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Research Article

Enzymatic Depolimerization of Chitosan for the Preparation of Functional Membranes

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This work reports the study of chitosan depolymerization through the synergy of the Celuzyme® XB enzyme complex; it is composed of cellulase, xylanase, and β-glucanase. The optimal conditions of temperature, pH, and concentration were determined to verify the depolymerization reaction. The specificity of the enzymes at the β (1-4) glycosidic link site was checked. Low molecular weight chitosan (64×10^3 g·mol^{-1}) with degree of acetylation 15% was obtained. The depolymerized chitosan products were characterized by infrared spectroscopy, the degree of acetylation was obtained by UV-Vis spectroscopy, and the determination of the molecular weight was obtained by capillary viscosimetry. With the depolymerized chitosan, membranes were formed and their antioxidant and antimicrobial functionality was determined; results show that these properties are dependent on the molecular weight and on the acetylation degree of chitosan.

1. Introduction

In recent years, the increasing use of the chitosan biopolymer has been observed in activities related to the preservation of the environment and the care of human health. Its importance lies in its outstanding properties such as biocompatibility, biodegradability, and solubility in aqueous media [1, 2]; particularly, its broad spectrum of antimicrobial and antifungal activity [3] makes it of vital interest for the preservation of food and emerging processes, thus being considered as an attractive biomaterial for biotechnological and industrial applications. It has been reported the development of chitosan films has been tested in food preservation and packaging technology because they exhibit a high activity against pathogens, such as fungi and bacteria, decreasing the deterioration of foods of animal and vegetable origin [4, 5]. However, the physicochemical properties of chitosan limit its applications due to several factors; depending on the origin, the extraction method, and the deacetylation process of chitin [6], high molecular weight chitosan is obtained which favors its insolubility in water at pH > 6 and a diminished biocide effect [7]. Another of the limiting factors of the biopolymer in some of these applications is its high viscosity in acidic media given by a high molecular weight and its low solubility at pH above 5 [8].

For this reason, the interest in obtaining low molecular weight chitosan to improve its functional properties has become an important area of research that attempts to obtain oligochitosans with adequate properties for direct application. The process of depolymerization of chitosan has been studied, finding that its lower molecular weight derivatives show greater solubility than its larger counterparts, are more environmentally friendly substances, and/or have excellent biocompatibility characteristics. These *N*-acetylglucosamine (NAcGlc) materials have also shown greater bioactivity in some specific applications, such as in food preservation, in agricultural applications, and in gene

therapy. Its low toxicity is also a factor to consider. To carry out the depolymerization of chitosan, physical methods, acid hydrolysis, or enzymatic methods have been used. Physical methods such as irradiation or ultrasound cause a rapid and random degradation of chitosan-generating products of great variability and instability in terms of its solubility [9]. In acid hydrolysis, high volumes of strong acids and high working temperatures are required to generate *N*-glucosamine (NGlc) residues and a smaller amount of hetero-oligosaccharides [10], as well as secondary toxic products. In the enzymatic depolymerization, the course of the reaction as well as the distribution of the obtained products is easier to control due to the specificity of the reaction; the products retain their initial biological properties since deacetylation does not occur and the conditions are milder [11].

The topic grows in interest because the different degradation pathways can generate materials with structures and therefore with different properties [12]. In this sense, the enzymatic depolymerization seems to be the best alternative because it allows obtaining chitosan and products derived from it with better functional properties [13]. The enzymes act in a specific manner on reactive sites by fragmenting the molecule into oligomers, or acting from one end releasing monomers or dimers sequentially; for this reason, the total depolymerization of the biopolymer is not complete. Based on the above, this work reports the process of depolymerization of chitosan with a system of hydrolytic enzymes to obtain low molecular weight chitosan. Its functionality is evaluated to propose alternatives for exploitation. The enzymes cellulase, xylanase, and β-glucanase are able to form a multienzyme complex, in which they establish strong and lasting interactions; these interactions increase the activity and stability of the individual enzyme, mainly due to the decrease in the diffusion loss of the intermediate product and substrate of the neighboring enzyme. Conformational changes are also inhibited that lead to a lower rate of denaturation of the same enzyme, which promotes a stable, efficient, and rapid reaction.

2. Materials and Methods

Sigma-Aldrich reagents of the highest purity (98.5–99.98%) were used. The enzymatic complex Celuzyme® XB was donated by the company ENMEX, S.A. de C.V. The microorganisms for the antimicrobial tests were donated by the Center for Research in Microbiological Sciences (CICM-BUAP). The chitosan (Qs) was obtained from shrimp skeletons collected in marine food establishments based on the methodology proposed in [14].

2.1. Determination of the Conditions for the Depolymerization of Qs. To perform the Qs depolymerization, the enzymatic system Celuzyme XB® food grade was used. It is an enzymatic extract consisting of cellulase (45,000 U/g), xylanase (34,000 U/g), and β-glucanase (12,000 U/g), obtained from the controlled fermentation of *Trichoderma longibrachiatum*, which can be used in food processes, fermentation of alcohol, and animal feed. Celuzyme® XB allows hydrolyzing a large amount of polysaccharides (not starch), including arabinoxylanase and β-glucans of cereals. The hydrolytic action of the three enzymes is specific on the β (1,4) bonds of the substrate Qs.

The enzyme capacity was evaluated by the determination of reducing sugars using carboxymethylcellulose (CMC) of low viscosity as a substrate. The reaction mixture was prepared with 500 μL of 0.5% substrate in 50 mM sodium citrate buffer, pH = 4.8, and 200 μL of the enzyme system. The reaction was incubated at 50°C for 60 min and stopped in an ice bath for 5 min. The supernatant was analyzed by the 3,5-dinitrosalicylic acid (DNS) method to determine the concentration of reducing sugars [15].

Stability as a function of pH was evaluated by exposing 25 mL of the enzyme system at different pH (3.5–6.5) for 24 h at 25°C. It was adjusted to pH = 5.3 using 0.5 N NaOH or 0.5 N HCl, depending on the case. The effect of the thermal stability of the enzymes was evaluated by incubating 25 mL of the enzyme system at different temperatures for 120 min. The pH range and reaction time were adjusted considering the optimal values of the isolated enzymes. The depolymerization reaction was carried out with 25 mL of Qs 0.5% solution (in a buffer of acetic acid-sodium acetate 0.2 M), pH 5.3, and 1.6 μL of Celuzyme XB®; it was incubated for 120 min at 51.4°C. The reaction was stopped by denaturation of the enzymes (60°C, 10 min).

2.2. Characterization of Chitosan

2.2.1. Infrared Spectroscopy. The structural analysis of the Qs was performed in a PerkinElmer Fourier-Transform Infrared (FTIR) spectrophotometer in attenuated total reflectance mode (ATR). Six sweeps with resolution 4 cm^{-1} were performed in the middle region of the IR at room temperature.

2.2.2. Acetylation Degree (AD). The AD of the Qs was determined following the method of Muzzarelli et al. [16]. A PerkinElmer UV-Vis double-beam spectrophotometer at 197 nm was used. A calibration curve of *N*-acetyl glucosamine with 10, 20, 30, 40, and 50 $mg \cdot L^{-1}$ concentrations in 0.01 M acetic acid solution was constructed.

2.2.3. Average Molecular Weight in Viscosity (Mv). An Ubbelohde capillary viscometer was introduced into a thermostatic bath. Solutions of Qs of different concentrations (0.08, 0.12, 0.16, 0.2, and 0.24 $g \cdot mL^{-1}$) were prepared in a buffer solution of acetic acid (0.30 M)/sodium acetate (0.20 M) at 25°C. The flow times of the pure solvent and the solutions of Qs were recorded to calculate the intrinsic viscosity. The viscosity average molecular weight was determined with the Mark–Houwink–Kunh–Sakurada equation (MHKS). Under these conditions, the parameters of the constants are known: $K_m = 7.4 \times 10^{-4}$ $cm^3 \cdot g^{-1}$ and $a = 0.76$, reported by Rinaudo et al. [17].

2.2.4. Mv and AD of the Depolymerized Chitosan (QsD). The QsD was filtered in a permeation unit adapted with nitrocellulose membranes in a Buchner porous glass funnel

conditioned to a vacuum system. The QsD was washed with two volumes of 15 mL of the starting buffer solution for depolymerization. The elimination of the salts was carried out by dialyzing the filtered residues in distilled water at 10°C for 8 h, changing the water every 90 min. The AD and the flow time were determined to calculate the Mν of the QsD following the methodology used with the Qs of origin.

2.2.5. Preparation of Membranes. The membranes were prepared with a 1% (w/v) solution of Qs and QsD, in 0.1 M acetic acid and 0.1% v/v glycerin. The system was kept under vigorous agitation on a Vortex Genie 2 instrument at 70°C for 2 h. The solution was deposited in glass Petri dishes. The membranes were formed by evaporating the solvent at 50°C for 18 h. After the heat treatment, the membranes were washed in 4% NaOH solution and washed finally with deionized water; additionally, they were degassed in an ultrasonic bath for 15 min. The thickness of the dry membranes was determined with a METROLOGY EM-9001N high-sensitivity digital outdoor micrometer; 15 measurements were made considering the entire surface of the membrane, and the average corresponds to the thickness.

2.2.6. Antioxidant Functionality. To evaluate the functionality of the Qs and QsD membranes, they were first homogenized in 80% methanol solution in a ratio of 1 : 10 (w/v), at 10°C for 12 min; they were centrifuged for 5 min, and the precipitate was collected on a cellulose filter paper (Whatman grade 595). The determination of the antioxidant activity was carried out by the method based on the capture of free radicals DPPH (1,1-diphenyl-2-picryl-hydrazyl) proposed by Chen et al. [18], applying small modifications. Ethanol/DPPH solutions were prepared with the extract of the membranes at different concentrations (0.0004, 0.0006, 0.0008, 0.001, and 0.0012 g·mL^{-1}) in a 0.1 M acetic acid solution, with constant stirring, and incubated at room temperature for 60 min. The antioxidant activity was determined as a function of the absorbance of the solutions at 520 nm by the reduction of the DPPH radical using

$$\text{antioxidant activity} = \frac{1 - A_{\text{sample}}}{A_{\text{control}}} \times 100, \quad (1)$$

where A_{sample} is the absorption of the sample of Qs and QsD and A_{control} is the absorption of the control sample of DPPH/CH_3COOH 0.1 M.

2.2.7. Antimicrobial Functionality. The antimicrobial activity was evaluated against different pathogenic microorganisms for humans and other phytopathogens: *Staphylococcus aureus*, *Pantoea ananatis*, *Pseudomonas aeruginosa*, *Raoultella planticola*, and *Escherichia coli*. The double layer technique was used to plant the microorganisms; they were grown in TESMA medium and in Luria–Bertani (LB) broth for 24 h at 30°C with constant agitation at 150 rpm [19]. The membranes of Qs and QsD were placed in a 1% acetic acid solution and placed in Petri dishes. The cultures of the microorganisms were adjusted to an optical density of 0.05 to 620 nm; 300 μL of the bacterial culture was inoculated in 5.7 mL of the TESMA medium and LB at 45°C for 24 h. They were homogenized and poured on the first agar layer where the Qs and QsD membranes were placed. 0.25% acetic acid was used as a negative control. To quantify the microbial populations, the preinoculum of each strain was made in TESMA liquid one day before. The cells were then washed with 10 mM $MgSO_4 \cdot 7H_2O$ solution. Three different treatments were carried out: inoculation of type strains in pure Qs solution, inoculation of type strains in QsD solution, and inoculation of type strains in 0.25% acetic acid solution. All strains were inoculated in test tubes with 4 mL of TESMA and LB broth and incubated at 30°C with constant shaking for 21 h. Finally, the count of the microbial populations was performed using serial dilutions [20, 21].

3. Results and Discussion

3.1. Depolymerization of Qs. The conditions for the Qs depolymerization reaction were determined and are listed in Table 1.

3.2. Optimal Temperature. The depolymerization was achieved at 51.4°C (Figure 1). An increase in the rate of degradation of the substrate is observed when the temperature is below this value. On the other hand, due to the protein nature of the enzymes, with the increase in temperature, the enzyme complex tends to decrease its activity gradually. This result agrees with that obtained by Liao et al. [22] for xylanase; for cellulase, Gao et al. [23] reported stability at 50°C for 2 h, maintaining close to 90% of their residual activity. Liu et al. [24] confirmed that the enzymatic activity of β-glucanase is stable in a range of 50–70°C for a long time.

At temperatures above 55°C, it is observed that the stability of the enzymes drops below 50% of the initial activity; this is due to the fact that their tertiary structure is modified, losing their active and functional conformation, at 50°C [25].

3.3. Optimum pH. It was observed that, at a pH close to neutral, the solubility of Qs was very low, so it was adjusted between 3.5 and 6.5 to promote homogeneous conditions and ensure the verification of the depolymerization. In Figure 2, it is shown that, at pH 4.8, the activity is maximum; as the pH increases, the performance of the system tends to decrease gradually.

This result coincides with that reported for β-glucanase; the purified enzyme is highly stable at pH 5 maintaining more than 90% of its initial activity [26]; Liao et al. [22] found the maximum stability for cellulase and xylanase in a pH range of 4 to 9. Favorably, the three enzymes are highly stable at acidic pH and, likewise, their efficiency decreases at a pH close to neutral. The loss of stability and functionality depends on its spatial structure. If the structure is modified by chemical changes such as pH, the bonds that make up the tertiary structure of the enzyme are altered and this causes the protein not to fulfill its catalytic function efficiently.

Table 1: Determined chemical conditions for the enzymatic depolimerization.

Celuzyme XB® concentration	2.15×10^{-3} mg·mL^{-1}
E : S quotient	1 : 10
pH	5.3
Temperature	51°C
Incubation time	120 min

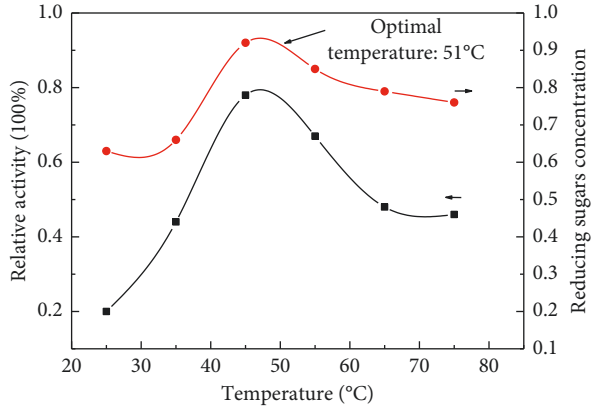

Figure 1: Enzyme complex activity as a function of temperature.

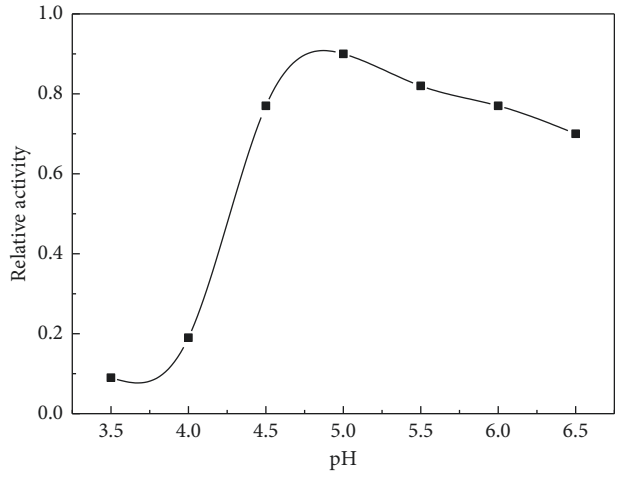

Figure 2: Relative activity of the enzyme system as a function of the pH. After pH = 4.8, the enzymatic degradation begins.

3.4. Optimal Concentration of Celuzyme XB®. At concentrations higher than 1 mg·mL^{-1} of the enzyme system, degradation is maximal, and up to a limit value of 2.75×10^{-3} mg·mL^{-1}, the synergistic effect of the enzyme system can be considered constant [27]; after this value, the concentration of reducing sugars is constant (Figure 3). A substrate concentration of 1 : 10 was maintained. The degradation of the biopolymer increases with the increase in the concentration of the enzyme system; the likelihood of binding and catalysis of the glycoside bond also increase.

3.5. Optimum Time. The ideal time to verify the depolymerization was determined as a function of the relative concentration of reducing sugars. In Figure 4, it is observed

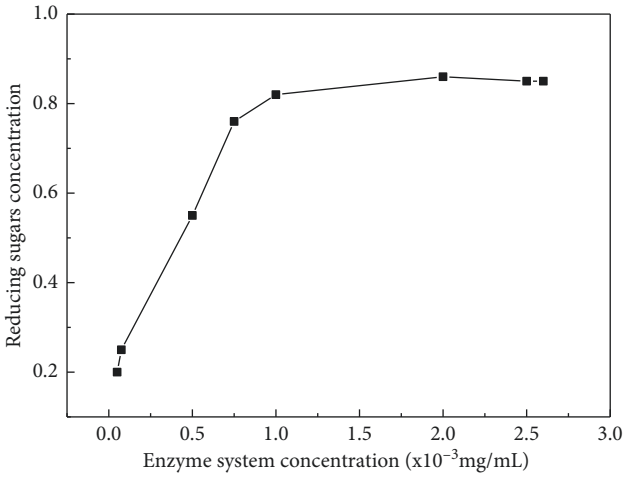

Figure 3: Reducing sugars concentration measures the synergic effect of the complex; up to 2.75×10^{-3} mg·mL^{-1}, there is no more effect.

that the concentration of sugars increased progressively up to 50% in the first 135 min. During this time, the viscosity decreases favoring a decrease of the Mv. After 4 h of reaction, the concentration of reducing sugars and Mv remain constant; this confirms that the diminution of enzymatic activity limits the depolymerization and the consequent obtaining of glycoside units [28].

3.6. FTIR Analysis. The characteristic bands of the functional groups of the Qs in the group frequencies region were identified, which confirms their chemical identity before and after depolymerization (Figure 5). At 3379 cm^{-1}, a band due to the stretching of the -OH group is observed, followed by the tension vibration at 3260 cm^{-1} of the NH bond; at 2877 cm^{-1}, the characteristic signals of the CH stretching are observed; at 1654 cm^{-1} appears the voltage vibration of the carbonyl group (C=O) of the amide; the signal at 1570 cm^{-1} corresponds to the flexion of the amine (NH), while the secondary absorption of the alkyl radical -CH_2 is found at the frequency of 1423 cm^{-1}. The absorption bands that complete the spectrum were identified in the region of the fingerprint: the CN voltage vibration at 1315 cm^{-1}, the symmetric COC stretch of the glycoside bond (1.079 cm^{-1}), and the pyranose ring vibrations (1023, 910, and 713 cm^{-1}). In both spectra, the characteristic signals are conserved without appreciable changes, confirming the conservation of the chemical phase of the QsD. Greater intensity is observed in the spectral signals of the QsD because the functional groups vibrate with greater freedom of movement because the steric hindrance of the polymer chain decreases. The increase in the intensity of the signal at 3379 cm^{-1} is due to a greater number of OH groups, while the intensity of the signal at 3260 cm^{-1} is associated with the greater number of NAcGlc units due to the increase of the AD [27]. The conservation of the signals of absorption of the glycoside bond and the pyranosic ring confirms that the mechanism of action of the enzyme complex is selective at the site of the β (1-4) glycoside bond. The synergy of the three enzymes highly specific to the substrates Qs and QsD is checked, which favors the obtaining of fragments of NAcGlc and NGlc.

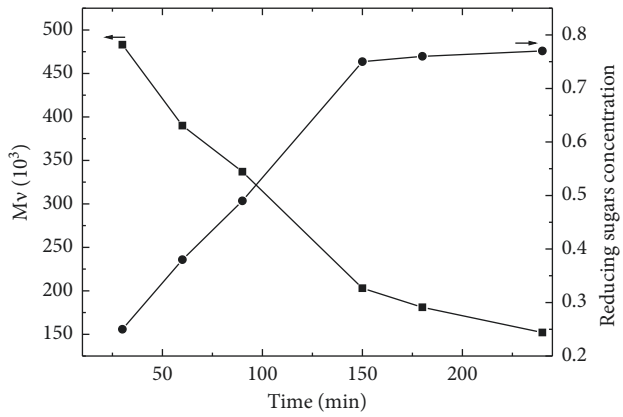

Figure 4: Effect of the time in the depolimerization developed by the enzyme system.

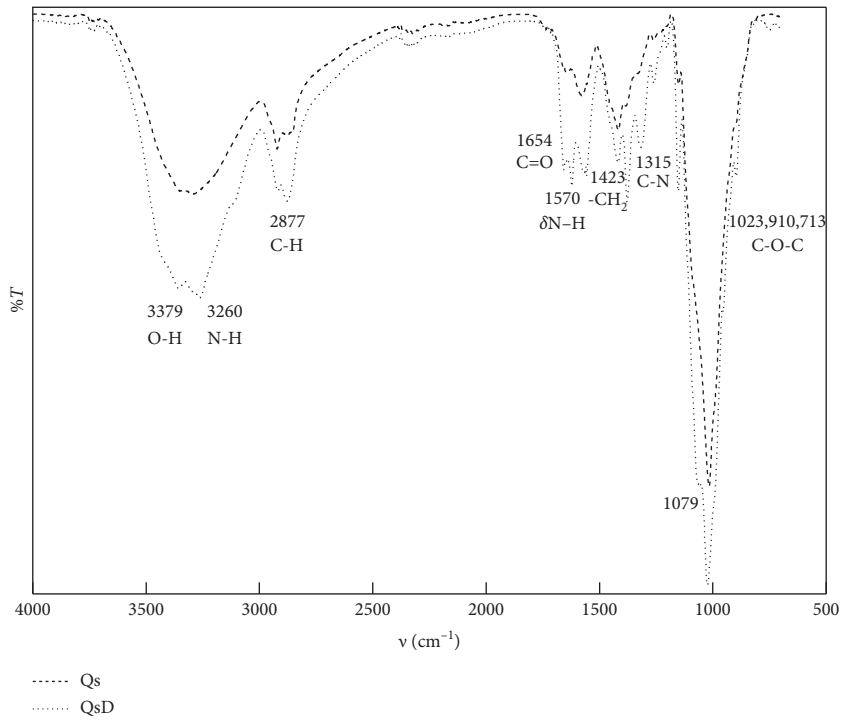

Figure 5: FTIR spectrum of Qs and QsD; in both, there exist the characteristic groups.

3.7. Mν and AD. The Mν of Qs and QsD was determined considering the polydispersity and the AD of the polymer. The molecular weights are the result of the contribution of short chains of units of NAcGlc and NGlc through the β-glycoside bond (1,4); the decrease in Mν is related to the change in the viscosity of the solutions after depolymerization, due to the reduction in the size of the polymer chain. The distribution of the small units of NAcGlc and NGlc in solution is randomized, which allowed accounting for a greater number of acetylated fragments (AD) in the QsD (Table 2). It is also possible to assume that QsD is formed by a heterogeneous mixture of oligosaccharides of different degrees of polymerization and these small chains present sequences with a greater number of acetylated units.

3.8. Functionality of Qs and QsD Membranes. Membranes were obtained with Qs and QsD of average thicknesses 28 and 17 μm, respectively.

3.8.1. Antioxidant Functionality. The antioxidant activity grows with the increase in the concentration of chitosan (Figure 6). In the Qs, the DPPH reduction barely exceeds 10%; its compact structure promotes the high viscosity in solution, and under these conditions, the binding force of intramolecular hydrogen weakens the activity of the hydroxyl and amine groups, limiting their reduction capacity [29]. On the contrary, in the QsD, the antioxidant activity is four times higher; the existence of a greater quantity of fractions of NGlc and NAcGlc increases the functionality

Table 2: Main characteristics of chitosan before and after the enzymatic hydrolysis.

Sample	AD (%)	$M\nu$ (10^3 g·mol^{-1})
Qs	11	152
QsD	15	64

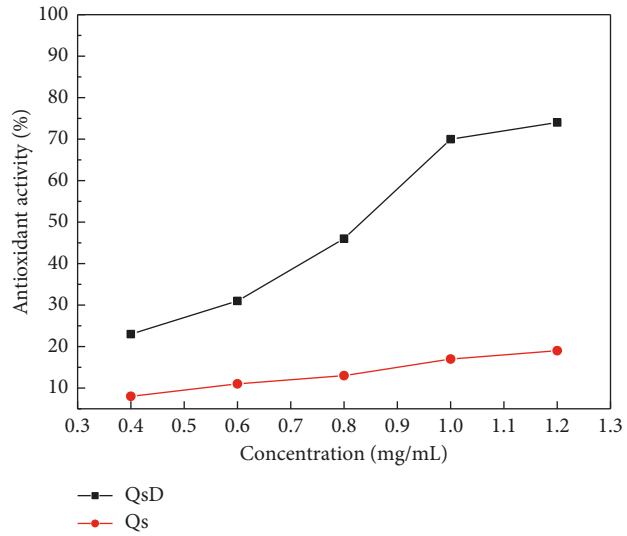

Figure 6: Antioxidant activity of Qs and QsD, which measures their capacity to trap the DPPH.

through the action of nitrogen that has a single pair of electrons and can be attached to a proton released from the acid solution to form NH_3^+ groups [30]. It is also possible to yield the proton of the hydroxyl groups of the glucopyranose ring since there are a greater number of fractions of lower $M\nu$ [31]. The use of the DPPH radical exhibits a reduction favored by some functional groups or atoms of the chitosan molecule that behaves as an electron donor. Based on the obtained results, it is suggested that the mechanism of antioxidant activity is related to hydroxyl groups. So, the contribution of a lower AD to the possibility of a greater ability to trap free radicals is not relevant; that is, it is not associated with the greater or lesser presence of amine groups that can yield their binding electrons. The mechanism of antioxidant activity in chitosan is not very clear at this time. What seems to be constant is the relationship of a lower $M\nu$ with a sensitive capacity to neutralize DPPH radicals [32], as shown in Figure 6.

3.8.2. Antimicrobial Functionality. Different hypotheses have been proposed about the mechanism of inhibition of microbial cells by chitosan and its derivatives. The most accepted correlates the ability to alter the outer and inner membranes of the cell mediated by the polycationic nature of chitosan and the electronegative charges on the surface of the cell wall of microorganisms. Qs interferes with bacterial metabolism due to the possible penetration into the phospholipid bilayer of the cytoplasmic membrane with consequent perturbation of it and imminent cell death [33]. It can be observed (Figure 7) that the treatments in the control

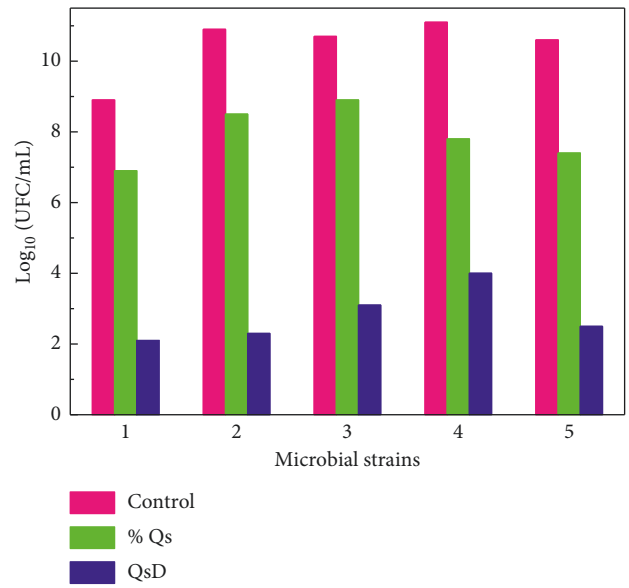

Figure 7: Antimicrobial activity of Qs and QsD against *Staphylococcus aureus* (1), *Pantoea ananatis* (2), *Pseudomonas aeruginosa* (3), *Rautella planticola* (4), and *Escherichia coli* (5).

sample with culture broth and absence of Qs did not present any inhibitory effect on the microorganisms, with values greater than 9E + 10 CFU mL^{-1} after 24 h of incubation in all cases.

In general, the results of the antimicrobial activity of QsD show an important ability to inhibit the growth of all bacteria. In the sample of Qs, inhibition in bacterial growth decreased two orders of magnitude (in UFC·mL^{-1}) against all microorganisms with respect to the control. The high molecular weight and poor solubility of Qs impede the activity of bacteria; based on this behavior, Benhabiles et al. [34] classified Qs as a bacteriostatic substance. *Staphylococcus aureus* revealed to be more susceptible to pure Qs than Gram-negative bacteria; this is due to the structural difference of its cell wall. Cells present an external barrier that the Qs would have to overcome [35], as well as a high number of negative charges on its wall surface that gives them greater hydrophobicity [36]. In addition, the number and order of fragments of NAcGl and NGlc could have an effect, as reported by Vishu Kumar et al. [37]. The high bacterial inhibition of QsD is associated with the AD of 14–19%, with the protonation of the amine groups. In *Staphylococcus aureus*, the cell membrane is covered by a wall formed by 30–40 layers of peptidoglycans containing NAcGlc and *N*-acetylmuramic acid as well as d- and l-amino acids, including isoglutamate and teicoic acid; these can bind to the amine groups in the QsD that is positively charged and promote a distortion-disruption of the cell wall, followed by exposure of the cell membrane to osmotic shock and exudation of the cytoplasmic content. In contrast, *Rautella planticola* showed greater resistance to the effect of inhibition at the same time and counting conditions, followed by *Pseudomonas aeruginosa*, *Pantoea ananatis*, and *Escherichia coli*; these microorganisms contain an outer membrane in which lipopolysaccharide

and proteins are maintained. The repetitive units of negatively charged oligosaccharides form an ionic bond with the amino groups of QsD (which are cationic in nature below pH 6.2), thus blocking the flow of nutrients with bacteria that lead to death upon exhaustion. The smaller molecular mass of QsD and its greater electronegativity facilitate the union of Gram-negative bacteria, blocking the flow of nutrients that leads to cell lysis. The QsD of lower molecular weight and higher DA has advantages as an antimicrobial agent due to its higher activity and because it is more soluble than the Qs of origin; therefore, a high percentage of free amino groups and the presence of acetylated sequences are necessary in these molecules to exert a good antibacterial capacity.

4. Conclusions

In this work, the study of the depolymerization of chitosan using the enzymatic complex Celuzyme XB® has been reported; it is composed of cellulase, xylanase, and β-glucanase. This enzymatic complex is currently on the market, and its immediate use is possible. The optimum conditions of temperature, pH, and concentration that give rise to the depolymerization reaction were determined. The products derived from the enzymatic depolymerization are quito-oligosaccharides formed by short units of NAcGlc and NGlc that exhibit a decrease in their viscosity reflected in the reduction of Mν. The functionality of the Qs and QsD samples was determined by Mν, viscosity, and AD. In turn, these characteristics affect the affinity between enzymes and Qs for their depolymerization to occur. The Qs of origin has the lowest reducing power compared to its depolymerized derivative, verifying the relationship of a lower Mν with a sensitive antioxidant functionality. The QsD has better antibacterial properties, so it could work as an effective combat agent to counteract the growth of at least these five microorganisms; thus, it would be of potential application in the agrifood and clinical area.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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