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Full Length Research Paper

Enhanced production of mycelial biomass and extracellular polysaccharides in caterpillar-shaped medicinal mushroom *Cordyceps sinensis* CS001 by the addition of palmitic acid

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To screen stimulators for cell growth and polysaccharides production of caterpillar-shaped Chinese medicinal mushroom, *Cordyceps sinensis*, the fungus was inoculated into the submerged culture media with and without supplementation of several fatty acids and ether extracts of Chinese medicinal insects. The results showed that palmitic acid at 1.0 g/l lead to significant increases in both biomass and extracellular polysaccharides (EPS) concentration (C) from 8.26 ± 0.34 to 11.10 ± 0.39 g/l and 353.1 ± 11.5 to 431.2 ± 13.8 mg/l, respectively. In addition, ether extract of medicinal insect, *Eupolyphaga sinensis* at 1.5 g/l was also of advantage to biomass and EPS production. There were no new components in the EPS obtained by the addition of palmitic acid.

Key words: *Cordyceps sinensis*, medicinal mushroom, extracellular polysaccharides, palmitic acid, submerged culture.

INTRODUCTION

The caterpillar-shaped Chinese medicinal mushroom ‘‘Winter-Worm-Summer-Grass’’, called ‘‘DongChong-XiaCao’’ in Chinese and ‘‘Tochukaso’’ in Japanese, originating from the entomopathogenic fungus *Cordyceps* sp., generally recognized as *Cordyceps sinensis*, has been used as one of the most valued traditional Chinese herbal remedy for centuries to maintain health (Hsu et al., 2002). *C. sinensis* infects the larvae of *Hepialus armoricanus*, found only in the highlands of the Himalayan region, and the larva hibernates underground through the winter (Bok et al., 1999). The fungus kills the infected host and grows throughout the cadaver, and in the summer, a rod-like stroma of the fungus grows out from the mummified shell of the dead host. *Cordyceps* species are generally known as the ‘caterpillar fungus' due to this characteristic parasitism of the living larvae of insects (Bok et al., 1999).

Modern chemistry studies show that polysaccharides, cordycepin and other antibacterial and antitumor adenosine derivatives, ophicordin (an antifungal agent), immunopotentiating galactomannan, and L-tryptophan are the major source of biological activity and therapeutic use of *C. sinensis* (Bok et al., 1999). The polysaccharide from *C. sinensis* has shown particularly strong results in immunomodulatory and antitumor effects (Kuo et al., 2007; Zhang et al., 2005, 2008; Yalin et al., 2005). The acid polysaccharide fraction (APSF) extracted from the mycelia of cultivated *C. sinensis* stimulated macrophage activities (Cheng et al., 2010). Extracellular polysaccharides

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Abbreviations: EPS, extracellular polysaccharides; **EES,** ether extract of *Eupolyphaga sinensis*; **EDH,** ether extract of *Dendrolimus houi*; **EMP,** ether extract of *Mylabris phalerata*.

(EPS) significantly elevated splenic TNF-alpha and IFN-gamma protein expressions and increased thymic TNF-alpha and IFN-gamma protein levels (Sheng et al., 2010). In addtion, a water-soluble polysaccharide named CPS1 from *C. sinensis* mycelium showed a high antioxidant effect, especially scavenging effect of hydroxyl radicals, the reducing power and $Fe^{(2+)}$-chelating activity (Wang et al., 2009). A water-soluble polysaccharide (CPS-2), isolated from the cultured could significantly relieve renal failure caused by fulgerizing kidney (Wang et al., 2010).

As *C. sinensis* is very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. Its cultivation has become essential to meet the increasing demands in the international markets. Submerged fermentation of *C. sinensis* is viewed as a promising alternative for production of mycelial biomass and polysaccharides (Hsieh et al., 2002, 2005; Cha et al., 2007). To accelerate mycelial growth and polysaccharides production, it has been a topic of concern to screen environmentally sound and economically feasible compounds that stimulate mycelial growth and polysaccharides production of medicinal mushroom (Liu and Zhang, 2007; Yang et al., 2004; Liu et al., 2010a), however, similar data on *C. sinensis* were scare. The objective of this research was to screen stimulators from several fatty acids and ether extracts of Chinese medicinal insects for mycelial growth and EPS production of *C. sinensis* in submerged culture.

MATERIALS AND METHODS

Chemical materials and preparation of ether extracts of medicinal insects

Tested fatty acids, stearic acid, oleic acid, linoleic acid and palmitic acid were purchased from Sigma (USA); Chinese medicinal insects, *Eupolyphaga sinensis* and *Mylabris phalerata* Pallas (MP) were purchased from Anhui Medicinal Materials Factory (Hefei, P.R. China); *Dendrolimus houi* (DH) was collected by our lab. All insect samples were air-dried and round to powder, then stored at 4°C.

For the preparation of the ether extracts, 50 g insect samples were separately extracted by circumfluence with 500 ml ether for 3 h, the obtained extracts were filtered, and then ether was removed under reduced pressure to obtain dry extracts.

Microorganism

The strain of *C. sinensis* CS001 was screened and collected by Strain Collection of Industrial Microorganisms (SCIM), Central South University of Forestry and Technology. It was maintained on potato-agar-dextrose slant subcultured every 4 weeks.

Culture conditions

The strain of *C. sinensis* CS001 was grown in a 250 ml flask containing 60 ml medium at 27°C for 7 days with shaking at 160 rpm. This was then inoculated at 12% (v/v) into the same medium now containing various fatty acids. The culture time (t) was 7 days. The culture medium was composed (g/l) of: glucose 30, yeast extract 3, peptone 2, KH_2PO_4 0.6, $MgSO_4 \cdot 7H_2O$ 0.4, vitamin B_1 0.01 g l^{-1}; the initial pH of the medium was adjusted to 6.5.

Determination of mycelial biomass

Biomass was obtained by centrifuging a sample at 8,000 rpm for 15 min, washing the precipitated cells for three times with distilled water, and drying at 60°C for a sufficient t to a constant weight (Liu et al., 2010a, b).

Measurements of EPS

For the determination of EPS, after the removal of mycelia by centrifugation, the crude EPS was precipitated with addition of 95 % (v/v) ethanol by four times of volume, then separated by centrifugation at 10,000 rpm. The insoluble components were suspended in 1 mol/l NaOH at 60°C for 1 h, and the supernatant was measured by phenol–sulfuric acid method (Liu et al., 2010b; Liu and Wang, 2007, 2009).

Analysis of EPS components

The EPS obtained with palmitic acid at 1.0 g/l and the control EPS were respectively collected as the method mentioned above, and then fractionated on ÄKTA Explorer (Sweden). 2 ml polysaccharide (about 9 to10 mg/ml) of each sample was eluted on a column (HiPrep 16/10 DEAE) with H_2O and followed stepwise by 0.1, 0.3, and 0.5 mol/l NaCl at 2 ml/min. Fractions (5 ml) were assayed by the phenol-sulphuric acid method. The main component of EPS, EPS-1 were repurified on a column of Superdex 200 HR 10/30 with 0.1 mol/l NaCl at 0.25 ml/min. Fractions (1 ml) were assayed by the phenol-sulfuric acid method (Liu and Zhang, 2007).

Statistical analysis

Incubations were performed at triplicate and data were analyzed using SPSS 10.0 version. The results were expressed as the mean ± SD. The significance of the mean difference between the control group and each treatment group was determined by Student's t-test. The level of $P < 0.05$ was used as the criterion of statistical significance.

RESULTS

Effects of fatty acids and medicinal insect extracts on production of biomass and EPS

Several ether extracts of Chinese medicinal insects and fatty acids (0.6 g/l) were added into the culture medium of *C. sinensis* to investigate their effects on the production of mycelial biomass and EPS. The results showed that both the ether extract of *E. sinensis* (EES) and palmitic acid at 55 mg lead to significant increase in biomass production from 8.26 ± 0.34 to 9.95 ± 0.34 g/l and 8.26 ± 0.34 to 10.47 ± 0.32 g/l, respectively (Table 1). Furthermore, palmitic acid and EES also showed stimulatory effects on EPS production, the EPS yield significantly increased from 353.1 ± 11.5 to 401.7 ± 14.7 mg and 353.1 ± 11.5 to 379.5 ± 15.5 mg/l, respectively.

Table 1. Effects of medicinal insect extracts and fatty acids at 0.6 g/l on the production of biomass and EPS of *C. sinensis*.

Samples	Biomass (g/l)	EPS (mg/l)
Control	8.26 ± 0.34	353.1 ± 11.5
Linoleic acid	4.49 ± 0.65**	203.4 ± 11.3**
palmitic acid	10.47 ± 0.32*	401.7 ±14.7*
Stearic acid	7.95 ± 0.43	361.5 ± 15.2
Oleic acid	8.51 ± 0.64	357.3 ± 16.2
EDH	8.81 ± 0.25	368.4 ± 12.5
EES	9.95 ± 0.34*	379.5 ± 15.5*
EMP	2.69 ± 0.22**	111.6 ± 5.3**

EDH, ether extract of *Dendrolimus houi*, EES, ether extract of *Eupolyphaga sinensis*, EMP, ether extract of *Mylabris phalerata*. Values with an asterisk are significantly different from that of the control group by Student's *t*-test, *P < 0.05;, **P < 0.01.

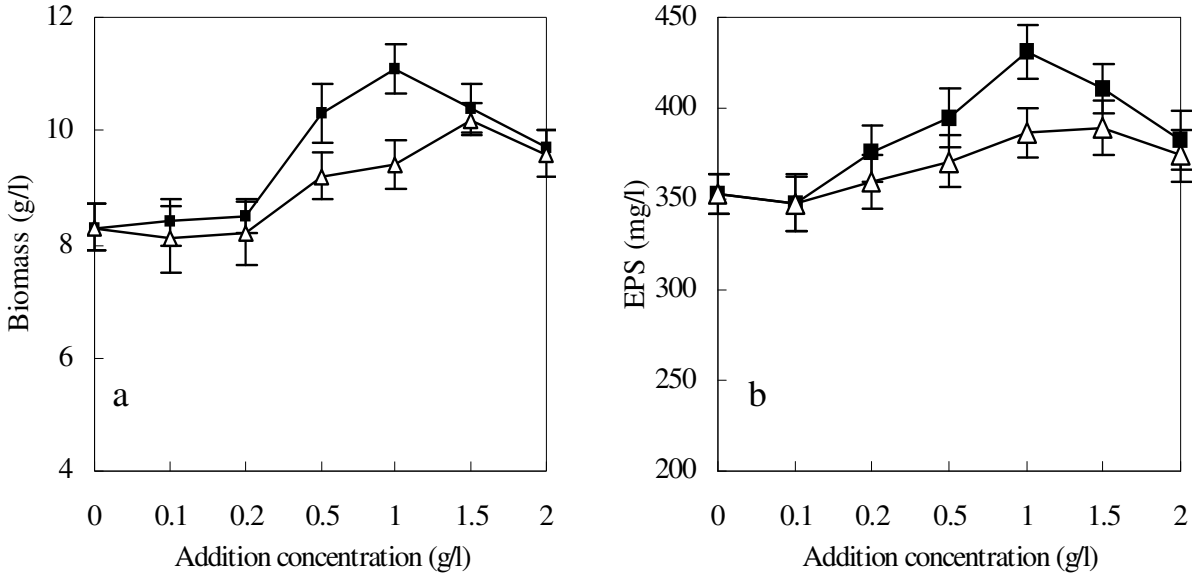

Figure 1. Effects of palmitic acid and EES levels on biomass (a) and EPS (b) production of *C. sinensis*. ■, palmitic acid; △, EES, ether extract of *E. sinensis*. *C. sinensis* cells were cultivated at 27°C for 7 d on a rotary shaker at 160 rpm.

However, stearic acid and oleic acid had no stimulatory effect on cell growth and EPS production (P > 0.05), and linoleic acid markedly inhibited both mycelial growth and polysaccharide production (P < 0.01) (Table 1).

Effects of palmitic acid and EES at different levels on biomass and EPS production

Table 1 shows that palmitic acid and EES could significantly stimulate the production of biomass and EPS, so it is necessary to study the effects of palmitic acid and EES at different concentrations on production of biomass and EPS. The effects of palmitic acid and EES at different levels on myceylial growth were shown in (Figure 1a). Palmitic acid in the range of 0.1 to 1.0 g/l showed a positive effect with an increase in the palmitic acid concentration (C), and the maximal yield was obtained at 1.0 g/l with a yield of 11.10 ± 0.39 g/l. This yield was significantly high compared to the control (8.26 ± 0.34 g/l) (P < 0.05). In addition, EES in the range of 0.2 to 1.5 g/l also displayed a positive effect, but the maximal yield was obtained at the level of 1.5 g/l with a yield of 10.21 ± 0.33 g/l.

When a selection of palmitic acid and EES levels was tested, the results of EPS yield are given in (Figure 1b). In the range of 0.1 to 1.0 g/l palmitic acid displayed a positive effect on EPS production, and at 1.0 g/l, a maximal yield (431.2 ± 13.8 mg/l) of EPS was obtained, which had significant difference compared to the control

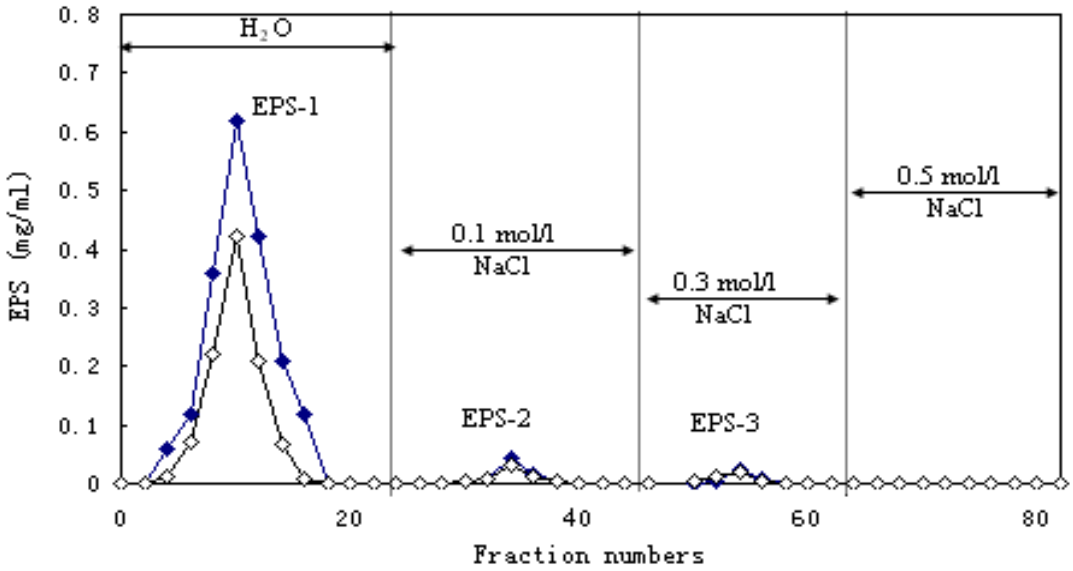

Figure 2. HiPrepTM 16/10 DEAE elution profile of EPS, the column was eluted stepwise with H_2O, 0.1, 0.3, 0.5 mol/l NaCl solutions. ◆, Sample of containing 1.0 g/l palmitic acid in medium; ◇, control.

group (353.1 ± 11.5 mg/l) ($P < 0.05$). While EES had the similar effect on the EPS production in the range of 0.2 to 1.5 g/l, but the maximal yield was obtained at the level of 1.5 g/l with a yield of 389.7 ± 12.2 mg/l.

Effect of palmitic acid on EPS components

The results in (Figure 1b) suggest that palmitic acid had the better stimulatory effect on EPS production than EES, so the EPS obtained with palmitic acid at 1.0 g/l and the control EPS were fractionated by the column chromatography as the method mentioned above. The results are shown in (Figure 2). Four EPS fractions were isolated in each EPS sample with EPS-1 as the main component. EPS-1 was further fractionated on the column of Superdex 200 HR 10/30: two components were separated, EPS-1-1 and EPS-1-2, however, there was no new EPS component produced by the addition of palmitic acid at 150 mg/l (Figure 3). These results suggest that palmitic acid did not change the main biosynthetic pathways of EPS though it significantly enhanced the EPS production.

DISCUSSION

Mushrooms have recently become attractive as healthy foods (physiologically functional) and as a source material for the development of drugs (Liu and Zhang, 2007; Liu et al., 2010a). The fruiting body of the entomopathogenic fungus *Cordyceps* is used as tonic herb in Chinese traditional medicine for a long t. During the past several decades, much interest has been shown in the polysaccharides produced by *Cordyceps* due to their various biological and pharmaceutical activities, and a number of bioactive constituents in *Cordyceps* species have been reported.

Even though physiologically active substances have been isolated from various *Cordyceps* species, few are being used commercially due to the difficulties of mass production. To overcome these difficulties, artificial solid media have been developed for mass production of *C. sinensis*. But comparing to the liquid culture, the yield of solid culture is not sufficient. In addition, exo-biopolymers, EPS, which have synergistic biological effects with mycelia, can be concurrently produced in liquid culture (Cha et al., 2007).

Recently, some stimulators that stimulate mycelial growth and polysaccharides production of medicinal mushroom in liquid culture have been reported. A study showed that ethanol was advantageous for EPS production of *Ganoderma lucidum* in submerged culture (Yang et al., 2004). Yang et al. (2000) reported that fatty acid, oleic acid at 1.5 g/l led to a significant increase in biomass production, and palmitic acid (2.0 g/l) was of great advantage to polysaccharides production of *G. lucidum*. In the present study, addition of palmitic acid at 1.0 g/l lead to significant increase in both biomass and EPS production, however, oleic acid had no stimulatory effects on cell growth and EPS production ($P > 0.05$). These differences may be due to the different strain species and culture condition. We further studied the effect of palmitic acid on EPS components of *C. sinensis*. The results showed that there were no new components in EPS obtained by the addition of palmitic acid,

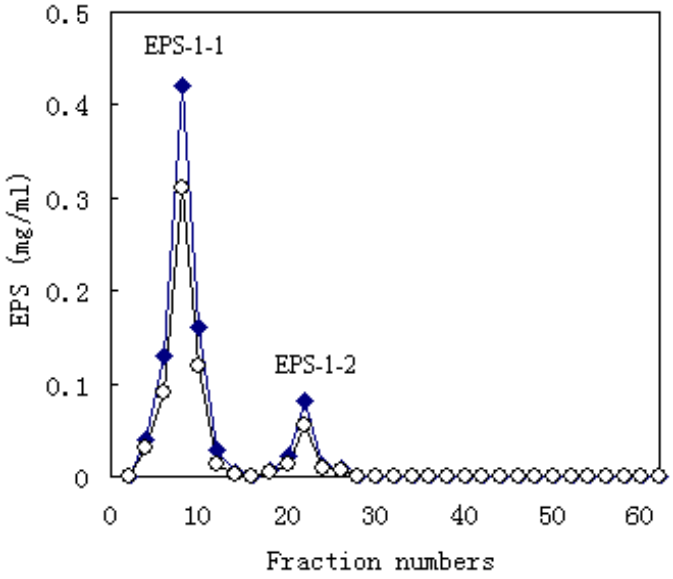

Figure 3. HiPrepTM 16/10 DEAE elution profile of EPS-1, the column was eluted stepwise with 0.1 mol/l NaCl solution. ◆, Sample of containing 1.0 g/l palmitic acid in medium; ◇, control.

suggesting the biosynthetic pathways of EPS had not been changed.

In addition, medicinal herbs or insects are sources of drugs. Studies have reported that many types of extracts from herbs displayed various pharmacological effects. In our previous work, we found that the ethyl acetate extracts from the medicinal insect *E. sinensis* and *Catharsius molossus* were useful to enhance the polysaccharides production of *G. lucidum* in submerged culture (Liu and Zhang, 2007), and in our another study, the ether extract of *E. sinensis* at a C of 60 mg/l lead to a significant increase in ganoderic acid yield from 187.6 ± 8.32 to 251.3 ± 11.27 mg/l (Liu et al., 2010a). In the present study, several ether extracts of Chinese medicinal insects were added into the media to screen new inducers for mycelial growth and polysaccharide production. The results show that the ether extract of *E. sinensis* (EES) was of great advantage to biomass and EPS production, but palmitic acid had better stimulatory effect that EES on both biomass and EPS production.

In conclusion, palmitic acid significantly enhanced both biomass and EPS production, it can be used as easily available stimulators for the biomass and polysaccharide production by submerged culture of *C. sinensis*.

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