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Production of recombinant *Bacillus subtilis* chitosanase, suitable for biosynthesis of chitosan-oligosaccharides

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HIGHLIGHTS

- ▸ Chitosanase (Csn) is used to convert chitosan from chitin waste to chitosan-oligosaccharides (COS).
- ▸ Csn from *B. subtilis* was cloned, efficiently expressed and secreted in *E. coli*.
- ▸ *B. subtilis* Csn thermostability is dependent on the substrate.
- ▸ Recombinant *B. subtilis* Csn is suitable for industrial application.
- ▸ Crude culture media containing secreted Csn was used to convert chitosan to COS.

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ABSTRACT

Chitosanases are enzymes that catalyse the hydrolysis of the β-1,4 glycosidic bond of chitosan. One of the most promising applications of this enzyme is for the bioconversion of chitosan into value-added chitosan-oligosaccharides (COS). GH46 chitosanase (Csn) from *Bacillus subtilis* 168 was expressed in *Escherichia coli* by fusing the gene encoding mature Csn to the *E. coli* OmpA signal peptide sequence. The recombinant enzyme was secreted into the culture supernatant. The recombinant Csn showed high specific activity and stability over a wide range of pH. The enzyme was >100 times more thermostable in the presence of the substrate, with a half-life time of activity ($\tau_{1/2}$) of approximately 20 h at 50 °C and pH 5.5. Efficient bioconversion of chitosan into different mixtures of COS, using crude culture supernatant containing secreted enzyme was demonstrated.

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1. Introduction

Chitosan *N*-acetylglucosaminohydrolase or chitosanases (EC 3.2.1.132) are enzymes that catalyse the hydrolysis of the β-1,4 glycosidic bond of chitosan, a partially deacetylated derivative of chitin, which comprises *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) residues (Dahiya et al., 2006). These enzymes can be found in a wide variety of microorganisms including Gram-positive and Gram-negative bacteria, yeast and fungi (Eijsink et al., 2010). The enzymes belong to glycoside hydrolase (GH) families 5, 7, 8, 46, 75 and 80, according to the Carbohydrate-Active Enzymes database (CAZy) (Cantarel et al., 2009). While families GH5, GH7 and GH8 contain a few chitosanases and other glycoside hydrolases, specifically, families GH46, GH75 and GH80 comprise exclusively chitosanases (Eijsink et al., 2010). Chitosanase from family 46, especially those from *Streptomyces* (Dubeau et al., 2011; Fukamizo and Brzezinski, 1997) and *Bacillus circulans* (Fukamizo et al., 2005) are presently the best-studied enzymes.

One of the most important and promising applications of chitosanases is the bioconversion of chitosan into chitosan-oligosaccharides (COS) (Aam et al., 2010). These polysaccharide oligomers have been shown to have potential medical applications (Aam et al., 2010; Khoushab and Yamabhai, 2010), including in gene delivery, as drugs against asthma, malaria and cancer, antibacterial and antifungal agents, ingredients for wound dressing and bone strengthening, as well as substances for lowering serum glucose in diabetics (Aam et al., 2010; Khoushab and Yamabhai, 2010). The advantage of using chitosanase to produce COS over physical methods such as the use of mirowaves, γ-rays, ultrasonication, or hydrothermal treatments is the more environmentally friendly process that produces more defined COS mixtures.

Chitosan can be prepared from chitin by homogeneous or heterogeneous deacetylation, resulting in polysaccharide polymers with various degrees of N-acetylation (DA), polymerisations (DP), and molecular weight distributions (Aam et al., 2010). Since the various biological activities of COS are dependent on the degree of poly-

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merisation and deacetylation (Hayes et al., 2008; Kim and Rajapakse, 2005), it is essential to produce a well-defined chitosan-oligosaccharide mixtures for specific uses, as well as for a more appropriate study of the structure–function relationship. Therefore, obtaining an efficient expression system for the production of enzymes that are suitable for bioconversion of chitosan will be highly beneficial for the chitin biotechnological industry.

Bacillus subtilis strain 168 was used as a source of the recombinant chitosanase in this study because this bacterium has been used extensively for the production of various enzymes and bioactive compounds for industrial purposes (Moszer, 1998). In addition, its genomic sequence has been published (Barbe et al., 2009) and various aspects of gene regulations and protein functions from this bacterium have been analysed (The Bacillus Genetic Stock Center, http://www.bgsc.org/). Previous report on the expression and partial purification of the native chitosanase, overproduced in *B. subtilis*, indicated that the csn gene was temporally regulated and showed similar properties to a *Streptomyces* chitosanase. However, the yield obtained from using native strain was extremely low (Rivas et al., 2000). The recombinant Csn in the present study was engineered to facilitate efficient secretion into *Escherichia coli* culture media, rendering much higher yield and more convenient purification procedure. *E. coli* was selected as the expression host for this enzyme because this bacterium is a standard host for the production of recombinant proteins either extracellulary or intercellulary (Mergulhao et al., 2005). Moreover, expression of recombinant enzymes in *E. coli* can be easily done in high-throughput format, allowing the application of directed evolution (DE) technique for the improvement of the enzyme properties (Alcalde et al., 2006; Songsiriritthigul et al., 2009). Recently, this expression system has been successfully used for efficient expression of several enzymes from *Bacillus* sp. (Songsiriritthigul et al., 2010a,b; Yamabhai et al., 2008).

In the present study, an efficient *E. coli* expression system for the expression, secretion and purification of chitosanase from *Bacillus subtilis* 168 was developed. Biochemical characterisation of the purified enzyme revealed that the enzyme is suitable for industrial applications. The ease of using the culture supernatant containing secreted enzyme directly for the bioconversion of chitosan into chitosan-oligosaccharides was also demonstrated.

2. Methods

2.1. Materials

Chitosan practical grade [product number 417963, ⩾75% degree of deacetylation (DDA)], low molecular weight [product number 448869 (75–85% DDA)] and medium molecular weight [product number 448877 (75–85% DDA)] were purchased from Sigma–Aldrich. Commercial chitosan solution from shrimp and prawn shells (>90% DDA) was purchased from Poodang168. Co., Ltd. (Thailand) and commercial chitosan powder from shrimp and prawn shells (85–90% DDA) was purchased from NNC products Co., Ltd. (Thailand). Various chitosan-oligosaccharides $(GlcN)_{2-6}$ or G2–G6 were purchased from Seikagaku (Tokyo, Japan). Glycol chitosan (product number G7753) was purchased from Sigma–Aldrich. Chitosan solutions (10 mg/ml) were prepared by adding 10 g of chitosan into 400 ml of distilled water and adding, 90 ml of 1M acetic acid with stirring. The chitosan solution was adjusted to pH 5.5 with 1M sodium acetate, and to a final volume of 1 L with distilled water.

2.2. Bacterial strains and plasmid

B. subtilis strain 168 (ATCC23857) was obtained from the American Type Culture Collection. The bacteria were grown on nutrient agar (NA) agar at 30 °C. *E. coli* DH5α (Life Technologies) and TOP10 (Invitrogen) was used as the cloning and expression host, respectively. Plasmid pMY202, which was used for the cloning and expression of the *B. subtilis* chitosanase gene, was constructed as previously described (Sinsuwan et al., 2012).

2.3. Cloning of chitosanase gene from B. subtilis strain 168

The gene encoding recombinant chitosanase, whose native signal peptide sequence was replaced with that of the *E. coli* OmpA signal peptide (OmpA-Csn) sequence, was cloned by a PCR-based method according to a previously published protocol (Songsiriritthigul et al., 2009, 2010a,b). The primers were designed from the published DNA sequence of the chitosanase gene from *B. subtilis* 168 (NCBI accession number: NC_000964 REGION: complement (2747984..2748817). The primers B.subCsnOmpAHindIIIFw: 5′ CTGTGCAAG CTT CGG CGG GAC TGA ATA AAG ATC AAA AGC3′and B.subCsnBamHIRv: 5′ GCA CAG GGA TCC TTT GAT TAC AAA ATT ACC GTA CTC GTT TGA AC 3′ were used in the PCR reaction. The PCR products were cut with *Hind* III and *Bam* HI and ligated with pMY202 that had been digested with *Nde* I and *Bgl* II. The recombinant construct was designated as CsnOmpApMY202. The integrity of the constructs was confirmed by automated DNA sequencing (Macrogen, Korea).

2.4. Enzyme expression and purification

The expression of recombinant *B. subtilis* chitosanase was done according to a previously published protocol (Juajun et al., 2011; Songsiriritthigul et al., 2010a,b). Briefly, a single colony of freshly transformed *E. coli* TOP10 harbouring appropriate constructs was grown in Luria Bertani (LB) broth containing 100 μg/ml ampicillin (LB-Amp) overnight at 37 °C. Then 2% of the overnight culture was added into 0.4–1 L of LB-Amp broth and grown at 37 °C, 250 rpm until the OD_{600} reached 0.6–0.7. Subsequently, isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture broth to a final concentration of 0.1 mM, and the incubation was continued at room temperature with vigorous shaking (180 rpm) for 20 h. The cells were harvested by centrifugation at 4000*g* for 30 min at 4 °C. Preparation of cell lysate was done as previously described (Songsiriritthigul et al., 2010a,b). The recombinant 10× His-tagged chitosanase was purified by immobilised metal affinity chromatography (IMAC), using Ni-NTA agarose (Qiagen), according to the manufacturer's instruction (Qiagen). Crude enzyme from cell lysate or culture supernatant was loaded into an Ni-NTA column that had been pre-equilibrated with equilibration buffer (30 mM Tris–HCl, 300 mM NaCl and 10 mM imidazole), and incubated at 4 °C with rotation for 2 h. The column was washed with wash buffer (30 mM Tris–HCl buffer, pH 8.0 and 300 mM NaCl and 20 mM imidazole) three times. The enzyme that bound with Ni-NTA resin was eluted with elution buffer containing 250 mM imidazole (30 mM Tris–HCl buffer, pH 8.0 and 300 mM NaCl and 250 mM imidazole). The eluted enzyme was passed through a Vivaspin6 column, M_r cutoff 10 kDa (GE Healthcare, Sweden), to remove imidazole and concentrate the protein. The purified enzyme was stored at 4 °C.

2.5. Determination of protein concentration

Protein concentration was determined by the method of Bradford using the Bio-Rad protein assay kit, and bovine serum albumin (BSA) as the standard. The standard calibration curve was constructed from 0.05 to 0.5 mg/ml of BSA.

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2.6. SDS–PAGE and zymogram analysis

Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli. Protein bands were stained with Coomassie brilliant blue R-250. A protein ladder (10–200 kDa) was purchased from Fermentas (St. Leon, Germany) and Bio-Rad. The protein samples were heated for 3 min in the loading buffer (Laemmli buffer) containing reducing agent at 100 °C, using a heat block.

For the zymogram analysis of chitosanase, the protein samples were mixed with loading buffer (Laemmli buffer) without reducing agent and heating. The proteins were electrophoresed in a 12.5% SDS–PAGE that was placed on ice. After electrophoresis, the gel was rinsed with de-ionised water, soaked in 100 mM sodium acetate buffer pH 5.5 containing 1% Triton X-100 for 3 h with shaking, on ice. After the gel was rinsed with 10 mM sodium acetate buffer pH 5.5 several times, the protein gel was laid on top of a polyacrylamide gel, containing 0.1% chitosan (practical grade). The gel was incubated in 10 mM sodium acetate buffer, pH 5.5, at 50 °C for 18 h. The chitosanase activity on the gel was visualised by staining the gel with 0.1% Congo Red, followed by de-staining with 1N NaCl.

2.7. Chitosanase activity assay

Standard chitosanase activity was determined using the dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction mixer consisted of 40 µl of dilute enzyme (0.4 µg) and 160 µl of 0.5% chitosan (in 200 mM sodium acetate buffer, pH 5.5, which was pre-incubated at 50 °C for 30 min. The reaction was incubated in a Thermomixer Comfort (Eppendorf AG, Hamburg, Germany) at 50 °C for 5 min, with mixing at 900 rpm. The reaction was stopped by adding 200 µl of DNS solution, and the mixture was centrifuged at 12000*g* for 5 min to remove the remaining chitosan. The colour in the supernatant was developed by heating at 100 °C for 20 min and cooling on ice. The reducing sugar in the supernatant was determined by measuring OD at 540 nm, using 1–5 µmol/ml D-glucosamine as standards. The reactions were done in triplicate and their mean and standard deviation values were reported. One unit of chitosanase was defined as the amount of enzyme that released 1 µmol of D-glucosamine per min under standard assay conditions.

2.8. Effect of pH on enzyme activity

The optimal pH of *B. subtilis* chitosanase was determined between pH 2.4–6.5 under standard assay conditions, using two buffer systems (250 mM each): glycine–HCl (pH 2.4–4.4) and sodium acetate buffer (pH 4–7.0). The reaction mixture consisted of 1.12 µg of purified enzyme and 0.5% chitosan (low molecular weight). It was not possible to determine the enzyme activity at pH > 7.0 because the chitosan substrates were insoluble at this condition.

To determine pH stability, 28 µg of purified enzyme in a total volume of 100 µl was pre-incubated in various buffers without substrate at 30 °C, for 24 h. The buffers used were 100 mM each of glycine–HCl (pH 2–3), sodium acetate (pH 4–7) and Tris–HCl (pH 7–9). After pre-incubation, the reactions were diluted 10 times in a total volume of 200 µl, then, 40 µl of the diluted samples were taken to determine the remaining activity under standard assay conditions.

2.9. Effect of temperature on enzymatic activity

The optimal temperature of *B. subtilis* chitosanase was measured by incubating 0.4 µg of the purified enzyme with 0.5% chitosan (practical grade) at temperatures ranging from 0 to 80 °C, for 5 min at pH 5.5. Thermal stability of the enzyme without substrate was determined by incubating 15 µg of the purified enzyme in 50 mM sodium acetate buffer, pH 6.0, at various temperatures ranging from 4 to 80 °C for 30 min. The remaining enzyme activity was measured under standard assay conditions.

To measure the thermal stability of *B. subtilis* chitosanase in the presence of substrates, 18 µg of the purified enzyme in a total volume of 50 µl was pre-incubated with 0.5% chitosan (low molecular weight) or 10 mM of chitosan oligosaccharide (G1–G6) in 50 mM sodium acetate buffer pH 5.5, at 50 °C for 30 min. After incubation, the reactions were diluted 30 times in a total volume of 300 and 40 µl of the diluted samples were taken to determine the remaining activity under standard assay conditions. In addition, the thermal inactivation kinetics at 50 °C in the presence of chitosan were measured by incubating 18 µg of the purified enzyme with 0.5% chitosan (low molecular weight) in 50 mM sodium acetate buffer pH 5.5, at 50 °C. After incubation at various time points (0, 0.5, 1, 6, 12 and 24 h), the reactions were diluted 10 times in a total volume of 300 and 40 µl of the diluted samples was taken to determine the remaining activity under standard assay conditions.

2.10. Kinetic analysis

The kinetic parameters were analysed using various concentrations of low molecular weight chitosan, ranging from 0.8 to 9.6 mg/ml. The reactions were performed at 50 °C for 5 min. The kinetic parameters were calculated by nonlinear regression, and the observed data were fitted to the Henri–Michaelis–Menten equation using Sigma Plot (SPSS Inc., Chicago, IL, USA).

2.11. Analysis of the hydrolytic products by thin layer chromatography (TLC)

Hydrolysis of chitosan-oligosaccharides (G2–G6) was carried out in a 50-µl reaction mixture containing 10 mM substrate and 0.16 µg of purified enzyme in 50 mM sodium acetate buffer, pH 5.5. The reaction mixture was incubated at 50 °C with shaking for 2, 5, 10, 30 and 60 min, and terminated by boiling for 10 min. To analyse the products by TLC, each reaction mixture was applied five times (1 µl each) onto a Silica gel 60 F254 aluminum sheet (6.0 × 10.0 cm) purchased from Merck (Damsladt, Germany) and chromatographed two times (2 h each) in a mobile phase containing 28–30% ammonium water:water:isopropanol (2:4:14)(v/v). The products were detected by wiping the TLC plate with a cotton ball soaked with 10% sulphuric acid in ethanol, followed by baking at 180 °C for 3 min. A mixture of 10 mM chitosan-oligosaccharides (G1–G2) and 5 mM chitosan-oligosaccharides (G3–G6) was used as standard.

To analyse the hydrolysis products using different chitosans as substrates, 0.5% of soluble chitosan, re-suspended in 200 mM sodium acetate buffer, pH 5.5 was pre-incubated for 30 min at 50 °C before 0.64 µg of purified enzyme was added and incubated at 50 °C. The actual reaction comprised 160 µl of 0.5% soluble chitosan in 200 mM sodium acetate buffer pH 5.5 and 40 µl of 0.016 mg/ml enzyme. The sample was taken for TLC analysis at various time points (2, 5, 10, 30 and 60 min).

3. Results and discussion

3.1. Cloning, expression and purification of recombinant chitosanase (Csn) from Bacillus subtilis 168

Amino acid sequence analysis revealed that the *B. subtilis* chitosan *N*-acetylglucosaminohydrolase (EC 3.2.1.132), commonly known as chitosanase (Csn) belongs to glycoside hydrolase family GH46, according to the CAZy (Carbohydrate-Active Enzymes) data-

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bank. The putative amino acid sequence deduced from the cloned *B. subtilis csn* gene was compared with amino acid sequences of other bacterial chitosanases from family GH46 (Fig. 1). The enzyme has highest identity to chitosanase from *B. amyloliquefaciens* (87%); followed by chitosanases from *Streptomyces cattleya* (67%); *Streptosporangium roseum* (28%); and *B. circulans* (14%). Amino acid sequence alignment (Fig. 1) suggested that the overall molecular folding of these enzymes is similar to *B. circulans* chitosanase (Saito et al., 1999), despite a very low amino acid sequence identity between them.

The recombinant *B. subtilis* Csn, expressed in *E. coli*, was purified to apparent homogeneity as illustrated by SDS–PAGE analysis (Fig. 2a). Chitosanase activity of the purified recombinant enzyme was demonstrated by in-gel activity staining (Fig. 2b) and by a standard chitosanase assay. Recombinant Csn showed a molecular mass of approximately 30 kDa on SDS–PAGE. These results correspond to the hypothetical molecular weight (MW) of the mature recombinant enzyme of 30.35 kDa, without the OmpA signal peptide. Routinely about 57000 U of total chitosanase activity from a 1-l shake flask culture were obtained. Approximately 45000 U came from culture supernatant, and 12000 U (14 mg/ml) was from cell extract (cytosol + periplasm). This yield is higher than those of most previous reports (see Supplementary data), except for a similar yield achieved for recombinant chitosanase from *Janthinobacterium* (Johnsen et al., 2010). The production of *B. subtilis* HD145 chitosanase in *Pichia pastoris* achieved a high yield of 7.2×10^6 U/L (Kang et al., 2012); however, the method that was used to measure enzyme activity in this report was different from the present study (30 min incubation instead of 5 min). It is worthwhile to note that there are many methods to measure chitosanase activities, based on the determination of reducing ends (see Supplementary data). These various assay procedures must be taken into consideration when comparing different enzymes. The most popular method for chitosanase assay is based on the determination of reducing end with 3,5 dinitrosalicylic acid (DNS); therefore, this method was used in this study, even though its accuracy has been questioned (Horn and Eijsink, 2004).

Bioinformatic analysis of the entire genomic sequence revealed that there was only one chitosanase gene in this bacterium. It is interesting to note that no chitosanase gene was detected in the genomes of the related *Bacillus licheniformis* DSM13 and DSM8785 strains (Veith et al., 2004); while an endo-chitinase gene

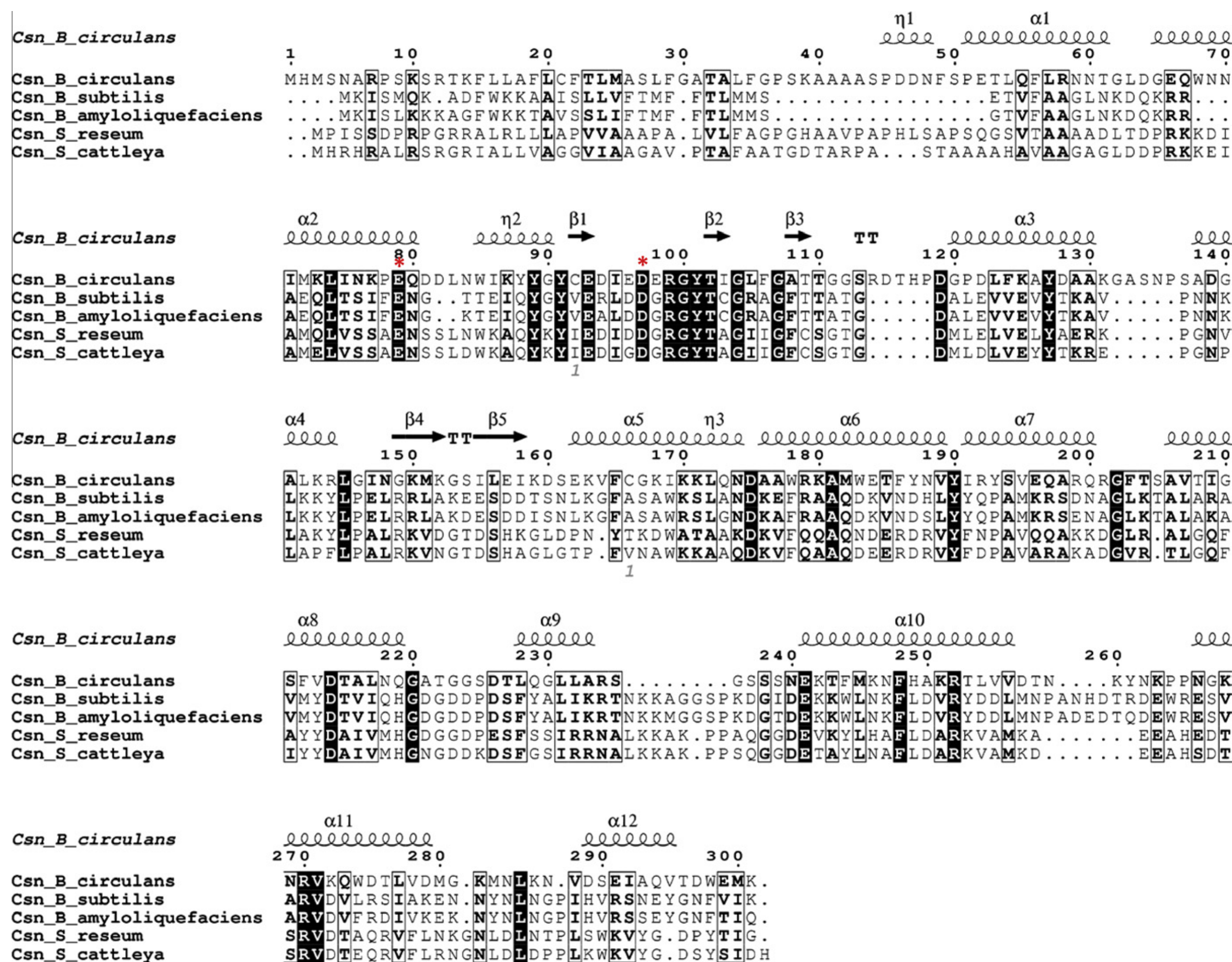

Fig. 1. Amino acid sequence alignment of Csn from *B. subtilis* and other bacterial chitosanases belonging to glycosyl hydrolase family 46 (GH46). α-Helices are displayed as squiggles; β-strands are rendered as arrows. A white character in a black box indicates strict identity, while a black character in a frame indicates similarity across a group. The two catalytic residues (Glu^{79} and Asp^{97}) are indicated by asterisks. Multiple sequence alignment was done by CLUSTAL W followed by ESPript (Gouet et al., 2003) to display the secondary structure of the Csn from *B. circulans*, PDB code; 1QGI. Key: Csn_B_circulans (Csn from *B. circulans*, UniProt accession number P33673); Csn_B_subtilis (Csn from *B. subtilis*, UniProt accession number O07921); Csn_B_amyloliquefaciens (Csn from *Bacillus amylofiquefaciens*, UniProt accession number Q9ET84); Csn_S_reseum (Csn from *Streptosporangium roseum*, UniProt accession number D2AW85) and Csn_S_cattleya (Csn from *Streptomyces cattleya*, UniProt accession number F8JQF6).

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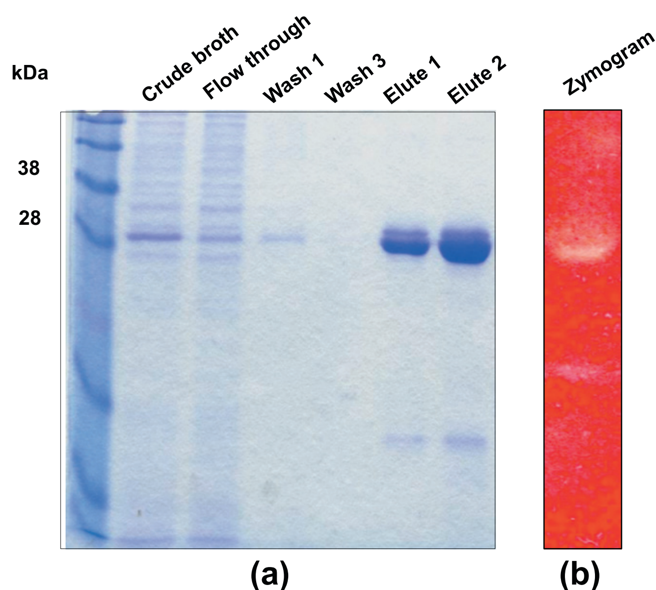

Fig. 2. Expression and purification of recombinant chitosanase from *B. subtilis* 168. Panel a illustrates SDS–PAGE analysis of recombinant chitosanase purified from culture supernatant at various steps. Fifteen microlitre of samples were loaded onto each lane, except for lanes from the 1st and 2nd elution (Elute 1 & 2), 10 µl of samples were loaded. The gel was stained by Coomassie brilliant blue. Panel b illustrated zymogram analysis of the purified enzyme. White band indicated chitosanase activity.

was found in *B. licheniformis* and *B. subtilis* (Songsiriritthigul et al., 2009, 2010b).

Recently, it has been shown that both native and *E. coli* OmpA signal peptide could be used to direct the secretion of bacillus extracellular (Songsiriritthigul et al., 2010a,b; Yamabhai et al., 2008, 2011), but not intracellular (Juajun et al., 2011; Sinsuwan et al., 2012) enzymes. Since bacillus chitosanase is an extracellular bacterial enzyme, therefore the gene encoding mature enzyme was fused with OmpA signal peptide for efficient secretion using *E. coli* expression system. Hence, this is a straightforward approach for the expression of a gene that has not been cloned and little is known about the structure–function relationship. For a large-scale production of chitosanase for food industry, *E. coli* K-12 must be used as an expression host, as this is the only strain that has been approved for "generally recognised as safe" (GRAS) status by USA-FDA (Olempska-Beer et al., 2006). In the next step, it will be worthwhile to exploit other bacterial expression system such as those of bacillus (Olempska-Beer et al., 2006) or lactobacillus (Peterbauer et al., 2011), as they are non-pathogenic and more suitable for food and feed applications. Nevertheless, these systems are still in a developmental process for the expression of enzyme from heterologous source in order to enable the reliable and inexpensive production of high yields of enzymes (Wenzel et al., 2011).

3.2. Effect of pH on catalytic activity

The optimal pH of recombinant chitosanase from *B. subtilis* 168 was 5.0–6.0 (Fig. 3a, solid line). The enzyme was more active in glycine buffer than in sodium acetate buffer. Notably, the chitosanase activity was stable within pH 2–9 after incubation at 30 °C for 24 h (Fig. 3a, dashed line). This is the widest range of pH stabilities of chitosanases that have been reported (see Supplementary data).

3.3. Effect of temperature on catalytic activity

The optimal temperature for *B. subtilis* Csn activity was between 40–50 °C under standard assay conditions (Fig. 3b, solid line). The enzyme was stable up to 45 °C after incubation for 30 min at pH 6.0, without substrate. Less than 10% of residual activity could be detected after incubation at 50 °C under these conditions (Fig. 3b, dashed line). However, when the enzyme was incubated for 30 min at 50 °C and pH 5.5, in the presence of 10 mM chitosan dimer $(GlcN)_2$, chitosan trimer $(GlcN)_3$, and COS equal or longer than tetramers, the residual activities of the enzymes were 38%, 50%, and ~80%, respectively (Fig. 4a). These results indicated that substrates could prevent thermal inactivation of the chitosanase activity. The enhancement of thermal stability by substrate was confirmed by the thermal inactivation experiment as shown in Fig. 4b. The recombinant *B. subtilis* Csn showed a half-life time of activity, $\tau_{1/2}$ of approximately 20 h at 50 °C, which was more than 100 times the stability of the enzyme in the absence of 0.5% chitosan (low molecular weight). Enhancement of chitosanase thermostability by the substrate has previously been reported for chitosanase of the GH family 8 from *Bacillus thuringienesis* (Kobayashi et al., 2011).

3.4. Enzyme activity and kinetic analysis

The specific activity of the purified enzyme was 900 U/mg under standard assay conditions. The relative activity of recombinant Csn from *B. subtilis* for various substrates was determined as shown in Table 1. The enzyme had no activity toward glycol chitosan and

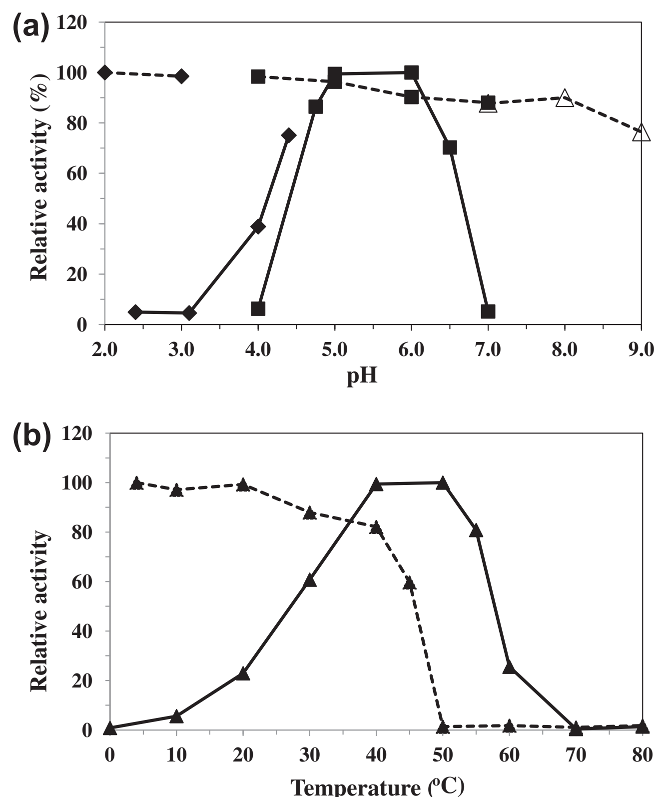

Fig. 3. Effect of pH (a) and temperature (b) on the activity (solid lines) and stability (dashed lines) of recombinant *B. subtilis* 168 chitosanase. The optimal pH was determined at 50 °C using 0.5% chitosan low MW in 250 mM of different buffers (a, solid line). The pH stability was determined by measuring the remaining activity after incubation at various pH values at 30 °C for 24 h (a, dashed line). The buffers (100 mM each) used were glycine–HCl buffer (black diamond) from pH 2–4; sodium acetate buffer (black square) from pH 4–7; Tris–HCl (triangle) from pH 7–9. The optimal temperature was determined using 0.5% chitosan (practical grade) in 200 mM acetate buffer, pH 5.5 (b, solid line). The temperature stability was determined by measuring the remaining activity after incubation without substrate at various temperatures at pH 6.0 for 30 min, using the standard assay (b, dashed line).

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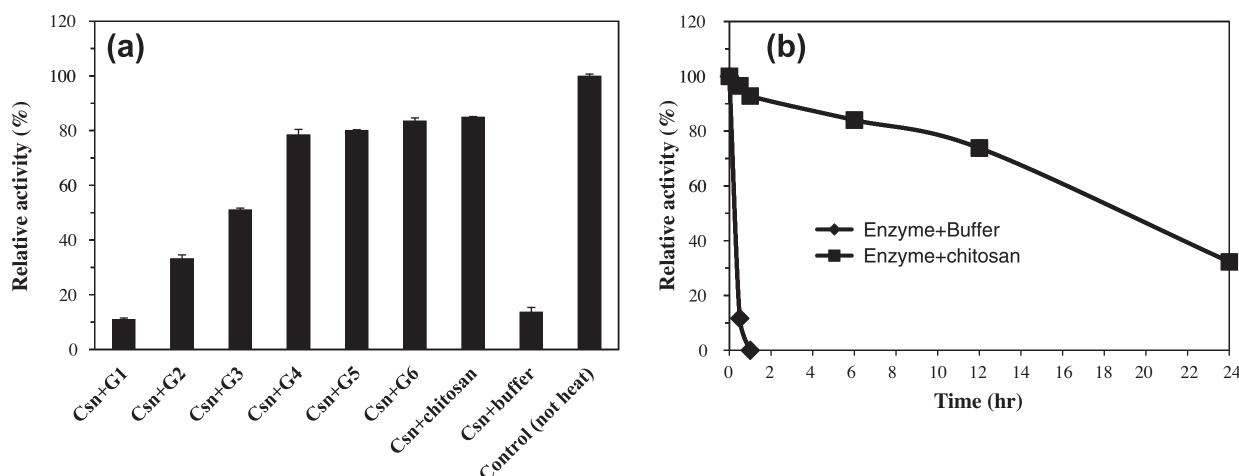

Fig. 4. Thermal stability in the presence of substrate. (a) The thermal stability was determined by measuring the remaining activity after incubation with 10 mM chitosan oligosaccharide (G1–G6) or 0.5% chitosan (practical grade) at 50 °C and pH 5.5, for 30 min. The relative activity was compare with that of the control, of which the Csn was not heated. (b) The remaining activity of *B. subtilis* chitosanase after incubation without (diamond) or with (square) 0.5% chitosan (practical grade) at 50 °C, at various time points (0, 0.5, 1, 6, 12 and 24 h).

Table 1
Substrate specificity of recombinant *B. subtilis* chitosanase.

Substrate	Relative activity (%)
Chitosan practical grade (⩾75% DDA)	112
Chitosan low molecular weight (75–85% DDA)	100
Chitosan medium molecular weight (75–85% DDA)	49
Chitosan commercial grade (85–90% DDA)	160
Glycol chitosan	nd
Colloidal chitin	nd

colloidal chitin. The activity towards low MW chitosan was higher than for medium MW chitosan but lower for the practical and commercial grades. Kinetic analysis revealed that K_m, V_{max}, and k_{cat} values of recombinant *B. subtilis* Csn were 1.57 ± 0.09 mg/mL, 530 ± 20 μmol sec^{-1} mg^{-1}, and $2.0 \times 10^5 \pm 1.2 \times 10^4\ s^{-1}$, respectively. The overall catalytic efficiency, k_{cat}/K_m of the enzyme was $1.9 \times 10^5 \pm 1.3 \times 10^4\ s^{-1}$/mg/mL. These results indicated that the recombinant *B. subtilis* chitosanase could be attractive for industrial applications because it has high specific activity and is relatively thermo- and pH-stable, when compared to the biochemical properties of other chitosanases (see Supplementary data).

3.5. Product analysis by thin-layer chromatography (TLC)

Product analysis by thin layer chromatography (TLC) using various substrates were performed to determine the hydrolytic activity of *B. subtilis* chitosanase (Fig. 5). When the enzyme was incubated with chitosan dimer $(GlcN)_2$ or G2 and chitosan trimer $(GlcN)_3$ or G3, no hydrolytic products could be detected, suggesting that G2 and G3 were not the substrates for this enzyme. When chitosan tetramer $(GlcN)_4$ or G4 was used as a substrate, G2 could be observed, indicating that the enzyme could cleave in the middle of G4. With chitosan pentamer $(GlcN)_5$ or G5 substrate, G2 and G3 were detected as early as 2 min after incubation. The hydrolysis of chitosan hexamer $(GlcN)_6$ or G6 initially yielded G3 and G4. Then, after 5 min of incubation, G4 was cleaved to G2, and after 1 h of incubation, all G6 substrate was converted to a mixture of G2, G3

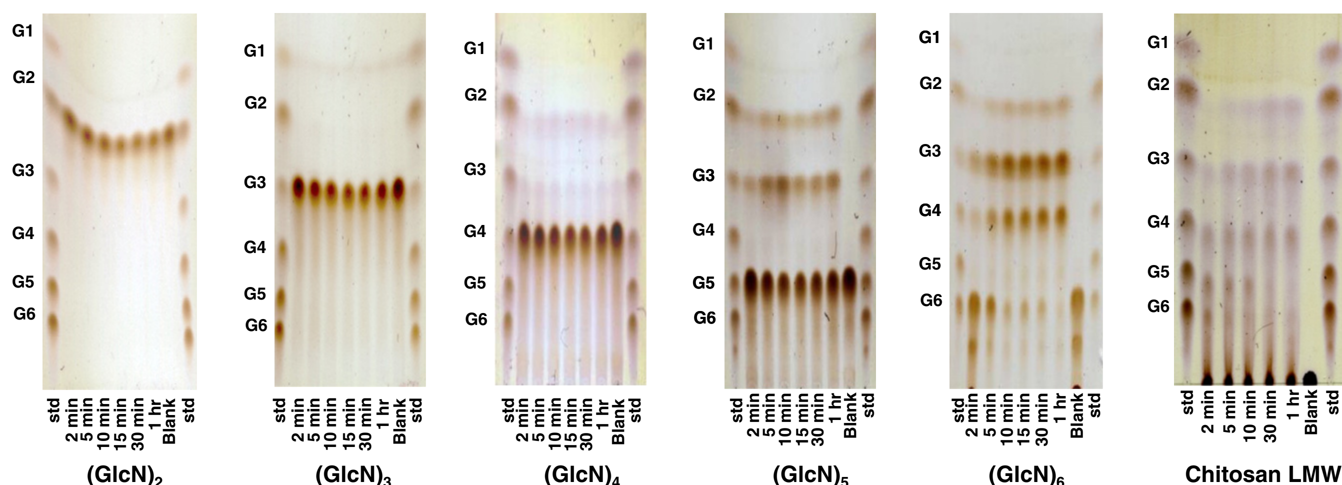

Fig. 5. Thin layer chromatography analysis of hydrolysis products. Hydrolysis products when using chitosan dimer $(GlcN)_2$ or G2, chitosan trimer $(GlcN)_3$ or G3, chitosan tetramer $(GlcN)_4$ or G4, chitosan pentamer $(GlcN)_5$ or G5, chitosan hexamer $(GlcN)_6$ or G6 and low molecular weight chitosan (chitosan LMW) are shown. The reaction products after incubation at various time points are illustrated. Std: a standard mixture of G1–G6; 2 min, 5 min, 10 min, 30 min and 1 h are the reaction products after incubation at 2 min, 5 min, 10 min, 30 min and 1 h, respectively; blank: enzyme blank.

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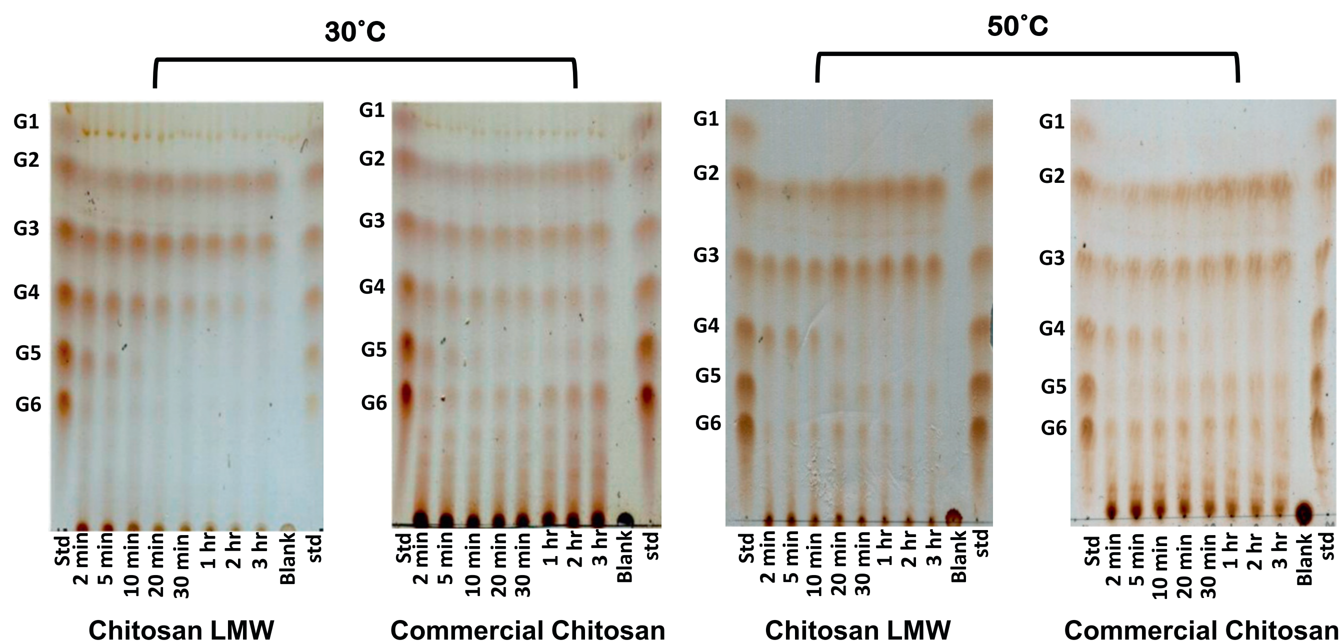

Fig. 6. Bioconversion of chitosans using crude secreted enzyme in culture broth. Thin layer chromatography analysis of hydrolysis products using chitosan low molecular weight (chitosan LMW) and commercial liquid chitosan as substrates. The substrates were incubated with 100 μl of culture supernatant containing crude enzyme, after an overnight cultivation. The reaction products after incubation at pH 5.5, for 2 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h and 3 h at 30 °C (left panel) and 50 °C (right panel) are illustrated.

and G4. These results suggested that the recombinant *B. subtilis* chitosanase prefers substrates longer than G3 and confirmed its endohydrolytic property to cleave GlcN–GlcN links, which is common to all known chitosanases.

3.6. Production of chitooligosaccharide using crude secreted enzyme from culture broth

To determine the applicability of the recombinant enzyme for the bioconversion of chitosan into chito-oligosaccharides (COS), the culture supernatant from an overnight culture of the *E. coli* containing the CsnOmpApMY202 expression vector was used directly in the experiments. One hundred microlitre of freshly prepared culture broth containing approximately 5 μg of recombinant enzyme was assayed with low MW and commercial grade chitosan (Fig. 6). Product analysis by TLC revealed that various chitosan-oligosaccharides, ranging from G2 to G6 and higher could be detected in different amounts, depending on the temperature and duration of incubation as well as the types of chitosan used. The hydrolysis products from commercial chitosan comprised higher amounts of G5, G6 and longer COS than those from low molecular weight chitosan. The product distribution at different temperatures was also varied. For both types of chitosans, G4 could be observed at every time point at 30 °C; while, at 50 °C, G4 disappeared after 20 min of incubation, and G5 and G6 accumulated instead. For low MW chitosan, G5 could be detected in the first 5 min after incubation at 30 °C, but not at 50 °C.

These results demonstrated the convenient application of secreted recombinant *B. subtilis* chitosanase for the production of COS. The results also confirmed previous observation that the pattern of hydrolytic COS products depend on the source of the chitosan starting material and the reaction conditions (Aam et al., 2010). Application of chitosanase for the hydrolysis of chitosan into chitosan-oligosaccharide is a highly attractive method for the synthesis of COS because chitosan is soluble in a slightly acidic condition. Moreover, it is also possible to combine chitosanase with chitinase and/or chitin binding protein in the degradation reaction in order to achieve the desired COS mixtures (Eijsink et al., 2008; Songsiriritthigul et al., 2010b). Biological effects of COS mixtures from various bioconversion reactions will be investigated in the future.

4. Conclusions

An efficient *E. coli* expression system for the expression, secretion and purification of a relatively thermo- and pH-stable chitosanase from *B. subtilis* was developed. The recombinant enzyme is suitable for various biotechnological applications. The ease of using culture supernatant containing recombinant enzyme directly for the synthesis of chitosan-oligosaccharide will significantly decrease their production costs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.09.130.

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