Carbohydrate Polymers 101 (2014) 57–67

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Optimization of the fermentation conditions of *Rhizopus japonicus* M193 for the production of chitin deacetylase and chitosan

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ARTICLE INFO

Article history:
Received 26 July 2013
Received in revised form 30 August 2013
Accepted 6 September 2013
Available online 14 September 2013

Keywords:
Chitin·chitosan
Chitin deacetylase
Rhizopus japonicus M193 fermentation
Plackett–Burman and Taguchi design

ABSTRACT

To improve the production of chitin deacetylase (CDA) for the bioconversion of chitin to chitosan with desirable functionality, the effect of the nutritional requirement on the CDA production from *Rhizopus japonicus* M193 fermentation was investigated under submerged conditions. Nutritional elements including glucose (g/L), inoculum level (%), and $MgSO_4 \cdot 7H_2O$ (g/L), as well as culture time (d) were identified as the most critical factors for the CDA production based on the results from Plackett–Burman design (PBD). Taguchi design with orthogonal array was further employed to optimize *R. japonicus* M193 fermentation conditions based on the results from PBD, in which 2.5% chitin, 5 g/L glucose, 5% inoculum level, 0.6 g/L $MgSO_4 \cdot 7H_2O$, and 5 d culture time were identified as the optimal fermentation conditions. Under this condition, the maximum CDA production, DDA and MM of produced chitosan were 547.38 ± 12.06 U/L, 78.85 ± 1.68%, and 125.63 ± 3.74 kDa, respectively. Obtained chitosan displayed similar physicochemical and structural properties to those of commercial chitosan extracted using chemical method based on the results from Fourier transform infrared spectrometer (FT-IR), thermogravimetric analysis (TGA)–differential scanning calorimetry (DSC), and nuclear magnetic resonance (NMR) assays, while the use of chemical reagents was significantly reduced.

1. Introduction

Chitin, a polymer of β-(1→4) linked N-acetyl glucosamine, is mainly used as a raw material to produce chitin-derived products, such as chitosan, chito-oligosaccharides, and glucosamine (Alishahi & Aïder, 2012). However, this vast biopolymer still remains industrially unexploited because of its high degree of crystallinity and insolubility in aqueous and organic solvents (Aranaz et al., 2009). Chitosan, the deacetylated form of chitin, is a polymer of β-(1→4) linked glucosamine residues. Chitosan can be further degraded to obtain chitosan oligosaccharides or other derivatives with reduced molecular weight, better solubility, and improved biomedical functions. Chitosan and its derivatives have been applied and/or studied in many fields such as medicine, food, and agriculture (Fan, Wei, Xu, & Ni, 2012; Uriarte-Montoya, Arias-Moscoso, & Plascencia-Jatomea, 2010).

Currently, the conversion of chitin into chitosan is mostly done by strong alkaline deacetylation of chitin, which has resulted in numerous problems, including seriously environmental pollution, tedious to control, and heterogeneous range of the resulted chitosan products (Chang, Gengia, Lee, & Fu, 1997). An alternative or complementary procedure to strong alkaline deacetylation is the use of chitin deacetylase (CDA) (EC 3.5.1.41) produced by microbial fermentation. Chitin can be converted to chitosan by N-deacetylation through the catalysis of CDA. This method is more controllable and can lead into the production of chitosan with superior quality (Tsigos, Martinou, Kafetzopoulos, & Bouriotis, 2000). More importantly, microorganism can be propagated rapidly, thus great application potential for saving cost. CDA was initially identified and partially purified from the mycelial extracts of *Mucor rouxii* (Araki & Ito, 1974), *Penicillium* sp. (Pareek, Singh, & Ghosh, 2011), *Aspergillus* sp. (Chen, Xia, & Yu, 2005) and *Rhizopus* sp. (Gauthier, Clerisse, Dommes, & Jaspar-Versali, 2008; Ghaouth, Arul, Grenier, & Asselin, 1992).

It has been well known that most of fungal strains observed so far can only produce intracellular enzymes with low activity and yield. To solve the problem, *Rhizopus japonicus* M193, possessing a high intracellular and extracellular activity, has been studied for producing CDA that can catalyze the deacetylation of N-acetyl-D-glucosamine residues under mild reaction conditions (Ghaouth et al., 1992; Zhao, Park, & Muzzarelli, 2010). CDA production by

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http://dx.doi.org/10.1016/j.carbpol.2013.09.015

microbial fermentation is affected by many factors including the nutritional sources and cultivation conditions. Therefore, optimization of the nutritional suppliers and the fermentation conditions is critical for not only improving the production yield and activity of CDA, but also rendering the cost of the process (Pareek, Singh, et al., 2011).

Up to now, no statistically based experimental design has been applied and reported for the bioconversion of chitin to chitosan using microbial fermentation. In conventionally experimental design, a single treatment factor is usually considered at a time while maintaining other variables consistent (Ghorbel-Bellaaj, Jellouli, et al., 2011). This method may lead to unreliable results and inaccurate conclusions because it is unable to detect any interactions among the treatment factors and cannot guarantee the determination of the optimal conditions. To overcome these limitations, response surface methodology (RSM) by considering difference treatment factors may be applied. RSM can not only reduce the number of experimental trials and identify the significant treatment factors, but can also be employed to optimize the treatment conditions and processes (Bas & Boyaci, 2007; Da Silva, Honorato, Franco, & Rodrigues, 2012). Plackett–Burman design (PBD) is one of the most frequently used RSM designs. Hence, to reduce the experimental time and ensure the accuracy of the experimental results, the most important treatment factors significantly contributing to the production of CDA can be identified first using PBD of RSM. Furthermore, the identified treatment factors can be optimized using Taguchi design (Jung & Zhao, 2011). These successive two-step optimization procedures can be applied to produce highly active CDA using microbial fermentation for the bioconversion of chitin to chitosan.

In this study, the production of CDA using *R. japonicus* M193 fermentation was investigated. PBD was initially applied to screen the most important factors that influence the production of CDA. Subsequently, the orthogonal arrangement of Taguchi design was applied to optimize the identified treatment factors for CDA production, which was then employed for the bioconversion of chitin to chitosan. In addition, the physicochemical and structural properties of chitin and chitosan prepared by microbial fermentation and the chemical extraction method were investigated using Fourier transform infrared spectrometer (FT-IR), thermogravimetric analysis (TGA)–differential scanning calorimetry (DSC), and ^{13}C nuclear magnetic resonance (^{13}C NMR) assays. Based on our best knowledge, no study has reported the deacetylation of chitin using *R. japonicus* M193 fermentation where all contributing factors were statistically considered.

2. Materials and methods

2.1. Materials

Shrimp shells of headless *Penaeus vannamei* were obtained from Nantong Xingcheng Biological Products Factory (Nantong, China) for preparing chitin using *Serratia marcescens* B742 and *Lactobacillus plantarum* ATCC 8014 successive two-step fermentation (see details in Section 2.5.1). *S. marcescens* B742, *L. plantarum* ATCC 8014, and *R. japonicus* M193 were all obtained from Shanghai Institute of Industrial Microbiology (Shanghai, China). Potato dextrose agar (PDA) was purchased from Shanghai Yayan Biotechnology Co., Ltd. (Shanghai, China). Methyl orange and methylene blue were prepared by dissolving them in 0.1% water solution and 95% ethanol solution, respectively. D-glucosamine·HCl standard was purchased from Sigma (St. Louis, USA). The 0.1 mol/L HCl and NaOH standard solutions were obtained from Shanghai Institute of Measurement and Testing Technology (Shanghai, China). All other chemical reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Production of chitin deacetylase (CDA)

For CDA production, 24 mL of PDA broth culture was evenly distributed into three 10 mL test tubes, inoculated with *R. japonicus* M193 strain (3.4×10^6 spores/mL), and then incubated at 30 °C for 24 h. Followed that, 20 mL of culture broth collected from above three test tubes and 400 mL of PDA broth culture were transferred into a 500 mL of Erlenmeyer flask and further incubated at 30 °C and 200 rpm/min in a constant temperature incubator for 5 d (Thermo 250B, Thermo Co., USA). The fermentation medium consisted of (g/L): glucose, 12; yeast extract, 3.0; peptone, 5.5; KH_2PO_4, 3.0; K_2HPO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.6; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; and $CaCl_2$, 0.5 with the pH adjusted to 6.0 ± 0.2 using 0.1 M HCl and NaOH. The fermentation broth including mycelia and culture supernatant was separated by Whatman No. 1 filter paper after 5 d. Culture supernatant was assayed for extracellular CDA activity, and the mycelia in the culture supernatant was filtrated and washed with 30 mL of cold distilled water (4 °C) and homogenized in a pestle and mortar (OMNI Prep., GA, USA) with 30 mL of 50 mM cold Tris–HCl buffer (pH 8.5, 4 °C). The mycelia suspension was then centrifuged (Thermo/Pico 17/2, Thermo Co., USA) at 4 °C and 10,000 rpm/min for 30 min and the resulting supernatant was assayed for intracellular CDA activity (Pareek, Singh, et al., 2011).

2.3. Plackett–Burman design (PBD) for identifying significant factors contributing to CDA production

PBD is a very powerful tool for screening critical treatment factors and has been successfully exploited by many researchers for the optimization studies (Kumar & Satyanarayana, 2007). Chitin was prepared using *S. marcescens* B742 and *L. plantarum* ATCC 8014 successive two-step fermentation as described below (Section 2.5.1) so that the final chitosan product in this study is completely produced using microbial fermentation method. Based on the previous report about the factors affecting the CDA production (Pareek, Vivekanand, & Dwivedi, 2011), eight variables including chitin (%), glucose (g/L), inoculum level (%), peptone (g/L), yeast extract (g/L), $(NH_4)_2SO_4$ (g/L), $MgSO_4{\cdot}7H_2O$ (g/L) and culture time (d), as well as three dummy variables of D_1, D_2 and D_3, generated automatically by the RSM program were evaluated on CDA production by *R. japonicus* M193 fermentation using the PBD in 12 experimental runs (Table 1). The effect of each variable was studied at two levels, high and low, denoted by (+1) and (−1) signs, respectively. The difference between the two values was large enough to ensure that the peak area for the highest enzyme production was included. All the experiments were conducted in triplicates. Design Expert Version 7.0 (Stat-Ease Inc., Minneapolis, USA) was used for the data analysis.

2.4. Taguchi design for the optimization of CDA production

The identified factors significantly contributing to the production of CDA from the PBD were further investigated (optimized) to find the most contributing factors and the suitable level of each contributing factor on CDA production using Taguchi experimental design with orthogonal array. The orthogonal array offered a simple and systematic approach to optimize the experimental conditions with significantly reduced numbers of treatment combinations when multiple factors were considered (Jung & Zhao, 2011; Zhang, Jin, Deng, Wang, & Zhao, 2012). Based on the results on CDA production from the PBD, five significant factors including chitin (A, % w/w), glucose (B, g/L), inoculum

Table 1
Plackett–Burman experimental design matrix for screening important variables for the production of chitin deacetylase.*

No	Chitin (%)	Inoculum level (%)	Glucose (g/L)	Peptone (g/L)	Yeast extract (g/L)	$MgSO_4{\cdot}7H_2O$ (g/L)	Culture time (day)	K_2HPO_4(g/L)	D_1	D_2	D_3	CDA production+ (U/L)	DDA++ (%)	MM+++ (kDa)
1	2	15 (+1)	14 (+1)	4 (-1)	6 (+1)	1.0 (+1)	9.0 (+1)	0.4 (−1)	−1	−1	+1	410.87[a]	50.35[a]	1041.05[l]
2	4	10 (−1)	14 (+1)	8 (+1)	6 (+1)	1.0 (+1)	5.0 (−1)	0.4 (−1)	+1	−1	+1	326.40[a]	51.86[ab]	756.56[g]
3	4	15 (+1)	14 (+1)	4 (−1)	2 (−1)	0.5 (−1)	9.0 (+1)	0.4 (−1)	+1	+1	−1	472.97[a]	51.12[ab]	437.73[e]
4	2	15 (+1)	14 (+1)	8 (+1)	2 (−1)	1.0 (+1)	5.0 (−1)	0.8 (+1)	−1	+1	+1	401.35[a]	51.41[ab]	315.72[a]
5	4	15 (+1)	6 (−1)	8 (+1)	6 (+1)	1.0 (+1)	9.0 (+1)	0.4 (−1)	−1	+1	−1	321.30[a]	61.52[b]	787.45[i]
6	4	15 (+1)	6 (−1)	8 (+1)	2 (−1)	1.0 (+1)	5.0 (−1)	0.8 (+1)	+1	−1	+1	369.77[a]	57.31[ab]	414.80[d]
7	4	10 (−1)	14 (+1)	8 (+1)	2 (−1)	1.0 (+1)	9.0 (+1)	0.8 (+1)	−1	−1	−1	409.24[a]	55.37[ab]	404.22[c]
8	2	15 (+1)	6 (−1)	8 (+1)	6 (+1)	0.5 (−1)	9.0 (+1)	0.8 (+1)	+1	−1	−1	361.24[a]	60.14[ab]	759.46[h]
9	4	10 (−1)	14 (+1)	4 (−1)	6 (+1)	0.5 (−1)	9.0 (+1)	0.8 (+1)	+1	−1	+1	413.27[a]	51.72[ab]	561.49[f]
10	2	10 (−1)	6 (−1)	8 (+1)	2 (−1)	1.0 (+1)	9.0 (+1)	0.4 (+1)	+1	+1	+1	417.60[a]	58.34[ab]	1698.55[l]
11	2	10 (−1)	6 (−1)	4 (−1)	2 (−1)	0.5 (−1)	5.0 (−1)	0.4 (−1)	−1	−1	−1	357.08[a]	55.69[ab]	1691.47[k]
12	2	10 (−1)	14 (+1)	4 (−1)	6 (+1)	1.0 (+1)	5.0 (−1)	0.8 (+1)	+1	+1	+1	396.34[a]	52.31[ab]	374.75[b]

* In the same column, values with the same superscript letter (a–l) were not significantly different ($P > 0.05$).
\+ Chitin deacetylase activity.
++ Degree of chitin deacetylation.
+++ Molecular mass

level (C, % v/w), $MgSO_4{\cdot}7H_2O$ (D, g/L) and culture time (E, d) were considered for the optimization of CDA production using *R. japonicus* M193 fermentation (Table 2), while other insignificant factors including the amount of peptone, yeast extract, KH_2PO_4, $(NH_4)_2SO_4$, NaCl, and $CaCl_2$ were fixed. The resultant media with the same volume were aerobically cultured at 30 °C for 4–7 d on a rotary shaker (160 rpm/min) (Shanghai Jing Hong Laboratory Instrument Co., Ltd., Shanghai, China), and the supernatants were collected for the measurement of the degree of deacetylation (DDA) and CDA activity (including both extracellular and intracellular activity).

To obtain chitosan with standard quality, 1 M NaOH and HCl were used to remove the fermented residues at room temperature for 24 h (Sini, Santhosh, & Mathew, 2007) and the remaining chitosan solids were dissolved in 2% acetic acid at 30 °C for 16 h and centrifuged at 12,000 rpm for 20 min, followed by precipitation by adding 40% NaOH until reaching pH 9.0 and then separated by centrifugation again at 12,000 rpm/min for 20 min. The precipitants were washed by demineralized water and 95% ethanol, respectively for three times, and then freeze dried (LABCONCO 7948030 Co., USA) to obtain dried chitosan.

2.5. The extraction of chitin and chitosan

2.5.1. Preparation of chitin using S. marcescens B742 and L. plantarum ATCC 8014 successive two-step fermentation

Same procedures applied in our previous study were applied (Zhang et al., 2012). Briefly, dried shrimp shells were pulverized with Waring blender (Shanghai Shibang Machinery Co., Ltd China), and then passed through a 0.75 mm-sieve to obtain shrimp shell powders (SSPs). *S. marcescens* B742 *and L. plantarum* ATCC 8014 successive two-step fermentation was employed to extract the chitin. The identified optimal fermentation conditions using *S. marcescens* B742 were 2% SSP, 2 h of sonication time, 10% inoculum level, and 4 d of culture time, while that of using *L. plantarum* ATCC 8014 fermentation was 2% SSP, 15% glucose, 10% inoculum level, and 2 d of culture time. Successive two-step fermentation using identified optimal fermentation conditions resulted in chitin yield of 18.9% with the final deproteinization (DP) and demineralization (DM) rate of 94.5% and 93.0%, respectively.

2.5.2. Chemical extraction of chitin and chitosan

The same shrimp shell powders were dried at 90 °C for 12 h, treated with 40% NaOH (1:40) at 100 °C for 1 h to remove proteins, and then soaked in 5% HCl (1:40) solution with rotation at 750 rpm/min for 24 h at room temperature to remove $CaCO_3$. The obtained chitin was depigmented using 10% (v/v) H_2O_2 at 100 °C for 24 h, followed with washing using distilled water and drying at 70 °C till constant weight. Chitin was then deacetylated using 50% (w/v) NaOH at the chitin: NaOH ratio of 1:40. The mixture was incubated in a 100 °C water bath for 1 h with constant stirring, and then deacetylated again under the same conditions. The remaining chitosan solids were treated using the same procedures as described above.

2.6. Determination of CDA activity

The spore suspension grown on PDA broth culture was cultivated at 30 °C and 200 rpm/min for 24 h on a rotary shaker. The growth of the strains was evaluated by determining the cell dry biomass through filtering the samples and drying the mycelia to a constant weight at 100 °C (Oliveira, Porto, & Tambourgi, 2006).

CDA activity including both intracellular and extracellular activities was assayed based on the method of Martinou, Kafetzopoulos, and Bouriotis (1995) using a mixture of 50 mM of 50 μL Tris–HCl

Table 2
The contributing factors for *R. japonicus* M193 fermentation using orthogonal arrangement from the Taguich design.

Rhizopus japonicus M193	Chitin (%) A	Glucose (g/L) B	Inoculum level (%) C	$MgSO_4 \cdot 7H_2O$ (g/L) D	Culture time (d) E
1	1	5	5	0.2	4
2	1.5	10	10	0.4	5
3	2	15	15	0.6	6
4	2.5	20	20	0.8	7

buffer at pH 7.5, 100 μg of hexa-N-acetylchitohexaose in 100 μL water, and 50 μL enzyme supernatant. The mixture was incubated at 55 °C for 15 min, and the reaction was terminated by adding 5% (w/v) $KHSO_4$ (250 μL) (Cai et al., 2006). A 5% (w/v) $NaNO_2$ (250 μL) was then added into the above solution in centrifuge tubes which were capped immediately and allowed to stand with occasional shaking for 15 min, followed by adding 12.5% (w/v) aqueous 3-methy1-2-benzothiazolinonehydrazone hydrochloride (250 μL) and heating at 100 °C for 3 min. After cooled to room temperature, 0.5% (w/v) $FeCl_3$ (250 μL) solution was added and the developed color was read after 30 min at 650 nm using a spectrophotometer (UNICO UV-2000, Shanghai UNICO Instrument CO., China). Standard curves were measured using D-glucosamine·HCl standard (0–6 μg). One unit of CDA activity was defined as the amount of the enzyme required to produce 1 μmol of acetate/min when incubated with hexa-N-acetylchitohexaose (Cai et al., 2006).

2.7. Determination of degree of deacetylation (DDA), viscosity and molecular mass (MM) of produced chitosan

DDA was measured using the acid–base titration method (Cai et al., 2006). A 0.3 g of chitosan sample was dried to constant weight in a 105 °C oven (Thermo Heraeus T 6030, Thermo Co., USA) and then dissolved in 30 mL of 0.1 mol/L HCl standard solution in a 250 mL of Erlenmeyer flask for 24 h at room temperature till dissolved completely. After adding 3 drops of methyl orange–methylene blue (1:1, v/v) indicator, the solution was titrated with 0.1 mol/L NaOH standard solution till the solution color changed to red. The DDA was calculated as:

$$\mathrm{DDA}(\%) = \frac{203 \times (C_1V_1 \times C_2V_2) \times 100}{42 \times (C_1V_1 - C_2V_2) + m(1 - W)} \quad (1)$$

where C_1 and V_1 were the concentration (mol/L) and volume (mL) of the standard HCl, respectively; C_2 and V_2 were the concentration (mol/L) and volume (mL) of the standard NaOH solution, respectively; m was the weight of sample, g; W was the moisture content of sample, %; 203 was the molar weight of chitin; and 42 was the molar weight of acetyl.

The intrinsic viscosity of chitosan was determined using a Capillary Viscometer (Zhejiang Taizhou Jiaojiang Glass Instrument Co., China) that has a capillary size of 0.38 mm in a water bath at 25 °C.

Table 3
Result of multiple regression analysis of Plackett–Burman experimental design for the determination of chitin deacetylase activity (CDA), degree of deacetylation (DDA) and molecular mass (MM).

Variables	Medium components	Coefficient	*F*-value	*P*-value
–	Intercept	296.82842	–	–
X_1	Chitin (%)	−0.0646	0.21	0.6674
X_2	Inoculum level (%)	0.45963	5.13	0.0534
X_3	Glucose (g/L)	0.46799	6.54	0.0338
X_4	Peptone (g/L)	−0.45287	4.05	0.0804
X_5	Yeast extract (g/L)	0.36571	1.12	0.3675
X_6	$MgSO_4 \cdot 7H_2O$ (g/L)	0.94895	81.46	<0.0001
X_7	Culture time (d)	0.63555	10.16	0.0128
X_8	K_2HPO_4 (g/L)	0.18640	0.01	0.9274

Viscosity average MM was calculated from the measured intrinsic viscosity according to the Mark–Houwink–Sakurada (MHS) equation (Jung & Zhao, 2011).

For each sample, four different concentrations of chitosan in a range of 0.33–1% were employed for measuring the viscosity of the samples. The intrinsic viscosity was determined by the intercept between the Huggins (reduced viscosity $(\eta_{sp}/c) \sim C$ and Kraemer relative viscosity $(\ln[\eta]_{rel})/c \sim C$ plots when the concentration was 0) (Jung & Zhao, 2011). Relative viscosity, reduced viscosity, and intrinsic viscosity were determined as:

$$\eta_{rel} = \frac{t}{t_0} \quad (2)$$

$$\eta_{sp} = \eta_{rel} - 1 \quad (3)$$

$$\eta_{red} = \frac{\eta_{sp}}{c} \quad (4)$$

$$\ln[\eta] = \left(\frac{\eta_{sp}}{c}\right)_{c\sim 0} + \left(\frac{(\ln[\eta]_{rel})/c}{C}\right)_{c\sim 0} \quad (5)$$

where t was the flow time measured for the sample solution at a given time t (s); t_0 (s) was the flow time of the solution (0.1 M HOAc) without chitosan sample; C was the concentration of chitosan sample in diluted solution (g/mL); and $[\eta]$ was intrinsic viscosity, mL/g. The viscosity–average molecular mass (MM) of chitosan was calculated by the MHS equation:

$$[\eta] = K(\mathrm{MM})^a \quad (6)$$

where K and a were the constants, $K = 1.81 \times 10^3$, $a = 0.93$ (Zhang et al., 2012), and $[\eta]$ was the intrinsic viscosity obtained from the Huggins and Kraemer plots.

2.8. Structural analysis of chitin and chitosan

2.8.1. SEM

Morphological studies of *R. japonicus* M193 strain were carried out using scanning electron microscopy (SEM, FEI SIRION-200, Holland). Samples were subjected to fixation using 3% (v/v) glutaraldehyde for 24 h. Following the primary fixation, samples were washed twice with double distilled water and then treated with the alcohol gradients of 30%, 50%, 70%, 80%, 90% and 100% for dehydration for 15 min. Samples were kept for 15 min each up to 70% alcohol gradient, thereafter treated for 30 min each for subsequent alcohol gradients. After treating with 100% alcohol, samples were lyophilized and scanned under SEM using a gold shadowing technique (Pareek, Vivekanand, et al., 2011).

2.8.2. FT-IR and TGA–DSC analysis

A Nexus 6700 Fourier transform infrared spectrometer (FT-IR) (ThermoFisher Co., USA) was used to record infrared spectra of samples between 4000 and 500 cm^{-1}. All samples were scanned for three times.

Thermogravimetric analysis (TGA)–differential scanning calorimetry (DSC) was carried out on the SDT Q600 simultaneous

DSC–TGA instrument (Netzsch Co., Germany). Approximate 6 mg of sample was weighed, placed onto an aluminum cup, and sealed with an empty cup used as reference. The samples were heated up to 650 °C at a heating rate of 10 °C/min under air flow of 23 mL/min. At least three runs for each sample were conducted and the mean values were reported.

2.8.3. X-ray diffraction (XRD) assay

A D8 ADVANCE XRD (BRUKER-AXS Co., Germany) was applied to detect the crystallinity of samples and their patterns based on the wide-angle X-ray diffraction (WAXD) analysis. 2θ was scanned from 5° to 50° at a coating time of 2 s with an angle step width of 0.05°.

The crystallinity index (Cr_{Ipeak}) was calculated as (Ghorbel-Bellaaj, Hmidet, et al., 2011)

$$Cr_{Ipeak} = \frac{(I_{110} - I_{am})}{I_{110}} \quad (7)$$

where I_{110} was the maximum intensity (arbitrary unit) of the (1 1 0) lattice diffraction pattern at $2\theta = 20°$ and I_{am} was the intensity of amorphous diffraction in the same unit at $2\theta = 16°$.

2.8.4. Nuclear magnetic resonance (NMR) analysis

For NMR analysis, 0.4 g of chitin and chitosan samples was dried till constant weight and ground into fine powders for the spectral analysis using 400 M NMR (AVANCE III 400, BRUKER Optics Co., Germany) (Ghorbel-Bellaaj, Jellouli, et al., 2011). The NMR spectra of chitin and chitosan samples were obtained by the ^{13}C cross-polarization, magic-angle-spinning (CP-MAS) spectroscopy using a BRUKER-ASX300 instrument (BRUKER ASX Co., Germany) and were recorded at a ^{13}C frequency of 75.5 MHz. CP-MAS sequence was used with the following conditions: 5 s of ^{13}C spin lattice relaxation time; 8 ms of contact time; and 8 kHz spun speed with samples placed in an alumina rotor used for the double air-bearing type MAS system.

2.9. Data analysis

All experiments were carried out in triplicate, and the mean values with standard deviation were reported. One-way ANOVA was carried out to determine the significant differences among different treatment factors and their levels, and the LSD test was conducted for multiple comparisons in the orthogonal arrangement from Taguchi design ($P < 0.05$) using the SPSS program (SPSS 17.0, IBM SPSS Institute, Inc., USA).

3. Results and discussion

3.1. Optimal conditions for deacetylation by R. japonicus M193 fermentation

3.1.1. CDA production based on the results from the Plackett–Burman design

The results obtained from the eight-factor-twelve-run experiments on the CDA production using PBD are reported in Table 1, showing the CDA production in the range of 321.30–472.97 U/L. The DDA and MM of obtained chitosan were 50.35–61.25% and 315.72–1698.55 kDa, respectively. No significant difference in DDA among treatment groups ($P > 0.05$) was

observed except the fifth run, but there were significant differences in MM ($P < 0.05$). Hence, a more effective optimization study was carried out using the Taguchi experimental design as discussed below.

The results of multiple regression analysis based on the PBD are shown in Table 2. The model "*F*-value" of 5.25 was obtained based on *F*-value test. Hence, any treatment factor having an *F*-value greater than 5.25 was considered significant. Meanwhile, based on the individual probability factor of failure (>*F*), a variable corresponding to a probability less than 0.1 was significant. Therefore, among the eight treatment variables, four of them, including glucose (g/L), inoculum level (%), $MgSO_4 \cdot 7H_2O$ (g/L) and culture time (d) were most significant in terms of the effect on the CDA production. Moreover, because chitin provides the rich carbon and nitrogen sources for the growth of *R. japonicus* M193, by considering the optimized ratio for the bioconversion of chitin to chitosan, chitin was considered as an important contributing factor to be introduced into the experimental design in the next stage.

Table 4
Chitin deacetylase activity (CDA, U/L), degree of deacetylation (DDA, %) and molecular mass (MM, kDa) of chitosan after *R. japonicus* M193 fermentation based on the orthogonal arrangement from the Taguchi design.

Experimental run	CDA production (U/L)	DDA (%)	MM (kDa)
1	478.89^{bc+}	60.33^{a}	806.52^{l}
2	366.13^{a}	62.42^{a}	557.17^{j}
3	484.84^{c}	64.70^{a}	116.29^{a}
4	411.34^{abc}	69.02^{a}	265.78^{g}
5	440.66^{abc}	56.41^{a}	1723.53^{o}
6	405.30^{abc}	56.59^{a}	131.25^{b}
7	452.96^{abc}	59.83^{a}	561.49^{k}
8	378.00^{ab}	55.42^{a}	2319.70^{p}
9	420.81^{abc}	53.66^{a}	263.19^{f}
10	454.54^{abc}	55.49^{a}	177.45^{d}
11	364.80^{a}	57.50^{a}	821.47^{m}
12	400.50^{abc}	55.31^{a}	227.29^{e}
13	471.69^{bc}	52.38^{a}	1402.60^{n}
14	407.10^{abc}	54.81^{a}	400.93^{i}
15	430.54^{abc}	53.24^{a}	168.43^{c}
16	550.97^{d}	67.61^{a}	386.13^{h}

Factors[*]	Levels	CDA production (U/L)	DDA (%)	MM (kDa)
A	K_{A1}	435.30^{a++}	64.12^{a}	436.44^{a}
	K_{A2}	419.23^{a}	57.06^{a}	1183.99^{a}
	K_{A3}	335.16^{a}	55.49^{a}	372.35^{a}
	K_{A4}	465.07^{a}	57.01^{a}	589.52^{a}
	R_i[+++]	129.91	8.63	811.64
B	K_{B1}	453.01^{a}	55.69^{a}	1048.96^{a}
	K_{B2}	408.27^{a}	57.33^{a}	316.70^{a}
	K_{B3}	336.53^{a}	58.82^{a}	416.92^{a}
	K_{B4}	360.20^{a}	61.84^{a}	799.72^{a}
	R_i	116.48	6.15	732.26
C	K_{C1}	449.99^{a}	60.51^{a}	536.34^{a}
	K_{C2}	334.46^{a}	56.84^{a}	669.10^{a}
	K_{C3}	422.69^{a}	57.15^{a}	775.03^{a}
	K_{C4}	447.63^{a}	59.18^{a}	601.83^{a}
	R_i	115.53	3.67	238.69
D	K_{D1}	359.86^{a}	57.57^{a}	499.06^{a}
	K_{D2}	395.15^{a}	56.93^{a}	1275.23^{a}
	K_{D3}	482.75^{a}	61.05^{a}	600.85^{a}
	K_{D4}	417.00^{a}	58.13^{a}	207.16^{a}
	R_i	122.89	4.12	1068.07
E	K_{E1}	435.49^{a}	56.12^{a}	868.02^{a}
	K_{E2}	447.72^{a}	60.88^{a}	441.99^{a}
	K_{E3}	365.58^{a}	57.25^{a}	469.36^{a}
	K_{E4}	405.97^{a}	59.43^{a}	802.93^{a}
	R_i	82.14	4.76	426.03
	Rank[++++]	A > D > B > C > E	A > B > E > D > C	D > A > B > E > C

[*] A, chitin (%); B, glucose (g/L); C, inoculum level (%); D, $MgSO_4 \cdot 7H_2O$ (g/L); E, culture time (d).

[+] In the same column, values with the same superscript letter (a–p) were not significantly different ($P > 0.05$).

[++] Means were not significantly different within individual factor ($P > 0.05$).

[+++] R_i was the difference between the highest and lowest K_{ij} values for each factor. K_{ij} was the average value of each measured functional parameter in level j ($j = 1, 2, 3, 4$) of each factor i (i = A, B, C, D, E).

[++++] Rank represented the order of R_i values.

3.1.2. CDA production (activity) based on the results from the Taguchi design

Based on the results from the PBD study as discussed above, five contributing factors including chitin (%), glucose (g/L), inoculum level (%), $MgSO_4 \cdot 7H_2O$ (g/L), and culture time (d) were further studied for the optimization of *R. japonicus* M193 fermentation conditions using orthogonal arrangement from the Taguchi design (Table 3). CDA activity (U/L), DDA (%) and MM (kDa) of chitosan, as well as the rank of each contributing factor on these three parameters are displayed in Table 4. For CDA activity (including both extracellular and intracellular activities), R_i value for culture time was the lowest among all contributing factors, while chitin (%), glucose (g/L), inoculum level (%), and $MgSO_4 \cdot 7H_2O$ (g/L) were ranked first, second, third and fourth, respectively. In this study, CDA activity was used as an indicator for the optimized treatment conditions as it represented the superiority of *R. japonicus* M193 fermentation and the efficacy for the bioconversion of chitin to chitosan. Therefore, 2.5% chitin, 5 g/L glucose, 5% inoculum level, 0.6 g/L $MgSO_4 \cdot 7H_2O$ and 5 d culture time were identified as the optimal treatment conditions for achieving the high CDA activity. Among the 16 experimental runs, the maximum CDA activity and DDA were 550.97 U/L and 69.02%, respectively, while the minimal MM was 116.29 kDa (Table 4). However, the highest MM was 2319.70 kDa, probably owning to the mild reaction conditions and long reaction time. These results were consistent with the previous study by Tolaimate, Desbrieres, Rhazi, Vincendon, and Vottero (2000), in which chitosan with higher MM was required for repeating deacetylation because of the high concentration of reagents, high temperature, and prolonged reaction time. The low MM (with the molecular mass < 180 kDa in this study) of chitosan obtained in the experimental run of 3, 6, 10, and 15 (Table 4) showed great application potentials of chitosan in multiple fields such as drug treatment, cancer therapy and others (Li et al., 2009; Huang et al., 2012). For instance, low molecular weight chitosan (LMWC)/shRNA nano-complexes could effectively inhibit VEGF expression in cancer cells and tumor tissues and suppress tumor growth in different HCC models (Huang et al., 2012). Moreover, the LMWC coating significantly modified the properties of liposome and brought a series of notable advantages for ocular drug delivery (Li et al., 2009). In addition, low MW chitosan are employed for the preparation of chitosan nanoparticles which have great potential in the applications of drug delivery and non-viral vector for gene delivery (Fan et al., 2012; Lavertu, Méthot, Tran-Khanh, & Buschmann, 2006).

Time courses of cell dry weight, extracellular, and intracellular activity of CDA using *R. japonicus* M193 fermentation are shown in Fig. 1. The observed curves reflected a time-course typical of diauxic growth: a first phase (0–3 d) corresponding to the use of a preferred carbon source; then a second, slower phase (3–9 d) corresponding to the use of less preferred carbon sources and, finally, a stationary phase (beginning at 8 d). The maximum extracellular and intracellular CDA activity reached 173.33 and 92.35 U/L at 5 d, respectively. A previous study reported that maximum CDA activity is 414.70 ± 6.32 U/L produced by *Penicillium oxalicum* SAE_M-51 (Pareek, Vivekanand, et al., 2011).

3.2. CDA activity, DDA and MM of chitosan under optimized R. japonicus M193 fermentation conditions

The optimized *R. japonicus* M193 fermentation conditions based on the results from the Taguchi design was employed to produce chitosan from chitin that was prepared from shrimp shell powders using the successive two-step fermentation procedures. Chitin and chitosan prepared from the biological method (fermentation) were compared with those extracted using chemical method in their structural and physicochemical properties (Table 5). CDA activity, DDA and MM of chitosan produced using optimized *R. japonicus* M193 fermentation conditions at 5 d of culture time were 547.38 ± 12.06 U/L, 78.85 ± 1.68% and 125.63 ± 3.74 kDa, respectively (Table 5). It was apparent that the CDA activity has been improved significantly under the optimized fermentation conditions. Compared with the microbial fermentation, the DDA and MM of chitosan obtained using chemical deacetylation method was 85.62 ± 2.18% and 1959.45 ± 59.56 kDa, respectively (Table 5). Based on these results, it might be concluded that the bioconversion of chitin to chitosan using microbial fermentation has a significant advantage over chemical extraction method in the preparation of low MM chitosan. While the multistage procedures in chemical extraction of chitosan using strong alkali reagent might be effective for increasing the DDA of chitosan, the microbial fermentation has the advantages of significant less usage of chemical reagents and resulted low MM products. Previous studies reported that low MM chitosan and chitosan oligosaccharides have improved biomedical application as a blood hemostasis agent (Yaghobi & Mirzadeh, 2004) and anti-inflammatory effect on lipopolysaccharide stimulated Cells (Qiao et al., 2010). Therefore, depending on the nature of applications, different preparation methods (chemical or biological method) may be employed to obtain chitosan and its derivatives.

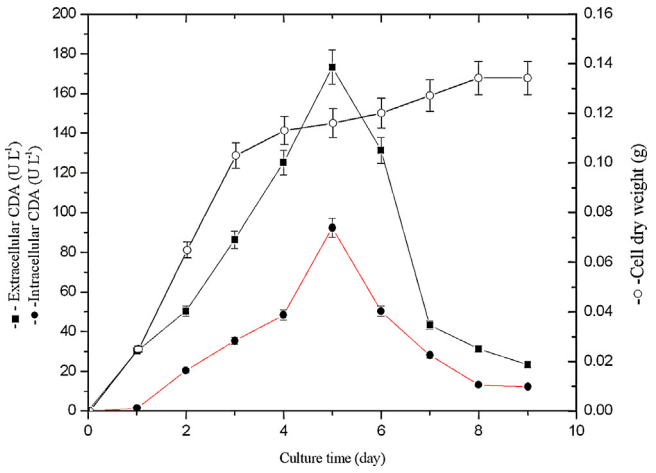

Fig. 1. Time course of cell dry weight, extracellular, and intracellular activity of chitin deacetylase (CAD) using *R. japonicus* M193 fermentation. Data were mean of three replications (-■-, extracellular; -●-, intracellular; -○-, cell dry weight).

3.3. Micrographs of R. japonicus M193 spores, chitin and chitosan samples

SEM micrographs of *R. japonicus* M193 spores, *R. japonicus* M193 fermentation at 5, 6 and 7 d, chitin from the successive two-step fermentation, and chitosan obtained by optimized *R. japonicus* M193 fermentation are shown in Fig. 2. The shape of *R. japonicus* M193 spores was oval with a maximum diameter of 5.32 μm (Fig. 2A). Note that the reason of selecting 5 d culture time was because the spores grown on chitin at 5 d was evenly distributed on the surface of fermentation medium, while the spores at 6, 7 and 8 d were gradually died (data did not shown) which might be related to the consumption of nutritional substances and fierce competition among them. The chitin obtained by the successive two-step fermentation displayed smooth microfibrillar crystalline structure (Fig. 2E), similar to what was reported in the previous study (Kjartansson, Zivanovic, Kristbergsson, & Weiss, 2006). The structure of chitosan obtained by the optimized *R. japonicus* M193 fermentation exhibited high fracture (Fig. 2F), which confirmed the existence of deacetylation and showed the promising potential of microbial fermentation for producing chitosan.

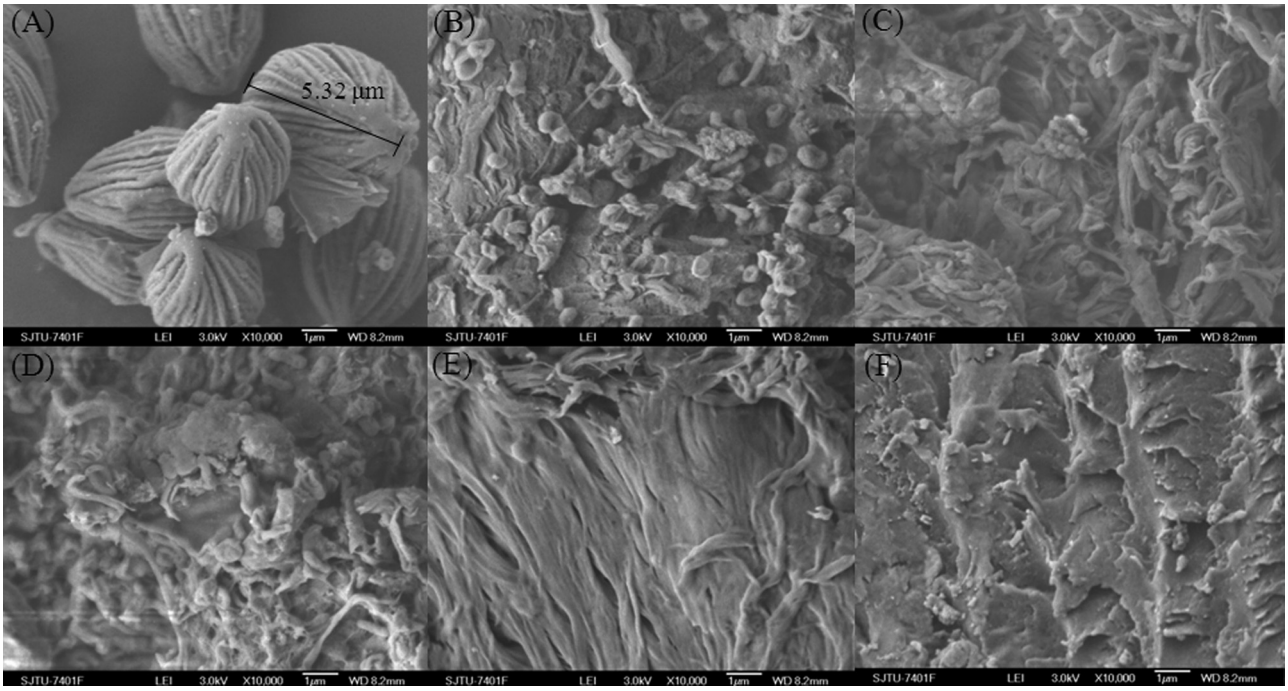

Fig. 2. SEM micrographs of *R. japonicus* M193 spores (A), images from *R. japonicus* M193 fermentation at 5 d (B), 6 d (C), and 7 d (D), images for chitin from successive two-step fermentation (E), and images for chitosan obtained by optimized *R. japonicus* M193 fermentation (F). All images were at10,000× magnification.

Table 5
DDA, MM, crystallinity index and thermal transitions of chitosan obtained by optimized *R. japonicus* M193 fermentation conditions at 5 d, chitin and chitosan obtained by chemical extraction method, and chitin obtained by successive two-step fermentation.

Sample[+]	CDA production (U/L)	DDA (%)	Crystallinity index (%)	MM (kDa)	Endotherm[+++]			
					T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
A	547.38 ± 12.06	78.85 ± 1.68^{c} [++]	14.36 ± 1.25^{a}	125.63 ± 3.74	184.78 ± 0.60^{a}	305.47 ± 0.83^{a}	429.95 ± 1.31^{a}	2788.01 ± 5.82^{b}
B	–	85.62 ± 2.18^{d}	27.15 ± 2.64^{b}	1959.45 ± 59.56	253.10 ± 0.81^{c}	315.83 ± 0.65^{a}	409.34 ± 1.25^{b}	2279.78 ± 5.34^{a}
C	–	32.56 ± 6.32^{a}	86.59 ± 1.26^{d}	–	215.33 ± 0.63^{b}	324.04 ± 0.74^{a}	428.53 ± 1.18^{a}	2829.67 ± 6.01^{c}
D	–	46.48 ± 0.67^{b}	79.23 ± 2.31^{c}	–	220.31 ± 0.51^{b}	335.37 ± 0.93^{a}	433.50 ± 1.25^{a}	3533.51 ± 7.63^{d}

[+] In the same column, values with the same superscript letter (a–d) were not significantly different ($P > 0.05$). Data were mean of three replications.
[++] A represented chitosan obtained by optimized *R. japonicus* M193 fermentation conditions at 5 d; B represented chitosan obtained by chemical extraction method; C represented chitin obtained by chemical extraction method; D represented chitin obtained by successive two-step fermentation.
[+++] T_o, onset temperature; T_p, peak temperature; T_c, completion temperature; ΔH (J/g, dry weight), peak enthalpy.

3.4. FT-IR spectra and thermal properties of chitin and chitosan samples

FT-IR spectra of chitin and chitosan prepared from chemical extraction method, chitosan from optimized *R. japonicus* M193 fermentation, chitin from the successive two-step fermentation are exhibited in Fig. 3 to compare the structural property of chitin and chitosan prepared by microbial fermentation and chemical extraction method. Absorption bands at 1384 (C-CH_3 amide stretching), 1321 (amide III and CH_2 wagging), 1157 (COC bridge stretching), and 1028 (C=O stretching) cm^{-1} were clearly distinguished in the spectrum (Muzzarelli et al., 2007). Compared to the chitosan (Fig. 3A and C), the band observed at 2976 cm^{-1} for chitin demonstrated an intensification of the peak (Fig. 3B and D) and further suggested the occurrence of deacetylation (Cahú et al., 2012). The more intensive the peak areas were, the higher the DDA was.

DDA of chitosan obtained by the optimized *R. japonicus* M193 fermentation at 5 d, chitosan obtained by chemical extraction method, chitin obtained by chemical extraction method and by the successive two-step fermentation were 78.85%, 85.62%, 32.56% and 46.48%, respectively (Table 5). Although the DDA of chitosan obtained by chemical extraction method was relatively higher

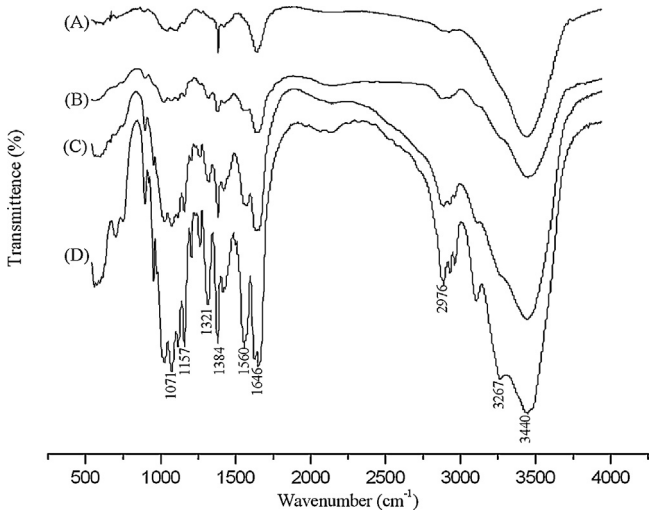

Fig. 3. FT-IR spectra of chitosan (A) and chitin (B) from chemical extraction, chitosan from optimized *R. japonicus* M193 fermentation (C), and chitin from successive two-step fermentation (D).

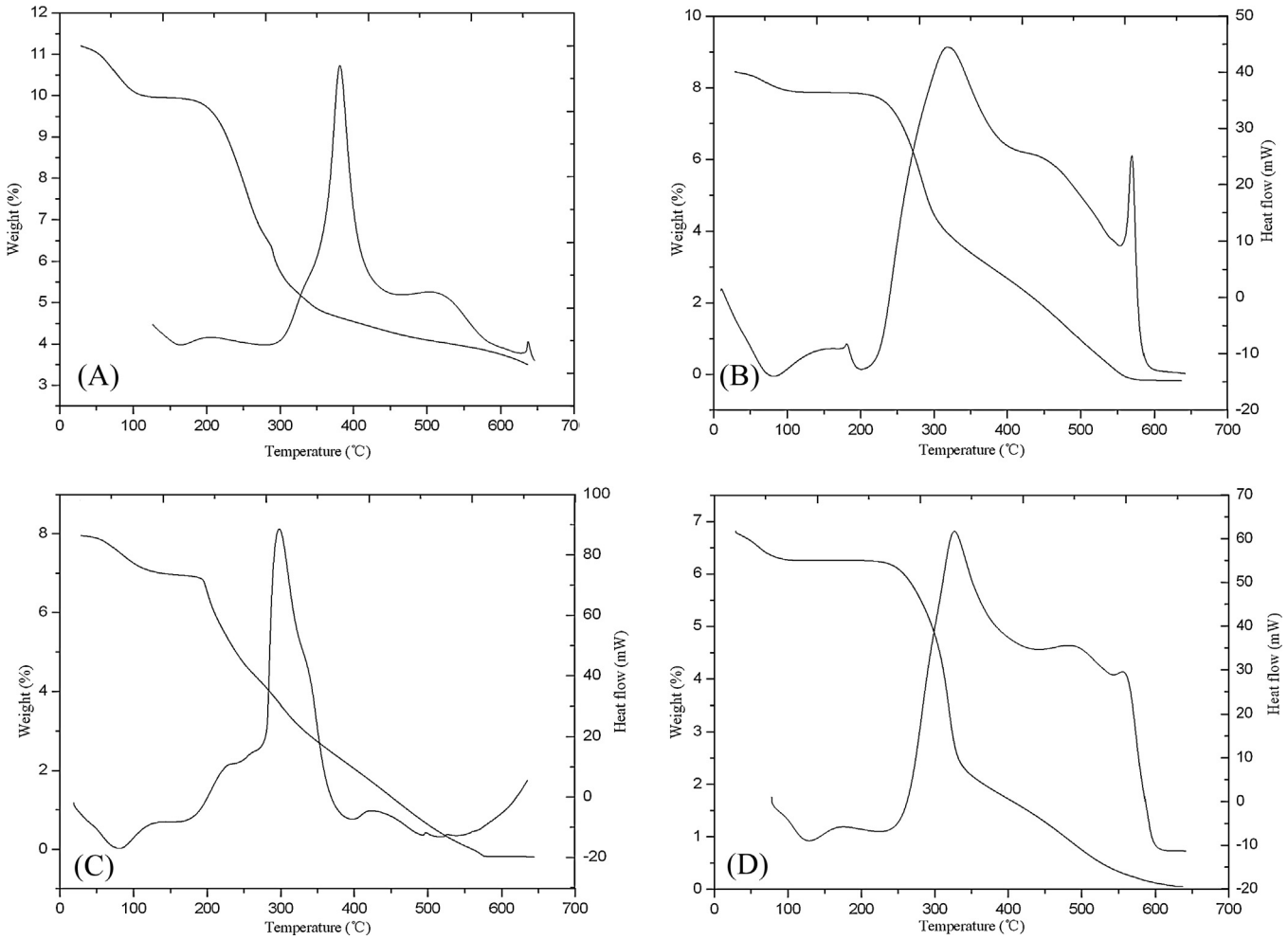

Fig. 4. Thermogravimetric analysis of chitin (A) and chitosan (B) from chemical extraction method, chitin from successive two-step fermentation (C), and chitosan from optimized *R. japonicus* M193 fermentation (D).

(85.62 ± 2.18%) than that of chitosan obtained by *R. japonicus* M193 fermentation (78.85 ± 1.68%), the chemical treatment displayed many disadvantages, such as uneven DDA and inconsistent physiological properties (Ghorbel-Bellaaj, Hmidet, et al., 2011; Chaussard & Domard, 2004). For improving deacetylation of chitosan prepared from microbial fermentation, CDA production using hypersecretory mutant strain was recently studied (Pareek, Vivekanand, et al., 2011), but the detailed experimental protocol was unknown.

The thermal properties of chitin and chitosan samples obtained from TGA–DSC at temperature range of 50–650 °C are illustrated in Fig. 4. All the samples showed similar trend, but chitin had higher thermal stability than the corresponding chitosan. DSC is an effective technique to evaluate the process of N-deacetylation and carboxymethylation (Kittur, Prashanth, Sankar, & Tharanathan, 2002). The transition temperatures and their associated enthalpies of chitin and chitosan samples are reported in Table 5. The onset temperatures were in the range of 184.78–253.10 °C with the ascending order of A < C < D < B. The completion temperatures were in the range of 409.34–433.50 °C, in the ascending order of B < A ∼ C < D. Apparently, chitin and chitosan extracted by the microbial fermentation had a wider temperature range than that of chitin and chitosan obtained from chemical extraction. However, for either extraction method, the thermal decomposition enthalpies (ΔH) of chitin were higher than that of chitosan. These results were similar to the previous report (Yen & Mau, 2007).

The endothermic peak areas of samples were in the range of 305.47–335.35 °C, higher for chitin and chitosan extracted by microbial fermentation than those obtained by chemical extraction. The endothermic peak area increased with increase in N-deacetylation and carboxymethylation, indicating that a definite correlation exists between the water holding capacity and chemical and supramolecular structure of these polymers (Yen & Mau, 2007). As shown in Fig. 4, the discrepancy in the peak temperature might be due to the diversity of chitin fiber aggregation in nature and the different preparation methods applied (Yen & Mau, 2007).

3.5. XRD analysis

The crystallinity indexes of chitin and chitosan from chemical extraction method, chitosan from optimized *R. japonicus* M193 fermentation, and commercial chitin were determined from the scattering intensity at two angles, one at $2\theta = 9–10°$ and another at $2\theta = 19–20°$ (Fig. 5). The results were consistent with the literature, in which the purified chitin had wide-angle X-ray diffraction pattern and showed two crystalline peaks at $2\theta = 9.3°$ and 19.17° (Yen & Mau, 2007). Similarly, previous study reported that fungal chitin displays two crystalline reflections at 9.03° and 19.17° (Wang et al., 2013). However, for the two angles of the chitosan extracted by *R. japonicus* M193 fermentation, one at about 9.03 disappeared and the other at about 20.04 showed narrow range, indicating that further purification is necessary to obtain satisfactory chitosan.

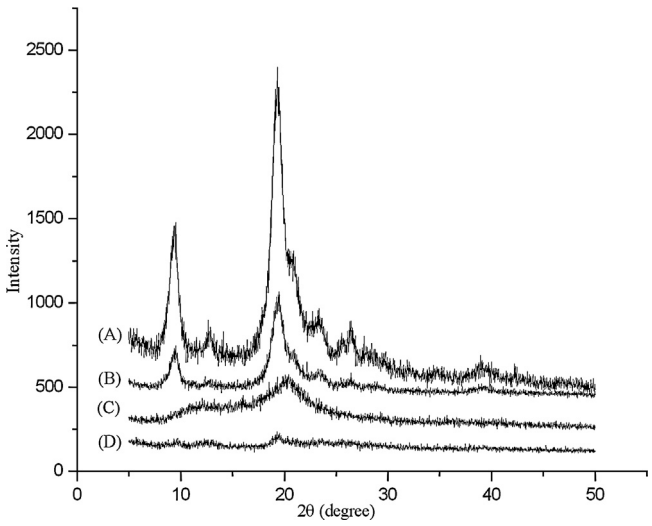

Fig. 5. X-ray diffraction graphs of chitin (A) and chitosan (B) from chemical extraction method, chitosan from optimized *R. japonicus* M193 fermentation (C), and chitin from successive two-step fermentation (D).

The crystallinity index of chitosan obtained by optimized *R. japonicus* M193 fermentation at 5 d, chitosan obtained by chemical extraction method, and chitin obtained by chemical extraction and by successive two-step fermentation were 14.36%, 27.15%, 86.59%, and 79.23%, respectively (Table 5). Overall, the crystallinity index of chitin was higher than that of chitosan, in which a lower crystallinity of polysaccharides indicates disruption of intra- and inter-molecular hydrogen bonds. The higher the crystallinity index, the higher the ΔH value, similar to the results from DSC analysis. The lower crystallinity index in chitin and chitosan produced by microbial fermentation might indicate their improved water solubility in comparison with chitin and chitosan prepared from the chemical extraction method, probably due to the more severe extraction conditions during the chemical extraction (Tolaimate et al., 2000).

3.6. NMR analysis

NMR is one of the most powerful tools in studying polysaccharide composition and sequential structure, making it possible to monitor reactions and other structural and physical properties. ^{13}C CP/MAS-NMR spectrums of chitin and chitosan samples prepared by microbial fermentation and chemical extraction method are shown in Fig. 6. NMR analysis showed similar peak patterns in chitin obtained from the successive two-step fermentation and the chemical extraction. There were eight signals for the eight carbon atoms of chitin including C-1–C-6 carbons of N-acetylglucosamine monomeric unit observed between 50 and 110 ppm, indicating high structural homogeneity. The carbonyl group is around 173 ppm, while the methyl group of the acetyl group produced a peak at around 23 ppm. The ^{13}C signals for C-3 (73 ppm) and C-5 (75 ppm)

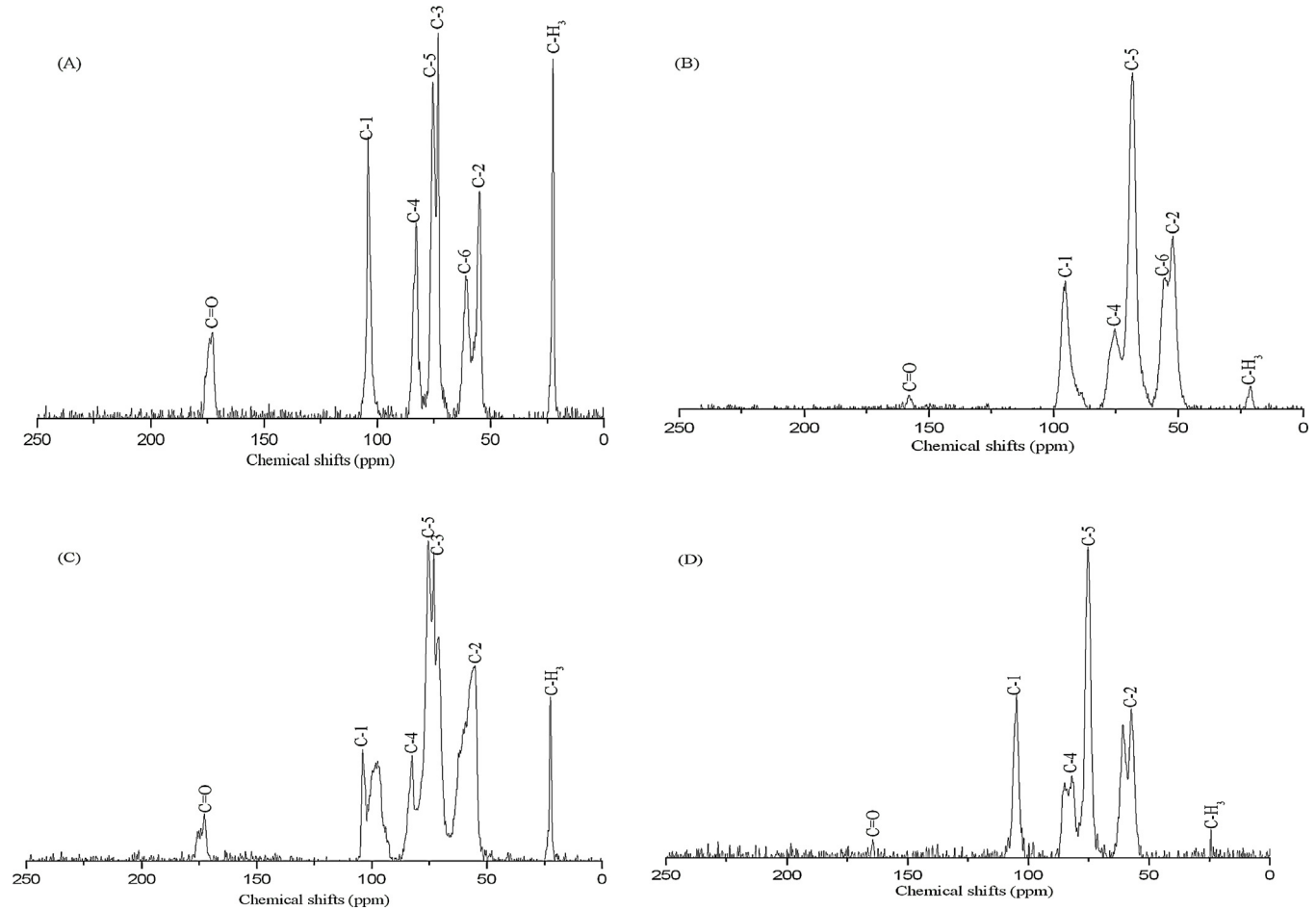

Fig. 6. ^{13}CP/MAS NMR solid-state spectra of chitin from successive two-step fermentation (A), chitosan from optimized *R. japonicus* M193 fermentation (B), chitin from chemical extraction method (C), and chitosan from chemical extraction method (D).

in the spectrum were clearly separated into two signals, similar to the α-chitin reported by Cárdenas, Cabrera, Taboada, and Miranda (2004).

Fig. 6 clearly shows the deacetylation of chitin extracted by microbial fermentation and chemical extraction, as the peak at 173 ppm disappears, and the peak at 23 ppm decreased, representing C=O and CH_3 of the acetyl group, respectively (Paulino, Simionato, Garcia, & Nozaki, 2006). Moreover, it was found that chitin and chitosan obtained by microbial fermentation have higher purity than that of chitin and chitosan obtained by chemical method because ^{13}C CP/MAS-NMR spectrums of chitin and chitosan obtained by chemical method had more miscellaneous peaks. Hence, the microbial fermentation is a much more promising method to extract chitosan than that of chemical method.

4. Conclusions

The successive two-step study using Plackett–Burman design based on the response surface methodology followed by the Taguchi design with orthogonal arrangement were proved to be effective to develop optimized fermentation conditions for producing highly active CDA using *R. japonicus* M193 fermentation for the bioconversion of chitin to chitosan. Results showed that the amount of chitin (%), glucose (g/L), and $MgSO_4{\cdot}7H_2O$ (g/L) as fermentation media, inoculum level (%), and culture time (d) most significantly impact the production of CDA. The optimized fermentation conditions at 5 d resulted in the maximal CDA production of 547.38 ± 12.06 U/L, which in turn produced chitosan with the highest DDA of ~79% and the lowest MM of ~126 kDa. This study enlightened the potential of utilizing *R. japonicus* M193 fermentation for the bioconversion of chitin to high quality chitosan. However, it should be noted that a large-scale production of CDA at current conditions may not be suitable. Hence, gene mutation and other means of enzyme engineering are necessary for improving the production of CDA, which is under the investigation in the authors' laboratory.

Acknowledgements

This research was financially supported by the "National Natural Science Foundation of China (No. 31271955)", "National Science and Technology Pillar Program during the 12th Five-year Plan Period (No. 2013BAD18B02)", "Key Discipline Talented Special Foundation of Shanghai Jiao Tong University (GKWS2107215001)", Shanghai Jiao Tong University – Qingpu Cooperation foundation (2010), "International Advanced Agricultural Science and Technology Introduction Program (948 Program) (No. 2013-S18) "and "Shanghai Natural Science Foundation (No. 11ZR1416100)".

References

Alishahi, A., & Aïder, M. (2012). Applications of chitosan in the seafood industry and aquaculture: A review. *Food and Bioprocess Technology*, *5*, 817–830.

Araki, Y., & Ito, E. (1974). A pathway of chitosan formation in *Mucor rouxii*: Enzymatic deacetylation of chitin. *Biochemical and Biophysical Research Communications*, *56*, 669–675.

Aranaz, I., Mengíbar, M., Harris, R., Paños, I., Miralles, B., Acosta, N., et al. (2009). Functional characterization of chitin and chitosan. *Current Opinion in Chemical Biology*, *3*, 203–230.

Bas, D., & Boyaci, I. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, *78*, 836–845.

Cahú, T. B., Santos, S. D., Mendes, A., Córdula, C. R., Chavante, S. F., Carvalho, L. B., Jr., et al. (2012). Recovery of protein, chitin, carotenoids and glycosaminoglycans from Pacific white shrimp (*Litopenaeus vannamei*) processing waste. *Process Biochemistry*, *47*, 570–577.

Cai, J., Yang, J. H., Du, Y. M., Fan, L. H., Qiu, Y. L., Li, J., et al. (2006). Purification and characterization of chitin deacetylase from *Scopulariopsis brevicaulis*. *Carbohydrate Polymers*, *65*, 211–217.

Cárdenas, G., Cabrera, G., Taboada, E., & Miranda, S. P. (2004). Chitin characterization by SEM, FTIR, XRD, and ^{13}C cross polarization/mass angle spinning NMR. *Journal of Applied Polymer Science*, *93*, 1876–1885.

Chang, K. L. B., Gengia, T., Lee, J., & Fu, W. R. (1997). Heterogeneous N-deacetylation of chitin in alkaline solution. *Carbohydrate Research*, *303*, 327–332.

Chen, X., Xia, W. S., & Yu, X. B. (2005). Purification and characterization of two types of chitosanase from *Aspergillus* sp. CJ22-326. *Food Research International*, *38*, 315–322.

Chaussard, G., & Domard, A. (2004). New aspects of the extraction of chitin from squid pens. *Biomacromolecules*, *5*, 559–564.

Da Silva, L. C. A., Honorato, T. L., Franco, T. T., & Rodrigues, S. (2012). Optimization of chitosanase production by *trichoderma koningii* sp. under solid-state fermentation. *Food and Bioprocess Technology*, *5*, 1564–1572.

Fan, W., Wei, Y., Xu, Z. S., & Ni, H. (2012). Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. *Colloids and Surfaces B: Biointerfaces*, *90*, 21–27.

Gauthier, C., Clerisse, F., Dommes, J., & Jaspar-Versali, M. F. (2008). Characterization and cloning of chitin deacetylases from *Rhizopus circinans*. *Protein Expression and Purification*, *59*, 127–137.

Ghaouth, A. E., Arul, J., Grenier, J., & Asselin, A. (1992). Effect of chitosan and other polyions on chitin deacetylase in *Rhizopus stolonifer*. *Experimental Mycology*, *16*, 173–177.

Ghorbel-Bellaaj, O., Jellouli, K., Younes, I., Manni, L., Salem, M. O., & Nasri, M. (2011). A solvent-stable metalloprotease produced by *Pseudomonas aeruginosa* A2 grown on shrimp shell waste and its application in chitin extraction. *Applied Biochemistry and Biotechnology*, *164*, 410–425.

Ghorbel-Bellaaj, O., Hmidet, N., Jellouli, K., Younes, I., Maalej, H., Hachicha, R., et al. (2011). Shrimp waste fermentation with *Pseudomonas aeruginosa* A2: optimization of chitin extraction conditions through Plackett–Burman and response surface methodology approaches. *International Journal of Biological Macromolecules*, *48*, 596–602.

Huang, Z., Dong, L., Chen, E. J., Gao, F. B., Zhang, Z. P., Chen, J. N., et al. (2012). Low-molecular weight chitosan/vascular endothelial growth factor short hairpin RNA for the treatment of hepatocellular carcinoma. *Life Science*, *91*, 1207–1215.

Jung, J., & Zhao, Y. Y. (2011). Characteristics of deacetylation and depolymerization of β-chitin from jumbo squid (*Dosidicus gigas*) pens. *Carbohydrate Research*, *346*, 1876–1884.

Kittur, F. S., Prashanth, K. V. H., Sankar, K. U., & Tharanathan, R. N. (2002). Characterization of chitin, chitosan and their carboxymethyl derivatives by differential scanning calorimetry. *Carbohydrate Polymers*, *49*, 183–185.

Kjartansson, G. T., Zivanovic, S., Kristbergsson, K., & Weiss, J. (2006). Sonication-assisted extraction of chitin from North Atlantic shrimps (*Pandalus borealis*). *Journal of Agriculture and Food Chemistry*, *54*, 5894–5902.

Kumar, P., & Satyanarayana, T. (2007). Optimization of culture variables for improving glucoamylase production by alginate-entrapped thermomucor indicae-seudaticae using statistical methods. *Bioresource Technology*, *98*, 1252–1259.

Lavertu, M., Méthot, S., Tran-Khanh, N., & Buschmann, M. D. (2006). High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials*, *27*, 4815–4824.

Li, N., Zhuang, C. Y., Wang, M., Sun, X. Y., Nie, S. F., & Pan, W. S. (2009). Liposome coated with low molecular weight chitosan and its potential use in ocular drug delivery. *International Journal of Pharmaceutics*, *379*, 131–138.

Martinou, A., Kafetzopoulos, D., & Bouriotis, V. (1995). Chitin deacetylation by enzymatic means: Monitoring of deacetylation processes. *Carbohydrate Research*, *273*, 235–242.

Muzzarelli, R. A. A., Morganti, P., Morganti, G., Palombo, P., Palombo, M., Biagini, G., et al. (2007). Chitin nanofibrils/chitosan glycolate composites as wound medicaments. *Carbohydrate Polymers*, *70*, 274–284.

Oliveira, L. A., Porto, A. L. F., & Tambourgi, E. B. (2006). Production of xylanase and protease by *Penicillium janthinellum* CRC 87M-115 from different agricultural wastes. *Bioresource Technology*, *97*, 862–867.

Pareek, N., Singh, R. P., & Ghosh, S. (2011). Optimization of medium composition for enhanced chitin deacetylase production by mutant *Penicillium oxalicum* SAEM-51 using response surface methodology under submerged fermentation. *Process Biochemisty*, *46*, 693–1697.

Pareek, N., Vivekanand, V., & Dwivedi, P. (2011). Penicillium oxalicum SAEM-51: A mutagenised strain for enhanced production of chitin deacetylase for bioconversion to chitosan. *New Biotechnology*, *28*, 118–125.

Paulino, A. T., Simionato, J. I., Garcia, J. C., & Nozaki, J. (2006). Characterization of chitosan and chitin produced from *silkworm crysalides*. *Carbohydrate Polymers*, *64*, 98–103.

Qiao, Y., Ruan, Y. Y., Xiong, C. N., Xu, Q. S., Wei, P., Ma, P., et al. (2010). Chitosan oligosaccharides suppressant LPS binding to TLR4/MD-2 receptor complex. *Carbohydrate Polymers*, *82*, 405–411.

Sini, T. K., Santhosh, S., & Mathew, P. T. (2007). Study on the production of chitin and chitosan from shrimp shell by using *Bacillus subtilis* fermentation. *Carbohydrate Research*, *342*, 2423–2429.

Tolaimate, A., Desbrieres, J., Rhazi, A., Vincendon, M., & Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer*, *41*, 2463–2469.

Tsigos, I., Martinou, A., Kafetzopoulos, D., & Bouriotis, V. (2000). Chitin deacetyalases: New, versatile tools in biotechnology. *Trends in Biotechnology*, *18*, 305–312.

Uriarte-Montoya, M. H., Arias-Moscoso, J. L., & Plascencia-Jatomea, M. (2010). Jumbo squid (*Dosidicus gigas*) mantle collagen: Extraction, characterization, and

potential application in the preparation of chitosan-collagen biofilms. *Bioresource Technology*, *101*, 4212–4219.

Wang, Y. C., Chang, Y. G., Yu, L., Zhang, C. Y., Xu, X. Q., Xue, Y., et al. (2013). Crystalline structure and thermal property characterization of chitin from Antarctic krill (*Euphausia superba*). *Carbohydrate Polymers*, *92*, 90–97.

Yaghobi, N., & Mirzadeh, H. (2004). Enhancement of chitin's degree of deacetylation by multistage alkali treatments. *Iranian Polymer Journal*, *13*, 131–136.

Yen, M. T., & Mau, J. L. (2007). Selected physical properties of chitin prepared from shiitake stipes. *LWT – Food Science Technology*, *40*, 558–563.

Zhang, H. C., Jin, Y. F., Deng, Y., Wang, D. F., & Zhao, Y. Y. (2012). Production of chitin from shrimp shell powders using *Serratia marcescens* B742 and *Lactobacillus plantarum* ATCC 8014 successive fermentation. *Carbohydrate Research*, *362*, 13–20.

Zhao, Y., Park, R. D., & Muzzarelli, R. A. A. (2010). Chitin deacetylases: Properties and applications. *Marine Drugs*, *8*, 24–46.