IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

EXTRACTION OF POLYMER FROM BACTERIA AND FUNGI AND ITS CHARACTERIZATION

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ABSTRACT:

In the present study, the chitosan biopolymer was extracted from the bacterial and fungal species- *Pseudomonas fluorescens*, *Aspergillus niger* and *Rhizopus oryzae*. The chitosan was characterized by chemical treatment with iodine and concentrated sulphuric acid, characteristic colour change was observed, it indicates a positive result. The Chitosan yield was maximum in bacteria (Chitosan from *Pseudomonas fluorescens* 63%) than fungi (*Aspergillus niger* 20% and *Rhizopus oryzae* 15%) comparatively after 5 days of fermentation. Characterization by FTIR also confirms similar chitosan functional groups presence in the microbially extracted chitosan. The extracted chitosan was subjected to antimicrobial activity, and it was found that the extracted Chitosan was active against the bacteria (*Bacillus, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*) and fungi (*Candida albicans*). In addition to the antimicrobial property of chitosan and drug, a combination of both chitosan and drug show higher antimicrobial activity when compared with the standard chitosan and drug. Thus, microbial chitosan could have potential in medical and agricultural applications. We can conclude that the strains used in the above work are promising sources for chitosan production. By recent developments in pharmaceutical biotechnology, these strains can be genetically manipulated and further optimized for getting better results in producing a quality and economical Chitosan.

INDEX TERMS:

Chitosan, *Pseudomonas fluorescens*, *Aspergillus niger*, *Rhizopus oryzae*, Chitosan extraction, Chitosan confirmation, FTIR, Antimicrobial activity.

I. INTRODUCTION:

Polymers are the larger macromolecules with repeating units of monomer. They are solid, higher molecular weight, non-metallic compounds (A. Ashwin Kumar *et.al*, 2011). The polymers are classified into synthetic polymers and biopolymers respectively (D.R Lu *et.al*, 2009). The biopolymers are classified into three main categories such as amino acid-based biopolymers, polysaccharide from bacteria and polyester (A.P. Bonartsev*et.al*, 2007). The polymer which is extracted in the present study was chitosan, an example of polysaccharide. Chitosan sometimes known as deacetylated chitin, is a natural polycationic linear polysaccharide derived from the partial deacetylation of chitin (Chandy.T *et.al*., 1990). Chitosan is composed of beta (1-4) linked d-glucosamine and N-acetyl D-glucosamine randomly distributed within the polymer (Venkatesan J *et.al*., 2010). The interesting characteristics of chitosan such as biocompatibility, non-toxicity, low allergenicity and biodegradability allow it to be used in various applications (Kumar M.N *et.al*., 2004). Besides, chitosan is reported to have other biological properties, such as antitumor, antimicrobial and antioxidant activities (Martins A.F *et.al*., 2014, Ngo D.H *et.al.*, 2014). On a commercial scale, chitin and chitosan were extracted from the exoskeleton of crustaceans employing various chemical process resulted in pollution and other environmental problems. To overcome this, recent advances in fermentation technology have attracted the researchers to extract the chitin and chitosan from various microbial sources (Jesteena Johney *et.al*., 2016). The chitosan can be extracted from the bacterial and fungal species like *Pseudomonas fluorescens, Aspergillus niger, Penicillium chrysogenum, Rhizopus oryzae, Trichoderma ressei, Mucor rouxii, Absidia glauca, Absidia coerulea, Gongronellabutleri, Phycomycesblakesleeanus, Absidia blakesleeana, Lentinus edodes* have been investigated for the production of chitin and chitosan (Chatterjee S *et.al*., 2004). The antimicrobial activity of chitosan against different groups of microorganisms, such as bacteria, yeast and fungi, has received considerable attention in recent years (Yang N, *et.al*., 2014, Mater Lett *et.al*., 2014). The chitosan binds with the microbial DNA, which leads to the inhibition of the mRNA and protein synthesis via., the penetration of chitosan into the nuclei of the microorganisms (Sebti *et.al*., 2005, Hadwiger *et.al*., 1981, Sudarshan *et.al*., 1992). In the present study, the chitosan was extracted from *Pseudomonas fluorescens*, *Aspergillus niger* and *Rhizopus oryzae.* It was characterized by using FTIR and subjected to antimicrobial activity along with the drug 5-Fluoro Uracil.

II. MATERIALS AND METHODS:

We focused on an eco-friendly method of extracting the chitosan from bacterial and fungal species namely, *Pseudomonas fluorescens*, *Aspergillus niger* and *Rhizopus oryzae*.

2.1 Isolation of microorganisms

The chosen organisms for the extraction of chitosan are:

- *Pseudomonas fluorescens*
- *Aspergillus niger*
- *Rhizopus oryzae*

2.1.1 *Pseudomonas fluorescens*

One gram of *Pseudomonas fluorescens* powder was taken and dissolved in 100 ml of sterile distilled water. About 1 ml of the inoculum was taken and inoculated into the sterile plates containing nutrient agar. The plates were then incubated at 37℃ for 24 h, serially diluted to isolate the colonies at 37℃ for 24 h. The inoculated colonies were incubated at 37℃ for 24 h.The colonies were observed after the incubation period. For the confirmation the organism is characterized by the following tests: Gram staining, Indole test, Methyl red test, Voges-proskauer test, Citrate test, Catalase test, Oxidase test.

2.1.2 Isolation of *Aspergillus niger*

Black rots present in the onion was taken with the help of inoculation needle and inoculated in the sterile petriplate containing sabouraud's dextrose agar (SDA). It is then incubated at 25℃ for 2-3 days. Pure culture was made by inoculating the fungal spores in the petriplate containing SDA. After incubation the result was observed and confirmed with the colony appearance.

2.1.2 Isolation of *Rhizopus oryzae*

Rhizopus oryzae was bought from the KMCH laboratory. It was sub cultured in SDA, incubated at 25℃ for 2-3 days. Pure culture was made by inoculating the fungal spores in the petri plates containing SDA. Incubate it at room temperature for 2-3 days. After incubation the results were observed and confirmed with colony appearance.

2.1.3 Confirmation test for fungal species - LPCB Staining

Using a dropper or pipette, add one or two drops of lacto phenol cotton blue solution on a clean microscopic glass slide. Add the fungal specimen to the drops of Lacto Phenol Cotton Blue (LPCB) stain using the inoculation needle and tease the fungal sample to ensure the sample mixes well with the stain. Carefully cover the stain with a clean sterile cover slip without making air bubbles to the stain. Examine the stain microscopically at high magnification, to observe the fungal spores and morphology.

2.2 Chitosan extraction from *Pseudomonas fluorescens*

2.2.1 Fermentation process

The 10 ml of seed culture was inoculated into the fermentation medium (Glucose – 20 g/l, Di ammonium sulphate ($(NH_4)_2SO_4$) – 2.0 g/l, Potassium dihydrogen phosphate (KH_2PO_4) – 13.3 g/l, Magnesium sulphate ($MgSO_4.7H_20$) – 1.2 g/l, Citric acid – 1.7 g/l, Manganese sulphate ($MnSO_4$)- 0.4 g/l, Ferrous sulphate ($FeSO_4.7H_20$) – 1.0 g/l, $CoCl_2.6H_2O$ – 0.2 g/l, Zinc chloride ($ZnCl_2$) – 0.2 g/l) which was incubated at 37° C, 72 h (Santhanam. A *et al*, 2010).

2.2.2 Extraction of biopolymer

Intracellular biopolymer was extracted by using Sodium hypochlorite extraction method. Initially 50 ml of incubated fermentation medium was taken and centrifuged at 6000 rpm for 15 min and

the supernatant was discarded. The cell pellets were suspended in l M of sodium hypochlorite and incubate for 1-2 hrs at 37°C for complete digestion of cell component except biopolymer, where by lipids and proteins were degraded. This mixture was centrifuged at 6000 rpm for 15 min and the supernatant was discarded. The sediment was washed twice with distilled water and centrifuged. The biopolymer granule was washed with acetone. The biopolymer was dissolved with boiling chloroform and was evaporated by air drying to yield dry powder of biopolymer (Aravindan. J *et.al*., 2012).

2.3 Chitosan extraction from *Aspergillus niger* and *Rhizopus oryzae*

The pure culture of the fungal species was inoculated into Sabouraud dextrose broth separately in another conical flask and incubated at room temperature. The broth culture from the previously inoculated broth was taken and inoculated in SDA in another separate conical flask. This process was repeated for five days by means of a continuous fermentation process at room temperature in the shaker at 150 rpm for a better yield.

2.3.1 Chitosan isolation

After cultivation, fungal mycelia were recovered by filtration (Whatman no 1), washed twice with distilled water until a clear filtrate was obtained and then dried at 65°C to a constant weight. Yeast cells were harvested by centrifugation at 8000 g for 30 min, washed twice with distilled water and dried at 65°C to a constant weight. Chitosan extraction was carried out by a modified method of Rane and Hoover (1993) and Crestini *et al*., (1996). Dry fungal mycelia and dry yeast cells were finely ground, suspended with 1 M NaOH solution and autoclaved at 121°C for 15 min. Alkali-insoluble fractions were collected after centrifugation at 12000 rpm for 15 min, washed with distilled water and re-centrifuged to a neutral pH (pH 7). The residues were further extracted using 2% acetic acid at 95°C for 8 h. The extracted slurry was centrifuged at 12000 rpm for 15 min and the insoluble acid discarded. The pH of the supernatant fluids was adjusted to 10, with 2 mol NaOH, the solution centrifuged at 12000 rpm for 15 min and the precipitated Chitosan was washed with distilled water, 95% ethanol and acetone separately and dried at 60°C to a constant weight.

2.4 Confirmation test for Chitosan

Add 2-3 drops of iodine/potassium iodide solution to the dried precipitate, mixed and the mixture was acidified with 2-3 drops of 1% H_2SO_4 (Kuldeep Kaur *et.al*.,2012).

2.5 Quantitative estimation of Chitosan

Once again, the precipitate of Chitosan was obtained. It was washed twice with distilled water (pH 7) and was resuspended in 1 ml of distilled water (pH 7). The weights of two dried, clean Petri plates were taken; 1 ml of Chitosan suspension obtained from the isolates was poured. Petri plates were kept at 55°C for 2–6 h for drying. After drying, plates were again weighed. (Kuldeep Kaur et al., 2012).

2.6 Dry weight calculation

The extracted Chitosan from *Pseudomonas fluorescence*, *Aspergillus niger*, *Rhizopus oryzae* were measured after air drying in a weighing balance machine in grams.

2.7 Yield determination of biopolymer

10 ml of seed medium was transferred aseptically to the production medium and incubated for 72 h for bacterial and 120 h for fungal in incubator shaker at 250 rpm. Meanwhile, the cells were subjected to chloroform-sodium hypochlorite extraction method and yield was calculated using the formula given below.

$$\text{Yield } \% = \frac{\text{Amount of biopolymer}}{\text{Amount of Biomass}} + 100$$

The results were tabulated Amount of Biomass

2.8 ANTIMICROBIAL SENSITIVITY TEST

Antimicrobial sensitivity test was done in order to see the antimicrobial activity against *Staphylococcus aureus, Bacillus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* which were purchased from KMCH. It is then sub-cultured and used for antimicrobial studies.

2.8.1 Preparation of microbial disk

- Control – Standard chitosan + drug 5-fluorouracil (0.01 gm)
- Sample 1 – Extracted chitosan from *Pseudomonas fluorescence* + drug (0.01 gm)
- Sample 2 – Extracted chitosan from *Aspergillus niger* + drug (0.01 gm)
- Sample 3 – Extracted chitosan from *Rhizopus oryzae* + drug (0.01 gm)
- Standard chitosan (0.02 gm)
- Standard drug (5-fluorouracil - 0.01 gm)
- The above components were dissolved in DMSO and sterile antibiotic Whatman filter paper disk was immersed overnight for a better loading efficiency.
- These disks were directly used for the antimicrobial studies.

2.8.2 Procedure

Select a pure culture plate of one of the organisms to be tested. Aseptically emulsify a colony from the plate in the sterile saline solution. Mix it thoroughly to ensure that no solid material from the colony is visible in the saline solution. Repeat until the turbidity of the saline solution visually match that of the standard turbidity. Take a sterile swab and dip it into the broth culture of *Staphylococcus aureus, Bacillus, Escherichia coli, Pseudomonas aeruginosa, Candida albicans*. Gently squeeze the swab against the inside of the tube in order to remove excess fluid in the swab. Take a sterile Mueller-Hinton agar (MHA) plate. Use the swab with the test organism to streak an MHA plate for a lawn of growth. After the streaking is complete, allow the plate to dry for 5 minutes. Overnight soaked disc can be placed on the surface of the agar using sterile forceps. Gently press the discs onto the surface of the agar using flame sterile forceps or inoculation loop. Carefully invert the inoculated plates and incubate for 24 h at 37°C. After incubation, use a metric ruler to measure the diameter of the zone of inhibition the sensitivity zone. Compare the measurement obtained from the individual antibiotics to the standard table to determine whether the tested bacterial species is sensitive. The zone of inhibition was observed and tabulated.

2.9 FTIR Analysis

Before FTIR analysis, the sample was prepared, for testing using either the attenuated total reflectance (ATR), Nujol or other technique. Enough samples are required to obtain an absorption spectrum. The FTIR spectrometer generates a graph in the form of absorbance spectra, which shows the unique chemical bonds

and the molecular structure of the sample material. This absorption spectrum will have peaks representing components present. These absorbance peaks indicate functional groups (e.g., alkanes, ketones, acid chlorides). Different types of bonds, and thus different functional groups, absorb infrared radiation of different wavelengths. The analytical spectrum is then compared in a reference library program to identify components or to find a "best match" for unknown material using the cataloged spectra.

III. RESULTS AND DISCUSSION:

3.1 EXTRACTION OF BIOPOLYMER

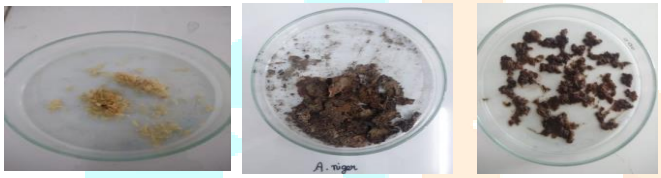


Fig 1: Polymer extracted from *Pseudomonas fluoroscens, Aspergillus niger* and *Rhizopus oryzae*

In *Pseudomonas fluorescens* the biopolymer was extracted by sodium hypochlorite method. In fungi (*Aspergillus niger* and *Rhizopus oryzae*) the biopolymer was extracted by means of a continuous culture method.

3.2 CHITOSAN CONFIRMATION

Fig 2: Chitosan extracted from *Pseudomonas fluoroscens, Aspergillus niger* and *Rhizopus oryzae*

On addition of iodine/potassium iodide solution, the precipitate change colour to dark brown and the solution becomes colourless and on addition of sulphuric acid, the dark brown colour turns to dark purple. This confirms that the precipitate extracted

S. No	**Test Organism**	**Drug (mm)**	**Commercially available Chitosan (mm)**	**Chitosan extracted from *Pseudomonas fluorescens*(mm)**	**Chitosan extracted from *Aspergillus niger* (mm)**	**Chitosan extracted from *Rhizopus oryzae* (mm)**
1	***Staphylococcus aureus***	10	42	38	40	37
2	***Bacillus***	30	43	38	44	35
3	***Escherichia coli***	25	28	20	20	25
4	***Pseudomonas aeruginosa***	25	39	35	35	35
5	***Candida albicans***	37	35	33	30	26

from *Pseudomonas fluorescens, Aspergillus niger* and *Rhizopus oryzae* is chitosan.

3.3 BIOMASS OF EXTRACTED CHITOSAN

Fig 3: Dried mass of chitosan extracted from *Pseudomonas fluoroscens, Aspergillus niger* and *Rhizopus oryzae*

The chitosan extracted from *Pseudomonas fluorescens* gave good result with a microbial mass of 15.6 g / 250 ml after 5 days of cultivation. Among the fungi *Aspergillus niger* gave the highest growth rate with a maximal mycelial biomass of 13 g / 250 ml after 5 days of cultivation, while *Rhizopus oryzae* grew very slowly with a maximal biomass of 7.26 g / 250 ml after 5 days of cultivation.

3.4 YIELD OF CHITOSAN:

Table 1:

Chitosan extracted from organisms	**Incubation hours**	**Biomass (g)**	**Biopolymer (g)**	**Yield in percentage**
Pseudomonas fluorescens	72 hours	9.4	3.58	38%
	120 hours	15.6	5.96	63.3%
Aspergillus niger	120 hours	13	2.57	19.7%
Rhizopus oryzae	120 hours	7.26	1.09	15.07 %

Chitosan extracted from *Pseudomonas fluorescens* was shown to give a maximal yield of about 5.96 g /250 ml which gain dry weight (63.3 % chitosan), where the *Aspergillus niger* results 2.57 g / 250 ml and *Rhizopus oryzae*results 1.09 g/ 250 ml. The chitosan content of fungal mycelia also depends on the chitosan extraction method (White et al. 1979). The yield % of chitosan obtained from *Pseudomonas fluorescens* was 63%, *Aspergillus niger* was 20% and *Rhizopus oryzae*was 15% (Table 1). The polymer extracted from *Pseudomonas fluorescens* by sodium hypochlorite method had a yield of about 55% (M.N. Priyadharshini 2015).

3.5 ANTIBIOTIC SENSITIVITY TEST

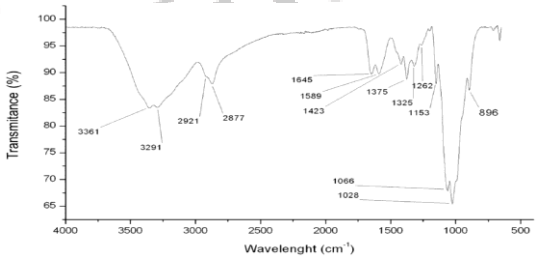
Table 2 and 3:

S.No	Test Organism	Drug (mm)	Commercially available Chitosan +Drug (mm)	Chitosan extracted from *Pseudomonas fluorescens* +Drug (mm)	Chitosan extracted from *Aspergillus niger* +Drug (mm)	Chitosan extracted from *Rhizopus oryzae* +Drug(mm)
1	***Staphylococcus aureus***	10	54	46	50	54
2	***Bacillus***	30	44	45	40	45
3	***Escherichia coli***	25	44	28	39	42
4	***Pseudomonas aeruginosa***	25	38	36	39	43
5	***Candida albicans***	37	45	37	32	40

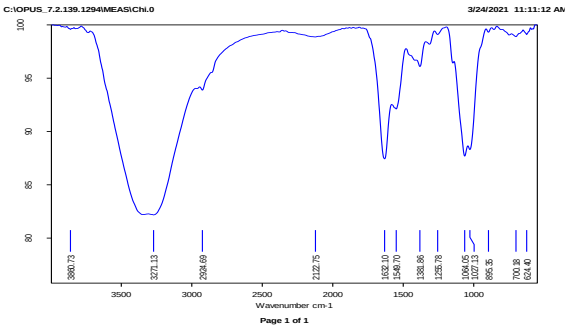
The diameter of the zone of inhibition produced by bacterial (*Pseudomonas fluorescens*) and two fungal (*Aspergillus niger*, *Rhizopus oryzae*) chitosan loaded with 5- fluorouracil on *Staphylococcus aureus* (46,50,54,54 mm), *Bacillus sp* (45,40,45,44 mm), *E. coli* (28,39,44,42 mm), *Pseudomonas aeruginosa* (36,39,43,38 mm) and *Candida albicans* (37,32,40,45 mm) and the diameter of the inhibition zones of bacterial (*Pseudomonas fluoroscens)* and two fungal *(Aspergillus niger*and *Rhizopus oryzae)* on *Staphylococcus aureus* (38,42,40,37 mm), *Bacillus sp*(38,43,44,35 mm), *Escherichia coli* (20,28,20,25 mm), *Pseudomonas aeruginosa* (35,39,35,35 mm), and *Candida albicans* (33,35,30,26 mm) confirmed that the extracted microbial chitosan has minimal antimicrobial activity (Table 2 and 3).

3.6 FTIR ANALYSIS:

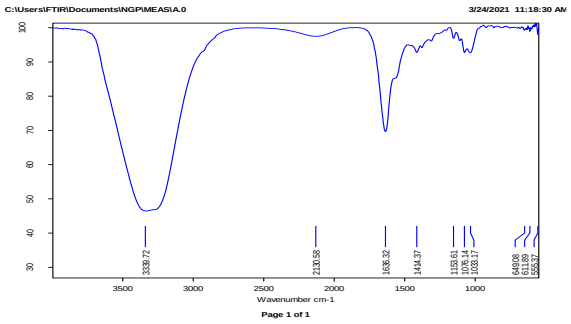
Graph 1: Commercially available chitosan



Graph 1 a: Chitosan extracted from *Pseudomonas fluorescens*



Graph 1 b: Chitosan extracted from *Aspergillus niger*



Graph 1 c: Chitosan extracted from *Rhizopus oryzae*

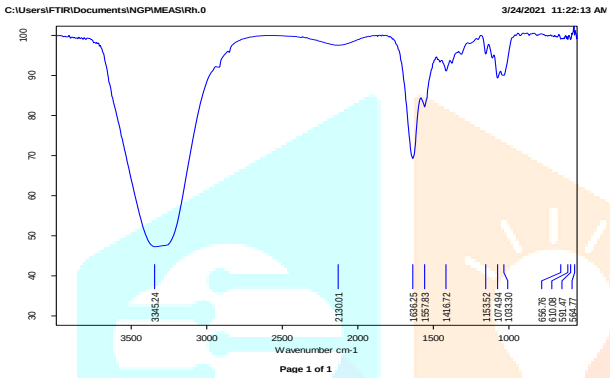


Table 4:

COMPOSITION OF POLYMER	**FUNCTIONAL GROUPS**	**WAVE NUMBER RANGES**
Standard chitosan	Alcohol, Carboxylic acid, Alkane, Imine/Oxime, Amine, Phenol, Aromatic ester, Tertiary alcohol, Primary alcohol, Sulfoxide	3361, 3291, 2921,2877, 1645, 1589, 1423, 1375, 1325, 1262, 1153, 1066, 1028
Chitosan extracted from *Pseudomonas fluoroscens*	Alcohol, Alkane, Azide, Imine/Oxime Nitro compound, Aromatic ester, Primary alcohol, Amine, Alkene	3371, 2924, 2122, 1632, 1549, 1381, 1255, 1064, 1027,895
Chitosan extracted from *Aspergillus niger*	Alcohol, Azide, Amine, Cyclic alkene, Tertiary alcohol, Primary alcohol, Sulfoxide	3339, 2130, 1636, 1414, 1153, 1076, 1033
Chitosan extracted from Rhizopus oryzae	Alcohol, Azide, Amine, Cyclic amine, Tertiary alcohol, Primary alcohol, Sulfoxide	3345, 2130, 1636,1557, 1416, 1153, 1074, 1033

The FTIR properties of bacterial and fungal chitosan were compared with those of a commercial chitosan derived from crab shells or shrimps. Several functional groups in all chitosan samples could be observed in FTIR spectra including hydroxyl stretching band at 3,450 cm^{-1}, amine (N–H) stretching band at 3,400–3,250 cm^{-1}, amide I band at 1,655–1,550 cm^{-1}, primary amine band at 1,630–1,550 cm^{-1} and amide III band at 1,320–1,315 cm^{-1}(Table 4). The results obtained from the extracted chitosan were also similar to the commercially available chitosan. Moreover, it was implied that extraction conditions would not affect the functional groups of bacterial and fungal chitosan.

I. SUMMARY AND CONCLUSION

The findings suggest that *Pseudomonas fluorescens*, *Aspergillus niger* and *Rhizopus oryzae* are the potential candidate to produce eco-friendly Chitosan in the development of drugs delivery system. The production of Chitosan from the shell waste produces large quantities of harmful effluents. Hence, microbial Chitosan provides an attractive alternative, and in addition the recent emphasis on green synthesis for sustainable development has necessitated the adoption of alternate sources of Chitosan. It can also be adapted by industries in producing a quality and economical Chitosan as an alternative to the shell fish derived product. Finally, we can conclude that

the strains used in the above work are promising sources for Chitosan production. Thus, microbial Chitosan could have potential in medical and agricultural applications. By recent developments in pharmaceutical biotechnology, these strains can be genetically manipulated and further optimized for getting better results in producing a quality and economical Chitosan.

II. ACKNOWLEDGMENT

The author thanks DBT star scheme, chairman and managing Trustee of Dr. N.G.P. Arts and Science College, Coimbatore, Tamil Nadu, for providing Necessary facilities for carrying out this research work. Author extended her thanks to Health care workers of KMCH hospital, Coimbatore for their help and support.

III. CONFLICTS OF INTEREST:

The author has no conflicts of interest to publish this Research article in this journal.

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