

## SYNTHESIS AND CHARACTERIZATION OF CHITOSAN FROM CRAB SHELLS VS BACTERIOLOGICAL BIOMASS

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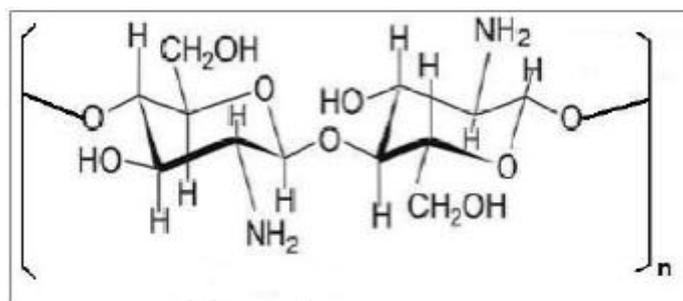
### ABSTRACT

A study was carried out to investigate the extraction and characterization of chitin and chitosan from crab shell and bacteriological biomass. Chitin and chitosan was extracted and characterized from crab shell found in Bay of Bengal, and by bacteria isolated from soil using the conventional methods of pretreatment, demineralization, deprotienization and deacetylation. The results obtained revealed that carbon nitrogen ratio of the chitosan extracted was 5.9 with a degree of deacetylation of 60.69% and 60.66% calculated from the elemental analysis and the FTIR spectra of chitosan respectively.

**KEYWORDS:** Characterization, Chitin, Chitosan, Extraction, Crab, soil microbes.

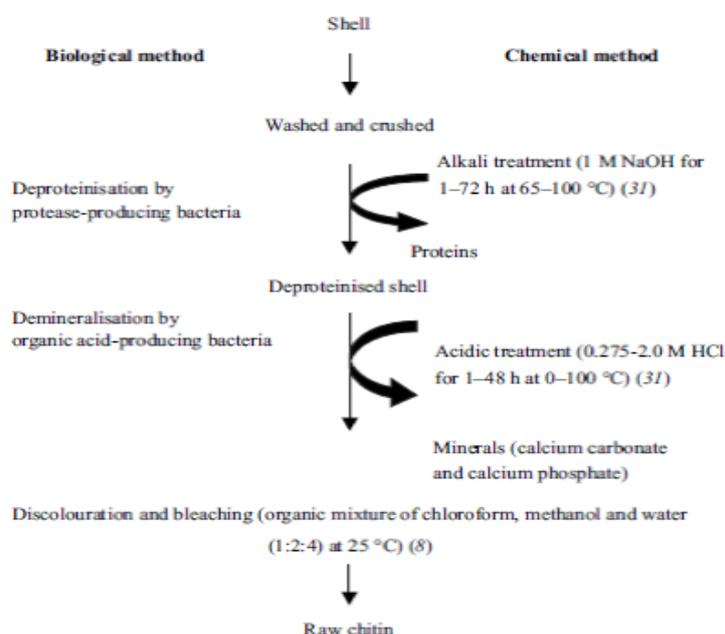
### INTRODUCTION

Chitosan, a natural polysaccharide, is being widely used as pharmaceutical excipients.<sup>[1]</sup> It is obtained by the partial deacetylation of chitin, natural polymer composed of randomly distributed  $\beta$ -(1-4)-linked D-glucosamine. It consists of two types of monomers; chitin-monomers and chitosan monomers. Chitin is a linear polysaccharide consisting of (1-4)-linked 2-acetamido-2-deoxy-b-D-glucopyranose. Chitosan is a linear polysaccharide consisting of (1-4)-linked 2-amino-2-deoxy-b-D-glucopyranose.<sup>[2&3]</sup>



**Figure 1: Structure of Chitosan (Lertsutthiwong *et al.*, 2002).**

Chitosan alone is biologically active which is efficient in managing dietary treatments and also exhibit pharmacology as antimicrobial, hypocholesterolemic and wound healing. Its unique structure makes opens its applicability as promising candidate not only as pharmaceutical aid but also in specific drug targeting and delivery. This research mainly focuses on the synthesis of pharmaceutical grade chitosan confining to predetermined quality attributes of its physiochemical properties such as gram molecular weight, viscosity, the extent of deacetylation, remanence after burnt, water log content, present of heavy metals etc. Additional studies conducted show that chitin extracted from crab exoskeleton and its derivatives can be useful for many studies. 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.<sup>[8]</sup> Crustacean shell waste consists mainly of 30–40% protein, 30–50% calcium carbonate and 20–30% chitin,<sup>[9-12]</sup> with species and seasonal variations.<sup>[13]</sup>



**Figure 2 Chitin recovery by chemical and biological methods.**

## MATERIALS AND METHODS

### Synthesis of chitosan from crab shell

#### Sample preparation

The crabs were obtained from Bay of Bengal coast; the shell and operculum are removed from the animal. The crabs exoskeletons collected are placed in Ziploc bags and refrigerated overnight. Approximately 1500 grams of crushed crab's exoskeletons wet samples were placed on foil paper and measured using a balance. The crab exoskeletons were crushed into smaller pieces using a meat tenderizer. The samples were oven-dried for 4 consecutive days at 65°C until constant weight. The obtained crab is made into 4 equal parts for efficient material handling.<sup>[14]</sup>

#### Extraction of chitin and chitosan

The chitin and chitosan sequence involves washing of crushed exoskeletons. Crushed crabs exoskeletons were placed in 1000 ml beakers and soaked in boiling sodium hydroxide (2 and 4% w/v) for one hour in order to dissolve the proteins and sugars thus isolating the crude chitin. 4% NaOH is used for chitin preparation, concentration used by the scientists at the Sonat Corporation.<sup>[15]</sup> After the samples are boiled in the sodium hydroxide, the beakers containing the crab shell samples are removed from the hot plate and allowed to cool for 30 minutes at room temperature.<sup>[16]</sup> The exoskeletons are then further crushed to pieces of 0.5-5.0 mm using a meat tenderizer.

#### Source of the Basidiomycetes

The mushrooms were collected from Tamil Nadu, India. These basidiomycetes were identified by their spore prints and, by comparing their morphological, anatomical and physiological characteristics with the standard descriptions.<sup>[3]</sup> The basidiomycetes were used for producing the grain spawn by the convenient method. The prepared spawn were stored at 5°C until using them for cultivation.

#### Growing Media and Cultivation

Mushroom, Hard wood sawdust and rice straw was used (1:1) for preparation of growing media as follows.

Sawdust + 1% CaCo + 1% sugar    Rice straw + 1% CaCo + 1% sugar

Sawdust + rice straw + 1% CaCo + 1% sugar

The moisture content of the aforementioned media formulae were adjusted to approximately 63 - 64%. Then each formula was filled in polypropylene bags (1kg each) and autoclaved at 121°C for 1 hour. After the sterilized media was cooled down, the bags were inoculated by the previously prepared grain spawn 2% (w/w), then being incubated at 22 - 27°C for spawn run (mycelium growth). At the end of incubation time (spawn run) the bags were opened and subjected to the fruiting conditions i.e. exposure to scattered light, watering by daily water spraying, good ventilation, adjusting relative humidity to 85-90% and temperature to 20-25°C. The crop was picked after 14-20 days from the end of incubation time in consecutive flushes at intervals of 15-20 days.

### **Synthesis of chitosan by bacteriological biomass**

#### **Soil Sampling and Analysis**

Soil samples were collected from different areas of Chennai, India using a sterile scalpel. Samples were stored individually in sterile polythene bags. Samples were analyzed for organic carbon, available phosphorus, and for microbial population. The organic C in the soil samples was 1.25%. Percentage availability of total phosphorus was 60. The pH of the soil was in the range 6.00– 6.50.

#### **Isolation of Bacterial Isolates**

The Cultivable bacterial strains were isolated from soil using initial screening in normal saline (0.9%). Population counts of soil samples were determined by dilution plating on nutrient agar plate with vortexing at every dilution step. Plates were incubated at 30 for 24 hrs. Colonies were counted and restreaked on nutrient agar. The cultures were identified by standard biochemical and staining methods. Pure cultures were preserved as glycerol stock and stored at  $-70^{\circ}\text{C}$ ,<sup>[27]</sup>

#### **Chitosan Extraction**

The bacterial biomass obtained dried in a freeze dryer (Thermo scientific) and weighed. Lyophilized bacterial biomass was then subjected to chitosan extraction protocol employing the standard method.<sup>[19]</sup> This extraction protocol includes an alkaline treatment which is followed by an acid treatment. In the optimization of the extraction protocol, freeze-dried fungal biomass were subjected to modified alkaline treatment followed by the acid treatment method of extraction. Mean while in the acid treatment, fungal biomass was subjected to the standard alkaline treatment prior to the modified acid treatment.<sup>[20]</sup>

### **Demineralization**

The grounded exoskeleton is demineralized using 1% HCl with four times its quantity. The samples were allowed to soak for 24 h to remove the minerals (mainly calcium carbonate).<sup>[17]</sup>

The demineralized crab shell samples were then treated for one hour with 50 ml of a 2% NaOH solution to decompose the albumen into water soluble amino-acids. The remaining chitin is washed with deionized water, which is then drained off. The chitin was further converted into chitosan by the process of deacetylation.<sup>[18]</sup>

### **Deacetylation**

The deacetylation process is carried out by adding 50% NaOH and then boiled at 100°C for 2 h on a hot plate. The samples are then placed under the hood and cooled for 30 min at room temperature. After-wards the samples are washed continuously with the 50% NaOH and filtered in order to retain the solid matter, which is the chitosan. The samples were then left uncovered and oven dried at 110°C for 6 h. The chitosan obtained will be in a creamy-white form.<sup>[5]</sup>

### **Recovery of Chitosan from Production Media**

The fermented broth from each flask was centrifuged at 12000 rpm for 15 minutes. The supernatant was discarded. The pellet contained mixture of bacteria, chitin and chitosan. To each of these pellets was added 10mL of 0.1N NaOH. The contents were mixed thoroughly and taken in separate clean test tubes that were autoclaved for 15 minutes. The tubes were then allowed to come to room temperature. Most of the cells were solubilized during the alkaline treatment. The tubes were again centrifuged at 12000 rpm for 15 minutes. The supernatants were carefully removed and pellets containing chitin, chitosan, and small amount of cell debris were mixed with 10mL of 2% acetic acid and mixtures were taken in clean test tubes that were left on a shaker overnight at room temperature to solubilize chitosan in 2% acetic acid. The contents of the above tubes were again centrifuged at 12000 rpm for 15 minutes. Pellet was discarded and 10mL supernatant was collected and the presence of chitosan in it was checked by the formation of white precipitate upon neutralization with 1N NaOH.<sup>[27]</sup>

## CHARACTERIZATION OF PREPARED CHITOSAN

### Molecular weight

Average molecular weight of chitosan is determined by determination of its Intrinsic viscosity by Brook-field viscometer.<sup>[23]</sup>

### pH

The pH measurements of the chitosan solutions will be carried out using a microprocessor pH meter.

### Ash value

The ash value of chitosan were determined by 2.0g of chitosan sample placed into previously ignited, cooled and tarred crucible. The samples are heated in a muffle furnace preheated to 650°C for 4 hr. The crucibles are allowed to cool in the furnace to less than 200°C and then placed into desiccators with a vented top. Percentage of ash value is calculated using the following.<sup>[25]</sup>

$$\% \text{ Ash} = \frac{(\text{Weight of residue, g})}{\text{sample weight, g}} \times 100$$

### Loss on drying

Loss on drying of the prepared chitosan will be determined by the gravimetric method. The water mass loss will be determined by drying the sample to constant weight and measuring the sample after and before drying. The water mass (or weight) will be the difference between the weights of the wet and oven dry samples.<sup>[26]</sup>

$$\% \text{ Loss on drying} = \frac{(\text{Wet weight} - \text{Dry weight})}{\text{Dry weight}} \times 100$$

### Fourier Transform - Infra Red spectroscopy (FT-IR)

The chitin, chitosan, water soluble chitosan, standard chitin and chitosan were determined using FT-IR spectrometer (Bio-Rad FTIS-40 model, USA).

## RESULTS AND DISCUSSION

The dry weights of the samples were determined to be 1269 grams.

### Isolation and Characterization of Isolates

In total, 20 bacteria were isolated from fresh soil samples. They exhibited wide morphological variation (Table 2). Bacterial morphotypes were selected on the basis of their

color, morphological characteristics, namely, colony morphology (shape, margin, elevation, and surface) and cell morphology (gram reaction, cell shape, and arrangement) according to Bergey's Manual of Systematic Bacteriology.<sup>[27]</sup>

It was observed that the yield of chitosan increased significantly with the increase of temperature and incubation period especially for 1hr and 3hrs incubation period. At 121°C, a raise of the incubation period from 3hrs to 5hrs yielded a significant increase of chitosan yield (41.29%).

Higher temperatures and longer incubation periods were necessary to enable effective interactions between NaOH and the constituents of the bacterial cell wall thus making it possible to extract higher levels of chitosan.<sup>[21]</sup> Longer incubation period also gave longer reaction time for NaOH to act on the chitosan structure in order to separate chitosan from other cell wall polysaccharides.<sup>[3]</sup> Thus it can be concluded that increasing the incubation period to 3hrs and maintaining the temperature at 121°C yielded high amounts of chitosan when alkaline treatment was used (Table 3).

In acid treatment, it was observed that the utilization of acetic acid as the extracting solution yielded higher amounts of chitosan as compared to hydrochloric acid. The highest chitosan yield was obtained with 6% Hydrochloric acid at incubation period of 12 hours at 95°C. This study also observed that the same incubation period, temperature and acid concentration rendered different effects and interactions when different acids were used as the extracting solution. (Table 4).

### **Degree of Deacetylation**

The increase of the alkaline concentration in the alkaline treatment increased the degree of deacetylation (DD) of the chitosan extracted (Table 6). This is in accordance with the previous study<sup>[3]</sup> which stated that the level or extent of deacetylation is influenced and controlled by alkaline concentration, temperature, incubation period, particle size and density.

Higher alkaline concentrations may cause alkaline hydrolysis to occur at a higher rate in the resulting in a higher degree of deacetylation.<sup>[22]</sup> Repetition of the standard alkaline treatments to the samples for up to 3 times also produced chitosan with a 3 higher DD as compared to chitosan extracted using the Industrial prospects for chitin and protein from standard alkaline treatment (Table 5).<sup>[22]</sup>

The degree of deacetylation (DD) for chitosan extracted by the various acid treatment were also determined (Table 5). The general trend observed was that DD increased with the increase in incubation temperature, incubation period and acid concentration. DD was also found to be significantly higher when hydrochloric acid was used as the extracting solution compared to acetic acid. Hydrochloric acid, being a strong acid in comparison to acetic acid, caused a greater extent of hydrolysis towards the acetyl moieties, in addition to the hydrolysis within the network of monomers in the chitosan polymer.<sup>[6]</sup> DD was found to be highest for all acids at 8% acid concentration. In the acid treatment, it was observed that varying individual factors (acid concentration, temperature and incubation period) did not seem to give consistent results whereas the varying/manipulation of particular parameter factor gave mixed results. (Table 5).

### FTIR

The Prolonged decalcification time, even during 24 h, results in a very slight drop in the ash content but can cause polymer degradation the calcium carbonate which demonstrates as ash value is very low. The FT-IR studies of the chitosan from standard commercial species. The major absorption band is observed between 1220 and 1020  $\text{cm}^{-1}$  which represents the free amino group ( $-\text{NH}_2$ ) at C2 position of glucosamine, a major group present in chitosan. Further the sample showed the absorption bands for the free amino group between 1026 and 1259  $\text{cm}^{-1}$  when the peak at 1374  $\text{cm}^{-1}$  represents the  $-\text{C}-\text{O}$  stretching of primary alcoholic group ( $-\text{CH}_2 - \text{OH}$ ). The absorbance bands of 3268, 2930, 2878, 1563 and 1418  $\text{cm}^{-1}$  indicated the N-H stretching, Symmetric  $\text{CH}_3$  stretching and asymmetric  $\text{CH}_2$  stretching, CH stretching, C=O stretching in secondary amide (amide I) and C-N stretching in secondary amide (amide II), respectively.

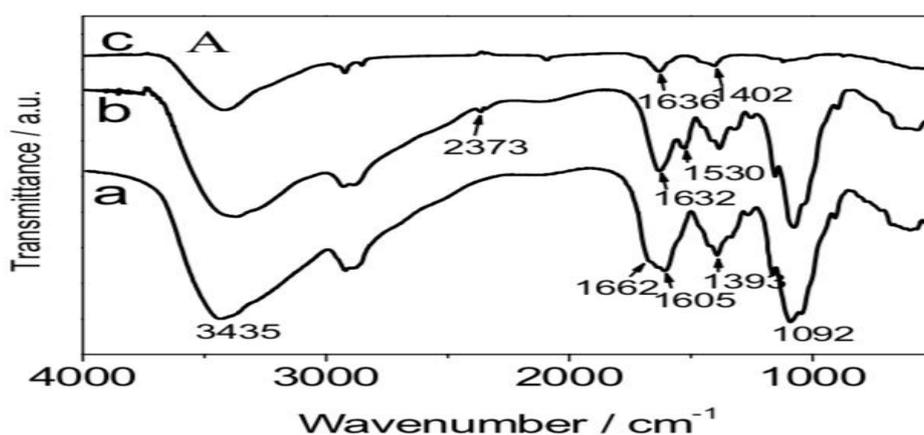
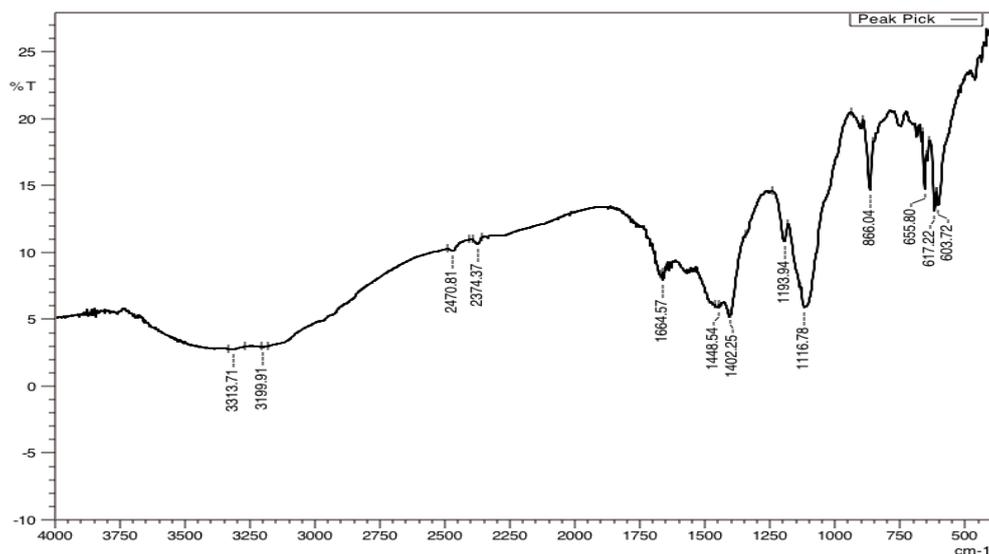
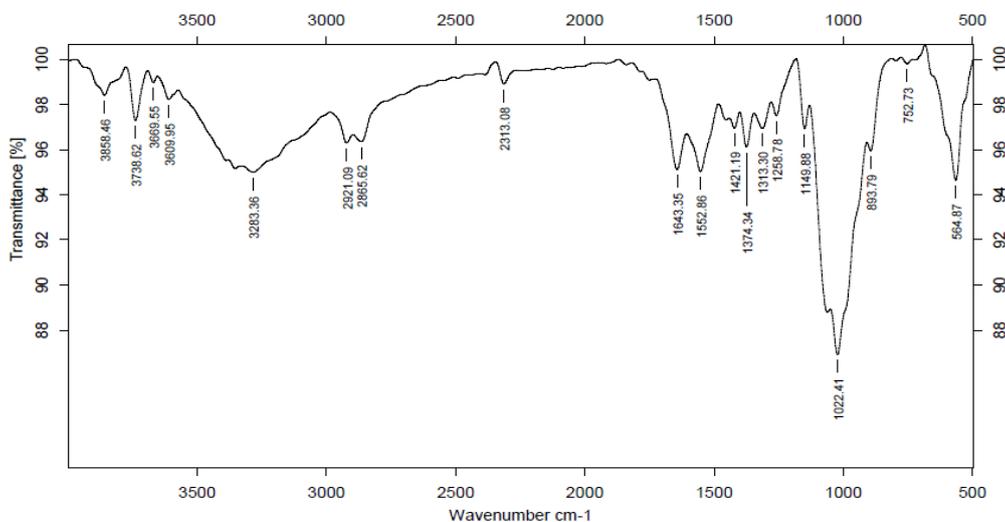


Figure 3: The FT-IR report of the standard chitosan.



### A. Isolation of chitosan from crab shell.



**Figure 4: Isolation of Chitosan from A. crab shell B. Bacteriological biomass.**

In the present study also the same absorbance bands were observed at 3283, 2921, 2865, 1643, 1552, 1421, 1022, 893 and 752  $\text{cm}^{-1}$  which confirms the structure of chitosan from table 2 and figure 3 and 4 A, 4 B.

### CONCLUSION

Drug delivery systems such as floating, intestinal and other gel forms require high molecular weight with high degree of deacetylation. Simultaneously vaccine, gene and enzyme drug delivery systems are suitable in high molecular weight chitosans which are highly deacetylated (>85%), different techniques are employed for these type of drug delivery systems such as adsorption, covalent linking, encapsulation, which require dense network,

potentially charged ends and long chains respectively. Chitosan is used in design of many different types of drug carriers for various administration routes like oral, parenteral, nasal, buccal, transdermal, vaginal, topical etc. It can be formulated as nanoparticles, microspheres, membrane sponge etc. For drug delivery, special preparation techniques are used to prepare chitosan drug carriers by taking care such parameters as cross linker concentration, chitosan molecules weight and processing conditions all these effect release rate of the loaded drug.

The recovery of chitin by chemical method using concentrated acids and bases in order to deproteinise and to demineralise shellfish waste (the most industrially exploited) at high temperature can deteriorate the physicochemical properties of this biopolymer and consequently its biological properties, which results in products of varying quality that are neither homogeneous nor reproducible. Biotechnology offers the opportunity to preserve the exceptional qualities of chitin and its derivatives. Nowadays, a new method based on the use of lactic acid bacteria and/or proteolytic bacteria has been used for chitin extraction. This method allows to produce a good quality chitin; it also leads to a liquid fraction rich in proteins which can be used for human and animal feed, and also produces pigments, mainly asthaxanthin. Although the biological method seems to be a promising approach for demineralisation and deproteinisation, the use of this method is still limited to laboratory scale because demineralisation and deproteinisation have not yet reached the desired yields if compared to the chemical method. The physicochemical conditions that influence the fermentation are the key factors of this bioprocess. Determination of the optimal conditions for biodeproteinisation and biodemineralisation of shells, the use of an effective bacterium and an inexpensive carbon source are the main factors which have to be considered to optimize chitin recovery from shellfish waste by fermentation.

**Table 1: Characteristics of chitosan obtained from Mushroom.**

Characteristics	Chitosan
Chitosan yield	41.29 %
Average Molecular weight	1,599,558
Degree of deacetylation	89.79 %
pH	8.5
Ash value	0.25 %
Loss on Drying	9.34 %

**Table 2: Colony morphology of recovered isolates of soil samples.**

Isolate	Form	Size	Color	Margin	Elevation	Surface	Opacity	Organism
S1	Circular	Small	Dirty white	Entire	Raised	Smooth	Opaque	<i>Staphylococcus sp</i>
S2	Circular	Small	Yellow	Entire	Flat	Smooth	Opaque	<i>Micrococcus sp</i>
S3	Circular	Small	White	Entire	Raised	Dry	Opaque	<i>Bacillus sp.</i>
S4	Circular	Small	Dirty white	Entire	Flat	Smooth	Opaque	<i>Streptococcus sp.</i>
S5	Irregular	small	Greyish	Senate	Raised	Smooth	Opaque	<i>Proteus sp.</i>
S6	Circular / convex	Large	White	Slightly curved	Flat with matted surface	Rough / Smooth	Opaque	<i>Erysipelothrix sp.</i>
S7	Circular	Small	L i g h t green	Entire	Raised	wrinkle	Opaque	<i>Pseudomonas sp.</i>
S8	Circular	Small	Yellow	Entire	Flat	Smooth	Opaque	<i>Micrococcus sp</i>
S9	Circular	Small	White	Entire	Raised	Dry	Opaque	<i>Bacillus sp.</i>

**Table 3: Effect of concentration of NaoH solution on the extraction yield of chitosan at temperature 121°C.**

Alkaline treatment molarity of NaoH	Incubation Time	Sample 1 Mushroom	Sample 2 Crab's shell
1 M	1 hr	0.174	0.104
2 M	3 hrs	0.071	0.113
3 M	5 hrs	0.458	0.118
4 M	8 hrs	0.656	0.122

**Table 4: Extraction of chitosan using HCl at different concentration and temperature.**

HCl	Incubation time	Chitosan extracted from Mushroom	Chitosan extracted from Crab's shell
2 %	12 hrs	0.071	0.064
6 %	12 hrs	0.095	0.074
10 %	12 hrs	0.084	0.071

**Table 5: Effect of concentration of NaoH solution of the degree of deacetylation of chitosan.**

Alkaline treatment Molarity of NaoH	Degree of deacetylation
1 M	0.82 %
2 M	0.85 %
3 M	0.88 %
4 M	0.93 %

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