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Antimicrobial effect of chitooligosaccharides produced by bioreactor

You-Jin Jeon, Pyo-Jam Park, Se-Kwon Kim*

Department of Chemistry, Pukyong National University, Pusan 608-737, South Korea

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Abstract

The antibacterial effect of three kinds of chitooligosaccharides with relatively higher molecular weights (HMWCOS), medium molecular weight (MMWCOS), and lower molecular weight (LMWCOS), respectively, was evaluated against various microorganisms. The oligosaccharides were prepared from chitosan and fractionated using ultrafiltration (UF) membrane in conjunction with an enzymatic bioreactor. The growth of most bacteria tested was inhibited by chitooligosaccharide treatments, in particular by HMWCOS, although chitosan treatment showed a better inhibitory effect. From the results here, the molecular weight of chitooligosaccharides is critical for microorganism inhibition and required higher than 10,000 Da. Generally, the chitooligosaccharides have more effective activity against pathogens than non-pathogens, except in the case of lactic acid bacteria. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Chitooligosaccharides; Ultrafiltration; Membrane reactor; Antimicrobial effect

1. Introduction

Chitosan is derived from chitin by deacetylation in the presence of alkali which is a copolymer consisting of β-(1 → 4)-2-acetamido-D-glucose and β-(1 → 4)-2-amino-D-glucose units with the latter usually exceeding 80% (Arvanitoyannis, Nakayama & Aiba, 1998).

Recent studies on chitin and chitosan have attracted interest for converting them to oligosaccharides, because the oligosaccharides are not only water-soluble but also possess versatile functional properties such as antitumor activity (Suzuki, Mikami, Okawa, Tokoro, Suzuki & Suzuki, 1986; Suzuki, Matsumoto, Tsukada, Aizawa & Suzuki, 1989; Tsukada et al., 1990), immuno-enhancing effects (Suzuki, 1996; Suzuki, Watanabe, Mikami, Matsumo & Suzuki, 1992), enhancement of protective effects against infection with some pathogens in mice (Tokoro, Kobayashi, Tayekawa, Suzuki & Suzuki, 1989; Yamada, Shibuya, Kodama & Akatsuks, 1993), antifungal activity (Hirano & Nagao, 1989; Kendra, Christian & Hadwiger, 1989), and antimicrobial activity (Hirano & Nagao, 1989; Uchida, Izume & Ohtakara, 1989). With respect to antibacterial activity, chitosan is superior to chitin since chitosan possesses a lot of polycationic amines which interact with the negatively charged residues of macromolecules at the cell surface of bacteria (Young & Kauss, 1983) and subsequently inhibit the growth of bacteria. The antibacterial effect of chitooligosaccharides has been shown to be greatly dependent on their degree of polymerization (DP) or molecular weight and requires glucosamine polymers with DP 6 or greater (Kendra & Hadwiger, 1984). In addition, the water-soluble chitooligosaccharides may be advantageous as antibacterial agents in in vivo system compared to water-insoluble chitosan.

We have previously reported (Jeon & Kim, 1999) that we could prepare chitooligosaccharides from chitosan by using an UF membrane in conjunction with enzymatic reactor to give chitooligosaccharides with three different molecular weight range such as a high molecular weight fraction (HMWCOS), a medium molecular weight fraction (MMWCOS), and a lower molecular weight fraction (LMWCOS). In the present study, the antimicrobial activity of these three fractions were examined against four Gram-negative, five Gram-positive and four lactic acid bacteria, to investigate the effect of molecular weight on the growth of microorganisms.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation: 89%, average molecular weight: 685,000) was donated by Kitto Life Co. (Korea). Chitosanase (694 units (U)/g protein) for the preparation of chitooligosaccharides was from *Bacillus pumilus* BN-262

* Corresponding author. Tel.: + 82-51-620-6375; fax: + 82-51-628-8147.

E-mail address: sknkim@mail.pknu.ac.kr (Se-Kwon Kim).

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Table 1
Characterization of chitooligosaccharides fractionated using UF membrane bioreactor

Chitooligo-Saccharide[a]	MWCO[b] of the membrane used (kDa)	Yield (%)	TRS[c] (mg/g chitosan)
HMWCOS	10	12.6	51.82 ± 3.58[d]
MMWCOS	5	9.4	74.56 ± 4.62
LMWCOS	1	78.0	229.54 ± 11.36

[a] HMWCOS: chitooligosaccharides with relatively higher molecular weights; MMWCOS: chitooligosaccharides with medium molecular weights; LMWCOS: chitooligosaccharides with lower molecular weights.
[b] Molecular weight cut-off.
[c] Total reducing sugars (TRS).
[d] TRS data are expressed as mean ± standard deviation of three determinations.

and purchased from Wako Chemical Industries, Ltd (Japan). UF membrane reactor system for production of chitooligosaccharides was from Millipore Co. (USA). The microorganisms tested for antimicrobial activity were from Korean Collection of Type Cultures (KCTC) and American Type Culture Collection (ATCC).

2.2. Preparation of chitooligosaccharides using an UF membrane bioreactor

Chitooligosaccharides were prepared by continuous hydrolysis of chitosan in an UF membrane reactor system connected to an immobilized enzyme column reactor in which chitosanase from *Bacillus* sp. was adsorbed on chitin as a carrier for immobilization, according to our previous method (Jeon & Kim, 1999). The three different UF membranes used in the system had molecular weight cut offs (MCWO) of 10, 5, and 1 kDa. One percent chitosan solution (pH 5.5) was passed through the packed column reactor containing the immobilized enzyme at an output flow rate of 5 ml/min to obtain partially hydrolyzed chitosan (PHC) solution. PHC was continuously added to the UF membrane reactor system for the enzymatic conversion of PHC to chitooligosaccharides. Three kinds of chitooligosaccharides (COS) prepared in the system were HMWCOS, these oligosaccharides passed through the 10 kDa MWCO membrane but not the 5 kDa membrane; MMWCOS, these passed through 5 kDa membrane but not the 1 kDa membrane and LMWCOS, these passed through the 1 kDa membrane. The yield and total reducing sugar content of the three oligosaccharides fractionated according to molecular weight were calculated by our previous method (Jeon, Park, Byun, Song & Kim, 1998).

2.3. Assays for antimicrobial activity

Antimicrobial activity of chitosan and three oligosaccharides was examined against various bacteria including four Gram-negative bacteria (*Esherichia coli* KCTC 1682, *Escherichia coli* O-157 ATCC 11775, *Salmonella typhi* KCTC 2424, *Pseudomonas aeruginosa* KCTC 1750), five Gram-positive bacteria (*Streptococcus mutans* KCTC 3065, *Micrococcus luteus* KCTC 10240, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* KCTC 1917, *Bacillus subtilis* KCTC 1028), four lactic acid bacteria (*Lactobacillus bulgaricus* KCTC 3188, *Lactobacillus casei* KCTC 3189, *Lactobacillus fermentum* KCTC 3112, *Streptococcus faecalis* ATCC 10541). The assays were carried out by colony count on incubated agar plates. The mixture of 0.5 ml of the cultured bacteria, 0.5 ml of the autoclaved sample solution and 4 ml of 0.05 M acetate buffer (pH 6.0) was incubated with shaking at 37°C for 1 h. In control samples, 4.5 ml of the acetate buffer was used. The mixture solution (1 ml) was diluted by 10-fold, added to Tryptic soy agar (TSA, Difuco) medium, plated on a plastic petri-dish, and then incubated at 37°C for 24 h. After incubation, the colonies were counted to indicate bactericidal activity which was calculated by the following equation: Bactericidal activity $(\%) = [(C - T)/C] \times 100$, where C is the colony numbers counted on the control and T is those on the sample plate tested.

Minimum inhibitory concentration (MIC) was tested by two-fold serial broth dilution as follows. Bacteria culture (10^5–10^6 colony/ml) grown in 5 ml Tryptic soy broth (TSB) which contained 1 ml of the test sample was incubated at 37°C for 18 h. MIC was defined as the lowest concentration of the tested sample at which the cell growth was not visible with naked eye or microscopy.

The inhibitory effects of chitosan and the oligosaccharides on the growth of *E. coli* were examined periodically by measuring the turbidity of the cultured medium at 640 nm. Either 5 ml of 0.05 M acetate buffer (pH 6.0) or 1% sample solution (pH 6.0) was added to the mixture of the cultured bacteria (0.5 ml) and TSB medium (44.5 ml) to give a final concentration of 0.1% and incubated at 37°C.

3. Results and discussion

Chitosan as well as its hydrolysates has shown antibacterial activity and the inhibitory levels have been shown to be significantly dependent on the DP (degree of polymerization) molecular weight (Hirano & Nagao, 1989; Kendra et al., 1989; Uchida et al., 1989; Ueno, Yamaguchi, Sakairi, Nishi & Tokura, 1997). We have successfully obtained three

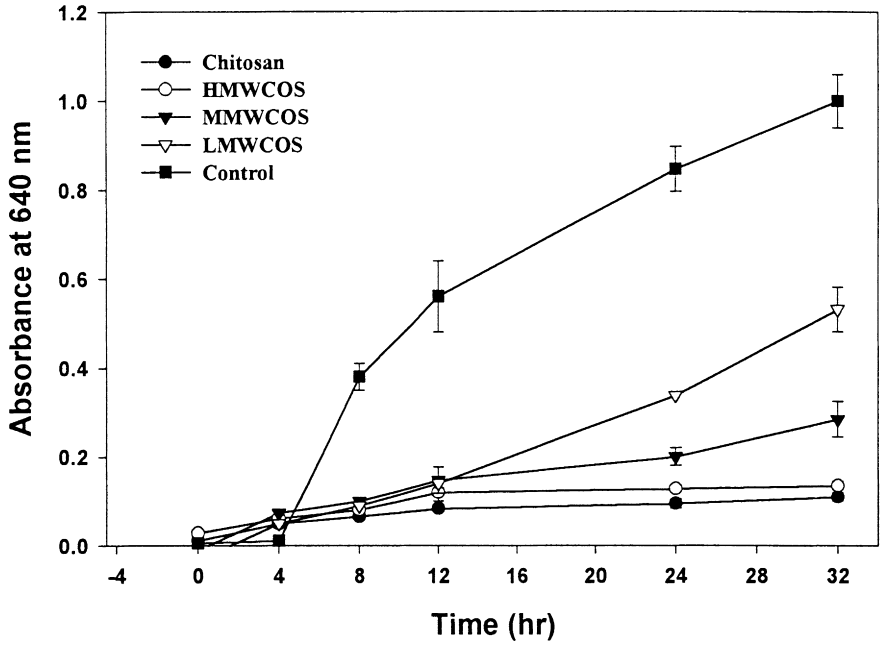

Fig. 1. Inhibitory effect of chitosan and chitooligosaccharides on *E. coli* growth as a function of incubation time. *E. coli* treated with 1% sample concentration was incubated at 37°C and the turbidity of the cultured medium was measured at 640 nm. Data are mean ± standard deviation of duplicate samples.

fractions of chitooligosaccharides, based on their molecular weights, using the UF membrane reactor in our previous study (Jeon & Kim, 1999) and their antibacterial activity was examined in this study. As indicated in Table 1, the yields obtained by weighing the dried products of HMWCOS and MMWCOS were 12.6 and 9.4%, respectively, while that of LMWCOS was 78.0%. Total reducing sugar, which provides information on the hydrolytic level of the oligosaccharides was determined. The content of LMWCOS (229 mg/g chitosan) was greatly higher than others (51 and 74 mg/g chitosan for HMWCOS and MMWCOS, respectively). From the results of the previous study (Jeon & Kim, 1999), the molecular weight distribution of HMWCOS and MMWCOS ranged from 24 to 7 kDa and 6 to 1.5 kDa, respectively. The profile of LMWCOS consisted of oligosaccharides with DP in the range from pentamer to heptamer. These results revealed that the enzymatic hydrolysis of chitosan using the UF membrane bioreactor was suitable for the production of chitooligosaccharides.

Bactericidal activity of chitosan and three oligosaccharides on the growth of *E. coli* as a function of incubation time

Table 2
Antimicrobial activity of chitosan and chitooligosaccharides (data are expressed as mean ± standard deviation of duplicate samples)

	Bacteria	Bactericidal activity (%)[a]			
		Chitosan	HMWCOS	MMWCOS	LMWCOS
Gram-negative bacteria	*Escherichia coli*	>99 ± 0	98 ± 0	62 ± 6	51 ± 7
	Escherichia coli O-157	>99 ± 0	71 ± 3	56 ± 4	60 ± 2
	Salmonella typhi	>99 ± 0	91 ± 2	88 ± 0	89 ± 0
	Pseudomonas aeruginosa	68 ± 3	47 ± 5	35 ± 5	22 ± 8
Gram-positive bacteria	*Streptococcus mutans*	100 ± 0	100 ± 0	99 ± 0	99 ± 0
	Micrococcus luteus	>99 ± 0	70 ± 0	67 ± 3	63 ± 7
	Staphylococcus aureus	100 ± 0	97 ± 3	95 ± 0	93 ± 9
	Staphylococcus epidermidis	>99 ± 0	82 ± 0	57 ± 3	23 ± 1
	Bacillus subtilis	98 ± 2	63 ± 5	60 ± 5	63 ± 7
Lactic acid bacteria	*Lactobacillus bulgaricus*	100 ± 0	98 ± 0	63 ± 0	57 ± 3
	Lactobacillus casei	100 ± 0	95 ± 0	63 ± 5	70 ± 4
	Lactobacillus fermentum	100 ± 0	>99 ± 0	100 ± 0	99 ± 1
	Streptococcus faecalis	100 ± 0	99 ± 0	99 ± 1	99 ± 0

[a] After incubation of the bacteria treated with 0.1% sample, bactericidal activity (%) was calculated by counting the colony formed on the medium, comparing to the control.

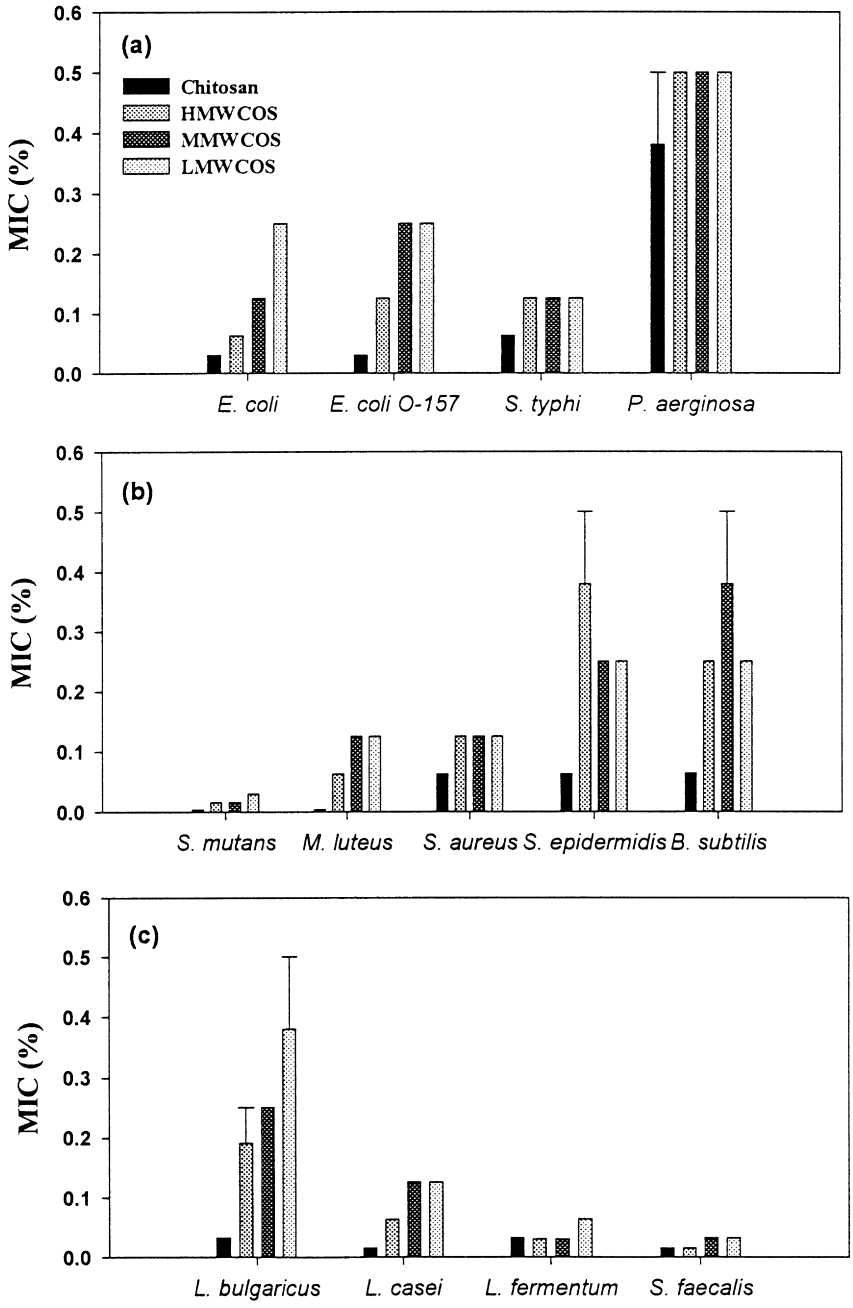

Fig. 2. MICs of chitosan and chitooligosaccharides against: (a) Gram-negative; (b) Gram-positive; and (c) lactic acid bacteria. Data are mean ± standard deviation of duplicate samples. *E. coli*, *Escherichia coli*; *E. coli*, O-157, *Esherichia coli* O-157; *S. typhi*, *Salmonella typhi*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. mutans*, *Streptococcus mutans*; *M. luteus*, *Micrococcus luteus*; *S. aureus*, *Staphylococcus aureus*; *S. epidermidis*, *Staphylococcus epidermidis*; *B. subtilis*, *Bacillus subtilis*; *L. bulgaricus*, *Lactobacillus bulgaricus*; *L. casei*, *Lactobacillus casei*; *L. fermentum*, *Lactobacillus fermentum*; *S. faecalis*, *Streptococcus faecalis*.

was shown in Fig. 1. All chitosan preparations of 0.1% concentration inhibited the growth of *E. coli*, as compared with the control. The efficacy increased with molecular weight. Treatment with HMWCOS and chitosan resulted in little or no growth after 32 h of incubation time but the inhibitory effects of MMWCOS and LMWCOS decreased after 24 and 12 h, respectively. Numerous studies have examined the effect of chitosan and chitooligosaccharide on the growth of *E. coli*. It was shown that chitosans were very effective for *E. coli* inhibition although their concentrations required for complete inhibition of *E. coli* growth varied according to the degree of acetylation, molecular weights and functional groups (Chang, Cho, Goo & Choe, 1989; Darmadji & Izumimot, 1994; Papineau, Hoover, Knoor & Farkas, 1991; Sudharshan, Hoover & Knorr, 1992; Wang, 1992).

Bactericidal effects of chitosan preparations against various microorganisms including Gram-negative,

Gram-positive and lactic acid bacteria were investigated in detail because the chitooligosaccharides obtained from the bioreactor exhibited antimicrobial activity against *E. coli*.

Antimicrobial acitivites of chitosan and chitooligosaccharides were summarized in Table 2. The MICs of those against various bacteria were shown in Fig. 2. Chitosan markedly inhibited the growth of most bacteria tested and its MIC values were less than 0.06% against Gram-negative bacteria, except for the case of *P. aeruginosa* (0.25%). The inhibitory effects were slightly varying according to the type of bacteria. Chitooligosaccharides also effectively blocked the growth of the bacteria although their effects were lower than that of chitosan. With Gram-negative bacteria, chitosan showed stronger bactericidal effects for *E. coli*, *E. coli* O-157 and *S. typhi* than *P. aeruginosa*, and their growth was completely inhibited in medium containing over 0.06%. Chitooligosaccharides were the most effective for *S. typhi*, which was inhibited approximately 90% at a concentration of 0.1%. Their MIC values were the same at 0.12%. Regarding *E. coli* and *E. coli* O-157, HMWCOS among the chitooligosaccharides displayed the highest antibacterial effects against the respective bacteria and the MICs were 0.06 and 0.12%, respectively. For *P. aeruginosa*, all chitooligosaccharides tested indicated a slight suppression of the growth and their bactericidal activities at a concentration of 0.1% were 50% or lower.

With regard to Gram-positive bacteria, all microorganisms tested were definitely inhibited at 0.1% chitosan, and the MICs were 0.008% for *S. mutans* and *M. luteus*, and 0.06% for *S. aureus*, *S. epidermidis* and *B. subtilis*. Chitosan showed more effective suppression against Gram-positive bacteria comparing to Gram-negative bacteria. Similar results were observed with chitooligosaccharides although their antimicrobial activities were lower than that of chitosan. In particular, no or little evidence of growth was detected in chitooligosaccharides-treated *S. mutans*. Their MICs for *S. mutans* were all below 0.03%.

From the results, MW of chitosan is an important factor governing the inhibition of Gram-negative and Gram-positive bacteria. For effective inhibition, it should be higher than or around 10,000 Da. In addition, chitosan showed an excellent antimicrobial activity for all the microorganisms examined (except *P. aeruginosa*), while the activity of chitooligosaccharides generally showed a tendency to be more effective against pathogens associated with human disease in comparison to non-pathogens.

Uchida et al. (1989) have observed that chitosan hydrolysate with 50 mg total reducing sugar per gram of chitosan and chitosan oligomer I composed of mainly components of tetramer, pentamer and hexamer possessed antimicrobial and antifungal effects, while chitosan oligomer II consisted of mainly trimer and tetramer showed no activity. Kendra and Hadwiger (1984) have reported that heptamer among the oligomers with seven units or smaller was maximal in both antifungal activity and formation of pisatin. Ueno et al. (1997) showed that a chitosan oligomer with average MW of less than 2200 Da was hard to suppress microbial growth, while those with MW of around 5500 Da suppressed the growth dependent on the concentration. In the present study, the chitooligosaccharides with MW of around 10,000 Da or larger seemed to be suitable for antimicrobial activity because HMWCOS with MW distribution of 24,000 Da to 7000 Da was significantly superior to others.

On the other hand, the antagonistic action of chitosan and its oligosaccharides against many fungi including plant and animal pathogens has already been reported elsewhere (Allan & Hadwiger, 1979; El Ghaouth, Arul, Grenier & Asselin, 1992; Kendra & Hadwiger, 1984; Lee, Uhm & Lee, 1996; Uchida et al., 1989). We also showed that chitooligosaccharides appeared to be effective for suppressing the growth of microbacterial pathogens. In the case of Gram-negative bacteria, for example, all oligosaccharides at a concentration of 0.1% showed bactericidal effects of around 90% or more for *S. typhi*, which causes typhoid fever. In Gram-positive bacteria, furthermore, *S. mutans*, which induces tooth decay was completely inhibited by 0.1% of the oligosaccharide. And the oligosaccharides indicated bactericidal effects of 93–100% against *S. aureus*, which causes pimples on the human skin and is one of the most common cause of food poisoning.

In the case of lactic acid bacteria, the microorganisms examined were completely inactivated by chitosan treatment and the MICs were less than 0.03%. The chitooligosaccharides also acted extensively and effectively against lactic acid bacteria. HMWCOS suppressed at least 95% or more at a concentration of 0.1%, and its MICs were below 0.03% except for *L. bulgaricus*. MMWCOS and LMWCOS also suppressed up to 99% for *L. fermentum* and *S. faecalis*. From the results, it was shown that lactic acid bacteria, among three different strains tested in this study, were effectively inactivated most sensitively by all chitosan preparations.

There are two contrary reports about the antimicrobial effect of chitosan against lactic acid bacteria. Austin, Brine, Castle and Zikakis (1981) reported that addition of up to 10% of chitin to the diet of chickens resulted in normal growth and increased the growth of *Bifidobacterium* in the gut. Sugawara, Oohisa and Kobayash (1997) explained that 0.05% or more chitosan showed antimicrobial effect and even lower MW chitosan of around 3000 Da could suppress the bacteria growth. The reason for this difference is unclear but, in our study, the oligosaccharides as well as chitosan exhibited better inhibitory effects against Gram-positive compared with Gram-negative bacteria. Thus, it might be reasonable that chitosans showed remarkable inhibitory effect against the growth of lactic acid bacteria which belong to Gram-positive bacteria.

4. Conclusions

Current attention on chitin and chitosan focuses to their

oligosaccharides because of their water-solubility and better absorption in vivo. The fraction of chtiooligosaccharides produced by the UF membrane bioreactor has been demonstrated to possess better antimicrobial effects against pathogens in comparison to non-pathogens and oligosaccharides with molecular weight over 10,000 Da were required for this activity.

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