FACTORS AFFECTING THE RECOVERY YIELD AND THE PURITY OF N-ACETYL GLUCOSAMINE FROM CHITIN HYDROLYSATE

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ABSTRACT

In this study, different ratio of ethanol to hydrolysates obtained by hydrolyzing chitin by chitinase as well as concentration of hydrolysates was investigated to find the optimal value. The result showed that the optimal ratio of absolute ethanol to hydrolysates was 9:1. The further increase of this ratio up to 11:1 resulted only in insignificant increase of N-acetylglucosamine (NAG) recovery yield from 74.47 % to 75.39 % and NAG purity from 93.25 % to 93.44 %. Concentration of hydrolysate also affected the NAG recovery yield and NAG purity. The impurity of crystallization by cooling down the hydrolysates appeared only when hydrolysate was concentrated at a certain level. The mass of crystallization impurity was increased sharply from 0 to 0.082 mg/ml when hydrolysate's concentration was increased significantly from 1 to 7.9 fold, then slightly from 0.082 to 0.093 mg/ml as its concentration further increased up to 15.7 fold. Higher concentration of hydrolysate resulted in lower recovery yield but purer product. According to HPLC analysis, 93.25 % of NAG purity with recovery yield 74.47% was obtained at optimal ethanol/hydrolysates ratio and optimal concentration of hydrolysate.

Keywords: N-Acetyl-D-glucosamine NAG, purification, crystallization, precipitation.

1. INTRODUCTION

N- Acetyl Glucosamine (GlcNAc or NAG), the monomer unit of polymeric chitin possesses therapeutic potential for treatment of osteoarthritis [1], anti-inflammatory bowel disease, gastritis, application in cosmetics.... [2]. Chitin represents the second most abundant natural biopolymer, which is found in the exoskeletons of crustaceans such as crab, shrimp shell... Although it would be desirable to utilize chitin extensively as a biomaterial, most chitin is not being utilized due to its insolubility. In order to exploit the application of chitin, it is important to develop the value-added functional substances derived from it such as NAG.

Compared to chemical method, enzymatic method of NAG production is preferred due to mild condition, enviroment- friendly, cost effective and relatively free from chemical residues. Many reports showed that NAG can be produced by crude chitinase from *Penicillium oxalicum* 20B [3], *Aeromonas* sp. GJ-18 [4], *A. hydrophila* H-2330 [5]... However the recovery and purification of NAG from hydrolysates were reported rarely. Setthakaset et al., 2008, using activated charcoal to decolorize hydrolysates with subsequent concentration and washing by absolute ethanol could produce NAG with recovery yield 65 % and purity above 70 % [6]. According to Aiba, after concentrated at 50 ^oC, NAG with purity 95 % and recovery yield 47 % could precipate from concentrated hydrolysates by ratio of absolute ethanol to concentrated hydrolysate 10:1 [7]. However how the concentration level as well as ratio of ethanol to concentrated hydrolysates affect the recovery yield and NAG purity was not studied. In this study, the factors affecting the recovery yield and purity of NAG product have been studied for further improvement of purification process.

2. MATERIALS AND METHODS

2.1. Materials

N-Acetyl-D-glucosamine (NAG) was purchased from Sigma Chemical Company (USA). Ethanol absolute was purchased from Duc Giang Detergent-Chemical SJC (Specification: Assay >= 99.7 %, evaporation residue ≤ 0.001 %, acidity ≤ 0.04 %, alkalinity ≤ 0.01 %, water content ≤ 0.25 %, iso-propanol ≤ 0.01 %). Chitin from slipper lobster shells was purchased from MTV Chitosan company (Kien Giang). *Penicillium oxxalicum* 20B was provided by Department of Biotechnology, School of Biotechnology and Food Technology, Hanoi University of Science and Technology.

Technical enzyme was prepared by ultrafiltration of crude enzyme. Briefly, crude enzyme was obtained by culturing *P. oxalicum* 20B at optimal conditions as described in previous study [8] The supernatant that collected by centrifuging at 6000 rpm for 15 min at 4 ^oC was filtrated by cross flow filtration on QuixStand^{TM} system at 12 ^oC at pump speed 30–35 rpm using membrane 10 kDa of MWCO with an effective surface area S = 1400 cm^2 under transmembrane pressure (TMP) of 0.758 bar. The ultrafiltration process was ended when the final volume of ¼ original was reached. The obtained technical enzyme had chitinase activity 0.239 U/ml.

2.2. Methods

2.2.1. Preparation of colloidal chitin

Colloidal chittin was prepared according to Lee et al. (2009) with small modification [9]. Briefly, colloidal chitin was prepared by dissolving shrimp shell chitin (5 g) in 100 ml of cold concentrate hydrochloric acid 37 % under vigorous magnetic stirrer at 4 ^oC for 18 h. The mixture was then added to 500 ml of ice-cold ethanol (96 %) and stirred at 4 ^oC for 24 h. The precipitate of colloidal chitin was collected by centrifugation at 6000 rpm for 10 min at 4 ^oC and was washed several times with distilled water until pH 5. About 18 - 22 g colloidal chitin was obtained from 1 g dry chitin and stored at 4 ^oC.

2.2.2. Preparation of hydrolysates

Technical chitinase was prepared as decribed above 2.1. The hydrolysis was carried out by incubation of technical chitinase with 40 % colloidal chitin solution at ratio 1:1 and at 40 ^oC, pH

5 for 24 hours. The reaction was terminated by boiling the solution for 15 min, then the reaction mixture was centrifuged at 6000 rpm for 10 min at 4 ^oC to collect the hydrolyzing supernatant.

2.2.3. Determination of N-Acetyl-D-glucosamine by HPLC

Sampling to analyse HPLC: Hydrolysates were centrifugated (6000 rpm for 10 minutes) and then filtered through a membrane filter with a filter size 0.2 μm. HPLC analysis was performed on equipment Agilent 1200 Infinity (column Aminex 87H; H_2SO_4 10 mM; pressure pump P_{max} = 100 bar, flow rate = 0.6 ml/min; injection, 0.02 mL; detection UV at 210 nm.

2.2.4. Determination characterization by FTIR

The characterization was done by Fourier transformed infrared (FTIR) spectroscopy (the Perkin Elmer Spectrum100 FTIR spectrometer was used) in the range of 400 to 4000 cm^{-1}.

2.2.5. Recovery and purification process

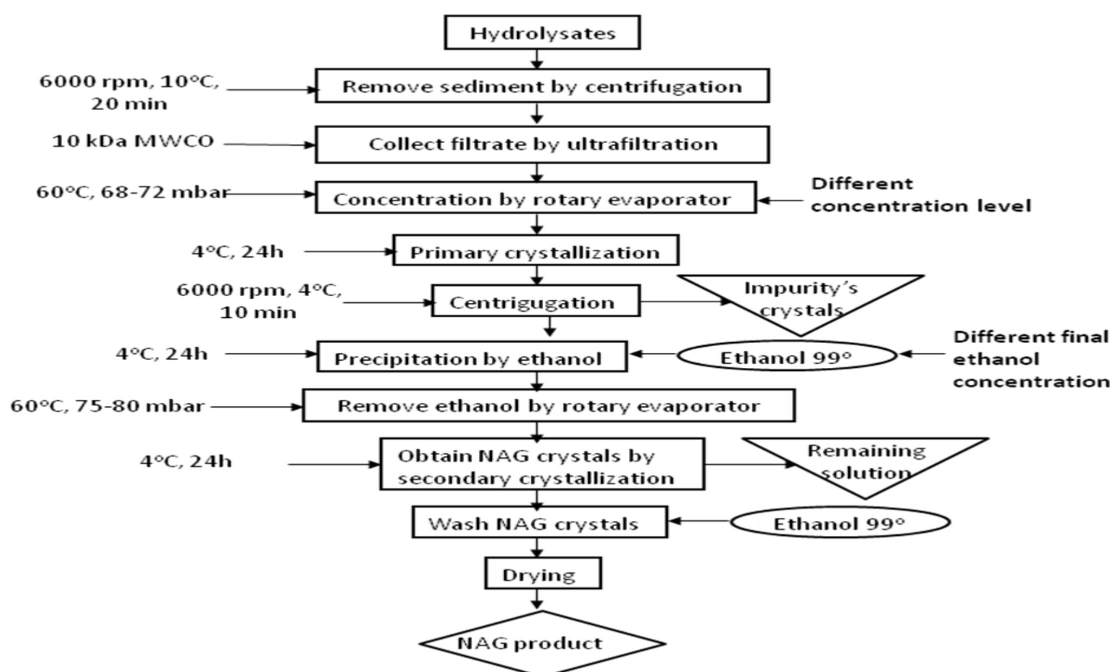

Figure 1. NAG purification process.

The hydrolyzed supernatant was cross flow filtrated on QuixStand^{TM} system using membrane 10 kDa of MWCO. For purification of NAG, the supernatant was concentrated by rotary evaporator under vacuum pressure (30 rpm, 60 ^oC, 68 - 72 mbar). The concentrate was kept 4 ^oC for 24 hours for primary crystallization and then centrigugated (6000 rpm, at 4 ^oC for 10 minutes) to remove impurity' crystals. After that ethanol was added to the concentrate and kept at 4 ^oC for 24 hours for the precipitation with subsequent centrifugation to collect supernatant. Ethanol was removed from supernatant by rotary evaporator under vacuum pressure (30 rpm, at 55 ^oC, in 75 - 80 mbar). NAG crystals formed when keeping supernatant at 4 ^oC for

24 hours (secondary crystallization). The forming NAG forming crystals was washed with ethanol and dried in dryer at 60 ^{o}C.

3. RESULTS AND DISCUSSION

3.1. Effect of ethanol concentration on recovery yield and purity of NAG

For this study, the hydrolysates after ultrafiltrating with NAG concentration of 9.756 mg/ml (as estimated by HPLC) was used (Fig. 2B). It could be seen that the hydrolysates contained only NAG and no $(NAG)_{2-3}$. After concentration by rotary evaporator to final volume, which was 15.7 fold reduction of original, the concentrated hydrolysates was used for the experiment.

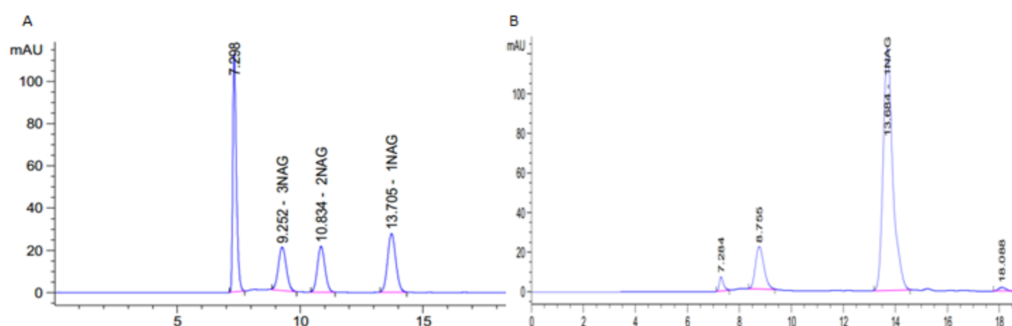

Figure 2. HPLC analysis of standard (A) NAG, 2NAG or (NAG)2, 3 NAG or (NAG)3, (B) hydrolysates after ultrafiltration.

Ethanol was added to the concentrated hydrolysates at different ratio 7/1, 8/1, 9/1, 10/1, 11/1 (v/v) i.e. the final concentration of Ethanol in concentrated hydrolysates increased from 87.5 % to 91.7 %. The obtained mixures were kept at 4 ^{o}C for 24 hours. The results from Figure 3 showed that with the increase of Ethanol ratio from 7/1 to 9/1, the precipitate mass increases sharply from 77.5 to 95.2 mg/ml and then only slightly increase to 96.3 mg/ml when the ratio further increased up to 11/1. The precipitate contained mainly impurity since the percentage of NAG in precipitate was from 3 - 11 %. The increase of ethanol concentration resulted in the increased amount of NAG in precipitate. It is explainable since NAG was poorly dissolved in absolute ethanol.

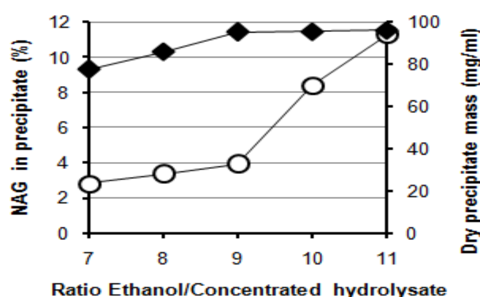

Figure 3. Effect of ethanol concentration on precipitate mass (closed square symbol) and coprecipitate NAG amount (open circle symbol).

The NAG in precipitate increased slowly from 2.8 - 3.9 % when ratio increased from 7 - 9 but sharply increased to 8.4 and 11.3 % at higher ratio 10 and 11. The results indicated that the

ratio of Ethanol/concentrated hydrolysates 9/1 was optimal for impurity's precipitation without the big loss of NAG into precipitate.

The NAG was purified further according to process described on Figure 1. The recovery yield as well as purity of NAG products was determined (Table 1). The results revealed that the higher ethanol concentration the lower uncrystallized NAG remained in solution. The lower amount of impurity precipitated from hydrolysates at lower ethanol concentration resulted in higher concentration of impurity. This might interfer with the NAG crystallization and thus cause the high amount of NAG remained in solution after NAG crystallization (secondary crystallization). Therefore despite higher lost NAG in precipitate (Fig. 3), the recovery NAG yield increased from 68.17 % to 75.39 % with increased Ethanol ratio from 7 to 11. In accordance with increased recovery yield, the purrity of NAG increased from 76.46 % to 93.44 %. The results from Table 1 also implicated that ratio 9 was optimal since there is no strong increase of recovery yield and purity of NAG at high ratio 10 and 11.

Table 1. Effect of ethanol concentration on recovery yield and purity of NAG.

Ratio ethanol/hydrolysates	Lost NAG into remaining solution after secondary crystallization (%)	Recovery yield of NAG (%)	Purity of NAG (%)
7	19.06	68.17	76.46
8	15.87	70.79	84.66
9	11.75	74.47	93.25
10	8.54	74.75	93.34
11	6.09	75.39	93.44

3.2. Effect of concentration on recovery yield and purity of NAG

Again for this study, the hydrolysates after ultrafiltrating with NAG concentration of 9.756 mg/ml were used. After concentrating of hydrolysates by rotary evaporator to different final volume, the obtained concentrated hydrolysates were kept at 4°C for 24 h for primary crystallization. From Figure 4, it could be seen that, the crystals mass from primary crystallization increased strongly from 0 to 0.082 mg/ml when the concentration increased from 1 to 7.9 but slowly (from 0.082 to 0.093 mg/ml) as concentration level increased further from 7.9 to 15.7. The crystals contained mostly impurity since the percentage of NAG was about 2%. However the increase of hydrolysate concentration resulted in strong increase of NAG in crystals i.e the NAG amount was doubled when its concentration level increased from 11.2 to 15.7.

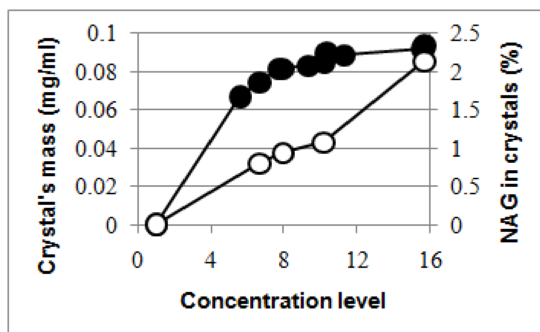

Figure 4. Effect of concentration level on removal of impurity by the primary crystallization (closed symbol) and NAG in crystals (open symbol).

The NAG was further purified according to process described on Fig. 1. The results from Table 2 indicated that when concentration factor was increased from 1.0 to 15.7 fold, besides the increase of NAG in crystals, the NAG in precipitate by ethanol and NAG in remained solution also increased. Therefore the recovery yield of NAG decreased from 86.93 % to 74.47 %. However, the purity of NAG product due to the increase of removal mass of impurities by primary crystallyzation, increased from 60.85 % to 93.25 %.

Table 2. Effect of concentration level on recovery yield and purity of NAG product.

Concentration level	**NAG in thanol precipitate (%)**	**NAG in crystals (%)**	**NAG in remaining solution (%)**	**Recovery yield of NAG (%)**	**Purity of NAG (%)**
1.0	0	0	0	86.93	60.85
6.7	1.48	0.812	6.50	81.99	85.25
7.9	1.53	0.947	8.22	80.36	88.45
10.2	1.75	1.090	9.91	78.11	89.78
15.7	2.43	2.148	11.75	74.47	93.25

High concentration factor thus resulted on purer product but with lower recovery yield. The NAG product at concentration 15.7 was analyzed by HPLC (Fig. 5).

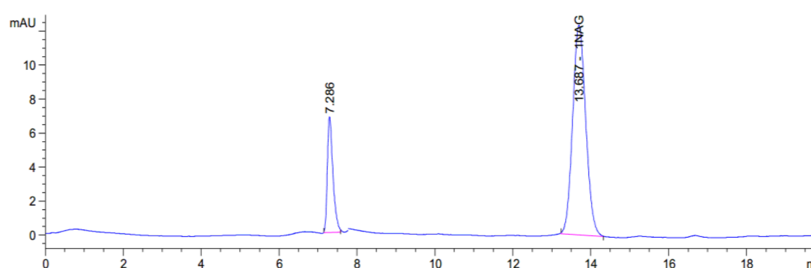

Figure 5. HPLC analysis of NAG product at 15.7 concentration level.

Results from Figure 5 indicated that there was only one peak NAG. The ratio of the band intensities at 1379.88 cm^{-1} and 2932.69 cm^{-1} (on M6_FTIR) and at 1380.33 cm^{-1} and 2932.78 cm^{-1} (on M7_FTIR) were 0.9676 and 0.9664 respectively (Fig. 6). According to Focher et al [10] as well as Wu et al [11] the purity of NAG product and of sigma NAG (99% purity) was almost the same.

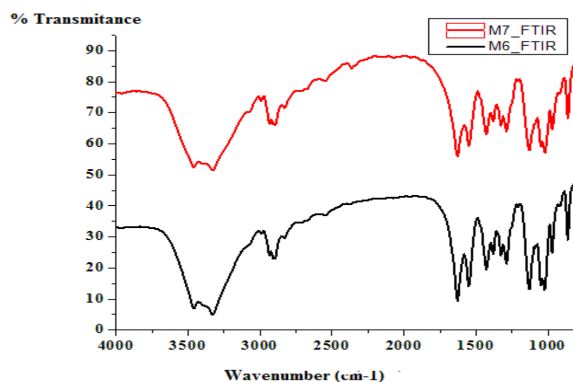

Figure 6. FTIR of NAG product (M6_FTIR) and sigma NAG (M7_FTIR).

4. CONCLUSION

Ethanol concentration and concentration are important factors which affected the recovery yield as well as purity of NAG. Optimal ratio of ethanol to concentrated hydrolysis was 9. Higher ethanol ratio resulted on very small increase of recovery yield and purity of NAG. Higher concentration factor resulted in lower NAG recovery yield but purer NAG product. At optimal ratio of ethanol to concentrated hydrolysis 9 and concentration factor 15.7, the recovery yield and purity of NAG were 74.47 % and 93.25 %, respectively.

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REFERENCES

1. Houpt J. B., McMilla R., and Paget-Dellio S. D. - Effect of Glucosamine Hydrochloride in the Treatment of Pain pf Osteoarthritis of the Knee, J. Rheumatol. **26** (1999) 2423-2430.
2. Xu Q., Liu J., and Yuan Z. - Use of N-acetylglucosamine as additive in milk products: WO patent NO.2004 093556 (2004).
3. Quỳnh D. T. N., Hường Đ. T., Thương B. T., and Hà L. T. - Ảnh hưởng của các yếu tố lên men đến sinh tổng hợp chitinase và N-acetyl-D-glucosaminidase từ chủng *Penicillium oxalicum* 20B, Tạp chí khoa học công nghệ thủy sản (2013a) Số đặc biệt.
4. Kuk J. H., W. J. Jung, Jo G. H., Ahn J. S., Kim K. Y., and Park R. D. - Selective preparation of N-acetyl-β-D-glucosamine from chitin using a crude enzyme preparation from *Aeromonas sp.*, Biotechnol Lett **27** (2005a) 7–11.
5. Pichyangkura R., Kudan S., Kuttiyawang K., Sukwattanasinitt M., and Aiba S. - Quantitative Production of 2-Acetoamodo-2-D-glucose from Crystalline Chitin by Bacterial Chitinase, Carbohydr. Res. **337** (2002) 557-559.
6. Setthakaset P., Pichyangkura R., Ajavakom A., and Sukwattanasinitt M. - Preparation of N-acetyl-D-Glucosamine Using Enzyme from Aspergillus sp., Journal of Metals, Materials and Minerals **18** (2) (2008) 53-57.
7. AIBA S. - Production of N-acetyl-D-glucosamine from Chitin Using Crude Enzyme. Preparations in Large Scale, Journal of Metals, Materials and Minerals **15** (1) (2005) 23-25.
8. Bon D. V., Tra D. T. X., and Ha L. T. - Improvement of chitinase biosynthesis by Penicillium oxalicum 20B for N-acetyl-D-Glucosamine production, Journal of science and technology **53** (4B) (2015) 118-125.
9. Lee Y. G., Chung K. C., Wic S. G., Lee J. C., and Bae H. J. - Purification and properties of a chitinase from Penicillium sp. LYG 0704, Protein Expresion and Purification **65** (2009) 244-250.
10. Focher B., Beltranme P. L., Naggi A., and Torri G. - Alkaline Ndeacetylation of chitin enhanced by flash treatments: reaction kinetics and structure modifications, Carbohydr. Polym. **12** (1990) 405-418.
11. Wu T., Zivanovic S., and Draughon F. A. - Physicochemical Properties and Bioactivity of Fungal Chitin and Chitosan, J. Agric. Food Chem. **53** (2005) 3888-3894.

TÓM TẮT

CÁC YẾU TỐ ẢNH HƯỞNG HIỆU SUẤT THU HỒI VÀ ĐỘ TINH SẠCH CỦA N-ACETYL GLUCOSAMINE THU TỪ DỊCH THỦY PHÂN CHITIN

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Trong nghiên cứu này ảnh hưởng của tỉ lệ cồn/dịch thủy phân cũng như mức độ cô đặc của dịch thủy phân đến hiệu suất thu hồi và độ tinh sạch của NAG đã được khảo sát. Kết quả cho thấy tỷ lệ tối ưu của cồn/dịch thủy phân là 9. Nếu tiếp tục tăng tỉ lệ này lên 11 cũng chỉ làm tăng lên không đáng kể hiệu suất thu hồi từ 74,47 % lên 75,3 9 % và độ tinh sạch của NAG từ 93,25 % lên 93,44 %. Mức độ cô đặc dịch thủy phân cũng ảnh hưởng đến hiệu suất thu hồi và độ tinh sạch NAG. Kết tinh tạp chất bằng cách làm lạnh dịch thủy phân chỉ xảy ra đối với dịch thủy phân được cô đặc. Khi mức độ cô đặc dịch thủy tăng từ 1 đến 7,9 lần, tạp chất kết tinh tăng nhanh từ 0 đến 0,082 mg/ml; sau đó tăng chậm từ 0,082 tới 0,093 mg/ml khi mức độ cô đặc dịch thủy phân tăng từ 11 đến 15,7 lần. Mức độ cô đặc dịch càng cao dẫn tới hiệu suất thu hồi càng giảm nhưng sản phẩm NAG tinh sạch hơn. Theo phân tích HPLC, sản phẩm NAG có độ tinh sạch 93,25 % và hiệu suất thu hồi 74,47 % thu được ở tỉ lệ cồn tối ưu và mức độ cô đặc 15,7 lần.

Từ khóa: tinh sạch, N-acetyl-D-glucosamine NAG, kết tủa, kết tinh.