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Extraction of Chitosan from White Shrimp (*Litopenaeus vannamei*) Processing Waste and Examination of its Bioactive Potentials

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Abstract: Fisheries are playing a key role in the changing profile of Indian economic growth. Export of processed and frozen crustacean products is the backbone of seafood export in India. The industrial processing of shrimps after peeling generates 3000 tonnes of wastes every year and it largely exists in wastes from the processing of marine food products. A part of this waste used for feed industry and maximum of this waste simply discarded which usually cause environmental nuisance. This study aims at the extraction of chitosan from shrimp shells and the use of this chitosan in successfully carrying out various applications in laboratory. At first, this waste can be utilized as an economic source of chitin. The obtained chitin was transformed into the more useful soluble Chitosan. These products were characterized by their biological activity as antimicrobial and antifungal properties. The results showed that tested chitosan markedly inhibited more of the gram negative bacteria and pathogenic fungal strains. Their antioxidant activity was investigated with 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity. The scavenging ability of Chitosan on DPPH radicals ranged from 10.08% to 14.72% at 0.25 to 2mg/ml. Chitosan, since a high antimicrobial activity against a wide variety of pathogenic and spoilage bacteria and fungi with antioxidative property it can be used very fast development in food application area.

Key words: Shrimp Processing Waste · Antibacterial · Anti Fungal · Antioxidant

INTRODUCTION

Shrimp processing for freezing normally involves removal of head and body carapace. Processing of shrimps generates large quantities of solid wastes. The solid shrimp waste contains head and body shell accounts approximately to 40-50% of whole shrimp weight [1]. About 40% of the waste is chitin, incrusted with calcium carbonate and astaxanthin and containing meat and a small amount of lipid residues. A small part of the waste is dried and used as chicken feed [2] while the rest is dumped into the sea, which is one of the main pollutants in coastal areas [3]. The waste generated from the worldwide production and processing of shellfish is a serious problem of growing magnitude and is a threat to the environment.

The utilization of shellfish waste has been proposed not only to solve environmental problems, but as a waste treatment alternative to the disposal of shellfish wastes [4]. Crustacean shell waste consists mainly of 30 - 40% protein, 30 - 50% calcium carbonate and 20-30% chitin [5-7] with species and seasonal variations [8]. The disposal of such an enormous amount of waste has become a serious environmental concern [9]. Although these wastes are biodegradable but the rate of degradation of a large amount of waste generated per processing operation is comparatively slow [10]. This results in accumulation over time and the ads to environmental concerns as they not only produce obnoxious smell but also attract pathogenic insects, flies and rodents, thus creating an unhygienic atmosphere.

The immediate solution to this problem seems to be quick recycling of the crustacean shells generated and extraction of commercially viable substances to be further used in other applications [11]. As we know the shell and head wastes of crustaceans contain chitin, proteins and minerals. So by demineralizing and deproteinizing the wastes chitin can be obtained. Moreover the chitin can be further deacetylated to produce Chitosan, a valuable chemical substance having a wide range of viable uses [12]. It is essential to convert shell waste into useful products such as chitin and chitosan etc., by recycling

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and reducing the waste as well as contribute towards gainful employment and economic benefits. Several methods have been reported for preparation of chitosan from chitin. Chitosan is the deacetylated form of chitin, which, unlike chitin, is soluble in acidic solutions. Application of chitinous products in foods and pharmaceuticals as well as processing aids has received considerable attention in last decades as exotic synthetic compounds are losing their appeal [13]. The major procedure for obtaining chitosan is based on the alkaline deacetylation of chitin with strong alkaline solution at high temperature [14] another important characteristic to consider for this polymer is the molecular weight; chitosan is a biopolymer of high molecular weight. Like its composition, the molecular weight of chitosan varies with the raw material sources and the method of preparation [15]. Chitosan is Generally Recognized as Safe (GRAS) by the US FDA [16]. The primary aim of this study was to extract chitosan from shrimp wastes and investigate the bioactive potentials.

The natural antibacterial and antifungal characteristics of chitosan have resulted in their use in commercial disinfectants. Chitosan have been shown to activate the defense system of a host and prevent the invasion of pathogens [17]. Mechanism of chitosan antibacterial action is involving a cross-linkage between polycations of chitosan and the anions on the bacterial surface that changes membrane permeability. Chitosan has been approved as a food additive in Korea and Japan since 1995 and 1983, respectively [18]. Many synthetic chemicals such as phenolic compounds are found to be strong radical scavengers; however, the use of synthetic antioxidants is under strict regulation due to their potential health hazards [19]. Therefore, the search for natural antioxidants as alternatives to synthetic product is of great importance. Recently, the antioxidant activity of chitosan and its derivatives attracted an increased attention [20]. Therefore, chitosan were extracted from white shrimp (*Litopenaeus vannamei)* and their proximate analysis of chitosan and characterized by biological activities such as anti-bacterial, anti-fungal and anti-oxidative activities.

MATERIALS AND METHODS

Production of Chitosan from Shrimp Wastes: Shrimp waste (Heads and scales) were collected from processing industry of Tuticorin. The waste was packed in plastic bags and stored in ice and transported to the laboratory. During the preparation of shrimp wastes for extraction the shrimp wastes were washed, dried at 50°C overnight and stored in dry place at room temperature until extraction. Chitin and chitosan were prepared from shrimp waste according to Gopalakannan *et al*. [21]. The chemicals used in the extraction process consist of 1. Hydrochloric acid (1.25N to 1.5N): for 100ml stock solution, 10.8ml HCl is measured and the volume made up to 100 ml with distilled water. 2. Sodium Hydroxide (0.5% w/v): 0.5 gm NaOH per 100 ml distilled water. 3. Sodium Hydroxide (3% w/v): 3g NaOH per 100 ml distilled water. 4. Sodium Hydroxide (42% w/v): 42g NaOH per 100 ml distilled water. 5. 1% Acetic Acid: 1ml acetic acid per 100 ml distilled water.

To extract Chitosan, 50 g of shrimp shell waste as raw material was collected. After washing it properly, the shrimp shells were under sunlight. Then the demineralization process is done by adding 1.5N HCl at room temperature for 1 hour. The spent acid was discarded and the shells were repeatedly washed with distilled water until the pH is neutral. The de-mineralized shells were then de-proteinized with 0.5% NaOH at 100°C for 30 minutes. This method helped to weaken the protein tertiary structure of the shells. Protein solution was removed and washed thoroughly with distilled water and the pH was checked. The de-proteinization process was again repeated for the removal of the remaining protein from the shells, for that 3% NaOH was added to the sample at 100°C for 30 minutes. After draining the residual proteins along with the effluents, the sample once again washed and the pH was observed till it was approximately near to neutral. This step also helped in decolourization of the shells. Hence the chitin slurry was obtained. The excess water was removed and chitin cake was formed. The Chitosan was prepared by deacetylation of chitin by treating with 42% aqueous NaOH at 95°C for 1.5 hour. After deacetylation the alkali was drained off and washed thoroughly with distilled water until the pH is less than 7.5 and then dried at ambient temperature (30±2°C).

Quality of the Chitosan Produced: Quality of the chitosan produced was checked by a solubility test with 1% Acetic Acid. Chitosan dissolves completely in 1% Acetic Acid. For this freshly prepared 15 mg chitosan sample was taken and put inside a clean beaker and 10 to 20 ml of 1% acetic acid was added to it. The solution was kept in BOD shaker for 30 to 40 minutes. Then the sample was taken out for checking solubility.

Purification of Chitosan: The chitosan was purified by dissolving in 1% acetic acid and precipitated by 4% NaOH at pH 10. The purified chitosan was freeze - dried.

Proximate Analysis: Moisture, ash, lipid and protein contents of shrimp waste and crude chitosan samples were determined according to AOAC [22].

Antibacterial Activity: Anti-bacterial activity of chitosan was determined by the method proposed by Varadharajan and Soundarapandian [23]. The human pathogens like *Escherichia coli, Staphylococcus aureus, S. epidermidis, Bacillus subtilis, Pseudomonas aeruginosa* and *Pseudomonas mirabilis* were used in the study. The method used for this particular experiment was the agar diffusion method using Zobell marine agar. In this method, first the media was prepared, poured into culture plates and left out for solidification. The bacterial species were taken as test strains and inoculated in agar plate and wells of 6mm diameter were dug into each plate and 12 µl of chitosan dissolved in 1% acetic acid were poured in each well. The plates were then sealed carefully using parafilm, marked accordingly and were incubated at 35°C for 24 hours. It was made sure that the plates were not being kept in an inverted position and kept erect instead. If there is any antimicrobial activity in the extracts then a zone of clearance will be formed around the corresponding well, which occurs due to diffusion of the extract through the agar. Streptomycin sulphate was used as the positive control. All plates were subjected for incubation for 2 hours. To prevent drying, the plates were covered with sterile plastic bags. The plates were later incubated at 37°C for 24 hours. The result was obtained by measuring the inhibition zone for each well and expressed in millimeter.

Antifungal Activity: Anti-fungal activity of chitosan was determined by the method proposed by Chien and Chouu [24]. The antifungal activity of chitosan has been tested by normal inoculation method. The 48 hours grown cultures on potato dextrose agar (PDA) were used for inoculation of fungal strain on PDA plates. The fungal cultures such as *Candida glabrata*, *Candida albicans*, *Candida parapsilensis, Candida kreusei*, *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus*, *Mucor* spp., *Aspergillus terreus, Trichoderma viride, Fusarium semitectum* were used. An aliquot of 20 µl inoculum was introduced to molten PDA and poured in to a petri dish by pour plate technique. After solidification, the appropriate wells were made on agar plate by using cork borer. In agar well diffusion method 12µl chitosan with 1% acetic acid were introduced into the wells. Incubation period of 24 - 48 hours at 28°C was maintained for the observation of antifungal activity of the chitosan. The antifungal activity was evaluated by measuring the zones of inhibition of fungal growth surrounding the chitosan. The complete antifungal analysis was carried out under strict aseptic conditions. The zones of inhibition were measured in diameter. The negative control was set without the chitosan and Nyastine was used as the positive control.

Antioxidant Properties of Chitosan

Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity: The ability to scavenge DPPH radical by chitosan was estimated by the method of Yamaguchi *et al*. [25]. 0.25 to 2 mg chitosan product at different concentrations in 1 ml 0.1 M Tris HCl buffer (pH 7.4) was mixed with 1 ml of DPPH (1,1-diphenyl-2-picrylhydrazyl) (250 µM) with vigorous shaking. The reaction mixture was stored in the dark at room temperature for 20 min and the absorbance was measured against blank samples at 517 nm. The scavenging activity was calculated by the following equation: Scavenging ability (%) = $[(A_{517}$ of control – A_{517} of sample) / A_{517} of control] × 100

RESULTS AND DISCUSSION

Chitosan [poly-β-(1>4) N-acetyl-D-glucosamine], is the N-deacetylated derivative of chitin. A precise nomenclature with respect to the degree of N-deacetylation has not been defined between chitin and chitosan [26, 27]. However, the chemical and biochemical reactivity of chitosan is higher than that of chitin since chitosan has free amino groups distributed regularly in its molecular chain [28]. Chitin and chitosan are manufactured commercially on a large scale from the outer shells of shrimps, lobsters and crabs [29].

In the present study the dry weight basis, 50 g of shrimp was treated with commercial grade hydrochloric acid to examine the effect of demineralization and for deprotenization the samples were further treated with commercial grade NaOH solution. The end product of the experiment was white in colour due to the removal of more protein, lipid, pigments and other inorganic acid. Rokshana [30] reported the end product of shrimp shell processing after treated with HCL and NaOH the product which contains more chitin, must be whiter in colour and the little brownish will be the lowest grade of end product which contain lowest chitin content due to incomplete demineralization and deprotenization. According to the colour of the end product, the best chitinous was determined. The products are completely soluble in 1% acetic acid. It indicates that the product (Chitosan) is of good quality.

Table 1: Proximate composition analysis of Chitosan

Proximate composition	Chitosan
Weight of raw material (Dry weight basis)	50g
Moisture (%)	7.82
Protein (%)	37.55
Lipid (%)	11.11
Ash (%)	2.41
Weight of chitin	42.0 g
Weight of Chitosan	23.50 g
Protein content of purified chitosan (%)	0.96
Ash (%)	0.98
Product appearance	White color
Smell	Shrimp powder
Solubility in acetic acid	Completely dissolved

Table 2: Antibacterial activity of Chitosan

Bacterial strains	Zone of inhibition
Escherichia coli	17mm
Staphylococcus aureus	8mm
S. epidermidis	8.5mm
Bacillus subtilis	12mm
Pseudomonas aeruginosa	14mm
Pseudomonas mirabilis	15mm
Bacillus cerevisea	-

The moisture, ash, protein, lipid and ash content of the shrimp waste were 7.82%, 37.55%, 11.11% and 2.41% respectively. The quality of chitin and chitosan produced from crustacean shell is partially dependent on the type of raw material used. As observed in this study, the proximate composition of shrimp wastes were high compared to the results of Rokshana [30] and it was equal to previous studies of Rao and Stevens [31] Aytekin and Elibol [32] and Jung *et al*. [33] and it may be due to the variation size of the shrimp samples used and the preparation method of the shell wastes in which all adhering meat was removed and washed. Furthermore, proximate composition of shrimps, crustaceans and other aquatic organisms has found to be varied due to the seasonal factors, climatic factors, geographic factors, habitat, developmental stage, sex and sexual maturation [34].

The purified chitosan were characterized and the results were presented in the Table 1. The chitosan shows good quality, the protein and ash content is less than 1% as required [35]. In the present study the ash and protein content was 0.98 and 0.96% respectively, indicating the effectiveness of the demineralization and deproteinization steps in removing minerals and protein. A high quality grade of chitosan should have less than 1% of ash content [36]. Initial weight of chitin was 42.0 g and after extraction final weight of chitosan was 23.50g.

The antibacterial activity of the extracted chitosan samples against gram negative and gram positive bacteria are shown in Table 2. The plates were checked for anti-bacterial activity after 24 hours of incubation. Data indicated that, chitosans markedly inhibited the growth of most of gram negative bacteria tested; however, the inhibitory effects differed depending on the types of chitosan and the tested bacteria. It could be reported that, good antibacterial activity was observed in the plates containing test strains *E. coli* (17mm), *Pseudomonas aeruginosa* (14mm) and *Pseudomonas mirabilis* (15mm). The bacterial pathogen *B. subtilis* for chitosan showed 12mm inhibition zone whereas the *B. cerevisea* showed no inhibition zone for chitosan. There was mild inhibition of *Staphylococcus aureus* and *S. epidermidis* by chitosan with the inhibition zone of 8 and 8.5 mm diameter. Chitosan showed appreciable inhibition for test strain *E. coli*. It was observed that that chitosan has profound antagonistic activity against gram negative bacteria as compared to gram positive. The results were agreed with the results of Abhrajyoti and Gargi [37]. Generally it could be observed that, anti- bacterial activity of tested chitosans has been influenced by its molecular weight, degree of deacetlyation and concentration of solution. The mechanism underlying the inhibition of bacterial growth that the cationically charged amino group may combine with anionic components such as N-acetylmuramic acid, sialic acid and neuraminic acid on the cell surface and may suppress bacterial growth by impairing the exchanges with the medium, chelating transition metal ions and inhibiting enzymes. Due to the positive charge on the C-2 of the glucosamine monomer below pH 6, chitosan is more soluble and has a better antimicrobial activity than chitin. The exact mechanism of the antimicrobial action of chitin, chitosan and their derivatives is still unknown, but different mechanisms have been proposed. Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents [38-41]. In antimicrobial assay, chitosan acted mainly on the outer surface of the bacteria. At a lower concentration (<0.2 mg/ml), the polycationic chitosan does probably bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations, the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension [17, 42]. In general, chitosan, being a cationic antibacterial agent, acts by interfering with the negatively charged residues of macromolecules at the bacterial cell

Table 3: Antifungal activity of Chitosan against fungal pathogens

Bacterial strains	Zone of inhibition
Candida glabrata	4mm
Candida albicans	4mm
Candida parapsilensis	2mm
Candida kreusei	3mm
Aspergillus niger	4mm
Aspergillus flavus	3mm
Aspergillus fumigatus	3mm
Mucor spp.	2mm
Aspergillus terreus	3mm
Trichoderma viride	1mm
Fusarium semitectum	2mm

Table 4: Antioxidant activity - DPPH Radical scavenging activity of Chitosan

Concentration mg/ml	Calculation of% scavenging activity
0.25	10.08
0.50	12.7
0.75	14.06
1.00	14.32
1.50	14.46
2.00	14.72

surface, thereby resulting in changes in cell permeability [17, 43]. According to Sudarshan *et al*. [17], the mechanism of antibacterial activity of chitosan involves binding of the amino group of chitosan to the surface components of bacteria, thereby inhibiting their growth.

The antifungal activity of chitosan has been reported by many investigators. This study has demonstrated that chitosan from crustacean sources exhibited antifungal activity against a large number of human pathogenic fungi. The tested chitin compound has a significant effect against pathogenic Candida species (Table 3). After 5 days of incubation, selected fungal strains showed the anti-fungal activity of chitosan. The experimental sets showed a decline in fungal growth as compared to the control set without chitosan. This verifies the claim that the presence of chitosan in experiment inhibited the growth of fungi and that the anti- fungal activity of Chitosan enhanced with the degree of deacetylation. The combination of chitosan and acetic acid was able to highly inhibit the fungal pathogens *Candida glabrata, Candida albicans, Aspergillus niger* showing an zone of inhibition of 4mm. The fungal pathogens such as *Candida kreusei, Aspergillus flavus, Aspergillus fumigates, Aspergillus terreus,* which produced an inhibition zone of 3 mm. Chitosan extract was also able to inhibit the fungal pathogens *Candida parapsilensis, Mucor* spp., *Fusarium semitectum* with the zone of inhibition of 2 mm. *Trichoderma viride* showed the 1mm zone of inhibition against chitosan extract and no one of the pathogen was not able to resist the chitosan. However the positive control of Nyastin was able to produce good antifungal activities against the tested fungal pathogens. The negative control did not show any antibacterial activity. Sagoo *et al.* [44] reported a similar sensitivity to chitosan for yeasts and moulds. Bostan and Mahan [45] revealed that yeast and mould counts in sausage treated with chitosan during cold storage were considerably lower than non- treated sausage at all sampling days. The growth rates of fungal hyphae have been shown to be sensitive to all factors in chitosan which influence intracellular calcium ions, including variations in extracellular calcium concentrations and the presence of calcium transport inhibitors [46]. Therefore, it is conceivable that chitosan limits the growth of filamentous fungi indirectly by making calcium and other essential minerals and nutrients inaccessible. Several authors have proposed that the antimicrobial action of chitosan against filamentous fungi could be explained by a more direct disturbance of membrane performance [47, 48].

Antioxidant activity of chitosan at different concentration was shown in Table 4. The scavenging abilities of different extracted chitosan samples on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals were increased with their concentration increased. It seems that scavenging abilities of chitosan increased with increasing the degree of deacetylation. These results agree with Yen *et al.* [49]. It is generally considered that the inhibition of lipid peroxidation by an antioxidant can be explained by various mechanisms. One is the free radical scavenging activity where DPPH is a stable free radical with a maximum absorbance at 517nm. When DPPH encounters a proton- donating substance such as antioxidant, the radical would be scavenged and the absorbance is reduced [50]. The scavenging ability of Chitosan on 1, 1-diphenyl-2-picrylhydrazyl radicals ranged from 10.08% to 14.72% at 0.25 to 2mg/ml. Based on this principle, the antioxidant activity of the substances can be expressed as its ability in scavenging the DPPH radical. Park *et al*. [51] suggested that chitosan may eliminate various free radicals by the action of nitrogen on the C-2 position of the chitosan. The chitosan extracted from *L. vannamei* had higher radical scavenging than the other products measured at the same concentration. The scavenging activity of chitosan may be due to the reaction between the free radicals and the residual free amino group to form stable macromolecule radicals and/or the amino groups can form ammonium groups by absorbing hydrogen ions from the solution and then reacting with radicals through an additional reaction [52].

During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics has led to the search of new antimicrobial agents mainly among chitin extracts with the goal to discover new chemical structures, which overcome the above disadvantages. Recent research on natural molecule and products primarily focuses on chitin since they can be sourced more easily and be selected based on their ethno medicinal uses. A holistic eco-friendly shell waste management strategy is outlined for preventing environmental pollution converting shrimp waste into used as a packaging material for the quality preservation of a variety of food products and it's recycling. So searching of chitosan which shows antimicrobial properties from shrimp is the right choice to solve many problems

CONCLUSION

Chitosan can be extracted from shrimp heads which is available in large amount from shrimp processing industry. The Chitosan produced by deacetylation of chitin was observed to have many important properties like antibacterial, antifungal and radical scavenging activity. Tested chitosans markedly inhibited growth of most bacteria tested; however, the inhibitory effects differed depending on the types of chitosan and the tested bacteria with greater antimicrobial activity against gram positive bacteria than gram negative bacteria. Chitosan inhibits the mould and yeast growth and extends the shelf life. We concluded that chitosan can be used as an alternative natural preservative in the food products. The antifungal and antibacterial activities of chitosan can be employed in production of biofertilizers and biopesticides of economical benefits. Likewise the radical scavenging or the anti-oxidant activity of chitosan is of great interest in food industries and its possible use as natural additives has lead to a great interest in replacing synthetic additives. The use of the antimicrobial activity of chitosan has been used for development of antimicrobial films intended for use in packaging materials for foods, medical supplies and so on, or as laminated coating on items for which surface colonization is undesirable. Chitosan used as coating on fruits and vegetables is almost as effective as the fungicide TBZ at preventing spoilage during storage at proper conditions. Chitosan truly is a very useful chemical with a great scope of industrial applications in diverse fields.

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