DEACETYLATION OF CHITIN ISOLATED FROM FERMENTED PRAWN WASTE TO PRODUCE CHITOSAN USING AUTOCLAVE METHOD

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Abstract

A partially purified chitin was isolated from fermented prawn waste. Chitin was further deacetylated using autoclave method under condition of 121 C and 101.kPa to form chitosan with the objective of reducing the deacetylation time and cost of production. The effect of NaOH concentrations and various steeping modes on the degree of deacetylation (DD) of chitosan were evaluated. Degree of deacetylation of chitosan was determined using FTIR spectroscopy. Chitosan film was not formed when chitin was deacetylated in 40% and 45% (w/w) NaOH solution. Effective chitosan film formation was observed only when 50% (w/w) NaOH was used. Result showed that chitin was successfully deacetylated using autoclave and reaction time of 20 minutes to form chitosan of up to 84% DD. The DD also increases with steeping time and a double steeping mode was able to increase up to 91% DD of chitosan.

***Keyword**: chitin, steeping time, deacetylation, chitosan, autoclave*

INTRODUCTION

The recent expansion of Malaysian shrimp industry due to global demand is contributing to the increase in shrimp waste production locally as nearly 50% of the whole prawn are discarded as waste [1-2]. Major compositions of prawn waste are chitin, protein and minerals. An economical way to minimize environmental impacts due to shrimp waste disposal is to extract chitin from the shrimp shell waste and put it to good use such as conversion to a more versatile derivatives chitosan. Chitin can be extracted from shrimp waste using chemical method through strong acid and alkali treatment. Alternatively, lactic acid bacterial fermentation has also been applied in our laboratory to extract protein [3] for aquaculture purposes and produces chitin by-product.

Chitin is often deacetylated under strong alkali and high temperatures to produce chitosan which is of higher economic value than its starting material chitin. Deacetylation of chitin can be done by boiling, autoclaving (boiling under pressure), or boiling by microwave method [4]. Degree of deacetylation (DD) is used as an indication of how much chitin has been converted to chitosan through the removal of acetyl group from chitin. It is envisaged that steeping or soaking prior to deacetylation process may assist in increasing the DD of chitosan [5]. Chitosan with higher DD will have more amine groups (more basic) and is therefore expected to be of higher reactivity. In this study, chitosan is produced by deacetylation of chitin using autoclave method at temperature of 121ºC, and pressure of 101.3 kPa and the effect of steeping time, mode of steeping and concentration of sodium hydroxide (NaOH) used are discussed. To date, no data has been reported on using chitin from fermented prawn waste to produce chitosan.

MATERIALS AND METHODS

Chitin source

Chitin was obtained from fermented tiger prawn waste from our laboratory (Biochemistry Laboratory, Faculty of Science, Universiti Teknologi Malaysia). After three days fermentation, chitin was separated from the proteinaceous liquor, washed and dried.

Effect of steeping on DD of chitosan

Steeping process prior to autoclaving to improve deacetylation process was evaluated. In this study, two steeping modes were conducted.

Single Steeping: Chitin (2.00 g) was mixed with NaOH (50% w/w), in an autoclave bottle (50mL) with solid to liquid ratio of 1:10. Samples were left soaking in the solution for various durations (1, 2, 3, 4 and 5 days) before being deacetylated in an autoclave.

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Double Steeping: The double steeping mode is similar to single steeping method. However, after steeping time was completed, NaOH solution was replaced with fresh NaOH (50% w/w) and again steeped for similar durations (1, 2, 3, 4 and 5 days respectively) before being deacetylated.

Deacetylation of chitin to chitosan using autoclave

Chitin which has been steeped in NaOH (50%, w/w) for the required durations, was deacetylated in an autoclave under pressure of 102.3 kPa, 121 °C for 20 minutes using Hirayama Autoclave (HVE-50). After autoclaving, the cooled mixture was centrifuged at 4500 rpm for 15 minutes (Hettich Zentrifugen) to separate the solid product. After thoroughly washing under running tap water to remove excess NaOH, the product was dissolved in a constantly stirred acetic acid solution (1%, v/v) in a ratio of 1:15 (w/v). The dissolved chitosan solution was centrifuged at 4500 rpm for 15 minutes for degassing and to separate impurities and unreacted chitin as chitin does not dissolve in acetic acid. Chitosan films were prepared according to the method of Mayachiew and Devahastin [6]. The obtained chitosan solution (20mL) was poured into a petri dish, and oven dried (Memmert) at temperature of 60°C for 24h. The thin chitosan film formed was further treated by immersing the chitosan film in NaOH (1% w/w) solution for 30 minutes to neutralize the acetic acid present in the chitosan film [7]. The treated chitosan film was then washed with distilled water to remove excess NaOH (1% w/w) followed by absolute ethanol and dried in desiccator for 2 days before analyses using FTIR spectrophotometer. The degree of deacetylation were calculated using three methods for comparioson, Domszy and Robert (1985), Baxter *et al.* (1992), and Sabnis & Block (1997) [8, 9, 10 respectively]. It is interesting to note that these three methods were chronologically developed and all of them had used the same values of OH and C=O of amide bonds.

Determination of degree of deacetylation

The treated chitosan film was analysed using FTIR spectroscopy and the DD was calculated by applying the formula which was proposed by Domszy and Robert (1985)(Eq. 1), Baxter *et al.* (1992) (Eq. 2), and Sabnis & Block (1997) (Eq. 3) for comparison.

$$\text{Degree of Deacetylation} = 100 - \frac{\frac{A_{1655}}{A_{3450}} \times 100}{1.33} \quad \text{(Eq. 1)}$$

$$\text{Degree of deacetylation} = 100 - \frac{A_{1655}}{A_{3450}} \times 115 \quad \text{(Eq. 2)}$$

$$\text{Degree of deacetylation} = 97.67 - (26.486 \times \frac{A_{1655}}{A_{3450}}) \quad \text{(Eq. 3)}$$

Where,

A_{1655} = the absorption band of amide at 1655 cm^{-1}

A_{3450} = the absorption band of hydroxyl at 3450 cm^{-1}

RESULTS AND DISCUSSION

Effect of concentration of NaOH solution on the formation of chitosan film

The result showed that 40% and 45% (w/w) NaOH solution were unable to form film when casted on a petri dish (Figure 1) which indicates that not enough chitosan was formed. Although several studies have indicated that DD of chitosan is proportionally related to the concentrations of NaOH solutions, most results also suggested that the minimum concentration of NaOH solution to produce chitosan from chitin is by using concentrations higher than 40% (w/w). In this work, the efficiency of NaOH to effect deacetylation is probably reduced further due to the presence of some impurities in the fermented chitin as reported by Zakaria *et al*., (1998) that chitin obtained through a 72h fermentation process of scampi waste had minimal protein left (6%) and small amount of minerals (20%) [11]. It may therefore be suggested that if chitin from fermented shrimp is to be used for deacetylation processes, minimal pre-treatment by acid and alkali treatment to remove remaining protein and impurities before undergoing deacetylation process would be highly recommended.

Determination of DD of chitosan by FTIR spectroscopy

Samples of chitosan formed were analysed using FTIR spectroscopy. All samples showed absorbance peaks at 1655cm^{-1} and 3450cm^{-1} (Table 1). The absorption band of N-H stretching (3270 cm^{-1}) was not observed as it

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overlaps with the broad absorption band for O-H stretching at a region of 3200-3400 cm^{-1}. Table 1 also shows the calculated DD using the three equations (Eq 1, 2 and 3). The results showed that equation 3 (Eq 3) by Sabnis and Block (1997) showed the highest DD while equation 2 the least.

Chitosan is a hygroscopic material and thus the hydroxyl (OH) groups of the chitosan are sensitive to atmospheric humidity. As a result, the absorption band for O-H stretching in FTIR spectrum may increase in intensity if the sample is not properly dried. Precautions had been taken to reduce the effect of water moisture by keeping the chitosan film in desiccators prior to FTIR analyses.

(a) no film formation (b) no film formation (c) film formed

Figure 1: Effect of concentration of NaOH solution on film formation. (a) 40% NaOH, (b) 45% NaOH, and (c) 50% NaOH.

Chitin with (1 → 4)-2-acetamido-2-deoxy-D-glucan (GlcNAc) as structural units, and (1 → 4)-D-glycoside linkages, forms a linear chain through the many inter- and intramolecular hydrogen bonds. The hydroxyl groups in chitin contribute strong intra- and intermolecular hydrogen bonds. Both hydrophobic and hydrophilic interactions may also occur between macromolecular chains. If these hydrogen bondings in chitin can be broken prior to deacetylation process, it will improve the degree of deacetylation of chitosan. Concentrations of NaOH solution used and steeping of the chitin in NaOH solution prior to hydrolysis by autoclaving is envisaged to promote higher degree of deacetylation of chitosan. It has also been suggested that cleavage of hydrogen bonds by acid can be done to improve DD of chitosan [12].

Effect of steeping on degree of deacetylation of chitosan

Single steeping time

Table 1 shows that DD in all three equations increases with steeping time. In equation 3, after 120h of steeping, the value of DD increases to 84% DD of chitosan. Hence, it can be suggested that various DD of chitosan can be easily produced by using appropriate steeping time. This is important as certain applications of chitosan may only require certain amount of purification of chitin. Efforts have been made in our laboratory to find usage for low grade chitin or chitosan for various applications since this work produces chitin as a by-product of fermentation of prawn waste for protein extraction. Low grade chitin or chitosan may potentially be used as fillers or reinforcing materials in forming biocomposites.

Table 1: Effect of duration of single steeping time on DD of Chitosan.

Single steeping time (h)	Absorbance		% DD of chitosan			
	1655 cm^{-1}	3450 cm^{-1}	Eq. 1[a]	Eq. 2[b]	Eq. 3[c]	Average
24	0.58	0.69	37.07	3.75	75.50	38.78
48	0.69	0.98	47.06	19.03	79.02	48.37
72	1.70	2.35	45.61	16.81	78.51	46.98
96	1.40	2.11	50.11	23.70	80.10	51.30
120	1.75	3.45	61.86	41.67	84.24	62.59

Reaction condition: NaOH (50%w/w), 101.3 kPa and 121°C. [a]Domszy and Roberts (1985), [b]Baxter et al.(1992), [c]Sabnis and Block (1997).

Visual observations on the colour of the chitosan produced from partially purified chitin changes from dark brown to light brown with increasing number of steeping days. This shows that NaOH (50% w/w) purifies the

protein residue from the partially purified chitin and the amount of protein removed increases as steeping time increases. This further demonstrates the necessity of minimal acid and alkali treatments prior to steeping and decetylation process if fermented chitin is to be used.

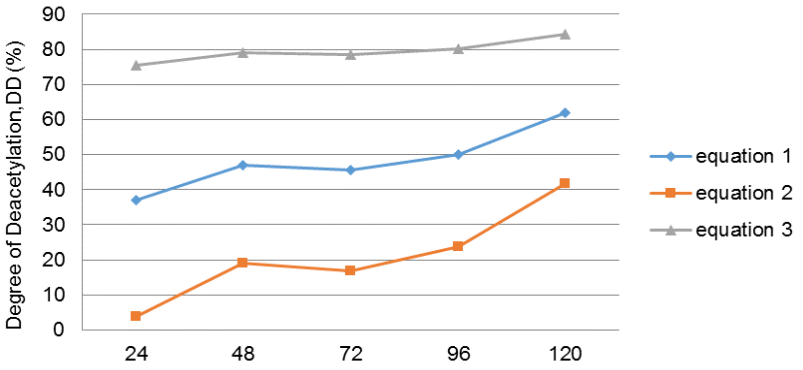

Figure 2: Effect of duration of single steeping time on DD of chitosan. Eq. 1: Domszy and Roberts(1985), Eq. 2:Baxter *et al*.(1992), Eq.3: Sabnis and Block

Double steeping time

The result shows that double steeping mode with 120h steeping in NaOH (50% w/w) produced high DD chitosan (average of 74.87%) (Table 2). Results using equation 3 shows that DD of chitosan are all above 80%. It is interesting to note that using a 120 days of double steeping had only increased the DD by 6% from 83% (at 24hr) to 89%(at 120hr) (equation 3) compared to an 8% increase (76% at 24hr to 84% at 120hr) when using single steeping for the same duration of reaction time (refer Table 1).

Table 2: Effect of double steeping time on DD of Chitosan

Double steeping time (h)	Absorbance		% DD of chitosan			
	1655 cm^{-1}	3450 cm^{-1}	Eq. 1	Eq. 2	Eq. 3	Average
24	1.75	3.07	57.14	34.45	82.57	58.05
48	1.59	3.60	68.67	52.08	86.63	69.12
72	1.33	2.34	60.63	39.78	83.80	61.40
96	0.00	4.22	100.00	100.00	97.67	99.20
120	0.59	1.75	74.65	61.23	88.74	74.87

Reaction condition: NaOH (50%w/w), 101.3 kPa and 121°C.

Similar to single steeping effect shown in Figure 2, results of equation 3 by Sabnis and Block (1997) shows the highest DD of chitosan throughout the 120h (Figure 3). Interestingly however, all equations showed a maximum DD of 98% - 100% after 96h of double steeping time which suggested that no further treatment is needed to enhance DD of chitosan beyond this point. However, beyond 96h of steeping time, chitosan showed only a slight decrease in DD. Similar trend was also noted by Baskar and Sampath Kumar (2009), during deacetylation of chitin by boiling method at 107°C whereby DD of chitosan decreases after 6h of boiling treatment [6]. They suggested that when DD is high or as the amino groups increases within the molecular chain of chitosan, it forms cations which combine with the water in the alkaline solution, rendering the solution to be more viscous. This hinders the stirring rate thus reducing the DD values.

Effect of steeping mode and steeping time on DD of chitosan

Figure 4 shows a comparison between the effect of mode of steeping (double and single steeping) and steeping time (24h to120 h) on DD of chitosan. In both modes of steeping, DD increases with time except for double steeping whereby DD decreased slightly after 96h.

Comparing the increase in DD when double mode was used shows that the increase in DD reduces with time. There is a 7% increase when steeped for 24h and only 4.5% increase when steeped for 120h (Table 3). This shows that although double steeping method may be useful in improving DD of chitosan, longer periods of double steeping is not economical. It is therefore appropriate to suggest that a double steeping mode is cost effective when used in shorter durations such as a 48h double steeping which had produced a DD of 86.63% (Table 3) which is similar to commercial chitosan of 85%. Yaghobi and Hormozi (2010) also suggested that a multistage deacetylation might be effective for increasing the degree of deacetylation of chitin [15].

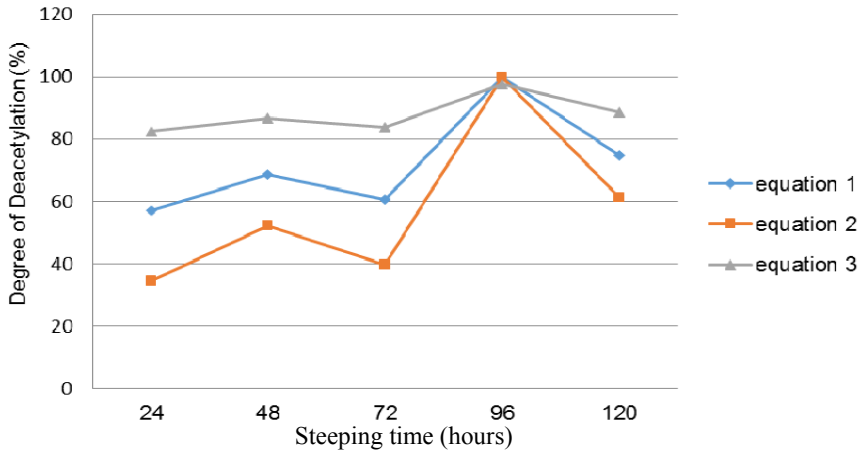

Figure 3: Effect of double steeping time on DD of chitosan. Eq.1: Domszy and Roberts (1985), Eq 2: Baxter et al.(1992), Eq.: Sabnis and Block (1997).

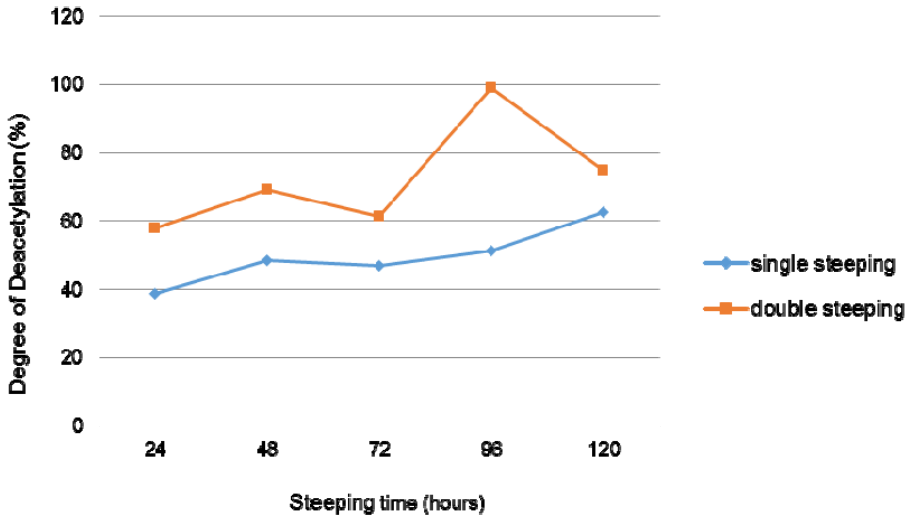

Figure 4: Effect of steeping mode on DD of chitosan.

Table 3: Comparison between the increase in DD by single and double steeping method using equation 3 of Sabnis and Block (1997) [**10**].

Steeping time (h)	**24**	**48**	**72**	**96**	**120**
Single steeping (%)	75.50	79.02	78.51	80.10	84.24
Double steeping (%)	82.57	86.63	83.80	97.67	88.74
Increase in DD (%)	**7.07**	**7.61**	**5.29**	**17.57**	**4.50**

CONCLUSIONS

Chitosan was produced from chitin isolated as by-products of fermented shrimp waste. Characterization of chitosan using FTIR spectroscopy showed that DD of chitosan increased with increasing number of steeping time and double steeping mode demonstrate better DD compared to single steeping mode. A limited double steeping mode shows a promising method to economically produce chitosan. This study which involved deacetylation using steeping and autoclave method together with using fermented chitin as a starting material could provide a more cost effective methods of producing high DD chitosan. However further investigation needs to be conducted to evaluate true cost of production.

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REFERENCES

1. Samin, S. (2002). The status of the Fish Marine Cage Culture Industry in Sabah, Department of Fishery Sabah.
2 Shahidul Islam, Md., Saleha Khan, Masaru Tanaka. (2004). Waste loading in shrimp and fish processing effluents: potential source of hazard to the coastal and nearshore environments. *Marine Pollution Bulletin*. 49, 103-110.
3. Nor, N. M. , Zakaria, Z. , Manaf, M. S. A. and Salleh, M. M .(2011). The Effect of Partial Replacement of Dietary Fishmeal with Fermented Prawn Waste Liquor on Juvenile Sea Bass Growth, *Journal of Applied Aquaculture*, Volume 23, Issue 1, 51-57.
4. Singh, S, Tripathi, D. N., Tiwari, A., Sanghi, R. (2006). Microwave synthesized chitosan-graft-poly(methylmethacrylate): An efficient Zn^{2+} ion binder. *Carbohydrate Polymers.* 65, 35-41.
5. Abdou, E.S., Nagy, K.S.A., Elsabee, M.Z.(2008). Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technol*., *99*, 1359-1367.
6. Mayachiew, P. and S. Devahastin (2010). Effects of drying methods and conditions on release characteristics of edible chitosan films enriched with Indian gooseberry extract. *Food Chemistry*. 118, 594-601
7. Baskar, D. and T. S. Sampath Kumar (2009). Effect of deacetylation time on the preparation, properties and swelling behavior of chitosan films. *Carbohydrate Polymers*. 78, 767-772.
8. Domszy, J. G. and G. A. F. Roberts (1985). Evaluation of infrared spectroscopic techniques for analysing chitosan. Die Makromolekulare Chemie. 186, 1671-1677.
9. **Baxter, A. Dillon, M, Taylor,** K.D.A**., Roberts, G.A.F.,** (1992). Improved method for i.r. determination of the degree of N-acetylation of chitosan. *International Journal of Biological Macromolecules.* 14, 166-169.
10. Sabnis, S. and L. Block (1997). Improved infrared spectroscopic method for the analysis of degree of N-deacetylation of chitosan*. Polymer Bulletin*. 39, 67-71.
11. Zakaria,Z., G.M. Hall and G. Shama. (1998). Lactic Acid Fermentation of Scampi Waste in a Rotating Horizontal Bioreactor for Chitin Recovery, *Process Biochemistry*, 33, 1-6.
12. Theruvathil K Sini, Sethumadhavan Santhosh, Paruthapara T Mathew. (2007). Study on the production of chitin and chitosan from shrimp shell by using Bacillus subtilis fermentation. *Carbohydrate Research*. 342, 2423-2429.
13. Yaghobi, N. and F. Hormozi (2010). Multistage deacetylation of chitin: Kinetics study. *Carbohydrate Polymers*. 81, 892-896.