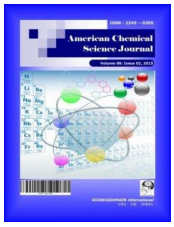

American Chemical Science Journal
11(4): 1-8, 2016, Article no.ACSJ.22842
ISSN: 2249-0205

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Integrated Process for β-glucan Concentrate from *Ganoderma lucidum* by Extraction and Micronization

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SM and Wahyudiono designed the study, managed analytical study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author SK did experiment and analysis. Authors HK and MG managed the literature searches and provided experimental and analytical apparatus. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ACSJ/2016/22842
Editor(s):
(1) Anonymous.
Reviewers:
(1) Meihua Mo, South China Agricultural University, Guangzhou, China.
(2) Mutemi Muthangya, South Eastern Kenya University, Kenya.
(3) Rosaria Costa, University of Messina, Italy.
Complete Peer review History: http://sciencedomain.org/review-history/12645

Original Research Article

Received 30th October 2015
Accepted 1st December 2015
Published 11th December 2015

ABSTRACT

Hydrothermal treatment is an environmentally friendly technique with a wide range of applications such as extraction, hydrolysis, and wet oxidation of organic compounds. In this work, water under hydrothermal conditions was used to extract β-glucan from *Ganoderma lucidum* (*G. lucidum*) at 433 K and 4.0 MPa using a semi-batch system. The extracts were then directly atomized and contacted with a hot inert gas to produce microsphere particles. When nitrogen was used as the inert gas, the moisture evaporated faster than when normal air was used at the same inlet gas temperature. Scanning electron microscopy (SEM) images showed that corrugated particles were obtained, with diameters varying from 3 to 20 microns. The β-glucan content in the particle products was 40 to 45% in weight, approximately.

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Keywords: G. lucidum; β-glucan; extraction; nitrogen; autohydrolysis.

1. INTRODUCTION

Plant biomass, including agricultural and forestry residues, large portions of municipal solid waste, and herbaceous and woody plants, is the most abundant renewable resource of the world [1]. The main components of plant biomass are cellulose (40-45 wt %), hemicellulose (25-35 wt %), and lignin (15-30 wt %) [2]. As a primary component of plant biomass, hemicellulose, which is mainly composed of xylans, provides an important source of interesting molecules such as xylose and xylo-oligosaccharides, which have potential applications in different fields, notably in the chemical, food, and pharmaceutical industries [3,4]. In addition, due to the presence of glucan functional groups in hemicellulose, polymeric or oligomeric hemicellulose can possibly be used in health applications [5-8]. To increase the value of biomass components, they need to be separated into their components. Hence, the fractionation of plant biomass components has become an important topic.

Recently, hydrothermal extraction has become a technique that has gained much attention as an important process and is the most widely used technology for extracting polysaccharides [8-13]. This technology is based on the use of water as an extractant at temperatures above its boiling point (373 K) and below its critical point (647 K) and at a pressure high enough to maintain the liquid state [14,15]. This work demonstrates that water soluble compounds containing β-glucan from hydrothermal extraction at 433 K and the microparticulate formation of them into powders from *G. lucidum* is a one step-process. Generally, hemicellulose is branched, with the degree of polymerization (DP) ranging from less than 100 to about 200 units [16]. Because of its structure and branched nature, hemicellulose is amorphous and relatively easy to hydrolyze to its monomer sugars compared with cellulose [17]. Therefore, water at 393 K can extract hemicellulose from wood [9].

In our previous work [13], microparticulate powders were directly generated during the extraction process to maintain or increase the concentration of β-glucan in the aqueous extract. The water evaporated instantaneously when the aqueous extract was co-currently contacted with hot air via a nozzle. However, this microparticle formation process exhibits extremely complex behaviours because many process and formulation variables need to be tuned correctly to achieve the desired result [18]. In order to develop a new technique, the normal air was replaced with nitrogen gas under similar conditions. It provides an inert atmosphere for long-term preservation once the drying process is complete. Therefore, this work focused only on the morphology of the particles formed.

2. MATERIALS AND METHODS

2.1 Materials

G. lucidum was obtained from Refarmer Co., Ltd. (Kumamoto, Japan) was used as a starting material. It was shredded by a laboratory mill to a particle size of < 2 mm and passed through 16-mesh sieves. The sample was then refrigerated at < 278 K. Distilled water obtained from a water distillation apparatus (Shibata Co., model PW-16, Japan) was used as a solvent. Potassium hydroxide (KOH, 85.0%), Sodium hydroxide (NaOH, 97.0%), Hydrochloric acid (HCl, 35.0~37.0%), and Acetic acid (CH_3COOH, 99.9%) were purchased from Wako Pure Chemicals Industries Ltd., Japan, and were used without further purification. Nitrogen (N_2) gas was purchased from Sogo Kariya Sanso, Inc. Japan.

2.2 Experimental Setup and Procedure

The apparatus consisted of an extraction unit and a precipitation unit [13]. The main apparatus for extraction unit consisted of: a high-pressure pump (LC–6AD Shimadzu, Japan); electric water heater (ESPEC ST-110, Japan); reactor (10 ml in volume; Thar Design Inc., USA); and back-pressure regulators (BPRs; AKICO, Japan). The precipitation unit consisted of an air compressor, heater, chamber, nozzle, and aspirator, and allowed the production of particles from liquid fraction products via atomization. The experiment was conducted at 433 K and 4 MPa. The inlet dry nitrogen gas temperatures of 408, 443, and 473 K corresponded to the outlet dry nitrogen gas temperatures of approximately 335, 342, and 349 K, respectively. These conditions were selected based on the previous report [13]. The fine powdery products were collected using a filter, which was placed before the aspirating pump. The morphologies of the powder products were observed using a scanning electron microscope (Hitachi S-4300, Japan). The powder particle diameters were determined from the SEM

images using the image analyser software Image J 1.42 (National Institutes of Health, USA).

To understand the β-glucan contents in the particle products, a mushroom and yeast β-glucan assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) was used following to the manufacturer's instruction. The absorbance of the solution samples was measured by using UV–vis spectrophotometry at 510 nm (UV–vis spectrophotometry V–550, Jasco Corporation, Japan). 5 mg of particles dissolved in water was suspended in 1.5 mL of concentrated HCl (37% v/v) and incubated at 303 K for 45 min; 10 mL distilled water was then added, and it was placed in a boiling water bath for 120 min. The pH was neutralized with 10 mL of 2 M KOH, followed by centrifugation for 10 min. 0.1 mL of the solution was digested with an aliquot of exo-1,3-β-glucanase (20 U/mL) plus β-glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5.0). The hydrolysates were incubated with 3.0 mL of glucose oxidase-peroxidase mixture (GOPOD) at 313 K for 1 h. The total glucan content (α- and β-) was observed by introducing this solution into UV-vis spectrophotometry at 510 nm. The content of α-glucan was also measured at the same wavelength. 5 mg of particles dissolved in water was suspended in 2 mL of 2 M KOH for 20 min and neutralized with 8 mL of 1.2 M sodium acetate buffer (pH 3.8). Then, the solution was centrifuged for 10 min and aliquots of amyloglucosidase (1630 U/mL) plus invertase (500 U/mL) were added to the 0.2 mL of solution, followed by incubation at 313 K for 30 min. The solution was incubated with 3.0 mL of glucose oxidase-peroxidase mixture at 313 K for 20 min. The amount of β-glucan was counted by subtracting α-glucan from the total glucan content.

3. RESULTS AND DISCUSSION

The particles form through a spray drying process, which consists of three operating steps: atomization, water evaporation, and powder collection. The feed (aqueous extract) is sprayed via a nozzle (atomizer) into a drying chamber. Aided by the large specific surface area of the droplets, the water evaporation in the drying chamber takes place in matter of seconds [19]. The dried powder is protected from overheating by the rapid removal of the solvent from the drying zone. Finally, the dry particles are carried into the cyclone and settle in the product collector. Instead of air, dry nitrogen gas was used as an inert gas to improve the drying process [20]. Fig. 1 shows the SEM images of the particles formed from water-soluble compounds extracted from *G. lucidum* and then directly contacted with the dry nitrogen gas to remove their water content. It is clear that the obtained particles had different morphologies under different conditions. The morphology of the particles produced via spray drying depends on the drying air inlet temperature, drying air outlet temperature, drying air flow rate, atomizing air flow rate, and residence time. Of these parameters, the drying air outlet temperature is the dominating factor in controlling the drying rate and important particle characteristics such as particle shape and moisture content. Therefore, when the water content of the droplet reaches a critical value, a dry crust is frequently formed at the droplet surface. If the droplet is dried very slowly, it is transformed into a fully dried particle. At high drying temperatures, cycles of repeated expansion and collapse of the particle occur due to the formation of an internal air bubble [21-23].

As shown in Fig. 1, surface corrugation of particles occurred at various drying gas inlet temperatures. In our previous work [13], at a low gas inlet temperature (408 K), the microparticles were successfully generated with spherical morphology and smooth surfaces with a particle size distribution between 0.5 and 6.0 μm. This phenomenon could be explained by the fact that the diffusional motion of the solutes is fast compared to the radial velocity of the receding droplet surface and is typically associated with spherical and solid particle formation. The surface corrugation of particles occurred when the gas inlet temperature increased at 443 and 473 K. It is obvious that the temperature had almost no effect on the morphology of the particles, which presented well-defined spherical smooth surfaces. In this case, the surface (skin) moves faster than the dissolved or suspended components. In this work, all the experimental results showed that corrugated particles have been formed. By using nitrogen as the inert gas, it was found that the moisture evaporated faster than when using normal air. The possible reason for this relates to the physical properties of the different gases; the specific heat capacity of nitrogen was higher than that of normal air under the same conditions [20]. Therefore, when using chambers of the same volume, nitrogen could absorb more heat from the heater (heat exchanger) and release more heat when flowing over the extract droplets in the chamber.

Hawlader et al. used nitrogen as the drying media and experimentally investigated the drying kinetics and quality of the heat pump dryer products [20]. They reported reduced browning, faster rehydration, and enhanced vitamin C retention in the final products and highlighted the great potential of the modified atmosphere heat pump drier in the food drying industry. In addition, the high drying rate of nitrogen also influenced the sizes of the particles formed.

Fig. 2 described the particle size distributions of the powder products, which had average diameters of (a) 4.7, (b) 9.3, and (c) 10.9 μm, respectively. The geometric diameter of particles that are not perfect spheres depends on their orientation. In this case, an average geometric diameter can be substituted, e.g., by averaging the Feret's horizontal and vertical diameters [18,24]. The result clearly showed that the use of a higher inlet air temperature led to the production of larger particles, which is related to the higher swelling caused by the higher temperature [25,26]. Tonon et al. explained that drying at higher temperatures results in faster drying rates, which leads to the early formation of a structure and does not allow the particles to shrink during drying [26]. When the inlet air temperature is low, the particle remains shrunk, and thus, retains its smaller diameter. In the current study, the heat exchanger attached to the apparatus was modified. This modification was expected to allow the use of various gases. Therefore, air, nitrogen, and other inert gases as atomizers could be applied as a mixture of gases or as a pure gas.

As informed above that the content of β-glucan in the particle products was analyzed by using a mushroom and yeast β-glucan assay kit. The result showed that the β-glucan content in the particle products was 40 to 45% in weight, approximately. Since the content of water-soluble β-glucan from *G. lucidum* was around 10 to 50 % [27], it could be said that this technique may extract and concentrate the most of water-soluble β-glucan from *G. lucidum*.

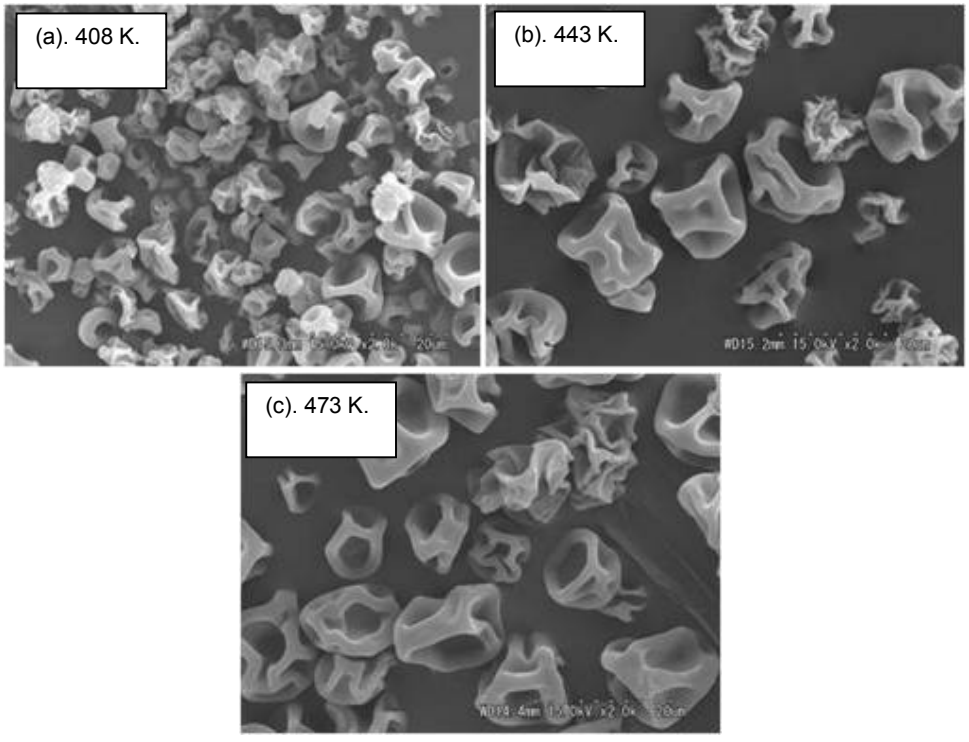

Fig. 1. SEM images of generated particles with different inlet nitrogen gas contact temperatures

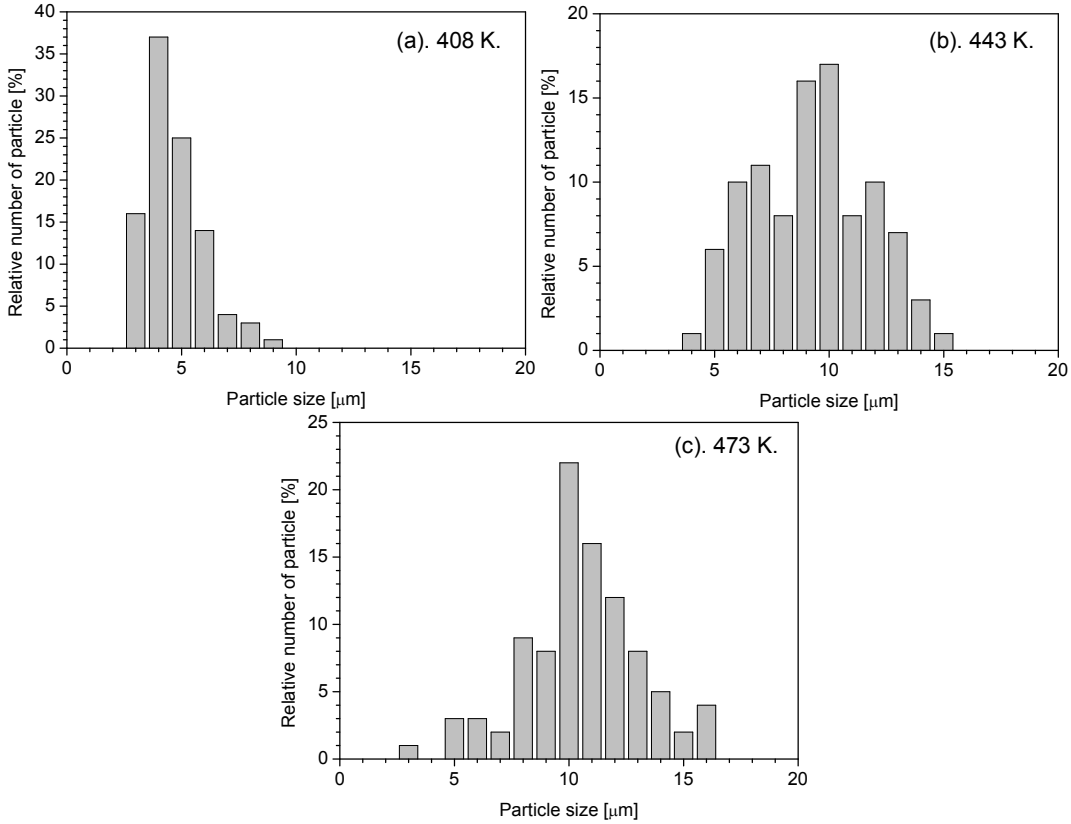

Fig. 2. Particle size distribution of powders produced with different inlet nitrogen gas contact temperatures

Hydrothermal extraction could be defined as an extraction process in which water at a temperature of between 373 K and 647 K (critical temperature) and a pressure higher than that of its vapor saturate, up to 22.1 MPa, to maintain the water in its liquid state [14,15]. This type of process was also called autohydrolysis. Under hydrothermal conditions, *G. lucidum* components underwent degradation reaction into its backbone units. Fig. 3 showed SEM images of *G. lucidum* before and after hydrothermal treatment at various temperatures. Before hydrothermal treatment, the morphologies of *G. lucidum* were pointed by some boundary sides clearly. They exhibited essentially regular and compact surface structure, and a highly fibrillar and intact morphology. After treatment by hydrothermal, the physical structures disruption of *G. lucidum* occurred and clearly found at each operating condition. Probably, the hydrothermal treatment allows to disrupt the structure of *G. lucidum* that contains carbohydrates, lignin, protein, and lipids as main components similar to plants biomass. As shown in Fig. 3, the physical structures of *G. lucidum* changed clearly with increasing hydrothermal extraction temperatures from 403 to 463 K. The structural breakdown of many fibers occurred via defibration process. At the higher temperatures of hydrothermal treatment, defibration of many fibers occurred extensively. The textures of *G. lucidum* after hydrothermal treatment seemed different from that of the original *G. lucidum* since some materials melted and resolidified. Some cracks were also apparent on the defibrated fibers surface. These showed the cell wall of *G. lucidum* was crushed, expressing the release of their components to dissolve in water at hydrothermal conditions.

Since the plants biomass such as *G. lucidum* consisted of the various components, the extraction of β-glucan from *G. lucidum* via degradation process at hydrothermal conditions seems extremely complex. Hence, the studying of biomass model compounds conversion is very useful to explain the hydrothermal extraction process [7,28-30]. Toor et al. explained that the degradation of plants biomass components in

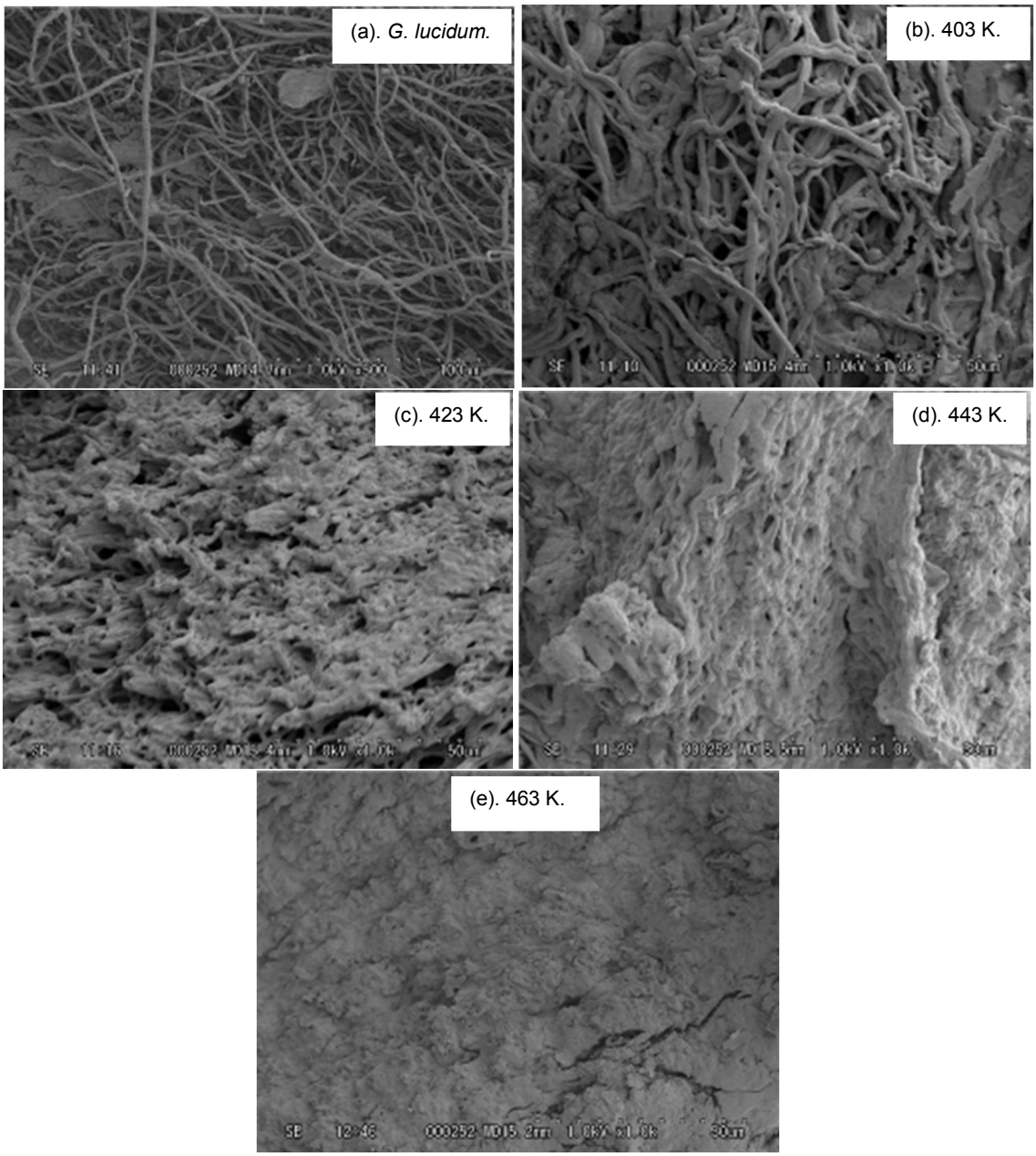

Fig. 3. SEM images of *G. lucidum* before and after treatment by hydrothermal

hydrothermal conditions differ; however, the basic reaction mechanisms can be listed as follows: first, depolymerization of the plants biomass following by decomposition of plants biomass monomers by cleavage, dehydration, decarboxylation and deamination [28]. Then the recombination and repolymerization of reactive fragments. Ruiz et al. described clearly the hydrolysis of cellulose to glucose in hydrothermal conditions [30]. They explained that initially the water as a major constituent of extraction system underwent auto-dissociation to produce hydrogen ion (H^+) and hydroxide ion (OH^-). At these conditions, the auto-dissociation of water molecules is believed 3 orders of magnitude larger than that at ambient conditions. The increased amount of ions from the water will make acid and base catalyzed reactions far more likely to occur at these conditions than at ambient. The generated H^+ ion interacts the

oxygen atom in the β–1,4-glycosidic bond to generate conjugate acid continued by the glycosidic bond split and leads to the two glucose units. The OH^- ion will attack at the anomeric carbon atom, renders the split of the O bridge and again yields the two glucose units. Water molecules and the glycosidic bond split simultaneously and form two glucoses again. By extension of these reactions, glucose monomers were generated.

4. CONCLUSION

Hydrothermal extraction of β-glucan from *G. lucidum* was conducted at 433 K and 4.0 MPa using a semi-batch system. Under these conditions, thermal softening of *G. lucidum* occurred, allowing the removal of *G. lucidum* components that were protecting its other constituents via dehydrogenation and deoxygenation reactions. Next, the micronisation of the extract solution was carried out using an inert gas as a drying medium. The results showed that the moisture evaporated faster than when using normal air at the same inlet gas temperature. As a result, particle surface corrugation occurred in all experiments. The β-glucan content in the particle products was around 40 to 45% in weight. Since the system is suitable for isolating β-glucan from *G. lucidum*, it is proposed that this process can be applied to the isolation of β-glucan from other types of materials as well.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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