

Optimization of deacetylation in the production of chitosan from shrimp wastes: Use of response surface methodology

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Abstract

The use of chitosan in diverse areas is directly related to the polymer's molecular weight and degree of deacetylation, which depends on the conditions of chitin deacetylation. The aim of the present study consisted of optimization of the deacetylation stage in the production of chitosan, using the response surface methodology for the polymer's molecular weight. Chitin was obtained from shrimp wastes and the study of deacetylation made through a factorial experimental design, where temperature and time were varied. The estimate of chitosan's intrinsic viscosity was made by linear regression with the values of reduced viscosity and concentration, using Huggins equation for polymers. The viscosity average molecular weight of chitosan was calculated for each experiment by the equation by Mark–Houwink–Sakurada that relates the intrinsic viscosity to the polymer's molecular weight. The optimum condition for the deacetylation reaction for molecular weight was observed at a temperature of 130 °C and in 90 min, and corresponded to a molecular weight of chitosan of about 150 kDa, and a deacetylation degree of 90%.

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1. Introduction

Chitosan, β -(1 \rightarrow 4)-D-glucosamine, is a partially deacetylated form of chitin, β -(1 \rightarrow 4)-N-acetyl-D-glucosamine, a substance found naturally in the exoskeletons of insects, shells of crustaceans, and fungal cell walls (Ravi Kumar, 2000).

Chitin can be converted into chitosan by enzymatic means or alkali deacetylation, this being the most utilized method. During the course of deacetylation, part of polymer N-acetyl links are broken with the formation of D-glucosamine units, which contain a free amine group, increasing the polymer's solubility in aqueous means (Chen & Tsaih, 1998).

The variations in preparation methods of chitosan result in differences in its deacetylation degree, the distribution

of acetyl groups, the viscosity and its molecular weight (Berger et al., 2005; Chen & Hwa, 1996). These variations influence the solubility, antimicrobial activity among other properties, being that commercial chitosan usually has a deacetylation degree varying from 70% to 95%, and a molecular weight ranging from 50 to 2000 kDa (Rege, Garimise, & Block, 2003).

Chitosan has many applications in agriculture, medicine, environment and food. However, in some fields, especially in medicine and the food industry, the application of this polysaccharide is limited by its high molecular weight resulting in its low solubility in aqueous media (Ilyina, Tikhonov, Albulov, & Varlamov, 2000). Chitosan is used in food as clarifying agent and enzymatic browning inhibitor in apple and pear juices and in potatoes, and as antioxidant in sausages. Chitosan can also be used as an antimicrobial film to cover fresh fruits and vegetables (Devlieghere, Vermeulen, & Debevere, 2004).

Chitosan's molecular weight distribution is influenced by factors such as time, temperature, reagent's concentration

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and atmospheric conditions of the deacetylation reaction (Tolaimate et al., 2000).

Although molecular weight can be determined by several methods, such as gel permeation chromatography (GPC) and light scattering (Jumaa, Furkert, & Müller, 2002; Methacanon, Prasitsilp, Pothsree, & Pattaraarchachai, 2003), viscometry is the most simple, fast and probably the most accurate determination method (Zhang & Neau, 2001). The viscosity average molecular weight of linear polymers, such as chitosan, can be determined using Mark–Houwink–Sakurada's empirical equation that relates the intrinsic viscosity to the polymer's molecular weight (Chen & Tsaih, 1998).

The aim of the present study consisted of optimizing the deacetylation stage in the production of chitosan from shrimp wastes using the response surface methodology, having as variables temperature and time of reaction, for the polymer's molecular weight.

2. Materials and methods

2.1. Raw material

The raw material used was shrimp wastes obtained from fish industries of the city of Rio Grande, RS, Brazil.

2.2. Obtainment of chitin

Chitin was obtained in pilot scale, according to the procedure of Soares, Moura, Vasconcelos, Rizzi, and Pinto (2003), through the stages of demineralization, that consists of the reduction of raw material's ashes; deproteinization, where there is a reduction of shrimp wastes' protein nitrogen; and deodorization, for the reduction of shrimp's characteristic odor. Chitin was dried in a tray drier until reaching commercial moisture content (5.0–6.0%, wet basis).

2.3. Study of the deacetylation reaction

Dried chitin for each carried out experiment was placed in a reactor, in small scale, under heating and stirring, where a sodium hydroxide solution (45°Bé) was added.

A complete factorial experimental design (3²), with two study factors and three variation levels, was used for the study of the deacetylation reaction, being analyzed by the response surface methodology, obtaining a statistical model of second order for the average viscometric molecular weight of chitosan (Myer, 1976).

The study factors were temperature (*T*) and reaction time (*t*). These factors were chosen for being two of the main factors of the experimental conditions for attainment of adequate molecular weights for chitosan. The variation levels in the codified form (*X_T*) and (*X_t*), based on literature data, are presented in Table 1 (Cervera et al., 2004; Chen & Tsaih, 1998; Prashanth, Kittur, & Tharanathan, 2002; Ravi Kumar, 2000; Tolaimate et al., 2000).

Table 1
Factors and levels used for the study of deacetylation reaction

Factors	Levels		
	−1	0	1
<i>T</i> (°C)	120	125	130
<i>t</i> (min)	90	120	150

2.4. Purification of chitosan

Chitosan, obtained from each experiment of the design matrix, was purified according to the following procedure: dissolution in dilute acetic acid, centrifugation for separation of not dissolved material, precipitation until pH 12.5, neutralization until pH 6.5 and centrifugation to separate the suspended substance.

2.5. Determination of chitosan's deacetylation degree

The deacetylation degree of chitosan was determined by the potentiometric titration method described by Broussignac, reported by Tolaimate et al. (2000). Chitosan was dissolved in a known excess of hydrochloric acid. From the titration of this solution with a 0.1 M sodium hydroxide solution, a curve with two inflexion points was obtained. The difference between the volumes of these two inflexion points corresponded to the acid consumption for the salification of amine groups and permitted the determination of chitosan's acetylation degree, through Eq. (1):

$$\% \text{NH}_2 = 16.1(V_2 - V_1) \times M_b/W \quad (1)$$

where (*V*₁) and (*V*₂) are the base volumes referred to first and second inflexion points, respectively, in mL, (*M*_b) is the base molarity in g/mol, and (*W*) is the original weight of the polymer in g.

2.6. Determination of chitosan's viscosity average molecular weight

Chitosan samples were dissolved in a solvent system, constituted of acetic acid (0.1 M), sodium chloride (0.2 M) and water. The viscosity of the samples was measured in a Cannon–Fenske capillary viscometer (Schott Geraete, model GMBH – D65719, Germany), in five different concentrations (0.001 and 0.012 g/mL), at a temperature of 25 °C.

The capillary was filled with 10 mL of the sample that passed through the capillary twice before the running time was measured. Each sample was measured three times. The running times of the solution and solvent through the capillary and capillary's nominal constant were used to calculate the cinematic viscosity (Chen & Tsaih, 1998). The relationship between cinematic and dynamic viscosity is shown in Eq. (2):

$$\nu = \frac{\eta}{\rho} \quad (2)$$

where (ν) is the kinematic viscosity in stokes, (η) is the dynamic viscosity in poise, and (ρ) is the density in g/mL. The solution and solvent viscosities were used to calculate the specific viscosity (η_{sp}), using Eq. (3):

$$\eta_{sp} = (\eta_{\text{solution}} - \eta_{\text{solvent}}) / \eta_{\text{solvent}} \quad (3)$$

Huggins equation (Eq. (4)) was used for the estimation of intrinsic viscosity, according to Simal (2002):

$$\frac{\eta_{sp}}{c} = [\eta] + k[\eta]^2 c \quad (4)$$

where (η_{sp}/c) is the reduced viscosity in mL/g, (c) is the solutions' concentrations in g/mL, (k) is a constant valid for each polymer in g/mL and $[\eta]$ is the intrinsic viscosity in mL/g.

A graph of (η_{sp}/c) in relation to the solution's concentration (c) supplies the intrinsic viscosity of the solution, by extrapolation of the straight line obtained by linear regression for $c = 0$, as presented by Alsarra, Betigeri, Zhang, Evans, and Neau (2002).

The viscosity average molecular weight of chitosan (M_v) was determined by Mark–Houwink–Sakurada's empirical equation, reported by Roberts and Domszy (1982), that relates the intrinsic viscosity to the polymer's molecular weight, in the form presented in Eq. (5):

$$[\eta] = KM_v^\alpha \quad (5)$$

where (K) in mL/g, and (α) dimensionless, are the constants that depend on the solvent–polymer system.

2.7. Statistical treatment

The response function's equation (viscosity average molecular weight, M_v), was in polynomial form, where the terms are constituted by the studied variables in the codified form, by combinations between them, by the coefficients of each term, and a constant that corresponds to the average of the values, and it is presented in Eq. (6):

$$M_v = A + B \cdot X_T + C \cdot X_t + D \cdot (X_T)^2 + E \cdot (X_t)^2 + F \cdot (X_T \cdot X_t) \quad (6)$$

The experimental procedure of chitosan's deacetylation reaction study followed the experimental design matrix, in the codified form, presented in Table 2.

3. Results and discussion

Fig. 1 presents the representative graph of reduced viscosity (η_{sp}/c) in relation to the five solution concentrations used for experiment number 3 from the design matrix presented in Table 2. The values obtained for the triplicates of each concentration value demonstrate the adequateness of the experimental design used for the determination of intrinsic viscosity.

The intrinsic viscosity estimation, using Huggins equation (Eq. (4)), for all the experiments from the factorial design matrix, is presented in Table 3. A good adjustment

Table 2
Factorial experimental design matrix for the study of deacetylation reaction

Experiment number	Codified temperature (X_T)	Codified time (X_t)
1	-1	-1
2	0	-1
3	1	-1
4	-1	0
5	0	0
6	1	0
7	-1	1
8	0	1
9	1	1

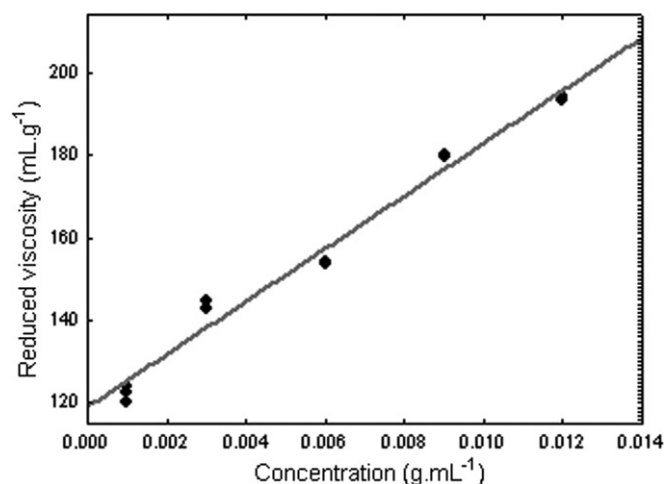


Fig. 1. Graph of the adjustment of Huggins for reduced viscosity values and solution concentration for experiment number 3 of the design matrix.

of the equation to the experimental data was verified through the high determination coefficient values obtained ($R^2 \geq 0.99$), as shown in Fig. 1 for experiment number 3.

The viscosity average molecular weight of chitosan was calculated using Eq. (5), taking the values of $K = 1.81 \times 10^{-3}$ mL/g and $\alpha = 0.93$, reported by Roberts and Domszy (1982), for a solvent system constituting of acetic acid 0.1 M, sodium chloride 0.2 M, at a temperature of 25 °C. The estimated values for the viscosity average molecular weight from each experiment of the factorial design matrix used are presented in Table 3.

A statistical analysis of the viscosity average molecular weight of chitosan obtained in each experiment (Table 3) for determination of deacetylation reaction's optimum conditions was carried out.

In Table 4 the results of the analysis of variance for the response viscosity average molecular weight of chitosan (M_v) are presented.

It is observed through p -values in Table 4 that the main effect of temperature (X_T) and the quadratic effect of time (X_t)² were the factors that most influenced the molecular weight's value, followed by the interaction effect between the main factors (X_T)(X_t). The main effect of time (X_t)

Table 3
Results of the experimental design matrix for Huggins equation's adjustment parameter $[\eta]$ and viscosity average molecular weight of chitosan (M_V)

Experiment	$[\eta]$ [mL/g]	M_V [kDa]
1	151.7	196.8
1'	153.6	199.4
2	140.7	181.4
2'	138.1	177.8
3	121.0	154.3
3'	119.7	152.5
4	162.1	211.3
4'	163.8	213.7
5	153.2	198.8
5'	151.7	196.8
6	144.4	186.6
6'	142.3	183.6
7	146.0	188.8
7'	144.4	186.6
8	128.7	164.8
8'	130.8	167.8
9	137.4	176.8
9'	138.5	178.4

('): replicate.

Table 4
Analysis of variance from chitosan's data for response average viscosity molecular weight

Parameters	Mean square	F-ratio	p-Value
X_T	2252.28	76.21	<0.001
X_I	0.08	<0.01	0.959
$(X_T)^2$	81.00	2.74	0.126
$(X_I)^2$	1823.29	61.69	<0.001
$(X_T)(X_I)$	598.58	20.25	0.001
Blocks	0.50	0.02	0.899
Error	29.55	–	–

$R^2 = 94\%$.

was not significant in the analysis, and it was unconsidered from the theoretical statistic model.

Fig. 2 presents the analysis of the main factors time and temperature, in the codified form, for the viscosity average molecular weight of the polymer. The effects temperature and time presented a nonlinear behavior, and the lower values of molecular weight are found in the combination of lower time (–1) and higher temperature (+1). In this condition, the substitution of acetyl groups for amino groups in the polymeric chains predominated, besides the depolymerization due to the conditions of reaction. According to Prashanth et al. (2002), the acetyl groups of chitin cannot be removed in the presence of alkalis without the degradation of polysaccharide chains, resulting in depolymerization, due to the reagent's high temperature and the reaction times required to obtain a complete deacetylation.

Eq. (6) represents the statistic theoretical model obtained from the regression analysis for the response viscosity average molecular weight of chitosan, considering the main effects of factors and second order interactions, in the

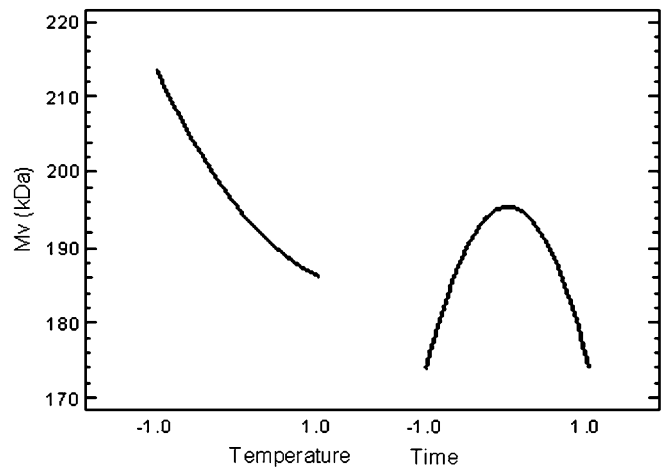


Fig. 2. Main effects in codified form for chitosan's molecular weight.

codified form. A good adjustment to this model was observed by the high determination coefficient ($R^2 = 94\%$).

$$M_V = 195.5 - 13.7 \cdot (X_T) - 4.5 \cdot (X_T)^2 - 21.4 \cdot (X_I)^2 + 8.7 \cdot (X_T) \cdot (X_I) \quad (6)$$

Fig. 3 presents the response surface for viscosity average molecular weight of chitosan, obtained from Eq. (6).

Fig. 3 shows the linearity of temperature and the nonlinearity of time. The optimum operation conditions were defined in the lower molecular weight region, because it is related to higher deacetylation degrees (Chen & Hwa, 1996) and depolymerization, once chitosan applications are limited by its high molecular weight, resulting in a low solubility in aqueous mean (Ilyina et al., 2000).

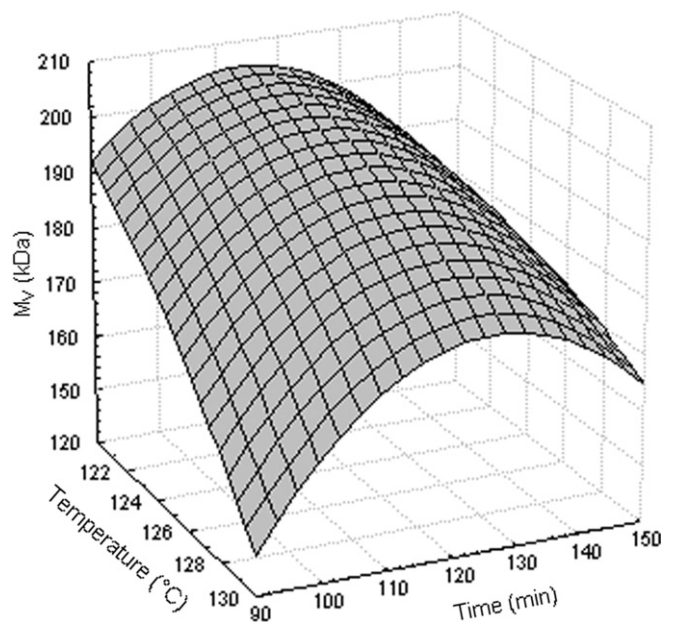


Fig. 3. Response surface for the polymer's viscosity average molecular weight.

Tolaimate et al. (2000) reported that a decrease of temperature from 120 °C down to 95 °C, keeping the same composition of the reaction medium, resulted in an increase in the viscosity molecular weight from 150 kDa to 490 kDa, thereby confirming the influence of temperature.

The condition that presented the lower molecular weight value was the one of higher temperature (130 °C) and lower time (90 min), with a molecular weight value of about 150 kDa, and a deacetylation degree of 90%, calculated by Eq. (2).

4. Conclusions

The experimental methodology used to calculate the intrinsic viscosity was evidently adequate, by the reproducibility of experiment's triplicates. The use of Huggin's equation for the estimation of intrinsic viscosity presented a good adjustment, with high correlations ($R \geq 0.99$).

Using the response surface methodology, great influence of the main effect of temperature and the quadratic effect of time were observed. Through the nonlinear regression analysis of the results obtained from the experiments for the determination of viscosity average molecular weight of chitosan, a second order statistical model with interactions was established, presenting a determination coefficient (R^2) of 94%. The optimum condition of the deacetylation reaction was obtained at a temperature of 130 °C and time of 90 min, by presenting a lower molecular weight value, about 150 kDa, with a deacetylation degree of 90%.

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References

- Alsarra, I. A., Betigeri, S. S., Zhang, H., Evans, B. A., & Neau, S. H. (2002). Molecular weight and degree of deacetylation effects on lipase-loaded chitosan bead characteristics. *Biomaterials*, *23*, 3637–3644.
- Berger, J., Reist, M., Chenite, A., Felt-Baeyens, O., Mayer, J. M., & Gurny, R. (2005). Pseudo-thermosetting chitosan hydrogels for biomedical application. *International Journal of Pharmaceutics*, *288*, 197–206.
- Cervera, M. F., Heinämäki, J., Räsänen, M., Maunu, S. L., Karjalainen, M., Nieto Acosta, O. M., et al. (2004). Solid-state characterization of chitosans derived from lobster chitin. *Carbohydrate Polymers*, *58*, 401–408.
- Chen, R. H., & Hwa, H. D. (1996). Effect of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical and permeability properties of prepared membrane. *Carbohydrate Polymers*, *29*, 353–358.
- Chen, R. H., & Tsaih, M. L. (1998). Effect of temperature on the intrinsic viscosity and conformation of chitosans in dilute HCl solution. *International Journal of Biological Macromolecules*, *23*, 135–141.
- Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, *21*, 703–714.
- Ilyina, A. V., Tikhonov, V. E., Albulov, A. I., & Varlamov, V. P. (2000). Enzymic preparation of acid-free-water-soluble chitosan. *Process Biochemistry*, *35*, 563–568.
- Jumaa, M., Furkert, F. H., & Müller, B. W. (2002). A new lipid emulsion with high antimicrobial efficacy using chitosan. *European Journal of Pharmaceutics and Biopharmaceutics*, *53*, 115–123.
- Methacanon, P., Prasitsilp, M., Pothsree, T., & Pattaraarchachai, J. (2003). Heterogeneous *N*-deacetylation of squid chitin in alkaline solution. *Carbohydrate Polymers*, *52*, 119–123.
- Myer, R. H. (1976). *Response surface methodology*. USA: Library of Congress.
- Prashanth, K. V. H., Kittur, F. S., & Tharanathan, R. N. (2002). Solid state structure of chitosan prepared under different *N*-deacetylating conditions. *Carbohydrate Polymers*, *50*, 27–33.
- Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*, 1–27.
- Rege, P. R., Garmise, R. J., & Block, L. H. (2003). Spray-dried chitinosans. Part I. Preparation and characterization. *International Journal of Pharmaceutics*, *252*, 41–51.
- Roberts, G. A. F., & Domszy, J. G. (1982). Determination of the viscosimetric constants for chitosan. *International Journal of Biological Macromolecules*, *4*, 374–377.
- Simal, A. L. (2002). *Estrutura e Propriedades dos Polímeros*. São Carlos, SP, Brasil: Gráfica UFSCar.
- Soares, N. M., Moura, C., Vasconcelos, S., Rizzi, J., & Pinto, L. A. A. (2003). Obtenção e purificação de quitosana a partir de resíduos de camarão em escala piloto. *Revista Univap*, São José dos Campos, SP, Brasil, *10*(18), 88–92.
- Tolaimate, A., Desbrières, J., Rhazi, M., Alagui, A., Vincendon, M., & Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer*, *41*, 2463–2469.
- Zhang, H., & Neau, S. H. (2001). In vitro degradation of chitosan by a commercial enzyme preparation: Effect of molecular weight and degree of deacetylation. *Biomaterials*, *22*, 1653–1658.