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Effects of Tween 80 and pH on mycelial pellets and exopolysaccharide production in liquid culture of a medicinal fungus

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Abstract This study investigated the effects of surfactant additives and medium pH on mycelial morphology and exopolysaccharide (EPS) production in liquid culture of a valuable medicinal fungus *Cordyceps sinensis* Cs-HK1. In the medium containing 20 g l^{-1} glucose and 6 g l^{-1} peptone as the sole nitrogen source, the Cs-HK1 fungal mycelia formed smooth and spherical pellets about 1.8-mm mean diameter. The mycelial pellets became less uniform at pH (4.0–5.0) lower than the optimum (pH 6.0) or turned to filamentous form at higher pH (8–9). Surfactants added to the medium inhibited pellet formation, resulting in smaller and looser pellets. Tween 80 exhibited a remarkable promoting effect on EPS production, increasing the EPS yield more than twofold at 1.5% (w/v), which was most probably attributed to the stimulation of EPS biosynthesis and release from the fungal cells by Tween 80.

Keywords *Cordyceps sinensis* · Liquid culture · Exopolysaccharide · Tween 80 · Mycelial morphology

Introduction

Polysaccharides from medicinal fungi or mushrooms represent an attractive class of nutraceutical and therapeutic compounds owing to their notable antitumor and immunomodulatory activities and other health-benefitting effects. Crude polysaccharide fractions extracted from several well-known medicinal mushrooms are widely applied in commercial health food products and some pure polysaccharides have been in clinical use or trials as immunotherapeutic agents or adjuvant drugs for cancer therapy [20]. The medicinal value of mushroom polysaccharides has attracted increasing research interest in the production of exopolysaccharide (EPS) by submerged or liquid fermentation of fungal mycelia.

EPS production by microbial fermentation has been applied in industry for a long time, providing several important commercial polysaccharides for food, pharmaceutical, and other applications [11, 17]. To the best of our knowledge, however, the production of bioactive EPS by liquid fermentation of edible or medicinal fungi is still a new area of research without much industrial application. The maximum EPS yields attained in most previous studies from various fungal species were below 10 g l^{-1}, significantly lower than the yields of existing industrial processes (50–100 g l^{-1} or higher). There is a need to enhance the EPS productivity through effective strategies of process intensification. The addition of chemical stimulants is a simpler and often more effective than other strategies such as optimization of the medium composition and culture conditions and alteration of the fermentation processes. Fatty acids, plant oils, and surfactants have been used as stimulants to promote metabolite production in various fermentation processes including EPS production by bacteria and fungi [5, 15, 16].

Tween 80 (polysorbate 80) is one of the most favorable surfactants for EPS production by most microorganisms including medicinal fungi such as *Grifola frondosa* [5] and *Schizophyllum commune* [4]. In addition to their effects on the growth and metabolite production, surfactants added to culture medium of fungal mycelia also influence the mycelial morphology. The morphological characteristics of

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fungal mycelia usually have a close relationship to the flow properties of culture broth, the mycelial growth, and product formation. A few recent studies have demonstrated the close association between EPS productivity and mycelial morphology and the influence by various culture factors such as nutrients, and medium pH [6, 12]. Fungal mycelia in a submerged culture present two major morphological forms—filamentous, freely dispersed filaments and pellets of aggregated mycelia—and the specific growth form in a fungal culture depends on numerous physiochemical factors [13].

Cordyceps sinensis (Berk.) Sacc., generally known as Cordyceps or DongChongXiaCao in Chinese, is a rare and precious medicinal fungus in Chinese medicine with a broad range of health benefits [9]. As natural Cordyceps is very limited and expensive, liquid fermentation has been widely explored for mass production of EPS with Cordyceps and other medicinal fungi [6, 14]. We have established the mycelial culture of a Cordyceps fungus Cs-HK1 and applied it to liquid fermentation for the production of mycelial biomass and EPS with tonic and medicinal value [7, 8, 20].

In our previous studies, a nutrient-rich medium was used for the Cs-HK1 mycelial culture, which was favorable for mycelial growth and EPS production [7, 8]. In this medium, however, the Cs-HK1 fungus retained the filamentous growth form of freely dispersed mycelia, producing a highly viscous broth. In the present study, a different medium was used for the Cs-HK1 mycelial culture to retain pellet morphology. Experiments were first performed to evaluate the effects of culture pH and surfactants, especially Tween 80, on pellet morphology and EPS production in the pellet growth medium.

Materials and methods

Fungal species and mycelial culture

The fungus Cs-HK1 strain was isolated from a natural Cordyceps fruiting body in our lab and identified as an anamorphic species of *Cordyceps sinensis* both morphologically and genetically [7]. The stock culture of Cs-HK1 mycelia was maintained on potato dextrose agar (PDA) medium at 4°C. For initiation of liquid culture, the stock culture on a petri dish was incubated at 20°C for about 3 weeks until sporulation. A loopful of the fungal spores was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of a nutrient-rich liquid medium and incubated at 25°C on a rotary shaker at 150 rpm for 7 days. This culture broth was used as the starter culture for all experiments in this study and the starter culture medium was composed of 40 g l^{-1} glucose, 15 g l^{-1} yeast extract (YE), 5 g l^{-1} peptone plus three inorganic salts (K, P, Mg) as reported previously [7].

Experiments on surfactants

The basal liquid medium for the experiments was composed of 20 g l^{-1} glucose and 6 g l^{-1} peptone plus the same inorganic salts as for the starter culture, and the medium pH was 6.0. As shown in the "Results", this medium favored pellet growth of the Cs-HK1 mycelia and was referred to as the pellet growth medium, but the nutrient-rich medium favored filamentous morphology. Three nonionic surfactants including Tween 80 (from Sigma-Aldrich), Tween 20 (from BDH), and Pluronic F68 (from Sigma Chemical Co.) were initially tested as medium additives for stimulation of EPS production, all at a fixed concentration of 1% (w/v) in the medium. The effect of initial medium pH was also tested in the range of 4.0–9.0, adjusted with HCl or NaOH.

All experiments were carried out in shake-flask cultures in 250-ml Erlenmeyer flasks placed on a rotary shaker running at 150 rpm and at 25°C for an overall culture period of 7–9 days. Each of the culture flasks was filled with 50 ml liquid medium and inoculated with 1 ml of diluted (10 times) seed culture broth. All experimental variables were tested in triplicate and the results are reported as mean ± standard deviation (SD).

Determination of biomass and EPS concentrations

Culture broth collected at various intervals during the incubation period was centrifuged at 12,000×*g* for 10 min. The supernatant was removed and collected for determination of EPS concentration; the pellet of mycelial biomass was washed thoroughly with distilled water and dried at 80°C for 12 h to constant weight for determination of biomass dry weight. The supernatant medium was filtered through a 0.45-μm membrane and the filtrate was mixed vigorously with 4 volumes of absolute ethanol and then left overnight at 4°C. The precipitate was collected, rinsed thoroughly with 80% (v/v) ethanol, and lyophilized as the crude EPS.

Image analysis of mycelial morphology

The morphological characteristics of mycelia were observed and measured by image analysis. The broth sample was placed in a petri dish and mounted on an optical microscope, and the mycelium images were captured, and processed with the Image-Pro Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). The average pellet diameter and size distribution were measured with representative samples taken from each flask. For each sample, at least 20 pellets were measured to attain the average pellet size and size distribution. For visualization of the

mycelium microstructures, the broth sample was mixed with an equal volume of fixative (13 ml 40% formaldehyde, 5 ml glacial acetic acid in 200 ml 50% ethanol). The fixed sample was stained with Evans Blue solution (0.25% w/v in 30% ethanol) for 2 min, placed on a slide, and covered with a coverslip for the image analysis.

The mass of pellets as a percentage of the total biomass in the culture was determined as follows. The mycelial broth sample was filtered through a 400-μm standard mesh sieve which retained pellets on the mesh and allowed the filamentous mycelia to pass through. The pellets on the mesh were washed thoroughly with deionized water, whereas the liquid passing through the mesh was filtered through a 0.45-μm filter paper to retain the filamentous mycelia. Both parts were dried to constant weight at 80°C.

Results

Effects of medium pH and surfactants on pellet formation

In the nutrient-rich medium used for the starter culture containing 40 g l^{-1} glucose, 15 g l^{-1} yeast extract, and 5 g l^{-1} peptone, the Cs-HK1 fungus grew in the filamentous form of well-dispersed mycelia, presenting a milky and viscous broth. In the pellet growth medium used for the experiments in present study with lower glucose (20 g l^{-1}) and peptone (6 g l^{-1}) as the sole nitrogen source (without YE), the Cs-HK1 mycelia formed smooth and spherical pellets, suspended in a clear culture broth. The pellets in the culture were relatively uniform with a narrow diameter range of 1.79 ± 0.3 mm (Table 1 at pH 6.0). The variation of medium pH resulted in significant changes in pellet formation and size distribution of the mycelia. At pH 4.0, i.e., much lower than the optimal (pH 6.0), the mycelia formed pellets with a wide diameter range from less than 1.0 mm to about 6.0 mm; at a higher pH (8.0), the mycelia turned to filamentous with a few loose pellets in the culture broth. As shown by the quantitative data in Table 1, in the low pH range of 4.0–6.0, pellets dominated the mycelial culture, accounting for 84–86% of the total biomass in the culture, whereas in the high pH range of 6.0–9.0, the proportion of pellets decreased rapidly with the increase of medium pH to less than 50% of the total biomass in the culture, and the mycelia formed small pellets entangled with dispersed filaments. The mean pellet diameter increased notably with the reduction of medium pH from 6.0 to 4.0 and decreased slightly with the increase of pH from 6.0 to 9.0.

Table 1 Effect of initial medium pH on biomass and EPS yields and pellet properties (in pellet growth medium for 7 days' culture)

pH	Pellet size (mm)	Pellet mass (wt%)	Biomass (g l^{-1})	EPS (g l^{-1})
4	2.30 ± 0.4	85.9 ± 2.6	8.80 ± 0.1	2.4 ± 0.3
5	1.91 ± 0.2	83.4 ± 4.1	11.7 ± 0.6	3.2 ± 0.3
6	1.79 ± 0.3	83.6 ± 1.8	11.9 ± 0.2	3.4 ± 0.2
7	1.78 ± 0.1	67.6 ± 3.9	11.3 ± 0.2	3.0 ± 0.1
8	1.72 ± 0.1	48.7 ± 1.7	10.8 ± 0.3	2.0 ± 0.2
9	1.73 ± 0.2	47.1 ± 2.3	10.8 ± 0.8	0.6 ± 0.1

Data values represent mean ± SD, $n = 3$

wt% weight percent of pellet in total biomass

The addition of Tween 80 to the pellet growth medium caused a notable reduction of the pellet size and presence of dispersed mycelia in the culture broth. Another surfactant, Tween 20, caused similar morphological changes to the mycelia pellets. According to the quantitative properties of pellet morphology in Table 2, the pellet size (mean diameter) as well as the total number of pellets in each culture flask (data not shown) increased rapidly from day 3 to day 5 and slowly from day 5 to day 7 in the medium with or without Tween 80. The mass ratio of pellets to the total biomass of mycelia in the culture broth with Tween 80 was significantly lower than that without throughout the culture period (~50% vs. 80–90%), and the average pellet diameter with Tween 80 was also notably smaller. The results suggest that Tween 80 and other surfactants in the culture medium inhibited the aggregation of mycelia to pellet formation and growth.

The optical microscopy images of Cs-HK1 mycelia in Fig. 1 reveal the morphological structures of mycelial filaments or pellets at different culture stages in various media. In the nutrient-rich medium, the mycelia were in the form of freely dispersed filaments or loosely connected clumps throughout the culture period (Fig. 1a). In the pellet growth medium (Fig. 1b), the mycelia appeared as dispersed filaments (24 h) and gradually formed a hairy pellet with a dense core surrounded by filaments, and the pellet core grew in diameter and became more and more compact with the culture period. In the medium containing 1.5% Tween 80 (Fig. 1c), the mycelia showed a similar course of morphology development and pellet formation over the culture period.

Effects of pH and surfactants on mycelial growth and EPS production

The application of a much lower or higher initial pH than the optimum (pH 6.0), i.e., 4.0, 8.0, or 9.0, resulted in poor biomass growth and EPS production, leading to lower biomass and EPS yields (Table 1). The results from the experiments on three different surfactants (Fig. 2a) showed that the biomass yield was increased with Tween 80 but decreased with the two other surfactants. The EPS yield was increased significantly with Tween 80, and slightly

Table 2 Changes of pellet morphology and biomass growth during the culture period in medium with 1.5% Tween 80 (Twn +) and without Tween 80 (Twn −)

Time (days)	Pellet size (mm)		Pellet wt% (w/v)		Biomass (g l^{-1})	
	Twn −	Twn +	Twn −	Twn +	Twn −	Twn +
3	1.45 ± 0.10	1.25 ± 0.10	90.6 ± 3.4	54.0 ± 2.7	n.d.	4.0
4	1.53 ± 0.08	1.36 ± 0.11	88.5 ± 3.2	54.1 ± 3.5	4.60	10.6
5	1.63 ± 0.09	1.56 ± 0.10	87.3 ± 2.8	51.2 ± 3.2	5.14	12.4
6	1.76 ± 0.10	1.63 ± 0.07	85.8 ± 2.5	48.7 ± 3.7	6.10	13.7
7	1.80 ± 0.09	1.67 ± 0.09	80.9 ± 3.2	47.1 ± 3.5	11.9	14.7

Data values represent mean ± SD, $n = 3$

n.d. not determined

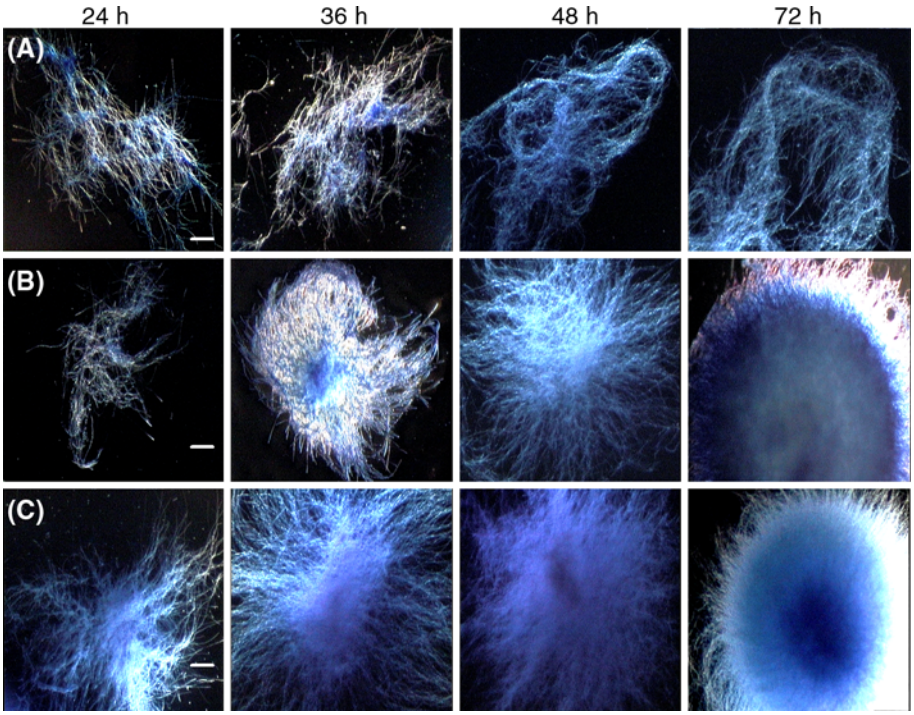

Fig. 1 Morphological changes of Cs-HK1 mycelia in different culture media over the culture period: **a** Nutrient-rich medium. **b** Pellet growth medium. **c** Pellet growth medium with 1.5% Tween 80. (*Bar* 0.2 μm)

with Pluronic 68 but decreased with Tween 20. Tween 80 as the most favorable additive was further tested at various concentrations from 0.25 to 2% (w/v) and the highest EPS yield was attained at 1.5% (Fig. 2b), 7.2 g l^{-1}, i.e., more than double the yield (3.3 g l^{-1}) from the mycelial culture without Tween 80.

Discussion

The influence of medium pH on mycelial morphology is most probably associated with changes in the surface charges of fungal filaments which generate electrostatic forces, attraction or repulsion, leading to pellet formation or dispersion [2, 10]. The surface properties of fungal cells, e.g., hydrophobicity, surface energy, and surface charge, are the intrinsic properties controlling mycelial morphology and the aggregation of mycelia to pellets [13]. These properties are dependent on the metabolic activities of the cells and the culture factors such as nutrients, pH, and additives. Our results from the Cs-HK1 mycelial culture showing that a lower, acidic pH favored pellet formation and size growth was also found for several other fungal cultures [18, 19]. As suggested by Papagianni [13], the cell wall surfaces of microorganisms at pH above 5.5 usually are negatively charged, which causes electrostatic repulsion, promoting the separation of cell aggregates. However, the specific pH range for pellet or filamentous growth can vary with different fungal cultures because the surface charge properties depend on the fungal species and the physiochemical conditions [2].

The effects of Tween 80 on mycelial morphology may be attributed in part to its surface-active properties, lowering

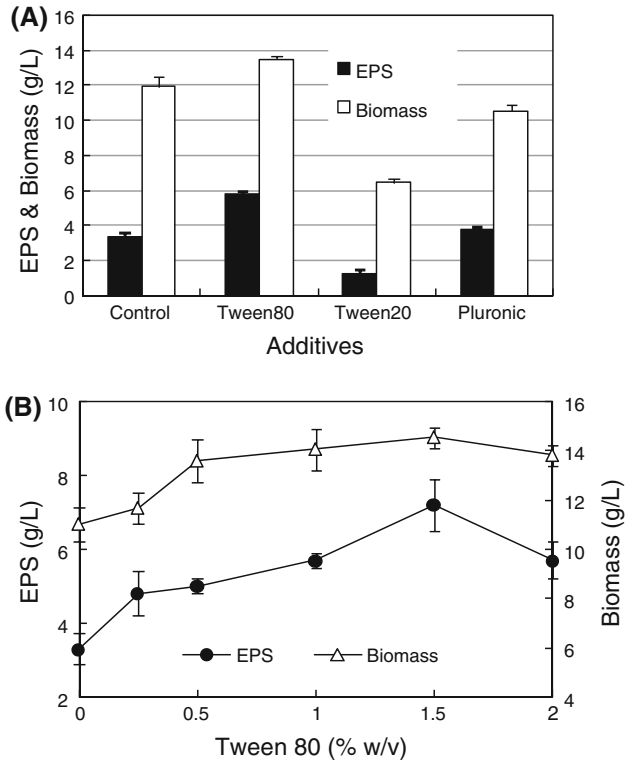

Fig. 2 Effects of surfactant additives on the biomass and EPS yields of Cs-HK1 mycelial culture (in pellet growth medium) with the addition of **a** various surfactants (at 1.0%) or **b** Tween 80 at various concentrations

the mycelium–liquid interfacial tension and thus the potential or tendency of mycelia to form aggregates. A decrease in the surface tension of the medium by a surfactant lowers the thermodynamic potential for the aggregation but favors the dispersion of mycelia. Similarly, Tween 80 inhibited pellet formation of other filamentous fungi such as a *Trichoderma reesei* strain in liquid cultures [3].

A more remarkable and desirable effect of Tween 80 on the Cs-HK1 mycelial culture is the dramatic enhancement of EPS production. A few previous studies have also shown the stimulating effect of Tween 80 on EPS production in submerged mycelial cultures of various fungi such as *S. commune* [4] and *Botryosphaeria rhodina* [15]. Some possible causes have been suggested, including the stimulation of biosynthetic activity for EPS production and the membrane permeability for EPS secretion, and the prevision of an alternative carbon source for EPS production [1, 3]. The stimulating effect of Tween 80 on EPS release is based on its putative function as a potent surfactant which can interact with the phospholipid bilayer membranes and can also solublize biomolecules, thereby facilitating the release of EPS into the extracellular medium.

In view of the similar effect of Tween 20 on the mycelial pellets but its adverse effect on EPS production in contrast to Tween 80, the dramatic enhancement of EPS production by Tween 80 should not be attributed significantly to the reduction of pellet size so as to facilitate the nutrient and oxygen transfer. The nutritional function of Tween 80 should not be a chief cause either, as judged from the much lower EPS yield in the nutrient-rich medium with sufficient carbon and nitrogen nutrients, and because Tween 80 also enhanced the EPS production in this medium as found in a separate experiment (data not shown). Likewise, nutrient function has been ruled out as a major cause for the enhanced EPS production by Tween 80 in some other fungal cultures *G. frondosa* [5] and *B. rhodina* [15]. A more possible cause is the stimulation of EPS biosynthesis in the fungal cells, though the stimulation mechanism is still unknown and needs further investigation.

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