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The effect of cellulase-hydrolyzed chitosan on the degree of deacetylation, solubility and viscosity of chitosan oligosaccharides

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Chitosan is a source of potential bioactive material with many uses in the food, health and agricultural sectors. However, the limitation to utilize the chitosan in biological applications, comprising poor solubility under physiological conditions. In order to overcome the limitations, chitosan is being partially hydrolysed to produce chitosan oligosaccharides (COS). The objective of this research was to determine the degree of deacetylation (DDA), solubility and viscosity of the chitosan and COS. In this study, chitosan was hydrolysed by the cellulase from *Trichoderma reesei* to produce COS. The DDA of chitosan and COS were determined by linear potentiometric titration. The physical characteristics including solubility and viscosity were assessed. The COS was characterized by the degree of polymerization (DP) and FTIR spectroscopy by examining the structural changes before and after the degradation of chitosan. COS showed a higher DDA (99.69 % ± 0.28) compared to chitosan obtained from Sigma Aldrich (77.54 % ±0.28). This enzymatic product, COS also showed high solubility in aqueous and acidic solutions as compared to the chitosan sample. The product of hydrolysis of chitosan mainly composed of COS, with the DP 1-2. The IR spectrum showed that there was no significant change of chitosan before and after the enzymatic hydrolysis confirming the conservation of the chemical phase of the chitosan. In conclusion, COS preparation using cellulase, a low-cost commercial enzyme could open a wide range of new potential applications to improve the quality of many foods.

Keywords: chitosan, chitosan oligosaccharides, solubility, degree of polymerization, degree of deacetylation

INTRODUCTION

Chitosan is the major derivative of chitin, an abundant renewable natural resources and the main source of crustacean waste (Mincea et al. 2012). The amino group (NH_2) is formed after an acetyl group (C_2H_3O) is being removed from the deacetylation process of chitin. The resulting copolymer of N-acetyl-glucosamines (GlcNAc) and glucosamine (GlcN) with the copolymer contain more than 50% GlcNAc units referred to as chitin. The degree of acetylation (DA) referred to the percentage of GlcNAc units in chitin polymers can vary from 50% to 100%. On the other hand, the copolymer contains more than 50% GlcN units is typically referred to as chitosan. For chitosan polymers, the degree of deacetylation (DDA) referred to as the percentage of GlcN units can vary from 50% to

100%. The DA and DDA are related so that DA + DDA = 100%. The high reactive amino groups in the chitosan polymer contribute to the high solubility of chitosan in weak acids and impart their use in textile, cosmetics, food, environmental, biomedical, and pharmaceutical applications (Seyam et al. 2020; Sivashankari & Prabaharan, 2017). Thus, chitosan can be characterised in terms of its quality and intrinsic properties, which include molecular weight (MW), purity, viscosity, and degree of deacetylation (DDA). However, the applications of chitosan are limited due to its physicochemical properties are influenced by several factors; the origin, the extraction method, and the deacetylation process of chitin (Dimzon et al. 2013). Besides, the high MW of chitosan has high viscosity in acidic media and low solubility at pH above 5 (Lin et al. 2009; Xia et al. 2008).

A few sensitive and precise approaches have been used to quantitatively determine chitosan and its DDA, such as Fourier transform infrared (FTIR) (Kasaai, 2008), the first derivative UV-spectrophotometric method (^{1}DUV) (Wu & Zivanovic, 2008), ^{1}H NMR methods (Lavertu et al. 2003) and potentiometric titration method (Zhang et al. 2011). The infrared spectroscopy method is a rapid and easy method, insensitive to most organic and inorganic impurities however, this method needs an extremely well-dried sample (Sweidan et al. 2016; Zhang et al. 2011). This technique can achieve the accuracy of the DDA measurement by about ±2% (Balázs & Sipos, 2007). The NMR technique is relatively high cost and not always available in laboratories. Potentiometric titration suggested first by Broussignac (1968) as a simple and low-cost technique for the determination of chitosan DDA. The biopolymer is dissolved in a known excess of hydrochloric acid (HCl) during chitosan titration. At that moment the solution is then titrated with sodium hydroxide (NaOH), whereas the pH of the solution is monitored with a calibrated pH-sensitive glass electrode. The result shows a titration curve with two equivalence points which is the first corresponds to the excess of HCl and the second to the chitosan that have been protonated. Thus, the difference between the two inflexion points yields the moles of H^+ required for the protonation of the free (deacetylated) amino groups that form the GlcN portion of the chitosan. The DDA value of the chitosan can readily be obtained by assuming the rest of the sample is GlcNAc (Balázs & Sipos, 2007).

The restriction to use the chitosan particularly in medicine and food industries due to the high MW, high viscosity and selective solubility to the acid. This limitation is due to the absence of enzymes in animal intestines, especially in the human gastrointestinal tract to degrade the β-glucosidic linkage in chitin and chitosan, such as chitinase and chitosanase. Thus, several methods have been used to hydrolysed the chitosan including the enzymatic method (Yusof & Ali, 2020), acidic hydrolysis (Tsao et al. 2011), oxidative degradation (Xia et al. 2013) and ultrasonic degradation (Liu et al. 2006). In the presence of hydrolysing agent (e.g enzyme), the weak glycosidic bonds in chitosan cleave and generate chitosan oligomers with various monomer units. Chitosan oligosaccharides (COS) is an oligomer of chitosan, with a degree of polymerization (DP) less than 50-55 and a smaller MW (less than 10 kDa) than low-molecular-weight chitosan (LMWC) (less than 30kDa) (Muanprasat & Chatsudthipong, 2017; Tian et al. 2015; Yu et al. 2017). Enzymatic processes have advantages over chemical reactions since the enzyme catalyse is more specific on reactive sites by fragmenting the molecule into oligomers, therefore produce the partial hydrolysis of the hydrolyzed product. Enzyme cellulase has been used for the hydrolysis of chitosan to produce COS instead of chitosanase because of its economy. Recently, an unspecific hydrolytic action of Celuzyme ® XB enzyme complex, composed of cellulase, xylanase, and β-glucanase on chitosan at the optimum condition of pH 5.3 and 51 °C was reported (Águila-Almanza et al. 2019). Xie et al. (2011) reported that complex enzyme-containing cellulase, pectinase and α-amylase acts on chitosan in endo-pattern, producing low MW chitosan ranged from 1000 to 4000 after enzymatic degradation for 2 hours at pH 5.3 and temperature 56℃.

COS can perform valuable biological activities at the cellular or molecular level due to readily soluble in aqueous solutions. Thus, COS has been reported to have a great number of effects and activities including prebiotics (Nurhayati et al. 2016); antitumor (Salah et al. 2013) and antibacterial (Lu et al. 2014). COS that has depolymerised into a smaller molecular size than that of pentasaccharides (DP 5) or hexasaccharide (DP 6) has the great solubility and rapid absorption in aqueous solutions. By contrast, COS that is larger than hexasaccharide (DP 6) is less soluble in aqueous solutions.

Although its high solubility, the functional properties of COS with smaller molecular size are limited. COS with pentameric (DP 5) or hexameric (DP 6) has a balanced physiological activity and solubility. Therefore, these two forms of COS have greater industrial value. Guan et al. (2020) identified the chitosanase CHIS5 from marine metagenome as an endo-type chitosanase that degraded chitosan into COS with DP of 2–5. COS with a DP 2–6 induces apoptosis in human colon carcinoma HCT116 cells. COS with DP 6 (hexamer) exerted definite effects on hydroxyl and ABTS radical scavenging (Zou et al. 2013). However, the effect of the cellulase from *Trichoderma reesei* on the chitosan has not been addressed, especially for the physicochemical properties of COS. Thus, this study aimed to determine the degree of deacetylation (DDA), solubility and viscosity of the chitosan and COS. The COS was characterized by the DP using Q-TOF MS and FTIR spectroscopy for examining the structural changes before and after degradation of chitosan.

MATERIALS AND METHODS

Materials

Chitosan produced from crab shell with a DDA of >75% was obtained from Sigma–Aldrich. Chitosan powder-PM 100 was obtained from Eastern Global (M) Sdn. Bhd. (Parit Buntar, Perak, Malaysia). A total of 1.5 L Celluclast® was purchased from Sigma Chemicals, USA. According to the product sheet of Sigma–Aldrich, Celluclast® 1.5 L was produced from *Trichoderma reesei ATCC* 26921, with a specific activity of 700 U/g and density of 1.2 g/mL at 25 °C. Acetic acid solution (99.8%) (Darmstadt, Germany), sodium acetate trihydrate, and sodium hydroxide (Merck, Germany) were used as pH buffer in the preparation of chitosan hydrolysis. Subjected for characterizations, a filter paper with a pore size of 20/25 µm (Advantec, No. 1, Japan), polyethersulfone, ultrafiltration (UF) membranes (Sartorius vivaflow 50 modules: Sartorius, Goettingen, Germany) with 10 kDa molecular weight (MW) cut-off were used in the purification of chitosan hydrolysis products.

Preparation of Samples by Enzymatic Hydrolysis

A total of 3% (w/v) chitosan was completely dissolved in 0.2 M acetate buffer with a pH of 4.5. The solution in the reaction vessel was placed in an incubator shaker (Ecotron Shaking Incubators: Infors-HT; Bottmingen, Switzerland) at 49.8 °C, and 25% (v/w) Celluclast® was added to initiate the reaction. After 24 h, the mixture was taken out and boiled for 10 min to stop the enzyme reaction. UF membranes with a 10 kDa MW cut-off were used to remove the enzyme. The filtrates were concentrated to about 1/20 with a rotary evaporator under reduced pressure. The precipitates were thoroughly washed with ethanol and then dried using a freeze dryer (FreeZone 4.5, Labconco; Kansas City, Missouri, USA).

Linear Potentiometric Titration

The determination of DDA by linear potentiometric titration was a modification of the method proposed by (Ke & Chen, 1990) which aimed to determine DDA by acid-base potentiometric titration. In their method, the titration of a strong acid with a strong base was represented by the titration of excessive hydrochloric acid with sodium hydroxide.

Chitosan (0.20 g to 0.25 g) was dissolved in 20 ml of standardised 0.10 N HCl (to yield a non-viscous solution) and diluted with 10 ml of distilled water. The pH of the solution was adjusted to 2, which was the start point, by adding standard 0.01 M NaOH, the titration was performed with a Sartorius pH meter PB-11 (Goettingen, Germany). Under continuous stirring, 1 ml of standard NaOH was added, the solution was allowed to equilibrate, and the pH was recorded. This sequence was repeated until the pH reached a value of 3. A value of *f(x)* of the corresponding volume of NaOH added was calculated using the following formula:

$$f(x) = \frac{(V_0 + V)}{N_B} \times ([H^+]-[OH^-])$$

Where V_0 is the volume of chitosan solution (ml)
V is the volume of NaOH added (ml)
N_B is the concentration of NaOH(N)
$[H^+]$ is the concentration of H^+ (M)
$[OH^-]$ is the concentration of OH^- (M)

The linear titration curve was obtained by plotting *f(x)* against the corresponding volume of NaOH. The volume of NaOH at the endpoint of the titration, V_e, was estimated by extrapolating the linear titration curve to the *x*-axis. The amount of the acid consumed between these two points was considered to correspond to the amount of the free amino groups in the solution (Tolaimate *et al.* 2000). The DDA of the chitosan sample was calculated using the following formula. Fives

replicates were performed for each sample.

DDA (%) = Ø/ [(W-161 Ø)/204+ Ø] x 100
Where Ø= (N_AV_A - N_BV_B) /1000
N_A is the concentration of HCl (N)
V_A is the volume of HCl (ml)
N_B is the concentration of NaOH (N)
V_e is the volume of NaOH at the endpoint (ml)
W is the sample mass (g)

Viscosimetry

The enzyme-catalyzed viscosity decrease of the highly viscous chitosan solutions with reaction time was continuously measured with a HAAKE Rotovisco I Rheometer (Thermo Scientific; Waltham, Massachusetts, United States). Measured values were normalized and plotted as the relative viscosity, and an enzyme-free chitosan solution was used as a control.

Solubility Test

The solubility of the sample was tested by dissolving 10 g of the sample in 200 ml of 0.2 M acetate buffer. The sample solution was stirred at room temperature for 1 h. Then, the sample was filtrated, dried in an oven at 80 °C for 8 h, and weighed. The solubility was determined by the following equation:

$$\text{Solubility} = 1 - \frac{\text{(Weight of insoluble part)}}{\text{(Total weight of sample)}} \%$$

Chromatography and Mass Spectrometry Condition

The components of COS were analysed by Synapt G2 HDMS Quadrupole Time-of-flight OF (TOF/MS) Mass Spectrometer (Waters, Milford Massachusetts, USA). The MS detections were performed with Positive (ES+) and the parameters in Table 1.

Table 1: Quadrupole Time-of-flight (QTOF/MS) working parameters.

Parameter	Value
Ionization mode	Positive (ES+)
Analyser	Sensitivity Mode
Capillary Volt (kV)	2.5
Sampling Cone (V)	15
Source Temperature ©	100
Desolvation Temperature (C)	150
Cone Gas Flow (L/Hr)	0.0
Desolvation Gas Flow (L/Hr)	800.0

FTIR

Infrared spectra were measured by a KBr-supported chitosan sample over the frequency range of 4000 cm^{-1} to 650 cm^{-1} at a resolution of 16 cm^{-1} using IRPrestige-21 (Shimadzu, Columbia, USA). Sixty co-added scans were taken for each sample. All powder samples of COS were compressed into KBr disks. The spectra were measured in triplicates and averaged to a spectrum using the IRsolution software.

RESULTS AND DISCUSSION

Degree of Deacetylation (DDA) of chitosan and COS

The acting of the enzyme on chitosan polymer as a mixture of monosaccharides was classified into two types which were the enzyme that can catalyze the cleavage of the GlcNAc reducing end (chitinase type) and the enzyme that can catalyse the cleavage of the GlcN reducing end (chitosanase type). The action of the enzyme on the chitosan polymer contributed by the DDA. The chitosan samples used and the DDA values obtained from the linear titration are presented in Table 2. As a reference, the chitosan sample from Sigma-Aldrich with a DDA of >75% was measured to test the accuracy of this linear potentiometric method. The DDA of the chitosans from Sigma–Aldrich and Eastern Global were 77.54% and 53.60%, respectively. The value of reference chitosan sample purchased from Sigma-Aldrich close to the value of DDA of the experimental chitosan, prove that the accuracy of the method with simple instrumentation and evaluation techniques. The commercial chiton has DDA lower than 95% and averages about 70–90%. The chitosan sample used for COS preparation was chitosan from Eastern Global. The DDA of COS was 99.69%. The huge difference in DDA value between the chitosan and COS since the biggest amount of free-NH_2 group is produced after hydrolysis and more positive charges in a solution (Zou et al. 2016).

.**Table 2: Degree of deacetylation of chitosan and COS samples**

Sample	Degree of Deacetylation (%)
Chitosan from Sigma-Aldrich	77.54±0.28
Chitosan from Eastern Global	53.60±1.48
Chitosan Oligosaccharides	99.69±0.28

Solubility of chitosan and COS

Chitosan sample from Eastern Global (DDA 53.60%) was used in this solubility measurement for comparison with COS. In this experiment, about 5% of the chitosan and COS resolved in an acetic acid solution that had the same concentration and solubility was determined (Table 3). The solubility of chitosan increased with increasing pH when the concentration of acetic acid was constant (0.2 M acetic acid). Thereafter, the solubility of chitosan solution reached a maximum value at pH 5 (46.338%) and a minimum value at pH 2 (27.853%). The solubility of the chitosan solution decreased with decreasing acid concentration. Chitosan is insoluble in distilled water (dH_2O), double-distilled water (ddH_2O), an alkaline solution and thus write as not determined (nd). After enzymatic degradation in the experiment, COS was completely soluble in distilled water (dH_2O) (100%) and double-distilled water (ddH_2O) (99.9%). The solubility of the COS at pH 5 was the highest (99.0%) and lowest at pH1 (68.580%). However, the gelation did not tend to occur in this strong acidic at pH1.

Roncal et al. (2007) reported that chitosan dissolves in dilute acidic aqueous solutions with a pH of below 6. In addition, the solubility of chitosan is low when the pH is above 7, in which precipitation or gelation tends to occur. The amount of protonated amino groups ($NH3^+$) in the polymer chain is associated with the chitosan solubility. By having a bigger number of amino groups, the greater electrostatic repulsion between the chains would be greater, as well as greater solubility in water. The solubility is a very difficult parameter to control due to the influence of the DDA, the pH, molecular weight (MW) and the acetyl groups distribution along the main chain as well as the preparation process (Tian et al. 2015). In addition, higher DDA results in higher solubility (Kumirska et al. 2009). The solubility of the chitosan in the water is higher by lowering the MW of the chitosan. The high solubility of low-molecular-weight chitosan cause the decrease of intermolecular interactions, such as van der Waals forces and hydrogen bonds. On the other hands, the low-molecular weight of chitosan that has low solubility corresponds to lower intermolecular attraction forces (Kubota et al. 2000).

Table 3: Solubility of chitosan and chitosan oligosaccharides (COS) at different pH

pH	Solubility (%)	
	Chitosan	COS
pH 1	nd	68.580±5.645
pH 2	27.853±7.052	74.907±2.571
pH 3	45.418±2.531	98.768±1.340
pH 4	33.298±8.752	97.772±0.817
pH 5	46.338±1.893	99.001±0.341
pH 6	43.738±4.941	97.420±0.262
pH 7	nd	96.764±0.095
pH 8	nd	96.934±0.230
pH 9	nd	96.129±0.648
pH 10	nd	96.216±0.321
dH_2O	nd	100.000±0.245
ddH_2O	nd	99.989±0.343

nd: not determined

Viscosity

In this study, the cellulase used was a mixture of endo- and exoenzymes. During the early hydrolysis stage, fast degradation was occurred due to endoenzyme activity. The first 2 hours of hydrolysis showed the greatest viscosity decreased due to the effect of enzyme action on the chitosan. The speed of viscosity decreased at the starting of hydrolysis, and the change of these parameters with time was monitored for 24 h of reaction (Figure 1). The main viscosity decreased in the first 4 h about 70% of relative viscosity. The viscosity reduced by about 15% from 4 to 8 h. This is due to the major action of the endo-type enzymes. The hydrolysis of the initial large chitosan chains caused a greater decrease in viscosity than the degradation of the subsequent shorter hydrolysis products (Roncal et al. 2007). The action of exoenzymes is by modifying the initial chitosan product into smaller fragments which is dimer and monomer of GlcNAc and GlcN with flanked on the reducing end of chitosan. The extensive degradation of chitosan in the reaction upon completion of chitosan digestion in 24 h was attributed to the nonspecific digesting behaviour of cellulase.

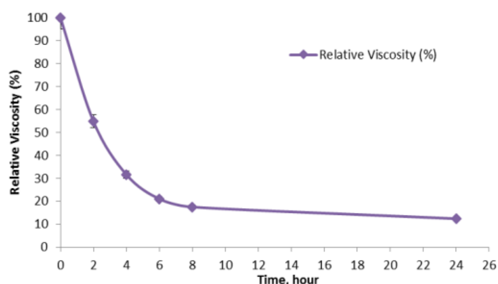

Figure 1: Time course of the viscosity decrease of a 3% (w/v) chitosan solution resulting from cellulase activity. The enzymes were assayed in 0.2 M acetate buffer with pH 4.5 at 49.8 °C and 25% (v/w) of Celluclast® (Mean± SD, n=3).

Mass Spectrometry Analysis of COS

After the hydrolysis, the produced product, COS was filtered using an ultrafiltration membrane to obtain MW less than 10kDa. The MW of 10 kDa was selected because the solubility of chitosan or COS improved with an MW in the range of 4-10 kDa while could retain all the beneficial properties of a polymer (Poshina et al. 2018).

QTOF MS was run in positive ion mode and the GlcN molecules usually form singly charged ions by adding H^+. Thus, the theoretical average *m/z* value of GlcN with a degree of polymerization (DP) value of *n* in QTOF MS calculated as follows: $[C_{6n}H_{13n}ON_5 + H]^+$, where n≥1. Thus, the theoretical *m/z* value of COS (DP = 1) was 180.9691 as shown in Table 4.

Fig. 2(a-b) shows that products were composed of monomers of GlcN and GlcNAc, especially of DP 1-2. Xie et al. (2009) conducted a study on the preparation of COS using the cellulase of *Aspergillus niger* with the result showed that cellulase can hydrolyse chitosan to yield COS with DP 3 to 11, especially with DP 6 to 8 in good yield. Prasertsung et al. (2012) reported that COS with the DP of 2–8 were also generated by solution plasma treatment. Chitosanase and some enzymes in the production of COS have been assessed using enzyme reactors both in batch and in continuous configurations. The result shows products with a high MW (>10 kDa) in most of the cases. However, when the low DP of oligosaccharides are obtained, the results show poor yield with the majority of monomers and smallest-sized species (2 to 4 residues). Chen et al. (2010) had done a study using enzyme BC487 to degrade the chitosan and observed the COS products with the composition of DP 5–12, mainly 7–10. The cellulases obtained from various microbial sources, including *Bacillus cereus* and *Streptomyces griseus* also can degrade the chitosan with a similar result, $(GlcNAc)_{2–4}$ as observed by several researchers (Kim et al. 2004; Tanabe et al. 2003). The stronger catalysing capability of cellulase than lysozyme and chitinase shows the nonspecificity of the cutting behaviour, instead of its high chitinolytic activity.

Table 4: Measured and calculated Mass Spectrometric data for COS

m/z		Types	DP	Ion Composition
measured	calculated			
180.9691	180.0937	[M+H]+	1	GlcN
222.9182	222.1047	[M+H]+	1	GlcNAc
402.9341	401.1824	[M+H]+	2	GlcN-GlcNAc

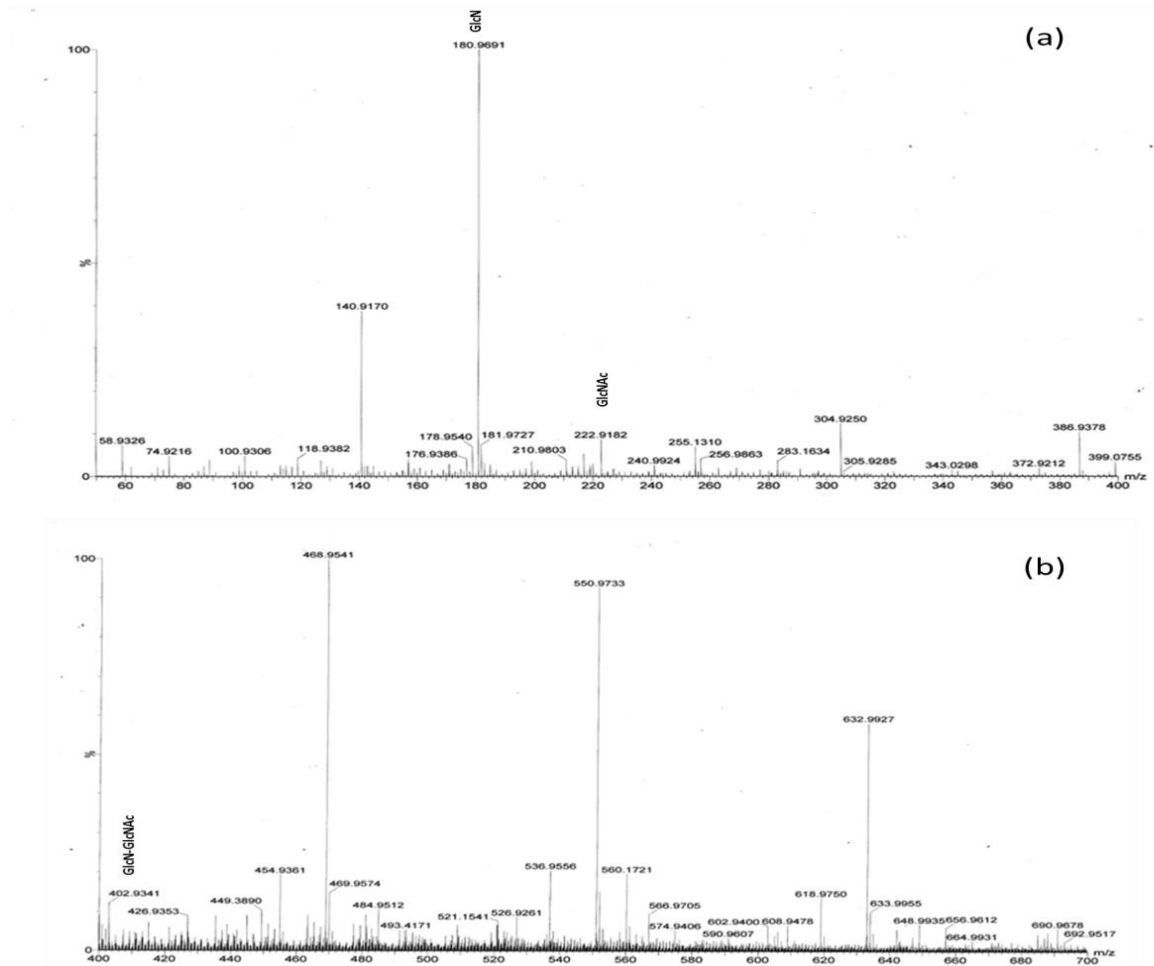

Figure 2: QTOF MS of products from hydrolysis of chitosan by cellulase

FTIR analysis

FTIR spectroscopy is important to study the physicochemical properties of polysaccharides. Figure 3 shows the IR spectra of the initial chitosan. The chitosan sample had a band at 3400 cm^{-1} due to the stretching of the -OH group, followed by the tension vibration at 3270cm^{-1} of the NH bond. The characteristic signals of the CH stretching are observed at 2875cm^{-1} (Águila-Almanza et al. 2019). The absorption bands at 1650 and 1550 cm^{-1} in the initial chitosan were attributed to the C=O–NH–CH_3 stretching of amide I (C=O stretch) and amide II (N–H bend) bands. The CN voltage vibration at 1320cm−1 and the symmetric C-O-C stretch of the glycoside bond (1.075cm−1). At 1075, and 1025 cm^{-1} (skeletal vibrations involving the C–O stretching) were characteristic of the saccharide structure. The vibrational band at about 1155 cm^{-1} corresponded to the ether bond in the pyranose ring. Figure 4 for the COS shows the O–H stretch of the intermolecular hydrogen bonding at 3300 cm^{-1}. Similar to the case of O–H stretching, the reduced of frequency N–H stretching is by hydrogen bonding, even though to a lesser degree. Overlapping in the position of N–H and O–H stretching frequencies is observed, which makes an unequivocal differentiation in structure sometimes impossible. In the IR spectra of COS, an overlap in the new absorption band appeared at 1575 cm^{-1}, referred to as amide I (C=O) and amide II (N–H). The peaks of C–N stretched at 1400 cm^{-1}. A greater intensity is observed in the spectral signals of the COS because the functional groups vibrate with greater freedom of movement due to the steric hindrance of the polymer chain decreases (Águila-Almanza et al. 2019). Even though the IR

spectrum suggests that no significant change in the residues of chitosan before and after the enzymatic hydrolysis, however, the DDA of the hydrolysis products increased. These data coincided well with the data of potentiometric determination of DDA.

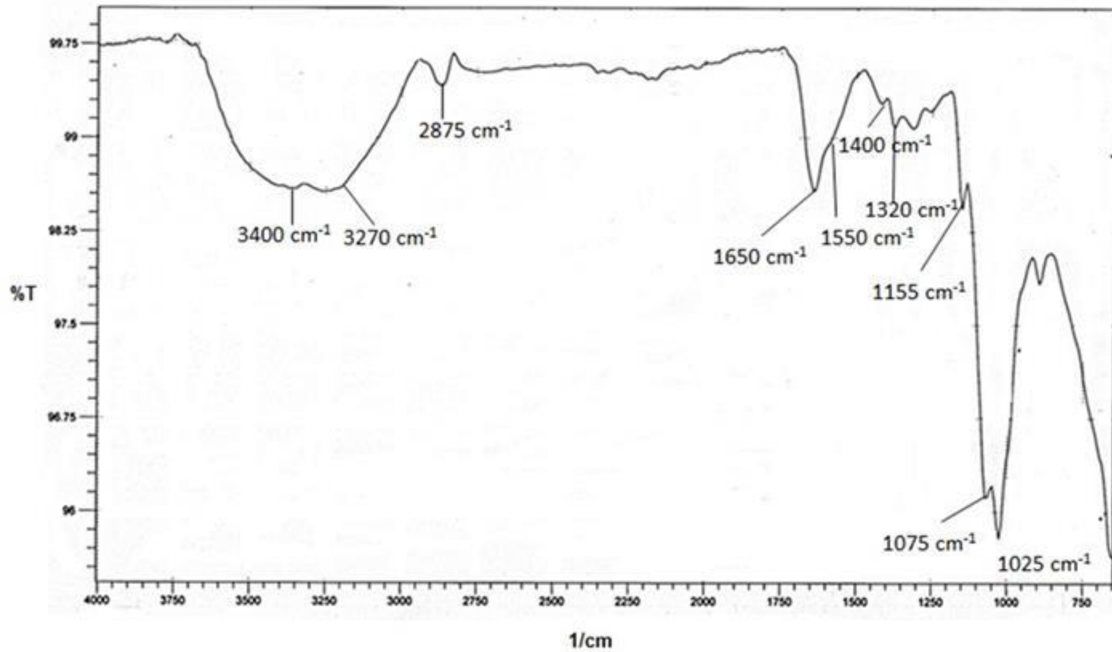

Figure 3: FTIR spectra for chitosan

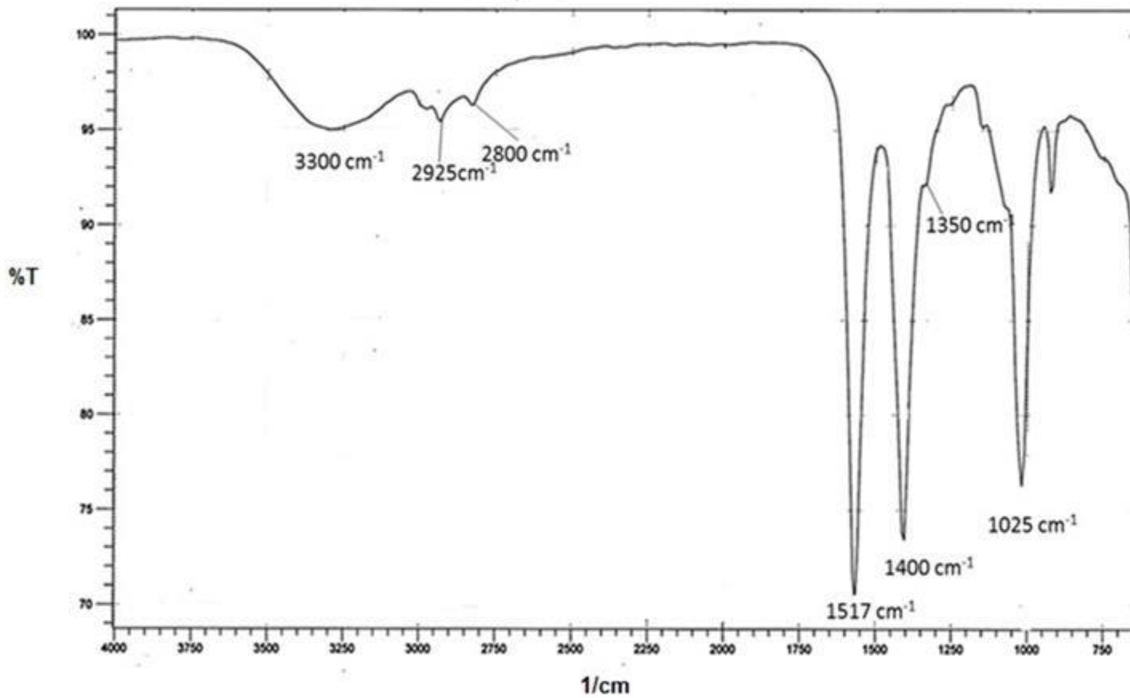

Figure 4: FTIR spectra of chitosan oligosaccharide

CONCLUSION

The result shows that the cellulase from *T. reesei* can hydrolyse chitosan to yield monomers and dimers of GlcN and GlcNAc with DP 1-2. IR spectra confirmed that the chemical structures of the COS were not modified. Overall, COS produced has a MW of less than 10kDa and low molecular weight compared to chitosan. The COS soluble in aqueous media at pH values close to neutrality and have a reduced viscosity that increases their bioavailability and opens a wide range of new potential applications in food, agriculture and pharmaceuticals.

CONFLICT OF INTEREST

The authors declared that the present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Nurhayati Y designed and perform the experiments, collected and analysed data, and lead the manuscript writing while Azman MA examined the QTOF MS analysis. Tang JYH and Abd Ghani A improve the experiments and reviewed the manuscript. Yusof N edited and formatted the manuscript. All authors have read and approved the final version of the manuscript.

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