

Enzymatic deacetylation of chitin using beta-glucosidase as a biocatalyst.

Desacetilación enzimática de quitina utilizando beta-glucosidasa como biocatalizador.

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Abstract

This study focuses on the enzymatic deacetylation of chitin using beta-glucosidase as a biocatalyst, as part of the search for sustainable alternatives. Chitin extracted from seafood waste is transformed into chitosan using traditional methods that raise environmental concerns due to their high energy and water consumption, as well as the possibility of depolymerization. Enzymatic deacetylation is a more environmentally friendly option that uses emulsin, a beta-glucosidase, to catalyze the process and achieve a homogeneous, soluble, and intact product. However, these enzymatic methods are limited in terms of time and cost. Experiments were carried out at various reaction times (1, 2, 4, 8 hours) and enzyme-substrate ratios (E/S, 0.5:1, 1:1 mg/g). Chitosan was characterized using potentiometric and viscometric methods to determine the degree of deacetylation and the molar mass, respectively. These results highlight the possibility of obtaining chitosan with varying degrees of deacetylation by adjusting the experimental parameters. It was observed that enzymatic treatments affected the degree of deacetylation but not the molar mass, indicating the possibility of optimizing the process by increasing the enzyme concentration and reaction time. In conclusion, this study emphasizes enzymatic deacetylation as an ecological method for obtaining chitosan and provides perspectives for optimizing enzymatic parameters.

Keywords. Beta-glucosidase; chitosan; emulsine; enzymatic deacetylation.

Resumen

Este estudio se enfoca en la desacetilación enzimática de quitina utilizando beta-glucosidasa como biocatalizador, como parte de la búsqueda de alternativas sostenibles. La quitina, obtenida de residuos de mariscos, se convierte en quitosano mediante métodos tradicionales que plantean preocupaciones ambientales debido a su alto consumo de energía y agua, así como la posibilidad de despolimerización. La desacetilación enzimática se presenta como una opción más ecológica, utilizando emulsina como beta-glucosidasa para catalizar el proceso y obtener un producto homogéneo, soluble e intacto. Sin embargo, estos métodos enzimáticos enfrentan limitaciones en términos de tiempo y costo. Se llevaron a cabo experimentos variando los tiempos de reacción (1, 2, 4, 8 horas) y las relaciones enzima-sustrato (E/S, 0.5:1, 1:1 mg/g). La caracterización del quitosano se realizó mediante métodos potenciométricos y viscométricos para determinar el grado de desacetilación y la masa molar, respectivamente. Los resultados resaltan la posibilidad de obtener quitosano con diferentes grados de desacetilación mediante la optimización de parámetros experimentales. Se observó que los tratamientos enzimáticos afectan el grado de desacetilación, pero no la masa molar, lo que sugiere estrategias para optimizar el proceso mediante el aumento de la concentración de enzima y el tiempo de reacción. En conclusión, este estudio subraya la

desacetilación enzimática como un método ecológico para la producción de quitosano, proporcionando perspectivas para mejorar los parámetros enzimáticos.

Palabras clave. Beta-glucosidasa; quitosano; emulsina; desacetilación enzimática.

Introduction

Polymers derived from synthetic sources are derived from non-renewable resources, posing environmental problems due to their non-biodegradability and the generation of waste and toxic compounds during synthesis. Consequently, there is a global effort to seek sustainable and eco-friendly polymers¹. To achieve this, the use of natural fibers and agricultural residues in combination with matrices created from fully renewable biomaterials or biopolymers has become increasingly widespread². These materials can meet the required performance and quality attributes for various applications.

Chitin, an abundant biopolymer after cellulose, is extracted from shellfish waste and is insoluble in most solvents. Chitosan, derived from chitin, is soluble in acidic aqueous solutions and is suitable for forming films and fibers³. Chitosan is valued for its versatility, and its applications depend on its origin, properties, and extraction method⁴. It is primarily used in agriculture, paper, textiles, and wastewater treatment, with potential applications in energy⁵ and the environment⁶.

Traditional chitosan production involves the use of strong alkaline systems at high temperatures, consuming significant energy and water⁷. More environmentally friendly methods are sought, such as enzymatic deacetylation of chitin, which reduces toxic effluents and prevents depolymerization^{8,9}. However, this method has limitations regarding hydrolysis time and process cost^{10,11,12}.

For standardized enzymatic activity, specific steps should be followed, varying based on the enzyme type and application¹³. This involves consulting regulations, solid separation, chromatography, deodorization, dilution, concentration determination, concentration reduction, and pressure control¹⁴. Factors such as pH, temperature, pressure, substrate concentration, and inhibitors can affect enzymatic activity¹⁵.

Chitin deacetylases (CDAs) modify chitin by removing acetyl groups, and while limited microorganisms have been studied for their CDAs, there is potential in others. Exploring new CDAs and different approaches remains crucial for achieving pure chitosan and chito-oligosaccharides (COS)¹⁶.

Emulsin catalyzes the hydrolysis of $\beta 1 \rightarrow 4$ bonds in chitin. Although it is not a chitin deacetylase, a study showed its capacity to deacetylate chitin using emulsin extracted from sweet almonds. This process occurs in a solid state and benefits from mild conditions, preserving the polymer's structure β -glucosidase is sustainably produced by microorganisms or genetically modified organisms^{17,18,19}. Enzymatic deacetylation of chitin involves selectively removing acetyl groups from GlcNAc units, altering the polymer's structure and properties. Beta-glucosidase catalyzes the hydrolysis of glucosidic bonds between GlcNAc units, releasing glucose and acetate¹⁶. This enzyme can act on acetyl groups attached to GlcNAc units, promoting polymer deacetylation. Various models have been proposed for the kinetics of chitin deacetylation, with researchers exploring the use of ions and ultrasound²⁰. The activity of chitin deacetylase has been evaluated under different conditions, revealing multi-step processes and modified Michaelis-Menten equations²¹.

While the exact mechanism of beta-glucosidase catalyzing chitin deacetylation is not fully understood, it is suggested that the enzyme interacts with acetyl groups, facilitating their removal. Enzymatic deacetylation using beta-glucosidase offers advantages over other methods, occurring under mild conditions and allowing controlled modification of chitin²². This process is compatible with gentle reaction conditions, preserving the polymer's structure and functional properties, making it particularly relevant in biomedical applications²³.

Beta-glucosidase can be sustainably sourced from microorganisms or genetically modified organisms. Its use has potential applications across various industries, including food, pharmaceuticals, regenerative medicine, cosmetics, and textiles, offering efficiency, selectivity, and sustainability^{24,25}. Further research on this enzyme presents opportunities for innovative

biotechnological processes. This study aims to explore the use of emulsin as a beta-glucosidase catalyst in enzymatic deacetylation for a more efficient and environmentally friendly production of chitosan²⁶.

Materials and methods

Materials

The shrimp exoskeleton was obtained at the Bilbosa packing plant in the city of Manta, Ecuador, and was kept frozen at -4 °C before its use. This material was treated using the process described by Cisneros Pérez et al. (2019) for enzymatic chitin extraction²⁷. The obtained chitin had a molar mass of 7.18×10^5 g/mol and a minimum degree of acetylation of 67.80%. The other reagents used were of analytical grade and were acquired from S. D. Fine Chemicals (Mumbai, India).

The enzymatic deacetylation process of chitin was carried out following the guidelines established by Sedano Torres (2020)²⁸. A 5% sulfuric acid solution was added to chitin in a 1:30 (w/v) ratio, maintaining the pH at 4, as a pretreatment to improve its solubility. To this chitin solution, beta-glucosidase enzyme (derived from sweet almonds, Type 2A, 10U/mg) was added in enzyme-substrate (E:S) ratios of 0.5:1 and 1:1 (mg/g). The mixture was refluxed and agitated at 40 °C for intervals of 1, 2, 4, and 8 hours (Table 1). Subsequently, the resulting chitosan was vacuum-filtered and dried for two hours at 50 °C in an oven. Finally, the enzyme was inactivated by raising the temperature to over 60 °C before its final characterization, where the chitosan's molar mass was determined using the viscosimetric method, and its degree of deacetylation was assessed using the potentiometric method, as indicated by Ramírez Márquez et al. (2016) y De la Paz et al. (2012)^{29,30}.

Table 1. Experimental design of chitin samples for enzymatic deacetylation.

Sample	Quantity of enzyme (mg) Beta-Glucosidase	Time (h)
Q1	0.5	1
Q2	0.5	2
Q3	0.5	4
Q4	0.5	8
Q5	1	1
Q6	1	2
Q7	1	4
Q8	1	8

Experiment Design

All experiments were conducted in triplicate, and average values were reported. An analysis of variance (ANOVA) was performed, and differences between variables were evaluated for significance using a one-way ANOVA with Tukey's post hoc test. The software GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego, CA, USA) was used. A statistical difference with $p < 0.05$ was considered significant.

Characterization of deacetylated chitin

Characterization focuses on two fundamental aspects: the determination of the molecular weight and the degree of deacetylation as indicated by Curbelo C., et al. (2021)³¹. The objective of determining the molecular weight is to ensure that enzymatic treatments are not so severe as to cause excessive depolymerization, which would be reflected in a decrease in molecular weight and, therefore, limit their applications. On the other hand, the degree of deacetylation serves to differentiate whether the product obtained is chitin or chitosan, which are mainly distinguished by their solubility, establishing the 50% deacetylation as the limit³².

Degree of deacetylation

As indicated, the degree of N-acetylation of chitin was determined by potentiometric titration³³. For this purpose, 0.5 g of chitin was dissolved in 20 mL of 0.3 M HCl and titrated with a solution of 0.1 M NaOH. The titration was carried out by measuring the pH change every 2 mL of added base until reaching a pH value of approximately 11. The degree of deacetylation (DD) was determined by obtaining the derivative of the pH as a function of the volume of NaOH used. These values are plotted, and two inflection points (V1 and V2) are obtained, which are applied in equation (1). These points represent the amount of acid necessary to protonate the amino groups of chitin and determine the degree of deacetylation of the sample. This value is subtracted from 100 to obtain the degree of acetylation (DA) of the chitin sample (equation 2).

$$DD = 16.1 * (V2 - V1) * f/w \quad (1)$$

$$DA = 100 - 16.1 * (V2 - V1) * f/w \quad (2)$$

f: molarity of the NaOH used in the titration (0.1M)

w: weight of the sample (chitin) used (0.5g)

Molecular weight by intrinsic viscosity

According to the modified method of Martínez Robinson (2012), 0.02 g of purified chitin was dissolved in 25 mL of a solution of dimethylacetamide (DMAc) with 5% lithium chloride for 24 hours³⁴. The parameters were determined using the Mark-Houwink equation, with the constants *a* and *K* being 0.69 and $2.4 \times 10^{-4} \text{ L g}^{-1}$, respectively.

The intrinsic viscosity ($[\eta]$) of the chitosan samples was measured using a Canon-Fenske 300 viscometer (ASTM D 445), using a solution of 0.3 M acetic acid and 0.2 M sodium chloride as the solvent and maintaining the viscometer in a thermostatic bath at $40 \pm 0.01 \text{ }^\circ\text{C}$. The drop times of the solvent (t_0) and the polymer solutions (t_i) at known concentrations were measured. From these values, the relative viscosity ($\eta_{\text{rel}} = t_i / t_0$) and specific viscosity ($\eta_{\text{spe}} = \eta_{\text{rel}} - 1$), as well as the reduced viscosity ($\eta_{\text{red}} = [\eta] = \eta_{\text{spe}} C/C$), were calculated. By extrapolating to zero concentration in a plot of reduced viscosity versus concentration, the intrinsic viscosity was obtained, and finally, the molar mass (*Mv*) was determined using the Mark-Houwink equation (equation 3).

$$Mv = ([\eta]/K)^{1/a} \quad (3)$$

Donde: *K* = 0.074 mL/g, *a* = 0.796 a 30 °C

Results

The molecular weight of the chitin used as raw material for enzymatic deacetylation, with a 95% confidence level, was measured at $7.18 \pm 0.15 \times 10^5$, and its degree of acetylation was $13.08 \pm 0.65 \%$. The chitosan with the highest degree of deacetylation was obtained with an enzyme/time ratio of 1:8 (mg/h). On the other hand, the sample with the lowest degree of deacetylation was obtained with an E/t ratio of 0.5:1 (mg/h).

Table 2. Degree of deacetylation of chitosan by potentiometric titration method.

Trial Beta-Glucosidase (mg/h)	weight (g)	Volume inflection (cm ³)	of 1	Volume inflection (cm ³)	of 2	Potentiometric titration %DD
0.5:1	0.5023	1.32		5.58		38.46
0.5:2	0.5011	1.58		5.32		44.98
0.5:4	0.5022	1.52		7.18		51.29
0.5:8	0.5011	1.50		7.20		51.41
1:1	0.5014	2.30		7.30		57.79

1:2	0.5023	2.38	7.24	57.69
1:4	0.5016	1.34	8.20	64.19
1:8	0.5013	1.34	8.12	64.23

In Table 2, it was also observed that as the reaction time increases, the degree of deacetylation increases, indicating that the reaction time can potentially be gradually increased, similar to what happens when increasing the enzyme concentration.

To determine the molecular weight after enzymatic treatments, some samples were grouped based on the similarity of their degrees of deacetylation, which were rounded. The assays were conducted in quintuplicate.

In Table 3, the five assays are presented for the samples whose previously determined degrees of deacetylation were approximately 38, 44, 51, 57, and 64%. Based on these data, their concentration was calculated, and their drop time was measured to determine the intrinsic viscosity and consequently the molecular weight.

Table 3. Molecular weight of chitosan as a function of the degree of deacetylation.

%DD	Dissolution	Drop Time	Concentration (g/cm ³)	Molecular Weight (g/mol)	Mv	Intrinsic Viscosity (cm ³ /g)	R ²
38	1	132.35	8.59E-04	1.39E+05	599.89	0.9939	
	2	127.15	7.97E-04				
	3	121.35	7.32E-04				
	4	115.9	6.68E-04				
	5	110.21	5.92E-04				
44	1	132.05	8.49E-04	1.24E+05	551.42	0.999	
	2	127.1	7.95E-04				
	3	121.3	7.31E-04				
	4	115.8	6.68E-04				
	5	110.1	5.96E-04				
51	1	131.32	8.19E-04	1.12E+05	510.16	0.9987	
	2	127.1	7.79E-04				
	3	122.02	7.24E-04				
	4	117.8	6.79E-04				
	5	111.2	6.03E-04				
57	1	133.4	8.44E-04	1.04E+05	480.05	0.9976	
	2	128.3	7.91E-04				
	3	123.2	7.42E-04				
	4	115.7	6.59E-04				
	5	110.0	5.96E-04				
64	1	134.4	8.49E-04	1.01E+05	599.89	0.9939	
	2	127.3	7.79E-04				
	3	122.2	7.30E-04				
	4	115.8	6.61E-04				
	5	110.2	5.96E-04				

AVERAGE

1.16±0.16E+05

In Table 3, it is also observed that the molecular weight is practically the same for all assays with an average value of $1.16 \pm 0.16 \times 10^5$ g/mol, being approximately 70% lower compared to the original value of chitin.

Discussion

According to Guzhñay Lozano (2022)³⁵, the values allow the characterized chitin (Table 2) to be considered industrial-grade chitin, with a molecular weight close to 10^5 . The sample with the lowest degree of deacetylation was obtained with an E/t ratio of 0.5:1 (mg/h). This result may be attributed to the use of a lower amount of enzyme compared to the fixed amount of substrate. Now, according to the study by Dima, Sequeiros, and Zaritzky (2013)³², for the product obtained to be considered chitosan, it must have a molecular weight of around 10^5 and a degree of deacetylation between 50 and 60%. These parameters are met in all samples except Q1 and Q2. This indicates that with an enzyme-substrate ratio E/S (0.5:1, mg/g) and more than two hours of reaction time, chitosan is obtained; while with the E/S ratio (1:1, mg/g), chitosan is obtained from the first hour of reaction. When comparing enzymatic treatment times with various authors, similarities with the obtained results are evident. Ramírez et al. (2016)²⁹ experimented under similar conditions with two hours of treatment, achieving chitosan with low dye absorption capacity (qualitative deacetylation test). Similarly, Tello Palma (2017)²⁵ treated "Rainbow" trout scales with emulsin for 45 minutes at temperatures of 35, 50, and 40 °C, obtaining deacetylation levels below 40%, attributing this result to the high ash content (75%). However, Dimas et al. (2017)³² using slightly higher E/S ratios than those used in this study at 40 °C for 45 minutes, achieved deacetylation levels above 90%.

Table 3 shows that, regardless of the treatment applied, the reduction in molar mass is similar, with no significant differences between treatments at a 95% confidence level. The difference in the average value of this molar mass compared to the initial value could be due to the pretreatment of chitin with sulfuric acid. It is estimated that sulfuric acid treatment can cause depolymerization in chitin with low ash content. In contrast, a high ash content can reduce the efficiency in chitosan production²⁵. As previously mentioned, each enzyme responds differently, necessitating a specific approach for its study and optimization³⁶.

Conclusions

Characteristics of chitin are similar to those reported for industrial chitins. However, its molecular weight was slightly low, which could be attributed to the pre-treatment with sulfuric acid applied to the raw material.

After the enzymatic treatments for the deacetylation of enzymatic chitin, was observed that the process can still be optimized by increasing the value of both factors.

Producing pure chitosan or chitosan oligosaccharides (COS) with specific degrees of polymerization (DP) and degree of acetylation (DA) remains challenging.

Future research should focus on discovering new chitin-acetylase enzymes (CDAs) with unique properties, understanding their catalytic mechanisms, and modifying chitinous substrates and CDAs for efficient catalysis.

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Conceptualization, Iván Cisneros; methodology, Iván Cisneros; software, Iván Cisneros; validation, Iván Cisneros; formal analysis, Iván Cisneros and Luzmila Burbano; investigation, Iván Cisneros; writing, Iván Cisneros and Luzmila Burbano; supervision, Caridad Curbelo. All authors have read and agreed to the published version of the manuscript.

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