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Production of high degree polymerized chitooligosaccharides in a membrane reactor using purified chitosanase from *Bacillus cereus*

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ABSTRACT

Crude chitosanase from *Bacillus cereus* NTU-FC-4 was separated by a cation exchanger to three fractions named CBCI, CBCII, and CBCIII. The CBCI hydrolyzed chitosan to yield dimers. The primary hydrolytic products of CBCII were low degree polymerized (DP) chitooligosaccharides. The CBCIII had the fastest reaction rate and yielded high DP chitooligosaccharides (heptamer and higher DP oligomers). When CBCIII was used in the ultrafiltration membrane reactor with enzyme/substrate ratio 0.06 unit/mg and 100 min of residence time (RT), the concentration of high DP oligomers was 9.78 mg/mL which occupied ca. 48% of total oligomers in the final product as compared to ca. 29% resulted from the crude enzyme. Decrease of RT to 50 min and 33 min, the high DP oligomers in the products were ca. 61% and 69%, respectively. This system could be operated for at least 24 h and kept a constant permeate flux and product output rate.

1. Introduction

Chitooligosaccharides, formed with 2 to 10 glucosamines by β-(1 → 4) glycoside linkage, can be manufactured by hydrolyzing chitosan with chitosanase. Chitosanases are produced by many microorganisms, including fungi, actinomycetes, and bacteria (Boucher, Dupuy, Vidal, Neugebauer & Brzezinski, 1992; Fenton & Eveleigh, 1981; Izume, Nagae, Kawagishi, Mitsutomi, & Ohtakara, 1992; Seino, Tsukud, & Shimasue, 1991; Yamasaki et al., 1993; Yoshihara, Hosokawa, Kubo, Nishiyama & Koba, 1993). The bacterial chitosanases are especially useful for industrial applications because the enzyme can be easily prepared (Akiyama et al., 1999). However, most chitosanases from microorganism tended to hydrolyze chitosan and yield dimers, trimers and tetramers, rather than high degree polymerized (DP) oligomers (Park et al., 1999). For instance, chitosanase from *Matsuebacter chitosanotabidus* 3001 was able to hydrolyze chitosan to yield monomer to trimer (Park et al., 1999) and chitosanase from *Acinetobacter sp.* strain CHB101 yielded dimer and trimer (Shimosaka, Nogawa, Wang, Kumehara, & Okazaki, 1995). But it is known that high DP oligomers, such as chitopentaose, chitohexaose and chitoheptaose are more biologically active than the low DP ones (Suzuki et al., 1986; Suzuki, Tokoro, Okawa, Suzuki & Suzuki, 1986; Tokoro et al., 1989).

Among the bacteria, the *Bacillus sp.* is particularly useful for the chitosanase production. Chitosanases with different activities and characteristics were produced by *Bacillus sp.* isolated from plants and soil, including *Bacillus cereus* S1 (Kurakake, Yo-u, Nakagawa, Sugihara, & Komaki, 2000), *Bacillus sp.* strain KFB-C108 (Yoon, Ha, Lim, & Cho, 1998), *Bacillus subtilis* KH-1 (Omumasaba, Yoshida, Sekiguchi, Kariya, & Ogawa, 2000), *B. subtilis* IMR-NK1 (Chiang, Chang, & Sung, 2003), *Bacillus sp.* strain KCTC 0377BP (Choi, Kim, Piao, Yun, & Shin, 2004), *B. subtilis* TKU007 (Wang & Yeh, 2008) and *B. cereus.* D-11 (Gao, Ju, Jung, & Park, 2008). The major concerns of selection of bacteria for the production of chitosanase are always the high specificity activity and good yield of high DP chitooligomers because they are very important for industrial application.

Besides selection of bacteria for chitosanase production, there are other methods which may be used to increase specific activity of enzyme and the yield of high DP chitooligomers. The crude enzyme can be purified to increase its specific activity and to remove the fractions which are not suitable for the production of high DP oligomers. In addition, a proper reactor which can control the progress of hydrolysis reaction may also facilitate the high DP oligomers production. Ming and coworkers used an immobilized enzyme bioreactor for high DP chitooligmers production. By removing the immobilized enzyme from the reaction mixture when the target pentamers and hexamers reached a maximum, the yield of high DP oligomers was increased in the batch reaction (Ming, Kuroiwa, Ichikawa, Sato, & Mukataka, 2006). Kuo, Chen, and Chiang (2004) demonstrated that chitooligomers could be produced by a continuous membrane reactor (Kuo et al., 2004). In the continuous membrane bioreactor, enzyme is recycled and reused while the product of enzymatic hydrolysis is continuously withdrawn as permeate. The membrane reactor separates the products (oligomers) from enzyme during processing thus preventing the high DP oligomers being further hydrolyzed by

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the enzyme. Therefore, the yield of high DP oligomer might be increased if the bioreactor was operated at a proper residence time.

This study aimed to investigate the characteristics of the chitosanases purified from the crude enzyme of *B. cereus* NTU-FC-4, and the possibility of using the purified enzyme in the membrane reactor for producing high DP chitooligosaccharides.

2. Materials and methods

2.1. Raw materials

Chitosan with 95% deacetylation was obtained from the Lytone Enterprise Inc. (Taipei, Taiwan). Both of chitin and glucosamine were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The soytone and yeast extract were from Difco Laboratories (Detroit, MI, USA). Acetonitrile was purchased from the Tedia Co. (Fairfield, OH, USA). Pure chitooligosaccharides, including dimer, trimer, tetramer, pentamer, and hexamer, were purchased from the Seikagaku Co. (Tokyo, Japan). CM-Sepharose Fast Flow gel was obtained from Pharmacia (Uppsala, Sweden).

2.2. Preparation of crude enzyme

The crude enzyme was extracted from the *B. cereus* NTU-FC-4, which was cultured in a pH 6.24 broth medium, containing 0.3% colloidal chitin, 0.5% yeast extract, 0.5% soytone, 0.1% sodium dihydrogen phosphate and 0.05% magnesium sulfate. After incubation at 30 °C for 2 days in a shake incubator, the culture was centrifuged at 4 °C and 6200 rpm for 30 min to remove bacteria and colloidal chitin. The crude enzyme was precipitated by 70% acetone and dried by lyophilization (FLEXI-DRY^{TM}, FTS system Co., New York, USA) (Kuo et al., 2004).

2.3. Separation of chitosanase by ion-exchange chromatography and gel electrophoresis

The crude enzyme was applied to a CM-Sepharose Fast Flow column (2.6 cm × 20 cm) which had been equilibrated with 20 mM acetate buffer (pH 5.0). The adsorbed proteins were eluted by a linear gradient of NaCl from 0 to 1 M in 20 mM acetate buffer (pH 5.0) at a flow rate of 66 mL/h. Fractions containing the major chitosanase activity were collected and dried by lyophilization. SDS–polyacrylamide gel electrophoresis using 12.5% acrylamide was performed and stained by Coomassie blue-R250. The sheets were destained with a destaining solution (acetic acid/methanol/water, 1/3/6, v/v). A pre-stained protein standard (SeeBlue Plus2, Invitrogen Co. Carlsbad, CA) was used during SDS–PAGE for determining the molecular weights of the separated proteins.

2.4. Enzymatic reaction pattern in bulk aqueous system

An amount of 15 unit of enzyme was dissolved in 1 mL acetate buffer solution (50 mM, pH 5.0) and mixed with 5 mL of 10 mg/mL chitooligomer standards which was dissolved in the same buffer. The mixture was incubated at 40 °C in a reciprocating shaker bath (100 rpm), and the NANOSEP (Pall Gelman Sci., MI, USA) was employed to remove the enzyme to stop the enzymatic reaction. At various time intervals, samples (1 mL) were taken, and then centrifuged for 10 min using NANOSEP micro-concentrator. The concentrations of chitooligomers in the filtrate were analyzed by HPLC.

2.5. Membrane reactor system

A hollow fiber UF membrane module (AG Technology, MA, USA) with molecular weight cut-off 3000 dalton and 0.042 m^2 effective membrane area was coupled with a 1 L jacked glass stirred tank to build the membrane reactor. A peristaltic pump was installed between membrane and reaction tank to circulate solution through the system. Warm water from a water bath flowed continuously through the jacked of tank to maintain a constant reaction temperature at 45 °C. For the substrate preparation, proper amount of chitosan was dissolved in 0.2 M acetate buffer at pH 5.0 to obtain a 20 mg/mL of chitosan solution. During operation, the chitosan solution was charged to the reaction tank along with proper amount of enzyme. The enzymatic reaction was first proceed for 5 min in the reaction tank to reduce the viscosity of the chitosan solution; then the pump was turned on to pump the mixture in the reaction tank through the membrane module. The permeate was diverted to a collection vessel and the retentate was returned to the reaction tank for further reaction. The substrate solution (chitosan solution) was continuously fed to the reaction tank from a substrate tank at a rate equals to the permeation rate to maintain a constant volume and substrate concentration of the mixture in the reactor (Kuo et al., 2004).

2.6. Enzyme assay methods

Chitosanase activity was determined by adding 1 mL of 1% (W/V) chitosan solution and 3.5 mL of 0.2 M acetic buffer at pH 5.0 to 0.5 mL of the enzyme solution. After shaking (100 rpm) the mixture at 45 °C for 30 min, the sample was boiled for 15 min to stop enzyme reaction and analyzed its reducing sugar content. One unit of chitosanase activity was defined as the amount of enzyme needed to hydrolyze 1% (W/V) chitosan solution and produce 1 μmol reducing sugar per minute at 45 °C. The method suggested by Imoto and Yagishita (1971) was used to determine the reducing sugar content and the glucosamine as standard. Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) was used to analyze the protein content for determining the specific activity of the enzyme.

2.7. HPLC analytical methods

An ICI HPLC system (LC1100 pump, Australia) equipped with RI detector was used for analyzing chitooligosaccharides content. The chitooligosaccharides were separated on a HYPERSIL HS APS column (Thermo Instrument Systems Inc., Runcorn, UK, 25 cm × 4.6 mm), eluted by acetonitrile and distilled water (60/40) mixture with a flow rate of 0.8 mL/min at 40 °C. The retention time and concentration of each individual peak in the sample was compared to the peak of standard chitooligosaccharides with DP 1–6. However, the pure compounds of heptamer and octamer were not available, therefore, the higher DP chitooligomers were identified by the sequence of peaks appeared in the HPLC chromatogram, and the concentrations of these chitooligomers were estimated based on the standard curve of hexamer.

3. Results and discussion

3.1. Purification of chitosanases from crude enzyme extract

For purification, the crude enzyme was first precipitated by acetone. The acetone precipitation could eliminate nearly 90% of the non-chitosanase protein, thus the specific activity of chitosanase increased from 0.46 unit/mg to 3.69 unit/mg and the yield of total enzyme activity was 81.37%. The crude enzyme was then separated by CM-Sepharose ion-exchange column using sodium chloride gradient from 0 to 1 M, and the three fractions obtained were named CBCI, CBCII and CBCIII (Fig. 1). It appeared that CBCII was a minor enzyme as compared to CBCI and CBCIII. The three fractions of

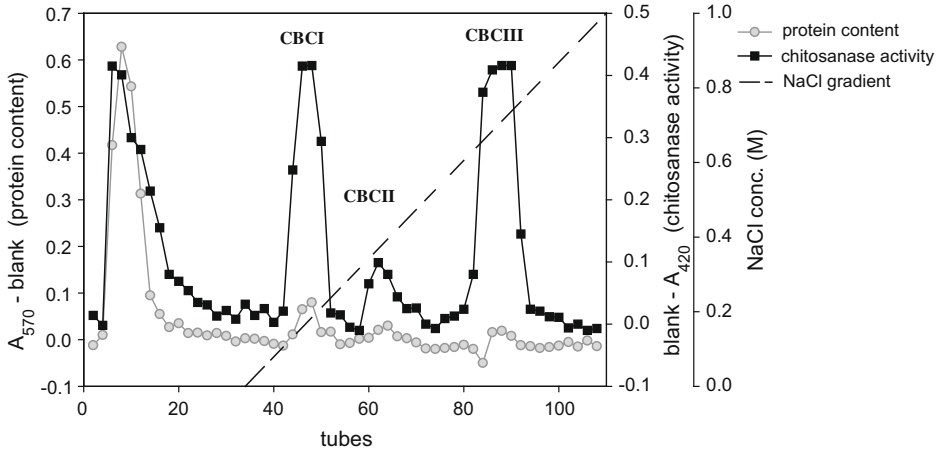

Fig. 1. The CM-Sepharose column chromatogram of chitosanase from *Bacillus cereus* NTU-FC-4. Every tube collected 6 mL elute solution.

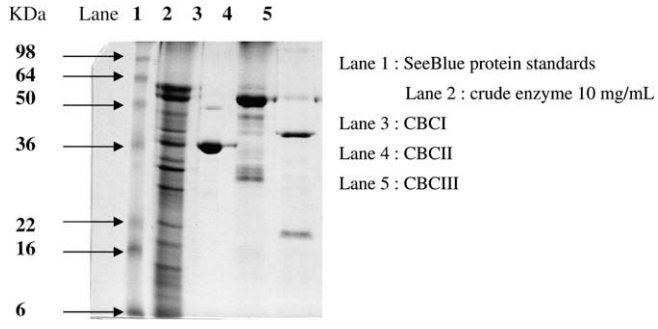

Fig. 2. SDS–PAGE of crude chitosanase and CBCI, II and III separated from CM-Sepharose column.

CBCI, CBCII and CBCIII were also analyzed by SDS–PAGE, the molecular weights of the major protein in each fraction were 41 kDa, 54 kDa and 46 kDa (Fig. 2). The molecular mass of CBCI, CBCII and CBCIII was obviously different from the other *Bacillus* chitosanases, such as those from *B. subtilis* TKU007 (25 kDa) (Wang & Yeh, 2008), and *B. subtilis* KH-1 (28 kDa) (Chiang et al., 2003). However, the molecular mass of chitosanase of *B. subtilis* IMR-NK1 (41 kDa) (Chiang et al., 2003) was the same as CBCI. The chitosanases of *B. cereus* S1 (45 kDa) (Kurakake et al., 2000) and *Bacillus sp.* KCTC 0377BP (45 kDa) (Choi et al., 2004) had the similar molecular mass as CBCIII. The specific activities of CBCI, CBCII and CBCIII were 10.19, 4.25 and 129.94 unit/mg, respectively (Table 1). It was noticed that CBCII not only had the lowest specific activity, but also the lowest yield (0.58%). The CBCIII had a specific activity (129.94 U/mg) approximately 281-fold of the crude extract, which is also much higher than the purified chitosanase from *B. subtilis* TKU007 (0.0603 U/mg) (Wang & Yeh, 2008). However, the yield of the purified chitosanase from *B. subtilis* TKU007 was 48% (Wang & Yeh, 2008), which is much higher than the yield of CBCIII (2.14%). Nevertheless, the CBCIII had the highest specific activity among the three fractions, and appeared to be the major component in the enzyme system responsible for the hydrolysis. The CBCI and CBCII might only play assisting role during hydrolysis.

3.2. Profiles of hydrolysates in batch reactor

In order to elucidate the characteristics of the enzyme fractions, the crude enzyme, CBCI, CBCII and CBCIII were used to hydrolyze 1% chitosan at 40 °C with reaction volume 10 mL and E/S ratio 0.03. The chitooligosaccharides profiles of the hydrolysates are shown in Fig. 3. The CBCI was found to yield dimer only (Fig. 3B). The primary hydrolytic products of CBCII were low DP chitooligosaccharides, such as dimer, trimer and tetramer (Fig. 3C). The CBCIII had the fastest reaction rate and might hydrolyze chitosan at some specific site to yield high DP chitooligosaccharides (Fig. 3D).

Since dimer is the only product of CBCI and the yield of CBCII was only 0.58%, both CBCI and CBCII were not used in the follow-up study. For the subsequent experiments, the crude enzyme and CBCIII were compared for their abilities of hydrolyzing chitosan to yield high DP chitooligosaccharides.

3.3. Reaction pattern of crude chitosanase and CBCIII

In order to understand the hydrolysis mechanism of crude chitosanase and CBCIII, we used pure chitobiose, chitotriose, chitotetraose, chitopentaose and chitohexaose as substrate. It was found that crude enzyme and CBCIII could not hydrolyze dimer or trimer, and only slightly hydrolyzed tetramer to dimer (data not shown). It was noteworthy that chitosan oligosaccharides pentamer was completely hydrolyzed to dimer plus trimer after 10 min of reaction by crude enzyme, but CBCIII must take approximately

Table 1
Effect of purification procedures on the chitosanase from the *Bacillus cereus* NTU-FC-4.

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	4050.1	8782.83	0.46	1.00	100
Precipitation by 70% (v/v) acetone	3295.5	893.53	3.69	8.00	81.37
CM-Sepharose					
CBCI	43.4	4.26	10.19	22.10	1.07
CBCII	23.5	5.52	4.25	9.22	0.58
CBCIII	86.8	0.67	129.94	281.78	2.14

*Crude extract from 1800 mL medium.

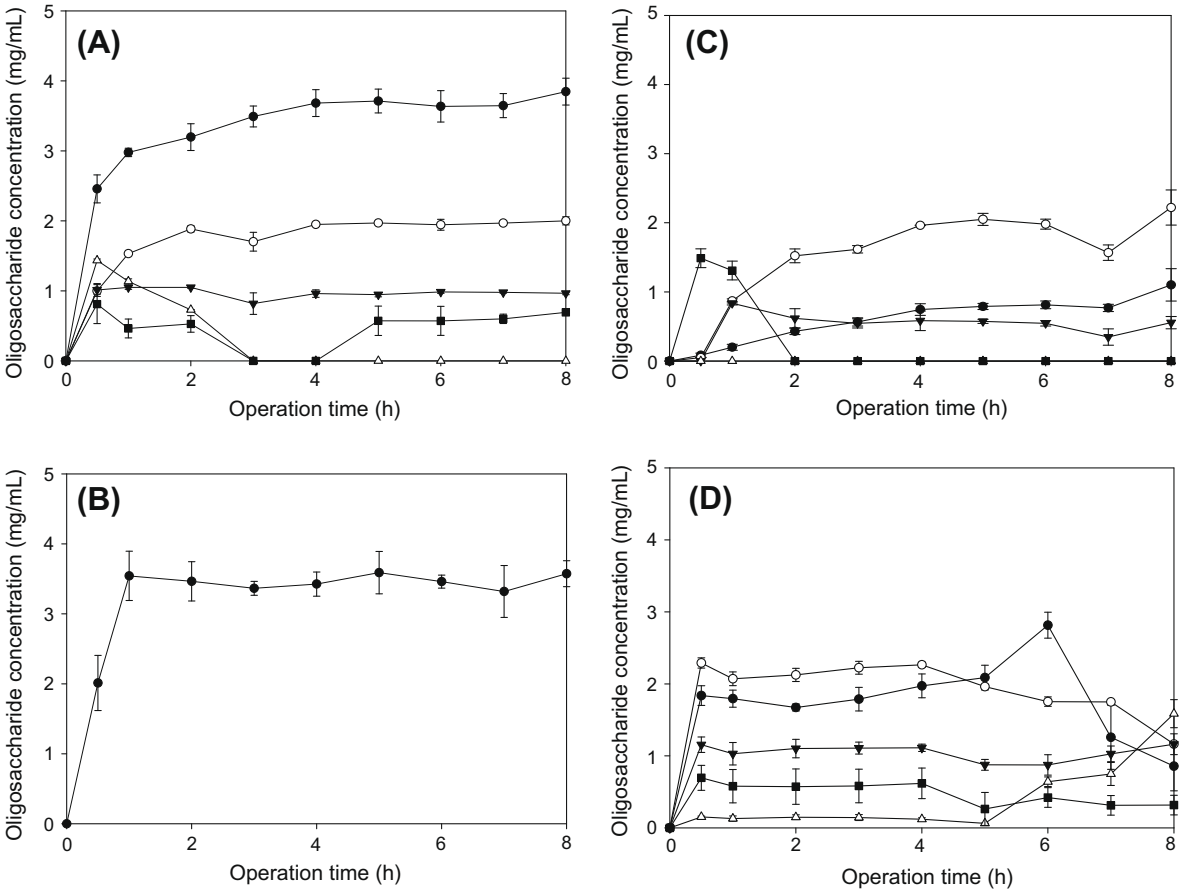

Fig. 3. The reaction patterns of chitooligosaccharides during hydrolysis of 1% chitosan by crude chitosanase (A), CBCI (B), CBCII (C) and CBCIII (D) for 8 h at 40 °C with reaction volume 10 mL and E/S ratio 0.03. ●, chitobiose; ○, chitotriose; ▼, chitotetraose; △, chitopentaose; and ■, chitohexaose.

Table 2
The products and hydrolysis time of chitopentaose and chitohexaose by crude enzyme and CBCIII in batch reactor with E/S ratio 0.3 unit/mL.

	Chitopentaose		Chitohexaose	
	Products	Hydrolysis time	Products	Hydrolysis time
Crude enzyme	Chitobiose and chitotriose	10 min	Chitotetraose chitotriose and chitotetraose	10 min
CBCIII	Chitobiose and chitotriose	90 min	Chitobiose, chitotriose and chitotetraose	50 min

90 min to completely hydrolyze the pentamer (Table 2). The hexamer was hydrolyzed mainly to trimer plus trimer and to a lesser extent to dimer plus tetramer as shown in Fig. 4, and the tetramer might be subsequently hydrolyzed to dimer. It was also observed that hexamer could be hydrolyzed completely in 10 min by crude enzyme, but it took 50 min to be completely hydrolyzed by CBCIII. From the cleavage patterns of the various pure chitooligosaccharides, it reveals that the pentamer and hexamer will be further hydrolyzed, and the rate of hydrolysis of pentamer and hexamer by crude enzyme is faster than that by CBCIII. Therefore, it would be ideal to use a reactor which could separate the hydrolyzed products from enzyme in a controlled time period for manufacturing high DP chitooligomers. To investigate the above idea, we used membrane reactor to observe the chitooligosaccharides produced by crude enzyme and CBCIII.

3.4. Hydrolysis of chitosan in a membrane reactor

The residence time (RT), defined as reactor volume divided by permeate flux, is the time period that the enzyme has chance to react with the substrate before the hydrolyzed products being removed from the reactor. When the membrane reactor was operated at E/S ratio 0.06 unit/mg, substrate concentration 20 mg/mL and residence time 100 min, the crude enzyme hydrolyzed chitosan and yielded product consisting of dimer to hexamer, and the concentration of high DP oligomers (pentamer and above) in the final product was only 3.56 mg/mL (Fig. 5A). However, when CBCIII was used, dimer did not exist in the hydrolyzed product but heptamer appeared. The concentration of high DP oligomers was 9.78 mg/mL (Fig. 5B). In addition, the high DP oligomers occupied ca. 48% of total oligomers in the final product of CBCIII, as compared to ca. 29% resulted from the crude enzyme. It appears that the CBCIII is indeed superior to the crude enzyme for manufacturing high DP chitooligomers, and membrane reactor, which can reduce the chance for high DP oligomers being further hydrolyzed to low DP oligomers, is a proper tool for carrying out the reaction.

To investigate the effect of residence time on the yield of high DP oligomers, enzymatic processes were also conducted at shorter residence time, 50 min and 33 min, and results are given in Fig. 6. Decrease of residence time, indeed, reduced the chance for high DP

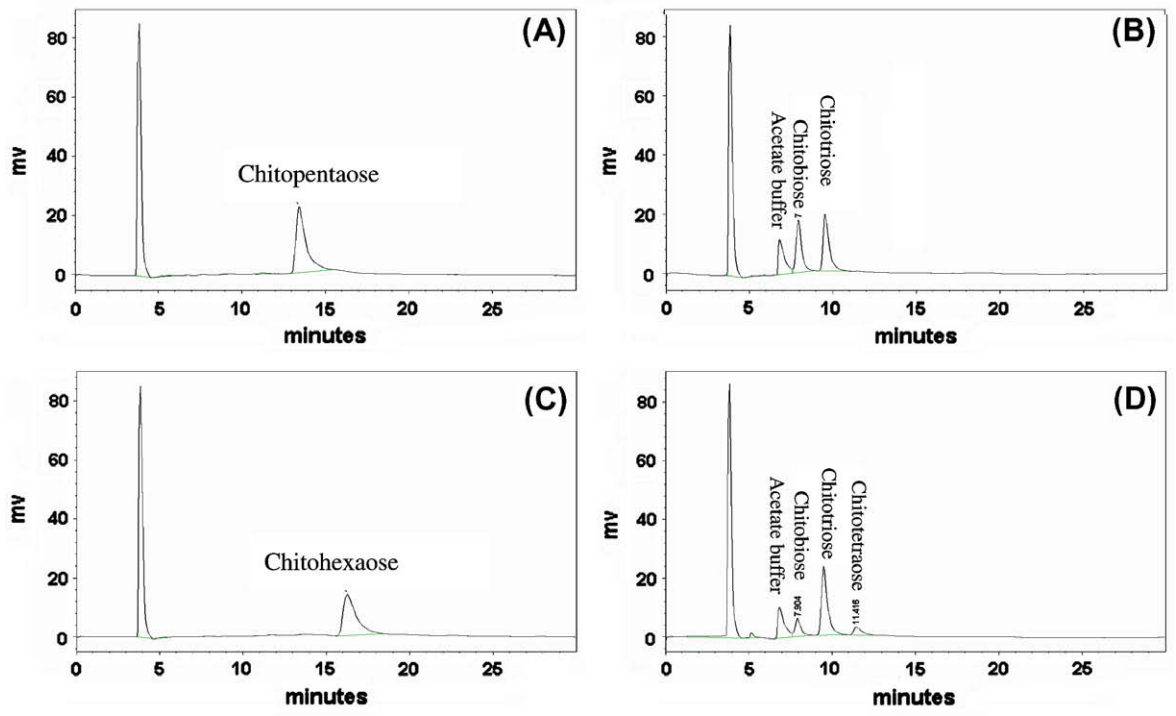

Fig. 4. The hydrolysis profile of chitopentaose and chitohexaose by chitosanase from *Bacillus cereus* NTU-FC-4. (A) The chitopentaose without hydrolysis, (B) the products of chitopentaose after hydrolysis, (C) the chitohexaose without hydrolysis and (D) the products of chitohexaose after hydrolysis.

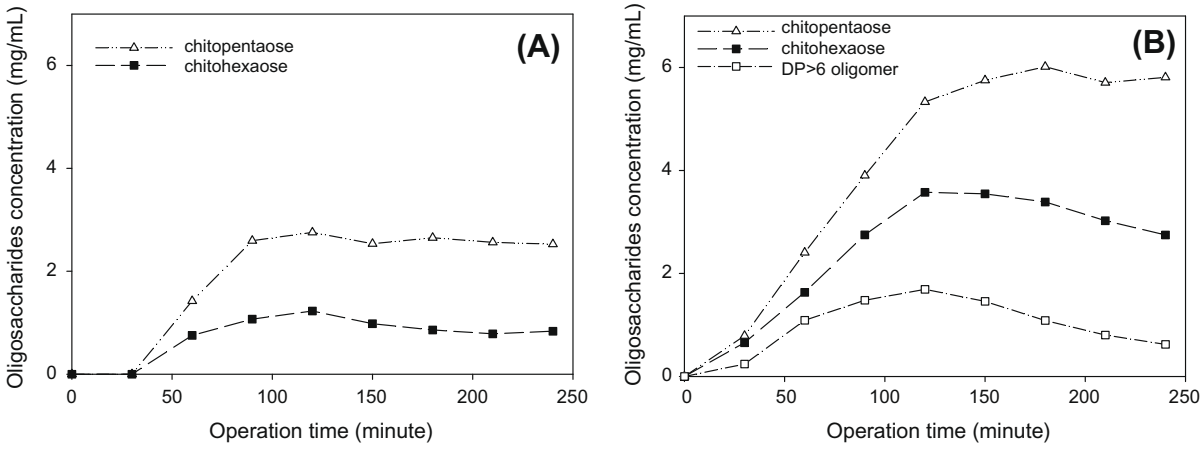

Fig. 5. Chitooligosaccharides with DP above 5 yielded from the enzymatic hydrolysis of chitosan by crude chitosanase (A) and CBCIII (B) in the membrane reactor. System was operated at E/S ratio 0.06 unit/mL; reactor volume 500 mL; flux 5 mL/min; residence time 100 min. △, chitopentaose; ■, chitohexaose; and □, DP > 6 oligomer.

oligomers being further hydrolyzed by CBCIII and resulted in the high percentage of high DP oligomers in the product. The average percentages of high DP oligomers in the products were ca. 61% and 69% for RT 50 min and RT 33 min, respectively, as compared with 48% in the product of RT 100 min. However, the RT shorter than 100 min did not provide enough reaction time for enzyme to fully hydrolyze the substrate, therefore, the total oligomer content as well as the high DP oligomers content in the product of RT 100 min was significantly higher than that produced at RT 50 min or RT 33 min. It was reported that the maximum yield of the target pentamer and hexamer was 22% when the hydrolysis of chitosan by chitosanase was conducted in an immobilized enzyme reactor, and 15% in the hydrolysis of chitosan by hydrochloric acid solution (Horowitz, Roseman, & Blumenthal, 1957). Ming et al. (2006) found that decreased the surface activity of immobilized chitosanase could increase the yield of target products (pentaose and hexaose) from 20% to 45% (Ming et al., 2006). In this study, when the residence time was 50 min, the average content of the high DP oligomers, pentamer and above, hydrolyzed by CBCIII was about 35% and the maximum content was 49% in the product. The average yield of the high DP oligomers, which was represented by the ratio of average concentration of high DP oligomers (5.5 mg/mL) in the product to initial chitosan concentration (20 mg/mL) in the feed, was approximately 28%.

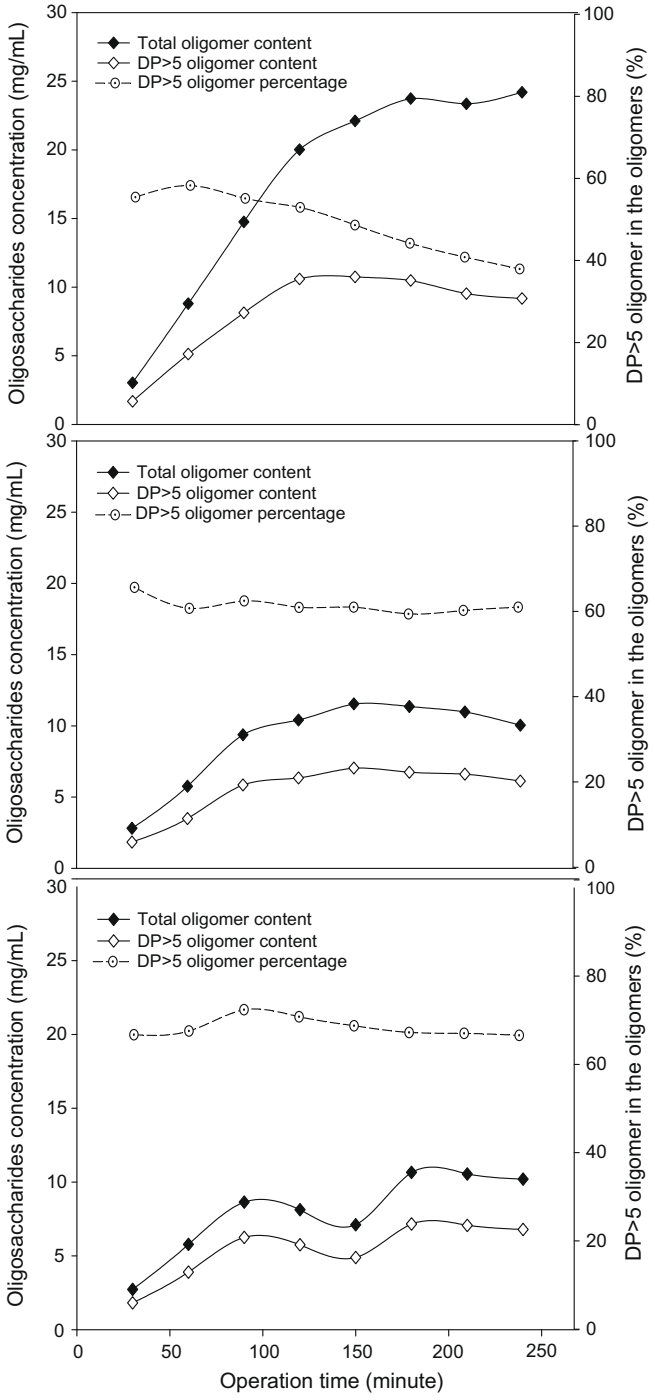

Fig. 6. The contents of total and high DP oligomers (pentamer–octamer) and the percentage of high DP oligomers in the product yielded from the enzymatic hydrolysis of chitosan by CBCIII in the membrane reactor. System was operated at E/S ratio 0.06 unit/mL; reactor volume 500 mL and residence time 100 min (A), 50 min (B) and 33 min (C). ♦, total oligomer content; ◇, DP > 5 oligomer content; and ⊙, the DP > 5 oligomer percentage.

3.5. Stability of the enzymatic membrane reactor

Since it is quite important for industrial application that the reactor can be operated at steady state for a long time, we further investigated process stability. For an enzymatic membrane reactor, enzyme may be denatured and the membrane may be fouled during operation. However, the stability of permeate flux as well as the composition of hydrolyzed product can reveal the stability and the performance in a system. We found that the membrane reactor with CBCIII could be operated for at least 24 h and still maintained stable output (Fig. 7).

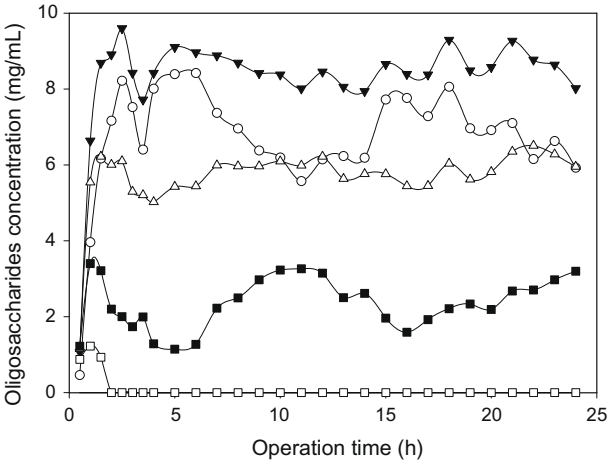

Fig. 7. The chitooligosaccharides yielded from the enzymatic hydrolysis of chitosan by CBCIII during 24 h of operation in the membrane reactor. System was operated at E/S ratio 0.06 unit/mL; reactor volume 500 mL; flux 10 mL/min; residence time 50 min.○, chitotriose; ▼, chitotetraose; △, chitopentaose; ■, chitohexaose; and □, DP > 6 oligomer.

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