



**HAL**  
open science

## $\beta$ -chitosan-clay films: Characterization and antibacterial study using response surface methodology

Mohamed Hajjaji, Abdelhakim Alagui, Nicolas Joly, Patrick Martin

### ► To cite this version:

Mohamed Hajjaji, Abdelhakim Alagui, Nicolas Joly, Patrick Martin.  $\beta$ -chitosan-clay films: Characterization and antibacterial study using response surface methodology. *Polymers from Renewable Resources*, In press, 10.1177/20412479221128967 . hal-03788046

**HAL Id: hal-03788046**

**<https://hal.science/hal-03788046>**

Submitted on 28 Sep 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



## **$\beta$ -chitosan-clays films: Characterization and antibacterial study using response surface methodology**

Journal:	<i>Polymers from Renewable Resources</i>
Manuscript ID	PRR-22-0014.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Hajjaji, Mohamed; Cadi Ayyad University Faculty of Sciences Semlalia, Chemistry; University of Cadi Ayyad Alagui, Abdelhakim; Cadi Ayyad University Faculty of Sciences Semlalia, Chemistry Joly, Nicolas; Artois University, Unité Transformations & Agroressources Martin, Patrick; Artois University, Unité Transformations & Agroressources
Keywords:	$\beta$ -chitosan-clay films, structure, physical/mechanical properties, antibacterial activity, response surface methodology
Abstract:	Properties of the chitosan films can be improved by incorporating clay minerals. So, solvent-cast films of the $\beta$ -chitosan containing stevensite-rich or kaolinitic-illitic clays (up to 50 mass %) were characterized for their structural and mechanical properties. The effects of molecular weight (MW) and deacetylation degree (DD) of chitosan, and the clay/chitosan mass ratio on the inhibition growth of <i>Escherichia coli</i> and <i>Staphylococcus aureus</i> were studied using the response surface methodology (RSM). The films consisted of exfoliated/intercalated or flocculated composites, and the electrostatic bonds formed between the functional moieties of the chitosan and the clay particles active sites essentially influenced their mechanical strength. The results of the study using RSM showed that the optimal value of MW required for the inhibition of the bacteria varied according to the film used, and high antibacterial activity necessitated high DD (89-97%).

SCHOLARONE™  
Manuscripts

1  
2  
3  **$\beta$ -chitosan-clays films: Characterization and antibacterial study**  
4  
5 **using response surface methodology**  
6  
7

8 Mohamed Hajjaji<sup>1\*</sup>, Abdelhakim Alagui<sup>1</sup>, Nicolas Joly<sup>2</sup>, Patrick Martin<sup>2</sup>  
9

10  
11 <sup>1</sup>Laboratoire des Sciences des Matériaux et Optimisation des Procédés, Faculté des Sciences  
12 Semlalia, Université Cadi Ayyad, B.P. 2390, Av. Pce My Abdellah, Marrakech 40001,  
13 Morocco.  
14

15 <sup>2</sup> Unité Transformations & Agroressources- ULR7519, Univ. Artois, UniLasalle, F-62408  
16 Béthune, France.  
17

18  
19 **Abstract**

20  
21 Properties of the chitosan films can be improved by incorporating clay minerals. So, solvent-  
22 cast films of the  $\beta$ -chitosan containing stevensite-rich or kaolinitic-illitic clays (up to 50 mass  
23 %) were characterized for their structural and mechanical properties. The effects of molecular  
24 weight (MW) and deacetylation degree (DD) of chitosan, and the clay/chitosan mass ratio on  
25 the inhibition growth of *Escherichia coli* and *Staphylococcus aureus* were studied using the  
26 response surface methodology (RSM). The films consisted of exfoliated/intercalated or  
27 flocculated composites, and the electrostatic bonds formed between the functional moieties of  
28 the chitosan and the clay particles active sites essentially influenced their mechanical strength.  
29 The results of the study using RSM showed that the optimal value of MW required for the  
30 inhibition of the bacteria varied according to the film used, and high antibacterial activity  
31 necessitated high DD (89-97%).  
32  
33  
34  
35  
36  
37  
38  
39  
40

41 **Keywords:**  $\beta$ -chitosan-clay films, structure, physical/mechanical properties, antibacterial  
42 activity, response surface methodology  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57

58 \*Correspondence: hajjaji@uca.ma  
59  
60

## 1. Introduction

Chitosan is a deacetylated derivative of the chitin, which is a constituent of shellfish and crustaceans exoskeletons, among others. It is a copolymer composed of  $\beta$ -(1-4)-2-amino-D-glucose and  $\beta$ -(1-4)-2-acetamido-D-glucose units. Three polymorphic forms ( $\alpha$ ,  $\beta$  and  $\gamma$ ) of chitosan are identified.  $\beta$ -chitosan is more soluble and more reactive than  $\alpha$ -chitosan,<sup>1</sup> which is the most abundant form. Chitosan is suitable for many practical uses<sup>2-6</sup> because of its non-toxicity, biodegradability, film-forming character, and high density of positive charges associated with the protonated amino-groups. The chitosan exhibited a good antibacterial activity. Referring to some authors, the antibacterial activity of  $\beta$ -chitosan was higher than that of  $\alpha$ -chitosan.<sup>1</sup>

The inhibition processes depend on chitosan characteristics, mainly its deacetylation degree (DD) and molecular weight (MW), as well as on bacteria strain.<sup>7-9</sup> It can be summarized that the higher the DD, the better the inhibition. This fact is linked to the abundance of protonated amino-groups, and to their ability to interact with the specific functional groups of cells membrane macromolecules.<sup>10</sup> The studies dealing with the influence of MW on the inhibition of bacteria growth, such as *Escherichia coli*, showed different results.<sup>10</sup> Disparate effects of MW on chitosan antibacterial properties were cited elsewhere.<sup>11</sup> According to Raoka et al.<sup>12</sup> the effect of MW on the antibacterial activity of chitosan is greater than that of DD.

Clay minerals, especially those of the smectite group, can be delaminated. So, nano-sized particles with high-aspect ratio are obtained. Compared to kaolinite and illite, smectite minerals have high cation exchange capacity (CEC) and surface area.<sup>13</sup> Owing to these characteristics and to their anionic character, these clay minerals are suitable materials for medical and cosmetic purposes.<sup>14</sup>

To improve mechanical and barrier properties of chitosan films for drug carrying and food packaging uses, the chitosan was mixed with limited amounts (up to about 5 wt%) of montmorillonite.<sup>15-17</sup> The addition of the montmorillonite (di-octahedral clay mineral of the smectite group) to chitosan resulted in the formation of nanocomposites with efficient bactericidal effects.<sup>16, 18, 19</sup> So far, no attention has been paid to the study of antibacterial activity of  $\beta$ -chitosan films containing stevensite or kaolinitic-illitic clays. Stevensite is a tri-octahedral swelling clay mineral, which belongs to the smectite group such as montmorillonite. So, chitosan association with limited amount of stevensite should result in intercalated and/or exfoliated nanocomposites formation. The lack of studies related to bacterial inhibition by chitosan-stevensite films could be due to stevensite scarcity. Kaolinite

1  
2  
3 71 and illite are non-expandable natural clay minerals,<sup>13</sup> and chitosan intercalation in the  
4 72 interlayer spaces of their pristine structures could not occur. Thereby, chitosan association  
5 73 with kaolinite or illite should lead to tactoids formation. In such a condition, kaolinite as well  
6 74 as illite behaves like conventional fillers. To obtain chitosan-kaolinite nanocomposites films,  
7 75 some authors prepared exfoliated kaolinite by mechano-chemical treatment.<sup>20</sup>

8  
9  
10  
11 76 The use of  $\beta$ -chitosan-montmorillonite films as bactericidal materials has been ignored  
12 77 in spite of the potential reactivity of  $\beta$ -chitosan, which consists of parallel chains with weak  
13 78 intermolecular bonds.<sup>21</sup> For the same amount of montmorillonite (up to 5 mass%),  $\beta$  chitosan-  
14 79 based films showed higher tensile strength and Young's modulus than those of  $\alpha$  chitosan-  
15 80 based films, and both films manifested similar water solubility.<sup>22</sup>

16  
17  
18  
19  
20 81 The influences of chitosan characteristic (DD and MW) and clay/chitosan ratio  
21 82 changes on the inhibition of pathogenic bacteria growth have not been investigated, probably  
22 83 because of the numerous experiences required. This drawback could be overcome by using  
23 84 the response surface methodology.<sup>23</sup>

24  
25  
26  
27 85 In this study, the structure of solvent-casted films composed of  $\beta$ -chitosan and  
28 86 stevensite-rich clay (RH) or kaolinitic-illitic clay (BN2) was examined. Moreover, the  
29 87 changes of the physical/mechanical properties of films according to clay additions were  
30 88 followed. In addition, the growth inhibition of the most prevalent bacteria (*Escherichia coli*  
31 89 and *Staphylococcus aureus*) according to chitosan DD, chitosan MW, and clay/chitosan ratio  
32 90 was assessed using the RSM.

33  
34  
35  
36  
37  
38 91

## 39 92 **2. Materials and Methods**

### 40 93 **2.1. $\beta$ -chitosan**

41  
42  
43 94 The  $\beta$ -chitosan was prepared from the chitin, extracted from the pens of local squid.  
44 95 For this purpose, finely ground squid pens were demineralized with HCl solution (0.55 N) and  
45 96 deproteinized with heated NaOH solution (80 °C, 0.33 N). The pens powder/solution ratio  
46 97 was 1:10 (w/v). To prepare chitosan, chitin was deacetylated for 24 h using NaOH solution  
47 98 (40 mass%) and standard reflux setup.<sup>24</sup> The X-ray diffraction pattern of the prepared chitosan  
48 99 is shown in Fig.1. Taking into consideration the latter trace, and using the Scherrer's equation  
49 100 and the crystallinity index determination method given in Focher et al.,<sup>25</sup> the chitosan  
50 101 crystallinity index and apparent crystal size were found to be 77 and 2.1 nm, respectively.

51  
52  
53  
54  
55 102 Referring to the thermal curve shown in Fig. 2, the chitosan was thermally stable up to  
56 103 about 266°C.

The molecular weight (MW) of the chitosan was calculated using the Mark-Houwink equation.<sup>26</sup>

$$[\eta] = K(MW)^a \quad (1)$$

$\eta$  is the intrinsic viscosity, K and a are determined constants. The deacetylation degree (DD) of the chitosan was determined by potentiometric titration following the experimental procedure in Tolaimate et al.<sup>24</sup>, and using the following equation.<sup>27</sup>

$$DD = \frac{203(V_2 - V_1)N}{m + 42(V_2 - V_1)N}$$

$V_1$  and  $V_2$  are the volumes of NaOH solution at the first and the second neutralization points.

$N$  is the strength of the alkali solution used.  $m$  is the weight of the chitosan sample.

## 2.2. Clays

Clays, referenced as RH and BN2, were taken from clay pits located at Jbel Rhassoul (Midelt province, Morocco) and Tetouan-Hoceima province (Morocco), respectively. They consisted of stevensite and an assemblage of kaolinite and illite, respectively. Quartz and carbonates were the main ancillary minerals found. The chemical compositions of RH and BN2 are given in Table 1, and their cation exchange capacities, determined by the cobaltihexamine chloride method<sup>28</sup> were 0.8 and 0.3 meq/g, respectively. The decarbonation of clays was done with a buffer acetic acid/Na-acetate solution (pH = 4.74). The carbonate-free clays (particles size < 80  $\mu\text{m}$ ) were sodium-saturated,<sup>24</sup> and oven dried at 105 °C.

## 2.3. Chitosan-clay films

Chitosan-clay films (clay/chitosan mass ratio  $\leq$  100%) were prepared with the solvent casting technique.<sup>30</sup> For this purpose, sample ( $\leq$  1 g) of the sodium-saturated clay was dispersed in 5 mL of acetic acid (1% v/v) solution, and stirred for 24 h. On the other hand, a portion of chitosan (1 g) was introduced in 100 mL of an acetic acid solution (1 %v/v), and stirred for 2 h. The limpid solution of chitosan was mixed with the clay dispersion, and the mixture was homogenized for 24 h. 25 mL of the mixture was poured in a Petri dish and kept at 30°C for 48 h, so to evaporate water. The film formed was retrieved and soaked with a solution of NaOH (0.5 M) for 12 h, rinsed with distilled water, and dried at 25 °C. The thickness of the films was of about 10  $\mu\text{m}$ .

## 2.4. Physical/mechanical properties of the film

The mass loss of films was determined by weighing disks (~3 cm diameter) before and after immersion in 50 mL of distilled water stirred at 350 rpm for 24 h. The tensile strength and the elongation at break of films were measured on strips according to the ASTM D882,<sup>31</sup> using an Instron universal machine operating with a crosshead speed of 50 mm/min. The measurements were done in triplicate.

## 2.5. Analysis techniques

Fourier-transform infrared (FT-IR) analyses were carried out on thin disks composed of 1mg sample and 99 mg KBr. The spectra were recorded with a Perkin Elmer 1725 spectrophotometer functioning in the range of 4000–400 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup>. The X-ray diffraction (XRD) analyses were performed on powder samples with a Philips X'Pert MPD diffractometer operating with a copper anode ( $\lambda_{K\alpha} = 1.5418 \text{ \AA}$ ). The step size ( $^{\circ}2\theta$ ) and the scan step time were set at 0.013 and 1 s, respectively.

## 2.6. Percentage inhibition of bacteria growth

To determine the percentage inhibition (PI) of bacteria growth, a bacteria suspension (considered as a blank suspension) was adjusted to 10<sup>5</sup>-10<sup>6</sup> CFU/mL using sterile saline water (0.9% w/v). A film sample (9 cm<sup>2</sup>) was introduced in a tube containing 5 mL of nutrient broth, and autoclaved at 120 °C for 20 min. Then, the tube was inoculated with 0.5 mL of the bacteria suspension, and incubated at 37 °C for 24 h. The optical density (OD) of the incubated and the blank suspensions were measured by using an UV-visible spectrophotometer operating at 620 nm.<sup>32</sup> These data were used to calculate PI:

$$PI (\%) = \frac{OD \text{ of the blank suspension} - OD \text{ of the incubated suspension}}{OD \text{ of the blank suspension}} \times 100 \quad (2)$$

## 2.7. Effects of the chitosan characteristics and the clay/chitosan ratio on the growth inhibition of the bacteria

The weights of the effects of clay/chitosan mass ratio (R), DD and MW on the PI of bacteria were assessed using the RSM and adopting the following model:<sup>33</sup>

$$Y = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{1 \leq i < j}^3 b_{ij} X_i X_j \quad (3)$$

Where Y = PI, and b<sub>0</sub>, b<sub>i</sub>, b<sub>ii</sub> and b<sub>ij</sub> are constant. X<sub>1</sub>, X<sub>2</sub> and X<sub>3</sub> are the coded variables: X<sub>1</sub> = (R-R<sub>0</sub>)/ΔR, X<sub>2</sub> = DD-(DD)<sub>0</sub>/Δ(DD) and X<sub>3</sub> = MW-(MW)<sub>0</sub>/Δ(MW). Considering the



1  
2  
3  
4 169 factors domains investigated:  $0 \leq R \leq 100\%$ ,  $70 \leq DD \leq 100\%$ ,  $50000 \leq MW \leq 400000$  g/mol,  
5 170 the factors values at the centers of these domains, and the variation steps were:  $R_0 = 0.5$ ,  
6 171  $(DD)_0 = 85\%$ ,  $(MW)_0 = 225000$  g/mol, and  $\Delta R = 0.5$ ,  $\Delta(DD) = 15\%$ ,  $\Delta(MW) = 175000$   
7  
8 172 g/mol. The Doehlert design and the least-square regression method were adopted for the  
9  
10 173 determination of the constants.<sup>34,35</sup> The experiments planned according to the Doehlert design  
11  
12 174 and the PI measured ( $Y_1$ ,  $Y_2$ ) are given in **Table 2**.

13  
14 175 The validity of the polynomial models was assessed on the basis of the analysis of  
15 176 variance (ANOVA) and checked by the probability plot of residuals.

16  
17 177

### 18 178 **3. Results and discussion**

#### 19 179 **3.1. Structural characterization of the chitosan-clay films**

20  
21  
22  
23 180 As can be deduced from the typical XRD patterns shown in **Fig. 3A**, the association of  
24  
25 181  $\beta$ -chitosan with BN2 clay did not have impact on the position of the basal reflexions of illite  
26  
27 182 ( $2\theta = 8.78$ ) and kaolinite ( $2\theta = 12.48$ ). So, the interlayer space of both clay minerals  
28  
29 183 remained unchanged, and consequently intercalated composites did **not** occur in this case. So,  
30  
31 184 **tactoid structure took place.<sup>36</sup> Considering that the points of zero charge of chitosan and**  
32  
33 185 **chitosan-clay films (Table 1) were somewhat similar, the surface charge densities of these**  
34  
35 186 **materials were comparable. So, BN2 particles were layered with chitosan.**

36  
37 187 Referring to **Fig. 3B**, the basal X-ray reflexion of the stevensite ( $2\theta = 6.02$ ) vanished  
38  
39 188 as a result of the association of RH clay with  $\beta$ -chitosan. This fact was associated to stevensite  
40  
41 189 delamination caused by excessive expansion of the interlayer due to the excessive insertion of  
42  
43 190 chitosan chains. Such a phenomenon was also observed in the case of mixing montmorillonite  
44  
45 191 (smectite clay mineral) with chitosan.<sup>37</sup> **Stevensite particles, as was the case with BN2**  
46  
47 192 **particles, were coated with chitosan because the  $pH_{PZC}$  of stevensite-containing films and**  
48  
49 193 **chitosan were close (Table 1). However, in this case exfoliated/intercalated structure was**  
50  
51 194 **formed.**

52  
53 195 Considering the FT-IR analyses shown in **Fig. 4A**, the spectrum of the film (b)  
54  
55 196 consisted of an additional band at  $1566\text{ cm}^{-1}$ , which is associated to the protonated amino-  
56  
57 197 groups of chitosan. In addition, the band at  $1417\text{ cm}^{-1}$ , which is attributed to the deformation  
58  
59 198 of the  $\text{CH}_2$  in the  $\text{CH}_2\text{OH}$  group of the chitosan, intensified. The bands related to the clay  
60  
199 199 minerals bonds were not modified. These results allowed the deduction that the functional



1  
2  
3 200 groups ( $-\text{NH}_3^+$  and  $\text{CH}_2\text{OH}$ ) of the chitosan were involved in its retention by clay particles  
4  
5 201 surfaces, which are negatively charged.

6  
7 202 The comparative examination of FT-IR spectra given in Fig. 4B showed that the band  
8  
9 203 at  $1420\text{ cm}^{-1}$  associated to the  $\text{CH}_2$  in the  $\text{CH}_2\text{OH}$  group of the chitosan was well distinguished  
10  
11 204 on the spectrum of the film (b), whereas the remaining bands were not affected. So, it was  
12  
13 205 believed that the primary hydroxyl group of chitosan developed hydrogen bonds with  
14  
15 206 stevensite particles, known as anionic clay mineral. These bonds played a key role in the  
16  
17 207 adsorption of the chitosan on the clay particles.

18  
19 208 SEM examinations of chitosan-BN2 films showed clay aggregates such as seen in Fig.  
20  
21 209 5A (micrograph a) together with embedded particles (Fig. 5A, micrograph b). In addition,  
22  
23 210 chitosan-rich zones were identified (Zone 1). These observations allowed the deduction that  
24  
25 211 chitosan-BN2 films consisted of segregated domains and the inter-aggregates spaces were  
26  
27 212 chitosan-rich zones. The SEM observations realized on chitosan-RH films revealed scarce  
28  
29 213 clay aggregates (Fig. 5B, micrograph b), and chitosan-coated clay particles such as shown in  
30  
31 214 Fig. 5B (micrograph b). The scarcity of clay aggregates was associated to the clay exfoliation  
32  
33 215 and the formation of exfoliated/intercalated composites, as previously mentioned.

## 32 216 33 217 **3.2. Physical/mechanical properties and thermal stability of the films**

### 34 218 **3.2.1. Films mass loss**

35  
36 219 The mass loss of films containing up to about 0.15 g of RH/g of chitosan was  
37  
38 220 somewhat constant ( $3.5 \pm 0.7\text{ mass}\%$ ) (Fig. 6, curve a). However, it increased almost  
39  
40 221 linearly with the increase of clay content, and the increment was of  $10.3x\text{Mc}$  (Mc : mass of  
41  
42 222 the clay added). Considering chitosan and clay characteristics (DD = 90%, CEC (cation  
43  
44 223 exchange capacity) =  $0.8\text{ meq/g}$ ), the estimated amount of glucosamine unit per one gram of  
45  
46 224  $\beta$ -chitosan was of  $5.56 \times 10^{-3}$  mole, and the quantity of clay active sites was of  $8 \times 10^{-4}\text{ mol/g}$ .  
47  
48 225 Hence, the portion of chitosan bonded to RH particles (0.15 g) was not significant ( $\sim 2\%$ ), and  
49  
50 226 the clay particles were thick-coated with chitosan. In the clay-rich films, a portion of clay  
51  
52 227 particles was scarcely coated with chitosan. So, in such a condition, the clay particles were  
53  
54 228 easily lost.

55  
56 229 The mass loss of chitosan-BN2 films, which consisted of up to 0.5 g BN2 per g  
57  
58 230 chitosan, was almost constant (Fig. 6, curve b). However, it increased linearly in the case of  
59  
60 231 clay-rich films. In this case, the mass loss per gram of clay was estimated to be 10.5.

61  
62 232 Taking into consideration the DD of chitosan and the CEC of BN2 clay ( $0.3\text{ meq/g}$ ),  
63  
64 233 together with the fact that the hydroxyls of chitosan were implicated in the association of film

1  
2  
3 234 constituents, the fraction of chitosan involved in the binding process was estimated to be 10%  
4 235 for the film containing 0.5 g BN2. Thus, a high amount of chitosan was stacked over the clay  
5 236 particles, and flocculated composite formed.

6  
7  
8 237 In view of the above results, the abundance of flocculated composites in chitosan-clay  
9 238 films contributed to the reduction of clay particles loss.

### 10 239 11 12 239 13 240 **3.2.2. Thermal stability of the films**

14  
15 241 The thermal curves of chitosan-BN2 and chitosan-RH films displayed endothermic  
16 242 peaks at 89 and 92°C, and exothermic effects at 277 and 280°C, respectively (Fig. 2). The  
17 243 endotherms were associated to the loss of physisorbed water. The exotherms were assigned to  
18 244 the chitosan decomposition. The relative thermal stability shown by clay-containing films  
19 245 could be associated to the clay characteristics (nanosized structure, high aspect ratio) and to  
20 246 their barrier effect.<sup>38</sup>

### 21 22 247 23 24 25 26 247 27 248 **3.2.3. Mechanical properties**

28  
29 249 Small additions of RH (< ~0.14 g/g chitosan, i.e. 12 mass%) to  $\beta$ -chitosan improved  
30 250 the Young's modulus as well as the tensile strength of films (Fig. 7A), but they led to the  
31 251 reduction of the elongation at break and the tensile energy absorption (Fig. 7B). Excessive  
32 252 additions (> 20 wt %) resulted in the decline of the films stiffness.

33  
34  
35  
36 253 The tensile strength of the chitosan-BN2 films containing up to about 0.25 g clay /g  
37 254 chitosan increased with respect to that of the chitosan film (Fig. 8A), whereas the Young's  
38 255 modulus (Fig. 8A), the elongation at break and the tensile energy absorption (Fig. 8B)  
39 256 fluctuated. For clay-rich films, the mechanical properties declined for the reasons given  
40 257 below.

41  
42  
43  
44 258 In overall, the mechanical properties of polymer-based composites are affected by the  
45 259 inherent characteristics of the components used, the amount and the distribution of fillers, the  
46 260 interfacial bonding as well as by the processing methods.<sup>39</sup> Considering chitosan-RH films,  
47  
48 261 the improvement of films mechanical resistance was essentially due to the formation of  
49 262 hydrogen bonds between the chitosan and the delaminated clay particles. In fact, the  
50 263 formation of tough interfaces clay/chitosan facilitated the load transfer across the film, and  
51 264 consequently the stress was homogeneously distributed.<sup>40</sup> However, as the volume fraction of  
52 265 RH exceeded 1.6% (v/v), the clay addition had a detrimental effect on film mechanical  
53 266 resistance. It was believed that because of the swelling character of RH clay, the clay-rich  
54  
55  
56 267 films were the object of microcracks due to the drying shrinkage. The formation of such

defects together with the uneven distribution of the clay particles could be responsible for the decline of mechanical strength.

For BN2-containing films, the presence of the aforementioned electrostatic forces seemed to play a main role in the improvement of mechanical resistance of films with clay fraction < 3.2% (v/v). Further clay additions resulted in a drop of mechanical strength, possibly because of the flocculation and/or the heterogeneous distribution of clay particles.

### 3.3. Kinetics of the growth inhibition of the bacteria

Referring to the kinetics curves given in Fig. 9A, PI of the *E. coli* growth by the films approximated 90 to 100% after 24 h. The kinetics of the inhibition of the bacteria growth followed the pseudo-first order equation:

$$(PI)_t = (PI)_e e^{-kt} \quad (4)$$

(PI)<sub>t</sub> and (PI)<sub>e</sub> are the instantaneous and the equilibrium percentages inhibition of the *E. coli* growth, respectively, k and t are the rate constant and time. The estimated values of k were of 3.12x10<sup>-5</sup> s<sup>-1</sup> and 3.45x10<sup>-5</sup> s<sup>-1</sup> for RH- and BN2- containing films, respectively. Based on the linear evolution of  $PI = f(\sqrt{time})$  curves, the kinetics of the inhibition process was chiefly controlled by the diffusion process,<sup>41</sup> which seemed to be affected by the closest environment of cells (boundary layer). The diffusion process likely represented the leakage of vital intracellular substances, as the interaction between the chitosan and the negatively charged phospholipid bilayer of the bacterium has a detrimental effect on membrane permeability.<sup>10,18,19</sup>

Taking into consideration the curves shown in Fig. 9B, the kinetics of the inhibition of the *S. aureus* growth by the RH-chitosan film was relatively slow, but for both films, the change of the PI versus time fitted better the following linear form of the pseudo-second kinetics equation:

$$\frac{t}{(PI)_t} = \frac{t}{(PI)_e} + \frac{1}{k_2(PI)_e^2} \quad (5)$$

k<sub>2</sub> is the rate constant. (PI)<sub>t</sub>, (PI)<sub>e</sub> and t kept the same meaning. The values of k<sub>2</sub> were determined to be 3.17x10<sup>-2</sup> and 5.88x10<sup>-2</sup> /s.(%) for RH- and the BN2-based films, respectively.

In view of the above results, the kinetics of inhibition process was mainly dependent on the interaction between microorganisms and film constituents, mainly chitosan. Indeed, according to some authors,<sup>10</sup> the lipoteichoic acid, which is a constituent of the cell wall of the

gram-positive bacteria, such as the *S. aureus*, can interact with chitosan, and results in the disturbance of the cell membrane functions. The built up of chitosan around the cell wall might impede the entrance of nutriment and the release of residue by the cell. Therefore, cell metabolism was drastically altered. On the other hand, the PI of the *E. coli* was controlled by diffusion, seemingly owing to the loss of the selective permeability of the cell membrane. This fact was attributed to the interaction between the anionic groups of bacterial membrane lipopolysaccharide and the protonated amino-groups of chitosan.<sup>10</sup>

### 3.4. Evaluation of the effects of the factors studied on the bacteria inhibition

The ANOVA results (Table 3) showed that the F-ratio was somewhat high and the statistical significance was smaller than 0.05, which is considered as the maximum limit to assert the validity of the proposed model.<sup>42</sup> So, the variations of PI ( $Y_i$ ) of the bacteria by both films as a function of the coded variables related to R, DD and MW were adequately described by the following equations:

$$Y_1 (\text{Chitosan-RH}/E. coli) = 45.3 + 5.0 X_1 + 27.7 X_2 - 21.2 X_3 + 36.5 X_1 X_2 - 34.7 X_1 X_3 - 50.2 X_2 X_3 - 40.6 X_1^2 - 25.8 X_2^2 - 15.6 X_3^2 \quad (6)$$

$$Y_2 (\text{Chitosan-RH}/S. aureus) = 43.8 - 1.0 X_1 + 28.4 X_2 - 21.3 X_3 + 53.6 X_1 X_2 - 22.5 X_1 X_3 - 61.0 X_2 X_3 - 29.0 X_1^2 - 10.4 X_2^2 - 29.8 X_3^2 \quad (7)$$

$$Y_1 (\text{Chitosan-BN2}/E. coli) = 95.2 - 42.9 X_1 + 27.6 X_2 + 13.7 X_3 - 35.7 X_1 X_2 - 53.8 X_1 X_3 + 7.6 X_2 X_3 - 45.5 X_1^2 - 66.8 X_2^2 - 62.2 X_3^2 \quad (8)$$

$$Y_2 (\text{Chitosan-BN2}/S. aureus) = 97.2 - 6.4 X_1 + 39.2 X_2 + 57.1 X_3 - 5.1 X_1 X_2 - 2.0 X_1 X_3 - 18.5 X_2 X_3 - 9.8 X_1^2 - 65.0 X_2^2 - 54.2 X_3^2 \quad (9)$$

The validity of these models was also assessed by the probability plot of residuals.<sup>35</sup> The almost linear distribution of the residuals, shown in Fig. 10, supported the adequacy of these polynomial models.

The comparison of the algebraic values of the linear terms  $b_2 X_2$  (equations 6-9) pointed out that the increase of DD, which is correlated to the increase of the chitosan amino-groups amount, improved the inhibition growth of both strains of bacteria. This result was in conformity with the above discussion that is the amino-groups were the main functional moieties involved in the inhibition of the bacteria growth. Considering the linear terms of the equations 6 and 7, the weights of the studied factors effects on PI followed the order:  $DD > MW > R$ . Moreover, PI using chitosan-RH films decreased with the increase of MW. In contrast, it increased with the use of chitosan-BN2 films (equations 8 and 9). These results supported the disparate effects of MW on the bactericidal activity.<sup>10</sup> Recalling the above

1  
2  
3 335 equations, the weight of the effect of R on PI was relatively low, except in the case of the  
4  
5 336 equation 8 where the increase of R resulted in the decline of PI.

6  
7 337 Taking into consideration the  $b_{ij}X_iX_j$  terms of the equations 6, 7 and 9, the most  
8  
9 338 important influencing interaction was that occurring between DD and MW. This interaction  
10  
11 339 was categorized as an antagonistic one because the simultaneous rise of DD and the decrease  
12  
13 340 of MW or vice-versa should result in the increase of PI. A typical response surface  
14  
15 341 representing the antagonistic effect of the interaction between DD and MW is shown in Fig.  
16  
17 342 11a. In the case of the equation 8, R and MW interacted antagonistically and their interaction  
18  
19 343 had the prominent effect on PI. The response surface showing the variation of PI against R  
20  
21 344 and MW is given in Fig. 11b.

22  
23 345 The optimal operating factors for high inhibition of the bacteria growth, which were  
24  
25 346 determined by using the desirability function approach, are given in Table 4. Based on these  
26  
27 347 results, the films composed of chitosan with high DD should inhibit the growth of both  
28  
29 348 bacteria strains, as previously documented. In addition, the films consisting of about 40  
30  
31 349 mass% RH of chitosan with low to moderate MW should be highly effective against the  
32  
33 350 tested bacteria. In the case of the chitosan-BN2 films, the use of chitosan with high MW is  
34  
35 351 one of the operating conditions for high inhibition of the bacteria growth.

36  
37 352 Considering the  $PI = f(\text{clay/chitosan})$  curves plotted on the basis of the equations 6-9  
38  
39 353 (Fig. 12), the antibacterial effect of the chitosan film was less important as compared to that  
40  
41 354 of the chitosan-RH films, which consisted of exfoliated/intercalated composites. In contrast, it  
42  
43 355 was almost equivalent or somewhat better than that of the chitosan-BN2 films, which were  
44  
45 356 composed of tactoids.

#### 43 357 44 358 **4. Conclusions**

45  
46 359 Stevensite-containing chitosan films consisted of exfoliated/intercalated  
47  
48 360 nanocomposites, and the association between the films components were presumably  
49  
50 361 achieved through hydrogen bonding. Limited additions of RH (up to 0.14 g /g chitosan)  
51  
52 362 improved films tensile strength and Young's modulus, but they had a negative impact on their  
53  
54 363 elongation at break.

55  
56 364 Chitosan films containing kaolinitic-illitic clay were essentially composed of  
57  
58 365 flocculated composites. The amino-groups together with the hydroxyls of chitosan were  
59  
60 366 involved in its association with the clay particles. The addition of BN2 clay (up to 0.25 g  
367  
BN2/g chitosan) improved the mechanical strength of films.

1  
2  
3 368 The results of the kinetics of the bacteria growth inhibition by the studied films  
4  
5 369 allowed the deduction that the inhibition process depended on the bacterial strain. In the case  
6  
7 370 of *E. coli*, the cell membrane seemed to be damaged due to its interaction with the protonated  
8  
9 371 amino-groups of chitosan, and the vital substances flowed out. For *S. aureus*, the nutrients  
10  
11 372 seemed to be impeded to get into the cells because of the stack of chitosan over their  
12  
13 373 membranes.

14 374 The polynomial models obtained by using the RSM allowed the prediction of the  
15  
16 375 effects of the factors studied and of their mutual interactions on PI. The rise of MW resulted  
17  
18 376 in the decrease of the PI of *E. coli*, but it increased that of the *S. aureus*. The increase of DD  
19  
20 377 yielded to the rise of PI, and the optimal value should be in the range of 89-97%. Based on the  
21  
22 378 RSM results, the antibacterial activity of the chitosan film was a good as that of the tactoid-  
23  
24 379 rich films. However, it was less significant as compared to that of the films composed of  
25  
26 380 exfoliated/interacted clay.

## 381 382 **Acknowledgement**

383 This work was supported by the CNRST (grant number PPR/26/2015).

## 384 385 386 **References**

- 387 1. Van Hoa N, Vuong NTH, Minh NC, et al. Squid pen chitosan nanoparticles: small  
388 size and high antibacterial activity. *Polymer Bulletin* 2021; 78: 7313–7324.
- 389 2. Choi C, Nam JP and Nah JW. Application of chitosan and chitosan derivatives as  
390 biomaterials. *J Ind Eng Chem* 2016; 33: 1–10.
- 391 3. Dutta PK, Dutta J and Tripathi VS. Chitin and chitosan: Chemistry, properties and  
392 applications. *J Sci Ind Res* 2004; 63: 20-31.
- 393 4. Islam S, Rahman Bhuiyan MA and Islam MN. Chitin and chitosan: Structure,  
394 properties and applications in biomedical engineering. *J Polym Environ* 2017; 25:  
395 854–866.
- 396 5. Mahmoud MG, El Kady E and Asker, MS. Chitin, chitosan and glucan, properties and  
397 applications. *World J. Agri. Soil. Sci.* 2019; 3(1). DOI:  
398 10.33552/wjass.2019.03.000553.



- 1  
2  
3 399 6. Rinaudo M. Chitin and chitosan: properties and applications. *Prog Polym Sci* 2006;  
4 400 31: 603–632.
- 5  
6  
7 401 7. Walke S, Srivastava G, Nikalje M, et al. Physicochemical and functional  
8 402 characterization of chitosan prepared from shrimp shells and investigation of its  
9 403 antibacterial, antioxidant and tetanus toxoid entrapment efficiency. *Int J Pharm Sci*  
10 404 *Rev Res* 2014; 26(2): 215-225.
- 11  
12  
13  
14 405 8. Wang W, Xue C and Mao X. Chitosan: Structural modification, biological activity and  
15 406 application. *Int J Biol Macromol* 2020; 164: 4532–4546.
- 16  
17  
18 407 9. Confederat LG, Tuchilus CG, Dragan M, et al. Preparation and antimicrobial activity  
19 408 of chitosan and its derivatives: A concise review, *Molecules* 2021; 26: 3694.
- 20  
21 409 10. Kong M, Chen XG, Xing K, et al. Antimicrobial properties of chitosan and mode of  
22 410 action: A state of the art review. *Int J Food Microbiol* 2010; 144: 51–63.
- 23  
24  
25 411 11. Li J and Zhuang S. Antibacterial activity of chitosan and its derivatives and their  
26 412 interaction mechanism with bacteria: Current state and perspectives. *Eur Polym J*  
27 413 2020; 138: 109984. DOI: 10.1016/j.eurpolymj.2020.109984.
- 28  
29  
30  
31 414 12. Rajoka MSR, Mehwish HM, Wu Y, et al. Chitin/chitosan derivatives and their  
32 415 interactions with microorganisms: a comprehensive review and future perspectives.  
33 416 *Critical Reviews in Biotechnology* 2020; 40(3): 365-379.
- 34  
35  
36 417 13. Murray HH. Applied clay mineralogy-occurrences, processing and applications of  
37 418 kaolins, bentonites, palygorskite-sepiolite, and common clays, Development in clay  
38 419 science 2, Elsevier, 2007.
- 39  
40  
41 420 14. Carretero MI and Lagaly G. Clays and health: An introduction. *Appl Clay Sci* 2007;  
42 421 36: 1–3.
- 43  
44  
45 422 15. De Azeredo HMC. Nanocomposites for food packaging applications. *J Int Food Res*  
46 423 2009; 42: 1240–1253.
- 47  
48  
49 424 16. Han Y-Su, Lee S-H, Choi KH, et al. Preparation and characterization of chitosan–clay  
50 425 nanocomposites with antimicrobial activity. *J Phys Chem Solids* 2010; 71: 464–467.
- 51  
52  
53 426 17. Qu B and Luo Y. A review on the preparation and characterization of chitosan-clay  
54 427 nanocomposite films and coatings for food packaging applications. *Carbohydrate*  
55 428 *Polymer Technologies and Applications* 2021; 2: 100102. DOI:  
56 429 10.1016/j.carpta.2021.100102.
- 57  
58  
59  
60



- 1  
2  
3 430 18. Chandrasekaran M, Kim KD and Chun SC. Antibacterial activity of chitosan  
4 nanoparticles: A Review. *Processes* 2020; 8: 1173. DOI:10.3390/pr8091173.  
5 431  
6  
7 432 19. Kassem A, Ayoub GM and Malaeb, L. Antibacterial activity of chitosan nano-  
8 composites and carbon nanotubes: A review. *Sci Total Environ* 2019; 668: 566–576.  
9 433  
10 434 20. Neji AB, Jridi M, kchaou H, et al. Preparation, characterization, mechanical and  
11 barrier properties investigation of chitosan-kaolinite nanocomposites. *Polymer Testing*  
12 435 2020; 84: 106380. DOI: 10.1016/j.polymertesting. 2020.106380.  
13 436  
14 437 21. Kurita K, Tomita K, Tada T, et al. Squid chitin as a potential alternative chitin source:  
15 Deacetylation behavior and characteristic properties. *J Polym Sci Part A-1:*  
16 438 *Polym Chem* 1993; 31: 485-491.  
17 439  
18 440 22. Mourak A, Hajjaji M, Alagui A, et al. Effects of geomaterial-originated fillers on  
19 microstructure and mechanical/physical properties of  $\alpha$ - and  $\beta$ -chitosan-based films.  
20 441 *Molecules* 2021; 26: 7514. DOI: 10.3390/molecules26247514.  
21 442  
22 443 23. Khalfaoui A, Hajjaji M, Kacim S, et al. Evaluation of the simultaneous effects of  
23 firing cycle parameters on technological properties and ceramic suitability of a raw  
24 444 clay using the response surface methodology. *J Am Ceram Soc* 2006; 89 (5): 1563–  
25 445 1567.  
26 446  
27 447 24. Tolaimate A, Desbrières J, Rhazi M, et al. A. Contribution to the preparation of chitins  
28 and chitosans with controlled physicochemical properties. *Polymer* 2003; 44: 7939–  
29 448 7952.  
30 449  
31 450 25. Focher B, Beltranme PL, Naggi A, et al. Alkaline N-deacetylation of chitin enhanced  
32 by flash treatments. Reaction kinetics and structure modifications. *Carbohydr Polym*  
33 451 1990; 12: 405–418.  
34 452  
35 453 26. Maghami GG and Roberts GAF. Evaluation of the viscometric constants for chitosan.  
36 *Makromol. Chem.* 1988; 189: 195-200.  
37 454  
38 455 27. Gupta KC and Jabrail FH. Effects of degree of deacetylation and cross-linking on  
39 physical characteristics, swelling and release behavior of chitosan microspheres.  
40 456 *Carbohydrate Polymers* 2006; 66: 43–54.  
41 457  
42 458 28. Aran D, Maul A and Masfaraud J-F. A spectrophotometric measurement of soil cation  
43 exchange capacity based on cobaltihexamine chloride absorbance. *C. R. Geoscience*  
44 459 2008 ; 340 : 865–871.  
45 460

- 1  
2  
3 461 29. Lagaly G. Colloid clay science. In: Bergaya, F.; Theng, BKG.; and Legally, G.(eds)  
4 462 Handbook of Clay Science-Developments in Clay Science. Elsevier, 2006, p.141.  
5  
6 463 30. Tang ZG, Black RA, Curran JM, et al. Surface properties and biocompatibility of  
7 464 solvent-cast poly[e-caprolactone] films. *Biomaterials* 2004; 25: 4741–4748.  
8  
9 465 31. ASTM D882-18. Standard Test Method for Tensile Properties of Thin Plastic Sheeting;  
10 466 ASTM International: West Conshohocken, PA, USA, 2018.  
11  
12 467 32. Muloiwa M, Nyende-Byakika S and Dinka M. Comparison of unstructured kinetic  
13 468 bacterial growth models. *S Afr J Chem Eng* 2020; 33: 141–150.  
14  
15 469 33. El Hafid K, and Hajjaji M. Geopolymerization of glass- and silicate-containing heated  
16 470 clay. *Constr Build Mater* 2018; 159: 598–609.  
17  
18 471 34. Ferreira SLC, dos Santos WNL, Quintella CM, et al. Doehlert matrix: a chemometric  
19 472 tool for analytical chemistry-review. *Talanta* 2004 ; 63 : 1061–1067.  
20  
21 473 35. Mathieu D. Phan Tan Luu, R. Software NEMROD. Université d’Aix, Marseille III,  
22 474 France, 1980.  
23  
24 475 36. Qu B and Luo Y. A review on the preparation and characterization of chitosan-clay  
25 476 nanocomposite films and coatings for food packaging applications. *Carbohydrate*  
26 477 *Polymer Technologies and Applications* 2021; 2: 100102.  
27  
28 478 37. Darder M, Colilla M and Eduardo Ruiz-Hitzky E. Biopolymer–clay nanocomposites  
29 479 based on chitosan intercalated in montmorillonite. *Chem Mater* 2003; 15: 3774–3780.  
30  
31 480 38. Leszczynska A, Njuguna J, Pielichowski K, et al. Polymer/montmorillonite  
32 481 nanocomposites with improved thermal properties. Part II: Thermal stability of  
33 482 montmorillonite nanocomposites based on different polymeric matrixes. *Thermochim.*  
34 483 *Acta* 2007; 454 : 1–22.  
35  
36 484 39. Tjong SC. Structural and mechanical properties of polymer nanocomposites. *Mater Sci*  
37 485 *Eng R Rep* 2006; 53: 73–197.  
38  
39 486 40. Rhim JW, Hong SI, Park HM, et al. Preparation and characterization of chitosan-based  
40 487 nanocomposite films with antimicrobial activity. *J Agric Food Chem* 2006; 54: 5814-  
41 488 5822.  
42  
43 489 41. Ismadji S, Edi Soetaredjo F and Aning Ayucitra A. Clay materials for environmental  
44 490 remediation. Springer, 2015, p.113.  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4 491 42. Myers RH, Montgomery DC and Anderson-Cook CM. Response surface  
5 methodology: process and product optimization using designed experiments, 4th  
6 492  
7 Edition Joh.  
8 493

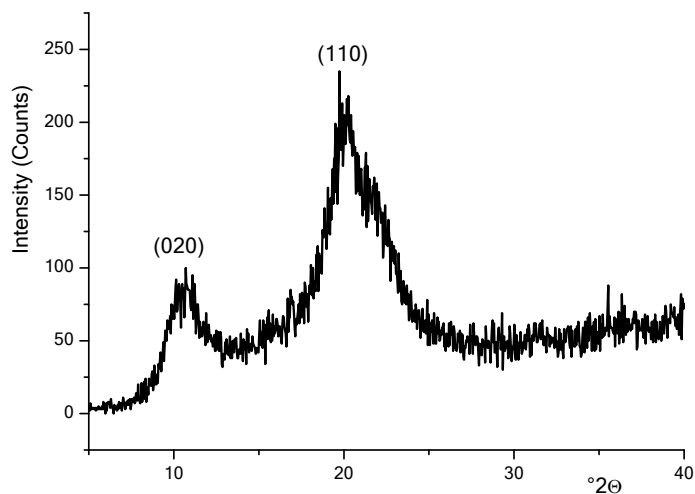
9 494

10 495

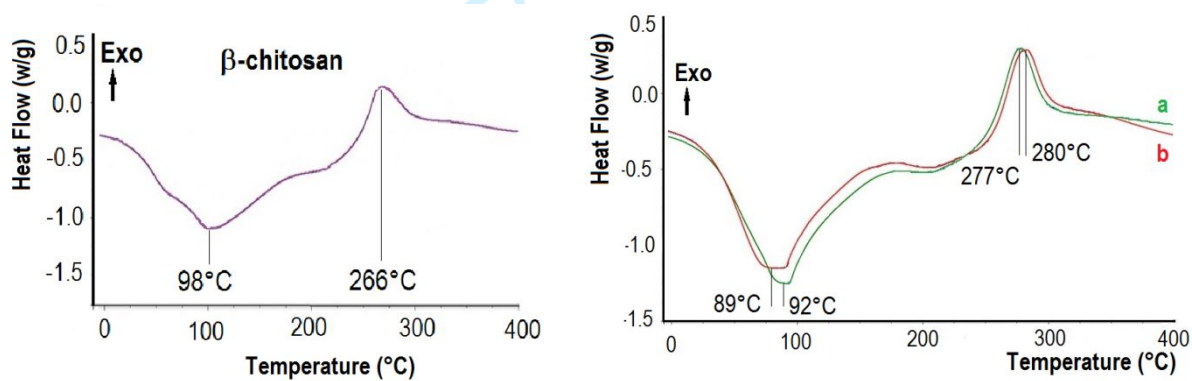
11 496

12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

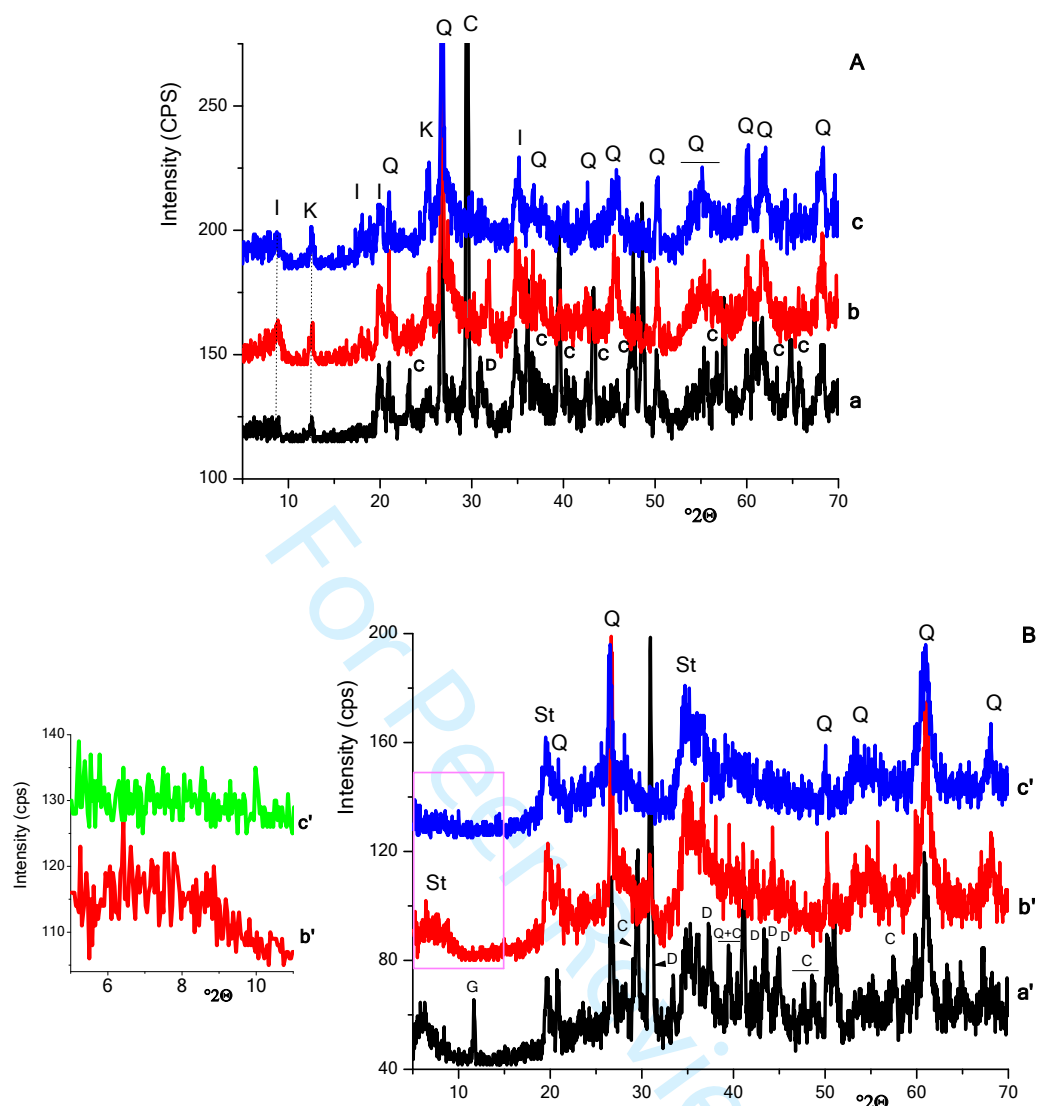
For Peer Review



**Figure 1.** X-ray diffraction trace of the prepared chitosan.

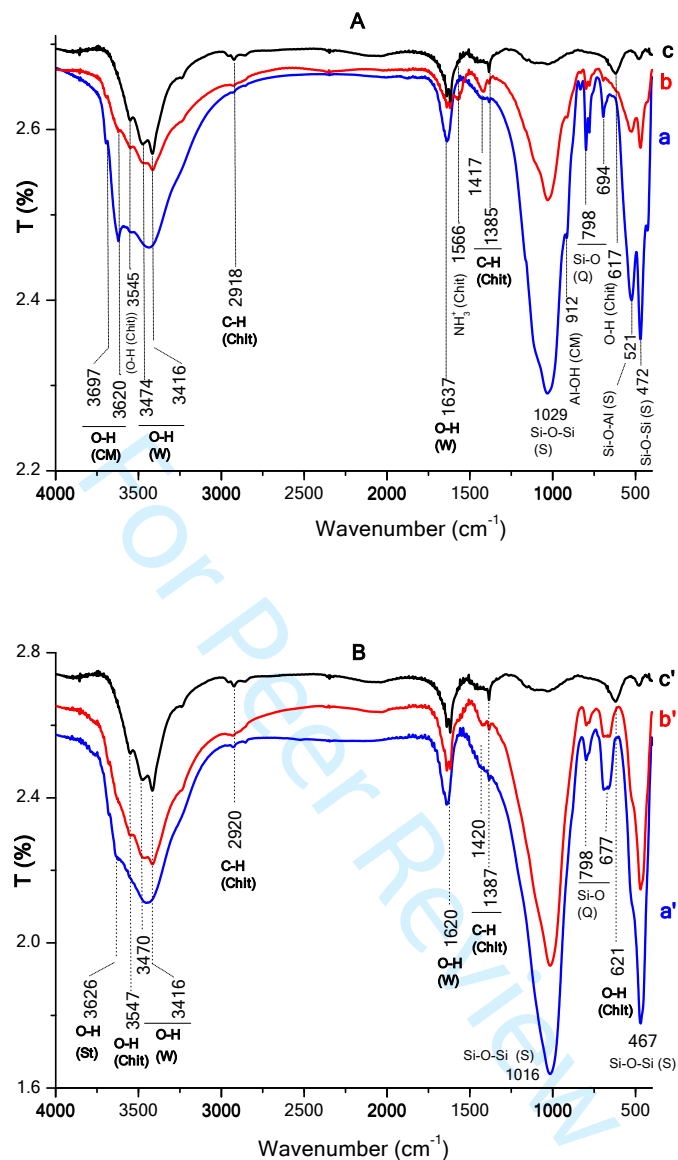


**Figure 2.** Thermal curves of the prepared chitosan, BN2- chitosan (a) and RH-chitosan (b) films. (clay/chitosan mass ratio: 0.25/1).



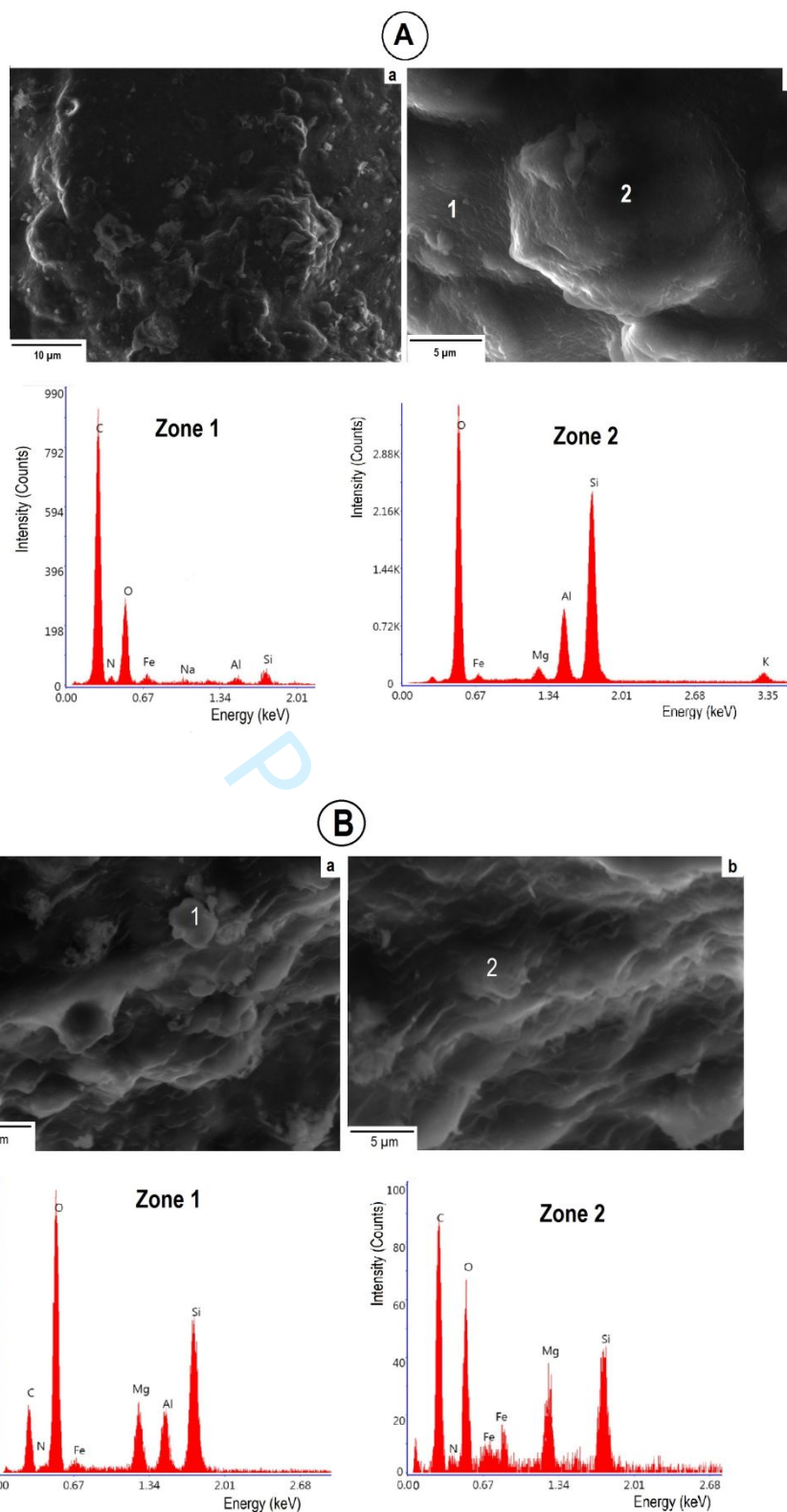
**Figure 3.** XRD patterns of the clays (**a**, **a'**), the sodium-loaded clay fractions (**b**, **b'**) and the chitosan-clay films (**c**, **c'**). (**A**) and (**B**) are associated to BN2 and RH, respectively. I: illite, K: kaolinite, Q: quartz, C: calcite, D: dolomite, St: stevensite, G: gypsum.

(clay/chitosan mass ratio: 1/1).



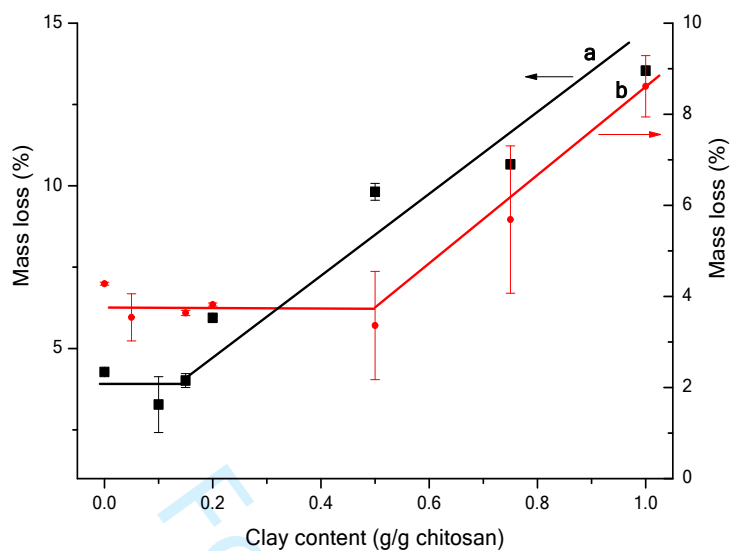
**Figure 4.** FT-IR spectra of the clay fractions (**a**, **a'**), the chitosan-clay films (**b**, **b'**), and the  $\beta$ -chitosan (**c**, **c'**). (**A**) and (**B**) are related to BN2 and RH, respectively. CM: clay minerals, Chit: chitosan, W: water, Q: quartz, S: silicates, St: stevensite.

(clay/chitosan mass ration: 1/1)

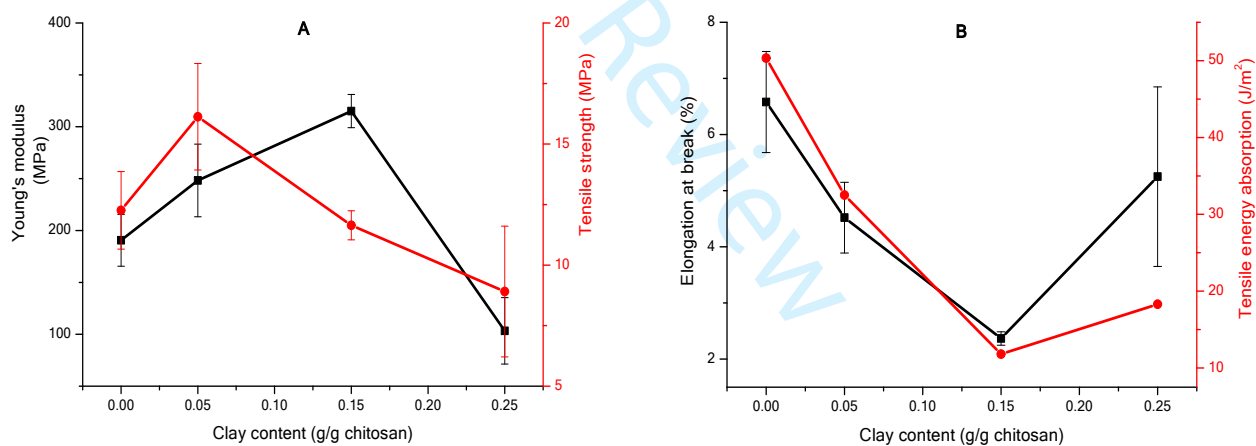


**Figure 5.** SEM micrographs of chitosan-BN2 (A) and chitosan-RH (B) films, and EDS spectra of different areas. Zone 1 (A) and zones 2 (B) are chitosan-rich domains. Zone 2 (A) and zone 1 (B) are clay-rich areas.

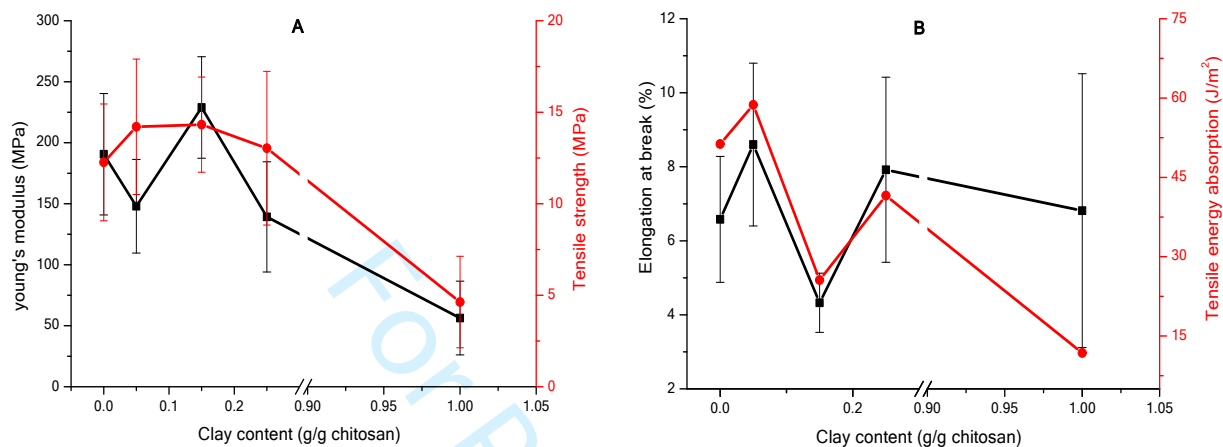




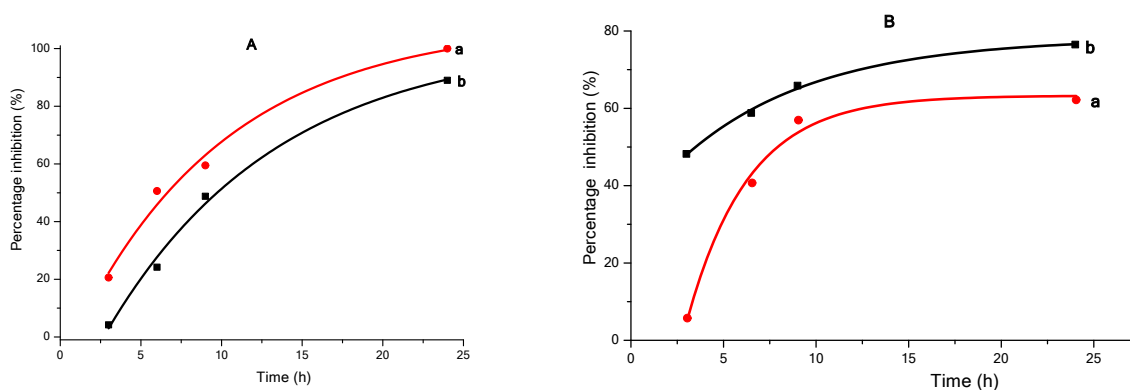
**Figure 6.** Variation of the mass loss of films vs. the clay content. (a) chitosan-RH film, (b) chitosan-BN2 film.



**Figure 7.** Variation of the mechanical properties of the chitosan-RH films as a function of the clay content. **A:** Young's modulus and tensile strength; **B:** Elongation at break and tensile energy absorption.



**Figure 8.** Evolution of the mechanical properties of the chitosan-BN2 films versus the clay content. **A:** Young's modulus and tensile strength; **B:** Elongation at break and tensile energy absorption.



**Figure 9.** Kinetics curves associated to the inhibition growth of *E. coli* (**A**) and *S. aureus* (**B**) by the chitosan-RH (**a**) and the chitosan-BN2 (**b**) films.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

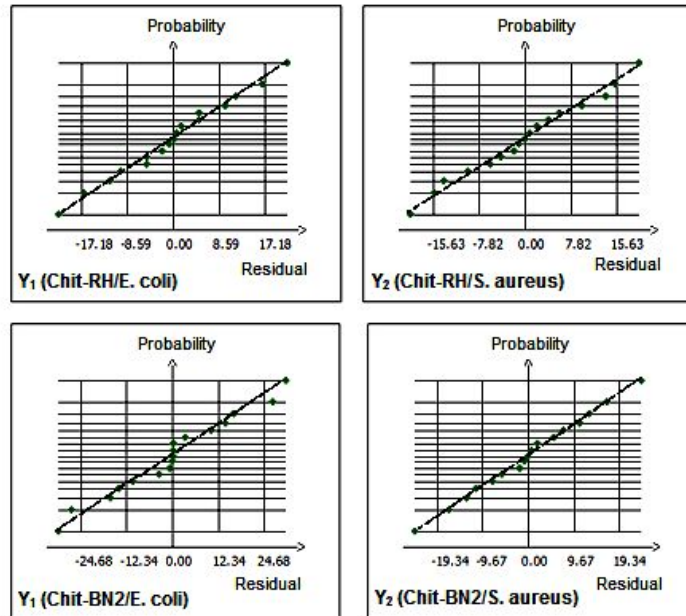
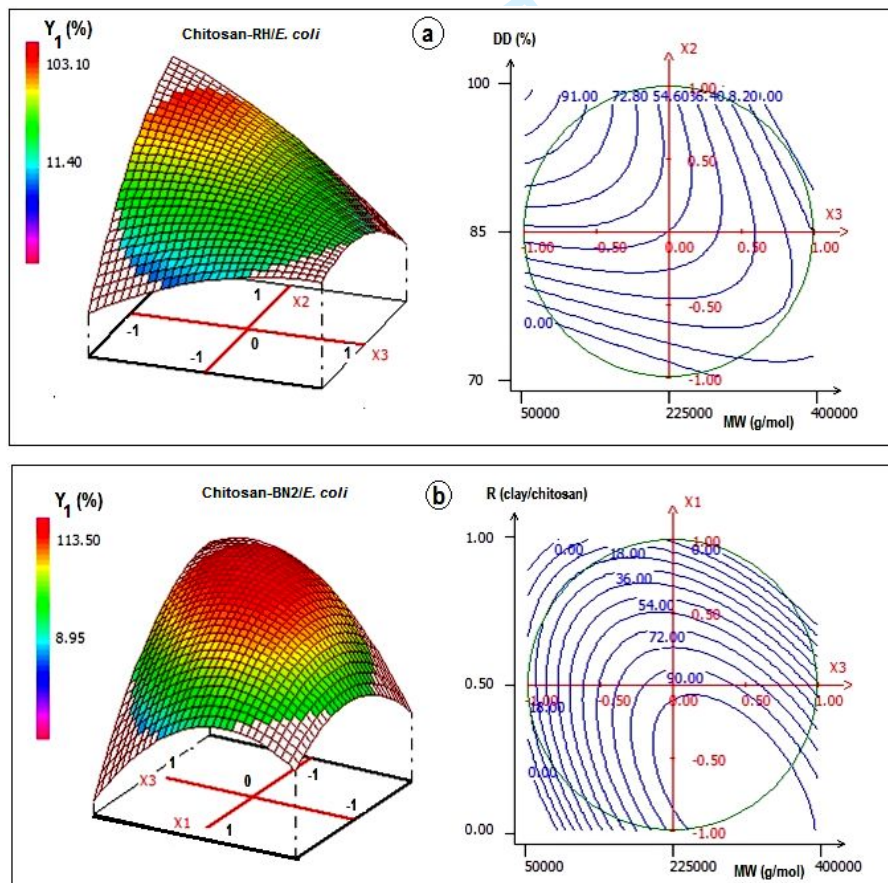
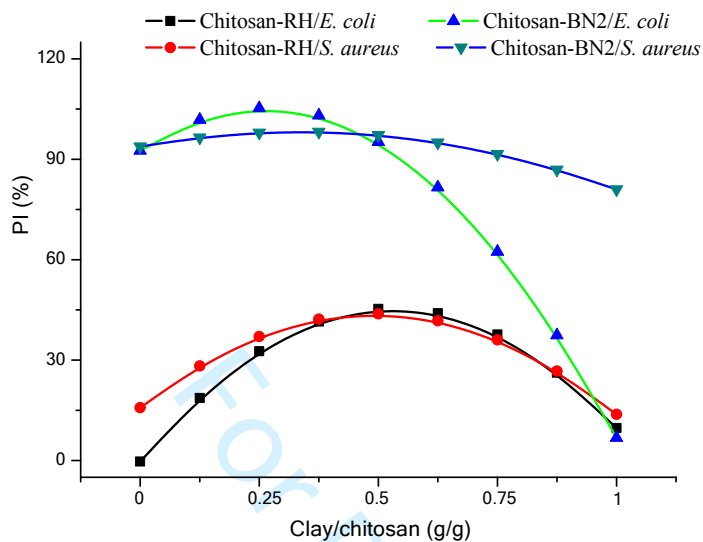


Figure 10. Probability plots of residuals related to the models used.



**Figure 11.** Variations of the percentage of inhibition of *E. coli* ( $Y_1$  (Chitosan-RH),  $Y_1$  (Chitosan-BN2)) against the factors studied. (a): clay/chitosan mass ratio ( $R$ ) = 0.5; (b): deacetylation degree (DD) = 85%.



**Figure 12.** Variation of the percentage inhibition of the bacteria growth by the prepared films versus clay/chitosan mass ratio. DD = 85%, MW = 225000 g/mol.

**Table 1.** Chemical compositions (mass%) of the raw clays used, and  $pH_{PZC}$  of the basic materials and the studied chitosan-clay films.

Chemical compositions							
	SiO <sub>2</sub>	Al <sub>2</sub> O <sub>3</sub>	Fe <sub>2</sub> O <sub>3</sub>	MgO	CaO	K <sub>2</sub> O	SO <sub>3</sub>
BN2	48.1	16.5	7.3	4.5	19.4	2.8	1.4
RH	58.1	4.8	2.3	22.6	6.6	1.1	4.5
$pH_{PZC}$							
Chitosan	BN2	RH	Chitosan-clay films				
6.0	9.1	8.0	6.6 ± 0.3				

**Table 2.** Experimental design matrix and the measured percentage inhibition ( $Y_i$ ) of the bacteria (*E. coli* and *S. aureus*) by the films prepared.

Run	$X_1$	$X_2$	$X_3$	Chitosan-RH film		Chitosan-BN2 film	
				$Y_1$ (%)	$Y_2$ (%)	$Y_1$ (%)	$Y_2$ (%)
				<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
1	1.0000	0.0000	0.0000	0.00	0.00	0.00	75.36
2	-1.0000	0.0000	0.0000	9.48	29.60	99.31	99.43
3	0.5000	0.8660	0.0000	62.93	80.17	96.54	87.68
4	-0.5000	-0.8660	0.0000	0.29	23.85	0.00	0.00
5	0.5000	-0.8660	0.0000	0.00	0.00	0.00	0.00
6	-0.5000	0.8660	0.0000	0.00	11.21	98.39	96.56
7	0.5000	0.2887	0.8165	0.00	0.00	0.00	94.56
8	-0.5000	-0.2887	-0.8165	3.74	0.00	22.81	0.00
9	0.5000	-0.2887	-0.8165	19.83	5.75	35.71	6.02
10	0.0000	0.5774	-0.8165	100.00	98.56	12.90	0.00
11	-0.5000	0.2887	0.8165	19.54	0.00	95.62	94.84
12	0.0000	-0.5774	0.8165	0.00	0.00	42.86	96.28
13	0.0000	0.0000	0.0000	44.64	44.64	94.47	99.14
14	0.0000	0.0000	0.0000	43.40	41.95	95.39	95.42
15	0.0000	0.0000	0.0000	45.48	42.82	95.39	97.99
16	0.0000	0.0000	0.0000	46.07	43.87	95.62	97.13
17	0.0000	0.0000	0.0000	46.98	45.98	95.16	96.56

**Table 3.** ANOVA results and statistical parameters related to the validity of the models adopted.

		Sources of variation	Sum of squares	of Degrees of freedom	Mean square	F-ratio	Significance
Chitosan- RH film	Y <sub>1</sub>	Regression	11555.4	9	1283.9	4.4	<0.01
		Residues	2046.3	7	292.3		
		Total	13601.7	16			
	Y <sub>2</sub>	Regression	12544.9	9	1393.9	5.1	<0.01
		Residues	1919.1	7	274.1		
		Total	14464.0	16			
Chitosan- BN2 film	Y <sub>1</sub>	Regression	27542.2	9	3060.2	11.9	< 0.01
		Residues	1793.1	7	256.2		
		Total	29335.3	16			
	Y <sub>2</sub>	Regression	28572.5	9	3174.7	8.9	< 0.01
		Residues	2499.4	7	357.1		
		Total	31071.9	16			

**Table 4.** Optimal operating factors for total inhibition of the bacteria growth determined by using the desirability function approach.

	X <sub>1</sub>	R	X <sub>2</sub>	DD (%)	X <sub>3</sub>	MW (g/mol)
Y <sub>1</sub> (Chitosan-RH/ <i>E. coli</i> )	0.3554	0.68	0.6404	95	-0.6808	105557
Y <sub>2</sub> (Chitosan-RH/ <i>S. aureus</i> )	0.4572	0.73	0.7726	97	-0.4405	147917
Y <sub>1</sub> (Chitosan-BN2/ <i>E. coli</i> )	-0.4861	0.26	0.6962	95	0.5282	317430
Y <sub>2</sub> (Chitosan-BN2/ <i>S. aureus</i> )	0.6617	0.83	0.2956	89	0.6890	354370