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Simultaneous increase of mycelial biomass and intracellular polysaccharide from *Fomes fomentarius* and its biological function of gastric cancer intervention

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ABSTRACT

In this work, the effects of submerged culture conditions and nutritional requirements on simultaneous production of mycelial biomass and intracellular polysaccharide (IPS) from a medicinal mushroom *Fomes fomentarius* were studied using desirability functions. Under the optimal culture condition, the production of mycelia and IPS reached 17.19 and 2.86 g l^{-1}, respectively in a 15l stirred tank bioreactor, which were about twice than that of the basal medium. Furthermore, the ethanol extract of mycelia (EEM) and IPS had a direct antiproliferative effect on human gastric cancer cell lines SGC-7901 and MKN-45 in a dose-dependent manner. In contrast, human normal gastric cell line GES-1 was less susceptible to EEM and IPS. These results suggest that *F. fomentarius* may represent a promising novel approach for gastric cancer intervention.

1. Introduction

Gastric cancer ranks as the second most common cause of cancer-related death in the world (Catalano et al., 2005). This aggressive disease is a major global threat to public health, with a high incidence reported in Asia, especially in China and Japan, while a much lower incidence has been observed in Western Europe and the United States (Catalano et al., 2009; Davis & Sano, 2001). Surgical resection represents the cornerstone of any curative treatment at early stages, but gastric cancer is often diagnosed in advanced and inoperable stages, the median survival for advanced gastric cancer is in the range of only 6–10 months, and 5-year survival rate is less than 10% (Catalano et al., 2009). Chemotherapy may play a key role in advanced gastric cancer because the majority of patients with gastric cancer develop metastases during the course of their disease (Rivera, Vega-Villegas, & Lopez-Brea, 2007). However, current therapies are limited due to considerable side effects (Bouche et al., 2005). It is therefore necessary to search for novel approaches to treat gastric cancer patients with less adverse effects.

Traditional Chinese medicine has been used for thousands of years, and their importance in the prevention and treatment of cancer is becoming increasingly apparent. In the last decades, experimental and clinical studies indicated that several Chinese herbs were effective in treating and preventing gastric cancer (Atten, Attar, Milson, & Holian, 2001; Chen, Zhao, Li, et al., 2008; Endo et al., 2006; Lin & Tan, 1994). Thus, there is a reason to consider the use of other medicinal herbs in the treatment of gastric cancer. *Fomes fomentarius*, also called "Mudi" in China, is a fungus of the polyporaceae family and is parasitic on broadleaf trees. This mushroom has been used as a traditional Chinese medicine for centuries in China for treating various diseases such as gastroenteric disorder, hepatocirrhosis, oral ulcer, inflammation, and various cancers. Previous studies of this fungus have revealed its various appealing biological activities, including antidiabetic, anti-inflammatory, antioxidant and anticancer (Ito, Sugiura, & Miyazaki, 1976; Lee et al., 2006; Park et al., 2004). In our previous study, the strain of *F. fomentarius* was isolated from the fruiting body of a wild *F. fomentarius* and identified by ITS-5.8S rDNA sequencing analysis. Then, we optimized the submerged culture conditions and nutritional requirements of exopolysaccharide (EPS) from *F. fomentarius*, and observed that EPS had a direct antiproliferative effect on SGC-7901 human gastric cancer cells (Chen, Zhao, Chen, & Li, 2008). However, we found that sometimes the properties of EPS were vulnerable to culture conditions in large-scale fermentation, which resulted in poor quality control. In comparison, the properties of mycelia and intracellular polysaccharide (IPS) were more stable. On the other hand, two major drugs from medicinal mushroom for cancer treatment in

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China are Huaier Granule and Bailing Capsule, whose raw materials are intracellular polysaccharide of *Trametes robinioplila* and mycelia of *Cordyceps sinensis*, respectively. Therefore, it is necessary to investigate the process of mycelia and IPS production, and study their potential effects on human gastric cancer. As mycelia and IPS belong to two-parameter products, the total amount of IPS cannot reach maximal even if the concentration in mycelium is maximal. In view of this, it is better using a kind of optimization method to achieve both production maximal simultaneously. One of such methods should be desirability functions, which combine multiple responses into a single objective function (Rueda, Sarabia, Herrero, & Ortiz, 2003; Safa & Hadjmohammadi, 2005). This work attempts to develop suitable media and culture condition to produce simultaneously and efficiently bioactive mycelia and IPS from submerged culture of *F. fomentarius* by using desirability functions, and investigate their potential efficacy for gastric cancer intervention.

2. Materials and methods

2.1. Microorganism, inoculum preparation and flask cultures

F. fomentarius was maintained and cultured as described previously (Chen, Zhao, Chen, et al., 2008). Briefly, *F. fomentarius* was maintained on potato dextrose agar (PDA) slant at 4 °C and transferred every 2 months. The stock culture was incubated at 25 °C or 7–8 d, and then stored at 4 °C before use. The fermentation medium was based on basal medium (glucose 3%, peptone 0.5%, yeast extract 0.2%, KH_2PO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, distilled water, initial pH 6.0) to monitor carbon and nitrogen sources, mineral element, etc. Unless otherwise specified, the flask culture experiments were performed under the following conditions – basal medium, cultural temperature: 25 °C, culture period: 6 d, impeller speed: 150 rpm, initial pH 6.0 – in a 500 ml flask containing 100 ml of the fermentation medium after inoculation with 10% (v/v) of the seed culture.

2.2. Bioreactor cultures

The fermentation medium was inoculated with 10% (v/v) of the seed culture and then cultivated at 25 °C in a 15-l autocontrol bioreactor (FUS-Xl, Shanghai Guoqiang Bioengineering Equipment, China) equipped with sensors for pH, dissolved oxygen (DO) and temperature. Unless otherwise specified, fermentations were conducted under the conditions of temperature 25 °C, aeration rate 1.0 vol of air per volume of liquid per minute (vvm), agitation speed 150 rpm, initial pH 6.0, and working volume 9 l.

2.3. Detection of mycelial growth and polysaccharide production

Mycelia of *F. fomentarius* collected at various intervals from shake flasks were centrifuged at 10,000 × *g* for 10 min, washed several times with distilled water and lyophilized to a constant weight. Isolation of mycelial polysaccharide was carried out according to previous reports with some modifications (Schepetkin et al., 2008). Briefly, the lyophilized mycelia of *F. fomentarius* were extracted three times with distilled water at 80 °C for 1 h in a water bath (mycelia/distilled water ratio: 1:100). The extracts were cooled and centrifuged at 10,000 × *g* for 10 min. Supernatants were collected and mixed with four times its volume of 95% ethanol, stirred vigorously, and left overnight at 4 °C. The precipitated was centrifuged at 10,000 × *g* for 10 min, with the supernatant discarded. The precipitate of IPS was lyophilized and the IPS content was measured by a phenol-sulphuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The concentration of residual sugar was estimated using 3,5-dinitryl-salicylic acid colorimetry assay (Cai & Yuan, 1982). Preparation of mycelial extract was carried out according to the previous study with some modifications (Mau, Chang, Huang, & Chen, 2004). Briefly, the lyophilized mycelia of *F. fomentarius* (10 g) were extracted three times with 100 ml of 95% ethanol under stirring at room temperature for 24 h. The extracts were then centrifuged at 3000 rpm for 15 min and filtered through Whatman no. 4 filter paper; the filtrate was then evaporated to dryness in a vacuum.

2.4. Desirability of mycelial yield and polysaccharide production

The mycelium formation and the polysaccharide production were considered as two product quality variables. The desirability function was used to combine these two responses into a single objective function. In this study, a higher mycelium yield and a higher polysaccharide production are the desirability. Therefore, the desirabilities of the mycelium yield (d_M) and the polysaccharide production (d_P) are both "larger-the-better" responses, which can be expressed as follows:

$$\begin{aligned} &d = 0, && y \le y_{\min} \\ &d = (y - y_{\min})/(y_{\max} - y_{\min}), && y_{\min} \le y \le y_{\max} \\ &d = 1, && y \ge y_{\max} \end{aligned}$$

where d is the desirability. The desirability lies between zero and one, which represents the closeness of a response to its ideal value (denoted as y_{max}). If a response (y) is better than its most desirable value, $d = 1$; If a response (y) is worse than its most undesirable value, $d = 0$; When a response is outside the above parameters, d lies between 0 and 1. For the two-response system in this study, the overall desirability (D) is defined as a geometric mean of the individual desirabilities: $D = (d_M \times d_P)^{1/2}$. If the two responses are all better than their most desirable values, d_M and d_P equal 1, thus associated D is also 1. likewise, if any response is worse than the most undesirable values, i.e. any $d = 0$, associated $D = 0$ also. If any one of the two responses is not better than its most desirability value, i.e. $d < 1$ for that response, associated D will range from 0 to 1. According to preliminary experiments, y_{max}, the most desirable values for mycelial yield and polysaccharide production, are assumed as 30 and 10 g l^{-1}, respectively. The y_{min} for the most undesirable mycelia yield and polysaccharide production values are all assumed to be 0 g l^{-1}. Associated D, in which the two responses (mycelia yield and polysaccharide production) simultaneously reach a certain desirable value, is calculated from the above definition.

2.5. Cell culture and reagents

Human gastric cancer cell lines SGC-7901, MKN-45 were obtained from Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). The human normal gastric epithelial cell line GES-1 was obtained from the Cancer Research Institute of Beijing, China (Chen, Zhao, Li, et al., 2008). Cells were maintained in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 100 units/ml penicillin, and 100 units/ml streptomycin in a humidified cell incubator with an atmosphere of 5% CO_2 at 37 °C. Doxorubicin (Dox), Cisplatin (CIS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma, all other chemicals were of high purity available.

2.6. Cell viability assay

Cell viability was measured by the MTT method as previous described, with some modifications (Chen, He, & Li, 2006; Chen, Zhao, Chen, et al., 2008). Briefly, cells were seeded into 96-well microtiter plates at a density of 5×10^3 cells/well. After 24 h of incubation in the appropriate medium, cells were treated with various concentrations of EEM, IPS, Dox or CIS. When incubated for 48 h,