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Full Length Research Paper

Effect of metal ions on the growth and metabolites production of *Ganoderma lucidum* in submerged culture

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The effects of several metal ions on the cell growth, production of polysaccharides by *Ganoderma lucidum* in submerged fermentation were studied. The results showed that 50 ppm Se^{2+} and 25 ppm Se^{2+} was identified to be the most favorable for biomass (11.103 ± 0.6 g/l) and polysaccharide production (IPS and EPS was 183 ± 10.2 and 248 ± 5.5 mg/l, respectively); 100 ppm of Fe^{2+} and 50 ppm of Zn^{2+} were suitable for growth (the biomass was 8.23 ± 0.67 and 8.01 ± 0.29 g/l, respectively) and under the concentration of 50 ppm of Zn^{2+} and Fe^{2+}, the production of polysaccharide was up to the most (EPS content: 263±4 and 254.3±8.0 mg/l; IPS content : 170±0.8 and 174±5 mg/l); Mg^{2+} had no obvious effect on biomass and polysaccharide production; Cr^{2+} was poisonous to the cell under the test concentration. The combination ($FeSO_4$, 50 ppm; $NaSeSO_3$, 25 ppm; $ZnSO_4$, 75 ppm) by A 9 ×3 replicates (27) experiments of L9 (3^4) orthogonal projects was tested optimal for the cell growth and polysaccharides production. Biomass, EPS and IPS production reached their good value of 14.7 ± 0.5 g/l, 369 ± 6 mg/l and 239 ± 4 mg/g, respectively under the combination, which were higher 130.7, 50 and 50%, respectively than in the basal fermentation medium without metal ions. The validation experiment showed the experimental values agreed with the predicted values well (error <1%).

Key words: *Ganoderma lucidum,* metal ions, biomass, polysaccharide, orthogonal projects.

INTRODUCTION

Ganoderma lucidum (Fr.) Krast, a basidiomycete belonging to the Polyporaceae, is called “Lingzhi”. Also known as, Lingzhi, meaning, “herb of spiritual potency” has been used for thousands of years in traditional Chinese medicine. It is considered particularly important in vegetarian diets and regarded as medicinal food that promotes longevity. Studies have indicated that Lingzhi has many pharmacological activities such as anti-microbial activity in mice, simulative effects on the production of monocytes, macrophages and cytokines, anti-viral and anti-oxidant (Chen et al., 2004; Jong et al., 1992; Berovic et al., 2003). Lingzhi contains several chemical constituents, including sterols, coumarin, mannitol, polysaccharides and triterpenoids.

Polysaccharides and triterpenoids are regarded as the key bioactive components. Hence, studies on the two compositions producing of Lingzhi have been intensified in recent years.

Many metals are essential for microorganism for example, K, Na, Mg, Ca, Mn, Fe, Co, Ni, Cu, Zn and Mo which can interact with microbial cells and may play an important function in cell growth and metabolism. Some of microorganisms can accumulate metals even when they are present in media at low levels, others do not do it even at high concentrations of metal ions, which depend on the type of microorganisms, metal ions, their concentrations and the type of media used (Tsezos, 1985). Zhou showed that trace metals in the cultivation media had significant effects on pellet formation and fumaric acid fermentation (Zhou et al., 2000). Strains of *G. lucidum* have the capability to produce ganoderic acid, polysaccharide, pectinase, amylogucosidase, etc. (Cao et al., 1996; Hang et al., 1989; Papagianni et al., 2004; Soccol et al., 1994). Many factors including nutrition factor and environment condition were studied on the effect of growth and metabolite of *G. lucidum* in submerged culture. Fang and Yang studied the effects of

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glucose concentration, peptone concentration, pH and some additives on the polysaccharide production and biomass formation of *G. lucid*um (Fang et al., 2002b; Yang et al., 1998). However, a comprehensive investigation of effects of medium composition on polysaccharide and growth is lacking. In comparison to other fermentation factors, our knowledge of the influence of metal ions on the growth and metabolites of *G. lucidum* in submerged fermentation is relatively poor.

The human health is related with some metal ions. Metal ion unbalance is one reason for cerebrovascular disease and cancer. For example, if Mg^{2+}, Se^{2+} and Zn^{2+} are deficient, then they can induce cerebrovascular disease. However, some heart diseases are also because of a disorder of Zn^{2+}, Cr^{2+} and Mn^{2+}. More so, a very low content of Mn^{2+} and Fe^{2+} can induce liver cancer in human body. These metal ions participate in metabolism in body. But the human body can not absorb the free ion, but bond ion. In order to obtain bond ion, the work will investigate the Se^{2+}, Mg^{2+}, Fe^{2+}, Cr^{2+}, Zn^{2+} and Cr^{2+} and their concentrations (under safe dosage in human body) on the cell growth and polysaccharide biosynthesis by the approach of 'one-variable-at-a-concentration'. To investigate the synergistic effect among various metal ions, orthogonal projects combined with only 9 × 3 replicates (27) experiments of L9 (3^4) was used to point out the relationships existing between the response functions and the process variables. The information obtained in this study is helpful for the hyper production of mycelia and polysaccharide by submerged cultivation of *G. lucidum* on a large scale and also can obtain the bond metal ion for human.

MATERIALS AND METHODS

Culture maintenance and preparation of inoculums for the pre-culture

*G. lucidum*127 (provided by Biomass Resource Lab in Southern Yangtze University) was maintained on potato dextrose agar (PDA) slopes and were transferred every 6 to 7 weeks. Then, mycelium was activated with growing on a PDA slope at 30°C for 2 days as inoculums, three 5 mm diameter disks of mycelium and agar were punched out from a culture by a self-designed cutter and spread on the surface of 80 ml of medium in 250 ml Erlenmeyer flasks. Culture medium composition were made up of the following components (in grams per liter): glucose, 20; corn powder (0.2 mm), 20; bran powder (0.2 mm), 10; bean cake powder (0.2 mm), 5; pH was naturally. The media were sterilized at 121°C for 30 min and cultured in the dark at 30°C on a rotary shaker at 150 rpm. 10 ml of broth with formed pellets about 7 to 8 day-old was used as seed inoculums for further cultivation in 150 ml fermentation medium.

Metal ion experiments

The experiments were carried out in 500 ml Erlenmeyer flasks containing 140 ml medium. The basal medium consisted of (in grams per liter): peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5; Vitamin B1, 0.01. Effects of metal ion on the cell growth, mycelia morphology and the production of polysaccharides including extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS) by *G. lucidum* cells were studied by using various mineral sources, that is, Na_2SeO_3, $ZnSO_4$, $MgSO_4$, $FeSO_4$, and $CrSO_4$. The initial concentration (25, 50, 75, 100, 150 and 200 ppm) of a certain kind of metal ion supplemented in the basal medium was also investigated. The control experiment was conducted in the fermentation medium without metal ion, the pH was naturally. After sterilization at 121°C for 30 min, fermentation mediums in Erlenmeyer flasks were inoculated with 10% (v/v) of the seed culture under asepsis, the fermentation was conducted in the dark at 30°C on a rotary shaker at 150 rpm. The cell growth and polysaccharide biosynthesis were determined after culture 5 days. Multiple flasks were run at the same concentration and three flasks were taken at each sampling point. Each data point was expressed by an average with an error bar (standard deviation from three independent samples).

Effect of combine metal ion on the growth and polysaccharide of *G. lucidum*

A 9 ×3 replicates (27) experiments of L9 (3^4) orthogonal project was conducted in the optimum vicinity to locate the optimum concentrations of Zn^{2+}, Se^{2+} and Fe^{2+} for the cell growth and polysaccharides production. The levels of variables for orthogonal experiment were selected according to the results of one-variable-at-a-concentration. The fermentation medium was composed of (in grams per liter): peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5; Vitamin B1, 0.01 and a certain combination of Zn^{2+}, Se^{2+} and Fe^{2+} was investigated.

Analytical methods

To measure the cell dry weight, 100 ml fermented broth from a sample was centrifuged and then washed the mycelia with a 0.45 mm mesh under flowing water. The fresh cells were dried at 60°C for sufficient time (48 h) until a constant dry weight was obtained. After centrifuging, the fermentation supernatant was stored at -20°C and then thawed for analyses of residual sugar. Residual sugar level was assayed by the DNS method. For the determination of extra-cellular polysaccharide (EPS), after removal of mycelia by filtration, 100 ml of the fermentation filtrate was precipitated with addition of 95% ethanol to make the ethanol concentration of solution up to 30% for removing a part of protein, after standing over night at 4°C and centrifuging, then adding ethanol to make the ethanol concentration of supernatant up to 60%, then standing over night at 4°C again. The precipitated polysaccharide was collected by centrifugation at 3000 rpm for 15 min and the insoluble components was re-suspended in the distilled water and de-protein by sevega's method. Total polysaccharide was determined by phenol-sulphuric acid assay according to Dubois's (Dubois et al., 1956). For the analysis of intracellular polysaccharide (IPS), the dried mycelia (100 mg) were extracted by 1 M NaOH at 60°C (1 h) and then the supernatant was assayed by phenol-sulfuric acid method.

RESULTS

The effect of addition of five kind of metal ions on growth of *G. lucidum*

The effect of various levels of metal ions on mycelia growth for 5 days were studied in freely suspended

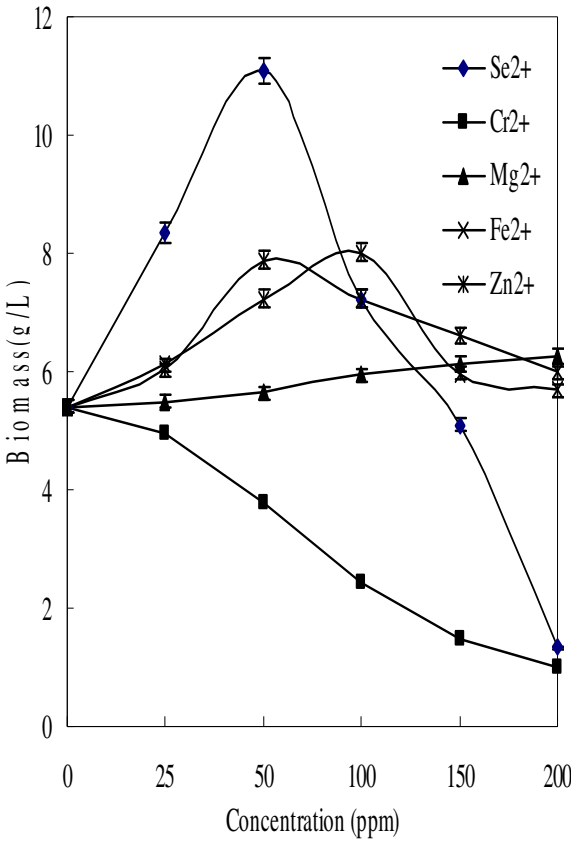

Figure 1. Effect of supplementing metal ion(Se^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+} and Cr^{2+}) in the medium on the biomass of *G. lucidum* in shake flask cultures(medium components: peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5; vitamin B1, 0.01; concentration of metal ion :25, 50, 75, 100, 150 and 200 ppm; temperature: 30℃; rotates: 150 r/min; inoculum: 10%; pH: natural; incubation period: 5 days).

cultures. The result in Figure 1 revealed that Zn^{2+}, Se^{2+} and Fe^{2+} showed a stimulatory effect under an extend concentration. Se^{2+} was the most impressive and with the optimum of 50 ppm for growth, mycelia concentration increased significantly from 5.393±0.2 g/l (without Se^{2+}) to 11.103 ± 0.6 g/l; the morphology of mycelia was mixed pellets and hyphae. But addition of high level of Se^{2+} (200 ppm) drastically suppressed mycelia growth and biomass was little and no pellets were observed, which suggested that high level of Se^{2+} was poisonous to the cell. Fe^{2+} and Zn^{2+} could stimulate the growth of *G. lucidum* with concentration from 0 to 150 ppm. The suitable concentration was 100 ppm for Fe^{2+} and 50 ppm for Zn^{2+} and the most biomass was respectively, 8.23±0.67 and 8.01±0.29 g/l, morphology of mycelia was small pellets (Table 1). For Mg^{2+}, the concentration here did not affect the growth *of G. lucidum*; it seemed that, Mg^{2+} was not necessary to the growth of *G. lucidum*, which is contrast to the convention idea that Mg^{2+} is important for the growth of fungi (Agrawal et al., 2004). Cr^{2+}, inhibited the growth, it was detrimental to cell under the concentration here. These data also indicated the correlation between growth stimulation and the extent of metal ions and the kind of metal ion had the effect on the growth of *G. lucidum*

Environmental conditions markedly influence the growth pattern of filamentous fungi, which can range from a pellet to a dispersed filamentous form, affecting in this way both the growth rate and product formation (Byrne et al., 1989a, b; Yang et al., 1998; Nielsen et al., 1992; Wagner et al., 2004). Table 1 suggested that the positive effect of Zn^{2+}, Se^{2+} and Fe^{2+} might also be related to the increase of mycelia branching. The presence of shorter and highly branched hyphae probably favors' the formation of pellet, improving the performance of the process. Under high concentration of 200 ppm, pellets formed were inhibited and the pellets were sparse and large. In the medium without metal ions (Blank, Table 1), the mycelia formation stages were not fulfilled. The pellets formed were less and irregular in form. As desired, fluffy loose and round pellets were formed by adding Se^{2+} 50 ppm, Zn^{2+} 100 ppm $^{+}$ and Fe^{2+} 100 ppm.

Effect of addition of metal ions on the EPS and IPS

When a selection of metal ions was tested, the results of polysaccharide production in suspended cultures are given in Figure 2a, b. It showed that Se^{2+} at the levels tested (below 50 ppm), had a positive effect on the formation of EPS and IPS, but the two polysaccharide did not increase proportionally with the amount of metal ions added. Se^{2+} showed the stimulation only at the lower concentration and the optimum at the level of 25 ppm. The production of IPS and EPS was 183±10.2 and 248±5.5 mg/l, respectively. Zn^{2+} and Fe^{2+} had the most effect on the EPS at the level of 50 ppm; the content of EPS was 263±4 and 254.3±8.0 mg/l. And the macro fungi produced the most IPS content (170±0.8 and 174±5 mg/l), also at the level of 50 ppm for Zn^{2+} and Fe^{2+}. Mg^{2+} had no function on the production of polysaccharide. In contrast, Cr^{2+} had a strong inhibitory effect on the polysaccharide formation and this was in agreement with the results of Zha (2007), in which EPS production by *Acremonium persicinum* was inhibited with some metal ion (Zha et al., 2007). Results shown here indicated that the synthesis of polysaccharide by *G. lucidum* can be substantially increased by the presence of certain metal ions in the medium. Although, metal ions have been reported to stimulate the production of other fungal metabolites (Zwicker et al., 1997), their effect on polysaccharide formation has only been examined in few studies. The mechanism of stimulatory effect has been proposed as directly affecting the level of synthesis of the enzymes involved in polysaccharide production and the penetration of cell membrane or affect transportation of

Table 1. Effect of concentrations of metal ions(Se^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+} and Cr^{2+}) on mycelia morphology by *G. lucidum* (medium components: peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5; Vitamin B1, 0.01;concentration of metal ion :25, 50, 75, 100, 150 and 200 ppm; temperature: 30°C; rotates: 150 r/min; inoculum: 10%; pH: natural; incubation period: 5 days).

Concentration of metal ion	Mycelia morphology				
	Se^{2+}	Fe^{2+}	Zn^{2+}	Mg^{2+}	Cr^{2+}
0	Mixed pellets and hyphae	Mixed pellets and hyphae	Mixed pellets and hyphae	Mixed pellets and hyphae	Mixed pellets and hyphae
25	Mixed pellets and hyphae	Mixed pellets and hyphae	Mixed pellets and hyphae	Mixed pellets and hyphae	No pellets and hyphae
50	Fluffy and small pellets	Mixed pellets	Mixed pellets	Large pellets	No pellets
100	Sparse and anomalous pellets	Small pellets	Small pellets	Large pellets	No pellets
150	Sparse and smooth pellets	Large pellets and elongated hyphae	Large pellets and elongated hyphae	Large pellets	No pellets
200	No pellets	Large broken pellets	Large pellets and elongated hyphae	Large pellets	No pellets

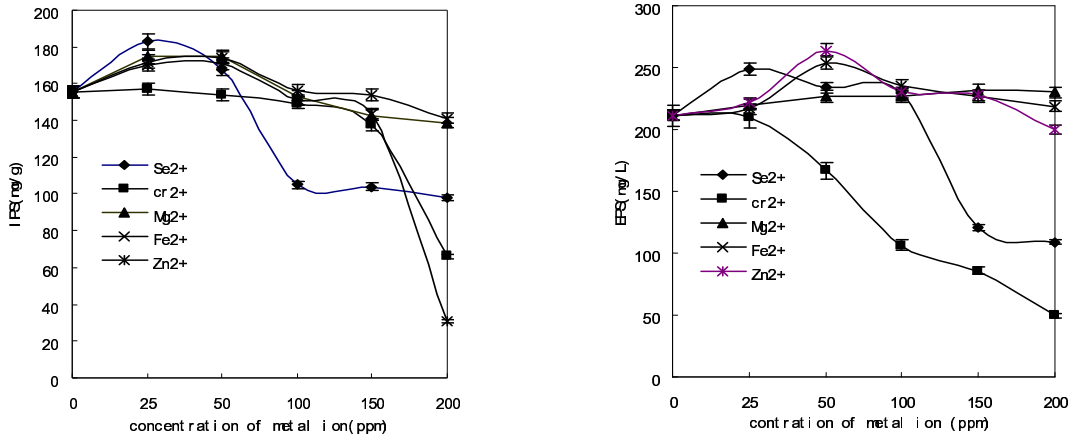

Figure 2. Effect of supplementing metal ion(Se^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+} and Cr^{2+}) in the medium on production of IPS and EPS from *G. lucidum* in shake flask cultures (medium components: peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5; vitamin B1, 0.01; concentration of metal ion :25, 50, 75, 100, 150 and 200 ppm; temperature: 30°C; rotates: 150 r/min; inoculum: 10%; pH: natural; incubation period: 5 days).

ion by the ion channels.

Combination of metal ions on the growth and production of polysaccharide of *G. lucidum*

To determine the optimal concentration combination of microelements, multi-factorial experiments were carried out. The orthogonal projects, as a result of the suitable design of factors, can give effective responses. They have been successfully applied to the improvement of cell culture media for metabolite production in fermentation. Thus, with only 9 ×3 replicates (27) experiments of L9 (3^4) orthogonal projects, the result in dry biomass, polysaccharide of *G. lucidum* under different conditions is shown in Table 2. Effects of these media on biomass and polysaccharides production were calculated and the results were also shown in Tables 3 to 5. According to the magnitude order of R (Max Dif), the

Table 2. Biomass in dry weight, IPS and EPS production of *G. lucidum* under different conditions (Se^{2}+: 25 to 75 ppm; Fe^{2+}: 25 to 75 ppm, Zn^{2+}: 25 to 75 ppm) using orthogonal projects (L9 (3^4)) (medium components: peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5; vitamin B1, 0.01 culture conditions: temperature: 30℃; rotates: 150 r/min; inoculum: 10%; pH: natural).

Run	Se^{2+} (ppm)	Fe^{2+}(ppm)	Zn^{2+}(ppm)	Biomass(g/l)	IPS(mg/l)	EPS(mg/l)
1	25	75	50	10.99 ±0.2	123±10	234±2.3
2	25	50	75	15.45±0.4	235±5	362±4.5
3	25	25	25	10.78±0.6	134±2	245±5
4	50	75	75	9.78±0.8	256±3	384±4
5	50	50	25	9.67±1.2	146±3	223±7
6	50	25	50	9.24±2.1	248±7	356±9
7	75	75	25	6.88±4.1	167±1	289±3
8	75	50	50	6.78±1.3	187±6	276±1
9	75	25	75	6.98±0.8	134±2	264±8

The arrangements of columns Se^{2+}, Fe^{2+} and Zn^{2+} were decided by orthogonal design for 3 (factor) 9 (run number); every row of run number represents one experimental replicate, every run was replicated twice. Values are mean ± S.D. of triple determinations. EPS, Extracellular polysaccharides; IPS, intracellular polysaccharides.

Table 3. Analysis of *G. lucidum* proliferation in submerged culture with orthogonal experiments.

Item	Biomass of *G.lucidum* in dry weight (g DWL^{-1})		
	Se^{2+}	Fe^{2+}	Zn^{2+}
K1	12.407	9.217	9.003
K2	9.563	10.633	10.523
K3	6.880	9.000	9.113
R	5.527*	1.633	1.410
Optimal level	1	2	2

K_i^{Se2+}=∑ (growth index) at Se^{2+}i; R^ai= maxK_{Se2+}-minK_{Se2+}; K_i^{Fe2+}=∑ (growth index) at Ai; R^ai= maxK_{Fe2+}-minK_{Fe2+}; K_i^{Zn2+}=∑ (growth index) at Zn^{2+}i; R^ai= maxK_{Zn2+}-minK_{Zn2+}; *: significance ($p < 0.5$).

Table 4. Analysis of IPS production by *G. lucidum* in submerged culture with orthogonal experiments.

Item	IPS of *G. lucidum* (g/g)		
	Se^{2+}	Fe^{2+}	Zn^{2+}
K1	164.000	182.000	186.000
K2	216.667	189.333	208.333
K3	162.667	172.000	149.000
R	54.000	17.333	59.333*
Optimal level	2	1	2

K_i^{Se2+}=∑(IPS index) at Se^{2+}i; R^ai= maxK_{Se2+}-minK_{Se2+};K_i^{Fe2+}=∑(IPS index) at Ai; R^ai= maxK_{Fe2+}-minK_{Fe2+}; K_i^{Zn2+}=∑(IPS index) at Zn^{2+}i; R^ai= maxK_{Zn2+}-minK_{Zn2+}; *:significance ($p < 0.5$).

Table 5. Analysis of EPS production by *G. lucidum* in submerged culture with orthogonal experiments.

Item	EPS of *G. lucidum* (g/l)		
	Se^{2+}	Fe^{2+}	Zn^{2+}
K1	280.333	302.333	288.667
K2	321.000	287.000	336.667
K3	276.333	288.333	252.333
R	44.667	15.333	84.334*
Optimal level	2	1	2

K_i^{Se2+}=∑(EPS index) at Se^{2+}i; R^ai= maxK_{Se2+}-minK_{Se2+}; K_i^{Fe2+}=∑(EPS index) at Ai; R^ai= maxK_{Fe2+}-minK_{Fe2+}; K_i^{Zn2+}=∑(EPS index) at Zn^{2+}i; R^ai= maxK_{Zn2+}-minK_{Zn2+}; *:significance ($p < 0.5$).

order of the three factors affecting growth was $Se^{2+} > Zn^{2+} > Fe^{2+}$ and the order of the three factors affecting EPS and IPS of *G. lucidum* was $Se^{2+}>Fe^{2+}>Zn^{2+}$. To obtain the optimization levels of each factor, the intuitive analysis based on statistical calculation using data in Table 2 demonstrated that the optimal concentration combination for growth of *G. lucidum* was determined as 25 ppm Se^{2+}, 50 ppm Zn^{2+}, 75 ppm Fe^{2+}(2^{nd} medium) and 50, 75 and 75 ppm (4^{th} medium) for production of IPS and EPS. The corresponding productions of biomass, EPS and IPS in the two medium were 15.45±0.4 g/l, 362±4.5 and 235±5 and 9.78±0.8, 384±4 mg/l and 256±3 mg/g, respectively. The difference of biomass at the two kinds of medium was significant, but IPS and EPS were contrary to the biomass, there was no significance of difference ($p<0.5$) in the two medium. So, we consider the 2^{nd} medium to be fit for the growth and polysaccharide production. To confirm these data, further experiments were carried out using these factor concentrations and 14.7 g/l biomass, 369±6 mg/l EPS and 239±4 mg/g IPS were obtained at the 2^{nd} medium which were good for the production of biomass, IPS and EPS (error<5%). Fe^{2+}, Se^{2+} and Zn^{2+} were considered as essential elements and act as

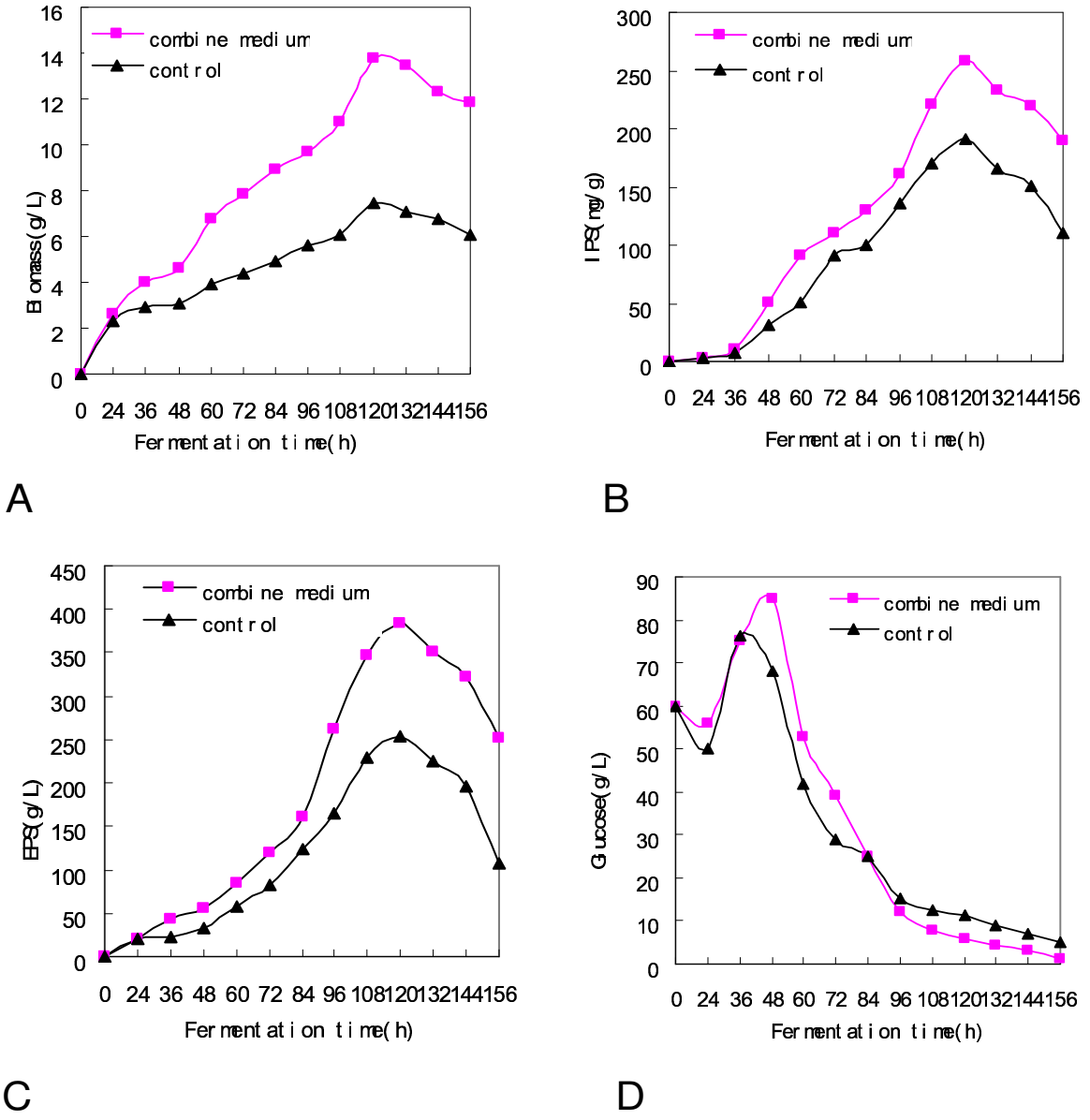

Figure 3. The dynamics of biomass, polysaccharide accumulation and reduce glucose in the optimum growth medium in shake flask (cultures medium components: peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5; vitamin B1, 0.01; FeSO4, 50 ppm; NaSeSO3, 25 ppm; ZnSO4, 75 ppm; temperature: 30°C; rotates: 150 r/min; inoculum: 10%; pH: natural).

nutrients. These must be present at low concentrations in all media used for in cultures. They also act as a metallo-enzyme or a cofactor for several enzymes such as anhydrases, dehydrogenases, oxidase and peroxidases and play an important role in regulating the metabolism.

Analysis of dynamics of biomass, polysaccharide accumulation and reduced glucose in the optimum growth medium

Based on the above experimental results, an optimized medium (peptone, 10; glucose, 60; $KH_2PO_4 \cdot H_2O$, 1.5;Vitamin B1, 0.01; $FeSO_4$, 50 ppm; $NaSeSO_3$, 25 ppm; $ZnSO_4$, 75 ppm) for polysaccharide was developed. Figure 3 showed the typical time courses of biomass, polysaccharide accumulation and reduced glucose resumption in the optimized growth medium and control medium. Figure 3a suggested that the biomass in the medium proliferated exponentially during the period of 2 to 5 days of culture and then slowly with culture time to reach a maximum biomass on the 5th day. For further culture, cellular lyses occurred and resulted in a turbid of medium, while biomass started to decrease slowly.

Growth curve was similar to that in the control medium,but the maximum biomass was twice of that of original medium. With regard to polysaccharide, the Figure 3b and c showed that EPS and IPS content increased rapidly after 5 day of culture and then followed dropped. EPS released in this culture condition was twicehigher than in control medium and the IPS was 0.5 times more than the control. For the residual sugar, Figure 3d suggested that the contents were decreased rapidly until 8 days of culture after which they were hardly detected. The phenomenon was similar to that in the control medium. Figure 3 showed that the metal ion could not alter dynamics direction, but could be accelerant.

DISCUSSION

The production of mycelia, polysaccharide by *G. lucidum* in submerged cultures has prospered in the Orient in recent years. In order to enhance the production efficiency, the control of environmental conditions or the modification of media composition and mode of culture would be vital (Fang et al., 2002a, b). But the productivity improved is limited because the researchers only think about the energy and nutrient, but not the metabolism path of polysaccharide. Metal ions have paid an important role on secondary metabolites during synthesizing (Liu et al., 1975; Solivery et al., 1988; Lee et al., 1997). In this study, metal ions show markedly effect on the mycelia growth and polysaccharide production of *G.lucidum*, suggesting metal ions exert effect in the metabolic pathway of polysaccharide; they may participate in regulation of related enzymes or as its constituents of the related enzymes for biosynthesis of polysaccharide of *G. lucidum.* Our experiments showed that metal ions indeed stimulate the synthesis of polysaccharide. Also, because the polysaccharide has many hydroxyl groups, these can bond metal ions, which may make it improve the pharmacological activity. Of course, that should be a further research.

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