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ORIGINAL PAPER

Improved simultaneous production of mycelial biomass and polysaccharides by submerged culture of *Hericium erinaceum*: optimization using a central composite rotatable design (CCRD)

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Abstract The aim of this study was to optimize the culture medium used for the mycelial growth and production of intracellular polysaccharides (IPS) and exopolysaccharides (EPS) in a submerged culture of *Hericium erinaceum*. Of the various factors examined, including carbon and nitrogen sources, vitamins, mineral elements, and initial pH, those that proved to have a significant effect were then tested using a 2^4 central composite rotatable design (CCRD). Under the optimal culture conditions, the maximal yield of biomass reached 14.24 ± 0.45 g l^{-1} and was 1.85-fold higher than in the basal medium. The kinetics of EPS biosynthesis in a bioreactor showed that although the highest yield of EPS (2.75 ± 0.27 g l^{-1}) could be obtained on day 8, the process of biosynthesizing high molecular weight polysaccharides proceeded until the depletion of the carbon source in the medium (after 14 days of cultivation). Our results could be very helpful in the large-scale production of bioactive polysaccharides from *H. erinaceum*.

Keywords Lion's Mane mushroom · Exopolysaccharides · Intracellular polysaccharides · Media optimization · Response surface methodology

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Introduction

For the past few decades, compounds of fungal origin have attracted special attention from researchers due to their various biological and pharmacological activities, including antitumor and immunostimulatory actions [5, 30]. Among these compounds, bioactive polysaccharides isolated from higher *Basidiomycota* are the best known, and appear to have the strongest anticancer activities among mushroom-derived substances [20, 23, 33]. *Hericium erinaceum* (Bull.: Fr.) Pers. of the *Bankeraceae* family, commonly called Lion's Mane mushroom, is an edible and medicinal mushroom that has been used for hundreds of years in traditional Chinese medicine [16]. A number of polysaccharides have been isolated from its fruiting body that have been found to display antitumor activities in in vivo tests on sarcoma 180 cancer cells [17]. The exopolysaccharide isolated from a submerged mycelial culture of *H. erinaceum* was also found to exhibit stimulatory activity towards neurons in an in vitro model, and also to lower low-density lipoprotein (LDL) cholesterol and total cholesterol levels [21, 32]. Antioxidant activity of *H. erinaceum* EPS has also been reported [14]. Therefore, both the cultivation of *H. erinaceum* on liquid artificial media and the isolation of polysaccharides that possess numerous favorable activities and can potentially become new anticancer agents or cancer-preventive nutraceuticals are highly desirable tasks.

In recent years there have been many reports on the optimization of cultivation conditions in submerged cultures of higher mushrooms in order to obtain a high level of mycelial biomass and EPS [1, 10, 31]. Traditional methods of optimizing the culture medium involve changing one independent parameter (e.g., a nutrient or pH) while keeping the others constant, so that it is

possible to establish the effect of each parameter on the course of the process. However, such one-dimensional methods are very laborious and time-consuming and require a lot of experimental data. Moreover, they do not provide any information on correlations between parameters, and in most cases they do not guarantee that the optimal conditions for the production of mycelial biomass and the desired metabolites are obtained. Currently, therefore, methods based on design of experiments (DOE) are used, where it is possible to minimize the number of experiments by employing, for example, the central composite rotatable design (CCRD). This experimental design allows the effects of many independent variables at different levels to be examined, such as the effects of several components of the culture medium at different concentrations. To identify the optimal level of each of the test variables, response surface methodology (RSM) is used, where the experimental responses are fitted to a predictive polynomial equation. Such multiple regression analysis provides the most complete information available on the effects of various parameters, even in the presence of mutual interactions [4, 11]. The RSM method was successfully applied to enhance biomass yield and polysaccharide production in submerged cultures of higher fungi of such species as *Schizophyllum commune* and *Pholiota squarrosa* [11, 29].

The aim of the present study was to optimize the cultivation conditions in a submerged culture that employed to simultaneously produce mycelial biomass, EPS and IPS via *H. erinaceum* using the one-factor-at-a-time method and a CCRD experiment. To the best of our knowledge, this is the first report on optimizing the cultivation conditions and the composition of the culture medium used in a submerged culture of *H. erinaceum* by means of CCRD. We have also examined the kinetics of the polysaccharide biosynthesis and the mycelial growth of *H. erinaceum* in a bioreactor with a culture medium selected during the CCRD experiment. As far as our literature survey could ascertain, there is no information on the changes in the elution profiles of EPS during the cultivation process of a higher basidiomycete. Therefore, in this study, the EPS obtained at regular time intervals during cultivation in a bioreactor were subjected to high-performance size exclusion chromatography (HPSEC) to establish their degree of homogeneity and their molecular weights. In this way, we were able to determine the changes in the proportions of polysaccharides of particular molecular weights, and also to establish the cultivation time needed to obtain the highest amount of the required product with a precisely defined molecular weight. This kind of information is extremely valuable because the size of a fungal biopolymer molecule may have a significant effect on its biological activity [18, 19, 28, 30].

Methods

Microorganism and culture conditions

Hericium erinaceum (Bull.: Fr.) Pers. mycelium was purchased from Fungi Perfecti (Olympia, WA, USA) and deposited in the Medicinal Mushroom Culture Collection of the Chair and Department of Drug Technology, the Medical University of Warsaw, Poland. The stock culture of *H. erinaceum* was cultivated on malt agar plates at 25°C for 14 days, and then stored at 4°C and transferred onto the new plate every three months. All culture media were sterilized at 121°C for 20 min. The inoculum volume was 10%.

Preparation of inocula

The basic nutrient medium, which consisted of glucose 50.0 g l^{-1}, yeast extract 10.0 g l^{-1}, casein peptone 10.0 g l^{-1}, and KH_2PO_4 1.0 g l^{-1}, pH 5.0, was inoculated with several 10-mm-diameter discs of mycelium that were punched out with a sterilized self-designed cutter from a 14-day-old colony growing on a malt agar. Cultivation was performed in 500-ml Erlenmeyer flasks containing 200 ml of nutrient medium on a rotary shaker at 110 rpm at 25°C for 14 days. The seed cultures obtained were used for inoculation.

Flask culture

Experimental cultures were grown in 500 ml flasks containing 200 ml of culture medium. Cultivation was performed on a rotary shaker at 110 rpm and 25°C for 21 days. After this time, the mycelial dry weight and the amounts of EPS and IPS were determined. The effects of the carbon sources, nitrogen sources, pH, vitamins, and minerals on the mycelial biomass yield and also on IPS and EPS production were examined by the one-factor-at-a-time method.

The results obtained allowed us to determine which of the components had a significant effect on biomass growth and polysaccharide production. Moreover, an experiment based on the CCRD method was performed to examine the dependence of different components of the culture medium and their concentrations on mycelial growth and polysaccharide biosynthesis. At least two replications were performed for each cultivation to ensure reproducibility.

Experimental design and data analysis

A full 2^4 factorial CCRD that contained eight star points ($\alpha = \pm 2$) with two replicates and six replications at the central points ($n_0 = 6$) was performed. This means that a

total number of 54 experiments were required for this procedure. Table 1 shows the ranges and the levels of the independent variables tested in the CCRD experiment. The following equation was used to code the test variables:

$$X_i = \frac{x_i - x_i^0}{\Delta x_i} \quad (1)$$

where X_i is the dimensionless coded value of the *i*-th test variable, x_i is the real value of the *i*-th test variable, x_i^0 is the real value of the *i*-th test variable at the center point, and Δx_i is the step change value.

The relationship between the independent variables (nutrient medium components) and the response (mycelial growth, EPS, and IPS production) was fitted to a predictive second-order polynomial equation:

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \sum \beta_{ij} X_i X_j \quad (2)$$

where Y_i is the predicted response, subscripts i and j take values from 1 to the number of variables, β_0 is a constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the cross-product coefficient, k is the number of factors, and X_i and X_j are the coded dimensionless values of the investigated variables. The software packages Statistica 8 (StatSoft Inc., Tulsa, OK, USA) and Design Expert 7 (Stat-Ease, Minneapolis, MN, USA) were used for the experimental design, the analysis of variance (ANOVA), and the graphical analysis of the data. The statistical significance of the quadratic model was assessed using an F-test, and the quality of fit was evaluated by the coefficient of determination R^2. The significances of the regression coefficients were tested by a t-test, and the P-values were used as a tool to check the significance of each coefficient.

The optimal concentrations of the culture medium components needed for simultaneous maximization of all responses (mycelial growth, EPS and IPS production) were established using the overall desirability function, which is the geometric mean of the individual desirabilities, and is described by the following equation:

$$D = (d_1 \times d_2 \times \cdots \times d_n)^{\frac{1}{n}} = \left(\prod_{i=1}^{n} d_i\right)^{\frac{1}{n}} \quad (3)$$

where d is the individual desirability and n is the number of responses. The individual desirability was calculated as follows: $d = 0$ if $Y < Y_{\min}$, $d = (Y - Y_{\min})/(Y_{\max} - Y_{\min})$ if $Y_{\min} < Y < Y_{\max}$, and $d = 1$ if $Y > Y_{\max}$, where Y is the predicted response, $Y_{\min}$ is the most undesirable response, and $Y_{\max}$ is the most desirable response.

Table 1 Actual and coded values of the independent variables tested in CCRD

Variables (g l^{-1})	Symbol		Coded levels				
	Uncoded	Coded	−2	−1	0	1	2
Malt extract	x_1	X_1	30.0	40.0	50.0	60.0	70.0
CSP	x_2	X_2	18.65	27.98	37.30	46.62	55.95
$CaCl_2$	x_3	X_3	0.50	0.75	1.00	1.25	1.50
$MgSO_4 \cdot 7H_2O$	x_4	X_4	0.50	0.75	1.00	1.25	1.50

Cultivation in the bioreactor

To examine the kinetics of mycelial growth and polysaccharide production, the cultivation was performed in a bioreactor on a nutrient medium selected on the basis of results obtained during the CCRD experiment, which showed which of the media were the most favorable for obtaining optimal biomass growth and optimal yield of polysaccharides.

Cultivation was performed in a fully equipped and computer-controlled 5-l bioreactor (Minifors, Infors HT, Reigate, UK) of working volume 3 l, which was equipped with a pH sensor and an oxygen electrode. Cultivations were carried out for 21 days at 25°C with aeration rate of 1 vvm and an agitation speed of 150 rpm. Cultivation parameters such as the temperature, pH, oxygen concentration and agitation speed were monitored online. The samples (150 ml) collected from the bioreactor at regular time intervals were analyzed for cell dry weight, the production of IPS and EPS, and residual sugar concentration. The obtained EPS were additionally analyzed using HPSEC with triple detection to determine the changes that had occurred in their elution profiles during the time course of cultivation.

Mycelial dry weight

After the cultivation had finished, the biomass was separated from the culture medium by filtering it through Whatman no. 2 filter paper under reduced pressure. The mycelium was washed thoroughly with 250 ml of distilled water, dried at 40°C, and then powdered, and the dry weight was measured at 105°C with a moisture analyzer.

Determination of polysaccharides

Four volumes of methanol containing an admixture of 0.1% acetic acid was slowly added to the remaining filtrate, and this mixture was stirred thoroughly and left overnight at 4°C. The precipitated crude EPS were centrifugated at 5,000×g for 15 min, redissolved in distilled water, recentrifugated to remove undissolved residue, and the EPS were

reprecipitated from the supernatant as above. The EPS were then washed with methanol, acetone and diethyl ether and dried over P_2O_5 until a fluffy powder was obtained. The polysaccharide concentration was measured by the phenol–sulfuric acid method with glucose used as a standard [3].

The content of IPS was determined as follows. Powdered mycelium was defatted with ethyl alcohol for 6 h in a Soxhlet apparatus to remove low-molecular weight substances. Next, 100 mg of defatted mycelium was extracted with 5 ml of water at 100°C for 3 h and centrifugated (5,000×*g*, 15 min). The supernatant (250 µl) was applied to a precalibrated column (1.2 × 6.5 cm, bed volume 4 ml) filled with Sephadex G-25 (exclusion limit of 5 kDa, Pharmacia, Uppsala, Sweden) and eluted with water. Eluate (2 ml) containing the IPS with molecular weights of over 5 kDa was collected, and the polysaccharide concentration was determined by the phenol–sulfuric acid method [3].

In the samples collected during the cultivation of *H. erinaceum* in a bioreactor, the residual sugar concentration was assayed by a 3,5-dinitrosalicylic (DNS) acid method [15]. The pH value of the culture broth was determined with a digital pH meter.

Molecular weight determination

The molecular weights of the EPS obtained from the bioreactor were determined by HPSEC with triple detection. Three TSK-GEL columns joined together were employed (G5000PWXL + G3000PWXL + G25000PWXL; 7.8 × 300 mm; Toshoh) with a Knauer K-501 pump, an RI (LDC) detector and a T60A double detector (viscometric and LS detectors, Viscotek, Houston, TX, USA). The analysis was performed at 26°C. An aqueous solution of NaN_3 (0.1%) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. Samples at a concentration of 5 mg ml^{-1} were filtered through 0.2 µm pore size membrane filters. The injection volumes of the sample solutions were 100 µl. The molecular weights of the EPS were determined by applying OmniSEC software (Viscotek) to the data.

Estimation procedures in cultivation kinetics

The specific growth rate μ (day^{-1}) was calculated by the following equation: $\mu = (1/X)\,(dX/dt)$, where X is the cell concentration (g l^{-1}) at time t (day). The specific consumption rate of the substrate (g g^{-1} day^{-1}) was calculated according to the equation: $Q_{S/X} = (dS/dt)\,(1/X)$, where S is the concentration of the carbon source (g l^{-1}) at time t (day). The specific production rate of EPS $P_{P/X}$ (g g^{-1} day^{-1}) was calculated according to the equation: $P_{P/X} = (dP/dt)\,(1/X)$, where P is the concentration of EPS (g l^{-1}) at time t (day). The yield of EPS on the substrate $Y_{P/S}$ (g g^{-1}) was calculated as follows: $Y_{P/S} = (dP/dt)/(dS/dt)$.

Statistical analysis

Each result is expressed as the mean of at least two replicates ± standard deviation (SD). The obtained data were subjected to a one-way analysis of variance (ANOVA) using Microsoft Excel Data Analysis ToolPack. Differences between means at the $P < 0.05$ level were considered significant.

Results and discussion

Effect of the carbon source

The carbon source in the culture medium plays a key role in mycelial growth and the production of metabolites, and is an important source of metabolic energy. According to general opinion, the mycelia of many species of fungi can use a wide range of carbon sources for their cell growth and the production of metabolites [10]. To establish the effect of carbon sources on the mycelial growth of *H. erinaceum* and polysaccharide production, 12 different carbon sources (50 g l^{-1}) were tested. The control medium contained no carbon sources.

The results showing the effects of various carbon sources on mycelial growth as well as EPS and IPS production are presented in Fig. 1a. Malt extract and fructose were found to have the best effects on mycelial growth. The good result obtained on malt extract is due to its high content of maltose, which was earlier found to stimulate biomass growth in various *Basidiomycota* species [22, 25]. A stimulatory effect of fructose on mycelial growth was previously noted in a study on *Tremella fuciformis* [2]. The lowest level of mycelial growth was observed in media containing sucrose, molasses, and in the control medium (which contained no carbon source). It is worth noting that the effect of sucrose on mycelial growth in various mushroom species is a controversial subject and findings seem to vary; according to some authors, sucrose has an inhibitory effect on biomass growth [22, 25], while others assert that it enhances mycelial growth [2].

The effect of the composition of the culture medium on IPS production in submerged cultures of higher mushrooms is little known. In our experiment, the highest IPS content was obtained from mycelium cultivated in the medium containing lactose and trehalose, whereas the smallest IPS content was found in mycelium grown in nutrient medium enriched with fructose and mannitol. The biosynthesis of IPS was also decreased in cultures with added sucrose and glycerol. It was obvious that ketosugars and sugar alcohols

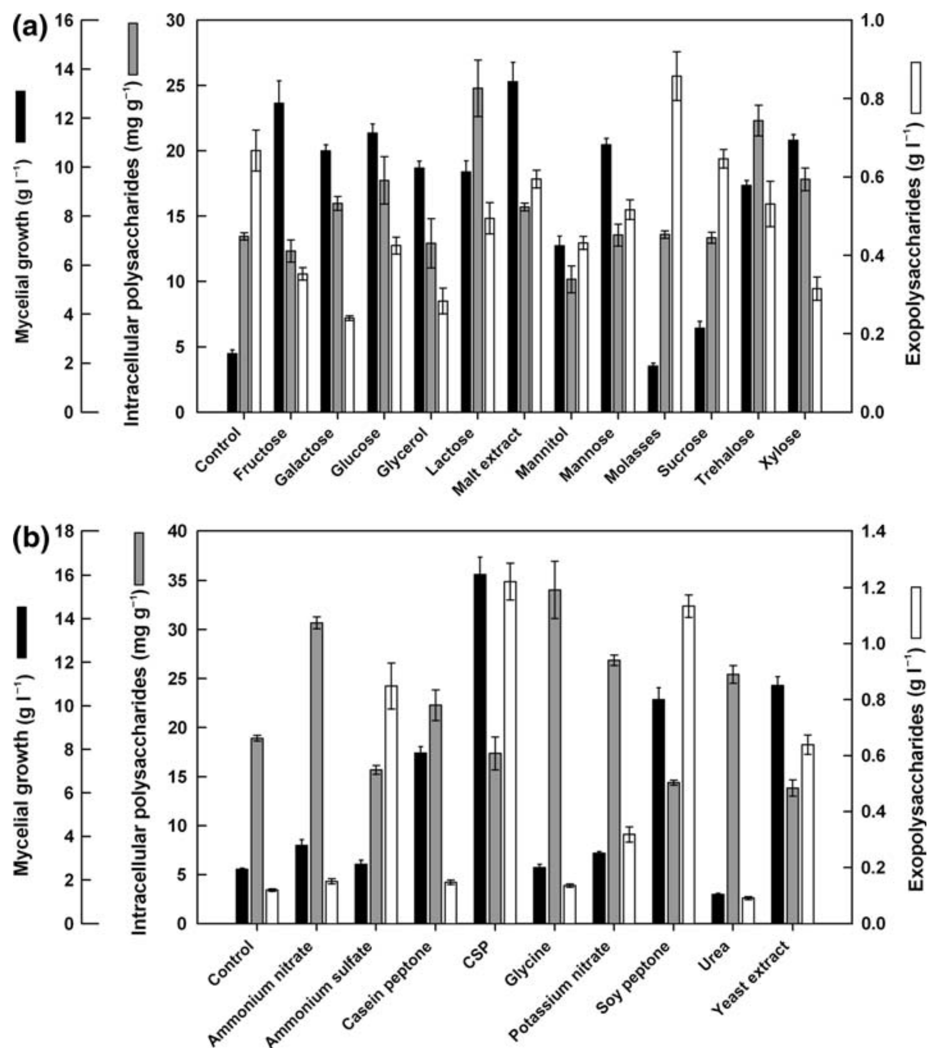

Fig. 1a–b Effect of carbon sources (**a**) and nitrogen sources (**b**) on mycelial growth and EPS and IPS production in shake-flask cultures of *H. erinaceum*. All experimental data are given as the mean ± SD of triplicate determinations. *Control* refers to the nutrient medium with no carbon or nitrogen source added

are utilized to a lesser degree for the synthesis of inner polysaccharides of *H. erinaceum* than other carbohydrates. Pokhrel and Ohga [22], in their experiments with *Lyophylium decastes*, found that the smallest IPS production was obtained when using fructose as the carbon source, which is similar to our findings. Contrary results were obtained by Shih et al. [25], who showed that fructose and maltose enhanced IPS biosynthesis by *Antrodia cinnamomea* mycelium, whereas the addition of sucrose clearly inhibited IPS production.

Out of the 12 tested carbon sources, molasses yielded the highest EPS production, whereas the lowest was obtained in the medium containing galactose. High EPS yields were also obtained in nutrient media with added sucrose or malt extract, and in the control medium that contained no carbon source. It should be noted that, of these, only malt extract did not also cause the simultaneous inhibition of mycelial growth. The high EPS yield obtained in the nutrient medium with sucrose is consistent with investigations on *A. cinnamomea* and *Ganoderma lucidum* [25, 27]. Those studies showed that sucrose is a good carbon source for EPS production, but that it inhibits the growth of mycelium. It may thus be concluded that various carbon sources have different catabolic repression effects on the secondary cell metabolism. This phenomenon was observed in submerged cultures of many mushroom species [1, 10, 25]. Moreover, the study of Huang et al. [8] showed that sucrose was one of the most preferable carbon sources for both EPS production and mycelial growth in the Chinese strain of *H. erinaceus* CZ-2. Thus, it seems that nutrition requirements can differ, even among various strains of the same mushroom species.

Effect of the nitrogen source

Nitrogen is a critical factor in the synthesis of some fungal enzymes involved in both primary and secondary metabolism. This element can be supplied to the culture medium in the form of ammonium or nitrate ions, or in organic form (such as amino acids or proteins). The omission of nitrogen

from the basic nutrient medium resulted in considerable inhibition of the growth and production of metabolites [10, 12].

The effect of the nitrogen source on biomass growth and polysaccharide biosynthesis was examined in a basic nutrient medium in which the tested nitrogen source (2.8 g l^{-1} calculated as nitrogen) was substituted for yeast extract and casein peptone.

Detailed results of the effects of various nitrogen sources on mycelial growth as well as EPS and IPS production are shown in Fig. 1b. Our investigations showed that the best nitrogen source was CSP, affording a mycelial yield of 16.0 ± 1.6 g l^{-1}. Considerably lower mycelial growth was observed in culture media containing some other complex nitrogen sources, such as yeast extract, soy peptone, and casein peptone. In comparison to organic nitrogen sources, the mycelial growth on media containing inorganic nitrogen sources was comparable to that obtained in the control medium without the addition of a nitrogen source. These results are consistent with those of Chen et al. [1] and Shih et al. [25], whose studies on the growth of *Fomes fomentarius* and *A. cinnamomea*, respectively, revealed that certain essential amino acids could barely be synthesized from inorganic nitrogen sources, and hence that the synthesis of proteins and mycelial growth were inhibited.

Of all the tested nitrogen sources, the highest yield of IPS was obtained with glycine, whereas the lowest was obtained with yeast extract. To our great surprise, as observed for the carbon sources, the best IPS yields were obtained in cultivations where mycelial growth was rather poor. Our results disagree with those obtained by Pokhrel and Ohga [22], who suggest that in the cultivation of *Lyophyllum decastes*, yeast extract is the best nitrogen source for IPS production. On the other hand, the results obtained in investigations on polysaccharide biosynthesis in *A. cinnamona* culture were similar to our results [25].

The highest EPS yield was achieved in culture media containing CSP and soy peptone as the nitrogen sources. This is consistent with the results obtained in the investigations on *A. cinnamomea* [25]. It is generally agreed that complex nitrogen sources are favorable for mycelial biomass and EPS production in submerged cultures of *Basidiomycota* [1, 10, 13, 26]. It can also be supposed that wood-inhabiting fungi prefer similar nitrogen sources for the synthesis of EPS under liquid culture conditions.

Effect of initial pH

The pH value of the culture medium may affect the functions of the cell membrane, the cell morphology and the cell structure, the uptakes of various nutritional sources, and the biosynthesis of metabolites [10]. In our experiment, we examined the effect of the initial pH of the culture medium on mycelial growth and polysaccharide production. As shown in Table 2, maximal mycelial growth was observed at pH 4.5, whereas the lowest mycelial yield was found at pH 7. The above results confirm a well-known hypothesis that higher fungi prefer more acidic conditions in submerged cultures [10, 26]. In our investigations, the optimal EPS production was also observed at pH 4.5. It can thus be supposed that in the submerged culture of *H. erinaceum* there is a positive relationship between the EPS production and biomass growth, depending on the initial pH value of the culture medium. For the other pH values tested, the EPS production was a little lower, while no statistically significant differences were observed between those values. As different authors have reported different optimal pH values for EPS production, it seems that changes in this environmental factor lead to certain changes in the EPS yield that vary depending on the mushroom species [2, 10, 22]. Furthermore, a study of the submerged culture of *H. erinaceus* CZ-2 revealed that a pH of between 5 and 6 is optimal for both mycelial growth and exopolymer production, which indicates that there are differences in the environmental requirements of various strains of this mushroom [8].

Table 2 Effects of initial pH, mineral elements and vitamins on mycelial growth and EPS and IPS production in shake-flask cultures of *H. erinaceum*

	Mycelial growth (g l^{-1})	EPS (g l^{-1})	IPS (mg g^{-1})
pH			
4.0	10.05 ± 0.53	0.58 ± 0.11	10.4 ± 1.4
4.5	**11.36 ± 0.41**	**0.92 ± 0.06**	**14.2 ± 1.6**
5.0	10.78 ± 0.44	0.560 ± 0.027	12.5 ± 1.2
5.5	10.56 ± 0.34	0.55 ± 0.06	11.2 ± 2.5
6.0	10.6 ± 0.8	0.520 ± 0.052	11.4 ± 1.2
7.0	8.2 ± 0.9	0.63 ± 0.06	9.6 ± 1.5
Minerals			
$MgSO_4 \cdot 7H_2O$	12.1 ± 0.6*	0.46 ± 0.06*	**18.4 ± 4.2**
$ZnSO_4 \cdot 7H_2O$	11.1 ± 0.9*	0.427 ± 0.027*	17.2 ± 0.8
$CaCl_2$	**13 ± 1***	0.404 ± 0.018	17.0 ± 1.1
$CuSO_4 \cdot 5H_2O$	9.14 ± 0.71	**0.55 ± 0.05***	16.7 ± 1.7
Control	7.6 ± 0.9	0.327 ± 0.023	17.7 ± 1.5
Vitamins			
Thiamine	**11.04 ± 0.33***	0.46 ± 0.06*	16.1 ± 1.3
Pyridoxine	10.60 ± 0.81*	0.41 ± 0.06	**16.3 ± 1.1**
Biotin	10.2 ± 1.5*	**0.551 ± 0.034***	8.57 ± 0.73*
Control	7.8 ± 0.8	0.33 ± 0.05	16 ± 2

All experimental data shown here are the mean ± SD of triplicate determinations

* $P < 0.05$ versus control. "Control" refers to the nutrient medium with no minerals or vitamins added

The investigations of the effect of the initial pH on the biosynthesis of the IPS revealed that, just as for the EPS, an initial pH of 4.5 was the most favorable and afforded the best yield of this polysaccharide. This shows that there is a clear positive relationship between mycelial growth and IPS biosynthesis at the same pH value (Table 2).

Effects of mineral elements and vitamins

Magnesium, calcium, zinc, and copper ions play a key role in the cells of mushrooms, as they are cofactors of enzymatic reactions, activate many enzymes, and induce the transcription of many enzymatic proteins [10]. In our investigations on the mycelial growth of *H. erinaceum* and the biosynthesis of polysaccharides, we examined the effects of various mineral salts in the culture medium, such as $CaCl_2$, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CuSO_4 \cdot 5H_2O$ at concentrations of 1.0, 1.0, 0.05, and 0.005 g l^{-1}, respectively. The minerals selected for investigation were used at concentrations required for the growth and development of microorganisms, because higher concentrations of mineral elements in the culture medium are toxic for microorganisms (for instance, copper at high concentrations behaves as an inhibitor and is a fungicide) [24]. As shown in Table 2, all of the tested minerals exhibited a favorable effect on mycelial growth. Most favorable for cell growth were calcium and magnesium. A positive effect of Ca^{2+} has been found in the investigations of many authors. This is because Ca^{2+} can change the cell membrane permeability by controlling the internal calcium gradient and the activities of some fungal enzymes involved in cell wall expansion [1, 9, 10]. The addition of Zn^{2+} and Cu^{2+} ions also improved mycelial growth. The clearly positive effect of all of the mineral elements tested was expected, considering their physiological functions in fungal growth.

None of the examined minerals notably affected the IPS content, but all of them except Ca^{2+} clearly enhanced the secondary metabolism connected with EPS biosynthesis. The best stimulatory effect on EPS production was obtained with Cu^{2+} ions. The rather low EPS yield obtained in the culture medium containing calcium salts, which was comparable to that obtained in the control (with no minerals added), may be due to the effect of Ca^{2+} accumulation, which could inhibit biopolymer synthesis, probably by affecting some enzymes such as β-glucan synthase [1, 10]. The enhanced EPS biosynthesis observed for Mg^{2+} and Zn^{2+} has been found by many authors for submerged cultures of different *Basidiomycota* species [1, 7, 10].

Vitamins are responsible for a growth response at very small concentrations; they usually perform catalytic functions in the cell in the form of coenzymes or coenzyme components [1, 13]. In our investigation, the following vitamins were examined for their effect on mycelial growth and polysaccharide biosynthesis: biotin (vitamin H or B_7), thiamine (vitamin B_1), and pyridoxine (vitamin B_6), each at a concentration of 1.0 mg l^{-1}.

As shown in Table 2, all of the vitamins examined were found to markedly stimulate mycelial growth to a similar degree. Neither thiamine nor pyridoxine exhibited any statistically significant effect on IPS biosynthesis; however, the addition of biotin resulted in an almost twofold inhibition of IPS biosynthesis. EPS production was found to increase considerably as a result of the addition of thiamine and biotin to the culture medium, whereas the application of pyridoxine showed no notable differences from the control. Similar results were obtained for a submerged culture of the tree mushroom *F. fomentarius* [1].

Optimizing the culture conditions with CCRD and RSM

The experimental projects performed by the RSM method are based on mathematical techniques that enable us to investigate the relationships between variables of medium components. The full-factorial 2^4 CCRD was used for the optimization of the components of the culture medium in submerged cultivation of *H. erinaceum*, which means that a total number of 54 experiments with four variables (i.e., components of the culture medium) and five levels (five different concentrations) were performed.

Based on the results obtained from the one-factor-at-a-time experiments for optimizing the culture medium, we selected four factors that exhibited the greatest effects on mycelial growth and polysaccharide production. These were: carbon source, nitrogen source, and two mineral elements. Among all the tested carbon sources, malt extract was chosen because of its most favorable effect on biomass growth in comparison with other carbon sources, along with its good EPS and IPS yields. Among the nitrogen sources, we chose CSP since this complex nitrogen source was found to clearly enhance both the growth of biomass and EPS production. Another argument in favor of CSP was the fact that it is a bothersome waste product that could be recycled in this way. The choice of Ca^{2+} and Mg^{2+} ions for the experiment was rather obvious in view of their stimulatory effects on both mycelial growth and EPS production. The other culture conditions were as follows: temperature 25°C, agitation speed 110 rpm, time course of cultivation 21 days. The pH of the culture medium was 4.5, which was selected as the optimal pH on the basis of the one-factor-at-a-time experiment. The concentration of KH_2PO_4 was fixed at 1 g l^{-1}.

Table 3 shows the design matrix for the CCRD experiment, together with the experimental results and the

Table 3 Central composite rotatable design (CCRD) matrix of independent variables and the corresponding experimental and predicted values for mycelial growth and EPS and IPS production by submerged culture of *H. erinaceum*

Run	Factor				Mycelial growth (g l^{-1})		EPS (g l^{-1})		IPS (mg g^{-1})	
	X_1	X_2	X_3	X_4	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	−1	−1	−1	−1	10.74	10.53	2.15	2.19	27.1	26.7
2	−1	−1	−1	1	10.61	10.69	2.21	2.07	28.1	28.3
3	−1	−1	1	−1	11.04	11.02	2.18	2.07	23.1	24.0
4	−1	−1	1	1	11.42	11.32	1.91	1.80	28.6	28.7
5	−1	1	−1	−1	9.40	9.53	2.34	2.32	29.7	29.1
6	−1	1	−1	1	10.43	10.40	2.24	2.19	28.7	28.4
7	−1	1	1	−1	11.39	10.96	2.18	2.12	29.8	28.7
8	−1	1	1	1	11.63	11.98	1.91	1.85	30.6	31.0
9	1	−1	−1	−1	12.83	12.30	2.37	2.37	28.5	28.0
10	1	−1	−1	1	12.46	12.60	2.32	2.39	29.3	29.7
11	1	−1	1	−1	11.76	12.09	2.24	2.23	25.1	25.9
12	1	−1	1	1	12.83	12.54	2.17	2.11	30.8	30.6
13	1	1	−1	−1	12.44	12.56	2.58	2.68	30.1	29.7
14	1	1	−1	1	13.54	13.57	2.61	2.69	30.2	29.0
15	1	1	1	−1	13.36	13.30	2.41	2.46	30.4	29.9
16	1	1	1	1	14.30	14.46	2.27	2.34	32.1	32.2
17[a]	−2	0	0	0	8.80	8.58	1.97	2.11	28.2	27.8
18[a]	2	0	0	0	12.91	12.82	2.83	2.77	29.7	30.3
19[a]	0	−2	0	0	11.32	11.48	1.84	1.90	29.1	28.3
20[a]	0	2	0	0	12.44	12.39	2.27	2.25	32.2	32.4
21[a]	0	0	−2	0	10.87	11.10	2.51	2.50	26.6	27.1
22[a]	0	0	2	0	12.52	12.47	1.93	2.02	27.9	27.7
23[a]	0	0	0	−2	12.23	12.38	2.41	2.32	26.0	26.2
24[a]	0	0	0	2	14.01	13.70	2.06	2.07	30.6	30.2
25[b]	0	0	0	0	14.29	14.29	2.37	2.40	29.7	29.7
26[b]	0	0	0	0	13.90	14.29	2.36	2.40	29.4	29.7
27[b]	0	0	0	0	14.31	14.29	2.48	2.40	30.4	29.7
28	−1	−1	−1	−1	10.63	10.53	2.23	2.19	27.2	26.7
29	−1	−1	−1	1	10.71	10.69	2.19	2.07	27.6	28.3
30	−1	−1	1	−1	10.96	11.02	2.10	2.07	23.4	24.0
31	−1	−1	1	1	11.31	11.32	1.76	1.80	28.6	28.7
32	−1	1	−1	−1	8.94	9.53	2.29	2.32	28.6	29.1
33	−1	1	−1	1	10.97	10.40	2.13	2.19	28.9	28.4
34	−1	1	1	−1	10.97	10.96	2.16	2.12	28.8	28.7
35	−1	1	1	1	11.30	11.98	1.90	1.85	30.3	31.0
36	1	−1	−1	−1	12.92	12.30	2.37	2.37	28.4	28.0
37	1	−1	−1	1	12.10	12.60	2.34	2.39	29.1	29.7
38	1	−1	1	−1	11.62	12.09	2.24	2.23	26.0	25.9
39	1	−1	1	1	13.06	12.54	2.17	2.11	30.4	30.6
40	1	1	−1	−1	12.39	12.56	2.70	2.68	29.6	29.7
41	1	1	−1	1	13.76	13.57	2.72	2.69	29.3	29.0
42	1	1	1	−1	13.38	13.30	2.36	2.46	30.1	29.9
43	1	1	1	1	14.11	14.46	2.41	2.34	31.6	32.2
44[a]	−2	0	0	0	8.54	8.58	1.97	2.11	27.9	27.8
45[a]	2	0	0	0	12.66	12.82	2.90	2.77	30.3	30.3
46[a]	0	−2	0	0	11.19	11.48	1.80	1.90	28.9	28.3

Table 3 continued

Run	Factor				Mycelial growth (g l^{-1})		EPS (g l^{-1})		IPS (mg g^{-1})	
	X_1	X_2	X_3	X_4	Observed	Predicted	Observed	Predicted	Observed	Predicted
47[a]	0	2	0	0	12.90	12.39	2.31	2.25	31.2	32.4
48[a]	0	0	−2	0	11.02	11.10	2.56	2.50	26.4	27.1
49[a]	0	0	2	0	12.82	12.47	1.96	2.02	28.6	27.7
50[a]	0	0	0	−2	12.40	12.38	2.27	2.32	25.5	26.2
51[a]	0	0	0	2	13.62	13.70	1.95	2.07	30.7	30.2
52[b]	0	0	0	0	14.63	14.29	2.42	2.40	28.9	29.7
53[b]	0	0	0	0	14.57	14.29	2.39	2.40	29.3	29.7
54[b]	0	0	0	0	14.04	14.29	2.40	2.40	30.6	29.7

X_1, malt extract; X_2, corn steep powder (CSP); X_3, $CaCl_2$; X_4, $MgSO_4 \cdot 7H_2O$

[a] Axial point

[b] Central point

predicted responses for mycelial growth and EPS and IPS production. The experimental values obtained from the CCRD were regressed by a quadratic polynomial equation, where only the significant terms were considered ($P < 0.05$), giving three empirical correlations:

$$Y_1 = 14.29 + 1.06X_1 + 0.23X_2 + 0.34X_3 + 0.33X_4 - 0.90X_1^2 - 0.59X_2^2 - 0.63X_3^2 - 0.31X_4^2 + 0.32X_1X_2 - 0.18X_1X_3 + 0.24X_2X_3 + 0.18X_2X_4 \quad (4)$$

$$Y_2 = 2.40 + 0.17X_1 + 0.086X_2 - 0.12X_3 - 0.062X_4 - 0.081X_2^2 - 0.035X_3^2 - 0.052X_4^2 + 0.044X_1X_2 + 0.035X_1X_4 - 0.034X_3X_4 \quad (5)$$

$$Y_3 = 29.72 + 0.62X_1 + 1.00X_2 + 1.00X_4 - 0.58X_3^2 - 0.38X_4^2 + 0.56X_2X_3 - 0.60X_2X_4 + 0.76X_3X_4 \quad (6)$$

where Y_1 is the mycelial growth (g l^{-1}), Y_2 is the EPS production (g l^{-1}), Y_3 is the IPS production (mg g^{-1}), and X_1, X_2, X_3 and X_4 are coded values of the independent variables (malt extract, CSP, $CaCl_2$, and $MgSO_4 \cdot 7H_2O$, respectively).

Table 4 presents the parameter estimates for the predicted response along with the corresponding *P*- and *F*-values. The analysis of variance (ANOVA) for the CCRD experiments showed that the model exhibits an excellent correlation with the experimental data, with high *F*-values (71.28, 33.16, and 30.48 for mycelial growth and EPS and IPS production, respectively) and $P < 0.001$ for each model. When we consider the *P* values and parameter estimates, we can see that malt extract has the largest effect on mycelial growth, followed by $CaCl_2$, $MgSO_4 \cdot 7H_2O$, and CSP. The order of effects on EPS and IPS production was as follows: malt extract > $CaCl_2$ > CSP > $MgSO_4 \cdot 7H_2O$ and CSP > $MgSO_4 \cdot 7H_2O$ > malt extract. The effect of $CaCl_2$ on IPS biosynthesis was insignificant.

The goodness of fit for the models was expressed by the coefficient of determination R^2, and was found to be 0.9624, 0.9234, and 0.9156 for mycelial growth, EPS, and IPS production, respectively, suggesting that the predicted values exhibit a good correlation with experimental data and that the model is suitable and practicable.

Figures 2, 3, and 4 present the three-dimensional response surfaces and their respective contour plots, which are the graphical representations of Eqs. 4, 5, and 6, respectively. Each plot shows the effect of two independent variables varying within the experimental range of mycelial growth, EPS, or IPS production, while the other two variables were fixed at their respective center point levels. This kind of graphical visualization allows the relationships between the experimental levels of each factor and the response to be investigated, and the type of interactions between test variables to be determined, which is necessary to establish the optimal composition of the culture medium. In contrast to the circular shapes of the contour plots, the elliptical nature of the curves indicates significant mutual interactions between variables. As can be seen from the shape of contour lines shown in Figs. 2 and 3, there is a drastic interactive effect of malt extract and CSP on mycelial growth and EPS. The mycelial growth is also strongly influenced by an interaction between CSP and $CaCl_2$, while EPS production is influenced by malt extract and $MgSO_4 \cdot 7H_2O$. The most prominent effects on IPS biosynthesis are exerted by the interactions between CPS and $MgSO_4 \cdot 7H_2O$ and between CPS and $CaCl_2$ (Fig. 4).

Taking into account the data from the 3D and 2D response surface plots and the equations obtained from multiple regression analysis, we can deduce the optimum concentrations of the medium components. The model predicts that the maximum mycelial growth (14.84 g l^{-1}) is located at $X_1 = 0.62$, $X_2 = 0.41$, $X_3 = 0.27$, and $X_4 = 0.36$. By putting the respective values of X_i into

Table 4 Analysis of variance (ANOVA) for the experimental results of the CCRD

Factor	Mycelial growth			EPS			IPS		
	Parameter estimate	*F*-value	*P*-value*	Parameter estimate	*F*-value	*P*-value*	Parameter estimate	*F*-value	*P*-value*
Intercept	14.29	71.28	<0.0001	2.40	33.16	<0.0001	29.72	30.48	<0.0001
X_1	1.06	461.2	<0.0001	0.17	196.9	<0.0001	0.62	42.91	<0.0001
X_1^2	−0.90	293.6	<0.0001	0.009	0.5321	0.4701	−0.17	2.927	0.0951
X_2	0.23	21.45	<0.0001	0.086	53.00	<0.0001	1.00	113.8	<0.0001
X_2^2	−0.59	126.2	<0.0001	−0.081	42.02	<0.0001	0.16	2.581	0.1162
X_3	0.34	48.35	<0.0001	−0.12	103.3	<0.0001	0.13	2.006	0.1646
X_3^2	−0.63	143.4	<0.0001	−0.035	7.850	0.0079	−0.58	34.13	<0.0001
X_4	0.33	44.32	<0.0001	−0.062	27.46	<0.0001	1.00	111.9	<0.0001
X_4^2	−0.31	35.63	<0.0001	−0.052	17.18	0.0002	−0.38	14.26	0.0005
X_1X_2	0.32	27.22	<0.0001	0.044	9.352	0.0040	−0.19	2.644	0.1120
X_1X_3	−0.18	8.414	0.0061	−0.002	0.0167	0.8978	0.15	1.692	0.2009
X_1X_4	0.036	0.3536	0.5555	0.035	5.818	0.0207	0.03	0.0470	0.8295
X_2X_3	0.24	15.32	0.0004	−0.019	1.670	0.2039	0.56	23.80	<0.0001
X_2X_4	0.18	8.901	0.0049	−0.001	0.0019	0.9659	−0.60	27.08	<0.0001
X_3X_4	0.037	0.3786	0.5419	−0.034	5.612	0.0229	0.76	43.73	<0.0001

X_1, malt extract; X_2, corn steep powder (CSP); X_3, $CaCl_2$; X_4, $MgSO_4 \cdot 7H_2O$

* $P < 0.05$ are significant

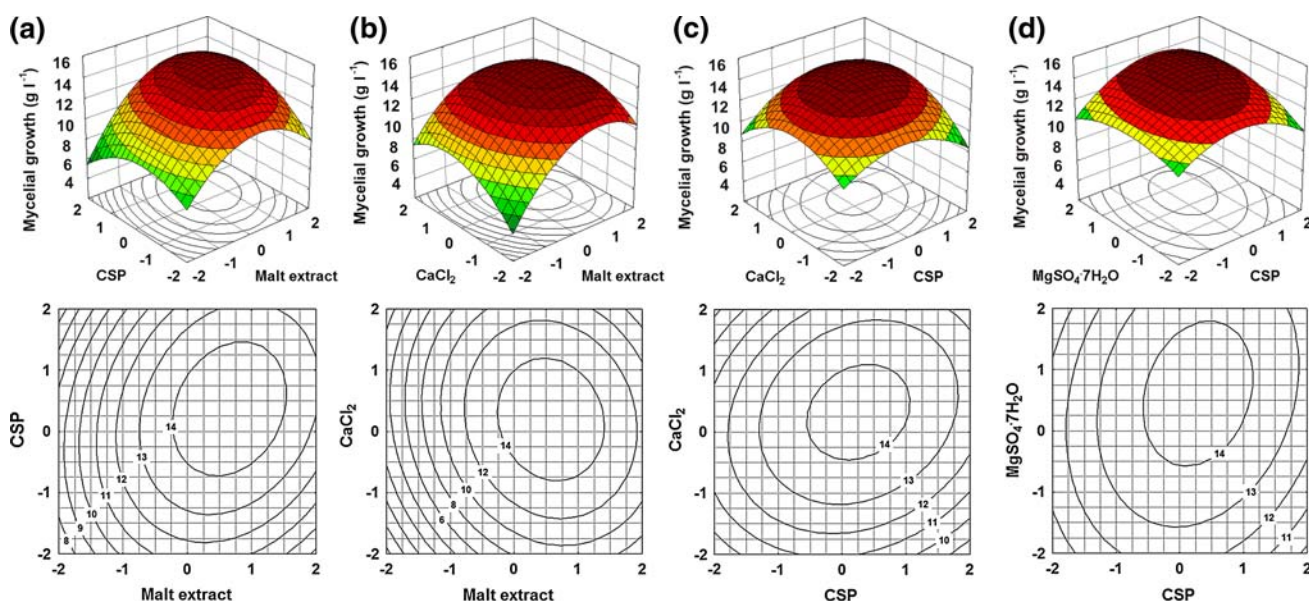

Fig. 2a–d Response surfaces and corresponding contour plots showing the combined effect of **a** malt extract and CSP; **b** malt extract and $CaCl_2$; **c** CSP and $CaCl_2$; **d** CSP and $MgSO_4 \cdot 7H_2O$ on the mycelial growth of *H. erinaceum*

Eq. 1, we have calculated the following actual values: malt extract 56.16 g l^{-1}, CSP 41.11 g l^{-1}, $CaCl_2$ 1.07 g l^{-1}, and $MgSO_4 \cdot 7H_2O$ 1.09 g l^{-1}. The predicted EPS production (3.00 g l^{-1}) reached its maximum at the coded values $X_1 = 1.99$, $X_2 = 1.53$, $X_3 = -1.95$, and $X_4 = -0.18$, corresponding to actual concentrations of malt extract of 69.85 g l^{-1}, CSP 51.54 g l^{-1}, $CaCl_2$ 0.51 g l^{-1}, and $MgSO_4 \cdot 7H_2O$ 0.96 g l^{-1}, while the IPS production reaches its maximum theoretical value (33.3 mg g^{-1}) in coded values at $X_1 = 1.26$, $X_2 = 1.64$, $X_3 = 1.46$, and $X_4 = 1.13$ (malt extract 62.59 g l^{-1}, CSP 52.57 g l^{-1}, $CaCl_2$ 1.37 g l^{-1}, and $MgSO_4 \cdot 7H_2O$ 1.28 g l^{-1}).

The predicted optimum levels of nutrient medium components for mycelial growth are rather different from

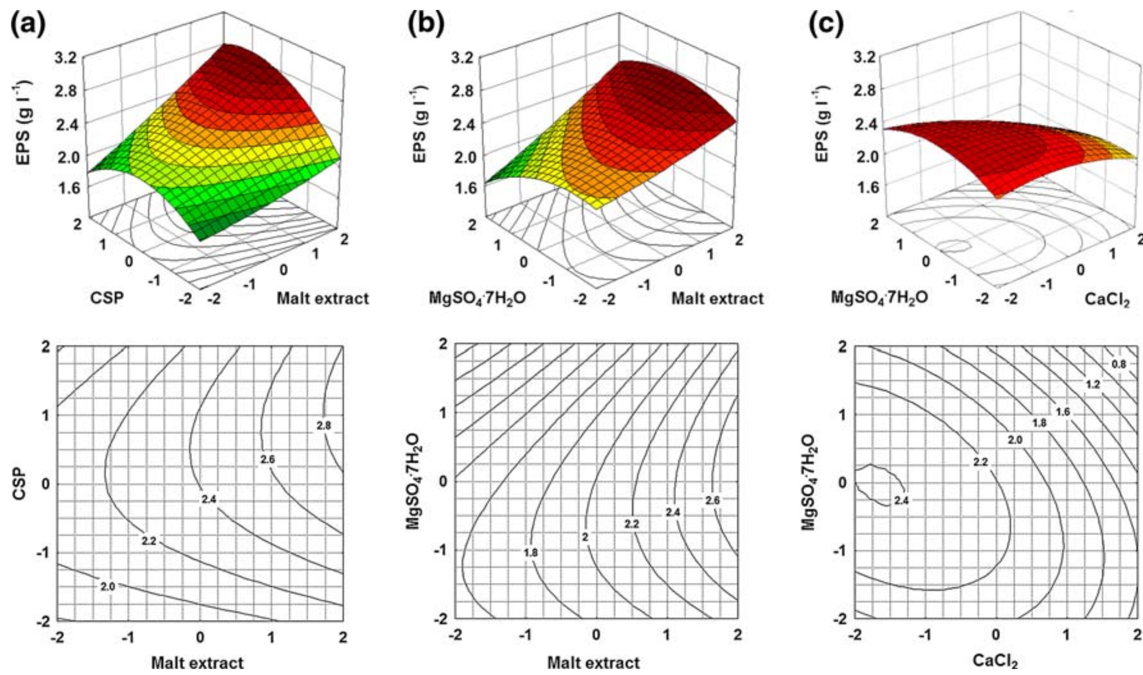

Fig. 3a–c Response surfaces and corresponding contour plots showing the combined effect of **a** malt extract and CSP; **b** malt extract and $MgSO_4{\cdot}7H_2O$; **c** $CaCl_2$ and $MgSO_4{\cdot}7H_2O$ on *H. erinaceum* EPS production

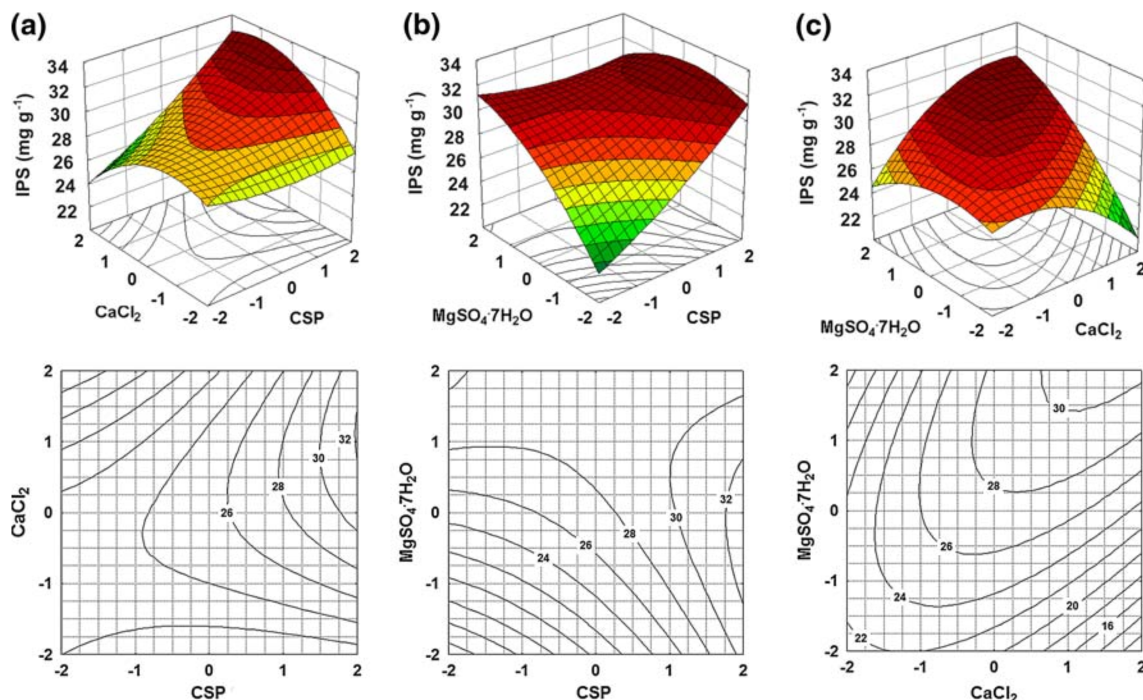

Fig. 4a–c Response surfaces and corresponding contour plots showing the combined effect of **a** CSP and $CaCl_2$; **b** CSP and $MgSO_4{\cdot}7H_2O$; **c** $CaCl_2$ and $MgSO_4{\cdot}7H_2O$ on *H. erinaceum* IPS biosynthesis

those needed for EPS or IPS production. A possible explanation for this is that IPS and EPS are products of different metabolic pathways. Intracellular polysaccharides are the major component of the cell walls of fungi, and like proteins are the main product of primary metabolism, whereas EPS are a group of mushroom polymers that are the products of secondary metabolic pathways [7]. The synthesis of those individual polysaccharide fractions may

be caused by different concentrations of culture medium components, resulting in different effects of catabolic repression [31].

Although mycelial growth requires moderate concentrations of nutrients, maximal EPS production can be achieved in the presence of a relatively high concentration of malt extract and a low concentration of $CaCl_2$, while high concentrations of both CSP and $CaCl_2$ should be used for optimal IPS biosynthesis. For instance, in the nutrient medium needed for maximal EPS production (3.00 g l^{-1}), the predicted mycelial growth and IPS biosynthesis are 9.59 g l^{-1} and 28.8 mg g^{-1}, respectively, which is not satisfactory. Thus, numerical optimization of the overall desirability function was performed to determine the best possible goals for each response simultaneously. The predicted optimal values for the variables are as follows: $X_1 = 1.57$, $X_2 = 1.08$, $X_3 = 0.13$, and $X_4 = 0.49$ (malt extract 65.73 g l^{-1}, CSP 47.34 g l^{-1}, $CaCl_2$ 1.03 g l^{-1}, and $MgSO_4 \cdot 7H_2O$ 1.12 g l^{-1}, respectively), whereas the predicted responses are: mycelial growth 14.08 g l^{-1}, EPS and IPS production 2.72 g l^{-1} and 31.5 mg g^{-1}, respectively. To test the validity of the optimal conditions predicted with the Design Expert software, four repeated experiments were conducted. The average mycelial growth (14.24 ± 0.45 g l^{-1}) and EPS and IPS production (2.64 ± 0.52 g l^{-1} and 32.0 ± 1.1 mg g^{-1}, respectively) were very close to the predicted values, which indicates that the CCRD model developed in this study is accurate and applicable for predicting the mycelial growth and polysaccharide production associated with the submerged cultivation of *H. erinaceum*.

Cultivation in a bioreactor

To investigate the kinetics of EPS production during cultivation in a bioreactor, the cultivation of *H. erinaceum* was performed on an optimized medium of the following composition: malt extract 65.73 g l^{-1}, CSP 47.34 g l^{-1}, $CaCl_2$ 1.03 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 1.12 g l^{-1}. This medium composition was chosen on the basis of the results obtained from the CCRD experiment in view of simultaneously high yields of biomass, EPS, and IPS. The concentration of KH_2PO_4 was 1 g l^{-1}, and the pH was fixed at 4.5. Figure 5 presents the changes in mycelial growth and the time courses of EPS and IPS production, while also taking into account the changes in pH and residual sugar concentration. The maximal biomass growth was equal to 15.3 ± 2.3 g l^{-1} on day 8 of cultivation with simultaneous depletion of the carbon source in the culture medium, which attained ca. 3.5% of its initial value on day 14. During the first days of cultivation, a major increase in the EPS yield was observed, which reached 2.75 ± 0.27 g l^{-1} after 8 days of cultivation and then gradually declined together with the biomass yield. The phenomenon of decreasing EPS production during the course of the fermentation has been observed by many authors in cultivations of higher fungi such as *F. fomentarius* [1] or *Cordyceps sinensis* [6]. It may be due to the secretion of polysaccharide-degrading enzymes (1,3-glucanase) [25]. The biosynthesis of IPS reached a maximum on day 2 of cultivation, when it was equal to 29.1 ± 3.5 mg g^{-1}, and then decreased rapidly to attain a constant value of about 13 mg g^{-1} after day 8 of cultivation. This result can be explained by a rapid transformation of the carbon source from the culture medium into IPS, which became an energy reserve that was gradually used up, accompanied by the growth of biomass and the depletion of the carbohydrates in the culture medium.

The resulting kinetic parameters were as follows: the specific growth rate $\mu = 0.58 \pm 0.31$ day^{-1}, the specific production rate of EPS $P_{P/X} = 0.92 \pm 0.06$ g g^{-1} day^{-1}, the specific consumption rate of substrate $Q_{S/X} = 0.117 \pm 0.022$ g g^{-1} day^{-1}, and the yield of EPS on substrate $Y_{P/S} = 8.08 \pm 1.85$ g g^{-1}.

During the cultivation in the bioreactor, the pH was found to increase from its initial value of 4.51 ± 0.18 to

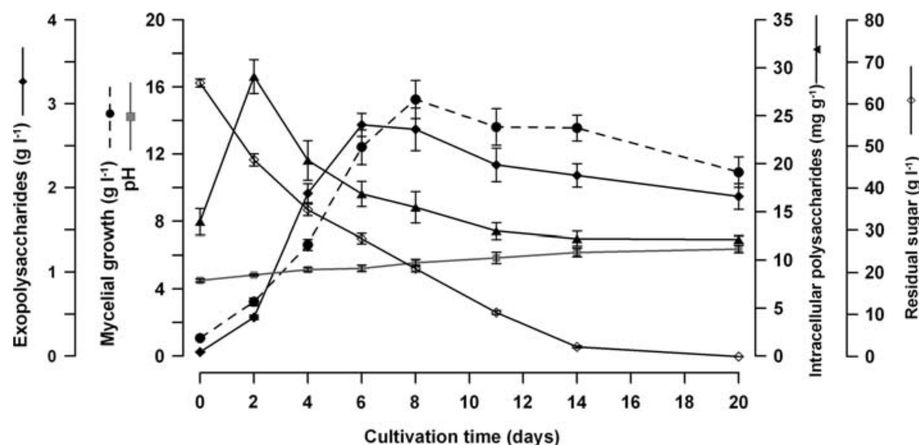

Fig. 5 Time courses of mycelial growth and EPS and IPS production of *H. erinaceum* in the optimized medium in a 5-l bioreactor. All experimental data correspond to the mean ± SD of triplicate determinations

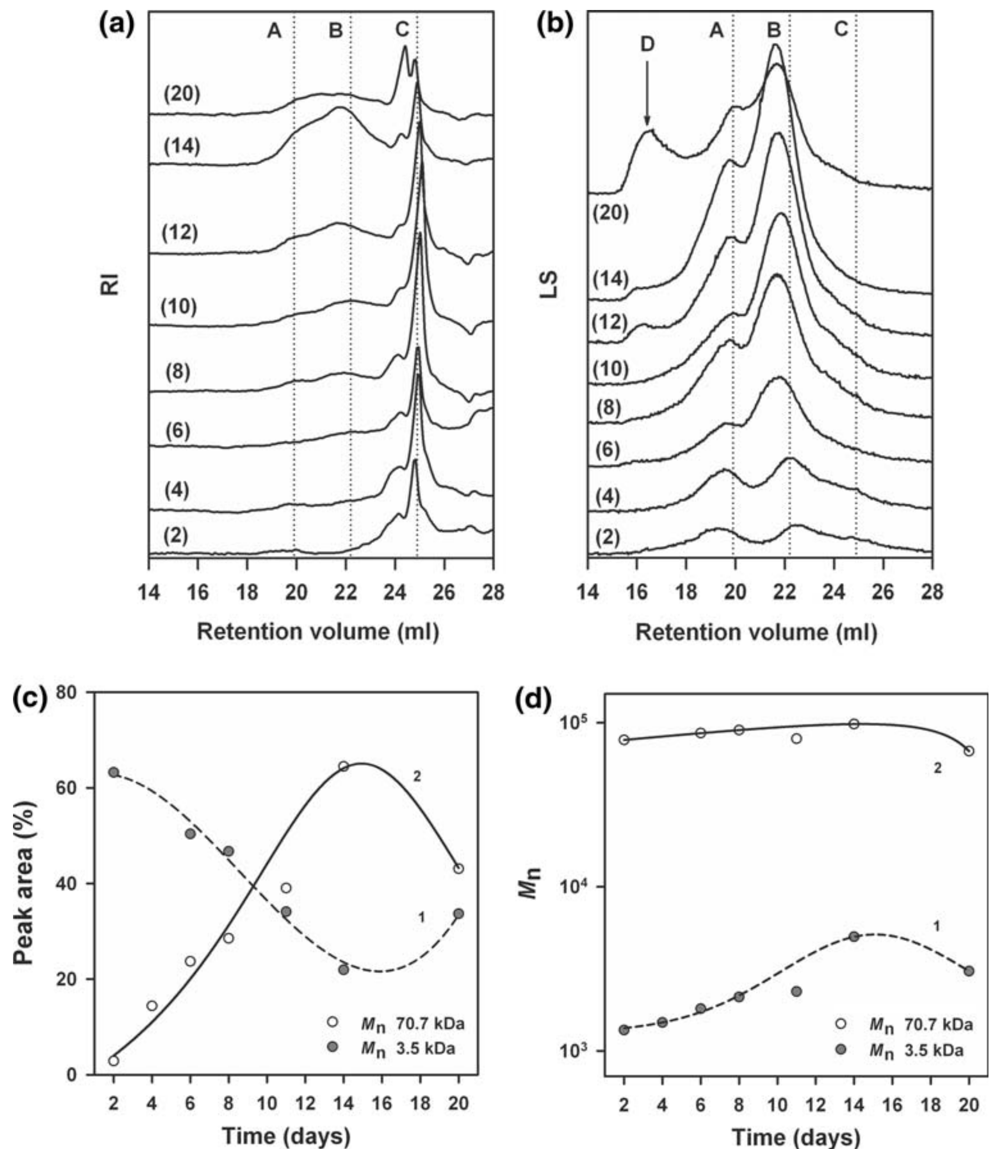

Fig. 6 **a** RI and **b** LS HPSEC chromatograms of EPS obtained at different time intervals from the submerged cultivation of *H. erinaceum*. *Dotted lines* denote the retention volumes corresponding to the respective molar masses: *A*: $M_n \approx 105$; *B*: $M_n \approx 70.7$; *C*: $M_n \approx 3.5$ kDa. *Numbers in parentheses* refer to days of cultivation. **c** The effect of cultivation time on the content of EPS with high molecular weight ($M_n \geq 70.7$ kDa) and low molecular weight ($M_n \geq 3.5$ kDa). **d** The effect of cultivation time on molecular weight changes of EPS during the submerged cultivation of *H. erinaceum*. *1*: $M_n \geq 3.5$ kDa; *2*: $M_n \geq 70.7$ kDa

6.38 ± 0.45 after 20 days of cultivation. During the course of cultivation in submerged cultures of fungi, a drop in pH is usually observed due to the secretion of organic acids into the culture medium [26]. However, there are a few reports of a gradual pH increase during mycelial cultivation of higher mushrooms in a bioreactor [9, 10].

Molecular weight determination of EPS during cultivation in a bioreactor

It is generally known that polysaccharides with high molecular weights always exhibit stronger anticancer and immunostimulatory activities than those with low molecular weights [19, 30]. To determine the changes in the proportions of polysaccharides with different molecular weights, the samples of EPS obtained during the 20-day cultivation in a bioreactor were analyzed by HPSEC with triple detection. Figure 6a and b present chromatograms for the EPS during submerged cultivation of *H. erinaceum* in a bioreactor. Signals A, B, and C derived from the RI and LS detectors correspond to $M_n \geq 105$, 70.7, and 3.5 kDa, respectively. As is shown in Fig. 6c, the amount of polysaccharide B with molecular weight $M_n \approx 70.7$ kDa increased rapidly during cultivation and reached a maximum on day 14. After that it gradually declined together with the total EPS content in the culture medium (from 2.29 ± 0.27 g l^{-1} on the 14th day of cultivation to 1.98 ± 0.31 g l^{-1} on day 20 of cultivation). This phenomenon is connected with the depletion of carbon source in the culture medium and the use of EPS as the energy source stored in the culture broth. The molecular weight of this polysaccharide remained at a constant level throughout the cultivation (Fig. 6d). The elution profile of EPS collected on the 20th day of cultivation shows that the

A and B signals, corresponding to polysaccharides of $M_n \geq 105$ kDa and $M_n \geq 70.7$ kDa, have lower intensities (Fig. 6a, b). At the same time, the relative intensity of signal C from low molecular weight polysaccharide increased. As shown in Fig. 6d, the molecular weight of polysaccharide C ($M_n \geq 3.5$ kDa) increased until the 14th day and then decreased at the end of cultivation. It could be supposed that the low molecular weight polysaccharide C is an intermediate product that takes part in the syntheses of both polysaccharides A and B and is formed as a result of their degradation. On the 20th day of cultivation, signal D appears in the LS traces, but is not registered by the RI detector, which indicates the existence of a new nonpolysaccharidic colloidal fraction of very high molecular weight. The presence of a small quantity of product D was observed on days 12 and 14. The above phenomenon can be explained by a gradual cell lysis of mycelium in the late stationary phase, an increase in the cell membrane permeability, and the release from cytosol to the culture medium of considerable amounts of high molecular weight protein, which can be both enzymatic and structural in nature. Our results suggest that in order to obtain polysaccharides of high molecular weight it is more favorable to continue the cultivation process until the depletion of the carbon source in the culture medium (i.e., for about 14 days). Although the maximal EPS yield can be obtained over a shorter time (after 8 days), it contains higher amounts of polysaccharides of lower molecular weight.

Conclusions

In our study, we found that by using a CCRD and a response surface methodology we can optimize the culture medium composition so as to obtain simultaneously high yields of *Hericium erinaceum* mycelial biomass, exopolysaccharides, and intracellular polysaccharides. When examining the kinetics of EPS biosynthesis in a bioreactor, we discovered that the process should be continued until nutritional sources in the culture medium are depleted in order to obtain polysaccharides of high molecular weight. These results can be widely applied to optimize the submerged cultures of other mushrooms, especially those producing bioactive polysaccharides and possessing industrial potential.

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