Synthesis of a Quaternary Ammonium Derivative of Chito-oligosaccharide as Antimicrobial Agent for Cellulosic Fibers

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ABSTRACT: A derivative of chito-oligosaccharide (COS), N-(2-hydroxyl)propyl-3-trimethyl ammonium chito-oligosaccharide chloride (HTACC), was synthesized using a reaction of glycidyltrimethylammonium chloride (GTMAC) and COS prepared by depolymerization of a fully deacetylated chitosan. COS and HTACC were applied to the cotton fabrics with a pad-dry-cure process using the reaction between the hydroxyl group of cellulose and terminal aldehyde group in COS and HTACC. Their minimum inhibition concentration (MIC) was evaluated, and the antimicrobial activity and durability to laundering of cotton fabrics treated with them were compared. The complete substitution of NH_2 groups in COS with GTMAC was obtained at a 4 : 1 mol ratio of GTMAC to NH_2 in 18 h at 80°C under the presence of acetic acid. MIC values of the 1.04 DS of HTACC and COS were 50 and 400 μg/mL, respectively. A cotton fabric treated with 0.2% of HTACC and 1.8% of COS exhibited 100% reduction of bacteria. At the 50th laundering cycle, 0.3% of HTACC and 2.4% of COS indicated 100% bacterial reduction.
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Key words: cellulosic fiber; antimicrobial activity; chito-oligosaccharide; N-(2-hydroxy)propyl-3-trimethyl ammonium chito-oligosaccharide chloride

INTRODUCTION

Chitin is the second most plentiful natural polymer and is attracting a lot of attention in agricultural, industrial, and medical fields.[1,2] Chitosan, a deacetylated derivative of chitin, is poly[β-(1 → 4)-2-amino-2-deoxy-D-glucopyranose] and is a nontoxic and biodegradable polymer. One of the most important characteristics of chitosan is its antimicrobial activity due to the amino group at the C-2 position of the glucosamine residue.

There are some reports about the utility of chitosan polymer to impart antimicrobial activity in textile finishing. For examples, (1) chitosan salt produced by organic acid was bound to the surface of textiles by a large amount of resin that formed crosslinks.[3] (2) A quaternary ammonium derivative of chitosan demonstrates a satisfactory use as an antimicrobial agent, but this derivative lacks durability to laundering, although the proper use of binder could improve the laundering durability.[4] Recently, we reported on the preparation of depolymerized chitosan, chito-oligosaccharide (COS), as an antimicrobial agent to overcome the difficulty of applying a high molecular weight chitosan polymer to a fabric. The fabric treated with COS exhibited durable antimicrobial activity without the need for a binding chemical as a crosslinker.[5]

One of the most important classes of antimicrobial agents is quaternary ammonium salts. It is expected that a quaternary ammonium derivative of COS could further improve its antimicrobial activity. In this study, we synthesized a quaternary ammonium derivative of COS, N-(2-hy-

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droxy)propyl-3-trimethyl ammonium COS chloride (HTACC), using a reaction of glycidyltrimethylammonium chloride (GTMAC) and COS. The COS and HTACC were used to treat cotton. Antimicrobial activity was evaluated to compare the COS and HTACC.

EXPERIMENTAL

Preparation of Chito-oligosaccharide (COS)

A solution of chitosan was prepared by adding 3 g of the fully deacetylated chitosan (99.3% degree of deacetylation), prepared by the method of Mima et al.[6] to 100 mL of a 2% (w/w) aqueous acetic acid solution. An aqueous solution containing 0.429 g of sodium nitrite, corresponding to 1/3 mol equivalent to a glucosamine unit of chitosan, was slowly stirred into the chitosan solution over a half-hour period. The reaction mixture was stirred for an additional 2.5 h at room temperature, then neutralized with dilute NaOH solution. Excess water was evaporated with a Shimazu rotary evaporator at 40°C while applying a vacuum to make a concentrated solution of 10% of total volume. To extract the COS, the solution was poured into excess methanol. The precipitate was collected by filtration and washed with methanol, then washed several times with acetone, dried in vacuum at 40°C, and kept in a refrigerator.

The IR spectrum of COS was obtained with a MIDAC Prospect FTIR spectrometer. ^{1}H-NMR spectrum was obtained with a Bruker AMX 500 spectrometer, and COS was dissolved in D_2O, which contained a small amount of CD_3COOD. The average degree of polymerization (DP) was determined by colorimetric titration. Terminal aldehyde groups of COS reacted with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH, Aldrich Chem. Co.) and ferric chloride (Showa Chemicals Inc., Japan) to yield an intense blue color. The absorbance at 650 nm was measured with a Shimazu UV-VIS scanning spectrophotometer, and the amount of the end groups was determined from the calibration curve between the concentrations of glucosamine and absorbance.[7,8]

Synthesis of HTACC

A 2-g quantity of COS was dispersed in 40 mL of distilled water, and GTMAC (Negese Co. Ltd., Japan) was stirred into the dispersion under N_2 atmosphere. After the reaction, the solution was poured into an acetone/ethanol (1/1, v/v) mixture. The precipitate was washed three times with an acetone/ethanol (1/1, v/v) mixture, filtered, dried in vacuum at 40°C, and kept in a desiccator.

The chlorine content in HTACC was measured by a combustion flask method to determine the degree of substitution (DS) of NH_2 groups in COS with GTMAC. The degree of substitution was calculated as follows:

$$DS = 645.48/[(10635/Y(\%) - 454.92)]$$

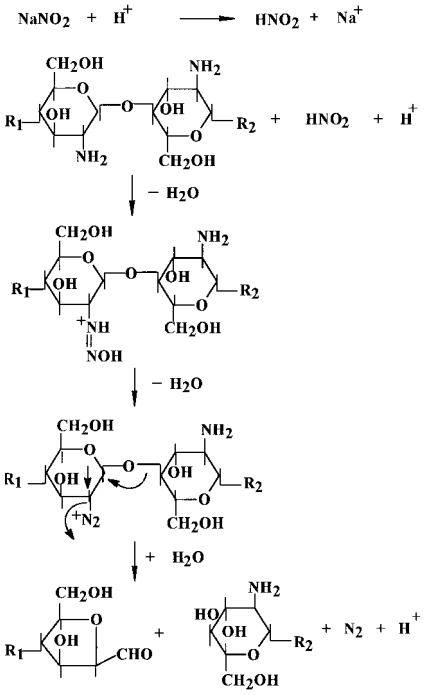
where Y is the percent chlorine content of HTACC. The IR spectrum of HTACC was obtained with a MIDAC Prospect FTIR spectrometer. The IR sample of HTACC was prepared by making a pellet with potassium bromide. The ^{1}H-NMR spectrum was obtained with a Bruker AMX 500 spectrometer and HTACC was dissolved in D_2O.

Treatment of COS and HTACC on Cotton Fabric

Scoured and bleached, 100% cotton fabrics were treated with COS and HTACC by a pad-dry-cure process. A pad bath consisted of COS; acetic acid, which was in mol equivalence to NH_2 in COS; $MgCl_2 \cdot 6H_2O$ in 0.6 mol equivalence to COS as a catalyst; 0.1% Triton X-100 as a penetrating agent; and 0.1% softening agent. The other pad bath consisted of HTACC; $MgCl_2 \cdot 6H_2O$ in 0.6 mol equivalence to HTACC as a catalyst; 0.1% Triton X-100 as a penetrating agent; and 0.1% softening agent. The pressure on the mangle was adjusted to produce 80% wet pickup. The padded sample was dried at 60°C for 3 min and then cured at 120°C for 3 min. The treated cotton samples were rinsed with tap water thoroughly for 30 min at 50°C and dried at room temperature.

Antimicrobial Activity

Minimum inhibition concentration (MIC) of COS and HTACC to a Gram-positive bacterium, *Staphylococcus aureus* (American type culture collection No. 6538) was determined according to the broth serial dilution method from NCCLS (National Committee for Clinical Laboratory Standards, USA). Muller Hinton broth (DIFCO Laboratories) was used for the dilution medium of the MIC test.



Scheme 1 Depolymerization mechanism of chitosan by sodium nitrite.

The AATCC Test Method 100-1993 was used for determining the antimicrobial activity of cotton fabric treated with COS and HTACC. *Staphylococcus aureus* was used to test antimicrobial activity. The number of swatches used per jar was four for each sample. The dilution medium was Nutrient broth (DIFCO Laboratories). The neutralizer was a dilute phosphate buffer solution (pH 7.2). The results were expressed as percent reduction of bacteria (R) by the following formula:

$$R = 100(C - A)/C$$

where A is the number of bacteria recovered from the inoculated-treated test specimen swatches in the jar incubated over the desired contact period; and C is the number of bacteria recovered from the inoculated untreated control specimen swatches in the jar immediately after inoculation (at "0" contact time). To determine if the finish washed off during laundering, the treated samples were washed under washing and drying condition, specified in 1-III-A-i of AATCC Test Method 135-1995.

RESULTS AND DISCUSSION

Preparation of COS

The almost 100% deacetylated chitosan was depolymerized into COS. The overall stoichiometry of the reaction between chitosan and HONO is shown in Scheme 1.[9] Nitrosating species originating from the HONO attack the amine groups and subsequently cleave the β-glycosidic linkages. Hence, a 2,5-anhydro-D-mannose unit is formed as the reducing end group of the cleaved polymer. It is reported[7,8] that MBTH reacts readily with 2,5-anhydrohexoses produced by the deamination of hexosamines, and exhibits a blue color when ferric chloride solution is added. From a calibration curve of the concentration of glucosamine vs. absorbance at 650 nm, the DP of the COS was 3. COS was confirmed by the spectrum band in the 1585 cm^{-1} and 1655 cm^{-1} connected with NH_2 scissoring and C═O stretching peaks, respectively, as shown in Figure 1. Figure 2 shows ^{1}H-NMR spectroscopy of COS. The protons of C(1), C(2), and C(3)–C(6) absorb at δ = 4.85, δ = 3.15, and δ = 3.5–4.0 ppm, respectively. The proton of the terminal aldehyde group absorbs at δ = 9.8 ppm. The above ^{1}H-NMR peaks could also verify the COS.

Synthesis of HTACC

The HTACC was synthesized from the reaction of GTMAC and NH_2 groups in COS as shown in Scheme 2. The COS has both NH_2 groups and OH groups, which could react with GTMAC. It is known that GTMAC mainly reacts with NH_2 groups in COS under acidic condition, while it reacts with OH groups under alkaline condition.[10] The DS of the HTACC depends on the reaction conditions. Therefore, reaction time, re-

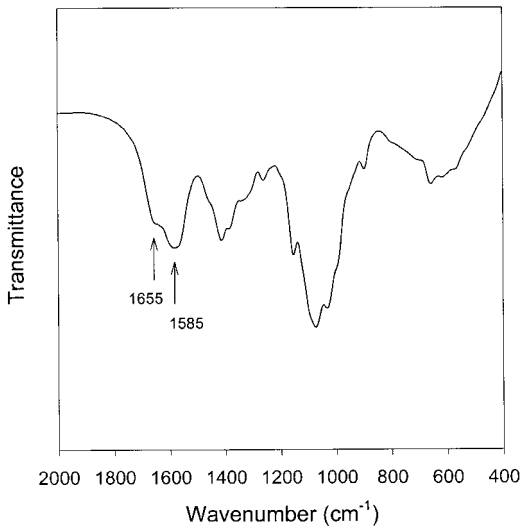


Figure 1 FTIR spectrum of COS.

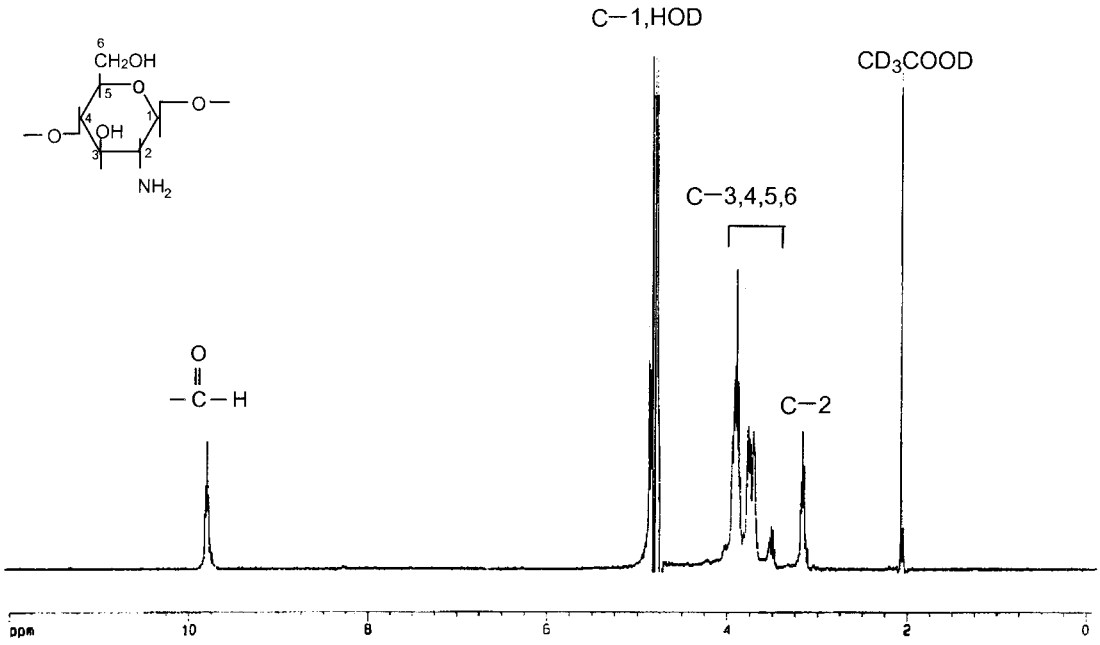
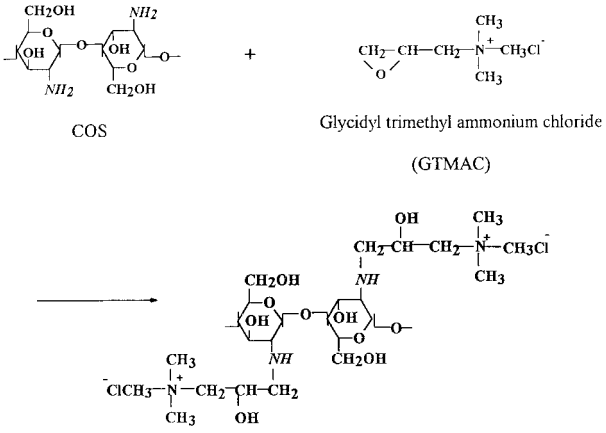


Figure 2 ^{1}H-NMR spectrum of COS.

action temperature, and mol ratio of GTMAC to NH_2 groups in COS were studied. Table I shows the effect of reaction time on the DS. Generally, with a longer reaction time, higher DS of the HTACC can be expected. With increasing reaction time from 0 to 18 h under the presence of acetic acid, the DS increased significantly. This is possibly due to the fact that acidic condition makes the epoxy ring of GTMAC more actively reactive with the NH_2 groups in COS. After 18 h, the DS showed a minimal increment, suggesting 18 h as optimal reaction time.

Figure 3 shows the effect of reaction temperature on the DS. As the reaction temperature increased up to 50°C, the DS increased almost proportionally. Above the 50°C level, only a minimal increase occurred. The DS reached 1.02 in 18 h at 80°C under 1% acetic acid, indicating the complete substitution of NH_2 groups in COS. Figure 4 shows the effect of the mol ratio of GTMAC to NH_2 groups in COS on the DS. The DS of HTACC increases with increasing the GTMAC amounts. The DS, at a six to one mol ratio, is 1.04, indicating the complete substitution of NH_2 groups in COS. The synthesis of HTACC could be verified by the disappearance of the NH_2 scissoring peak



Scheme 2 Reaction between COS and GTMAC.

Table I Effect of Reaction Time on the DS with or without Acid Catalyst

Catalyst	Reaction[a] Time (h)	DS
None	6	0.19
	12	0.36
	18	0.43
	24	0.47
	30	0.49
Acetic acid	6	0.53
	12	0.79
	18	0.86
	24	0.90
	30	0.92

[a] Other reaction conditions: reaction temp.; 50°C, mol ratio of GTMAC to NH_2 in COS; 4.

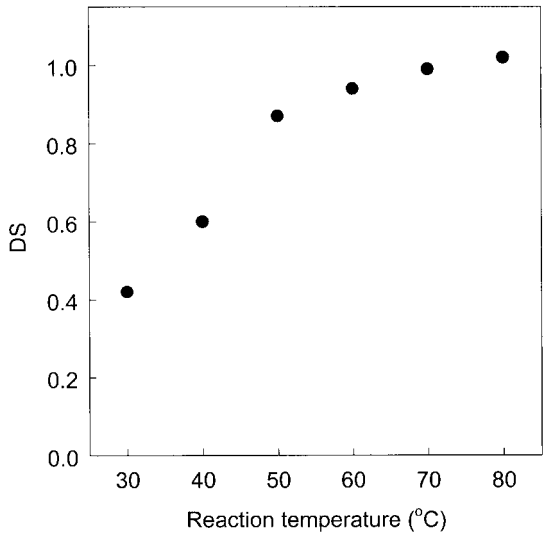


Figure 3 Effect of reaction temperature on the DS. Reaction conditions: cat; 1% acetic acid, GTMAC: GlcN; 4 : 1 mol ratio, reaction time; 18 h.

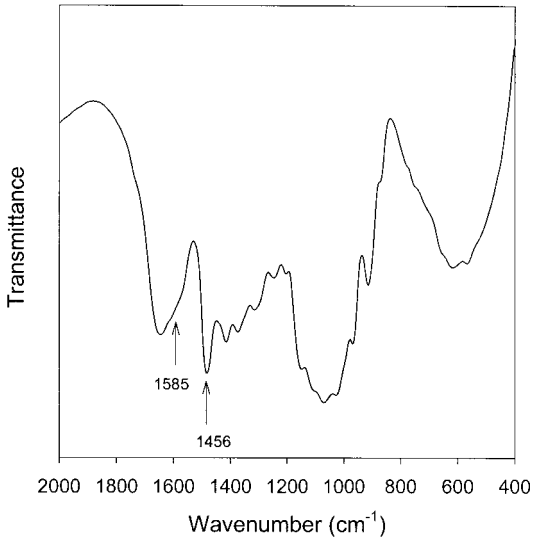


Figure 5 FTIR spectrum of HTACC (DS 1.04).

at 1585 cm^{-1} and appearance of the methyl band at 1456 cm^{-1}, as shown in Figure 5. Figure 6 shows the ^{1}H-NMR spectrum of the synthesized HTACC. The protons of C(1)–C(6) in the repeating unit of glucosamine in COS are at the same positions as shown in Figure 2. The CH peak at the (b) position, the CH_2 peak at the (c) position, and the CH_2 peak at the (a) position of secondary amine showed at $\delta = 4.60$ ppm, $\delta = 3.38$ ppm, and $\delta = 2.85$ ppm, respectively. Remarkably, there is a new intense CH_3 peak at $\delta = 3.1$ ppm, indicating the synthesized HTACC. Additionally, the terminal aldehyde group of HTACC could be verified from the proton peak at $\delta = 9.50$ ppm.

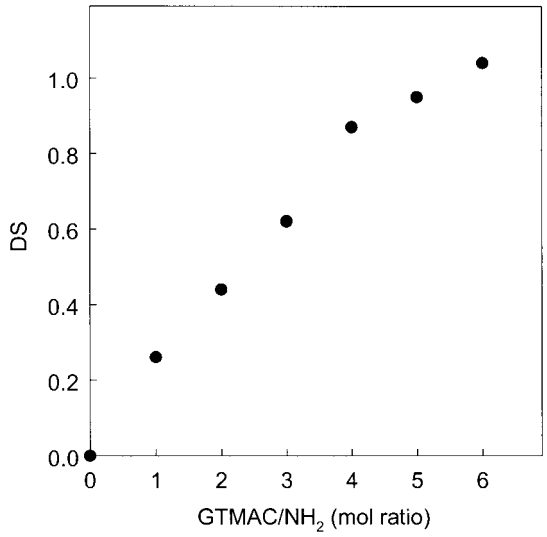


Figure 4 Effect of mol ratio of GTMAC to NH_2 in COS on the DS. Reaction conditions: cat; 1% acetic acid, reaction temp; 50°C, reaction time; 18 h.

Antimicrobial Activity

MIC means the minimum inhibition concentration of agent needed to inhibit bacterial growth; therefore, the lower the MIC value of an agent, the higher the antimicrobial activity that can be expected. Table II shows the MIC values of COS and HTACC having various DS. The MIC value of COS was 400 μg/mL. On the other hand, the MIC value decreased from 300 μg/mL for the 0.25 DS of HTACC to 50.0 μg/mL for the 1.04 DS of HTACC. A significant reduction occurred due to the quaternary ammonium group from the substitution of NH_2 groups in COS with GTMAC.

Table III shows the effect of various concentrations of COS and HTACC on the percent reduction of bacteria. The percent reduction of bacteria increased as the concentration of HATCC and COS increased from 0.05 to 0.20% and from 0.4 to 1.8%, respectively. Above the 0.20 and 1.8% levels of concentration of HTACC and COS, they showed a 100% reduction of bacterial values thereafter. HTACC exhibited antimicrobial activity superior to COS, although the concentration of HTACC is nine times lower than that of COS. This is again attributed to the quaternary ammonium group from the substitution of NH_2 groups in COS with GTMAC.

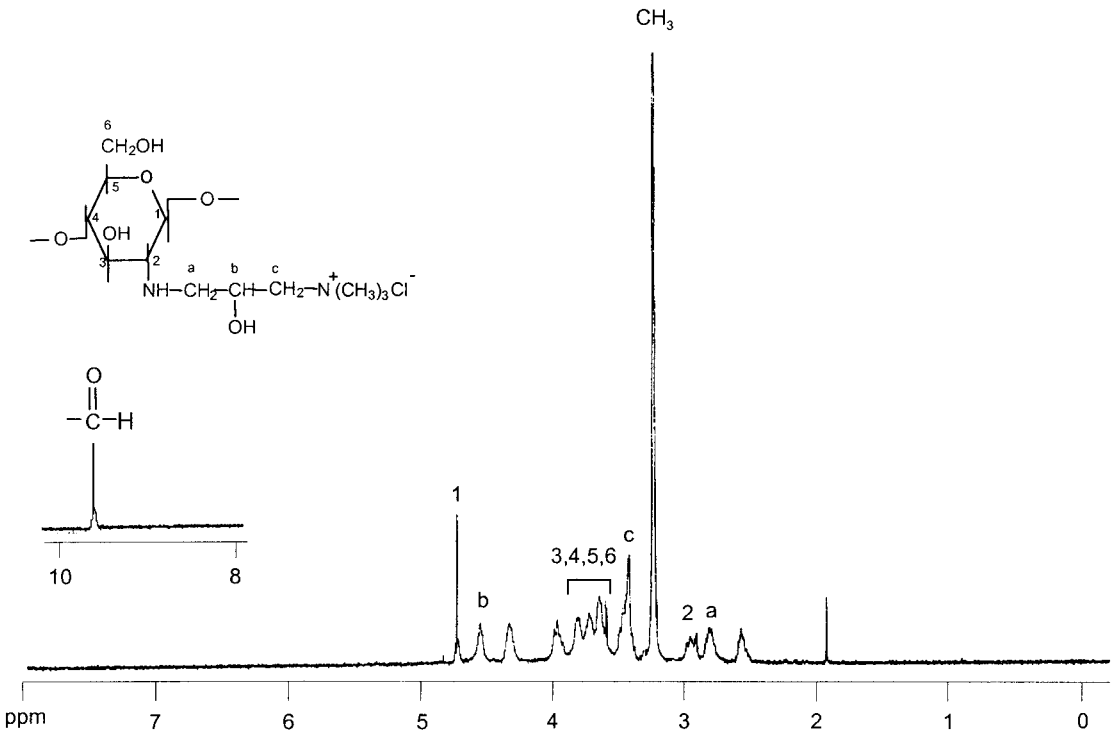


Figure 6 ^{1}H-NMR spectrum of HTACC (DS 1.04).

Table IV shows the effect of repeated laundering of COS on the percent reduction of bacteria. The cotton fabric treated with 2.4% COS was used in this durability test. The COS retained 100% reduction of bacteria up to the 30th laundering cycle, and exhibited a 95% reduction of bacteria at the 50th laundering cycle. Figure 7 shows the percent reduction of bacteria at various concentrations of HTACC at the 50th repeated laundering. The percent reduction of bacteria increased as the concentration of HATCC increased from 0.05 to 0.30%. Above the 0.30% level of concentration of HTACC, it showed a 100% reduction of bacteria values thereafter.

Table II MIC Value of COS and HTACC Having Various DS

Antimicrobial Agent	DS	MIC (μg/mL)
COS	—	400.0
	0.25	300.0
	0.45	200.0
HTACC	0.64	125.0
	0.87	87.5
	1.04	50.0

CONCLUSIONS

Chito-oligosaccharide (COS) was prepared by depolymerization of a fully deacetylated chitosan with sodium nitrite. To enhance antimicrobial activity in COS, HTACC was synthesized using a

Table III Effect of Various Concentrations of COS and HTACC on the Percent Reduction of Bacteria

Antimicrobial Agent	Concentration of Bath (%)	Reduction of Bacteria (%)
	0.4	59
	0.8	72
	1.2	86
COS	1.6	97
	1.8	100
	2.0	100
	2.4	100
	0.05	64
	0.10	81
	0.15	92
HTACC	0.20	100
	0.25	100
	0.30	100

Table IV Effect of Repeated Laundering of Cotton Fabric Treated with COS on the Percent Reduction of Bacteria

Number of Launderings	Reduction of Bacteria (%)
10	100
20	100
30	100
40	96.5
50	95.7

reaction between GTMAC and COS. COS and HTACC were confirmed by FTIR and ^{1}H-NMR. The degree of substitution (DS) of HTACC was calculated from chlorine content as a function of reaction time, reaction temperature, and mol ratio of GTMAC to NH_2 in COS. The complete substitution of NH_2 in COS with GTMAC was obtained at a four to one mol ratio of GTMAC to NH_2 in COS in 18 h at 80°C in the presence of acetic acid.

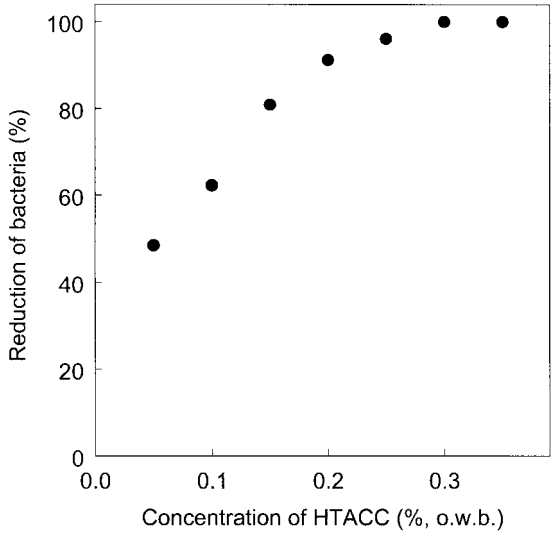


Figure 7 Percent reduction of bacteria after the 50th laundering of cotton fabrics treated with different concentrations of HTACC.

In comparing antimicrobial activity for COS and HTACC, HTACC showed superior antimicrobial activity to COS due to the quaternary ammonium group from the substitution of NH_2 in COS with GTMAC. MIC values of COS and the 1.04 DS of HTACC were 400 and 50 μg/mL, respectively. At the low concentration of 0.2%, HTACC shows a 100% reduction of bacteria, whereas 1.8% of COS exhibits 100% reduction of bacteria. For durability of laundering, 0.30% of HTACC shows 100% bacterial reduction, while 2.4% of COS shows 96% bacterial reduction after the 50th wash cycle.

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