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Carboxymethyl chitin and chitosan derivatives: Synthesis, characterization and antibacterial activity

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ABSTRACT

Water-soluble carboxymethyl chitin (CMCT) **1a**–**b**, and chitosan (CMCS) **2a**–**b** derivatives were synthesized and evaluated for antibacterial activity. The synthesized compounds were characterized by Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD). Thermal properties of the synthesized compounds were studied by thermogravimetric analysis (TGA) and their surface morphologies examined by scanning electron microscopy (SEM). Antibacterial activity of the chitosan (CS) **2** and the synthesized derivatives were tested against both gram-negative (Shigella flexneri, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Vibrio paraheamolyticus) and gram-positive (Staphylococcus aureus, Bacillus subtilis, Bacillus cereus) bacterial strains. CS **2** shows antimicrobial activity against all tested pathogenic bacteria, whereas CMCS **2a** shows antimicrobial activity against Shigella flexneri, Bacillus cereus, and Bacillus subtilis. CMCS **2b** shows antimicrobial activity against Pseudomonas aeruginosa, Klebsiella pneumoniae and Staphylococcus aureus. CMCT **1a** only shows antimicrobial activity against Bacillus subtilis and **1b** shows antimicrobial activity against Vibrio paraheamolyticus and Bacillus cereus.

1. Introduction

In recent years, there is much research underway globally which is concerned with producing new materials in a sustainable manner, including those derived from natural resources such as sustainable biopolymers which are biodegradable, are environmentally-friendly and are renewable with lower energy consumption. Chitin (CT) **1** and its *N*-deacetylated derivative chitosan (CS) **2** at the C-2 are such promising natural biopolymers (Fig. 1) which have been widely used in pharmaceutical and biomedical applications (Silva et al., 2017). The major industrial source of biomass for the large-scale production of CT and CS are mainly from the shell waste of prawn shrimp and crab (Fu et al., 2013; Ramamoorthy et al., 2018). CT **1** is structurally identical to cellulose, but it has acetamide groups (–$NHCOCH_3$) at the C–2 position. On the other hand, CS **2** is a deacetylated derivative of CT **1**, which is a cationic amino-polysaccharide with a linear chain consisting of β-(1, 4)-linked 2-amino-2-deoxy-β-D-glucopyranose moieties and completely and also partially-deacetylated 2-acetamino-2-deoxy-β-D-glucopyranoses (Fig. 1) (Vasiliu et al., 2009).

CT and CS are bio-renewable, environmentally friendly, show no toxic effects on human cells and as a result these properties have made them scientifically attractive to chemists (Kumar et al., 2004). The biological activities of chitin and chitosan derivatives have made them promising biopolymeric agents for drug delivery applications which can control the specific release of the drug over a long period of time (Prabaharan, 2008). Notably, CT and CS are nontoxic biopolymers having

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antimicrobial activity, wound-healing properties, and can decrease the cholesterol levels in humans (Denkbaş & Ottenbrite, 2006; Harish Prashanth & Tharanathan, 2007; Islam et al., 2011b; Islam et al., 2011c; Roller & Covill, 1999). Due to the poor solubility of chitin and chitosan in water or in organic solvents, however, their utilization for specific applications in industry and medicine are very limited. The chemical modification of the amino or hydroxyl groups in the side chains of CT and CS afford new derivatives for promising biological activities and physiochemical properties. By utilizing the acetamido, amino and hydroxyl groups of chitin and chitosan as modification sites, many derivatives have been obtained via substitution, chain modification, and depolymerization (Harish Prashanth & Tharanathan, 2007). These modified chitin and chitosan derivatives may enhance their solubility in water and organic solvents Scheme 1, 2, 3.

CS **2** has three types of reactive functional groups, namely, a primary amino group at C–2 and two hydroxy (primary and secondary) groups at the C–3 and C–6 positions which allow for chemical modification (Fig. 1) (Aranaz et al., 2010). The chemical modification of chitin and chitosan to produce derivatives such as *N,N,N*-trimethylchitosan, *N, O*-acetylchitosan, *N*-acetylated-chitosan and *N*-carboxymethylchitosan reported by many researchers, have been synthesized to improve the solubility of chitosan derivatives (Xu S. et al., 2020) Among other modifications or derivatives, chitosan gel formation *via* a chitosan-epichlorohydrin adduct has been shown to have significant cell adhesion properties; cross-linking chitosan with glutaraldehyde can improve the chemical and mechanical resistance of chitosan; *N*-[(2-*hydroxy*-3-*trimethylammonium*)*propyl*]chitosan chloride has been synthesized to produce good antibacterial activity (Fangkangwanwong et al., 2006; Kim & Je, 2015; Kumirska et al., 2011; Lim & Hudson, 2004; Lu et al., 2004; Muzzarelli et al., 1988; Muzzarelli et al., 1994; Sashiwa et al., 2002; Vieira & Beppu, 2005). Jayakumar et al., have reported that alkyl, or carboxymethyl functional groups introduced at the side chains of chitin and chitosan can significantly increase their solubility as well as their potential application in different fields without affecting their original structure (Jayakumar et al., 2010).

The shrimp industry is one of Bangladesh's leading exports and it contributes to a significant part of it's economy. It is the second largest export industry after garment production. However, the resulting rapid expansion of shrimp farming has created a series of negative environmental impacts, including ecological imbalance, environmental pollution and some cases of disease outbreaks. Thus, shrimp farming in Bangladesh is facing management-related difficulties which lead to greater concerns about its sustainability. During the processing of shrimp the meat is mostly separated and taken and used, while the shell and head portions generate a large amount of waste, presenting financial and environmental challenges to the waste management practices for the shrimp processors. Instead of dumping the shrimp waste into landfills or into the sea, the waste could be chemically converted to chitin and its derivative chitosan. Therefore, for both economic and environmental reasons it is necessary and desirable to develop a low-cost suitable technology to convert the waste biomaterials into valuable products such as chitin and its chitosan derivatives. In our laboratory, we are now focusing on the synthesis of such valuable derivatives of chitin and chitosan, determining their biological properties and developing composites for water purification and other potential applications (M. Islam, Masum, Rahman, & Shaikh, 2011d; M.M. Islam et al., 2011a; M.M. Islam, Masum, & Mahbub, 2011b; M.M. Islam, Masum, R, & Haque, 2011c; Kabiraz et al., 2016; Siraj et al., 2012).

The main objective reported in the present study was the preparation of CS **2** via CT **1** from indigenous sources, and to form their water-soluble derivatives such as carboxymethyl chitin (CMCT) and carboxymethyl chitosan (CMCS) as well as study their physicochemical and antibacterial activity.

2. Experimental

2.1. Materials and methods

Prawn scientific name is *Penaeus monodon* (in Bengali: *Bagda*), head peels were collected from the Satkhira shrimp firm, located in the southern part of Bangladesh. Prawn head peels were scraped free of loose tissue, washed with cold water and dried in the sunlight (Temperature 30–33 °C) in the open for 2 days (48 h). All reagent grade chemicals such as HCl (37%, Merck), NaOH (98%, Daejung), Monochloro acetic acid (>99, Merck), Isopropyl alcohol (99.7%, Active Fine Chemicals), Methanol (99.8%, Active Fine Chemicals) were purchased from local market and used without further purification.

2.2. Preparation of chitin 1 (CT)

Dried prawn head peel (500.0 g) was mixed with 5% hydrochloric acid (100 mL) in an Erlenmeyer flask and the mixture was stirred for 24 h at room temperature. The reaction mixture was then filtered and the residual head peel was washed with distilled water and dried in the sun. The de-mineralized head peel was mixed with aqueous 5% sodium hydroxide solution (50 mL) and stirred at 70 °C for 3 h. The deproteinized peel was washed with distilled water and dried in the sun (30–33°C) for 2 days (48 h). The dried product is chitin **1** (115.0 g). IR (KBr), ν_{max} (cm^{-1}): 3430 (O–H stretch), 3262 (N–H stretch), 2918 (C–H stretch), 1738 (C=O bend, amide I), 1655 (C=O bend, amide I), 1619 (C=O bend, amide I), 1569 (N–H bend, amide II), 1416 (C–H stretch), 1376 (C–H stretch), 1312 (C–N stretch, amide III), 1115 (bridge O stretch) and 1063, 1009 (C–O stretch).

2.3. Preparation of chitosan 2 (CS)

Chitin **1** (115.0 g) was mixed with aqueous 70% sodium hydroxide (50 mL) and stirred at 80 °C for 4 h. The mixture was then cooled and filtered. The residue was washed with distilled water and dried in sun. The dried product is chitosan **2** (90.0 g). IR (KBr), ν_{max} (cm^{-1}): 3359

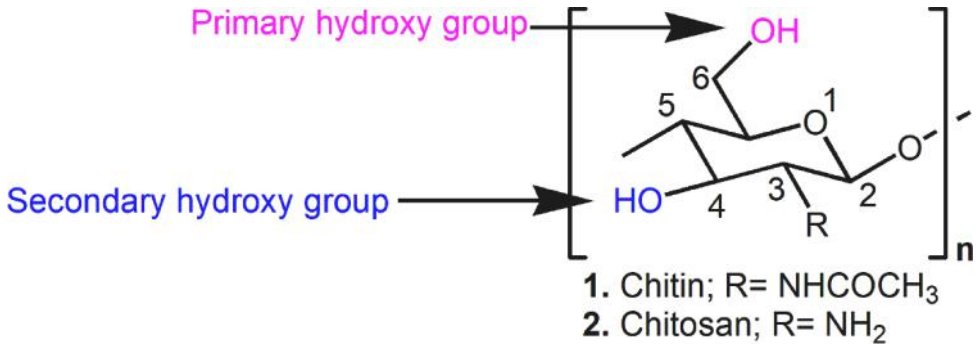
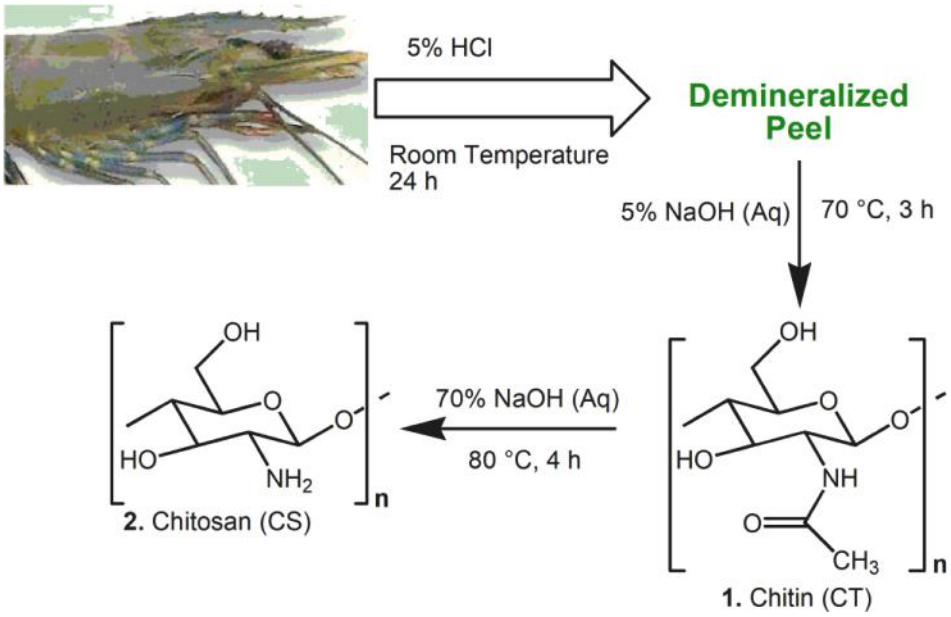
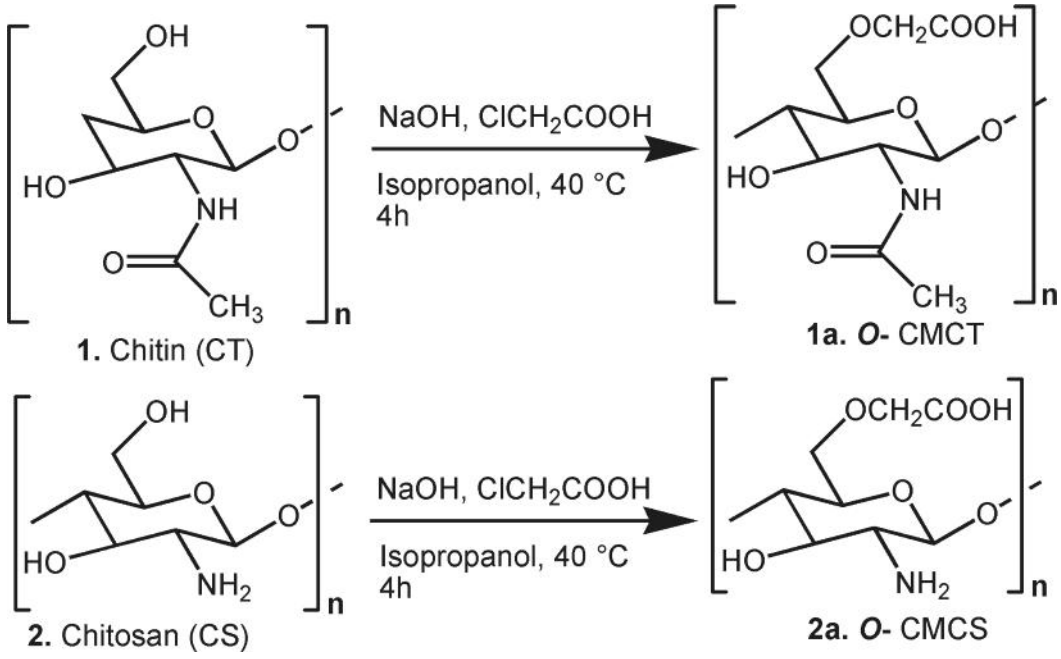


Fig. 1. Basic structural units of chitin **1** and chitosan **2**.



Scheme 1. Preparation of chitosan **2** via chitin **1**.



Scheme 2. Preparation of carboxymethyl derivatives of chitin **1a**–**b**.

(O–H stretch), 2879 (C–H stretch), 1578 (N–H bend), 1420 (C–H stretch), 1375 (C–H stretch), 1312 (C–N stretch, amide III), 1146 (bridge O stretch), and1056, 1030 (C–O stretch).

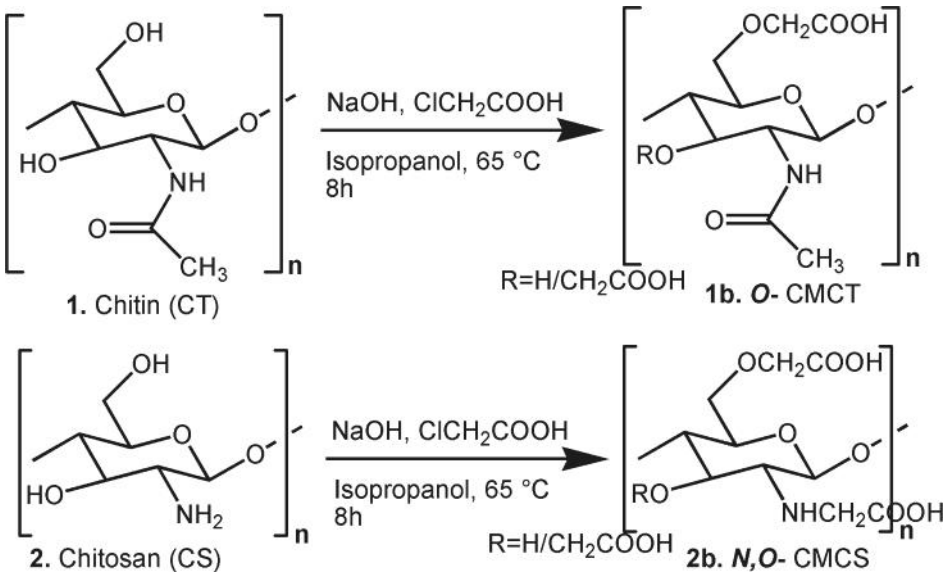
2.4. Preparation of O-carboxymethyl chitin 1a (O—CMCT)

Chitin **1** (5.0 g) was mixed thoroughly with aqueous 40% w/v NaOH (40 mL) and kept overnight (12 h) at -20 °C. Isopropanol (100 mL) was added into the thawed CT **1** slurry and monochloroacetic acid (28.8 g) in 100 mL IPA was added dropwise to the reaction mixture under constant stirring using a magnetic stirrer and then the reaction mixture was stirred at 40 °C for a further 6 h. The reaction mixture was cooled to room temperature (25 °C) and neutralized with 10% hydrochloric acid. The mixture was filtered, and the solid was collected and washed using 80% (v/v) methanol/water.

The product was dried in an oven at 50 °C for 8 h to give 5.5 g of *O*-carboxymethyl chitin **1a** (*O*—CMCT). IR (KBr), ν_{max} (cm^{-1}): 3357 (O–H stretch), 3282 (N–H stretch), 2895 (C–H stretch), 1736 (C=O bend), 1571 (C=O, COOH, anti-symmetric stretching), 1470 (N–H bend), 1416 (C=O, COOH, symmetric stretching), 1360 (C–H stretch), 1311 (C–N stretch), 1135, 1119 (bridge O stretch), and 1055, 923 (C–O stretch).

2.5. Preparation of O-carboxymethyl chitosan 2a (O—CMCS)

Chitosan **2** (5.0 g) was mixed thoroughly with aqueous 40% NaOH (w/w, 40 mL) and kept overnight (12 h) at -20 °C. Isopropanol (100 mL) was added into the thawed CS **2** slurry and monochloroacetic acid (28.8 g) in 100 mL IPA was added dropwise to the reaction mixture with under



Scheme 3. Preparation of carboxymethyl derivatives of chitosan **2a**–**b**.

stirring with a magnetic stirrer and the reaction mixture was stirred at 40 °C for a further 6 h . The the reaction mixture was then neutralized with 10% hydrochloric acid. The mixture was filtered, and the solid was collected, washed using 80% (v/v) methanol/water and the product was dried in the oven at 50 °C for 8 h to give 5.2 g of dried *O*-carboxymethyl chitosan **2a**. IR: ν_{max} (KBr)/cm^{-1}: 3257(O–H, N–H stretch), 2865(C–H stretch), 1726 (C=O bend), 1579 (C=O, COOH, anti-symmetric stretching), 1411 (N–H bend), 1310 (C–N stretch), 1246 (bridge O stretch) and 1125, 1056 (C–O stretch).

2.6. Preparation of O–carboxymethyl chitin 1b (O—CMCT)

Chitin **1** (2.0 g) was added to 50 mL isopropyl alcohol in a round-bottom flask (500 mL) and the reaction mixture was stirred using a magnetic stirrer, at room temperature (25 °C) for 2 h. Aqueous 60% NaOH solution (w/v, 80 mL) was then added into the reaction mixture which was then heated at reflux at 65 °C for 8 h. Monochloroacetic acid solution in 100 mL IPA was then added in five equal parts over a period of 10 min. The reaction mixture was heated with stirring, at 65 °C for a further 8 h. The reaction mixture was then neutralized using hydrochloric acid (4.0 M). After removal of the insoluble residue by filtration, the resulting carboxymethyl chitin **1b** was precipitated by adding methanol. The product was filtered, washed several times with a mixture of methanol/water (v/v, 1:1) and dried in the oven at 50 °C for 8 h to give *O*—CMCT **1b** (2.2 g). IR: ν_{max} (KBr)/cm^{-1}: 3372 (O–H, N–H stretch), 2865(C–H stretch), 1724 (C=O stretch), 1630 (C=O, COOH, anti-symmetric stretching), 1377 (CH_2 stretch), 1228 (C–N stretch), 1054 (C–O stretch) and 899 (C–C stretch).

2.7. Preparation of N,O–carboxymethyl chitosan 2b (N,O—CMCS)

Chitosan **2** (2.0 g) was added to 50 mL isopropyl alcohol in a round-bottom flask (500 mL) and the reaction mixture was stirred at room temperature for 2 h, using a magnetic stirrer,. Aqueous 60% w/v NaOH (80 mL) was then added into the reaction mixture which was then heated at 65 °C for 8 h. Aqueous monochloroacetic acid solution in 100 mL IPA was then added in five equal parts over a period of 10 min. The reaction mixture was heated with stirring, at 65 °C for a further 8 h. The reaction mixture was then neutralized using hydrochloric acid (4.0 M). After removal of the insoluble residue by filtration, the resulting carboxymethyl chitin **2b** was precipitated by adding methanol. The product was filtered, washed several times with a mixture of methanol/water (v/v, 1:1) and dried in an oven at 50 °C for 8 h to give 2.4 g of *N,O*—CMCS **2b**. IR: ν_{max} (KBr)/cm^{-1}: 3410 (O–H, N–H stretch), 1737(C=O, COOH stretch), 1436, 1375 (CH_2stretch), 1417 (COO^-stretch), 1267, 1216 (C–N stretch), 1133 (bridge O stretch), and 1066, 1033 (C–O stretch), 899 (C–C stretch).

2.8. Characterization of chitin 1, chitosan 2 and their respective derivatives, 1a–b and 2a–b

2.8.1. Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) was performed using a Perkin Elmer Universal ATR spectrophotometer (UATR-FT-IR, USA) equipped with a ZnSe crystal for the FT-IR spectroscopy. Transmittance was measured as the function of the wave number between 4000 and 650 cm^{-1} with their solution of 4 cm^{-1} and the number of scans equal to 12. The degree of deacetylation (DD) of chitosan **2** was calculated from Eq. (1) (Kaya et al., 2015; Kaya & Baran, 2015):

$$DD\% = 100 - [(A_{1655} / A_{3450}) * 100 / 1.33] \quad (1)$$

where A_{1655} is the amide-I C=O absorbance at 1655 cm^{-1} which corresponds to the *N*-acetyl group content in the sample. A_{3450} is the absorbance at 3450 cm^{-1} of the hydroxyl band and is taken as an internal standard for correcting for film thickness. The factor 1.33 denotes the value of the ratio of A_{1655}/A_{3450} for fully *N*-acetylated chitosan.

2.8.2. Acid-base titration

A sample of CS **2** (0.10 g) was dissolved in 25 mL hydrochloric acid (0.10 M) and then titrated with a standard aqueous 0.10 M NaOH solution. The titrant was added dropwise until the pH reached 11.5. The pH values of the mixture were recorded and a curve with two inflection points was obtained. The average degree of deacetylation (DD) of chitosan samples were determined by using Eq. (2) (Abdel-Rahman et al., 2015; Kaya, Baran et al., 2014), where c_{NaOH} is the concentration of the NaOH solution; (V_2−V_1) is the difference between the two inflection points; 161 is the molecular mass unit of chitosan; m is the mass of chitosan sample:

$$DD\,(\%) = C_{NaOH} \times (V_2 - V_1) \times 161/m \quad (2)$$

2.8.3. Thermogravimetric analysis (TGA)

Approximately 15–20 mg of each sample was weighed into an aluminum pan and subjected to thermogravimetric analysis (TGA model

6300 Japan). Samples were heated in a nitrogen atmosphere (50 mL/min) from room temperature to 600 °C at a rate of 20 °C/min. A representative TGA result showing weight change data for chitin and chitosan samples plotted against temperature can be seen in Fig. 3.

2.8.4. Determination of degree of substitution for 1a–b and 2a–b

The degree of substitution (DS) of **1a**–**b** and **2a**–**b** was determined by using a titrimetric method as reported (Ge & Luo, 2005). In brief, 0.10 g samples of each derivative (**1a**–**b** and **2a**–**b**) were dissolved in distilled water (20 mL). The pH of the solutions was adjusted to pH < 2 by adding standard 0.10 M hydrochloric acid. The solutions were then titrated with 0.050 M standard aqueous NaOH, the pH values being simultaneously recorded under non-stop stirring conditions. The degree of substitution (DS) of **1a** and **1b** were determined using Eq. (3), and the DS of **2a** and **2b** were determined using Eq. (4):

$$\mathrm{DS} = 203A/(m - -58A) \tag{3}$$

$$\mathrm{DS} = 161A/(m - -58A) \tag{4}$$

where, $A = V_{\mathrm{NaOH}} X C_{\mathrm{NaOH}}$, V_{NaOH} is the difference in the volumes (L) of NaOH solution recorded between the two inflection points, C_{NaOH} is the molarity of the aqueous NaOH (0.050 mol/L) and m is the mass of samples (**1a, 1b** and **2a, 2b**) used; 203, 161 and 58 are the molecular weights of N-acetylglucosamine (CT skeleton unit), glucosamine (CS skeleton unit) and a carboxymethyl group, respectively.

2.8.5. Scanning electron microscopy (SEM)

SEMs of all samples were taken using a field emission scanning electron microscope (JSM-7610F, JAPAN); under the following conditions: 10 kV; working distance of 3.4–7.3 mm; display mode: secondary electrons; high vacuum and room temperature. The pictures were made with 55–90,000-X magnification.

2.8.6. X-ray diffraction (XRD)

The X-ray diffraction patterns of water-soluble carboxymethyl chitin (CMCT) **1a**–**b**, and carboxymethyl chitosan (CMCS) **2a**–**b** were collected on a EMMA GBC, Australia, X-ray Diffractometer. The scan was completed at room temperature from 10 °C to 60 °C (2θ) in 0.02 °C steps, with a counting time of 4 °C per minute.

2.8.7. Solubility test in distilled water

The solubility of synthesized derivatives in water were determined by naked eye observation at 25 °C. At first, 20 mg amounts of the synthesized derivatives were added in 100 mL distilled water then mixed using a vortex mixer (Model: VM-1000, Brand: Digisystem Origin: Taiwan). When a clear solution was observed, additional 10 mg amounts of the product was added to the clear solution until precipitation was observed. This total amounts of product added was considered as the solubility limit level.

2.9. Antimicrobial activity determination of chitosan 2, 1a–b and 2a–b

2.9.1. Preparation of solutions for antimicrobial testing

For the preparation of a 2.4% (w/v) CS **2** solution, CS **2** was dispersed in aqueous 1.0% (v/v) acetic acid. The other prepared derivatives **1a, 1b, 2a** and **2b** were dispersed in distilled water. After stirring overnight, all solutions were autoclaved at 120 °C for 15 min (the thermostability of the compounds under these conditions had been previously checked).

2.9.2. Microorganisms

The antibacterial activities of CS **2** and the other prepared derivatives were tested against eight bacterial strains. The five gram-negative bacteria used were *Shigella flexneri* ATCC 12,022, *Enterococcus faecalis* ATCC 29,212, *Pseudomonas aeruginosa* ATCC 27,853, *Klebsiella pneumoniae* ATCC 13,883, *Vibrio paraheamolyticus* ATCC 17,802, and gram-positive three were *Staphylococcus aureus* ATCC 9144, *Bacillus subtilis* ATCC 11,774, *Bacillus cereus* ATCC 10,876.

2.9.3. Determination of antibacterial activity

The bacterial inoculums were prepared using the Clinical and Laboratory Standards Institute (CLSI) guidelines. The bacterial cultures were emulsified in normal saline and their turbidities were matched with 0.5 McFarland turbidity standards. The agar cup method was followed to investigate the antibacterial activity of the extracts. The TSB (0.1 mL) broth culture of the test organisms was firmly seeded over Mueller-Hinton Agar (MHA) plates (Barry, 1980). The chitosan solution was added to the different wells in the plate by using a micropipette and the plates were kept at low temperature (4 °C) for 2–4 h and then incubated at 37 °C for 24 h. After the incubation period, the formation of zones around the wells confirmed the antibacterial activity of the respective compounds.

3. Result and discussion

The distinction between CT **1** and CS **2** is somewhat unclear; but it has been scientifically estimated that chitosan **2** consists of >50% of deacetylated CT **1**, and whereas CS **2** is soluble in aqueous 1% acetic acid, chitin **1** is insoluble (Peter MG, 1995). Due to its relative simplicity, FT-IR spectroscopy is one of the most important techniques for characterization of CT **1** and CS **2** (Ng et al., 2006). The IR-spectra of α- and β-chitin display a series of narrow absorption bands, typical of crystalline polysaccharide samples. The C=O stretching region of the amide moiety, between 1700 and 1500 cm^{-1} yields different signatures for α- and β-chitin. For α-chitin, the amide **I** band is split into two components at 1655 and 1619 cm^{-1} (due to the influence of hydrogen bonding or the presence of an enol form of the amide moiety (Focher et al., 1992), whereas for β-chitin it is at 1619 cm^{-1} (Fig. 2: *top*). The amide **II** band is observed in 1569 cm^{-1} for β-chitin. Infrared spectra of β-chitin reveal two additional bands for CHx deformations at about 1416 and 1376 cm^{-1} and a greater number of narrower bands in the C–O–C and C–O stretching vibration region (1150–950 cm^{-1}) observed in β-chitin. The FT-IR spectrum of the CT **1** (Fig. 2: *top*) isolated from prawn head confirmed the finding that this chitin resembles β-chitin more closely than α-chitin (Kumirska et al., 2010).

The production efficiency of CS **2** by the *N*-deacetylation of CT **1** was evaluated using FT-IR analysis. During the *N*-deacetylation of CT **1**, the band at 1619 cm^{-1} gradually decreased, while that at 1578 cm^{-1} increased (Fig. 2: *bottom*), indicating the prevalence of NH_2 groups. The band at 1578 cm^{-1} displayed a greater intensity than the one at 1619 cm^{-1} and demonstrated the effective deacetylation of CT **1**. The formation of a new band at 1578 cm^{-1} and the disappearance of the band at 1619 cm^{-1} are due to the NH_2 deformation, which predominates over the band at 1736 cm^{-1} (Fig. 2: *bottom*). This latter band is associated with the carbonyl (C=O) groups, that tends to decrease as the degree of deacetylation of CS **2** increases. The disappearance of the two bands between the regions 3430 and 3262 cm^{-1}, as already mentioned, is related to deacetylation of the group $NHCOCH_3$, transforming the amide into the primary amine. The degree of deacetylation (DD) of CS **2** was calculated from both the FT-IR spectra (Eq. (1)) and the titrimetric method by using (Eq. (2)) and found to be 80% and 78%, respectively. The average DD of CS **2** is therefore assumed to be 79%. The FT-IR absorption bands (cm^{-1}) of product *O*–CMCT **1a** (SI, Figure. S2) were 3357 (O–H stretching) and 3282 (N–H stretching), 2895 (C–H stretching), 1736 (C=O). 1571 (C=O of –COOH antisymmetric stretching), 1416 (C=O of –COOH symmetric stretching), 1311 (C–N stretching), and 1055 (C–O stretching).

Compared with the CT **1** spectrum, the new absorption bands of –COOH are strong, and the O–H and N–H bands become narrow and weak, both indicating a high carboxymethylation of the –OH group. Meanwhile, the bands at 1571 cm^{-1} intensify significantly, thus

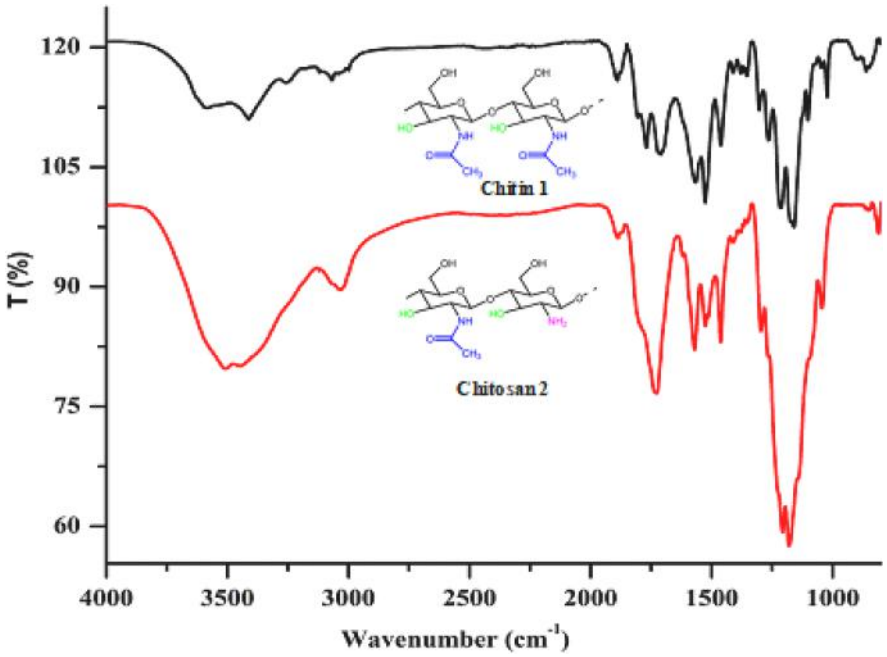


Fig. 2. FT-IR spectrum of chitin **1** (*top*) and chitosan **2** (*bottom*).

indicating that carboxymethylation has occurred on the –OH groups of the CT **1**. The FT-IR absorption bands (cm^{-1}) of the product *O*–CMCT **2a** (SI, Figure. **S3**) are 3257 (O–H stretching) and 2865 (C–H stretching), 1579 (C=O of –COOH antisymmetric stretching), 1310 (C–N stretching), and 1056 (C–O stretching). Compared with the CS **2** spectrum, the new –COOH absorption bands of are strong, and the O–H and N–H bands become narrow and weak, both indicating a high carboxymethylation on the –OH group. These phenomena are similar to those observed with compound **1a**. The broad peak of *O*–CMCT **1b** (SI, Figure. **S4**) at 3372 cm^{-1} is due to the –OH stretching vibrations. The sharp absorption bands at 1377 cm^{-1} correspond to the CH_2 bending vibration. The band at 899 cm^{-1} is attributed to the C–C stretching vibration. The peak at 1724 cm^{-1} in the FT-IR spectrum can be assigned to the C=O vibrational mode. Compared with the CT **1** spectrum, the new –COOH absorption bands are strong, and the O–H and N–H bands become narrow and weak, both indicating a high carboxymethylation of the –OH group at the C-3 position. The broad peak of *N,O*–CMCS **2b** (SI, Figure. **S5**) at 3410 cm^{-1} is due to–OH stretching vibrations. The sharp absorption bands at 1436 cm^{-1} correspond to the CH_2 bending vibration. The peak observed at 1375 cm^{-1} is due to the CH_2 wagging vibration. The band at 899 cm^{-1} is attributed to the C–C stretching vibration. The peak at 1737 cm^{-1} in the FT-IR spectrum can be assigned to the C=O vibrational mode. The absorption band at 680 cm^{-1} is assigned to the out-of-plane bending vibration of the carboxylate group. Compared with the CS **2** spectrum, the new –COOH absorption bands are strong, and the O–H and N–H bands become narrow and weak, both indicating a high carboxymethylation of the –OH or $–NH_2$ groups. Meanwhile, the bands at 1737 cm^{-1} and 1216 cm^{-1} intensify significantly, thus indicating that the carboxymethylation has occurred on both the amino and the hydroxyl groups of CS **2** (Kaya, Baran et al., 2014). In addition, the bands corresponding to C=O of NH–C=O stretching and N–Bending are overlapped with the much stronger C=O of COOH antisymmetric stretching. The degree of substitution (DS) of **1a**–**b** and **2a**–**b** were calculated by using the Eqs. (3 and 4) are 0.66, 0.75, 0.68 and 0.89, respectively.

The thermal parameters obtained from the TGA curves (Fig. 3) are listed in Table 1. The initial weight loss (below 120 °C), observed in all

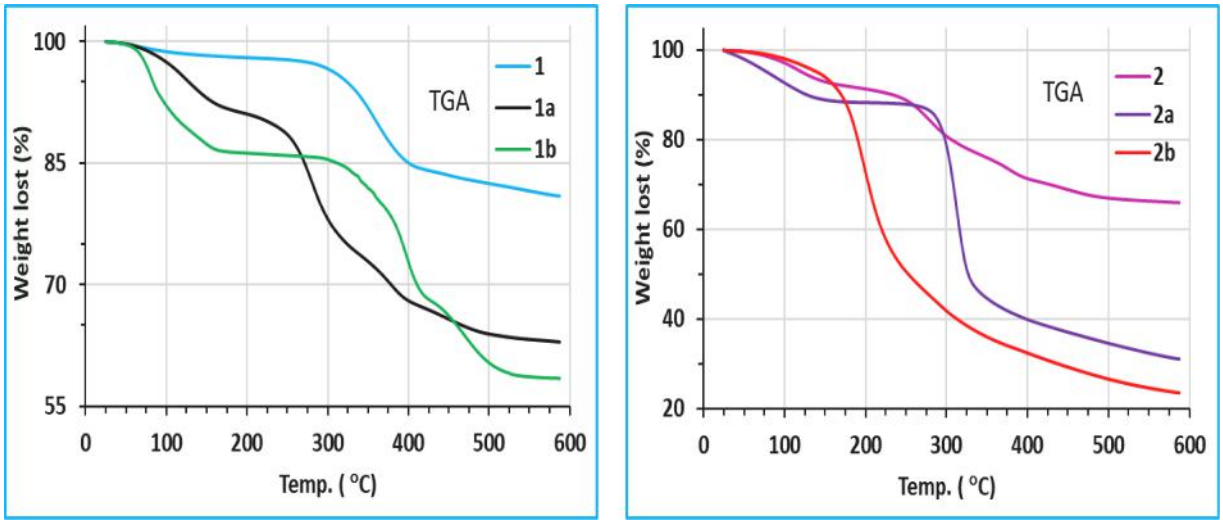


Fig. 3. Thermogravimetric analysis (TGA) of **1, 1a-b, 2, 2a-b** samples weight lost (%) data with temperature (°C).

Table 1
Results of thermogravimetric analysis of **1, 2, 1a–b** and **2a–b**.

Compound	1st Stage Temp range (°C)	Mass loss (%)	2nd Stage Temp range (°C)	Mass loss (%)	3rd Stage Temp range (°C)	Mass loss (%)	Residue at 600 °C (%)
1	25–200	2	290–410	16	—-	—-	81
2	25–200	11	280–420	59	—-	—-	32
1a	25–120	8	250–320	17	360–400	32	63
2a	25–150	8	220–350	24	360–410	30	66
1b	25–110	11	360–430	32	—-	—-	58
2b	25–120	2	120–300	57	—-	—-	23

compounds, can be attributed to the loss of moisture, since polysaccharides usually have a strong affinity for water and therefore may be easily hydrated. The second (main) step includes both decomposition and oxidation reactions of the prepared compounds.

In the last stage, there is almost a complete degradation of intermediates generated earlier at lower temperatures. It can be seen from the TGA curve in Fig. 3 that the decomposition stage starts at 260 °C for most of the samples. However, in the case of **2b**, we observed an exceptional second stage from 150 °C. This may be due to the change of the structure of the material and a change in the mechanism of its thermal degradation process.

Scanning electron microscopy was used to investigate the surface morphology of CT **1**, CS **2** and their derivatives **1a**–**b** and **2a**–**b**. The surface morphology of CT **1** (Fig. 4: *Top left*) shows a more compact, denser structure, with layers of crumbling flakes without porosity. CMCT **1a** exhibits an irregular, rough and wrinkled surface without any smear layer and ice melting type (Fig. 4. *Top middle*). CMCT **1b** shows a prominent arranged microfibrillar crystalline structure with the porous surface (Fig. 4. *top right*). The surface morphology of CS **2** (Fig. 4. *Bottom left*) shows a non-homogenous and non-smooth surface with straps and shrinkage. CMCS **2a** has the non-smooth porous surface (Fig. 4. *Bottom middle*). The surface morphology of **2b** showed an irregular non-smoothed surface (Fig. 4. *bottom right*). From these SEM results, we can conclude that the incorporation of the acetyl group affects the surface morphology and the physicochemical characteristics of the polymer.

The degrees of substitution (DS) for **1a, 2a, 1b,** and **2b** were calculated using Eqs. (3 and 4). The DS of **1a** (0.66) is almost equal to that of **2a** (0.68) indicating that under the same conditions only the C–6 primary hydroxyl hydrogen might be substituted by the carboxymethyl group. In the case of **1b** (0.75) and **2b** (0.89), at a higher temperature (65 °C), for **1b** both C–3, C–6 hydroxyl hydrogens have a chance to be substituted by carboxymethyl groups. For **2b** both the C-2 -NH_2 group and the C–3 and C–6 hydroxyl hydrogens could be substituted by carboxymethyl groups and result in a higher value of DS (0.89).

X-ray diffraction analysis was applied to detect the crystallinity of the isolated CT **1** and CS **2**. CT **1** (Fig. 5: *Left side*) shows strong reflections at 2θ around 19–20°. CS **2** (Fig. 5: *right side*) shows reflections at 2θ around 20–21°. The XRD patterns of CT **1** and CS **2** are both in good accordance with the literature data from different sources of CT **1** and CS **2** (Kaya & Baran, 2015; Kumari et al., 2015; Kumirska et al., 2010; Sagheer et al., 2009).

The X-ray patterns of all compounds reported herein are shown in the Supporting Information. For carboxymethyl chitin **1a** (Figure. S6) peaks can be seen on angle 2θ = 26.8°, 33.5°, 29.3°, 42.2°, 54.3°, 58.1°. The X-ray patterns of carboxymethyl chitosan **2a** shown in Figure. S7, peaks can be seen on angle 2θ = 32.2°, 45.8°, 56.2°, 75.6°. Figure. S8 shows the XRD patterns of carboxymethyl chitin **1b** with peaks on angle 2θ = 31.9°, 45.8°, 57.1°, 77.9° (SI,). However, Figure. S9 reveals no crystalline peaks for **2b**. It is clear therefore, that the carboxymethylation of CT **1** and CS **2** forced important changes in the array of the polymer chains in the solid state. In fact, the spectra of **1a, 1b** and **2a, 2b** exhibit poorly defined and less intense peaks when compared to those of their respective parents, CT **1** and CS **2**. This may be due to the presence of the carboxymethyl moieties which replace the hydrogen atoms of the hydroxyl and amino groups of the CT **1** and CS **2**. Thus, since the carboxymethyl groups are much larger than the hydrogen atoms, an important excluded volume effect occurs and a polyelectrolyte effect must also be considered due to the presence of charged groups in the chains of **1a, 1b** and **2a, 2b** which lead to the deformation of the strong hydrogen bonds in CT **1** and CS **2**. This result means that the carboxymethyl groups of **1a**–**b** and **2a**–**b** are more amorphous than chitin **1** and

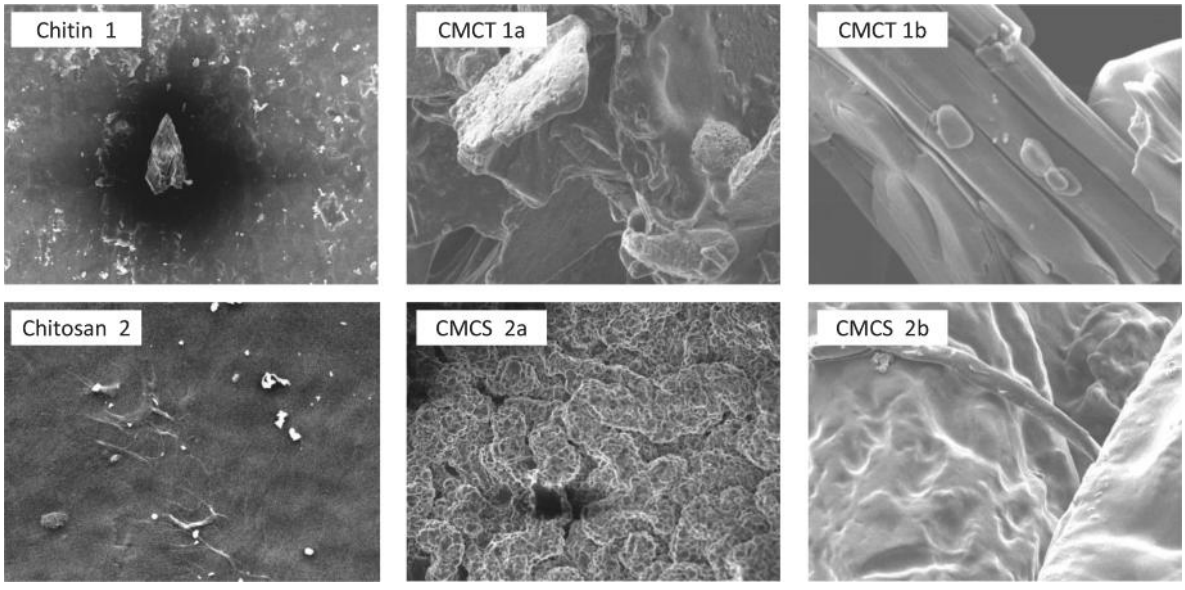


Fig. 4. SEM images spectrum of the Chitin **1,** CMCT **1a**, CMCT **1b**. Chitosan **2**, CMCS **2a** and CMCS **2b**. (Scale 20 μm and 700X).

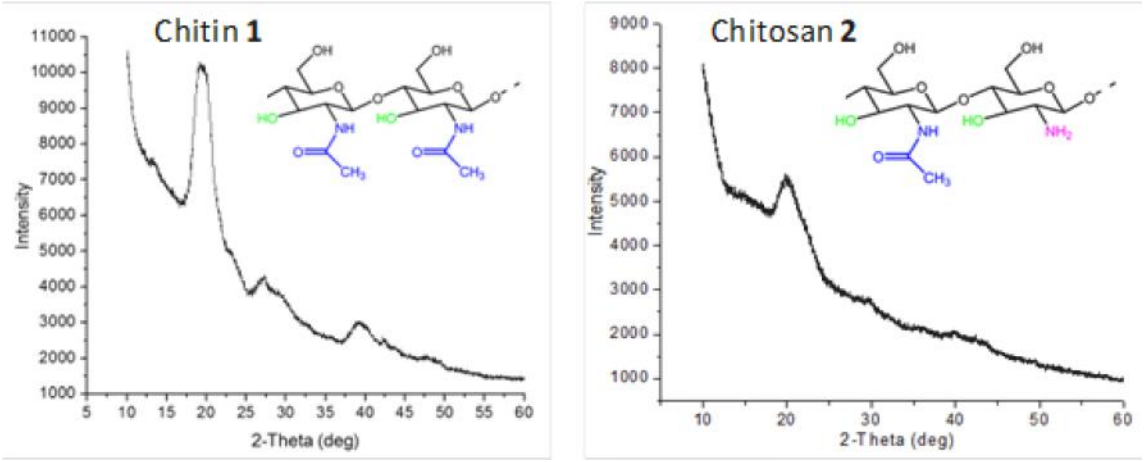


Fig. 5. X-Ray diffraction pattern of Chitin 1 and Chitosan 2.

chitosan **2.** Kim et al. observed similar phenomena in the case of water-soluble chitin derivatives and they reported that trimethylaminoethyl–chitin (TEAE–chitin) did not show any crystalline peak (Mohammed et al., 2013).

3.1. Solubility test of derivatives 1a, 1b, 2a and 2b in distilled water

From the solubility tests conducted, chitin products **1a** and **1b** are both ~0.2% by weight soluble in distilled water. Chitosan product **2a** is ~0.6% and **2b** is ~0.3% soluble by weight in water. The photographs on the left in Fig. 6(A) show the saturated (homogeneous) solution of our synthesized derivatives in water at room temperature 25 °C, and on the right-side (B) that a higher mass loading that the solubility limit in water has been reached.

3.2. Antibacterial activity of the solutions of CS 2 and the derivatives 1a, 1b, 2a and 2b

The antibacterial activity of CS **2**, and the derived products were tested against eight bacterial strains according to the reported procedures, and are listed in Table 2 (Islam, Masum, & Mahbub, 2011b; Islam, Masum, R, & Haque, 2011c). The five gram-negative bacteria *Shigella flexneri* ATCC 12,022, *Enterococcus faecalis* ATCC 29,212, *Pseudomonas aeruginosa* ATCC 27,853, *Klebsiella pneumoniae* ATCC 13,883, *Vibrio paraheamolyticus* ATCC 17,802, and three gram-positive *Staphylococcus aureus* ATCC 9144, *Bacillus subtilis* ATCC 11,774, *Bacillus cereus* ATCC 10,876 in Muller–Hinton (M–H) broth were tested. This study was conducted to assess the inhibitory effects of CS **2**, derivatives **1a**–**b** and **2a**–**b**, as measured by macro and micro broth dilution techniques and the results are presented in Table 2. CS **2** exhibited activity against all eight pathogens and the highest zone of inhibition was observed against *Staphylococcus aureus* (35 mm).

Table 2
Antimicrobial activity and zone of inhibition of chitosan **2, 1a**–**b** and **2a**–**b**.

Test organism	Concentration (ppm) growth in peptone broth (175 ppm)				
	2	1a	2a	1b	2b
	Zone of inhibition in diameter(mm)				
S. flexneri	34	–	11	–	–
E. faecalis	30	–	–	–	–
P. aeruginosa	31	–	–	–	14
K. pneumoniae	26	–	–	–	12
V. paraheamolyticus	31	–	–	14	–
B. cereus	18	–	12	12	–
S. aureus	35	–	–	–	12
B. subtilis	17	13	12	–	–

The carboxymethyl derivatives of chitin (*O*—CMCT) **1a** only show antimicrobial activity against *Bacillus subtilis*, whereas *O*—CMCT **1b** shows antimicrobial activity against two pathogens, *Vibrio paraheamolyticus*, and *Bacillus cereus*. The carboxymethyl methyl derivatives of CS, *O*—CMCS **2a** show antimicrobial activity against three pathogens *Shigella flexneri, Bacillus cereus,* and *Bacillus subtilis*. The prepared derivative of CS, **2b** also shows antimicrobial activity against three pathogens such as *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and

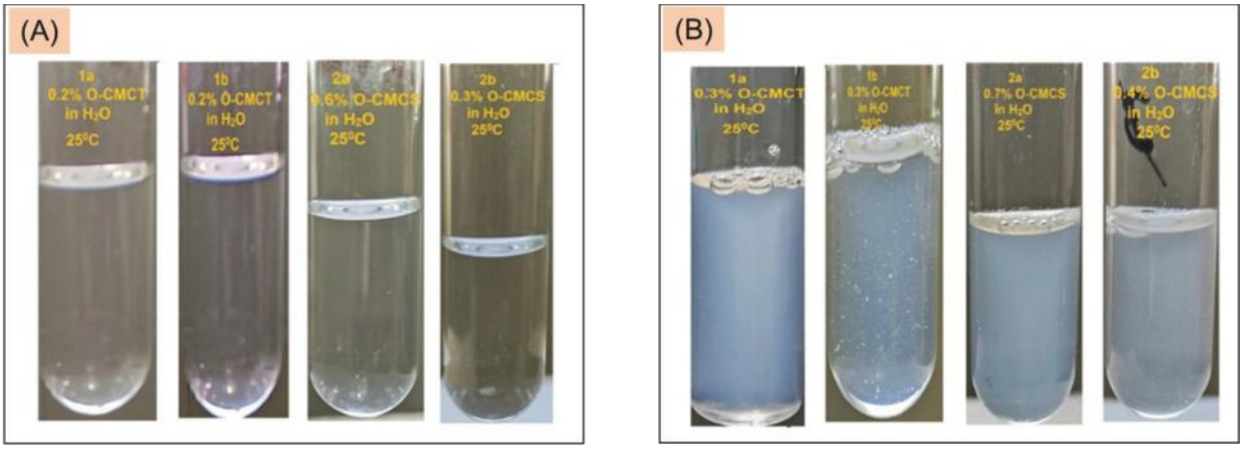


Fig. 6. Solubility photograph of synthesized chitin and chitosan derivatives in water at 25 °C.

Staphylococcus aureus.

In the case of the CS **2** derivatives, due to their having free amino groups, antibacterial activity against three pathogens were observed. Similar phenomena have also been reported for the decrease of antibacterial activity of carboxymethyl chitosan relative to CS (Kim et al., 1997). The antimicrobial activity of CS is associated with the degree of deacetylation (DD); consequently, the antimicrobial activity of CS depends on its positive charge number (Kaya, Cakmak et al., 2014; Leuba & Stossel, 1986; Young et al., 1982) and it is reported that CS with a higher DD, and thus a higher positive charge, would be expected to have greater antimicrobial activity (Tsai & Su, 1991). Park et al. studied the susceptibility of both gram-negative and gram-positive bacteria against CS with a different DD and observed that CS with 75% DD was more effective than 90 or 50% deacetylated chitosan (Park et al., 2004). Mohamed *et al.* reported the antimicrobial activity of carboxymethyl chitosan against *Bacillus subtilis, Staphylococcus aureus*, and *Escherichia coli* (Mohamed & Abd El-Ghany, 2012). However, in the present study CS **2** showed greater antibacterial activity than the carboxymethyl derivatives (**1a–b** and **2a–b**). Its application however may only be effective in an acidic medium due to its low solubility in neutral and basic media Therefore, chemical modifications of CS **2** are required to enhance its solubility and broaden the field of its applications.

4. Conclusion

The carboxymethyl chitin (CMCT) **1a**–**b**, and carboxymethyl chitosan (CMCS) **2a**–**b** derivatives were successfully synthesized from prawn head shell via CT **1** using relatively mild chemical methods with minor modification. The synthesized compounds were characterized by Fourier transform infrared spectroscopy (FT-IR) and X-ray diffraction (XRD). The thermal properties were studied by thermogravimetric analysis (TGA) and the surface morphology examined by scanning electron microscopy (SEM) of the synthesized compounds. The synthesized carboxymethyl derivatives of chitin and chitosan showed potential antimicrobial activity against the tested microorganisms. From these results it could be concluded that the chemical methods reported here have suitable potential for the extraction of chitosan via chitin from prawn head shell wastes. Furthermore, this approach could help to develop an environmentally-friendly waste management system to minimize environmental pollution from prawn head shell wastes and has the potential to earn a significant amount of foreign currency to Bangladesh by exporting chitin and chitosan, or save the currency spent by reducing their imports. Synthesis of various derivatives of chitin and chitosan under varying conditions are in progress in our laboratory to improve their solubility in different solvents and investigate their resulting biological properties.

CRediT authorship contribution statement

Md. Monarul Islam: Investigation, Validation, Formal analysis, Funding acquisition, Conceptualization, Data curation, Methodology, Project administration, Writing – original draft. **Rashedul Islam:** Formal analysis, Investigation, Data curation. **S M Mahmudul Hassan:** Formal analysis, Investigation. **Md.Rezaul Karim:** Formal analysis, Investigation. **Mohammad Mahbubur Rahman:** Formal analysis, Investigation. **Shofiur Rahman:** Formal analysis, Investigation. **Md. Nur Hossain:** . **Dipa Islam:** Formal analysis. **Md. Aftab Ali Shaikh:** Supervision, Writing – review & editing. **Paris E. Georghiou:** Writing – review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2023.100283.

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