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Enzymatic hydrolysis of thermally pre-treated chitin and antimicrobial activity of *N,N'*-diacetylchitobiose

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Abstract

BACKGROUND: *N,N'*-diacetylchitobiose ($GlcNAc_2$) is known to be highly functional and offers a wide range of applications, especially as an antimicrobial agent. In this study, a thermal pre-treatment process using steam under pressure in an autoclave, has been employed to facilitate subsequent enzymatic hydrolysis of chitin with chitinase from *Streptomyces griseus*.

RESULTS: Pre-treatment of chitin with 0.05 M sodium acetate buffer (pH=6.0) at 121 ºC for 60 min, followed by enzymatic hydrolysis involving 24 h incubation, was found to be the best condition for producing the $GlcNAc_2$. The $GlcNAc_2$ obtained was tested regarding its antimicrobial activity against Gram-negative and Gram-positive strains

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and showed minimum inhibitory concentrations (MIC) at 5 and 10% w/v against *Escherichia coli* K-12 and *Listeria monocytogenes* 10403S, respectively.

CONCLUSION: The extent of swelling and crystallite size of chitin increased with the pre-treatment residence time, and enhanced the rate of subsequent hydrolysis using chitinase.

Keywords: Thermal pre-treatment; enzymatic hydrolysis; $GlcNAc_2$; antimicrobial activity.

INTRODUCTION

Chitin is a polymer consisting of *N*-acetyl-D-glucosamine (GlcNAc) monomeric units that form covalent β-(1→4)-linkages and is the second most abundant polysaccharide in nature after cellulose. It is a dominant component of the cell walls in most fungi, crustaceans and insect exoskeletons, and offers a wide range of structural and protection functionality.[1] *N,N'*-diacetylchitobiose ($GlcNAc_2$) is an oligomer composed of two GlcNAc units, which can be produced by the acidic or enzymatic hydrolysis of chitin. The oligomers, that are composed of approximately ten or less GlcNAc residues, have received considerable attention because they offer excellent solubility in water, nontoxicity, biocompatiblity and numerous biological properties such as antimicrobial and antitumor activities.[1,2] It has also been reported previously that the oligomers inhibit the growth of Gram-positive (*B. cereus, L. monocytogenes* and *S. aureus*) and Gram-negative bacteria (*B. fragilis, E. coli, P.*

aeruginosa, P. melaninogenica, Shigella disenteriae and *V. cholera*) more effectively than chitin and chitosan.[3–5]

Hydrolysis of chitin, using either acid or enzyme, is normally restricted due to its highly crystalline structure caused by the strong dipole–dipole interactions between polysaccharide chains.[3] Several chitin pre-treatment methods, such as gamma irradiation, grinding, microwave irradiation, steam explosion, supercritical water and ultrasonication, have been proposed to assist the hydrolysis.[1] These pre-treatment methods have been reported to reduce the chitin crystallinity, particle size and molecular weight, as well as increase the crystallite size and extent of deacetylation. In addition to such physical pre-treatment, the use of ionic liquid as solvent has been studied in polysaccharides for removing their native recalcitrant such as cellulose and lignocellulose[6,7] Ionic liquids have also been used for chitin pre-treatment, which is reported to have induced greater enzyme accessibility to the substrate during hydrolysis with chitinases.[8,9] It is interesting to note that the pre-treatments reported so far are fairly complex and difficult to scale up. To date, the effectiveness of simple autoclaving as a pre-treatment process has not been explored. Therefore, the aim of this study was to investigate the effectiveness of thermal pre-treatment of chitin under autoclaving conditions (121 ºC, 204.7 kPa) in order to assist the production of $GlcNAc_2$ by enzymatic hydrolysis, and assess the antimicrobial activity of the product against Gram-negative and Gram-positive organisms.

MATERIALS AND METHODS

Commercial chitin from shrimp shells (practical grade powder) and chitinase from *Streptomyces griseus* were obtained from Sigma Aldrich, UK. Gram-negative bacterium *Escherichia coli* K-12 and Gram-positive bacterium *Listeria monocytogenes* 10403S were cultivated in-house (Department of Food and Nutritional Sciences, University of Reading, UK). The chitin was sieved through a 125 μm sieve to produce particles in the narrow size range of less than 125 μm and was stored in opaque plastic bottles at ambient temperature until further use. All other reagents used in this study were of analytical grade and commercially available.

Pre-treatment of chitin

One percent w/v chitin in 0.05 M sodium acetate buffer at pH 6.0 was thermally pre-treated in an autoclave at 121 ºC (204.7 kPa) for time durations ranging between 15 and 60 min. Immediately after pre-treatment, the sample was rapidly cooled under running tap water, and separated into an aqueous solution and a non-soluble solid fraction by centrifugation at 5000 rpm for 15 min.[10] The solid fraction was thoroughly washed with distilled water and then left to dry at 60 ºC for 10 h. The aqueous solution was stored at 4 ºC prior to analysis.[11]

Characterisation of chitin

Reducing sugars

The amount of reducing sugars produced during pre-treatment in the aqueous solution were determined by the dinitrosalicylic acid (DNS) method. The DNS reagent was prepared by mixing 10 g of DNS powder with 300 g of sodium potassium tartrate (Rochelle salt) and 2 N NaOH in 800 mL of distilled water. The mixture was gently heated to dissolve the solids, and the volume was made up to 1 L with distilled water. This method was proposed by Saqib and Whitney[12] but the concentrations of NaOH solutions used in this work were different. Prior to the analysis, the sample was prepared by mixing the aqueous solution with distilled water and DNS reagent at a ratio of 1:1:3 and boiled in a water bath for 10 min.[11] After boiling, the sample was allowed to cool under running tap water. Colour formation was determined by measuring absorbance against blank at a wavelength of 530 nm through UV-Vis spectrophotometer (Cecil CE 7400, Cambridge, UK). The defined absorbance value of the sample was converted to the amount of reducing sugars produced during the reaction using a GlcNAc standard curve.

Crystallinity index (I_{CR}) and crystallite size (nm)

Crystallinity of the dried pre-treated chitin was determined by X-ray powder diffraction (Bruker D8 Advance, Karlsruhe, Germany) with an incident radiation CuKα and λ = 1.5418 Å in the range of 2θ = 4.5-50 with steps of 0.02º (CAF, University of Reading, UK). The method described was identical to the one reported by Villa-Lerma et *al.*[13]

Crystallinity index (I_{CR}) is a quantitative indicator of the crystalline fraction in the chitin material.[14,15] I_{CR} of the samples was determined using intensities of the peaks at [1 1 0] around 2θ = 20º (corresponding to the maximal intensity, I_{110}) and at 2θ = 16º (amorphous diffraction, I_{am}).[2,13]

$$I_{CR}\ (\%) = \left(\frac{I_{110} - I_{am}}{I_{110}}\right) \times\ 100 \qquad (1)$$

Crystallite size (nm) of the chitin at [1 1 0] lattice was calculated by using the Scherrer equation.[2]

$$L = \frac{0.9\lambda}{(H\ cos\ \theta)} \qquad (2)$$

where *L* is the crystallite size perpendicular to the plane; *H* is the full-width at half-maximum of the deflection peak; and *θ* is the Bragg's angle.

CP/MAS ^{13}C-NMR spectroscopy

Structure of the pre-treated solid chitin was determined by carbon nuclear magnetic resonance (^{13}C-NMR) spectroscopy in an AV500 two-channel NMR instrument (Bruker Advance III 500 MHz, Germany) operating at Larmor frequency of 125.77 MHz with the use of 4 mm MAS probe spun at 10 kHz rate (CAF, University of Reading, UK). The spectra were obtained over 1024 number of scans with 10 s relaxation delays at ambient temperature. The CP contact time was 2.0 ms, and the 90º pulse width was 3.7 µs at 38 W power level. All spectra were referenced to

external adamantane signal as a secondary reference (frequency peak at 38.5 ppm with respect to tetramethylsilane (TMS)).

Enzymatic hydrolysis of pre-treated chitin

Enzymatic hydrolysis was conducted by mixing the pre-treated chitin solution, i.e. 1.0% (w/v) chitin in 0.05 M sodium acetate buffer at pH 6.0, with 0.1% (w/v) of enzyme chitinase from *Streptomyces griseus* (Sigma Aldrich, UK). The reaction mixture was incubated in a shaker water bath at 40 ºC for different time intervals. The reaction was stopped by boiling for 10 min to denature the chitinase and centrifuged for 15 min at 5000 rpm. The supernatant was collected and filtered with 0.22 µm pore size of PVDF membrane syringe filter and stored at 4 ºC for HPLC analysis.[2] The filtrate was lyophilized and stored at -20 ºC until further use.

$GlcNAc_2$ composition

The $GlcNAc_2$ composition was determined using high pressure liquid chromatography by an Agilent 1100 Series HPLC System (Agilent Technologies, Italy) equipped with a UV detector, at a wavelength 205 nm. The product was identified through a Nucleosil® NH2 HPLC column, with 5 µm particle size (L × I.D. 25 cm × 4.6 mm), using 70% acetonitrile and 30% ultra-pure water as the mobile phase, at a flow rate of 1.0 mL/min at 50 ºC. A calibration curve was constructed by measuring the peak area of standard $GlcNAc_2$ (Sigma Aldrich, UK). The concentration of the $GlcNAc_2$ in the enzymatic reaction mixture was estimated by

comparison with the calibration standard, and the yield was calculated by the following equation.[2,16]

$$\text{Product yield (\%)} = \frac{\text{Weight of } GlcNAc_2 \text{ produced}}{\text{Weight of chitin used}} \times 100 \quad (3)$$

Mass spectrum of the $GlcNAc_2$ was obtained by liquid chromatography-mass spectrometry (LC-MS) using a Thermo Scientific Accela HPLC system coupled with a Thermo Scientific LTQ Orbitrap XL mass spectrometer. The mass spectrum (m/z) of $GlcNAc_2$ was theoretically calculated by the following equation:[17]

$$\text{Mass spectrum (m/z)} = ([DP \times C_8H_{13}O_5N] + H_2O) + \text{adduct ions} \quad (4)$$

where DP is the degree of polymerisation, $C_8H_{13}O_5N$ is the chitin molecular weight, H_2O is the water molecule attached to the $GlcNAc_2$ after hydrolysis and Na^+ or H^+ the cationised adduct ions transferred from the solvent (0.1% formic acid in 30% water and 70% acetonitrile).

Antimicrobial activity determination

The antimicrobial activity of $GlcNAc_2$ was evaluated following the broth microdilution methodology as described previously.[18] Antibacterial susceptibility tests were performed against the Gram-negative bacterium *E. coli* K-12 and Gram-positive bacteria *L. monocytogenes* 10403S. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of $GlcNAc_2$, in which bacteria where not able to grow after 24 h of incubation.[19]

E. coli and *L. monocytogenes* were grown on Tryptic Soy (TS) and Brain Heart Infusion (BHI) agar respectively, at 37 ºC for 24 h. Agar plates were then stored at 4 ºC for up to 3 weeks prior to use. For the bacterial suspension preparation, 2-3 colonies were picked from each agar plate using a sterile plastic loop. Colonies of *E. coli* and *L. monocytogenes* were transferred to sterile Mueller Hinton (MH) and Tryptic Soy (TS) broth respectively and incubated at 37 ºC for 16-18 h. The optical density (OD) of the suspension was then adjusted to 0.5 McFarland using a UV-Vis spectrophotometer (Orion AquaMate 8000, Thermo Scientific, UK) at 620 nm, which corresponds to approximately 1.5×10^8 CFU/mL. Then, the suspensions were diluted to give a final colony count of 1.5×10^5 CFU/mL and used as inoculum for the determination of the MIC determination in the presence of $GlcNAc_2$.

GlcNAc2 was dissolved in the respective growth medium (MHB or TSB), and the maximum concentration tested was 10% w/v. Two-fold dilutions were performed and 100 µL of each dilution were placed in each column of a flat bottomed 96-well plate (Cellstar, Greiner bio-one Ltd., UK). Column 11 of the 96-well plate contained only the growth medium and, upon inoculation, served as a growth control while column 12 contained 200 µL of the $GlcNAc_2$ tested at its maximum concentration (10 % w/v) and served as a sterility control. In all columns, apart from column 12, 100 µL of each bacterial suspension were added and 96-well plates were incubated at 37 ºC for 24 h. The optical density of the cultures was assessed at 620 nm (OD_{620nm}) after 24 hours using a FLUOstar Omega multi-detection microplate reader (BMG Labtech,

UK) in order to detect the MIC for each case. All experiments were performed in triplicate.

Statistical analysis

Minitab® 18 (USA) statistical software was used for analysis of the difference in means by Tukey's multiple range test. The results were determined by one-way analysis of variance (ANOVA) at a significance level of 0.05 (P<0.05). The pre-treatment was undertaken in duplicate and each replication was run for analysis of crystallite size, crystallinity index, reducing sugar contents and $GlcNAc_2$ yield. The experiments on antimicrobial activity were run in triplicate regardless of pre-treatment replication due to the lyophilisation of $GlcNAc_2$ from the hydrolysate that accumulated after hydrolysis.

RESULTS AND DISCUSSION

Effect of thermal pre-treatment on chitin

The crystallite size is referred to as the size of individual crystal of lattice [1 1 0] inside a chitin particle. The changes in crystallite size of the chitin after pre-treatment is shown in Figure 1. It is evident that the crystallite size did not significantly change when the chitin was exposed to the pre-treatment between 15 and 45 min. The crystallite size of the control, and that after 60 min of pre-treatment, were not significantly different from the chitin that underwent pre-treatment for 15 to 45 min. However, after 60 min of exposure to the pre-treatment, the crystallite size changed

significantly (P<0.05) from 7 nm (control sample) to 9 nm. This increase may be attributed to swelling of the chitin during autoclaving. This observation is consistent with an earlier study which showed that crystallite size increased by almost 30 to 40% when chitin was pre-treated between 300 and 400 ºC for times up to 5 min.[2,20] These authors reported that the crystallite size increased with autoclaving time initially, but then went through a turning point. They also noted that chitin swelling occurred due to increased spacing of the [1 1 0] planes of the individual cell lattice.[2]

The crystallinity index (I_{CR}) was used to quantify the relative amount of crystalline material in chitin.[21] The I_{CR} of chitin, reported in Table 1, shows no significant differences between control and various pre-treated samples. The absence of decrystallisation suggests that the chitin structure survives 60 min of autoclaving. In a previous study, by Osada et *al*.[20], it has been reported that the decrystallisation of the crystalline region did not occur even at higher temperature between 180 and 400 ºC unless the chitin was subjected to mechanical grinding and the I_{CR} was reduced to 27%. Nishino et *al*.[22] also reported that the molecular chain of *α*-chitin was mechanically stable against heating at 150 ºC.

The reducing sugar content of chitin aqueous solution seems to increase very marginally after pre-treatment at 121 ºC between 15 and 60 min as shown in Table 1. No reducing sugar was detected in the starting sample. This suggests that the pre-treated aqueous solution may contain some partially degraded chitin polymers. The oligomers ($GlcNAc_{1\text{-}10}$) were not detected in the aqueous solution by HPLC

(data not shown), indicating that the chitin degraded to some extent due to the action of free aldehyde group at reducing end (carbon C1) accepting hydrogen atoms from the water molecule during pre-treatment.[23,24] Villa-Lerma et *al*.[13] observed that the highest reducing sugar contents of chitin in the water was detected after 1 h exposure to the steam explosion at 240 ºC. In addition to steam explosion, there are a few studies on chitin pre-treatment that employed high temperatures between 180 and 400 ºC and have concluded that these methods could lead to partial hydrolysis of chitin.[2,13,20,25]

Solid state ^{13}CP/MAS NMR shown in Figure 2 was performed in order to observe the changes occurring in chitin structure during pre-treatment. There are eight signals for eight carbon atoms of *N*-acetyl-D-glucosamine residue observed between 20 and 110 ppm which correspond to C1-C6, C=O and CH_3. The spectra shows that the samples possess high structural homogeneity as the peaks C1 (104 ppm), C2 (55.1 ppm), C3 (73.4 ppm), C4 (83.2 ppm), C5 (75.6 ppm) and C6 (60.8 ppm), which remain unchanged with autoclaving. On the other hand, the C=O and CH_3 peaks at 173.6 and 22.6 ppm respectively, were less pronounced above 15 min of autoclaving, which shows enhanced deacetylation while transforming to chitosan. It has been reported previously that, the higher the deacetylation, the lower the intensity of C=O and CH_3 peaks due to removal of the acetyl group.[26,27] Kasai[27] reported that the peaks of carbonyl and methyl groups of the chitin obtained by ^{13}CP/MAS NMR became less pronounced as the degree of acetylation (DA) decreased from 1.0 to 0.58. According to Villa-Lerma et *al*.[25], pre-treatment with

steam explosion at 180 ºC for 8 min was sufficient to decrease the degree of acetylation of the chitin from 98 to 92 %. Furthermore, it has been reported that the use of elevated temperature and pressure also enhanced deacetylation as these conditions promote acidic hydrolysis by the formation of acetic acid from acetyl groups.[13,28]

In this present study, the pre-treatment time employed has been limited to 60 min in order to avoid significant formation of chitosan by deacetylation, which can potentially lower the yield of $GlcNAc_2$ after hydrolysis.

Enzymatic hydrolysis of chitin

Enzymatic hydrolysis of chitin, with chitinase from *Streptomyces griseus*, mainly produced $GlcNAc_2$ and a relatively small amount of GlcNAc which is shown in Figure 5. Given the relatively small concentrations of the monomer, further discussion and indeed figures will only focus on the $GlcNAc_2$ levels as this compound is the dominant product of hydrolysis. As shown in Figure 3, the HPLC chromatogram of the standard $GlcNAc_2$ was comparable with the hydrolysate sample obtained by 24 h hydrolysis at 40 ^{o}C of the chitin autoclaved for 60 mins (i.e. PC-60 referred earlier). The retention time of the standard $GlcNAc_2$, as well as the sample obtained in this work, corresponded to the peak visible after 4.2 min. The chromatogram of the chitin hydrolysate detected by HPLC was also confirmed by the LC/MS mass spectra shown in Figure 4. The $GlcNAc_2$ spectrum detected at m/z 425.17 and 447.15 corresponds to the chitin dimer cationised by proton (H^+) and sodium (Na),

respectively. Moreover, the spectrum shows that the GlcNAc (222.09 m/z) and $GlcNAc_2$ were the only compounds detected in the hydrolysate of the chitin pre-treatment PC-60, and the absence of the higher oligomers. These results are consistent with an earlier study by Osada et *al*.[2], who employed a similar chitinase, i.e from *Streptomyces griseus*, to hydrolyse chitin into monomer and dimer of GlcNAc. Thus, the present, and indeed previous studies, suggest that the hydrolysis occurred by the following steps: (1) random cleaving to form the intermediate oligomers, i.e trimer ($GlcNAc_3$) and tetramer ($GlcNAc_4$) and (b) breaking down of the intermediate oligomers into GlcNAc and $GlcNAc_2$.[29]

The concentration of GlcNAc and $GlcNAc_2$ obtained by the hydrolysis of the control and PC-60 pre-treated chitins were monitored between 12 and 48 h by HPLC. Figure 5 shows that the concentration of $GlcNAc_2$ in the hydrolysate obtained from PC-60 reached a maximum value after 24 h, whereas the concentration in the hydrolysate obtained from the control sample reached similar values after 36 h. The higher hydrolysis rate observed in the case of the pre-treated chitin may be attributed to the partial degradation and swelling of the chitin caused during pre-treatment, which also improves enzyme accessibility. Osada et *al*.[2] also reported swelling of chitin pre-treated at 400 ºC for 1 min which resulted in a significantly higher formation of $GlcNAc_2$ after hydrolysis. Thus, thermal pre-treatment involving autoclaving in steam at a pressure of 204.7 kPa at 121 ºC for 60 min, accelerated chitin hydrolysis with its crystalline structure preserved.

Effect of $GlcNAc_2$ on antimicrobial properties

Growth of *E.coli* and *L. monocytogenes* were significantly inhibited by the presence of $GlcNAc_2$ as shown in Figure 6 and Figure 7, respectively. The growth of these bacteria was found to be completely inhibited with the addition of 5% and 10% w/v of $GlcNAc_2$ respectively, which represent their respective MIC.

The inhibitory effect on Gram-negative and Gram-positive bacteria seems to increase with the concentration of $GlcNAc_2$. It has to be noted here that there has been only one study that reported the antimicrobial activity of the chitin dimer $GlcNAc_2$, compared to studies performed on the mixture of chitin and chitosan oligomers.[30] For example, molecules composed of dimer to hexamer (GlcNAc—GlcN)$_{2\text{-}6}$, which have molecular weights greater than monomer and dimer, are more likely to exert antimicrobial activity.[5,30–32] Benhabiles et *al*.[3] and Raut et *al*.[4] found that *E. coli* was completely inhibited when treated with 0.1% (w/v) chitin and chitosan oligomers as well as chitin monomer. Low molecular weight chitosan oligomers have also been reported to effectively inhibit the bacteria.[33] No et *al*.[33]observed that 1.0% w/v of 1 kDa chitosan oligomers were more effective in inhibiting Gram-negative bacteria, including *E. coli*, compared to higher molecular weight oligomers. Benhabiles et *al*.[3] also reported that the chitin and chitosan oligomers possess higher antibacterial activity with the MIC values being much lower than their native compounds. However, it has been argued by Kittur et *al*.[5] that the chitosan monomer, GlcN, has lower antimicrobial activity than its hexamer, $GlcN_6$. This

indicates that the antimicrobial activity of the oligomers is dependent on their molecular weight. The higher the molecular weight of the oligomers, the greater its antimicrobial activity in the case of Gram-negative bacteria.

Studies on the inhibitory effect of the chitin against *L. monocytogenes* are very limited because of its functionality on the chitinolytic system. This bacteria possesses two chitinases, ChiA and ChiB, which can convert the chitin oligomers into monomers.[34,35] Chitosan has been reported to be more active than its oligomers in inhibiting *L. monocytogenes* Scott A, having an MIC at 0.1% (w/v) in the presence of acetic acid after 24 h of incubation.[33] In that study, 1% acetic acid had been used as the chitosan solvent and was proven effective in inhibiting the growth of several Gram-negative and Gram-positive bacteria, apart from lactic acid bacteria that were more effectively suppressed when 1% lactic or formic acid was used. Chitin and chitosan are insoluble in water, therefore, strong inorganic and organic acids respectively, are required to dissolve these compounds.[3,4,33] According to Bjarnsholt et *al*.[36], 0.5% acetic acid in AB minimal medium resulted in a pH equal to 4.33 that led to complete killing *Pseudomonas aeruginosa* PAO1. It was also revealed that the pH had a significant influence on the effectiveness of acetic acid as an antimicrobial agent.

In the present study, the mechanism of bacterial inactivation may be attributable either to (1) its ability to bind to and weaken the barrier function of the cell wall, followed by the destruction of the cell membrane; or to (2) the deposition of the

$GlcNAc_2$ on the cell wall thereby blocking the flow of nutrients into the cell.[5,37,38] In addition, the MIC of the $GlcNAc_2$ against both Gram-negative and Gram-positive bacteria obtained in the present study cannot be directly compared to previously reported studies, in terms of the antibacterial activity of chitin and its derivatives, due to the variations between the degree of polymerisation, degree of acetylation and the presence of other functional group.[32]

CONCLUSION

In this study, the accessibility limitation of the β-glycosidic bonds in the crystalline chitin for enzymatic reactions was overcome by employing thermal pre-treatment in an autoclave prior to hydrolysis with chitinase from *Streptomyces griseus*. Pre-treatment of chitin at 121 ºC for 60 min was found to be effective for substrate modification prior to enzymatic hydrolysis. The crystallite size of chitin increased and deacetylation occurred as the pre-treatment residence time increased. The hydrolysis of pre-treated chitin with chitinase was completed within 24 h, showing that the proposed condition for pre-treatment accelerated the enzymatic reaction. The dominant $GlcNAc_2$ produced has advantages regarding its application as an antimicrobial agent since it is more readily soluble in water. The $GlcNAc_2$ produced in this study exhibited strong antimicrobial activity against *E. coli* and *L. monocytogenes*, showing MIC values of 5 and 10% w/v, respectively.

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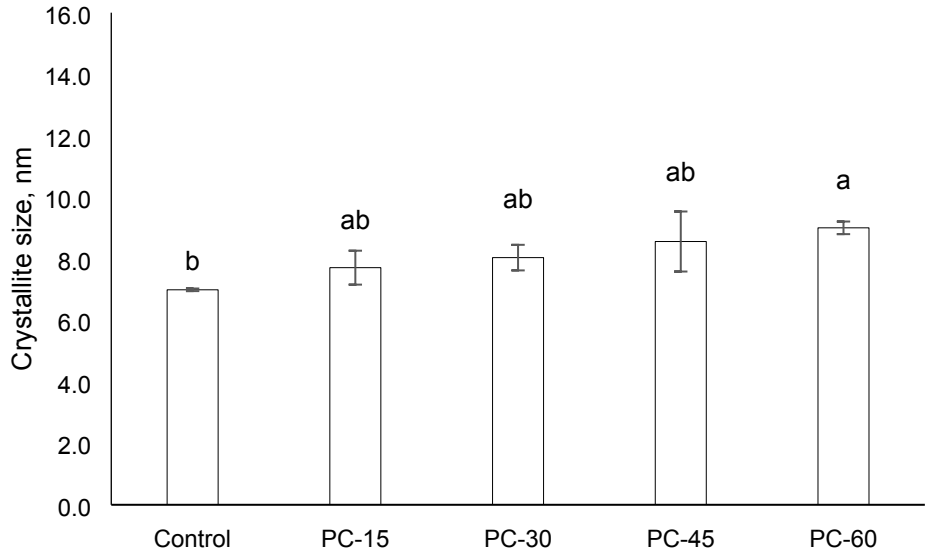

Figure 1. Crystallite size (nm) of chitin in sodium acetate buffer (pH 6.0) after autoclaving at 121 °C for time durations between 15 and 60 min. Control represents non-autoclaved chitin in the same solution. Different letters above each bar indicate significant differences (P<0.05) between pre-treatment conditions.

Table 1. Effect of autoclaving chitin at 121 ºC (204.7 kPa) for various times on the reducing sugar content and crystallinity index (I_{CR}).

Sample	Time (min)	Reducing sugars (mg/mL)	I_{CR} (%)
Control	-	ND	74.534 a
PC-15	15	0.005 b	75.394 a
PC-30	30	0.003 b	73.933 a
PC-45	45	0.006 b	74.182 a
PC-60	60	0.010 a	74.894 a

*ND, not detected
** Different letters in each column indicate significant differences (P<0.05) among pre-treatment conditions

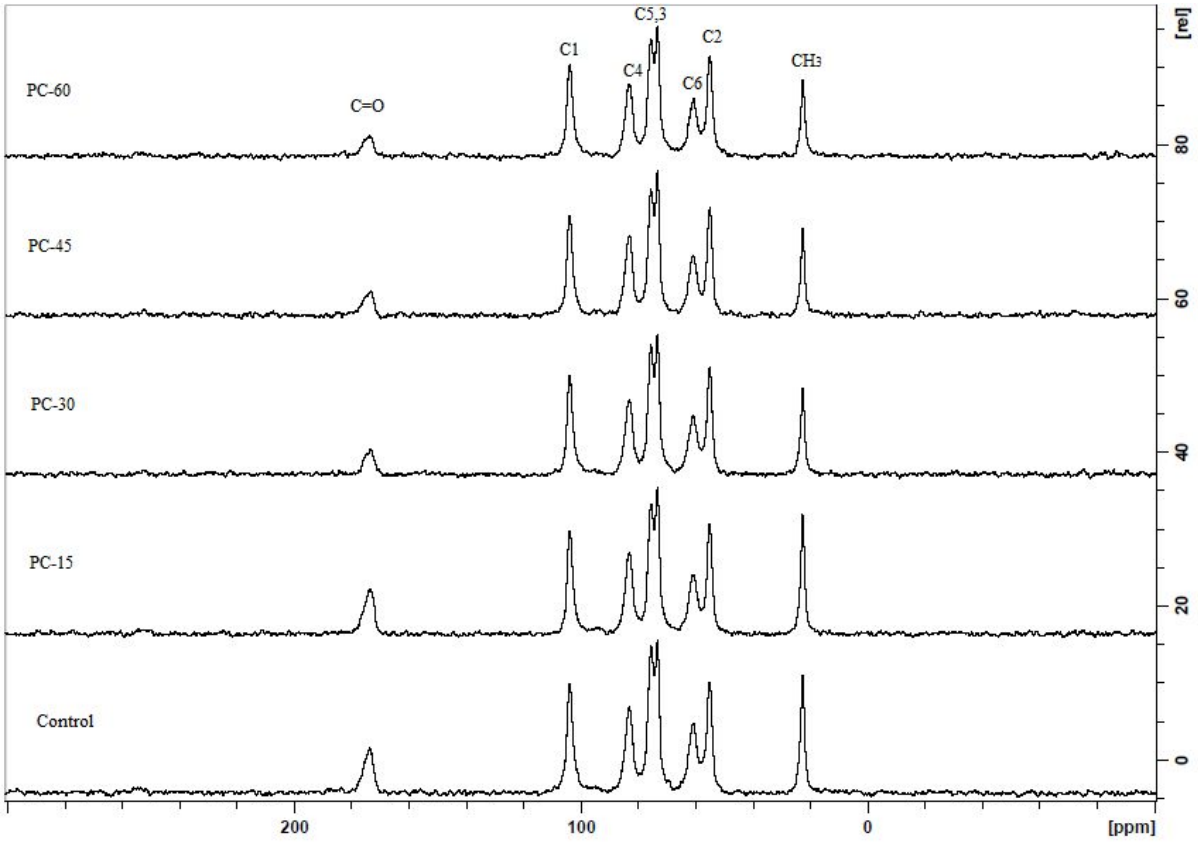

Figure 2. ^{13}CP/MAS NMR spectra of control and thermal pre-treated chitin in sodium acetate buffer with pH 6.0 at 121 ºC between 15 and 60 min.

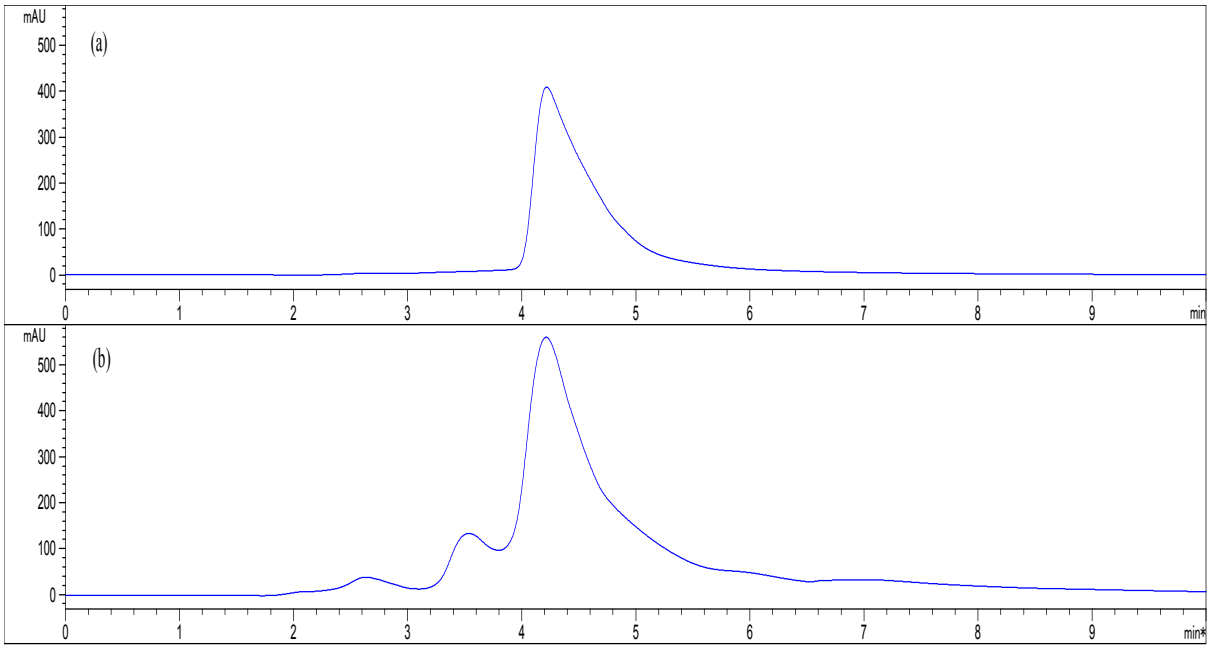

Figure 3. HPLC chromatography of (a) standard $GlcNAc_2$ and (b) sample $GlcNAc_2$ obtained in this study (i.e. hydrolysate obtained by reacting pre-treated chitin sample, i.e. PC-60, with chitinase from *Streptomyces griseus* at 40 ºC for 24 h).

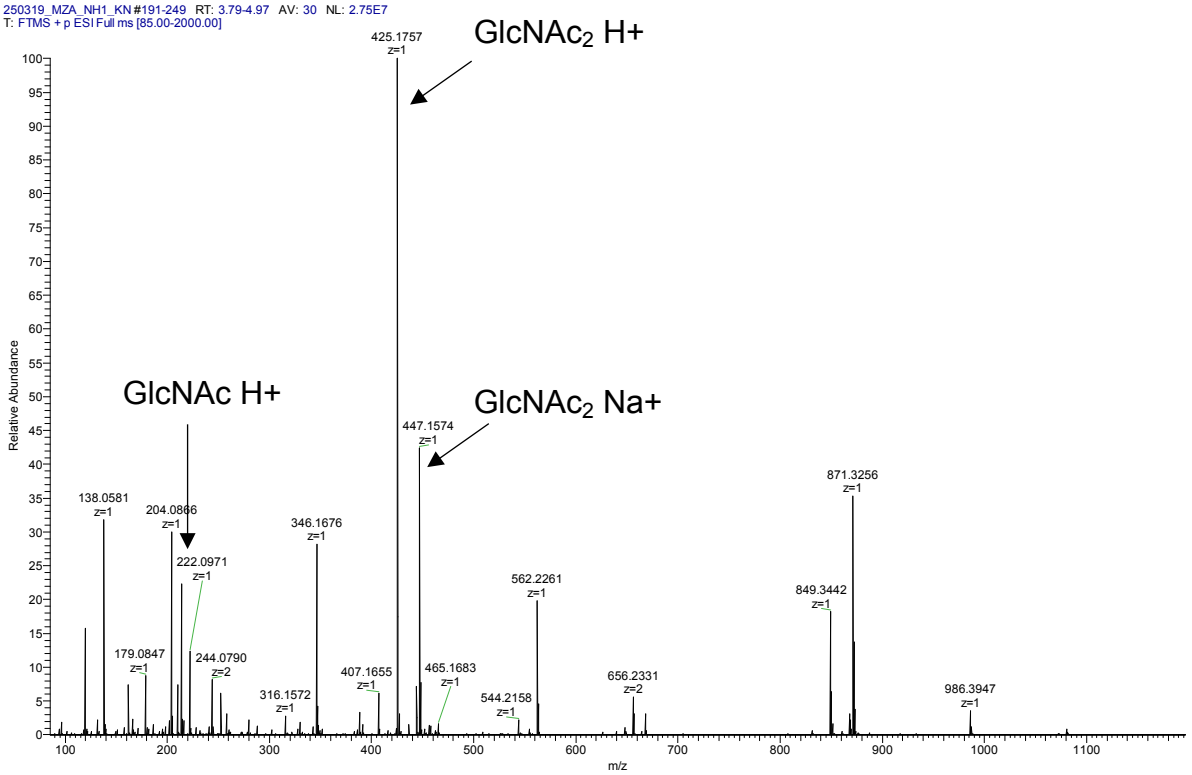

Figure 4. Mass spectra obtained by LC/MS of the hydrolysate formed from PC-60 after hydrolysis at 40 ºC for 24 h by chitinase from *Streptomyces griseus*.

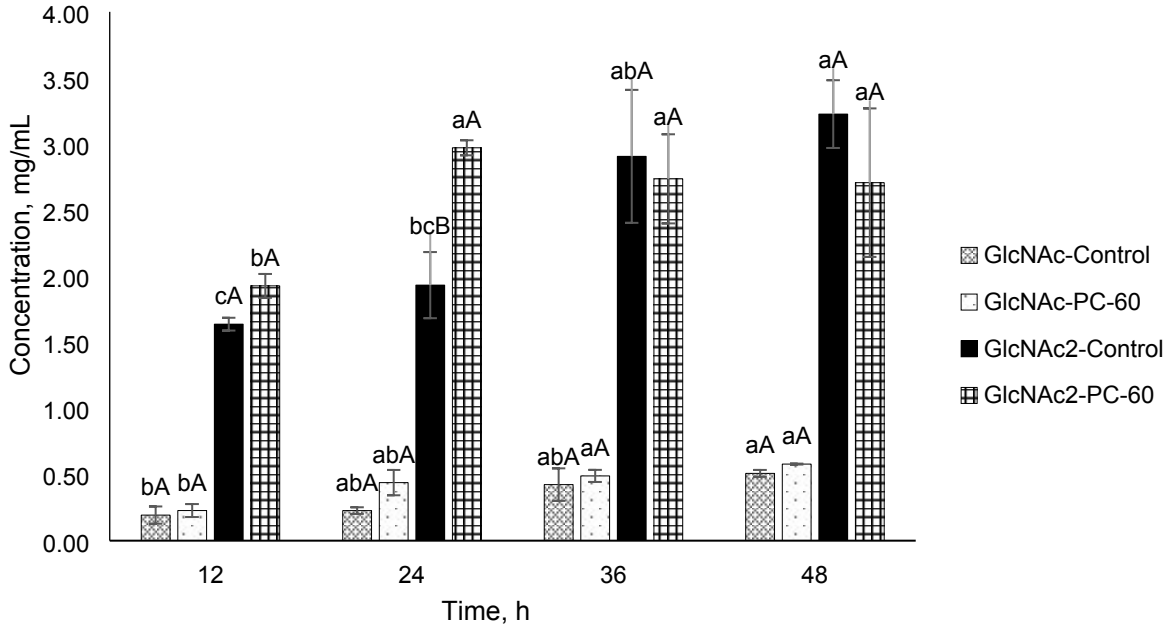

Figure 5. Time course of enzymatic hydrolysis of pre-treated (PC-60) and untreated (control) chitin with chitinase from *Streptomyces griseus* at 40 ºC. Different small and capital letters indicate significant differences (P<0.05) between incubation time (h) and pre-treatment conditions of each compound, respectively.

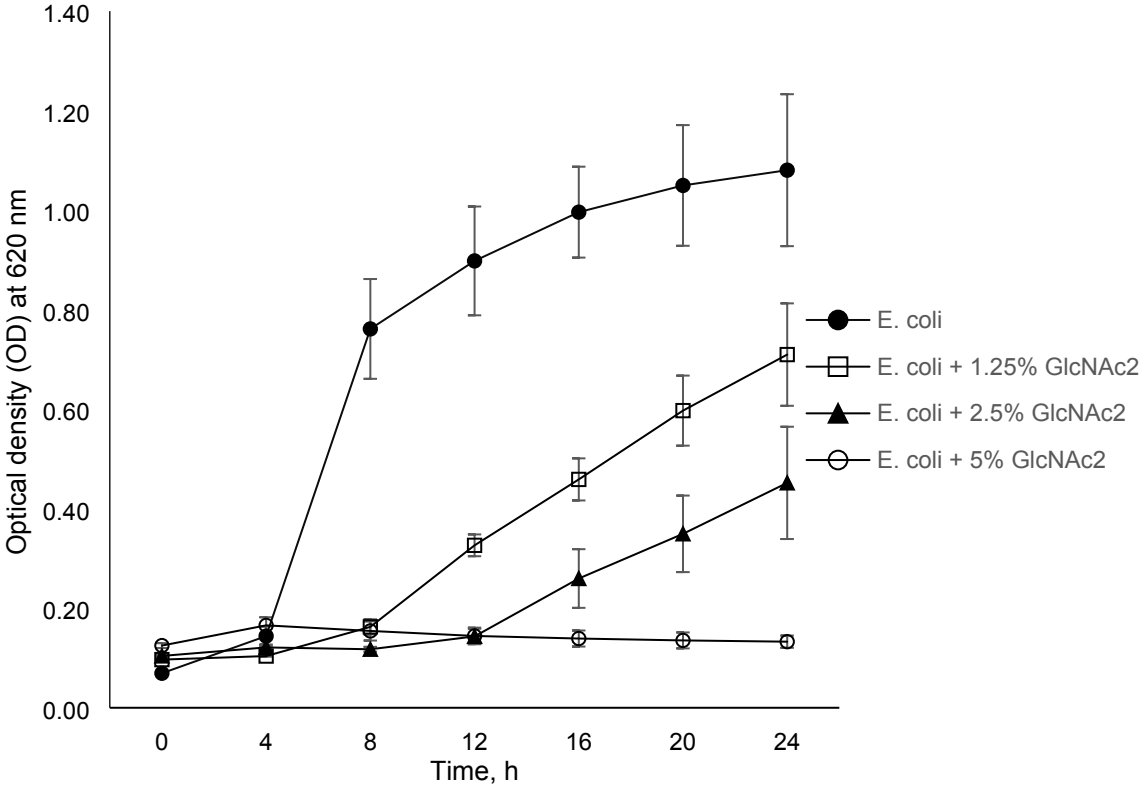

Figure 6. Effect of $GlcNAc_2$ on growth of *E. coli* K-12 at 37 ºC for 24 h.

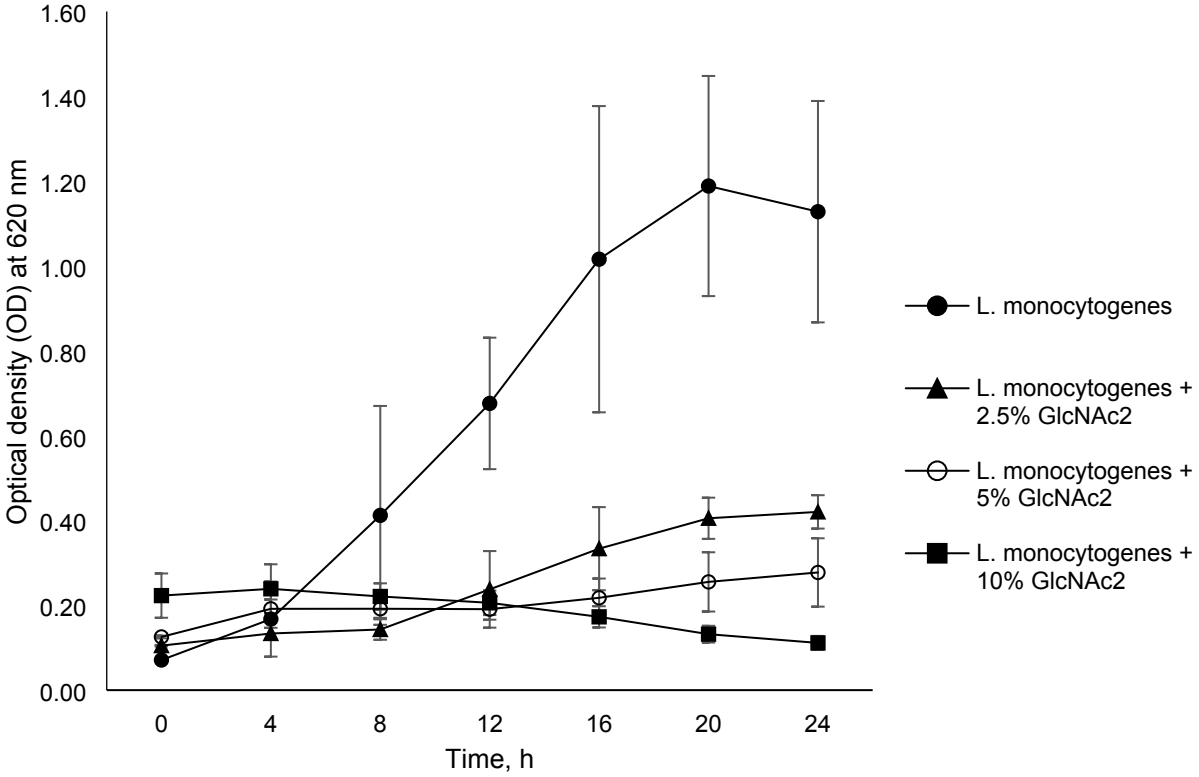

Figure 7. Effect of $GlcNAc_2$ on the growth of *L. monocytogenes* 10403S at 37 ºC for 24 h.