Carbohydrate Polymers 85 (2011) 369–375

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

Wei Chen [a,b,*], Zhao Zhao [a], Yongquan Li [a,**]

[a] College of Life Science, Zhejiang University, Hangzhou 310058, China
[b] College of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310035, China

ARTICLE INFO

Article history:
Received 6 July 2010
Received in revised form 30 January 2011
Accepted 22 February 2011
Available online 1 March 2011

Keywords:
Fomes fomentarius
Intracelluar polysaccharide
Desirability functions
Submerged culture
Gastric cancer

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

* Corresponding author at: 149 JiaoGong Road, Hangzhou 310035, China. Tel.: +86 571 88071024x7589; fax: +86 571 88056656.
** Corresponding author at: 388 Yuhangtang Road, Hangzhou 310058, China. Tel.: +86 571 88206632; fax: +86 571 88208569.
E-mail addresses: zjgsuchenwei@gmail.com, zjgsuchenwei@zjgsu.edu.cn (W. Chen), lyq@zju.edu.cn (Y. Li).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.02.035

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,

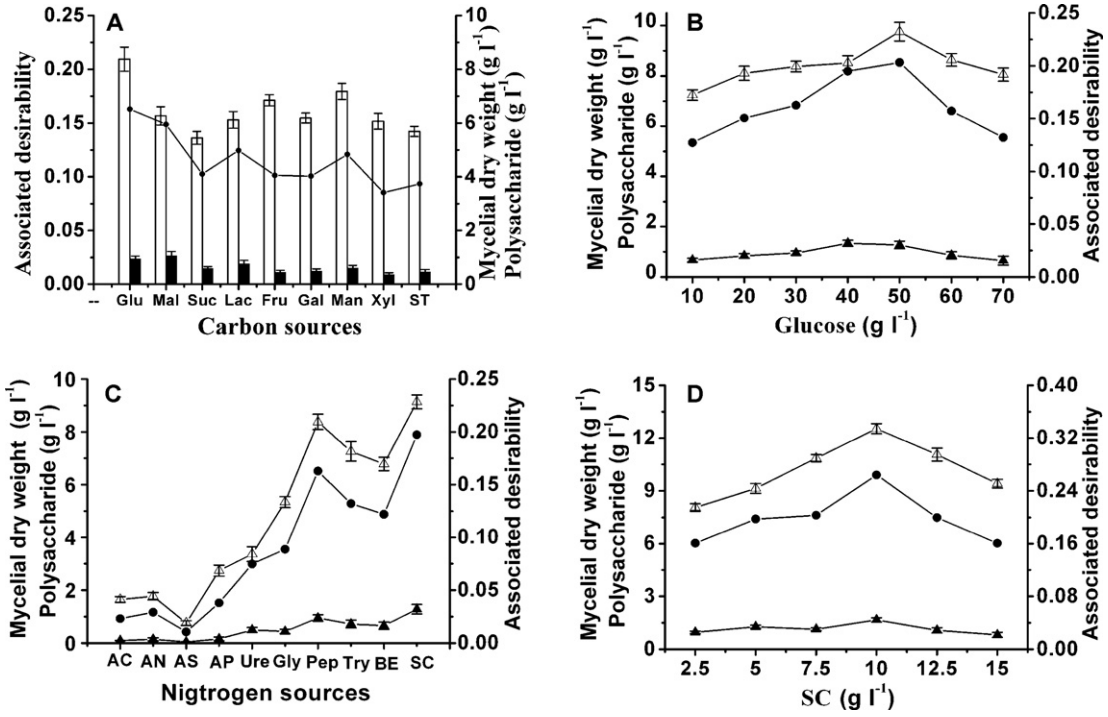

Fig. 1. Effects of carbon (A: various carbon sources; B: various levels of glucose) and nitrogen sources (C: various nitrogen sources; D: various levels of SC) on simultaneous production of mycelial and polysaccharide in shake flask cultures of *F. fomentarius*. All experimental data are mean ± SD of triplicate determinations. White bar (mycelial dry weight) and black bar (polysaccharide) in (A), (△) mycelial dry weight, (▲) polysaccharide, (●) associated desirability for production of mycelia and polysaccharide. Glu, Mal, Suc, Lac, Fru, Gal, Man, Xyl and Sol represent glucose, maltose, sucrose, lactose, fructose, galactose, mannitol, xylose and soluble starch. AC, AN, AS, AP, Ure, Gly, Pep, Try, BE and SC represent ammonium chloride, ammonium nitrate, ammonium sulfate, ammonium phosphate, urea, glycine, peptone, tryptone, beef extract and silkworm chrysalis.

cells were incubated with MTT (0.5 mg ml^{-1}) for 4 h. The formazan precipitate was dissolved in 150 μl DMSO, and the absorbance was detected at 490 nm with a model ElX800 microplate reader (Bio-Tek Instruments). Each test was performed in triplicate experiments.

2.7. Statistical analysis

Unless otherwise stated, data were expressed as means ± SD and analyzed statistically by one-way ANOVA using SPSS (version 16.0). $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Effect of medium compositions

The effects of various medium compositions on the cultivation of *F. fomentarius* were examined. As shown in Fig. 1A, a high level of mycelial biomass and IPS was obtained when glucose, mannitol, maltose was used as the carbon source. Among the carbon sources tested, the maximum mycelial biomass (8.38 g l^{-1}) was obtained in the glucose medium, whereas maximum IPS production (1.06 g l^{-1}) was achieved in the maltose medium. As indicated in this study, the profile of polysaccharide production was not totally consistent with mycelial biomass. This is a common phenomenon in fermentation kinetics of higher fungi (Hwang, Kim, Xu, Choi, & Yun, 2003; Kim & Yun, 2005), thus, it is necessary that associated *D*, involving mycelial biomass and IPS production, was analyzed using desirability functions. The highest associated *D* value was 0.16 when glucose medium was used in shake-flask culture. Further experiments demonstrated that the maximal associated *D* value was obtained in the 50 g l^{-1} glucose medium (Fig. 1B), with the corresponding mycelial and IPS were 9.76 and 1.27 g l^{-1}, respectively. Although mycelia and IPS yields were not the highest values in this experiment, they may be the best selection for synchronously achieving higher mycelial biomass and IPS production during cultivation. Therefore, among the carbon sources tested, glucose was considered the best for mycelial growth and polysaccharide production by *F. fomentarius*, with an optimal concentration of 50 g l^{-1}.

To investigate the suitable nitrogen source for the IPS production and mycelial growth in *F. fomentarius*, various nitrogen sources were separately provided at 5 g l^{-1} instead of peptone employed in the basal medium. As shown in Fig. 1C, The maximal associated *D* value was 0.19 when silkworm chrysalis (SC) instead of peptone was used as nitrogen source, and the corresponding mycelia and IPS production were 9.14 and 1.28 g l^{-1}, separately. In comparison with organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelial biomass and IPS. This is consistent with some previous studies, because certain essential amino acids in organic nitrogen are absorbed and biosynthesized directly, but those from an inorganic nitrogen source are more difficult to synthesize in the cultivation of higher fungi (Shih, Pan, & Hsieh, 2006). After testing six levels of SC for mycelial growth and IPS production (Fig. 1D), the optimal concentration was found to be 10 g l^{-1}.

To examine the effect of mineral sources on the production of mycelial biomass and IPS, various mineral elements were added to the basal medium at a concentration of 0.05%. As shown in Table 1, among the mineral sources examined, the mycelial growth and IPS production reached the highest levels in the media containing KH_2PO_4 and $MgSO_4$, which was different from the previous reported result that $CaCl_2$ was better for exopolysaccharide production (Chen, Zhao, Chen, et al., 2008). Previous studies reported that external Ca^{2+} has a double-action in fungal growth: (1) it could change cell membrane permeability by controlling the internal Ca^{2+} gradient and the activity of some enzymes involved in cell

Table 1
Effect of mineral elements and vitamins on simultaneous production of mycelial and polysaccharide of *F. fomentarius* in shake flask culture.

	Mycelial dry weight ($g\,l^{-1}$)	Polysaccharide ($g\,l^{-1}$)	Associated *D* value
Mineral elements			
$MgSO_4$	8.07 ± 0.77	0.86 ± 0.08*	0.15
KH_2PO_4	7.86 ± 0.83	0.79 ± 0.07	0.14
$CaCl_2$	7.51 ± 0.45	0.48 ± 0.05*	0.11
$FeSO_4{\cdot}7H_2O$	3.98 ± 0.32*	0.25 ± 0.04*	0.06
$MnSO_4$	2.92 ± 0.28*	0.33 ± 0.06*	0.06
$KH_2PO_4 + MgSO_4$	8.38 ± 0.79	0.95 ± 0.08*	0.16
Control (no minerals)	7.43 ± 0.61	0.68 ± 0.05	0.13
Vitamins			
Thiamine (VB_1)	7.68 ± 0.56	0.63 ± 0.06*	0.13
Riboflavin (VB_2)	6.96 ± 0.83	0.52 ± 0.04	0.11
Pyridoxine (VB_6)	3.96 ± 0.52*	0.43 ± 0.03	0.07
D-Biotin (VH)	7.87 ± 0.63	0.61 ± 0.06*	0.13
Myoinositol	5.32 ± 0.41*	0.43 ± 0.03	0.09
Folic acid	7.86 ± 0.64*	0.69 ± 0.04*	0.13
Yeast extract	8.38 ± 0.79*	0.95 ± 0.08*	0.16
All vitamins	8.13 ± 0.48*	0.81 ± 0.07*	0.15
Control (no vitamins)	6.85 ± 0.61	0.67 ± 0.06	0.12

Experiments were carried out in flasks for 6 d at 25 °C with initial pH 6.0. Data are mean ± SD of triplicate determinations.
* $p < 0.05$ versus control.

wall expansion (Kim & Yun, 2005); (2) it could inhibit biopolymer synthesis through the internal Ca^{2+} gradient, leading to affect protein and neutral sugar content (Papagianni, 2004). Therefore, the outcome was enhancement of exopolysaccharide production and inhibition of intracellular polysaccharide accumulation during fermentation of *F. fomentarius*.

It is suggested that vitamins generate a growth response at very low concentration and typically have a catalytic function in the cell as coenzymes or constituents of coenzymes (Garraway & Evans, 1984). In this study, vitamins of thiamine (VB_1), riboflavin (VB_2), pyridoxine (VB_6), D-biotin (VH), folic acid and myoinositol were selected. They were added to the media with yeast removed from the basal medium at 200 $\mu g\,l^{-1}$, except VH and myoinositol which were added at 5 $\mu g\,l^{-1}$ and 5 $mg\,l^{-1}$, respectively (Dong & Yao, 2005). Media with yeast extract removed from the basal medium or by all the vitamins tested served as controls (Liu & Chen, 2002; Satchuthananthavale & Cooke, 1967). Among the vitamins examined, the maximum mycelial biomass and IPS production were both obtained by the medium containing yeast extract, even higher than the medium with all vitamins (Table 1). Thus, yeast extract was the most efficient growth factor for production of mycelia and IPS by *F. fomentarius*, which was consistent with exopolysaccharide production by *F. fomentarius* (Chen, Zhao, Chen, et al., 2008). Further study indicated that the optimal concentration of yeast extract was found to be 5 $g\,l^{-1}$.

3.2. Effect of culture conditions

To obtain the optimal temperature for mycelial growth and IPS production, *F. fomentarius* was cultivated at various temperatures ranging from 15 to 35 °C. Both maximum mycelial biomass (8.38 $g\,l^{-1}$) and IPS (0.95 $g\,l^{-1}$) were observed at 25 °C (Fig. 2A), which were consistent with *F. fomentarius* production of mycelia and exopolysaccharide (Chen, Zhao, Chen, et al., 2008), and comparable to most kinds of mushrooms that have relatively low temperature optima (e.g. 20–25 °C) in their submerged cultures (Bae, Sinha, Park, Song, & Yun, 2000). The medium pH may affect cell morphology and structure, cell membrane function, the uptake of various nutrients, and product biosynthesis (Shu & Lung, 2004). In this work, maximum IPS concentration (0.95 $g\,l^{-1}$) was obtained at an initial pH 6.0, whereas maximum biomass concentration (8.87 $g\,l^{-1}$) was obtained at an initial pH of 5.0, The optimal associated *D* value was found at initial pH 6.0, but mycelial biomass and IPS production approximated optimal values in the initial pH range 5–7 and declined sharply outside this range (Fig. 2B). These results were similar to some previous reports in other higher fungi (Fang & Zhong, 2002).

Medium capacity is closely related to oxygen supply during the high fungi cultivation processes, and oxygen supply could significantly influence the formation and accumulation of bioactive metabolites in submerged cultivation of medicinal fungi (Tang & Zhong, 2003). Therefore, medium capacity might play an important role for the production of mycelia and IPS. In the present study, *F. fomentarius* was cultivated in basal medium for 6 d at 25 °C in a 500-ml flask containing various medium capacity ranging from 50 to 300 ml with agitation rate 150 rpm. The mycelial biomass and IPS production had no evident change under a medium capacity range from 100 to 150 ml and declined sharply above this range (Fig. 2C). Accordingly, the mycelial biomass and IPS production have no significant differences under the range from 120 to 180 rpm and declined sharply out of this range (Fig. 2D).

Among several fungal physiological properties, the slant age and inoculum may play an important role in fungal development (Glazebrook, Vining, & White, 1992). To examine the effect of slant age and inoculum volume, *F. fomentarius* was grown on PDA slants for three different time periods (4, 8, and 12 d) varying the inoculum volume (2.5, 5, 10, and 15%) in basal medium. The colony developed in each slant was separately used as the inoculum in flask cultures. The mycelial biomass and IPS production fluctuated between 5.23 and 8.38 $g\,l^{-1}$ and between 0.36 and 0.95 $g\,l^{-1}$, respectively, when a culture of 4–8 d slant age was employed (Table 2). But using the culture of 12 d slant age, the corresponding mycelial biomass and IPS production were lower than that of other samples. These results indicated that 5–10% of inoculum size was fit for the mycelial growth and enhanced IPS production. The medium capacities and

Table 2
Effect of slant age and inoculum volume on simultaneous production of mycelial and polysaccharide of *F. fomentarius* in shake flask culture.

Slant age (d)	Inoculum volume (%, v/v)	Mycelial dry weight ($g\,l^{-1}$)	Polysaccharide ($g\,l^{-1}$)	Associated *D* value
4	2.5	5.23 ± 0.51	0.36 ± 0.04	0.08
4	5	6.67 ± 0.58	0.61 ± 0.02	0.12
4	10	7.14 ± 0.81	0.83 ± 0.06	0.14
4	15	5.75 ± 0.67	0.72 ± 0.03	0.12
8	2.5	6.13 ± 0.38	0.47 ± 0.07	0.10
8	5	7.93 ± 0.18	0.75 ± 0.08	0.14
8	10	8.38 ± 0.26	0.95 ± 0.05	0.16
8	15	7.86 ± 0.25	0.81 ± 0.03	0.15
12	2.5	4.36 ± 0.54	0.38 ± 0.04	0.07
12	5	5.27 ± 0.64	0.56 ± 0.06	0.10
12	10	6.36 ± 0.18	0.75 ± 0.07	0.13
12	15	4.81 ± 0.13	0.63 ± 0.01	0.10

Fermentations were carried out in flasks for 6 d at 25 °C with initial pH 6.0. Values are mean ± SD of triple determinations.

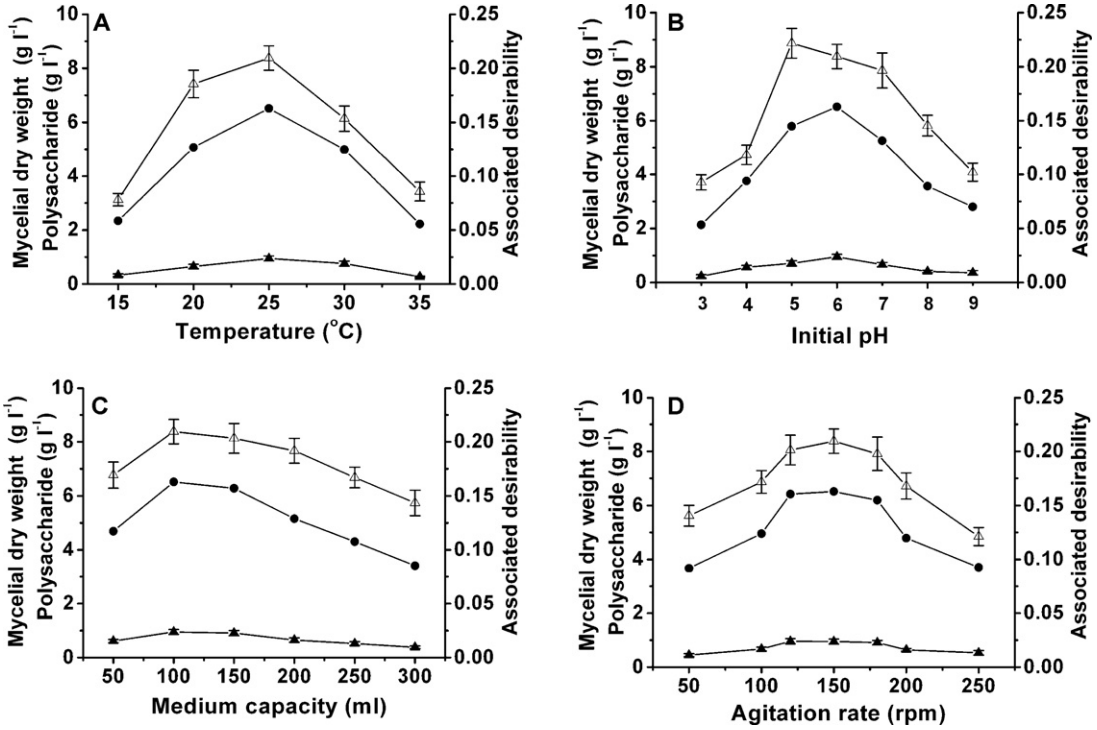

Fig. 2. Effects of culture conditions (A: temperature; B: initial pH; C: medium capacity; D: agitation rate) on simultaneous production of mycelial and polysaccharide in shake flask cultures of *F. fomentarius*. All experimental data are mean ± SD of triplicate determinations. (△) mycelial dry weight, (▲) polysaccharide, (●) associated desirability for production of mycelia and polysaccharide.

inoculum sizes are in accordance with previous results reported in *F. fomentarius* (Chen, Zhao, Chen, et al., 2008).

3.3. Time profile of optimized culture

To examine the kinetics mode on the mycelial biomass and IPS production, *F. fomentarius* was cultivated in 500-ml flask using a rotary shaker incubator under the basal culture medium (glucose 30 g l^{-1}, peptone 5 g l^{-1}, Yeast extract 2 g l^{-1}, KH_2PO_4 1 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}) and optimal culture medium (50 g l^{-1} glucose, 10 g l^{-1} silkworm chrysalis, 5 g l^{-1} yeast extract, KH_2PO_4 1 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}), other conditions such as initial pH, temperature, medium capacity, agitation rate, slant age, and inoculum size, were 6.0, 25 °C, 100 ml, 150 rpm, 12 d and 10% (v/v), respectively. As shown in Fig. 3A, under the basal culture condition, the maximum associated *D* value for mycelial biomass and IPS production reached 0.18 after 7 d culture, and the corresponding production of mycelia and IPS were 8.05 and 1.18 g l^{-1}, respectively.

Meanwhile, under the optimal culture condition, the highest associated *D* value for mycelial biomass and IPS production reached 0.38 after 7 d culture, with the production of mycelia and IPS were 16.21 and 2.67 g l^{-1}, respectively (Fig. 3B), which were about two fold greater than that at the basal culture condition. Hence, a 7-day period was suitable for simultaneous higher production of mycelia and IPS in submerged culture of *F. fomentarius*, while morphological characteristics of mycelial pellets also indicated it was a suitable termination date (data not shown).

Further study in a 15-l stirred-tank bioreactor (culture conditions: temperature 25 °C, aeration rate 1.0 vvm, agitation speed 150 rpm, initial pH 6.0, and working volume 9 l, inoculum size 10%) indicated the similar results: the production of mycelia and IPS were 17.19 and 2.86 g l^{-1} after 7 d culture under the optimal culture condition, compared to 8.49 and 1.34 g l^{-1} under the basal culture condition.

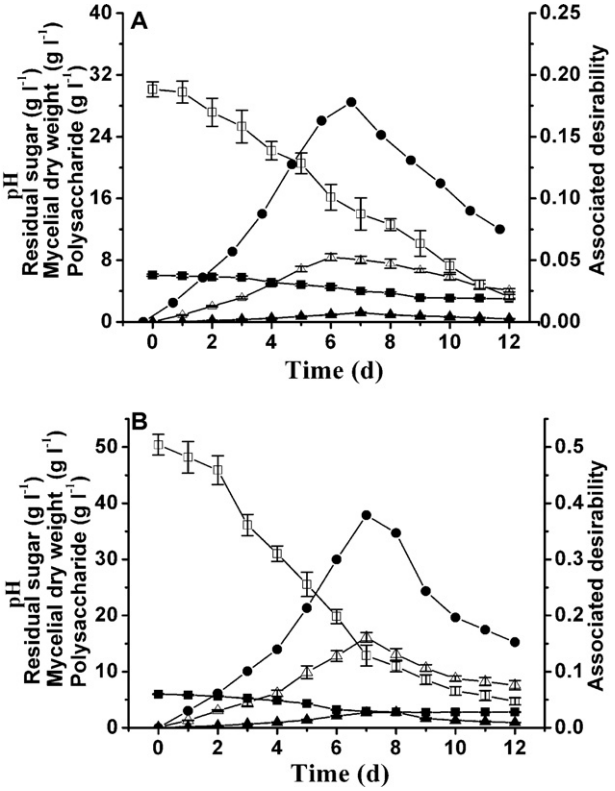

Fig. 3. Time profiles of mycelial growth and polysaccharide production of *F. fomentarius* in a rotary shaker incubator under the basal medium (A) and the optimized medium (B). All experimental data are mean ± SD of triplicate determinations. (△) mycelial dry weight, (▲) polysaccharide, (□) residual sugar concentration, (■) pH, (●) associated desirability for production of mycelia and polysaccharide.

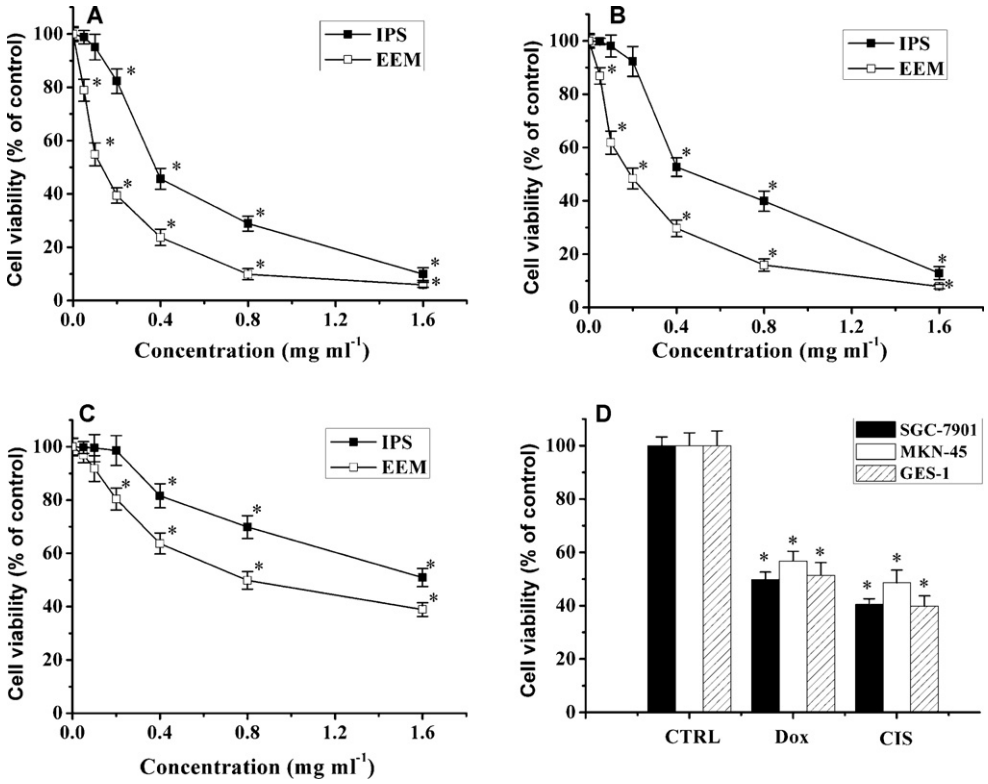

Fig. 4. Effect of EEM and IPS on the cell growth inhibition of human gastric cancer cell lines SGC-7901 (A), MKN-45 (B), and normal gastric cell line GES-1 (C). Cell viability was determined by MTT assay after 48 h treatment as described in the text. EEM, IPS represent the ethanol extract of mycelia, intracellular polysaccharide separately. Dox (1 μg ml^{-1}), CIS (10 μg ml^{-1}) represent doxorubicin and cisplatin, respectively (D). Data represent mean ± SD of three independent experiments (*$p < 0.05$ versus untreated cells).

3.4. Anticancer effect in vitro

In recent years, some studies reported that ethanol extract and polysaccharides from mushrooms can inhibit the growth of cancer cells directly (Fullerton et al., 2000; Gu & Belury, 2005; Hu, Zhang, Lei, Yang, & Sugiura, 2009; Li et al., 2004). In present study, to examine the effect of growth inhibition, various concentrations of IPS (0.05–1.6 mg ml^{-1}) and ethanol extract of mycelia (EEM, 0.05–1.6 mg ml^{-1}) were added to gastric cancer and normal cells for 48 h. As shown in Fig. 4, EEM and IPS both inhibited the growth of gastric cancer cell lines SGC-7901 (Fig. 4A) and MKN-45 (Fig. 4B) in a dose-dependent manner. The antiproliferative effect of EEM on SGC-7901 and MKN-45 cells was much stronger than that of IPS. In contrast, human normal gastric cell line GES-1 was less susceptible to EEM and IPS (Fig. 4C). Moreover, there is no different effect on cell viability between gastric cancer cells and normal cells after treatment with 1 μg ml^{-1} Dox or 10 μg ml^{-1} CIS (Fig. 4D). These results suggest that human gastric cancer cells are more susceptible to EEM and IPS from *F. fomentarius* compared to normal GES-1 cells, which might make *F. fomentarius* more safe for gastric cancer intervention.

4. Conclusions

In this study, a simultaneous higher production of mycelia and IPS in *F. fomentarius* was obtained using desirability functions, which could have a wide application in other microbial fermentation processes. Furthermore, the EEM and IPS from *F. fomentarius* had a direct antiproliferative effect on SGC-7901 and MKN-45 human gastric cancer cells 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. Further investigation in mouse models will contribute to the additional understanding of its in vivo activity toward malignant cells and its potential toxicity toward normal tissues.

Acknowledgements

This work was partially supported by grants from the National High Technology Research and Development Program of China (No. 2007AA021506), the Natural Science Fund of Zhejiang Province (No. R207609), the National Natural Science Foundation of China (No. 31000775), the Key Project of Chinese Ministry of Education (No. 210086), Research Fund for the Doctoral Program of Higher Education of China (No. 20103326120006), and Zhejiang Gongshang University Starting Seed Grant. We thank Dr. Anderson from Virginia Polytechnic Institute and State University for helpful advice and English revision.

References

Atten, M. J., Attar, B. M., Milson, T., & Holian, O. (2001). Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism. *Biochemical Pharmacology*, *62*(10), 1423–1432.

Bae, J. T., Sinha, J., Park, J. P., Song, C. H., & Yun, J. W. (2000). Optimization of submerged culture conditions for exo-biopolymer production by *Paecilomyces japonica*. *Journal of Microbiology and Biotechnology*, *10*(4), 482–487.

Bouche, O., Ychou, M., Burtin, P., Bedenne, L., Ducreux, M., Lebreton, G., et al. (2005). Adjuvant chemotherapy with 5-fluorouracil and cisplatin compared with surgery alone for gastric cancer: 7-year results of the FFCD randomized phase III trial (8801). *Annals of Oncology*, *16*(9), 1488–1497.

Cai, W. C., & Yuan, H. J. (1982). *Common chemoanalytic methods of biosubstances*. Peking, China: Science Press. Vol. 322, pp. 8–9, 59–60.

Catalano, V., Labianca, R., Beretta, G. D., Gatta, G., de Braud, F., & Van Cutsem, E. (2005). Gastric cancer. *Critical Reviews in Oncology/Hematology*, *54*(3), 209–241.

Catalano, V., Labianca, R., Beretta, G. D., Gatta, G., de Braud, F., & Van Cutsem, E. (2009). Gastric cancer. *Critical Reviews in Oncology/Hematology*, *71*(2), 127–164.

Chen, W., He, F. Y., & Li, Y. Q. (2006). The apoptosis effect of hispolon from *Phellinus linteus* (Berkeley & Curtis) Teng on human epidermoid KB cells. *Journal of Ethnopharmacology*, *105*(1–2), 280–285.

Chen, W., Zhao, Z., Chen, S. F., & Li, Y. Q. (2008). Optimization for the production of exopolysaccharide from *Fomes fomentarius* in submerged culture and its antitumor effect in vitro. *Bioresource Technology*, *99*(8), 3187–3194.

Chen, W., Zhao, Z., Li, L., Wu, B., Chen, S. F., Zhou, H., et al. (2008). Hispolon induces apoptosis in human gastric cancer cells through a ROS-mediated mitochondrial pathway. *Free Radical Biology and Medicine*, *45*(1), 60–72.

Davis, P. A., & Sano, T. (2001). The difference in gastric cancer between Japan, USA and Europe: What are the facts? What are the suggestions? *Critical Reviews in Oncology/Hematology*, *40*(1), 77–94.

Dong, C. H., & Yao, Y. J. (2005). Nutritional requirements of mycelial growth of *Cordyceps sinensis* in submerged culture. *Journal of Applied Microbiology*, *99*(3), 483–492.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Endo, S., Nishida, T., Nishikawa, K., Nakajima, K., Hasegawa, J., Kitagawa, T., et al. (2006). Dai-kenchu-to, a Chinese herbal medicine, improves stasis of patients with total gastrectomy and jejunal pouch interposition. *American Journal of Surgery*, *192*(1), 9–13.

Fang, Q.-H., & Zhong, J.-J. (2002). Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. *Process Biochemistry*, *37*(7), 769–774.

Fullerton, S. A., Samadi, A. A., Tortorelis, D. G., Choudhury, M. S., Mallouh, C., Tazaki, H., et al. (2000). Induction of apoptosis in human prostatic cancer cells with beta-glucan (Maitake mushroom polysaccharide). *Molecular Urology*, *4*(1), 7–13.

Garraway, M. O., & Evans, R. C. (1984). *Fungal nutrition and physiology*. New York: Wiley., pp. 401.

Glazebrook, M. A., Vining, I. C., & White, R. I. (1992). Growth morphology of *Streptomyces akiyoshiensis* in submerged culture: Influence of pH, inoculum, and nutrients. *Canadian Journal of Microbiology*, *38*, 98–103.

Gu, Y.-H., & Belury, M. A. (2005). Selective induction of apoptosis in murine skin carcinoma cells (CH72) by an ethanol extract of *Lentinula edodes*. *Cancer Letters*, *220*(1), 21–28.

Hu, H., Zhang, Z., Lei, Z., Yang, Y., & Sugiura, N. (2009). Comparative study of antioxidant activity and antiproliferative effect of hot water and ethanol extracts from the mushroom *Inonotus obliquus*. *Journal of Bioscience and Bioengineering*, *107*(1), 42–48.

Hwang, H. J., Kim, S. W., Xu, C. P., Choi, J. W., & Yun, J. W. (2003). Production and molecular characteristics of four groups of exopolysaccharides from submerged culture of *Phellinus gilvus*. *Journal of Applied Microbiology*, *94*(4), 708–719.

Ito, H., Sugiura, M., & Miyazaki, T. (1976). Antitumor polysaccharide fraction from the culture filtrate of *Fomes fomentarius*. *Chemical & Pharmaceutical Bulletin*, *24*(10), 2575.

Kim, H. O., & Yun, J. W. (2005). A comparative study on the production of exopolysaccharides between two entomopathogenic fungi *Cordyceps militaris* and *Cordyceps sinensis* in submerged mycelial cultures. *Journal of Applied Microbiology*, *99*(4), 728–738.

Lee, J. S., Lim, M. O., Cho, K. Y., Cho, J. H., Chang, S. Y., & Nam, D. H. (2006). Identification of medicinal mushroom species based on nuclear large subunit rDNA sequences. *Journal of Microbiology*, *44*(1), 29–34.

Li, G., Kim, D. H., Kim, T. D., Park, B. J., Park, H. D., Park, J. I., et al. (2004). Protein-bound polysaccharide from *Phellinus linteus* induces G2/M phase arrest and apoptosis in SW480 human colon cancer cells. *Cancer Letters*, *216*(2), 175–181.

Lin, W. C., & Tan, T. W. (1994). The role of gastric muscle-relaxation in cytoprotection induced by San–Huang–Xie–Xin–Tang in rats. *Journal of Ethnopharmacology*, *44*(3), 171–179.

Liu, X. Z., & Chen, S. Y. (2002). Nutritional requirements of the nematophagous fungus *Hirsutella rhossiliensis*. *Biocontrol Science and Technology*, *12*, 381–393.

Mau, J. L., Chang, C. N., Huang, S. J., & Chen, C. C. (2004). Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia. *Food Chemistry*, *87*(1), 111–118.

Papagianni, M. (2004). Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances*, *22*(3), 189–259.

Park, Y. M., Kim, I. T., Park, H. J., Choi, J. W., Park, K. Y., Lee, J. D., et al. (2004). Anti-inflammatory and anti-nociceptive effects of the methanol extract of *Fomes fomentarius*. *Biological & Pharmaceutical Bulletin*, *27*(10), 1588–1593.

Rivera, F., Vega-Villegas, M. E., & Lopez-Brea, M. F. (2007). Chemotherapy of advanced gastric cancer. *Cancer Treatment Reviews*, *33*(4), 315–324.

Rueda, M. E., Sarabia, L. A., Herrero, A., & Ortiz, M. C. (2003). Optimisation of a flow injection system with electrochemical detection using the desirability function – Application to the determination of hydroquinone in cosmetics. *Analytica Chimica Acta*, *479*(2), 173–184.

Safa, F., & Hadjmohammadi, M. R. (2005). Simultaneous optimization of the resolution and analysis time in micellar liquid chromatography of phenyl thiohydantoin amino acids using Derringer's desirability function. *Journal of Chromatography A*, *1078*(1–2), 42–50.

Satchuthananthavale, V., & Cooke, R. C. (1967). Vitamin requirements of some nematode-trapping fungi. *Transactions of the British Mycological Society*, *50*, 221–228.

Schepetkin, I. A., Xie, G., Kirpotina, L. N., Klein, R. A., Jutila, M. A., & Quinn, M. T. (2008). Macrophage immunomodulatory activity of polysaccharides isolated from *Opuntia polyacantha*. *International Immunopharmacology*, *8*(10), 1455–1466.

Shih, I.-L., Pan, K., & Hsieh, C. (2006). Influence of nutritional components and oxygen supply on the mycelial growth and bioactive metabolites production in submerged culture of *Antrodia cinnamomea*. *Process Biochemistry*, *41*(5), 1129–1135.

Shu, C.-H., & Lung, M.-Y. (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorata* in batch cultures. *Process Biochemistry*, *39*(8), 931–937.

Tang, Y.-J., & Zhong, J.-J. (2003). Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of Ganoderma polysaccharide and ganoderic acid. *Enzyme and Microbial Technology*, *32*(3–4), 478–484.