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Changes in some components of soymilk during fermentation with the basidiomycete *Ganoderma lucidum*

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ABSTRACT

Soymilk was fermented with the basidiomycete *Ganoderma lucidum* WZ02 and the changes in the contents of polysaccharide, sugars, crude protein, B-vitamins, free amino acids and isoflavones were analyzed. Polysaccharide and crude protein were increased by the fermentation of *G. lucidum* while most free amino acids were reduced. The flatulence factor (e.g. stachyose and raffinose) was significantly decreased and stachyose was not detected after 72 h of fermentation. The contents of thiamin, riboflavin, and niacin were increased during the fermentation. Most of isoflavone glycosides were converted to aglycones and the contents of daidzein and genistein were increased by the fermentation of *G. lucidum*. The results suggested that fermentation by *G. lucidum* could improve the acceptability and health properties of soymilk.

1. Introduction

Ganoderma lucidum (Fr.) Karst (Polyporaceae), a medicinal fungus called "Lingzhi" in China and "Reishi" in Japanese, is one of the most famous traditional Chinese medicines. In regions of China and other Asian countries, Lingzhi has been used as a remedy to promote health and longevity (Shiao, 2003). Modern pharmaceutical researches show that Linzhi has many interesting bioactivities, including antitumor, immuno-modulating, hepato-protection, hypoglycemia, and platelet-aggregation inhibition effects (Chen et al., 2004; Lin, 2005; Wang et al., 1997). Bioactive compounds from fruiting body and mycelia of *G. lucidum* include polysaccharides, triterpenoids, steroids, alkaloids, nucleotides, lactones, and fatty acids (Shiao, 2003). Because of its perceived health benefits, Lingzhi has gained wide popularity as a health food in China, Japan, Korea and Taiwan.

Soymilk, the water extract of soybeans, is an inexpensive source of protein and calories for human consumption and is seen as a low-cost substitute for dairy milk for the poor in the developing countries. Being free of cholesterol, gluten and lactose, soymilk is also a suitable food for lactose-intolerant consumers, vegetarians and milk-allergy patients (Chou & Hou, 2000). However, the presence of raw bean flavour and indigestible oligosaccharides, such as stachyose and raffinose, limits the wide consumption of soymilk and other soybean products (Girigowda & Mulimani, 2006). To overcome these limitations, fermentation of soymilk with various microorganisms, especially lactic acid bacteria and bifidobacteria, has been attempted (Beasley, Tuorila, & Saris, 2003; Chou & Hou, 2000; Garro, de Valdez, Oliver, & de Giori, 1998; Scalabrini, Rossi, Spettoli, & Matteuzzi, 1998; Wang, Yu, & Chou, 2002).

As a white-rot fungus, *G. lucidum* could use various substrates for mycelial growth and metabolite production (Han, An, & Yuan, 2005). Fermented soymilk (with *G. lucidum)* has become a health protein beverage in China (Shao & Tang, 2002). However, few reports have paid attention to the changes in the components of soymilk by the fermentation with *G. lucidum*. In this work, Soymilk was fermented with the basidiomycete *G. lucidum* and the changes in the contents of polysaccharide, sugars, crude protein, B-vitamins, free amino acids and isoflavones during the fermentation were examined.

2. Materials and methods

2.1. Preparation of soymilk

Soybeans were purchased from retail markets in Wenzhou, China. Care was taken to ensure that good quality and mould-free soybeans were selected. Whole soybeans were first washed and soaked overnight in 0.5% sodium bicarbonate at 30 °C, and then blanched in boiling water for 30 min. The blanched soybeans were dehulled and blended with 10 times their weight of distilled water for 3 min in a blender. The resultant slurry was then filtered

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through a double-layered cheesecloth to yield soymilk. Soymilk was dispensed into containers and autoclaved for 15 min at 121 °C.

2.2. Microorganism and fermentation

The strain of *G. lucidum* WZ02 was screened and collected in the Laboratory of Fermentation Engineering, Wenzhou University (Wenzhou, China). The strain was maintained by bimonthly subcultivation on potato-agar-dextrose slants. Slants were incubated at 30 °C for 7 days and then stored at 4 °C.

Actively growing mycelia were obtained from a newly prepared agar-plate culture after it was incubated for 7 days at 30 °C. Around 0.3 cm × 0.3 cm sections of the mycelia were then transferred into a 500 ml flask that contained 100 ml of seed culture media, which consisted of the following components (g l^{-1}): glucose, 35; peptone, 5; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.75; vitamin B_1, 0.01. Initial pH of the media was adjusted to 6.5 before sterilization, where glucose was separately sterilized. The seed culture was carried out at 30 °C on a rotary shaker incubator at 160 rpm for 7 days. The soymilk was then inoculated with 10% (v/v) of the seed culture and then cultivated in a 10 l (working volume) stirred fermenter (East biotech Co. Ltd., China). The fermentations were performed under the following conditions: temperature, 30 °C; aeration rate, 1 vvm (volume(air)/volume (fermentation broth)/minute); agitation speed, 150 rpm.

2.3. Analytical methods

For measurement of mycelia biomass, the mycelia were obtained by centrifugation of a sample at 5000 *g* for 20 min, washing the precipitate three times with water, and drying the mycelia to constant weight at 60 °C. The concentration of fungal biomass was determined gravimetrically. After removal of mycelia, the crude polysaccharide was precipitated by adding 4 vol.95% (v/v) ethanol. The precipitated polysaccharide was collected by centrifugation at 5000 *g* for 20 min, and washed with 80% (v/v) ethanol three times, then dried to remove residual ethanol at 60 °C. Total exopolysaccharide was determined by the phenol/sulphuric acid assay.

The crude protein content of the samples was calculated by multiplying the nitrogen (N) content by a factor of 6.25, which was determined by the Kjeldahal method (KDN-08A, Shanghai Xinjia Electron Co. Ltd., China). Amino acids were analyzed using a Hitachi Amino Acid Analyser, Model Hitachi 835-50 (Tokyo, Japan). Samples were filtered (0.45 μm) to remove the sediments, and the supernatant was injected into the amino acid analyzer for the determination of the amino acid composition. The amino acids were separated on an ion-exchange resin 2619 Column (2.6 × 150 mm) using sodium citric acid buffer at pH 2.2, a flow rate of 0.225 ml min^{-1}, a column temperature of 85 °C and postcolumn reaction with ninhydrin (0.3 ml min^{-1} ninhydrin flow rate), followed by photometric detection at 570 nm according to the procedure described by Li, Beta, Sun, and Corke (2006). Individual amino acids were quantified on the basis of amino acid standard (AAS18, Sigma Chemical Co., USA).

The contents of niacin, riboflavin and thiamin were determined by an HPLC method as described by Hou, Yu, and Chou (2000). Five-gramme samples were mixed with 50 ml of 0.1 N H_2SO_4 and were autoclaved for 30 min at 121 °C. After cooling, they were adjusted to pH 4.5 with 2.0 M acetate and mixed with 5.0 ml Takadiastase (1 M) and 5.0 ml papain. The contents were incubated overnight at 35 ± 1 °C, then diluted to 100 ml with distilled deionized water and filtered through 0.45 μm membrane and subjected to HPLC analysis. The HPLC equipment used was Agilent 1100 fitted with a 4.6 × 250 mm Diamonsil C-18 column. The mobile phase was prepared by mixing 390 ml of HPLC grade methanol, 600 ml of distilled deionized water and 10 ml of glacial acetic acid. Twenty-five milligrams each of the PIC-5 and PIC-7 ion partitioning reagents was added and the solution was filtered through a 0.45 μm membrane and deaerated by vacuum. The flow rate and column temperature were 1 ml min^{-1} and 30 °C, respectively. The wavelength for the detector was 260 nm.

Sugar contents were measured by HPLC according to Hou et al. (2000). The HPLC equipment used was a Waters 600/2410 system equipped with a 6.5 × 300 mm Sugur-Pak™ column. The mobile phase was distilled deionized water. The flow rate and column temperature were 0.5 ml min^{-1} and 85 °C, respectively. Eluted sugars in the effluent were quantified with an RI detector. Sugar standards, including sucrose, stachyose and raffinose, were all obtained from Sigma Chemical Company.

Isoflavones in the broth were quantitative analyzed by HPLC using the method of Hoeck, Fever, Murphy, and Grace (2000). The HPLC equipment used was Agilent 1100 equipped with a 6.0 × 250 mm lichrospher C-18 column. A linear HPLC gradient was employed: solvent A was 1% glacial acetic acid in distilled water, and solvent B was absolute methanol. Following the injection of the sample, solvent B was increased from 15% to 100% over 20 min, then held at 100% for 30 min. The flow rate and column temperature were 1 ml min^{-1} and 35 °C, respectively. The wavelength for the detector was 250 nm.

3. Results and discussion

3.1. Changes of polysaccharide and crude protein in soymilk during fermentatiom

In this work, soymilk was fermented by *G. lucidum*. As shown in Fig. 1, the maximal mycelial biomass of *G. lucidum* (10.85 g l^{-1}) was obtained at 72 h. The polysaccharide in soymilk was assimilated by *G. lucidum*, and the concentration was decreased in 24 h from 0.16 g l^{-1} to 0.1 g l^{-1}. Then, exopolysaccharide was secreted with the growth of *G. lucidum* and the highest polysaccharide concentration reached 0.32 g l^{-1} at 84 h. Polysaccharides from *G. lucidum* have many interesting biological activities, including immunostimulating, antitumor, and free radical scavenging effects (Chen et al., 2004; Lin, 2005; Wang et al., 1997); fermentation resulting from the increasing of polysaccharide could enhance the nutritive value of soymilk. Moreover, The contents of crude protein in soymilk fermented by *G. lucidum* increased from 2.14 g l^{-1} to 3.21 g l^{-1}after 96 h of fermentation. The crude protein from the mycelia of *G. lucidum* may contribute to the increased content of crude protein.

3.2. Changes of free amino acid content in soymilk during fermentation

As shown in Table 1, the total content of amino acid was decreased from 7.51 mg ml^{-1} at the beginning to 4.71 mg ml^{-1} after 96 h of fermentation by *G. lucidum*. The contents of individual free amino acids changed little in 48 h, but most free amino acids were reduced after 96 h of fermentation. Of all the 17 amino acids determined, the levels of serine, tyrosine, phenylalanine, leucine, and lysine were decreased by about 50%, and the levels of aspartic acid, histidine, glycine, threonine, and proline were slightly reduced. As reported by Chang, Tsai, and Houng (2006), amino acids were good for nutrition of *G. lucidum* (Chang et al., 2006). The free amino acids in soymilk could be assimilated by *G. lucidum* for growth and metabolite production.

3.3. Changes of B-vitamin content in soymilk during fermentation

Some researches have revealed that fermentation of food could improve the nutritional value by increasing the content of

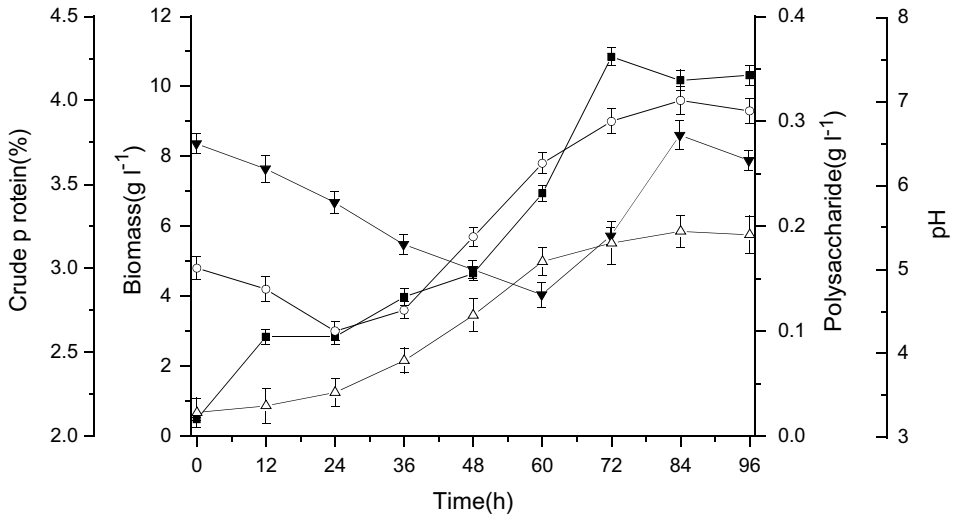

Fig. 1. Time courses of soymilk fermentation by *G. lucidum* in 15 l fermenter under the following conditions: temperature, 30 °C; aeration rate, 1 vvm (volume (air)/volume (fermentation broth)/minute); agitation speed, 150 rpm, and experiments were performed in triplicate. ■, biomass of *G. lucidum*; ○, polysaccharide; ▼, pH; △, crude protein.

Table 1
Changes of amino acid contents in soymilk during the fermentation by *G. lucidum*[a]

Amino acids content (mg ml^{-1})	Fermentation time (h)				
	0	24	48	72	96
Aspartic acid	0.85 ± 0.037	0.85 ± 0.039	0.88 ± 0.043	0.80 ± 0.028	0.67 ± 0.027
Glutamic acid	1.65 ± 0.051	1.67 ± 0.060	1.68 ± 0.062	1.40 ± 0.048	0.96 ± 0.041
Serine	0.41 ± 0.019	0.39 ± 0.022	0.39 ± 0.023	0.31 ± 0.014	0.22 ± 0.010
Histidine	0.21 ± 0.013	0.20 ± 0.011	0.21 ± 0.011	0.20 ± 0.010	0.20 ± 0.010
Glycine	0.36 ± 0.019	0.36 ± 0.014	0.37 ± 0.015	0.35 ± 0.012	0.28 ± 0.012
Threonine	0.26 ± 0.011	0.26 ± 0.012	0.27 ± 0.012	0.25 ± 0.011	0.24 ± 0.012
Arginine	0.60 ± 0.022	0.55 ± 0.024	0.51 ± 0.023	0.36 ± 0.013	0.25 ± 0.013
Alanine	0.33 ± 0.014	0.32 ± 0.013	0.32 ± 0.014	0.27 ± 0.012	0.20 ± 0.012
Tyrosine	0.27 ± 0.012	0.21 ± 0.010	0.21 ± 0.009	0.17 ± 0.008	0.13 ± 0.008
Cystine	0.06 ± 0.003	0.06 ± 0.003	0.07 ± 0.004	0.06 ± 0.003	0.06 ± 0.002
Valine	0.36 ± 0.012	0.34 ± 0.013	0.35 ± 0.014	0.34 ± 0.014	0.25 ± 0.013
Methionine	0.02 ± 0.002	0.02 ± 0.001	0.03 ± 0.002	0.04 ± 0.002	0.06 ± 0.003
Phenylalanine	0.42 ± 0.018	0.40 ± 0.019	0.39 ± 0.013	0.33 ± 0.015	0.26 ± 0.013
Isoleucine	0.37 ± 0.014	0.35 ± 0.014	0.35 ± 0.014	0.33 ± 0.011	0.24 ± 0.011
Leucine	0.61 ± 0.022	0.55 ± 0.016	0.53 ± 0.017	0.42 ± 0.012	0.28 ± 0.015
Lysine	0.45 ± 0.019	0.43 ± 0.020	0.39 ± 0.011	0.29 ± 0.009	0.22 ± 0.009
Proline	0.28 ± 0.011	0.29 ± 0.016	0.28 ± 0.014	0.24 ± 0.009	0.19 ± 0.010
Total	7.51 ± 0.294	7.25 ± 0.265	7.23 ± 0.213	6.16 ± 0.187	4.71 ± 0.226

[a] Measurements were performed in triplicate.

vitamins. Tamine, Marshall, and Robinson (1995) reported that cultured dairy products had higher contents of folic acid, niacin, biotin, vitamins B6 and B12 than had milk. Hou et al. (2000) reported that thiamin and riboflavin contents were increased in soymilk fermented with bifidobacteria. Table 2 showed the changes of B-vitamin content in soymilk during fermentation, and the contents of niacin, riboflavin and thiamin were all increased by fermentation with *G. lucidum*. The content of riboflavin showed the largest magnitude, increasing from 0.17 μg ml^{-1} at the beginning to 2.62 μg ml^{-1} after 96 h of fermentation.

Table 2
Changes of B-vitamin content in soymilk during the fermentation by *G. lucidum*[a]

Fermentation time (h)	Content (μg ml^{-1})		
	Niacin	Riboflavin	Thiamin
0	0.86 ± 0.037	0.17 ± 0.009	16.5 ± 0.62
24	2.64 ± 0.148	1.23 ± 0.041	25.3 ± 0.87
48	2.58 ± 0.129	2.25 ± 0.035	26.9 ± 0.72
72	2.08 ± 0.112	2.87 ± 0.043	25.3 ± 0.58
96	2.89 ± 0.106	2.62 ± 0.057	33.1 ± 0.69

[a] Measurements were performed in triplicate.

3.4. Changes of sugar content in soymilk during fermentation

Soybean oligosaccharides have prebiotic effects and studies have shown that their consumption is related to several health benefits, such as lowering of blood cholesterol, increased absorption of minerals, and prevention of some types of cancer (Mussatto & Mancilha, 2007; Roberfroid, 2007). However, one factor for the low consumer acceptability was the presence of high levels of non-digestible oligosaccharides (e.g. stachyose and raffinose). These oligosaccharides were degraded by bacteria in the human intestine to carbon dioxide, hydrogen and methane, which cause flatulence and abdominal pain (Girigowda & Mulimani, 2006). There were reports that these carbohydrates could be hydrolyzed by some microorganisms, such as lactic acid bacteria and bifidobacteria, to produce highly nutritious soymilk foods (Mital & Steinkraus, 1975; Hou et al., 2000; Leblanc, Garro, & de Giori, 2004). Tang and Zhong (2002) reported that *G. lucidum*

possessed high galactosidase activity and exhibited high hydrolyzing activity toward *o*-nitrophenyl glycosides. Table 3 showed that the contents of raffinose and stachyose were reduced significantly during the fermentation by *G. lucidum*, and stachyose was not detected, under the determination conditions, after 72 h of fermentation. That could be attributed to being hydrolyzed and being used as a carbon source for mycelial growth and metabolite production. The decreases of stachyose and raffinose by the fermentation with *G. lucidum* could enhance the acceptability of soymilk.

3.5. Changes of isoflavone content in soymilk during fermentation

Soybeans have high concentrations of isoflavones. In many studies, these soy isoflavones have been shown to have some health-enhancing properties, such as the prevention of certain cancers (Farina, Pomies, Alonso, & Gomez, 2006; Miura et al., 2002), lowering the risk of cardiovascular diseases (Goodman-Gruen & Kritz-Silverstein, 2001), an improvement of bone health (Weaver & Cheong, 2005). Soybean contains three 'families' of isoflavones as four distinct chemical structures, and most of the isoflavones in natural food materials exist in glycosylated form. However, the effective biological moieties of isoflavones are their aglycones, such as daidzein and genistein (Chien, Huang, & Chou, 2006; Choi, Kim, & Rhee, 2002; Izumi et al., 2000).

It has also been revealed that the β-glycosidic bonds of isoflavone glucosides could be hydrolyzed during fermentation of soybean by a number of microorganisms, including *Rhizopus oryzae*, *R. oligosporus*, *Saccharomyces rouxii*, *Bacillus subtilis*, *B. natto*, lactic acid bacteria and bifidobacteria, and isoflavone aglycones are abundant in fermented soy products, such as miso, natto, and tempeh (Chien et al., 2006; Choi et al., 2002; Miura et al., 2002). β-Glucosidase is widely distributed in microorganisms and plants, and in some basidiomycete mushrooms. Miura et al. (2002) reported that *G. lucidum* could convert isoflavone glycosides to aglycones by its β-glucosidase activity.

To examine whether isoflavone aglycones were increased by the fermentation by *G. lucidum*, the contents of daidzein and genistein were determined. As shown in Table 4, the contents of daidzein and genistein were increased from 1.89 and 2.34 μg ml^{-1} at the beginning to 16.7 and 17.1 μg ml^{-1} after 96 h of fermentation.

Table 3
Changes of sugar content in soymilk during the fermentation by *G. lucidum*[a]

Fermentation time (h)	Content (mg ml^{-1})		
	Stachyose	Raffinose	Sucrose
0	0.29 ± 0.016	0.48 ± 0.016	5.12 ± 0.064
24	0.16 ± 0.021	0.45 ± 0.017	3.93 ± 0.059
48	0.04 ± 0.003	0.29 ± 0.013	3.19 ± 0.071
72	-	0.06 ± 0.002	1.22 ± 0.033
96	-	0.07 ± 0.001	1.18 ± 0.042

[a] Measurements were performed in triplicate.

Table 4
Changes of isoflavone content in soymilk during the fermentation by *G. lucidum*[a]

Fermentation time (h)	Content (μg ml^{-1})	
	Daidzein	Genistein
0	1.89 ± 0.033	2.34 ± 0.042
24	2.40 ± 0.071	4.07 ± 0.195
48	7.26 ± 0.184	8.86 ± 0.158
72	15.3 ± 0.195	14.4 ± 0.317
96	16.7 ± 0.273	17.1 ± 0.395

[a] Measurements were performed in triplicate.

4. Conclusions

When fermented by *G. lucidum*, some components of soymilk were changed. The contents of crude protein, polysaccharide and B-vitamins were increased; the contents of free amino acids and sugars (e.g. sucrose, raffinose and stachyose) were decreased; isoflavone glucosides could be hydrolyzed and the contents of daidzein and genistein were increased. As a whole, the results suggested fermentation by *G. lucidum* could improve the health properties of soymilk.

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