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Laboratory scale production of ^{13}C labeled chitosan by fungi *Absidia coerulea* and *Gongronella butleri* grown in solid substrate and submerged fermentation

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ABSTRACT

Nowadays, chitin and chitosan are applied in many medical and pharmaceutical products. However little is known about the metabolism of chitin and chitosan *in vivo*. In the human body, lysozyme will degrade chitin and chitosan into chito-oligosaccharides. ^{13}C labeled chitosan is an essential prerequisite for the further study of the fate of chito-oligosaccharides *in vivo*. To fulfill this requirement, chitosans were extracted from mycelia of fungi, *Absidia coerulea* ATCC 14076 and *Gongronella butleri* USDB 0201 and ATCC 42618 grown in solid substrate fermentation (SSF) and submerged fermentation (SMF) to select the best fungus and fermentation method for the production of ^{13}C labeled chitosan. Based on the production yield of chitosan, the SSF method is the best method for the production of fungal chitosan when compared with SMF methods (i.e., fed-batch fermentation and batch fermentation). However synthesis of ^{13}C labeled chitosan in cell wall of *G. butleri* grown in SSF medium coated with 1-^{13}C-glucose was not observed. Alternatively, fungus *A. coerulea* was grown in SMF medium containing 2-^{13}C-glucose. The successful synthesis of ^{13}C labeled glucosamine from 2-^{13}C-glucose was observed in mycelia of *A. coerulea* grown in SMF medium containing 2-^{13}C-glucose in a yield of about 13 g/100 g mycelia.

1. Introduction

Chitin is a copolymer of mainly *N*-acetyl-D-glucosamine and a small number of D-glucosamine units linked by β-(1-4) glycosidic bonds. The deacetylated form of chitin is called chitosan. Chitin and chitosan can be found as supporting material in aquatic organisms, in terrestrial organisms and in microorganisms (Tokura & Tamura, 2007). Nowadays, commercially, chitins and chitosans are produced from biowaste obtained from aquatic organisms. The quality and quantity of chitosan depend on its biological source and the extraction procedure used (Nwe & Stevens, 2008a; Nwe, Furuike, & Tamura, 2009). Chitosans isolated from crustacean sources such as shells of shrimps and crabs and bone plates of squids have a high molecular weight with low polydispersity, DA below 20% and a 1% solution viscosity of 500–1700 cps, fungal chitosan has a low molecular weight with high polydispersity, DA lower than 15% and a 1% solution viscosity of 10–15 cps (Nwe & Stevens, 2002a, 2008a). In which, the physico-chemical characteristics of chitosan obtained from crustacean sources are batch to batch variable due to seasonal and variable supply of raw materials, as well as variability and difficulties in the process conditions (Crestini, Kovac, & Giovannozzi-Sermanni, 1996; Nwe & Stevens, 2008a). To overcome those problems, terrestrial organisms: insects, terrestrial crustaceans, mushrooms and fungi are choosen as alternative sources for the production of chitin and chitosan.

Among the terrestrial organisms; silkworms, honey bees, mushrooms and fungi have been cultured in laboratory and industrial scale to produce valuable products for human consumptions (Nemtsev, Zueva, Khismatullin, Albulov, & Varlamov, 2004; Nwe & Stevens, 2002a; Nwe, Furuike, & Tamura, 2010; Paulino, Simionato, Garcia, & Nozaki, 2006; Synytsya et al., 2009; Wu, Zivanovic, Draughon, & Sams, 2004; Yen & Mau, 2007). These production processes result in massive amount of waste materials. Some of

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these materials have been used for the production of chitin and chitosan. The yield of purified chitin and chitosan obtained from these sources was lower than that of aquatic crustacean sources (Nwe et al., 2010). In some of these sources chitin is complexed with other compounds such as melanin in insect, and glucan and other cell wall components in mushroom and fungi (Nemtsev et al., 2004; Nwe, Stevens, Montet, Tokura, & Tamura, 2008; Yen & Mau, 2007). This makes it difficult to extract and purify the chitin and chitosan from these sources. Therefore, there is almost no attempt to make commercialized production of chitin and chitosan from biowaste of insects, mushrooms and fungi (Nwe et al., 2010).

Within last 11 years, our research has been focused on (1) investigation of the bonding between chitosan and glucan in the cell wall of fungus *G. butleri*, (2) comparison the yield of chitosan from mycelia of fungi, *G. butleri* and *A. coerulea*, and (3) applications of fungal chitosan in agriculture and medical sectors. In this research, it was found out that chitosan in the fungal cell wall exists in two forms, free chitosan and chitosan bounded to glucan (Nwe & Stevens, 2002a). The linkage between chitosan and β-glucan in the chitosan–glucan complex has been successfully cleaved using a heat stable α-amylase and the resultant chitosan and β-glucan polymeric moieties have been purified and characterized. Data from elementary analysis, IR and ^{13}C NMR spectroscopy, and the results of various enzymatic treatments and reducing sugar analysis confirmed that the β-linked chitosan and β-linked glucan are linked with α-(1-4) glycosidic bond (Nwe & Stevens, 2002b; Nwe, Stevens, Tokura, & Tamura, 2008). Based on this observation, enzymatic chitosan extraction method has been developed to obtain high yield fungal chitosan in very easy way (Nwe & Stevens, 2002a). This method has been proposed for the large-scale production of fungal chitosan. An effective chitosan extraction procedure is essential for an economical production of fungal chitosan.

Pure chitosan is non-toxic, free of antigenic effects, biocompatible, biodegradable and polar (Kim, Jeon, Byun, & Park, 2001; Shigemasa, Morimoto, Saimoto, Okamoto, & Minami, 1998; Tokura et al., 1994; VandeVord et al., 2002). Chitosan has been used to prepare a variety of forms such as powder, solution, hydrogel, fiber, membrane, macro- and micro-bead and porous scaffold. These materials have been tested in many biological and medical applications, e.g., as a metal absorption agent in waste water treatment, as a component in medical, cosmetic, food and feed products, as a scaffolding material in tissue engineering, as a growth stimulator in agriculture (Madihally & Mattew, 1999; Nwe & Stevens, 2008a, 2008b; Tamura, Nagahama, & Tokura, 2006). For agriculture and tissue engineering applications, it was found that fungal chitosan, which has low molecular weight and low degree of acetylation is better than chitosan with high molecular weight obtained from shells of shrimps and crabs and bone plates of squids (Nge, Nwe, Chandrkrachang, & Stevens, 2006; Nwe et al., 2006, 2009). Now, the question is how do chito-oligosaccharide materials (i.e., the products from degradation of chitosan in plant and animal systems) metabolize in plant and animal systems?

For agriculture and medical applications little is known about the metabolism of degradation products of chitin and chitosan *in vivo* and *in vitro*. ^{13}C labeled chitosan is an essential prerequisite for study the metabolic pathway of chito-oligosaccharides, which are degradation products of chitin and chitosan by lysozyme, chitinase and chitosanase. Therefore in this research, fungi *G. butleri* and *A. coerulea* were grown in solid substrate and submerged fermentation media containing ^{13}C-glucose. Mycelia were collected and chitosan was extracted. The substitution of ^{13}C labeled glucosamine and ^{13}C labeled *N*-acetylglucosamine in the extracted chitosan was confirmed by ESI-mass spectrometry analysis.

2. Experimental

G. butleri USDB 0201 and ATCC 42618 and *A. coerulea* ATCC 14076 belonging to the class of Zygomycetes, were obtained from the Department of Biological Sciences, National University of Singapore, Singapore and the American Type Culture Collection, Rockville, MD, USA respectively. D-Glucose (1-^{13}C, 99%) and D-glucose (2-^{13}C, 99%) were purchased from Cambridge Isotope Laboratories, Inc., USA. Termamyl, Type LS enzyme, was kindly provided by Siam Modified Starch Co. Ltd. Thailand and NovoNordisk, Thailand and Japan (density 1.2 g/ml, activity 120 k Novo α-amylase unit/g). The other chemicals were of analytical grade.

2.1. Preparation of spore suspension for inoculation in solid substrate and submerged fermentation

Fungi were maintained on 3.9% potato dextrose agar (PDA) slants at 4 °C. Spores from each fungus were inoculated on each PDA plate and incubated for 10 days in an incubator at 30 °C for *G. bulteri* and for 7 days in an incubator at 25 °C for *A. coerulea*. Spores from the culture of *G. butleri* and *A. coerulea* were harvested by flooding the sporangiophores in sterilized distilled water. The spores from sporangia were gently scrapped off with the help of a sterilized glass spreader. Each spore suspension was collected in each sterilized 500 ml Erlenmeyer flask and the number of spores per ml of suspension was counted using a haemocytometer.

2.2. Cultivation of G. butleri USDB 0201 in solid substrate fermentation

Peeled sweet potatoes (*Manihot esculenta*) were cut into 1–1.5 cm × 4–6 cm pieces and washed with water. Mineral solution was prepared with 1 L distilled water containing 5 g $(NH4)_2SO_4$, 1 g K_2HPO_4, 1 g NaCl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, supplemented with 7.2 g urea and adjusted to pH 4.5 with 0.5 M H_2SO_4 (Nwe & Stevens, 2004, 2006). Sweet potato pieces (850 g, wet based) were mixed with 850 ml mineral solution and sterilized by autoclaving at 121 °C for 20 min. After cooling, the free solution was decanted (about 650–700 ml). The sweet potato pieces were inoculated with the fungal spore suspension (1.8×10^9 spores/kg of solid substrate). An airtight steamer solid substrate fermenter was used with 3 trays of perforated aluminium, tray volume about 9.6×10^3 cm^3 and tray-working volume about 1.6×10^3 cm^3 each. The fermenter was sterilized at 165 °C for 3 h and cooled to room temperature. The inoculated sweet potato solid substrate was mounted in the tray-fermenter and incubated under a constant supply of filtered and humidified air at a flow rate 0.8 L/min. The outside temperature of the fermenter was maintained at 29 ± 2 °C (Nwe & Stevens, 2006). Fermentation was carried out for 7 days. The mycelia biomass obtained was dried at 45 °C. The dried mycelia were used for extraction of chitosan.

2.3. Cultivation of G. butleri USDB 0201 in solid substrate fermentation medium coated with 1-^{13}C-glucose

Sweet potatoes pieces (850 g) mixed with 850 ml of mineral solution (same as above) and sterilized by autoclaving at 121 °C for 20 min. The preparation was cooled to room temperature. The free solution was removed by decantation. 1-^{13}C-glucose, 0.1603 g was dissolved in 10 ml of distilled water and then sterilized using filtration method. The sweet potato pieces were inoculated with 28.76 ml of *G. butleri* spore suspension plus sterilized 1-^{13}C-glucose solution by gently mixing with the help of a stainless steel spatula (kitchenware). The inoculum size was adjusted to 1.8×10^9 spores/kg sweet potato pieces. An airtight steamer solid state fermentor was used with only one tray of perforated alu-

minium, tray volume about 9.6×10^3 cm^3 and tray-working volume about 1.6×10^3 cm^3. The fermentor was sterilized by heat sterilization at 165 °C for 3 h and cooled to room temperature. The inoculated sweet potato solid substrate was spreaded out in the tray of the fermentor and incubated under a constant supply of filtered and humidified air at a flow rate 0.8 L/min. The outside temperature of the fermentor was maintained at 28 ± 2 °C. Fermentation was carried out for 7 days. The mycelial biomass obtained was dried at 45 °C in an oven. The dried mycelia were used to extract ^{13}C labeled chitosan.

2.4. Cultivation of Absidia coerulea ATCC 14076 and Gongronella butleri ATCC 42618 in submerged fermentation medium

Erlenmeyer flasks of 500 ml, each flask containing 200 ml of medium (20 g/L glucose; 10 g/L peptone; 1 g/L yeast extract; 5 g/L $(NH_4)_2SO_4$; 1 g/L K_2HPO_4; 1 g/L NaCl; 5 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 g/L $CaCl_2 \cdot 2H_2O$ in distilled water at pH 5.5), were sterilized in an autoclave at 121 °C for 15 min. After cooling, each fermentation medium was inoculated with each spores solution, 3×10^6 spores/L of fermentation medium and incubated in a shaking incubator at 30 °C, 200 rpm. After 5 days cultivation, fungal mycelia were harvested from growth media, washed with distilled water and freeze-dried. The dried mycelia were used to extract chitosan.

2.5. Cultivation of Absidia coerulea ATCC 14076 in submerged fermentation medium containing 2-^{13}C-glucose

Erlenmeyer flasks of 500 ml, containing 200 ml of medium without glucose (22.5 g/L peptone; 1 g/L yeast extract; 5 g/L $(NH_4)_2SO_4$; 1 g/L K_2HPO_4; 1 g/L NaCl; 5 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 g/L $CaCl_2 \cdot 2H_2O$ in distilled water at pH 5.5) was sterilized in an autoclave at 121 °C for 15 min. After cooling, non-labeled glucose or 2-^{13}C-glucose, 1 g was dissolved in 10 ml of medium and sterilized by filteration method (Disposable filter unit FP 30/0,2 CA-S, 0.2 μm, cellulose acetate sterilized filter, Whatman®) and then added to each 200 ml of sterilized fermentation medium. Each fermentation medium was inoculated with 3.27×10^6 spores/L of fermentation medium and incubated in a shaking incubator at 30 °C, 200 rpm. After 5 days cultivation, fungal mycelia were harvested from the growth medium, washed with distilled water and freeze-dried. The dried mycelia were used to extract chitosan.

2.6. Extraction of free chitosan

Free non-complex-bound chitosan was isolated according to the method described in Nwe and Stevens (2002a) and Nwe, Stevens, Montet, et al. (2008). Briefly, mycelia, 1 g were treated with 10–40 ml of 1 M NaOH at 45 °C for 13 h. After treatment, alkaline insoluble material, AIM was collected and washed with water up to neutral pH and then dried. The dried AIM, 1 g was treated with 200 ml of 0.35 M acetic acid at 95 °C for 5 h. After cooling, acid extracted solution was collected by centrifugation and chitosan in the solution was precipitated by adjusting the pH of the solution to 9. The chitosan precipitate was collected by centrifugation and washed with distilled water up to neutral pH and freeze-dried. The dried chitosan was weighted and stored at room temperature for further analysis.

2.7. Extraction of total chitosan

Total chitosan, free chitosan plus chitosan bounded to glucan was isolated according to the method described in Nwe and Stevens (2002a) and Nwe, Stevens, Montet, et al. (2008). Briefly, dried mycelia, 1 g were treated with 10–40 ml of 11 M NaOH at 45 °C for 13 h. After treatment, alkaline insoluble material, AIM was collected and washed with distilled water up to neutral pH and dried. The dried AIM, 1 g was treated with 200 ml of 0.35 M acetic acid at 95 °C for 5 h. The resultant AIM suspension was cooled to room temperature and adjusted to pH 4.5 with 1 M NaOH and then treated with 4% (v/v) Termamyl, Type LS (α-amylase) in a shaking water-bath at 65 °C, 200 rpm for 3 h. Enzyme treated suspensions were centrifuged at $1600 \times g$ for 15 min to obtain a clear chitosan solution. Chitosan was precipitated by adjusting the pH of the supernatant to 8–9 with 1 M NaOH solution. The chitosan precipitate was washed with distilled water up to neutral pH and freezed–dried. The dried chitosan was weighted and stored at room temperature for further analysis.

2.8. Acid digestion of chitosan samples and analysis of digested samples by thin layer chromatography (TLC) and electrospray ionization (ESI) mass spectrometry

The chitosan samples, 30 mg were digested with 6 ml of 6 M HCl at 110 °C for 10–13 h to obtain monomers of chitosan. The digested samples were precipitated with acetonitrile (sample: acetonitrile, 1:50, v/v) at room temperature for overnight. The precipitate was collected by centrifugation and re-dissolved again in distilled water and then re-precipitated with acetonitrile to obtain samples with low acid content. The samples were analyzed using thin layer chromatography and electrospray ionization mass spectrometer to observe the incorporation of ^{13}C labeled glucosamine and ^{13}C labeled *N*-acetylglucosamine in the chitosan molecules.

TLC analysis was carried out with the modification of the method described by Cabrera and Cutsem (2005). The hydrolyzed samples and standards, glucosamine and *N*-acetyl glucosamine were spotted on TLC plate (silica gel plates, MERCK 60. GF-254) and separated the chito-oligosaccharides in hydrolyzed samples by migration of the solvents (*n*-propanol:water:concentrated ammonia 7:2:1, v/v) in a tank according to the conventional TLC method. After that the plate was taken out from the tank and dried. Spots were visualized by charring with 10% H_2SO_4 in ethanol and each spot was identified by comparison with standard samples.

For mass spectrometry analysis, the ESI-MS experiments were performed with a TSQ7000 System (ThermoQuest, USA). The sample solution was injected into the electrospray source at a flow rate of 10 μl/min and electrosprayed by infusion. The electrospray source was set in the positive ionization mode and operated using nitrogen gas under 40 psi (sheath gas). The electrospray voltage was 4.5 kV and the heated capillary temperature was 180 °C. Qualitative analysis of ^{13}C labeled chito-oligosaccharides in hydrolyzed sample of chitosan extracted from mycelia grown in fermentation medium with ^{13}C-glucose was done by comparing the molecular ion chromatogram with the chromatogram of hydrolyzed control sample, i.e., non-labeled chitosan sample extracted from mycelia grown in fermentation medium with non-labeled glucose.

2.9. Characterization of chitosan

The degree of deacetylation of chitosan was determined by first derivative UV spectrophotometry with the modification of Tan, Khor, Tan, and Wong (1998) and Muzzarelli and Rocchetti (1985). Relative molecular weight was determined by gel permeation chromatography (GPC) using a Waters HPLC equipped with Ultrahydrogel 2000, 1000 and 500 columns and a WatersTM 410 Differential Refractometer Detector. The solvent used was 0.2 M acetic acid/0.1 M sodium acetate buffer at a flow rate of 0.6 ml/min. Dextrans of various molecular weights ranging from 9.9×10^3 to 2×10^6 Da were used as standards.

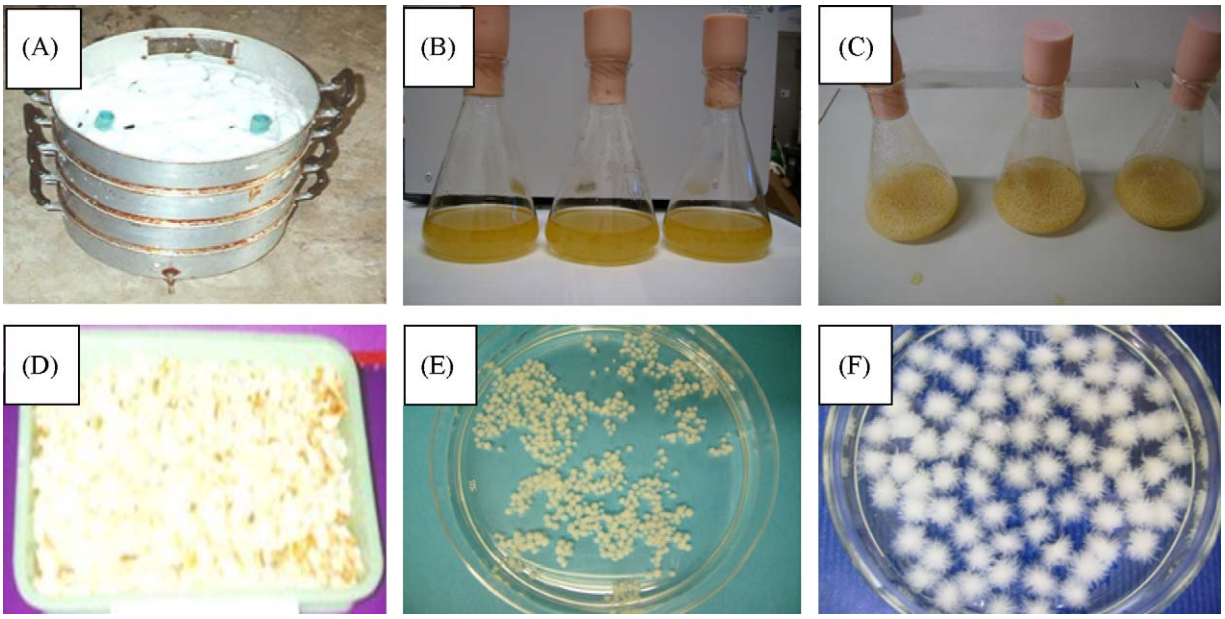

Fig. 1. (A and D) Mycelia of fungus *Gongronella butleri* USDB 0201 grown in SSF, (B and E) mycelia of fungus *Absidia coerulea* ATCC 14076 grown in SMF, and (C and F) mycelia of fungus *Gongronella butleri* ATCC 42618 grown in SMF.

3. Results and discussion

3.1. Laboratory scale production of fungal chitosan from fungi, Absidia coerulea ATCC 14076 and Gongronella butleri USDB 0201 and ATCC 42618 by solid substrate and submerged fermentation

In order to select the fungus species and fermentation method for the production of ^{13}C-chitosan, fungi *A. coerulea* ATCC 14076 and *G. bulteri* USDB 0201 and ATCC 42618 were selected based on our previous data and published literature (Muzzarelli, Ilari, Tarsi, Dubini, & Wenshui Xia, 1994; Nwe & Stevens, 2004, 2006; Nwe et al., 2002, 2008; Tan, Tan, Wong, & Khor, 1996; Wang et al., 2008). Fungus *G. butleri* USDB 0201 was grown on sweet potato pieces in a tray-type solid substrate fermenter under humidified air supply and fungi *A. coerulea* ATCC 14076 and *G. butleri* ATCC 42618 were grown in SMF media (Fig. 1). For SSF, sweet potato pieces were used as carbon and energy source. Sweet potato is the most suitable as substrate and solid support for growth of fungus *G. butleri*, because it can keep its water activity for a long time, and fungal mycelia could be easily removed from the substrate since weight of remained sweet potato pieces was heavier than weight of fungal mycelia. Moreover the fungal mycelia grew on the surface of the substrate, it did not penetrate into the solid matrix. This makes it easy to separate the mycelia from the substrate at the end of the fermentation. Fermentation conditions for the growth of fungus also have been optimized using tray-type fermenter to obtain high yield mycelia and chitosan (Nwe & Stevens, 2004, 2006). The design of fermenter makes it easy to operate and is suitable for scaling up with minor investment (Nwe & Stevens, 2002a). At the end of each fermentation, mycelia were harvested and dried. The yield of mycelia obtained from *G. butleri* in SSF was higher than that obtained from *A. coerulea* and *G. butleri* in SMF (Table 1). Similar results have been reported by Crestini et al. (1996) and Nwe et al. (2002).

In the fungal cell wall, chitosan occurs in two forms, as free chitosan and chitosan covalently bound to β-glucan (Muzzarelli et al., 1994; Nwe & Stevens, 2002a, 2002b; Nwe, Stevens, Montet, et al., 2008). The formation of the chitosan–glucan complex chains results in a rigid cross-linked network in the cell wall and causes considerable problems for the extraction of intact chitosan and glucan (Nwe, Stevens, Montet, et al., 2008). The free chitosan in the cell wall was extracted after treatment of fungal mycelia with 1 M NaOH (Nwe & Stevens, 2002a; Nwe, Stevens, Montet, et al., 2008). Total chitosan, i.e., free chitosan plus chitosan bounded glucan was extracted by enzymatic method. The purity of the chitosan extracted using the enzymatic method has been confirmed by elementary analysis, IR and ^{13}C NMR spectroscopy (Nwe, Stevens, Tokura, et al., 2008). The yield of free chitosan extracted from mycelia of *G. butleri* grown in SSF was lower than that from mycelia of *G. butleri* and *A. coerulea* grown in SMF (Table 1). In SMF, the yield of free chitosan extracted from mycelia of *A. coerulea* was higher than that of *G. butleri*. The reason might be the morphologies of mycelia obtained from both fermentation methods were different (Figs. 1 and 2). According to these observations together with our previous data, it can be con-

Table 1
Yields of mycelia and chitosan obtained from fungi, *Absidia coerulea* ATCC 14076 and *Gongronella butleri* ATCC 42618 grown in submerged fermentation (SMF) and *Gongronella butleri* USDB 0201 grown in solid substrate fermentation (SSF).

Compositions	*A. coerulea*	*G. butleri*	
Type of fermentations	SMF	SMF	SSF
Yield of mycelia (g/L of SMF medium)	4–5	5–6	–
Yield of mycelia (g/kg for SSF)	–	–	30–40
Free chitosan g/100 g of mycelia	8–9	5–6	1.5–2
Total chitosan g/100 g of mycelia	9–10	13–14	8–10
Production yield of chitosan (g/L of SMF medium)	0.4–0.5	0.3–0.8	–
Production yield of chitosan (g/kg of SSF medium)	–	–	3.0–4.0

SSF (Nwe & Stevens, 2002a, 2004, 2006; Nwe, Stevens, Montet, et al., 2008).

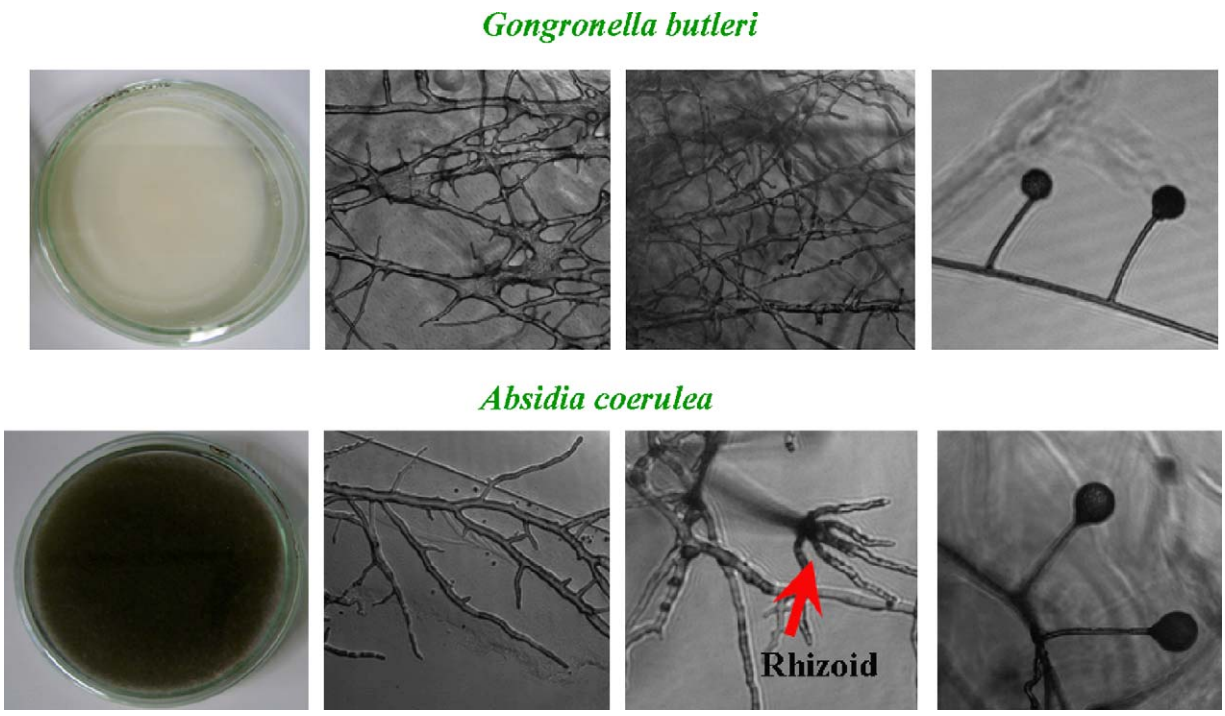

Fig. 2. Macroscopic and microscopic observations of *Gongronella butleri* and *Absidia coerulea*.

firmed that mycelia matrices of *G. butleri* grown in SSF and SMF have higher cross-linked points than *A. coerulea* grown in SMF (Table 1; Figs. 1 and 2; Nwe, Stevens, Montet, et al., 2008). The yield of total chitosan obtained from mycelia of *G. butleri* grown in SMF was higher than that of *G. butleri* grown in SSF and *A. coerulea* grown in SMF. The cell wall of *A. coerulea* composed more chitosan in free form and *G. butleri* synthesized more chitosan in complex form under SSF and SMF (Table 1; Nwe, Stevens, Montet, et al., 2008). The production yields of chitosan obtained from mycelia of *G. butleri* grown in SSF was higher than that of *G. butleri* and *A. coerulea* grown in SMF, because the yield of mycelia obtained from SSF was higher than that from SMF (Table 1).

The chitosan obtained from mycelia of *G. butleri* grown in SSF has molecular weight 20–70 kDa and DA 13%. The mycelia of *A. coerulea* show with root like rhizoids but *G. butleri* does not (Fig. 2). Therefore the best fermentation method for *A. coerulea* and *G. butleri* is SMF and SSF respectively. Therefore fungus *G. butleri* USDB 0201 and solid substrate fermentation were selected for the production of ^{13}C labeled chitosan.

3.2. Biosynthesis of ^{13}C labeled chitosan in the cell wall of fungus *Gongronella butleri* USDB 0201 by solid substrate fermentation

For the synthesis of ^{13}C labeled chitosan, *G. butleri* was grown on sweet potato pieces coated with 1-^{13}C-glucose. Here 1-^{13}C-glucose is the glucose molecule labeled with ^{13}C at carbon number 1. At the end of fermentation, fungal mycelia was harvested from the SSF medium and dried. The dried mycelia, 38.94 g/kg of solid substrate were obtained after 7 days of fermentation. The dried mycelia were treated with 11 M NaOH at 45 °C for 13 h. The alkaline insoluble material (AIM) was collected. The chitosan was extracted from the dried AIM. Dried AIM was obtained from dried mycelia in a yield of 26 g/100 g. Dried chitosan could be extracted from fungal mycelia in a yield of 9.8 g/100 g. The molecular weight of the chitosan was about 76 kDa.

In order to observe the synthesis of ^{13}C labeled glucosamine and *N*-acetylglucosamine in chitosan molecules, the extracted chitosans obtained from mycelia of fungus *G. butleri* grown in SSF medium coated with 1-^{13}C-glucose and in SSF medium without coated with 1-^{13}C-glucose were digested with 6 M HCl at 110 °C for 10–13 h. The digested samples were treated with acetonitrile to obtain precipitates of digested materials. The precipitates were dissolved in distilled water and then analyzed by TLC. For ESI-mass spectrometry analysis, the precipitates were dissolved in distilled water and further diluted in water/acetonitrile (1:1, v/v). The analysis results showed that both chitosans hydrolyzed to monomers of chitosan (Fig. 3). Both spectra show the characteristic glucosamine peaks at *m*/*z* 179.6 and 179.8 and the fragment ion of glucosamine peaks at *m*/*z* 161.6 and 161.5. These two peaks are absence in blank spectrum. Roda et al., 2006 reported that the ion mass spectrum of glucosamine showed the protonated molecular ion of glucosamine at *m*/*z* 180 and the loss of one water molecule from glucosamine at *m*/*z* 162.

The chitosans have about 13% degree of deacetylation. *N*-acetylglucosamine peak is not observed in the spectra of chitosan samples (Fig. 3B). The reason might be the low concentration of *N*-acetylglucosamine in the digested samples or the hydrolysis of the acetyl groups from *N*-acetylglucosamine units during the acid hydrolysis reaction. The similar observation has been reported by Holme, Foros, Pettersen, Dornish, & Smidsrød, 2001. They assumed that hydrolysis of *N*-acetyl bond may occur in addition to the hydrolysis of the glycosidic bond during acid hydrolysis reaction. The synthesis of ^{13}C labeled chitosan in cell wall of *G. butleria* grown in SSF medium coated with 1-^{13}C-glucose was not observed (Fig. 3B). One might expect that in case all ^{13}C-glucose would be incorporated in the synthesis of chitosan that about 0.16 g labeled glucose is present in 3.32 g chitosan, i.e., 48 mg ^{13}C-chitosan per g of chitosan. The molecular weight of chitosan is 76 kDa (=457 residues), so every chitosan molecule will carry 23 molecules of ^{13}C-glucosamine and ^{13}C-*N*-acetylglucosamine.

For the SSF, sweet potato pieces were treated with mineral solution and then mixed with 1-^{13}C-glucose solution and spore solution. Here one can make three assumptions (Fig. 4) that may interfere with labeling of chitosan: (1) In lag phase to early exponential growth phase, fungus might use 1-^{13}C-glucose on the top of

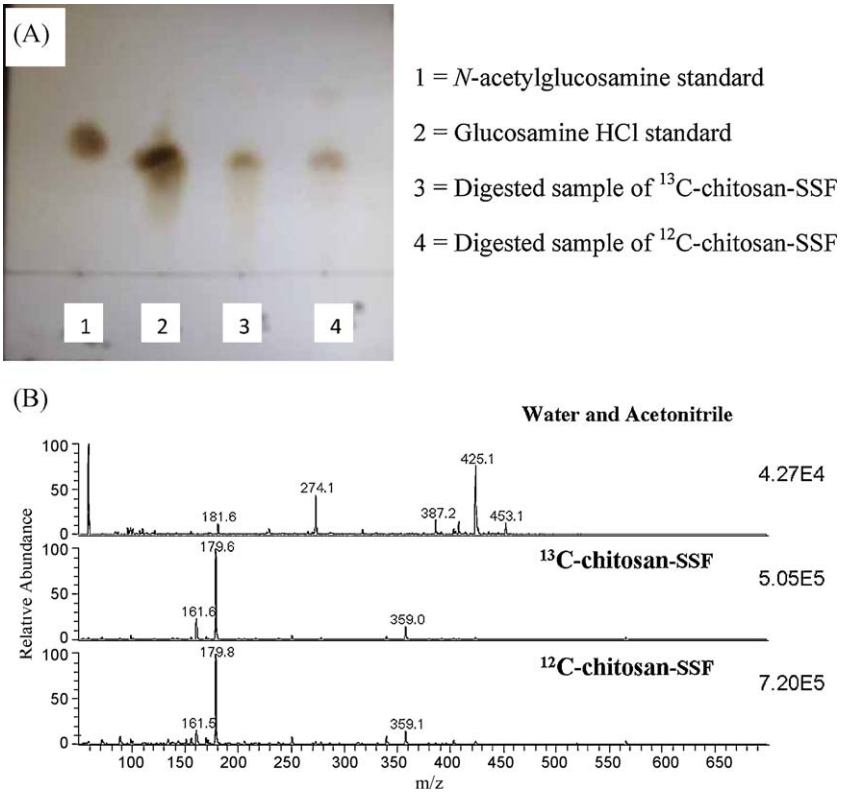

Fig. 3. (A) TLC chromatogram of acid digested chitosans samples. (B) Mass spectra of chitosan samples digested with 6 M HCl (^{13}C-chitosan-SSF = chitosan obtained from mycelia of fungus *G. butleri* USDB 0201 grown in SSF medium coated with 1-^{13}C-glucose; ^{12}C-chitosan-SSF = chitosan obtained from mycelia of fungus *G. butleri* USDB 0201 grown in SSF medium without 1-^{13}C-glucose coating).

the sweet potato pieces and then it might use glucose (non-labeled) from sweet potato starch to grow in exponential phase. So that chitosan produced from the mycelia grown in lag phase to early exponential growth phase might have ^{13}C-glucosamine substitute in the chitosan chain and it might degradate in the late exponential phase of fungus. In the growth curve of *G. butleri*, the yield of chitosan in the cell wall of fungus decreased after 6 days of fermentation (Nwe & Stevens, 2002a). However the mechanism of the decrease amount of chitosan is still unknown. (2) The labeled glucose is diluted by the non-labeled glucose generated from the sweet

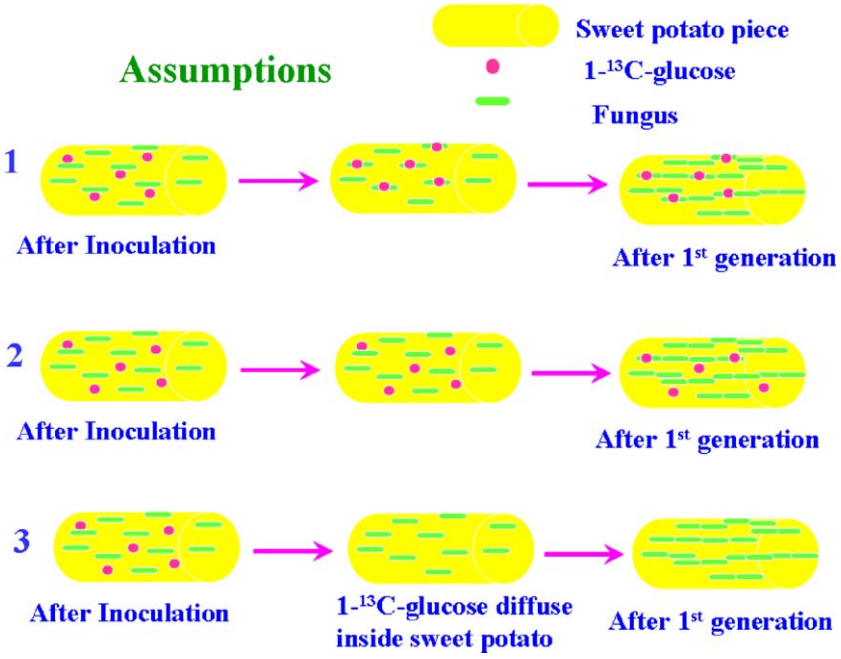

Fig. 4. Possible ways of interaction between 1-^{13}C-glucose and fungal hyphae.

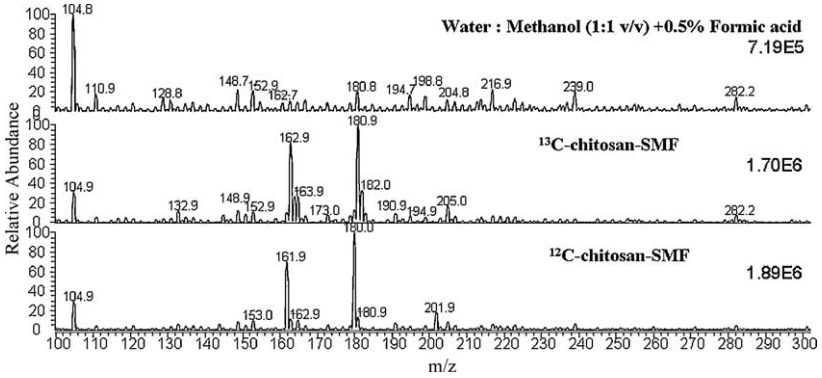

Fig. 5. Mass spectra of chitosan samples digested with 6 M HCl. (^{13}C-chitosan-SMF = chitosan obtained from mycelia of *A. coerulea* ATCC 14076 grown in SMF medium containing 2-^{13}C-glucose; ^{12}C-chitosan-SMF = chitosan obtained from mycelia of *A. coerulea* ATCC 14076 grown in submerged fermentation medium containing non-labeled glucose).

potato material during growth of fungus. (3) The labeled glucose is diffused away from the mycelium before it is absorbed. According to these assumptions together with the above mentioned results, it can be assumed that there is a need to supply sufficient ^{13}C-glucose in the fermentation medium throughout the cultivation period. Therefore SMF might be better than SSF to synthesize ^{13}C labeled chitosan in the cell wall of fungus. *A. coerulea* ATCC 14076 was selected for the synthesis of ^{13}C labeled chitosan in the cell wall of fungus by submerged fermentation.

3.3. Synthesis of 2-^{13}C-chitosan in cell wall of fungus Absidia coerulea ATCC 14076 by submerged fermentation

The fungus, *A. coerulea* ATCC 14076 was grown in submerged fermentation medium containing ^{12}C glucose or 2-^{13}C-glucose (i.e., glucose molecule labeled with ^{13}C at carbon number 2) at 30 °C, 200 rpm for 5 days. There is no non-labeled glucose in the fermentation medium containing 2-^{13}C-glucose. The resultant fungal mycelia, about 4 g/L of fermentation medium were used to extract chitosan. The fungal mycelia were treated with 11 M NaOH solution. The alkaline insoluble material (AIM), about 22 g/100 g of dried mycelia was obtained. The yield of chitosan was in both cases about 13 g/100 g of dried mycelia. The chitosan was digested in 6 M HCl at 110 °C for 10–13 h to obtain monomers of chitosan. The methods of sample digestion and preparation are critical points to obtain a perfect sample for mass spectrometry analysis. Glucosamine dissolves in acetonitrile: water (65:35) and it precipitates afterward. The best condition for precipitation of the glucosamine in digested samples were treatment with acetonitrile (digested sample: acetonitrile, 1:50, v/v) at room temperature for overnight. The glucosamine precipitate was washed with acetonitrile and dissolved in distilled water. For ESI-MS analysis, glucosamine solution was diluted in 0.5% formic acid in methanol solvent (methanol: water, 1:1, v/v) and then analyzed using an ESI-mass spectrometer. The chitosan obtained from the fungal mycelia grown in fermentation medium containing 2-^{13}C-glucose showed two glucosamine peaks at m/z 180.9 and 162.9 and that in the fermentation medium containing non-labeled glucose showed two glucosamine peaks at m/z 180 and 161.9 (Fig. 5). The peaks at m/z 162.9 and 161.9 are the fragments of glucosamine, which fragmented during mass spectrometry analysis. The peaks corresponding to *N*-acetylglucosamine could not be observed in both spectra. According to these data, ^{13}C labeled glucosamine was incorporated in the chitosan molecules synthesized in cell wall of fungus *A. coerulea* grown in SMF medium containing 2-^{13}C-glucose. Further experiments need to perform to observe the localization of ^{13}C in ^{13}C labeled glucosamine molecules.

4. Conclusion

Fungi consumed glucose from fermentation medium and synthesized chitosan in their cell wall under solid substrate and submerged fermentation conditions. The production yield of chitosan produced from fungus grown in solid substrate fermentation is higher than that in submerged fermentation. However solid substrate fermentation medium and conditions did not support to produce ^{13}C labeled chitosan in cell wall of fungus. The best fermentation method to produce ^{13}C labeled chitosan is submerged fermentation method. The production yield of ^{13}C labeled chitosan, 0.5 g/L of fermentation medium could be produced by fungus *A. coerulea* grown in submerged fermentation medium. The resultant ^{13}C labeled chitosan is proposed to be use for study the metabolic pathway analysis of chitosan in plant and animal systems.

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