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Low-cost production of chitosanolytic enzymes from *Lentzea* sp. strain OUR-I1 for the production of antimicrobial substances against food-borne pathogens

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Abstract

Bioactive compounds derived from chitosan are exploited in various applications, and enzymatic hydrolysis of chitosan substrate using chitosanases is a promising approach to prepare these compounds. However, low yield and high cost of enzyme production still limit its use. The use of agricultural residues is an alternative to reduce the enzyme production costs. The present work aimed to use shrimp shell as a cheap substrate for the production of chitosanase, and to apply the obtained enzyme in producing antimicrobial compounds. The isolated *Lentzea* sp. strain OUR-I1 was able to use shrimp shell waste as a nutrient source and exhibited high chitosanase activity. Although shrimp shell powder (1.5%, w/v) could be used as the main carbon source in producing chitosanase, the addition of chitosan powder (0.05%, w/v) as an inducer substantially increased chitosanase activity. Other factors involved in the production of chitosanase were also optimised. Under these optimised conditions, the obtained amount of chitosanase increased by about 17-fold from 0.14 ± 0.006 to 2.4 ± 0.038 U/mL. Additionally, the partially purified chitosanase could effectively hydrolyse insoluble forms of chitosan into a hydrolysate mixture which in turn exhibited antimicrobial activities against a broad spectrum of food-pathogenic bacteria. The present work proposes a cost-efficient and environmentally friendly approach to produce chitosanase by a newly isolated bacterial strain, and demonstrates high potential of the obtained enzyme in the preparation of antimicrobial compounds.

Introduction

Chitosanases are hydrolytic enzymes that cleave β-1,4-glycosidic bonds in chitosan. The enzymes could be classified as endo-chitosanases (EC 3.2.1.132) and exo-chitosanases (EC 3.2.1.165) based on the mechanism of cleavage and the end-products of hydrolysis. While the former randomly splits chitosan to generate chitooligosaccharides (COS), the latter continually hydrolyses chitosan substrate from the non-reducing ends to release D-glucosamine (GlcN) (Thadathil and Velappan, 2014). Recently, chitosanases have attracted extensive interest due to their potential applications in preparing hydrolysis products such as COS and GlcN that are well-known bioactive compounds.

There is a huge demand for chitosan-derived bioactive compounds in various applications (Kim and Rajapakse, 2005) . For instance, COS has been used as an immune activator (Tokoro *et al.*, 1989), a food additive (Kim and Rajapakse, 2005), an antioxidant compound (Sinha *et al.*, 2012), and an antimicrobial agent (Wu, 2012; Li *et al.*, 2014). In addition, GlcN has also attracted much attention owing to its beneficial effects, including healing of skin wounds (McCarty, 1996), having antibacterial effects (Lippiello, 2003; Rozin, 2009), and promoting bone regeneration (Uğraş *et al.*, 2013). A cost-effective and high-quantity method for industrial production would be desirable to meet this demand for these bioactive compounds.

Unfortunately, the current production methods have several limitations. At industrial scale, the degradation of chitosan to obtain bioactive

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compounds, COS and GlcN, could be achieved by a chemical method through acid hydrolysis. This is cost-effective and preferable for large-scale production. However, regarding sustainable development, the strong acids involved in processes of hydrolysis might be toxic and harmful to the environment (Nidheesh *et al.*, 2015a). Meanwhile, the enzymatic hydrolysis of chitosan can be easily carried out by microbial chitosanases under mild conditions with high yields of specific products (Sinha *et al.*, 2016), but this requires a reliable method for the isolation and purification of chitosanase.

Several microorganisms have been isolated to generate chitosanases, including bacteria such as *Bacillus* sp. (Lee *et al.*, 2006; Su *et al.*, 2006; Wee *et al.*, 2009; Liang *et al.*, 2015), *Paenibacillus* sp. (Zitouni *et al.*, 2013), *Streptomyces* sp. (Jiang *et al.*, 2012; Sinha *et al.*, 2014), and fungi such as *Aspergillus* sp. (Zhang *et al.*, 2015) and *Penicillium* sp. (Nguyen *et al.*, 2014; Nidheesh *et al.*, 2015a). In addition, some intracellular chitosanases have been found in *Mucor* sp. (Reyes *et al.*, 1985; Struszczyk *et al.*, 2009). These microorganisms commonly utilise soluble (Chen *et al.*, 2005) or colloidal chitosan (Shimosaka *et al.*, 1995; Zhou *et al.*, 2008; Pagnoncelli *et al.*, 2010) as the major carbon sources for inducing enzyme synthesis. Nevertheless, the preparation of chitosan inducers is an expensive and time-consuming process. Furthermore, it has been widely reported that the process of converting shellfish wastes to chitosan requires high concentration of chemicals and energy to remove minerals, proteins and acetyl groups (Du *et al.*, 2009; Benhabiles *et al.*, 2012). This highlights the need for a low-cost and environment-friendly approach to produce microbial chitosanase.

Various studies have used bio-waste as a nutrient source for chitosanase production, bypassing the direct use of chitosan. For example, shrimp shell has been used as nutrition source to generate chitosanase enzymes from *Pseudomonas* sp. TKU015 (Wang *et al.*, 2008b), *Bacillus cereus* TKU018 (Wang *et al.*, 2009a), *Serratia marcescens* subsp. *sakuensis* TKU019 (Liang *et al.*, 2010), and *Purpureocillium lilacinum* CFRNT12 (Nidheesh *et al.*, 2015b). However, many aspects involved in these methods need to be addressed. These include isolating and screening potential microorganisms that possibly utilise by-products as nutrient elements, as well as optimising the bioprocess to produce chitosanases efficiently.

The present work aimed to screen and isolate microorganisms that could produce chitosanase on a bio-waste medium. Among the isolated strains, the strain OUR-I1, which was identified as a member of the genus *Lentzea*, generated the highest amount of chitosanases in the medium supplemented with by-product, and was selected for further studies. The conditions for the production of chitosanase by this strain were optimised. Using insoluble chitosan as substrate, the obtained enzyme was also exploited to produce bioactive compounds, which were further examined for their antimicrobial effects against food-pathogenic bacteria.

Materials and methods

Materials

D-glucosamine HCl (GlcN), chitosan powder (> 75% deacetylated), N-acetyl-β-D-glucosamine (GlcNAc), 3,5-dinitrosalicylic acid (DNS), and L-tyrosine were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Shrimp shells (*Penaeus monodon*) were obtained from Chotiwat Manufacturing Co. Ltd. (Songkhla, Thailand). The food-borne pathogens, including *Bacillus cereus* DMST 5040, *Escherichia coli* DMST 4212, *Staphylococcus aureus* DMST 8840, *Salmonella Typhimurium* DMST 15674, and *Salmonella enteritidis* DMST 15676, were kindly provided by the Department of Industrial Biotechnology, Prince of Songkla University. All other reagents were of analytical grade.

Preparation of shrimp shell powder (SSP)

Shrimp shells were cleaned with water twice to get rid of impurities. After drying at 55°C for 12 h, the shrimp shells were blended and subsequently sieved through a 22-mesh sieve (Suresh, 2012). This shrimp shell powder (SSP) was directly utilised as a nutrient source in chitosanase production.

Preparation of chitosan substrates

One gram chitosan powder (CP) was dissolved in 70 mL 0.1 M acetic acid, and constantly stirred until it was completely dissolved. Soluble chitosan (SC) and colloidal chitosan (CC) solution were obtained by adjusting the pH to 5.5 (Choi *et al.*, 2004) and to 7.0 (Kurakake *et al.*, 2000), respectively, using 6 M NaOH. Then, the solutions were topped up to 100 mL with distilled water.

Isolation and screening of chitosanase-producing microorganisms

Soil samples collected from different areas in Thailand and Vietnam were used as the sources to isolate microorganisms. Each soil sample (1 g) was individually mixed into 10 mL sterilised NaCl (0.85%, w/v). Subsequently, 0.5 mL of the suspension

was transferred into chitosan powder mineral medium (CPMM), which contained CP (0.25%, w/v), $(NH_4)_2SO_4$ (0.1%, w/v), KH_2PO_4 (0.1%, w/v), K_2HPO_4 (0.1%, w/v), $MgSO_4 \cdot 7H_2O$ (0.03%, w/v), and NaCl (0.07%, w/v). Initial pH of the medium was adjusted to 5.5 in order to isolate fungi and actinomycetes; and to 7.0 in order to isolate bacteria. Colloidal chitosan mineral medium (CCMM) and soluble chitosan mineral medium (SCMM) were prepared with similar components to CPMM, but the CP was replaced by CC or SC, respectively. The chitosan-assimilating microorganisms were enriched at room temperature for 1 w. Then, 0.1 mL of the culture medium was diluted with sterilised NaCl (0.85%, w/v) and spread onto CPMM plate, followed by 5-day incubation at room temperature. The colonies that grew on CPMM plates were sub-cultivated on CPMM agar plates until a single colony was obtained.

In a primary screening, the obtained isolates were examined for their abilities to produce chitosanases by examining chitosanolytic activity on soluble chitosan mineral medium (SCMM) plate. The isolates that express chitosanolytic activity would form clear zones around their colonies; hence, such isolates were selected for a secondary screening.

Each isolate chosen from the primary screening was inoculated into 5 mL CCMM broth for the secondary screening. After a 3-day incubation, the supernatant of cell culture was separated by centrifugation at 10,000 *g* for 10 min and used as crude enzyme for the determination of chitosanolytic activity. The strains that produced a comparatively high level of chitosanase activity were selected for the next step of examining chitosanase productivity using by-products as the nutrient sources. In this stage, the selected strains were grown in a medium containing SSP (0.25%, w/v) as the carbon source, at room temperature for 3 d. Then, the supernatants were harvested by centrifugation and used to assay chitosanase activity. The isolate that produced the highest chitosanase activity on the by-product medium was chosen for further study.

Identification of the strain OUR-I1

The selected strain at final screening step, named OUR-I1, was identified by its morphological characteristics under a microscope (Nikon Eclipse E100). Briefly, this strain was cultivated on YM plate containing glucose (1.0%, w/v), peptone (0.5%, w/v), yeast extract (0.3%, w/v), malt extract (0.3%, w/v), and agar (1.5%, w/v) at room temperature for 2 d. Subsequently, a colony was Gram-stained and examined under the microscope. In addition, this strain was further identified by analysing its 16S ribosomal DNA (16S rDNA). The primer pairs used for PCR reaction were UFUL (5'-GCC TAA CAC ATG CAA GTC GA-3') and 802R (5'-TAC CAG GGT ATC TAA TCC-3'). DNA sequences of the purified PCR product were analysed by Automate DNA Sequencer (3100-Avant Genetic Analyzer, ABI). Bioedit software was used to edit the DNA sequences. A homology search for the 16S rDNA sequences was done with the Basic Local Alignment Search Tool (BLAST). Alignment similarities and phylogenetic tree were created by ClustalX.

Preparation of inoculum

The strain OUR-I1 was grown on YM plate at room temperature for 3 d and subsequently transferred into 25 mL YM broth. Then, the strain was cultivated under shaking (150 rpm) at room temperature for 30-36 h. The cell culture was used as inoculum when its optical density at 600 nm (OD_{600}) reached 1.0. Inoculum (1%, v/v) was inoculated into the medium for enzyme production.

Optimisation of chitosanase production by Lentzea sp. strain OUR-I1

Cell culture at OD_{600} of 1.0 was used as inoculum. Inoculums of various sizes (1 to 10%, v/v) were inoculated into 100 mL of medium containing 0.5% (w/v) carbon sources, 0.1% (w/v) nitrogen sources, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) K_2HPO_4, 0.03% (w/v) $MgSO_4 \cdot 7H_2O$, and 0.07% (w/v) NaCl. Apart from varying the size of inoculum, the effects of different carbon sources and nitrogen sources were also explored. The carbon sources used were CC, CP, SSP, and SSP+CP, while the nitrogen sources were $(NH_4)_2SO_4$, KNO_3, NH_4Cl, urea, tryptone, peptone, and yeast extract. In addition, the pH of culture medium was varied from 4.5 to 7.0. Moreover, the effects of SSP at various concentrations (0.5-2%, w/v) as the major carbon source, and of CP (0.005-0.1%, w/v) as the inducer, were investigated. The activity of chitosanase in the supernatant under these conditions was assayed after 5-day incubation at 30°C on orbital shaker at 150 rpm.

Production and partial purification of chitosanolytic enzyme from Lentzea sp. strain OUR-I1

Chitosanase was produced by strain OUR-I1 under earlier optimised conditions. The supernatant obtained after centrifugation at 10,000 *g* for 10 min was precipitated by chilled acetone with the volume ratio 3:1 of acetone to crude enzyme. After incubation at -20°C for 6 h, the precipitated enzyme was dissolved in chilled acetate buffer (10 mM, pH

5.0), and dialysed against the same buffer at 4°C (Sinha *et al.*, 2016). The obtained dialysate was used as partially purified chitosanase for the hydrolysis of chitosan.

Enzymatic hydrolysis of chitosan

Chitosan powder and colloidal chitosan were used as substrates to study the hydrolytic activity of partially purified chitosanase. The reaction mixture contained chitosan substrates, partially purified enzyme (1 U/mL), and acetate buffer (50 mM, pH 5.5). The hydrolysis reaction was carried out at 37°C under shaking at 100 rpm. Aliquots were sampled at different time-points. The hydrolysis reaction was terminated by boiling for 10 min. After centrifugation at 10,000 *g*, 4°C for 10 min, the amount of reducing sugar generated in the supernatant was determined by the DNS method (Miller, 1959).

Optimisation of chitosan hydrolysates production

The production of chitosan hydrolysates was performed by using chitosanolytic enzymes generated by the *Lentzea* sp. strain OUR-I1. The variables that influence the activity of chitosanase could directly affect the yield of chitosan hydrolysates. Therefore, the variables involved in the enzymatic reaction were optimised to maximise the yield of hydrolysates. To assess the effects of temperature on hydrolytic reaction, the reaction mixture of CP (0.5%, w/v), partially purified chitosanase (1 U/mL), and acetate buffer (50 mM, pH 5.5) were incubated at various temperatures from 30 to 60°C for 1 h.

The effects of pH on hydrolytic reaction were also investigated. To this end, the pH of the reaction mixture was adjusted to different values using different buffers, including acetate buffer (for pH 4.0 to 5.0), sodium citrate buffer (for pH 5.0 to 6.0), and potassium phosphate buffer (for pH 6.0 to 8.0). Then, the reaction mixture was incubated in 50 mM buffers at the optimised temperature obtained from the experiment described above for 1 h. Chitosan concentration was also varied (0.5-4.0%, w/v). The products of chitosan hydrolysis were harvested by centrifugation at 10,000 *g* for 15 min, freeze-dried, and kept at -20°C until further use. The extent of chitosan hydrolysis is correlated with the quantity of reducing sugars produced. Thus, the yield was calculated with a previously described equation (Sinha *et al.*, 2016).

$$\text{Production yield}(\%) = \frac{\text{Released reducing sugar (g)}}{\text{Chitosan substrate (g)}} \times 100 \quad \text{(Eq. 1)}$$

Antibacterial activity of chitosan hydrolysate

The lowest concentration of antimicrobial compound that inhibits the growth of a test microorganism by 90% after 20-24 h incubation was considered as the minimum inhibitory concentration (MIC_{90}) in the present work. Microdilution method was used to vary the concentration of chitosan hydrolysis products from 0.5 to 10 mg/mL. The test pathogens were cultivated on Tryptone Soy broth at 37°C overnight. An inoculum of pathogenic bacteria was monitored to be equivalent to a turbidity of 0.5 McFarland standard. Subsequently, 100 µL of the test bacteria suspension was transferred into each well that contained 100 µL of serially diluted chitosan hydrolysis products. The cell growth after 24 h incubation at 37°C was monitored by measuring the OD_{600} of the culture media. The wells exhibiting no visible turbidity were spread onto Müeller Hinton agar (MHA) plates. After further incubation at 37°C for 24 h, the number of colonies on the plate was counted to determine the minimum bactericidal concentration (MBC). The MBC was defined as the lowest concentration of antimicrobial compound that inhibits the growth of a test microorganism, resulting in no colony on the MHA plates. Sterilised NaCl solution (0.85%, w/v) was used as the negative control, while GlcN and GlcNAc were also used to test the antimicrobial effects of monomeric GlcN and GlcNAc, respectively.

Thin layer chromatography (TLC)

A TCL analysis of the hydrolysates was carried out by spotting on silica plate (Silica gel 60; Millipore) and developing with a mixture of n-propanol and 25% ammonia (volume ratio of 2:1). The compositions of hydrolysates were visualised by staining with 0.2% (w/v) ninhydrin reagent dissolved in butanol, and heating at 105°C for 5 min.

Mass spectrometry (MS)

The compositions of hydrolysates were also analysed by mass spectrometry (AccuTOF™ JMS-T100 LC, Jeol, Japan). Briefly, the hydrolysates were directly injected into the mass spectrometer equipped with an ESI source and a time-of-flight (TOF) mass analyser in positive ion mode. The source voltage was set at 2,000 V, the orifice voltage at 85 V, the desolvation chamber temperature at 250°C, and the orifice temperature at 80°C. MS scans were acquired over the m/z range 100-1,000.

Assay of chitosanase activity

The activity of chitosanase was measured based on the method of Miller (1959). Briefly, the reaction

mixture contained CC (0.5%, w/v), acetate buffer (50 mM, pH 5.5), and an appropriate amount of enzyme. Then, the reaction was allowed to take place at 37°C for 1 h, and terminated by immersing in boiling water for 10 min. The insoluble particles were removed by centrifugation at 10,000 *g* for 10 min, and the supernatant was used to measure the amount of reducing sugar generated by incubating with 1% (w/v) DNS reagent (Miller, 1959). Finally, the absorbance of the reducing sugar was read at 540 nm. Heat-deactivated enzymes were used as the control. One unit of chitosanase was defined as the amount of enzyme that liberates one µmole of reducing sugar (as GlcN) per minute.

Determination of chitinase and protease activities

Chitinase activity was measured using colloidal chitin as substrate and GlcNAc as standard, following the previously published method (Miller, 1959). Briefly, the reaction mixture of chitinase assay contained colloidal chitin (0.5%, w/v), acetate buffer (50 mM, pH 5.5), and an appropriate amount of enzyme. The amount of reducing sugar generated was measured after 1 h incubation at 37°C. One unit of chitinase activity was defined as the amount of enzyme that releases one µmole of reducing sugar (as GlcNAc) per minute.

Protease activity was also determined by using casein as substrate, following previously published method (Anson, 1938). In brief, the reaction mixture contained casein (0.65%, w/v), potassium phosphate buffer (50 mM, pH 7.0), and an appropriate amount of enzyme and was incubated at 37°C for 10 min. One unit of protease activity was defined as the amount of enzyme that generates one µmole of L-tyrosine equivalent per minute.

Quantification of protein

Concentration of protein was quantified by the Lowry method (Lowry *et al.*, 1951). Bovine serum albumin (BSA) was used to construct a standard curve.

Scanning electron microscopy (SEM) analysis

Chitosan hydrolysate sample was adjusted to 1 mg/mL and subsequently used to treat the test bacteria at 37°C for 6 h. Sterilised NaCl solution (0.85%, w/v) was used as the control. The treated cells were sputter coated with gold-palladium under vacuum. Subsequently, cellular morphology was observed and photographed with SEM (Quanta SEM 400, FEI Company, Hillsboro, Oregon, USA) at 20 kV.

Statistical analysis

Each experiment was performed in triplicate. The obtained data were subjected to Analysis of Variance (ANOVA), where $p \leq 0.05$ was considered as statistically significant. The graphs were made using by Origin 2017 software.

Results and discussion

Selection and identification of chitosanase-producing strains

In the primary screening, chitosan powder mineral medium (CPMM) containing chitosan powder as the carbon source was used as the selective medium to screen the chitosan-assimilating microbes. There were 119 isolates obtained from this screening step. Of these, 42 isolates were able to form clear zones on soluble chitosan mineral medium (SCMM) agar, indicating their abilities to produce chitosanases. These were selected for further screening based on ability to produce extracellular chitosanase in colloidal chitosan mineral medium (CCMM) broth. Results from assaying chitosanase activity showed that the level of chitosanase varied from 0.008 to 0.06 U/mL. However, only four strains generated chitosanase above 0.02 U/mL, and these were selected for the third stage of screening. At this stage, shrimp shell powder (SSP) was utilised as the carbon source for the chitosanase production. Of these four isolates, the one that generated the highest level of chitosanase activity (0.14 ± 0.006 U/mL) was chosen for further studies and was named OUR-I1.

OUR-I1 was a rod-shaped Gram-positive bacterium that formed aerial hyphae. The analysis of its 16S rDNA sequence showed high similarity (99%) of the strain OUR-I1 to *Lentzea* genus, including *L. waywayadensis*, *L. albida*, and *L. kentuckyensis*; thus, it was identified as *Lentzea* sp. strain OUR-I1 (GenBank accession no. KY888167). To the best of our knowledge, this is the first report on *Lentzea* genus regarding the ability to produce chitosanolytic enzymes.

Production of chitosanolytic enzyme by Lentzea sp. strain OUR-I1

For industrial scale production, fermentation using inexpensive nutrients is a promising approach to reduce the costs of enzyme production by microorganisms. Thus, we explored the use of a cheap carbon source to produce chitosanase by using this newly isolated *Lentzea* sp. strain OUR-I1.

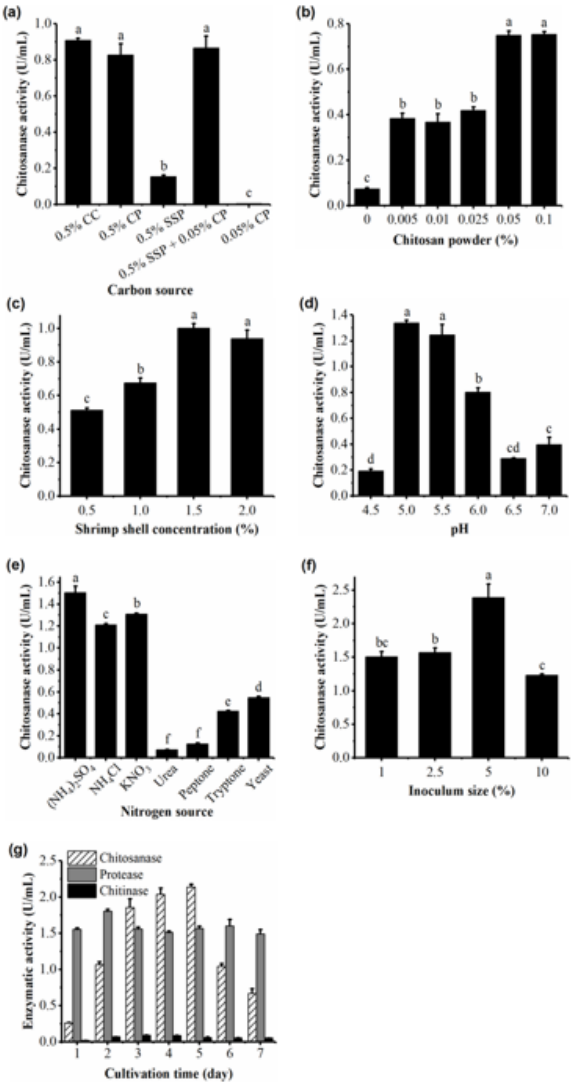

Figure 1. Effects of (a) carbon source, (b) chitosan powder, (c) shrimp shell concentration, (d) pH of medium, (e) nitrogen source, (f) inoculum size, and (g) cultivation time on the production of chitosanase (and protease and chitinase in figure g) by *Lentzea* sp. strain OUR-I1. Abbreviations: colloidal chitosan (CC), chitosan powder (CP), and shrimp shell powder (SSP). The letters on the top of the bars indicate statistically significant ($p < 0.05$) differences in chitosanase activity as a control parameter is varied, with the letter "a" representing the highest activity. Each data point represents mean ± standard deviation (SD) from triplicate ($n = 3$) experiments.

As shown in Figure 1a, the medium supplemented with chitosan powder (CP, 0.5%, w/v) or colloidal chitosan (CC, 0.5%, v/v) generated the highest levels of chitosanase, 0.83 and 0.9 U/mL, respectively. In contrast, the level of chitosanase obtained from the medium containing SSP (0.5%, w/v) was less than 0.2 U/mL (Figure 1a). It is well-known that almost all chitosanases are inducible enzymes and the synthesis of these enzymes could be induced by inducers such as soluble and colloidal chitosan (Sun *et al.*, 2007; Da Silva *et al.*, 2012). Among the chitosan substrates, CP is widely used as an inducer due to its relative ease of preparation. Taking these points into account, we supplemented CP (0.05%, w/v) into the SSP medium and explored its effects as inducer of the production of chitosan. Interestingly, the addition of CP into the SSP medium resulted in a substantial increase in chitosanase activity, up to comparable levels with that in media containing chitosan substrates (Figure 1a). It was noteworthy that the chitosan activity in the medium containing CP at 0.05% was negligible, implying that CP at 0.05% was not sufficient as the main carbon source for chitosanase production (Figure 1a). Taken together, these results indicate that CP at low concentrations played a role as inducer and SSP serves as the main carbon source for chitosanase production.

We varied the concentrations of CP (0-0.1%, w/v) in SSP medium and observed its concentration-dependent effects on the production of chitosanase (Figure 1b). Chitosanase activity increased with CP concentration and reached the highest level of 0.75 ± 0.01 U/mL at 0.05% of CP. Further increase of CP (to 0.1%, w/v) did not significantly increase chitosanase activity (Figure 1b).

We also optimised the factors involved in fermentation process to produce chitosanases by this strain. As shown in Figure 1c, chitosanase activity increased with the concentration of SSP and peaked at around 1 U/mL at 1.5% and 2% of SSP. However, we observed that the culture medium was too viscous at 2% of SSP, which made the separation of enzyme and culture medium more difficult. Hence, 1.5% of SSP was chosen as the optimal concentration for the production of chitosanase. Similarly, culture media with initial pH 5.0 and pH 5.5 resulted in the highest levels of chitosanase activity (Figure 1d). However, we also observed that CP and SSP were partly dissolved at pH 5.0, making them more accessible to microorganisms; hence pH 5.0 was chosen as the optimal condition. In addition, 0.1% (w/v) of $(NH_4)_2SO_4$ (Figure 1e) and 5% (v/v) of inoculum size (Figure 1f) were the optimal conditions for producing chitosanase with this strain. The chitosanase activity was also found to peak at day 5 of incubation (Figure 1g). Under these optimised conditions, the chitosanase level was increased by about 17-fold, from the initial 0.14 ± 0.006 to 2.4 ± 0.038 U/mL. This level was 3 to 120 times higher than chitosanase activities obtained from *Gongronella* sp. JG (0.8 U/mL) (Zhou *et al.*, 2008), *Serratia marcescens* TKU011 (0.02 U/mL) (Wang *et al.*, 2008a), or *Metarhizium anisopliae* (0.06 U/mL) (de Santana *et al.*, 2015). However, chitosanase activity obtained in the present work was lower than that in some other reports with chitosanase activities from 21.85 to 118

U/mL (Choi *et al.*, 2004; Sun *et al.*, 2007; Wee *et al.*, 2009; Zhang and Zhang, 2013).

It is well-documented that the simultaneous presence of protease and chitosanase is advantageous for the deproteinisation of shellfish to chitin, facilitating the access of enzyme to chitin molecules that might contribute to the production of COS (Oh *et al.*, 2000; Wang *et al.*, 2009a). Therefore, apart from chitosanase activity, we also monitored the activities of protease and chitinase enzymes that may be produced during the degradation of SSP. As shown in Figure 1g, protease activity was comparable to chitosanase activity during the cultivation. However, the activity of chitinase was relatively low (< 0.1 U/mL, Figure 1g). The high level of protease activity observed is consistent with previous studies (Oh *et al.*, 2000; Wang *et al.*, 2009b), confirming beneficial effects of protease on the production of COS.

Partial purification of chitosanase

We partially purified chitosanase enzyme from the crude supernatant to remove unwanted proteins and to concentrate our protein of interest. The activities of chitosanase, protease, and chitinase were measured during the process of purification. Total protein was lost significantly after acetone precipitation and dialysis, but the activity of chitosanase increased from 0.83 to 7 U/mg with 64% recovery rate. Specific activities of protease and chitinase also increased, but less than chitosanase activity (data not shown).

Production and analysis of chitosan hydrolysis product

Hydrolysing chitosan to produce bioactive compounds is a promising application of chitosanase. Therefore, we further explored the ability of partially purified chitosanase to hydrolyse chitosan substrates (CC or CP). Under the optimised conditions described earlier, the partially purified enzyme was able to degrade both CC and CP, generating reducing sugar up to the levels of 4.86 ± 0.155 and 4.62 ± 0.23 mg/mL, respectively (Figure 2a). In general, soluble and colloidal forms of chitosan are favourable for hydrolysis, because chitosanolytic enzymes have limited access to the exposed surfaces of insoluble chitosan. In the present work, we found that our partially purified chitosanase effectively hydrolysed even insoluble chitosan. This finding offers a convenient alternative in the production of chitosan hydrolysates, as the current method to prepare soluble and colloidal chitosan is labour-intensive and expensive. Moreover, our approach also avoids the use of other reagents such as strong acids that affect the antimicrobial activity of chitosan hydrolysates.

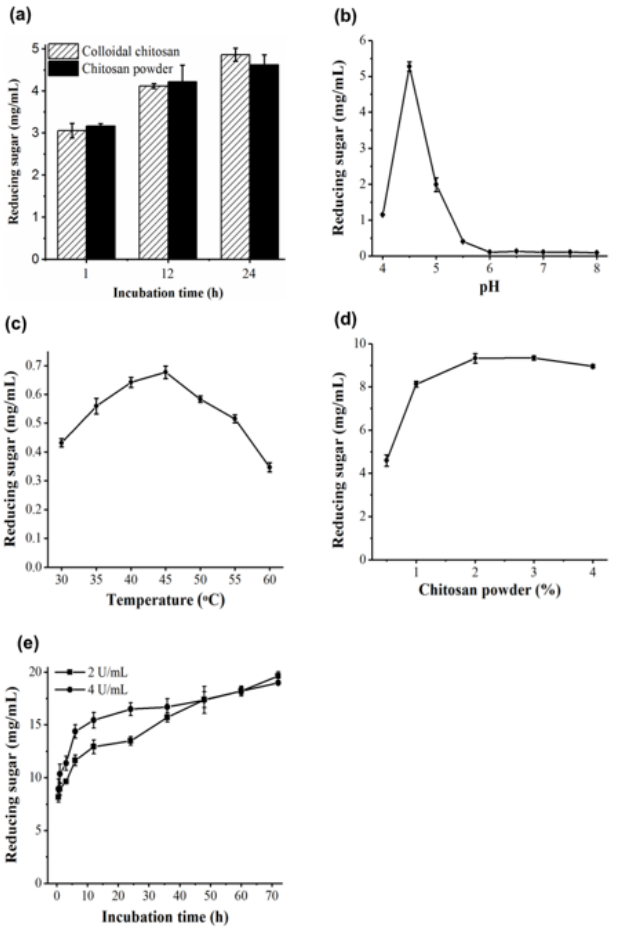

Figure 2. Enzymatic hydrolysis of chitosan under the effect of (a) types of substrate, (b) pH, (c) temperature, (d) chitosan powder concentration, and (e) concentration of enzyme at various incubation periods. The reaction was stopped by immersion in boiling water for 10 min, and the content of reducing sugar was measured by the DNS method. Each data point represents mean ± standard deviation (SD) from triplicate (n = 3) experiments.

Numerous factors affect the enzymatic reactions that hydrolyse chitosan powder, including pH, temperature, substrate concentration and enzyme concentration. Therefore, we further optimised these variables to increase the yield of hydrolytic products. We observed that the highest amount of reducing sugar was obtained in acetate buffer at pH 4.5 (Figure 2b), at 45°C (Figure 2c), using 2% (w/v) of substrate (Figure 2d), 2 U/mL of enzyme, and with incubation for 72 h (Figure 2e). Under these optimised conditions, the hydrolysis yield was 98% (Figure 2e).

The TLC analysis revealed that monomers (GlcN and GlcNAc) and putative COS with different degrees of polymerisation (DP) were the major end-products of the hydrolysis of chitosan powder (Figure 3a). Similarly, ESI-MS analysis showed that the chitosan hydrolysates obtained under optimal

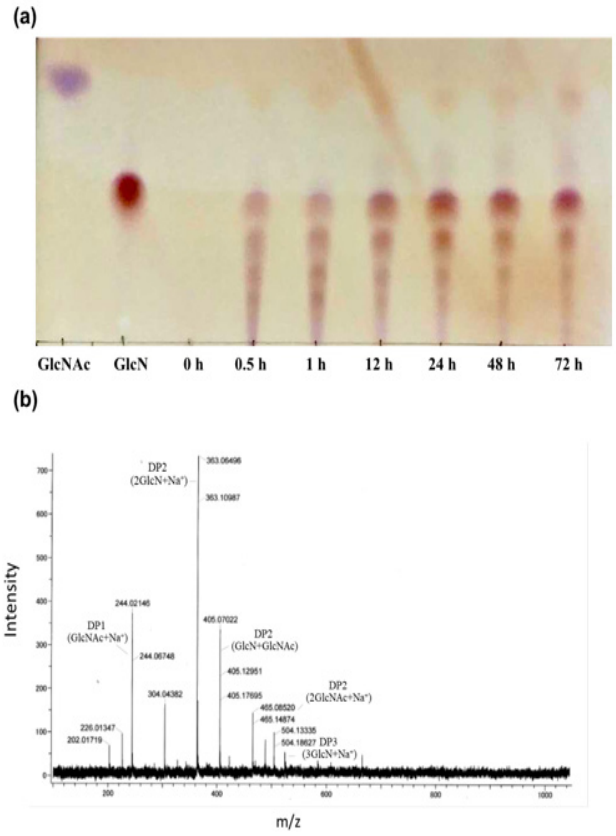

Figure 3. (a) TLC analysis of chitosan hydrolysate obtained at various hydrolysis times, and (b) ESI-MS analysis of chitosan hydrolysate at the optimal condition (72 h). Glucosamine (GlcN) and N-acetyl-β-D-glucosamine (GlcNAc) were used as the reference standards.

conditions contained GlcNAc monomers and dimers (DP2) of $(GlcN)_2$, (GlcN-GlcNAc), and $(GlcNAc)_2$ as the major products, and a trimer (DP3) of $(GlcN)_3$ as a minor product (Figure 3b). However, GlcN was not observed in this analysis, which might be due to signal interference by the matrix (Liang *et al.*, 2015).

Antimicrobial activity of chitosan hydrolysates

As mentioned earlier, chitosan hydrolysate obtained from the optimised conditions mainly contained a mixture of monomeric and oligomeric GlcN and GlcNAc. This mixture was tested for its antimicrobial activities against food-borne bacteria, including *E. coli*, *B. cereus*, *S. Typhimurium*, *S. aureus*, and *S. enteritidis*. The antimicrobial activities were expressed as minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), and are summarised in Table 1. The antibacterial activity of this monomeric-oligomeric mixture varied, depending on the bacterial strain. For example, *E. coli*, *S. aureus* and *B. cereus* were more susceptible to actions of the monomeric-oligomeric mixture with MIC_{90} (minimum inhibitory concentration that inhibits the growth by 90% of a bacterial strain) of 2.5 mg/mL, whereas the MIC_{90} against *S. enteritidis* and *S. Typhimurium* was 10 mg/mL (Table 1). In addition, the chitosan hydrolysates had the MBC of 2.5 mg/mL against *S. aureus*, while MBC of 5 mg/mL was found against *E. coli* and *B. cereus*. However, the MBC of 10 mg/mL or higher was required to completely kill *S. enteritidis* and *S. Typhimurium*, respectively.

The antibacterial activities of commercial GlcN and GlcNAc were examined in the same way as positive controls. As shown in Table 1, the MIC_{90} of GlcN against *S. aureus* and *B. cereus* was 20 times higher than that of the chitosan hydrolysate mixture (50 versus 2.5 mg/mL). In addition, there was no obvious inhibitory effect against *E. coli*, *S. enteritidis*, or *S. Typhimurium* observed at the tested concentrations of GlcN (Table 1). No bactericidal activity of GlcN was observed at the highest tested concentration of 50 mg/mL, except for *B. cereus* (Table 1). Similarly, GlcNAc showed very low inhibitory effects against all tested strains, and less than 50% of all strains were inhibited at all the highest concentration of GlcNAc (50 mg/mL, Table 1). It appears that higher concentrations of GlcN and GlcNAc would be required to completely inhibit these bacterial strains. However, we did not further investigate the concentration-dependent antimicrobial effects of GlcN and GlcNAc on these bacterial strains. Taken together, the results indicate that the chitosan hydrolysate contained both monomer and oligomer had stronger antibacterial activity than GlcN or GlcNAc. This also suggests that this mixture could be used as an antibacterial agent without further purification.

Since chitosan hydrolysate mixture had considerable antimicrobial effects, we also observed cellular morphologies of test bacteria under SEM to elucidate the mechanisms of inhibition. Two pathogenic strains, *E. coli* and *B. cereus*, were used as the bacterial models and treated with 1 mg/mL chitosan hydrolysate. Before treatment with chitosan hydrolysate, the cell morphologies of *B. cereus* (Figure 4a) and *E. coli* (Figure 4b) appeared intact and smooth. However, numerous dents were formed on the cell walls of *B. cereus* (Figure 4a) and *E. coli* (Figure 4b) following treatment with the hydrolysate. This could be due to the penetration of small COS and monomers through the microbial cell wall to cytoplasmic membrane, which made the cell membrane permeable (Benhabiles *et al.*, 2012; Laokuldilok *et al.*, 2017). These observations are in good agreement with a previous report, in which a chitooligomeric and monomeric mixture caused the cell walls of *B. cereus* to become leaky, leading to apoptosis (Vishu Kumar *et al.*, 2005).

Table 1. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the monomeric-oligomeric mixture (chitosan hydrolysate), glucosamine (GlcN), and N-acetyl-β-D-glucosamine (GlcNAc).

Microorganism	Chitosan hydrolysate			GlcN			GlcNAc		
	[a]MIC90 (mg/mL)	[b]MBC (mg/mL)	[c]Inhibitory activity (%)	MIC90 (mg/mL)	MBC (mg/mL)	Inhibitory activity (%)	MIC90 (mg/mL)	MBC (mg/mL)	Inhibitory activity (%)
Escherichia coli	2.5	5.0	99.9 ± 0.1	> 50.0	> 50.0	31.2 ± 3.3	> 50.0	> 50.0	38.0 ± 2.9
Salmonella enteritidis	10.0	10.0	99.7 ± 1.7	> 50.0	> 50.0	43.2 ± 1.6	> 50.0	> 50.0	37.3 ± 3.7
Salmonella Typhimurium	10.0	> 10.0	98.8 ± 0.5	> 50.0	> 50.0	51.0 ± 1.2	> 50.0	> 50.0	38.4 ± 2.9
Staphylococcus aureus	2.5	2.5	99.1 ± 0.9	50.0	> 50.0	99.2 ± 0.5	> 50.0	> 50.0	43.3 ± 2.4
Bacillus cereus	2.5	5.0	99.8 ± 0.9	50.0	50.0	100.0 ± 0.0	> 50.0	> 50.0	30.6 ± 0.7

[a]MIC90: minimum inhibitory concentration that inhibits visible growth by 90% of a bacterial strain.
[b]MBC: minimum bactericidal concentration that completely inhibits the growth of bacteria.
[c]Inhibitory activity of chitosan hydrolysates was calculated by using the formula: Inhibitory activity = [(C-T)/C]*100, where C and T are the optical densities measured at a wavelength of 600 nm of control and hydrolysates samples, respectively.

Numerous factors have been reported to affect the antimicrobial activity of chitosan hydrolysate, such as its molecular weight, degree of polymerisation (DP), and degree of deacetylation (DD) (Jeon *et al.*, 2001; Sinha *et al.*, 2016). The antibacterial activity of chitosan hydrolysate was dependent on COS preparation method, its concentration and the type of bacteria used (Benhabiles *et al.*, 2012). For example, Wang *et al.* (2007) reported that COS with DP4, which was obtained by hydrolysis of colloidal chitosan with chitosanase from *Pseudomonas* CUY8, exhibited its highest inhibitory effect against *S. aureus*. Meanwhile, Li *et al.* (2014) demonstrated that the strongest antimicrobial effect of COS was achieved against the same bacteria with DP > 12, when generated by acid hydrolysis of highly N-deacetylated chitosan. Apart from its antimicrobial activity against *E. coli* (Rozin *et al.*, 2009), synergistic inhibitory activity of GlcN monomer and COS has been observed against food-borne pathogens (Vishu Kumar *et al.*, 2005). GlcN in COS solution has been found to enhance the solubility of COS at pH above its critical point, contributing to its antimicrobial activity (Blagodatskikh *et al.*, 2013).

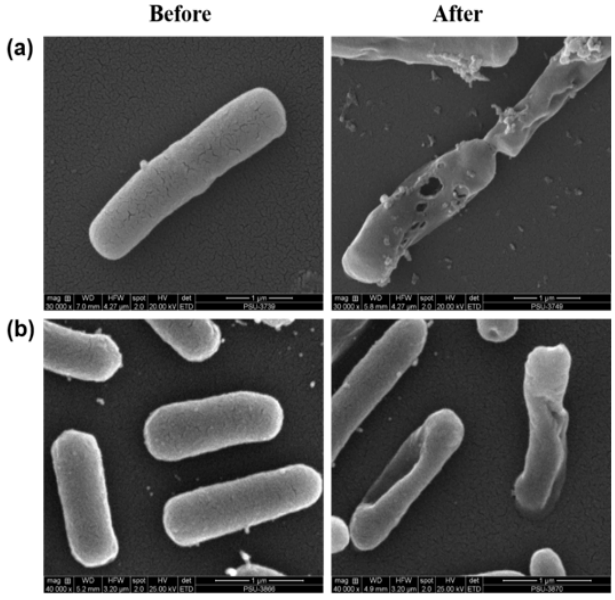

Figure 4. Scanning electron microscopy of (a) *Bacillus cereus,* and (b) *Escherichia coli* before and after treatment with the chitosan hydrolysate, at 30,000× magnification.

However, the contributions of COS and monomers to the overall inhibitory activity against pathogenic bacteria, as observed in the present work, remain unclear. There might be effects of COS alone, or synergistic effects in the mixture. Therefore, a future study is warranted to separate and characterise these compounds to better elucidate their mechanisms of microbial inhibition.

Conclusion

In the present work, a novel chitosanase-producing *Lentzea* sp. strain OUR-I1 was isolated, and a cost-efficient and environment-friendly approach to produce chitosanases from an inexpensive nutrient source, shrimp shell waste, which is a by-product of food processing, was proposed. The partially purified chitosanase could effectively degrade insoluble chitosan into antibacterial compounds. Although the individual effects of chitosan hydrolysates on pathogenic bacteria should be further studied, our results highlight the potential of this newly isolated strain to produce bioactive compounds. With its antimicrobial activity, the chitosan hydrolysate obtained could potentially serve as an antimicrobial agent against food-borne pathogens in food industries.

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