academicJournals

Vol. 7(20), pp. 2259-2268, 14 May, 2013
DOI: 10.5897/AJMR12.1185
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

*Full Length Research Paper*

# Preparation and antimicrobial activity of *O*-(benzoyl) chitosan derivatives against some plant pathogens

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Accepted 29 March, 2013

***O*-(benzoyl) chitosan derivatives were synthesized and evaluated as antimicrobial agents against some plant pathogens. The chemical structures were characterized by infra-red (IR) and nuclear magnetic resonance (NMR) spectroscopy and the data confirmed that the acylate reaction took place at *O*-position of chitosan. The antimicrobial activity was investigated against bacteria of *Agrobacterium tumefaciens* and *Erwinia carotovora* and fungi of *Alternaria alternata*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum*. Among the derivatives, *O*-(3,5-dinitrobenzoyl) chitosan was the most active against bacteria of *A. tumefaciens* and *E. carotovora* (MIC = 3275 and 3125 mg/L, respectively). However, *O*-(2-chloro-6-flourobenzoyl) chitosan was the most active in fungal growth inhibition ($EC_{50}$ = 3040, 1526 and 3301 mg/L for *A. alternata*, *F. oxysporum* and *S. sclerotiorum*, respectively). On the other hand, the derivatives caused significant reduction in spore germination of *A. alternata* and *F. oxysporum* with complete inhibition at 1000 mg/L for *O*-(2-nitrobenzoyl) chitosan and *O*-(4-chloro-3,5-dinitrobenzoyl) chitosan.**

**Key words:** Antimicrobial activity; *O*-(benzoyl) chitosan derivatives; degree of substitution; IR and NMR spectroscopy; spore germination.

## INTRODUCTION

Numerous works have been published on the chemical modification of chitosan; this polymer is still being modified, leading to various derivatives with improved properties. With regard to its unique properties such as biocompatibility, biodegradability and no toxicity to mammals, it is widely used in fields like biotechnology, pharmaceutics, cosmetics and agriculture. In particular, the antimicrobial activities of chitosan and its derivatives have aroused considerable recent interest (Badawy, 2010; Badawy and Rabea, 2012; Kim et al., 2007; Muzzarelli, 2009; Pires et al., 2013; Rabea et al., 2009; Sajomsang et al., 2009).

The chitosan derivatives mentioned in the literatures showed that one can differentiate specific reactions involving the -$NH_2$ group at the C-2 position or nonspecific reactions of -OH groups at the C-3 and C-6 positions, especially, esterification and etherification (Anitha et al., 2009; Badawy et al., 2005). Therefore, acylation can occur on the hydroxyl groups to obtain chitosan ester and occur on the amino groups to obtain acylamide. Much attention has been paid on the synthesis of *N*-aryl chitosan, and *N*-benzylation of chitosan is often used as interim outcome to make some other derivatives relying on the strongpoint of the high rate of condensation and production, also the reactive condition and process of the synthesis appears not to be

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so strict and complicated, in addition, *N*-benzylation of chitosan is easily reverted. The number of *O*-derivatives of chitosan is much lesser. In many cases, modification of chitosan through hydroxyl groups has an advantage because of free amino groups in the products and possible less influence on the fundamental skeleton.

Chitosan has a certain antibacterial strength and anti-bacterial spectrum. The hydroxyl groups of chitosan at the C-6 position have a certain activity, so chitosan can theoretically react with aromatic acids. The reaction not only improved the solubility of chitosan, but also reserves the original amino groups that contribute to enhancing the antimicrobial activity (Abdelaal et al., 2013; Badawy and Rabea, 2011, 2012; Liu et al., 2013; Taghizadeh and Bahadori, 2013; Wang and Wang, 2011).

Herein, we reported the preparation of *O*-(benzoyl) chitosan derivatives by reaction of chitosan with benzoic acid derivatives in the presence of $H_2SO_4$. The chemical structure of the derivatives was characterized by Fourier transform infrared (FTIR), $^1$H- and $^{13}$C-NMR techniques. The antimicrobial activities of these derivatives were evaluated against plant pathogenic bacteria *Agrobacterium tumefaciens* and *Erwinia carotovora* and fungi of *Alternaria alternata*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum*. These compounds are important since no real screening has been performed yet on chitosan derivatives in order to attribute the possible activity to these compounds. The detection of improved biological activity of one of the compounds could open an interesting agricultural application to crop protection.

## MATERIALS AND METHODS

### Materials and tested microorganisms

Chitosan with average molecular weight of $3.60 \times 10^5$ Da was purchased from Sigma-Aldrich Co. (USA). The degree of deacetylation (DDA) was found to be 89% by $^1$H NMR spectroscopy. Benzoic acid, 2-nitrobenzoic acid, 4-nitrobenzoic acid, 3,5-dinitrobenzoic acid, 4-chloro-3,5-dinitrobenzoic acid, 2-chloro-6-flourobenzoic acid, citrus pectin and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Co. (USA), Nutrient Agar (NA), nutrient broth (NB), potato dextrose broth (PDB) and potato dextrose agar (PDA) media were purchased from Oxoid Ltd., (Basingstoke, Hampshire, UK). All commercially available solvents and reagents were used without further purification.

Bacteria of *A. tumefaciens* (Family: Rhizobiaceae; Class: Alpha Proteobacteria), the causal agent of crown gall disease and *E. carotovora* (Family: Enterobacteriaceae; Class: Gamma Proteobacteria), the causal agent of soft mold and fungi of early blight *A. alternata*, the causal agent of leaf spots, rots and blights, (Family: Pleosporaceae; Class: Dothideomycetes), *F. oxysporum,* the causal agent of root rot, (Family: Tubercularіaceae; Class: Deuteromycetes) and *S. sclerotiorum* (Family: Sclerotiniaceae; Class: Leotiomycetes), the causal agent of white mold, were provided by the Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

### Synthesis of *O*-(benzoyl) chitosan derivatives

*O*-(benzoyl) chitosan derivatives were synthesized according to the method of Fischer and Speier (1895) esterification with some modifications as follows (Figure 1): 18 mmol of chitosan (3 g calculated as glucosamine units) was suspended in 50 mL of $H_2SO_4$ (5M). To this solution, the acid derivative (1-1 equivalent/ glucosamine unit of chitosan) was added. The mixture was refluxed for 4 h and was cooled subsequently to room temperature. The pH was adjusted to 7.0 by neutralization with $NaHCO_3$. The desired compound was precipitated in acetone, filtered and washed with acetone to remove the unreacted acid. Finally, the precipitants were soxhlet-extracted with acetone for two days and then oven-dried overnight at 60°C, giving the titled compounds.

### *Spectroscopic characterizations of O-(benzoyl) chitosan derivatives*

$^1$H- and $^{13}$C-NMR measurements were performed on a JEOL A-500 NMR spectrometer (Faculty of Science, Alexandria University, Alexandria, Egypt) under a static magnetic field of 500 MHz at 25°C. For these measurements, 20 mg of chitosan sample was introduced into 5 mm Φ NMR tube, to which 0.5 mL of 1% $CD_3COOD/D_2O$ solution was added, and finally the tube was kept at room temperature to dissolve the polymer. IR spectra were recorded with KBr discs in the range of 4000 to 400 $cm^{-1}$ with resolution of 4.0 $cm^{-1}$ on Nicolet RXIFT-IR Spectrophotometer.

### *$^1$H-NMR spectral data of chitosan*

δ 2.09-2.12 (br s, NHCO$\underline{CH_3}$), 3.15-3.30 (br m, H-2 of GlcN residue), 3.57-4.10 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit) and 4.88-5.00 (m, H-1 of GlcN and GlcNAc units).

### *$^1$H-NMR spectral data of O-(benzoyl) chitosan (1)*

δ 1.99-2.06 (br s, NHAc), 3.09-3.29 (br s, H-2 of GlcN residue), 3.59-4.09 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.62 (br s, H-1 of GlcNAc residue), 4.98 (br s, H-1 of GlcN residue), 7.56-7.59 (2H of the pheny ring), 7.71-7.73 (1H of the phenyl ring), 8.01-8.13 (2H of the phenyl ring) (Figure 2A).

### *$^1$H-NMR spectral data for O-(2-nitrobenzoyl) chitosan (2)*

δ 1.99-2.05 (br s, NHAc), 3.10-3.30 (br s, H-2 of GlcN residue), 3.45-4.16 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.88-4.90 (br s, H-1 of GlcN residue), 7.75-7.80 (3H of the phenyl ring), 8.01-8.04 (1H of the phenyl ring).

### *$^1$H-NMR spectral data for O-(4-nitrobenzoyl) chitosan (3)*

δ 1.94-2.14 (br s, NHAc), 3.07-3.33 (br s, H-2 of GlcN residue), 3.45-4.13 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.87-5.06 (br s, H-1 of GlcN residue), 8.11-8.27 (2H of the phenyl ring), 8.8.28-8.38 (2H of the phenyl ring).

### *$^1$H-NMR spectral data for O-(3,5-dinitrobenzoyl) chitosan (4)*

δ 2.05-2.10 (br s, NHAc), 3.08-3.33 (br s, H-2 of GlcN residue), 3.54-4.15 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.61 (br s, H-1 of GlcNAc residue), 4.78-4.89 (br s, H-1 of GlcN residue), 8.0-8.10 (1H of the phenyl ring), 8.60-8.68 (1H of the phenyl ring), 8.96-9.02 (1H of the pheny ring).

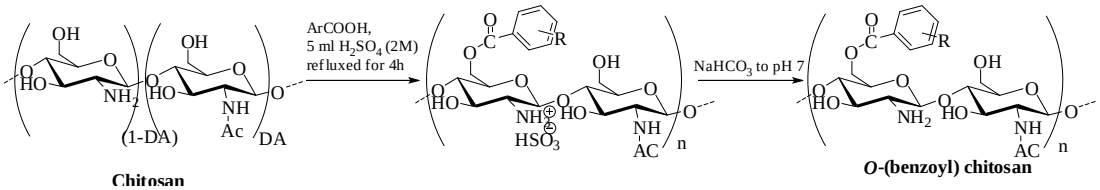


**Figure 1.** Synthetic route to *O*-(benzoyl) chitosan derivatives. DA is the degree of acetylation and Ac is the acetyl group.

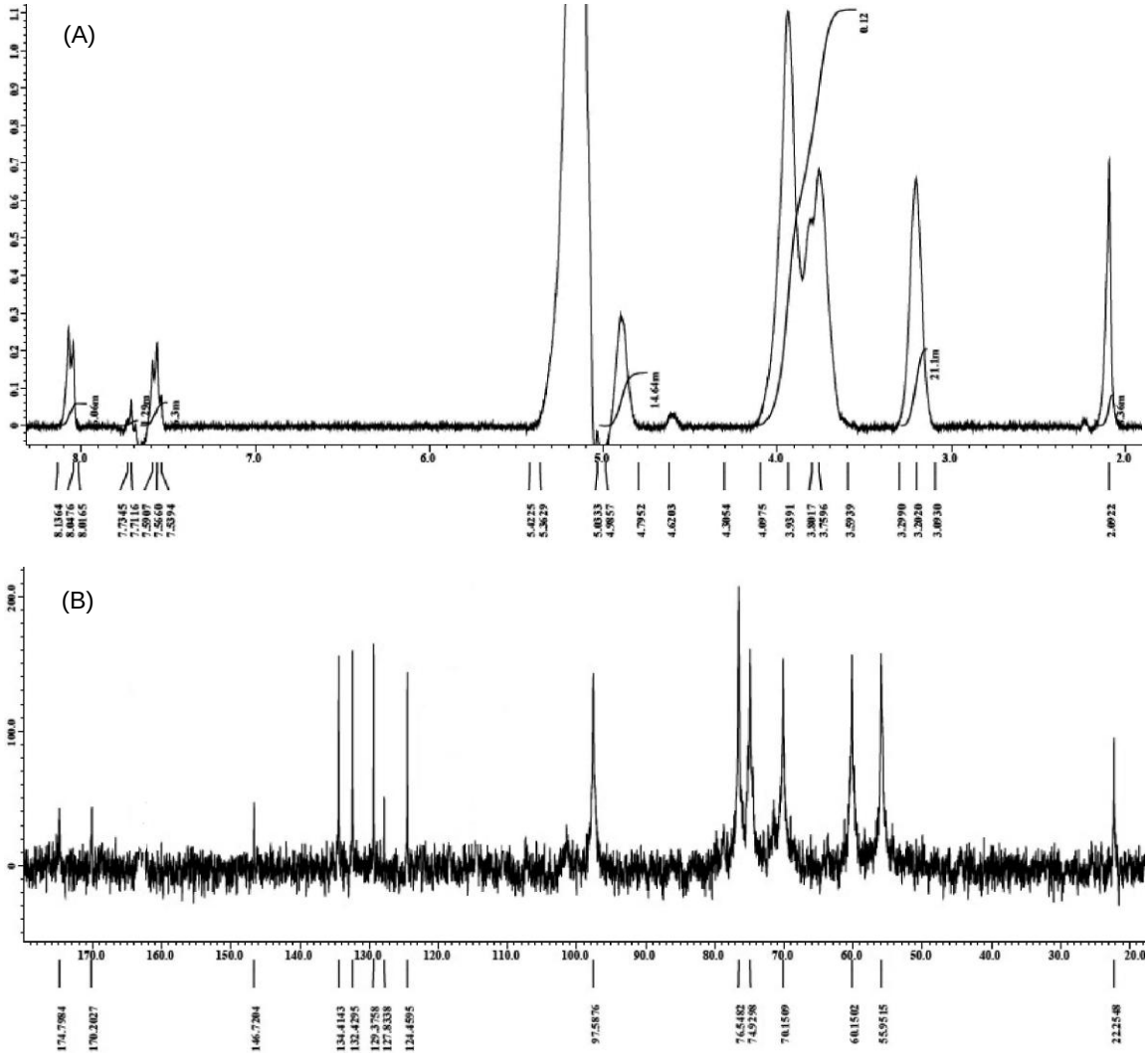


**Figure 2.** $^{1}$H-and $^{13}$C-NMR spectra of *O*-(benzoyl) chitosan (A and B, respectively) in 1% $CD_3COOD/D_2O$ at 25°C.

***$^{1}$H-NMR spectral data for O-(4-chloro-3,5-dinitrobenzoyl) chitosan (5)***

δ 2.00-2.17 (br s, NHAc), 3.06-3.30 (br s, H-2 of GlcN residue), 3.47-4.15 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 5.13-5.18 (br s, H-1 of GlcN residue), 8.75-8.78 (2H of the phenyl ring).

***$^{1}$H-NMR spectral data for O-(2-chloro-6-fluorobenzoyl) chitosan (6)***

δ 2.05-2.15 (br s, NHAc), 3.07-3.32 (br s, H-2 of GlcN residue), 3.46-4.30 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.58-4.70 (br s, H-1 of GlcNAc residue), 4.79-4.98 (br s, H-1 of GlcN resi-

due), 7.20-7.60 (3H of the phenyl ring).

***$^{13}$C-NMR spectral data of chitosan and O-(benzoyl) chitosan derivatives***

$^{13}$C-NMR of chitosan: δ 22.08 (NH(CO)<u>C</u>H$_3$), 56.59 (C-2), 61.02-61.28 (C-6), 70.59-70.76 (C-3), 75.15-75.44 (C-5), 77.78 (C-4), 98.24 (C-1) and 174.79 (<u>C</u>(O)CH$_3$). $^{13}$C-NMR of *O*-(benzoyl) chitosan derivatives: 22.00-22.80 (NH(CO)<u>C</u>H$_3$), 55.00-55.95 (C-6), 60.00-61.00 (C-2), 70.00-70.80 (C-3), 74.92-75.50 (C-5), 76.00-76.60 (C-4), 97.50-98.50 (C-1), 170.00-173.00 ppm corresponded to C=O of –OCO-Ph group, 174.00-175.50 (<u>C</u>(O)CH$_3$) and the other signals showing at 124 to 146.00 ppm attributed to the phenyl ring (Figure 2B).

**The antibacterial assay**

The bactericidal tests of chitosan compounds against *A. tumefaciens* and *E. carotovora* were performed in accordance with the National Committee for Clinical Laboratory Standards (NCCLS, 2000) to determine the minimum inhibitory concentration (MIC) values. The bacterial culture was obtained by growing the bacteria overnight at 37°C in NB. A series of concentrations (200 to 5000 mg/L) were prepared in 0.5% (v/v) aqueous trifluoroacetic acid and mixed with NA medium. The pH was adjusted to 5.5 to 6.0 with 1 M NaOH and solutions were then poured into autoclaved Petri dishes. One loopful of bacterial suspension was spotted on the surface of NA medium (ten spots per plate) then incubated at 37°C for 24 h. Each concentration was tested in triplicate. The MIC was defined as the lowest concentration of the tested sample at which the bacterial colonies were not visible with naked eye within 24 h.

**The antifungal assay**

***Mycelial growth inhibition assay***

The antifungal activity of chitosan compounds on the mycelial growth of *A. alternata*, *F. oxysporum* and *S. sclerotiorum* was tested using radial growth technique. Chitosan compounds were dissolved in 0.5% (v/v) aqueous acetic acid and the pH was adjusted to 5.5 to 6.0 with 1M NaOH. Different concentrations of chitosan compounds (250 to 6000 mg/L) were, respectively, added to sterilized PDA medium immediately before pouring into the Petri dishes. Each concentration was tested in triplicate. Parallel controls were maintained with water and aqueous acetic acid (0.5%, v/v) mixed with PDA medium. The discs of mycelial culture (0.5 cm diameter) of fungi, taken from eight-day-old cultures on PDA plates, were transferred aseptically to the centre of the Petri dishes. The plates were incubated in the dark at 26°C. The colony growth diameter was measured when the fungal growth in the control had completely covered the Petri dishes. Inhibition percentage of mycelial growth was calculated as follows:

$$\text{Mycelial growth inhibition (\%)} = \left[\frac{(DC - DT)}{DC}\right] \times 100$$

Where, DC and DT are average diameters of fungal colonies of control and treatment, respectively. Inhibiting concentration of 50% of a mycelial growth ($EC_{50}$) and its corresponding 95% confidence limits were estimated by probit analysis (Finney, 1971).

***Spore germination assay***

*A. alternata* and *F. oxysporum* spores were harvested from two-weeks-old PDA culture. Aliquots of 50 µL of a spore suspension ($1.0 \times 10^6$ conidia/mL) were placed in Eppendorf tubes containing 500 µL of PDB medium with a compound concentration. Tests were performed at concentrations of 250, 500 and 1000 mg/L. The tubes were incubated at 26°C for 24 h. The samples were placed on both chambers of a hemocytometer by carefully touching the edges of cover slip with the pipette tip and allowing capillary action to fill the counting chambers, and observed under the microscope at 40x. The numbers of germinated and non-germinated conidia were recorded and inhibition of spore germination (%) was calculated. All experiments were conducted in three replicates (Griffin, 1994).

**Statistical analysis**

Statistical analysis was performed using SPSS 12.0 software program (Statistical Package for Social Sciences, USA). The effective concentration that cause a 50% reduction of mycelial growth ($EC_{50}$) and corresponding 95% confidence limits were estimated by probit analysis (Finney, 1971). The data of spore germination and enzymes activity were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Student-Newman-Keuls (SNK) test and differences at $P \leq 0.05$ were considered as significant.

## RESULTS AND DISCUSSION

### Characterizations of *O*-(benzoyl) chitosan derivatives

*O*-(benzoyl) chitosan derivatives were obtained with moderate degree of substitution (DS) values ranging from 0.04 to 0.12 and a high DS (0.12) was obtained with *O*-(benzoyl) chitosan (Table 1). $^1$H-NMR, analysis was employed for further estimation of the degree of acetylation (DA), degree of deacetylation (DDA), formula weight (FW) and yield (%) of chitosan derivatives according to the method of Hirai et al. (1991) and Sashiwa and Shigemasa (1999). DDA was estimated from δ 3.20 ppm (x) which was attributed to H-2 of GlcN unit vs. 3.40-4.40 ppm (6-x) which was attributed to H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit. DA was estimated from δ 1.94-2.10 that was assigned to the protons of residual $CH_3$ in acetyl group vs. 3.20-4.40 ppm. DS value was based on the ratio between the areas of the protons in the phenyl substituent and the protons of the pyranose unit. FW for chitosan was 166 and for *O*-(benzoyl) chitosans ranging from 180 to 198 depending on DA, DDA and DS. The results also show that *O*-(benzoyl) chitosan derivatives were isolated with 51 to 71% yields. The results obtained from the NMR analysis indicated that the benzoyl group was introduced into the backbone of chitosan by the acylation and the reaction mainly occurred on the hydroxyl group rather than the amino group. Although, the selective *O*-acylation of chitosan in presence of $H_2SO_4$ (owing to the salt formation of the primary amino group with $H_2SO_4$) was reported previously (Badawy et al., 2005) with aliphatic acids with high DS, the *O*-(benzoyl) chitosan derivatives was obtained in the present study with low DS values. Further evidence for confirmation of the chemical structure was obtained from $^{13}$C-NMR spectroscopy. The carbon

**Table 1.** Chemical structure and properties of *O*-(benzoyl) chitosan derivatives.

| Compound | R | DA[a] | DDA[b] | DS[c] | FW[d] | Yield[e] (%) |
|---|---|---|---|---|---|---|
| Chitosan | - | 0.11 | 0.89 | - | 166 | - |
| 1 | H | 0.11 | 0.89 | 0.12 | 198 | 71 |
| 2 | 2-$NO_2$ | 0.12 | 0.90 | 0.07 | 192 | 68 |
| 3 | 4- $NO_2$ | 0.12 | 0.90 | 0.04 | 182 | 64 |
| 4 | 3,5-di $NO_2$ | 0.13 | 0.88 | 0.07 | 193 | 63 |
| 5 | 4-Cl-3,5-di $NO_2$ | 0.12 | 0.90 | 0.04 | 183 | 53 |
| 6 | 2-Cl-6-F | 0.12 | 0.89 | 0.04 | 180 | 51 |

[a]DA is a degree of acetylation; [b]DDA is a degree of deacetylation; [c]DS is a degree of substitution; [d]FW is a formula weight and calculated as follows: FW = 161×DDA + 203×DA + MW of *O*-(benzoyl) glucosamine unit×DS. Where, 161 and 203 are the FW of GlcN unit and GlcNAc, respectively. [e]Yield was determined by weight recovery in accordance with the change in FW (Sashiwa and Shigemasa, 1999).

**Table 2.** Antibacterial activity of chitosan and *O*-(benzoyl) chitosans against *A. tumefaciens* and *E. carotovora.*

| Compound | R | MIC[a] (mg/L) | |
|---|---|---|---|
| | | ***A. tumefaciens*** | ***E. carotovora*** |
| **Chitosan** | - | > 5000 | > 5000 |
| **1** | H | 4800 | 4500 |
| **2** | 2-$NO_2$ | 4400 | 4250 |
| **3** | 4- $NO_2$ | 3775 | 3610 |
| **4** | 3,5-di $NO_2$ | 3275 | 3125 |
| **5** | 4-Cl-3,5-di $NO_2$ | 4500 | 4350 |
| **6** | 2-Cl-6-F | 4710 | 4475 |

[a]MIC minimum inhibitory concentration.

peaks due to C=O and $CH_3$ were found at 174.97 and 22.08 ppm in chitosan, respectively. When we grafted *O*-(benzoyl) in the chitosan molecule, new chemical shifts at 170.20 ppm corresponded to C=O of –OCO-Ph group, and the other signals showing at 124.50, 127.57, 129.37, 132.42, 134.41 and 146.72 ppm phenyl ring were attributed to the structures of *O*-(benzoyl) chitosan derivative (Figure 2B). The $^{13}$C-NMR spectrum again confirmed that the chitosan derivative was synthetized successfully.

The characteristic FTIR pattern of chitosan exhibited the absorption band at wave number band 3372 $cm^{-1}$ corresponding to the contribution of -OH and -NH stretching vibration, as well as inter- and extra-molecular hydrogen bonding of chitosan molecules (Figure 3A). The weak absorption bands at 2868 and 2919 $cm^{-1}$ represent -CH- stretching vibration of chitosan. The absorption bands at wave numbers of 1657 and 1377 $cm^{-1}$ corresponded to the C=O and C–O stretching of amide group, respectively. In addition, the absorption band at wave number 1596 $cm^{-1}$was due to the N–H deformation of amino groups, while the absorption band at wave numbers 1152, 1075 and 1024 $cm^{-1}$ corresponded to the symmetric stretching of the C–O–C and involved skeletal vibration of the C–O stretching, respectively (Brugnerotto et al., 2001). The FTIR spectra of *O*-(benzoyl) chitosan derivatives were similar to that of chitosan except the additional absorption bands at wave numbers 1455 and 747 $cm^{-1}$. These bands were assigned to the C=C stretching and C–H deformation (out of plane) of the aromatic group, respectively as shown in Figure 3B for compound 1.

## The antibacterial activity of *O*-(benzoyl) chitosan derivatives

The antibacterial activities of *O*-(benzoyl) chitosan derivatives against *A. tumefaciens* and *E. carotovora* are shown in Table 2. It was found that chitosan gave a less inhibitory effect on the tested bacteria. *O*-(3,5-dinitro-benzoyl) chitosan (4) was the most active with MIC = 3275 and 3125 mg/L against *A. tumefaciens* and *E. carotovora,* respectively. However, *O*-(benzoyl) chitosan

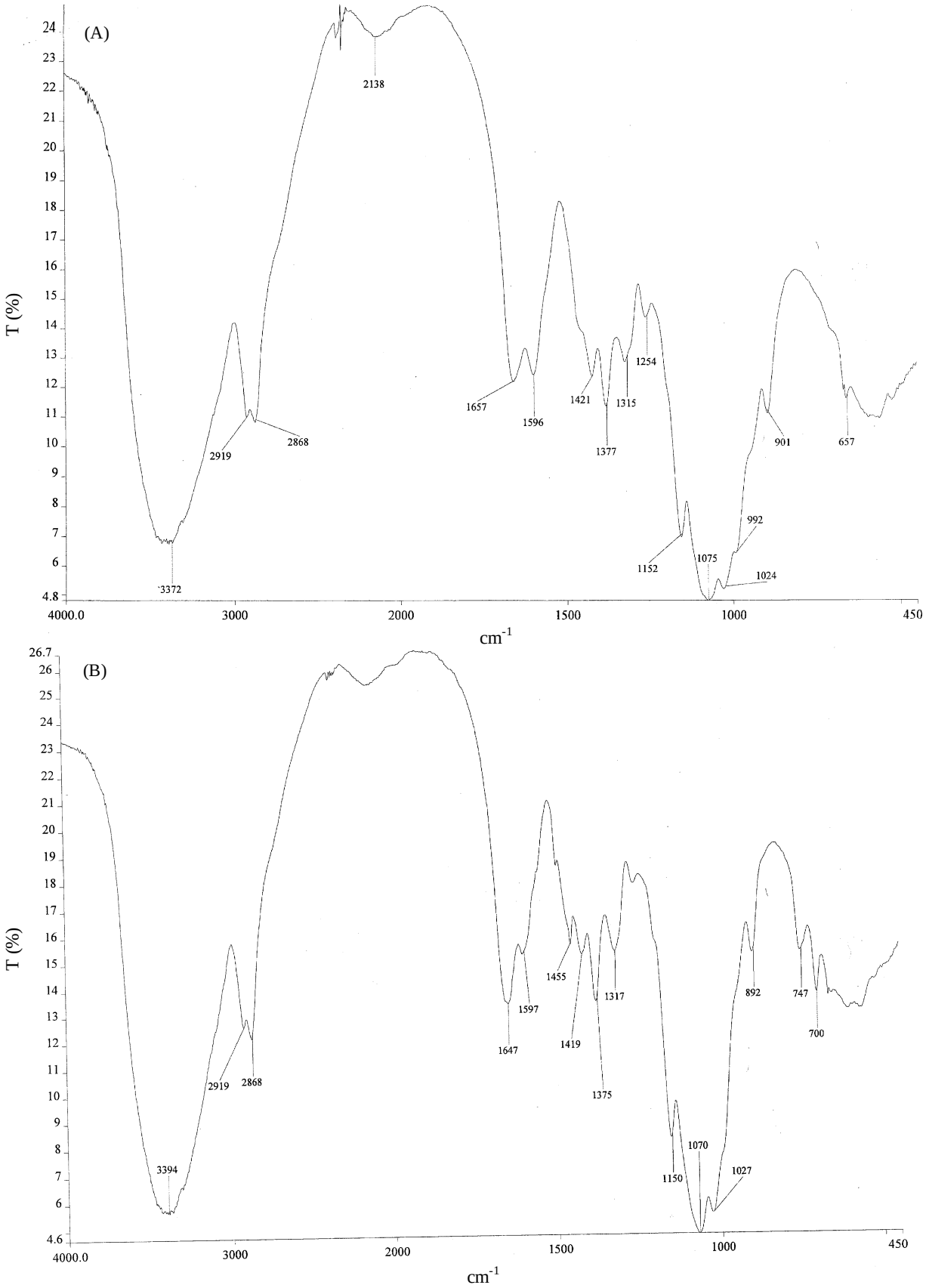


**Figure 3.** The FTIR spectra of chitosan (A) and *O*-(benzoyl) chitosan derivative (compound 1, B).

**Table 3.** Antifungal activity of chitosan and *O*-(benzoyl) chitosan derivatives against *A. alternata*, *F. oxysporum* and *S. sclerotiorum*.

| Compound | $EC_{50}$[a] (mg/L) | 95% confidence limits (mg/L) | | Slope[b] ± SE | Intercept[c] ± S.E | $(\chi^2)$[d] |
|---|---|---|---|---|---|---|
| | | Lower | Upper | | | |
| ***A. alternata*** | | | | | | |
| Chitosan | > 6000 | - | - | - | - | - |
| 1 | 3374 | 2541 | 5328 | 1.26±0.20 | -4.45±0.66 | 2.51 |
| 2 | 5467 | 3738 | 11064 | 1.23±0.21 | -4.60±0.70 | 0.802 |
| 3 | 3854 | 2837 | 6452 | 1.25±0.20 | -4.60±0.70 | 1.185 |
| 4 | 3649 | 2767 | 5663 | 1.37±0.21 | -4.88±0.68 | 3.219 |
| 5 | 5113 | 3607 | 9481 | 1.30±0.22 | -4.85±0.71 | 0.001 |
| 6 | 3040 | 2484 | 4103 | 1.95±0.26 | -6.81±0.85 | 3.25 |
| ***F. oxysporum*** | | | | | | |
| Chitosan | > 3000 | - | - | - | - | - |
| 1 | 1696 | 893 | 4881 | 2.57±0.26 | -8.32±0.84 | 6.579 |
| 2 | 1989 | 1686 | 2447 | 1.87±0.24 | -6.19±0.77 | 3.534 |
| 3 | 1750 | 1500 | 2092 | 1.96±0.24 | -6.36±0.76 | 2.581 |
| 4 | 1706 | 1423 | 2116 | 1.62±0.23 | -7.22±0.77 | 3.442 |
| 5 | 1862 | 1592 | 2245 | 1.95±0.24 | -6.40±0.77 | 2.188 |
| 6 | 1526 | 1251 | 1905 | 1.48±0.22 | -4.72±0.70 | 0.415 |
| ***S. sclerotiorum*** | | | | | | |
| Chitosan | > 6000 | - | - | - | - | - |
| 1 | 3872 | 3491 | 4443 | 4.64±0.61 | -16.66±2.15 | 0.450 |
| 2 | 5842 | 4539 | 8920 | 2.39±0.36 | -9.01±1.24 | 0.87 |
| 3 | 4604 | 3564 | 6844 | 1.79±0.24 | -6.58±0.81 | 2.788 |
| 4 | 4321 | 3413 | 6147 | 1.88±0.24 | -6.86±0.81 | 3.434 |
| 5 | 4660 | 3813 | 6297 | 2.50±0.33 | -9.18±1.11 | 3.070 |
| 6 | 3301 | 2704 | 4456 | 2.13±0.28 | -7.50±0.93 | 1.007 |

[a]The concentration causing 50% mycelial growth inhibition; [b]slope of the concentration-inhibition regression line ± standard error; [c]intercept of the regression line ± standard error; [d]Chi square value.

(1) possessed a weak antibacterial activity against *A. tumefaciens* and *E. carotovora* (MIC = 4800 and 4500 mg/L, respectively). It was observed that *di*-substitution of nitro group was very effective in decreasing the viable growth for both bacteria than the mono-substitution (see compound 4 versus 2 and 3). It was also shown that introducing the chlorine atom into the *di* nitro-substitution (5) caused lower inhibition of viable growth of both test organisms.

Considering the susceptibility of the microorganisms, it was noticed that *E. carotovora* was more susceptible to these compounds than *A. tumefaciens* which may be attributed to their different cell walls (Badawy and Rabea, 2012; Xie et al., 2002). The fact may be attributed to the cell wall of *A. tumefaciens* and *E. carotovora*, which are typical Gram-negative bacteria. The cell wall of Gram-negative bacteria is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Due to the of the bilayer structure, the outer membrane is a potential barrier against foreign molecules (Ratledge and Wilkinson, 1988).

## The antifungal activity of *O*-(benzoyl) chitosan derivatives

The antifungal activity of *O*-(benzoyl) chitosan derivatives towards the three plant pathogenic fungi *A. alternata*, *F. oxysporum* and *S. sclerotiorum* was investigated *in vitro* and the results are shown in Table 3. In general, all the modified derivatives were more active than the unmodified chitosan. Compound *O*-(2-chloro-6-fluoro benzoyl) chitosan (6) exerted significantly prominent antifungal activity with $EC_{50}$ of 3040, 1526 and 3301 mg/L against *A. alternata*, *F. oxysporum* and *S. sclerotiorum,* respectively. However, *O*-(2-nitrobenzoyl) chitosan (2) was the lowest active one with $EC_{50}$ of 5467, 1989 and 5842 mg/L against *A. alternata*, *F. oxysporum* and *S. sclerotiorum*, respectively. With regards to the effect of the substituent and the position on the phenyl moiety, it

**Table 4.** Effect of chitosan and *O*-(benzoyl) chitosan derivatives on spore germination of *A. alternata* and *F. oxysporum.*

| Compound | R | Concentration (mg/L) | Inhibition of spore germination (%) ± SE | |
|---|---|---|---|---|
| | | | ***A. alternata*** | ***F. oxysporum*** |
| **Control** | - | 0 | $0.00^{i}$±0.00 | $5.28^{h}$±3.06 |
| | | 250 | $14.38^{hi}$±5.44 | $18.06^{gh}$±3.23 |
| **Chitosan** | - | 500 | $25.00^{fgh}$±8.33 | $33.80^{fg}$±7.19 |
| | | 1000 | $47.50^{de}$±7.25 | $58.63^{de}$±6.03 |
| | | 250 | $17.50^{hi}$±3.06 | $28.81^{fg}$±3.14 |
| **1** | H | 500 | $41.67^{defg}$±4.81 | $43.75^{ef}$±6.25 |
| | | 1000 | $60.83^{cd}$±3.94 | $69.05^{cd}$±1.37 |
| | | 250 | $25.00^{fgh}$±8.33 | $30.00^{fg}$±7.58 |
| **2** | 2-$NO_2$ | 500 | $44.64^{def}$±5.36 | $59.17^{de}$±3.44 |
| | | 1000 | $85.42^{ab}$±8.59 | $95.00^{ab}$±5.00 |
| | | 250 | $17.74^{hi}$±1.39 | $19.64^{gh}$±3.09 |
| **3** | 4- $NO_2$ | 500 | $45.83^{def}$±4.17 | $56.67^{de}$±4.08 |
| | | 1000 | $72.08^{bc}$±7.56 | $80.87^{bc}$±1.70 |
| | | 250 | $18.99^{hi}$±2.32 | $31.25^{fg}$±2.08 |
| **4** | 3,5-di $NO_2$ | 500 | $50.00^{de}$±0.00 | $55.71^{de}$±4.29 |
| | | 1000 | $83.33^{ab}$±9.62 | $90.83^{ab}$±5.34 |
| | | 250 | $21.31^{ghi}$±2.54 | $34.58^{fg}$±3.56 |
| **5** | 4-Cl-3,5-di $NO_2$ | 500 | $63.33^{cd}$±1.92 | $63.33^{d}$±1.92 |
| | | 1000 | $100.00^{a}$±0.00 | $100.00^{a}$±0.00 |
| | | 250 | $17.71^{hi}$±2.62 | $18.33^{gh}$±6.87 |
| **6** | 2-Cl-6-F | 500 | $33.63^{efgh}$±3.65 | $39.17^{f}$±3.94 |
| | | 1000 | $62.50^{cd}$±4.17 | $69.94^{cd}$±2.03 |
| *F* | | | 26.64 | 38.05 |
| df | | | 21, 66 | 21, 66 |
| *P* | | | < 0.0001 | < 0.0001 |

Data are averages of four replicates ± SE. Values within a column bearing the same letter are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls (SNK) test. *F*: F ratio, df: degree of freedom, *P:* p value (significance of the F ratio).

was observed that *para* substitution with nitro group was more effective in decreasing the fungal mycelial growth for the tested fungi than the *ortho* substitution (see compound 3 versus 2). With regards to the susceptibility of the three tested fungi, it can be noticed that fungous of *F. oxysporum* was more susceptible than *A. Alternata* and *S. sclerotiorum* to *O*-(benzoyl) chitosan derivatives. In addition, the effect of chitosan and its derivatives on spore germination of *A. alternata* and *F. oxysporum* are shown in Table 4. The result shows that a complete inhibition (100%) of the fungal spores was observed with *O*-(2-chloro-6-fluorobenzoyl) chitosan (6) at high concentration (1000 mg/L).

Based on our experiments and those from literature, we believed that the use of natural compounds to control plant pathogens may lead to a reduction in the use of fungicides. Chitosan is already known to interfere with the growth of several phytopathogenic fungi including *Botrytis cinerea*, *Pythium debaryanum* and *Rhizoctonia solani* (Badawy, 2010; Badawy and Rabea, 2012; Rabea et al., 2009) but the mechanism by which it affects the growth of the pathogen is still unclear. It is known that chitosan

antimicrobial activity is influenced by a number of factors that act in an orderly and independent fashion. Because of the positive charge on the C-2 of the glucosamine monomer below pH 6.0, chitosan is more soluble and has a better antimicrobial activity by interfering with the negatively charged residues of macromolecules exposed on the fungal cell surface, and thereby changes the permeability of the plasma membrane (Rabea et al., 2003). Our data indicates that the chemical modification of chitosan molecule by esterification reaction led to enhancing its antifungal activity against the tested fungi. The compounds inhibited both spore germination and mycelial growth of *A. Alternata* and *F. oxysporum* as shown in Tables 3 and 4. This result is interesting if we consider that some commercial antifungal agents act only on spore germination or on mycelial growth. However, it is still not known how chitosan derivatives inhibited spore germination and blocked the mycelial growth. On the other hand, many fungicides have little or no effect on spore germination but strongly inhibit mycelial growth. Consequently, comparison of the potency of chitosan derivatives as an inhibitor of spore formation with its activity in a mycelial growth assay can provide preliminary information on its mode of action.

In general, chitosan inhibits spore germination and radial growth of *B. cinerea* (El-Ghaouth et al., 1992) However, this author did not achieve complete inhibition even at a concentration of 6000 mg/L, indicating that chitosan is more fungistatic rather than fungicidal. This finding is in agreement with our results, reporting that the $EC_{50}$ of chitosan is higher than 6000 mg/L for *A. alternata* and *S. sclerotiorum*. According to this fact, several research groups have started to modify chitosan molecule to produce high antimicrobial active compounds. For example, we have previously prepared some hydrophobic chitosan derivatives through the reductive amination reaction with various aldehydes. We noted that *N*-alkylation or -arylation of chitosan with aliphatic or aromatic aldehydes, respectively, effectively enhanced the antifungal activity of chitosan against *B. cinerea*, *F. oxysporum* and *Pythium debaryanum,* respectively (Badawy et al., 2005; Rabea et al., 2005; 2006, 2009). However, *N,N,N*-dimethylalkyl chitosans as water soluble derivatives that was recently prepared in our laboratory enhanced the antibacterial activity against *A. tumefaciens* and *E. carotovora* and *N,N,N*-dimethylpentyl chitosan was the most active with MIC 750 and 1225 mg/L, respectively. However, both *N,N,N*-dimethylpentyl chitosan and *N,N,N*-dimethyloctyl chitosan were significantly the highest in fungal mycelial growth inhibition of *B. cinerea*, *F. oxysporum* and *P. debaryanum* (Badawy, 2010).

## Conclusion

Esterification of chitosan could be accomplished successfully in one pot reaction. The established procedure enables a facile preparation of *O*-(benzoyl) chitosan derivatives which are evaluated as biological active materials against the plant pathogenic bacteria and fungi. The chemical structures were defined by FT-IR, $^{1}$H-NMR and $^{13}$C-NMR spectra and confirmed that the acyl group was selectively acylated onto the hydroxyl group of chitosan. The derivatization improved the antimicrobial activity of chitosan and expanded the antimicrobial spectrum against bacteria of *A. tumefaciens* and soft mold *E. carotovora* and fungi of *A. alternata*, *F. oxysporum* and *S. sclerotiorum* when compared with that of chitosan. These results suggest that *O*-(benzoyl) chitosans have the potential of becoming alternatives for plant protection instead of some harmful microbicides, because of its higher antimicrobial activity.

## REFERENCES


Abdelaal MY, Sobahi TR, Al-Shareef HF (2013). Modification of chitosan derivatives of environmental and biological interest: A green chemistry approach. Int. J. Biol. Macromol. 55:231-239.

Anitha A, Divya Rani VV, Krishna R, Sreeja V, Selvamurugan N, Nair SV, Tamura H, Jayakumar R (2009). Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, *O*-carboxymethyl and *N,O*-carboxymethyl chitosan nanoparticles. Carbohydr. Polym. 78:672-677.

Badawy MEI (2010). Structure and antimicrobial activity relationship of quaternary *N*-alkyl chitosan derivatives against some plant pathogens. J. Appl. Polym. Sci. 117:960-969.

Badawy MEI, Rabea EI (2011). A biopolymer chitosan and its derivatives as promising antimicrobial agents against plant pathogens and their applications in crop protection. Int. J. Carbohydr. Chem., ID 460381, 29 p. doi:10.1155/2011/460381.

Badawy MEI, Rabea EI (2012). Characterization and antimicrobial activity of water-soluble *N*-(4-carboxybutyroyl) chitosans against some plant pathogenic bacteria and fungi. Carbohydr. Polym. 87:250-256.

Badawy MEI, Rabea EI, Rogge TM, Stevens CV, Steurbaut W, Höfte M, Smagghe G (2005). Fungicidal and insecticidal activity of *O*-acyl chitosan derivatives. Polym. Bull. 54:279-289.

Brugnerotto J, Lizardi J, Goycoolea FM, Argüelles-Monal W, Desbrières J, Rinaudo M (2001). An infrared investigation in relation with chitin and chitosan characterization. Polymer 42:3569-3580.

El-Ghaouth A, Arul J, Grenier J, Asselin A (1992). Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. Phytopathology 82:398-402.

Finney DJ (1971). ProbitAnalysis, 3$^{rd}$ ed. CambridgeUniversity Press, Cambridge. pp. 318.

Fischer E, Speier A (1895). Darstellung der Ester. Chemische Berichte, 28:3252-3258.

Griffin DH (1994). Spore dormancy and germination In: Fungal physiology, 2nd ed. New York, NY: John Wiley & Sons; p. 375-98.

Hirai A, Odani H, Nakajima A (1991). Determination of degree of deacetylation of chitosan by $^{1}$H-NMR spectroscopy. Polym. Bull. 26:87-94.

Kim TH, Jiang HL, Jere D, Park IK, Cho MH, Nah JW, Choi Y-J, Akaike T, Cho C-S (2007). Chemical modification of chitosan as a gene carrier *in vitro* and *in vivo*. Progress in Polym. Sci. 32:726-753.

Liu Y, Zhao D, Wang JT (2013). Preparation of *O*-carboxymethyl chitosan by Schiff base and antibacterial activity. Adv. Mat. Res. 647:794-797.

Muzzarelli RAA (2009). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. Carbohydr. Polym. 77:1-9.

National Committee for Clinical Laboratory Standards. (2000). Methods for dilution antimicrobial susceptibility tests for bacteria that grow

aerobically; Approved standard. 5th ed. NCCLS document M7-A5, Wayne, Pennsylvania.
Pires, NR, Cunha PLR, Maciel JS, Angelim AL, Melo VMM, de Paula RCM, Feitosa JPA (2013). Sulfated chitosan as tear substitute with no antimicrobial activity. Carbohydr. Polym. 91:92-99.
Rabea EI, Badawy MEI, Rogge TM, Stevens CV, Smagghe G, Höfte M, Steurbaut W (2005). Insecticidal and fungicidal activity of new synthesized chitosan derivatives. Pest Manag. Sci. 61:951-960.
Rabea EI, Badawy MEI, Rogge TM, Stevens CV, Steurbaut W, Höfte M, Smagghe G (2006). Enhancement of fungicidal and insecticidal activity by reductive alkylation of chitosan. Pest Manag. Sci. 62:90-97.
Rabea EI, Badawy MEI, Steurbaut W, Stevens CV (2009). *In vitro* assessment of *N*-(benzyl) chitosan derivatives against some plant pathogenic bacteria and fungi. Eur. Polym. J. 45:237-245.
Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W (2003). Chitosan as antimicrobial agent: Applications and mode of action. Biomacromolecules. 4:1457-1465.
Ratledge C, Wilkinson SG (1988). An overview of microbial lipids. In: Ratledge, C., Wilkinson, S.G. (Eds.), Microbial Lipids, vol. 1. Academic Press, London. pp. 3-22.
Sajomsang W, Gonil P, Saesoo S (2009). Synthesis and antibacterial activity of methylated *N*-(4-*N*,*N*-dimethylaminocinnamyl) chitosan chloride. Eur. Polym. J. 45:2319-2328.
Sashiwa H, Shigemasa Y (1999). Chemical modification of chitin and chitosan 2: preparation and water soluble property of *N*-acylated or *N*-alkylated partially de-acetylated chitins. Carbohydr. Polym. 39:127-138.
Taghizadeh MT, Bahadori A (2013). Preparation, characterization and adhesive properties of di- and tri-hydroxy benzoyl chitosan nanoparticles. Chinese J. Polym. Sci. 31:649-659.
Wang J, Wang H (2011). Preparation of soluble *p*-aminobenzoyl chitosan ester by Schiff's base and antibacterial activity of the derivatives. Int. J. Biol. Macromol. 48:523-529.
Xie W, Xu P, Wang W, Liu Q (2002). Preparation and antibacterial of a water-soluble chitosan derivative. Carbohydr. Polym. 50:35-40.