J. Chitin Chitosan 23(3), 152-157 (2018)
DOI http://dx.doi.org/10.17642/jcc.23.3.2

Environmental Friendly Deproteinization and Saccharification of Industrial Fungal Biomass by Enzymatic Processing

Cecilia Perez-Cruz[1], Carlos N. Cano-Gonzalez[1], Jose Fuentes[1], Nagamani Balagurusamy[2], Carolina E. Vita[3], Roque A. Hours[3], Cristobal N. Aguilar[1], Sebastian F. Cavalitto[3], and Juan C. Contreras-Esquivel[1,4]*

[1]*School of Chemistry, Universidad Autonoma de Coahuila, Saltillo City 25280, Coahuila State, Mexico*
[2] *School of Biological Science, Universidad Autonoma de Coahuila, Torreon City 27104, Coahuila State, Mexico*
[3]*Center for Research and Development of Industrial Fermentation (CINDEFI), School of Science, National University of La Plata. 47 y 115 (B1900ASH), La Plata, Buenos Aires, Argentina*
[4]*Research and Development Center, Coyotefoods Biopolymer and Biotechnology Co., Saltillo City 25000, Coahuila State, Mexico*

ABSTRACT

Aspergillus niger biomass, an industrial by-product of citric acid fermentation is an emergent source of glycoderivatives with applications in biofuel, cosmetics, feed, energy, food, medicine, and nanotechnology. In this study, the effect of purified neutral protease for deprotenization of fungal biomass studied at various levels (0, 5, 10, 20 and 40 U/100 mg of biomass) and the saccharification of fungal biomass was evaluated with amylolytic enzymes and chitosanases. The efficiency of deproteinization of fungal biomass was based on the enzyme concentration and contact time. Protease at a concentration of 20 U/100 mg of dry biomass and with a contact time of 8 h achieved 30% final deproteinization. No effect on saccharification of *A. niger* biomass was observed by treatment with purified amylolytic enzymes. Meanwhile, the endo- and exo-chitosanases treatment yielded 54 g of g reducing sugars (equivalent to amino sugars)/ kg of fungal biomass, which can be employed for tailor-made carbohydrate production.

Keywords: Fungal biomass, Chitin, Chitosan, Glucan, Amino sugars, Citric-acid, Biofuels

Introduction

The search for feasible petroleum substitutes among renewable resources has become a global priority as atmospheric carbon dioxide levels continue to rise. Industrial biorefineries have been identified as the most promising alternative for biomass based energy production (1,2). The biorefinery concept implies the use of alternative carbon sources mainly derived from agro industrial activities, such as the residues from sugarcane, wheat or corn processing as well as oilseeds. However, the search for alternative carbon sources is not limited and the abundant availability of seaweed, crustacean or fungal biomasses offers much scope since they have not been studied yet for this purpose.

Aspergillus niger biomass is a waste product of the citric acid fermentation process, and this fungal biomass has a uniform composition. The biomass of *A. niger* has been used as cattle feed and as a source of extraction of chitin and chitosan (3,4). *A. niger* biomass contain a complex mixture of polysaccharides (chitin-chitosan-glucan complex) and peptidoglycans, some which appear in layers or tiers in the hyphal architecture (4,5). It is difficult to be hydrolysed, and requires specific and severe conditions for its saccharification. It can be hydrolysed either chemically or enzymatically for the production of different monosaccharides (glucose, glucosamine and N-acetyl glucosamine), and peptides.

Corrosive chemical agents such as NaOH, H_2SO_4, and HCl are widely employed at high temperature for chemical deproteinization and hydrolysis of *A. niger* biomass and this results in environmental pollution (6). Whereas, the enzymatic method is environmental friendly and the use of proteases is particularly suitable for *A. niger* biomass deproteinization (7). Proteases have high stability and activity under harsh conditions such as high temperature, extreme pH values and in the presence of surfactants or oxidizing agents (8).

Endo- and exo-chitinases/chitosanases are known to hydrolyze chitin and chitosan contained in *A. niger* biomass into chitooligosaccharides or chitosanoligosaccharides, or their monomeric forms such as N-acetyl glucosamine and glucosamine (9-12). These products have significant economic value due to their importance in various biotechnological processes. Polymer (chitosans), or its oligomeric forms (chitin- or chitosan-oligosaccharides), are used in different technologies due to their polyelectrolyte characteristics, in addition to the presence of reactive functional groups, their gel-forming ability, high adsorption capacity, biodegradability as well as their bacteriostatic, fungistatic, and antitumour properties (13). Further, N-acetyl glucosamine and glucosa-

*To whom all correspondence should be addressed
Tel & Fax: +52-844-416-9213; E-mail: carlos.contreras@uadec.edu.mx / coyotefoods@hotmail.com

mine can be used as substrates for the production of bioethanol, one of the favoured substitutes for petroleum (14).

In this study, the physical-chemical characters of industrial *A. niger* biomass was studied and enzymatic deproteinization and saccharification was evaluated using neutral proteases, amylases, and exo-and endo-chitosanases.

Materials and Methods

Chemicals and reagents

A. niger biomass was provided by SucroAL Co. (Valle de Cauca, Colombia). The details of biomass and the enzymes used in this study are described in Table 1. The chitosanolytic enzyme mixture (exo and endo activities) from *Trichoderma viride* was prepared as previously published (15) at 2.5 U/ mL. Glucose-oxidase/peroxidase system (Gluc-Rap R1 kit lot 1764GL) was purchased from Randox Laboratories Ltd. (Crumlin, Antrim, UK). Bovine serum albumin, 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) regent, glucosamine hydrochloride ≥ 99% crystalline and D-(+)-glucose ≥ 99.5% were purchased from Sigma Aldrich Co (St. Louis, MO, USA). All other regents used were analytical grades.

Characterization of fungal biomass

Compositional analysis

Moisture, ash, protein and fat content were determined according to the AOAC official methods. To quantify the sugars presents in *A. niger* biomass, a dried sample (10 mg) was hydrolyzed with concentrate H_2SO_4 (0.3 mL) for 1 h at room temperature, then diluted with 2.7 mL of distilled water and kept at 100ºC for 3 h. The resulting solution was neutralized with $Ba(OH)_2$ solution. Then, glucosamine content was determined according the method of MBTH (16) and the glucose content was estimated by employing the glucose-oxidase method (Randox Laboratories, Ltd, Antrim, UK).

Particle size analysis and FT-IR spectroscopy

Particle size of *A. niger* biomass was analysed using different meshes (30, 40 50, 80, 100 and 120) in a Rotap equipment (RX-29, W.S. Tyler, Mentor, Ohio, USA). Analysis was carried out in triplicate and average of these values is presented. In addition, *A. niger* biomass was characterized using FTIR spectrometer (Spectrum GX, PerkinElmer, Waltham, Massachusetts, USA), equipped with a Golden-gate single reflection attenuated total reflectance (ATR) and in the spectral range of 670-4000 cm^{-1}.

Enzymatic deproteination of fungal biomass

The effect of neutral protease on the deproteinization of *A. niger* biomass was evaluated using a single factor design. Different concentrations of enzyme (0, 5, 10, 20, 40 U/ 100 mg of dry biomass) was added to the biomass (600 mg) suspended in 100 mM Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0 (30 ml) with sodium azide 0.02% (w/v). Samples were incubated in a shaker (INOVA 44, New Brunswick Scientific Co., Inc. New Jersey, USA), at 40°C with agitation (150 rpm) for 4 h. After 4 h, the insoluble material was removed by filtration and the filtrate was used for protein determination according to the method of Lowry *et al*. (17). Bovine serum albumin was used as standards for the quantification of hydrolysates in the samples. The residual insoluble biomass kept in the filter was recovered and dried in the oven at 60°C until constant weight.

Similarly, the effect of contact time on the enzymatic hydrolysis of *A. niger* biomass was evaluated using a single factor design with seven levels (0, 4, 8, 16, 24, 32 and 48 h). The protease concentration was maintained at constant level (20 U/ 100 mg dry biomass). Six hundred mg of biomass was suspended in 30 mL of 100 mM phosphate buffer pH 7 with 0.02% sodium azide and added with enzyme to initiate the reaction. The incubation conditions were as described above. On completion of the treatment time, the samples were filtered; the recovered solid was washed and brought to constant weight. The protein concentration in the filtrate was determined by the method of Lowry et al. (17).

Enzymatic saccharification of fungal biomass

Saccharification studies of fungal biomass was carried out with amylolytic and chitosanolytic enzymes. In the case of amylolytic enzymes a mixture of α-amylase (1.5 U/100 mg biomass), amyloglucosidase (1.6 U/100 mg biomass) and pullulanase (0.2 U/ 100 mg biomass) ware used. Biomass (10 mg) was suspended in 20 mM sodium acetate buffer with

Table 1. Details of *A. niger* industrial biomass and description of commercial enzymes used in this study

Material	Company	State, Country	Lot #	Source	Enzymatic activity* (U/mL)
Biomass	SucroAL Co	Valle del Cauca, Colombia	G41D06BG2D	*Aspergillus niger*	-
Neutral protease	Megazyme	Wicklow, Ireland	90701	*Bacillus licheniformis*	8.0
α-amylase (thermo stable)	Megazyme	Wicklow, Ireland	61101	*Bacillus licheniformis*	3.0
Pullulanase M2	Megazyme	Wicklow, Ireland	20101	*Bacillus licheniformis*	0.4
Amyloglucosidase	Megazyme	Wicklow, Ireland	51001	*Aspergillus niger*	3.3

*Declared volumetric activity by manufacturer.

50 mM $CaCl_2$ (pH 5.00) and the amylolytic mixture was added. The samples were incubated in a thermostatic water bath (Shel lab Co., Inc. Model 1211, Cornelius, Oregon, USA) at 40°C for 16 h. After 16 h, the supernatants were analysed for glucose by enzymatic-colorimetric method by using glucose-oxidase/peroxidase system (Gluc-Rap R1 kit, Randox Laboratories Ltd.) and glucose was employed as a standard.

In the case of treatment with chitosanolytic enzymes, a mixture of endo- and exo-chitosanases were employed for saccharification of fungal biomass. A two factor design was employed, where enzyme at two concentrations (0 and 0.625 U/100 mg dry biomass) was evaluated at 7 different contact time (0, 0.5, 1, 2, 4, 8 and 16 h). The biomass (600 mg) was suspended in 30 ml of 50 mM sodium acetate buffer pH 5 and added with the mixture of chitosanases and incubated in a shaker (New Brunswick Scientific Co., Inc. INOVA 44, New Jersey, USA) at 40°C for 0 to 16 h at 150 rpm. After the incubation period, the mixture was filtered and the filtrate was used for reducing sugars determination by using the protocol described by Somogyi-Nelson (18,19). A standard curve was prepared by using glucosamine was used to calculate the equivalent sugar content in enzymatically hydrolysed samples. The residual biomass was recovered and dried in the oven at 60°C for 12 h and then weighed.

Statistical analysis

All the treatments were performed in three replicates and the analysis of variance (ANOVA) of the data obtained was determined by using SAS system (SAS Institute Inc., Cary, NC, USA), followed by Tukey's multiple range test for mean comparison.

Results and Discussion

Physico-chemical characterization of *A. niger* biomass

The composition of *A. niger* biomass is presented in Table 2. Ash, lipid and water were present at small concentrations of 1.24±0.02, 1.93±0.06 and 4.9±0.12%, respectively. The maximum percentage of the fungal biomass is comprised of carbohydrate (80% of dry weight) and is similar to the results of Bardalaye and Nordin (20) and Vries and Visser (21). The protein content of the biomass of *A. niger* was 13.00± 0.05% on dry basis. The proteins are generally found in complex with polysaccharides such as chitin, chitosan and glucan. It is therefore important to remove the protein for the extraction of the poly-, oligo-or monosaccharides. Glucose and glucosamine are the main components of cell wall (60.18± 9.30 and 20.92±2.57% respectively). These values are consistent with the previous report of Camarillo-Contreras *et al.* (22).

The particle size analysis of the fungal biomass showed the distribution of particles in meshes 100 and 120 (29.9±6.1), which suggested that the biomass is a very fine material (Fig. 1A). This indicated the availability of greater contact surface for the enzymatic hydrolysis. IR spectrum analysis of *Aspergillus niger* biomass (Fig. 1B) showed the presence of characteristic bands from chitosan, at 1540 cm^{-1}, which indicated the presence of amino groups in the mode of deformation. The peaks near 1650 and 1325 cm^{-1} are characteristic bands of the primary acetamides (C=O) and tertiary amides (CN).

Table 2. Composition of *A. niger* biomass (dry basis)

Component	Content (%)
Proteins	13.09 ± 0.05
Lipids	1.93 ± 0.06
Ash	1.24 ± 0.02
Carbohydrates	
Glucose	62.76 ± 1.96
Aminosugars	22.11 ± 2.57

Effect of neutral protease on deproteinization of fungal biomass

The effect of different enzyme concentrations on deproteinization is presented in Fig. 2. It was observed that the rate of deproteinization gradually increased until the addition of 20 U enzyme/ 100 mg dry mycelium, beyond which there was very little change in the deproteinization rate. A maximum of 30.5±2.6% deproteinization of *A. niger* mycelial biomass was observed with 20 U enzyme/ 100 mg of dry biomass and is statistically different than other concentrations tested. Consequent to the deproteinization, a decrease in the fungal material was also observed (Fig. 2). This behaviour is inversely proportional to deproteinization rate, and there was about 13.28±2.28% decrease in fungal biomass. On contrary to enzymatic deproteinization, Synowiecki and Al-Khateeb (23) obtained a maximum protein extraction of 60.1% from dry mycelium using 2% (w/v) NaOH (1:30 w/v, 90°C, 2 h). Although the deproteinization rate by chemical method was greater than enzymatic method, the wastes and by-products of this method cause serious environmental pollution. While enzymatic deproteinization is more environmental friendly, the efficiency has to be increased. Cai (7) obtained 59.9% of deproteinization by employing a pretreatment to break the cell wall of *A. niger* biomass.

The effect of different contact times on deproteinization with the 20 U of enzyme/ 100 mg of dry biomass is presented in Fig. 3. It was observed that a contact time of 8 h showed statistically significant results than other periods tested. Results also showed that the highest percentage of weight loss occurred between 8 and 16 hours, which was 17±1.78% loss with respect to the initial biomass.

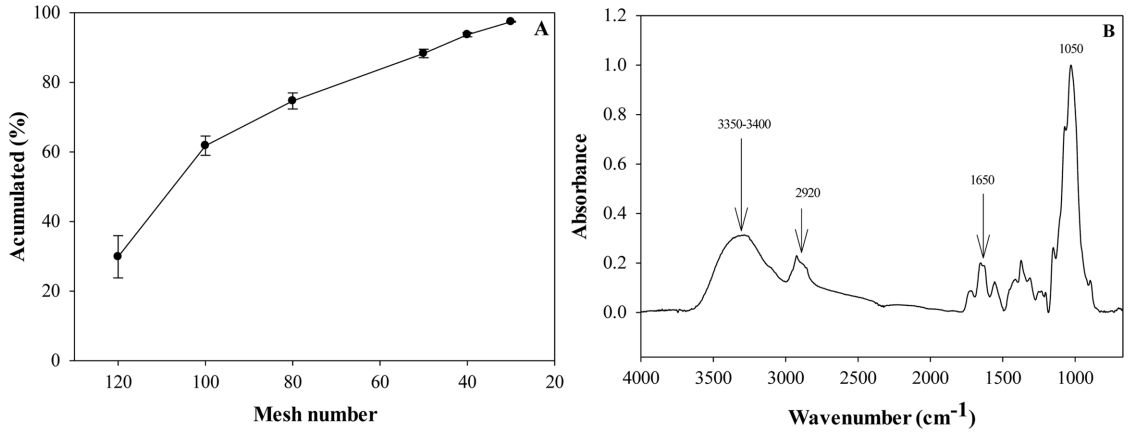

Fig. 1. Particle size analysis curve (A) and infrared spectrum (B) of *Aspergillus niger* biomass.

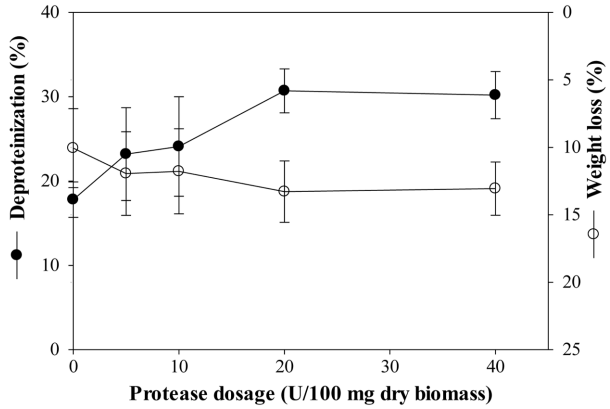

Fig. 2. Effect of neutral protease concentration on deproteinization and weight loss of *A. niger* biomass.

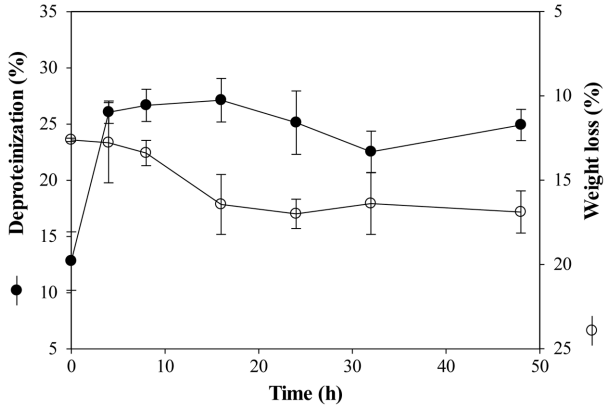

Fig. 3. Effect of contact time of protease on deproteinization and weight loss of *A. niger* biomass.

Enzymatic saccharification of fungal biomass

Amylolytic enzyme treatment resulted only in 0.02% of glucose release, which indicated low amount of glycogen and starch in the biomass. In our study, it can be concluded that use of purified amylolytic enzymes for hydrolysis of fungal industrial biomass from *A. niger* is not a viable option. Nwe and Stevens (24), employed α-amylase to hydrolyze the fungal cell wall from *Gongronella butleri* USDB 0201.

Aspergillus niger biomass saccharification was also carried out with a mix of endo- and exo-chitosanases under conditions described above. Results are presented in Fig. 4A. It was observed that enzyme concentration and contact time had an effect on the release of glucosamine from fungal biomass. The enzymatic hydrolysis is specific. It was found out that in the absence of enzyme, the glucosamine concentration remained constant (about 1%). But the addition of endo- and exo-chitosanases mixture at 0.625 U/ 100 mg dry mycelium yielded 5% glucosamine after 16 h of treatment. Previous study on the enzymatic hydrolysis of chitin obtained 4.1±0.2 g/l of N-acetyl-glucosamine from 50 g/l of chitin powder after 18 days (20). The time of reaction is very high when compared with the results of this work, although *A. niger* biomass matrix is very complex and difficult to break. The degradation behaviour of the biomass as a function of hydrolysis time and chitosanase activity is presented in Fig. 4B and can be observed that it is inversely proportional to the production of glucosamine. This could be attributed to the fibrous nature of *A. niger* biomass, which is difficult to degrade and thereby resulting in less weight loss. It is recommended to increase the contact time of the enzyme in order to increase the yield of glucosamine which can be used as a substrate for ethanol fermentation.

Conclusion

The exploitation of fungal biomass, fermentation industry waste is a promising alternative for biorefineries because of the availability of high quantity, quality biomass with uniform

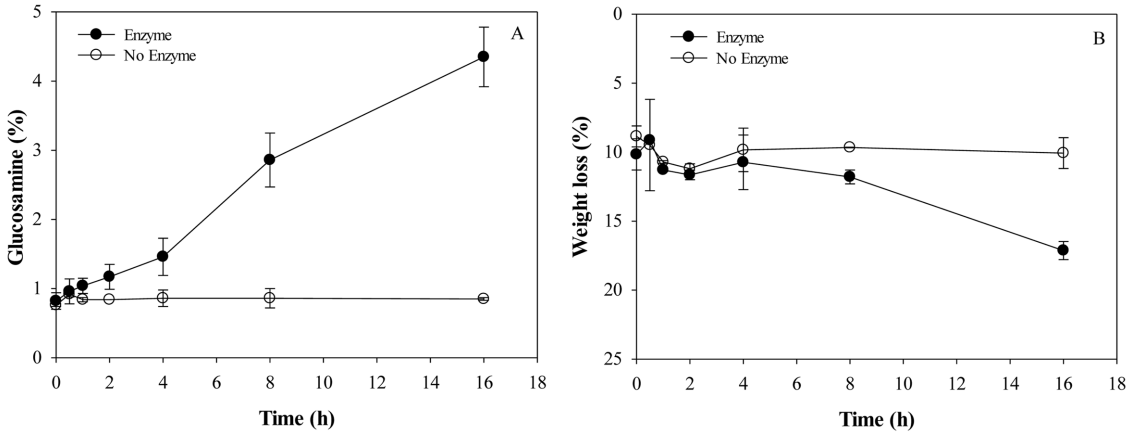

Fig. 4. Glucosamine yield (A) and weight loss (B) of *A. niger* biomass after endo- and exo-chitosanase treatment. Equivalent glucosamine related to reducing sugars evaluated by Somogyi-Nelson method (18,19).

composition. In order to design environmentally friendly bioprocess, proteases and chitosanases have been proposed for the enzymatic hydrolysis of *A. niger* biomass, instead of the common chemical treatment. Results of this study demonstrated that enzymatic hydrolysis is effective for deproteinization and saccharification of *A. niger* biomass to produce amino sugars as a carbon source for further fermentation to ethanol. In this study, enzyme deproteinization (20 U/100 mg of dry weight *A. niger* biomass) achieved a maximum percentage of 30.5±2.6% using a neutral protease after 8 h of treatment. Saccharification by endo- and exo-chitosanases resulted in the reducing sugars (equivalent to amino sugars) yield of 54 g/kg dry weight biomass. However it is recommended to increase the concentration of endo- and exo-chitosanase and reaction time to increase the yield of glucosamine from *A. niger* biomass.

Acknowledgements

The authors thank National Council for Science and Technology (CONACYT-Mexico) for supporting this research through the Graduate Scholarship to C. Perez-Cruz and C.N. Cano-Gonzalez.

References

1. Goshadrou, A., Karimi, K., and Teherzadeh, M. J.: Ethanol and biogas production from birch by NMMO pretreatment. *Biomass Bioenergy*, **2013**, *49*, 95-101.
2. Aburto, J., Martínez T., and Murrieta, F.: Technical and economical evaluation of bioethanol production from lignocelulosic residues. *Rev. Inst. Mexicana Ing. Quim.* **2008**, *23*, 23-30.
3. Spanelová, M., Machovic, V., and Brezina, M.: Characterization and sorption properties of *Aspergillus niger* waste biomass. *Cent. European J. Chem.* **2003**, *1*, 192-200.
4. Balanta, D., Grande, C. D., and Zuluaga, F.: Extraccion, identificacion y caracterizacion de quitosano del micelio de *Aspergillus niger* y sus aplicaciones como material bioadsorbente en el tratamiento de aguas. *Rev. Iberoamericana Polim.* **2010**, *11*, 297-316.
5. Ruiz-Herrera, J.: Cell wall composition. In: Fungal cell wall structure, synthesis, and assembly. Second ed. Boca Raton Florida: CRC Press, **2012**, pp. 7-28.
6. Valdez-Peña, A., Espinoza-Perez, J., Sandoval-Fabian, G. C., Balagurusamy, N., Hernandez-Rivera A., De-la-Garza-Rodriguez, I. M., and Contreras-Esquivel, J. C.: Screening of industrial enzymes for deproteinization of shrimp head for chitin recovery. *Food Sci. Biotechnol.* **2010**, *19*, 553-557.
7. Cai, J., Yang, J., Du, Y., Fan, L., Qiu, Y., Li, J., and Kennedy, J. F.: Enzymatic preparation of chitosan from the waste *Aspergillus niger* mycelium of citric acid production plant. *Carbohydr. Polym.* **2006**, *64*, 151-157.
8. Abidi, F., Limam, F., and Nejib, M. M.: Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. *Process Biochem.* **2008**, *43*, 1202-1208.
9. Hung T. H., Chang Y. M., Sung, H. Y., and Chang, C. T.: Purification and characterization of hydrolase with chitinase and chitosanase activity from commercial stem bromelain. *J. Agric. Food Chem.* **2002**, *50*, 4666-4673.
10. Cruz-Camarillo, R., Sanchez-Perez, O., Rojas-Avelizapa, N. G., Gomez-Ramirez, M., and Rojas-Avelizapa, L. I.: Chitosanase activity in *Bacillus thuringiensis*. *Folia Microbiol.* **2004**, *49*, 94-96.
11. Mourya, V. K., Inamdar, N. N., and Choudhari, Y. M.: Chitooligosaccharides: Synthesis, characterization and applications. *Polym. Sci. Ser. A*, **2011**, *53*, 583-612.
12. Yan, X. and Evenocheck, H. M.: Chitosan analysis using acid hydrolysis and HPLC/UV. *Carbohydr. Polym.* **2012**, *87*, 1774-

1778.
13. Shahidi, F., Arachchi, J. K. V., and Jeon Y. J: Food applications of chitin and chitosans. *Trends Food Sci. Technol*. **1999**, *10*, 37-51.
14. Inokuma, K., Takano, M., and Hoshino, K.: Direct ethanol production from N-acetylglucosamine and chitin substrates by *Mucor* species. *Biochem. Eng. J*. **2013**, *72*, 24-32.
15. Flores-Lopez, M. L., Espinoza-Perez, J. D., Charles-Rodriguez, A. V., Montanez, J. C., Aguilar, C. N., Rodriguez-Herrera, R., Renovato-Nuñez, J., and Contreras-Esquivel, J. C.: Huitlacoche (*Ustilago maydis*) as a new source of chitin and chitosan. In: Advances in Chitin Science and Technology, Kim, S. K., No, H. K., and Park, R. D. (Eds.). Hanrimwon (ed.), Korea, pp. 12-14.
16. Tsuji, A., Kinoshita, T., and Hoshino, M.: Analytical chemical studies on amino sugars. II. Determination of hexosamines using 3-methyl-2- benzothiazolone hydrazone hydrochloride. *Chem. Pharm. Bull*. **1969**, *17*, 1505-1510.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265-275.
18. Somogyi, M.: Notes on sugar determination. *J. Biol. Chem*. **1952**, *195*, 19-23.
19. Nelson, N.: A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem*. **1944**, *153*, 375-380.
20. Bardalaye, P. and Nordin, J. H.: Galactosaminogalactan of from cell walls of *Aspergillus niger*. *J. Bacteriol*. **1976**, *125*, 655-669.
21. Vries de, R. P. and Visser, J.: *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol. Mol. Biol. Rev*., **2001**, *65*, 497-522.
22. Camarillo-Contreras, K., Balvantin-Garcia, C., Barajas, L., Balagurusamy, N., and Cavalitto, S. F., Contreras-Esquivel, J. C.: Microwave assisted extraction of fungal biopolymer from *Aspergillus kawachii* biomass. *Asian Chitin J*. **2009**, *5*, 15-18.
23. Synowiecki, J. and Al-Khateeb, N. A. A. Q.: Mycelia of *Mucor rouxii* as a source of chitin and chitosan. *Food Chem*. **1997**, *60*, 605-610.
24. Nwe, N. and Stevens, W.: Chitosan isolation from the chitosan-glucan complex of fungal cell wall using amylolytic enzymes. *Biotechnol. Lett*. **2002**, *24*, 1461-1464.

Received	December	31	2017
Revised	July	10	2018
Accepted	July	24	2018