RESEARCH
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ISOLATION AND PRODUCTION OF CHITOSAN PHOSPHORYLATION FROM MARINE SQUID PEN AND INHIBIT THE GROWTH OF CLINICAL PATHOGENS

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Abstract

AIM: Characterisation and evaluation of the antimicrobial activity of phosphorylated chitosan isolated from *Sepioteuthis lessoniana* gladius

INTRODUCTION: Gladius (exoskeleton) of *Sepioteuthis lessoniana* (marine squid) is considered a waste product in seafood processing industries. Chitin is a semi transparent material which is present in the crustacean exoskeleton which is further demineralized and deproteinated.

MATERIALS & METHODS: Chitin is deacetylated in strong alkali and additionally phosphorylated using orthophosphoric acid. Characterisation of the chitosan is done by Fourier Transform Infra Red (FT-IR) Spectroscopy to identify the functional groups. SEM imaging is performed for cross sectional analysis. X Ray Diffraction (XRD) analysis is performed to analyze the morphology. Antimicrobial analysis is done using bacterial and fungal pathogens.

RESULTS: The peaks obtained in the FTIR analysis show the presence of -OH AND -CH2 functional groups. The XRD analysis shows the porous gel network of chitosan. The SEM analysis shows the diamond or crystal amorphous morphology of phosphorylated chitosan. The agar diffusion assay shows that phosphorylated chitosan has potential antimicrobial activity at 100 % concentration against *S.mutans* and *E.coli .*

DISCUSSION : Chitosan phosphorylation is done. FTIR analysis suggests that the hydroxyl as well as methylene groups may be causing antimicrobial action against clinical pathogens. XRD analysis shows a very broad peak at 2θ = 20° suggesting that chitosan has good biocompatibility. The SEM imaging shows the cross section of randomly oriented grains suggesting the possibility of better interaction of the chitosan with the tissue structure and the pathogens as well . The antibacterial action observed was significant whereas anti fungal action was not observed suggesting that the chitosan produced can be used as potential anti bactericide .

CONCLUSION : The study concluded that the S.lessoniana based gladius used for making phosphorylated chitosan , can be utilized as an antibacterial agent , whereas more research is required to analyze the anti fungal properties of this material .

KEYWORDS : Chitosan , phosphorylated chitosan , Squid pen , S.lessoniana , XRD , FTIR , SEM analysis , Antibacterial assay , agar disc diffusion

INTRODUCTION -

Cephalopoda includes Nautilus , cuttlefish , squids and octopods etc. These species are specifically marine , diverse in structures and also in form , size and nature . The gladius is exclusively present in the exoskeleton of marine animals . It contains chitin which is the primary constituent of the gladius . This material is disposed of as the waste product during the processing of the aquatic fauna (1). Chitosan is the second most abundant polysaccharide after cellulose . Furthermore , it is regarded as a natural renewable resource with various unique properties including anti microbial , non toxic and biocompatible action towards the human body (2) , (3) Due to these properties this material can be utilized in biotechnology , pharmaceuticals , waste water treatment etc. (4)

With respect to previous research , Chitosan has a wide spectrum of antimicrobial action towards gram positive and negative bacteria , and also yeast and molds . But due to its poor solubility it couldn't be utilized as a polysaccharide drug , therefore , in order to improve solubility and bioactive potential chitosan derivatives are processed by consolidating phosphate , sulfate , carboxyl groups etc. (5) , (6)

The primary objective of this research is to do characterisation of the chitosan with the help of FTIR , XRD analysis and SEM imaging and evaluate the inhibitory potential of phosphorylated chitosan against clinical pathogens. This involves testing its efficacy against a panel of pathogenic microorganisms, including bacteria and fungi. The technique employed is the diffusion assay method.

The clinical significance of this study lies in its potential to address the escalating issue of antibiotic resistance. Traditional antibiotics are becoming increasingly ineffective against certain pathogens due to their ability to develop resistance mechanisms. New, innovative antimicrobial agents are urgently needed. Phosphorylated chitosan, with its unique chemical composition, presents a promising alternative. If proven effective, it could be integrated into medical applications such as wound dressings, coatings for medical devices, or even systemic treatments.(7) , (8)

In conclusion, the investigation into isolating and producing phosphorylated chitosan from marine squid pen holds significant promise in addressing the challenge of clinical pathogen growth inhibition. By tapping into the marine environment's wealth of resources, this research has the potential to introduce a novel antimicrobial agent with broad applications in medicine and beyond.(9), (10)

As the world faces the growing threat of antibiotic resistance, innovative solutions rooted in sustainable practices become all the more crucial. This study exemplifies the merging of scientific inquiry, resourcefulness, and environmental consciousness in the pursuit of improving human health and well-being.

MATERIALS AND METHOD :

2.1. Collection of sample:

Gladius squid S. lessoniana was obtained from a seafood processing plant at Thondi (Lat. 9144′N; Long. 079102′ E).Gladius were packed in plastic bags and stored at −20 1C before and during transportation to the laboratory. The gladius were washed with distilled water, dried and pulverized using pestle and mortar. Standard chitosan and ortho-phosphoric acid were purchased from Sigma Chemical Co. (St. Louis, MO).

All other chemicals used were of analytical grade.

2.2. Extraction of chitin and chitosan from gladius:

Chitin was extracted from the pulverized sample by demineralization and deproteinization. The powder of gladius was treated with 2 N HCl for 24 h to remove the mineral content and then treated with 1 N NaOH at 80 1C for 24 h to remove protein (Takiguchi, 1991a, Chap. 1). Chitin was deacetylated into chitosan in 40% NaOH and purified by precipitation in 10% acetic acid (Takiguchi, 1991b, Chap. 2).

2.3. Phosphorylation of chitosan:

Phosphorylated chitosan was prepared by dissolving 2 g of chitosan powder with 30 g of urea and 50 ml of DMF. Then 5.2 ml of ortho-phosphoric acid was added to the chitosan solution. The mixture was reacted at 150 1C for 1 h. After cooling, the reaction mixture was precipitated and washed thoroughly with methanol and then the residue was re-dissolved in distilled water. The pH was adjusted to 10–11. The solution was dialyzed against distilled water for 48 h using a 12,000 Da MW cut-off dialysis membrane. Then the product was lyophilized to get phosphorylated chitosan.

2.4. FT-IR spectral analysis:

FT-IR spectroscopy of solid samples of standard chitosan and phosphorylated chitosan from S. lessoniana was relied on an AVATAR 330 Spectrometer. Sample (10 mg) was mixed with 100 mg of dried Potassium Bromide (KBr) and compressed to prepare salt discs (10 mm diameter) for reading the spectrum.

2.5 XRD analysis :

X-ray diffractograms were recorded according to a powder method with a Mac science M3X (model no. 1030) diffractometer using Cu K□ radiation. A Perkin–Elmer 2400 carbon–hydrogen analyzer was used for elemental analysis. The solubility of the polymers was tested in various polar and non-polar solvents by taking 10 mg of polymers in 2 mL of different solvents in a closed test tube and set aside for one day. The solubility of the polymers was noted after 24 h. The phosphorus content was determined spectrometrically by the Kjeldahl method [22]. The degree of substitution was calculated as previously reported methods [23].

2.6 SEM Imaging :

Samples are cleaned in a reagent grade solvent and rinsed with a reagent grade isopropyl alcohol (IPA). Samples are placed in a nitrogen-filled, resealable container and mounted on the instrument stub. The sample is oriented to the degree that the longitudinal axis of the sample curvature, if applicable, is aligned with the axis of the secondary detector. The magnification is increased and the image is captured.

2.7 Antibacterial activity of Phosphorylated chitosan :

Three bacterial strains (clinical isolates) (Gram-positive: *Streptococcus mutans* ; Gram-negative: *E. coli*, *Pseudomonas aeruginosa*,) , one fungal strain (*Candida tropicolis*) were used as test organisms. Nutrient broth was prepared and sterilized in an autoclave at 15 lbs pressure for 15 min. Individual species of bacteria and fungi were inoculated in the sterile nutrient broth and incubated at 37 °C for 24 h. Mueller Hinton Agar (MHA, Himedia) and Sabaurad agar medium was prepared, sterilized in an autoclave at 15 lbs pressure for 15 min and poured into sterile petri dishes and incubated at 37 °C for 24 h. The antibacterial activity of the individual bacterial strains was tested using Agar well diffusion method . 24 h old nutrient broth cultures of test bacteria were aseptically swabbed on sterile nutrient agar plates. Wells of 5 mm diameter were made

aseptically in the inoculated plates. The different concentrations of chitosan (Stock—5 mg/ml in 0.2% water) and phosphorylated chitosan (Stock-5 mg/ml in distilled water), from this stock solution four different concentrations viz ; 50 and 100% prepared were loaded in the respective wells. Standard (Streptomycin , ciprofloxacin , chloramphenicol and Fluconazole 1 mg/ml) and Control (0.2% water) were also loaded into the respectively labeled wells. The plates were incubated at 37 °C for 24 h in upright position. The experiment was carried out in triplicate and the zone of inhibition was recorded.

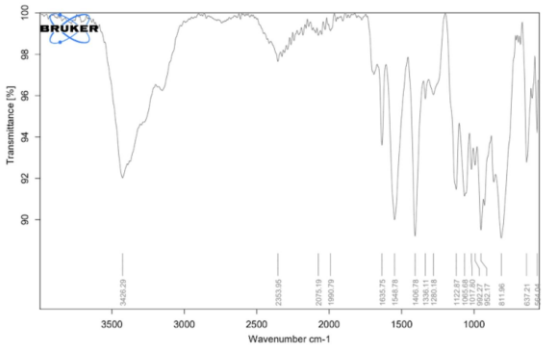
RESULTS :

The results of this study indicate promising outcomes in terms of phosphorylated chitosan and its inhibitory effects on clinical pathogens .

1. Isolation and production of phosphorylated Chitosan :

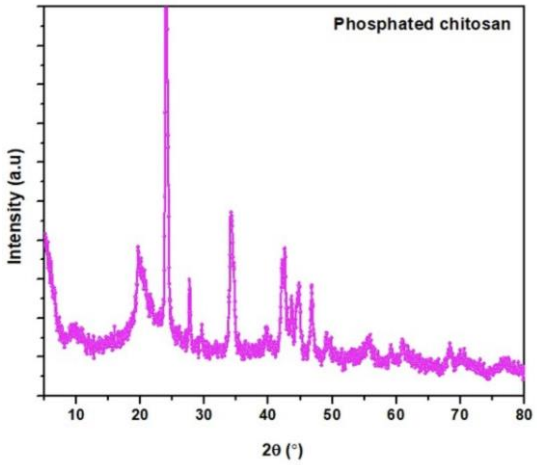
The study successfully isolated chitosan from marine squid pen waste through a series of cleaning , deproteinization and demineralization steps. This provides a source of chitin , the precursor to chitosan .Subsequently , the chitosan was phosphorylated , introducing phosphate groups to its structure . Various characterization techniques , such as FTIR , XRD and SEM analysis , confirm the successful production of phosphorylated chitosan.

1.1 FTIR ANALYSIS :



The intensity of bands around 3370 - 3385 cm ^-1 in the IR spectrum is due to hydroxyl stretching vibration of phosphorylated chitosan . The weak absorption at about 2955-2965 cm^ -1 was related to the C- H stretching vibration of CH 2 groups . At 1595-1610 cm ^-1 is assigned due to deforming vibrations of C-H bond . The absorbance of phosphorylated chitosan at 950-1200 cm ^-1 were where the C-O-C and C-O-H band positions.

1.2 XRD ANALYSIS :



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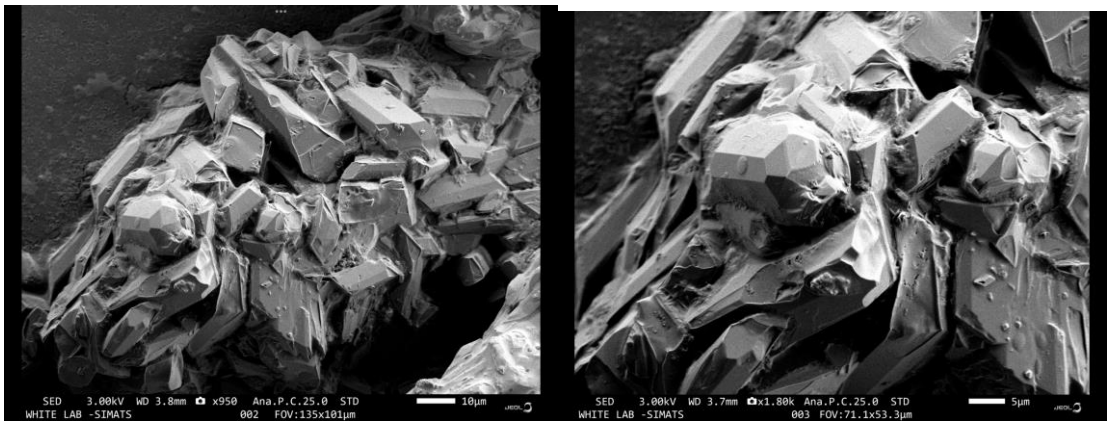
The phosphorylated chitosan displayed two weak peaks at around 2θ of 20°and 35°. However, the peak observed for chitosan at 2θ = 10°disappeared and the very broad peak at 2θ = 20°became weak in phosphorylated chitosan .These results suggest that chitosan has good compatibility, which leads to the formation of a porous xerogel network. The XRD pattern also indicated that the phosphorylated chitosan displays an amorphous form, which may participate in biomedical applications.

1.3 SEM ANALYSIS :



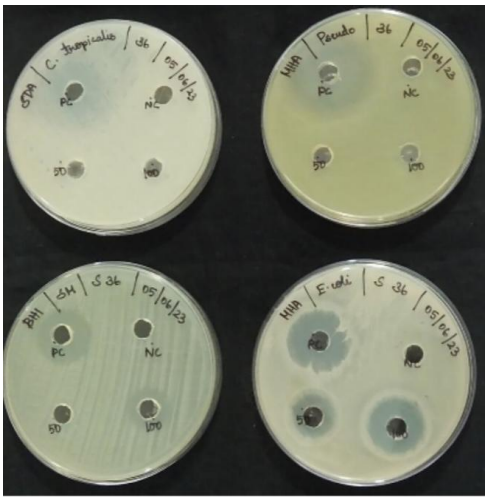
The electron micrographs of phosphorylated chitosan exhibit the cross section of randomly oriented grains and also give an image of the upper part of the bread slice . Phosphorylated chitosan have nearly diamond / crystal morphology .

2. Antimicrobial efficacy of chitosan towards various pathogens :

S. No	Bacterial/Fungal Strains	Phosphorylated chitosan from the gladius of *Sepioteuthis lessoniana*		+C (mm)	-C (mm)
		50% (mm)	100% (mm)		
1	*Candida tropicolis*	-	-	34±0.82	-
2	*Streptococcus mutans*	-	17±1.53	23±1.53	-
3	*Pseudomonas aeruginosa*	-		30±2.25	
4	*Escherichia coli*	20±2.25	24±2.25	25±1.75	

+Control (1. Fluconazole, 2. Streptomycin, 3. Ciprofloxacin, 4. Chloramphenicol);
-Control (H_2O)

The Zones of Inhibition exhibited by the Bacterial strains (S.mutans , P.aeruginosa , E. coli) and fungal strain (Candida tropicolis) with positive controls/+C(Streptomycin , Ciprofloxacin , Chloramphenicol and Fluconazole) and negative control / - C (H2O)



ANTI - MICROBIAL ANALYSIS (Mueller Hinton Agar / Sabouraud agar)

The antimicrobial efficacy of phosphorylated chitosan was evaluated against a panel of clinical pathogens , including bacteria and fungi .The evaluation of the bacterial strains such as *Escherichia coli* , *Streptococcus mutans* , *Pseudomonas aeruginosa* and fungal strain such as *Candida tropicolis* . The positive controls used for Chloramphenicol , Streptomycin , Ciprofloxacin and Flucanozole ; with the negative control as water .

DISCUSSION :

Chitosan is characterized by its potential implications for anti microbial research , sustainable resource utilization , and the broader understanding of marine derived compounds . The results and the findings of this study contribute to various dimensions , highlighting both the scientific and practical aspects of research . It is a non toxic and biocompatible cationic polysaccharide produced by partial deacetylation of chitin.(11), (12)

From this study the feasibility of repurposing marine waste materials to obtain valuable bioactive compounds . The process of isolating chitosan from squid pen demonstrates the feasibility of utilizing a readily available byproduct of the seafood industry as a source of chitin .(13) Furthermore , the phosphorylation process introduces a chemical modification that could enhance the chitosans' anti microbial properties , potentially making it a more potent inhibitor of clinical pathogens . (14), (15)

The characterization of the chitosan is done using XRD , SEM and FTIR analysis . The FTIR analysis of chitosan from *S. lessoniana* recorded that intensity bands around 3370 - 3385 cm ^-1 in the IR spectrum which is due to the stretching vibration of the hydroxyl group . The weak absorption levels seen at 2955 - 2965 cm ^-1 were related to the C-H stretching vibrations of the CH2 group . At 1595 - 1610 cm ^ -1 is assigned due to the deforming vibrations of C-H bond . The absorbance of phosphorylated chitosan at 950 - 1200 cm ^-1 were where the C-O-C and C-O-H bands are positioned . The highest peaks observed with the hydroxyl and the -CH2 group suggested that the anti microbial action will be more significant due to presence of these functional groups .

The XRD analysis suggested that the phosphorylated chitosan displayed two weak peaks at around 2θ of 20°and 35°. However, the peak observed for chitosan at 2θ = 10°disappeared and the very broad peak at 2θ = 20°became weak in phosphorylated chitosan .These results suggest that chitosan has good compatibility, which leads to the formation of a porous xerogel network. The XRD pattern also indicated that the phosphorylated chitosan displays an amorphous form, which may participate in biomedical applications.

The SEM imaging provided that the electron micrographs of phosphorylated chitosan exhibit the cross section of randomly oriented grains and also give an image of the upper part in the

form of bread slices . Phosphorylated chitosan have nearly diamond / crystal morphology . With the help of this morphology it can be understood that the interaction between the phosphorylated chitosan and the clinical pathogen or any external particle will be considerable , suggesting that there is a direct relation between the antimicrobial actions of the synthesized chitosan and the microbial strains .

The antibacterial activity was analyzed using the agar well diffusion assay , in this three species of bacterial stains were used (gram positive strain - *Streptococcus mutans* , gram negative strains - *Eschrichia coli* , *Pseudomonas aeruginosa*) ; the fungal strain studied is *Candida tropcolis* . From the results obtained it is observed that the antibacterial activity is concentration dependent . At the same time the activity was absent in negative control (water) . It was observed that at 100% concentration of the chitosan , the maximum inhibition was observed for *Escherichia coli* (24 mm) and then *Streptococcus mutans* (17 mm) . No significant inhibition was observed by *Candida tropicolis* and *Pseudomonas aeruginosa* .

The previous study suggested that the highest inhibition of a 17 mm diameter clear zone with *P. aeruginosa* also , good activity with *E. coli* was observed . The conclusion of that investigation revealed that it inhibits the growth of all the tested pathogenic bacterial strains when compared to the phosphorylated chitosan which indicates that the chitosan may contain a broad range of antibacterial activity. Additionally, the study brings out the possibility of using the internal bone of cuttlefish that is thrown as waste at home and seafood processing industries are very good and promising sources for antibacterials.(16)

Another study suggested that chitosan can showcase both antibacterial and anti-inflammatory action and evaluating its role against periodontal pathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* at 5 mg/mL. Chitosan exerts a predominantly anti-inflammatory activity by modulating PGE2 levels through the JNK pathway, which was considered useful in the prevention or treatment of periodontal inflammation.(17)

CONCLUSION -

The present study demonstrates the structure and antimicrobial action of the phosphorylated chitosan against *Streptococcus mutans* , *Escherichia coli* , *Pseudomonas aeruginosa* were of significance , whereas the action was non significant towards *Candida albicans* (fungal strain) . The results of this investigation proved that phosphorylated chitosan possesses good antibacterial properties , whereas notable research is required towards the antifungal action . Furthermore , research in animal models shall pave the way for its use as a potent antibacterial material in the future .

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest to disclose.

ETHICAL APPROVAL

As this is an in vitro study, the need for ethical approval is void.

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