Chitin, Chitosan and Chitosan-Silver nanocomposite solutions at the cconcentrations of chitin (8mg/ml), Chitosan (4mg) and nanocomposite (2mg/ml) as Minimal Inhibitory Concentration (MIC) and placed onto the cultured Nutrient agar plates. Inoculated plates were incubated at 37°C for 24 hrs. On the second day, plates were read by taking measurement of zone of inhibition around each disc. The diameter of zone of inhibition of bacteria was recorded in millimeters. Commerical ampicilin was used as positive control. The assay was done in triplicates and checked with the control plate.

Characterization of Chitosan Silver Nanocomposite

After the extraction of Chitosan-Silver nanocomposites, the obtained composites were subjected to sonication which leads to formation of fine powdery material. The fine powder of the nanocomposite was characterized by using three analytical techniques viz., SEM, XRD and DLS.

Results

In vitro inhibition of bacterial pathogens

Antimicrobial susceptibility test was evaluated against *Bacillus thuringenesis* (Mulberry silkworm pathogenic) and *Bacillus cereus* (Human pathogenic) showed the inhibition of both pathogens by chitin, chitosan and nanocomposites. Higher inhibition of *B. thuringenesis* and *B. cereus* was showed by nanocomposite followed by chitosan and chitin (Table 1& Fig. 2).

Characterization of chitosan-silver nanocomposites

Scanning Electron Microscope

The surface morphology of the synthesized silver Chitosan nanocomposite was analyzed using this technique. The SEM images show that the nanocomposites are in the form of cubic shaped particle. It is observed that the Chitosan and silver nanocomposites are embedded to each other to form a confined nanosized range. The SEM image showed 164nm and 241nm sizedparticle (Fig. 3).

X-Ray Diffraction studies

The structural properties of the synthesized silver Chitosan nanocomposite were analyzed using the XRD technique. The presence of Chitosan as well as silver peaks was observed from the graph pattern. Silver peaks were obtained at 2Θvalues of 1.9^0 and 2.3^0 and Chitosan peaks showed at $3.7^0$and $3.9^0$with no impurity (Fig. 4).

Dispersive Light Scattering method

The particle size distribution of the silver Chitosan nanocomposite was analyzed using DLS technique. The graph of nanocomposite showed presence of only two different peaks (Fig. 5) with negative charge under -3.4mv zeta potential at neutral pH (Fig. 6).

Discussion

Aspergillus molds are found throughout the world and are the most common type of fungi in the environment. The fungus cell wall comprises chitin which is the natural polysaccharide after cellulose. But not only the fungal cell, natural sources such as crustaceans, insects, annelids, molluscs are also found in wide range. The isolation of chitin form the fungus was found to be 8.46% and Chitosan with 9.83% wherein the increase in the biomass showed increase in the productivity. In contrast to our study, the strains of *A.niger* in the increase in the biomass showed increase in the yield of Chitosan (3).

In gram positive bacteria, cell membrane is covered by cell wall consisting of layer of peptidoglycans which contains acetylmuramic acid as well as D-and L-amino acid and techoic acid (4) to which the positively charged amino groups of Chitosan binds, result in cell wall distortion, disruption and expose cell membrane to osmotic shock and exudation of cytoplasmic contents (5).

Chitin and Chitosan are the important by products obtained from the cell of the fungus which is not only examined with the *A. niger* but with few of its strains and especially with *A. terreus* (6). Inhibition zone valves were obtained from the synthesized samples aganist *Bacillus thuringienesis* (Mulberry silkworm pathogen) and *Bacillus cereus* (Human pathogen). The table 1 showed that Chitosan silver nanocomposite and Chitosan showed high antibacterial activity against *B. thuringienesis* and *B. cereus*. This showed that the particle size of the nanocomposite interacted with the bacteria which in turn generated an electronic effect that improved the reactivity of the silver chitosan nanocomposite. The average size of inhibition zone varied from 0.5cm for chitin, 0.7cm for Chitosan and 1.2cm for silver Chitosan nanocomposite aganist *B. thuringienesis.* Similarly, zone of inhibition in *B. Cereus* showed 0.6cm for chitin, 0.7 cm for Chitosan and 1.3cm for Chitosan silver nanocomposite. Figure 2 showed the clear inhibition of bacterial pathogens by chitin, chitosan and nanocomposites.

The structural morphology of the Chitosan silver nanocomposite was characterized by scanning electron microscope (SEM) wherein it focuses the sample surface topography and composition by the interaction of electrons with atoms in the sample. The sample of silver Chitosan nanocomposite showed the size of having 165nm and 241nm. It is observed that the sliver nanocomposite is embedded in the matrix of Chitosan and also the nanocomposite is confined to the nano range. The shape of the particle was found to be cubic shaped in its morphology. The SEM images were found to have porous structure and smooth surface morphology. In XRD, the patterns of silver Chitosan nanocomposite were obtained and the sharpness of the peaks was high enough in both Chitosan and silver (7). This case of study allows us to understand the sample purity. Similar techniques were performed with respect to crude chitin extracted from the fish scale (8). In DLS, the particle size can be determined with respect to its diameter and width as well as the charge of the particle. From our part of study the Silver peaks were obtained which showed diameter of 1553nm and width 940nm and Chitosan showed 78.8nm in diameter and 8.36nm width. On the basis of zeta potential analysis, nanocomposite was found to be negatively charged.In contrast to earlier studies, the lower molecular weight chitosan is more effective against gram negative bacteria(9), inresults revealed that, due to high molecular weight, Chitosan was more effective over the gram positive bacteria *B. thuringienesis* and*B. cereus*.

Conclusion

Based on the results of the present study, chitosan based nanocomposites can be used for the management of the bacterial diseases of silkworm and medically important pathogens infecting humans. The chitosan derived from *A. niger* can be used for the base material for the effective drug delivery system against bacterial pathogens.

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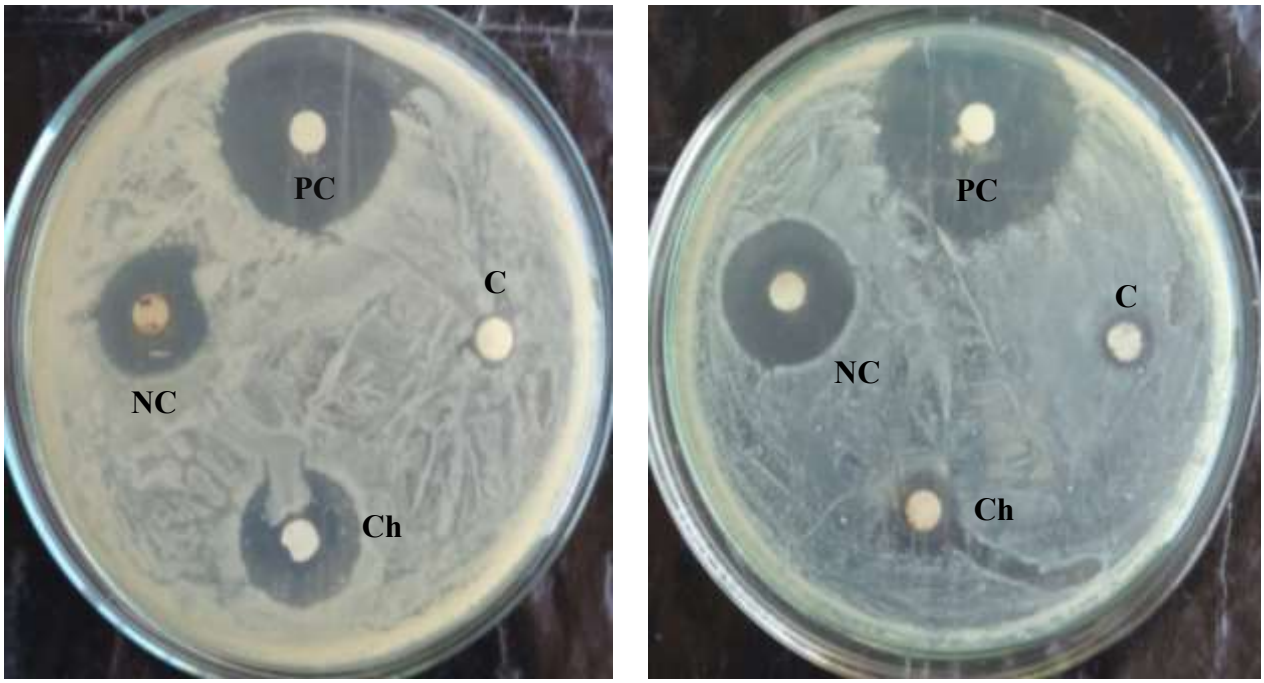
Table 1. The inhibitory potential of chitin, chitosan and chitosan-silver nanocomposites against *B. thuringienesis* and *B. cereus.*

Treatments	Growth inhibition (in Cms)	
	B. subtilis	B. cereus
Chitin	0.5± 0.73	0.6± 0.41
Chitosan	0.7± 0.47	0.7± 0.57
Nanocomposite	1.2± 0.57	1.3± 0.43
Positive control	1.9± 0.33	2.0± 0.37

The values are the means of three replicates ± SE.

Figure 1. The Potato Dextrose Agar (PDA) mediashowing growth of Aspergillus *niger*.



B. thuringenesis ***B. cereus***

Fig.2. Inhibition of bacterial pathogens by chitin, chitosan and chitosan-silver nanocomposites. C-Chitin, Ch-Chitosan, NC- chitosan-silver nanocomposites and PC-Positive control.

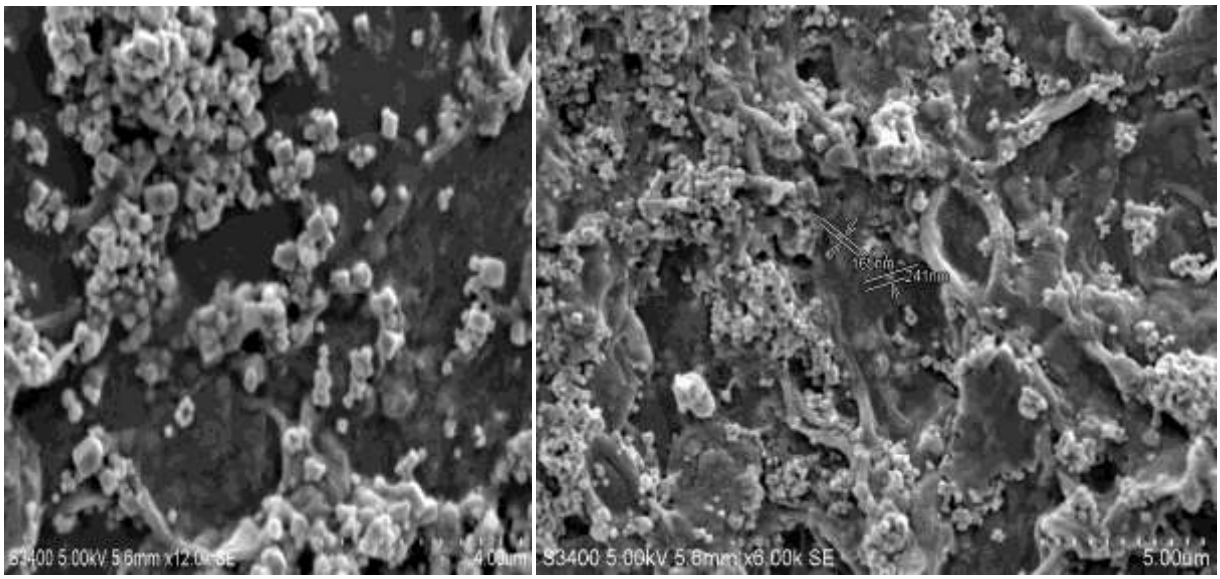


Fig. 3. Scanning Electron Microscope photographs of chitosan-silver nanocomposites

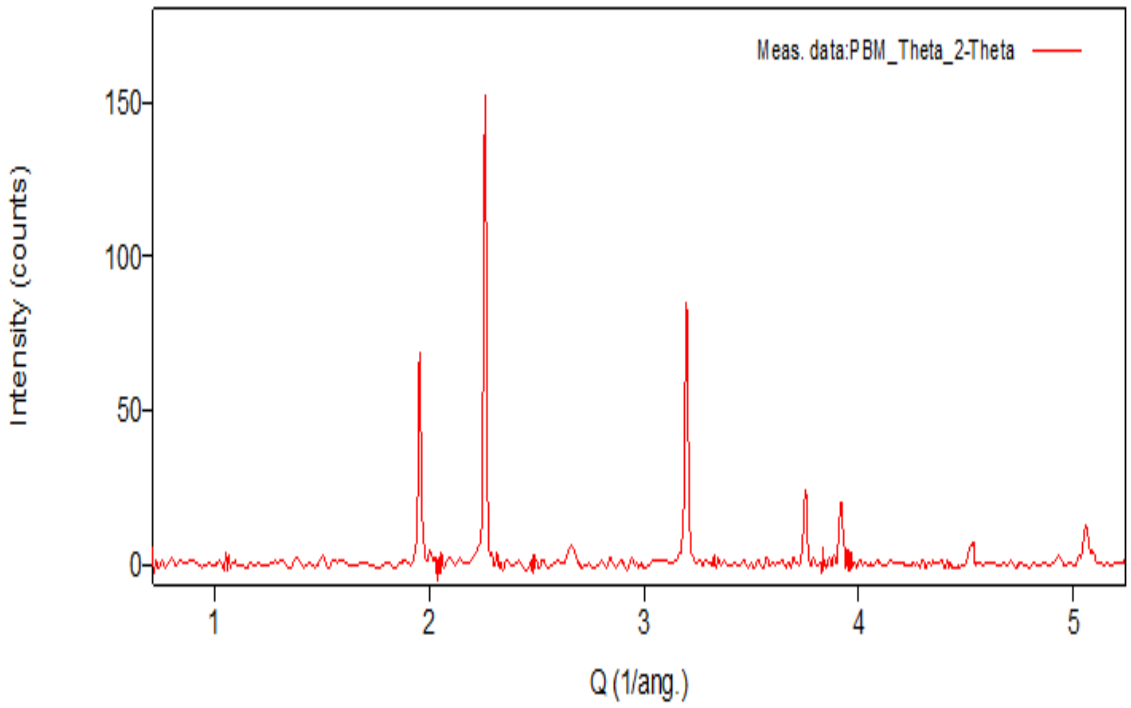
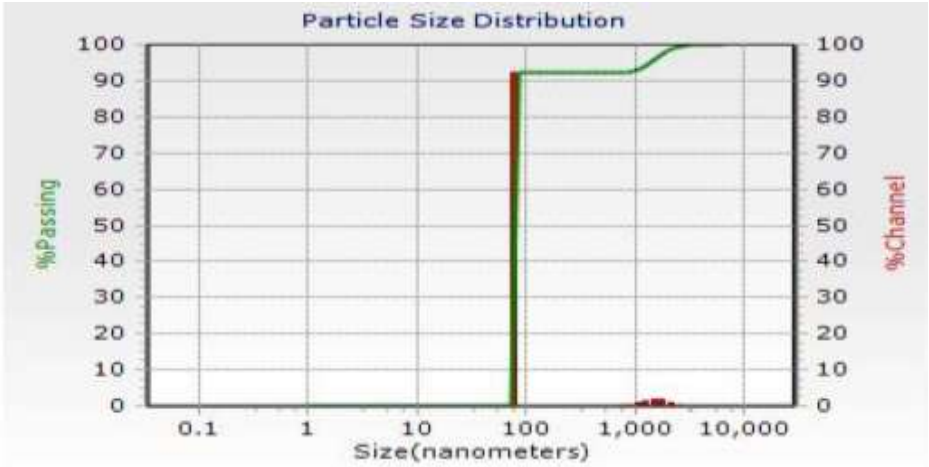


Fig. 4. X-Ray diffraction spectra of chitosan-silver nanocomposites.



Peaks Summary		
Dia(nm)	Vol%	Width
1,553	7.7	940
78.8	92.3	8.36

Fig. 5. Dynamic Light Scattering of Chitosan-Silver Nanocomposites.

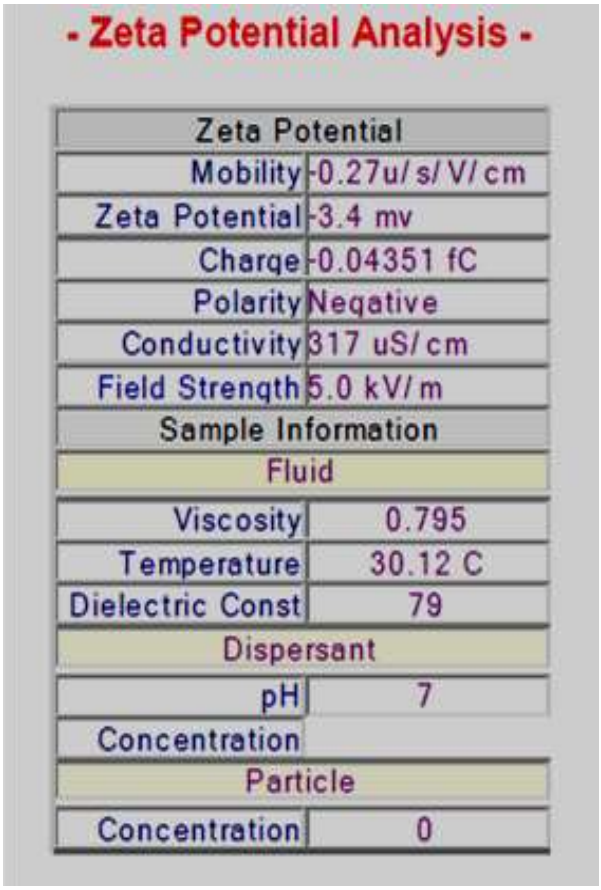


Fig. 6. Zeta potential of silver chitosan nanocomposite.