Journal of Advanced Zoology
ISSN: 0253-7214
Volume 45 Issue 1 Year 2024 Page 282-290

# Study on the extraction and purification of Chitin and Chitin based derivatives from Exoskeleton of Fresh water prawns

**Vinod Kumar[1], Abhishek Mathur[2*]**

*1Research Scholar, Dept. of Microbiology, Himalayan University, Arunachal Pradesh, India;*
*2*Dept. of Research and Development, Prathista Industries Limited, Telangana State, India*

***Corresponding author:** Abhishek Mathur*
Email: *drabhishekmathur123@gmail.com*

***Abstract***

Crustacean's shells constitute the traditional and current commercial source of Chitin. Chitin and its derivatives as a potential resource as well as multiple functional substrates have generated attractive interest in various fields such as biomedical, pharmaceutical, food and environmental industries. In the present investigation, chitinous wastes were collected from the fresh water areas of Dehradun and Rishikesh of Uttarakhand State. The bacterium, *Bacillus* sp. isolated from soil produces chitinase enzyme responsible for degradation of chitin obtained from chitinous wastes. Further the chitinases enzyme was utilized to degrade the chitinous wastes into chito-oligosaccharides. The chitin active molecule present in the chitinous waste at another stage was deacetylated to chitosan. Further the antibacterial activity of chitinases, chitin, chitosan and chito-oligosaccharides were determined *in vitro* by well diffusion method. The enzyme purified showed potent activity against the bacterial cultures, but no activity was observed against the fungal test cultures. Amongst the test bacterial cultures the chitinase showed maximum inhibition against *Micrococcus luteus* (diameter of zone of inhibition: 21 mm) followed by multi-drug resistant *Staphylococcus aureus* (diameter of zone of inhibition: 20 mm) and *Salmonella abony* (diameter of zone of inhibition: 17 mm). Further, chitin, chitosan and chito-oligosaccharide were subjected to antimicrobial activity against the similar strains and the results were found to be very satisfactory as the chitin and chitin-based derivatives were equally antimicrobial in nature. Further the antifungal activity of chitinases, chitin, chitosan and chito-oligosaccharides was determined *in vitro* by well diffusion method against *Aspergillus niger* and *Candida albicans*. Amongst the test fungal cultures, the chitinase showed maximum inhibition against *Aspergillus niger* (diameter of zone of inhibition: 24 mm) followed by *Candida albicans* (diameter of zone of inhibition: 14 mm). Deacetylated form of chitin i.e chitosan showed potent antifungal activity against *Candida albicans* (diameter of zone of inhibition: 24 mm) followed by *Aspergillus niger* (diameter of zone of inhibition: 18 mm). The chitin extracted showed almost similar antifungal activity against *Aspergillus niger* and *Candida albicans* (diameter of zone of inhibition: 15 mm) respectively. The low molecular weight derivatives viz. chito-oligosaccharide showed significant antifungal activity against *Aspergillus niger* (diameter of zone of inhibition: 14 mm) but no activity was found against *Candida albicans*.

## Introduction

Chitin, a polysaccharide of animal origin, is obtained from waste material of seafood industries. It occurs in the skeletal material of crustaceans such as crabs, lobsters, shrimps, prawns and crayfish. Chitin is also the important component of exoskeleton of Arthropods. Chitin is also forming the important composition of fungus. Chitosan is the deacetylated product formed by treatment of chitin with concentrated (50%) caustic alkali. The regulatory and toxicological status of Chitosan has already been established. Chitin and Chitosan are being used as the food supplement that effectively lowers blood cholesterol concentration and controls obesity. In some researches it was found that chitin is beneficial to cure various disorders such as arthrosclerosis, hypertension, macular degeneration, cancer, diabetes, osteoporosis etc. The chitin and chitosan extracted from crustaceans and fungus were meant to be highly beneficial in medical and health sciences as depicted from researches carried out from past years. The present study is thus localized on extraction and purification of chitin and its based derivatives from fresh waste crustaceans in order to understand the antimicrobial properties against different microbial pathogens [1-12].

## Materials and Methods

All the materials, reagents and media used for the study were procured from Ranchem, CDH and Hi-Media, India.

### Collection of chitinous wastes

The chitinous wastes of fresh water crustaceans (fresh water crabs and prawns) were collected from Lachhiwala area of Dehradun. The chitinous wastes were washed properly in order to remove the sand debris present on the surfaces. The chitinous wastes were then after air dried and powdered material obtained was used as chitin.

### Demineralization of chitinous wastes

The demineralization of chitinous wastes was performed as per the modified method [3-6]. The chitinous wastes were treated with 1.75 N acetic acid at room temperature for about 12-15 hours. The ratio of waste to solvent were maintained (1:15 w/v). The demineralized material obtained were recovered by filtration and rinsing with de-ionized water and will be dried in forced hot air oven at $65^0$C.

### Deproteinization and removal of lipids

The new and advanced methodology for deproteinization of proteins from demineralized chitinous wastes was designed by using deproteinization agents. This process can be performed either by using proteolytic enzymes such as proteinase-K dissolved in buffer containing 0.05 M Tris-base (pH, 6.5-9.1) in a ratio 1:20 (w/v) in flasks at various temperatures in incubator-shaker for about 72 h and adding mixture of solvents (phenol: chloroform, 1:1 ratio) again and again to the residue obtained and centrifuging the mixture until the residue gives no test for the presence of protein content. After repeating the procedure for 3-4 times, finally the residue was treated with 2N sodium hydroxide (1:25 w/v) at $70^0$C for 1 hour. The lipid content gets dissolved in phenol: chloroform mixture and was removed from the chitinous wastes. Greese spot test was performed in order to determine qualitatively the presence of lipid content if any present in the residual material [6].

### Preparation of Chitosan

The demineralized and deproteinized chitin material was subjected to concentrated sodium hydroxide at 40% w/v. The deacetylated forms of chitosan obtained were solubilized in 2 M dilute acetic acid.

### Determination of antibacterial activity of chitin and chitin-based derivatives and chitosan against pathogenic and drug-resistant bacterial strains

The antimicrobial activity of chitosan produced from *Bacillus* strain and chitin-based derivatives were screened for its antibacterial activity against some standard bacterial strains viz. *E.coli, Lactobacillus plantarum, Salmonella abony, Micrococcus luteus, Drug resistant Staphylococcus aureus, Drug resistant Acinobacter* by well diffusion method [3-6]. The pure cultures of test microorganisms were procured from National Chemical Laboratory (NCL), Pune, Maharashtra, India. Nutrient agar medium/broth was used for the growth of bacterial

cultures. The wells were punctured in the agar plates with sterile borer and $10^5$ Cfu/ml of the bacterial and fungal cell suspension were introduced in the plates separately. The enzyme supernatant, chitosan and its based nanoparticles was introduced in the wells in each of the bacterial plates. The plates were left free for the thorough diffusion of the enzyme supernatant within the medium plates and were kept for 18-24 h and 72 h at $37^0$ C for bacterial and fungal cultures respectively. The diameter of zone of inhibition observed was recorded.

**Results and Discussion**

In the present investigation, the chitin was extracted from exoskeleton of fresh water crustaceans. The processing of exoskeletons was performed by demineralization, deproteinization and removal of lipids in order to obtain the pure form of chitin. Further the chitin yield was determined and derivatives of chitin in form of chitosan were obtained after deacetylation process. The chitin was extracted from fresh water crustaceans according to the methodology designed. The percent yield of chitin extracted from fresh water prawn (*Palaemon* sp) was comparatively more in comparison to fresh water crab (*Potamon* sp). The yield of chitin extracted from fresh water prawn and crab was found to be 80 and 70 % respectively (Mathur *et al*., 2011). The results of percent yield of chitin extracted are reported in **Table 1.** The results were found to be very significant. Deacetylated form of chitin i.e chitosan showed potent antifungal activity against *Candida albicans* (diameter of zone of inhibition: 24 mm) followed by *Aspergillus niger* (diameter of zone of inhibition: 18 mm). The chitin extracted showed almost similar antifungal activity against *Aspergillus niger* and *Candida albicans* (diameter of zone of inhibition: 15 mm) respectively. The low molecular weight derivatives viz. chito-oligosaccharide showed significant antifungal activity against *Aspergillus niger* (diameter of zone of inhibition: 14 mm) but no activity was found against *Candida albicans.* The results are shown in **Table 2 and Figure 1.**

**Determination of *in vitro* antifungal activity of chitin-based derivatives**

In the present investigation, the antifungal activity of chitin-based derivatives was determined. The results were found to be very significant. Deacetylated form of chitin i.e chitosan showed potent antifungal activity against *Candida albicans* (diameter of zone of inhibition: 24 mm) followed by *Aspergillus niger* (diameter of zone of inhibition: 18 mm). The chitin extracted showed almost similar antifungal activity against *Aspergillus niger* and *Candida albicans* (diameter of zone of inhibition: 15 mm) respectively. The low molecular weight derivatives viz. chito-oligosaccharide showed significant antifungal activity against *Aspergillus niger* (diameter of zone of inhibition: 14 mm) but no activity was found against *Candida albicans.* The results are shown in **Table 3** and **Figure 2.**

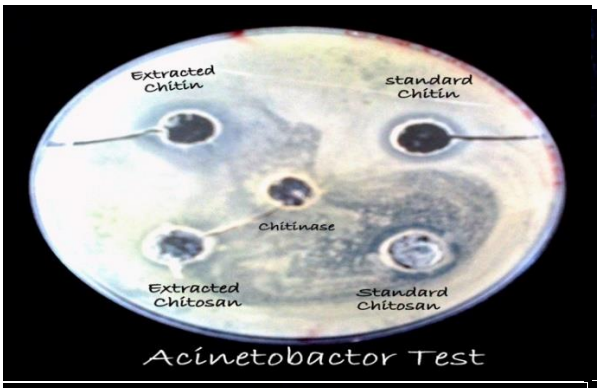
**Table 1:** Percent yield of Chitin extracted from fresh water crustaceans

| S.No. | Sample | % Yield |
|---|---|---|
| 1. | Fresh water prawn (*Palaemon* sp.) | 80.0 |
| 2. | Fresh water crab (*Potamon* sp.) | 70.0 |

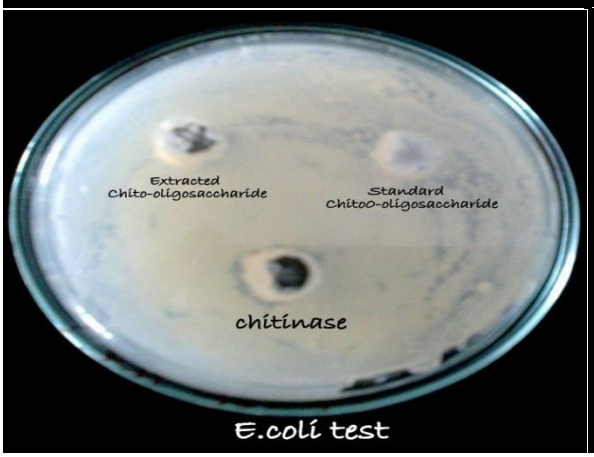
**Table 2: Determination of *in vitro* antibacterial activity of chitin-based derivative - Chitosan**

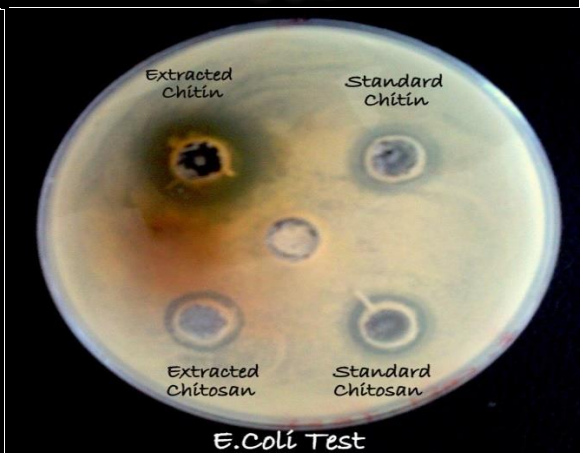
| Bacterial Strains | Diameter of Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | Standard Chitin | Extracted Chitin | Standard Chitosan | Erythromycin | Negative Control (0.1% NaOH) |
| *E. coli* | 13.0 | 13.0 | 14.0 | 25.0 | NA |
| *L. plantarum* | NA | 12.0 | 10.0 | 25.0 | NA |
| *S. abony* | 20.0 | 12.0 | 12.0 | 18.0 | NA |
| *Micrococcus* | NA | NA | NA | 33.0 | NA |
| *Acinetobacter* | 18.0 | 18.0 | 14.0 | NA | NA |

*NA, No activity


Extracted Chitin
standard Chitin
Chitinase
Extracted Chitosan
Standard Chitosan
Acinetobactor Test

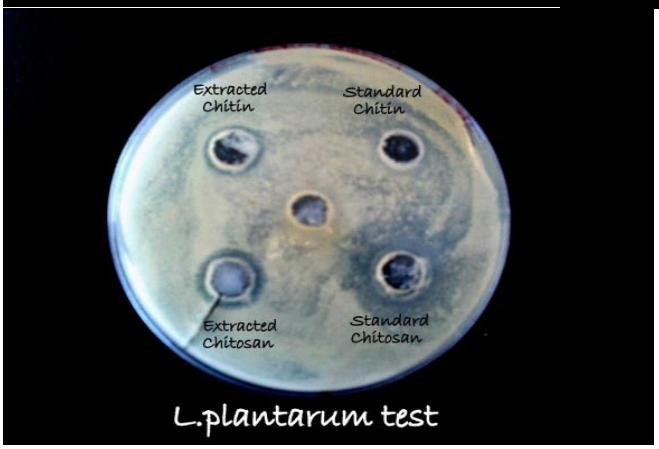

Erythromycin
Chloramphenicol
0.1% NaOH
Acinetobactor control


Extracted Chito-oligosaccharide
Standard Chitoo-oligosaccharide
chitinase
E.coli test


Extracted Chitin
Standard Chitin
Extracted Chitosan
Standard Chitosan
E.Coli Test


Chloramphenicol
Erythromycin
0.1% NaOH
L.plantarum Control


standard chito-oligosaccharide
Extracted Chito-oligosaccharide
chitinase
L.plantarum test


Extracted Chitin
Standard Chitin
Extracted Chitosan
Standard Chitosan
L.plantarum test

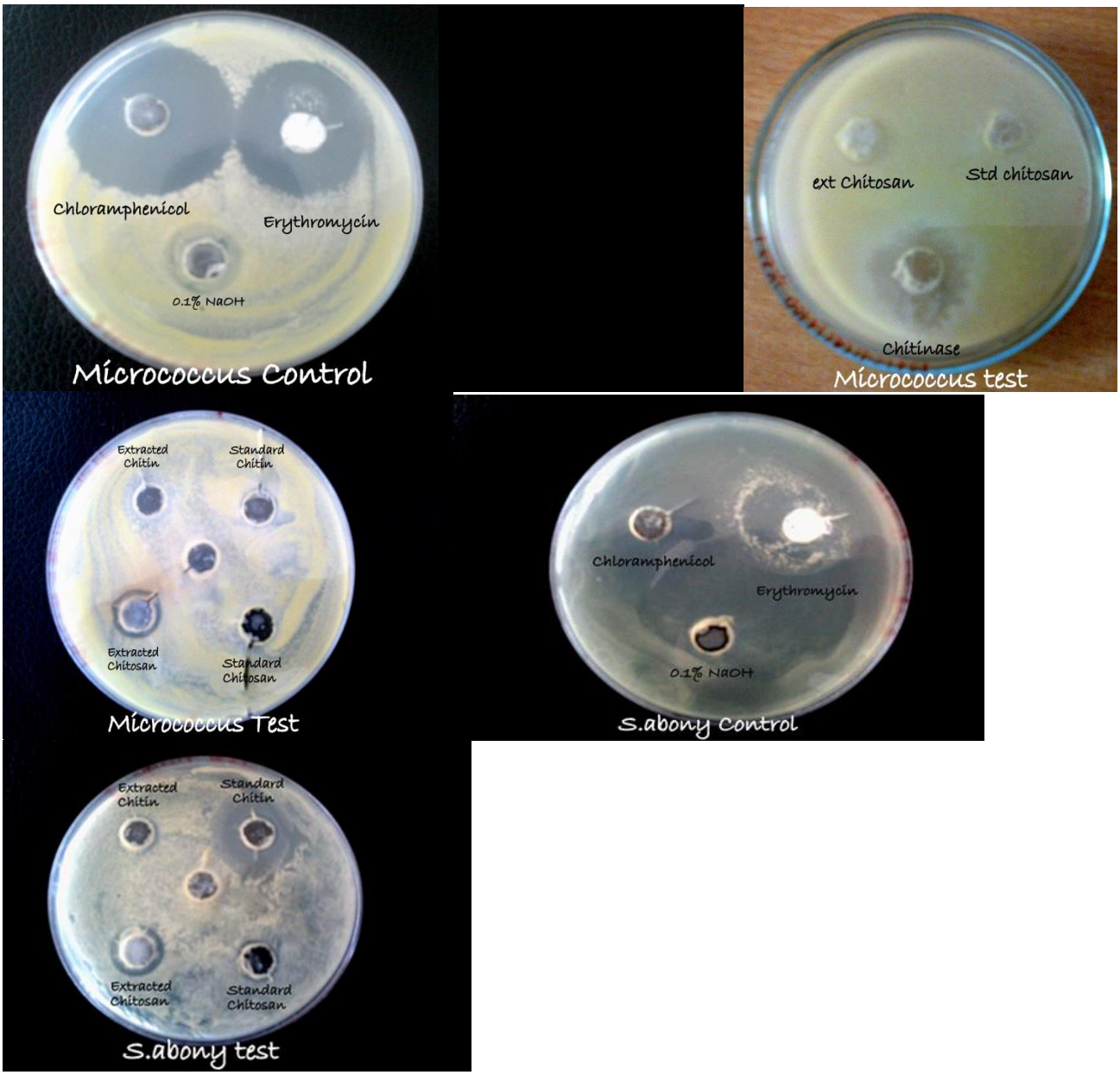



**Figure 1:** Antibacterial activity of Chitin based derivatives against pathogenic and drug resistant strains

**Table 3:** Anti-fungal activities of Chitin and Chitin Based Derivatives

| **Fungal Strains** | **Diameter of Zone of Inhibition (mm)** | | | | |
|---|---|---|---|---|---|
| | **Standard Chitin** | **Extracted Chitin** | **Standard Chitosan** | **Flucan azole** | **Negative Control 0.1% NaOH** |
| *Aspergillus niger* | 12.0 | 15.0 | 14.0 | 16.0 | NA |
| *Candida albicans* | 12.0 | 15.0 | 17.0 | 29.0 | NA |

*NA, No activity

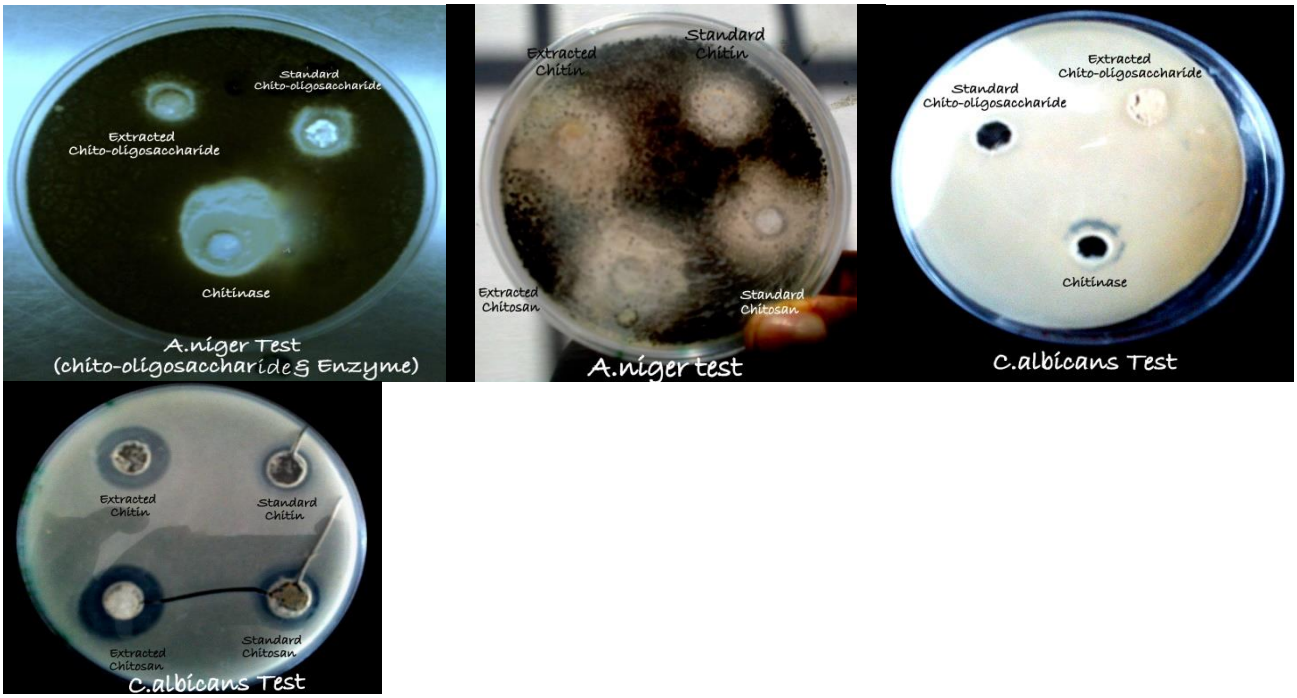



**Figure 2:** Anti-fungal activities of Chitin and Chitin Based Derivatives against *A. niger* and *C. albicans*

In the present investigation, the chitin active molecule present in the chitinous waste at another stage was deacetylated to chitosan. Further, chitin, chitosan and chito-oligosaccharide were subjected to antibacterial activity against the similar strains and the results were found to be very satisfactory as the chitin and chitin-based derivatives were equally antimicrobial in nature. The results were found to be in accordance with the previous researches performed [13-20]. The antifungal activity of chitinases, chitin, chitosan fused silver nanoparticles and chito-oligosaccharides was determined *in vitro* by well diffusion method against *Aspergillus niger* and *Candida albicans*. Deacetylated form of chitin i.e chitosan based silver nanoparticles showed potent antifungal activity against *Candida albicans* (diameter of zone of inhibition: 24 mm) followed by *Aspergillus niger* (diameter of zone of inhibition: 18 mm). The chitin extracted showed almost similar antifungal activity against *Aspergillus niger* and *Candida albicans* (diameter of zone of inhibition: 15 mm) respectively. The low molecular weight derivatives viz. chito-oligosaccharide showed significant antifungal activity against *Aspergillus niger* (diameter of zone of inhibition: 14 mm) but no activity was found against *Candida albicans*. The results were found to be similar with the previous studies performed [21-48].

## Conclusion

In the present study, the objectives of research are fulfilled in a corelative manner. Chitin after extraction in pure form was deacetylated to produce chitosan. These molecules, chitin, and chitosan were utilized to screen different properties viz. antibacterial, antifungal, plant-growth regulation activity and anti-diabetic activity. Thus the aim of the study directs to reveal the nature and miracle properties of chitin and chitin-based derivatives. The study reveals the pharmacological nature of chitn and chitn-based derivatives viz. chitosan and chito-oligosaccharides as an effective antibacterial, antifungal agents. Thus these molecules thus can be utilized to formulate or can be utilized as an ingredient in the preparation of antimicrobial agent.
The future prospects of research work are:

- Chitosan and chito-oligosaccharides (COS) can be utilized for screening of more other pharmacological properties.
- Further, the work can be carried in order to isolate and identify the gene of interest responsible for expression of chitinase enzyme.
- The chitinase gene can be expressed in the plants and the effect can be observed on pathogenecity of different funagal pathogens invading the plants.
- The studies can be utilized in order to investigate the nature of chitin-binding proteins in the compositional structure.

- The studies can be performed in order to determine the mechanism of action of these derivatives on the growth of plants viz. induction of synthesis of chlorophyll, induction of plant harmones such as auxins and cytokinins etc.
- Despite major progress in the past decade, the production of pure CHOS with defined DP, FA and PA is still a challenge. However, it is now fully possible to carry out controlled and reasonably well understood enzymatic production processes that yield CHOS preparations that are enriched for certain known compounds.
- The outcome of such processes can be controlled by controlling the enzyme, the starting chitosan (primarily FA), and the extent to which the degradation reaction is allowed to develop.
- Further refinement of the production step may be achieved by using engineered/genetically-modified enzymes.
- Thus, the studies suggested that, chitin, chitosan and chito-oligosaccharides can be utilized as a natural, organic, sanitization and disinfectant that can be utilized to kill surface borne pathogens.

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