Phytase Production by Grifola Frondosa and Its Application in Inositol-Enriched Solid-State Fermentation Brown Rice

Shih-Jeng Huang
Department of Health Food, Chung Chou University of Science and Technology, Taiwan, ROC

Chieh-Han Chen and Shu-Yao Tsai
Department of Food Nutrition and Health Biotechnology, Asia University, Taichung, Taiwan, ROC
Email: sytsai@asia.edu.tw

***Abstract*—Mushrooms can produce a variety of enzymes and therefore have many biotransformation capacities. Phytic acid is a ubiquitous anti-nutritional factor in many plant foods, which results in a lack phytase for monogastric animals (humans, pigs, and chickens) reducing digestion and absorption of metal elements, protein, starch and lipid. Phytase is used commercially to maximize phytic acid degradation and to increase inositol in animals. We analyzed the phytase activity based on 24 kinds of mushroom fruiting bodies. The results showed *G. frondosa* has higher phytase activity (1.85 μmol/min of tissue). In liquid-state fermentation, *G. frondosa* has high phytase activity of 2.46 μmol/min at 12th grown day. The increase of inositol content in *G. frondosa* solid-state fermentation brown rice was 176-fold as compared to that in the un-fermentation controls. The inositol-enriched *G. frondosa*-fermented brown rice demonstrated greater antioxidant properties and contained the highest antioxidant components than un-fermented brown rice.**

***Index Terms*—phytase, mushroom, inositol, solid-state fermentation, antioxidant**

I. Introduction

Phytases (such as *myo*-inositol hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8), belong to a family of enzymes that catalyze the hydrolysis of phytic acid and generate inositol phosphates, *myo*-inositol and inorganic phosphate [1]-[3]. The commercial phytase are commonly used as a food and feed additive that enhances the uptake of proteins, phosphorus and vital divalent ions [4], [5]. Inositol is regarded as an essential element in many organisms, and is classified as a member of the vitamin B-complex (often called vitamin B_8) [6]. In animal nutrition, inositol plays a vitamin-like function and its deficiency causes incomplete development, fatty liver, and alopecia [7]. Inositol is contained abundantly in vegetables and fruits and is also present in mushrooms.

Grifola frondosa [(Dickson: Fries) Gray], also called the king of mushrooms and the hen of the woods, is one of the specialty mushrooms commercially available in Taiwan [8]. From mushrooms, the studies reported are limited to enzyme activity achieved during cultivation such as liquid-state and solid-state fermentation, for example, on the phytase from *Agaricus bisporus* [9], *Flammulina velutipes* [10], *Ganoderma* sp. [11], *Grifola frondosa* [9], *Lentinus edodes* [12], *Pleurotus ostreatus* [9], *Schizophyllum commune* [13] and *Volvariella volvacea* [14].

Solid-state fermentation (SSF) involves the growth and metabolism of microorganisms on solid materials, which generally have a low water content to reduce the risk of contamination [8]. There are several recent publications describing the development of SSF for utilizing such raw materials for the production of food matrixes and value-added fine products such as enzymes, flavonoids, phenols, amino acids, organic acids, and biologically active secondary metabolites, among others [8].

Rice is an important staple food in Asian countries. In rural areas it is also a major source of micronutrients. Unfortunately, the bioavailability of minerals, e.g., zinc from rice, is low because it is present as an insoluble complex with food components such as phytic acid [15]. Brown rice contains more phytic acid than any other grain, even oats: 1250 mg/100 g of phytic acid and oats have 1170 mg/100 g.

So this study aimed to obtain high phytase activities by *Grifola frondosa* under liquid-state and solid-state fermentation. The antioxidant properties of solid-state fermentation brown rice, including its reducing power, scavenging ability of radicals, and chelating ability of ferrous ions, were determined and compared with those of the unfermented samples. The flavonoids and total phenols of potential antioxidant components were also determined.

II. Materials and Methods

A. Mushroom Fruiting Bodies Collection

Totally, 21 species of mushroom fruiting bodies were used and categorized into edible and medicinal mushrooms, including *Agaricus bisporus*, *Agaricus blazei*, *Ganoderma tsugae*, *Agrocybe cylindracea*,

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Auricularia auricular, *Flammulina velutipes*, *Grifola frondosa*, *Hericium erinaceum*, *Hypsizigus marmoreus*, *Lentinula edodes*, *Pholiota nameko*, *Pleurotus citrinopileatus*, *Pleurotus cystidiosus*, *Pleurotus eryngii*, *Pleurotus ferulae*, *Pleurotus eryngi* var. *ferulae*, *Pleurotus ostreatus*, *Pleurotus nerbrodensis*, *Pleurotus salmoneostramineus*, *Tremella fuciformis*, and *Volvariella volvacea* were obtained from Q-Yo Bio-Technology Farm, Pusin, Chunghua, Taiwan. Three strains of P. ostreatus, including Japan, Korea and Taiwan strains, are commercially available. Normal and white strains of H. marmoreus were also used. We selected the highest levels of phytase activity of fruiting bodies, performing cultured mycelia.

B. Liquid-State Fermentation of G. Frondosa

Mycelia cultures were obtained from the inner living tissues of the *G. frondosa* fruit bodies in potato dextrose agar (Difco Laboratories, Sparks, MD, USA) medium at 25 ℃. Each of 5 mL of G. frondosa cultures was inoculated into 100 mL of liquid medium in sterilized 250-mL Erlenmeyer shake flasks at an inoculation ratio of 5% (v/v) and incubated at 25 ℃ and 125 rpm. The liquid medium (1 L) consisted of 20 g of glucose, 5 g of yeast extract, 0.068 g of KH_2PO_4, and 2.3 g of $MgSO_4 \bullet 7H_2O$. Samples taken at specified times were assayed for the measurement of biomass, pH, and phytase activity. We selected the highest levels of phytase activity of *G. frondosa*, performing cultured solid-state fermentation of brown rice.

C. Solid-State Fermentation of Brown Rice by G. Frondosa

This *G. frondosa* seed culture containing mycelia was ready to serve as inoculum for brown rice fermentation. The brown rice (15 g) was soaked with 45 mL water for 2 h and then sterilized at 121 ℃ for 15 min. The culture was homogenized for ten seconds in a Waring blender (7011G; Torrington, CT, USA). Then, 5 mL of the homogenized seed culture was inoculated into the autoclaved brown rice, mixed thoroughly and placed in a 90 × 15 mm Petri dish, incubated at 25 ℃ for 30 days. After fermentation, the mycelia and *G. frondosa* solid-state fermentation brown rice (GFBR) were freeze-dried and ground into powder using a Retsch Ultracentrifugal Mill and Sieving Machine (Haan, Germany) to obtain fine powder (40 mesh) for further analysis. Unfermented brown rice (BR) was also freeze dried as control.

D. Mushroom Tissue Extract for Phytase Activity Analysis

Samples were homogenized in five parts of ice-cold 20 mM sodium acetate buffer (pH 5.5) containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) in a Waring blender for 60 s [9]. Samples were centrifuged for 30 min at 25000 g at 4 ℃, and the supernatants were filtered through Whatman No. 4 filter paper. After filtering, samples were assayed for phytase activity.

E. Analytical Methods

Phytase activity of mushroom tissue extract, extracellular cultured liquid and solid-fermentation extract was measured by using a modified method [11] based on the amount of phosphate released from sodium phytate. Enzyme activity, U, was defined as the average number of l mole/min of phosphate released by phytase from fortified sodium phytate at 37 ℃ for 65 min. The amount of phosphate in the clear, upper portion was determined by measuring the absorbance at 415 nm.

The samples of pH were measured immediately after removal from production by diluting 5 g samples with 30 mL water. Biomass (ergosterol) was determined according to Huang *et al.* [8] with some modification. Ergosterol was quantified by monitoring absorbance at 282 nm and comparing the peak area of the sample to peak areas obtained from different concentrations of the authentic standard. Phytic acid content in the extract was quantified by using spectrophotometric method [16]. The method was calibrated with standard phytic acid solutions for each set of analysis. Inositol was extracted and analyzed as described by Huang *et al.* [8].

F. Preparation of Extracts for Analysis of Antioxidant Properties and Components

Ethanolic extraction was performed using 10 g sample powder, and 100 mL 95% (v/v) ethanol was put into a glass beaker and shaken by rotary incubator shaker with extraction time at 25 ℃ and 150 rpm for 24 h and filtered. The filtrate was then evaporated on a rotary evaporator at 40 ℃ to dryness. The dried ethanolic extracts were stored at -20 ℃ until the analyses were carried out. Reducing power of extracts was determined, which followed the method described by Oyaizu [17]. The ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich) radicals was determined based on the work of Shimada *et al.* [18] The scavenging ability assayed is the ability of the extracts to react with DPPH radicals and to reduce most DPPH radical molecules. Chelating ability was determined according to the method described by Dinis *et al.* [19]. Chelating ability is the ability of the extracts to inhibit the complex formation of ferrozine with ferrous ions. The value (mg extract/mL) at a half-maximal effective concentration (EC_{50}) is the concentration at which the absorbance was 0.5 for reducing power; DPPH radicals were scavenged by 50%, ferrous ions were chelated by 50%. The EC_{50} values were obtained by interpolation from linear regression analysis.

Flavonoids of extracts were determined according to the method of Zhishen *et al.* [20] The absorbance was measured at 510 nm, with rutin (Sigma-Aldrich) used as standard. Results were expressed as milligrams of rutin (RE) equivalents per gram of extract. Total phenols of extracts were determined according to the method of Taga *et al.* [21]. The absorbance was measured at 750 nm, with gallic acid (Sigma-Aldrich) used as standard. Results were expressed as milligram of gallic acid equivalents (GAE) per gram of extract.

G. Statistical Analysis

All data were subjected to an analysis of variance using the Statistical Analysis System v. 94 (SAS Institute, Inc., Cary, NC, USA). When a significant difference was found among treatments, Duncan's multiple range tests

were performed and mean values at the level of $\alpha = 0.05$. The EC_{50} values were obtained from linear regression analysis.

III. Results and Discussion

A. Mushroom Fruit Body Screening

There were 24 mushroom fruit bodies which can produce phytase, mentioned in Fig. 1. Phytase activity ranged from 0.94-1.85 μmol/min of 24 kinds of fruit body tissue extract. *G. frondosa* had highest levels of phytase activity (1.85 μmol/min of tissue). *Pleurotus ostreatus* ranked second in level of phytase activity (1.80 μmol/min of tissue). There were 11 kinds of fruit body tissue extract with phytase activity between 1.70-1.79 μmol/min. This study represents the first survey of phytase activity in many popular edible mushrooms in Taiwan. Phytase activity was detected in all mushrooms, *G. frondosa* had highest level of phytase activity. Although the phytase levels detected in mushroom are relatively low, degradation of phytic acid from cereal by mushroom phytase does appear to be a good candidate for direct use in the human diet.

B. Growth Curve and Phytase Production of Liquid-State Fermentation by G. Frondosa

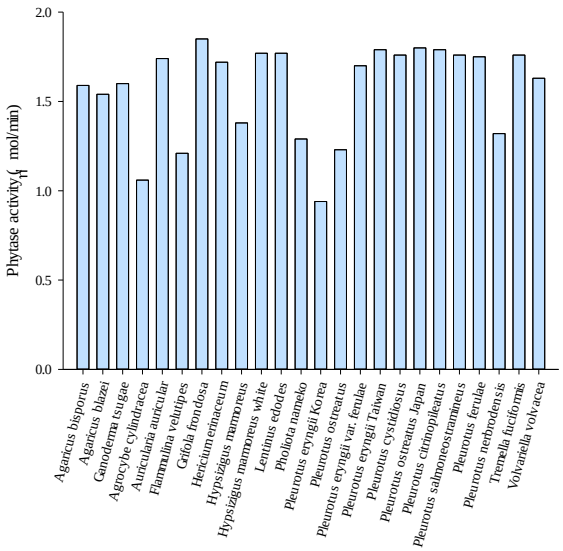

Figure 1. Phytase activity of various mushrooms fruiting body.

Liquid-state fermentation has become a promising alternative for efficient production of valuable mushroom metabolites. The time course of phytase production and growth in liquid-state fermentation by *G. frondosa* is shown Fig. 2. According to the results, phytase production was directly associated with biomass. Maximum phytase activity (2.46 μmol/min) was obtained after 12 days of fermentation, and biomass (0.52 g/100 mL of culture) was also at a maximum at this point. Phytase production from mushroom mycelia has the additional advantage of utilizing low value substrates or waste materials as carbon source. We selected the maximum days of phytase activity to from *G. frondosa* mycelia, brown rice degradation phytic acid, and then bio-transformation to inositol. The highest number of days of *G. frondosa* mycelium phytase activity was selected. Mycelia was used to degrade phytic acid from brown rice and convert it to inositol.

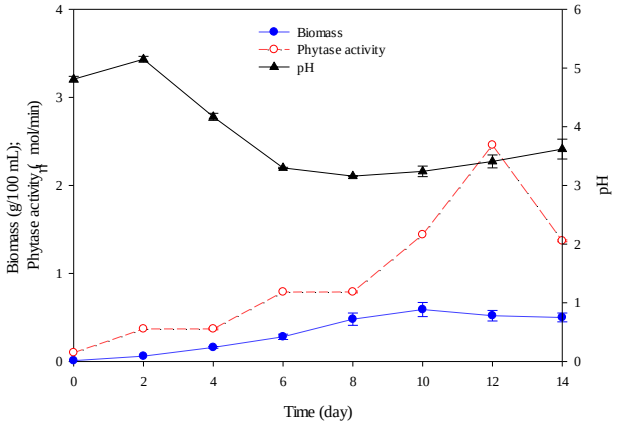

Figure 2. Profile of fermentation for phytase production in Erlenmeyer flasks using optimized medium (biomass, phytase activity and pH) in liquid-state fermentation by *Grifola frondosa*.

C. Growth Curve, Inositol Production and Phytic Acid Degradation of Solid-State Fermentation Brown Rice by G. Frondosa

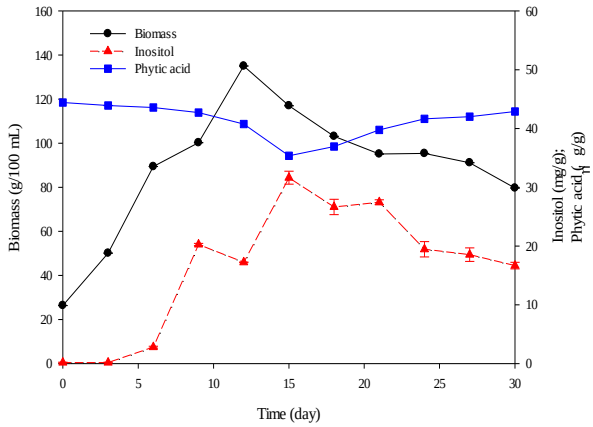

Figure 3. Profile of fermentation for inositol production in petri dish using optimized medium (biomass, inositol and phytic acid) in solid-state fermentation brown rice by Grifola frondosa.

Phytic acid is the major storage form of phosphate in cereal grains, which are the principal components of animal feeds. Phytase acts on phytic acid, an antinutrient factor present in animal feeds, and releases inorganic phosphate and inositol. We optimized the production parameters for inositol production and phytic acid degradation using *G. frondosa*, by solid-state fermentation. The time course of inositol production, phytic acid degradation and growth in solid-state fermentation is shown in Fig. 3. Inositol production and phytic acid degradation was directly associated with biomass. Maximum inositol content (31.64 mg/g) and minimal phytic acid content (35.36 μg/g) were obtained after 15 days of fermentation, and biomass (117.02 mg/g of culture) was at the second highest at this point. The increase of inositol content in *G. frondosa* solid-state fermentation brown rice was 176-fold as compared to that

in the unfermented controls. Furthermore, the degradation of phytic acid content in *G. frondosa* solid-state fermentation brown rice was 20-fold as compared to that in the unfermented controls. Overall, the use of *G. frondosa* strains of solid-state fermentation of brown rice has the ability to degrade phytic acid and then produce inositol.

D. Antioxidant Properties and Antioxidant Component of Inositol-Enriched Solid-State Fermentation Brown Rice by G. Frondosa

The antioxidant properties assayed herein are summarized in Table I, and the EC_{50} values (mg ethanolic extracts per mL) were calculated for comparison. The effectiveness of antioxidant properties correlates inversely with their EC_{50} values. The EC_{50} values from GFBR in terms of antioxidant properties (3.39-5.51 mg/mL) were lower than BR (4.08-9.83 mg/mL). For the reducing power and chelating ability assays, the EC_{50} values of GFBR demonstrated greater effectiveness than those from the BR and mycelia samples. In addition, for the scavenging ability assays, the EC_{50} values of mycelia demonstrated greater effectiveness than those from the GFBR and BR samples. Overall, the ethanolic extracts from inositol-enriched *G. frondosa*-fermented brown rice and mycelia demonstrated greater antioxidant properties than un-fermented substrates. Many flavonoids are shown to have antioxidative activity, free radical scavenging capacity, are hepatoprotective and anti-inflammatory. Phenols such as BHT (butylated hydroxytoluene) and gallate are known to be effective antioxidants. GFBR contained the highest flavonoid and total phenol content (Table I). A high amount of total phenol and flavonoids in ethanol extracts can explain its higher reducing ability and DPPH free radical scavenging ability. Due to their free radical scavenging abilities and ferrous ion chelating abilities, phenols may possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases. Overall, inositol-enriched *G. frondosa* can enhance flavonoid and total phenolic contents by solid state fermentation.

TABLE I. EC50 VALUES OF ANTIOXIDANT PROPERTIES, FLAVONOIDS AND TOTAL PHENOLS CONTENTS OF BR, GFBR AND MYCELIA

	BR[a]	GFBR[a]	Mycelia
Reducing power EC_{50} value (mg extract/mL) [b]	7.98±0.07a[c]	5.51±0.02c	5.54±0.09b
Scavenging ability EC_{50} value (mg extract/mL) [b]	4.08±0.02a	3.39±0.06b	1.27±0.01c
Chelating ability EC_{50} value (mg extract/mL) [b]	9.83±0.03a	4.20±0.02c	4.37±0.04b
Flavonoids (mg/g)	0.01±0.00c	1.56±0.02a	0.28±0.04b
Total phenols (mg/g)	0.34±0.00c	0.97±0.00a	0.74±0.01b

[a] BR, brown rice; GFBR, *G. frondosa* fermented brown rice.
[b] EC_{50} value: the absorbance of 0.5 for reducing power; 50% of 2,2-diphenyl-1-picrylhydrazyl radicals scavenged; and 50% of ferrous ions chelated. The EC_{50} value was obtained by interpolation from linear regression analysis.
[c] Different small letters within a row indicate significant differences ($p<0.05$) in the sample.

IV. CONCLUSIONS

G. frondosa was found to contain the highest phytase activity. Significant antioxidant properties took place during *G. frondosa* fermentation, allowing for the preparation from brown rice. The *G. frondosa* fermented brown rice with higher content inositol had greater flavonoids and total phenols than the unfermented control. Based on the results, mushrooms are safe to eat and rich in phytase, the culture media can not only use phytase to break down phytic acid into inositol. The mushroom having physiologically active substances to increase the added value. Therefore, the use of mushrooms to decompose phytic acid can not only reduce the damage of phytic acid, but inositol content also is increased, while improving the physiological activity of the fermentation products. Further studies on the preference and health-promoting activity of the cereal-based food treated by phytase preparation and inositol production with mushroom are now in progress.

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Shu-Yao Tsai was born in Chiayi, Taiwan. She is currently a professor in the Department of Food Nutrition and Health Biotechnology at Asia University in Taiwan. She received her Ph. D. degree in Department of Food Science and Biotechnology from National Chung-Hsing university in 2006. Her main research interests include aroma chemical and edible and medicinal mushroom.