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Enhancing exopolysaccharide antioxidant formation and yield from *Phellinus* species through medium optimization studies

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ABSTRACT

The study was conducted to enhance exopolysaccharide (EPS) antioxidant formation (as measured by Trolox-Equivalent Antioxidant Capacity, TEAC) by *Phellinus* sp. P0988 through medium studies, while jointly increasing EPS yield. Out of seven medium components, four significant variables were identified based on linear models with statistical significance ($p < 0.05$), namely rutin, FeSO4, and aspartate, which influenced TEAC, and malt extract, which influenced yield. Based on regression analysis of the experimental results from central composite design in response surface models, two second-order polynomial models adequately representing the relationship between these variables and EPS TEAC and yield were obtained, and the optimum concentration of these variables were determined and validated. Variable combinations for high EPS TEAC and yield were optimized through a desirability function test. The maximum EPS TEAC and yield reached 8.49 ± 0.32 mM and 10.92 ± 0.68 g/L, respectively, in a 7 L bioreactor under the optimum variable combinations.

1. Introduction

Antioxidants have many applications in food and chemical industries, as well as in health maintenance and in the prevention of chronic and degenerative diseases (Goiris, De Vreese, De Cooman, & Muylaert, 2012). Additionally, many bio-based antioxidants originating from medicinal fungi or plants such as polysaccharides and flavonoids are claimed to have beneficial effects on health, and as such are increasingly being used as nutraceuticals in healthy maintenance and food industry (Ding, Hou, & Hou, 2012; Yang et al., 2009; Zhang, Mills, & Nair, 2003). Currently, there is an increasing interest in natural antioxidants due to the consumer's demand for bio-based products and a potential to replace chemically synthetic antioxidants with those of a natural origin, particularly in food applications and health maintenance. Bio-based antioxidants have been recognized to be a promising group of antioxidant compounds due to their phototrophic nature (Wu & Hansen, 2008), providing an attractive alternative to synthetic chemical antioxidants for applications in food and medicinal fields.

Polysaccharides isolated from medicinal fungal fruiting bodies are reported to possess various important biological properties such as antitumor activity (Zheng et al., 2011), immunomodulation (Kim et al., 2007) and specifically antioxidant (Ding et al., 2010; Yang et al., 2009; Ye, Hou, & Zhang, 2007) with fungal antioxidants demonstrating low toxicity and good safety profile, compared to synthetic antioxidants (Diamantopoulou et al., 2012). In recent years, bioactive polysaccharides have been isolated from fungi such as *Pholiota adiposa* SX-02 (Deng et al., 2010), *Boletus speciosus Forst* (Ding et al., 2012) and *Tricholoma matsutake* (Ding et al., 2010), and the free radical scavengers and antioxidants found in these fungi have attracted much attention in the fields of biochemistry and pharmacology (Yang et al., 2009). However, it is known that the time required for obtaining either naturally harvested or farmed fruiting bodies of most medicinal fungal species is considerably lengthy, while product quality is also difficult to control in mushroom farming (Shih, Chou, Chen, Wu, & Hsieh, 2008). Encouragingly, many different exopolysaccharides (EPS) produced by medicinal fungi in submerged culture are also reported to possess antioxidant properties (Wu & Hansen, 2008), and many studies have focused their attention on medium optimization to enhance yield by the submerged fermentation of fungal mycelia, to address their antioxidant activity and structural analysis (Shih

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et al., 2008; Zheng et al., 2011). However, little attention has been given to the systematic enhancement of EPS bioactivity formation during bioproduction (Cui & Qiu, 2012; Hao et al., 2010; Liu & Wu, 2012; Zou, Guo, & Sun, 2009).

Phellinus sp. (*Basidiomycota*), a well-known pharmacodynamic fungus in the family of *Hymenochaetaceae* (Zhu, Kim, & Chen, 2008), has been used as a traditional Chinese medicine and interestingly, *Phellinus* mushrooms have been reported to possess antioxidant activity (Ye et al., 2007), with extracted compounds from fruiting bodies showing free radical-scavenging activity (Kim et al., 2007) and antioxidant activity (Park et al., 2004). However, previous polysaccharide bioproduction in submerged culture has been mainly aimed on the enhancement of EPS yield and mycelial growth (Zou et al., 2009), which may be problematic as the biological activity of polysaccharides are also of primary importance and are generally ascribed to the pharmacophore of these compounds (Yoon et al., 2012; Zheng et al., 2011).

As the continuation of our investigation of the influence of several factors on the synthesis of EPS by *Phellinus* sp. P0988 (Ma, Zhang, & Fam, 2013), the current study was conducted to enhance EPS antioxidant formation along with yield by this species through response surface method (RSM) and durability function.

2. Materials and methods

2.1. Microorganism and inoculum

The strain of *Phellinus* sp. P0988 employed in this study was deposited in the Chinese Type Culture Collection Center (CCTCC M 2012080). Analytical grade chemicals were used to formulate the basal medium of shake flask culture with a composition of (g/L): glucose 50, Glu (Glutamic acid) 4, $(NH_4)_2SO_4$ 4, MgSO4 1, KH_2PO_4 1. The pH was adjusted to 6.5 by addition of either 1 N NaOH or 1 N HCl. The inoculum culture was grown in a 250 mL Erlenmeyer flask containing 50 mL of basal medium on a rotary incubator at 28 °C and 160 rpm for 6 days. Rutin, which has been identified for the first time in our laboratory to have significantly influenced fungal EPS yield and TEAC (Ma et al., 2013), was obtained commercially (Batch No.: 20120509, Shaanxi, China). Chemicals such as aspartate were analytical grade and supplied by Sigma-Aldrich unless specified otherwise.

2.2. Optimization procedure and experimental design

Statistical experimental designs were applied in two steps to enhance EPS TEAC formation and yield. The first step was to separately identify the significant components for enhanced EPS bioactivity (TEAC) and yield using Plackett–Burman design (PB) design, and the second was to optimize these significant factors together using a central composite design (CCD) in RSM. The experimental designs and statistical analysis of the data were conducted by Stat-Ease Design-Expert 7.1.3 software (trial version 7.1.6, Stat-Ease Inc., Minneapolis, USA). Experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL medium on a rotary shaker at 28 °C and 150 rpm for 6 days after inoculating with 10% (v/v) inoculum.

2.2.1. Identifying significant variables using Plackett–Burman design

PB design serves as a valuable tool to determine the factors that have significant contributions in a small number of experiments (Plackett & Burman, 1946) by a linear model, indicating how each factor affects the production process for further optimization (Goswami, Chakraborty, Chaudhuri, & Dutta, 2012). To obtain a proper linear regression model for the evaluation of potential medium components affecting bioactive EPS production by *P.* sp. P0998, malt extract, mannitol, $CaCO_3$, rutin and aspartate, $FeSO_4$ and Tween 80, plus three central points at two levels each were chosen and their concentrations were determined based on the PB designs as shown in Table 1, while other components were maintained at the fixed concentration as that in the basal medium throughout the experiments.

PB experimental design is based on the first order polynomial model shown as standard equation Eq. (1), where Y is the response variable (EPS yield or TEAC), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable.

$$Y = \beta_0 + \sum_{i=1} \beta_i X_i \tag{1}$$

The effect of individual medium components on EPS TEAC and yield was determined based on the main effect Eq. (2).

$$\text{Effect} = \frac{\text{total response at high level} - \text{total response at low level}}{\text{number of trials}} \tag{2}$$

2.2.2. Response surface methodology

RSM involves three important steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. In this study, CCD in RSM performed by the Design Expert software was used to optimize the concentrations of the screened significant variables, to find a set of multiple experimental runs with several replicated center points. The screened variables, significantly affecting the EPS TEAC and yield, were coded according to Eq. (3).

$$\chi_i = \frac{X_i - X_0}{\Delta X_i} \tag{3}$$

where X_i represents the real value of variables; X_0, the real value of the X_i at the center point; ΔX_i, step change in X_i; and χ_i, the coded value of the variable, $i = 1, 2, 3$. The screened variables were studied at three different levels, low (−1), medium (0) and high (+1). The screened significant medium components with combinations of their individual concentrations were determined by CCD procedure in Design of expert software. All the experiments in shake flasks were carried out in triplicate and the results of EPS TEAC and yield obtained were taken as the response (Y, EPS TEAC or yield). The data was fitted into a second-order polynomial model Eq. (4) by multiple regression procedure in order to correlate the response to the independent variables.

$$Y_{\text{yield or TEAC}} = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \beta_{ij} X_i X_j \tag{4}$$

where β_0, β_i, β_{ii}, and β_{ij} represent the constant process effect in total, the linear, quadratic effect of X_i and the interaction effect between X_i and X_j on EPS production, respectively.

2.3. Analytical methods

Biomass quantification was performed on aqueous samples of known volume via centrifugation at 5000 rpm for 20 min, then washed and weighed to obtain the wet cell weight per 100 mL.

Glucose was measured using glucose oxidase kit from Sigma Chemical Co. (St. Louis, Missouri, USA).

For determination of EPS content and TEAC in broth, 1 mL of broth samples collected at various culture conditions from shake flasks or bioreactor was precipitated overnight with 3 volumes of ethanol at 4 °C and centrifuged at 5000 rpm and 4 °C for 15 min. The resulting precipitate was washed twice with acetone and ethanol, respectively, and was dried to obtain crude EPS. The precipitated EPS was re-dissolved in 8 mL distilled water as the solution of

Table 1
Plackett–Burman experimental design in actual and coded values with corresponding responses.

Run	Malt (g/L)	Rutin (g/L)	$FeSO_4$ (g/L)	$CaCO_3$ (g/L)	Mannitol (g/L)	Tween 80 (g/L)	Aspartate (g/L)	EPS (g/L)	TEAC (mM)
1	8 (−1)	1 (−1)	0.1 (−1)	0.1 (−1)	0.5 (−1)	3 (−1)	1 (−1)	3.6 ± 0.1	1.6 ± 0.3
2	8 (−1)	4 (+1)	0.1 (−1)	0.1 (−1)	3.5 (+1)	15 (+1)	4 (+1)	2.6 ± 0.1	6.6 ± 0.2
3	8 (−1)	1 (−1)	0.4 (+1)	0.4 (+1)	0.5 (−1)	15 (+1)	4 (+1)	3.5 ± 0.3	1.7 ± 0.2
4	14 (+1)	4 (+1)	0.4 (+1)	0.1 (−1)	0.5 (−1)	15 (+1)	1 (−1)	6.3 ± 0.5	2.9 ± 0.1
5	8 (−1)	4 (+1)	0.1 (−1)	0.4 (+1)	0.5 (−1)	3 (−1)	1 (−1)	5.1 ± 0.1	6.0 ± 0.4
6	14 (+1)	1 (−1)	0.1 (−1)	0.4 (+1)	3.5 (+1)	3 (−1)	4 (+1)	6.8 ± 0.8	4.0 ± 0.2
7	8 (−1)	1 (−1)	0.4 (+1)	0.4 (+1)	3.5 (+1)	15 (+1)	1 (−1)	3.4 ± 0.6	0.1 ± 0.2
8	14 (+1)	1 (−1)	0.1 (−1)	0.1 (−1)	3.5 (+1)	15 (+1)	1 (−1)	5.9 ± 0.2	1.7 ± 0.1
9	14 (+1)	4 (+1)	0.1 (−1)	0.4 (+1)	0.5 (−1)	15 (+1)	4 (+1)	6.0 ± 0.1	6.7 ± 0.4
10	14 (+1)	1 (−1)	0.4 (+1)	0.1 (−1)	0.5 (−1)	3 (−1)	4 (+1)	5.0 ± 0.1	3.9 ± 0.4
11	8 (−1)	4 (+1)	0.4 (+1)	0.1 (−1)	3.5 (+1)	3 (−1)	4 (+1)	3.7 ± 0.1	4.6 ± 0.1
12	14 (+1)	4 (+1)	0.4 (+1)	0.4 (+1)	3.5 (+1)	3 (−1)	1 (−1)	7.1 ± 0.3	5.5 ± 0.1

EPS represents exopolysaccharide. TEAC is Trolox-Equivalent Antioxidant Capacity. The (−1) indicates the low level, (+1) represents the high level in coded term. Low actual value corresponds to the coded low level (−1), and high actual value corresponds to the coded high level (+1). Data are expressed as mean ± standard deviation (SD). Experiments were performed at least in triplicate to ensure reproducibility.

crude EPS. This solution was treated by 2% tannic acid and then centrifuged for 15 min at 5000 rpm to remove protein in the solution. The pH of the solution was adjusted to 8 with 28% $NH_3{\cdot}H_2O$ and 10% (v/v) H_2O_2 was then added into the solution, which was kept at 55 °C for 6 h to remove the color of this EPS solution. The obtained solution was filtered through a membrane filter (0.45 μm, Millipore) and was supplemented with distilled water to obtain a 100 mL EPS solution. The EPS content and bioactivity in this solution were separately defined as the EPS yield (Luo et al., 2009) and bioactivity per 1 mL broth sampled at various culture conditions. The EPS bioactivity and EPS yield were measured as described previously (Ma et al., 2013).

Additionally, the precipitation at 4 °C during the above preparation of crude EPS solution was observed to prevent malt extract and glucose from dissolving in ethanol, which in turn reduced the extent to which these medium components interfered with EPS content determination, and this may be ascribed to the reduced solubility of malt extract and glucose in ethanol.

2.4. Validation of experimental model

The statistical models were verified with respect to all the determined variables within the design space. The experimental and predicted values based on the models were compared in order to determine the validity of the models. In this study, the combinations of the determined medium constituents at varied concentrations were tested to determine the EPS bioactivity and yield, and the results of these experiments were compared with the predicted values. The validation experiments at shake flask scale were conducted in triplicates.

For the validation experiment at bioreactor scale, 500 mL of inoculum was added to a 7 L stirred fermenter containing 5.0 L of optimized medium composition. The fermenter was equipped with a dissolved oxygen monitor and a pH meter for process control. Culture temperature, and agitation rate were controlled at 28 °C, and 80–100 rpm, while aeration was controlled at 3 L/min to prevent oxygen limitation in the system.

2.5. Statistical analysis

Unless otherwise stated, data were expressed as mean ± standard deviation (SD) and analyzed statistically by ANOVA methodology. *P*-values below 0.05 were regarded as statistically significant. The experimental data were analyzed using multiple regressions and the significance of regression coefficients was evaluated by *T*- or *F*-test. Modeling was started with a first order or quadratic polynomial model including linear, squared and interaction terms and the model adequacies were checked in terms of the values of R^2, adjusted R^2 and prediction error sum of squares (PRESS). The significant terms in the model were found by ANOVA for each response and ANOVA tables were generated.

3. Results

3.1. Factors significantly affecting EPS production

A total of seven medium components were screened through a number of twelve experimental runs designed by the PB method for bioactive EPS production by *P.* sp. P0998 and the results are shown in Table 1. The main effects of each medium component tested based on the results in Table 1 are presented in Fig. 1, indicating the difference between the averages of main effect made at the high level (+1) and at the low level (−1) of each variable (Rajendran & Thangavelu, 2007). It can be seen in Fig. 1 that the supplemented medium components significantly affecting EPS TEAC at positive levels are rutin, aspartate and $FeSO_4$ ($p < 0.05$), in which rutin has a statistically significant effect within a range of the tested concentrations. Tween 80 was observed to have a negative effect on EPS TEAC within the tested concentrations, for which its high concentration was 15 g/L. However, this concentration substantially exceeded a previously reported Tween 80 concentration of 4 g/L, which showed remarkable and positive effect on EPS yield and TEAC by this fungus (Ma et al., 2013). In addition, based on the main effect of medium components on EPS yield (Fig. 1), malt extract most significantly affected EPS yield at positive levels ($p < 0.05$), followed by $CaCO_3$ and aspartate ($p > 0.05$), and while the presence of $FeSO_4$ and Tween 80 in media negatively affects EPS yield, in which the concentration of Tween 80 at the high level was well above 4 g/l. Based on the influence of Tween 80 on EPS TEAC and yield in this study and our previous report (Ma et al., 2013), Tween 80 was supplemented at a concentration of 4 g/L in all the following experiments, unless specified otherwise. The minimal effect of mannitol and $FeSO_4$ at

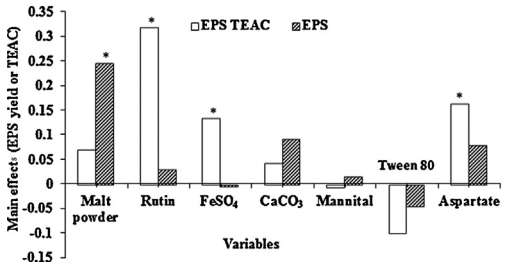

Fig. 1. Main effects of the medium constituents for exopolysaccharide (EPS) yield and TEAC based on the Plackett–Burman experimental results. "*" represents statistically significant constituents (*p*-values below 0.05).

positive or negative levels on EPS yield suggested that an exogenous source of these components in the media is not necessarily needed, if high EPS yield is the sole goal.

Two separate first-order polynomial models for EPS TEAC and yield in term of coded form and model summary statistics were obtained and shown in Table 2. The statistical summary output (Table 2) indicated that the linear models for EPS production are statistically highly significant (Goswami et al., 2012). The Model t and p values implied that the models separately using EPS TEAC and yield as response variables were significant. Furthermore, multiple R^2 coefficients of these models suggested that these two models may well correlate with experimental values. Model summary statistics also showed that these two linear models were found to have maximum "Adjusted R^2" and the "Predicted R^2" values, respectively. The adjusted R^2 statistics for the models of the EPS TEAC and yield further verified the adequacy of these models, as the adjusted R^2 only increases when significant terms are added to the model, while adding a variable to a model will always increase R^2 regardless of whether the additional variable is statistically significant or not (Sultania, Rai, & Srivastava, 2011). In addition, the models using EPS TEAC and yield as response variables possessed a very low PRESS value and a R^2 (predicted) of close to 1 implied that the regression models will perform well in predicting new data and these models can be expected to explain about most of variability in predicting new observations.

Based on the individual medium components showing significantly enhanced EPS TEAC and yield, alongside the experimental results of the stepwise regression method (data not shown), malt extract, $FeSO_4$, rutin, and aspartate were selected for further optimization by CCD, and their concentrations were set at 12 g/L, 0.25 g/L, 2.5 g/L and 2.5 g/L and determined as the center points of these factors in CCD, respectively.

3.2. Optimization of significant medium components using central composite design

CCD in the response surface method was applied to further investigate the individual and interactive effects of the selected significant variables (malt extract, rutin, aspartate, $FeSO_4$). Experimental runs with response variables (EPS TEAC and yield) were shown in Table 3. Multiple regression analysis was used to analyze the data, and two second-order polynomial equations with EPS TEAC and yield as response variables were derived from regression analysis, as shown by Eqs. (5) and (6), respectively.

$$\begin{aligned} Y_{\mathrm{TEAC}} = {} & 6.6 - 0.43X_1 - 0.38X_2 + 0.29X_3 + 0.65X_4 - 0.64X_1^2 \\ & - 0.79X_2^2 - 0.42X_3^2 - 0.11X_4^2 - 0.45X_1X_2 - 0.053X_1X_3 \\ & - 0.011X_1X_4 - 0.42X_2X_3 + 0.45X_2X_4 - 0.0024X_3X_4 \end{aligned} \quad (5)$$

$$\begin{aligned} Y_{\mathrm{Yield}} = {} & 5.9 + 1.2X_1 - 0.0097X_2 - 0.36X_3 + 0.32X_4 - 0.64X_1^2 \\ & - 0.22X_2^2 - 0.079X_3^2 - 0.082X_4^2 - 0.29X_1X_2 - 0.32X_1X_3 \\ & + 0.023X_1X_4 - 0.46X_2X_3 + 0.39X_2X_4 + 0.27X_3X_4 \end{aligned} \quad (6)$$

Y_{TEAC} represents EPS TEAC (mM) and Y_{Yield} represents EPS yield (g/L), while X_1, X_2, X_3 and X_4 represent the respective concentrations of malt extract, rutin, aspartate and $FeSO_4$ in coded form.

As shown in Table 4, the summary statistics indicate that the equations fitting EPS synthesis by *P.* sp. P0988 adequately represent the actual relationship between the independent variables and responses. For using EPS TEAC and yield as response variables, statistical parameters of the models are shown in Table 4. The model F value for these two models showed that the models were significant and also indicated that there was only a very low chance ($p < 0.0001$) that this could occur due to noise. A R^2 of close to 1 indicates that most of the sample variation was attributed to the variables. The adjusted R^2 value of close to 1 for EPS TEAC and yield also indicated the models' goodness of fit (Sultania et al., 2011). Also, the models separately possessed a very low PRESS value and a R^2 (predicted) of close to 1 and which suggests that these regression models can expect to explain a high percentage (close to 1) of variability in predicting new observations (Luo et al., 2009). In addition, lack of fit F value for response variables and lack of fit p-value (>0.5) also depicted that the lack of fit was insignificant relative to the pure error due to noise and these models performed well. These above results suggested that the proposed experimental design models were acceptable for simulation of the response for EPS TEAC and yield within the range of variables employed.

From Eq. (6), the optimal concentrations of medium components for the maximum EPS TEAC were calculated to be 0.5051, 0.4242, 0.1414, and 2.0 for X_1, X_2, X_3 and X_4 in term of coded form, respectively, with corresponding actual values were 10.9 g/L, 0.25 g/L, 2.7 g/L, 3.5 g/L for malt extract, $FeSO_4$, rutin, aspartate. Furthermore, the results of triplicate verification tests yielded TEAC values of 7.3 ± 0.48 mM under the specified optimum culture condition, which was very close to the predicted value of 7.7 mM obtained from Eq. (5). However, the validation tests just obtained 6 g/L EPS under the optimum conditions for EPS TEAC.

In addition, from Eqs. (3) and (6), the coded optimal values of X_1, X_2, X_3 and X_4 for maximum EPS yield were estimated to be $X_1 = 0.9801$, $X_2 = 1.8808$, $X_3 = -1.7866$, $X_4 = 1.045$, while their actual optimum values were 14 g/L, 0.3 g/L, 3.4 g/L and 1.6 g/L for malt extract, $FeSO_4$, rutin, aspartate, respectively. In order to validate the results from the analysis of RSM, three additional experiments for EPS production by *P.* sp. P0988 were performed randomly with these components at the optimum concentration levels prepared in the basal medium. As a result, the mean value of EPS yield was 8.2 ± 0.1 g/L, which was very close to the theoretical predicted value (8.2058 g/L) based on Eq. (6) and this actual yield was 6.3 times than that produced in basal medium alone, which only produced 1.32 g/L. However, a bioactivity of 3.9 ± 0.2 mM (TEAC) was obtained under the optimized culture conditions for maximum EPS yield.

3.3. Desirability functions test

It can be seen from above results that the desired combination of the highest EPS TEAC together with the highest EPS yield could not be obtained simultaneously. To find an optimal point or conditions simultaneously for both responses, a desirability function test was performed using an optimizer procedure in Minitab software (Tahkoniemi, Helmja, Menert, & Kaljurand, 2006). The desirability approach is a popular method that assigns a given score to a set of responses and then a factor setting that maximizes the overall score would be chosen. The following formula (7) describes how the overall desirability is expressed by individual desirabilities by calculating a geometric mean from individual response variable desirability. This desirability function test searches for a combination of the levels of several factors that jointly optimizes a set of responses by satisfying the requirements for each response in the design. It was then possible to find a set of optimum conditions for all responses together. By specifying the goals and boundaries required for each response, the individual desirability (d) was obtained (Maran & Manikandan, 2012).

$$D = \frac{d_1, d_2, d_3, \ldots, d_n}{n} \quad (7)$$

where D is the overall desirability or composite desirability, d_i is the normalized desirability function of the i^{th} response and n is the number of responses.

Table 2
Model summary statistics and statistical data obtained from ANOVA.

Response and models[a]	PRESS	R^2 (predicted, %)	R^2 (%)	R^2 (adjusted, %)	Model p
$Y_{yield} = 0.39 + 0.41X_1 + 0.10X_2 - 0.15X_3 + 3.1X_4 + 0.05X_5 - 0.04X_6 - 0.26X_7$	0.11	98.24	99.9	99.72	0.03
$Y_{TEAC} = 0.03 + 0.12X_1 + 1.1X_2 - 4.5X_3 + 1.4X_4 - 0.023X_5 - 0.08X_6 + 0.54X_6$	0.07	99.97	99.9	99.27	0.018

[a] Only significant coefficients with $p < 0.05$ are included. Factors are in term of coded levels. Y represents the experimental response, and $X_1 - X_7$ correspond to the independent variables of malt extract, rutin, $FeSO_4$, $CaCO_3$, mannitol, Tween 80 and aspartate, respectively. PRESS: the prediction error sum of squares.

Table 3
The central composition design and responses variables for optimization of medium composition for the EPS production by *P.* sp. P0988.

Run	Malt extract (g/L)	$FeSO_4$ (g/L)	Rutin (g/L)	Aspartate (g/L)	EPS yield (g/L)	EPS TEAC (mM)
1	14 (1)	0.3 (1)	3 (1)	2 (−1)	3.6 ± 0.1	2.0 ± 0.2
2	16 (2)	0.25 (0)	2.5 (0)	2.5 (0)	5.8 ± 0.3	3.5 ± 0.2
3	14 (1)	0.2 (−1)	3 (1)	3 (1)	6.3 ± 0.2	5.9 ± 0.1
4	12 (0)	0.25 (0)	2.5 (0)	2.5 (0)	6.1 ± 0.4	6.9 ± 0.3
5	8 (−2)	0.25 (0)	2.5 (0)	2.5 (0)	1.1 ± 0.1	4.7 ± 0.5
6	12 (0)	0.25 (0)	2.5 (0)	2.5 (0)	5.9 ± 0.2	6.8 ± 0.2
7	14 (1)	0.2 (−1)	2 (−1)	2 (−1)	6.9 ± 0.3	4.1 ± 0.2
8	12 (0)	0.25 (0)	1.5 (−2)	2.5 (0)	6.4 ± 0.3	4.6 ± 0.3
9	12 (0)	0.25 (0)	2.5 (0)	2.5 (0)	5.8 ± 0.5	6.8 ± 0.1
10	12 (0)	0.25 (0)	2.5 (0)	2.5 (0)	5.9 ± 0.3	6.1 ± 0.2
11	10 (−1)	0.3 (1)	2 (−1)	3 (1)	4.8 ± 0.2	5.9 ± 0.2
12	12 (0)	0.15(−2)	2.5 (0)	2.5 (0)	4.9 ± 0.2	3.9 ± 0.2
13	10 (−1)	0.2 (−1)	3 (1)	3 (1)	4.1 ± 0.1	5.9 ± 0.1
14	14 (1)	0.2 (−1)	3 (1)	2 (−1)	6.1 ± 0.3	5.7 ± 0.1
15	10 (−1)	0.2 (−1)	3 (1)	2 (−1)	3.8 ± 0.2	5.9 ± 0.3
16	14 (1)	0.2 (−1)	2 (−1)	3 (1)	6.5 ± 0.3	4.4 ± 0.2
17	12 (0)	0.25 (0)	2.5 (0)	1.5 (−2)	4.9 ± 0.4	4.7 ± 0.1
18	12 (0)	0.25 (0)	2.5 (0)	2.5 (0)	5.8 ± 0.4	6.1 ± 0.2
19	14 (1)	0.3 (1)	3 (1)	3 (1)	5.7 ± 0.3	4.1 ± 0.2
20	12 (0)	0.25 (0)	2.5 (0)	3.5 (2)	6.3 ± 0.2	7.7 ± 0.2
21	14 (1)	0.3 (1)	2 (−1)	3 (1)	7.3 ± 0.3	4.5 ± 0.2
22	12 (0)	0.25 (0)	2.5 (0)	2.5 (0)	5.9 ± 0.2	6.8 ± 0.1
23	10 (−1)	0.3 (1)	3 (1)	3 (1)	4.4 ± 0.2	6.5 ± 0.2
24	12 (0)	0.25 (0)	3.5 (2)	2.5 (0)	4.9 ± 0.1	5.3 ± 0.4
25	12 (0)	0.25 (0)	2.5 (0)	2.5 (0)	6.1 ± 0.2	6.8 ± 0.2
26	14 (1)	0.3 (1)	2 (−1)	2 (−1)	6.4 ± 0.2	2.4 ± 0.2
27	10 (−1)	0.3 (1)	3 (1)	1.5 (−1)	2.4 ± 0.3	3.9 ± 0.2
28	10 (−1)	0.3 (1)	2 (−1)	2 (−1)	4.2 ± 0.3	4.2 ± 0.2
29	10 (−1)	0.2 (−1)	2 (−1)	3 (1)	2.7 ± 0.1	4.8 ± 0.3
30	10 (−1)	0.2 (−1)	2 (−1)	2 (−1)	3.3 ± 0.1	4.1 ± 0.2
31	12 (0)	0.35 (2)	2.5 (0)	2.5 (0)	5.3 ± 0.3	2.9 ± 0.1

In this study, the individual response desirability was given a value of 1 and to jointly optimize the response variables including EPS TEAC and yield, a weight factor of 1 was chosen for all individual desirability. An optimization plot was then created and the composite desirability values and individual desirabilities values against four factors are shown in Fig. 2, from which it is possible to search for input variable settings with a higher composite desirability, allowing joint optimum conditions for these two responses to be found. The predicted responses included EPS TEAC and yield separately with a maximized value of 6.6256 mM and 7.0014 g/L, which were obtained where the concentrations of malt extract, rutin, aspartate and $FeSO_4$ were 0.8283, 0.2569, −0.1579 and 2 in term of coded form, with corresponding actual concentrations of 13.6 g/L, 2.6 g/L, 2.4 g/L, 0.35 g/L, respectively.

Table 4
Analysis of variance for response surface optimization of medium composition for EPS synthesis and the adequacy of the models tested.

Source	*Df*	Seq SS	Adj SS	Adj MS	*F*	*p*
For EPS TEAC						
Regression	14	58.86	58.86	4.204	35.31	<0.0001
Linear	4	20.28	20.28	5.069	42.57	<0.0001
Square	4	29.19	29.19	7.299	61.30	<0.0001
Interaction	6	9.389	9.389	1.565	13.14	<0.0001
Residual	16	1.905	1.905	0.1191		
Lack of fit	10	1.047	1.047	0.1047	0.73	0.685
Pure error	6	0.8585	0.8585	0.1431		
Corr. Total	30	60.77				
For EPS yield						
Regression	14	62.11	62.11	4.436	210.6	<0.0001
Linear	4	39.84	39.84	9.961	472.8	<0.0001
Square	4	12.27	12.27	3.069	145.7	<0.0001
Interaction	6	9.992	9.992	1.665	79.05	<0.0001
Residual	16	0.3371	0.3371	0.02107		
Lack of fit	10	0.2655	0.2655	0.02655	2.230	0.170
Pure error	6	0.0715	0.0715	0.01192		
Corr. Total	30	62.45				

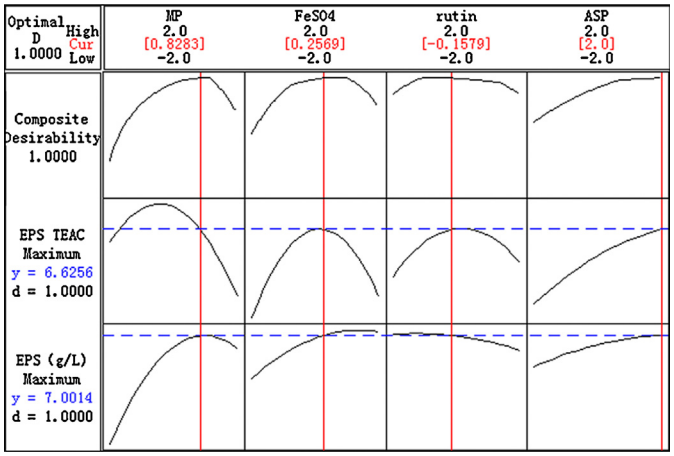

Fig. 2. Minitab's interactive optimization plot. MP and ASP represent malt extract and aspartate, respectively. Minitab calculates an optimal solution and draws a plot. The optimal solution serves as the starting point for the plot.

The optimized concentrations of these significant factors together with the remaining factors in the basal medium and the above determined concentration of Tween 80 configured an optimum medium composition for enhanced EPS TEAC formation together with high yield by *P.* sp. P0988.

3.4. Validation of the results of desirability function test at shake flask and bioreactor scare

Triplicate sets of experiments for EPS production by *P.* sp. P0988 were performed at shake flask scale, and the results from these validation experiments indicated that the mean experimental value of the highest EPS TEAC and yield were 6.9 ± 0.43 mM and 7.5 ± 0.37 g/L, respectively, which was clearly very close to the above predicted value. These results suggested that the models developed in this study through desirability function tests combined with RSM prove adequate for predicting antioxidant formation and EPS yield.

In addition, as shown in Fig. 3, the time courses of the EPS production by *P.* sp. P0998 in a 7 L bioreactor under the above specified optimal conditions was monitored for 162 h. EPS production as indicated by EPS yield and bioactivity increased significantly at 103 h, as well as wet cell weight, while the residual glucose decreased sharply. The maximal EPS TEAC in the bioreactor reached 8.49 ± 0.32 mM with EPS yield of 10.92 ± 0.68 g/L at 127 h, well above the results in shake flasks, which may be attributed to the improvement in cultivation conditions at the bioreactor scale. These results further confirmed the adequacy of the finally determined EPS production models including the EPS antioxidant formation and yield at reactor scale.

4. Discussion

The EPS TEAC formation was, for the first time, given attention during EPS bioproduction by fungi and the medium composition for enhanced bioactivity formation was optimized jointly with the enhancement of EPS yield using statistically based experimental designs. This study presented hereby provided more substantial information about the effect of separately tested medium components on EPS bioactivity formation and yield compared to our previous reports (Ma et al., 2013).

It is important to note that the experimental conditions that maximize the yield of bioactive metabolite (i.e. EPS) might not be consistent with the conditions favoring bioactivity formation, which has been in part confirmed in our previous study (Ma et al., 2013). However, in such situations, the optimization of medium composition or production conditions for several response variables of interest becomes laborious and complex (Vera, de Atauri, Cascante, & Torres, 2003). In this study, through use of desirability functions to transform a multivariate optimization problem into a univariate scenario, two responses were combined into one measurement, representative of both EPS antioxidant formation and yield simultaneously, despite different scaling. In addition, to date, no reports are available in the literature regarding the optimization of bioproduction conditions for both EPS bioactivity formation and yield by *Phellinus* genus or other fungal strains. Thus, our findings presented herein have provided new information for increasing bioactivity of metabolites produced by fungal species.

To date, many investigators have studied the influence of medium components on EPS production or optimized the production conditions for enhanced EPS yield or mycelial growth (Liu & Wu, 2012; Luo et al., 2009; Shih et al., 2008; Yoon et al., 2012). However, relatively few reports have been published concerning the enhanced bioactivity of extracellular polysaccharides, and it is still not clear which cultivation conditions are critical for the development of total antioxidant activity in fungal submerged culture. The influence of supplemental components on EPS bioactivity may be ascribed to their effect on the EPS structure development, which in turn may affect activity during bioproduction, but this still requires further research. In addition, EPS demonstrating high antioxidant activity, as produced by the approaches proposed in this study, may prove more attractive as a naturally antioxidant agent for application in food and medicinal fields. Furthermore, data on the EPS yield and bioactivity reported here will be useful in providing information for the development

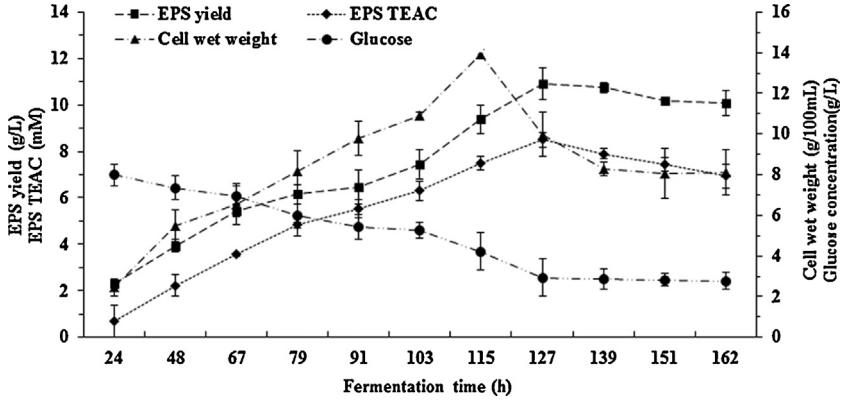

Fig. 3. The time course of the EPS production by *P.* sp. P0988 during liquid fermentation in a 7 L bioreactor. Data are given as means ± SD, $n = 3$.

of further mathematical models describing EPS synthesis by fungi for use in subsequent scale-up of production.

Also, the EPS total reactivity (activity/volume) and content per volume of culture (yield, g/L) at harvest were jointly considered in this study. Alternatively, these two responses can be considered as one term such as specific activity (reactivity/quantity of product) and could be optimized as one response. However, this would simply maximize the ratio of the two quantities measured, rather than both of the two responses simultaneously, and overall EPS production would also be important.

Additionally, the underlying cellular or molecular mechanism by which the optimal combination of medium components influences fungal morphology development, physiology and bioactivity formation is not the focus of this study. However, a beneficial role of some components supplemented in medium such as rutin in enhancing cell survival in submerged culture and in morphological development has been verified previously (Ma et al., 2013). The mechanism by which the different combinations of medium components favored EPS synthesis, morphological development and cell survival requires further investigation.

5. Conclusion

Statistically based experimental designs proposed in this study proved to be effective tools to simultaneously enhance EPS antioxidant formation and yield under submerged culture, demonstrating important considerations previously unexamined. Specifically, if fungal antioxidants are to be produced for use in the health industry, the bioactive strength of the product alongside overall production is important, and this study makes sound advances to improving the quality of fungal EPS antioxidant products. Through optimization experiments and model validations, the maximum EPS antioxidant and yield reached 8.49 ± 0.32 mM and 10.92 ± 0.68 g/L, respectively, at the bioreactor scale, which is amongst the highest performance values reported in the literature to date for *Phellinus* or other medicinal fungi. Further studies will be focused on the purification of crude EPS and the characterization of the detailed structure, function, and possible commercial benefit of these polysaccharides produced by this fungus.

Competing interests

The authors declare that they have no conflict of interest.

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