OPTIMIZATION OF PROCESS CONDITIONS FOR THE PREPARATION OF CHITOSAN FROM SHRIMP SHELL

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Abstract

Natural polymers viz., chitin and its deacetylated derivative form, chitosan consist of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitin and chitosan were prepared from shrimp shell by demineralization, deproteinization and deacetylation. Effects of demineralization, deproteinization and deacetylation conditions on their preparations were studied. The X-ray Diffraction (XRD) method was used to study the correlation between degree of deacetylation (DD) and crystallinity of the extracted chitin and chitosan. The functional groups and the effect of deacetylation of the extracted samples were studied by Fourier Transform Infrared (FTIR) spectroscopy. The viscosity average molecular weight of chitosan was determined depending on the different deacetylation time. The changes in surface morphology between the extracted chitin and deacetylated chitosan were studied by SEM techniques.

Keywords: chitosan, degree of deacetylation, crystallinity, functional groups, SEM

Introduction

After cellulose, chitin and chitosan are the second most available biopolymer. They are sourced mainly from exoskeleton of crustaceans such as crab and shrimp, but are also available from other sources such as fungi and some insect's wing (Al-Sagheer *et al.*, 2009). Shrimp shells are necessary first to be converted into chitin. Generally, extraction of chitin from shrimp shells consists of three steps, including demineralization, deproteinization and decolorization. After that, chitin can be converted into chitosan by deacetylation process which partially removed the acetyl group from the molecular chain of chitin. Chitin is widely distributed natural biopolymer composed of N-acetyl-D-glucosamine (GluNAc) subunits linked with β-1,4-glucosidic linkage. Chitosan is a nontoxic, deacetylated product of chitin, which is a linear heteropolysaccharide and contains N-acetyl-D-glucosamine, same linkages as in chitin (Shahidi & Abuzaytoun, 2005).

Chitosan contains the reactive amino group at C-2 and the hydroxyl group at C-3 and C-6. This chitosan is widely used in various industries such as pharmaceutical, biochemistry, biotechnology, cosmetics, biomedicine and paper industry. Beside that, chitosan can be used as an emulsifier, coagulant, chelating agent, and thickener emulsion. In addition, chitosan can be used as a substitute for formalin to preserve food that serves a relatively safe for consumption (Muzzarelli, 1985).

The use of chitin and chitosan in various fields depends on quality characteristics such as degree of deacetylation, solubility, viscosity and molecular weight. Quality of chitosan is mainly determined by the degree of deacetylation, where the deacetylation degree is dependent on materials and conditions process such as concentration of alkali solution, temperature, and time (Suhardi, 1993).

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In Myanmar, crustacean shell wastes are abundantly produced annually and these wastes create the environmental pollution problem in the form of bad odor and aesthetically damage the environment as well as health problem. By utilizing these wastes as value added products, the problems could be reduced. The objective of the present study was to obtain the processing conditions of demineralization, deproteinization and deacetylation for chitin and chitosan from shrimp shell waste.

Materials and Methods

Materials

Shrimp

Family : Penaeidae
Genus : *Penaeus*
Scientific Name : *Penaeus Monodon* (Fabricius, 1978)
Common Name : Puzun Kyar
Local Name : Giant Tiger Prawn (or) Asian Tiger Shrimp

(Giant Tiger Prawn)

Shrimp shells were collected from Yuzana Fishery Co., Ltd. Kwin Kyaung Street, Ahlone Township, Yangon Region. Required chemicals such as hydrochloric acid (specific gravity 1.16), sodium hydroxide and ethanol (Analar Grade) were purchased from Super Shell Chemical Shop, 27th Street, Pabedan Township, Yangon Region.

Methods

Extraction of Chitin from Shrimp Shell

About (100) g of shrimp shell powder was demineralized with 1 M of hydrochloric acid solution with shell powder to hydrochloric acid solution ratio (1:20 w/v) at ambient temperature (30 ± 4ºC). The mixture was continuously stirred by a 1.5-inch oblong stir-bar on a magnetic stirrer for 18 hr. Then, the solid and liquid portions were separated by filtration and the solid portion was washed with distilled water until neutral pH was achieved. The solid portion was dried at 60ºC for 6 hr. The dried solid portion was deproteinized with 2 M sodium hydroxide solution with shell powder to sodium hydroxide solution ratio (1:10 w/v) at 95ºC for 2 hr with constant stirring. Then, the deproteinized shell was washed with distilled water until neutral pH was obtained. The alkaline treatment was repeated four times. After neutralization of deproteinized shell, they were dried at 60ºC for 4 hr. Then, the crude chitin was purified with ethanol (1:10 w/v) for 30 min and dried at 50ºC for 3 hr. Then, the chitin was obtained.

Preparation of Chitosan

The chitin (100) g was deacetylated with 50 % (w/v) NaOH solution at 90ºC with a chitin to NaOH solution ratio (1:10 w/v) for 11 hr by refluxing. Then, the residue was washed with distilled water (70 ºC) until neutral pH was achieved. Finally, the obtained chitosan was dried at 60ºC for 3 hr.

Figure 1 Chitin from Shrimp Shell

Figure 2 Chitosan from Shrimp Shell

Characterization of Chitin and Chitosan

Determination of Moisture Content (AOAC 2011, 930.15)

About (3) g of sample was weighed accurately in a clean, dry and previously weighed moisture dish. The sample was dried at 105ºC until constant weight was obtained. After the drying was completed, sample was removed from the oven and placed in a desiccator for about 30 min and weighed accurately and the moisture content was calculated by equation (1).

$$\text{Moisture}(\%) = \frac{(B - C)}{A} \times 100 \qquad (1)$$

Where, A = sample weight in gram, B = weight of dish + sample prior to drying, C = weight of dish + sample after drying.

Determination of Ash Content (AOAC 2011, 942.05)

About (3) g of sample was weighed in a clean and previously weighed porcelain crucible and burnt in a muffle furnace at 600ºC for 6 hr. The crucible containing the ash was cooled in a desiccator and weighed. Heating, cooling and weighing were repeated until a constant weight was obtained. According to the equation (2), the total ash content was calculated as follows:

$$\text{Ash}\,(\%) = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100 \qquad (2)$$

Determination of Degree of Deacetylation of Chitosan (Sabins and Block, 2000)

2 g of chitosan sample was completely dissolved in 100 cm^3 of freshly prepared 0.2 M HCl solution and 100 cm^3 of concentrated HCl was then added to the homogeneous chitosan solution with vigorous stirring to precipitate the hydrochloride salt. The resultant solution was centrifuged for 15 min and the supernatant was discarded. The chitosan hydrochloride salt was then filtered off and washed several times with methanol. Residual moisture in the chitosan hydrochloride salt was removed by stirring for 6 hr in acetone. Then, the precipitate was dried in a vacuum desiccator for 12 hr to obtain white chitosan chloride salt.

The resulting salt was divided into two portions; one portion was used for the determination of moisture content, while the other was used for titration. An accurately weighed (approximately 0.2 g) chitosan chloride salt was dissolved in distilled water, volume made up to 100 cm^3 in the volumetric flask. The resulting solution 25 cm^3 was titrated against 0.1 M standard NaOH solution by using phenolphthalein as an indicator. The degree of deacetylation of chitosan products were calculated by equation (3).

$$DD(\%) = \frac{M_1 V_1}{1000} \times \frac{V_0}{V_2} \times MW \times \frac{100}{W(1 - \%MC/100)} \quad (3)$$

Where,
M_1 = molarity of NaOH solution
V_1 = volume of titrated NaOH
V_0 = total volume of chitosan solution
V_2 = volume of titrated chitosan solution
MW = molecular weight of one monomer unit of chitosan
W = weight of chitosan
%MC = moisture content

Determination of Molecular Weight of Chitosan (Sabins and Block, 2000)

2 g of chitosan sample was dissolved in 0.1 M acetic acid solution containing 0.2 M sodium hydroxide solution and stirred. Dilution was made using this homogeneous solution to obtain different concentrations of chitosan solution (0.002 to 0.01 %). Different concentrations of chitosan solution were filled in the viscometer by placing pipetting bulb over the capillary arm, the chitosan solutions were drawn up to a point above the upper mark. The bulb was removed and the time taken for the lowest point of the meniscus to pass from the upper to the lower mark was recorded. The procedure was repeated three times for each chitosan sample. The efflux times of the solvent and prepared chitosan solutions were also determined by stop watch. Molecular weight of chitosan was calculated by using Mark-Houwink-Sakurada equation.

$$[\eta] = K M_v^{\ a} \quad (4)$$

Where,
$[\eta]$ = intrinsic viscosity
K = constant (1.81×10^{-3} dLg^{-1})
a = constant (0.93)
M_v = viscosity average molecular weight (Da)
t_0 = flow time of solvent
t = flow time of solution

* K and a are constants for given solute-solvent system and temperature.

Determination of Solubility of Chitosan **(Fernandez-Kim, 1991)**

The solubility of the chitosan sample was determined according to the reported procedure. 0.1 g of chitosan powder in triplicate was placed in a centrifuge tube and then dissolved with 10 ml of 1% (v/v) acetic acid for 30 min using an incubator shaker operating at 240 rpm and 25ºC. The chitosan solution was then immersed in a boiling water bath for 10 min, cooled down to room temperature and centrifuged at 10,000 rpm for 10 min and the supernatant was decanted. The undissolved particles were washed with distilled water (25 ml) and the centrifugation process was repeated. The supernatant liquid was removed and undissolved particles were dried at 60ºC for 24 hr. Then, the particles were weighed and the percentage solubility was determined using following equation.

$$\text{Solubility}(\%) = (A - B) \times (A - C) \times 100 \quad (5)$$

Where,
A = Initial weight of the centrifuge tube with chitosan (g)
B = Final weight of the centrifuge tube with chitosan (g)
C = Initial weight of the centrifuge tube (g)

X-ray Diffraction (XRD)

X-ray diffraction is a very powerful, nondestructive tool for analyzing materials and a variety of information can be deduced from the obtained diffraction pattern. The X-ray diffraction analysis was applied to detect the crystallinity of the extracted chitin and chitosan and their patterns were recorded in the 2θ range of 5º to 70º using RIGAKU, Smart Lab X-ray Diffractometer with CuK_α radiation, λ = 1.54056 Å at the Universities' Research Center, University of Yangon. The crystallinity index (CrI) was obtained by using the following equation:

$$\text{CrI}\ (\%) = \frac{I_{max} - I_{am}}{I_{max}} \times 100\% \qquad (6)$$

where I_{max} is the maximum intensity of the (110) diffraction peak at $2\theta \approx 20°$ and I_{am} is that of the amorphous diffraction signal at $2\theta \approx 16°$.

Fourier-Transform Infrared (FTIR) Spectroscopy

To study the effect of deacetylation and the various functional groups contained in chitin and chitosan, the FTIR spectra were recorded at room temperature using PC-controlled SHIMADZU FTIR-8400 Spectrophotometer at the Universities' Research Center, University of Yangon.

Morphological Analysis by SEM

The change in morphology from chitin to chitosan was investigated by using JEOL JSM-5610LV SEM at Universities' Research Centre, University of Yangon.

Results and Discussion

The physico-chemical characteristics of shrimp shell used in this research are shown in Table (1). The calcium carbonate content of shrimp shell was 47.15 % (w/w) which closely matched with the literature value. The protein content of shrimp shell was 25.25 % (w/w) while the literature value was 30-40 %. The results in Table (2) indicate that 1 M hydrochloric acid concentration was adequate for demineralization of shrimp shell. It was noted that if hydrochloric acid concentration was higher than 1 M, the ash content was slightly decreased. The ash content indicates the effectiveness of demineralization process. The deproteinization ability of shrimp shell was studied by using different concentrations of sodium hydroxide and it is shown in Table (3). The total nitrogen of chitin from shrimp shell by using different alkali treatment varied from 6.30 - 6.36 % (w/w), respectively. According to AOAC 1975, protein nitrogen is the difference of total nitrogen and chitin nitrogen. Then, it was found that the more alkali concentration was used, the less protein content was found. For the deacetylation process, it was observed that the less protein content in chitin was more effective than the more protein content. The less protein contents in chitin obtained from 2 M and 2.5 M were the 0.56 and 0.50 % (w/w), respectively. From the economic point of view, 2 M was chosen as the suitable deproteinization concentration because the two values were not significantly different and matched with the literature ("marine-bio-resources / CHITIN & CHITOSAN", n.d.).

Table 1 Physico-chemical Characteristics of Shrimp Shell Powder (- 32 mesh)

Sr. No	Composition	Shrimp Shell	Literature Value # (Crustacean Shells)
1	Moisture (% w/w)	10.97 ± 0.01	-
2	Ash (% w/w)	48.9 ± 0.1	-
3	Protein (% w/w)	25.25	30-40
4	$CaCO_3$ (% w/w)	47.15 ± 0.01	30-50
5	Nitrogen Content (% w/w)	4.04	-

(Knorr, 1984)

Table 2 Effect of Hydrochloric Acid Concentration on the Demineralization of Shrimp Shell Powder (- 32 mesh)

Weight of Sample = 100 g
Demineralization Time = 24 hr
Demineralization Temperature = Ambient Temperature (30 ± 4ºC)
Weight of Shell and HCl Solution Ratio = 1:20 (w/v)

Sr. No	HCl (Conc:) (M)	Ash (% w/w)	$CaCO_3$ (% w/w)
1	0.5	20.38±0.02	17.73±0.1
2	1.0*	0.98±0.01	ND
3	1.5	0.97±0.02	ND
4	2.0	0.97±0.01	ND
5	2.5	0.97±0.02	ND

*Most suitable condition, ND = Not Detected

Table (4) shows the effect of deacetylation time on the physico-chemical properties of chitin. It was observed that 11 hr and 13 hr gave the higher deacetylation degree with 50 % (w/v) sodium hydroxide solution at 90ºC. In addition, it can be seen that the increasing of the deacetylation degree decreases the molecular weight.

Table 3 Effect of Sodium Hydroxide Concentration on the Deproteinization of Demineralized Shrimp Shell Powder (-32 mesh)

Weight of Sample = 100 g
Deproteinization Temperature = 65ºC
Deproteinization Time = 2 hr
Weight of Shell and NaOH Solution Ratio = 1:10 (w/v)

Sr. No	NaOH (Conc:) (M)	Total Nitrogen (% w/w)	Chitin Nitrogen (% w/w)	Residual Protein (%w/w)
1	0.5	6.36	6.18	1.13
2	1.0	6.33	6.20	0.81
3	1.5	6.31	6.21	0.63
4	2.0*	6.30	6.21	0.56
5	2.5	6.30	6.22	0.50

*Most suitable condition
Protein Nitrogen = Total Nitrogen – Chitin Nitrogen
Protein Content = Protein Nitrogen × 6.25

Table 4 Effect of Deacetylation Time on the Properties of Chitosan

Weight of Chitin = 100 g Deacetylation Temperature = 90ºC
NaOH Concentration = 50 % (w/v) Chitin and 50 % (w/v) NaOH Solution Ratio = 1:10 (w/v)

Sr. No	Time (hr)	Properties	
		DD (%)	M_v (Da)
1	5	83.94 ±0.4	1.01×10^6
2	7	88.4 ± 0.3	6.04×10^5
3	9	93.05 ± 0	3.31×10^5
4	11*	96.67 ± 0.09	1.04×10^5
5	13	97.66 ± 0	1.04×10^5

*Most suitable condition
DD = Deacetylation Degree
M_v = Molecular Weight

The solubility of chitosan increases with decreases in molecular weight. In this research, the deacetylation degree in shrimp shell chitosan for 11 hr and 13 hr are 96.67 % and 97.66 %, respectively. Deacetylation degree and molecular weight are the critical factors for chitosan's quality. The chitosan's quality was increased with increasing deacetylation degree and solubility of chitosan in 1 % (w/v) acetic acid. In this research, the values of deacetylation degree and molecular weight obtained from deacetylation times (11 hr and 13 hr) were not significantly different. According to deacetylation degree and molecular weight, 11 hr was suitable deacetylation time. Table (5) shows the physico-chemical characteristics of prepared chitosan and commercial standard chitosan. It was found that the prepared chitosan was compared well with standard chitosan.

X-ray Diffraction Analysis

The XRD stacked pattern of the chitin and chitosan extracted form shrimp shells are shown in figure (3). The chitosan behaves the semi-crystalline structure because the characteristic peaks appeared at around 2θ values 10º and 20º corresponding to the crystallographic planes (020) and (110) and minor reflections at higher 2θ values. When compared with chitin, the diffraction angle's intensity of chitosan was weakened significantly. The crystallinity of the chitin was 80.24 % and this value was reduced to 60.88 % when the chitin was treated with strong alkali to prepare chitosan. The crystalline structure of chitin was destroyed and the crystallinity was decreased as a result of strong alkali treatment during deacetylation process.

Table 5 Physico-chemical Properties of Prepared Chitosan

Sr. No	Characteristics	Experimental Value (Shrimp Shell)	Literature Value# (Shrimp Shell)
1	Colour	White	White or Buff
2	Appearance	Powder	Powder
3	Moisture (% w/w)	2.97 ± 0.03	< 10
4	Ash (% w/w)	0.32 ± 0.05	< 0.5
5	Deacetylation Degree (%)	96.67 ± 0.09	-
6	Molecular Weight (Da)	1.04×10^5	-
7	Crystallinity Index (%)	60.88	-
8	Solubility in 1 % (w/v) acetic acid solution	100 ± 0	-
9	Yield (%)	14.5	20-30

(ZHANG *et al*., 2011)

FITR Spectroscopic Analysis

Figure (4) shows FTIR stacked spectra of chitin and chitosan extracted from shrimp shell wastes. In the FTIR spectrum of extracted chitin, absorption peaks at 3443.05 and 3261.74 cm^{-1} represent the stretching vibrations of the –OH and $–NH_2$ groups. The most important signals in the spectrum of chitin, peaks of amide bands I, II, III were observed at 1662.69, 1558.54, 1315.50 cm^{-1} in shrimp chitin which compared well with the amide peaks at 1668.66, 1558.51, 1314.40 cm^{-1} reported by Varun *et al*., 2017. NH– stretching peak was also found in the extracted chitin at 3105.50 cm^{-1}. The absorption peaks appeared at 1074.39 and 1020.38 cm^{-1} in both spectra are due to the stretching vibrations of –C–O–C– of the glucosamine ring. The peak appeared at 898.86 cm^{-1} is ring stretching a characteristic band for β-1, 4 glycosidic bonds.

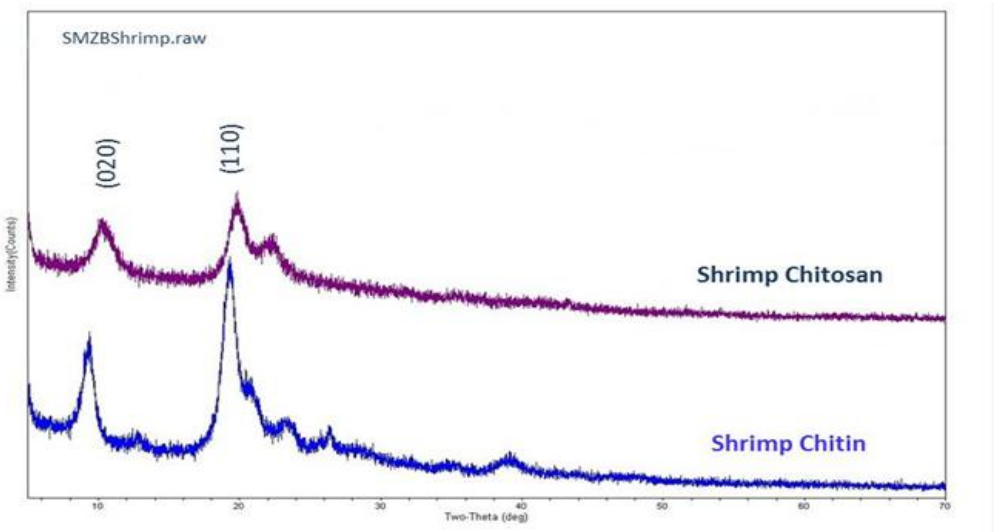


Figure 3 XRD Stacked Pattern of Prepared Chitin and Chitosan Extracted from Shrimp Shell

In the FTIR spectrum of extracted chitosan, stretching vibration of –OH group, $–NH_2$ group and hydrogen bonding showed peak at 3385.18 cm^{-1} and peak of amide I bond was indicated at 1656.91 cm^{-1}. Peak at 1591.33 cm^{-1} in extracted chitosan showed the presence of bending vibration of amide band II (N–H). The vibrational characteristics of CH_3 group of $NHCOCH_3$ (amide bond) was pointed at 1383.01 cm^{-1}. The absorption peaks appeared at 1151.54 cm^{-1} and 895.00 cm^{-1} were due to the stretching vibrations of oxygen in glycosidic linkage and Pyranose ring, respectively. Dahmane *et al*., (2014) reported that after the deacetylation of chitin, disappearance of peak 1556 cm^{-1} and appearance of new peak occurred at 1595 cm^{-1} which represents $–NH_2$ bending. Compared with the present research, the same process was found with disappearance of absorption peak at 1558.54 cm^{-1} (as in chitin) and appearance of new peak at 1591.33 cm^{-1} in chitosan and it can be seen in stacked FTIR spectra of chitin and chitosan. So, it can be said that the process of deacetylation was successfully performed in the present study. The characteristic of the asymmetric bridge O_2 stretching of the glycosidic linkage was indicated by the absorption peak appeared at 1151.54 cm^{-1}. The existence of CH_3, CH_2 and CH groups as well as the primary and secondary –OH groups attached to the Pyranose ring, is represented by the spectra between 1422 and 603 cm^{-1}.

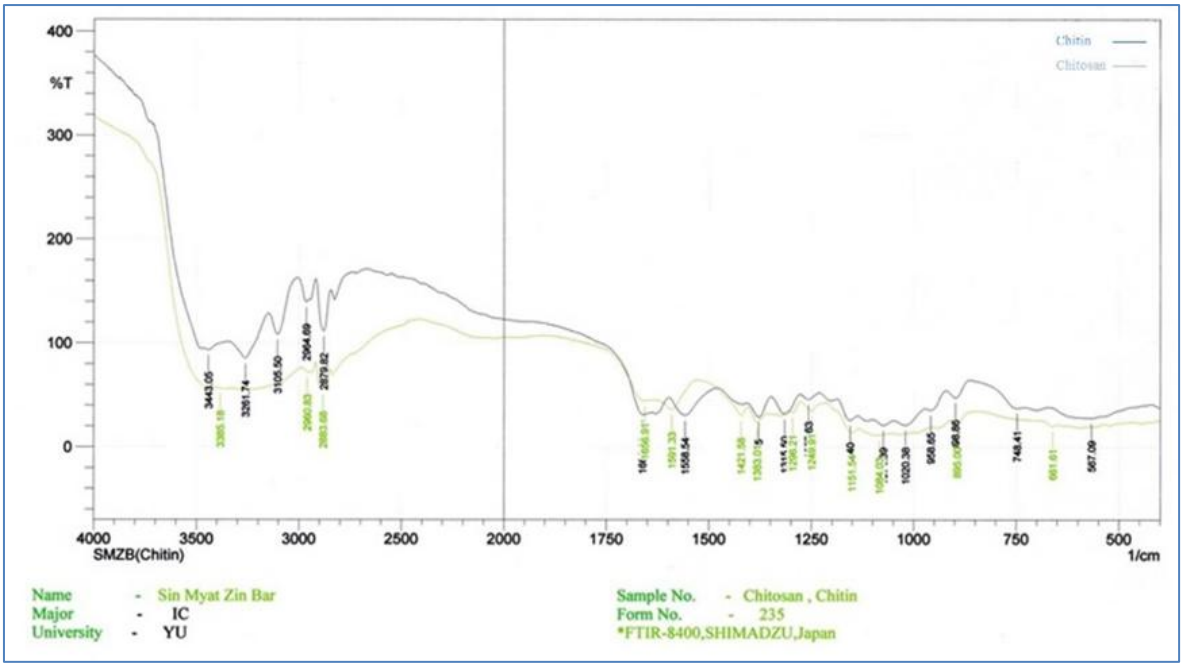
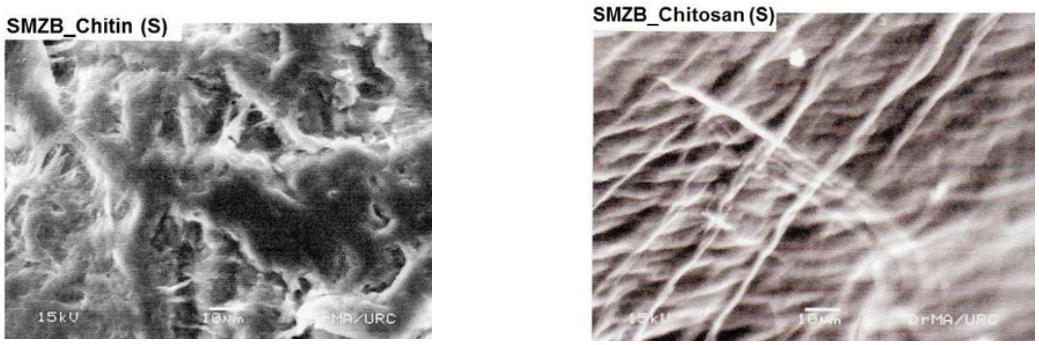


Figure 4 FTIR Stacked Spectra of Chitin and Chitosan Extracted from Shrimp Shell

Scanning Electron Microscopic Analysis

The surface morphology of demineralized chitin characterized by SEM is shown in Figure 5(a). The SEM micrograph of the chitin extracted from shrimp shell showed the smooth surface with some straps and shrinkages. The smoother microanalysis showed the disappearance of calcium carbonate after the process of demineralization with hydrochloric acid. Figure 5(b) showed the SEM micrograph of chitosan prepared from shrimp shells. In this microstructure, it was clearly seen the lesser microfibrillar structure with rough surface morphology than in chitin. Moreover, the microstructure of shrimp chitosan behaves no smoothing surface with straps and shrinkages. Rough microcrystalline surface of chitosan was due to the treatment of chitin with a stronger sodium hydroxide concentration on deacetylation reaction. So, it can be assumed that the deformation of the acetylated structure is associated with the differences in the morphology between chitin and chitosan of the obtained microstructures.



(a) (b)

Figure 5 SEM micrographs of (a) Shrimp Chitin (b) Shrimp Chitosan

Conclusion

In the present research, chitosan was successfully extracted from shrimp shell as a white powder with adequate physico-chemical properties such as ash content 0.32 % and completely soluble in 1 % (w/v) acetic acid. The deacetylation degree obtained was about 96.67 % and the crystallinity index was 60.88 %. The molecular weight and yield percent of the extracted chitosan were 1.04×10^5 Da and 14.5 % (w/w), respectively. Optimum values for chitin production were found to be 1 M HCl for demineralization and 2 M NaOH for deproteinization processes. 11 hr deacetylation time was used as optimum condition for the deacetylation of the chitosan preparation.

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