Synthesis and Antimicrobial Activity of Schiff Base of Chitosan and Acylated Chitosan

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ABSTRACT: Chitosan, a biocompatible, biodegradable, nontoxic polymer, is prepared from chitin, which is the second most naturally occurring biopolymer after cellulose. Schiff base of chitosan, sorbyl chitosan, and *p*-aminobenzoyl chitosan were synthesized working under high-intensity ultrasound and their antimicrobial properties were analyzed against *Escherichia coli*, *Staphylococcus aureus*, and *Aspergillus niger*. The structures of the derivatives were characterized by FTIR spectroscopy and elemental analysis. The results of antimicrobial activities indicated that the antimicrobial activities of the derivatives increased with increasing the concentration. The antibacterial activity of schiff base of chitosan against *E. coli* was stronger, while acylated chitosan had better inhiting effect on *S. aureus* than others. It was also found that the antifungal activities of the derivatives were stronger than that of chitosan, and schiff base of chitosan was obviously superior to acylated chitosan. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 3242–3247, 2012

Key words: chitosan; Schiff base; acylated chitosan; high-intensity ultrasound; antimicrobial activity

INTRODUCTION

Chitin is a natural polysaccharide which is usually obtained from shells of crustaceans such as crab, shrimp, and crawfish.[1] It is a copolymer of *N*-acetylglucosamine (GluNAc) and *N*-glucosamine (GluN) units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer.[2] Chitosan is a partially N-deacetylated derivative of chitin. The term chitosan is usually used when GluN units predominate or the polymers become soluble in a dilute acid solution. Conversely, the term chitin is used.[3]

As a natural renewable resource, chitosan possesses unique properties such as biocompatibility, biodegradability, nontoxicity, and excellent film-forming ability, and has important applications in the biomedical, agriculture, functional food, wastewater purification, environmental protection, biotechnology, and cosmetics domains.[4–6]

Although chitosan should be useful for even more numerous applications, its use suffers severe limitations because it is insoluble in neutral or alkaline media owing to its rigid and compact crystalline structure and strong intra- and intermolecular hydrogen bonds.[7,8]

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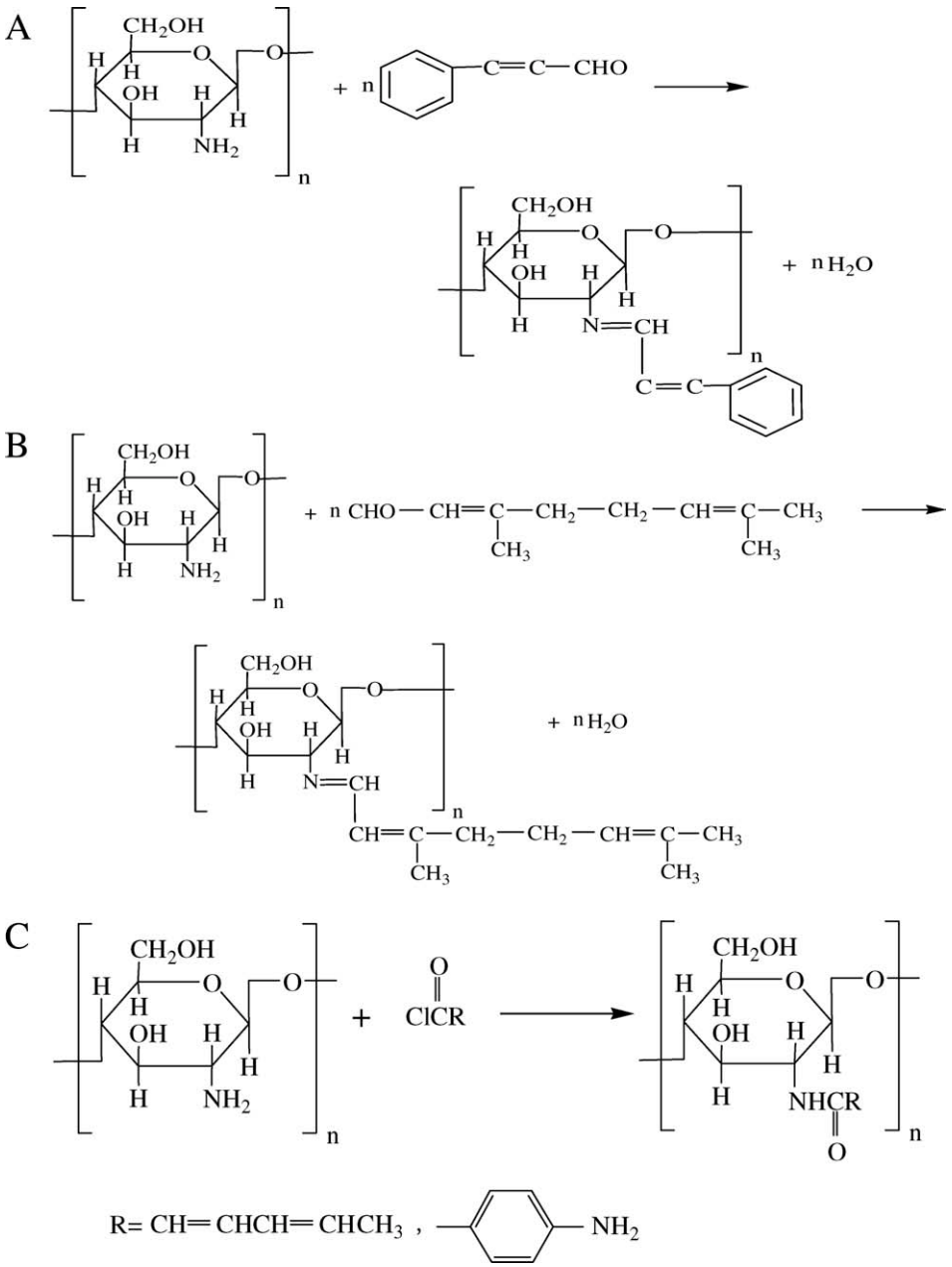
Chitosan has both reactive amino and hydroxyl groups that can be used to chemically alter its properties under mild reaction conditions.[9] The presence of amino groups leads to the possibility of a several chemical modifications, including the preparation of Schiff base (-RC=N-) by reaction with aldehydes and ketones and acylation using acid chloride.

This study included an efficient method to synthesize the Schiff base of chitosan with cinnamaldehyde (or citral), sorbyl chitosan, and *p*-aminobenzoyl chitosan working under high-intensity ultrasound, on the basis of which the antimicrobial activities against *Escherichia coil*, *Staphylococcus aureus*, and *Aspergillus niger* were investigated.

EXPERIMENTAL

Materials

Chitosan was purchased from Qingdao Haihui Bioengineering Co., Ltd. (Qingdao, China). The degree of deacetylation (DD) was 97% and the molecular weight was 1.0×10^5. Cinnamaldehyde was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Citral was obtained from Shanghai Aibi Chemistry preparation Co., Ltd (Shanghai, China). Sorbic acid was purchased from Tianjin Bodi Chemical Co., Ltd (Tianjin, China). *P*-aminobenzoic acid and all other reagents are analytical grade provided by Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). *E. coli*, *S. aureus*, and *A. niger* were



Scheme 1 The synthesis of Schiff base of chitosan and acylated chitosan.

supplied by Microbiology Laboratory of Ocean University of China.

Synthesis of Schiff base of chitosan

Chitosan was dispersed in 50 mL methanol in the three-necked flask for 30 min. Then superfluous cinnamaldehyde (or citral[10]) dissolved in anhydrous ethanol (20 mL) was added drop-wise to the solution under high-intensity ultrasound at 40°C for 8 h. When the reaction ended, the product was filtered and put in the Soxhlet apparatus with anhydrous ethanol, keeping extracting for 12 h, thus the unreacted cinnamaldehyde (or citral) could dissolved in the anhydrous ethanol and was removed. The Schiff base of chitosan was obtained after drying at 50°C for 24 h. Scheme 1(A,B) shows the synthesis pathway of the derivatives.

Synthesis of acylated chitosan

The solution of acid (sorbic acid or *p*-aminobenzoic acid) in ether (30 mL) was prepared in a three-necked flask and superfluous thionyl chloride was simultaneously added under stirring at 30°C for 15 min. Then chitosan dissolved in acetic acid was added in chloride solution under high-intensity ultrasound for 1 h at 25°C. The product was precipitated into 50 mL acetone, filtered off, and dipped in a mixture of ethanol and ether (1 : 1 v/v) for 24 h. The precipitate was collected by

TABLE I
The Elemental Analysis Results and Degree of Substitution of Derivatives of Chitosan

Samples	C (%)	N (%)	C/N	Degree of substitution
Chitosan	44.82	8.63	5.19	
Schiff base from chitosan and cinnamaldehyde	61.72	5.27	11.71	0.84
Schiff base from chitosan and citral	59.23	5.04	11.75	0.82
sorbyl chitosan	53.17	5.68	9.36	0.81
p-aminobenzoyl chitosan	55.39	10.03	5.52	0.79

filtration and dried in vacuum at 50°C for 6 h. The derivatives were synthesized according to Scheme 1(C).

Microorganisms and *in vitro* antimicrobial assays

Bacteria

E. coli and *S. aureus* were used as the test organisms. A representative microbe colony was picked off with a wire loop, placed in nutrient broth, and then incubated in air bath shaker at 37°C for 24 h. By appropriately diluting with sterile normal saline (0.9%) solution, the cultures of *E. coli* and *S. aureus* containing $\sim 10^7$ CFU/mL were prepared and used for the antibacterial test.

Antibacterial assay

The antibacterial activities of chitosan and derivatives against *E. coli* and *S. aureus* were carefully measured optically at 620 nm.[11] The bacterial suspension 0.2 mL was inoculated under aseptic conditions into 100 mL liquid peptone medium (1% peptone, 0.3% beef extract, and 0.5% NaCl) containing chitosan or derivatives that had been sterilized under 121°C for 20min. The control only contained nutrient broth without chitosan. All of the samples were incubated at 37°C with shaking. During incubation, turbidity of the medium was measured at 620 nm every 4 h with an UV spectrophotometer (UV-2550, SHIMADZU).

The inhibition rate of chitosan and derivatives on growth of *E. coli* and *S. aureus* was determined using agar plates. The peptone culture plates were prepared, in which 0.1 mL solution of bacterial suspension was first added and then 0.1 mL solution of chitosan with different concentrations were added. Both of them were spread uniformly. A blank without chitosan was prepared for comparison. All the plates were incubated at 37°C for 24 h. Then the plates were taken out and the inhibition rate was calculated.[12]

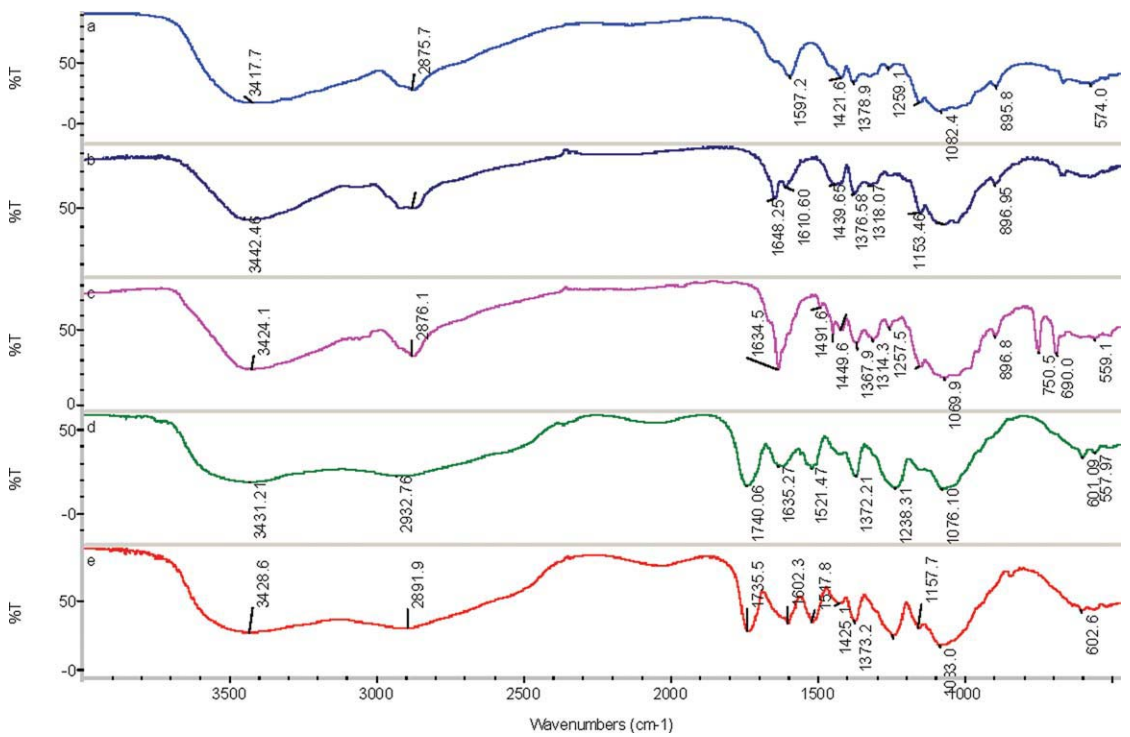


Figure 1 FTIR spectra of chitosan (a), Schiff base from chitosan and citral (b), Schiff base from chitosan and cinnamaldehyde (c),sorbyl chitosan (d), and *p*-aminobenzoyl chitosan (e). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

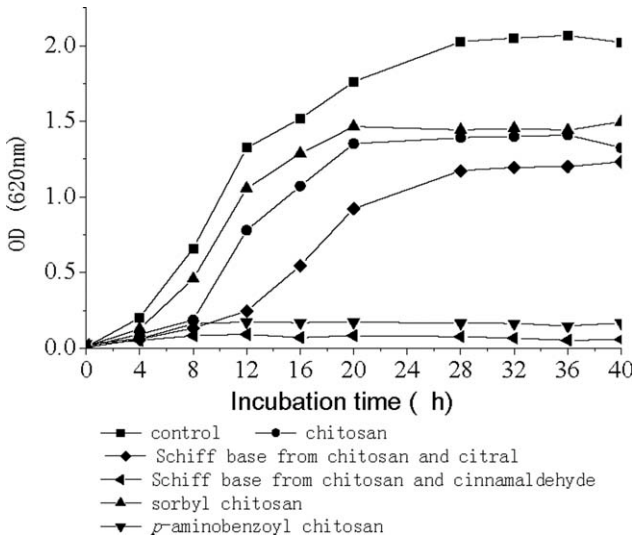


Figure 2 Inhibitory effects of chitosan and chitosan derivatives on growth of *E. coli*.

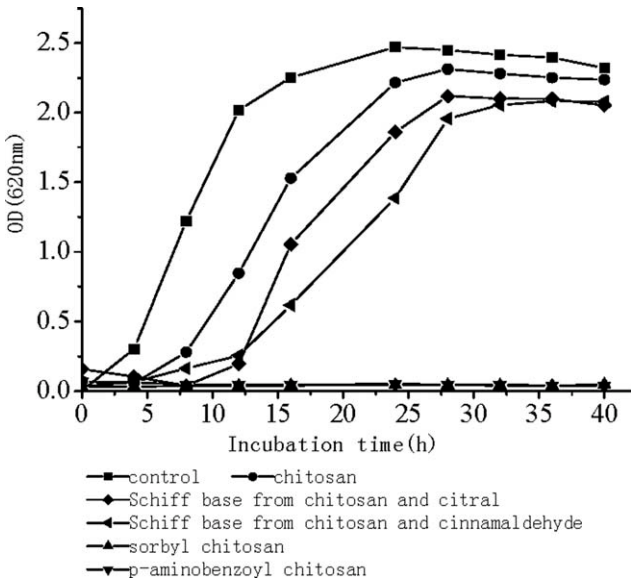


Figure 3 Inhibitory effects of chitosan and chitosan derivatives on growth of *S. aureus*.

The inhibition rate was defined as

$$\eta = (N_1 - N_2)/N_1 \times 100\%$$

here N_1 and N_2 mean the number of colony on the plates before and after inhibition, respectively.

The antibacterial activities of the derivatives of chitosan were investigated with the same method.

Fungi

A. niger was used as the test organism. A representative microbe colony was picked off with a wire loop, placed in liquid Sabouraud medium, and then incubated in air bath shaker at 28°C for 7 days.

Antifungal assay

The inhibition of fungi growth was evaluated by comparison of the dry cell weight with the normal growth in the control culture medium.[13] The microbe suspension 1 mL was inoculated under aseptic conditions in 100 mL liquid Sabouraud medium (1% peptone, 4% glucosse) containing chitosan or derivatives that had been sterilized under 121°C for 20min. The control only contained liquid Sabouraud medium without chitosan. All of the samples were incubated at 28°C with shaking. The cultures were filtered every 12 h and the pellet was washed with distilled water and dried at 80°C overnight, then mensurated the dry cell weight.

In the determination of the inhibition rate of chitosan and derivatives on growth of *A. niger*, the microbe suspension 1 mL was inoculated into 100 mL liquid Sabouraud medium containing chitosan with different concentrations. A blank without chitosan was prepared for comparison. All the samples were incubated at 28°C for 48 h. The cultures were filtered and the pellet was washed with distilled water and dried at 80°C to constant weight, then mensurated the dry cell weight and the inhibition rate was calculated.

The inhibition rate was defined as

$$\eta = (W_1 - W_2)/W_1 \times 100\%$$

where W_1 is the dry cell weight of the *A. niger* in the control culture medium and W_2 is the dry cell weight of the *A. niger* in the chitosan culture medium, respectively.

TABLE II
The Inhibition Rate of Chitosan and Chitosan Derivatives on Growth of *E. coli* (%)

Sample	Concentration (%) (w/v)							
	0.5	0.25	0.1	0.08	0.05	0.025	0.01	0.005
chitosan	100	100	97	91	87	73	67	57
Schiff base from chitosan and cinnamaldehyde	100	100	100	100	97	92	86	74
Schiff base from chitosan and citral	100	100	100	93	90	86	70	63
sorbyl chitosan	100	100	96	90	84	72	67	58
p-aminobenzoyl chitosan	100	100	100	96	93	89	83	72

TABLE III
The Inhibition Rate of Chitosan and Chitosan Derivatives on Growth of *S. aureus* (%)

Sample	Concentration (%) (w/v)							
	0.5	0.25	0.1	0.08	0.05	0.025	0.01	0.005
chitosan	100	100	98	93	89	76	69	61
Schiff base from chitosan and cinnamaldehyde	100	100	100	97	94	90	86	68
Schiff base from chitosan and citral	100	100	100	96	94	89	81	63
sorbyl chitosan	100	100	100	100	98	95	91	83
p-aminobenzoyl chitosan	100	100	100	99	97	93	90	72

The antifungal activities of the derivatives of chitosan were investigated with the same method.

RESULTS AND DISCUSSION

Characterization of the derivatives

The elemental analysis (C, N) of samples was performed on an Elemental Analyzer PE-2400II (Perkin–Elmer, USA). The results of elemental analysis and the degree of substitution of the derivatives are listed in Table I. It shows that the content of carbon in chitosan derivatives tended to be larger than that in chitosan but the degrees of substitution seemed to be of little difference. We estimated the degree of substitution based on the ratio of carbon and nitrogen. FTIR spectra were recorded in powder form in KBr discs in the range of 4000–500 cm^{-1} on an AVATAR360 FTIR spectrophotometer from Nicolet instrument Co. Figure 1 demonstrates the IR spectra of the starting chitosan (a), Schiff base from chitosan and citral (b), Schiff base from chitosan and cinnamaldehyde (c), sorbyl chitosan (d), and *p*-aminobenzoyl chitosan (e). All spectra exhibited the absorption peaks at 1154, 1082, 1022, and 895 cm^{-1}, which could be assigned to the saccharide moiety. Among the bands characteristic to chitosan, in the FTIR spectra of the Schiff base, new absorption peaks appeared at 1633.94 cm^{-1} corresponding to the C=N vibrations characteristic of imines and the 1491.35, 1449.52, 750.06, and 690.08 cm^{-1} assigned to the characteristic absorbance of phenyl group. The broad peak at around 3417.71 cm^{-1} corresponds to the stretching vibration of *N*—H and O—H bond shifted to higher frequency. In addition, the characteristic absorption peak at 1597.23 cm^{-1} almost disappeared, representing a decrease of -NH_2 group content, which implied that amino groups on chitosan reacted with cinnamaldehyde (or citral) to form a Schiff base. In comparison with the chitosan IR spectrum, the sorbyl chitosan and *p*-aminobenzoyl chitosan spectra both display a new absorption peak at 1740.06 cm^{-1} and 1735.52 cm^{-1}, respectively, corresponding to the C=O stretching mode of the carboxyl-functionalized substituents.

Antimicrobial assays

Antibacterial activity

Figures 2 and 3 demonstrate curves of optical density (OD) versus incubation time for chitosan and derivatives against *E. coli* and *S. aureus*, respectively. Because the bacterial cell is opaque, the medium became turbid as the bacteria propagated. Therefore, the optical density can be used as a criterion measuring the antibacterial activity.[14] As shown in the figures, the values of OD of derivatives are much less than that of control analysis and chitosan, which shows that chitosan derivatives have high antibacterial activity.

The effect of concentration on the antibacterial activity of chitosan and derivatives of chitosan against *E. coli* and *S. aureus* is shown in Tables II and III. The results indicated that the chitosan and derivatives had a significant inhibiting effect on the *E. coli* and *S. aureus*. It could be seen that with increase of the concentration of chitosan and derivatives, the antibacterial activity also increased. It was also found that the antibacterial activity of the derivatives was stronger than that of chitosan. The minimal inhibitory concentration (MICs) of Schiff base

TABLE IV
The Inhibition Rate of Chitosan and Chitosan Derivatives on Growth of *A. niger* (%)

Sample	Concentration (%) (w/v)									
	2	1	0.5	0.25	0.1	0.08	0.05	0.025	0.01	0.005
chitosan	–	–	–	–	–	–	–	–	–	–
Schiff base from chitosan and cinnamaldehyde	100	100	100	98	95	90	86	74	65	59
Schiff base from chitosan and citral	100	100	100	96	84	80	78	75	69	52
sorbyl chitosan	100	93	87	72	66	60	52	46	34	33
p-aminobenzoyl chitosan	100	100	95	81	73	69	55	51	38	33

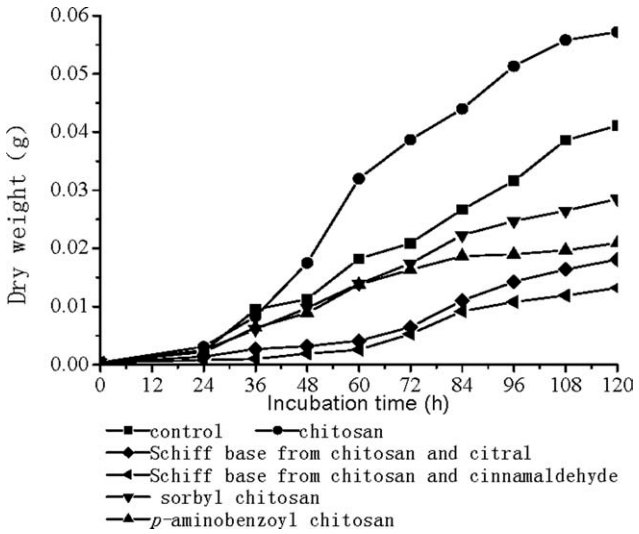


Figure 4 Inhibitory effects of chitosan and chitosan derivatives on growth of *A. niger*.

from chitosan and cinnamaldehyde (or citral), sorbyl chitosan, and *p*-aminobenzoyl chitosan were 0.08%, 0.1%, 0.25%, 0.1% (w/v) against *E. coli*. While the MICs were 0.1%, 0.1%, 0.08%, and 0.1% against *S. aureus*

Antifungal activity

The capabilities of chitosan and derivatives in inhibiting the growth of the *A. niger* are listed in Table IV. No inhibition rate was observed for the chitosan against the *A. niger*. In contrast, derivatives showed antifungal activity to some extent. When the concentrations achieved 0.5%, 2%, 1% (w/v), respectively, almost all *A. niger* were killed. Therefore, the MICs of Schiff base from chitosan and cinnamaldehyde (or citral), sorbyl chitosan and *p*-aminobenzoyl chitosan were 0.5%, 0.5%, 2%, 1% (w/v) against *A. niger*.

According to Figure 4, the derivatives show better antifungal activity than chitosan, which not only had no inhibition effect against the *A. niger*, but also contributed to the growth of the microbe. The possible reason is that *A. niger* belongs to fungi category while the fungal cell wall contains chitosan. Therefore, the *A. niger* has certain resistance to the antifungal performance of chitosan.[15] The lower values of dry cell weight of derivatives demonstrates that derivatives of chitosan have better antifungal activity of chitosan and expand the antimicrobial spectrum.

CONCLUSIONS

Schiff base of chitosan with cinnamaldehyde(or citral), sorbyl chitosan, and *p*-aminobenzoyl chitosan were synthesized working under high-intensity ultrasound. The antimicrobial activities of chitosan and derivatives were investigated against *E. coli*, *S. aureus*, and *A. niger*. The results indicated that the derivatives had better antimicrobial activities than chitosan. The antimicrobial activity of the derivatives increased with increasing the concentration. As novel chitosan derivatives, Schiff base of chitosan, sorbyl chitosan, and *p*-aminobenzoyl chitosan improve the antimicrobial activity of chitosan and expand the antimicrobial spectrum compared with that of chitosan.

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