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Enzymatic preparation of chitosan from the waste *Aspergillus niger* mycelium of citric acid production plant

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Abstract

A new route had been devised for obtain chitosan with stable properties and good quality suitable for medical, cosmetic and other purposes. Waste *Aspergillus niger* mycelia from a citric acid production plant were used as a source of chitosan. The extraction of chitosan was operated with lysozyme, snailase, neutral protease and novel chitin deacetylase from *Scopulariopsis brevicaulis* at the optimum condition of every enzyme. The optimum dosage of neutral protease and chitin deacetylase were 0.17 g (5100 units) per 100 g fresh mycelia and 1200 units per 100 g fresh mycelia, respectively. The deproteinization rate was 59.9%. The recovery rate of glucosamine was 50%. Weight-averaged molecular weight, degree of deacetylation of chitosan and the content of glucosamine were 267.97 kDa, 73.6 and 84.4%, respectively. Compared with chemical extraction methods, the weight-average molecular weight was three times higher whilst other parameters were very similar. The results of FT-IR, X-ray and elemental analysis proved that the structure of chitosan of enzymatic extraction was very similar to those of shrimp chitosan.

Keywords: Chitosan; Enzymatic preparation; Aspergillus niger mycelia; Chitin deacetylase; Citric acid

1. Introduction

Chitin, a linear polysaccharide composed of (1→4)-linked *N*-acetyl-β-D-glucosamine units is an extremely insoluble material and so far has been found very little large-scale industrial use. Chitin can be converted to chitosan by deacetylation. Chitosan is soluble in acid solutions, and has a wide range of use being employed in the cosmetics, pharmaceuticals, agricultural and food industries. It can be used for clarification of juices (Imeri & Knorr, 1998) and production of biodegradable packaging films (Mayer, Wiley, Henderson, & Kaplan, 1989). Its use as a component of toothpaste, hand and body creams, shampoo, cosmetics and toiletries, as well as pharmaceuticals, for lowering of serum cholesterol has been documented. Furthermore, its application in enzyme (Synowiecki, 1986) and cell immobilization, as a drug carrier, a material for production of contact lenses or eye bandages, as well as seed coats etc, have all been reviewed (Muzzarelli, 1989; Sandford, 1989). In addition, it can be applied to deliver drugs to the target (Onishi, Nagai, & Machida, 1997) and used as flocculating and chelating agents (Wan, Petrisor, Lai, Kim, & Yen, 2004).

The traditional source of chitin is from shrimp and crab processing waste (Shahidi & Synowiecki, 1991). However, the industrial isolation of the polymer is restricted due to the problems of seasonal, limited supply in some countries and environmental pollution while collecting large amounts of shell waste. Moreover, the conversion to chitosan at high temperature causes variability of product properties and chitosan quality, for example molecular weight, and increases the processing costs. At the same time, the waste liquid which contain base, proteins and protein degradation products from the industrial processing leads to the environmental pollution.

Recently, some other sources, such as fungi, have begun to be employed to obtain chitosan. The mycelia of various fungi including *Aspergillus niger* (Suntornsuk, Pochanavanich, & Suntornsuk, 2002; Zhao & Wang, 1999), *Mucor rouxii* (Synowiecki & Al-Khateeb, 1997), *Absidia coerulea* (Wu, Huang, Wang, & Chen, 2001), *Rhizopus oryzae* (Tan, Tan, Wang, & khor, 1996) are alternative sources of chitin and chitosan.

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In *A. niger*, the contents of chitosan reported range from 20 to 22% (Zhao & Wang, 1999) in dry mycelium. *A. niger* has often been used to produce citric acid. The final fermentation mash can contain 20 g/L mycelium. If the chitosan content of the mycelium was calculated according to 20%, the chitosan production rate would reach to 4.0 g/L. So, chitosan derived from the waste mycelium of citric acid production plants could have wider availability. It is not only regarded as resource of chitosan, but also opens a new path for the integration application of the waste mycelium of citric acid production plant.

The extractions of chitosan from the waste *A. niger* mycelium of citric acid production plant have mostly employed the traditional acid–alkali method. However, the chitosan obtained using this method was of poor quality and this technology can bring the environmental pollution.

In this study, chitosan was prepared by treating the waste mycelium of citric acid with neutral protease, lysozyme, snailase and novel chitin deacetylase from *Scopulariopsis brevicaulis* in order to utilize the waste resource, decrease the environmental pollution, and improve the quality of product. In addition, the characteristics of the products from different resource and extracting method were investigated and compared.

2. Materials and methods

2.1. Materials and reagents

Waste mycelia were provided by Hunan Yinhai Petrochemicals Group Co., LTD (Ningxiang, China). Neutral protease, lysozyme and snailase were purchased from Sino-American Biotechnology Company (Luoyang, China). Chitin deacetylase was prepared by our method (Cai, Yang, Du, Fan, Qu, & Li, in press). The strain is *S. brevicaulis* and the activity is 120 U/ml. D-glucosamine hydrochloride and chitosan of shrimp (Mw 284 kDa; DD 87%) were provided by Zhe Jiang Aoxing Biotechnology Co. Ltd (Taizhou, China). All other chemical reagents were of the highest purity commercially available

2.2. Enzymatic extraction of chitosan

Enzymatic extraction of chitosan from the waste mycelium was based on the chemical procedure from the literature (Synowiecki & Al-Khateeb, 1997). The procedure involved four distinct processes: cell disruption, deproteination, deacetylation and extraction of chitosan, was modified. Acid and alkali were replaced with lysozyme, snailase, neutral protease and chitin deacetylase.

2.2.1. Cell Disruption

The waste *A. niger* fresh mycelia (400 g) were suspended in 0.2 M Na_2HPO_4–NaH_2PO_4 buffer pH 6.4 (120 ml) containing lysozyme (1 g) and snailase (1 g). After stirring continuously at 50 °C for 5 h, the solid was collected with a centrifuge at 12,000 rpm for 20 min. The solid was washed with demineralized water and used in the next procedure.

2.2.2. Deproteination

The solids of cell disruption were suspended in Na_2HPO_4–NaH_2PO_4 buffer pH 7.0 (1000 ml) and 5100 U per 100 g fresh mycelia solid neutral protease was added, stirred continuously at 55 °C for 3 h and then the solid was collected with a centrifuge at 12,000 rpm for 20 min. The solid was washed with demineralized water and was used as the substrate of the next procedure.

2.2.3. Deacetylation

The solid of deproteination were suspended in 50 mM Tris–HCl buffer pH 7.5 (800 ml) and chitin deacetylase was added, stirred continuously at 55 °C for 8 h and the solid was collected with a centrifuge at 12,000 rpm for 20 min. The solid was washed with demineralized water and was used as the material of the next procedure.

2.2.4. Chitosan extraction

After deacetylation and washing, chitosan was extracted from the remaining solids using 2% acetic acid at 30 °C for 16 h and separated with centrifuge at 12,000 rpm for 20 min. The chitosan was precipitated from the acetic acid solution by adding 40% NaOH until pH 9.0 and separated with centrifuge at 12,000 rpm for 20 min. The precipitation was washed with demineralized water and 95% ethanol three times and freeze-dried.

2.3. Chemical extraction of chitosan

The waste *A. niger* mycelium (400 g) was suspended in 2.5% HCl (1200 ml) and refluxed at 100 °C for 1 h; then the solid was collected with a centrifuge at 12,000 rpm for 20 min, washed with demineralized water to neutrality; 4% w/v NaOH (100 ml) was added and the whole stirred continuously at 100 °C for 20 min. Then, the solid was suspended in 5% w/v NaOH (800 ml), heated at 125 °C for 2 h, and separated by vacuum filtration. The solid was washed with demineralized water to neutrality and then 2%w/v acetic acid was added and the mixture maintained at 30 °C for 12 h to extract the chitosan, separated by vacuum filtration and washed three times with 200 ml 2% w/v aqueous acetic acid. 40% w/v Sodium hydroxide (40 ml) was added to the solution to precipitate the chitosan. The precipitate was washed with demineralized water (100 ml) and 95% ethanol (100 ml) three times and freeze-dried.

2.4. Analysis

2.4.1. Protein

Crude protein in the sample was extracted (in three replicates) with 10% (w/v) NaOH solution for 2 h at 90 °C. Then, the insoluble matter in NaOH was removed by filtration on a Coarse's sintered-glass funnel, and the filter liquor was diluted to 100 ml with distilled water. The extract (10 ml) was used for protein determination ($N \times 6.25$) according to the Kjieldahl procedure (Johnson, 1971).

2.4.2. Glucosamine

Samples (20 mg) of lyophilized mycelia, deproteinized mycelia and chitosan were hydrolysed in of 4 M HCl (3.0 ml) in sealed ampoules (10 ml) for 18 h at 105 °C. The pH of the hydrolysates diluted with distilled water (40 ml) was adjusted to 7.0 with 4 M NaOH solution. The neutralized solutions were diluted to 100 ml in volumetric flasks and a further 1:5 dilution was made before analysis. The concentration of D-glucosamine in the investigated samples was determined using the Elson–Morgan procedure modified by Johnson (1971). The calibration curve was constructed with D-glucosamine hydrochloride as standard.

2.4.3. Degree of deacetylation

Chitosan (0.1 g) was dissolved in a known excess of 0.1 M HCl (10 ml). The pH was recorded in the titration process of adding 0.1 M NaOH by using a DELTA-320-S pH meter. A curve with two inflexion points was obtained. The amount of acid consumed between these two points was considered to correspond to the amount of the free amino groups in the solution (Tolaimate et al., 2000).

2.4.4. Measurement of molecular weight

Weight-average molecular weight was measured by gel permeation chromatography (GPC). The (GPC) equipment consisted of the connected column (TSK G3000-PW and TSKG5000-PW) (Tosoh Corp., Tokyo, Japan), TSP p100 pump, and RI 150 refractive index detector (Thermo Separation products, USA). The eluent was 0.1 M sodium acetate in 0.2 M acetic acid buffer solution, pH 4.3. The flow rate was maintained at 1.0 ml/min, the temperature of the column was maintained at 30 °C and the eluent was monitored with the RI 150 refractive index detector. The standards used to calibrate the column were TOSOH Pullulan (Tosoh Corp., Tokyo, Japan) of defined Mw ranging from 2.7 to 788 kDa. All data provided by the GPC system were collected and analyzed using the Jiangshen Workstation software package.

2.4.5. Other Characterization

IR spectra of chitosan from *A. niger* mycelium and chitosan from shrimp were taken as KBr discs on a Nicolet FT-IR 360 spectrophotometer (USA). The X-ray diffraction of chitosan from difference resource was recorded by a Rigaku X-ray diffractometer (D/max IIIA, Japan) using Ni-filtered Cu Kα radiation at 30 kV and 30 mA. The sample was scanned from 5 to 40° of 2θ.The elemental analysis was taken with elemental analyzer-MOD1106 after the samples had been reduced to powders.

3. Results and discussion

3.1. Deproteination.

The protein content of fresh mycelium was found to be 3.46%. The moisture content of the fresh mycelium was 85%. The protein content of dry mycelium was 23.1%. After the cell wall of mycelium was destroyed using lysozyme and snailase

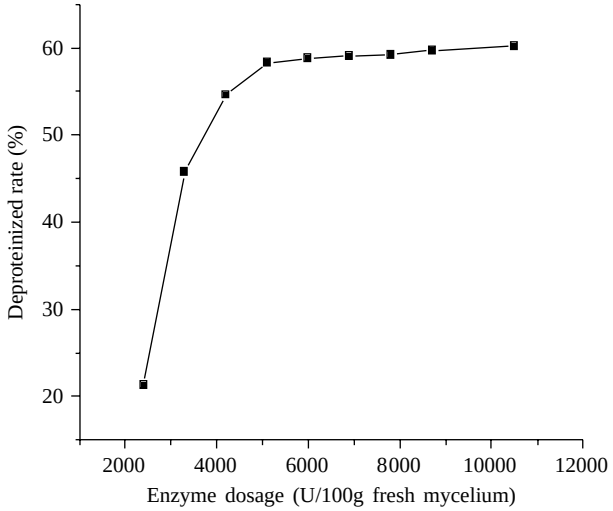

Fig. 1. Effect of neutral protease dosage on deproteinization rate.

treatment, the protein was removed by hydrolysis using the neutral protease. The effect of neutral protease dosage on deproteinization rate (Fig. 1) was to increase the rate gradually. After the neutral protease dosage had been added to 5100 U/100 g fresh mycelium, the deproteinization rate showed little change following the increase of enzyme dosage. 59.9% of the protein in the waste *A. niger* mycelium was removed during the deproteination process. So, the optimum neutral protease dosage was determined to be 5100 U/100 g fresh mycelium.

The enzymatic deproteinization was compared with chemical deproteinization (hot and cold 4% w/v NaOH) (Table 1). The deproteinization rate was highest using the hot 4% NaOH (62.3%), but the solution became dark brown and purification by-products can cause serious environment pollution. However this deproteinization rate (59.9%) was little different from that of the enzyme regime. So, the enzymatic deproteinization can replace the chemical methods.

The protein content of mycelia on a dry basis was decreased to 9.3% after enzyme deproteinization. The result was similar with that of Synowiecki and Al-Khateeb (1997), who achieved the maximum protein extraction from dry mycelium (60.1%) using 2% w/v NaOH (1:30 w/v, 90 °C, 2 h).

3.2. Deacetylation and molecular weights

In the cell wall of *A. niger* mycelium, the content of chitosan was less than that of chitin (Synowiecki & Al-Khateeb, 2003). In order to obtain the chitosan with high yield, the conversion

Table 1
Deproteinization rate of waste *Aspergillus niger* mycelium using chemical and enzymatic methods

Deproteinized rate (%) for a		
Chemical method		Enzymatic method
4% Hot NaOH	4% Cold NaOH	
62.3	34.7	59.9

Deproteinization rate = (A − B/A), where: A, total protein of mycelium; B, the protein content of mycelium after deproteinization.

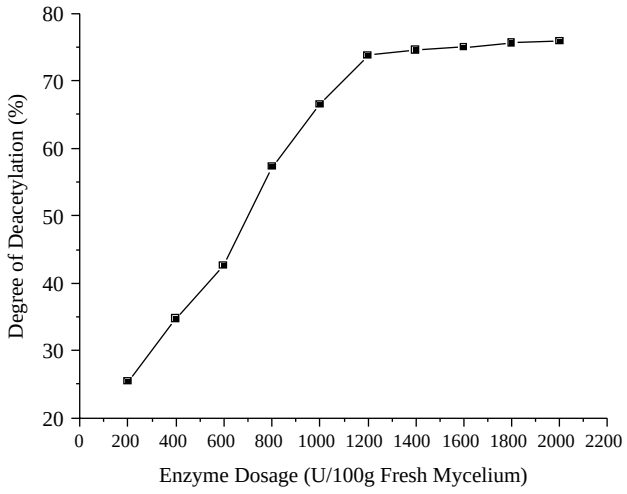

Fig. 2. Effect of chitin deacetylase dosage on degree of deacetylation.

of chitin to chitosan was necessary. At the same time, the molecular weight and degree of acetylation of chitosan are important criteria for its use in applications, since they have strong effects on the physical–chemical properties and bioactivity of chitosan. Thus, it was important to select an appropriate method to retain the molecular weight and to control the degree of acetylation. Chitin deacetylase was used to remove the acetyl groups in the chitin (Fig. 2) and it was shown that 73.6% of the degree of deacetylation could be reached, when the chitin deacetylase dosage was 1200 U/100 g fresh mycelium. After the enzyme dosage increased, the degree of deacetylation had little change. So, the optimum chitin deacetylase dosage was 1200 U/100 g fresh mycelium.

In the comparison of the enzymatic deacetylation with chemical deacetylation (50% w/v NaOH at 125 °C for 2 h) both the degree of deacetylation and molecular weight were determined (Table 2). The degree of deacetylation of chitosan obtained with two methods had little difference. The degree of deacetylation was higher than that reported by Alfonso, Nuero, Santamaria and Reyes, (1995). They detected extracellular chitin deacetylase during natural autolysins of *Aspergillus nidulans* and purified the enzyme to determine its properties and studied its role in cell wall degradation.

But the weight average molecular weight of chitosan obtained using enzymatic method (267.97 kDa) was far higher than that of chemical method chitosan (84.04 kDa), although chitosan could be degraded in the presence of the neutral protease (Li, Du, Yang, Feng, & Li, 2005). Also chitosan with 54% of degree of deacetylation was treated with the chitin deacetylase. The result showed chitin deacetylase did not have any effect on the molecular weight. It was in accord with Tokuyasu's conclusion (Tokuyasu, Ono, Ohnishi-Kameyama, Hayashi, & Mori, 1997).

Table 2
Comparison of chemical and enzymatic methods for deacetylation

Method	Degree of deacetylation (%)	Molecular weight (kDa)
Chemical method	76.8	84.04
Enzymatic method	73.6	267.97

Table 3
Glucosamine recovery during the different isolation phases of waste *A. niger* mycelium

Isolation phase	Weight of fraction (g)	Glucosamine as % dry weight basis of mycelia per phase	Weight of glucosamine (g)	Recovery yield[a] (%)
Waste mycelia	58.76	20.46	12.02	100.00
The dry product after cell disruption	32.47	34.25	11.12	92.51
The dry product after deproteinization	23.32	40.57	9.46	78.70
The dry product after deacetylation	16.28	47.36	9.17	76.29
Chitosan (dry weight basis)	7.13	84.43	6.02	50.05
Residue insoluble in acetic acid	8.86	–	3.06	–

[a] Recovery yield $=(C_P/C_T)\times 100\%$. Where C_T is amount of glucosamine in the dry waste mycelia and C_P is amount of glucosamine in the dry product of per phase. The recovery rate of chitosan with the chemical method was 41.7%.

3.3. Glucosamine recovery during isolation

The amount of glucosamine recovered from the mycelium after deproteinization and deacetylation were about 78.70 and 72.29%, respectively (Table 3). The yield rate of chitosan was about 50%. In the waste *A. niger* mycelium of citric acid production plant, there were also cellulose, pectic acid and protein. Thus, the yield rate (50.05%) was higher that that reported in the literature (48.4%) (Synowiecki & Al-Khateeb, 1997). Compared with the results of chemical method, the chitosan quality of this technology was better and the recovery rate is higher (Table 4).

The main components of the final insoluble residues in acetic acid were glucans, galactomannan and different acidic

Table 4
Comparison of chemical and enzymatic methods of chitosan extraction from waste *A. niger* mycelium for chitosan quality and recovery rate

Parameter	Chemical method	Enzymatic method
Degree of deacetylation (%)	76.8	73.6
Molecular weight (kDa)	84.04	267.97
Content of glucosamine (%)	82.7	84.4
Ash (%)	0.370	0.283

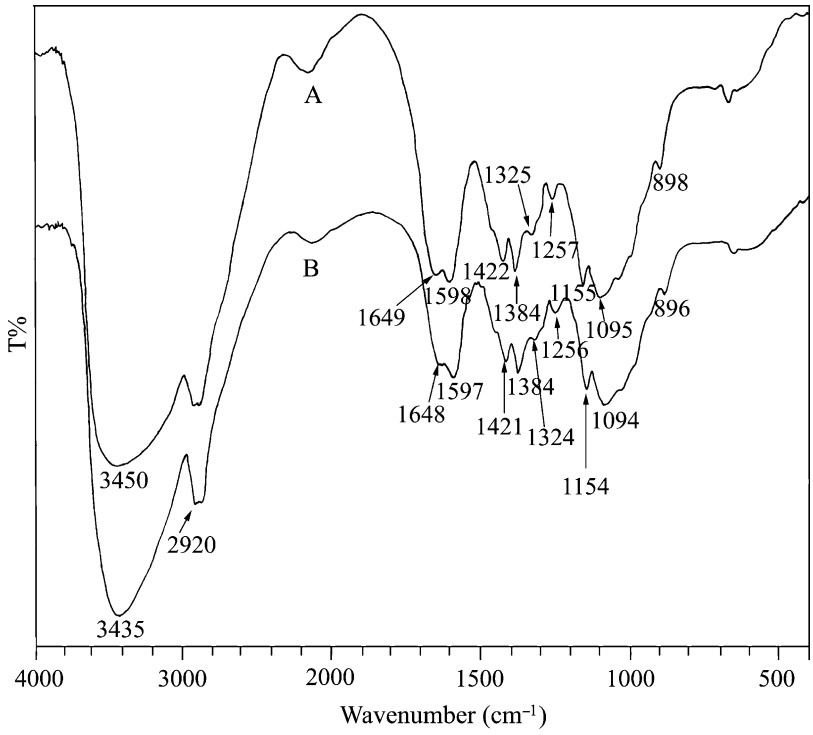

Fig. 3. FT-IR spectra of chitosan from enzymatic method extraction (B) and chitosan of shrimp (A).

polysaccharides in addition to a small amount of glucosamine-containing material (Azuma, Kimura, Hirao, Tsubura, & Yamamura, 1969). This could be because part of the chitin was covalently linked to glucan to form chitin–glucan complex in the cell wall of *A. niger*. The major component of cell wall is a (1→3)-β-D-glucan with some (1→6)-β-D-linked side chains. *N*-acetylglucosamine at the reducing terminus of the chitin chain is bound to the glucose units in the side chains either by (1→4)- or (1→2)-linkages (Chorvatovičová & Šandula, 1995; Machová, Kogan, Chorvatovičová, & Šandula, 1999; Tereshina, Memorskaya, Feofilova, Nemtsev, & Kozlov, 1997). Chitin–glucans contain about 70% β-D-glucan and 30% chitin (Šandula, Kogan, Kačuráková, & Machová, 1999).

3.4. Characterization

The IR spectrum of chitosan of enzymatic method extraction was similar to that of the chitosan from shrimp (Fig. 3) and similar to that of chitosan from *M. rouxii* (Synowiecki & Al-Khateeb, 1997). Thus, their molecular structures should be basically same. The absorbance band at 1598 cm^{-1} is characteristic of the amino deformation mode (Brugnerotto, Goycoolea, Arguelles-Monal, Desbrieres, & Rinaudo, 2001). The peaks at about 1649 and 1325 cm^{-1} are the amide I band (ν C=O) and the amide III band (δ C–N), respectively. The amide II band had disappeared in the FT-IR spectrum mainly because it overlapped with the band of the amino deformation vibration. However, the absorbance of the amide I band was still strong. This was because the band overlapped with band of O–H deformation vibration of H_2O at ~1640 cm^{-1}. The band at about 898 cm^{-1} was attributed to the β-anomer.

The degree of deacetylation value for chitosan isolated from the waste *A. niger* mycelium of citric acid production plant with chemical method extraction and enzymatic method extraction are 76.8 and 73.6%, respectively. The degree of deacetylation determined was 78–91% in different microbial chitosans (Shimahara, Takiguchi, Kobayashi, Uda, & Sannan, 1989). The results of lower degree of deacetylation of products could be because the deacetylation rate was low in the

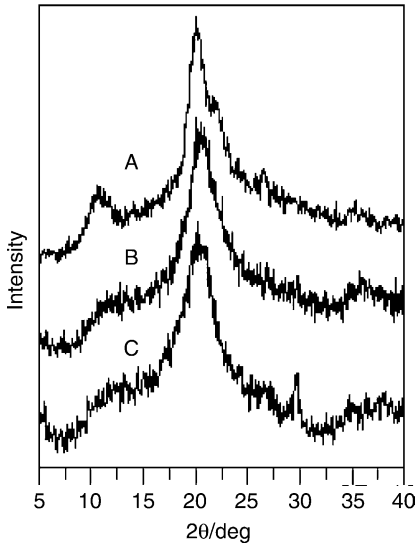

Fig. 4. X-ray diffraction patterns of shrimp chitosan (A), chitosan of chemical method extraction (B) and chitosan of enzymatic method extraction (C).

Table 5
Elemental analysis data of the chitosans produced from different extraction methods

Sample	Nitrogen%	Carbon%	Hydrogen%	Sulphur%	N/C
Shrimp chitosan	8.470	41.155	6.878	0	0.206
Chitosan of chemical method extraction	7.312	45.008	6.499	0	0.163
Chitosan of enzymatic method extraction	8.585	40.570	7.017	0	0.212

conversion of chitin to chitosan. However, degree of deacetylation values of chitosan produced from shellfish chitin by deacetylation with concentrated NaOH solutions ranged from near 50–100% with variable degrees of deacetylation (Roberts, 1992). The recovery rate of chitosan with chemical method and enzymatic method are 41.7 and 50.0%, respectively (Table 3).

The X-ray diffraction patterns (Fig. 4) of shrimp chitosan (A), chitosan of chemical method extraction (B) and chitosan of enzymatic method extraction (C), showed diffraction angle ranging from 5 to 40°. The diffraction pattern of shrimp chitosan showed the characteristic peaks around 10.5, 20.0, 26.5 and 35.3°. The chitosan of chemical method extraction and enzymatic method extraction had similar characteristic peaks, but the intensity of this peak was less than of the peak of shrimp chitosan around 20°. This indicated that the chitosan obtained from the waste *A. niger* mycelium had lower crystallinity.

The data from elemental analysis of the chitosan of shrimp, the chitosan of chemical method extraction are listed in Table 5. The mass ratio of N/C was in the order of chitosan of enzymatic extraction > shrimp chitosan > chitosan of chemical extraction. The data in Table 4 showed that the glucosamine content of chitosan obtained with enzymatic method was higher than that of chitosan obtained with chemical method. The low N/C or glucosamine content of chitosan from the chemical method could be related to the degradation of chitin—glucans or chitosan–glucans in the cell wall of mycelium. Polysaccharides including glucan, chitin and chitosan were degraded easily in the presence of strong alkali (Knill & Kennedy, 2003; Whistler & BeMiller, 1958). Thus the chitosan–glucans with low molecular weight, which were formed in the process of alkali treatment, could be dissolved in the acetic acid and existed in the final products. However, the solid chitin–glucan complex was difficult to degrade using the neutral protease, and the chitin deacetylase had no effect on the molecular weight of polysaccharide. So, chitosan obtained using the enzymatic method had higher purity and its N/C value was also higher.

4. Conclusion

For obtaining chitosan from the waste *A. niger* mycelium from a citric acid production plant, the Synowiechi's procedure (Synowiecki & Al-Khateeb, 1997) modified was used, in which acid and alkali were replaced with lysozyme, snailase, nutral protease and chitin deacetylase. This can avoid the environmental pollution of chemical method. Then, the chitosan from use of this technology has high purity and N/C value, so its quality was better than that of quality of the chitosan derived from classical chemical methods and the recovery rate of enzymatic method is higher than chemical method. Especially, compared with chemical method, the molecular weight of chitosan obtained was over three times as high. All these results showed that the enzymatic method was very good for the preparation of chitosan from *A. niger* mycelium.

Acknowledgements

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