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Optimization for production of exopolysaccharides with antitumor activity *in vitro* from *Paecilomyces hepiali*

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ABSTRACT

In the present study, optimal medium for the growth of mycelia and the production of exopolysaccharides from *Paecilomyces hepiali* HN1 (PHEPS) in submerged culture was investigated. As a result, the maximum production of mycelia (12.98 ± 0.14 g/L) and PHEPS (5.33 ± 0.11 g/L) were achieved under the optimal medium of sucrose 46.08 g/L, yeast extract 4.71 g/L, $(NH_4)_2SO_4$ 5.72 g/L, KH_2PO_4 1.70 g/L, $CaCl_2$ 0.50 g/L, $MgSO_4$ 0.50 g/L, potato extract 1% and malt extract 1%. Furthermore, the antitumor activity of PHEPS *in vitro* was evaluated by using three cell lines of human liver tumor HepG2 cells, breast cancer MCF-7 cells and cervical cancer Hela cells. It was found that PHEPS exhibited relative higher anti-proliferative activity against HepG2 cells than MCF-7 cells and Hela cells. At a concentration of 500 μg/mL and 72 h treatment, the inhibition rate of PHEPS on HepG2 cells reached to 62.58%. All these results suggested that PHEPS could be explored as novel natural antitumor agent with great potential application.

1. Introduction

Hepatocellular carcinoma (HCC) or malignant hepatoma, a type of liver cancers, is one of the most common tumors in the world (Jemal et al., 2011). In the past decades, due to the obesity epidemic and the rise in Hepatitis C virus infection, the incidence of liver cancer is increasing in many countries including United States, central Europe and a large part of Asian countries (Sarasin, Giostra, Mentha, & Hadengue, 1998). Currently, transplantation and chemotherapy are the popular treatments of liver cancer, but after treatments the patients have to take drugs to help suppress the immune system and prevent the body from rejecting the growth of new organ. Mostly, these drugs have their own risks and side effects, especially inducing serious infections, which may decrease patient's quality of life (Clavien, Petrowsky, DeOliveira, & Graf, 2007; Meng et al., 2012). Therefore, it is necessary to search for novel approach to avoid the side effects of cancer treatments.

Cordyceps sinensis (Berk.) Sacc., known as Chinese caterpillar fungus or "DongChongXiaCao" (summer-plant, winter-worm), is one of the most valuable and effective Chinese medicinal herbs with a broad spectrum of health promoting on the kidney, lung, liver and immune functions (Zhu, Halpern, & Jones, 1998). Especially, the polysaccharides, a major class of active compounds present in *Cordyceps*, have high medicinal value with antioxidant, immunomodulatory, hypoglycemic and anticancer activities (Zhang, Cui, Cheung, & Wang, 2007). However, the natural resource of this fungus is quite limited due to its strict host-specificity on moth insects, confined geographical distribution (Qinghai-Tibetan Plateau with >3000 m high altitude) and over exploitation by humans in recent years. Therefore, mycelia fermentation has become a major and economical source of *Cordyceps* materials. Moreover, exopolysaccharides (EPS) with anticancer and immunologically stimulating properties can be simultaneously produced and released into the fermentation medium during the submerged culture (Cha et al., 2007; Santos Arteiro et al., 2012; Wang et al., 2011).

Paecilomyces hepiali and *Hirsutella sinensis* are the two important fungi produced during *C. sinensis* maturation (Zhu et al., 2010). It has been reported that the chemical composition of cultivated *P. hepiali* is similar to that of wild *C. sinensis* (Thakur et al., 2011). Although there are some reports on dynamic profiles of EPS produced by submerged cultivation of *C. sinensis*, only few studies have conducted on *P. hepiali* HN1. Furthermore, there are large differences in nutrient requirement for various anamorph strains of *C. sinensis* (Guan, Zhao, Feng, Hu, & Li, 2011; Kocharin, Rachathewee, Sanglier, & Prathumpai, 2010; Kwon, Lee, Shin, Lee, & Hong, 2009). In order to make this strain be available for the production of mycelium and EPS by liquid fermentation, it is essential to optimize the culture medium. In the present study, therefore, single factor and statistical designs were firstly applied to optimize the medium for the

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production of EPS from *P. hepiali* HN1 (PHEPS). Then, the antitumor activities *in vitro* of PHEPS against human liver cancer HepG2 cells, breast cancer MCF-7 cells and cervical cancer Hela cells were evaluated.

2. Materials and methods

2.1. Microorganism, tumor cells and culture

The strain of *P. hepiali* HN1 was provided by Shanxi Institute of Microbiology (Xi'an, China). The stock culture was maintained on synthetic potato dextrose agar (PDA) at 4 °C and sub-cultured every two months. The submerged culture was performed in 250 mL flasks containing 100 mL of basal medium (glucose 30 g/L, yeast extract 5 g/L, KH_2PO_4 1.5 g/L, $MgSO_4$ 0.5 g/L, and initial pH 6.5) at 17 °C without shaking for 12 h and then with a shaking of 100 rpm for 120 h. Human liver cancer HepG2 cells, breast cancer MCF-7 cells and cervical cancer Hela cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China) and maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified 5% CO_2 incubator (Thermo Fisher Scientific Inc., MA, USA) at 37 °C.

2.2. Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FBS and RPMI-1640 medium were purchased from Gibco/Invitrogen (Grand Island, NY, USA). Corn powder and soybean meal were purchased from a local market (Nanjing, China) and filtrated with 60 screen mesh for use. All other regents were of analytical grade.

2.3. Determinations of mycelium biomass and PHEPS yield

After fermentation, the mycelia cells were harvested by centrifugation (5000 rpm, 20 min), washed with distilled water three times, dried at 50 °C to a constant weight and weighted (Kwon et al., 2009), affording the mycelium biomass (g/L). The resulting supernatant was treated for PHEPS production according to the reported method (Luo et al., 2009) with some modifications. Briefly, the supernatant from centrifugation was filtered through a Whatman filter paper and concentrated to one-third of its original volume with a rotary evaporator under reduced pressure, and the resulting residue was mixed with three times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitates were collected by centrifugation (5000 rpm, 30 min), washed three times with 80% (v/v) ethanol, acetone and ether, respectively, and dried at 50 °C to a constant weight, affording crude PHEPS. All analyses were carried out in triplicate to minimize errors.

2.4. Desirability of mycelium biomass and PHEPS yield

In the present study, mycelium biomass and PHEPS yield were considered as two responses. The mycelium biomass (d_m) and PHEPS yield (d_p) are both 'higher-the-better' responses. As described previously, let y_{min} and y_{max} be the most undesirable and desirable values, respectively, of a response for mycelium biomass or PHEPS yield. Assuming that desirability (d, ranging from 0 to 1) increases linearly from y_{min} to y_{max}, the value of d contributed by the response was calculated as follows:

$$d = 0 \quad \text{for } y < y_{min} \tag{1}$$

$$d = (y_{max} - y)/(y_{max} - y_{min}) \quad \text{for } y_{min} \le y \le y_{max} \tag{2}$$

$$d = 1 \quad \text{for } y > y_{max} \tag{3}$$

For the two-response system in this study, the associated d is defined as the geometric mean of the desirability of mycelium biomass and PHEPS yield as follows: $d = \sqrt{d_m \times d_p}$ (Chen, Zhao, & Li, 2011). For the sake of clarity, the associated d-value was multiplied 10-fold ($D = d \times 10$) in the present study. According to preliminary experiments, the most desirable values (y_{max}) for mycelium biomass and PHEPS yield were assumed as 30 and 15 g/L, respectively, while the most undesirable values (y_{min}) for mycelium biomass and PHEPS yield were all assumed to be 0 g/L. Associated D, in which the two responses (mycelium biomass and PHEPS yield) simultaneously reached a certain desirable value, was calculated from the above definition.

2.5. Medium optimization

2.5.1. One-factor-at-a-time experiments

One-factor-at-a-time method was used to investigate the factors affecting mycelium biomass and PHEPS yield. As shown in Table 1, eight carbon sources (glucose, fructose, mannose, sucrose, lactose, maltose, corn powder and soluble starch), seven nitrogen sources (beef extract, peptone, yeast extract, soybean meal, urea, $(NH_4)_2SO_4$ and KNO_3), nine mineral elements (KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, $CaCl_2{\cdot}2H_2O$, KCl and NaCl), six vitamins (VB_1, VB_2, VB_6, VH, myoinositol and folic acid) and two natural extracts (potato extract and malt extract) were added to the basal medium while the concentrations of other ingredients were kept constant. All experiments were performed in triplicate.

2.5.2. Fractional factorial design (FFD)

Based on the results of one-factor-at-a-time study, a FFD was employed in the present study to screen the most significant factors affecting D-value. As shown in Table 2, eight factors (sucrose, yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$, potato extract and malt extract) were examined at low level (coded as −1), high level (coded as +1) and a center point (coded as 0) for evaluating the linear and curvature effects of variables. As a result, a total 20 experiments including four replications were conducted. The analysis of variance (ANOVA) for the experimental data and the model coefficients was carried out (Table 3).

2.5.3. Central composite design (CCD)

According to the results of FFD, the center points and parameters of four significant factors (sucrose, yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4) were chosen for further optimization by CCD. The experimental design was carried out using the 'Stat-Ease Design-Expert' software (version 8.0.6, Stat-Ease Inc., Minneapolis, MN, USA). The four independent factors were investigated at five levels (−2, −1, 0, +1 and +2), and the experimental design is shown in Table 4. Briefly, the variables were coded according to Eq. (4):

$$x_i = (X_i - X_0)/\Delta X_i \tag{4}$$

where X_i is the real value of variable, X_0 is the real value of the X_i at the center point, ΔX_i is the step change value in X_i, x_i is the coded value of the variable, and $i = 1, 2, 3$. The experimental levels for these variables were selected from our preliminary work, which indicated that an optimum value could be found within the level of parameters studied. The behavior of the system was explained by the following second-degree polynomial equation (5):

$$Y = \beta_0 + \sum \beta_i X_j + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \tag{5}$$

Table 1
Effects of carbon sources, nitrogen sources, mineral elements and vitamins or natural extract on the production of mycelium and PHEPS.

Item	Mycelium biomass (g/L)	PHEPS (g/L)	Final pH	*D*-value
Carbon source (30 g/L)				
Glucose	8.54 ± 0.25bc	1.47 ± 0.22d	5.12	1.67 ± 0.11e
Fructose	8.86 ± 0.17b	1.54 ± 0.16d	4.68	1.74 ± 0.08 cd
Mannose	9.77 ± 0.15a	2.04 ± 0.17b	4.65	2.10 ± 0.08ab
Sucrose	8.99 ± 0.22b	2.43 ± 0.28a	4.83	2.20 ± 0.12a
Lactose	8.83 ± 0.24b	1.51 ± 0.23d	6.07	1.70 ± 0.11e
Maltose	8.75 ± 0.14b	1.75 ± 0.17c	5.76	1.85 ± 0.07c
Corn powder	7.71 ± 0.23d	2.07 ± 0.13b	6.02	1.88 ± 0.08c
Soluble starch	7.46 ± 0.15d	1.85 ± 0.24c	6.08	1.75 ± 0.09 cd
Control (none)	0.15 ± 0.04e	0.08 ± 0.03e	6.15	0.05 ± 0.02f
Nitrogen source (5 g/L)				
Beef extract	8.65 ± 0.24c	1.15 ± 0.22e	4.15	1.49 ± 0.11d
Peptone	8.55 ± 0.12c	1.12 ± 0.18e	5.12	1.46 ± 0.07d
Yeast extract	9.15 ± 0.25b	1.49 ± 0.04d	5.05	1.74 ± 0.05c
Soybean meal	7.98 ± 0.21d	1.69 ± 0.15c	5.15	1.73 ± 0.08c
Urea	6.88 ± 0.23f	1.61 ± 0.22c	8.23	1.57 ± 0.11d
KNO_3	7.70 ± 0.14de	1.63 ± 0.23c	6.05	1.67 ± 0.08c
$(NH_4)_2SO_4$	8.89 ± 0.12b	2.31 ± 0.16b	3.85	2.14 ± 0.07b
Yeast extract:$(NH_4)_2SO_4$ = 1:1	10.85 ± 0.16a	3.43 ± 0.03a	4.07	2.88 ± 0.03a
Control (none)	0.18 ± 0.04 g	0.04 ± 0.02f	4.65	0.04 ± 0.01e
Mineral elements (0.5 g/L)				
KH_2PO_4	9.48 ± 0.25ab	2.52 ± 0.17a	4.82	2.30 ± 0.10a
$MgSO_4{\cdot}7H_2O$	9.86 ± 0.27a	2.14 ± 0.16b	4.76	2.17 ± 0.10ab
$CuSO_4{\cdot}5H_2O$	7.77 ± 0.18de	1.84 ± 0.07c	4.54	1.78 ± 0.05d
$FeSO_4{\cdot}7H_2O$	7.99 ± 0.22d	1.46 ± 0.28d	4.73	1.61 ± 0.12e
$ZnSO_4{\cdot}7H_2O$	7.83 ± 0.24d	1.51 ± 0.13d	5.07	1.62 ± 0.08e
$MnCl_2{\cdot}4H_2O$	7.11 ± 0.11e	1.47 ± 0.21d	6.12	1.52 ± 0.07f
$CaCl_2{\cdot}2H_2O$	9.26 ± 0.25ab	2.05 ± 0.24b	5.89	2.05 ± 0.12ab
NaCl	8.57 ± 0.15c	1.89 ± 0.19bc	5.75	1.90 ± 0.08c
KCl	8.46 ± 0.12c	1.95 ± 0.11bc	5.52	1.91 ± 0.05c
Control (none)	8.15 ± 0.24 cd	2.08 ± 0.09b	6.11	1.94 ± 0.07c
Vitamins (0.1 g/L) or *natural extract (1%, v/v)*				
Thiamine (VB_1)	9.02 ± 0.04c	2.04 ± 0.12b	4.68	2.02 ± 0.03b
Riboflavin (VB_2)	8.73 ± 0.18 cd	1.15 ± 0.15e	4.75	1.49 ± 0.08e
Pyridoxine (VB_6)	8.82 ± 0.22 cd	1.12 ± 0.03e	5.02	1.48 ± 0.04e
d-Biotin (VH)	9.05 ± 0.15c	1.49 ± 0.24d	4.85	1.73 ± 0.09 cd
Myoinositol	8.68 ± 0.11d	1.69 ± 0.18c	5.22	1.81 ± 0.07c
Folic acid	8.88 ± 0.21 cd	1.75 ± 0.08c	5.23	1.86 ± 0.06c
Potato extract	9.97 ± 0.26ab	1.83 ± 0.22bc	6.05	2.01 ± 0.11b
Malt extract	10.89 ± 0.23a	2.57 ± 0.28a	4.85	2.49 ± 0.12a
Control (none)	8.58 ± 0.16d	1.93 ± 0.13b	5.17	1.92 ± 0.07b

The data are represented as mean value ± standard deviation ($n = 3$). Significance at a $p < 0.05$ level was determined by ANOVA followed by Duncan's multiple comparison tests. Different alphabet (a, b, c, d, e, f, g) in right of value indicates there is significant difference in groups of carbon sources, nitrogen source, mineral elements and vitamins or natural extract ($p < 0.05$). The same alphabet suggests difference between groups is not significant ($p > 0.05$).

where Y is the predicted response, β_0 is the intercept, β_i is the linear coefficient, β_{ij} is the interaction coefficients, β_{ii} is the squared coefficients, X_i and X_j represent the independent variables which influence the response variable Y.

Statistical analysis of the model was performed to evaluate the ANOVA. This analysis included the F-test (overall model significance), its associated probability (P), correlation coefficient (R) and determination coefficient (R^2) which measures the goodness of fit of the regression model. For each variable, the quadratic model was represented as contour plots (2D) and response surface curves (3D) generated by using 'Stat-Ease Design-Expert' software mentioned above.

2.6. Assay of inhibition activity in vitro of PHEPS on tumor cell proliferation

The inhibitory activities *in vitro* of PHEPS against HepG2 cells, MCF-7 cells and Hela cells were evaluated as described by Mosmann (1983) using MTT-based colorimetric method. Briefly, HepG2, MCF-7 or Hela cells in a density of 1×10^5 cells/mL in the RPMI-1640 medium supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL) were pipetted into 96-well flat-bottom plate (100 μL/well). After 12 h of incubation at 37 °C in a humidified 5% CO_2 incubator, the non-adherent cells were removed by washing three times with RPMI-1640 medium. Then, fresh medium (100 μL/well, control group) or test sample (100 μL/well, PHEPS at a final concentration of 31.25, 62.5, 125, 250, 500 and 1000 μg/mL) was added to each well, and the cells were incubated for 24, 48 and 72 h, respectively. After incubation, MTT solution (10 μL/well, 5 mg/mL) was added to each well, and the plate was incubated for an additional 4 h at 37 °C. Finally, 100 μL of dimethyl sulfoxide was added to each well and the plate was kept for 4 h for the dissolution of formazan crystals. The absorbance (Abs) at 570 nm of each well was then measured by an ELISA plate reader (BioTek Instruments Inc., Winooski, VT, USA). The inhibition rate was calculated according to the formula below:

$$\text{Inhibition rate}\,(\%) = (1 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}) \times 100$$

2.7. Statistical analysis

Data were analyzed by SPSS 17.0 (SPSS Inc., Chicagao, IL, USA) and expressed as mean ± standard deviation (SD) for at least three replicates. Significance at a $p < 0.05$ level was determined by ANOVA followed by Duncan's multiple comparison tests.

Table 2
The fractional factorial design for screening of significant factors affecting the production of mycelium and PHEPS.

Run	Factors								Mycelium biomass (g/L)	PHEPS (g/L)	*D*-value
	A[a]	B[b]	C[c]	D[d]	E[e]	F[f]	G[g]	H[h]			
1	−1	1	−1	−1	1	1	−1	1	8.79 ± 0.11	2.32 ± 0.14	1.82
2	0	0	0	0	0	0	0	0	11.89 ± 0.17	2.78 ± 0.27	2.71
3	1	1	−1	−1	−1	1	1	−1	9.57 ± 0.09	2.03 ± 0.08	1.97
4	0	0	0	0	0	0	0	0	11.95 ± 0.16	2.85 ± 0.32	2.75
5	−1	−1	1	1	1	−1	−1	1	8.89 ± 0.27	2.12 ± 0.24	2.16
6	−1	−1	−1	1	−1	1	1	1	8.87 ± 0.22	1.84 ± 0.15	1.90
7	1	−1	−1	−1	1	−1	1	1	10.62 ± 0.19	2.43 ± 0.18	2.08
8	0	0	0	0	0	0	0	0	12.05 ± 0.06	2.81 ± 0.32	2.74
9	1	1	1	−1	1	−1	−1	−1	9.84 ± 0.15	1.82 ± 0.12	2.31
10	1	−1	1	1	−1	−1	1	−1	12.58 ± 0.24	3.08 ± 0.29	2.93
11	−1	−1	−1	−1	−1	−1	−1	−1	7.27 ± 0.12	0.95 ± 0.28	1.24
12	0	0	0	0	0	0	0	0	12.05 ± 0.26	2.88 ± 0.32	2.78
13	1	1	1	1	1	1	1	1	13.79 ± 0.11	3.42 ± 0.14	3.24
14	−1	−1	1	−1	1	1	1	−1	9.87 ± 0.27	1.95 ± 0.25	2.07
15	1	−1	1	−1	−1	1	−1	1	11.72 ± 0.16	2.88 ± 0.08	2.70
16	1	−1	−1	1	1	1	−1	−1	11.95 ± 0.22	3.78 ± 0.32	3.71
17	−1	1	−1	1	1	−1	1	−1	9.79 ± 0.17	2.12 ± 0.24	2.25
18	−1	1	1	−1	−1	−1	1	1	12.07 ± 0.27	2.24 ± 0.33	2.29
19	−1	1	1	1	−1	1	−1	−1	10.67 ± 0.29	2.27 ± 0.29	2.16
20	1	1	−1	1	−1	−1	−1	1	11.82 ± 0.16	3.86 ± 0.22	2.62

[a] Sucrose at a low level (−1) of 30 g/L and a high level (+1) of 50 g/L.
[b] $CaCl_2$ at a low level (−1) of 0.50 g/L and a high level of 1.50 g/L.
[c] Yeast extract at a low level (−1) of 3 g/L and a high level of 5 g/L.
[d] $(NH_4)_2SO_4$ at a low level (−1) of 4 g/L and a high level of 6 g/L.
[e] KH_2PO_4 at a low level (−1) of 0.50 g/L and a high level of 1.50 g/L.
[f] $MgSO_4$ at a low level (−1) of 0.50 g/L and a high level of 1.50 g/L.
[g] Potato extract at a low level (−1) of 10 mL/L and a high level of 30 mL/L.
[h] Malt extract at a low level (−1) of 10 mL/L and a high level of 30 mL/L.

3. Results and discussion

3.1. Medium optimization for the production of mycelium and PHEPS

3.1.1. Effects of carbon sources, nitrogen sources, mineral elements, vitamins and natural extract

Culture medium is very important for any fermentation product, especially, carbon and nitrogen sources play significant role because these nutrients are directly related to cell proliferation and metabolite biosynthesis. To identify the effects of carbon sources on mycelium biomass and PHEPS yield, different carbon sources, *i.e.* glucose, fructose, mannose, sucrose, lactose, maltose, corn powder and soluble starch, were investigated. As shown in Table 1, the medium with sucrose as carbon source exhibited the highest *D*-value (2.20) with a PHEPS yield of 2.43 ± 0.28 g/L, followed by mannose. In addition, it was observed that mannose could promote the mycelium growth, affording the highest mycelium biomass (9.77 ± 0.15 g/L). These results are in accordance with the previous reports for the submerged culture of *C. sinensis* (Cha et al., 2007), but Kocharin et al. (2010) reported that glucose was the best carbon source for the submerged culture of *Ophiocordyceps*

Table 3
Statistical analysis of fractional factorial design for screening the significant variables.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Prob > *F*
Model	5.43	15	0.36	166.94	0.0007[a]
A	2.10	1	2.10	967.04	<0.0001[a]
B	3.91E−03	1	3.91E−03	1.80	0.2719
C	0.36	1	0.36	164.77	0.001[a]
D	1.33	1	1.33	613.04	0.0001[a]
E	0.18	1	0.18	84.35	0.0027[a]
F	0.015	1	0.015	7.11	0.0530
G	1.06E−03	1	1.06E−03	0.49	0.5353
H	1.56E−04	1	1.56E−04	0.072	0.8057
AB	0.41	1	0.41	187.57	0.0008
AC	0.019	1	0.019	8.73	0.0598
AD	0.39	1	0.39	181.73	0.0009
AE	5.26E−03	1	5.26E−03	2.43	0.2172
AF	0.15	1	0.15	69.3	0.0036
AG	0.28	1	0.28	130.87	0.0014
AH	0.045	1	0.045	20.84	0.0197
Curvature	0.56	1	0.56	256.67	0.0005[a]
Residual	0.013	4	0.003		
Lack of fit	0.006	3	0.003	1.457	0.3613[b]
Pure error	6.50E−03	3	2.17E−03		
Correction total	5.99	19			

R^2(predict) = 0.9457 and R^2(adjust) = 0.8426.
[a] 5% significance level.
[b] Not significant relative to the pure error due to noise.

Table 4
Coded levels and real values (in the parentheses, g/L) for the experimental design and results of CCD for the production of myceliu and PHEPS.

Run	Code level and actual values				Mycelium biomass (g/L)	PHEPS (g/L)	*D*-value
	X_1: Sucrose (g/L)	X_2: Yeast extract (g/L)	X_3: $(NH_4)_2SO_4$ (g/L)	X_4: KH_2PO_4 (g/L)			
1	2(60)	0(5)	0(5)	0(1.5)	11.68 ± 0.18	4.87 ± 0.32	3.55
2	0(40)	2(7)	0(5)	0(1.5)	10.67 ± 0.25	4.25 ± 0.22	3.17
3	1(50)	−1(4)	−1(4)	1(2.0)	12.78 ± 0.28	2.35 ± 0.19	2.58
4	1(50)	−1(4)	−1(4)	−1(1.0)	12.95 ± 0.12	5.16 ± 0.29	3.85
5	0(40)	0(5)	−2(3)	0(1.5)	11.24 ± 0.16	4.26 ± 0.23	3.26
6	−2(20)	0(5)	0(5)	0(1.5)	11.13 ± 0.13	4.34 ± 0.17	3.28
7	1(50)	1(6)	1(6)	1(2.0)	12.98 ± 0.21	4.88 ± 0.28	3.75
8	1(50)	1(6)	−1(4)	−1(1.0)	10.23 ± 0.15	2.19 ± 0.33	2.23
9	0(40)	0(5)	0(5)	0(1.5)	11.36 ± 0.26	5.08 ± 0.27	3.58
10	−1(30)	−1(4)	−1(4)	1(2.0)	11.39 ± 0.17	2.76 ± 0.21	2.64
11	1(50)	1(6)	−1(4)	1(2.0)	12.05 ± 0.28	3.12 ± 0.25	2.89
12	−1(30)	−1(4)	−1(4)	−1(1.0)	11.16 ± 0.08	4.25 ± 0.12	3.25
13	0(40)	0(5)	2(7)	0(1.5)	9.77 ± 0.23	2.09 ± 0.26	2.13
14	0(40)	0(5)	0(5)	2(2.5)	10.66 ± 0.16	3.22 ± 0.19	2.76
15	0(40)	0(5)	0(5)	0(0.15)	10.52 ± 0.14	2.25 ± 0.24	2.29
16	−1(30)	−1(4)	1(6)	−1(1.0)	13.11 ± 0.08	4.09 ± 0.23	3.45
17	1(50)	−1(4)	1(6)	−1(1.0)	10.21 ± 0.13	2.18 ± 0.33	2.22
18	1(50)	−1(4)	1(6)	1(2.0)	12.98 ± 0.16	5.21 ± 0.25	3.88
19	0(40)	0(5)	0(5)	0(1.5)	11.41 ± 0.06	2.37 ± 0.11	2.45
20	−1(30)	−1(4)	1(6)	1(2.0)	11.95 ± 0.22	3.11 ±0.13	2.87
21	1(50)	1(6)	1(6)	−1(1.0)	11.23 ± 0.14	4.32 ± 0.21	3.28
22	0(40)	0(5)	0(5)	0(1.5)	12.42 ± 0.21	4.73 ± 0.23	3.61
23	−1(30)	1(6)	−1(4)	−1(1.0)	9.58 ± 0.15	2.89 ± 0.22	2.48
24	0(40)	−2(3)	0(5)	0(1.5)	11.58 ± 0.23	3.18 ± 0.09	2.86
25	−1(30)	1(6)	1(6)	−1(1.0)	9.22 ± 0.24	3.18 ± 0.18	2.55
26	0(40)	0(5)	0(5)	0(1.5)	11.42 ± 0.17	2.98 ± 0.34	2.75
27	−1(30)	1(6)	1(6)	1(2.0)	12.33 ± 0.09	4.68 ± 0.15	3.58
28	0(40)	0(5)	0(5)	−2(0.5)	10.29 ± 0.36	2.06 ± 0.28	2.17
29	−1(30)	1(6)	−1(4)	1(2.0)	10.84 ± 0.15	2.75 ± 0.24	2.57
30	0(40)	0(5)	0(5)	0(1.5)	11.56 ± 0.11	2.62 ± 0.24	2.59

dipterigena BCC 2073. As the reason, it might be due to the difference between the two strains. On the basis of the nitrogen sources examined, we found that yeast extract produced the highest amount of mycelia and $(NH_4)_2SO_4$ improved the PHEPS synthesis (Table 1). The pH drop by the addition of $(NH_4)_2SO_4$ might be beneficial to the production of PHEPS, and similar observations were reported by Pavlova, Koleva, Kratchanova, and Panchev (2004) in the EPS production by yeast. Therefore, the balance between yeast extract and $(NH_4)_2SO_4$ was beneficial to production of both mycelium and PHEPS. When the ratio of yeast extract to $(NH_4)_2SO_4$ was 1, the mycelium biomass (10.85 ± 0.16 g/L) and PHEPS yield (3.43 ± 0.03 g/L) reached maximal values with the highest *D*-value of 2.88.

Mineral elements and vitamins at very low concentrations are essential factors for mycelium growth and EPS production (Ma, Zhang, & Fam, 2013). The metal ions and vitamins are essential coenzymes for enzyme-mediated reactions, especially mannosylglycerate synthase or glycosyltransferases that are involved in the synthesis of EPS (Nielsen et al., 2011; Jorgensen, Grimm, Sindhuwinata, Peters, & Palcic, 2012). To examine the effects of mineral elements and vitamins on mycelium biomass and PHEPS yield, nine mineral elements, six vitamins and two natural extracts

Table 5
Variance analysis of response surface quadratic model for *D*-value.

Source	Sum of squares	Degree of free	Mean square	*F*-value	*p*-Value
Model	8.058	14	0.576	15.749	<0.0001[a]
X_1	1.368	1	1.368	37.432	<0.0001[a]
X_2	0.502	1	0.502	13.728	0.0021[a]
X_3	1.029	1	1.029	28.161	<0.0001[a]
X_4	0.242	1	0.242	6.623	0.0212[a]
X_1X_2	2.256E−003	1	2.256E−003	0.062	0.8071
X_1X_3	0.334	1	0.334	9.125	0.0086[a]
X_1X_4	0.025	1	0.025	0.679	0.4229
X_2X_3	7.656E−003	1	7.656E−003	0.210	0.6537
X_2X_4	7.563E−004	1	7.563E−004	0.021	0.8875
X_3X_4	0.064	1	0.064	1.745	0.2064
X_1^2	2.446	1	2.446	66.936	<0.0001[a]
X_2^2	1.466	1	1.466	40.098	<0.0001[a]
X_3^2	1.296	1	1.296	35.469	<0.0001[a]
X_4^2	1.096	1	1.096	29.989	<0.0001[a]
Lack-of-fit	0.454	10	0.045	2.42	0.1707[b]
Pure error	0.094	5	0.019		
Residual	0.55	15	0.037		
Corrected total	8.606	29			

R^2(predict) = 0.9363 and R^2(adjust) = 0.8769.
[a] 5% significance level.
[b] Not significant relative to the pure error due to noise.

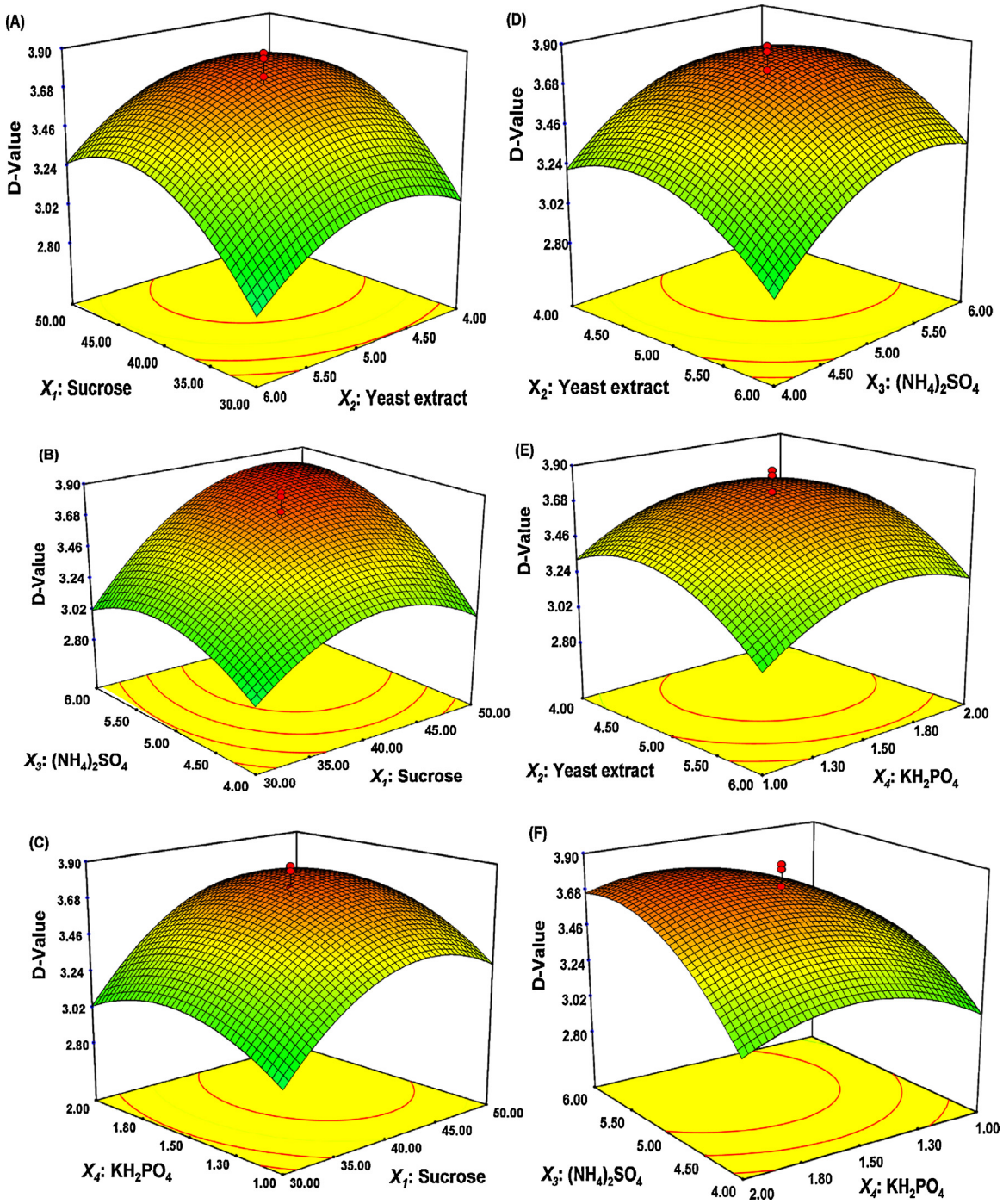

Fig. 1. 3D-response surface plots showing the interactive effects of concentrations of sucrose and yeast extract (A), sucrose and $(NH_4)_2SO_4$ (B), sucrose and KH_2PO_4 (C), yeast extract and $(NH_4)_2SO_4$ (D), yeast extract and KH_2PO_4 (E), $(NH_4)_2SO_4$ and KH_2PO_4 (F) on *D*-value.

were examined in the present study. Compared with the control group, higher mycelium biomass and PHEPS production were observed in the medium containing $MgSO_4$, $CaCl_2$, KH_2PO_4 or malt extract. Similar results were reported by Kim et al. (2002). However, Cui and Zhang (2012) reported that Ca^{2+}, Mg^{2+} and Mn^{2+} were favorable to the mycelium growth of *C. militaris*, but Ca^{2+} and K^+ showed no significant role in the production of EPS.

3.1.2. Screening of parameters using FFD

FFD is an efficient method for screening significant factors. In the present study, 20 runs were carried out to investigate the effects of the relative significance of eight nutritional factors including sucrose, yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2$, $MgSO_4$, potato extract and malt extract. Notably, the mycelium biomass and PHEPS yield varied greatly from 7.27 to 13.79 and 1.84 to 3.86 g/L, respectively, under different combinations of the media components (Table 2). Moreover, statistical analysis of the responses and the main effects of the selected variables were evaluated and the results are shown in Table 3. The *F*- and *p*-values for the model were found to be 166.94 and 0.0007, respectively, which implied that the model was significant. The determination coefficient R^2 of the model was 0.9457, indicating that 94.57% of the variability in the response could be explained by the model.

The "Curvature *p*-value" of 0.0005 implied that there was significant curvature in the design space and the optimum value was inside the experimental design. The results showed that four variables (sucrose, yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4) significantly influenced the *D*-value ($p < 0.05$) and were selected for further evaluation and optimization.

3.1.3. CCD and response surface analysis

According to the results of FFD, the four key factors and their central points chosen for CCD were sucrose (X_1) 40 g/L, yeast extract (X_2) 5 g/L, $(NH_4)_2SO_4$ (X_3) 5 g/L and KH_2PO_4 (X_4) 1.5 g/L, respectively. Table 4 shows the CCD and the corresponding experimental data. The experimental results were analyzed by multiple regression analysis and the following second-order polynomial Eq. (6) was established to explain *D*-value:

$$Y = -10.932 + 0.185X_1 + 2.084X_2 + 1.505X_3 + 15.846X_4 - 1.18750E-003X_1X_2 + 0.014X_1X_3 + 0.079X_1X_4 + 0.022X_2X_3 + 0.138X_2X_4 + 1.263X_3X_4 - 2.98646E-003X_1^2 - 0.231X_2^2 - 0.217X_3^2 - 79.958X_4^2 \quad (6)$$

where *Y* represents *D*-value, X_1, X_2, X_3 and X_4 represent the concentrations of sucrose, yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4, respectively. The *F*-test for ANOVA was conducted to test the significance of Eq. (6) for the experimental data as shown in Table 5. The model *F*-value of 15.749 implied that the model was significant ($p < 0.0001$), indicating that there was only a 0.01% chance that the model *F*-value could occur due to noise. The fitness of the model was examined by determining the coefficient R^2, which was calculated to be 0.9363, indicating that 93.63% of the variability in the response could be explained by the model. Additionally, the value of lack of fit for regression Eq. (6) was not significant ($p > 0.05$), indicating that the model equation was adequate for predicting *D*-value under any combination of values of the variables.

The interactions and optimum levels of the variables were determined by plotting the response surface curves as shown in Fig. 1, which depict the interactions between two variables by keeping the other variables at their zero levels for *D*-value. It is evident that the respective contour plots in 3D-response surface graphs provide a visual interpretation of the interaction. Among the tested variables, the interactions of sucrose and $(NH_4)_2SO_4$ were significant. The model predicted that the optimal values of X_1, X_2, X_3 and X_4 were 46.08, 4.71, 5.72 and 1.70 g/L for sucrose, yeast extract, $(NH_4)_2SO_4$ and KH_2PO_4, respectively. Under the optimum conditions, the maximum predicted *D*-value was 3.90. To validate the adequacy of the model equation, five additional experiments under the optimum medium were performed. The mean value of mycelium biomass and PHEPS yield were 12.98 ± 0.14 and 5.33 ± 0.11 g/L, respectively, and *D*-value was 3.92, which are in good agreement with the predicted values. Compared with normal culture medium, this optimization procedure caused the increases of 1.44 and 2.19 times for the production of mycelium and PHEPS, respectively. The results suggested that *P. hepiali* NH1 could be a promising EPS producer with potential industrial applications.

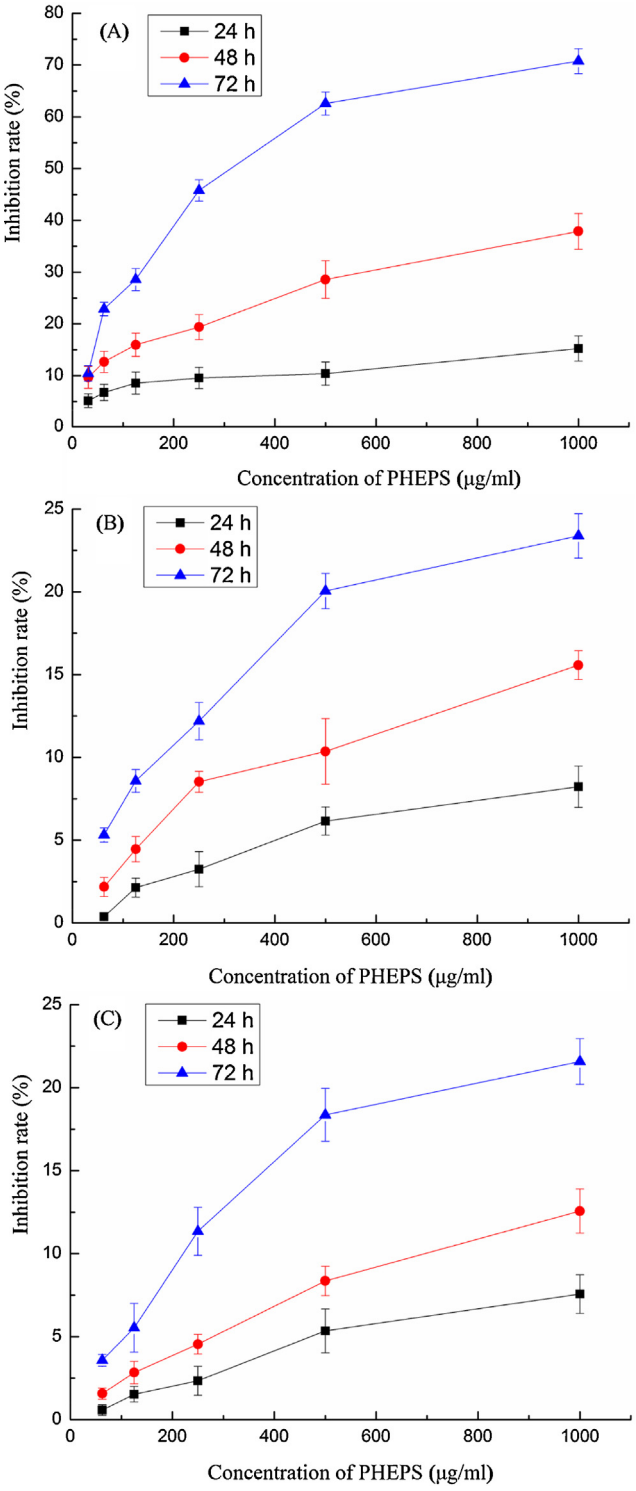

Fig. 2. Inhibitory effects of PHEPS on liver tumor HepG2 cells (A), cervical cancer Hela cells (B) and breast cancer MCF-7 cells (C).

3.2. Inhibitory effects of PHEPS on tumor cells

Fig. 2 shows the inhibitory effects *in vitro* of PHEPS on HepG2 cells, MCF-7 cells and Hela cells. PHEPS exerted dose and time-dependent inhibition effect against HepG2 cells. After 24 h of treatment, the inhibition rate of PHEPS at concentration of 1000 μg/mL was lower than 15%, indicating that the cytotoxicity of PHEPS was low. However, after 72 h of treatment, the inhibition rate of PHEPS at a concentration of 500 μg/mL reached to 62.58% (Fig. 2A), which was much higher than that of the inhibition rate for Hela cells (Fig. 2B, 20.05%) or MCF-7 cells (Fig. 2C, 18.36%). Similar results were reported by Xu et al. (2007) that a mannose-binding lectin from the rhizomes of *Aspidistra elatior* Blume exhibited an anti-proliferative activity of 56% on HepG2 cells. It has also been reported that EPSs from various mushrooms such as *Pleurotus abalones* (Li et al., 2012), *P. pulmonarius* (Xu, Huang, & Cheung, 2012) and *Phellinus linteus* (Wang et al., 2012) have significant anti-proliferative activities on HepG2 cells. As concerned about the

differences of PHEPS exhibited on the three cancer cells, it is probably due to the specific conformation and high affinity of PHEPS with the receptors on HepG2 cells. Previously, Ouchi, Matsumoto, Ihara, and Ohya (1997) reported that galactomannan showed a specificity to integrate the galactose receptor on HepG2 cellar surface, but the receptor is not present on the surface of MCF-7 and Hela cells. Furthermore, it is believed that the higher antitumor activity of mushroom polysaccharides is related to their physicochemical properties, including relatively high molecular weight, expanded chain, specific conformation and complicated monosaccharide composition which make the mushroom polysaccharides to have more chances to collide and bind with the receptors on the surface of tumor cells (Ren, Reynisson, Perera, & Hemar, 2013).

In the past decade, various antitumor mechanisms for mushroom polysaccharides were published. Among them, inducing apoptosis of tumor cells is considered as one of the important ways. Several polysaccharides from mushroom species such as *C. militaris* (Park et al., 2009), *Agaricus blazei* (da Silva et al., 2013), *Inonotus obliquus* (Song, Liu, Kong, Chang, & Song, 2013), *Ganoderma lucidum* (Liu, Shen, Xia, Zhang, & Park, 2012) have been demonstrated to induce the apoptosis of cancer cells. It has also been reported that the recognition of polysaccharides with the receptor on cell surface and the resulting signals can trigger the cascade reaction by MAPK pathway which is modulated by anti-apoptotic and pro-apoptotic effectors involving a great deal of proteins such as the Bcl-2 family (Yang, Kao, Huang, Wang, & Lin, 2012). Other possible mechanisms related to fungi polysaccharides, such as cell cycle arrest (OuYang et al., 2013), necrosis (Price, Wenner, Sloper, Slaton, & Novack, 2010), anti-angiogenesis (Ho, Konerding, Gaumann, Groth, & Liu, 2004) and inhibition of cellular adhesion (Kim et al., 2009), further confirmed the means and opportunities of polysaccharides against cancer cells. Certainly, the mode of action and pharmacokinetics are not still clear for polysaccharides and the anti-proliferation mechanisms of PHEPS against HepG2 cells needs to be further studied.

4. Conclusion

In the present study, sucrose and $(NH_4)_2SO_4$ were found to be important factors for the production of mycelia and PHEPS, and the optimal medium was determined to be sucrose 46.08 g/L, yeast extract 4.71 g/L, $(NH_4)_2SO_4$ 5.72 g/L, KH_2PO_4 1.70 g/L, $CaCl_2$ 0.50 g/L, $MgSO_4$ 0.50 g/L, potato extract 1% and malt extract 1%. In shake flask culture, the maximum production of mycelia and PHEPS were 12.98 ± 0.14 and 5.33 ± 0.11 g/L, respectively. The information is quite useful for efficient production of mycelium biomass and PHEPS on a large scale. Moreover, PHEPS exhibited relatively higher anti-proliferative activity and lower cytotoxicity against human liver tumor HepG2 cells. After 72 h treatment, the inhibition rate of PHEPS at a concentration of 500 μg/mL reached to 62.58%. These results suggested that the PHEPS production could be extended on industrial scale and it might be a new source of natural antitumor agent with high potential value to replace synthetic chemicals.

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