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Full Length Research Paper

The effect of exopolysaccharide biosynthesis and related enzyme activities of *Grifola frondosa* by the addition of ethanol extracts from traditional Chinese medicine, *Gastrodia* tuber

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***Grifola frondosa* is an edible medicinal polypore mushroom and its exopolysaccharide (EPS) has demonstrated immunomodulatory effects in many previous studies. After adding the *Gastrodia* tuber components in culture medium of *G. frondosa*, both EPS and biomass production were significantly increased. As a result, we detected two enzymes which are related to EPS synthesis. Our results suggested that the activity of α-phosphoglucomutase (α-PGM) increased, while the activity of phosphoglucose isomerase decreased. Mixture components of ethanol extract of *Gastrodia* tuber was more effective than pure gastrodine. A maximum dry cell weight of 138.5 mg l^{-1} and the EPS at 0.606 g l^{-1} were obtained when the unprocessed *Gastrodia* tuber culture was added.**

Key words: Exopolysaccharide, phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), *Grifola frondosa*, *Gastrodia* tuber.

INTRODUCTION

Grifola frondosa, commonly known as Sheep's head, Ram's head, Hen of the woods and Maitake, is an edible medicinal polypore mushroom, and its exopolysaccharide (EPS) has demonstrated immunomodulatory effects in many previous studies. Nanba (1993) reported that the EPS of *G. frondosa* is able to activate various immune cells. Adachi et al. (1998) observed that the EPS of *G. frondosa* can activate the macrophages and cytotoxic T cells in mice. In addition, it has also been shown that it possess anti-tumor, anti-inflammatory, anti-oxidant, hypoglycemic, hypocholesterolemic, anti-HIV and immunostimulating effects (Suzuki I et al., 1989; Keiko et al., 1994; Inoue et al., 2002; Nadeem et al., 2002; Lee et al., 2003; Gary et al., 2009). Although the use of EPS of *G. frondosa* has been so seriously, there is no much effective and safe method to improve EPS production.

Looijesteijn et al. (1999) reported that the phosphoglucose isomerase (PGI) which is responsible for lactate formation and the α-phosphoglucomutase (α-PGM) which is involved in EPS formation are important at the branch point between the Embden–Meyerhof–Parnas (EMP) pathway and the later part of EPS biosynthesis. Fungal synthesis of EPS is also primarily through the EMP and gluconeogenesis pathways. The glucose-6-phosphate (G-6-P) is the common precursor of these pathways and is related to the two important enzymes, PGI and α-PGM. Based on these, we detected the activity of two key enzymes which could explore the effects of the components of *Gastrodia* tuber on metabolism of *G. frondosa*.

Gastrodia tuber, a common valuable traditional Chinese medicine, has been used for thousands of years in the history of Chinese medicine. It is a good analgesic agent, tranquilizer and has an obvious effect on the

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Abbreviations: EPS, Exopolysaccharide; **α-PGM,** α-phosphoglucomutase.

central nervous convulsions. The components of *Gastrodia* tuber include gastrodin (*p*-hydroxymethylphenyl-*β*-D-glucopyranoside), *p*-hydroxybenzyl alcohol, vanillyl alcohol, parishin, gastrodioside, vanillin and so on. Among them, gastrodin are considered as the major active component (Liu et al., 2002). Many Chinese herbals need to grow with fungi, for example, *Gastrodia* tuber must grown with the fungus *Armillaria*. Recently, many Chinese scholars have proposed new ideas of fermenting traditional Chinese medicine in development of new technologies in traditional Chinese medicine processing. They believe that fermenting Chinese herbal medicine with fungus can significantly enhance the immune activities of these medicines. Yi et al. (2007) named "the bi-directional fermentation". Gao-Qiang et al. (2007) reported that the extracts of *Eupolyphaga sinensis* and *Catharsius molossus* can be used as easily available stimulators for the biomass or polysaccharide production by submerged fermentation with of *Ganoderma lucidum.* In addition, they also reported that the constituents of extracts responsible for the stimulatory effect on EPS production are currently unclear. There are many reports demonstrating that microbial fermentation of traditional Chinese medicine significantly improves their medicinal value and reduces toxicity, although the mechanisms of interaction between traditional Chinese medicine and metabolism of medicinal fungi still lack in-depth and systematic studies.

Our research group has studied the effects of adding a variety of traditional Chinese medicine to a fermentation medium of *G. frondosa* and remarkable improvement of EPS production was successfully achieved with *Gastrodia* tuber addition. In this work, we chose two kinds of *Gastrodia* tuber, one was bought from the pharmacy which was deeply processed and named *Gastrodia* tuber I, the other one was bought from the place of *Gastrodia* tuber origin without deep processing and named *Gastrodia* tuber II. Then, we compared the enzymes activity, EPS production and biomass when *Gastrodia* tuber I and II were added, respectively. And we also investigated the effect of adding mixture components of ethanol extract of *Gastrodia* tuber and the pure gastrodine.

MATERIALS AND METHODS

G. frondosa and growth

G. frondosa 51616 (purchased from Agricultural Culture Collection of China), grown on medium consisting of 30 g glucose, 5.5 g peptone, 1.5 g KH_2PO_4 and 0.75 g $MgSO_4$ per liter was inoculated with 15% (v/v) preculture broth. The fermentation was conducted at 30°C, 150 r/min (Zong-Yi and Tian-Xiang, 2011).

Pretreatment of *Gastrodia* tuber

For the preparation of the ethanol extracts, the powder of *Gastrodia* tuber I and II were soaked in ethanol (75%) for 48 h. The obtained extracts were filtered and then ethanol was removed under reduced pressure to obtain dry extracts. In the final step, the dry extracts were dissolved in water and the ethanol extract was obtained. The *Gastrodia* tuber I were purchased form Tong-Ren-Tang pharmacy and the *Gastrodia* tuber II were purchased from the place of *Gastrodia* tuber origin in GuiZhou Province, China.

Preparation of cell extracts

Samples, freshly taken from the fermentation broth, were centrifuged at 4000 r/min for 15 min. Then, the cell pellet were washed with PBS buffer (0.1 M, pH 7.2) and ground into pulp at 2 to 4°C. The pulp was centrifuged at 13000 r/min, 4°C for 15 min. The supernatant (the cell extract) was used as a source of enzymes.

Assay of α-PGM and PGI activities

α-PGM and PGI activity were defined as one mg protein in the enzymatic conversion of 1 μmol NAD(P)H in one minute as a unit. Therefore, the enzyme activity unit is $\mu molNADPH \cdot min^{-1} \cdot mg^{-1}$. The formation or consumption of NAD(P)H was determined by measuring the absorbance change at 340 nm (Qian and Grant, 1994).

The protein concentration of the cell extracts was determined by the method of Bradford and compared with a bovine serum albumin standard. The α-PGM reaction mixture contained 0.1 M TEA-HCL (pH 7.6), 1.98 mmol EDTA, 0.127 mmol β-NADP, 5 mmol $MgCl_2$, 1 U glucose 6-phosphate dehydrogenase and the cell extract. The reaction was initiated by adding 1.06 mmol α-glucose 1-phosphate.

The phosphoglucose isomerase reverse reaction mixture contained 0.1 M potassium phosphate (pH 7.2), 5 mmol $MgCl_2$, 0.127 mmol β-NADP, 1 U glucose 6-phosphate dehydrogenase and the cell extract. The reaction was initiated by adding 1.06 mmol fructose 6-phosphate. β-NADP, TEA-HCL, EDTA, glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co.

Determination of lactate concentration

Lactate concentration in the culture broth was determined by using a conventional enzymatic method (Chang-Xian and Xun, 2005). The lactate reaction mixture contained 0.15 M Tris-HCL(pH 9.2), 1.5 M hydrazine hydrate, 0.05 M EDTA, 1.7 mmol β-NAD, 10 U lactate dehydrogenase and fermentation broth. β-NAD, lactate dehydrogenase and Tris-HCL were purchased from Sigma Chemical Co.

EPS measurement and determination of dry cell weight

95% ethanol was added in a certain amount of fermentation supernatant to the ethanol content of 30% and it was left to stand at 4°C for 24 h. The precipitate was removed by centrifugation (4000 r/min, 15 min). Then, 95% ethanol was added into the supernatant and the ethanol content of 70%. Standing it at 4°C for 24 h, the supernatant was decanted by centrifugation (4000 r/min, 15 min). Then, the precipitate was dried and dissolved in water. The supernatant was measured by conventional phenolsulfuric acid method (Liang and Tian-Xiang, 2008). Dry cell weight was measured by gravimetrical method.

RESULTS AND DISCUSSION

Enhanced EPS production

Figure 1 shows the kinetics of EPS production of *G*

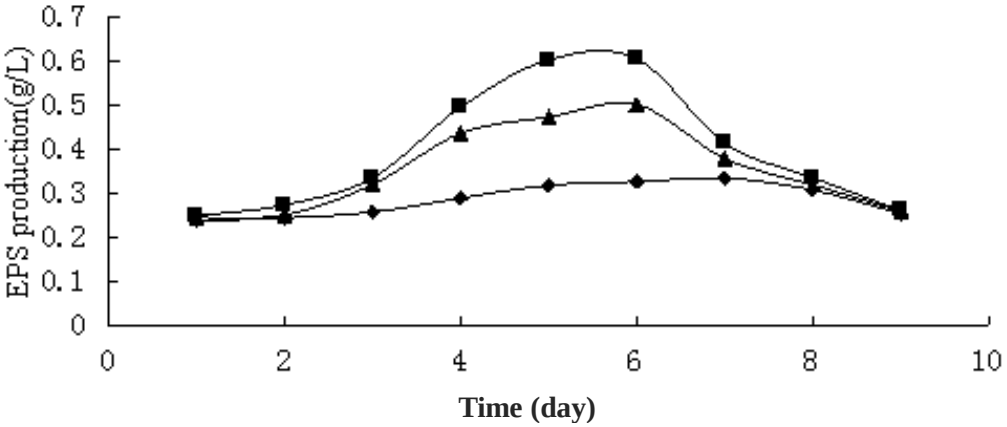

Figure 1. Time courses of exopolysaccharide (EPS) production with the addition of ethanol extracts of the *Gastrodia* tuber II, *Gastrodia* tuber I and the control group of *G. frondosa*. Symbols: Square, the *Gastrodia* tuber II; triangle, the *Gastrodia* tuber I; diamond, the control group.

Table 1. Comparison of EPS production of different strains of *G. frondosa* and enhancement of EPS production with different treatments.

***G. frondosa* strain**	**EPS production before optimization (g l^{-1})**	**EPS production after optimization (g l^{-1})**
G. frondosa 51616	0.334	0.606
G. frondosa from Korea (Bum et al., 2004)	4.000	5.300
G. frondosa from Taiwan (Ing-Lung and Bi-Wen, 2008)	3.300	3.880
G. frondosa from Fujian (Lei and Wei-guo, 2010)	**----**	0.111
G. frondosa TARI 619908 (Chienyan et al., 2008)	1.813	2.520

frondosa in 9 days. The highest EPS production was 0.334, 0.606 and 0.501 g l^{-1} in the control group when the ethanol extracts of the *Gastrodia* tuber I and II was added, respectively. The experimental group EPS production increased significantly in 2 to 4 days.

The EPS production in our research is lower than that of previous reports (Bum et al., 2004; Ing-Lung et al., 2008; Chienyan et al., 2008) but higher than that of Lei et al. (2010) (Table 1). The reasons for this difference may be due to the different EPS producing capability of different strains of *Gastrodia* tuber. Meanwhile, we noted that Bum et al. (2004) and Ing-Lung et al. (2008) used fermentation tank in their research which may also be one of the reasons for the above results. However, the enhancement of EPS production in our research is much higher than those of previous reports.

Cell growth

The highest dry cell weight of *G. frondosa* was 138.5 mg l^{-1} when the ethanol extracts of the *Gastrodia* tuber I was added and 123.3 mg l^{-1} when *Gastrodia* tuber II was added, respectively (Figure 2), which were much higher than the highest dry cell weight of the control group (59.6 mg l^{-1}). For all fermentations, the exponential growth phase was 2 to 3 days in the experimental group and 3 to 5 days in the control group. The maximum amount of the EPS accumulation was at the end of the exponential growth phase. These results are in agreement with previous report for *Streptococcus thermophilus* (Degeest and De Vuyst, 2000).

Many reports (Guo-hong et al., 2006; Yan-qun et al., 2003; Sai-jin et al., 2005) mentioned that the Chinese traditional medicines can stimulate the growth of the higher fungus cells. Our results suggest that the addition of *Gastrodia* tuber may advance the exponential growth phase, which means that the EPS of *G. frondosa* accumulated earlier.

Enhanced α-PGM activity

Compared with the control group, the experimental groups with the addition of ethanol extracts of the *Gastrodia* tuber I and II caused an increase of the α-PGM activity. The maximum α-PGM activity with the addition of ethanol extracts of the *Gastrodia* tuber II was 2-fold of

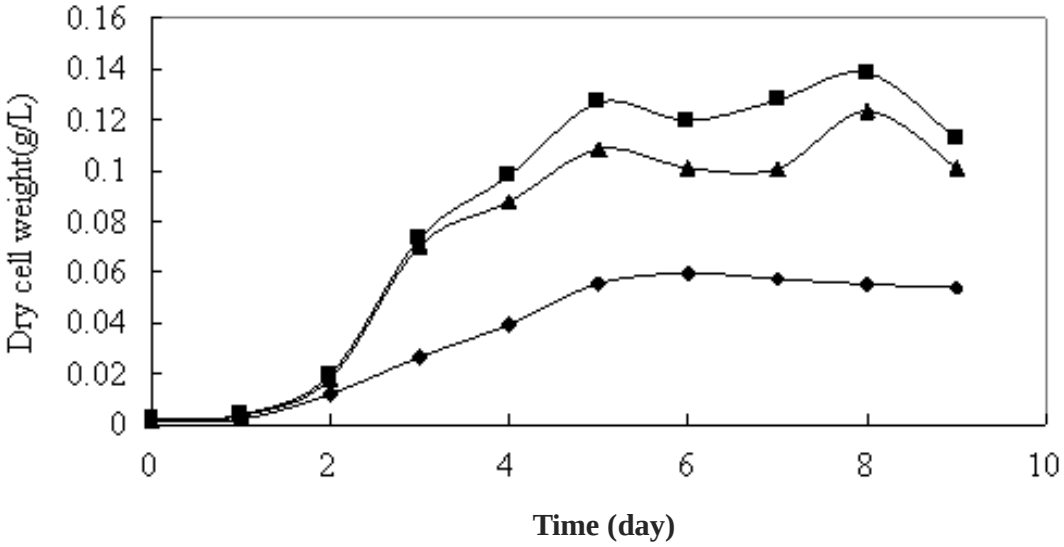

Figure 2. Time profile of dry cell weight with the addition of ethanol extracts of the *Gastrodia* tuber II, *Gastrodia* tuber I and the control group of *G. frondosa.* Symbols: Square, the *Gastrodia* tuber II; triangle, the *Gastrodia* tuber I; diamond, the control group.

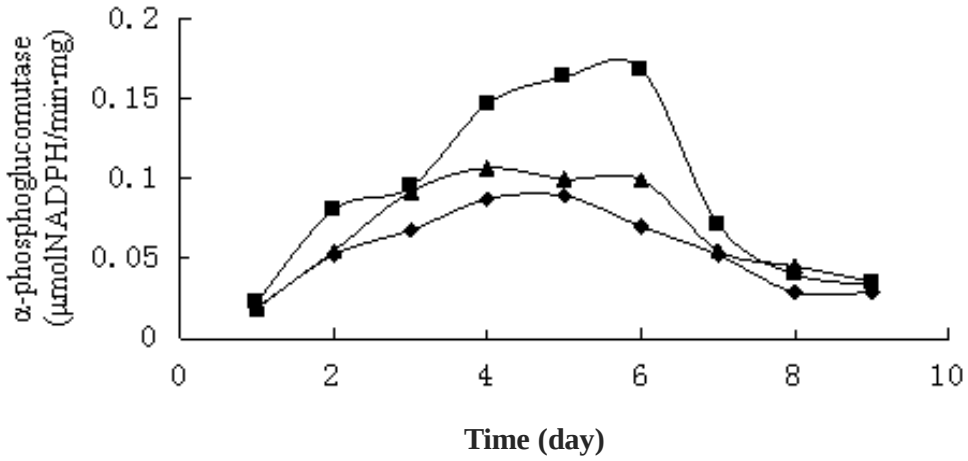

Figure 3. Time courses of α-phosphoglucomutase (α-PGM) activity with the addition of ethanol extracts of the *Gastrodia* tuber II, *Gastrodia* tuber I and the control group of *G. frondosa*. Symbols: Square, the *Gastrodia* tuber II; triangle, the *Gastrodia* tuber I; diamond, the control group.

that in the control group and 1.6-fold of that with *Gastrodia* tuber I addition, respectively. The results are shown in Figure 3, which is similar to the EPS production. Compared with others, higher EPS production with the addition of *Gastrodia* tuber II coincided with its higher α-PGM activity.

Degeest and De Vuyst (2000) reported that the activity of α-PGM will evidently increase the carbon flux via phosphoglucomutase to a sufficiently high level to improved EPS production. For the higher fungus, Ya-Jie and Jiang-Jiang (2002) showed that there may exist a correlation between the activity of α-PGM and EPS biosynthesis in *G. lucidum.* Our results suggest the same conclusion, and we also found that the addition of *Gastrodia* tuber may disturb higher fungal cell metabolism by affecting the activity of α-PGM. In addition, the addition of the ethanol extracts of the *Gastrodia* tuber II was more effective than the *Gastrodia* tuber I.

Changes in the activity of PGI and lactate accumulation

The activity of PGI in the experimental groups was

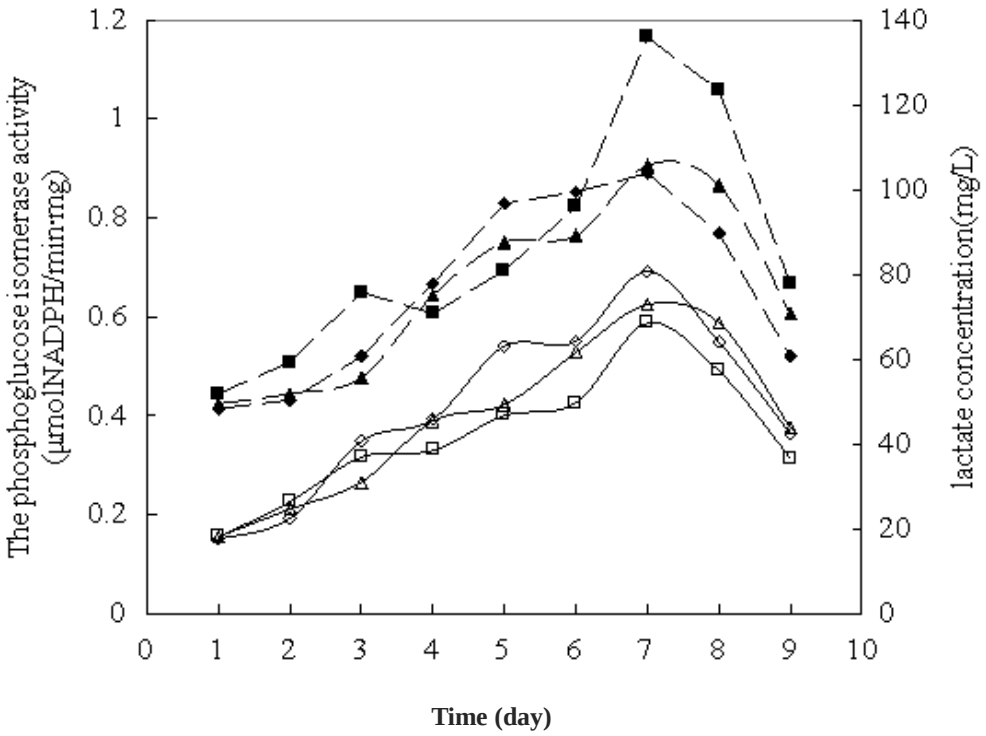

Figure 4. Kinetics of phosphoglucose isomerase (PGI) activity and lactate concentration with the addition of ethanol extracts of the *Gastrodia* tuber II, *Gastrodia* tuber I and the control group of *G. frondosa.* Symbols: Open symbol, PGI activity; dark symbol, lactate concentration; square, the *Gastrodia* tuber II; triangle, the *Gastrodia* tuber I; diamond, the control group.

generally lower than the control group (Figure 4). Ya-Jie and Jiang-Jiang (2002) reported that lactate the end-product of EMP pathway was related to the activity of PGI.

As reported previously (Rungrassamee and Liu, 2008), PGI contributes to the generation of reduced equivalents via the gluconeogenesis pathway. With the activity of α-PGM, our results suggest that the addition of *Gastrodia* tuber lead to more carbon flux flow to gluconeogenesis which forms glycolysis.

Effect of ethanol extract from *Gastrodia* tuber and gastrodine

To determine the effects of the mixed components or the single component of *Gastrodia* tuber, we contrasted the enzymes activity, EPS production and the dry cell weight in the same fermentation conditions but added the ethanol extract of *Gastrodia* tuber II and the pure gastrodine. The amount of the pure gastrodine was equal to the gastrodine in the ethanol extract of *Gastrodia* tuber II. The control group used the same culture medium but *Gastrodia* tuber components were not added.

Figure 5 shows the enzymes activity, EPS production and the dry cell weight in the three conditions. The addition of ethanol extracts show the highest EPS production, dry cell weight and the maximum α-PGM activity. When compared with the control group, the pure gastrodine addition has no significant effects on α-PGM activity or EPS production. Previous study of Yang et al. (2000, 2004) showed that fatty acid, oleic acid at 1.5 g l^{-1} led to a significant increase in biomass production and palmitic acid showed that ethanol was of great advantage to EPS production of *G. lucidum*. These reports are single component that stimulate the mycelial growth or polysaccharides production. This shows that single component cannot stimulate neither the mycelial growth nor polysaccharides production. However, Gao-Qiang et al. (2007) reported that the extracts of *Eupolyphaga sinensis* can significantly increase the production of both biomass and intracellular polysaccharides which is similar with our report.

In conclusion, the positive response of *G. frondosa* metabolism, EPS and biomass accumulation by *G. frondosa* to *Gastrodia* tuber components addition was demonstrated in this work. From these experiments, it was obvious that *Gastrodia* tuber II was more effective than deep processed *Gastrodia* tuber I. In addition, the mix components of the ethanol extracts of the *Gastrodia* tuber II was also more effective than the pure gastrodine. However, one or more compounds of the ethanol extracts of the *Gastrodia* tuber were the major active components responsible for the stimulatory effect on EPS and

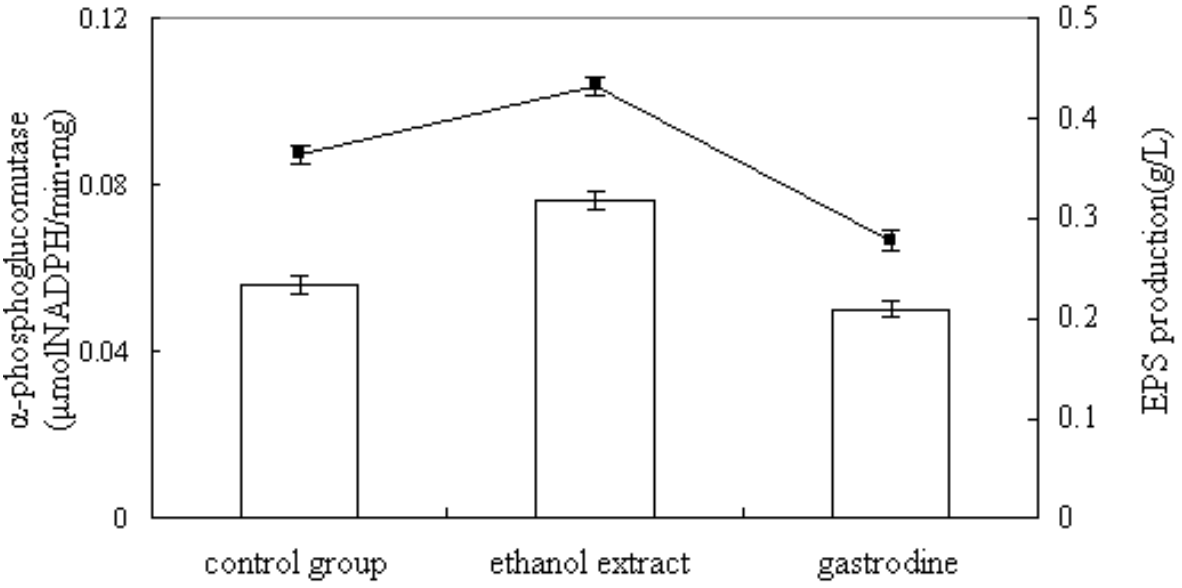

Figure 5A. Comparison of the α-phosphoglucomutase activity and EPS production with the addition of ethanol extract of *Gastrodia* tuber II, the pure gastrodine and the control group at the 7th day. Line chart: EPS production, column chart: the α-phosphoglucomutase activity.

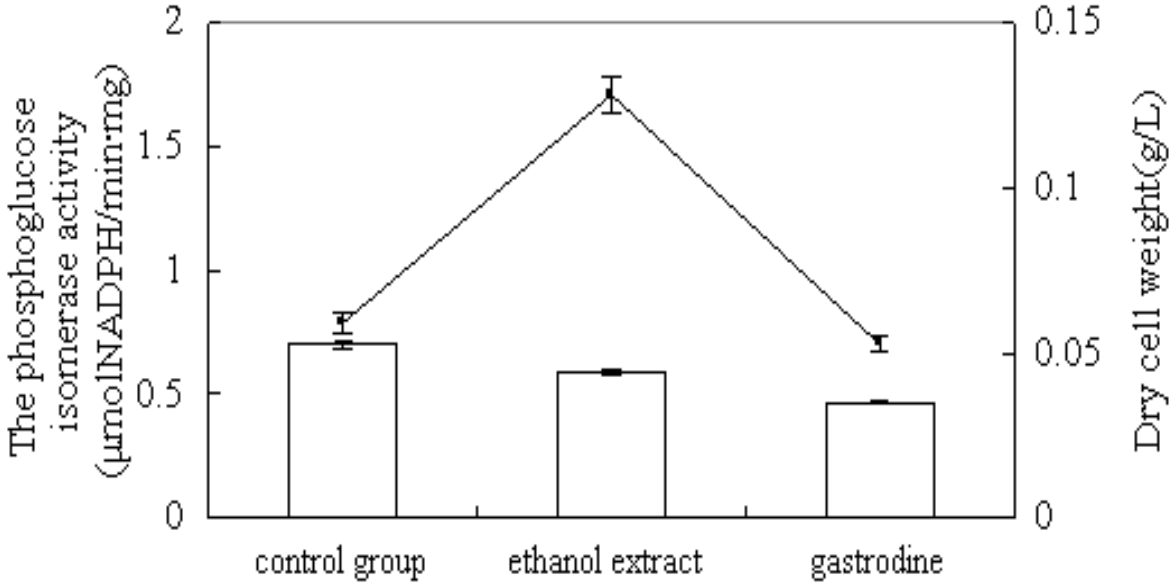

Figure 5B. Comparison of the phosphoglucose isomerase (PGI) activity and dry cell weight with the addition of ethanol extract of *Gastrodia* tuber II, the pure gastrodine and the control group at the 7th day. Line chart: Dry cell weight, column chart: the PGI activity. Error bars are the corresponding standard deviation.

biomass accumulation are currently unclear. The specific role of compounds of the ethanol extracts of the *Gastrodia* tuber in the *G. frondosa* metabolism process is not clear. Therefore, it is suggested that further works should be performed on the isolation and identification of one or more key components of the ethanol extracts of the *Gastrodia* tuber. Then, we will use these components fermentation with *G. frondosa*, observe and provide results.

This work may be beneficial for further research on the mechanism of "the bi-directional fermentation" and new processing method of the Chinese traditional medicines.

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