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Synthesis, characterization and antifungal activity of quaternary derivatives of chitosan on *Aspergillus flavus*

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ABSTRACT

Two series of new chitosan derivatives were synthesized by reaction of deacetylated chitosan (CH) with propyl (CH-Propyl) and pentyl (CH-Pentyl) trimethylammonium bromides to obtain derivatives with increasing degrees of substitution (DS). The derivatives were characterized by ^{1}H NMR and potentiometric titration techniques and their antifungal activities on the mycelial growth of *Aspergillus flavus* were investigated *in vitro*. The antifungal activities increase with DS and the more substituted derivatives of both series, CH-Propyl and CH-Pentyl, exhibited antifungal activities respectively three and six times higher than those obtained with commercial and deacetylated chitosan. The minimum inhibitory concentrations (MIC) were evaluated at 24, 48 and 72 h by varying the polymer concentration from 0.5 to 16 g/L and the results showed that the quaternary derivatives inhibited the fungus growth at polymer concentrations four times lower than that obtained with deacetylated chitosan (CH). The chitosans modified with pentyltrimethylammonium bromide exhibited higher activity and results are discussed taking into account the degree of substitution (DS).

1. Introduction

Chitosan is a polysaccharide usually obtained from deacetylation of chitin, which after cellulose is the second most abundant natural biopolymer found in nature. It may be extracted from various sources, particularly from exoskeletons of arthropods of crustaceans, fungi, insects, annelids, mollusks and coelenterata. The structures of chitin and chitosan correspond to those of poly[β(1→4)-2-acetamide-2-deoxy-D-glucopyranose] and poly[β(1→4)-2-amino-2-deoxy-D-glucopyranose], respectively (Ravi Kumar 2000). The homopolymer is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0 and is therefore insoluble at neutral and alkaline pH values. In acidic mediums, the amine groups will be positively charged, conferring to the polysaccharide a high charge density. Due to its polycationic nature, chitosan, after being dissolved in aqueous acid solutions, can be easily molded and used as membranes, beads, microparticles and gels (Rinaudo 2006; Ravi Kumar 2000). Also, its functional properties such as biodegradability and low toxicity (Domard 2011; Kean and Thanou 2010) have driven the research and applications of chitosan to medicine (Rinaudo 2006; Shi et al. 2006; Jayakumar et al. 2010), foods additives and preservatives (Shahidi et al. 1999) as well as in the paper industry and for the treatment of industrial wastewater (Ngah and Teong 2011).

One of the most attractive features of chitosan is its antibacterial, antiviral and antifungal activity (Zhang et al. 2011; Jayakumar et al. 2011). Recently the utilization of chitosan as a food preservative or adjuvant in agriculture to protect or stimulate the defense of different crops has increased (Zhang et al. 2011; Jayakumar et al. 2011). It is well known that pesticide residues are toxic for humans and animals and many of them are not biodegradable, what can cause serious environmental problems such as contamination of the water and soil (Satpathy et al. 2011). Chitosan and its oligomers have emerged as a promising source for many applications since it can be used to produce biodegradable fungicides to regulate the growing of plants and to protect seeds (Alburquenque et al. 2010).

The antifungal activity of chitosan is believed to occur from the interaction between the cationic chain and the negatively charged residues of macromolecules exposed on the fungal cell surface, leading to leakage of intracellular electrolytes and other constituents (Muzzarelli et al. 2001). It is believed that chitosan may affect the morphogenisis of the cell wall interfering directly on the activity of enzymes responsible for the growing of the fungi (El Ghaouth et al. 1992). Recently Li et al., based on confocal laser

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scanning microscopy of fluorescein-labeled chitosans, showed that low molecular weight chitosans could enter into the hypha of *Fulvia fulva*, suggesting that the growth of *F. fulva* could be inhibited by chitosan from the inside of the cell (Li et al. 2011).

The fungus *Aspergillus flavus* is considered one of the most serious problems in the production and consumption of grains in the world as it is related to the production of mycotoxins, such as aflatoxins, which are produced as secondary metabolites by some strains of *A. flavus* (Binder et al. 2007; Zhang et al. 2009). Fajardo et al. (1994) reported that chitosan limited *A. flavus* growth and subsequent aflatoxin production by inducing susceptible tissues to produce more phenolic compounds. Recently, Zhang et al. prepared chitosan-based blend films using chitosan, soybean trypsin inhibitor extracts and glycerol solutions (Zhang et al., 2009). The authors showed that the germination and growth of *A. flavus* were strongly inhibited by films prepared from soybean trypsin inhibitor extract (STI)/wild soybean trypsin inhibitor extract (WTI) and glycerol (Gly) solutions (chitosan-STI/WTI-Gly), indicating that the films could be useful as potential bio-control packaging against *A. flavus* during the peanut's and other cereal's storage. The study of new biomolecules having antifungal activities has emerged as a new subject and the modification of the chitosan structure aiming to improve its activity is a promising way to achieve effective biofungicides (Kenawy et al. 2005; Másson et al. 2008) and bactericides (Xu et al. 2011).

The aim of the paper is to report a simple and reliable method to prepare chitosans derivatives with improved capabilities of inhibiting the growth of *A. flavus*. The synthesis, characterization and antifungal activity of chitosan derivatives against the fungus *A. flavus* are described. A series of chitosan derivatives was obtained by the reaction of the amino groups of chitosan with propyl and pentyltrimethyl ammonium bromides. The results of the antifungal activity of these derivatives were presented and discussed, taking into account the degree of substitution of the substituted derivatives.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation (DD) 85%) was purchased from Polymar Co., Brazil, (3-bromopropyl) trimethylammonium bromide, (5-bromopentyl)trimethylammonium bromide, sodium hydroxide, sodium acetate, and acetic acid were purchased from Sigma Aldrich Chemical Co., Brazil. Spectra/pore membranes (Spectrum) were employed for dialysis. All solvents were of reagent grade and used as received. Water was deionized using a Gehaka water purification system.

2.2. Instrumentation

^{1}H NMR spectra were recorded on a Bruker ARX-500 500 MHz spectrometer. UV/Vis spectra were measured with a Cary 100 spectrophotometer equipped with a Peltier system. pH values of the solutions were determined using an Digimed pH-meter.

2.3. Preparation of the alkyltrimethylammonium-modified chitosans

Chitosan was deacetylated as described earlier (Tiera et al. 2006). The resulting polymer was purified by dialysis against water for 3 days and isolated by lyophilization. Next, chitosans with varying amount of grafted alkyltrimethylammonium bromides were prepared in aqueous NaOH solutions and the procedure is described below (Fig. 1). The degree of substitution was varied by setting the initial molar ratio as described in Table 1. A suspension of deacetylated chitosan (1.5 g, 9.3 mmol) in aqueous (20 mL) was prepared and the pH was adjusted to 9.0 by adding NaOH 0.1 M from an adapted funnel to a round-bottomed reaction flask equipped with a magnetic stirrer. Further (3-bromopropyl) trimethylammonium bromide (1.0 g) dissolved in water (20 mL) was added with stirring. Stirring was continued at 60 °C for 72 h and the pH was continuously monitored during the reaction time. The mixture was then dialyzed (membrane of MWCO 12–14,000 g/mol) to remove the unreacted alquiltrimethylammonium bromide, first against water for 2 days, then against aqueous NaOH (0.05 M) for 1 day, and finally against water for 2 days. The product was isolated by lyophilization and characterized by ^{1}H NMR and potentiometry.

2.4. Viscosity measurements

Viscosity measurements were carried out in water thermostated bath with a capillary calibrated viscosimeter for dilution Cannon-Ubbelohde 9722M-50 (Cannon Instr. Co.) at pH 4.5 acetic acid (0.3 M)/sodium acetate (0.2 M) buffer. The viscosimeter was immersed in a thermostatic bath at 298.15 ± 0.05 °C and the samples were allowed to equilibrate for 10 min in the bath before measurements. Measurements at each concentration were repeated and the reproducibility was better than ±0.01 s. The results of the viscosity measurements were expressed as reduced viscosity values calculated from

$$\eta = \frac{(t - t_o)/t_o}{c}$$

where t is the measured efflux time of the polymer solution, t_o is the efflux time of the pure solvent, and c is the polymer concentration (g/L). The mean viscosimetric molecular weight of chitosan and its derivatives were determined by using the Mark–Houwink equation, with constants $a = 0.796$, $K = 0.079$ mL g^{-1} (Roberts and Wang 1996).

2.5. Pathogen and cultures

The microorganism chosen to test the antifungal activity of chitosan and its derivatives was *A. flavus*. The strain was kindly provided by Brazilian Collection of Microorganisms from the Environment and Industry – CBMAI, Campinas – São Paulo, Brazil, and it was maintained on potato dextrose agar (PDA) (potato infusion from 200 g/L, 20 g/L dextrose, and 15 g/L agar) in the dark at 25 ± 2 °C.

2.6. Antifungal assays

The antifungal activity of deacetylated and commercial chitosan was compared to those of the alkyltrimethylammonium derivatives modified with a degree of substitution varying from 0.5 to 65%. The polymers solution were prepared at pH 5.5 in acetic acid and added at concentrations of 0.0 (control plate) 0.1, 0.5 and 1.0 g/L to the melted culture medium, which contained 10.0 mL of 10% potato dextrose broth and then transferred to Petri dishes. After solidification, the mixtures were inoculated with a 1 mm in diameter mycelium fungus *A. flavus* at the center of Petri dishes and these were incubated in an oven for 7 days at 25 ± 2 °C. Inhibition index of the fungus by the polymers was determined by the radial growth of the colony with a caliper on the 3rd, 5th and 7th days of cultivation, with the result of the 7th day used for comparative purposes.

The antifungal index was calculated as follows:

$$\text{Antifungal index } (\%) = \frac{1 - D_a}{D_b} \times 100,$$

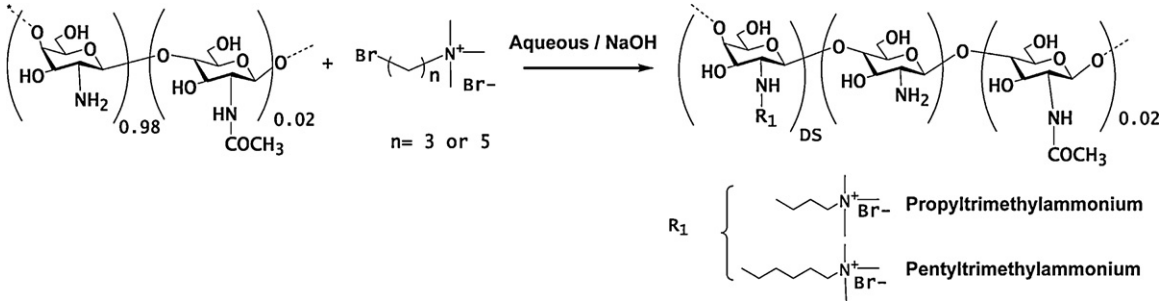

Fig. 1. Schematic representation of the synthesis of the quaternary derivatives of chitosan.

where D_a is a diameter of the growth zone in the test plates and D_b is growth zone in the control plate, according to Guo et al. (2006).

Each experiment was performed in quadruplicate, and the data were averaged. The student *T*-test and the Kruskal–Wallis test with Dunn's multiple comparison were used to evaluate the differences in antifungal index in antifungal tests. Results with $P < 0.05$ were considered statistically significant.

2.7. Minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) were determined using the micro dilution assay in potato dextrose agar (PDA), following the standard method applied to filamentous fungi (CLSI 2008). The chitosan derivatives were dissolved in aqueous acetic acid at pH 5.5 to obtain stock solutions of 16.0 g/L. These solutions were subsequently diluted to obtain decreasing polymer concentrations (16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 g/L). Thereafter 100 μL of the polymer solutions was transferred on 96-well plates and 2 μL of the fungus suspension at a density of 10^6 conidia/mL were inoculated at each well. The experiments were carried out in triplicate and the MICs were determined by visual inspection of the wells. The lowest concentration that gave complete growth inhibition in all three replicates was recorded as the minimum inhibitory concentration (MIC) after 24, 48 and 72 h.

3. Results and discussion

3.1. Preparation of the antifungal derivatives

To achieve the functionalization of chitosan with quaternary amino groups, we used nucleophilic substitution of the C-2 amine groups, thus leaving intact all the hydroxyl groups which play an important part in the biological activity of chitosan derivatives (Fig. 1). The synthetic strategy was successively carried out by using the alquiltrimethylammonium bromides. The transformation was carried out starting from a deacetylated chitosan, obtained *via* deacetylation of a commercial chitosan sample with a nominal degree of deacetylation (DD) of 85%, following a previously described procedure (Tiera et al. 2006). The deacetylation was carried out under continuous bubbling of nitrogen in order to prevent degradation of the polymer. The degree of deacetylation, expressed in $-NH_2$ mol%, determined from the ^{1}H NMR spectra of the starting material and the deacetylated sample, were 86.1 and 98.7 mol%, respectively. These values were obtained from the areas of the doublet at 5.5 ppm, due to the resonance of the anomeric proton (H1) and of the singlet at 2.7 ppm attributed to the acetamido methyl protons (Fig. 2a). Potentiometric titration conducted on a solution of CH confirmed that the deacetylation occurred with high efficiency, yielding a DD value of 98.5.

The degree of substitution was varied by setting the initial molar ratio of *N*-alkyltrimethylammonium to glucosamine units to values ranging from 1.2 to 8.0. The ^{1}H NMR spectra of the chitosan derivative obtained from reaction with (3-bromopropyl) trimethylammonium bromide exhibited a singlet at 3.67 ppm, which was attributed to the resonance of the trimethylammonium protons of the *N*-alkyltrimethylammonium moiety and also exhibited a signal at δ 2.81 ppm corresponding to the resonance of the methylene protons $CH_2\boldsymbol{CH_2}CH_2N^+(CH_3)_3$ (Fig. 2a). The attachment of alkyltrimethylammonium groups to the chitosan framework brings further changes to the ^{1}H NMR spectrum of chitosan, most notably in the resonances of the protons in close proximity to the substitution site: the anomeric proton H-1 and the proton H-2 linked to the C2 of the glucosamine unit. The anomeric proton signal undergoes a downfield shift, from δ 5.40 ppm to δ 5.56 ppm, while the peak at δ 3.67 ppm shifts to 3.85 ppm. The same pattern was also observed in the ^{1}H NMR spectra of the pentyltrimethylammonium derivatives, however the pentyltrimethylammonium bromide exhibited a triplet from 2.00 to 2.50 ppm attributed to the methylene protons from pentyl hydrocarbon chain. Similar deshielding of H-1 and H-2 upon C-2 derivatisation of chitosan has been reported in the case of chitosan carrying oligosaccharide branches (Tømmeraas et al. 2002).

The degree of substitution (DS) was determined from the areas of the signal at δ 2.81 ppm, which corresponds to the resonance of

Table 1
Properties and DS (degree of substitution) of the chitosan derivatives.

Derivative	Molar ratio[a] R/NH_2	DS (potentiometry)	DS (^{1}H NMR)	Mv (kDa)
CH-C	–	86.1%	85.9%	32.8
CH[b]	–	98.5%	98.7%	24.4
CH-Propyl-10	1.2	11.2%	10.7%	30.3
CH-Propyl-15	1.5	14.3%	14.7%	11.9
CH-Propyl-20	2.3	23.4%	18.1%	18.8
CH-Propyl-40	3.8	39.1%	38.4%	–
CH-Pentyl-25	1.5	–	25.1%	17.4
CH-Pentyl-30	2.5	–	30.0%	–
CH-Pentyl-65	8.0	–	64.9%	20.1

[a] Molar ratio of alkyltrimethylammonium bromide (R) to amino groups ($-NH_2$) used in the substitution reaction.
[b] Deacetylated chitosan.

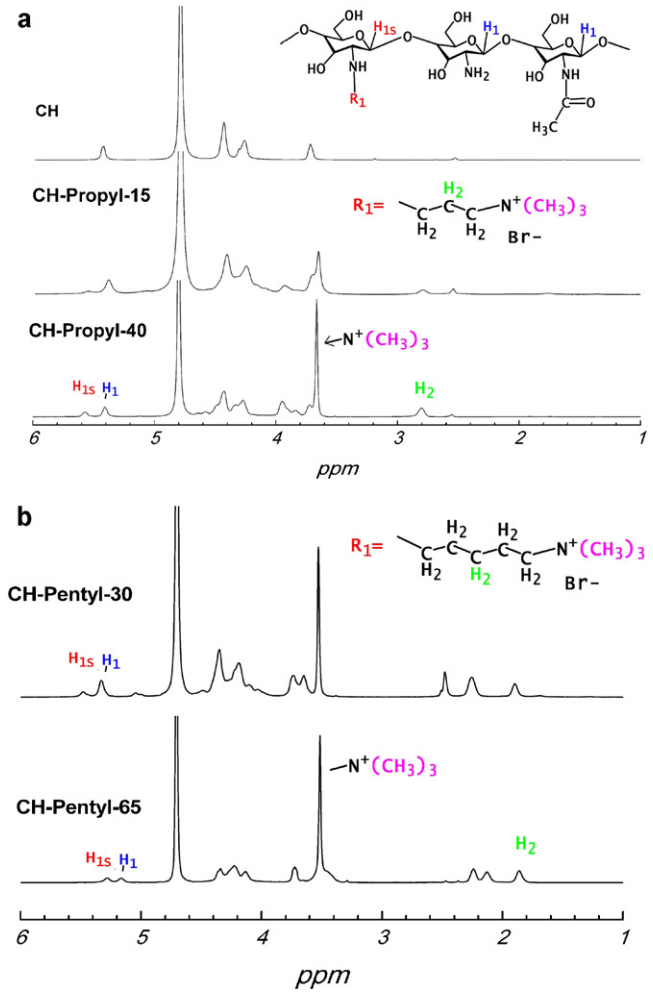

Fig. 2. ^{1}H NMR spectra of deacetylated chitosan and their (a) propyltrimethylammonium and (b) pentyltrimethylammonium derivatives of chitosan. The resonance of the methylene protons (green color) equidistant from the nitrogen atoms was used to determine DS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the methylene protons $CH_2\boldsymbol{CH_2}CH_2N^+(CH_3)_3$ (Fig. 2a) and from the signals due to the anomeric protons of propyltrimethylammonium substituted and unsubstituted glucosamine residues, H_{1S} and H_1, respectively (Fig. 2a) using Eq. (1):

$$DS = \frac{(1/2)I_{CH_2}}{I_{H_1} + I_{H_{1S}}} \quad (1)$$

The same equation was used to determine DS for the derivatives obtained with pentyltrimethylammonium, however the signal at δ 2.05 ppm, which corresponds to the resonance of the methylene protons $CH_2CH_2\boldsymbol{CH_2}CH_2$ $CH_2N^+(CH_3)_3$, was utilized for this purpose (Fig. 2b). All degrees of substitution (DS) are shown in Table 1.

3.2. Antifungal activity of commercial chitosan and its deacetylated and quaternary derivatives

The antifungal activity against *A. flavus* was previously tested for commercial (CH-C) and deacetylated (CH) chitosans (Table 1). The concentration range was varied from 0.1 to 1.0 g/L and inhibition percentages were calculated as described in the previous section (Section 2.6). The inhibition decreases with time, which is due to the adaptation of the *fungus* to its new environment, for instance the inhibition index obtained with deacetylated chitosan is 8.1, 6.5 and 5.1 in the 3rd, 5th and 7th day respectively

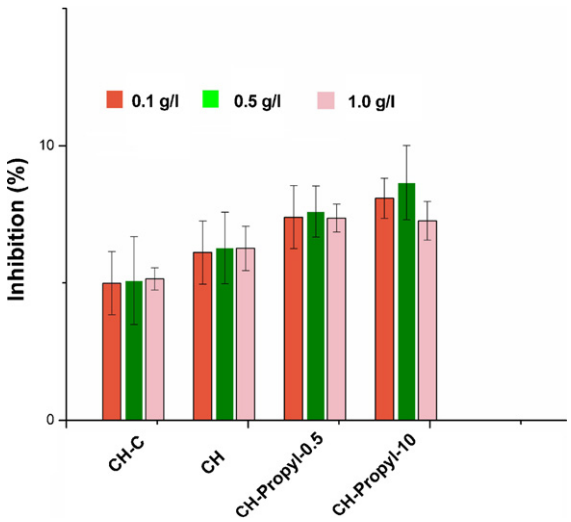

Fig. 3. Inhibition percentages for CH-C and CH at different polymer concentrations. The differences between the groups were not statistically significant with $P < 0.05$.

and the same pattern was observed for the other derivatives. As can be seen from Fig. 3 the inhibition percentage obtained for CH-C and CH remained around 5% and increased slightly from 0.1 to 1.0 g/L for the derivative substituted with 10% of propyltrimethyl ammonium groups (CH-Propyl-10), which is due to the low the degree of substitution. This result is similar to that observed by Li et al. investigating the antifungal activities of chitosans on *F. fulva*. These authors showed that inhibition percentages of low molecular weight chitosans, measured by the mycelial radial growth, exhibited modest increases in the concentration range from 0.1 to 1.0 g/L (Li et al. 2011). Although unexpected, in this work the fungal adaptation to the new environment was rapid and as the time of incubation was 7 days the effect of concentration was not clearly observed. However, the determination of the minimum inhibitory concentrations (MICs), discussed ahead in the paper, makes clear that derivatives having the higher degrees of substitution exhibit a significant inhibition increase.

Although increasing concentrations of chitosan may improve the inhibition percentage on many pathogens this trend depends on several factors such as molecular weight of chitosan, targeted pathogen and pH (Yang et al. 2005). The activity of chitosan has been explained as being based on the electrostatic interaction of the charged amino groups of chitosan with negatively charged cell wall surface of the targeted microorganisms, which can lead to the disruption of the cell wall and therefore to the death of the cell. Another possibility for antifungal activity of chitosan accounts for its chains to cross the cell membrane inhibiting the cell growing from inside (Guo et al. 2007). However, for deacetylated chitosan (CH) and the derivatives having low degrees of substitution the mechanism on *A. flavus* and the interaction with cell surface may form an impermeable layer around the cell, thus blocking the transport of essential solutes into the cell (Eaton et al. 2008).

In this work, we hypothesized that, the modification of chitosan by introducing permanently charged quaternary groups could improve the antifungal activity of chitosan. The addition of quaternized chitosan to the BDA medium inhibited mycelial growth of *A. flavus* significantly at all concentrations tested. A representative experiment comparing the effect of CH and CH-Propyl-40 is shown in Fig. 4. As shown in Fig. 5 the results obtained in microbiological assays showed that the capability to inhibit fungus growth *in vitro* was clearly increased for the higher degrees of substitution DS for the two series tested (CH-Propyl and CH-Pentyl). The Student *t*-test was applied to independent samples to a level of

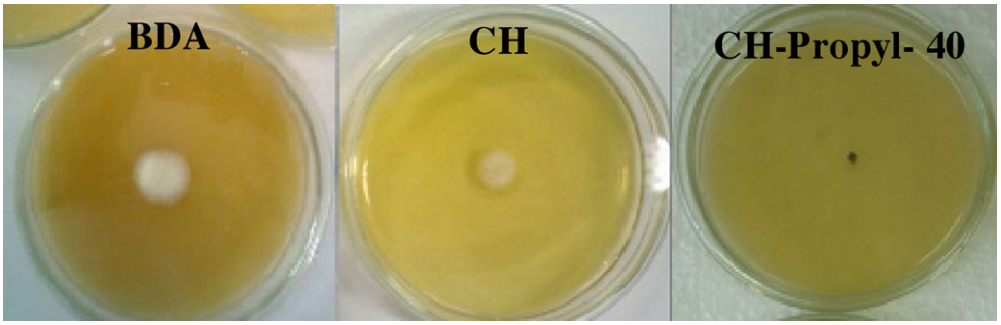

Fig. 4. Comparative effect of the quaternization of chitosan on the inhibition index at 0.5 g/L after 3 days of incubation: from left to right BDA; CH and CH-Propyl-40.

significance of 0.05 aiming to verify the existence of significant differences between results obtained for the inhibition index with 0.1 and 0.5 g/L. The descriptive statistics showed that no significant differences were observed regarding the 0.1 and 0.5 g/L, since the obtained *P* values were always higher than 0.5, which confirm that, in this concentration range, the inhibition does not change. A comparison between the inhibition indexes of the different chitosan derivatives was also performed. The Kruskal–Wallis test with Dunn's multiple comparison was utilized to the level of significance 0.05 and the descriptive statistics showed that *P* values found for 0.1 g/L were smaller than 0.001, indicating that structural changes on the chitosan chain significantly increases the antifungal activity.

The most substituted derivative from CH-Propyl series (DS = 38.5%) showed an inhibition index three times higher than that observed with deaceylated chitosan (CH).

However, the highest inhibition index was obtained for CH-Pentyl derivative having with DS 65%, which exhibited an inhibition index almost six times higher than CH. As shown by other authors, this activity can be attributed to a higher number of cationic groups available on the polymer chain, which allows a stronger interaction between the polycation chains and the cell wall, increasing the antifungal activity. However by comparing the efficiencies of both series it becomes clear that the less substituted CH-Pentyl derivative (DS = 25%) exhibited a higher inhibition than that obtained using the more substituted derivative from CH-Propyl series (DS = 38.5%). Since both the series present the same quaternary group, this higher activity for CH-Pentyl series may be attributed to the longer hydrocarbon chain of the grafted pentyl groups, which could favor a hydrophobic and stronger interaction with the cell membrane.

3.3. Minimum inhibitory concentrations

The results obtained from MICs determination confirmed that the cationic derivatives synthesized exhibit increasing efficiencies with the degree of substitution by the cationic groups, with MICs in the range of 1.0–4.0 g/L. As can be seen from Table 2 in the first 48 h, the derivatives CH-Pentyl-30, CH-Propyl-40, CH-Pentyl-25 and CH-Pentyl-65 inhibited completely the growth of the fungus at 1.0 g/L, *i.e.*, a concentration four times smaller than those needed for commercial (CH-C) and deacetylated chitosan (CH) (Table 2). Moreover, after 72 h no growth was observed in the presence of CH-Pentyl-65

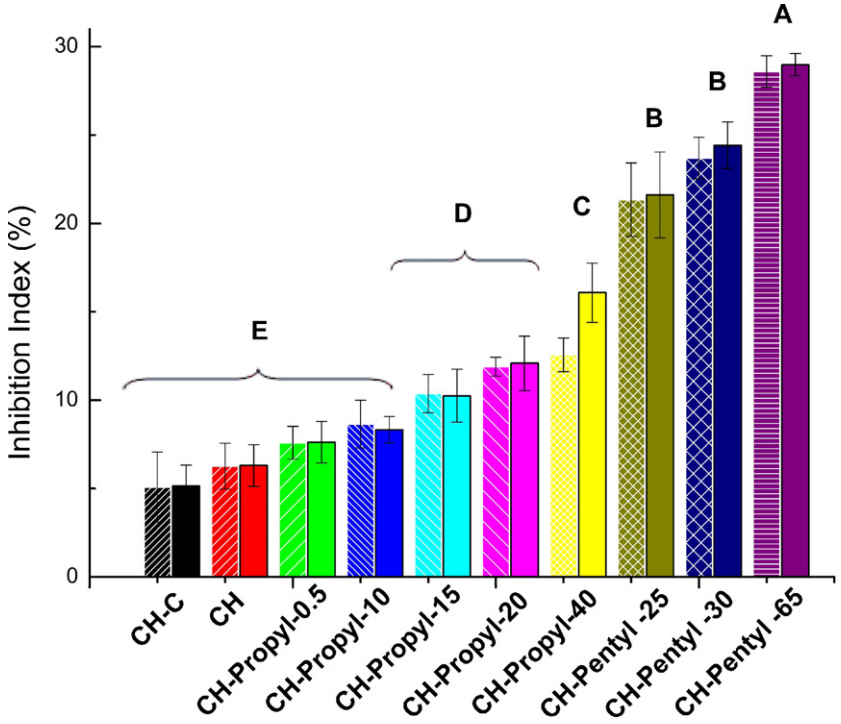

Fig. 5. Effect of the degree of substitution of the chitosan derivatives on the inhibition percentage of *Aspergillus flavus* "*in vitro*" at 0.1 and 0.5 g/L (mean ± SD, $n = 4$). Lighter color bars (left side) correspond to 0.1 g/L and the differences between the groups which share letters are not statistically significant with $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Minimum inhibitory concentrations of chitosan and its derivatives.

	MIC (g/L)		
	24 h	48 h	72 h
CH-C	4.0	4.0	4.0
CH	4.0	4.0	4.0
CH-Propyl-10	1.0	2.0	4.0
CH-Propyl-15	0.5	2.0	4.0
CH-Propyl-20	1.0	2.0	4.0
CH-Propyl-40	0.5	1.0	2.0
CH-Pentyl-25	0.5	1.0	2.0
CH-Pentyl-30	0.5	1.0	2.0
CH-Pentyl-65	0.5	1.0	1.0

at concentration of 2.0 g/L. The MICs for CH-C and CH was 4.0 g/L and this value is within of the range reported in the literature, which varies from 10 to 5000 ppm depending on molecular weight, degree of acetylation and type of fungi (Sajomsang et al. 2012). Similar responses were observed by other authors and the MICs of quaternized chitosans decrease with degree of substitution, which can be explained based on the interaction of these groups with negatively charged cell surface membrane, preventing nutrients from entering the cell (Guo et al. 2007; Yang et al. 2005).

4. Conclusions

The synthesis and characterization of new quaternary derivatives of chitosan were carried out and their antifungal activities against *A. flavus* were tested. The results have shown that the antifungal activity of chitosan can be improved by increasing the degree of substitution (DS) of alkyltrimethylammonium groups on the polymer chain. The inhibition indexes for both synthesized series (propyl and pentyltrimethylammonium) increased with DS and the most substituted derivatives (CH-Propyl-40 and CH-Pentyl-65) exhibited inhibition values three and six times respectively higher than those obtained with chitosan. The higher activity exhibited by CH-Pentyl derivatives can be attributed to the longer hydrocarbon chain of this substituent, which allows a stronger interaction with the cell membrane. The synthesis and characterization of new derivatives, having longer hydrocarbon chains, are ongoing in our laboratory aiming to obtain derivatives with higher antifungal activities.

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