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Growth inhibitory effect on bacteria of chitosan membranes regulated with deacetylation degree

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ABSTRACT

Antibacterial activity of chitosan membranes was investigated by a conductimetric assay using a Bactometer. The purpose of this investigation was to produce a practical, high-performance membrane for separation engineering. The antibacterial activity of powdered chitosan membrane was evaluated by the minimal inhibitory concentration (MIC). The MIC for *Escherichia coli* was almost 200 (mg-chitosan/ml-bacterial suspension), and for *Staphylococcus aureus* it was 40 (mg-chitosan/ml-bacterial suspension). Growth of the gram-positive sample (*S. aureus*) was more strongly inhibited by chitosan than the gram-negative sample (*E. coli*). This inhibitory effect was recognized as a bactericidal effect. Antibacterial activity was also observed and depended on the shape and the specific surface area of the powdered chitosan membrane. The influence of the deacetylation degree (DD) of the chitosan on inhibiting the growth of *S. aureus* was investigated by two methods: incubation using a mannitol salt agar medium, and a conductimetric assay. By both methods, chitosan with a higher DD successfully inhibited growth of *S. aureus*. Our findings regarding the dominant role of the DD of chitosan will be useful for designing long-life, hygienic, membrane-based processes.

1. Introduction

For years, material development focused on growth inhibition of bacteria was expected to lead to long-life, hygienic, membrane-based processes. The growth of microorganisms and the accumulation of colloids or organic compounds were major causes of membrane fouling, generally called "biofouling" [1]. Membrane fouling negatively influenced the permeation flux and some aspects of membrane performance (e.g., reduced salt rejection and elevated operational pressure [2–5]).

The development of membrane materials with antibacterial activity is important from both the economic viewpoint and for the hygienic management of practical membrane processes. Chitosan produced from crustacean shells is an attractive material for reducing biofouling. The authors previously reported a typical molecular characteristic of chitosan membranes, namely, the water permeability of these membranes when used to control the deacetylation degree (DD) [6]. An examination of the practical aspects of using chitosan membranes is necessary for developing membrane processing techniques.

Chitosan and its resolvent inhibit the growth of mold with plant pathogenicity [7,8]. In contrast, chitin does not inhibit the growth of mold [9]. The effect of chitosan concentration on the growth of mold (*Fusarium solani*, *Fusarium oxysporum*) was investigated. Mold growth was completely inhibited by 0.1% chitosan. A higher DD has a stronger growth inhibitory effect on mold [10].

According to Uchida [10], chitosan inhibited bacterial growth not only of gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) but also of gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*). For example, after 4 days of incubation, bacterial growth was completely inhibited in broth containing a chitosan concentration of 0.02% at pH 6.0. Lower viscosity (i.e., lower molecular weight) chitosan had a stronger growth inhibitory effect on bacteria.

According to No et al. [11], antibacterial activities of six chitosans and six chitosan oligomers with different molecular weights were examined against four gram-negative (including *E. coli*) and seven gram-positive bacteria (including *S. aureus*). Chitosans showed higher antibacterial activities than chitosan oligomers,

Abbreviations: BHI, brain heart infusion; BPU, Bactometer Processing Unit; CFU, colony-forming unit; DD, deacetylation degree; DT, detection time; MIC, minimal inhibitory concentration; MPCA, modified plate count agar; MW, molecular weight; PEG, polyethylene glycol; PVS-K, potassium polyvinyl sulfate; PVC, polyvinylidene chloride; SEM, scanning electron microscope.

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Nomenclature

A	additive volume of acetic anhydride (μl/50 g-chitosan solution of 2 wt%)
CFU	colony-forming unit (−)
DD	deacetylation degree (%) referred from Ref. [20]
DT_0	detection time at an antibacterial agent concentration of zero (h)
DT_i	detection time at an antibacterial agent concentration of i (h)
MIC	minimal inhibitory concentration (mg-chitosan/ml-bacterial suspension)

and markedly inhibited growth of most of the bacteria tested. Chitosan generally showed stronger bactericidal effects towards gram-positive bacteria than gram-negative bacteria. In addition, antibacterial activity of chitosan was inversely affected by pH (in the range tested, pH 4.5–5.9), with higher activity at lower pH values. These results were reported within the context of potential applications of liquid-soluble chitosan.

Various rapid examination techniques for antibacterial activity have been suggested, including turbidimetry [12], an ATP assay [13], calorimetry [14], an impedance assay [15], and conductimetric assays [16–19]. The present study employed the conductance method to evaluate the antibacterial activity of chitosan membranes; the method was based on the detection time of electric conductance.

Bacteria are generally classified according to gram dyeing. *E. coli* 745 (gram-negative) and *S. aureus* 9779 (gram-positive) were employed to evaluate the antibacterial activity of chitosan. These bacteria are representative of the bacterial species that are important in public sanitation and food hygiene. The antibacterial activity of chitosan membranes as a solid system for regulating the deacetylation degree was investigated by a direct conductimetric assay using a Bactometer. In this paper, chitosan was examined as a solid, not as a solute in solution as in previous papers. This approach will contribute to the design of a biopolymer membrane with high performance and long life for use in practical separation processing applications.

2. Materials and methods

2.1. Preparation of chitosan membranes

Chitosan (low molecular weight, Sigma–Aldrich Japan K.K., Tokyo) and polyethylene glycol (MW7500, Wako, Osaka) were dissolved in 10% acetic acid. The chitosan solution was diluted to 2% (w/w) with methanol. Acetic anhydride (97.0%, Wako, Osaka) was added to the chitosan solution after vacuum filtration. The resultant casting solution was dried in a petri dish for 12 h at 333 K and subsequently gelled by immersing it in 4% NaOH. The resultant product was washed with distilled water, and the membrane was obtained by putting it into hot water (353 K).

The DD of the chitosan membrane was analyzed by the colloidal titration method. The terminal point of each titration was clearly confirmed by a change in color from blue to claret. Potassium polyvinyl sulfate solution was used as the titrant, and toluidine blue was used as the indicator. Colloidal titration is a method of measuring free amino groups in a chitosan solution, and DD provides the molar percentage of glucosamine in the molecular chains of chitosan.

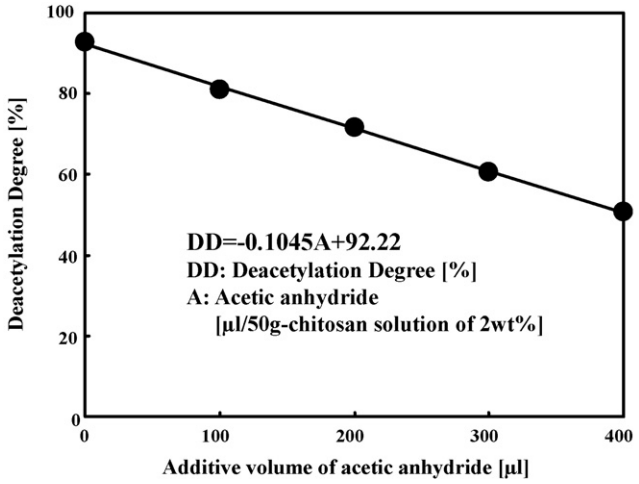

Fig. 1. Effect of acetic anhydride on *N*-acetylation of chitosan. Acetic anhydride was added to 2 wt% chitosan solution (50 g). Original data are quoted from one of the authors' previous publications [6].

During preparation of the membranes, the deacetylation degree (DD) can be regulated by changing the volume of acetic anhydride added (A (μl)). Fig. 1 depicts the linear relationship between the DD and A. The correlation equation (1) has been reported previously by the authors [6].

$$\mathrm{DD} = -0.1045A + 92.22 \quad (1)$$

We successfully employed empirical equation (1) for controlling the DD of chitosan membranes.

2.2. Preparation of chitosan powder from membranes

The water contained in a chitosan membrane was displaced by organic solvents in the order: ethanol aqueous solution, ethanol, and acetone. The membrane was then dried in a thermostatic oven at 333 K for 24 h. The dried membrane pieces were powdered by using a grinder (MILLSER IFM-77G, Iwatani Int. Co., Tokyo). The powder was classified into four classes according to the size of the particles: between 37 and 63 μm, between 74 and 105 μm, between 250 and 297 μm, and between 420 and 500 μm. These classified chitosan powders were stored in a glass vessel in a desiccator.

2.3. Microorganisms

E. coli 745 and *S. aureus* 9779 were obtained from the Tokyo Metropolitan Institute of Public Health. The bacterial strains were stored at 193 K. They were thawed and incubated in brain heart infusion broth (BHI) (Eiken Chemicals, Tokyo) at 310 K for 20 h. The cells were in stationary phase and were washed once with sterile saline (0.85%, w/v) and then resuspended in saline at approximately 10^3 CFU/ml.

2.4. Conductimetric assay

The antibacterial activity of the powder samples was judged by measuring the change in electrical conductivity with bacterial growth. In this paper, the direct conductimetric assay was employed [17]. The Bactometer Microbial Monitoring System, Model 64 (bioMerieux, Tokyo) was used for measuring the electric conductivity of broth (Fig. 2). The Bactometer consisted of a monitor, a computer, and a Bactometer Processing Unit (BPU, Fig. 2a). It has a dedicated module. One BPU can accommodate four modules. The standard module for the Bactometer was divided into