Optimization of Cultivating Conditions for Triterpenoids Production from Antrodia cinnmomea

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Indian Journal of Microbiology
The Official Publication of the Association of Microbiologists of India

ISSN 0046-8991

Indian J Microbiol
DOI 10.1007/s12088-012-0290-y

Springer

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DOI 10.1007/s12088-012-0290-y

ORIGINAL ARTICLE

Optimization of Cultivating Conditions for Triterpenoids Production from *Antrodia cinnmomea*

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Received: 20 December 2011 / Accepted: 3 July 2012

Abstract The submerged cultivating conditions for triterpenoids production from *Antrodia cinnamomea* were optimized using uniform design method and the one-factor-at-a-time method was adopted to investigate the effect of plants oils and glucose supply on triterpenoids production and mycelia growth. Corn starch and culturing time were identified as more significant variables for triterpenoids production. The optimal conditions for triterpenoids production was 20.0 g/L corn starch, 20.0 g/L wheat bran, 1.85 g/L MgSO4, initial pH 3 and 16 days of cultivation. In addition, investigation of plant oils and glucose supply showed that 0.3 % (v/v) olive oil supply at the beginning of fermentation stimulated mycelia growth and significantly increased triterpenoids production; 0.2 % (w/v) glucose supplement at 10th day enhanced production of triterpenoids with slight effect on biomass, which is reported for the first time. The triterpenoids production experimentally obtained under the optimal conditions was 7.23 % (w/w). The uniform design method may be used to optimize many environmental and genetic factors such as temperature and agitation that can also affect the triterpenoids production from *A. cinnamomea*.

Keywords *Antrodia cinnamomea* · Total triterpenoids · Optimization · Uniform design

Y.-C. He · K.-Z. He (✉) · Q. Pu
Key Laboratory of Environmental and Applied Microbiology, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China
e-mail: hekz@cib.ac.cn

Y.-C. He · J. Li · Z.-J. Zhao
Shenzhen CASW Technology CO., Ltd, Shenzhen 518000, China

Introduction

Antrodia cinnamomea Chang and Chou [1], sp. Nov. (zhan-ku family polyporaceae, Aphyllophorales) is a new and exclusive fungus parasitic on the inner wall of the endemic species *cinnamomum kanehira* Hay. It is well known as "Niu-chang-chih" and widely used as a traditional medicine for the treatment of food and drug intoxications, diarrhea, abnormal pains, hypertension and liver cancer in Taiwan [2]. Chemical compounds found in *A. cinnamomea* including polysaccharide, benzenoids, succinic and maleic derivatives, ergosterol and triterpenoids [3]. Triterpenoids was considered as one of the most biologically active components in recently years. For example, Zhankuic acid A exhibited a cytotoxic activity against P-388 murine leukemia cells and Zhankuic acid B showed weak anticholinergic and antiserotonergic activities [4]. Gao reported that triterpene compound B isolated from *A. cinnamomea* can reduce blood glutamate pyruvate transaminase level in mice which have acute liver abnormality induced by tetrachloride methane [5]. Methyl antcinate K promoted mouse bone marrow derived dendritic cells maturation and activated it to induced Ag-specific T cell proliferation and facilitate Th2 differentiation [6]. Some new triterpenoids exhibited anti-inflammatory NO-production inhibition activity with IC50 values of less than 5 μM, and exerts anti-inflammatory effect via mimicking glucocorticoids [7, 8].

Antrodia cinnamomea grows very slowly in the wild environment and it usually takes several months to cultivate fruiting body with some difficulty to control the product quality during cultivation [9]. Submerged fermentation is a rapid and alternative method of optimal production with consistent quality and used to producing effective substance from cultured mycelia [10]. In the

Published online: 14 July 2012

Table 1 Application of U_{10} (10^5) for optimization of biomass (g/L), triterpenoids production (w/w, %)

No.	Factors					Response *Y*	
	X_1 (g/L)	X_2 (g/L)	X_2 (g/L)	X_4 (g/L)	X_5 (d)	Biomass (g/L)	Triterpenoids production (w/w, %)
1	1 (20.00)	2 (23.00)	3 (0.80)	5 (4.20)	7 (13)	5.89 ± 0.51	4.41 ± 0.36
2	2 (23.00)	4 (29.00)	6 (1.25)	10 (5.70)	3 (9)	6.81 ± 0.50	2.95 ± 0.84
3	3 (26.00)	6 (35.00)	9 (1.70)	4 (3.90)	10 (16)	7.09 ± 0.39	5.23 ± 0.33
4	4 (29.00)	8 (41.00)	1 (0.50)	9 (5.40)	6 (12)	8.58 ± 0.18	3.16 ± 0.40
5	5 (32.00)	10 (47.00)	4 (0.95)	3 (3.60)	2 (8)	10.46 ± 0.75	2.43 ± 0.83
6	6 (35.00)	1 (20.00)	7 (1.40)	6 (5.10)	9 (15)	9.16 ± 0.79	3.98 ± 0.74
7	7 (38.00)	3 (26.00)	10 (1.85)	8 (3.30)	5 (11)	10.82 ± 0.44	3.13 ± 0.46
8	8 (41.00)	5 (32.00)	2 (0.65)	7 (4.80)	1 (7)	10.63 ± 0.77	1.61 ± 0.65
9	9 (44.00)	7 (38.00)	5 (1.10)	1 (3.80)	8 (14)	13.84 ± 0.65	3.53 ± 0.90
10	10 (47.00)	9 (44.00)	8 (1.55)	6 (4.50)	4 (10)	12.64 ± 0.65	1.82 ± 0.19

Symbols X_1–X_5 represent factors of corn starch, wheat bran, $MgSO_4$, initial pH and cultivating time, respectively. Symbols 1–10 represent the levels of each factor

submerged cultures, Biosynthesis of fungal metabolites during fermentation process varies with medium components, operation conditions, initial pH, and so on [11]. Nutritional components and incubating conditions influenced triterpenoids production [12–14]. Chang reported that triterpenoids content could be increased to 3.18 % in flask cultures by means of the control of cultural conditions and the modification of medium composition based on the RSM [13]. The triterpenoids production experimentally obtained using the ANN–GA designed medium was 64.79 mg/L [15]. Uniform design (UD), as a common method for optimization of experimental conditions [16], is a collection of mathematical and statistical techniques for designing experiments, building models, searching optimum conditions of factors for desirable responses, and evaluating the relative significance of several affecting factors even in the presence of complex interactions. In order to maximize the production of intracellular triterpenoids from *A. cinnamomea*, the uniform design method was used to optimize cultivation conditions. After optimization, according to possible practical fermentation process and biosynthesis pathway of triterpenoids, we investigated influences of plant oils and glucose supply.

Materials and Methods

Microorganism and Inoculums Preparation

A.cinnamomea CCRC35396 was obtained from the American Type Culture Collection (ATCC), USA. The strain was maintained on malt extract agar (MEA) plate. The plate was incubated at 25 °C for 21 days and then stored at 4 °C. A seed medium constituents included glucose 20.0 g/L malt extract 20.0 g/L, and peptone 1.0 g/L, with initial pH 5, followed by autoclaving at 121 °C for 15 min. The mycelium of *A. cinnamomea* was transferred from stock plate to the 250 ml Erlenmeyer flask with 100 ml seed medium and incubated at 25 °C for 7 days by shaking at 120 rpm. The mycelium obtained from liquid medium was used as the inoculums source.

Culturing Conditions

Each shaking-flask culture with 10 ml seed inoculums was carried out in a 250 ml Erlenmeyer flask containing 100 ml of medium and incubated at 25 °C by shaking at 120 rpm. For cultivation conditions optimization, experiments were carried out according to experimental design matrix (Table 1).

Determination of Mycelia Dry Weight

For measure of mycelia dry weight, the mycelia from a culture flask were filtered through pre-weighted filter-paper under suction, washed with distilled water, then collected and dried at 55 °C to a constant dry weight.

Assay of Triterpenoids

Triterpenoids were quantified by colorimetric method with vanillin–acetic acid system [17]. Mycelia powder (1.0 g) were regurgitated three times with 30 ml of absolute ethanol at 70 °C water bath for 1.5 h, collected all the solvent and determined volume to 100 ml. The supernatant (0.2 ml) was added to a tube and heated solvent to evaporation in a water bath, 0.4 ml newly mixed 5 % (w/v) vanillin–acetic acid solution and 1.6 ml perchloric acid

were added, mixed and incubated at 70 °C for 15 min. The tubes were taken out and cooled to room temperature in running water. Then 4.0 ml ethyl acetate was added and cooled to room temperature. The absorbance was scanned using a spectrophotometer at 560 nm with a blank solution as reference. Olenolic acid (0.2 g/L) was used as standard to prepare the standard curve, which was used as benchmark for the yield determination of triterpenoids.

Experimental Design

Based on the results of screening out with mono-factor experiments, the experiment for optimization of cultures conditions was arranged 5 factors, i.e., the concentration of corn starch (X_1), wheat bran (X_2), MgSO4 (X_3), respectively, initial pH(X_4), and cultivating time (X_5) by design, each at ten levels. The UD table U_{10} (10^5) was applied to arrange the experiments (Table 1) and each trial was performed in triplicate. The evaluated response Y was content of biomass or triterpenoids. Effects of plant oils on *A. cinnamomea* culture were also studied using shake flask culture. Plant oils such as camphor oil, sesame oil and olive oil were supplemented in optimum medium for triterpenoids production, all at volume fractions of 0.5 %. Olive oil concentration, as favorite oil on triterpenoid production, was determined. Effect of glucose, with concentration (0.1–0.5 %) (w/v) adding into cultures at 10th day, when triterpenoids production declined sharply, was tested under the optimal conditions for triterpenoids.

Statistical Analysis

The statistical and stepwise regression analyses of data were carried out using DPS software (Version 9.50 by Hangzhou Refine Information Teach., Co., Ltd, China). Experimental designs resulted in a mathematical expression.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_{ij} \quad (1)$$

where Y is the predicted response (biomass or triterpenoids production), β_0, β_i, β_{ii} andβ_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively; X_i and X_j represent the independent variables in the Table 1. The model was evaluated R^2 and P value.

Results and Discussion

UD Experiment Results

The uniform design of the experiments and the results are shown in Table 2. The equation relating the coefficients obtained for biomass and triterpenoids production to the experimental variable is as follows:

Table 2 Results of the stepwise regression analysis for optimization of biomass

	Parameters		
	Significance P	t Test	Coefficient
X_1	0.0000	246.2653	1.0000
X_2	0.0002	65.2705	−0.9998
X_2X_2	0.0002	73.3951	0.9998
X_4X_4	0.0001	104.9544	0.9999
X_1X_3	0.0013	27.5392	−0.9987
X_1X_4	0.0000	147.7642	−1.0000
X_3X_5	0.0355	5.1644	0.9645

$$Y_1 = 1.35 + 0.544X_1 - 0.273X_2 + 0.005X_2^2 + 0.186X_4^2 - 0.013X_1X_3 - 0.066X_1X_4 - 0.005X_3X_5 \quad (2)$$

$$Y_2 = 4.176 - 0.059X_1 + 0.015X_5^2 - 0.0002X_1X_2 + 0.004X_1X_3 - 0.021X_4X_5 \quad (3)$$

In Eq. 2, Y_1 is the response of biomass, with $R = 0.999$, F-value $= 57190.5$, standard error $S = 0.0012$, significance $p < 0.001$. In Eq. 3, Y_1 is the response of triterpenoids production, with $R = 0.999$, F-value $= 1807.3$, standard error $S = 0.036$, significance $p < 0.0001$. For testing the goodness of fit of the regression equation, R and F-value were evaluated. The closer the value of R is to 1, the better the correlation between the obtained and predicted values. The values of R were 0.999 for biomass, 0.999 for production of triterpenoids, which suggested that the experimental data be in good agreement with predicted values. Generally, the calculated F value, as a ration of the mean square from regression to the mean square from the real error, should be several times greater than tabulated F value, if the model is a good prediction of the experimental results and the estimated factor effects are real. Here, the computed F values of 57190.5 for biomass and 1807.5 for triterpenoids production were greater than the tabulated F value, revealing that this regression was statistically significant ($p < 0.001$) at the 99 % confidence level.

The results of the regression analysis listed in Tables 2, 3. Table 2 showed that X_1 linear coefficient, which constitutes the most significant term, i.e., corn starch concentration, had a significant effect on biomass ($P < 0.01$). The coefficients of the quadratic terms of wheat bran broth and initial pH and the interaction between variables appeared to be very significant ($P < 0.05$) effect on biomass. Table 3 indicted that X_1 linear coefficient had highly significant negative effect on triterpenoids production. The coefficients of the quadratic terms of cultivating time and the interaction between factors

Table 3 Results of the stepwise regression analysis for optimization of triterpenoids production

	Parameters		
	Significance p	*t* Test	Coefficient
X_1	0.0000	21.2295	−0.9956
X_5X_5	0.0000	58.4832	0.9994
X_1X_2	0.0068	5.1467	−0.9321
X_1X_3	0.0091	4.7310	0.9211
X_4X_5	0.0001	16.5751	−0.9928

had a significant effect on yield of triterpenoids ($P < 0.001$). The constituent of medium had effect on cell growth and triterpenoids [13], however, initial pH showed a significant effect on biomass and metabolites [18]. On the basis of condition optimization evaluated from the model, the optimal values of the tested variables obtained by DPS are as follows: (1) for high biomass, the optimum combination was 47.00 g/L corn starch, 47.00 g/L wheat bran broth and 0.5 g/L $MgSO_4$, with initial pH 3 and incubating for 7 days; (2) for high triterpenoids production, the optimum culture condition was 20.00 g/L corn starch, 20.00 g/L wheat bran broth and 1.85 g/L $MgSO_4$, with initial pH 3 and incubating 16 days. Under these conditions, the predicted biomass and triterpenoids production were 15.42 g/L and 5.98 % (w/w) respectively, where the corresponding experimental response was 15.64 g/L for biomass and 6.03 % for triterpenoids production. The experimental values of biomass and triterpenoids yield were in good agreement with those of the predicted value.

Time Course of Cultivation

After we obtained the optimal conditions for triterpenoids production by uniform design, we tested time courses of cell growth and triterpenoids production. Figure 1 displays the kinetics of mycelial growth and total triterpenoids accumulation in submerged fermentation. The mycelia concentration had slight changes from 4 to 16 days, A slowly increase of production of triterpenoids was observed from 4 to 10 days, however, triterpenoids production suddenly reduced from 10 to 12 days. It's interested that it increased rapidly from 12 to 16 days and achieved the highest point. From 16 to 18 days, these values were decreased significantly with terminal pH rebounding slightly (data not shown). Consequently, we concluded biosynthesis of triterpenoids, as a secondary metabolite, fell behind mycelia growth. The possible reason is that the growth of cell supplied enough substrates and enzymes for biosynthesis of trirterpenoids.

Effect of Plant Oils

Plant oils, which were usually used as antifoam agents in fermentation, had been reported to be beneficial for the mycelial growth of several medicinal mushrooms, and to increase the production of bioactive metabolites [12, 19]. In this research, the effects of camphor, sesame and olive oils were investigated in submerged fermentation of *A. cinnamomea*, all at volume fractions of 0.5 %. As shown in Fig. 2, all oils tested enhanced biomass, but olive oil significantly improved accumulation of triterpenoids. The concentration of olive oil had different effect on mycelial growth and triterpenoids production. The maximal production of triterpenoids was obtained when 0.3 % of olive oil was supplied, while the high olive oil supply slight inhibited production of triterpenoids but simulated cell growth. The stimulation of cell growth by oils was attributed to a partial incorporation of lipids in the cell membrane, thereby facilitating the uptake of nutrients from the

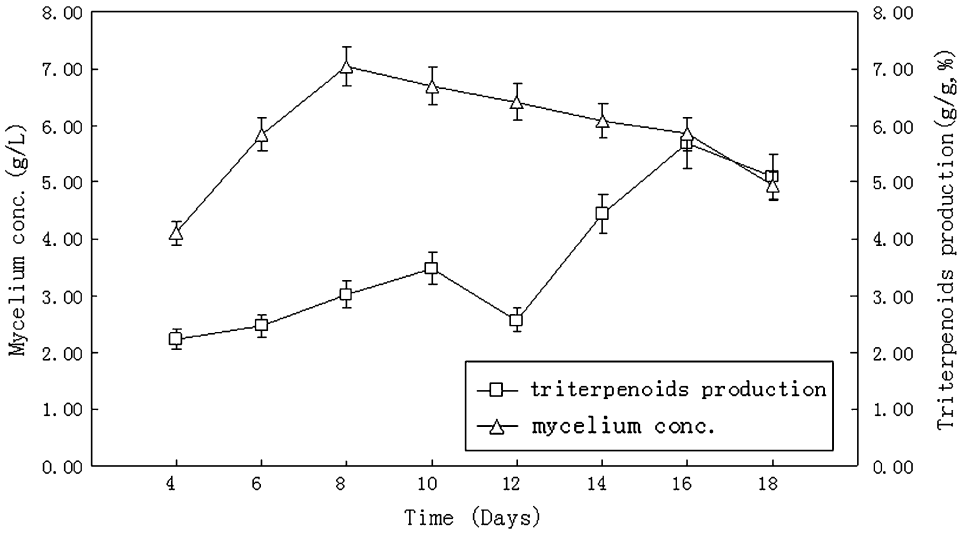

Fig. 1 The time courses of mycelium growth and triterpenoids production

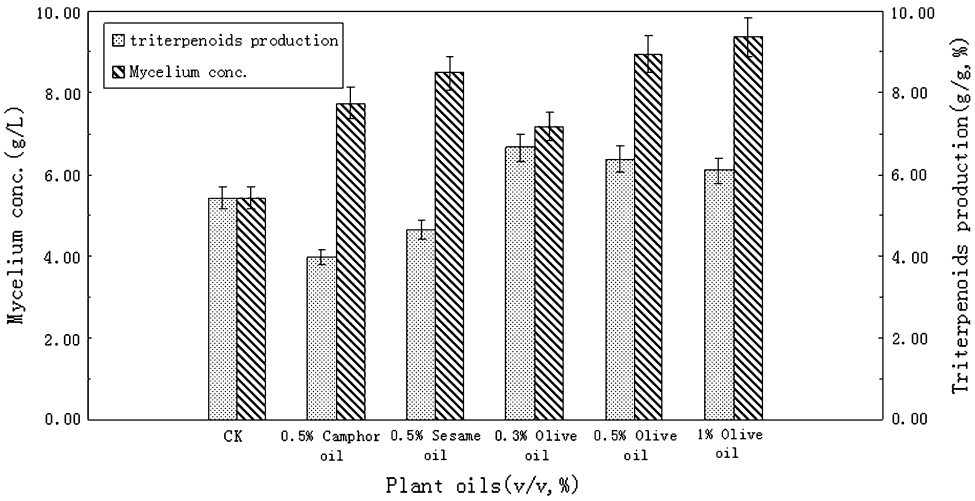

Fig. 2 The effects of plant oils on mycelium growth and triterpenoids production

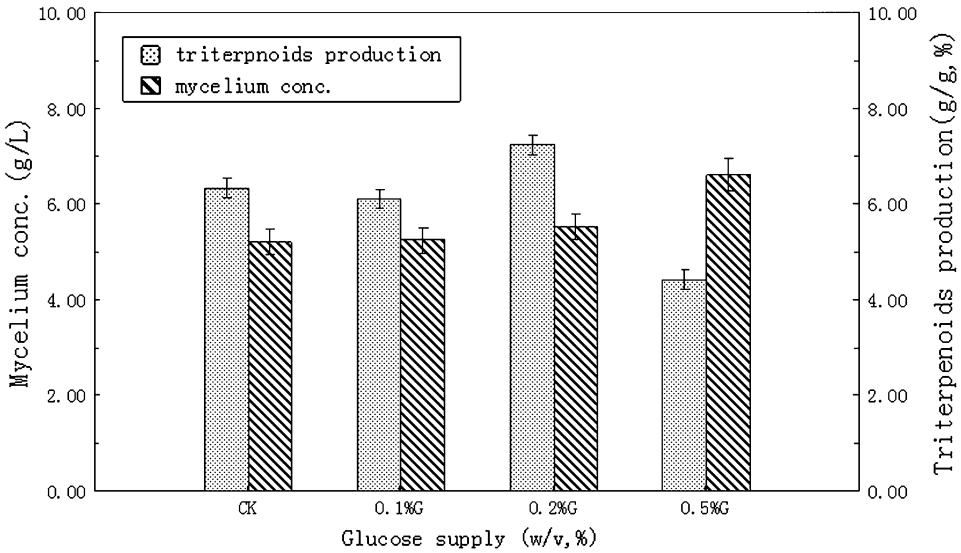

Fig. 3 The effects of glucose on mycelium growth and triterpenoids production

medium [20]. Olive oil supply significantly increased production of triterpenoids, especially adding into 0.3 % (v/v) at the beginning of fermentation, partly contributing to squalene in the olive oil [21]. The mechanism by which the oils affect the cell growth and triterpenoids production of *A.cinnmomea* is yet to be determined.

The Result of Glucose-fed Batch Fermentation

The effect of glucose supply before triterpenoids production decline by *A. cinnamomea* was studied in submerged culture, namely added different concentration of glucose at 10th day, at weight fractions of 0.1–0.5 % (w/v). Figure 3 suggested that the high concentration of glucose supply was beneficial to biomass but significantly inhibited triterpenoids biosynthesis. The highest biomass achieved when 0.5 % glucose supplied, however, the maximum of triterpenoids production was obtained when 0.2 % glucose supply. Glucose, the basic material on biosynthesis of metabolites, could be conversed squalene via MEA and DOXP/MEP pathway, which is the directed precursor of triterpenoids [22]. Glucose supplied at 10th day in submerged fermentation, when biosynthesis of triterpenoids declined, the triterpenoids production would be improved at fermentation terminal. The higher biomass under high glucose supply may be attributed to fast-growth of cell in glucose-rich environment. The high glucose supply significantly inhibited the production of triterpenoids, partly due to inhibitory effect of glucose on enzyme. In conclusion, the higher triterpenoids production would be obtained in glucose-fed batch fermentation.

Acknowledgments This work was supported by CASW Technology, CO., Ltd.

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