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Synthesis and antimicrobial activity of the Schiff base from chitosan and citral

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ABSTRACT

Chitosan, a biocompatible, biodegradable, non-toxic polymer, is prepared from chitin, which is the second most naturally occurring biopolymer after cellulose. The Schiff base of chitosan was synthesized by the reaction of chitosan with citral working under high-intensity ultrasound. The effect of the molar ratio of chitosan to citral, reaction time, and temperature on the yield has been investigated. The optimal conditions were a temperature of 50 °C, a molar ratio of chitosan to citral of 1:6, and a reaction time of 10 h. The maximum yield achieved was 86.4% under optimum conditions. The structure of the Schiff base was characterized by FTIR spectroscopy, elemental analysis, and X-ray diffraction studies. The strong peaks at 1648.3 and 1610.6 cm^{-1} are due to C=N and C=C stretching vibrations. The results confirmed that amino groups on chitosan reacted with citral to form the Schiff base. The antimicrobial activities of chitosan and Schiff base of chitosan were investigated against *Escherichia coil*, *Staphylococcus aureus*, and *Aspergillus niger*. The results indicate that the antimicrobial activity of the Schiff base increases with an increase in the concentration. It was also found that the antimicrobial activity of the Schiff base was stronger than that of chitosan.

Chitin is a natural polysaccharide that is usually obtained from shells of crustaceans such as crab, shrimp, and crawfish.[1] It is a copolymer of 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-glucosamine, GluNAc) and 2-amino-2-deoxy-D-glucose (*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 glucosamine 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, non-toxicity, 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]

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 several chemical modifications, including the preparation of Schiff bases (–RC=N–) by reaction with aldehydes and ketones. The reaction of chitosan with aromatic aldehydes to produce the corresponding Schiff bases has been described.[10–12] The possibility of forming a Schiff base of chitosan with citral and it demonstrating antimicrobial activity, of which there is no previous report, seemed attractive.

This study includes an efficient method to synthesize the Schiff base by the reaction of chitosan with citral working under high-intensity ultrasound. Antimicrobial activities of the product against *Escherichia coil*, *Staphylococcus aureus*, and *Aspergillus niger* were investigated (see Scheme 1).

1. Experimental

1.1. 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. Another chitosan sample, designated as CS1, was obtained through ultrasonic degradation as follows: Chitosan was dispersed in 50 mL of MeOH and was then ultrasonically degraded at 50 °C for 10 h. The DD determined by titration[13] was 97%, and the molecular weight calculated by Mark–Houwink equation[14] was 8.9×10^4. Citral was obtained from Shanghai Aibi Chemistry Preparation Co. Ltd. (Shanghai, China). All other reagents were of analytical grade and were provided by Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). *Escherichia coli*, *S. aureus*, and *A. niger* were supplied by Microbiology Laboratory of the Ocean University of China.

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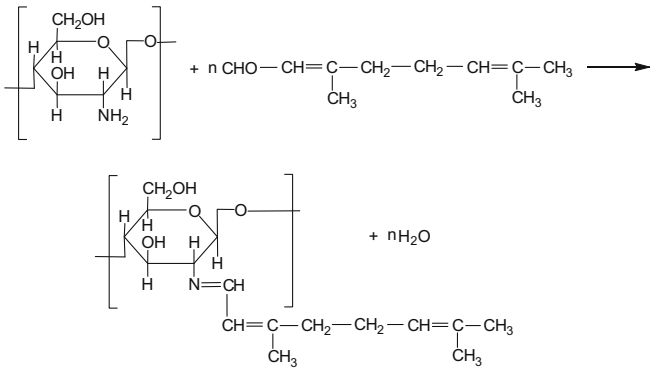

Scheme 1. The synthesis of Schiff base of chitosan.

1.2. Synthesis of a Schiff base of chitosan

Chitosan was dispersed in 50 mL of MeOH in a three-necked flask. Then citral dissolved in anhyd EtOH (20 mL) was added dropwise to the solution under high-intensity ultrasound at 30–50 °C for 6–10 h. When the reaction ended, the product was filtered, and the unreacted citral was extracted in a Soxhlet apparatus with anhyd EtOH for 12 h. The resulting Schiff base of chitosan was dried at 50 °C for 24 h.

The optimal conditions for preparing the Schiff base of chitosan were determined on the basis of orthogonal tests.

The yield was calculated from the following equation[15,16]:

$$P\% = m_2/M_2 \times M_1/m_1 \times 100\%$$

where m_1 is the quantity of chitosan (g); m_2 is the quantity of Schiff base (g); M_1 is the molecular weight of chitosan unit (161 g/mol); and M_2 is the molecular weight of Schiff base unit (g/mol).

1.3. Microorganisms and in vitro antimicrobial assays

1.3.1. 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 an 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.

1.3.2. Antibacterial assay

The antibacterial activities of chitosan and Schiff base against *E. coli* and *S. aureus* were carefully measured optically at 620 nm.[17] 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 Schiff base that had been sterilized at 121 °C for 20 min. The control contained only nutrient broth without chitosan. All the samples were incubated at 37 °C with shaking. During incubation, the turbidity of the medium was measured at 620 nm every 4 h with an UV spectrophotometer (UV-2550, Shimadzu).

The inhibition rates of chitosan and the Schiff base on growth of *E. coli* and *S. aureus* were 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. 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 of the incubator, and the inhibition rate was calculated.[18]

The inhibition rate was defined as

Table 1
Table designed for the $L_9\ 3^3$ orthogonal experiment

Levels	Factors		
	(A) Temperature (°C)	(B) Time (h)	(C) Ratio
1	30	6	1:4
2	40	8	1:5
3	50	10	1:6

Table 2
Test data of the $L_9\ 3^3$ orthogonal experiment

Experiment	Temperature (°C)	Time (h)	Ratio	Yield (%)
1	30	6	1:4	56.76
2	30	8	1:5	60.03
3	30	10	1:6	62.22
4	40	6	1:6	60.03
5	40	8	1:4	48.03
6	40	10	1:5	54.58
7	50	6	1:5	68.77
8	50	8	1:6	67.67
9	50	10	1:4	68.77
K_1	179.01	185.56	173.56	
K_2	162.64	175.73	183.38	
K_3	205.21	185.57	189.92	
k_1	59.67	61.85	57.85	
k_2	54.21	58.58	61.13	
k_3	68.40	61.86	63.31	
$R(k_{max} - k_{min})$	14.19	3.28	5.46	
Optimum conditions	50	10	1:6	

K_1, K_2, K_3 is the summation of yield of each factor level; k_1, k_2, k_3 is the average of K_1, K_2, K_3, respectively; R is the level difference.

Table 3
The elemental analysis results and the color of Schiff base of chitosan

Sample	C (%)	N (%)	C/N	Color
Chitosan	44.82	8.63	5.19	White
CS1	43.56	8.41	5.17	White
1	55.09	5.59	9.86	Deep yellow
2	60.24	5.61	10.74	Deep yellow
3	60.15	5.42	11.10	Deep yellow
4	59.23	5.04	11.75	Deep yellow
5	56.02	4.99	11.23	Deep yellow
6	58.06	5.10	11.38	Deep yellow
7	57.97	5.26	11.02	Deep yellow
8	56.28	4.87	11.56	Deep yellow
9	58.75	5.20	11.30	Deep yellow

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

Here N_1 and N_2 are the number of colonies on the plates before and after inhibition, respectively.

The antibacterial activity of the Schiff base of chitosan was investigated with the same method.

1.3.3. 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 an air-bath shaker at 28 °C for 7 d.

1.3.4. Antifungal assay

The inhibition of fungal growth was evaluated by comparison of the dry cell weight with the normal growth in the control culture medium.[19] The microbe suspension, 1 mL, was inoculated under aseptic conditions in 100 mL of liquid Sabouraud medium (1% peptone, 4% glucose) containing chitosan or Schiff base that had been sterilized under 121 °C for 20 min. The control contained only liquid Sabouraud medium without chitosan. All the samples were incubated at 28 °C with shaking. The cultures were filtered every

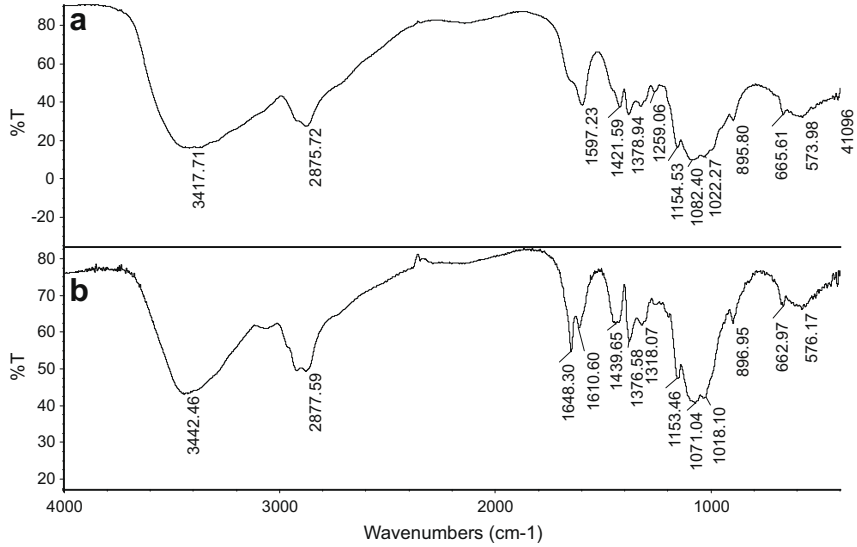

Figure 1. FTIR spectra of chitosan (a) and Schiff base (b).

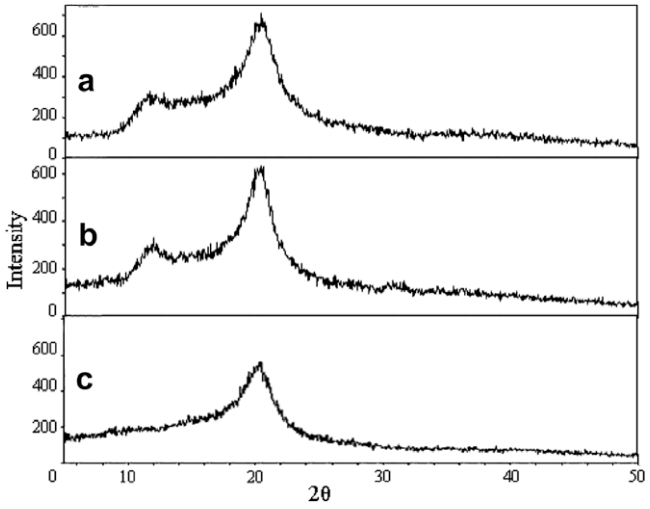

Figure 2. X-ray powder diffraction patterns of chitosan (a), CS1 (b) and the Schiff base (c).

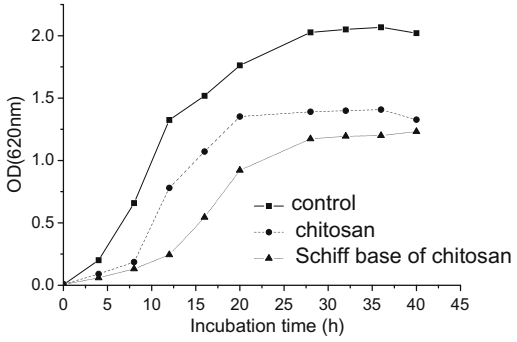

Figure 3. Inhibitory effects of chitosan and Schiff base on growth of *E. coli*.

12 h, and the pellet was washed with distilled water and dried at 80 °C overnight. The dry cell weight was then determined.

In the determination of the inhibition rate of chitosan and Schiff base on growth of *A. niger*, the microbe suspension, 1 mL, was inoculated into 100 mL of liquid Sabouraud medium containing chitosan with different concentrations. A blank without chitosan was

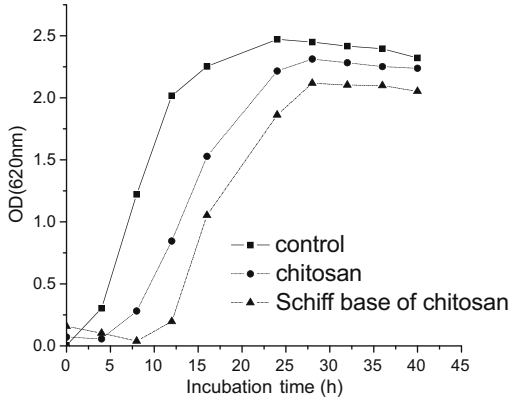

Figure 4. Inhibitory effects of chitosan and Schiff base on growth of *S. aureus*.

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. The dry cell weight was then determined, 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.

The antifungal activity of the Schiff base of chitosan was investigated using the same method.

2. Results and discussion

2.1. Synthesis of Schiff base of chitosan

The optimal conditions for preparing the Schiff base of chitosan were studied by the orthogonal test [$L_9(3^3)$]. Three controllable variables, reaction time, temperature, and molar ratio of chitosan to citral, were selected, each at three levels. The investigated variables and their test levels are listed in Table 1. The test results are listed in Table 2. As results indicated, the order of influence of each variable on the yield is reaction temperature > ratio of chitosan to

Table 4
The inhibition rate of chitosan and Schiff base on *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 of chitosan	100	100	100	93	90	86	70	63

Table 5
The inhibition rate of chitosan and Schiff base on *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 of chitosan	100	100	100	96	94	89	81	63

Table 6
The inhibition rate of chitosan and Schiff base on *A. niger* (%)[a]

Sample	Concentration (%) (w/v)							
	0.5	0.25	0.1	0.08	0.05	0.025	0.01	0.005
Chitosan	—	—	—	—	—	—	—	—
Schiff base of chitosan	100	96	84	80	78	75	69	52

[a] '—' means no activity.

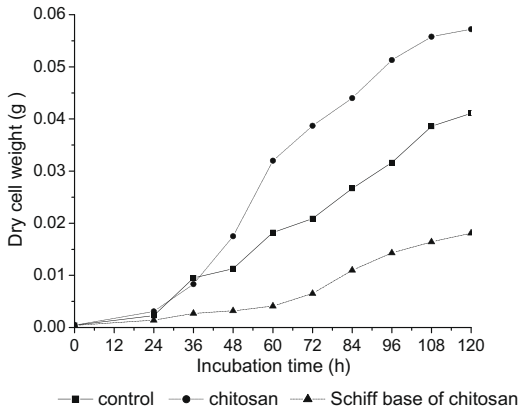

Figure 5. Inhibitory effects of chitosan and Schiff base on growth of *A. niger*.

citral > reaction time. Thus, the optimum reaction conditions were determined as follows: temperature of 50 °C; the reaction time of 10 h; and a molar ratio of chitosan to citral of 1:6. The maximum yield achieved was 86.4% under optimum conditions.

2.2. Characterization of the Schiff base

The results of the elemental analysis and the color of the derivatives are listed in Table 3. IR spectroscopy was used to confirm the structure of the Schiff base of chitosan. Figure 1 shows the IR spectra of the starting chitosan (a) and that of the Schiff base (b). Both spectra exhibit the absorption peaks at 1154, 1082, 1022, and 895 cm^{-1}, which can be assigned to the saccharide moiety. Among the bands characteristic to chitosan, in the FTIR spectra of the Schiff base, new absorption peaks appear at 1648.3 and 1610.6 cm^{-1} corresponding to the C=N vibrations characteristic of imines and C=C stretching vibrations. The broad peak at around 3417.7 cm^{-1} corresponds to the stretching vibration of N–H and O–H bonds shifted to higher frequency. In addition, the characteristic absorption peak at 1597.2 cm^{-1} almost disappears, representing a decrease in $-NH_2$ group content, which indicates that the amino groups on chitosan reacted with citral to form a Schiff base under these experimental conditions.

Figure 2 shows the X-ray powder diffraction patterns of chitosan and the Schiff base. The pattern of chitosan showed the characteristic peak at $2\theta = 10°$, 20°,which coincides with the pattern of the 'tendon hydrate polymorph' of chitosan.[9] For the Schiff base, the peak at $2\theta = 10°$ disappears, and most importantly, the characteristic peak at $2\theta = 20°$ is much wider and weaker than that of chitosan. This result indicates that the Schiff base is of poor crystallinity compared to chitosan. The poor crystallinity of the Schiff base is attributed to the deformation of the strong hydrogen bond in the chitosan backbone with the substitution of citral group on the N atoms of chitosan.

2.3. Antimicrobial assays

2.3.1. Antibacterial activity

The capabilities of chitosan and CS1 in inhibiting the growth of the tested microbes are similar. Figures 3 and 4 show curves of optical density (OD) versus incubation time for chitosan and the Schiff base 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 for measuring the antibacterial activity.[20] As shown in the figures, the values of OD of the Schiff base are much less than those of the control analysis and chitosan, which shows that this chitosan derivative has high antibacterial activity.

The effect of concentration on the antibacterial activity of chitosan and the Schiff base of chitosan against *E. coli* and *S. aureus* is shown in Tables 4 and 5. The results indicate that both the chitosan and Schiff base have a significant inhibiting effect on *E. coli* and *S. aureus*. It could be seen that with an increase in the concentration of chitosan and Schiff base, the antibacterial activity also increased. The minimum inhibitory concentrations (MICs) of the Schiff base against *E. coli* and *S. aureus* are 0.1% (w/v) and 0.1% (w/v). It was also found that the antibacterial activity of the Schiff base was stronger than that of chitosan.

2.3.2. Antifungal activity

The capabilities of chitosan and Schiff base in inhibiting the growth of the *A. niger* are listed in Table 6. No inhibition rate was observed for the chitosan against the *A. niger*. In contrast, the Schiff base sample showed antifungal activity to some extent. When the concentration reached 0.5% (w/v), almost all *A. niger* were killed. Therefore, the MIC of Schiff base is 0.5% (w/v) against *A. niger*.

According to Figure 5, the Schiff base shows 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 a group of fun-

gi whose cell walls contain chitosan. Therefore, *A. niger* has a certain resistance to the antifungal performance of chitosan.[21] The lower values of dry cell weight of the Schiff base demonstrate that the Schiff base of chitosan could enhance the antifungal activity of chitosan and expand its antimicrobial spectrum.

3. Conclusions

The Schiff base of chitosan was synthesized by the reaction of chitosan with citral working under high-intensity ultrasound. The optimal conditions were a temperature of 50 °C, a molar ratio of chitosan to citral of 1:6, and a reaction time of 10 h. The maximum yield achieved was 86.4% under optimum conditions. The antimicrobial activities of chitosan and the Schiff base of chitosan were investigated against *E. coli*, *S. aureus*, and *A. niger*. The results indicate that the Schiff base of chitosan has better antimicrobial activities than chitosan. The antimicrobial activity of the Schiff base increases with an increase in concentration. The MICs of the Schiff base against *E. coli*, *S. aureus*, and *A. niger* are 0.1% (w/v), 0.1% (w/v), and 0.5% (w/v), respectively. As a novel chitosan derivative, the Schiff base of chitosan improves the antimicrobial activity of chitosan and expands the antimicrobial spectrum compared with chitosan itself.

Acknowledgments

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