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Research Article

Biological Extraction of Chitosan from Aquatic Biowaste–A Low Cost Technology

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Abstract: The shrimp industry generates a huge amount of shell waste which usually cause environmental pollution. This waste can be utilized as an economic source of chitin and chitosan. Chitin is the second most abundant renewable natural source following cellulose and the main source of chitin is crustacean waste. Chitosan which is a derivative of chitin after the process of deacetylation has multiple of commercial and possible medical uses based on its degree of deacetylation. Keeping in view of its significance, the present study is aimed to extract chitosan by using chemical and biological methods from aquatic waste like shells of shrimp, crab and fish scales and to characterize the chitosan quality which includes parameters like ash, moisture, protein and lipid content and degree of deacetylation (DDA). Biological method of chitosan extraction from crustacean shells is an advanced and new eco-friendly technique which involves extraction of long chain carbohydrate polymer chitin by using marine fungi and it produces a good quality end product. Among the three aquatic biowaste materials selected, maximum quantity of chitin (474.66±25.02%) and chitosan (441.00±26.52%) were obtained from shrimp shell waste through biological extraction and consist of relatively low contents of protein (7.9±0.44%), fat (3.2±0.09%), moisture (1.7±0.08%) and ash (1.2±0.02%) on a dry basis compared to chitosan obtained from chemical extraction. Biologically extracted shrimp chitosan appeared to have superior quality than chitosan derived from crab shells and fish scales. Further, utilisation of shrimp shell waste for the production of these kind of valuable biopolymers give more economical and biological advantages along with reduction of environmental pollution.
Keywords: Chitin, Chitosan, Aquatic biowaste, Biological extraction, Quality parameters.

1. Introduction

The shell fish industry is operative among all the costal countries and contributes hugely to the food delicacies. During the processing of prawns, shrimps and lobsters mostly the meat is taken, while the shell and head portions are generated as wastes. This results in the generation of a huge amount of waste throughout the world. It is estimated that the shell-fish industry in India generates 125,000 to 150,000 tons of shell waste per year (Ramyadevi, 2012). The disposal of such an enormous amount of waste has become a serious environmental concern (Sagheer *et al.,* 2009). Chitin, a homopolymer of N-acetyl-D-glucosamine is the most abundant renewable natural resources and the main source of it is crustacean waste. Usually, crustacean shell wastes contain mainly chitin, proteins and minerals. So by demineralising and deproteinizing the wastes, chitin can be obtained (Jiang, 2003). Chitosan, the deacetylated product of chitin is a nontoxic biopolymer (Abdulkarim, 2013) and it is commercially produced from the crustacean shell wastes through different degrees of deacetylation, which attribute to a variety of properties (Rinaudo, 2006). Chitin and its

derivative chitosan are of commercial interest due to their excellent biocompatibility, biodegradability, nontoxicity, chelating and adsorption power (Szymanska, 2015).

The commonly practiced commercial process for chitosan extraction from crustacean waste is based upon demineralization by acid treatment and deproteinization by alkali treatment and that affect the physical and chemical properties of chitin and chitosan, and the effluents harm the environment. A different method for extraction of chitosan by using proteolytic microorganisms or fungi or purified microbial enzymes has been introduced which is a low cost technology and eco-friendly (Yadav *et al.,* 2019). The major target is to obtain quality final product, which is a function of the molecular mass (average and polydispersity) and the degree of acetylation (DA) with as low process cost as possible. With this background, it is proposed to study the biological extraction of chitosan from aquatic biowaste available abundantly from this region, using marine fungi of Nellore coastal zone. The present investigation has been taken up to evaluate the difference in yield % and in the quality parameters among the chitin and chitosan extracted from crab, fish scales and shrimp waste through chemical and biological extraction methods and partial characterization.

2. Methodology

2.1. Collection and processing of Aquatic bio waste

The shell waste (crab, shrimp and fish scales) was obtained from nearby processing industries located at Rajupalem area of Nellore District. The samples were washed with tap water to remove any insoluble material on the shell then dried at 50°C in oven for 24h and homogenized in a laboratory mixer before using for further processing. The yield of dried shell was determined by weighting after being dried (Khanafari, 2008).

2.2. Chemical extraction of Chitin and Chitosan

A. Deproteinisation: The obtained crushed waste (100g) was placed separately in 500 ml beakers and soaked in 100 ml sodium hydroxide (4%) for 24 hrs in room temperature in order to dissolve the proteins and sugars, thus isolating the crude chitin. (Kumari and Rath, 2014). Then solution was filtered and the samples were washed with distilled water. The shells were then further crushed to pieces of 0.5-5.0 mm using a meat tenderizer.

B. Demineralisation: The grounded shells were demineralised using 1% HCl with four times its quantity. The samples were allowed to soak for 12 h to remove the minerals (mainly calcium carbonate) (Puvvada *et al.*, 2012). The demineralized 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 was washed with deionized water, which was then drained off.

C. Deacetylation: Chitosan was obtained from extracted chitin through deacetylation method (Kumari, 2014). The extracted chitin was dissolved in 100ml of NaOH (50%) at 60°C for 8h to obtain crude chitosan. After filtration, the residue was obtained, washed three times with hot distilled water at 60°C. The chitosan was obtained by drying in a hot air oven at 50°C overnight.

2.3. Biological extraction of Chitin/Chitosan

A. Isolation of marine fungi

Totally four sampling stations at Bay of Bengal (Thummalapenta, Mypadu, Kothakodur, Nelaturu) were selected for the collection of water samples of the SPSR Nellore District. Sampling was done over a period of three months from February, 2015 to April, 2015. After

sampling, within 24 hrs the water samples from each station were subjected to appropriate dilutions (10^{-2} to10^{-9}) and 0.1 ml of sample was aseptically transferred into the plates containing modified Malt extract agar (3% Malt extract, 5g Peptone, 15g Agar, 37% Artificial sea water, pH-8) by Spread plate method. The plates were incubated at room temperature (28^0C) for 4-5days. Control plates were also maintained. The incubated plates were observed for the development of colonies from third day onwards.

B. Screening of Marine fungi

a) **Protease:** Pure cultures of fungal isolates were subjected to primary screening for extracellular protease production by plate assay using skim milk agar plate. Fungi from their respective slants were inoculated separately in skim milk agar plate and were incubated at 28˚C for 3 days. The clear zones were detected around fungal colonies at the end of incubation period (Abdel Galil, 1992).

b) **Organic acid:** The fungal isolates were screened for acid production using acid indicator medium (AIM) containing 0.04% of bromocresol purple (Das and Roy, 1998). A loopful of fungal inoculum was inoculated on to Czapek-Dox agar medium and incubated for five days for the formation of yellow coloration of the medium which indicated the production of acid.

c) **Chitin deacetylase:** Screening for chitin deacetylase producing fungi was carried out in chitin deacetylase screening plates. After 2-3 days of incubation, the clear zones were detected and confirmed as chitin deacetylase producers and used for further studies (Gao *et al.*, 1995).

C. Identification of fungi

The isolated fungi were sub cultured on Malt extract agar medium and pure cultures were used in the identification studies. Slide culture method was followed for the preliminary identification of fungi. The fungi grown on the slide cultures were stained using lacto-phenol cotton blue (Nizamydeen *et al.*, 2014). Morphology of the fungal hyphae, sporulation patterns and mycelial structures were studied using compound microscope (400 X). The fungal keys used in the study were Barnett and Hunter, (1972); Ainsworth *et al.*, (1973); Kohlmeyer and Kohlmeyer, (1979) and Bisby's Dictionary of the Fungi (Kirk *et al.*, 2008).

D. Deprotenisation of Aquatic waste

A loop full of fungal spores (*A. fumigatus*) were inoculated into the mineral salt medium for production of protease. Incubation was carried out at 30^o C for 5 days in a shaker incubator operated at 150 rpm. After incubation the production medium was centrifuged at 12,000 rpm for 15 minutes to separate the cells. The supernatant was collected as it contained the crude enzyme and stored at 4^0 C until further use (Oseni, 2011). The protease activity was assayed by the method of Lovrien *et al.*, (2010). Shell waste homogenate (100g) was prepared with 180 ml distilled water. The supernatant contain crude protease enzyme was mixed with Shell waste and incubated for 72h at 37^0C. The mixture was centrifuged at 3000 rpm for 5min. The recovered solids were washed thoroughly several times using deionized-distilled water then dried in hot air oven to obtain solid cake (Khanafari *et al.*, 2007).

E. Demineralisation of deprotenised shells

A loop full of fungal spores was inoculated in to Czapek-Dox broth medium and incubated for five days for the production of acid. The fungal isolate (*A .fumigatus*) was further assessed for quantitative acid production. The total acidity of the culture filtrates was

estimated by titration method, by taking 10ml of fermented broth against 0.1N NaOH (standard alkaline solution) using phenolphthalein as indicator (Peppler, 1967; El-Ktatney, 1978). The Supernatant was collected and added to the deprotenised shells were placed in the beaker. The beakers were kept for 24 hours with constant stirring at 40°C. The mixture was centrifuged at 3000 rpm for 5min. The filtrate was then dried in oven at 60º C to obtain chitin. This chitin is subjected to deacetylation further.

F. Deacetylation of chitin

Spore suspension of the fungal strain was inoculated in 50 mL CDA production medium and incubated at 30°C and 200 rpm for 96 hr. The culture sample was aseptically collected, centrifugation was done at 10,000 rpm for 10 min at 4°C, and the supernatant was used for the CDA assay. Acetate released by the action of CDA on its substrate, hexa-N-acetylchitohexaose was determined by a modified hydroxamate assay (Pareek *et al.*, 2011). The Supernatant was collected and added to the Chitin and kept for 24 hours with constant stirring at 40°C. The mixture was centrifuged at 3000 rpm for 5min. The filtrate was then dried in oven at 60º C to obtain chitosan.

2.4. Yield of Chitosan

The chitosan yield (%) was calculated as the dry weight of the chitosan flakes relative to the wet weight of shell waste (Nouri *et al.*, 2015).

Chitosan extraction yield (%) =
Dried extracted chitosan weight (g)/Shell waste (g) x 100%

2.5. Proximate analysis

Proximate analysis of extracted chitosan was carried out to determine moisture content, ash content, protein and lipid content. The samples were dried to a constant weight at 60 º C in an oven and the weight loss gives the amount of moisture in the samples. Samples were burned in a furnace at temperature of 555ºC and weighed to determine the ash content. The lipid and protein content were determined by standard method (AOAC, 2009).

2.6. Degree of deacetylation

Degree of deacetylation in chitosan was determined by potentiometric titration. A homogenous chitosan solution was prepared by using HCl containing 0.01 M/L and it was titrated against 0.1 M NaOH. The end point was detected by the inflection of the pH values. Two inflections were mainly noted out of which first one corresponds to neutralization of HCl and second one to the neutralization of ammonium ions for chitosan chain. The difference between two points give the amount of the amino group in the chitosan chain degree of deacetylation (DOD) (Zhanga *et al.*, 2010).

2.7. Fourier Transform-Infra Red spectroscopy (FT-IR)

Extracted chitosan sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare a salt disc. The FT-IR spectra were taken on an (FT-IR-8300 instrument (Shimadzu) (Szymanska-Chargot and Zdunek, 2013) accessory in the 400 to 4000cm-1 and repeated for three replicates. Standard chitosan was obtained from Himedia, Mumbai.

2.8. Statistical analysis

The tabulated values were analyzed by using SPSS 11.5 Computer based software programme.

3. Results and Discussion

3.1. Chemical extraction of chitin and chitosan

A total of three aquatic bio-waste like shrimp shell, fish scales and crab shell were selected for the present study for the extraction of chitin and chitosan. The yield of chitin/ chitosan using chemical extraction method was shown in Table 3.1. Among the three aquatic biowaste materials selected, maximum quantity of chitin (459.66±25.25mg/gm) and chitosan (418.33±28.32mg/gm) were obtained from shrimp shell waste whereas minimum quantity of chitin (305.00±23.28mg/gm) and chitosan (268.33±25.17mg/gm) were obtained from fish scales. The crab shell has given reasonably good production of chitin (380.16±24.21mg/gm) and chitosan (315.00±22.57mg/gm) than fish scales.

3.2. Biological extraction of chitin and chitosan

A total of 10 fungal isolates were obtained from the marine water samples from various places mentioned from Nellore coast. All the isolates were screened for the production of protease, organic acid and chitin deacetylase production (Plate 3.1 and 3.2). Protease producing fungi was identified as *Aspergillus niger* (VSM5) isolated from Mypadu coast. Organic acid producing fungi was identified as *Aspergillus terrus* (VSM1) isolated from Mypadu coast. Chitin deacetylase enzyme producing fungi was identified as *Aspergillus flavus* (VSK2) isolated from Koduru coast. The culture filtrates were used as enzyme source in the process of extraction of chitin and chitosan at deproteinisation, demineralisation and deacetylation steps. Among the three aquatic biowaste selected i.e shrimp shell, fish scales and crab shell, maximum chitin and chitosan were produced from shrimp shell through biological extraction. Among the three aquatic biowaste selected, maximum quantity of chitin (474.66±25.02 mg/gm) and chitosan (441.00±26.52 mg/gm) were obtained from shrimp shell whereas minimum quantity of chitin (307.33±22.14 mg/gm) and chitosan (285.00±21.21 mg/gm) were obtained from fish scales. The crab shell has given reasonably good production of chitin (458.33±23.43 mg/gm) and chitosan (426.66±23.83 mg/gm) than fish scales (Table 3.1).

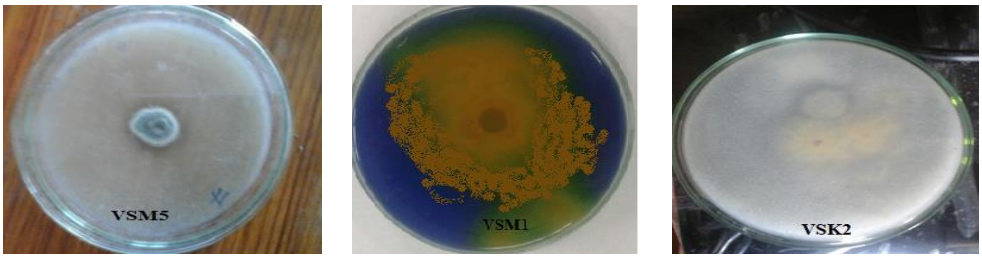

Plate 3.1. Screening for protease, organic acid and chitin deacetylase producing fungi on different media

Plate 3.2. Lactophenol cotton blue mounts of screened fungi

VSM5 = *A. niger* isolated from Mypadu coast,VSM1= *A. terrus* isolated from Mypadu coast and VSK2= *A. flavus* isolated from Koduru coast

Table 3.1. Amount of chitin/chitosan from different aquatic bio waste using chemical and biological extraction

Source of bio waste	Chitin (mg/gm) (Mean±SD)		Chitosan (mg/gm) (Mean±SD)	
	Chemical method	Biological method	Chemical method	Biological method
Shrimp shell	459.66±25.25	474.66±25.02	418.33±28.32	441.00±26.52
Fish scales	305.00±23.28	307.33±22.14	268.33±25.17	285.00±21.21
Crab shell	380.16±24.21	458.33±23.43	315.00±22.57	426.66±23.83

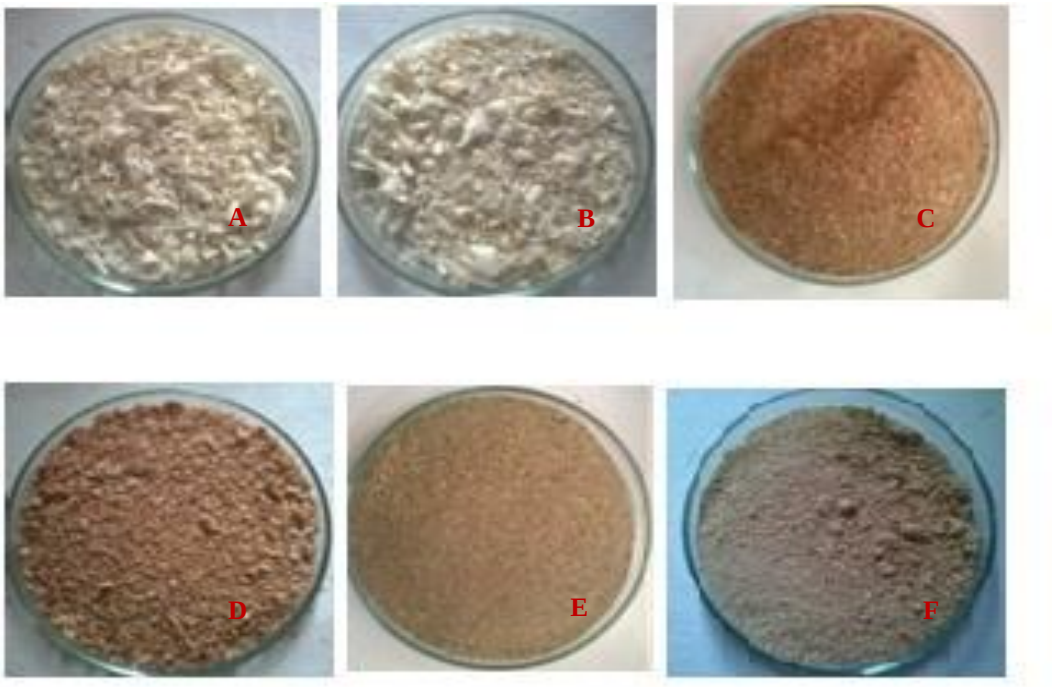

Plate 3.3. Chitosan from different sources extracted by chemical/ biological method

A- Chitosan extracted from fish scales (Chemical), B-Chitosan extracted from fish scales (Biological), C-Chitosan extracted from crab shell waste (Chemical), D-Chitosan extracted from crab shell waste (Biological), E-Chitosan extracted from shrimp waste (Chemical), F-Chitosan extracted from shrimp waste (Biological)

3.3. Proximate analysis

Both chemically derived and biologically derived chitosan samples were subjected for proximate analysis and the results obtained were presented in Table 3.2. In chemical extraction, the ash content was 1.4±0.05% (shrimp shell), 1.8±0.02% (fish scales) and 1.6±0.03% (crab shell). In biological extraction the ash content was 1.2±0.02% (shrimp shell), 1.7±0.01% (fish scales) and 1.4±0.05% (crab shell).The ash content is relatively low (1.2±0.02%) in chitosan isolated from shrimp biowaste using biological extraction than in the chemical extraction (1.4±0.05%). Similarly more ash content was seen in chitosan extracted chemically from fish scales (1.8±0.02%) followed by chitosan from crab shell by same method (1.6±0.03%). The moisture content of chemically extracted chitosan from shrimp waste, fish scales and crab waste was 2.0±0.06%, 3.1±0.09% and 4.0±0.09% respectively. Moisture content of biological extracted chitosan from shrimp shell, fish scales and crab was 1.7±0.08%, 3.0±0.07% and 3.4±0.07% respectively. The moisture content was found to be relatively low in chitosan extracted from shrimp shell by both methods and is relatively high

in chemically extracted chitosan (2.0±0.06%) compare to biologically derived chitosan (1.7±0.08%). High moisture content was observed in crab chitosan derived by chemical extraction (4.0±0.09%) followed by biological extraction (3.4±0.07%).

The fat content is high in chitosan from crab shell (4.7±0.06 %) than the fish scales (4.2±0.05 %) followed by chitosan from shrimp shell (3.4±0.08%). The fat content in shrimp shell isolated by chemical extraction is 3.4±0.08% which is more when compared with the chitosan extracted biologically that is 3.2±0.09% from the same source. There is no considerable difference in the fat contents of fish scale isolated chitosan from both the methods. The protein content present in chemically extracted chitosan is 7.9±0.51% (shrimp shell), 8.8±0.31% (fish scales) and 8.1±0.62% (crab shell**)**. In biological extraction the protein content found to be 7.9±0.44% (shrimp shell), 8.4±0.25% (fish scales) and 8.0±0.54% (crab shell). The protein content is relatively low in chitosan isolated from shrimp biowaste using biological extraction than in the chemical extraction. Similarly more protein content was seen in chitosan extracted chemically from fish scales followed by chitosan from crab shell by same method. The biologically extracted shrimp chitosan showed superior quality with DD value 70±1.79% than fish scales (67±2.34%) and crab shell (62±2.01%).

Table 3.2. Proximate analysis of chitosan extracted from different sources using chemical and biological methods

Constituents	Chemically extracted chitosan			Biologically extracted chitosan		
	Shrimp shell	Fish scales	Crab shell	Shrimp shell	Fish scales	Crab shell
Ash content (%) (Mean ± SD)	1.4±0.05	1.8±0.02	1.6±0.03	1.2±0.02	1.7±0.01	1.4±0.05
Moisture content (%) (Mean ± SD)	2.0±0.06	3.1±0.09	4.0±0.09	1.7±0.08	3.0±0.07	3.4±0.07
Fat content (%) (Mean ± SD)	3.4±0.08	4.2±0.05	4.7±0.06	3.2±0.09	4.0±0.04	4.5±0.08
Protein (%) (Mean ± SD)	7.9±0.51	8.8±0.31	8.1±0.62	7.9±0.44	8.4±0.25	8.0±0.54
Degree of deacetylation (%) (Mean ± SD)	65±2.25	61±2.22	59±1.89	70±1.79	67±2.34	62±2.01

3.4 Characterization of chitosan by FTIR analysis

In the present study biologically derived shrimp chitosan showed superior quality than chitosan extracted from crab and fish scales (Fig. 3.1 and 3.2). FTIR spectrum of biologically extracted chitosan from shrimp waste showed peaks at 3418cm^{-1} that indicated stretching vibration of - hydroxyl group, -NH_2 group of amines and hydrogen bonding which was comparable to spectrum peak of standard, i.e., 3420cm^{-1}. 1646cm^{-1} peak in extracted shrimp chitosan indicated the vibrations of carbonyl group (amide band I) and standard had this peak at 1654cm^{-1}. As the deacetylation process occurred, there was a variation in the intensity of carbonyl group at 1655/cm^{-1} and amide band peak at 3449/cm^{-1}.

Glycosidic linkage indicated by peak at 1151/cm^{-1} in shrimp chitosan which overlapped with standard chitosan at 1155/cm^{-1}. The presence of CH_3,CH_2 and CH groups as well as the primary and secondary-OH groups which are attached to the pyranose ring are represented by the spectra between 1422/cm^{-1} and 603/cm^{-1} .The presence of the entire band stretching in the extracted chitosan compared with standard band stretching depicts that extracted material was chitosan. The FT-IR analysis also confirmed that the product formed after biological extraction was chitosan.

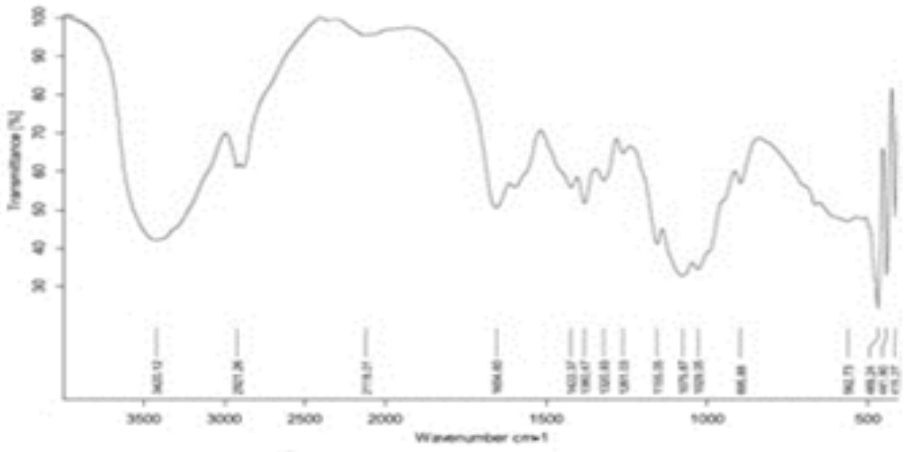

Figure 3.1. FTIR spectrum of standard chitosan (Himedia)

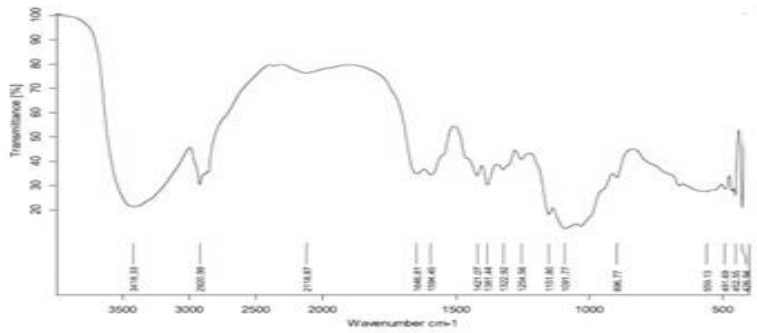

Figure 3.2. FTIR spectrum of shrimp chitosan (biologically extracted)

4. Conclusion

The shell fish processing industry generates a huge amount of shell waste which usually cause environmental nuisance. Alternatively this waste can be utilized as an economic source of chitin and its derivative chitosan. It is a good waste management practice leading to additional economic benefit by producing valuable biopolymers, for upliftment of socioeconomic status of coastal people. In addition, chitin and chitosan are biodegradable products therefore, it helps to maintain the environmental sustainability. Further, there is a tremendous scope of enhancing the functional properties of chitosan extracted particularly from crustacean waste. Biological and chemical methods are mainly employed for the production of chitin and chitosan. In this study an attempt is made to introduce a complete

biological method for the extraction of chitin by using proteolytic marine fungi and chitosan through deacylation by using enzymes obtained from marine fungi. This is an eco-friendly technology and is a viable alternate for extraction method for bio-polymer from crustacean shell. The obtained chitosan had high deacetylation degree (DD),which has greater scope in various applications such as agriculture and horticulture, water and wastewater treatment, food industry and other industrial uses.

Conflicts of interest: There is no conflict of interest of any kind.

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