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Degree of deacetylation of chitin in Sri Lankan prawn shells (*Penaeus semisulcatus*) under different treatment methods

Nayani Wanni Arachchi, Marliya Ismail, Sanja Gunawardena
Department of Chemical and Process Engineering, University of Moratuwa, Sri Lanka
Corresponding Author: Sanja Gunawardena

----------ABSTRACT----------
Chitin and chitosan are renewable natural biopolymers having extensive applications in food and many other industries due to their low toxicity, biocompatibility, biodegradability, etc. The main industrial raw material for chitin production is waste from seafood industries that consist of exoskeleton remains of crustaceans.
Isolation of chitin from prawn shells involved demineralization, deproteinization and purification processes. Chitin was converted to chitosan through deacetylation using 45% NaOH by different physical treatments; steeping at ambient temperature, thermal heating and microwave radiation. Chitosan produced from chitin was characterized using FTIR analysis.
Prawn shells contain mainly chitin, protein and minerals; the chitin content was approximately 22 %. Among the different physical treatments for deacetylation, conventional thermal heating and microwave radiation methods gave Degree of De-Acetylation (DDA)values in the range of 69 – 99 % whereas steeping at ambient conditions did not give significant DDA value even after 10 days. Microwave radiation was the most energy, time and cost-effective method for deacetylation as it reduced reaction time significantly. This study ultimately addressed a major concern in Sri Lankan seafood industry by adding value to a waste product.
KEYWORDS;-*Chitin, Chitosan, Degree of De-Acetylation*

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I. INTRODUCTION

Bio polymers are promising materials that possess important properties such as lack of toxicity, biocompatibility and biodegradability (Younes and Rinaudo. 2015; Gryczka et al, 2009). Over the last few years, considerable research has been conducted to develop and apply bio-based polymers made from a variety of waste materials from food and agro based industries. Such biopolymers include starches, cellulose derivatives, chitin/chitosan, gums, proteins (animal or plant-based) and lipids (Maria et al, 2009).

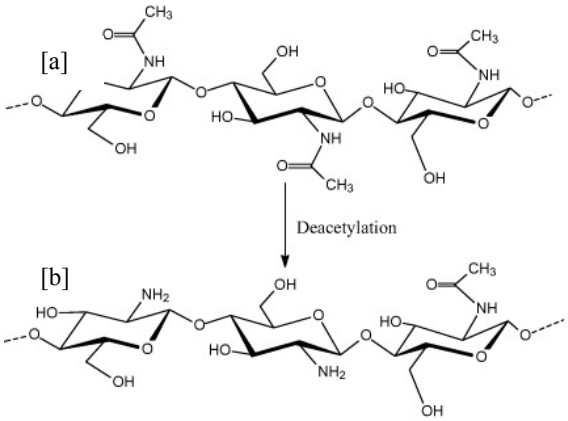

Figure 1. Structures of Chitin [a] and chitosan [b] (Dai Lam Tran et al 2011)

Chitin, (poly β-(1-4)-N-acetyl-D-glucosamine), next to cellulose, is the most abundant polysaccharide on earth. However, it still remains as an under-utilized resource mainly due to its compact polymeric structure (Kumar et al, 2000; Pillai et al., 2009). Therefore, chemical modification is required to alter its structure and thereby to enhance its applicability. Most common derivative of chitin is chitosan, derived by partial deacetylation

of chitin (Dutta et al., 2009; Maria et al., 2009; Weinhold et al., 2009). Both chitin and chitosan polymers have immense structural possibilities for chemical and mechanical modifications to generate novel properties, functions and applications (Al-Manhel et.al, 2016; Younes and Rinaudo. 2015; Pillai et al., 2009).

Deacetylation of chitin to chitosan is carried out in solid state under alkaline conditions using concentrated NaOH(Abdou et al, 2008; No & Meyer, 1989) or by enzymatic hydrolysis in the presence of chitin deacetylase (Jaworska, M., 2012).The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin leaving behind a complete amino group (NH2) as shown in Figure 1 (Baskar et al., 2009). Chitosan versatility depends mainly on this high degree of chemically reactive amino group (Clermont, 2005).

Degree of De-Acetylation (DDA) in chitosan is one of the most important chemical characteristics as it influences the performance of chitosan in many of its applications (He, et al., 2015; Liu et al., 2012; Baskar et al., 2009). It has been found to influence not only its physical, chemical properties and biological activities but also its biodegradability and immunological activities (Mohlous et al., 2007; Sagheer et al., 2009). It is therefore essential to characterize chitosan by determining its DDA prior to its utilization at the developmental stage (Baskar et al., 2009). Many researchers used FTIR methods to evaluate DDA and according to Brugnerotto et al (2001), the absorption ratio A1320/A1420 shows superior agreement between the absolute and estimated values of DDA.

Effectiveness of deacetylation process depends on the alkali concentration, temperature, reaction time and solid to solvent ratio (Mohlous et al., 2007). In this research, the effect of thermal treatment and consequently the temperature and the exposure time while keeping the concentration of NaOH constant on the degree of deacetylation was studied using steeping, thermal heating and microwave radiation.

II. MATERIALS AND METHODS

Raw Material Preparation

Flowery prawn heads (*Penaeus semisulcatus*) of marine species were obtained from a commercial prawn manufacturer in Sri Lanka. The prawn shells were separated from their heads and were washed with tap water and hot water to remove meat residues and other contaminants. Then they were dried in the oven (Memmert UFE 400) at 105°C to a constant weight. After drying, the shells were grounded and sieved to obtain particle size less than 250µm. To extract chitin, the resulted prawn shell powder was subjected to demineralization, deproteinization and purification as shown in Figure 2.

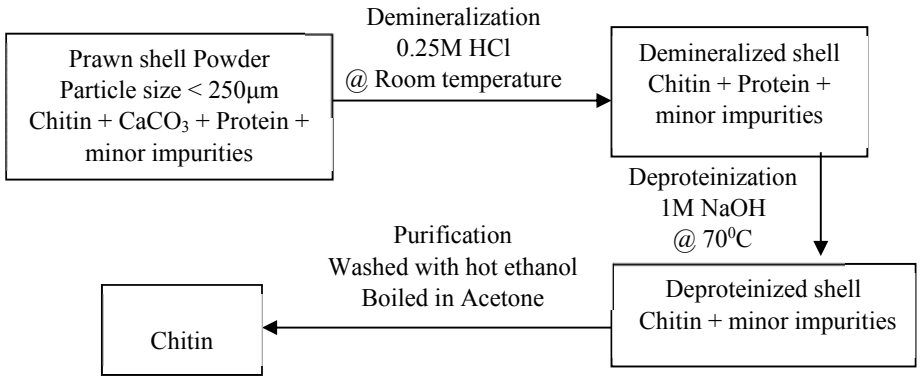

Figure 2: Isolation of chitin

Demineralization

Prawn shell powder was treated with 0.25 M HCl solution at ambient temperature with a solution-to-solid ratio of 40 ml/g to dissolve mineral in acid solution. The resulting solid was washed several times with distilled water until neutral to remove dissolved minerals and excess HCl. Then, the demineralized samples were dried in an oven at 105°C to a constant weight.

Deproteinization

Deproteinization of chitin was carried out using 1.0 M NaOH at 70°C with a solution-to-solid ratio of 20 ml/g solution. The treatment was repeated several times until the medium became colourless which indicate the absence of protein. The resulting solid was then washed to neutrality to remove dissolved proteins and excess NaOH. Then, the samples were dried at 105°C to a constant weight.

Purification

To remove other minor impurities present in the prawn shells, the resulting powder from the deproteinization process was washed with hot ethanol (10 ml/g) and then boiled in acetone (10 ml/g). Purified chitin was washed twice with distilled water and then dried in the oven at 105°C to a constant weight.

Determination of the Composition of Prawn Shells

Mineral, protein and chitin contents were determined from the weight differences of the raw materials and that of the resulted solids obtained after acid and alkaline treatments.

Preparation of Chitosan (Deacetylation)

Four different methods were used to prepare chitosan from chitin; steeping only (S), thermal heating (H), thermal heating followed by steeping (HS), and microwave radiation (MW).

i) **Steeping in 45% Sodium Hydroxide (S)**
Chitin was steeped in 45% NaOH solution (15 ml/g) at room temperature for different time intervals (2, 4 and 10 days).

ii) **Thermal Heating with 45% Sodium Hydroxide (H)**
Chitin was treated with 45% NaOH (15 ml/g) at 100 °C for 6, 5.5 and 5 h and the resulting products were referred to as H-6, H-5.5 and H-5.

iii) **Thermal Heating followed by Steeping with 45% Sodium Hydroxide (HS)**
Chitin was treated with 45% NaOH (15 ml/g) at 100 °C for 7 h followed by 16 h steeping in the same solution at room temperature. The resulting solid was referred to as H-7 S-16.

iv) **Microwave Radiation with 45% Sodium Hydroxide (MW)**
Chitin was treated with 45% NaOH (15 ml/g) and subjected to microwave radiation for 5 and 10 min at 60% and at 80% power level of 900 W microwave oven (Model LG MS-304A).

Then all these samples were washed with distilled water until neutral and were dried in the oven (Memmert UFE 400) at 105 °C until constant weight.

Degree of Deacetylation

Infrared spectrums of chitin and chitosan were measured over the frequency range 600–4000 cm^{-1} using FTIR spectrometer (Model: Bruker). Further, FTIR spectrums were measured over the frequency range 1250-1500 cm^{-1} to determine the degree of deacetylation of chitosan. The Degree of Deacetylation was calculated using equation (1) (Mahlous et al., 2009):

$$\frac{A_{1320}}{A_{1420}} = 03822 + 0.3133(100 - \mathrm{DDA}) \quad (1)$$

Where, A_{1320} is the characteristic band of –OH, $–NH_2$ and –CO groups and A_{1420} is the reference band.

III. RESULTS AND DISCUSSIONS

Composition of prawn shells

Sri Lankan Flowery Prawn shells used in the present study for the production of chitosan was analysed for its composition and the results indicates that the chitin content of the marine origin prawn type is 22.96 %. This is in agreement with previous work. Abdou et al (2008) and Nessaa et al (2010) mentioned that, depending on the type and species of crustacean, their exoskeleton composition could vary and may consist of 20-30% chitin, 30-40% protein and 30-50% minerals.

FTIR Spectrum of Chitin and Chitosan

FTIR spectra of chitin and chitosan measured over the frequency range 600–4000 cm^{-1} is shown in Figure 3. There were notable similarities as well as differences in the FTIR spectrum of chitin and chitosan. Occurrence of glucosamine units were attributed to spectral similarities between chitin and chitosan involved in this study.Palpandiet.al. (2009) mentioned that the main absorbance peaks that are common for both spectra are at around 3439, 3268, 2878, 1661,1418, and 1026 cm^{-1} which indicate O-H stretching, N-H stretching, C–H stretching, secondary amide I band, C–H deformations, CO stretching respectively. However, the differences in spectra were observed in the form of shifts in bands that was attributed to the loss of acetyl groups in chitosan.

In more detail, the intense band located at 3449 cm-1 in both spectra of Figure 3 can be attributed to O-H stretching frequency. This was also shown by several other researchers as the band at 3447 cm^{-1} (Zhimei et al., 2009), 3448.5 cm^{-1} (Mudasir et al., 2008) and 3450 cm^{-1} (Baskar et al., 2009).

Peaks at 3258 cm^{-1} and 3109 cm^{-1} could be due to N-H stretching and it is more evident in the spectrum of chitosan than in chitin. Mudasir et al., (2008) have reported that the bands observed at 3271 cm^{-1} and 3109cm^{-1} belong to asymmetric and symmetric stretching vibration of N-H group from acetamide ($-NHCOCH_3$), whereas Sagheer et al., (2009) have reported that 3264 $cm^{-1}$and 3107 cm^{-1} represent the stretching vibration of N-H group. Further, absorption peak at 2875cm^{-1} is from –C-H stretching and the doublet at 1652 and 1622 is typical of amide I band. The peak at 1418 cm^{-1} is the characteristic bending vibration for CH_3 and this has been also reported 1420 cm^{-1} by Basker et al (2009). Moreover, absorption at 1156 cm^{-1} belongs to the –C-O vibration of polymer and that observed at 1066 cm^{-1} could be the stretching vibration for –C-O-C– of the glucosamine ring. These observations confirmed the polysaccharide nature of chitin and its derivative chitosan used in the study.

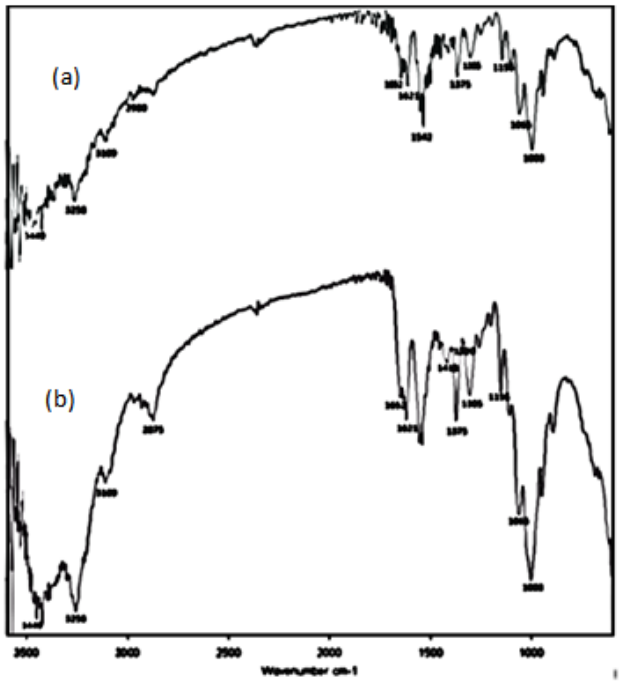

Figure 3: FTIR spectrum of (a) chitin and (b) chitosan prepared from prawn shells

Degree of deacetylation

Infrared spectra of chitosan and chitin that were measured over the frequency range 1250-1500 cm^{-1} (Figure 4) together with equation 1 were used to determine the degree of deacetylation. According to equation (1), ratio of absorbance bands at 1320 cm^{-1} and 1420 cm^{-1} measures the extent of deacetylation (Mahlous et al., 2009; Sagheer et al., 2009; Brugnerotto et al., 2001, Kumar et al, 2000). The presence of 1320 cm^{-1} peak in chitosan and the absence of it in chitin is a confirmation that deacetylation has occurred in the chitosan sample.

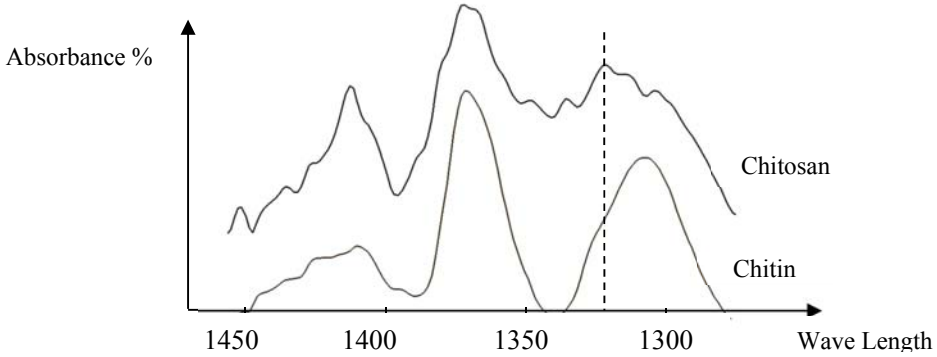

Figure 4. Infrared spectra of chitosan and chitin over the frequency range 1250-1500 cm^{-1}

i) Conversion of chitin through steeping in 45% NaOH solution

Figure 5 shows the FTIR spectrum over the frequency range 1500–1250 cm^{-1} of samples that were steeped for durations of 2, 4 and 10 days duration at ambient temperature.

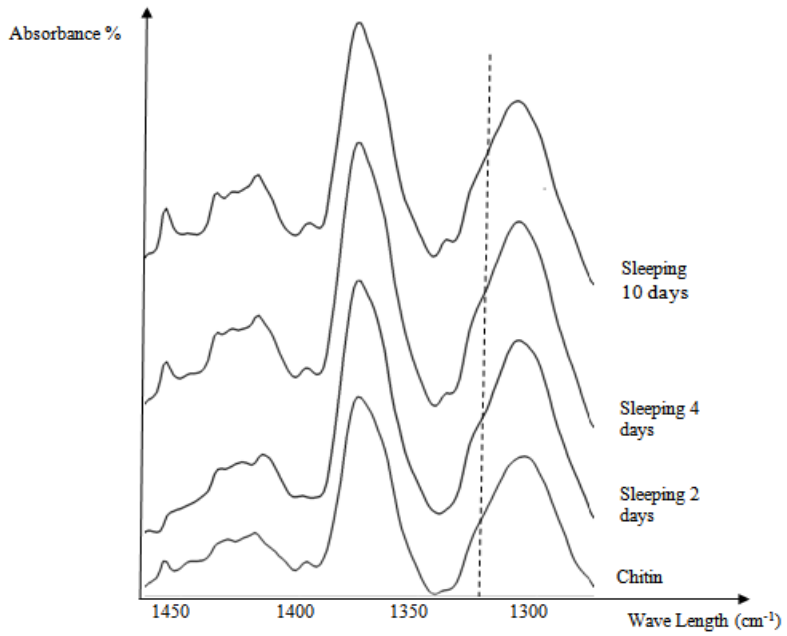

Figure 5. FTIR spectrums of chitin that was steeped in 45% NaOH solution for different time intervals

There is no observable peak at 1320 cm^{-1} wave length even after 10 days of steeping, even though a tendency for developing a peak could be observed with the increase of steeping time. The absence of peak and consequently no significant DDA could be due to the fact that the kinetics of the reaction is considerably low at ambient temperatures and at 45% NaOH concentration due to insufficient swelling of chitin particles. Steeping for more than 10 days to achieve higher DDA values is not economical to convert chitin to chitosan. Nessa et al (2010) carried out studies at ambient temperature, using 60% concentrated NaOH with a solid to solvent ratio of 1:15 (w/v) and obtained DDA values of 45, 71, 75 and 73 % when the duration of deacetylation was 45, 55, 65 and 72 h respectively. This shows increase of NaOH concentration increases the DDA up to an optimum with exposure time but thereafter there has been a tendency to decrease the DDA may be due to the degradation of chitin polymer. Moreover, high NaOH concentration will make chitosan purification difficult and it will generate large amount of alkali wastewater. The results show that there is an optimum combination of time and temperature for a specific concentration of NaOH to get a considerable conversion of chitin to chitosan. Therefore, other methods of conversion were considered in this work.

ii) Conversion of chitin by heating followed by steeping

IR spectra of chitosan H7-S16 (7 h heating at 100°C in 45% NaOH and steeping for 16 h and H5, H5.5, H6 (heating for prescribed timings at 100°C in 45% NaOH) compared with chitin over the frequency range 1500–1250 cm^{-1} are shown in Figure 6. According to fig 6, it is clear that increasing time at 100° C, shows a tendency in developing a peak at 1320 cm^{-1} which is an indication of conversion of chitin to chitosan. The conversion results for different treatment conditions through the calculation of DDA values are represented in Figure 7. It clearly shows that DDA depends on the duration of the treatment and higher the treatment time higher would be the DDA value. Therefore, by increasing the deacetylation time at a constant temperature and at a constant NaOH concentration, the degree of deacetylation can be increased.Moreover, as shown in Figure 6, DDA value of 98.9% was achieved by heating at 100 °C for 7 h followed by steeping in the same solution for further 16 h. Increase in contact time with NaOH after heating for 7 h has enhanced the removal of acetyl groups from chitin further.

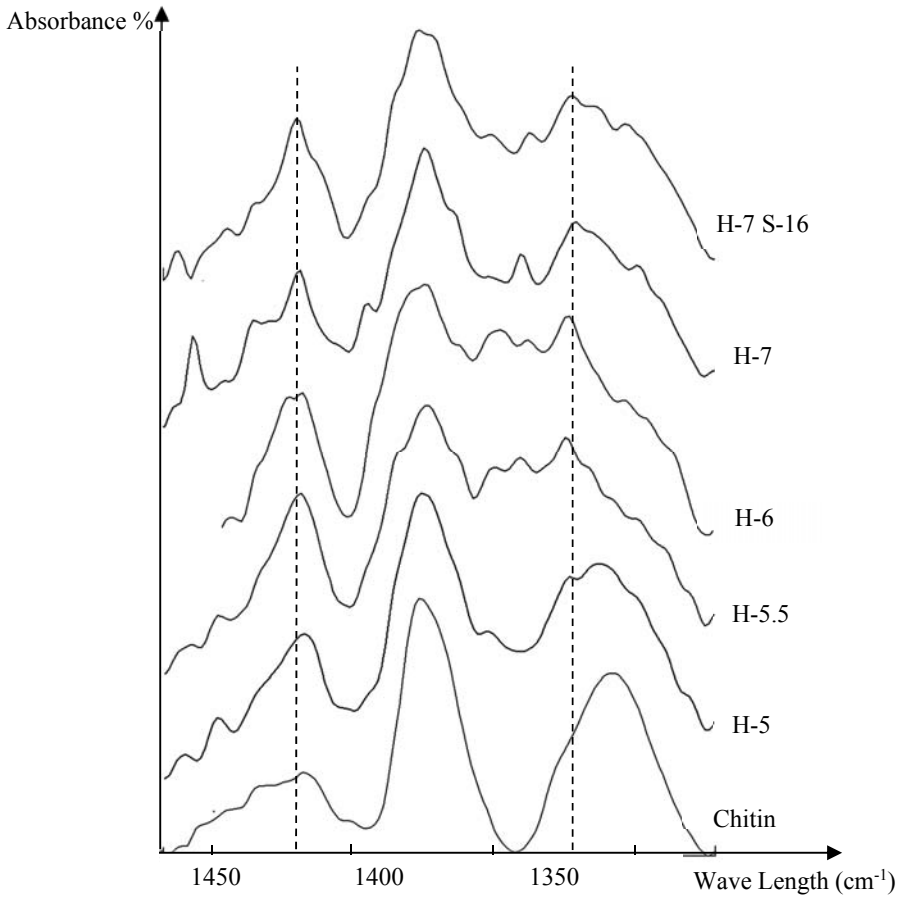

Figure 6: IR spectrums of chitosans prepared by thermal heating method

DDA values of 83 and 86 % obtained in this work for 5.5 and 6 h are comparable with the results of Sagheer et al., (2009). They have studied the patterns of deacetylation of chitin with reaction time using 45% NaOH with a solid to solution ratio of 1:15 at 110°C. According to their results, DDA values of chitosan prepared from prawn shell chitin were 79, 80, 82.5, 87 and 87% for reaction times of 2, 4, 6, 8 and 10 h respectively.Sagheer et al., (2009) reasoned out that the increase of DDA initially could be due to the bulk acetamido groups getting reduced to form amino groups (i.e. Chitosan) but subsequently these free amino groups within the molecular chain of chitosan form cations combine with the water in the alkaline solution and increase the viscosity. Thereby hinders the stirring rate of alkaline solution resulting in the reduction of DDA increment as the duration of treatment in increased.

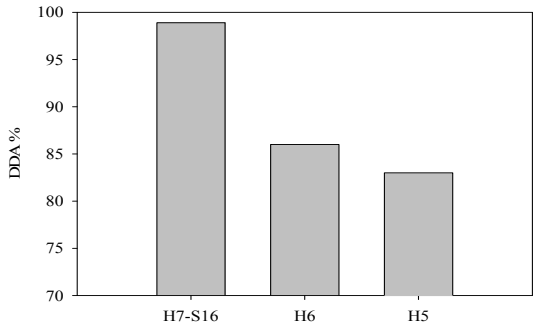

Figure 7: Degree of deacetylation of chitosan prepared by different thermal heating method

Further, studies have shown that considerably high DDA values at lesser time could be obtained by temperature increment. Morteza et al., (2009) have reported that with a 50% NaOH concentration, the DDA value was 75 % at 40°C and it could be increased to 87 % by heating at 65°C for 20 h. Maria et al., (2009) have reported that the DDA value of 94% was accomplished by immersing in a 50% (w/w) NaOH solution at 100°C for 60 min. The DDA values are also highly affected by the analytical methods employed (Tanveer et al., 2002) and therefore, the slight deviations of results may be due to the different methods used to determine the DDA value.

iii) Microwave Radiation with 45% Sodium Hydroxide

In the present work, investigations were also made with microwave radiation as an alternative method to decrease the long processing times in achieving the sufficient DDA values in chitin to chitosan conversion at high temperatures.

IR spectrums of deacetylated chitosan under microwave heating at 60% power level for 5 and 10 min durations in a 900W MW oven is shown in Figures 8(a).

According to Figure 8(a), there is no observable peak at 1320 cm^{-1} wave length when treated with MW.

However, a tendency to develop a peak at 1320 cm^{-1} wave length can be observed with the increase of exposure time. Therefore, further investigations were made with increasing the power level to 80 % while keeping the exposure time limit to 10 minutes as shown in Figures 8 (b).

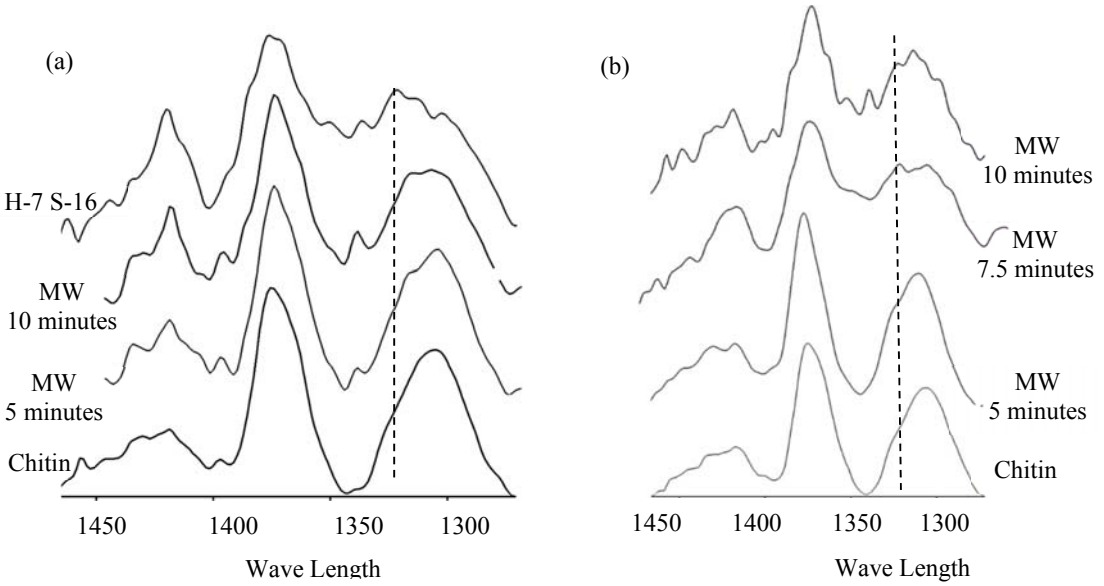

Figure 8: IR spectrums of chitin that was exposed to micro wave radiation at power level (a) 60% for different time intervals and (b) 80% for different time intervals

In the IR spectrum of MW at 80 % power level irradiated chitin sample for 5 min, there is only a tendency to develop a peak at 1320 cm^{-1} wave length. Whereas in the other two samples treated for 7.5 and 10 minutes, a clear peak at 1320 cm^{-1} wave length can be observed and their calculated DDA values are shown in Figure 9.

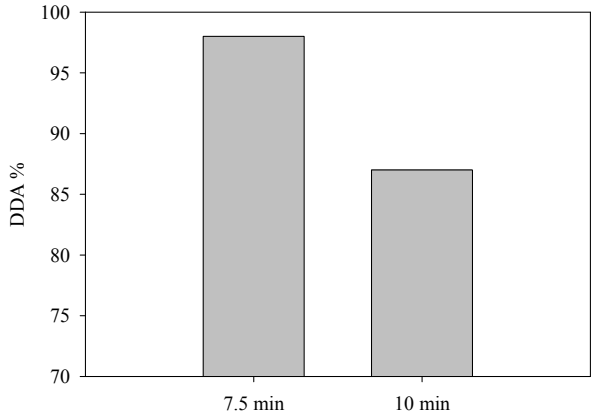

Figure 9: Degree of deacetylation of chitosan prepared by MW radiation method at 80% power level

Sample exposed to 7.5 min has a DDA value that is comparable to the DDA value of chitosan sample which was prepared by thermal heating at 100 ˚C for 7 h followed by steeping for 16 h i.e. 98.9% DDA. This shows that deacetylation time can be reduced considerably with microwave heating.

On the other hand, the DDA value ofMW radiated sample for 10 min has a lower DDA value compared to the 7.5 min sample. This may be due to the degradation of the polymer and the fragmentation into water-soluble, non-retainable fractions with the increase of exposure time. This effect is similar to the degradation that was observed in the method of thermal heating with long exposure times. However, further research need to be carried out to investigate the optimum time and power level with MW.

IV. CONCLUSIONS

Prawn shells discarded by local sea food industry were used for the isolation of chitin after subjecting them to demineralization and deproteinization processes. Chitin was converted to chitosan in different process conditions and was analysed using their DDA value. DDA values of greater than 80 % were obtained at 100 °C for 45 % NaOH concentration with 5 -7 h exposure time. These values were improved by steeping for long hours. However, microwave heating proved to give similar results by reducing time of exposure levels to 7.5 min.

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