

Controlling Molecular Weight and Degree of Deacetylation of Chitosan by Response Surface Methodology

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Response surface methodology (RSM) was used for controlling molecular weight (MW) and degree of deacetylation (DOD) of chitosan in chemical processing. In a reduced model, MW of chitosan is $y = 1736166.406 - 250.745X_1X_2 - 265.452X_2X_3$, with $R^2 = 0.86$, and DOD of chitosan is $y = 30.6069 + 0.3396X_1 + 0.4948X_2 + 0.0094X_3^2$, with $R^2 = 0.89$. MW of chitosan depends on the crossproduct of temperature and NaOH concentration and the crossproduct of NaOH concentration and time, and DOD depends linearly on temperature and NaOH concentration, and quadratically on time. Chitosan was widely depolymerized in a range from 1,100 kDa to 100 kDa and deacetylated from 67.3 to 95.7% by NaOH alkaline treatment. MW and DOD of chitosan were drastically decreased and increased, respectively, with increase of temperature, reaction time, and NaOH concentration. Furthermore, the rate of MW decrease and DOD increase of chitosan gradually decreased with prolonged reaction time.

KEYWORDS: Chitosan; response surface methodology; molecular weight; degree of deacetylation

INTRODUCTION

Chitosan, a copolymer derived from the abundant natural polymer chitin, is composed of 2-amino-2-deoxy-D-glucose and 2-acetoamido-2-deoxy-D-glucose units. In recent years, both chitin and chitosan have received great attention as biologically active substances. Chitosan and its derivatives have been used in a wide variety of applications, but the effectiveness of these materials has been found to be dependent upon their molecular weight (MW) and degree of deacetylation (DOD). Ikeda et al. (1) reported that chitosan hydrolysates with low MWs of 10 kDa and 20 kDa effectively lowered plasma and liver cholesterol concentration. Tokura et al. (2) reported that 9.3 kDa of chitosan inhibited the growth of *E. coli*, but 2.2 kDa of chitosan promoted the growth. Hirano et al. (3) described that an enhanced chitinase activity was found in the seedling growth of black-pine seeds coated with a depolymerized chitosan (MW of 3–5 kDa) and the activity was 1.3–1.5 times higher than that of uncoated

seeds. Tomihata and Ikada (4) revealed reduction in biodegradation of chitosan films in vivo and vitro with increased DOD.

Chitin can be converted to chitosan or chitoooligosaccharide by chemical process (5, 6) or enzymatic preparation (7–10). Chemical methods have several defects such as low product yields and chemical modifications of glucose ring, but are used extensively for commercial purpose of chitosan preparation because of low cost and suitability to mass production (6). Enzymatic methods can minimize alterations in the chemical nature of the reaction products, but few acceptable enzymatic methods have been described (11). Chemical process in chitosan preparation has been developed by many researchers. Kurita et al. (6) prepared 95% DOD of chitosan with 40% aqueous sodium hydroxide solution at 130 °C for 3 h under atmospheric state. Tolaimate et al. (12) reported that DOD and MW of the chitosan were greatly affected by several reaction conditions such as temperature and repetition of alkaline steps. Mima et al. (13) found that effective deacetylation was attained by intermittently washing the intermediate product in water two or more times during the alkali treatment. The average MW of the product was about 500 kDa with DOD at nearly 100% and insignificant degradation of the molecular chain. Wu and Bough (14) studied the effects of time and alkali concentration on deacetylation. The deacetylation proceeded rapidly to about 68% during the first hour of alkali treatment (50% NaOH) at 100 °C, and reached about 78% gradually in 5 h. Pelletier et al.

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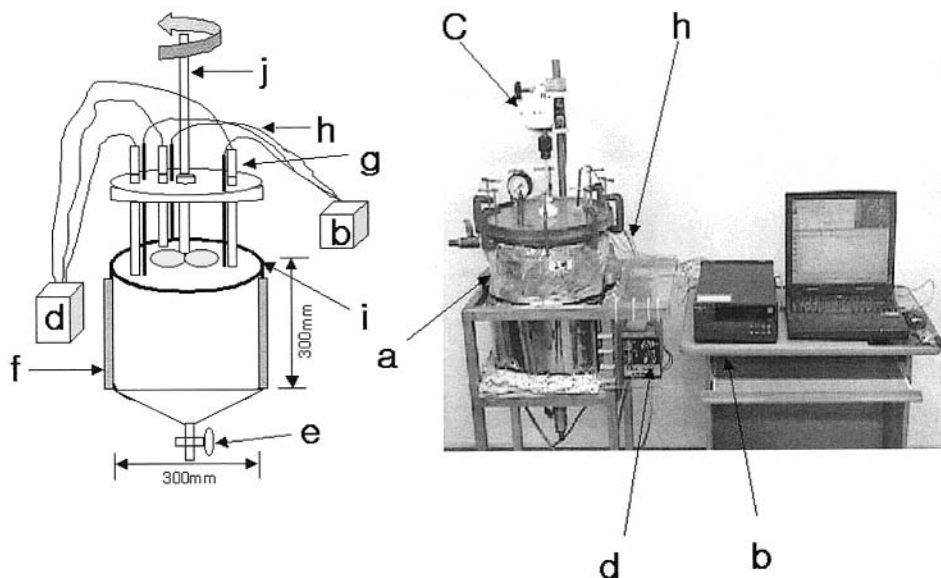


Figure 1. Schematic presentation of reactor apparatus: (a) reactor; (b) data logger; (c) mechanical stirrer; (d) heat controller; (e) drain valve; (f) heater; (g) temperature detector for heat controller; (h) thermocouple; (i) Teflon-coated inner wall; (j) impeller.

Table 1. Levels of Various Independent Variables at Coded Values of RSM Experimental Design

coded values	X_1	X_2	X_3
	temperature (°C)	NaOH concentration (%)	time (hours)
-2.68	40	32	2
-1	65	40	12
0	80	45	18
1	95	50	24
2.68	120	58.4	34

Table 2. Assignment of ^1H NMR Chemical Shifts of Partially *N*-acetylated in D_2O containing CF_3COOD

solvent	residue	H-1	H-2	H-3	H-4,5,6	<i>N</i> -acetyl
$\text{CF}_3\text{COOD}/\text{D}_2\text{O}$	GlcN	4.73	2.92	3.87	3.3–3.8	–
	GlcNAc	4.63	3.65	3.52	3.3–3.8	1.82

(15) applied thermo–mechano–chemical technology in chitin decetylation and found that efficient decetylation occurred at

temperatures exceeding 200 °C with a complete decetylation at 230 °C. Ottøy et al. (16) reported that 550 kDa MW chitosan from chitin was prepared with 50% (w/v) caustic solution at 75 °C under N_2 atmosphere with the ratio between the solution and chitin at 3.2:1. Knaul et al. (17) prepared 70.8% DOD chitosan with 50% NaOH concentration at 100 °C for 1 h under N_2 atmosphere. Tsaih and Chen (18) also prepared chitosan

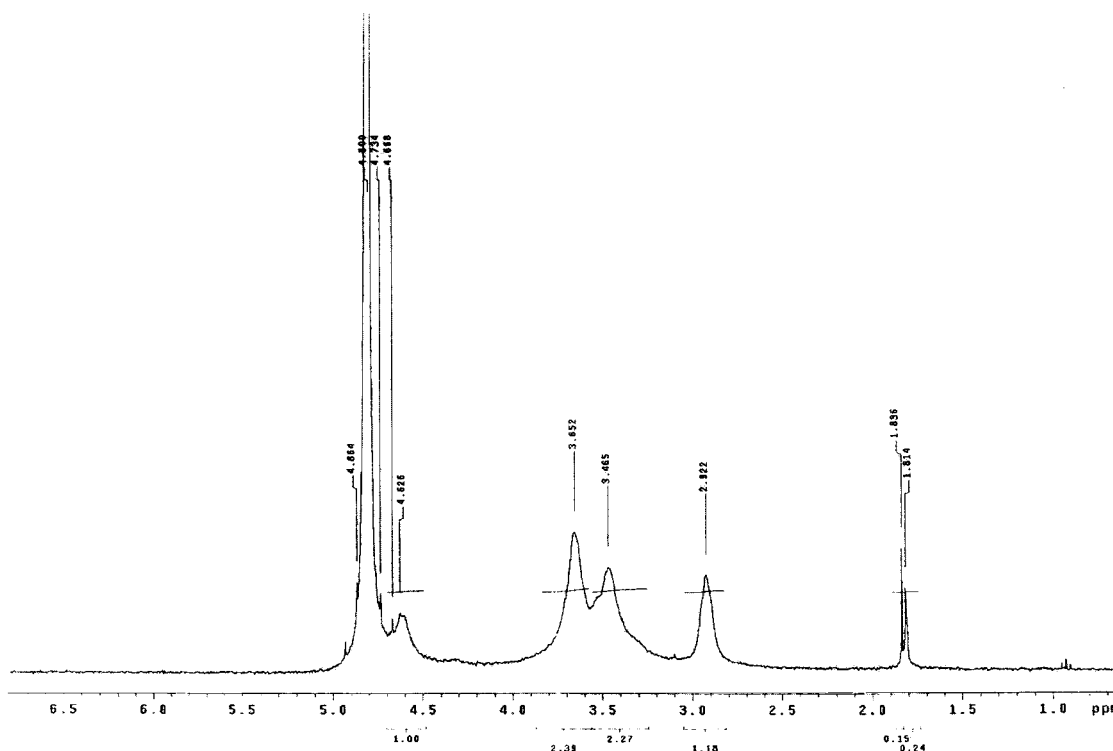


Figure 2. ^1H NMR spectrum of chitosan in D_2O containing CF_3COOD (at 25 °C and 300 MHz).

Table 3. Experimental Design and Results from Various Trials

experiment trial	independent variables			dependent variables	
	X_1	X_2	X_3	DOD (%)	MW (Da)
1	-1	-1	-1	76.3	864,000
2	-1	-1	1	80.2	816,000
3	-1	1	-1	83	786,000
4	-1	1	1	80.8	846,000
5	1	-1	-1	88	679,000
6	1	-1	1	90.9	679,000
7	1	1	-1	88.9	496,000
8	1	1	1	90.2	322,000
9	0	0	0	81.5	472,000
10	0	0	0	80.1	502,000
11	0	0	0	81.4	525,000
12	-2.68	0	0	67.3	1,105,000
13	2.68	0	0	95.7	106,000
14 ^a	0	-2.68	0	-	-
15	0	2.68	0	91.2	184,000
16	0	0	-2.68	78.2	873,000
17	0	0	2.68	92.4	321,000

^a MW and DOD of the sample at design point 14 could not be determined because of its high MW and low DOD.

Table 4. Analysis of Variance and Regression Coefficients for the Second Order Polynomial Model^a for Two Response Variables (DOD and MW)

regression coefficient	DOD (%)	MW (Da)
β_0	-35.237235 ^b	-1626012 ^b
linear		
β_1	0.892426 ^b	27025 ^b
β_2	2.010122 ^b	90141
β_3	1.087925	17396
interaction		
β_{12}	-0.011833	-820
β_{13}	0.003472	-258.333333
β_{23}	-0.032083	-275
quadratic		
β_{11}	-0.000518	19.728218
β_{22}	0.000099269	-445.162437
β_{33}	-445.162437	94.889529
R^2	0.9066	0.8607
F	7.548	4.119
probability of F	0.0071	0.0495

^a Model on which X_1 = temperature, X_2 = NaOH concentration (%), X_3 = time (hours) is $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$. ^b Significant at 1% level.

(MW of 91.4×10^4 Da) by alkaline treatment with 50% NaOH concentration at 100 °C for 3 h. Rege and Block (19) investigated the influence of reaction temperature, processing time, and mechanical shear on MW of chitosan and found that the reaction temperature and processing time had a significant impact on the MW and DOD of chitosan. These reports indicate that MW and DOD of chitosan are mainly affected by NaOH concentration, reaction time, and temperature. Additional factors are reaction reagents, particle size, atmosphere, chitin and chitosan solvent ratio, and source of raw material (6, 12, 20, 21). These multiple factors pose a challenge to understanding and controlling the outcome of the products. Therefore, there is a need for process optimization.

Response surface methodology (RSM) is a very useful statistical technique for optimization of complex chemical, biochemical, and food processes (22–25) and thus may be used in investigating chitosan production while controlling MW and DOD. Chang and Tsai (26) introduced RSM to optimize the deproteinization and demineralization process of chitin from pink shrimp shell powder. Chang et al. (21) reported the

Table 5. Analysis of Variance and Regression Coefficients from the Reduced Model^a for MW

source	regression coefficients	sum of squares	prob > F
intercept	1736166.41	2.15767E+12	0.0001
$X_1 * X_2$	-250.745	77727196055	0.0001
$X_2 * X_3$	-265.452	1.2213E+11	0.0124

^a X_1 = Temperature, X_2 = NaOH concentration, X_3 = time; regression prob > F = 0.0001; R^2 = 83.83%.

Table 6. Analysis of Variance and Regression Coefficients from the Reduced Model^a for DOD

source	regression coefficients	sum of squares	prob > F
intercept	30.6069	182.3026	0.0003
X_1	0.3396	579.4543	0.0001
X_2	0.4948	134.3041	0.0012
X_3^2	0.0094	101.0629	0.0033

^a X_1 = Temperature, X_2 = NaOH concentration, X_3 = time; regression prob > F = 0.0001; R^2 = 88.86%.

Table 7. MW and DOD of Chitosan with Various Chemical Processes

	condition	DOD	MW ($\times 10^5$)
in this study ^a	air, 50% NaOH, 95 °C, 24 h	88.9	4.96
	air, 50% NaOH, 95 °C, 24 h	90.9	6.79
	air, 50% NaOH, 95 °C, 24 h	90.2	3.22
	air, 50% NaOH, 95 °C, 24 h	88	6.79
Kurita et al. (1977)	air, 40% NaOH, 130 °C, 3 h	95	
Wu and Bough (1978)	air, 50% NaOH, 100 °C, 1 h		11.42
	air, 50% NaOH, 100 °C, 5 h		6.67
Tsaih and Chen (1997)	air, 50% NaOH, 100 °C, 3 h		9.14
	air, 50% NaOH, 100 °C, 1 h	94.5	3.02
Knaut et al. (1998) ^b	air, 50% NaOH, 100 °C, 5 h	95.3	2.69
	air, 50% NaOH, 100 °C, 4 h, m.s	87	
Rege and Block (1999) ^c	air, 50% NaOH, 100 °C, 4 h, m.s	83.5	
	air, 50% NaOH, 60 °C, 4 h, m.s	79.6	
	N ₂ , 40% NaOH, 80 °C, 3 h	75	
	N ₂ , 40% NaOH, 80 °C, 3 h ^d	99	5

^a Started from air state. ^b Initial DOD of the chitosan sample was 70.8%. ^c Mechanical shear on the process. ^d Repeated 3 h step process.

influence of alkaline concentration, temperature, and solution-to-chitin ratio and found that DOD was increased with increasing temperature or NaOH concentration. However, there has been no report on the optimization process for the chitosan production using RSM in relation to the major factors that control the MW and DOD of chitosan. The objective of this study was to investigate the effect of NaOH concentration, temperature, and reaction time using RSM.

MATERIALS AND METHODS

Materials. The following materials were used to prepare chitosan from chitin: NaOH (YoungJin Chemical, Korea); acetic acid, 99.7% (Showa Chemical Company, Japan); D₂O, 99.9 atom % D and CF₃-COOD, 99.5 atom % D (Aldrich Chemical Co., Milwaukee, WI), and chitin (Biotech Company, Korea).

Reactor Design. A schematic and photographs of two laboratory scale reactors are shown in **Figure 1**. The reactor and impeller were Teflon-coated for protection from NaOH corrosion. Furthermore, the reactor was surrounded by a wrap-around type heating-block on its outer surface. Temperature of the reactor was controlled and recorded by a FLUKE data logger system (model 2640A, Everett, WA). Each reactor was filled with 12 L of NaOH solution per 600 g of chitin. The ratio of chitin and NaOH solution was 1:20 (w/v). The contents of the reactors were agitated at 30 rpm (19).

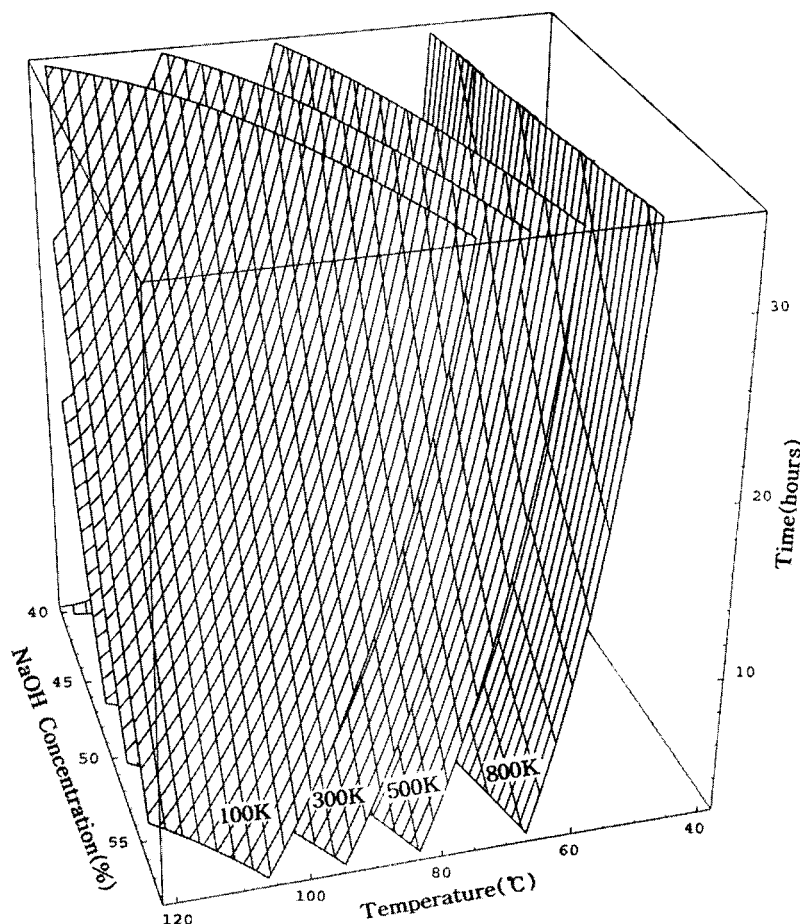


Figure 3. Response surface for the effect of temperature ($^{\circ}\text{C}$), NaOH concentration (%), and time (hours) on MW of chitosan.

Statistical Design. The experimental design was a modified central composite design for three variables at five levels (27). Temperature (X_1), NaOH concentration (X_2), and reaction time (X_3) were three independent variable factors considered in the preparation of chitosan. The MW and DOD were dependent variable responses. The coded values for the independent variables were -2.68 (lowest level), -1 , 0 , 1 , and 2.68 (highest level). The actual values and the corresponding coded values of three factors (X_1 , X_2 , and X_3) are given in **Table 1**. The complete design consisted of 17 experimental points which included three replications at the center point. The 17 samples were prepared in random order. Data were analyzed to fit the following second-order equation to all dependent Y variables:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where β s are constant regression coefficients and X_i s are coded independent variables. The SAS program (28) was used for analysis of variance and regression coefficient calculations. The models were modified for generating a four-dimensional response surface that was generated by the Mathematica program (29).

Test Methods. Determination of Molecular Weight (MW). A Ubbelohde-type capillary viscometer was used to measure the passage time of solutions flowing through the capillary at $25\text{ }^{\circ}\text{C}$. Seventeen different viscosity solutions of chitosan were used at various concentrations ranging from 0.00125 to 0.15% in 0.1 M acetic acid– 0.2 M NaCl solutions. The capillary viscometer was filled with 12 mL of sample and was equilibrated in a water bath (KOMA Company, Korea) at $25 \pm 0.1\text{ }^{\circ}\text{C}$ for 20 min . The sample was passed through the capillary once before the running time was measured. Three measurements were made on each sample. The solution and solvent flow times were measured to calculate relative viscosity, following which reduced viscosity was estimated. The intrinsic viscosity was obtained by extrapolating η_{rel}/C to zero concentration, where C is the chitosan

concentration in g mL^{-1} (30). The Mark–Houwink–Sakurada equation given below provides the relation between intrinsic viscosity $[\eta]$ and molecular weight and was used in calculating MW.

$$[\eta] = K \cdot \text{MW}^a \quad (1)$$

For this equation K and a are empirical constants: 3.04×10^{-5} and 1.26 , respectively (31).

Determination of Degree of Deacetylation (DOD). NMR spectroscopy was used for determining of DOD of chitosan (32–35). ^1H NMR spectra were recorded on a Bruker ARX 300 spectrometer (Billerica, MA) at $25\text{ }^{\circ}\text{C}$. Chitosan samples were twice freeze-dried from D_2O ($>99.9\%$) solution to minimize the HOD signal. All samples were dissolved in D_2O using 5-mm diameter tubes, and the sample concentrations were $20\text{ mg}\cdot\text{mL}^{-1}$. For water-insoluble samples, $\text{CF}_3\text{-COOD}$ (99.9%) was added. The chemical shifts were presented on the δ scale. The measurement conditions were as follows: spectral window of 8064 Hz , data points of 32k , pulse width of $3\text{ }\mu\text{s}$, acquisition time of 2.03 s , and 32 scans with delay of 1 s between scans (**Table 2**, **Figure 2**). DOD of chitosan was calculated from eq 2 by using the integral intensity, I_{Ac} , of the CH_3 residue, and the sum of integral intensities, $I_{\text{H-2,3,4,5,6}}$ protons.

$$\text{DOD}(\%) = \{1 - ((I_{\text{Ac}}/3)/(I_{\text{H-2,3,4,5,6}}/6))\} \times 100 \quad (2)$$

RESULTS AND DISCUSSION

Statistical Analysis. We used a response surface methodology (RSM) and ANOVA to investigate the effect of MW and DOD of chitosan on independent factors. Duncan method were used for the pairwise comparison of mean response outcomes with respect to each of 17 experimental designs which were composed of independent factors with 5 levels. **Table 1** shows independent factors according to each of the 5 levels. The

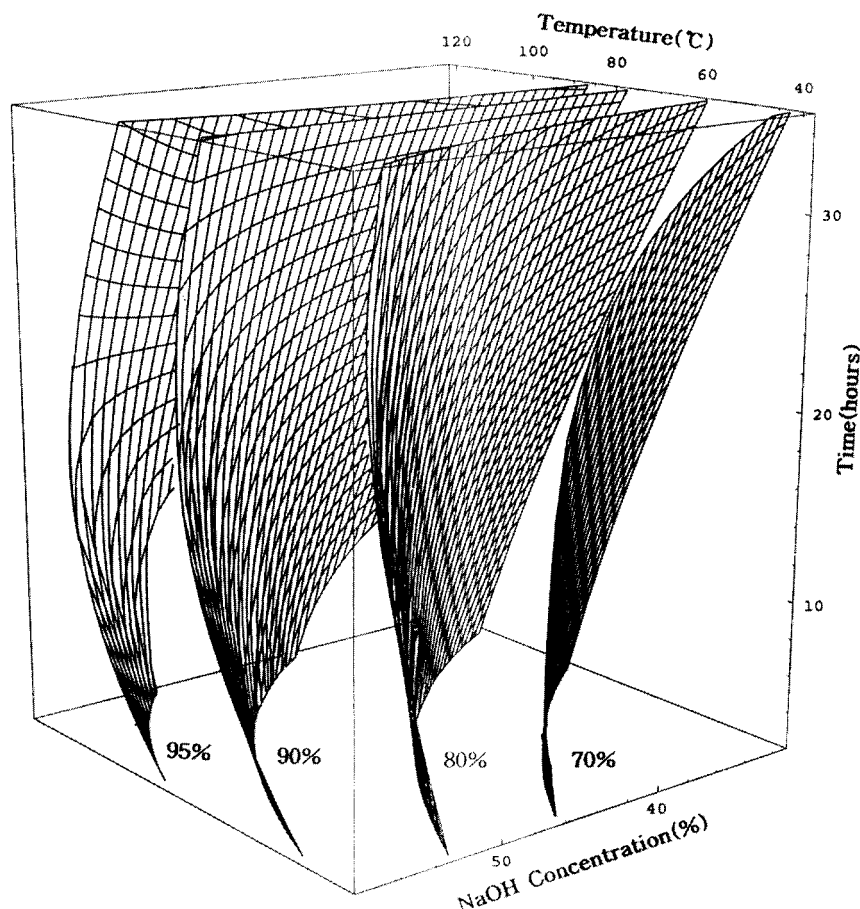


Figure 4. Response surface for the effect of temperature ($^{\circ}\text{C}$), NaOH concentration (%), and time (hours) on DOD of chitosan.

experimental outcomes of MW and DOD of chitosan are shown in **Table 3** for each of the 17 experimental trials. **Table 4** shows estimated regression coefficients (β) from RSM and the result of goodness-of-fit test for the full models using ANOVA. Our models for MW and DOD of chitosan were found to be significant ($p < 0.05$ and $R^2 = 0.86$ for MW, and $p < 0.01$ and $R^2 = 0.91$ for DOD of chitosan). However, only the linear component in our models is significant ($p < 0.05$). In addition to this, all regression coefficients shown in **Table 4** are also not acceptable even for the significant level. This implies that the full models in **Table 4** suffer from multicollinearity among independent factors. Therefore, we applied variance inflation factors (VIF) to detect multicollinearity. A value of VIF that is 5.0 or more indicates severe multicollinearity (36). The values of VIF for each independent factor in our full models were greater than 10, even greater than 300. To avoid these severe multicollinearities of input factors, we investigated a reduced model in which the difference between R^2 of the reduced model and that of our full model was as small as possible. We used the stepwise method to maximize R^2 for each fixed number of independent factors. The final selected model for MW is given in **Table 5**. As shown in **Table 5**, the regression coefficients on X_1X_2 and X_2X_3 are all significant under ($p < 0.01$) with $R^2 = 0.84$ which is slightly lower than the $R^2 = 0.86$ of the full model, and the reduced model for MW of chitosan is $y = 1736166.406 - 250.745X_1X_2 - 265.452X_2X_3$ (see **Table 5**). There is no multicollinearity problem in the reduced model, as VIF values for X_1X_2 and X_2X_3 are near to 1, which indicates no multicollinearity. Therefore, MW of chitosan depends on the crossproduct of temperature and NaOH concentration and the crossproduct of NaOH concentration and time.

Similarly, the reduced model for DOD of chitosan is $y = 30.6069 + 0.3396X_1 + 0.4948X_2 + 0.0094X_3^2$, with $R^2 = 0.89$ (see **Table 6**). Again, no multicollinearity problem for this reduced model occurred as all values of VIF are near 1. DOD depends linearly on temperature and NaOH, and quadratically on time.

Molecular Weight (MW) and Degree of Deacetylation (DOD). Results of the DOD and MW of chitosan obtained from this study (selected processing conditions) are compared with those from other studies (**Table 7**). The predicted models were used to create four-dimensional response surfaces within the experimental region using a Mathematica program (Mathematica v 3.0.1., Wolfram Research, IL). Four-dimensional surfaces obtained from the predictive reduced models for the MW and DOD are shown in **Figure 3** and **Figure 4**. Chitosan was widely depolymerized ranging from 1,100 kDa to 100 kDa, and deacetylation ranged from 67.3 to 95.7% by NaOH alkaline treatment. The MW and DOD of chitosan significantly decreased and increased, respectively, with increase of temperature, reaction time, and NaOH concentration (**Figures 3** and **4**). Furthermore, the rates of MW decrease and DOD increase gradually decreased with increased time. Similar observations were reported by several researchers (**Table 7**). Wu and Bough (14) reported that MW of chitosan was 1,142 kDa at the first 1 h of alkali treatment with 50% NaOH at 100 $^{\circ}\text{C}$ and then decreased to 667 kDa after 5 h of treatment under the same conditions. They reported that the MW and DOD decreased rapidly in the first hour and gradually decreased afterward. Rege and Block (19) investigated the influence of temperature, processing time, and mechanical shear on depolymerization and the DOD of chitosan and found that only the reaction temper-

ature and processing time had a significant impact on the MW and DOD of the resultant polymer. Chang et al. (21) studied the influence of alkaline concentration, temperature, and solution-to-chitin ratio on the DOD of shrimp chitin and reported that the DOD increased primarily with increasing temperature or NaOH concentration. Mima et al. (13) observed that decreasing alkali concentration increased the time required to obtain soluble chitosans with a less viscous product, but increasing alkali concentration to saturation had little effect on deacetylation and viscosity. They also reported that prolonged time also increased the percentage of deacetylation and reduced molecular size.

CONCLUSION

Response surface methodology (RSM) was a very useful statistical technique for investigation of the effect of the MW and DOD of chitosan on major independent factors such as temperature, NaOH concentration, and time. RSM results showed that only linear component in our models was significant and showed multicollinearity of the MW and DOD. To circumvent strong multicollinearity, a reduced model was investigated. The reduced model for the MW of chitosan is $y = 1736166.406 - 250.745X_1X_2 - 265.452X_2X_3$, and the reduced model for the DOD of chitosan is $y = 30.6069 + 0.3396X_1 + 0.4948X_2 + 0.0094X_3^2$ with $R^2 = 0.89$. No multicollinearity problem was noted in the reduced model, as VIF values for all factors were near 1.0. Chitosan was depolymerized in a wide molecular range from 1,100 kDa to 100 kDa, whereas deacetylation ranged from 67.3 to 95.7% by NaOH alkaline treatment. Increase of temperature, reaction time, and NaOH concentration decreased MW and increased DOD. Furthermore, the rate of MW decrease and DOD increase gradually decreased with prolonged reaction time. The four-dimensional surface model was useful for controlling the MW and DOD of chitosan and may be used for controlling other complex chemical and biochemical processes.

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