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Note

Chitooligosaccharides—preparation with the aid of pectinase isozyme from *Aspergillus niger* and their antibacterial activity

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Abstract—An isozyme of pectinase from *Aspergillus niger* with polygalacturonase activity caused chitosanolysis at pH 3.5, resulting in low-molecular weight chitosan (86%), chitooligosaccharides (COs, 4.8%) and monomers (2.2%). HPLC showed the presence of COs with DP ranging from 2 to 6. Charcoal–Celite chromatography and re-*N*-acetylation of the COs followed by CD, IR, MALDI-TOF-MS and FAB-MS analyses revealed an abundance of chitobiose, chitotriose and chitotetraose. The COs-monomeric mixture showed a bactericidal effect towards *Bacillus cereus* and *Escherichia coli* more efficiently than native chitosan. Among the chitooligomers, the hexamer showed maximum antibacterial effect followed by the penta-, tetra-, tri- and dimers. Of the two monomers, only GlcN showed slight bacterial growth inhibition. SEM revealed bactericidal action patterns of COs-monomeric mixture towards *B. cereus* and *E. coli*.
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Keywords: Chitosan; Pectinase; Chitooligosaccharides; MALDI-TOF-MS; FAB-MS; Antibacterial activity

Chitosan, a linear polymer composed of β-1,4-linked glucosamine residues with various degrees of *N*-acetylation, is a partially deacetylated derivative of chitin, extracted from an abundant source of shellfish exoskeletons.[1] The depolymerization products of chitosan (low-molecular weight chitosan (LMWC), homo-/hetero-chitooligosaccharides (COs) and GlcN/GlcNAc) are of special interest in agriculture and medicine. They have activities as elicitors of plant defence,[2] antibacterial[3,4] and antitumour agents.[5] Chitotriose exhibits inhibitory effect towards angiotensin converting enzyme (ACE) and hence could be used as an antihypertensive agent,[6] whereas COs are useful in milk preservation.[7] Because of their potential biological activities, many methods (chemical[8] and enzymatic[9]) have been developed to prepare chitosan degradation products. Due to rigorous control over the course of the reaction, enzymatic methods are preferred. During our studies on chitosanolysis by purified food grade enzymes, we identified a pectinase isozyme from *Aspergillus niger* with polygalacturonase activity, hydrolyzing chitosan to LMWC, COs and monomers, the former being the major degradation product,[10] was further structurally characterized.[11] Here, the purification, structural characterization and antibacterial activity of COs-monomeric mixture as well as of individual oligomers and monomers towards *Bacillus cereus* (Gram-positive) and *Escherichia coli* (Gram-negative) are presented.

The isozyme of *A. niger* pectinase associated with polygalacturonase activity,[10] showed optimum chitosanolysis

Abbreviations: GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; FT-IR, Fourier transform infrared spectroscopy; CD, circular dichroism; MALDI-TOF-MS, matrix assisted laser desorption time of flight mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; DP, degree of polymerization; DA, degree of acetylation; SEM, scanning electron microscopy.

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at pH 3.5 and 47 °C. As the isozyme lost 30% activity at 47 °C after 2 h of incubation, and also showed substrate inhibition above 1.0%, for further studies, chitosanolysis was done in acetate buffer (chitosan concn, 1.0%, pH 3.5, 37 °C) at an enzyme: chitosan ratio of 0.003% (w/w) for 6 h. The LMWC was separated by adjusting the pH to 7.0 with 2 M NaOH followed by centrifugation. The supernatant containing COs and monomers (in addition to salt and heat-denatured isozyme) were practically impossible to purify on Sephadex G-10 without the loss of mono- and disaccharides, as also observed by Ohtakara and Mitsutomi.[12] To overcome this, it was subjected to charcoal–Celite chromatography, wherein salts and GlcN/GlcN-rich oligomers were removed in the water wash (Fraction IA) and the adsorbed molecules (uncharged, GlcNAc/GlcNAc-rich oligomers) were desorbed with aqueous ethanol (Fraction II). Fraction IA, after *N*-acetylation, was rechromatographed and purified (Fraction I). GlcNAc-oligosaccharides produced by acid hydrolysis of chitin, as well as those generated in a reaction catalyzed by a chitosanase from *B. pumili* were similarly separated.[7,13]

A sudden drop in the viscosity of chitosan solution after the addition of pectinase isozyme was indicative of its *endo*-action, whereas the appearance of mono- to hexamers was due to its *exo*-action. Fractions I and II upon analytical HPLC showed oligosaccharides (DP, 2–6) with trisaccharide in abundance followed by tetra- and pentasaccharides. Interestingly, the oligomeric product profile of pectinase resembled that of a chitosanase from *Bacillus* sp. No.7-M.[14] Presence of GlcNAc in Fraction II and its absence in Fraction I (re-*N*-acetylated fraction) was indicative of action of pectinase isozyme on –GlcN–GlcNAc bond. Hexosaminidase treatment did not show any change in the HPLC pattern of Fraction II, indicating the presence of GlcNAc at the reducing end and/or at intermediate positions of the oligomers, thus suggesting the action of pectinase isozyme also on –GlcNAc–GlcN linkage in chitosan.

To elucidate the structure of individual oligosaccharides in Fraction I, they were fractionated into four Peaks (I–IV, corresponding to di-, tri-, tetra- and pentamers) by preparative HPLC in yields of 8.8, 19.0, 8.0 and 12 mg, respectively (combined yield, 4.8%), starting from 1 g of chitosan. Analytical HPLC on Lichrosorb-NH_2 column revealed Peaks I, II and III to be homogeneous, whereas Peak IV contained, in addition, considerable amount of Peak III, probably due to post column mixing during the later stages of elution and no attempts were made to purify this.

Since the oligosaccharides were re-*N*-acetylated prior to charcoal–Celite chromatography, CD spectroscopy was used to confirm the uniformity of *N*-acetylation, which showed a peak, corresponding to $n\rightarrow\pi^*$ transition at 211 nm (Fig. 1). This peak, indicating uniformly *N*-acetylated GlcN residues, is independent of α- and β-anomeric equilibrium, the chain length beyond two residues, ionic strength and pH. A fully deacetylated chitosan exhibits only one CD band, centred near 185 nm, which disappears in the acidic media, when only $-NH_3^+$ form is present, instead it shows a single peak centred around 211 nm.[15] Peaks II and III corresponding to chitotriose and chitotetraose, however showed additional peaks at 209 and 219 nm for reasons yet unknown.

FT-IR showed characteristic amide I and amide II bands at 1656 and 1564 cm^{-1} for di- and trimers (Fig. 2).[16,17] Tetramer (Peak III), however, showed amide I band split into well-resolved peaks at 1656 and 1621 cm^{-1}, may be due to inter-chain interactions. In the case of α-chitin the splitting of amide I band is still a highly contentious issue, wherein the band near

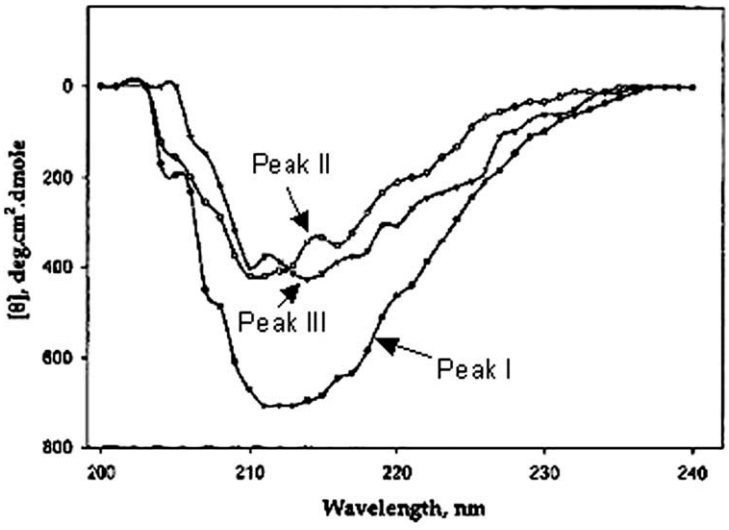

Figure 1. CD spectra of purified oligosaccharides (Peaks I–III of Fraction I corresponding to di-, tri- and tetramer, respectively).

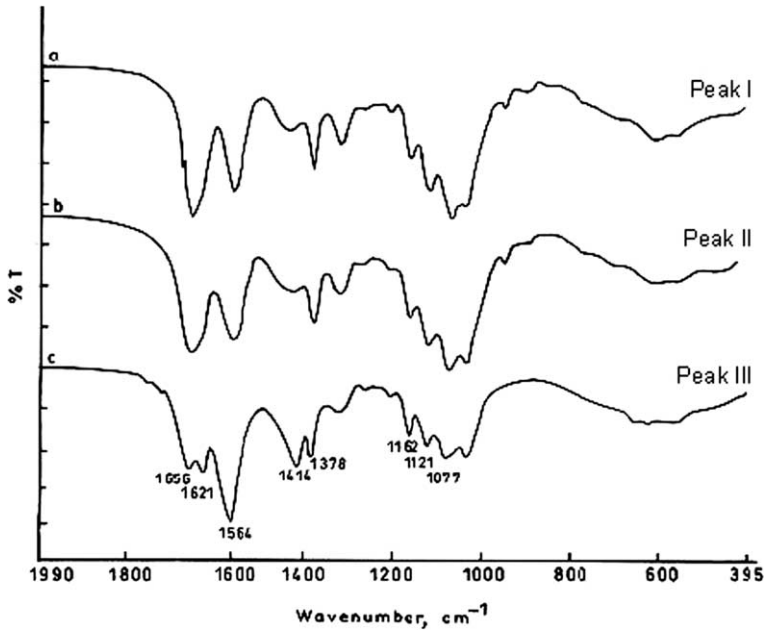

Figure 2. FTIR spectra of purified oligosaccharides (see Fig. 1).

1660 cm^{-1} is assigned to –C═O groups hydrogen bonded only to $–NH_3^+$ groups, whilst that near 1623 cm^{-1} is ascribed to –C═O groups bonded with a bifurcated hydrogen both in $–NH_3^+$ and $–CH_2OH$ groups.[18] It is likely that the oligomeric chain in Peak III may assume a structure similar to that of α-chitin, although its chain length is considerably smaller. The DA, deduced from the ratio of absorbance at 1655 cm^{-1}/3450 cm^{-1} was 1.0, indicating that all the GlcN residues were *N*-acetylated.

To elucidate their structures, the purified re-*N*-acetylated oligosaccharides were subjected to MALDI-TOF-MS (Fig. 3), the later being a soft ionization technique, is suitable for the analysis of biological molecules. Molecular ions at *m/z* 447, 650 and 853 could be assigned to $[M+Na]^+$ forms of di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and tetra-*N*-acetylchitotetraose, respectively. Nevertheless, the data revealed cross contamination of these oligomers. This was eliminated by repeated HPLC purification of the oligomers. FAB-MS of the latter (Fig. 4) showed sodiated $[M+Na]^+$ molecular ions at *m/z* 650 and 853, confirming trimeric and tetrameric structures, respectively. Since these oligosaccharides were re-*N*-acetylated after isolation, the original oligosaccharides in the chitosan hydrolyzate therefore, were chitobiose, chitotriose and chitotetraose.

For antibacterial assay, the oligomeric–monomeric samples obtained after Sephadex G15 chromatography were used as they were in the native form. Although it is reported that desalting is not preferred owing to co-elution of monomers and salts,[12] the objective was to obtain higher oligomers in the native form, as charcoal–Celite chromatographic separation of oligomers required them in the acetylated form, which in turn may affect their bio-functionality.

The COs showed antibacterial activity towards all the indicator bacteria screened (i.e., *B. cereus* F4810, *E. coli* MTCC 118, *Listeria monocytogenes* Scott A, *Yersinia enterocolitica* MTCC 859, *Staphylococcus aureus* FRI 722 and *Bacillus licheniformis* CFR 1621) and their growth inhibitory effect was better than native chitosan. However, for detailed study, *B. cereus* and *E. coli*, one each from Gram-positive and Gram-negative bacteria, respectively, were selected. The inhibitory effect of the COs was more towards *B. cereus* as against *E. coli*. From Table 1, it was clear that increase in the concentration of chitosan or chitooligomeric mixture did not show linear inhibitory effect and 100% inhibition was not obtained at any concentration, on either side there was a decrease in the growth inhibitory effect, as also observed for chitosan.[19] This could be due to the fact that at lower concentrations, entry of chitooligosaccharides into the bacterial cells facilitates their action as activator of various physiological functions, whereas at higher concentrations, in addition their binding to microbial cell surface provides a net positive charge, thus segregating the individual cells and hence, without any inhibitory activity. When the individual monomers and oligomers (separated on Biogel P2) were used as antibacterial agents, surprisingly, GlcN showed some inhibition whereas GlcNAc did not. Increase in the oligomer DP resulted in increased growth inhibitory activity (Table 2). From these observations, it may be concluded that an unsubstituted amino group (in the form of $–NH_3^+$) is vital for the antibacterial activity, which is

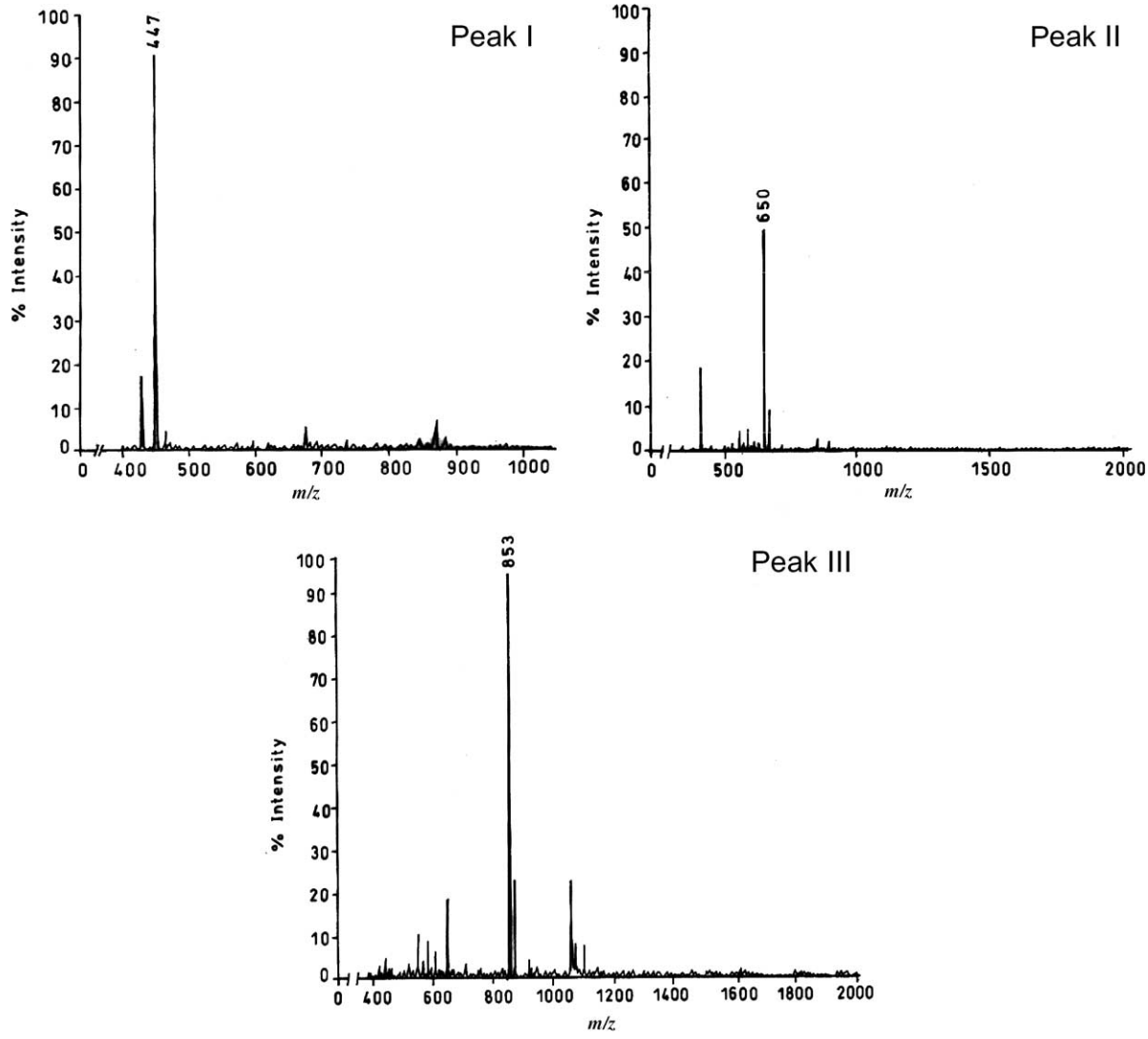

Figure 3. MALDI-TOF-MS of Peaks I–III (see Fig. 1).

also dependent on the molecular size (DP) of the oligomeric species.

The exact mechanism of antibacterial activity of chitosan still needs to be elucidated,[20] whereas so far there are no reports on the antibacterial activity of COs of DP 2–6 and GlcN/GlcNAc. In the present study, SEM clearly indicated pore formation and permeabilization of the cell surface, leading to lyses of *B. cereus* (Fig. 5A and B), whereas deposition of COs-monomers and blockage of the nutrient flow were the reasons for growth inhibition of *E. coli* (Fig. 5C and D).

In *B. cereus*, the cell wall is made up of proteoglycan, which contains in addition to other components, negatively charged *N*-acetyl muramic acid and D-amino acids including D-aspartic/glutamic acid, to which $-NH_3^+$ of COs-monomers bind, distorting cell wall structure, exposing cell-membrane to osmotic shock and ultimately cell death. In *E. coli*, the negatively charged oligosaccharide chains (O-antigen) of lipopolysaccharides on the outer membrane bind to $-NH_3^+$ of COs-monomers by ionic interactions and further due to their aggregation by hydrogen bond network and blockage of nutrient flow, cell lyses takes place. SEM showed irregularities on the cell surface (Fig. 5D), in support of the above hypothesis.

In conclusion, an isozyme of *A. niger* pectinase with polygalacturonase activity caused depolymerization of chitosan resulting in the formation of ~7% chitooligomeric mixture, the DP of which was 2–6 with trimer in abundance. Their antibacterial activity was different towards Gram-positive and Gram-negative bacteria. The results showed value addition to an otherwise waste material found in the offals of marine food processing industry and use of an inexpensive, easily available enzyme (i.e., pectinase), for better biofunctionalities.

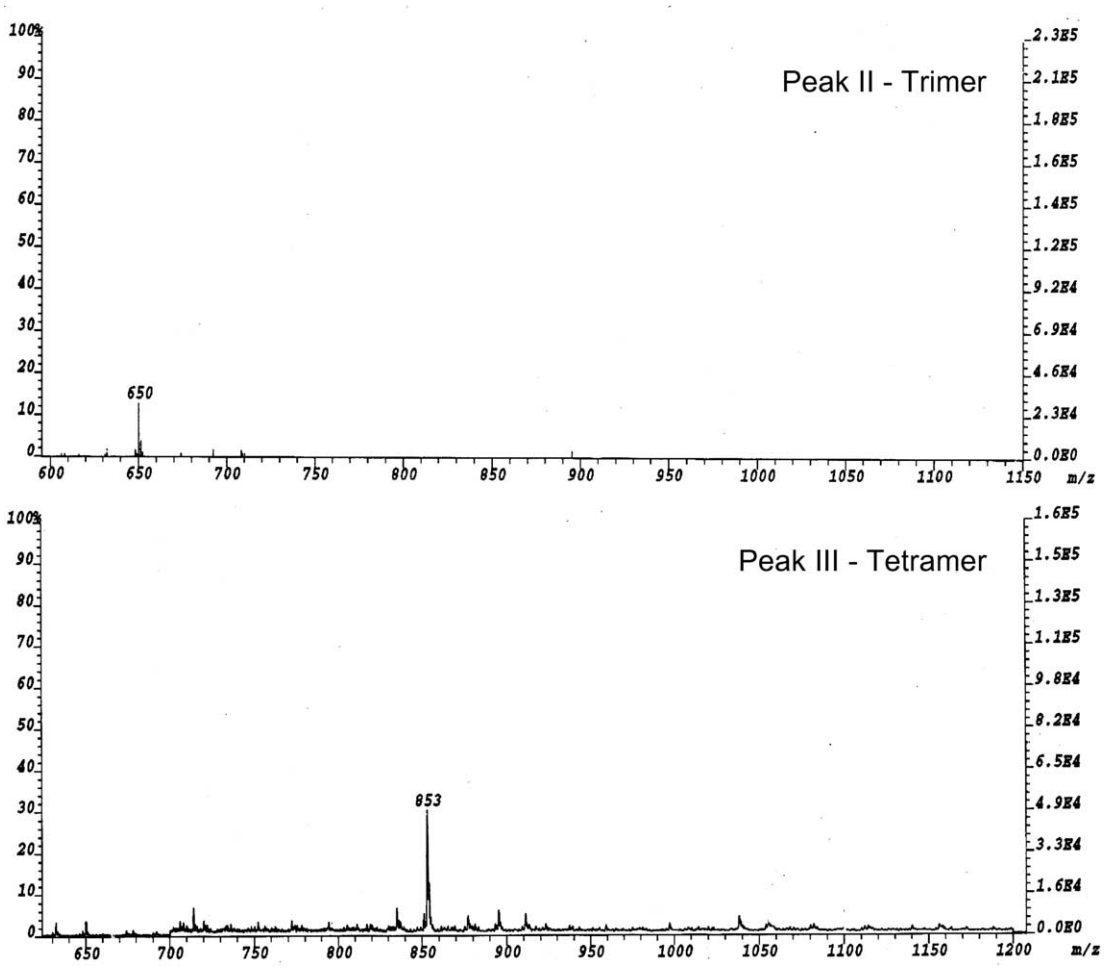

Figure 4. FAB-MS of Peaks II and III (see Fig. 1).

Table 1. Inhibitory effect of chitosan and COs-monomeric mixture towards *B. cereus* and *E. coli*

Compound	Concentration (mg %)	Indicator bacteria (10^6 CFU)	
		B. cereus F4810	*E. coli* D21
		Inhibition (%)	
Native chitosan	0.1	0	0
	0.2	0	0
	0.3	20	0
	0.5	10	0
COs-monomers	0.1	65	33
	0.2	79	74
	0.3	94	52
	0.4	82	34
	0.5	60	18

Table 2. Inhibitory effect of individual chitooligomers and monomers towards *B. cereus* and *E. coli*

Compound	Concentration (mg %)	Indicator bacteria (10^6 CFU/tube)	
		B. cereus F4810	*E. coli* D21
		Inhibition (%)	
GlcN	0.05	10	5
	0.10	14	8
GlcNAc	0.05	—	—
	0.10	—	—
Dimer	0.05	15	8
	0.10	19	12
Trimer	0.05	20	14
	0.10	26	17
Tetramer	0.05	28	20
	0.10	32	25
Pentamer	0.05	28	19
	0.10	30	23
Hexamer	0.05	40	32
	0.10	48	38

1. Materials and methods

1.1. Materials

Pectinase from *A. niger* was from Sigma Chemical Co., St. Louis, MO, USA (EC 3.2.1.15, Activity, 5 Units per mg protein). Chitin was obtained from CFTRI Regional Centre, Mangalore, India. All other chemicals used were of highest purity available. Isolation of pectinase isozyme with both pectinase and chitosanase activities

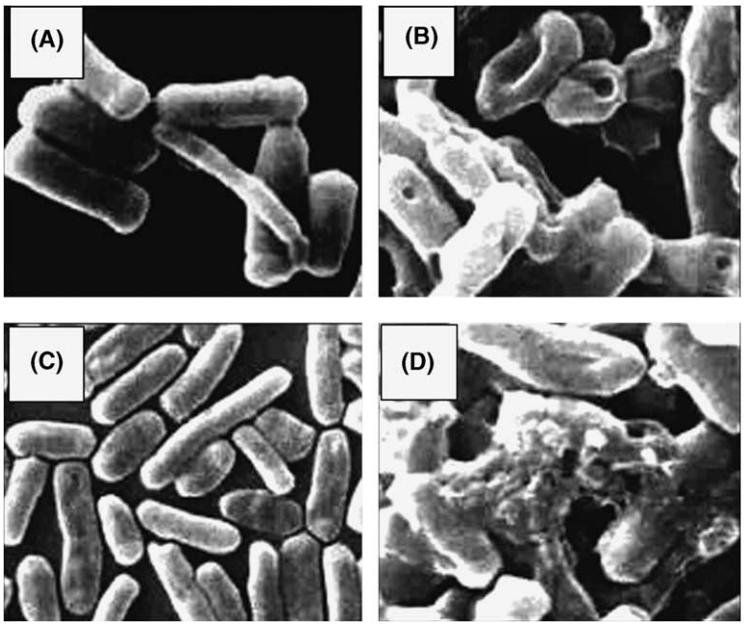

Figure 5. SEM of *B. cereus* and *E. coli*—before (A and C) and after (B and D) treatment with COs-monomeric mixture.

and chitosan preparation were done as described before.[10]

1.2. Chitosanolysis

Purified pectinase isozyme was added to 200 mL of 1.0% (w/v) chitosan solution, pH 3.0 at an enzyme–chitosan ratio of 0.003 (w/w), and incubated at 37 °C for 6 h with constant stirring. After hydrolysis, the enzyme was inactivated at 100 °C for 5 min, pH was adjusted to 7.0 with 2 M NaOH followed by centrifugation (1000*g*, 10 min). The supernatant containing COs, monomers as well as the heat-denatured enzyme, was divided into two halves, of which one was passed through Sephadex G15 (0.9×60 cm) and the other, through charcoal–Celite column.

1.3. Charcoal–Celite chromatography

On charcoal–Celite column (1.6×35 cm), the unadsorbed saccharides (GlcN and GlcN-rich oligomers) and heat-denatured enzyme were eluted with distilled water (Fraction IA), and the adsorbed saccharides (GlcNAc and GlcNAc-rich oligomers) were recovered using 60% ethanol (Fraction II). Fraction IA was re-*N*-acetylated[21] with acetic anhydride under alkaline condition and purified by rechromatography (Fraction I). The eluted oligosaccharide fractions were pooled, evaporated under reduced pressure and stored at 4 °C.

1.4. HPLC

The oligomeric fractions were dissolved in deionized water and loaded onto Lichrosorb-NH_2 column (4.0×250 cm) connected to a Shimadzu LC-3A HPLC system (Shimadzu Corp., Kyoto, Japan). Elution was performed with 70:30 acetonitrile–water (v/v) as the mobile phase at room temperature at a flow rate of 0.8 mL/min and using a RI detector. Fractions were assayed for reducing sugar[22] and quantitatively recovered by preparative HPLC on Bondapak-NH_2 column using acetonitrile–water (70:30, flow rate—5.0 mL/min), and their purity was reconfirmed by analytical HPLC.

1.5. Structural characterization of COs

IR, CD and MALDI-TOF-MS analyses were done as described elsewhere.[10,11] FAB-MS was performed with a VG Auto Spec M spectrometer (Micromass UK limited, Manchester, UK). Oligosaccharides were dissolved in aqueous acetic acid (5 μg in 1.0 μL) and loaded with a drop of glycerol (~2.0 μL) over the probe target. The atom gun was operated at 8 kV with xenon as the bombarding gas. Positive spectra were recorded in the scanned mass range of *m*/*z* 200–2000 amu.

1.6. Antibacterial assay

The indicator bacterial cultures (*B. cereus* F4810, *E. coli* MTCC 118, *L. monocytogenes* Scott A, *Y. enterocolitica* MTCC 859, *S. aureus* FRI 722 and *B. licheniformis* CFR 1621) were obtained from the culture collection maintained in the Food Microbiology Department of this Institute. The cultures were maintained at 6 °C on brain heart infusion (BHI) agar slants and sub-cultured at 15-day intervals.[23] Prior to use, the culture was successively

propagated twice in BHI broth at 37 °C. Cell suspension of the culture, individually, were prepared from 20 h old BHI culture broth with appropriate dilution in 0.85% saline, giving individual counts of 10^2–10^6 colony forming units (CFU) mL^{-1}.

The antibacterial activity of chitooligosaccharides was studied in nutrient broth following the method of Chen et al.[3] Briefly, to 10 mL aliquots of nutrient broth (HiMedia, Mumbai, India) supplemented with 0.5% dextrose, a cell suspension of specific bacterial strain was added giving individual cell number of 10^2–10^6 CFU/tube contents. To these tubes were added individually 0.1–0.5 mg % (w/v) of chitooligomers, incubated for ~20 h at 37 °C and tested for turbidity at 660 nm followed by pour plating with BHI agar (HiMedia, Mumbai, India), incubation for 24 h at 37 °C and observation for bacterial colonies. As positive controls, 0.1–0.5 mg % chitosan in 1% acetic acid, 1% acetic acid and 100 μg pectinase isozyme were used. The contents of tubes were mixed well and the inhibition rate (%) was calculated by $(N_1 - N_2/N_1) \times 100$, where N_1 and N_2 are the number of colonies on the plates before and after inhibition, respectively.[24]

After 20 h incubation of the test cultures with COs, 0.5 mL aliquots were transferred to micro-centrifuge tubes followed by centrifugation. The pellets obtained were treated with phosphate buffer (pH 7.0, 0.3 M), fixed with glutaraldehyde (1%) for 1 h at 4 °C and further treated with 10–96% alcohol in a sequential manner. The dried samples were spread on a double-sided conducting adhesive tape pasted onto a metallic stub, subjected to gold covering (~100 Å) and observed under SEM (LEO 435 VP, LEO Electron microscopy Ltd., Cambridge, UK) at 20 kV.

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