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METHODOLOGY FOR THE SYNTHESIS OF CHITOSAN DERIVATIVES TO BE APPLIED IN CULTURAL HERITAGE

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Abstract

The biological growth and microbial activity in heritage artefacts, promotes and accelerates their deterioration, constituting a reason of great concern for its desirable preservation. The aim of this work is to establish a simple and efficient methodology for the synthesis of new chitosan derivatives, to be applied in the production of new antibacterial biocides. The chitosan derivatives were evaluated for their antimicrobial activity using several bacterial strains isolated from heritage context. The results of the microbial activity allow us to conclude that the chitosan derivatives with N-acyl fatty acids are promising products for the short-term development of new biocides.

Keywords*: Chitosan; Biocides; Cultural Heritage; Biological Activity*

Introduction

The biological growth and microbial activity in heritage artefacts, promotes and accelerates their deterioration, constituting a reason of great concern for its desirable preservation. This problem imposes ways of acting to avoid the deterioration. The application of biocides is one of the most used ways for the biodegradation prevention of Cultural Heritage artefacts and its application is aimed at preventing or controlling the microbial growth [1-3].

In this context, the development of new chitosan derivatives can be a potential solution to avoid the biodegradation of Cultural Heritage artefacts.

Chitosan, a random copolymer of β-(1,4)-2-acetoamido-2-deoxy-D-glucose and β-(1,4)-2-amino-2-deoxy-D-glucose units, is a polysaccharide generally produced from deacetylation of chitin. This biopolymer present in the exoskeleton of crustaceans, arthropods, fungi, insects, annelids, molluscs and coelenterate, is the second most abundant found in nature, after cellulose [4].

Chitosan, is a biocompatible, biodegradable, and nontoxic polymer, but although very abundant, their real application in many biomedical, agricultural and industrial fields, has been developed only over the last 3 decades [5-8].

Actually, chitosan and its derivatives has attracted the interest of many research groups due their biocide properties against a wide range of microorganisms such as bacteria (Gram-positive and Gram-negative), yeast and filamentous fungi [9-17]. In this sense, there is a

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growing interest in the development of new chitosan derivatives that allow increase their bioactive properties and also its solubility at physiological pH.

In this work we establish a simple and efficient methodology for the synthesis of new chitosan derivatives, to be applied in the production of antimicrobial biocides. The chitosan derivatives were evaluated for their antimicrobial activity using several bacterial strains isolated from heritage context.

Experimental

General procedure for chitosan alkylation and benzylation reactions

The synthetic routes followed for the preparation of the novel chitosan derivatives CD1 to CD7, are shown in figure 1.

A mixture of 200mg of chitosan (low molecular weight), sodium iodide (2.64mmol, 3.0 equiv.) and alkyl/benzyl halide (13.2mmol, 15.0 equiv.) was stirred, in a mixture of DMF (8.0mL) and 15% sodium hydroxide solution (3.52mmol, 4.0 equiv.), at 60ºC for about 1h. The product was precipitated in ethanol (10mL), washed with diethyl ether (4 x 10mL) and evaporated to dryness. In a second step, the previously reaction procedure was repeated for the *N*-alkyl/benzyl chitosan iodide obtained in the first step. Subsequently, the product, prepared as described above, was dissolved in 10mL of a 10% NaCl aqueous solution, and stirred at room temperature for a period of 18h to exchange the iodide to chloride. The *N*-alkyl/aryl chitosan chloride was precipitated in ethanol (10mL), washed with diethyl ether (4 x 10mL) and evaporated to dryness. In the final step, was added hydrogen chloride [3M] in cyclopentyl methyl ether (1.2mL) to the dried product in diethyl ether (1.4mL). The reaction mixture was stirred at room temperature for a period of 18h and dried, yielded a water-soluble powder.

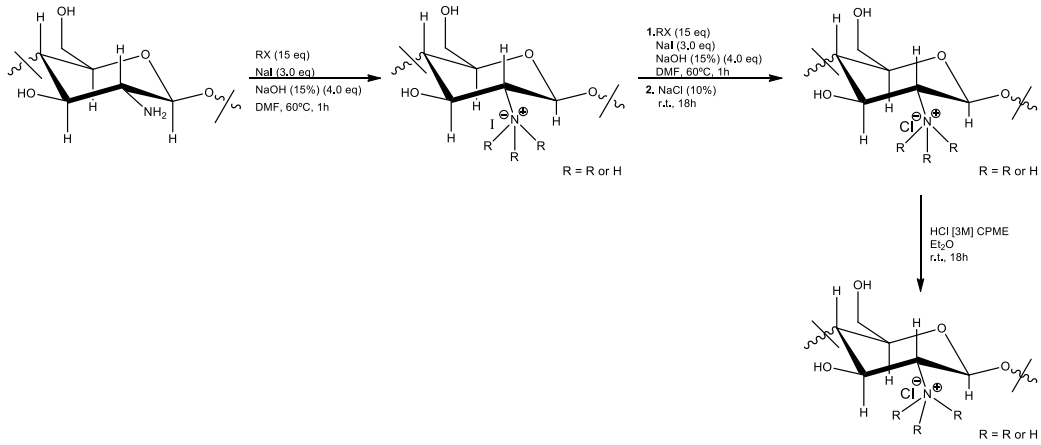

Fig. 1. Synthetic route for the preparation of the novel chitosan derivatives, CD1 to CD7

General procedure for chitosan acylation with fatty acid methyl esters

The synthetic routes followed for the preparation of the novel chitosan derivatives CD8 to CD12, are shown in figure 2.

A mixture of 200mg of chitosan (low molecular weight), sodium methoxide (0.874mmol; 1.0 equiv.) and fatty acid methyl ester (1.76mmol; 2.0 equiv.) in DMF (3mL) was stirred at 60ºC for about 24h. The reaction mixture was filtered under reduced pressure and the product was washed with water (4 x 10mL), ethanol (4 x 10mL) and hexane (4 x 10mL). In the final step, was added hydrogen chloride [3M] in cyclopentyl methyl ether (1.2mL) to the dried

product in diethyl ether (1.4mL). The reaction mixture was stirred at room temperature for a period of 18h and dried, yielded a water-soluble powder.

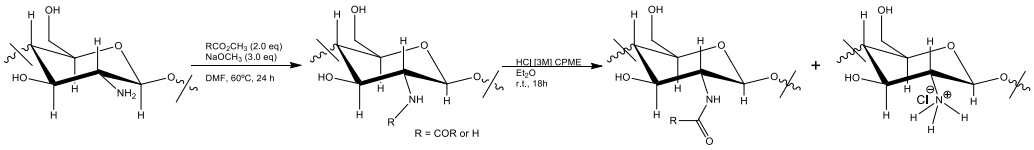

Fig. 2. Synthetic route for the preparation of the novel chitosan derivatives, CD8 to CD12

Screening of antibacterial activity of chitosan derivatives

The antimicrobial activity was assayed against a set of bacteria of the Culture Collection Laboratory of Biodegradation HERCULES (CCLBH): *Arthrobacter* sp. CCLBH-BP301; *Arthrobacter* sp. CCLBH-BP302; *Bacillus* sp. CCLBH-BP101; *Bacillus* sp. CCLBH-BP102 and *Rubrobacter* sp. CCLBH-BP401. The strains were maintained in Nutrient Broth (NB)-agar slants (1g/L glucose, 15g/L peptone, sodium chloride 6g/l, yeast extract 3g/L, 15g/L agar) and stored at 4°C. All bacterial strains were activated in Nutrient Agar at 37°C for 24h before testing.

A suspension of each tested microorganism was prepared with sterilized physiological saline solution (0.9% w/v), adjusted at 0.5 McFarland standard turbidity and spread evenly on the surface of the Mueller-Hinton (beef infusion solids 2.0 g/l, casein hydrolysate 17.5g/L, starch 1.5g/L, 15g/L agar) agar plates. Sterile filter papers discs with 12.0mm of diameter (Macherey-Nagel) were used. These discs were processed, to contain 30µL of the tested compounds using a total concentration of 20mg/mL. As positive controls or reference standard drugs tetracycline and amoxicillin were used for comparing the sensitivity of tested bacteria. These discs were then impregnated in the inoculated agar. Negative controls (disc without drugs) were also prepared. The inoculated plates were incubated for 1-3 days at 30°C. Finally, the antimicrobial activity of each compound was determined by measuring the inhibition halos around each disc. The assay was performed in triplicate (n = 3).

Results and discussion

Chemical characterization of chitosan derivatives by FTIR-ATR

With the objective to develop new antimicrobial biocides, was established a simple and efficient methodology for the synthesis of new chitosan derivatives with *N*-linear aliphatic chains and *N*-acyl fatty acids. In table 1 are presented the derivatization reagents used to synthesize the novel chitosan derivatives (CD1 to CD12).

Table 1. Derivatization reagents used to synthesize the novel chitosan derivatives CD1 to CD12

Derivatization reagents	Chitosan derivatives
Iodoethane	CD1
1-Bromobutane	CD2
1-Bromo-3-methylbutane	CD3
1-Bromooctane	CD4
1-Bromooctadecane	CD5
Bromomethylcyclohexane	CD6
Benzyl bromide	CD7
Methyl octanoate	CD8
Methyl stearate (methyl octadecanoate)	CD9
(*Z*)-methyl octadec-9-enoate	CD10
(9*Z*,12*Z*)-methyl octadeca-9,12-dienoate	CD11
(9*Z*,12*Z*,15*Z*)-methyl octadeca-9,12,15-trienoate	CD12

The FTIR-ATR spectrum of pure chitosan (Fig. 3) shows bands at approximately 891 and 1149cm^{-1} corresponding to the saccharide structure. Strong bands at 1022cm^{-1} (cyclic ethers) and 1060cm^{-1} are characteristic of the stretching vibration of the C-O group of chitosan. The small bands at 1374 and 1418cm^{-1} are attributed to the symmetrical deformation of the -CH group. The absorption bands at 1316 and 1259cm^{-1} are characteristic of the C-N bonds of the amine group type I and II, respectively. The bands at 1512 to 1648cm^{-1} can be attributed to the flexion vibration of the NH-group and the band at 1648cm^{-1} is due to the absorption of the –NH of the –NH_2 group. The strong absorption band at 1648cm^{-1} is also attributed to the stretching vibration of the C=O amide, due to partially acetylated amino groups, -$NHCOCH_3$ [18-21].

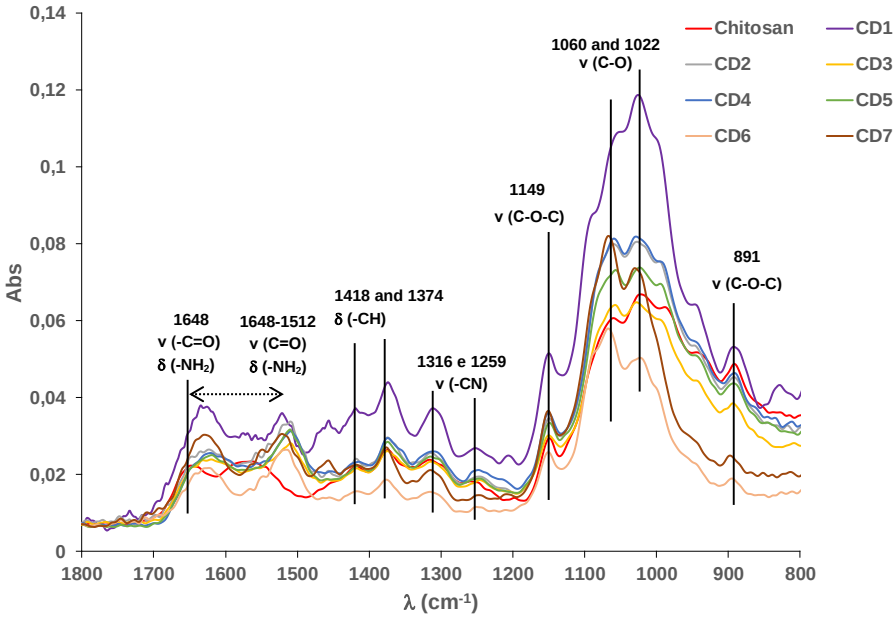

Fig. 3. FTIR-ATR spectrum of chitosan and chitosan derivatives CD1 to CD7

The FTIR spectra of chitosan derivatives, CD1 to CD6 (Fig. 3) after the alkylation reactions reveals substantial differences when compared to the pure chitosan spectrum, which suggests the effectiveness of this kind of reactions, namely: decrease in intensity of the band at 1520 to 1600cm^{-1}, attributed to the –NH group deformation vibrations and the presence of strong bands between 1490 and 1650cm^{-1}, confirm the alkylation of these amino groups; bands between 1440 and 1475cm^{-1} are attributed to the asymmetrical deformation of methyl and methylene groups from alkyl substituents.

The FTIR spectra of CD7 (Fig. 3) presents also characteristic bands at 1490 to 1650cm^{-1} and at 1440 to 1475cm^{-1} due the substitution reactions in the amino group. Additionally, these spectra present a strong band between 1050 and 1070cm^{-1}, attributed to C-H group in-plane deformations from a mono-substituted benzene ring, who confirms the benzylation reaction.

The FTIR-ATR spectra of CD8 to CD12 (Fig. 4) exhibit similar patterns to the other spectra, which confirms the chitosan acylation reactions. Additionally, the largest band between 1600 and 1660cm^{-1}, associated to the carbonyl group stretching vibrations of amides, reinforces the previously referred.

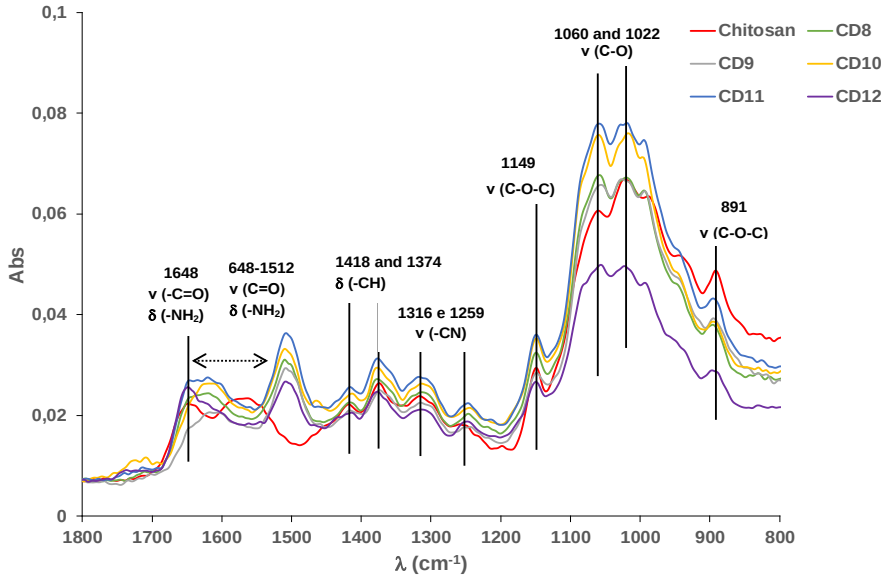

Fig. 4. FTIR-ATR spectrum of chitosan and chitosan derivatives CD8 to CD12

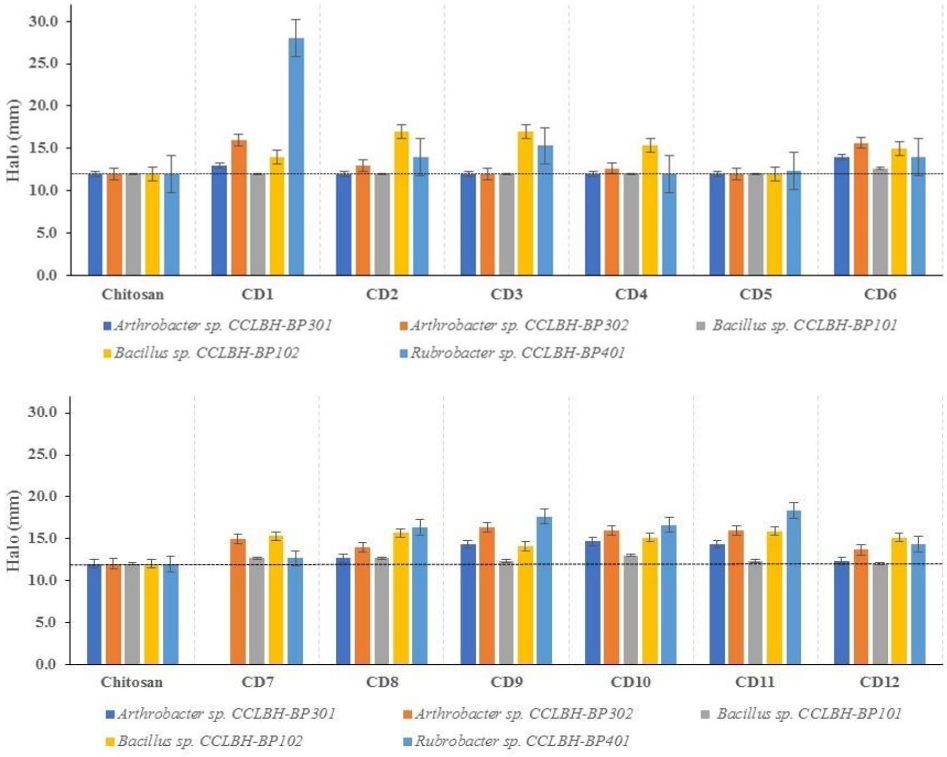

Fig. 5. Antibacterial activity of chitosan and their derivatives CD1 to CD12, against *Arthrobacter* sp. CCLBH-BP301; *Arthrobacter* sp. CCLBH-BP302; *Bacillus* sp. CCLBH-BP101; *Bacillus* sp. CCLBH-BP102 and *Rubrobacter* sp. CCLBH-BP401

Antibacterial activity of chitosan derivatives

The spectrum of action was determined against the bacterial stains *Arthrobacter* sp. CCLBH-BP301; *Arthrobacter* sp. CCLBH-BP302; *Bacillus* sp. CCLBH-BP101; *Bacillus* sp. CCLBH-BP102 and *Rubrobacter* sp. CCLBH-BP401.

According to the determination of inhibition zones produced in the agar plates, all studied CDs showed a broad spectrum of antibacterial activity presenting inhibition halos higher than 12.0mm in diameter (Fig. 5).

CD8 to CD11 showed the high ability to inhibit the growth of the selected bacteria. In fact, the presence of fatty acids associated to the chitosan structure, in particular with saturated, mono and di-unsaturated chains, seems to conferee a lipophilic character of these chitosan derivatives that potency the interaction with the bacterial cell membrane, making the antibacterial effect more effective. Furthermore, the synergistic interactions between CDs compound can potentiate their natural antimicrobial effect.

Conclusions

Chitosan derivatives with *N*-acyl fatty acids, in particular with saturated and mono and di-unsaturated chains, are promising products for the short-term development of new biocides with a broad spectrum of antibacterial activity against the selected heritage biodeteriogenic Gram-positive bacteria.

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