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Optimization of extracellular glucan production from *Pleurotus eryngii* and its impact on angiogenesis

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ABSTRACT

Pleurotus eryngii, an edible mushroom with therapeutic potential was optimized using response surface methodology of four-factor Box–Behnken design for maximum mycelial biomass and extracellular glucan (EPS) production. The model predicts to gain a maximal mycelial biomass and extracellular polysaccharide at 39.4 g/l; 36.04 g/l of glucose, 8.27 g/l; 7.51 g/l of yeast extract, pH 6.99; 7.07 and temperature 26.2 °C; 25.84 °C, respectively. The validation experiments showed that the model was significant and in close agreement with the model prediction. The evaluation of extracellular polysaccharide on angiogenesis by *ex vivo* CAM assay showed that there was significant inhibition in neo-vascularization.

1. Introduction

Pleurotus eryngii, (is also known as king trumpet mushroom, French horn mushroom, king oyster mushroom) an edible mushroom is considered to be the largest species in the genus, *Pleurotus.* It is originated from Europe and also grown in parts of Asia, Middle East and North Africa [1]. *P. eryngii* grows as a facultative biotroph on the roots and submerged regions of the stem of certain plant [2]. They can be easily grown in various substrates [3] and found to be the best tasting among the oyster mushrooms [4].

Mushrooms were used as food and medicine by humans for centuries [5,6], contains biologically active polysaccharides in the fruiting bodies and bioactive compounds in submerged broth. Recent reports on *Pleurotus* species showed that it has a number of therapeutic activities, such as anti-tumor, immunomodulatory, antioxidant, anti-inflammatory, antimicrobial and antiviral activities [7,8]. Similarly, *P. eryngii* also contains various antioxidative compounds such as polyphenols and flavonoids and murine splenocytes inhibitory protein called eryngeolysin [9].

Polysaccharides from mushrooms are the most promising compounds as they exerts antitumor effects by activating the host immune responses [6,10], less toxic to normal cells and lack of apparent side effects in patients. For example, lentinan from *Lentinus edodes*, krestin (PSK) from *Trametes versicolor* and schizophyllan from *Schizophyllum commune* have been used for cancer immunotherapy in Japan [11]. The bioactive polysaccharides such as glucans, proteoglycans, *etc.*, with good bioactive properties [12] can be isolated from many mushrooms including *Pleurotus* species. The studies on the *P. eryngii* show that it contains glucan [13,14] with potential biological activities such as activation of the phagocytic response of macrophages [15], proliferation of $CD4^+$ and $CD8^+$ cells [15], elevation of natural killer cell mediated cytotoxicity in mice [16].

The discovery and evaluation of novel polysaccharides from various medicinal mushrooms is a hotspot research as the compound is safe for functional foods or medicine. The bioactive polysaccharides can be obtained from the fruit bodies, mycelium of the mushroom [12], but it is time consuming to harvest and requires tedious purification steps [17]. Submerged culture has a number of advantages including higher mycelium and polysaccharide production in a more compact space and shorter time [18] and as an alternative for efficient production of polysaccharide with similar biological activity [19].

Angiogenesis, the formation of new blood vessels plays a key role in tumor growth and metastasis [20]. Inhibition of angiogenesis is a promising strategy for the treatment of cancer [21]. Several specific factors are known to regulate angiogenesis, including vascular endothelial growth factor (VEGF) [22]. Polysaccharides from few mushrooms suppressed angiogenesis of endothelial cell *in vitro* by inhibition of VEGF receptor signaling pathway [23].

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Table 1
Variables and experimental design levels for response surface.

Variables	Symbols		Coded levels		
	Uncoded	Coded	−1	0	1
pH	X_1	*A*	4.0	6.5	9.0
Temperature	X_2	*B*	20.0	27.85	35.7
Carbon source	X_3	*C*	2.5	21.25	40.0
Nitrogen source	X_4	*D*	1.0	5.5	10.0

Hence in the present paper, the most suitable conditions for effective mycelial growth and extracellular polysaccharide production were investigated in *P. eryngii* using the Box Behnken design of four-factor-at-a-time. The optimum fermentation conditions obtained from design experiment were verified using validation experiments. This study also investigated the effects of extracellular polysaccharide on angiogenesis using *ex vivo* CAM assay.

2. Material and methods

2.1. Microorganism and culture conditions

Edible mushroom *P. eryngii* MUBL 3701 (king oyster) was obtained from the fungal culture collection, Centre for Advanced Studies in Botany, University of Madras. The culture was maintained in potato dextrose agar (PDA) at 4 °C and sub cultured every three months. Cultivation was performed in two stages: the seed culture medium consisted of the following components: glucose – 20.0 g, yeast extract – 2.0 g, KH_2PO_4 – 1.0 g, K_2HPO_4 – 1.0 g and $MgSO_4{\cdot}7H_2O$ – 1.5 g. The medium was autoclaved at 121 °C for 15 min. The flask culture experiments were performed in 250 ml flasks containing 50 ml of fermentation medium, which was inoculated with 10% (v/v) of the seed culture. The flasks were inoculated and incubated at 25 °C for 10 days in a static condition.

2.2. Response surface methodology for optimizing the selected medium components

Response surface methodology (RSM) was used to determine the optimum concentration of the variables (pH, temperature, concentration of glucose and yeast extract) for maximum mycelial growth and EPS production. The experimental design was a Box–Behnken design with four key factors (Table 1).

The behavior of the system is explained by the following quadratic equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2 + \beta_{44} {x_4}^2 \quad (1)$$

where Y is the predicted response; β_0 is model constant; x_1, x_2, x_3 and x_4 are independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are cross product coefficients; and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. The fitted polynomial equation is expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions [24]. The analysis of variance, the regression coefficients of individual linear, quadratic and interaction terms were also determined. The regression coefficients were used to generate three dimensional and contour maps from the regression models. Design expert (Version 7.1.6, USA) software package was used to analyze the experimental data. The P values less than 0.05 were considered to be statistically significant.

2.2.1. Estimation of dry cell weight of the mycelium and extraction of EPS

The biomass was measured by filtering the fungal culture through a Whatman No. 4 filter paper until a clear filtrate was obtained. The obtained mycelium was washed twice with distilled water and dried overnight at 50 °C and weighed. EPS was precipitated from the culture filtrate mixing with four volumes of 95% (v/v) ethanol. This was left to stand overnight at 4 °C to precipitate crude EPS. The precipitated EPS was pelleted by centrifugation at 11,712 × *g* for 40 min at 4 °C and the supernatant was discarded. The precipitate was then re-suspended in an equal volume of 75% ethanol [25] to remove oligosaccharides and centrifuged again. The precipitated EPS was dried at 40 °C to remove residual ethanol.

2.2.2. Assay of polysaccharide and sugar composition in EPS

The polysaccharide content of lyophilized EPS was estimated by the phenol–sulfuric acid colorimetric assay [26]. Briefly, 10 mg EPS dissolved in 10 ml of double distilled water and mixed evenly. To 1 ml of the above solution, 1 ml of phenol and 5 ml of sulfuric acid was added and the polysaccharide content of the mixture was determined spectrophotometrically at 490 nm by using glucose as standard. The sugar composition in the EPS was determined by thin layer chromatography technique, a modified method of Hardy et al. [27]. A portion (100 mg) of the EPS was added to 5 ml of 2.0 M trifluoroacetic acid and hydrolyzed at 95 °C for 16 h and analyzed with thin layer chromatographic technique.

2.3. Chorioallantoic membrane (CAM) assay

Ex vivo anti-angiogenic activity of extracellular polysaccharide was measured by CAM assay as described by Li et al. [28] with minor modifications. A group of twenty 7-day-old fertilized eggs were incubated at 37.5 °C and 55% relative humidity. On day 8, 1 cm^2 window was carefully created on the broader side of the egg and candle the egg to assure the existence of embryonic blood vessels. Extracellular polysaccharide was dissolved in double distilled water (20 μl containing 200, 500 or 1000 μg/egg), it was then applied on a filter paper disk and placed onto chorioallantoic membrane of the egg. The window was closed immediately with a permeable sticky tape and incubated for 3 days (until day 11). After incubation, the egg shell was pushed aside around the window and the blood vessels were photographed. The anti-angiogenic effect of extracellular polysaccharide on chorioallantoic membrane was quantified by counting the number of blood vessel branch points, which were marked using artistic software on the photos.

2.4. Statistical analysis

Values are expressed as means ± S.D. and analyzed using one-way for comparisons of means. The statistical analysis was performed using SPSS version 10 for Windows, (SPSS, Inc.); a P-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Sugar composition

The EPS subjected to trifluroacetic acid digestion and thin layer chromatographic analysis showed that the extracellular polysaccharide was made of polymeric units of glucose (Fig. 1). The results obtained coincide with the observation made by Carbonero et al. [13] that the intracellular polysaccharide extracted from *P. eryngii* contains a branched β-glucan, with a main chain linked with glucopyranosyl residues. Moreover, previous studies on the polysaccharide from the different species of *Pleurotus* showed that it consisted predominantly of D-glucose [29]. These observations