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Characterization and potential antimicrobial effect of novel chitooligosaccharides against pathogenic microorganisms

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ABSTRACT

Chitosan has antimicrobial activities against a wide range of food-borne microorganisms. It was poorly soluble in neutral solution, which makes them difficult to use in food and biomedical applications. While chitooligosaccharides were fairly soluble in aqueous solution and consequently facilitated their utilization in industrial application. Therefore, enzymatic production of chitooligosaccharides from chitosan, previously, was carried out using immobilized pepper chitosanase. Chitooligosaccharides I, II, III and IV groups were prepared with molecular weight> 100, 100-10, 10-1 and < 1 KDa, respectively. Their particles were compared with chitosan by scanning electron microscope (SEM). Their degree of fissure and cracking were increased with decrease the molecular weight. The rod shape appear in IV group, indicated to the presence of disaccharides (chitobioses). Ultraviolet spectrum (UV) showed that IV group had different absorbance at wavelength ranged from (309-348 nm) than that of the other groups. The main difference in Fourier transform infra red spectroscopy (FT-IR) of the four groups and native chitosan were observed in the range of the wave number 3750-3000 Cm-1. Potential antimicrobial activity (in vitro)of chitooligosaccharides groups were evaluated against some selected pathogenic microorganism and were compared with that of chitosan. They were measured using the standard method of diffusion disc plates on agar (50 µg/ml). Our results clearly indicated that the antimicrobial activities depend mainly on the type of microorganism tested. Chitooligosaccharides had antimicrobial activity as follows: groups (I, II, III) against *B. cereus*, group (I, III, IV) against *B. subtilis*, group (II, IV) against *Staphylococcus aureus*, group (II) against *Pseudomonas aeruginosa*, group (I, II, IV) against *Candida albicans* and group (III) against *Saccharomyces chevalieri*.

INTRODUCTION

Side effect of the used famous antibiotics led to the search for new compounds with antimicrobial activity. Therefore, this study was undertaken to focus on the *in vitro* antimicrobial effects of the four prepared chitooligosaccharides groups. Chitooligosaccharides were composed of glucosamine and acetylated glucosamine rings connected with each other byβ 1,4-glycosidic bonds. They reacted with cell's receptors in living organisms through their hydroxyl, amino and acetylated amine groups, causing a cascade of interconnected reactions lead to different biological activities such as antimicrobial, antidiabetic, anti-inflammatory and antioxidant (Ngo *et al.*, 2009, Eun *et al.* 2010 and Ju *et al.*, 2010 and Fernandes *et al.*, 2010). The antimicrobial effect of chitooligosaccharides against wide range of microorganism, made them potentially used as a natural food preservative. (Devlieghere *et al.*, 2004). *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* strains are recognized as the most common causes of bacterial food-borne diarrhoeal disease throughout the world. Chitooligosaccharides have antimicrobial activity and have the advantage of not producing major side effect as is found in case of usual synthetic food preservative.

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They could be prepared by chemical, physical and enzymatic hydrolysis of chitosan (Tishchenko *et al.,* 2011). Enzymatic hydrolysis received more attention for its mild reaction conditions. Low molecular weight chitosan and chitooligosaccharides were readily soluble in water (Jeon *et al*., 2001). The good solubility of chitooligosaccharides made them especially attractive in an important number of useful applications. Hu *et al*. (2007) and Chi *et al*. (2007) were studied the antimicrobial activity of chitooligosaccharides.

As a part of our continuous search for new biological activities, chitooligosaccharides (natural products) were prepared as described in our previous reported (El-Sayed *et al.,* 2011and 2012). Chitosan was depolymerized by enzymatic hydrolysis using immobilized pepper chitosanase (El-Sayed *et al.,* 2016a). Prior to analysis, the reaction media containing chitooligosaccharides and low molecular weight chitosans at different portions also were fractionated by ultrafiltration to four groups according to the molecular weights, the procedure developed by us (El-Sayed *et al.,* 2016b). This study aimed to characterize of chitosan depolymerization products (chitooligosaccharides) by the use of scanning electron microscope (SEM),Fourier transform infra red spectroscopy (FT-IR) and of ultraviolet analysis (UV).Here,we also investigated the *in-vitro* effect of native chitosan and its splitting products (chitooligosaccharides) on some pathogenic microorganisms (Gram positive and negative bacteria, yeast and fungi).

MATERIALS AND METHODS

Chitosan with molecular weight 300,000 were purchased from Merck chemical Co, Germany. Chitooligosaccharides were prepared from chitosan using immobilized pepper chitosanase and were fractionated as previously described (El-Sayed *et al*., 2016a&b).All chemicals were of analytical grade.

Test microorganisms

Gram positive bacteria (*Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), yeasts (*Candida albicans* and *Saccharomyces chevalieri* and fungi (*Macrophomina phaseolina*and *Aspergillus niger*) were obtained from MIRCIN culture collection of the faculty of Agriculture, Ain Shams University, Egypt.

Characterization of the prepared chitooligosaccharides groups and comparing it with the native chitosan

Determination of the prepared chitooligosaccharides

Concentration of the prepared chitooligosaccharides and their fractions were estimated by dinitrosalicylic acid method (Miller, 1959) and calculated as mg D-glucosamine.

Scanning electron microscopy (SEM)

Dry chitooligosaccharides groups (I, II, III and IV) and native chitosan were mounted on SEM stubs with double-sided adhesive tape. Scanning electron micrographs were taken using Quanta 250 FEG. The accelerating voltage and the magnification are shown on the micrograph.

Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra of chitooligosaccharides groups (I, II, III and IV) and native chitosan were recorded using a spectrophotometer (National Research Centre, spectra department). The scan-spectra were recorded in the range of wavenumber 4000-400 cm^{-1}.

Ultraviolet absorption analysis (UV)

The absorbance behaviour of the water soluble chitooligosaccharides goups (I, II, III and IV) were studied at 200-380 nm using CECIL instruments CE595 U.V spectrophotometer.

Determination of antimicrobial activity of chitosan and chitooligosaccharides groups (I, II, III and IV):

Preparation of microorganisms for experiment

The pure cultures of organisms were sub-cultured in nutrient broth. They were inoculated separately into nutrient broth and kept at 37ºC for 24 h. then, they were kept at 4ºC until use.

Nutrient broth media

Nutrient agar medium for bacteria growth consists of 20.0 g agar, 3.0 g beaf extract, 5.0 g peptone and 3.0 g sodium chloride in1000.0 ml distilled water at pH 7.0. Czapek's dox agar medium for fungi growth consists of 20.0 g agar, 10.0 g sucrose, 2.0 sodium nitrate, 0.5 g magnesium sulphate, 0.5 g potassium chloride and 0.01 g ferrous sulphate in 1000.0 ml distilled water.

Preparation of Mueller-Hinton Agar

Freshly prepared Mueller-Hinton Agar medium was autoclaved, cooled to 50 °C and poured immediately into Petri dishes. They were used in the same day after cooling to room temperature or put in plastic bag and stored at -4 °C for seven days only.

Filter paper discs method

Antimicrobial activities of chitooligosaccharides groups (I, II, III and IV) and chitosan were measured using the standard method of diffusion disc plates on agar (Singh and Sharmila, 2015). Whitman filter paper (No.1) was used to prepare sterilized discs approximately 4 mm in diameter saturated with samples (50 µg chitooligosaccharides/disc). Saturated discs were transferred to the surface of agar media plates, previously incubated with the test organisms. The antimicrobial activities were estimated by detecting the clear inhibition zone surrounding the filter paper disc after 24 h for bacteria and yeasts and 48 h for fungi. Data are expressed as the mean of at least three experiments.

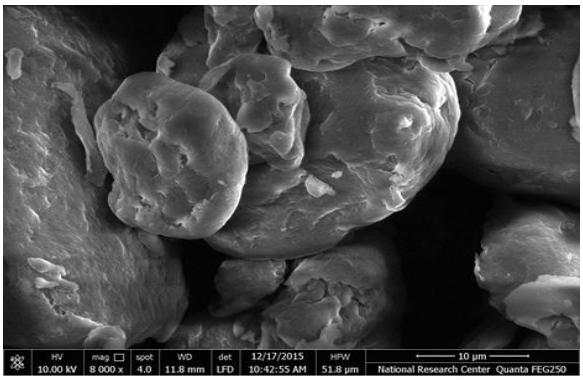
RESULTS AND DISCUSSION

Chitooligosaccharides have many biological activities useful for the industrial application. The most important one is its

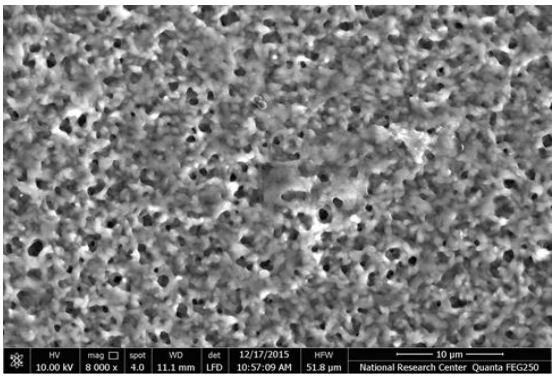
potential use as a food preservative of natural origin due to its antimicrobial activities against a wide range of food-borne microorganisms.

Therefore, chitooligosaccharides were produced previously, by enzymatic hydrolysis of chitosan using our immobilized pepper chitosanase. It was carried out at pH 5.8 and 40°C for 60 min. The production of chitooligosaccharides was maximized by recycling the incubation of immobilized chitosanase for four repetitions. Chitooligosaccharides syrup (72% concentration) were prepared from chitosan with relative production value 55.97%. They were separated according to the molecular weight by ultrafiltration technique. Four groups I (>100KDa), II (100-10 KDa), III (10 - 1 KDa) and IV (< 1. KDa) were separated from the prepared chitooligosaccharides. The yield of II and III (33.5 and 31.2%, respectively) were higher than in I and IV (18.8 and 16.5%, respectively).

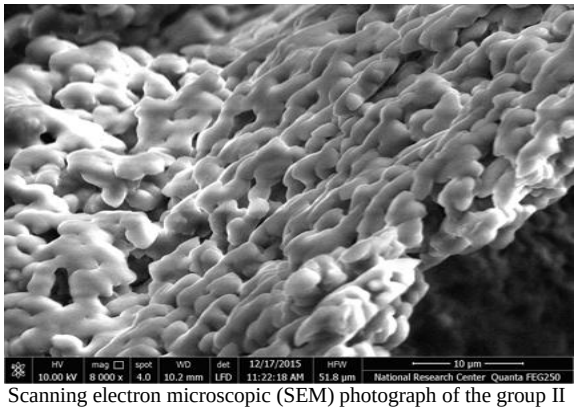
This study was undertaken to focus on the physical characterization of the four chitooligosaccharides groups and on their *in vitro* antimicrobial effect on some pathogenic microorganisms. Chitooligosaccharides groups were odourless and freely soluble in water, while chitosan was insoluble in water. The solubility facilitate their industrial utilization. The changes in the shape of the prepared chitooligosaccharides groups were studied by using scanning electron microscope (SEM).The shape and size of the obtained chitooligosaccharides were significantly different, compared with that of chitosan (Figure 1).



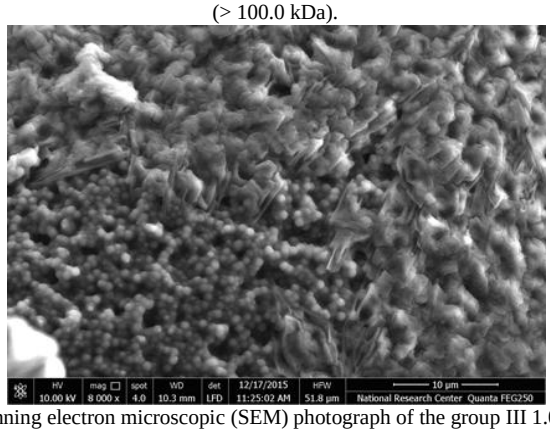
Scanning electron microscopic (SEM) photograph of the chitosan.



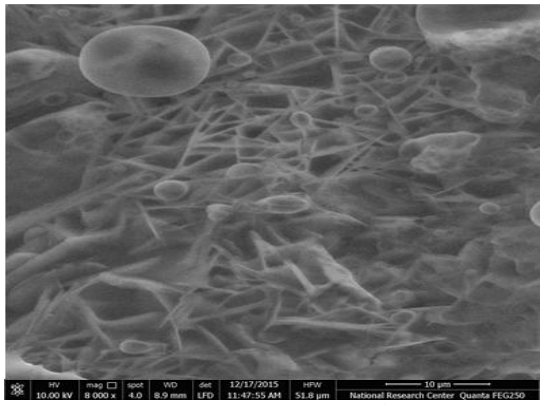
Scanning electron microscopic (SEM) photograph of the group I (> 100.0 kDa).



Scanning electron microscopic (SEM) photograph of the group II (10-100 kDa).



Scanning electron microscopic (SEM) photograph of the group III 1.0-10 kDa).



Scanning electron microscopic (SEM) photograph of the group IV (<1.0 kDa).

Fig. 1: Scanning electron microscopic (SEM) photograph of chitosan and chitooligosaccharides groups.

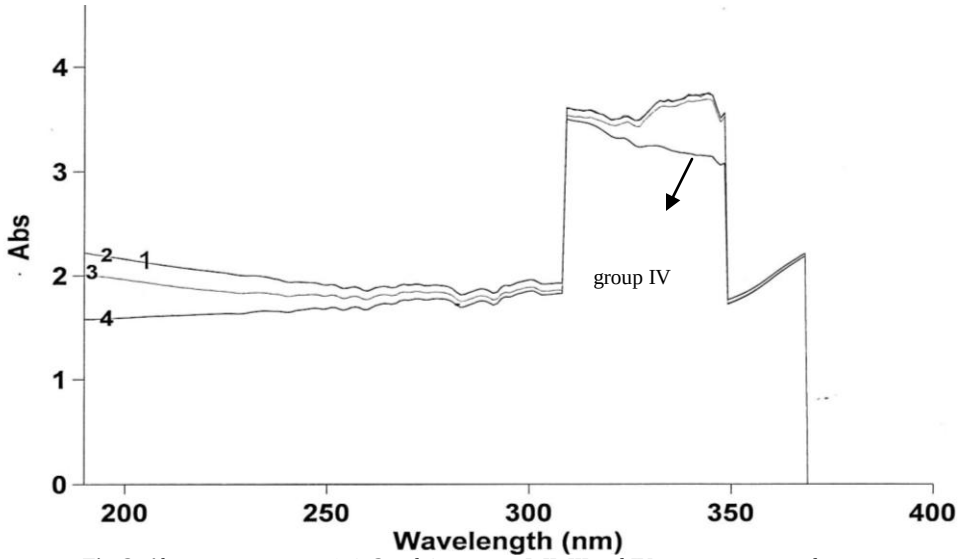


Fig. 2: Absorption spectrum 1, 2, 3 and 4 represent I, II, III and IV groups, respectively.

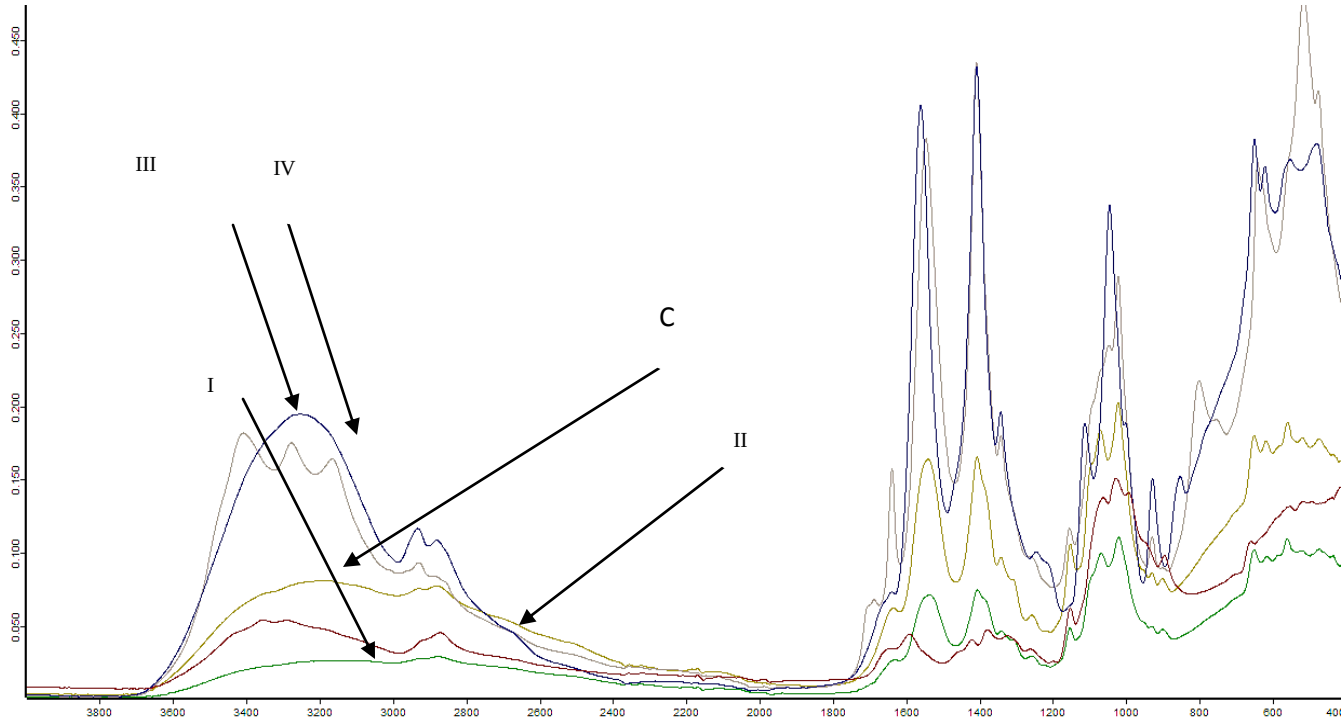


Fig. 3: Fourier transform infrared (FT-IR) spectra of the native chitosan and chitooligosaccharides (I, II, III and IV groups).

The native chitosan granules appeared with unregularly shape and smooth surface. group I granules appeared with many fissures on the surface. The degradation increased in group II and the granules appeared smaller than in group I. Granules of group III appeared spherical in shape and granules of group IV appeared different and smaller with many rod shape. The rod shape indicated to the presence of disaccharides (chitobioses).In comparison with the SEM of native chitosan, we noticed that there are many differences in the granules shape of four fractions. The increase in the destruction of the chitooligosaccharides granules indicate to decrease in the molecular weight. These results gave a good evidence that ultrafiltration membrane techniques was successful method for separation. Similarly, Yang *et al*.(2005) used SEM for characterization of xylooligosaccharides prepared from xylan by enzymatic hydrolysis with xylanase. The ultraviolet absorbency profile of the four groups dissolved in distilled water was demonstrated in figure (2). Ultraviolet spectrum pattern of chitooligosaccharides indicated that group IV only had different absorbance at wave length ranged from (309-348) nm. There is no any addition difference between them. Figure (3) showed FT-IR spectra of the four groups(I, II, III and IV). In order to fully characterize the starting materials, a spectrum of native chitosan

was also recorded. Some new bands appeared and some disappeared in the chitooligosaccharides groups spectra compared with that in the native chitosan. The main bands appearing in that spectra were due to stretching vibrations of OH groups in the range from 3750 cm^{-1} to 3000 cm^{-1}, which are overlapped to the stretching vibration of N-H; and C–H bond in $–CH_2$.Various absorption bands within the 4000-400 cm^{-1} range were recorded in the FTIR spectra of chitosan (Table 1). These bands were compared with those of chitooligosaccharides groups (I, II, III and IV). Different stretching vibration bands were observed in the range 3750-3000 cm^{-1} related to ν(O-H) overlapped to the $ν_S$(N-H) (Mano *et al*., 2003). New band appeared at 2925 cm^{-1} in four chitooligosaccharides fractions related to $ν_{as}(CH_2)$ in CH_2OH group (Zvezdova, 2010). In COSI fraction, new four bands appear at 2537, 2357, 2324 and 2321 cm^{-1} with no significant vibrations. The OH and CH deformation ring vibrations (1418 cm^{-1}) for chitosan disappeared in chitooligosaccharides fractions and new absorption band without characteristic vibrations appeared at 1405 cm^{-1}. Also the CH symmetrical deformation bending vibrations (1376 cm^{-1}) for chitosan disappeared in chitooligosaccharides fractions and new absorption band without characteristic vibrations appeared at 1340 cm^{-1}. The $ν_{as}$(C-O-C) vibrations (1068 cm^{-1}) disappeared in group III and IV fractions (Kolhe and Kannan, 2003).

Table 1: Characterization absorption bands in the FTIR spectra of chitosan and chitooligosacchrides groups.

Wave number, ν (cm-1)					Vibration modes	References
Chitosan	I group	II group	III group	IV group		
3355	3346	3201	3408	3253	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
3289	3340	3190	3277	2932	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
2871	3221	3182	3165	2880	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1644	3189	3111	2929	1637	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1638	3183	2928	2894	1559	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1589	3178	2881	2886	1405	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1418	3167	1633	1686	1340	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1376	3143	1539	1637	1243	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1321	3130	1404	1544	1110	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1259	3121	1340	1406	1043	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1197	3107	1308	1339	999	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1150	3099	1255	1261	925	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1060	3073	1217	1152	850	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
1025	3003	1150	1045	647	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
990	2989	1068	1019	619	ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
893	2924	1019	926		ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
657	2877	927	898		ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
	2537	899	799		ν(O-H) overlapped to the νS(N-H)	Mano *et al*., 2003
	2357	648	751		ν(O-H) overlapped to the νS(N-H)	-
	2324	616	638		-	Zvezdova, 2010
	2321				$ν_{as}(CH_2)$ in CH_2OH group	Zvezdova, 2010
	1628				ν (C-H) in pyranose ring	Zvezdova, 2010
	1533				ν (C-H) in pyranose ring	-
	1403				-	-
	1338				Amide I band	Tian *et al*, 2004
	1308				Amide I band	Tian *et al*, 2004
	1255				Amide I band	Tian *et al*, 2004
	1151				NH_2 deformation	Tian *et al*, 2004
	1065				NH_2 deformation	Brugnerotto *et al*., 2001
	1018				OH and CH deformation ring	-
	927				-	Brugnerotto *et al*., 2001
	898				CH symmetrical deformation bend	-
	648				-	-
	613				-	-
					-	Paluszkiewicz *et al*., 2011
					ν(C-O-H)	-
					-	-
					-	Kuptsov and Zhizhin, 1998
					Amine rocking	Kolhe and Kannan, 2003
					νs(C-O-C)	-
					-	Kolhe and Kannan, 2003
					νas(C-O-C)	Kolhe and Kannan, 2003
					ν (C-O) in primary OH group	Kolhe and Kannan, 2003
					ν (C-O) in primary OH group	-
					-	Roeges, 1994
					CH_3 rocking	Roeges, 1994
					CH_3 rocking	Pearson, 1960
					C-O ring stretching	Rumengan *et al*., 2014
					NH_2	Rumengan *et al*., 2014
					NH_2	Rumengan *et al*., 2014
					NH_2	Tian *et al*., 2004
					δ (NH) out of plane	
					-	
					δ (OH) out of plane	

Table 2: Antimicrobial activities of chitosan and chitooligosaccharides groups by disc filter paper assay.

Sample	Bacteria					Yeast		Fungi	
	Gram positive			Gram negative					
	B.Cereus	***B.Subtilis***	***S.aureus***	***E.coli***	***P.aeruginos***	***C.albicans***	***S.chevalieri***	***M.phaseolina***	***A.niger***
Chitosan	0	12	0	0	0	7	20	0	0
Group I	7	7	0	0	0	7	0	0	0
Group II	7	0	7	0	7	7	0	0	0
Group III	7	7	0	0	0	0	7	0	0
Goup IV	0	7	7	0	0	15	0	0	0

The diameters of the inhibition zone was measured to nearest millimeters. The inhibition zone was recorded after 24 h for bacteria and yeast and 48 h for fungi.

New band appear at 1019 cm^{-1} in three groups (I, II and III). Without characteristic vibrations. The C-O ring vibrations disappeared in IV group. The NH_2 vibrations appeared only in III and IV groups at range (850-750 cm^{-1}) (Rumengan *et al*., 2014). The δ (NH) vibrations in chitosan disappeared in four chitooligosaccharides groups and new bands appeared at 613, 616 and 619 cm^{-1} in I, II and IV that attributed to the occurrence of δ (OH) vibrations. The antimicrobial activities of chitooligosaccharides groups (I>100, II= 100-10, III=10-1KDa and IV<) and chitosan with molecular weight (100-300 kDa) were examined against pathogenic microorganisms(Table 2). Results indicated that chitosan had higher activity against *S. chevalieri* and *B. subtilis* than other chitooligosaccharides groups. For Gram negative bacteria, *P. Aeruginosa* growth, was inhibited only by group II while Gram negative *E. coli* was not inhibited by chitosan or by chitooligosaccharides. Group IV showed higher inhibitory effect against *C.albicans* than other groups and chitosan. On the other hand, Gram positive bacteria and yeast showed different growth inhibition were obtained independent on molecular weight. Chitosan and chitooligosaccharides showed no antimicrobial activity against *M. phaseoli* and *A. niger* fungi. Antimicrobial activity and molecular weight of the chitooligosaccharides didn't have an obvious relation. It depends on the microorganism used. The same relationship between the molecular weight of chitooligosaccharides and their antimicrobial activity was reported previously (Jeon *et al*., 2001; No *et al*., 2002 and Gerasimenko *et al*., 2004).

CONCLUSION

Immobilized pepper chitosanase could be used successfully for industrial production of antimicrobial chitooligosaccharides from chitosan. Solubility of chitooligosaccharides in water facilitate their industrial application. Their antimicrobial activities depend on the type of the microorganism used and not on their molecular weights. Chitooligosaccharides can be used as natural food preservative. They have the advantage of not producing major side effect as is found in case of usual synthetic food preservative.

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Conflict of Interests: There are no conflicts of interest.

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