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Influence of rutin, $FeSO_4$, Tween 80, aspartate and complex vitamins on synthesis of fungal exopolysaccharide

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ABSTRACT

The influence of several components on exopolysaccharide (EPS) production and antioxidative activity (TEAC, Trolox-Equivalent Antioxidant Capacity) as well as their effects on the morphological development and cell viability of *Phellinus* sp. P0988 was determined. Rutin, $FeSO_4$, Tween 80 and complex vitamins were found to impose a stronger influence on EPS production and TEAC compared to their effects on the mycelia growth of *Phellinus* sp. P0988. The relative effects of these components on EPS activity were found to be different from that on EPS yield. Rutin and aspartate significantly affected EPS TEAC ($P < 0.05$), while $FeSO_4$ and Tween 80 significantly influenced EPS production ($P < 0.05$). These results yielded the optimum culture medium composition, with an EPS yield and TEAC of 6.2 ± 0.2 g/L and 5.5 ± 0.1 mM, respectively.

1. Introduction

Production of exopolysaccharide (EPS) from medicinal fungi or mushrooms has received considerable research interest due to many beneficial biological functions of these compounds such as antitumor, hypoglycemic and immunomodulatory activities (Ajith & Janardhanan, 2003; Bae, Jang, & Jin, 2005). Crude polysaccharide fractions extracted from fruit body of several well-known medicinal fungi or artificial culture such as *shiitake* are utilized in the formulation of commercial health food products. Pure polysaccharides such as *Lentinan* have also been used for cancer therapy or tumor-postoperative recovery. However, the time required for the growth of either naturally harvested or artificially cultivated fruit body of most medicinal fungi species is considerably long and product quality is also difficult to be controlled in artificial solid culture (Shih, Chou, Chen, Wu, & Hsieh, 2008). Also, artificial cultivation techniques such as solid culture have not been developed adequately to meet the current and promising market of these polysaccharides. Encouragingly, EPS produced in submerged culture exhibits almost similar biological effects to those extracted from medicinal fungal fruit body, and hence many studies have focused their attention on producing these polysaccharides by the submerged fermentation of fungal mycelia (Guo, Zou, & Sun, 2009; Lim & Yun, 2006; Liu & Wu, 2012).

Several studies have aimed to increase the EPS yield and mycelial growth by media design and optimization of the cultivation process (Hao et al., 2010; Liu & Wu, 2012). However, high EPS yield and mycelial growth may not necessarily result in the increased yield of active components, more specifically when the products considered are secondary metabolites such as polysaccharide (Lo, Tsao, Wang, & Chang, 2007). Previous reports have not considered polysaccharide activity simultaneously with its yield (Hwang et al., 2008), and hence the physiological activity of these products is not verified during fermentation production and not guaranteed for application. The majority of approaches in enhancing EPS production reported previously have been primarily concerned with the evaluation of the effect of operating conditions (temperature and pH) and nutritional factors (carbon, nitrogen and mineral sources, and vitamins) on mycelial growth and polysaccharide production of different fungal species in submerged culture (Lee et al., 2006; Lin & Sung, 2006; Zeng, Wang, & Su, 2008). The biological activity of polysaccharide is of primary importance since it is generally ascribed to the pharmacophore of these compounds. In addition, under standard laboratory conditions, gene clusters of secondary metabolism of most filamentous fungi are often silenced, which may result in the activity decrease of these secondary metabolites (Brakhage & Schroeckh, 2010). EPS production by medicinal fungi has been thought to be synchronously associated with the production of mycelial biomass, and hence EPS production strategies reported previously have been primarily focused on EPS yield, biomass growth and on strain improvements

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(Domingues, Queiroz, Cabral, & Fonseca, 2000; Guo et al., 2009; Hwang, Kim, Xu, Choi, & Yun, 2003). However, there is still a lack of knowledge concerning the effect of nutritional conditions on EPS activity and on morphological development.

Phellinus Quél. (*Basidiomycota*) is a well-known pharmacodynamic fungus of the genus *Phellinus* in the family of *Hymenochaetaceae*. It has been used as a traditional Chinese medicine in China for a considerably long time with its fruiting body called "Sanghuang" in Chinese. It is also highly prized in Korea and Japan for its medicinal properties since ancient times. Polysaccharides or crude extracts isolated from some *Phellinus* species have been confirmed to possess medicinal properties such as stimulating antitumor immunity and inhibiting tumor growth (Park et al., 2004). They also have been shown to have free radical-scavenging activity in addition to anti-hepatotoxin (Kim et al., 2007) and antioxidant activity (Park et al., 2004). Recently, polysaccharides from the submerged cultivation of *P. linteus* have been shown to possess a favorable high potential in the development of anti-hyperlipermia drugs due to the high anti-hyperlipemia activity obtained from these compounds (Zou, Guo, & Sun, 2009). The increasing reports of the potential medicinal properties of *Phellinus* species have attracted considerable research interest in their biopharmaceutical application and bioproduction. However, the effect of nutritional conditions on EPS bioactivity and the cell development of this species has not been previously investigated, although relatively high yield of EPS and mycelial growth has been achieved in the submerged culture of *Phellinus* (Guo et al., 2009; Zou et al., 2009).

The *Phellinus* habitats such as the mulberry tree are often rich in components such as rutin (also called rutoside, $C_{27}H_{30}O_{16}$), Fe^{2+}, aspartate and vitamins (Jia, Tang, & Wu, 1999; Kumari, Srivastava, & Srivastava, 2009). In addition, Tween-80 has been observed to be one of the most favorable surfactants for EPS production by *Cordyceps sinensis* Cs-HK1 (Liu & Wu, 2012), *Grifola frondasa* (Hsieh, Wang, Chen, Hsu, & Tseng, 2008) as well as *Schizophyllum commune* (Hao et al., 2010). The influence of these components, however, on the activity of the polysaccharide, has not been previously investigated. Also, rutin has not been employed previously in any cell culture of fungi. The present study was aimed to investigate the effect of rutin (antioxidant) and Tween-80 (surfactant) supplementations, as well as the presence of specific nutrients ($FeSO_4$, aspartate and vitamins) in the culture medium of *Phellinus* sp. P0988 on EPS production and total antioxidative activity. Fungal morphological development and cell growth in response to the presence of these components in the course of EPS production were also explored. The relative effects of these components on EPS production and on EPS activity was evaluated by an orthogonal experimental design, a serviceable experimental design for the evaluation of the relative effects between different factors (Xu & Yun, 2003), and also minimizes the number of experiments required for investigating the effect of these factors. Identification of the influence of these components and their relative effects on EPS yield and activity, and on the cell development in *Phellinus* is critical to enhancing the production and the pharmacophore of these types of secondary metabolites, and can aid in providing a deeper understanding of the synthesis mechanisms involved in the production of secondary metabolites by fungi.

2. Materials and methods

2.1. Microorganism and inoculum

The strain of *Phellinus* sp. P0988 was isolated in our laboratory and was also deposited in the Chinese Type Culture Collection Center (CCTCC M 2012080). The stock culture was maintained on potato-dextrose-agar slants, and transferred to a fresh agar plate every month for a new stock culture. The stock slant of this strain was grown at 28 °C for approximately two weeks for obtaining new slant seeds. Analytical grade chemicals were used to formulate the basal medium of flask culture with a composition of: glucose 40 (g/L), glutamic acid (Glu) 4 (g/L), $(NH_4)_2SO_4$ 4 (g/L), $MgSO_4$ 1 (g/L), and KH_2PO_4 1 (g/L). The pH was adjusted to 6.5 by the addition of either 1 N NaOH or 1 N HCl. The cultures were grown in shake flasks with a 10% (v/v) inoculation and incubated in an orbital shaker at 28 °C and 160 rpm (revolutions per minute) for 12 days. Chemicals such as aspartate and others were analytical grade supplied by Sigma–Aldrich unless specified otherwise.

2.2. Effect of components on the fermentation of Phellinus sp. P0988

In order to investigate the influence of specific components on EPS yield and activity by *Phellinus* sp. P0988, rutin (Batch No.: 20120509, Shaanxi, China), $FeSO_4$, aspartate, complex vitamins (Shanghai, China) and Tween 80 with different concentrations were added separately to the basal medium. Experiments were performed at least in triplicate to ensure reproducibility.

An orthogonal layout L_9 (3^4) was used to investigate the relative effects of the factors (namely, the concentrations of rutin, Tween 80, $FeSO_4$, and aspartate) on EPS yield and activity. According to the relative magnitude of the *R* value obtained (*R* represents the difference between the maximum and minimum of results at different level of a factor in this study), the relative effect of components and optimum medium composition for EPS production and bioactivity were also determined.

2.3. Morphological observation and fluorescence microscopy of mycelial pellets

The pellets of *Phellinus* sp. P0988 in submerged culture were observed using an automatic colony analyzer (Hangzhou, China) as described previously (Cox & Thomas, 1992) with a modification. Broth samples from *Phellinus* sp. P0988 were allowed to stand for 20 min and the supernatant of each sample was discarded. The precipitated pellets were washed with distilled water (Millipore, USA) twice and were then placed on coverslips in a petri dish with a small amount of distilled water to ensure that the filaments of pellets were naturally stretched. The pellets on the coverslips were subsequently observed, photographed with the automatic colony analyzer, and the photos were further analyzed for pellet size and morphology.

Fluorescence staining was performed following the Hoechst Stain Kit guide with a modification (Shanghai, China). First, the precipitated pellets were carefully placed on coverslips in a petri dish and the residual supernatant in the petri dish was carefully aspirated by a pipette, only leaving enough liquid to barely cover the coverslips. Then, a sufficient amount of fixative was added into the petri dish to cover the pellets. The pellets were allowed to be fixed for 12 h or overnight at 4 °C. After removing the supernatants/fixative, the coverslips were then air-dried and 1.0 mL of 6 times Hoechst stain dilution was added on the coverslips, allowing pellets to stain for 2–3 h. After staining, the stain solution was removed from the petri dish. The pellets on the coverslips were washed three times in distilled water at room temperature and then air-dried. The stained pellets were subsequently observed using a confocal microscope (TCS SP5, Leica, Germany).

2.4. Analysis of cell dry weight, EPS production and total antioxidative activity

The cell dry weight (CDW) and EPS production of *Phellinus* sp. P0988 were determined as described previously (Liu & Wu, 2012)

with a little modification. Broth samples were collected at various intervals during cultivation and were centrifuged at 12,000 rpm for 20 min. The pellets of mycelial culture were washed thoroughly with distilled water and dried at 60 °C for 12 h or overnight until constant weight was reached for the determination of CDW. The supernatant was collected for the determination of EPS yield and antioxidative activity.

For the preparation of crude EPS, the culture supernatant (1 mL) was mixed vigorously with 3 volumes of 95% (v/v) ethanol, stored at 4 °C for 12 h to facilitate precipitation, and then centrifuged at 12,000 rpm for 5 min. The precipitate was then separated, rinsed thoroughly in 100% (v/v) ethanol, lyophilized with a freeze dryer (ALPHA1-2 Christ, Osterode German), then re-suspended in ethanol (75%, v/v), and centrifuged as described above to harvest crude EPS. The crude EPS was subsequently dried at 60 °C to remove residual ethanol. Finally, the crude EPS was dissolved in distilled water for the determination of EPS yield and antioxidative activity.

The EPS content was measured by a microplate colorimetric assay developed in our laboratory based on the phenol/sulfuric acid method. Briefly, to each sample well of a 96-well microplate (Applied Biosystems, USA), the following chemicals were added consecutively: 30 μL of glucose or EPS sample, 80 μL of concentrated sulfuric acid (Fisher Scientific, UK Ltd.) and 15 μL of 5% (w/w) phenol solution (Fisher Scientific, UK Ltd.). After shaking for 30 min, the mixture in the sample wells was heated at 90 °C for 5 min in a static water bath and then cooled at room temperature for 5 min. The absorbance of the mixture in the microplate was measured at a wavelength of 498 nm using a spectrophotometer (BioTek Epoch, Winooski, USA). A standard curve was determined using glucose with a determination coefficient (R^2) of over 0.9991.

2.5. EPS total antioxidative activity

The physiological activity of EPS was evaluated based on the EPS total antioxidative activity, which was in this study displayed as the molar concentration of Trolox, namely, Trolox-Equivalent Antioxidant Capacity (TEAC). TEAC was determined using a T-AOC Assay Kit (Total Antioxidant Capacity Assay Kit, Beyotime Biotechnology Company, China). This kind of kit is based on the reduction of $ABTS^{\bullet+}$ radicals (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, ABTS) (Dudonne, Vitrac, Coutiere, Woillez & Merillon, 2009) by antioxidants in the sample. ABTS solution from the kit was diluted with deionized water and allowed to react with potassium persulfate (2.45 mM). The reaction mixture was allowed to stand in the dark at room temperature for 12–16 h before use. This ABTS mixture was then diluted by a factor of 100 in phosphate buffered saline (PBS) to obtain an absorbance of 0.7 ± 0.05 at a wavelength of 734 nm (subtracting blank absorbance value), and this solution was then used as the ABTS working solution. For the spectrophotometric assay, 200 μL ABTS working solution and 10 μL EPS sample were added to the each of the sample wells of a 96-well microplate, respectively. The samples were mixed by a microplate shaker and the absorbance was then determined at 734 nm. The absorbance for different samples was corrected by the absorbance of an ABTS blank. A standard curve was determined using Trolox as a standard with a determination coefficient (R^2) of over 0.9994. All assays were carried out in triplicate.

2.6. Statistical analysis

The results were analyzed for statistical significance by a one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) program. All data were expressed as mean ± S.E. ($P < 0.05$). Group means were considered to be significantly different at $P < 0.05$, as determined by the least significant difference (LSD).

3. Results and discussion

3.1. Effect of rutin on the fermentation of Phellinus sp. P0988

The generation of active oxidative species may be induced by some factors such as shear stress in submerged culture and which may further cause eukaryotic cells apoptosis or growth-inhibition (Lorin, Dufour, & Sainsard-Chanet, 2006; Osiewacz & Stumpferl, 2001). Rutin, a ketone antioxidant, was employed in the basal medium to investigate its influence on cell morphology and viability, mycelial growth, EPS production and TEAC by *Phellinus* sp. P0988. As shown in Fig. 1A, there is a considerable difference in EPS production and TEAC with different supplementations of rutin. Specifically, the concentration of 2 g/L rutin was observed to be significantly desirable in achieving maximum EPS production and TEAC (Fig. 1A). CDW was shown not to be affected considerably with change in rutin concentrations from 1 to 8 g/L. The aggregated pellets cultivated for 12 days in the medium with 2 g/L rutin were observed to have a relatively smaller diameter, as well as fewer and shorter filaments (Fig. 1C) compared to that of the control cells (Fig. 1B). Fig. 1E shows that the nuclei cultivated with rutin for 12 days appear to have tidy edges, exhibiting a normal Hoechst staining which is indicative of healthy growth compared with the faint staining in control cells that display abnormal labeling with nucleus condensation (inset, Fig. 1D). From these observations, the presence of rutin with appropriate concentration in the medium has imposed a favorable effect on the cell survival of *Phellinus* sp. P0988 in submerged culture.

Rutin has been shown to improve the antioxidant ability of diabetic rat tissues (Kamalakkannan & Stanely Mainzen Prince, 2006). However, the influence of rutin on EPS synthesis in addition to the survival and growth of fungi has not been studied before. Since rutin had an inconsiderable influence on cell growth, its effect on the TEAC and production of EPS may occur in a secondary metabolism regulative way. The effect of rutin on EPS production and activity may be attributed in part to its role in the support of the cell viability of fungi in submerged culture, which is possibly associated with its antioxidant potential during the fermentation of *Phellinus* sp. P0988. The application of rutin in the EPS production in fungal fermentations has not been reported before. By supplementation of rutin with appropriate concentration in the culture medium, the production and antioxidant activity of EPS by *Phellinus* sp. P0988 was significantly increased, revealing for the first time a potential application of rutin in the enhanced production of polysaccharides by macrofungi. In addition, mycelial morphology of fungi in submerged culture affects the flow properties of broth and hence may impose a strong influence on EPS productivity by filamentous fungi (Kim et al., 2006). Also, fungal mycelial morphology was shown to be related to secondary metabolite production (Keller, Turner & Bennett, 2005). Therefore, the role of rutin in the enhancement of EPS production and activity may be in part connected to its influence on mycelia morphological development, as these results presented herein suggest that rutin may affect cell morphology and which may in turn lead to the enhancement of EPS synthesis. The underlying cellular or molecular mechanism by which rutin influences EPS synthesis and cell morphology development is not the focus of this study. However, a beneficial role of rutin in enhancing cell survival in submerged culture and in morphological development can be suggested. The mechanism by which rutin favored EPS synthesis, morphological development and cell survival requires further investigation.

3.2. The influence of aspartate and $FeSO_4$

Fig. 2A shows that the EPS yield and TEAC increase upon the addition of aspartate concentration up to 2 g/L in the basal medium,

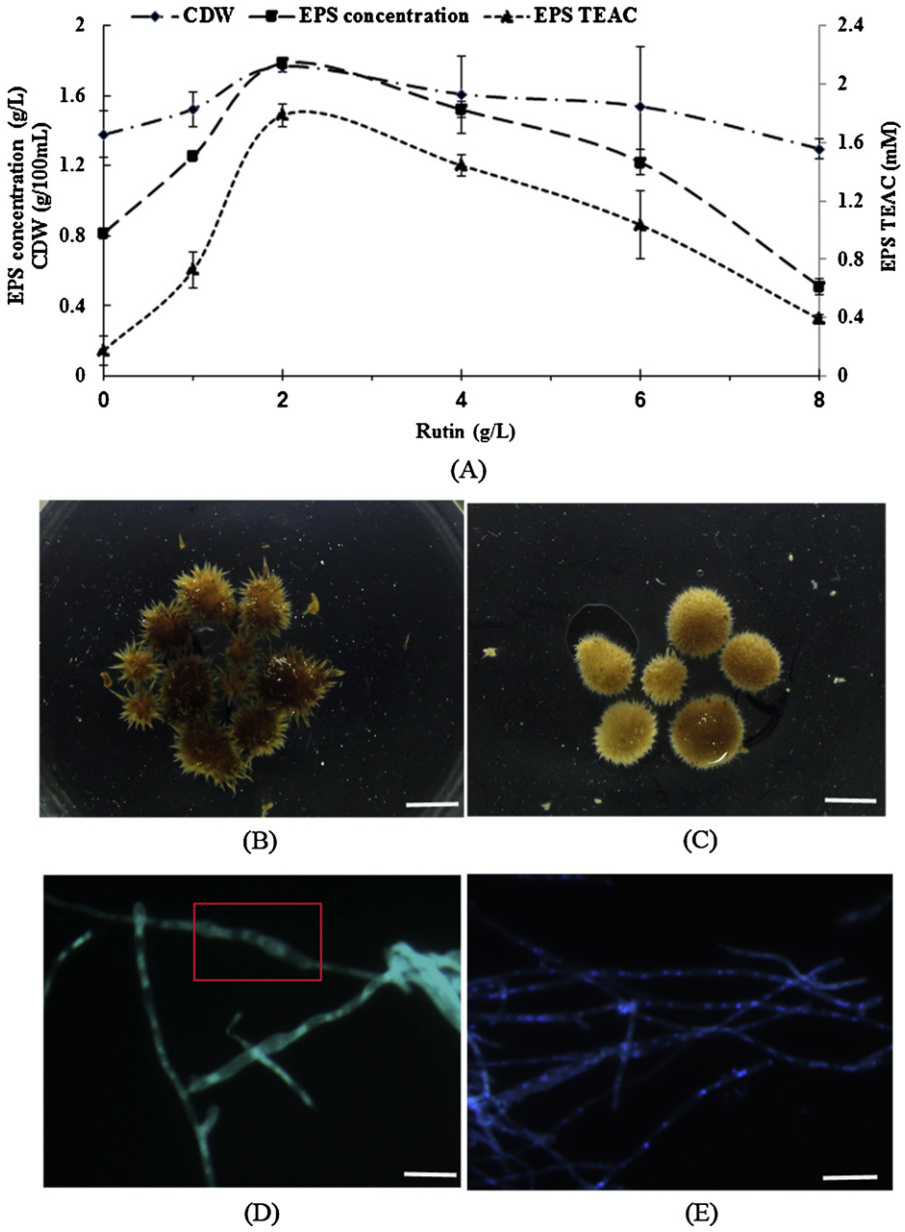

Fig. 1. Effect of rutin on the fermentation of *Phellinus* sp. P0988 in submerged culture. (A) The effect of rutin concentration on exopolysaccharide (EPS) production, mycelial growth (cell dry weight, CDW) and EPS antioxidant capacity (Trolox-Equivalent Antioxidant Capacity, TEAC). (B) The morphology of the aggregated pellets of *Phellinus* sp. P0988 grown in the basal medium. (C) The morphology of the aggregated pellets in medium containing rutin. (D) Hoechst staining of control cells. "Inset" shows nuclei condensation. (E) Hoechst staining of cells in medium with rutin. Scale bar, 200 μm. *The samples of mycelial pellets for morphological observations are from the control or the cultures containing an optimum concentrations of components or for EPS production and TEAC, unless specified otherwise.

reaching their maximum of 3.2 g/L and 2.4 mM at 2 g/L aspartate, respectively, and at a concentration above 2 g/L, aspartate was observed to suppress EPS production and TEAC. However, the influence of aspartate concentration on CDW was not as pronounced as its effect on EPS production and activity (Fig. 2A). The aggregated pellets in cell culture with 2 g/L aspartate appeared to be somewhat similar to those obtained when rutin was supplemented, having relatively smaller hairy filaments than those of the control cells (Figs. 1B and 2C). In Fig. 2E, the fluorescent staining of cells cultivated for 12 days with 2 g/L aspartate shows possible fragmentation of the nuclei.

Conflicting results were reported previously with respect to the influence of $FeSO_4$ on mycelial growth and on EPS production by fungi (Lin & Sung, 2006). In the study presented herein, the effect of $FeSO_4$ concentration on EPS production and activity in relation to the morphological development and cell viability of *Phellinus* sp. P0988 was explored. As observed in Fig. 2B, the enhanced EPS production and TEAC was achieved when $FeSO_4$ was supplemented at a concentration of up to 0.2 g/L, with a maximal EPS production and TEAC of 1.8 g/L and 1.6 mM with 0.2 g/L $FeSO_4$ supplementation, respectively. Above 0.2 g/L $FeSO_4$, the EPS production and TEAC declined with increase in $FeSO_4$ concentration (Fig. 2B). However, the CDW was nearly constant with change in $FeSO_4$ concentration. In addition, when cells were cultivated in medium with 0.2 g/L $FeSO_4$ for 12 days, as seen in Fig. 2D, the aggregated pellets in the medium were observed to be a little smaller, possessing relatively fewer filaments (Fig. 2D) compared to the control cells (Fig. 1B), and, the nuclei of the cells displayed a normal Hoechst staining (Fig. 2F), with neat and tidy edges.

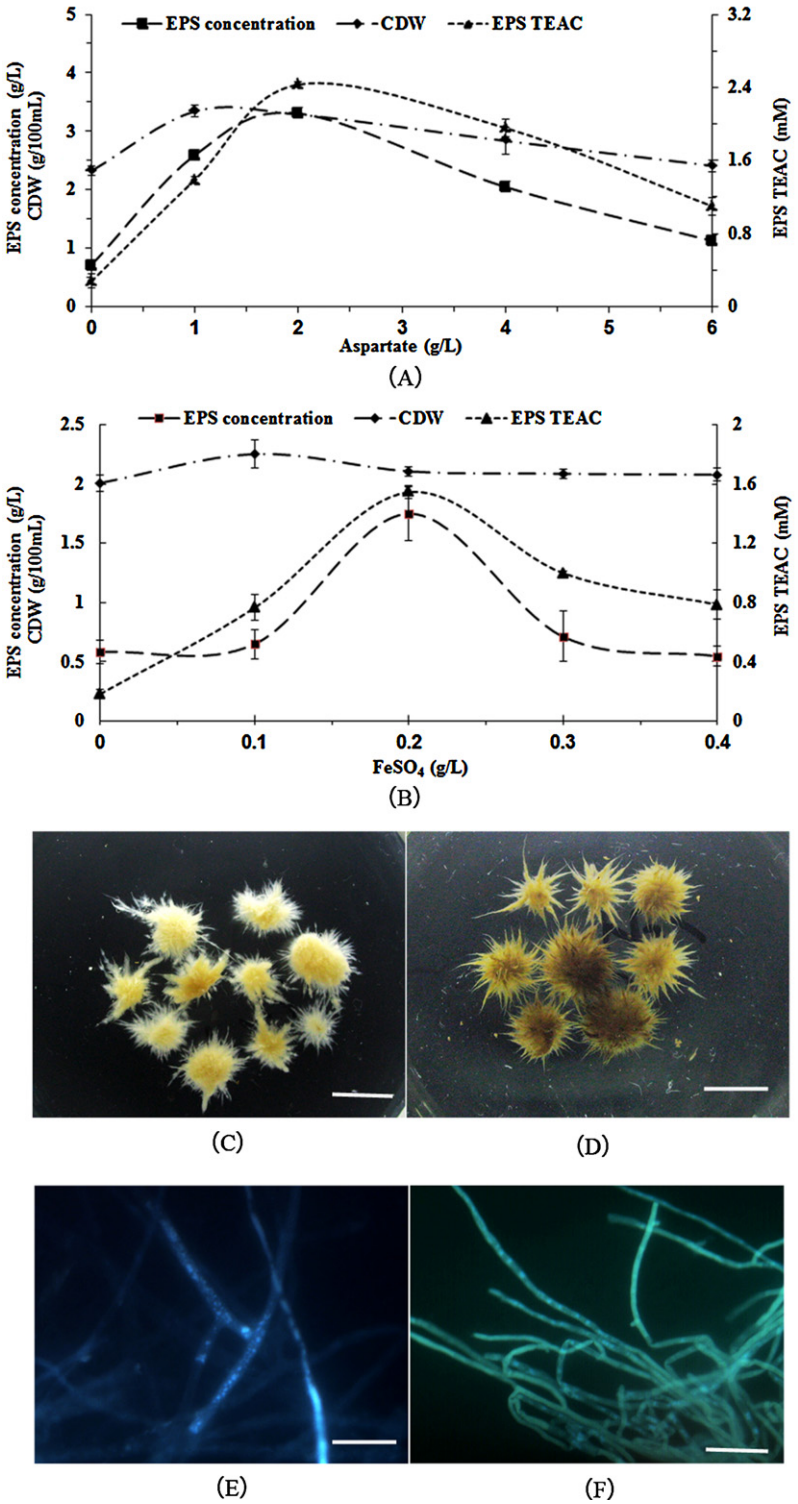

Fig. 2. The effect of aspartate and $FeSO_4$ on the fermentation of *Phellinus* sp. P0988. (A) The effect of aspartate on the EPS TEAC, CDW and EPS production. (B) The effect of $FeSO_4$. (C) The morphology of the aggregated pellets in cell culture with aspartate. (D) The morphology of the aggregated pellets with $FeSO_4$. (E) Hoechst staining of nuclei in cell culture with aspartate. (F) Hoechst staining of nuclei in culture with $FeSO_4$. Scale bar, 200 μm.

The Fe ion is a necessary nutrient in the cultivation of fungi such as *Pleurotus ostreatus* and *Phellinus igniarius* (Guo et al., 2009). Supplementation with $FeSO_4$ or other minerals enhanced the cell growth and EPS production by fungi (Hwang et al., 2008). The significant influence of the Fe ion (in the form of $FeSO_4$) on EPS production and total antioxidant activity as well as on cellular morphological development in addition to its minimal impact on the cell growth of *Phellinus* shown herein, suggest that the effect of $FeSO_4$ on EPS synthesis may be associated with its influence on the secondary metabolism of *Phellinus*. This is because the biosynthesis of secondary metabolites in filamentous fungi is usually linked with cell morphological development and is independent of cell growth (Calvo, Wilson, Bok, & Keller, 2002). The influence of $FeSO_4$ was confirmed to not only influence EPS production, but also have an effect

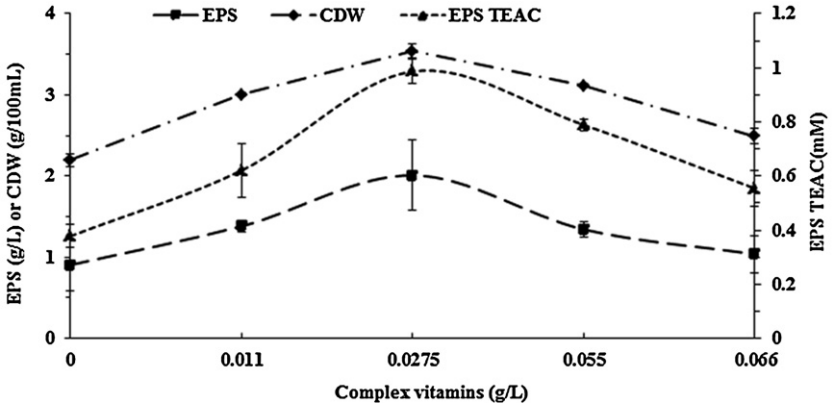

Fig. 3. Effect of complex vitamins on the fenmention of *Phellinus* sp. P0988.

on EPS total antioxidant activity in addition to the cell morphological development of *Phellinus*. The importance of this finding is that this can be applied to the enhanced production of EPS by fungi. The underlying cellular or molecular mechanism by which Fe ion influences EPS synthesis and cell morphological development has not been investigated in this study, although the role of $FeSO_4$ in affecting the secondary metabolism of *Phellinus* may be suggested.

3.3. Effect of complex vitamins

In order to satisfy the nutritional requirements of *Phellinus* sp. P0988 for a diverse range of vitamins, complex vitamins available at low cost were chosen to examine their influence on EPS production and TEAC as well as mycelial growth. In the study presented herein, the effect of vitamin supplementation on the activity of EPS produced by fungi was investigated for the first time. Fig. 3 shows that the EPS yield and TEAC increase with increasing concentration of complex vitamins of up to 0.0275 g/L in the basal medium. A concentration of 0.0275 g/L complex vitamins was found to be the optimum for EPS production and EPS TEAC. However, the effect of complex vitamins on mycelial growth (CDW) was relatively minimal (Fig. 3). In addition, the effect of complex vitamins on cell morphology and Hoechst staining of the nuclei was investigated, however, no significant changes relative to control cells were observed.

These results with respect to the influence of complex vitamins on cell growth are consistent with the previous report involving *Antrodia cinnamomea* in submerged culture (Lin & Sung, 2006) where supplementation with vitamins was found not to be an absolute requirement for cell growth. A possible reason for these observations is that some vitamins may only be involved in the secondary metabolism and may not directly affect the cell growth of fungi. For the first time, the role of complex vitamins in enhancing the activity of EPS by *Phellinus* is elucidated.

3.4. The influence of Tween 80

Different concentrations of Tween 80 were added in the basal medium to investigate its effect on the fermentation of *Phellinus* sp. P0988. As seen in Fig. 4A, Tween 80 exhibits a remarkable effect on EPS production and TEAC, increasing the EPS yield more than almost twofold at a concentration of 4 g/L than that of the control. However, above 4 g/L, inhibition for the EPS production and TEAC was observed, while the CDW was not influenced significantly with change in Tween 80 concentration. These results provide some new information about the effect of Tween 80 on EPS TEAC for the first time and are very important to the application of Tween 80 in the polysaccharide production by fungi. Also, when cultivated in submerged culture for 7 days (Fig. 4C), the cells appeared to be large multi-filamentous mycelium pellets compared to the smaller hairy pellets in the control cells (Fig. 4B). It should be noted that the most significant difference in morphology compared to the control cells was obtained at 7 days of cultivation. Beyond 7 days of cultivation, no significant difference in morphology was observed. These results suggest that the influence of Tween 80 on EPS yield and TEAC may not be directly growth dependent but rather possibly related to mycelial morphological development of *Phellinus* sp. P0988 (Fig. 4A and C). In addition, the staining results showed that the nuclei in the medium with Tween 80 exhibited normal Hoechst staining with neat and tidy edges compared to the faint staining in control cells (Figs. 1E and 4D). These results suggest that the presence of Tween 80 with an appropriate concentration in the medium leads to an enhancement in cell viability or at least has no significant unfavorable effect on cell viability. These results for the first time provide evidence on the influence of Tween 80 on cell viability and are important for the application of Tween 80 in the high yield production of EPS.

Surfactants such as Tween-80 affected mycelial growth, cell development and EPS production in fungal cultures such as *C. sinensis*, *Collybia maculata TG-1* and *Botryosphaeria rhodina* MAMB-05 (Lim & Yun, 2006; Liu & Wu, 2012; Silva, Dekker, Silva, Silva, & Barbosa, 2007). Possible mechanisms involved in the enhancing effect of Tween 80 are thought to involve enhancing cell membrane permeability for EPS secretion and the stimulation of biosynthetic activity for EPS production (Angelova & Schmauder, 1999; Liu & Wu, 2012). However, in the fungal cultivation of *C. maculata*, different results were obtained, in which Tween 80 did not result in any increase in EPS production (Lim & Yun, 2006). The role of Tween 80 in stimulating EPS production in this study is in good agreement with the previous report (Liu & Wu, 2012), however, the influence of Tween 80 on EPS TEAC was elucidated for the first time. These results are significant for the enhanced bioproduction by fungal species where Tween 80 can be applied to obtain high EPS activity and yield. The inhibition of pellet formation by Tween 80 was not observed with different supplementations as reported previously (Liu & Wu, 2012). On the contrary, the presence of Tween 80 in the medium favored the dispersion of mycelia, causing mycelia to be considerably longer and more dispersed compared to the mycelia of the pellets of the control cells (Fig. 4B and C). In addition, the study presented herein suggests that the stimulation of Tween 80 on EPS production may not possibly result from its influence on the cell growth, but may rather be linked to the secondary metabolism of fungi, as the secondary metabolite production is often connected with morphological development in filamentous fungi (Calvo et al., 2002) and is independent of cell growth. Tween 80 supplemented in the medium was not observed to have an unfavorable influence

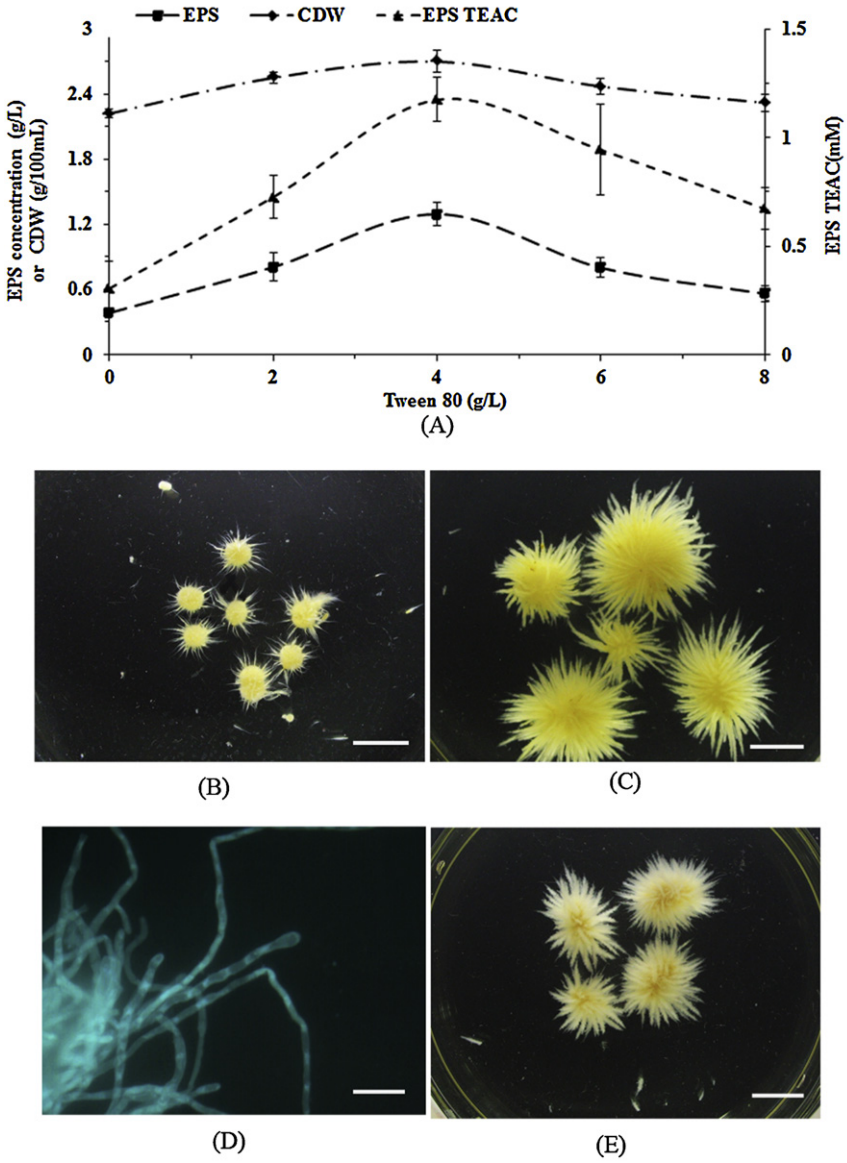

Fig. 4. The effect of Tween 80 on the fermentation of *Phellinus* sp. P0988 and the cell morphology in the optimized medium. (A) The effect of Tween 80 on EPS production, CDW and EPS TEAC. (B) The morphology of the aggregated pellets of controlling cells after 7 days cultivation. (C) The morphology of the aggregated pellets of cells in medium with Tween 80 after 7 days cultivation. (D) Hoechst staining of nuclei in cell culture with Tween 80 after 12 days cultivation. (E) The morphology of the aggregated pellets in optimized medium after 12 days cultivation. Scale bar, 200 μm.

on nucleus staining of *Phellinus* sp. P0988. This result is important to the application of Tween 80 in EPS production by fungi because it elucidated for the first time the influence of Tween 80 on cell viability in fungal submerged culture.

3.5. The relative effects of various components tested

The orthogonal matrix L_9 (3^4) and the fermentation results are shown in Table 1. Based on the magnitude order of the *R* value, the relative effects of the components tested on EPS yield were $FeSO_4$ > Tween 80 > rutin > aspartate, with optimum concentrations of 0.15 g/L, 7 g/L, 3.5 g/L, and 2.5 g/L, respectively, while the relative effects of components on EPS TEAC were aspartate > rutin > FeSO4 > Tween 80, with optimum concentrations of 3.5 g/L, 2.5 g/L, 0.35 g/L, and 7 g/L, respectively. Based on the results of the analysis of statistical significance of the effects of the components (ANOVA), rutin and aspartate had a statistically significant effect on EPS TEAC ($P < 0.05$), while $FeSO_4$ and Tween 80 had a statistically significant effect on EPS yield ($P < 0.05$). The elucidation about the different relative effects of the components tested on EPS production and TEAC is very important because these results are helpful to the establishment of an efficient production strategy for EPS production with high activity.

In addition, the obtained optimum concentrations of these tested components in addition to the content of nutrients in the basal medium formed the optimum medium compositions for EPS production and TEAC, with EPS yield and activity 6.2 ± 0.2 g/L and 5.5 ± 0.1 mM, respectively. The relative effects of complex vitamins with other chemicals have not been studied as it contains several vitamins with very low concentrations. In addition, the microscopy images of *Phellinus* sp. P0988 mycelia (Fig. 4E) indicated that the morphological structures of mycelial filaments or pellets in the optimized medium after 12 days cultivation were in a form of freely dispersed filaments or loosely connected clumps. During the pellet growth, the mycelia initially appeared as dispersed filaments (after 24 h) and gradually formed hairy pellets with a dense core

Table 1
The orthogonal matrix L_9 (3^4) and the results of EPS synthesis of *Phellinus* sp. P0988.

No.	$FeSO_4$ (g/L)	Rutin (g/L)	Aspartate (g/L)	Tween 80 (g/L)	EPS yield (g/L)	EPS TEAC (mM)
1	0.15	1.5	1.5	3	4.6 ± 0.2	4.5 ± 0.1
2	0.15	2.5	2.5	5	5.6 ± 0.2	5.3 ± 0.2
3	0.15	3.5	3.5	7	6.2 ± 0.1	5.5 ± 0.0
4	0.25	1.5	2.5	7	4.5 ± 0.9	4.9 ± 0.6
5	0.25	2.5	3.5	3	3.3 ± 0.1	5.5 ± 0.1
6	0.25	3.5	1.5	5	4.4 ± 0.4	5.0 ± 0.3
7	0.35	1.5	3.5	5	3.9 ± 0.2	5.3 ± 0.2
8	0.35	2.5	1.5	7	3.7 ± 0.2	5.2 ± 0.5
9	0.35	3.5	2.5	3	3.4 ± 0.2	5.4 ± 0.3
R_1	1.8	0.5	0.3	1.0		
R_2	0.2	0.4	0.6	0.1		

*R represents the difference between the maximum and minimum of production or TEAC at different level of a factor. R_1 represents the R of EPS production. R_2 represents the R of EPS TEAC.

surrounded by filaments. The mycelial morphology depicted in Fig. 4E may be favorable for the polysaccharide production and activity development of *Phellinus* sp. P0988. The optimum medium compositions for EPS production and TEAC is very important because these results can be used in the high yield of EPS with high antioxidative activity which can guarantee the biological functions of EPS at least in antioxidative activity.

The effect of nutrients on EPS production has been documented previously (Liu & Wu, 2012), however, the influence of components or nutrients in the medium individually and their relative effects on EPS activity during fermentation has not been investigated before. It is still not clear which components are critical for the EPS activity development such as total antioxidant activity in fungal submerged culture. For the first time, in the study presented herein, the relative effects of components investigated on EPS production were elucidated to be different from their effect on EPS activity. This is very important for the establishment of enhanced bioproduction strategy of polysaccharide such as EPS with high activity. Additionally, these components that significantly influenced EPS yield and TEAC were identified for the first time. The importance of these findings is that these appropriate chemicals at an identified concentration in medium can be employed to yield polysaccharide with high activity and that these results are helpful to guarantee the antioxidative activity and other bioactivity of EPS in biopharmaceutical application. The further optimization of process by response surface method (RSM) and the study of polysaccharide spectroscopy by *Phellinus* sp. P0988 are currently underway in our laboratory, as RSM has previously been shown to significantly enhance the bioproduction of polysaccharide by *Phellinus baumii Pilát* (Luo et al., 2010).

4. Conclusions

This present study has for the first time addressed the individual and the relative effects of medium components such as rutin, aspartate, $FeSO_4$, complex vitamins and Tween 80 on EPS biotechnological activity, as well as on the cell growth and morphological development of *Phellinus* sp. P0988 in submerged culture. Components such as rutin and $FeSO_4$ affected EPS production and bioactivity possibly through their effect on cell viability and morphological development. Thus, these components should be used in the EPS bioproduction by fungi. These findings are significant for obtaining EPS with high antioxidant activity which is critical for their pharmaceutical application.

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