Carbohydrate Polymers 92 (2013) 2141–2148

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Applied development of crude enzyme from *Bacillus cereus* in prebiotics and microbial community changes in soil

Tzu-Wen Liang [a,b], Chin-Pei Liu [b], Chunhung Wu [b], San-Lang Wang [a,b,*]

[a] *Life Science Development Center, Tamkang University, New Taipei City 25137, Taiwan*
[b] *Department of Chemistry, Tamkang University, New Taipei City 25137, Taiwan*

ARTICLE INFO

Article history:
Received 8 October 2012
Received in revised form
28 November 2012
Accepted 30 November 2012
Available online 10 December 2012

Keywords:
Chitosanase
Chitosan oligosaccharides
Depolymerization
Bacillus cereus
Prebiotic
PCR-DGGE

ABSTRACT

The chitosanase and chitinase activity were revealed in the culture supernatant of *Bacillus cereus* TKU027 with shrimp head powder (SHP) as the sole carbon/nitrogen source. The chitosan with 60% degree of deacetylation (DD) was depolymerized by TKU027 crude enzyme. The low DP oligomers stimulated the growth of *Lactobacillus paracasei* BCRC12193 and *Lactobacillus kefir* BCRC14011 in a MRS broth supplemented with low DP oligomers for 12 h. Conversely, the high DP oligomers (0.1%) had potent inhibitory effects against *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 for 48 h. Besides, the study also investigated the effects of *B. cereus* TKU027 on degradation of SHP and the survival conditions of bacteria in mangrove river sediment of Tamsui River. The 5 weeks-incubation sample of SHP and *B. cereus* TKU027-amended mangrove river sediment showed the highest amounts of biomass, reducing sugar and total sugar, and some variance of bacterial community compositions existed in the soils.

1. Introduction

Chitosan is a linear heteropolysaccharide composed of β-1,4-linked-D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) in varying proportions. Recent studies on chitosan have attaracted interest for converting these species into oligosaccharides because the oligosaccharides not only are water-soluble but also possess versatile functional properties such as antitumor activity and antimicrobial activity (Liang, Chen, Yen, & Wang, 2007; Wang, Lin, Yen, Liao, & Chen, 2006; Wang et al., 2006). Traditionally, the oligosaccharides were processed using chemical methods in industries. Many problems existed in these chemical processes, such as the production of a large amount of short-chain oligosaccharides, low yields of oligosaccharides, the high cost of separation and environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost and reproducibility, enzyme hydrolysis has become more popular in recent years (Wang et al., 2008).

The application of probiotic bacteria in food products is increasing due to potential health benefits associated with the consumption of these bacteria (Liang et al., 2010). The most well-known prebiotics are inulin and fructooligosaccharide; these selectively stimulate the bifidobacteria (Gibson, 1999; Kruse, Kleessen, & Blaut, 1999). Up to now, few of previous literatures reported the effects of chitosan oligosaccharides on lactic acid bacteria, and the hydrolysed chitosans of previous reports were not clearly defined in terms of DP and deacetylation. Consequently, one of the main objectives in this study was to investigate the feasibility of the growth stimulatory effect of chitosan oligosaccharides on lactic acid bacteria.

Tamsui mangroves, located at the mouth of the Tamsui River, are the most northern mangroves in Taiwan. These mangroves are habitats of an enormous variety of organisms. Among them, shrimps and crabs (such as mangrove crabs), which are rich in chitin and protein in their shells, are particularly abundant (Ewel, 2008; Yin, Gu, & Wan, 2005). If added useful microorganisms such as *B. cereus* TKU027, which are able to use chitin-containing wastes as the sole carbon/nitrogen source for the production of chitinase/chitosanase, it would be helpful for riverbed soil improvement as well as environmental bioremediation applications.

In this study, we prepared and characterized defined chitosan oligosaccharides mixtures enriched in oligomers with DP 4–9. Chitosan was degraded by enzymatical hydrolysis using the crude enzyme from *B. cereus* TKU027 and a selective precipitation of the degradation products was evaluated using MALDI-TOF mass spectrometry. The use of crude enzyme has advantage of omitting the procedure for purifying enzymes and helping decrease the cost of chitosan oligosaccharides production. This study reported in detail on the production of chitosan oligosaccharides using a non-purified

* Corresponding author. Present address: No. 151, Yingchuan Rd., Tamsui Dist., New Taipei City 25137, Taiwan. Tel.: +886 2 2626 9425; fax: +886 2 2620 9924.
E-mail address: sabulo@mail.tku.edu.tw (S.-L. Wang).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.11.097

enzyme preparation. The prebiotic potential of chitosan oligosaccharides was tested in terms of growth and growth rate on lactic acid bacteria. The above technologies facilitate the potential use of this process in industrial applications and functional foods. Besides, added *B. cereus* TKU027 and/or SHP in the mangrove river sediment of Tamsui River, and then used PCR-DGGE to analyze the changes of bacterial community in the soils. In addition, the biomass, reducing sugar and total sugar amount of the 5 weeks-incubation samples were also analyzed and compared.

2. Materials and methods

2.1. Materials

B. cereus TKU027, a chitinase-producing strain, could produce two chitinases with shrimp head powder (SHP) as the sole carbon/nitrogen source (Wang, Liu, & Liang, 2012). The SHP used in these experiments was prepared as described previously (Wang et al., 2006). The shrimp heads were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). During the preparation of the SHP, the shrimp heads were washed thoroughly with tap water and then dried. The dried materials obtained were milled to powders for use as the carbon source for enzymes production. Chitosan with 60% DD was from Kiotec Co., Hsinchu, Taiwan. All other reagents used were of the highest grade available.

2.2. Preparation of crude enzyme

B. cereus TKU027 was isolated from soils by using SHP as the sole carbon/nitrogen source. For the production of enzymes with chitinase and chitosanase activity, *B. cereus* TKU027 was grown in 50 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SHP, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$. One milliliter of the seed culture was transferred into 50 mL of the same medium and grown in an orbital shaking incubator for 2 days at 37 °C and pH 7.2 (the pH after being autoclaved was 7.5). After incubation, the culture broth was centrifuged (4 °C and 12,000 × *g* for 20 min) and ammonium sulfate was slowly added to the supernatant to 80% saturation and the mixture was stored at 4 °C overnight. The precipitate was collected by centrifugation at 4 °C for 20 min at 12,000 × *g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7) and dialyzed against the buffer. The resultant dialysate was measured the chitinase and chitosanase activity.

2.3. Measurement of chitinase and chitosanase activity

The colloidal chitin (1.3% in 50 mM phosphate buffer) was used as the substrate for the measurement of chitinase activity. The mixture of enzyme solution (0.5 mL) and substrate (1 mL) was incubated at 37 °C for 30 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita (1971) with *N*-acetylglucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min.

Chitosanase activity of the enzyme was measured by incubating 0.2 mL of the enzyme solution with 1 mL of 0.3% (w/v) chitosan (Kiotec Co., Hsinchu, Taiwan; with 60% deacetylation) in 50 mM phosphate buffer, pH 7 at 37 °C for 30 min. The reaction was stopped by heating it at 100 °C for 15 min. The amount of reducing sugar produced was measured by the method of Imoto and Yagishita (1971) with glucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of reducing sugars per min.

2.4. Enzymatic production of the chitosan oligosaccharides

For preparation of chitosan oligosaccharides, chitosan with 60% deacetylation (0.5% (w/v) in 50 mM phosphate buffer) was used as the substrate. The mixture of TKU027 crude enzyme solution (1 mL) with chitinase activity (0.24 U/mL) and chitosanase activity (0.20 U/mL) and substrate (1 mL) was incubated at 37 °C. Samples were withdrawn at 0, 2, 4, 6, 12, 24, 48, and 72 h from reaction mixtures for further preparation of the chitosan oligosaccharides. These solutions were concentrated to about 1/5 of the original volume with a rotary evaporator under diminished pressure and followed by adding methanol with final methanol concentration of 90% (v/v). Yellow agglomerates were formed in the solution. The agglomerates were concentrated with a rotary evaporator under diminished pressure and were collected after drying in vacuum. The supernatant was concentrated to about 1/10 of the original volume with a rotary evaporator under diminished pressure. Then, it was precipitated by adding acetone with final acetone concentration of 90% (v/v). The precipitates were collected after drying in vacuum.

2.5. Measurement of total sugars

To evaluate total sugars, the phenol-sulfuric acid method was used (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Briefly, 25 μL of 5% phenol was added to 1 mL of sample. After shaking, 2.5 mL of concentrated H_2SO_4 was added. The mixture was left to stand for 10 min and absorbance was read at 490 nm. Pure D-glucose was employed as standard.

2.6. MALDI-TOF MS analysis

An amount of 1 μL of the sample solution (2 mg/mL) was mixed on the target with 1 μL of a solution of 2,5-dihydroxybenzoic acid as a matrix (15 mg/mL) in H_2O–ACN–TFA solution (50/50/0.1%, v/v/v). Positive ion MALDI mass spectra were acquired with MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser emitting at 337 nm operating in linear mode. Each mass spectrum was the accumulating data of approximately 30–50 laser shots. External 3-points calibration was used for mass assignment.

2.7. Prebiotic effect of the chitosan oligosaccharides on lactic acid bacteria

The test strains of *Lactobacillus paracasei* subsp *paracasei* BCRC12193 and *Lactobacillus kefir* BCRC14011 were obtained from Bioresource Collection and Research Center (BCRC), Food Industrial Research Institute, Shin Chu, Taiwan. MRS broth base free of fermentable carbon/nitrogen sources was used to investigate the ability of the test strains to grow on prebiotic chitosan oligosaccharides. The tested chitosan oligosaccharides were added to the medium at 0.1% (w/v) and 0.5% (w/v), respectively. Additionally, growth of the strains was examined on 60% DD chitosan. The organism was grown in triplicate experiments in 10 mL culture tubes under anaerobic conditions at 37 °C for 12-48 h. The growth of the bacteria was monitored throughout the fermentations by measuring the culture O.D. at 660 nm.

2.8. Sampling and soil treatment

Mangrove river sediment (Tamsui, Taiwan) was collected as the soil samples. The soil samples were sieved through a 2 mm mesh screen to remove gravel, plant matter and other debris. Four samples were performed at 30 °C in 250 mL-Erlenmyer flask containing (1) Group Soil: 100 g soil (moisture content 50%), (2) Group S: 100 g soil (moisture content 50%) and 1 g SHP, (3) Group 027: 100 g soil

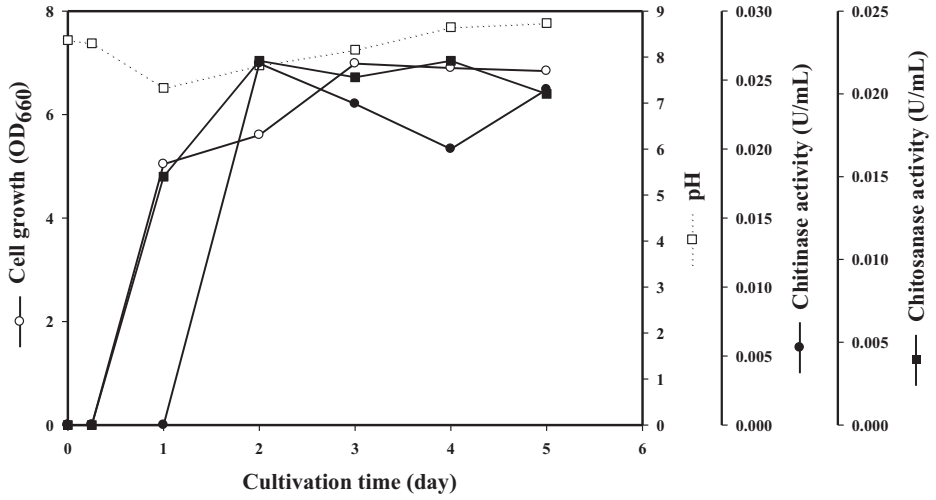

Fig. 1. Time courses of the chitosanase and chitinase production in a culture of *B. cereus* TKU027 on shrimp head-containing media: (●) chitinase activity (U/mL); (■) chitosanase activity (U/mL); (○) cell growth; (□) pH.

(moisture content 50%) and 1 mL (10^8 CFU) culture broth of *B. cereus* TKU027, (4) Group B: 100 g soil (moisture content 50%), 1 g SHP and 1 mL (10^8 CFU) culture broth of *B. cereus* TKU027, respectively. During the experiment, all the Erlenmyer flask of these four groups were covered with aluminum foils and the moisture content of the samples was adjusted to a certain value with sterilized water every day.

2.9. DNA extraction

Total genomic DNA was extracted using and UltraClean® Soil DNA Isolation kit (MO BIO laboratories, Inc.). Three replicates of DNA samples were retrieved from each of the treatment. DNA samples were stored in −20 °C.

2.10. PCR-DGGE and band recognition

PCRs were performed with a thermal cycler 9700 (Applied Biosystems, USA). The PCR reaction mixture contained 2× Taq PCR Master Mix (Ginomics BioScience and Technology Co. Ltd.), 0.4 μM of primers of 968F and 1401R, with a GC-clamp was attached to the 5′-end of 968F and 1 μL of template DNA which were mixture from three replicates of DNA samples. The 16S rDNA was amplified as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 2 min, 72 °C for 30 s and with a final extension at 72 °C for 10 min. The amplified product was confirmed by electrophoresis through a 1% (w/v) agarose gel in 1× TAE buffer, followed by staining with ethidium bromide. DGGE analysis was performed with a D-Code universal mutation detection system (Bio-Rad, Hercules, Calif). PCR product (25 μL) was load onto a 7% (w/v) denaturing gradient polyacrylamide gel consisting of 40–60% of formamide and urea. The electrophoresis was run at 60 °C in 1× TAE for 12 h at a constant voltage of 75 V. After the electrophoresis, polyacrylamide gel was stained with SYBR® Green I nucleic acid gel stain and visualized on an ultraviolet (UV) transilluminator.

2.11. Statistical analysis of DGGE patterns

DGGE profiles were recognized and analyzed with Quantity One® software (Bio-Rad, USA). The similarity matrices of each DGGE bands were calculated by coefficient from dice. Afterward, the phylogenic dendrograms were generated by unweighted pair-group method using arithmetic averages (UPGMA).

3. Results and discussions

3.1. Production of extracellular chitinase/chitosanase by B. cereus TKU027

In the previous study, *B. cereus* TKU027 could produce two chitinases and the two enzymes could hydrolyze chitin and chitosan (Wang et al., 2012). Thus the chitinase and chitosanase activity were measured respectively during 5 days of TKU027 cultivation in the medium with SHP as the sole carbon/nitrogen source. The 50 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing 1% SHP was the most suitable for the production of the enzymes with chitinase and chitosanase activity by strain TKU027 at 37 °C. After a 6-h lag phase, exponential growth was observed for 3 days, and stationary phase was reached after 3 days. As shown in Fig. 1, maximum activities of chitinase (0.026 U/mL) and chitosanase (0.022 U/mL) were detected in the culture on the second day and then decreased gradually. The enzymes production was closely related to the cell growth. This result indicated that the production of chitinase/chitosanase is cell growth dependent and *B. cereus* TKU027 is a promising chitinase/chitosanase producer.

3.2. Production of chitosan oligosaccharides

The chitosan oligosaccharides were produced from 60% DD chitosan with *B. cereus* TKU027 crude enzyme. The course of degradation of the chitosan sample was conveniently studied by measurement of total sugar and reducing sugar. Fig. 2 shows the total sugar and reducing sugar of the sample as a function of reaction time. The total sugar and reducing sugar on the chitosan sample resulted in similar pattern (Fig. 2). The total sugar and reducing sugar increased and the recovery of the chitosan sample decreased dramatically in the early reaction stage, which can be attributed to an endo-type degradation process. The fresh crude enzyme had been added into reaction solution after 72 h, but it did not cause any improvement in the total sugar and reducing sugar increase. To obtain low DP oligomers, a selective precipitation respectively in 90% methanol and acetone solutions was used as described earlier (Liang et al., 2007). By MALDI-TOF analysis of the precipitation in 90% acetone solution, it appeared that the chitosan oligosaccharides with DP up to 9 (Fig. 3). The higher DP chitooligomers were precipitated as a light yellow powder, leaving in the methanol solution. The MALDI-TOF MS of the low DP oligomers fraction reveals pronounced differences between the chitooligosaccharides

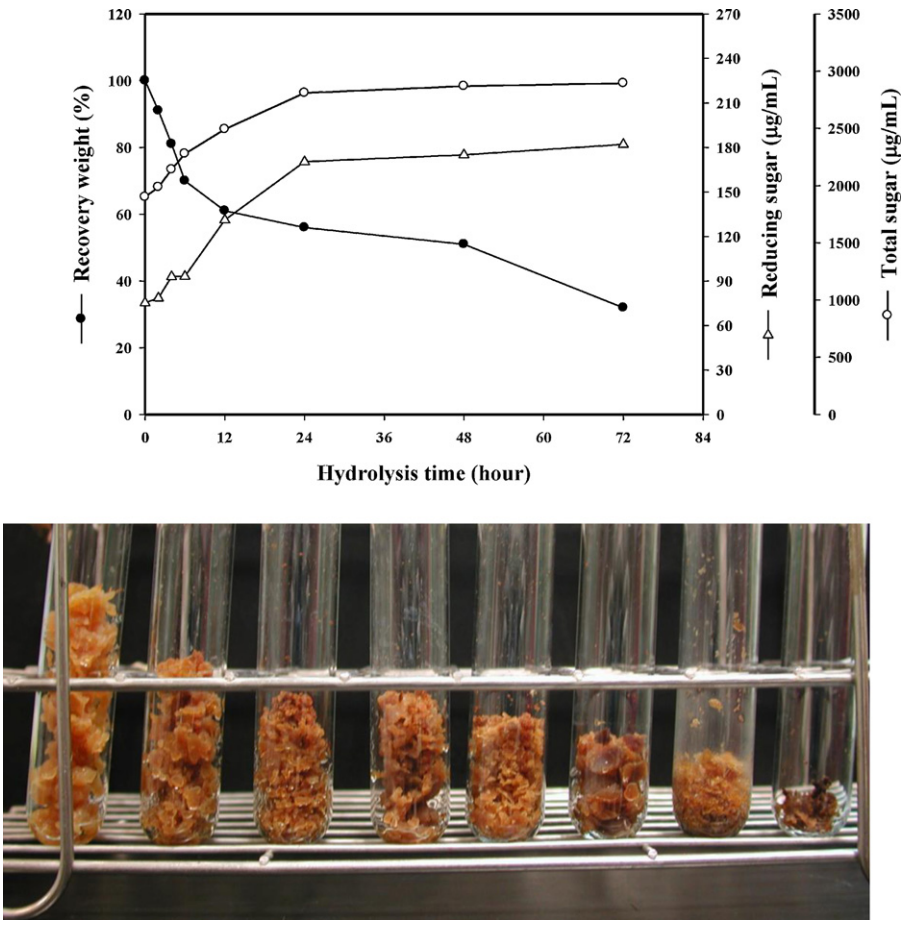

Fig. 2. Time course measurement of reducing sugar, total sugar, and the residual chitosan depolymerized with the crude enzyme from *B. cereus* TKU027. The tubes show the residual chitosan at various hydrolysis time (0, 2, 4, 6, 12, 24, 48, and 72 h, respectively, from left to right).

generated by the crude enzyme, as shown for the depolymerization of chitosan in Fig. 3. The hydrolysates ions present in the mass spectra were identified as sodium adducts, $[M+Na]^+$. Since MALDI-TOF analysis is limited to molecular weights higher than 500 Da due to interference of the matrix signals, the DP <2 oligomers could not be determined by this method. More information about the assigned structure of each signal at different times of hydrolysis is given in Table 1. The product from the reaction with TKU027 crude enzyme is a mixture of hetero-chitooligosaccharides of DP 4–9, containing $(GlcN)_3GlcNAc$ (short notation: D_3A_1), D_2A_2, DA_3, D_3A_2, D_2A_3, D_4A_2, D_3A_3, D_2A_4, D_4A_3, D_3A_4, D_5A_3, D_6A_3, and D_5A_4 as the major components of each of DP 4–9, respectively (Table 1). These products generated by hydrolysis of 60% DD chitosan with TKU027 crude enzyme are not a series of fully deacetylated oligomers of GlcN. The crude enzyme hydrolyzed the 60% DD chitosan by cleavage of glycosidic bonds of the type –A|A– and –D|D–, whereas –D|A– and –A|D– are not, or only very slowly, susceptible to hydrolysis. After 72 h, the appearance of chitooligosaccharides of DP >6 would mean in this case that those do not contain any sequence that could be susceptible to hydrolysis, e.g. the sequence of D_4A_3 must be composed mostly of alternating D and A units. We assume that the persistence of the oligomers of alternating D and A units, including D_2A_2, D_3A_2,

Table 1
Assigned ion composition of MALDI-TOF-MS spectra of chitosan oligosaccharides with DP below 9 prepared by enzymatic hydrolysis for the indicated time and fractionated by selective isolation by 90% methanol and acetone.

m/z	Ion composition	DP	Types	Hydrolysis time (h)						
				2	4	6	12	24	48	72
727	$(GlcN)_3$–GlcNAc	4	$[M+Na]^+$	+	+	+	–	–	–	–
769	$(GlcN)_2$–$(GlcNAc)_2$	4	$[M+Na]^+$	+	+	+	+	+	+	+
811	GlcN–$(GlcNAc)_3$	4	$[M+Na]^+$	+	+	+	–	–	–	–
930	$(GlcN)_3$–$(GlcNAc)_2$	5	$[M+Na]^+$	+	+	+	+	+	+	+
972	$(GlcN)_2$–$(GlcNAc)_3$	5	$[M+Na]^+$	+	–	–	–	–	–	–
1091	$(GlcN)_4$–$(GlcNAc)_2$	6	$[M+Na]^+$	+	+	+	+	+	+	+
1133	$(GlcN)_3$–$(GlcNAc)_3$	6	$[M+Na]^+$	+	+	+	+	+	+	+
1175	$(GlcN)_2$–$(GlcNAc)_4$	6	$[M+Na]^+$	+	+	+	–	–	–	–
1294	$(GlcN)_4$–$(GlcNAc)_3$	7	$[M+Na]^+$	+	+	+	+	+	+	+
1336	$(GlcN)_3$–$(GlcNAc)_4$	7	$[M+Na]^+$	+	+	+	–	–	–	–
1455	$(GlcN)_5$–$(GlcNAc)_3$	8	$[M+Na]^+$	+	+	+	+	+	+	+
1616	$(GlcN)_6$–$(GlcNAc)_3$	9	$[M+Na]^+$	–	–	–	+	+	–	–
1658	$(GlcN)_5$–$(GlcNAc)_4$	9	$[M+Na]^+$	+	+	+	–	–	–	–

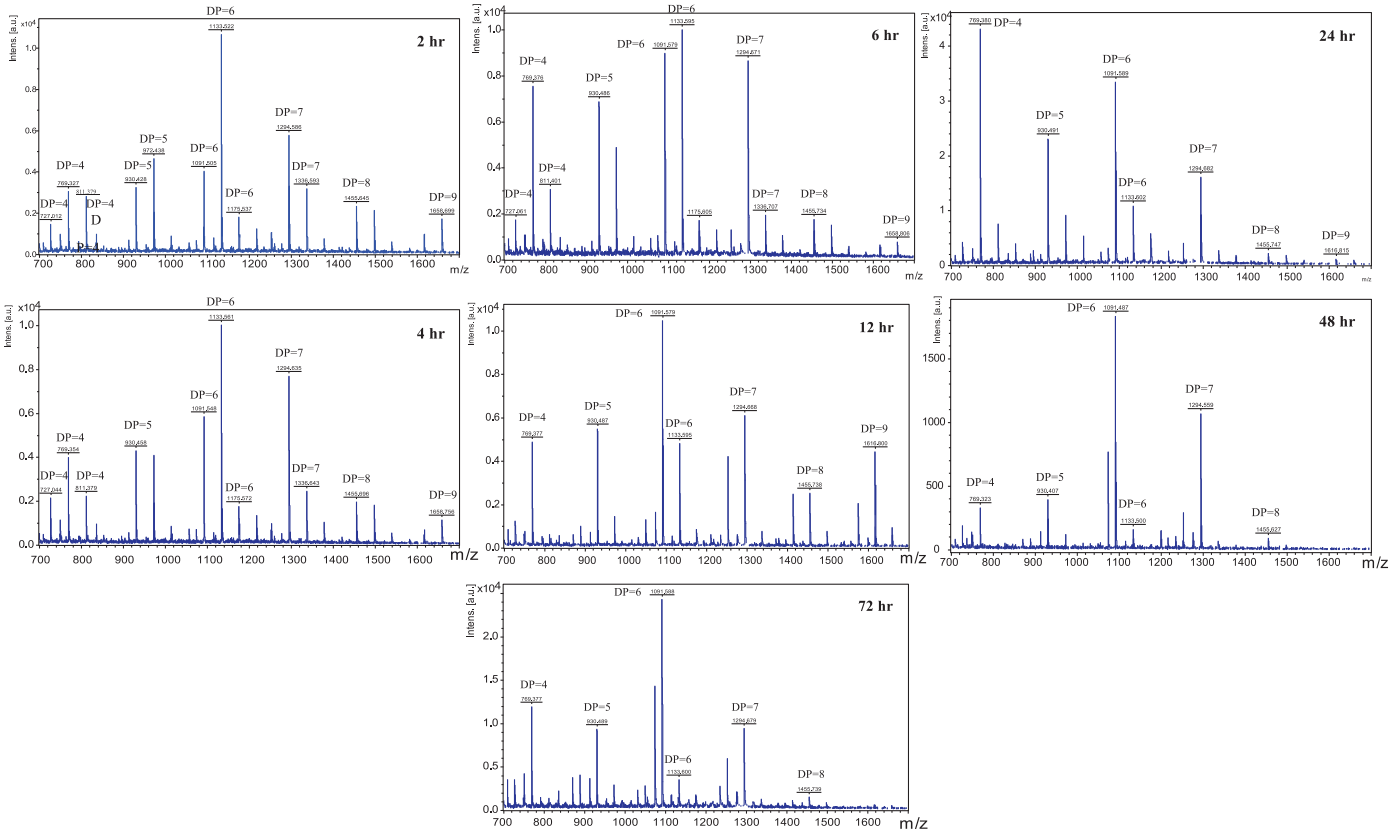

Fig. 3. MALDI-TOF-MS of the chitosan oligosaccharides mixtures obtained during the 60% DD chitosan hydrolysis with TKU027 crude enzyme. The proportion of low molecular weight oligomers was reduced by precipitation in 90% methanol soluble/90% acetone insoluble fraction. Identified peaks are labeled as DP, where DP indicated degree of polymerization. The hydrolysis time is labeled in each spectrum.

D_4A_2, D_3A_3, D_4A_3, and D_5A_3, is due to product inhibition or to a steady state of degradation and transglycosylation.

3.3. Effect of chitosan oligosaccharides on lactobacilli growth

In order to study the effect of the chitosan oligosaccharides generated by TKU027 crude enzyme on the growth of lactic acid bacteria, the bacteria were cultured in a MRS broth supplemented with 0.1% chitosan oligosaccharides for 48 h at 37 °C (Table 2). When the bacteria were cultured for 12 h, the low DP oligomers obtained after 24 h hydrolysis enhanced the growth of *L. paracasei* BCRC12193 most obviously (324%), followed by low DP oligomers after 72 h hydrolysis (201%). The two types of chitosan oligosaccharides from MALDI-TOF analysis indicated the only presence of D_6A_3 at 24 h hydrolysis, not at 72 h hydrolysis (Table 1). Thus, it is postulated that D_6A_3 presented in the chitosan oligosaccharides is responsible for enhancing the growth of *L. paracasei* BCRC12193. As shown in Table 2, the tested low DP oligomers showed a growth stimulatory effect on the lactic acid bacteria and had a larger growth stimulatory effect on *L. paracasei* BCRC12193 than on *L. kefir* BCRC14011. These results indicate that low DP oligomers from TKU027 crude enzyme are excellent growth stimulators of *L. paracasei* BCRC12193 and *L. kefir* BCRC14011. It is interesting to note that *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 were able to grow better in MRS broth with the low DP oligomers added than in MRS broth alone.

To test the effects of chitosan oligosaccharides on the growth rates of *L. paracasei* BCRC12193 and *L. kefir* BCRC14011, cells were cultivated in MRS broth supplemented with 0.1% low DP oligomers, and bacterial growth was measured at different time (Table 2). Cells reached a larger number after 12 h of cultivation in MRS broth with 0.1% low DP oligomers added than in MRS broth alone. While, after 48 h of cultivation, the tested lactic acid bacteria grew in 0.1% low DP oligomers as well as in MRS broth. So, the growth rate increased in MRS broth containing 0.1% low DP oligomers. The low DP oligomers had a substantial growth stimulatory effect on *L. paracasei* BCRC12193, but only a slight growth stimulatory effect on *L. kefir* BCRC14011.

3.4. Effect of chitosan oligosaccharides DP on lactobacilli growth

The higher DP chitosan oligosaccharides prepared from hydrolysates of TKU027 crude enzyme by precipitating in the methanol solution were supplemented to a MRS broth to investigate the growth of lactic acid bacteria. The cell growth effects of the high DP oligomers on lactic acid bacteria were compared with the low DP oligomers (Table 2). The high DP oligomers were found to have potent inhibitory effects at concentration of 0.1% against *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 for 48 h. While, *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 were not inhibited in a MRS broth supplemented with 0.1% low DP oligomers (Table 2). These findings indicate that the low DP oligomers do not inhibit the health benefits of enteric bacteria at the concentration of 0.1%, but the high DP oligomers may inhibit the growth of lactic acid bacteria.

Some reports exist which claim that chitosan oligomer has a more potent antimicrobial activity than chitosan polymer (Uchida, Izume, & Ohtakara, 1989). However, the DP or molecular weight (Mw) of the chitosan oligomer in previous reports was not defined clearly. Therefore, the chitosan oligomers might be mixed with the higher DP chitosan oligomers. It is known that the amine moiety and the DP or Mw are important for the biological activity of chitosan oligomer, especially, in terms of antimicrobial activity

Table 2
Effect of the chitosan oligosaccharides obtained from different hydrolysis time on the growth of lactic acid bacteria.

Supplement	Medium	Relative cell growth (%)			
		L. paracasei 12193		*L. kefir* 14011	
		12 h	48 h	12 h	48 h
Low DP oligomers	MRS broth alone (control)	100	100	100	100
	MRS broth + 0.1% oligomers (12 h hydrolysis)	182	102	174	103
	MRS broth + 0.1% oligomers (24 h hydrolysis)	324	100	166	104
	MRS broth + 0.1% oligomers (48 h hydrolysis)	184	100	149	100
	MRS broth + 0.1% oligomers (72 h hydrolysis)	201	100	152	103
High DP oligomers	MRS broth + 0.1% oligomers (12 h hydrolysis)	114	98	143	104
	MRS broth + 0.1% oligomers (24 h hydrolysis)	123	95	166	103
	MRS broth + 0.1% oligomers (48 h hydrolysis)	132	92	129	96
	MRS broth + 0.1% oligomers (72 h hydrolysis)	124	98	118	91

(Mengíbar et al., 2011). Currently, the number of primary amino groups is dependent on degree of acetylation and DP or Mw of chitosan oligomers. Also it had been observed that the antibacterial activity tends to increase upon the increase in the DD of chitosan oligomers. The DP or Mw of chitosan oligomers also influenced its antibacterial activity (Mengíbar et al., 2011). A relationship between the Mw of chitosan oligomers and their antimicrobial activity has been reported by several researchers (Mengíbar et al., 2011; No, Park, Lee, & Meyers, 2002). The Mw between 1 and 10 kDa (approximately DP 5-50) is critical for inhibition of microorganisms and the antimicrobial capacity increases with the Mw (Jeon, Park, & Kim, 2001). Although there is not a general consensus about the most effective Mw, chitosan oligomers ranging in an average from 5 to 27 kDa (approximately DP 25–135) are generally observed to be effective in suppressing bacterial growth (Gerasimenko, Avdienko, Bannikova, Zueva, & Verlamov, 2004). In the present study, chitosan oligomer of DP 4–9, did not inhibit the growth of *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 (Table 2), although the high DP oligomers had antimicrobial activity against *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 at a low concentration (0.1%). These results showed that the chitosan oligomers with DP below 9 (Mw below 1.7 kDa) could not inhibit the growth of *L. paracasei* BCRC12193 and *L. kefir* BCRC14011. This is in accordance with previous reports which claim that chitosans with an average Mw below 3 kDa (approximately DP 15) could be not capable of suppressing the microbial growth (Kim & Rajapakse, 2005). The low DP oligomers could be further developed into a functional beverage and TKU027 crude enzyme from the bioconversion of SHP to produce a valuable product could also reduce economical and environmental liabilities.

3.5. Effect of low DP chitosan oligomers concentration on lactobacilli growth

L. paracasei BCRC12193 and *L. kefir* BCRC14011 were cultured for 48 h in a MRS broth supplemented with various concentrations (0%, 0.1%, and 0.5%) of the low DP oligomers produced from the 24 h hydrolysis of TKU027 crude enzyme, relative cell growth was measured (Table 3). For 12 h of cultivation, the growth of *L. paracasei* BCRC12193 increased as 0.1% low DP oligomers, and reached to a steady state as the concentration of the low DP oligomers rose to 0.5%. The growth of *L. kefir* BCRC14011 in MRS broth supplemented with the low DP oligomers increased, but relative cell growth decreased slightly with the increase of the low DP oligomers concentration (Table 3). We also tested the effect of different cultivation time on cell growth (Table 3). The growth rate of *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 increased in MRS broth supplemented with the low DP oligomers. *L. paracasei* BCRC12193 in MRS broth containing 0.1% or 0.5% low DP oligomers showed a larger growth rate after 12 h cultivation compared with the 48 h of cultivation while the growth rate and the growth of *L. kefir* BCRC14011 increased slightly. These results indicate that 0.1% low DP oligomers more potently stimulate the growth on *L. paracasei* BCRC12193 than on *L. kefir* BCRC14011.

3.6. Biodegradation upon SHP amended in mangrove river sediment

To investigate biodegradation upon SHP amended in mangrove river sediment, four soil samples were carried out including the original soil (Soil), SHP amended soil (S), *B. cereus* TKU027 amended soil (027) and SHP and *B. cereus* TKU027 amended soil (B). The following properties of these four soil samples of 5 weeks-incubation were analyzed: (1) total viable count with nutrient agar (NA) containing 3% NaCl, (2) reducing sugar and total sugar in water extracts of the samples. As shown in Table 4, SHP and *B. cereus* TKU027-amended mangrove river sediment (sample B) had the largest number of bacteria. Additionally, sample B also had the maximum sugar amount (reducing sugar and total sugar).

PCR-DGGE analysis was used to measure the composition of the bacterial community in SHP and/or *B. cereus* TKU027-amended soils obtained from mangrove river sediment. In the DGGE gel, the position of band 5 represented *B. cereus* TKU027. As shown in Fig. 4a, the DGGE bands showed much variation in SHP contained soils, e.g. band 1, 2, 4 and 7. band 3, 6 and 8 revealed bright bands in the *B. cereus* TKU027-amended soils and original soils. Some DGGE bands in lane 027 and lane Soil were common in the processes, indicating that there were high similarities of the populations of bacteria between them (Fig. 4b). Many DGGE bands were detected in lane S and lane B, and these band positions were unique. These differences

Table 3
Effect of the concentration of the chitosan oligosaccharides after 24 h hydrolysis on the growth of lactic acid bacteria at different cultivation times.

Supplement	Concentration of supplement (%)	Relative cell growth (%)			
		L. paracasei 12193		*L. kefir* 14011	
		12 h	48 h	12 h	48 h
	0	100	100	100	100
Low DP oligomers	0.1	324	100	166	104
	0.5	330	105	120	102

Table 4
Total counts and sugar contents of the four samples after 5 weeks incubation.

	Soil	SHP (S)	*B. cereus* TKU027 (027)	SHP+ TKU027 (B)
Total count (CFU/g dried soil)	1×10^5	3×10^6	1×10^6	6×10^7
Sugar amount (μg/g dried soil)				
Reducing sugar	0	1443.4	0	1481.5
Total sugar	959.0	3168.6	1001.9	3511.4

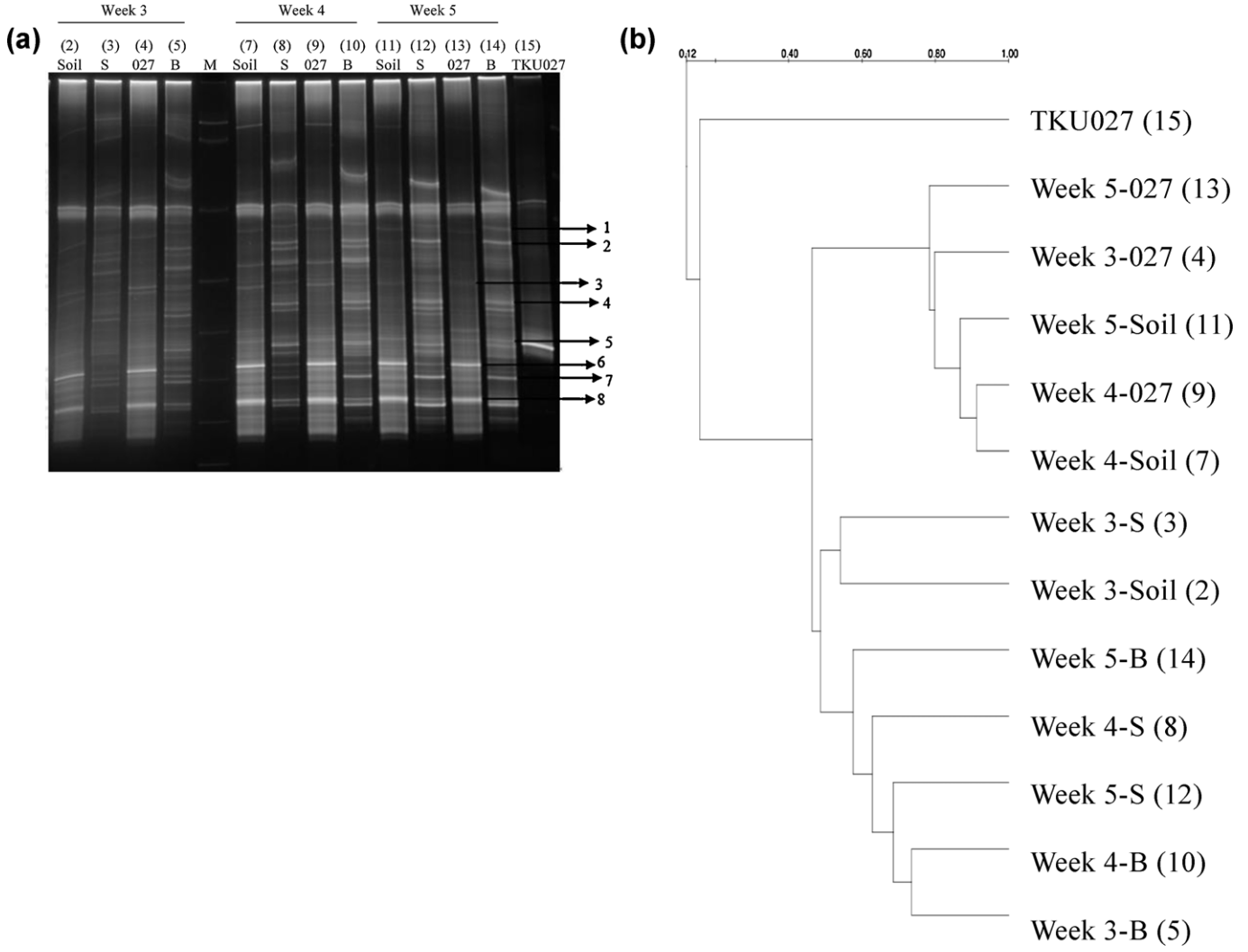

Fig. 4. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profile of 16S rDNA fragments (a) and unweighted pair-group method using arithmetic averages (UPGMA) dendrogram for analyzing the profile (b). Capital letters in the figure indicate: M, marker (The marker, DGGE marker II, which contains 10 fragments was purchased from Nippon Gene Co., Ltd., Japan); soil, mangrove river sediment; S, shrimp head powder amended in mangrove river sediment; 027, *B. cereus* TKU027 amended in mangrove river sediment; B, both shrimp head powder and *B. cereus* TKU027 amended in mangrove river sediment; TKU027, *B. cereus* TKU027 alone. Numbers in the parentheses indicate the lane orders in the profile.

indicated that some variance of bacterial community compositions existed in these soils. Consequently, it is inferred that the biodegradation of SHP by the chitinase/chitosanase of *B. cereus* TKU027 is conducive to the growth of *B. cereus* TKU027 and the co-existing bacteria. According to the results, we assumed that the amendment of *B. cereus* TKU027 can enhance the biodegradation of SHP in the seawater containing mangrove river sediment. We hope that these findings may provide some useful information for the reclamation of chitin-containing wastes in our environment.

4. Conclusions

These results show that the crude enzyme preparation from *B. cereus* TKU027 can hydrolyze 60% DD chitosan to yield chitosan oligosaccharides with DP 4–9, and it is cheaper than such enzymes specific as chitinase, chitosanase and lysozyme. The multi-chitinousolytic enzyme complex produced by *B. cereus* TKU027 is effective in the production of chitosan oligosaccharides. By fractionation of the hydrolyzates with methanol/acetone, products of DP below 9 and DP above 9 were separated out. The method is a useful procedure for preparing chitosan/chitin oligosaccharides. This work indicates that the crude enzyme preparation from *B. cereus* TKU027, a low-cost enzyme, could be a valid alternative to purified chitinase and chitosanase which are expensive and unavailable in bulk quantity for the production of chitosan oligosaccharides. Besides, the low DP chitosan oligomers stimulate the growth of *L. paracasei* BCRC12193 and *L. kefir* BCRC14011 and that low DP chitosan oligomers have potential use

as a prebiotic health-food. Adding the chitinase-producing strain *B. cereus* TKU027 and SHP in the soil collected from the Tamsui River showed a significant number of bacteria. These results may be useful for biological applications in relation to enzyme, bioactive materials production and environmental bioremediation.

Acknowledgment

This work was supported in part by a grant of the National Science Council, Taiwan (NSC101-2621-M-032-005- and NSC101-2811-M-032-015-).

References

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Ewel, K. C. (2008). Mangrove crab (*Scylla serrata*) populations may sometimes be best managed locally. *Journal of Sea Research*, *59*, 114–120.

Gerasimenko, D. V., Avdienko, I. D., Bannikova, G. E., Zueva, O. Y., & Verlamov, V. P. (2004). Antibacterial effects of water-soluble low-molecular-weight chitosans on different microorganisms. *Applied Biochemistry and Microbiology*, *40*, 253–257.

Gibson, G. R. (1999). Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. *The Journal of Nutrition*, *129*, 1438S–1441S.

Imoto, T., & Yagishita, K. (1971). A simple activity measurement of lysozyme. *Agricultural and Biological Chemistry*, *35*, 1154–1156.

Jeon, Y. P., Park, P. J., & Kim, S. K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. *Carbohydrate Polymers*, *44*, 71–76.

Kim, S. K., & Rajapakse, N. (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers*, *62*, 357–368.

Kruse, H. P., Kleessen, B., & Blaut, M. (1999). Effects of inulin on fecal bifidobacteria in human subjects. *The British Journal of Nutrition*, *82*, 375–382.

Liang, T. W., Chen, Y. J., Yen, Y. H., & Wang, S. L. (2007). The antitumor activity of the hydrolysates of chitinous materials hydrolyzed by crude enzyme from *Bacillus amyloliquefaciens* V656. *Process Biochemistry*, *42*, 527–534.

Liang, T. W., Wu, Y. Y., Huang, T. Y., Wang, C. Y., Yen, Y. H., Liu, C. P., et al. (2010). Conversion of squid pen by a novel strain *Lactobacillus paracasei* subsp *paracasei* TKU010, and its application in antimicrobe and antioxidant. *Journal of General and Applied Microbiology*, *56*, 481–489.

Mengíbar, M., Ganan, M., Miralles, B., Martínez-Rodriguez, A. J., Carrascosa, A. V., Peter, M. G., et al. (2011). Antibacterial activity of products of depolymerization of chitosans with lysozyme and chitosanase against *Campylobacter jejuni*. *Carbohydrate Polymers*, *84*, 844–848.

No, H. K., Park, N. Y., Lee, S. H., & Meyers, S. P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International Journal of Food Microbiology*, *74*, 65–72.

Uchida, Y., Izume, M., & Ohtakara, A. (1989). Preparation of chitosan oligomers with purified chitosanase and its application. In G. Skjåk-Bræk, T. Anthonsen, & P. Sandford (Eds.), *Chitin and chitosan: Sources, chemistry, biochemistry, physical properties and applications* (pp. 372–382). Barking, UK: Elsevier Applied Science.

Wang, S. L., Liu, C. P., & Liang, T. W. (2012). Fermented and enzymatic production of chitin/chitosan oligosaccharides by extracellular chitinases from *Bacillus cereus* TKU027. *Carbohydrate Polymers*, *90*, 1305–1313.

Wang, S. L., Lin, H. T., Liang, T. W., Chen, Y. J., Yen, Y. H., & Guo, S. P. (2008). Reclamation of chitinous materials by bromelain for the preparation of antitumor and antifungal materials. *Bioresource Technology*, *99*, 4386–4393.

Wang, S. L., Lin, T. Y., Yen, Y. H., Liao, H. F., & Chen, Y. J. (2006). Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase. *Carbohydrate Research*, *341*, 2507–2515.

Yin, B., Gu, J. D., & Wan, N. (2005). Degradation of indole by enrichment culture and *Pseudomonas aeruginosa* Gs isolated from mangrove sediment. *International Biodeterioration & Biodegradation*, *56*, 243–248.