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Determination of Glucosamine in Fungal Cell Walls by High-Performance Liquid Chromatography (HPLC)

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ABSTRACT: Glucosamine (GlcN) is a major and valuable component in the cell wall of zygomycetes fungi. In this study, a time independent and accurate method was developed for the determination of GlcN. In this method, the cell wall was treated via a two-stage sulfuric acid process, and chitin and chitosan were fully deacetylated, partially depolymerized, and converted to GlcN oligosaccharides. Then, the oligosaccharides were deaminated to 2,5-anhydromannose using nitrous acid. Finally, 2,5-anhydromannose was analyzed by high performance liquid chromatography (HPLC). The determinations of pure GlcN solutions were stable at least for 10 days, while those of the conventional colorimetric method were not stable for more than one hour. The alkali insoluble material (AIM) of biomass of purely yeast-like, mostly yeast-like, and filamentous forms of the fungus *Mucor indicus* was analyzed by the developed method. The respective GlcN content of AIM of the fungus was 0.232, 0.204, and 0.458 (g/g).

KEYWORDS: *Mucor indicus, fungal cell wall, chitin, chitosan, glucosamine (GlcN), N-acetyl glucosamine (GlcNAc), morphology, HPLC*

INTRODUCTION

Chitin and chitosan are natural copolymers containing randomly distributed glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) monomers. Chitosan is distinguished from chitin by its solubility in dilute organic acid solutions.[1,2] Despite the extensive availability of chitin in nature, due to extremely poor solubility in aqueous solutions, this biopolymer has received restricted applications. In contrast, chitosan is readily soluble in acidic aqueous solutions and can be used in a variety of applications, e.g., in medicine, food industry, chemical industry, and agronomy.[3,4] Nowadays, chitosan is often commercially produced by chemical deacetylation of chitin present in shellfish wastes.[5] However, cell wall materials of zygomycetes fungi are alternative sources for the production of chitosan. In this family of fungi, chitosan is produced enzymatically from chitin, stored in the cell wall, and can be separated by acid extraction.[6]

The chitosan content in the cell wall of zygomycetes fungi strongly depends on the strain as well as the cultivation conditions. An easy, accurate, and reproducible technique is therefore, necessary to compare the chitosan production ability of different strains prepared under various conditions. Recently, a new method was developed for the determination of chitin and chitosan contents of fungal cell wall by measurement of GlcN and GlcNAc contents.[7] After alkali extraction of mycelium, to prepare alkali-insoluble material (AIM), a two-step hydrolysis with concentrated sulfuric acid at room temperature followed by hydrolysis with dilute acid at 120 °C, results in complete deacetylation and partial depolymerization of chitin and chitosan and formation of acetic acid and GlcN oligosaccharides. A further treatment with nitrous acid ends up with complete deamination and depolymerization of the oligosaccharides to 2,5-anhydromannose. After the hydrolysis steps, the concentration of 2,5-anhydromannose (as a representative for total GlcN and GlcNAc residues) and acetic acid (as a representative for GlcNAc residues) are measured by a colorimetric method[8] and high performance liquid chromatography (HPLC),[7] respectively. In the colorimetric method, 2,5-anhydromannose is converted to a blue complex by a reaction with 3-methyl-2-benzothiozolone-hydrazone-hydrochloride and ferric chloride. This complex has a maximum absorption at 650 nm.[8]

In preliminary experiments of the current study, the time dependent stability of the blue complex was investigated, and the results showed that the absorbance of the complex prepared from a 0.1 g/L solution of pure glucosamine was declined by 1.5- and 7.8-fold after 30 and 150 min, respectively. Therefore, the accuracy of the results was significantly dependent on the duration of the analysis. Plassard et al.[8] reported that sulfuric acid solutions with extremely low pH have negative effects on the blue complex formation. Therefore, they suggested a pH adjustment step prior to the formation of the complex. However, the pH adjustment, especially for a high number of samples, is a time-consuming process and can reduce the accuracy of the measurement. This article describes a time independent method for the analysis of GlcN.

High performance liquid chromatography was evaluated for the measurement of GlcN. Furthermore, as an example of application, GlcN was measured in cell wall preparations from three different morphologies of *M. indicus*, using this new method.

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MATERIALS AND METHODS

Microorganism, Cultivation, and Preparation of Alkali Insoluble Material (AIM). *M. indicus* CCUG 22424 was obtained from Culture Collection University of Gothenburg (SE 413 46 Gothenburg, Sweden). The fungus was cultivated at 32 °C for 6 days on agar slants containing 40 g/L glucose, 20 g/L agar, and 10 g/L soy peptone. Afterward, the fungus was cultivated in liquid cultures either aerobically or anaerobically. All cultivations were performed in 100 mL working volumes in 250 mL Erlenmeyer flasks in a shaker incubator at 31 °C and 120 rpm for 48 h. The medium contained 50 g/L glucose, 3.5 g/L KH_2PO_4, 7.5 g/L $(NH_4)_2SO_4$, 0.75 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L $CaCl_2{\cdot}2H_2O$, and 5 g/L yeast extract. The cultivation media were autoclaved for 20 min at 121 °C, and after cooling to room temperature, the pH was adjusted to 5.5 ± 0.2 before being inoculated with the fungal spores. Morphology of the growing fungal cells was manipulated by adjusting the number of spores in the inoculum.[9,10] Under anaerobic conditions, inoculation with a high number of spores ($6 \pm 3 \times 10^6$ spores/mL) resulted in purely yeast-like cells, while mostly yeast-like cells were obtained with the similar number of spores under aerobic conditions. At lower spore numbers ($3 \pm 1 \times 10^4$ spores/mL) and aerobic conditions, the growth developed into a filamentous form (Figure 1).

The anaerobic cultures were purged with pure nitrogen at the beginning of cultivations. After 48 h of cultivation, the grown biomass was harvested by centrifugation at 4000*g* for 10 min, washed three times with water, and dried at 45 °C. Afterward, in order to prepare the cell wall materials, 30 mL of 0.5 M sodium hydroxide was added to each g dried mycelium, and the mixture was autoclaved at 121 °C for 20 min. The alkali insoluble material (AIM) of biomass was then separated by centrifugation (10 min, 4000*g*), washed 10 times with distilled water to obtain neutral pH, dried at 45 °C, weighed, and stored at room temperature.[7]

Production of 2,5-Anhydromannose from AIM and Pure GlcN. To prepare 2,5-anhydromannose, 10 mg of AIM was placed in a 10 mL screw cap tube, and 0.3 mL of sulfuric acid (72% v/v) was added. The suspension was mixed every 15 min for 90 min at room temperature. Then, 8.4 mL of water was added and autoclaved at 121 °C for 1 h. Afterward, a 0.5 mL sample was taken as soon as the temperature went down to about 100 °C. After cooling to room temperature, 0.5 mL of 1 M $NaNO_2$ was added to the sample, and the cap was closed tightly, mixed, and left for 6 h at room temperature. It was then opened and left overnight under a laminar air flow hood. This step converted chitin and chitosan contents of AIM to 2,5-anhydromannose. Finally, 0.5 mL of ammonium sulfamate (12 wt %) was added to neutralize the excess nitrous acid, and after 4 min mixing, the sample was centrifuged (10 min, 4000*g*).[7] Solutions of pure GlcN (Sigma-Aldrich) with concentrations of 0, 0.125, 0.25, 0.5, and 1 g/L in 2.48% (v/v) sulfuric acid were also prepared and subjected to the process described above after the hot sampling step to the end.

Determination of 2,5-Anhydromannose. The concentration of 2,5-anhydromannose was measured by HPLC with an ion exchange Aminex column (HPX-87H, Bio-Rad, Richmond, CA) at 60 °C with 0.6 mL/min eluent of 5 mM sulfuric acid with an RI detector (Jasco International Co., Tokyo, Japan).

To measure the GlcNAc, the concentration of acetic acid in cold sulfuric acid hydrolysates was measured concomitantly by HPLC. Moles of acetic acid in each sample were considered to be equal to moles of GlcNAc, and the concentration of GlcN in samples was calculated.[7]

Reproducibility of the measurements was investigated by keeping the samples obtained from pure GlcN solutions in a refrigerator and repeating the analysis at different times (i.e., 1, 3, 7, and 10 days). Calibration curves were calculated for each measurement and compared. Finally, the concentration of 2,5-anhydromannose was measured in samples prepared from AIM. All experiments were performed at least in duplicate, and the results are presented as averages.

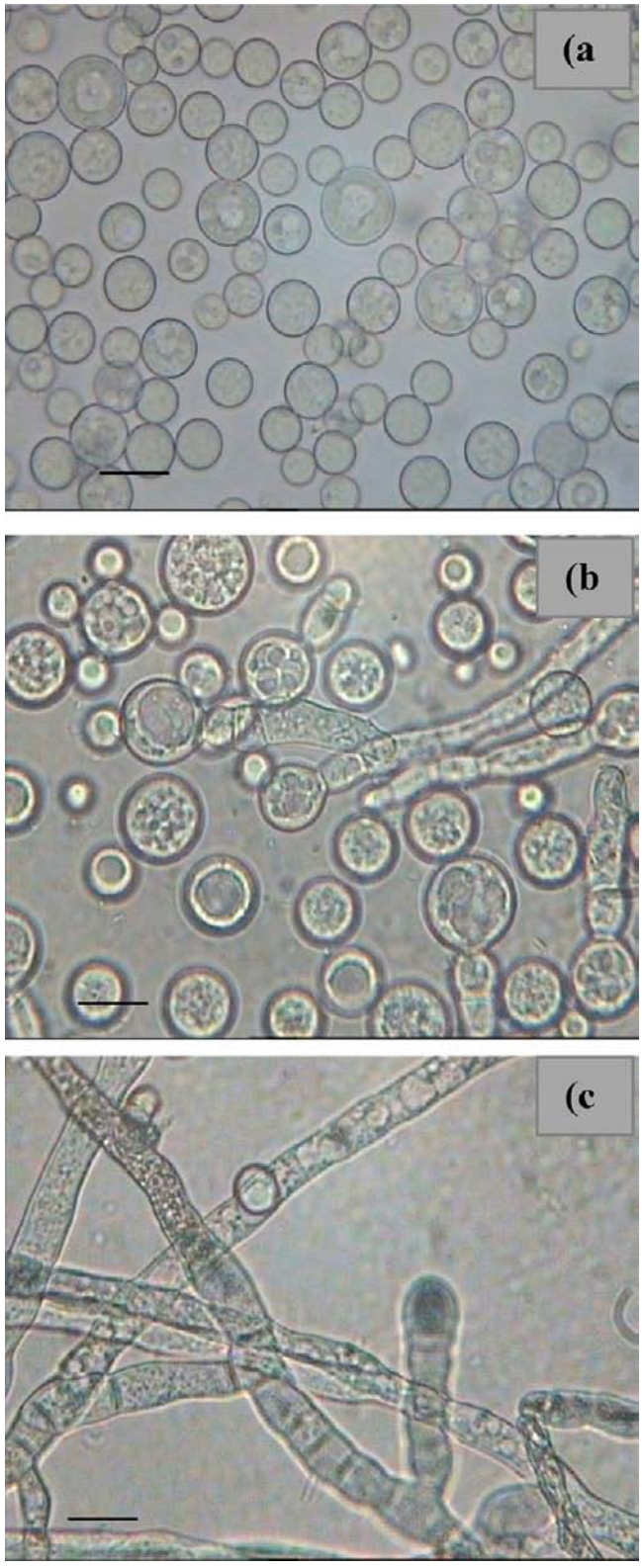

Figure 1. Microscopic observation of different morphologies of *M. indicus*, i.e., yeast-like (a), mostly yeast-like (b), and filamentous (c), after 48 h of cultivation. The bars correspond to 16 μm.

RESULTS

Determination of 2,5-Anhydromannose Concentration. Preliminary experiments proved the instability of the blue complex prepared from 2,5-anhydromannose under the

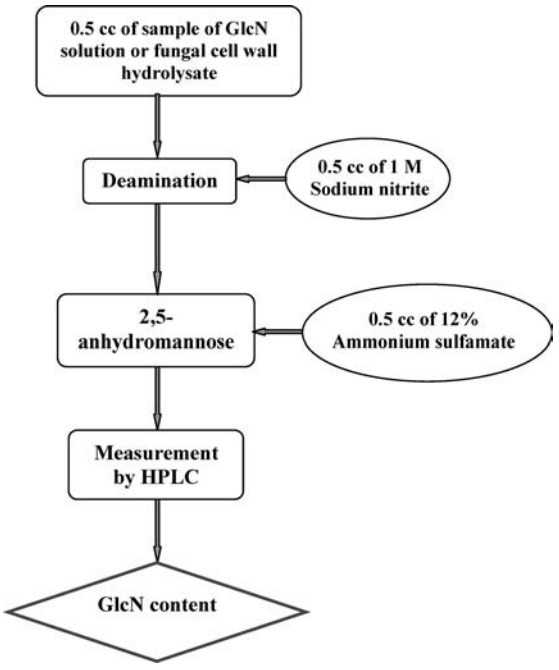

Figure 2. Procedure of treatment of GlcN solutions or cell wall hydrolysate and analysis with HPLC.

conditions used in the previous method.[7] In order to eliminate the negative effects of the very low pH of sulfuric acid, the solution of GlcN in sulfuric acid was first diluted 100 times after hot sampling and prior to the addition of $NaNO_2$. However, in this investigation, the blue complex was not formed. Thus, in the next step, the samples were diluted 2−10 times after the addition of MBTH, and absorbance of the blue complex was scanned over time. The results indicated that even at high dilutions levels, the colored complex was not stable, and hence, the method was highly time dependent (data not shown). Since all trials confirmed the unstable nature of the blue complex, in the next step, the goal was to investigate the stability of 2,5-anhydromannose, before conversion to the colored complex, at low pH. To reach the goal, performance of HPLC with an anionic ion exchange column for the detection of 2,5-anhydromannose was first evaluated. As shown in Figure 3, no peak appeared after 9 min in the chromatogram of sulfuric acid solution. In contrast, the chromatogram of a 0.0125 g/L GlcN solution in sulfuric acid, after conversion to 2,5-anhydromannose, showed a peak at about 9.5 min (Figure 3). In the next step, pure GlcN solutions of 0, 0.125, 0.25, 0.5, and 1 g/L in sulfuric acid were subjected to the reaction with nitrous acid and ammonium sulfamate and analyzed with HPLC. Results showed a linear relationship between the area of the peak related to 2,5-anhydromannose and the concentration of GlcN (Figures 4 and 5). Accordingly, HPLC was chosen as

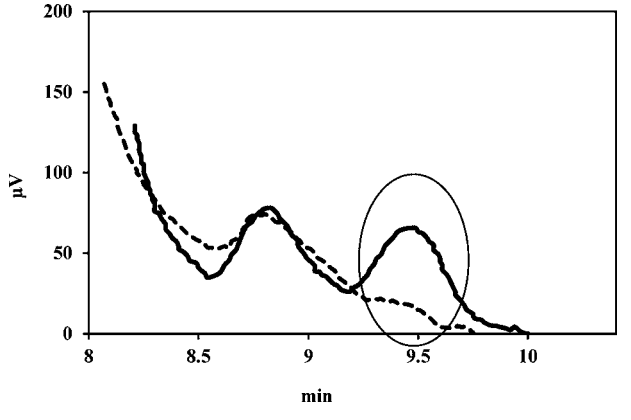

Figure 3. HPLC chromatograms: blank solution (sulfuric acid solution, with no dissolved GlcN) (---) and solution of 0.125 g/L pure GlcN (−).

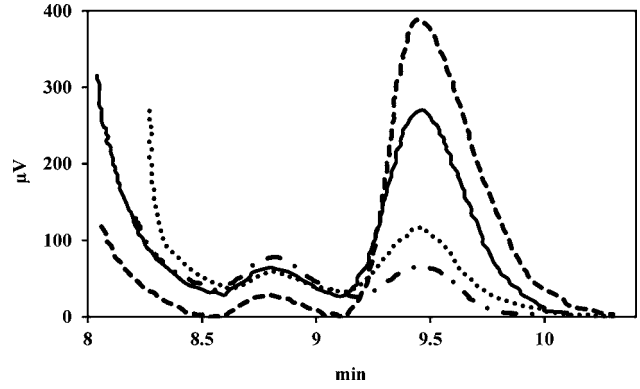

Figure 4. HPLC chromatograms of GlcN solutions with different concentrations (g/L): 0.125 (··-), 0.25 (···), 0.5 (—), and 1 (--).

the analysis method for the determination of 2,5-anhydromannose. To investigate the reproducibility of the results over time, the HPLC analyses were repeated after 3, 7, and 10 days, and the results proved the stability of 2,5-anhydromannose at low pH and the reproducibility of the HPLC analysis (Figure 5 and Table 1). Consequently, analysis with HPLC was suggested as a promising method for reproducible measurement of GlcN with high accuracy, and the method of GlcN determination in fungal cell walls was performed according to Figure 2. In the modified method, the pure GlcN solutions in sulfuric acid or the AIM hydrolyzates (prepared by two-step sulfuric acid hydrolyses) were reacted with nitrous acid to form 2,5-anhydromannose, the excess nitrous acid was neutralized with ammonium sulfamate, and finally 2,5-anhydromannose was analyzed by HPLC.

Analysis of the Cell Wall of Different Morphologies of *M. indicus*. The new method was used for the characterization of the cell wall of different morphologies of *M. indicus*. According to Table 2, the biomass yields of 0.097(±0.008), 0.106(±0.001), and 0.100(±0.001) g/g sugar were obtained after 48 h cultivation for purely yeast-like, mostly yeast-like, and filamentous cells, respectively. The respective AIM yields (as a representative for the cell wall) were 0.165(±0.005), 0.167 ± (0.010), and 0.173(±0.005) g/g biomass. Among the three studied morphologies, the filamentous form had the highest GlcN concentration (0.46 g/g AIM). Changing the morphology from filamentous to purely yeast-like and mostly yeast-like was accompanied by significant reductions of GlcN concentration by 49 and 55%, respectively. Besides the GlcN concentration, that of GlcNAc was also measured (Table 2). Again, the highest GlcNAc content belonged to the filamentous morphology (0.18 g/g). Concentrations of GlcNAc in purely and mostly yeast-like forms were somewhat lower (0.10 and 0.13 g/g AIM, respectively). The sum of the GlcN and GlcNAc contents, as a representative of total chitin−chitosan content of the cell wall, was 0.64, 0.34, and 0.33 g/g AIM, respectively, for filamentous, purely yeast-like, and mostly yeast-like cells.

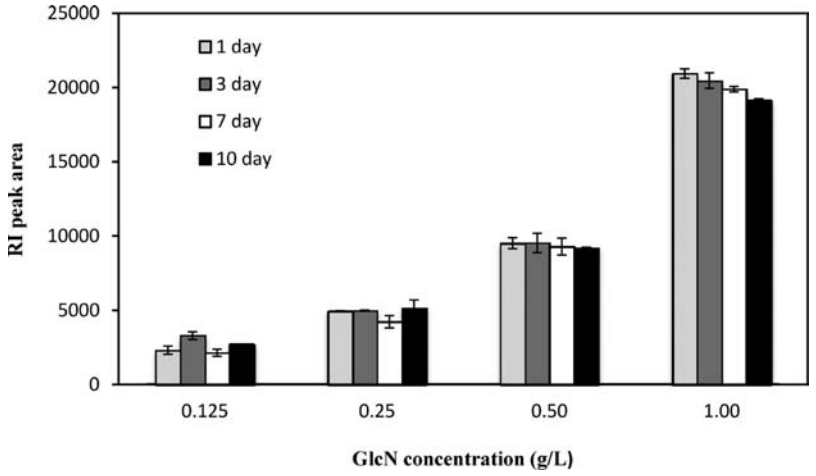

Figure 5. Linear correlations between the area under the peak related to 2,5- anhydromannose in the HPLC chromatogram and the concentration of GlcN at various analyzing times.

Table 1. Slopes and R^2 Values of Calibration Curves of GlcN Solutions Analyzed by HPLC during 1−10 Days

Time of analysis	1 day	3 days	7 days	10 days
Slope	0.000016	0.000016	0.000017	0.000017
Coefficient of determination (R^2)	0.995574	0.994737	0.994995	0.997791

Table 2. Biomass, AIM, GlcN, and GlcNAc Yields in Cultivation of *M. indicus* with Different Morphologies

Morphology	Fermentation conditions	Biomass yield (g/g sugar)	AIM yield (g/g biomass)	GlcN content (g/g AIM)	GlcNAc content (g/g AIM)
Yeast-like form	anaerobic	0.097	0.165	0.232	0.103
Mostly yeast-like form	aerobic	0.106	0.167	0.204	0.126
Filamentous form	aerobic	0.100	0.173	0.458	0.181

DISCUSSION

A simple and accurate method was presented for analysis of GlcN and GlcNAc in chitin−chitosan containing materials such as fungal cell walls. The method combines the hydrolysis procedure of a recently presented method[7] with HPLC analysis. Another advantage is the stability of the readings for at least 10 days.

Use of HPLC has been previously reported for the measurement of the amount of GlcN in hydrolysates obtained from chitin−chitosan containing materials.[11] In nearly all of the studies, HCl has been used for hydrolysis of chitin and chitosan, while a part of the sample stayed in the solid form without hydrolysis and depolymerization.[12−14] Zamani et al.[7] reported that complete hydrolysis of these biopolymers cannot be achieved unless the hydrolysis is performed under harsh conditions at prolonged times. Consequently, side reactions may occur during the hydrolysis and therefore, complete recovery of GlcN is rarely possible. This study took advantage of the high performance of using sulfuric acid and nitrous acid in the hydrolysis of chitin and chitosan to 2,5-anhydromannose and acetic acid as well as time independent analysis measurement of these materials by HPLC. Therefore, the current work represents a more accurate method for the analysis of chitin and chitosan with milder sample preparation conditions and time independent measurements compared to the previous methods.

The dimorphism of *M. indicus* has been reported by several studies.[9,10] Sharifia et al.[9] and Lennartsson et al.[10] manipulated the morphology of this fungus by changing the spore concentration and got three different growth forms of purely yeast-like, mostly yeast-like, and filamentous. Nowadays, *M. indicus* has been introduced as a promising ethanol producer microorganism.[9] All of the three forms of *M. indicus* are able to ferment different sugars to ethanol; however, generally the highest ethanol yield is obtained by yeast like cells. In this work, the effect of morphology on chitosan production by the fungus was investigated. The results showed a higher tendency of the fungus to produce chitin and chitosan in filamentous form in comparison to the other morphologies. However, the yield of AIM was not significantly affected by the morphology. Therefore, when the morphology is yeast-like or mostly yeast-like, probably some other component is present in the cell wall together with chitin and chitosan. Further investigations are necessary in order to evaluate this hypothesis.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Kurita, K. Chemistry and application of chitin and chitosan. *Polym. Degrad. Stab.* **1998**, *59* (1−3), 117−120.

(2) Rinaudo, M. Chitin and chitosan: Properties and applicacion. *Prog. Polym. Sci.* **2006**, *31* (7), 603−632.

(3) Tharanathan, R. N.; Kittur, F. S. Chitin- the undisputed biomolecule of great potential. *Crit. Rev. Food Sci.* **2003**, *43* (1), 61−87.

(4) Zamani, A.; Taherzadeh, M. J. Production of low molecular chitosan by hot dilute sulfuric acid. *BioResources* **2010**, *5* (3), 1554−1564.

(5) Zamani, A.; Edebo, L.; Sjöström, B.; Taherzadeh, M. J. Extraction and precipitation of chitosan from cell wall of zygomycetes fungi by dilute sulfuric acid. *Biomacromolecules* **2007**, *8* (12), 3786−3790.
(6) Zamani, A.; Edebo, L.; Niklasson, C.; Taherzadeh, M. J. Temperature shifts for extraction and purification of zygomycetes chitosan with dilute sulfuric acid. *Int. J. Mol. Sci.* **2010**, *11* (8), 2976−2987.
(7) Zamani, A.; Jeihanipour, A.; Edebo, L.; Niklasson, C.; Taherzadeh, M. J. Determination of glucosamine and N-acetyl glucosamine in fungal cell walls. *J. Agric. Food Chem.* **2008**, *56* (18), 8314−8318.
(8) Plassard, C. S.; Mousain, D. G.; Salsac, L. E. Estimation of mycelial growth of basidiomycetes by means of chitin determination. *Phytochemistry* **1982**, *21* (2), 345−348.
(9) Sharifia, M.; Karimi, k.; Taherzadeh, M. J. Production of ethanol by filamentous and yeast-like forms of *Mucor indicus* from fructose, glucose, sucrose, and molasses. *J. Ind. Microbiol. Biotechnol.* **2008**, *35* (11), 1253−1259.
(10) Lennartsson, P.; Karimi, K.; Edebo, L.; Taherzadeh, M. J. Effects of different growth forms of *Mucor indicus* on cultivation on dilute-acid lignocellulosic hydrolyzate, inhibitor tolerance, and cell wall composition. *J. Biotechnol.* **2009**, *143*, 255−261.
(11) Yan, X; Evenocheck, H. M. Chitosan analysis using acid hydrolysis and HPLC/UV. *Carbohydr. Polym.* **2012**, *87*, 1774−1778.
(12) Chen, W.; Chiou, R. Y. Y. A modified chemical procedure for rapid determination of glucosamine and its application for estimation of mold growth in peanut kernels and koji. *J. Agric. Food Chem.* **1999**, *47* (5), 1999−2004.
(13) Zhu, X. L.; Cai, J. B.; Yang, J.; Su, Q. D. Determination of glucosamine in impure chitin samples by high-performance liquid chromatography. *Carbohydr. Res.* **2005**, *340* (10), 1732−1738.
(14) Wu, T.; Zivanovic, S.; Draughon, F. A.; Sams, C. E. Chitin and chitosan-value-added products from mushroom waste. *J. Agric. Food Chem.* **2004**, *52* (26), 7905−7910.