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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

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Statistical optimization of medium components for the production of *Antrodia cinnamomea* AC0623 in submerged cultures

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Abstract The nutritional medium requirement for biomass and triterpenoid production by *Antrodia cinnamomea* AC0623 strain was optimized. Box–Behnken was applied to optimize biomass and triterpenoid production. According to response surface methodology (RSM), the optimum concentrations of N-source were determined. The results indicate that when a submerged culture in shake flasks was operated at 28°C, initial pH 5.5, and rotation speed 105 rpm, the biomass and triterpenoid content in dry basis could be increased to 3.20% (w/w) and 31.8 mg/g, respectively. The experiments were further scaled up to 100- and 700-l fermentors. Higher content of triterpenoids (63.0 mg/g) was obtained in 700-l fermentations by means of the control of cultural conditions and the modification of medium composition based on the RSM.

Introduction

Antrodia cinnamomea, a native species in Taiwan, grows naturally on *Cinnamomum kanehirai* Hay, a kind of camphor tree native to Taiwan only and is well known as "Niu-chang-chih". This fungus forms fruit body that causes brown rot on *C. kanehirai* Hay (Andersson et al. 2001; Chang and Chou 1995; Wu et al. 1997). Aborigines living in Taiwan's mountain range have used this fungus as traditional herb for the treatment of food and drug intoxications, diarrhea, abnormal pains, hypertension, and liver cancer in Taiwan folk medicine (Chang and Chou 1995; Cherng and Chiang 1995). The current price of fresh fruiting body of *A. cinnamomea* is estimated to be US $1,500 per kilogram. Therefore, it has become the most expensive wild fungus in the market. Chemical compounds found in *A. cinnamomea* include polysaccharide, ergosterol, and triterpenoids (Cherng and Chiang 1995; Chang et al. 2006; Chiang et al. 1995). Triterpenoid was considered as one of the most biologically active components in recent years. For example, the oxygenated triterpenoids found in fungi which were isolated from *Ganoderma lucidium* have biological activities including inhibition of cholesterol synthesis, inhibition of angiotensin-converting enzyme, and inhibition of platelet aggregation (Wang et al. 1991). Four novel ergostane-type triterpenoids were isolated from the fruiting body of the fungus *A. cinnamomea* by Cherng et al. (1996). Gao (1992) has reported studies of triterpene compound B isolated from *A. cinnamomea*. It was found that such compound can reduce blood glutamate pyruvate transaminase level in mice which have acute liver abnormality induced by tetrachloride methane. Dai et al. (2003) reported that both mycelium and sporocarp of *A. cinnamomea* protect against acute liver damage induced by ethanol.

In biotechnology culture condition, optimizing through factorial design and response surface methodology (RSM) is a common practice (Chen 1996; Cordenunsi et al. 1985; Rao et al. 1993). RSM has some advantages that included less experiment numbers, suitability for multiple factor experiments, search for relativity between factors, and finding of the most suitable condition and forecast response. Wang et al. improve the monacolin K, γ-aminobutyric acid and citrinin production of *Monascus purpureus* NTU 601 by using RSM method (Wang et al. 2003). For example, Yang et al. (2003) had applied RSM on *A. cinnamomea* and got an eightfold increase of mycelium yield of 2,164.2 mg/100 ml. In this research, the effect of cultured media on the mycelium growth and the triterpenoid content were investigated. RSM was applied to determine the optimum cultivation conditions.

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Table 1 Process variables and level in the three-factor, three-level response surface design of biomass and triterpenoids. Actual value of factors as per Box–Behnken designs

Factors	Symbol	Coded variable level		
		−1	0	1
Malt extract (%)	X_1	0	1.5	3
Peptone (%)	X_2	0	1.5	3
Yeast extract (%)	X_3	0	1.5	3

Materials and methods

Strain

The strain AC0623 was isolated from the fruiting body of *Antrodia cinnamomea* collected at Liou Kuei in Kao-hsiung County, Taiwan (Chang et al. 2006). The strain was maintained on M25 agar plate (Merck, Germany) at 26°C.

Inoculum preparation

The mycelium of *A. cinnamomea* was transferred from stock slant to Petri dish containing M25 medium and incubated at 26°C for 3 weeks for mycelium growth. Conidiospores obtained from the edge of 21-day-old colonies were used as the inoculum source.

Table 2 Process variables and level in the three-factor, three-level response surface design of biomass and triterpenoids. Observed experimental data

Run	Independent variables (coded level)			Observed experimental data	
	Malt extract (%)	Peptone (%)	Yeast extract (%)	DCW[b] (%, w/w)	Triterpenoids (mg/g)
1	3 (1)	3 (1)	1.5 (0)	2.59	8.0
2	3 (1)	0 (−1)	1.5 (0)	1.89	18.7
3	0 (−1)	3 (1)	1.5 (0)	2.22	10.8
4	0 (−1)	0 (−1)	1.5 (0)	1.12	9.5
5	3 (1)	1.5 (0)	3 (1)	3.54	16.7
6	3 (1)	1.5 (0)	0 (−1)	1.26	19.0
7	0 (−1)	1.5 (0)	3 (1)	2.32	15.8
8	0 (−1)	1.5 (0)	0 (−1)	1.05	21.5
9	1.5 (0)	3 (1)	3 (1)	3.42	11.8
10	1.5 (0)	3 (1)	0 (−1)	2.05	9.0
11	1.5 (0)	0 (−1)	3 (1)	2.41	28.8
12	1.5 (0)	0 (−1)	0 (−1)	0.49	6.7
13[a]	1.5 (0)	1.5 (0)	1.5 (0)	2.18	28.0
14[a]	1.5 (0)	1.5 (0)	1.5 (0)	2.39	28.1
15[a]	1.5 (0)	1.5 (0)	1.5 (0)	2.18	27.9

[a]Runs 13–15 were three replications
[b]*DCW* Dry cell weight of biomass

Table 3 Effect of carbon sources on the biomass and triterpenoid production

Carbon source	Final pH	DCW (%, w/w)[a]	Triterpenoids (mg/ml)
Glucose 1%	4.65	0.96	0.17
Glucose 2%	4.63	1.40	0.30
Glucose 3%	4.54	1.52	0.35
Sucrose 1%	7.96	0.75	0.14
Sucrose 2%	7.83	0.86	0.19
Sucrose 3%	7.96	1.06	0.22
Fructose 1%	7.28	1.00	0.10
Fructose 2%	6.58	1.26	0.19
Fructose 3%	6.24	1.37	0.20

[a]*DCW* Dry cell weight of biomass

Reagents

Yeast extract, malt extract, and bacto-peptone were purchased from Difco (Detroit, MI, USA). Reagent grade ethyl acetate was purchased from Merck (Damstadt, Germany). Other chemicals were analytical grade.

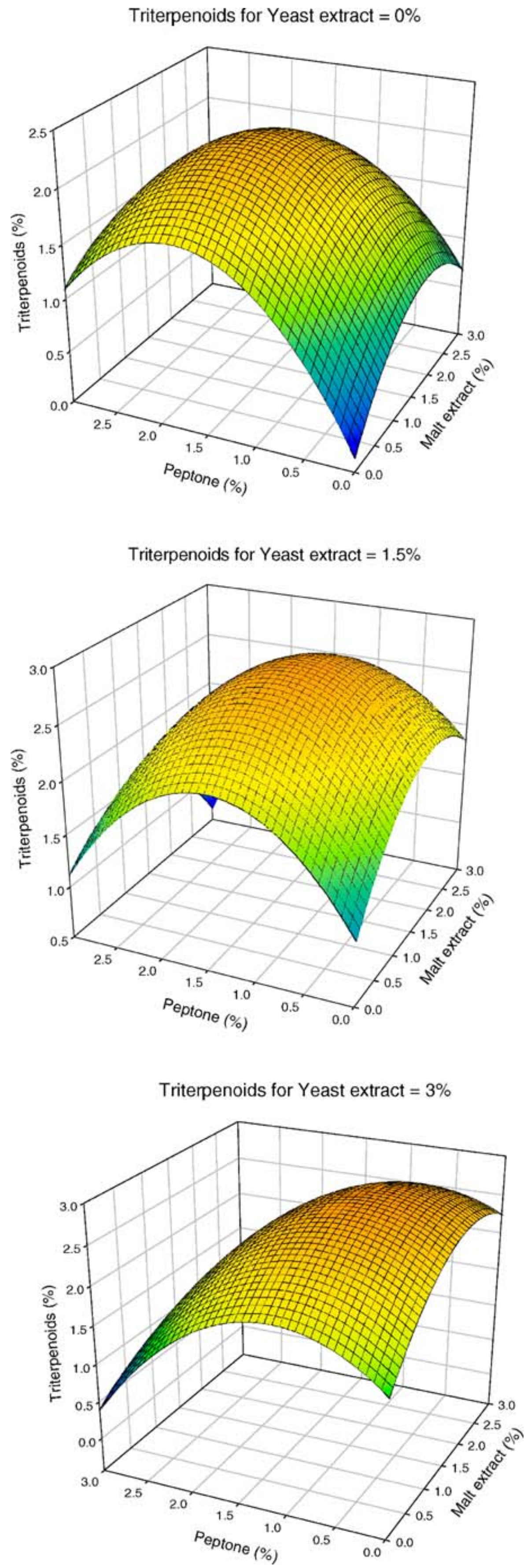
Shaking flask cultures and medium optimization through RSM

A shaking flask culture was performed in a 500 ml Hinton flask with a cotton plug, which contained 100 ml of the medium. The cultured medium is shown as following: glucose 2%, bacto-peptone 1.3%, malt extract 2%, $MgSO_4 \cdot 7H_2O$ 0.03%, KH_2PO_4 0.03%, and K_2HPO_4 0.03%, pH 5.5. The pH was measured with a digital pH meter (Model 720P, Istek, South Korea). Media were sterilized at 121°C for 15 min, and glucose was autoclaved separately. The flasks were incubated on a Firstek Scientific orbital rotary shaker (stroke 7 cm) under the conditions of 105 rpm and 28°C for 7 days. The effect of the carbon source (glucose, sucrose, and fructose), nitrogen source (yeast extract, malt extract, and peptone) on biomass, and triterpenoid production in submerged cultures was analyzed.

Table 4 Analysis of variance for the production of biomass and triterpenoids and various culture conditions[a]

Source	Biomass		Triterpenoids	
	df[b]	Sum of squares	df	Sum of squares
Regression	9	9.5865	9	7.5504
Residual	5	0.1225	5	1.4683
Pure error	2	0.1565	2	0.5419
Variability explanation (γ^2)	98.74		83.72	

[a]Analysis of variance from SAS statistics systems
[b]Degree of freedom



◀ **Fig. 1** The response surface for the production of triterpenoids at various concentrations of malt extract and peptone

Jar fermentor cultures and large-scale production

The experiments were carried out in 5-l fermentors (Modular Microferm Bench Top Fermentor, Model BIO-FLO 3000, New Brunswick Scientific, NJ, USA) at 28°C. The cultured media of *A. cinnamomea* strain are based on the optimum conditions from shaking flask cultures. The entire fermentor with 3-l culture medium was autoclaved at 121°C for 30 min. Three hundred milliliters of 7 days old shaking flask with the medium described previously was used as inoculum to each fermentor. The inoculum size was 9% (v/v) of the final working volume (3.3 l).

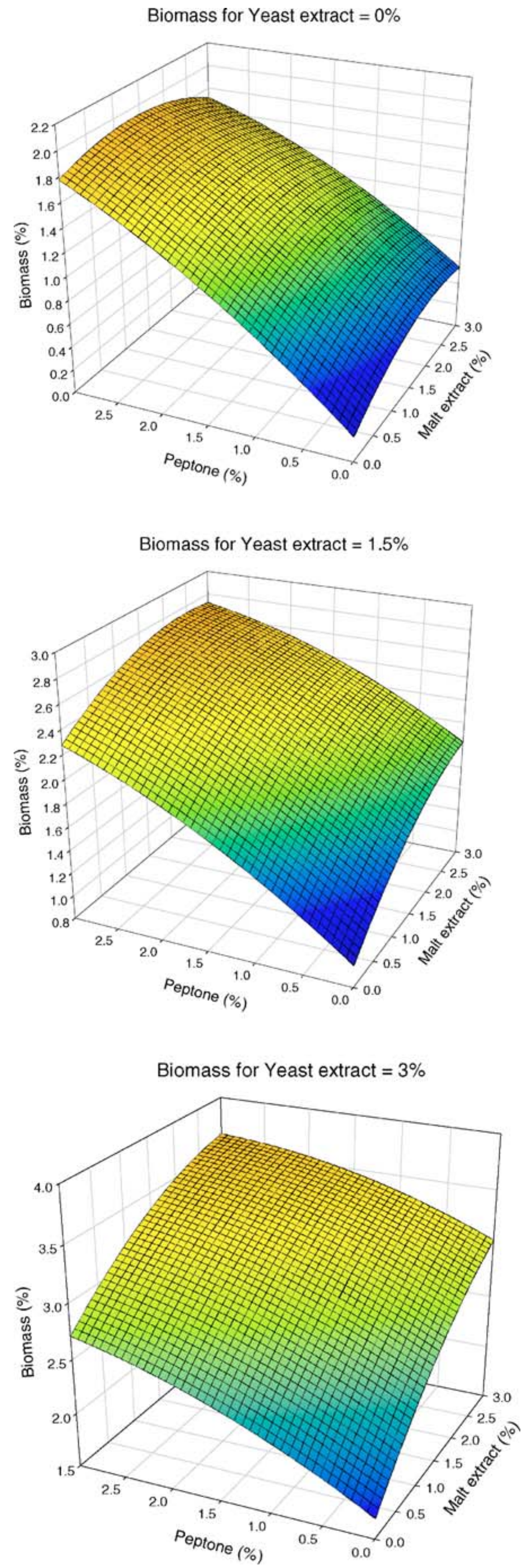
The fermentation conditions were set at 100 rpm of agitation and 1 vvm of aeration rate in bench-top scale fermentor. The experiments were further scaled up to 100- and 700-l fermentors, with some modifications on culture conditions primarily based on the dissolved oxygen value measured by DO probe during fermentation processes. The culture conditions of 100-l fermentor were described as follows: working volume, 60 l; temperature, 28°C; agitation, 70 rpm; aeration, 1 vvm; back pressure, 0.2 kg/cm^2. The culture conditions of 700-l fermentor were described as follows: working volume, 400 l; temperature, 28°C; agitation, 60~70 rpm; aeration, 1~1.2 vvm; back pressure, 0.3 kg/cm^2.

Cell mass determination (Chang et al. 2005)

Cell mass was determined by measuring the dry cell weight of 10 g suspension taken from the shaking flask or the fermentor. The mycelia were collected by centrifugation at 5,200 ×*g* for 20 min and washed once with distilled water. Cells were dried at 105°C to a constant weight.

Analysis of triterpenoids (Chen et al. 1999)

Ten grams of dry mycelia of *A. cinnamomea* was immersed in 150 ml ethanol and incubated in 65°C water bath for 1 h. The hot solvent extract was filtered with Whatman No. 2 filter paper to collect the solvent. The solvent was then concentrated by vacuum rotary drier to remove ethanol. The concentrated solvent was mixed with 50 ml ethyl acetate in a 125 ml separated funnel to extract triterpenoid content. The solvent extract in the separated funnel was violently mixed in 1 to 2 min with 50 ml distilled water. After extraction, the separation funnel was setting for 10 to 30 min. The upper layer was collected through filtration with Whatman No. 5 filter paper. Filtrates were dried at 105°C to a constant weight.



◀**Fig. 2** The response surface for the production of biomass at various concentrations of malt extract and peptone

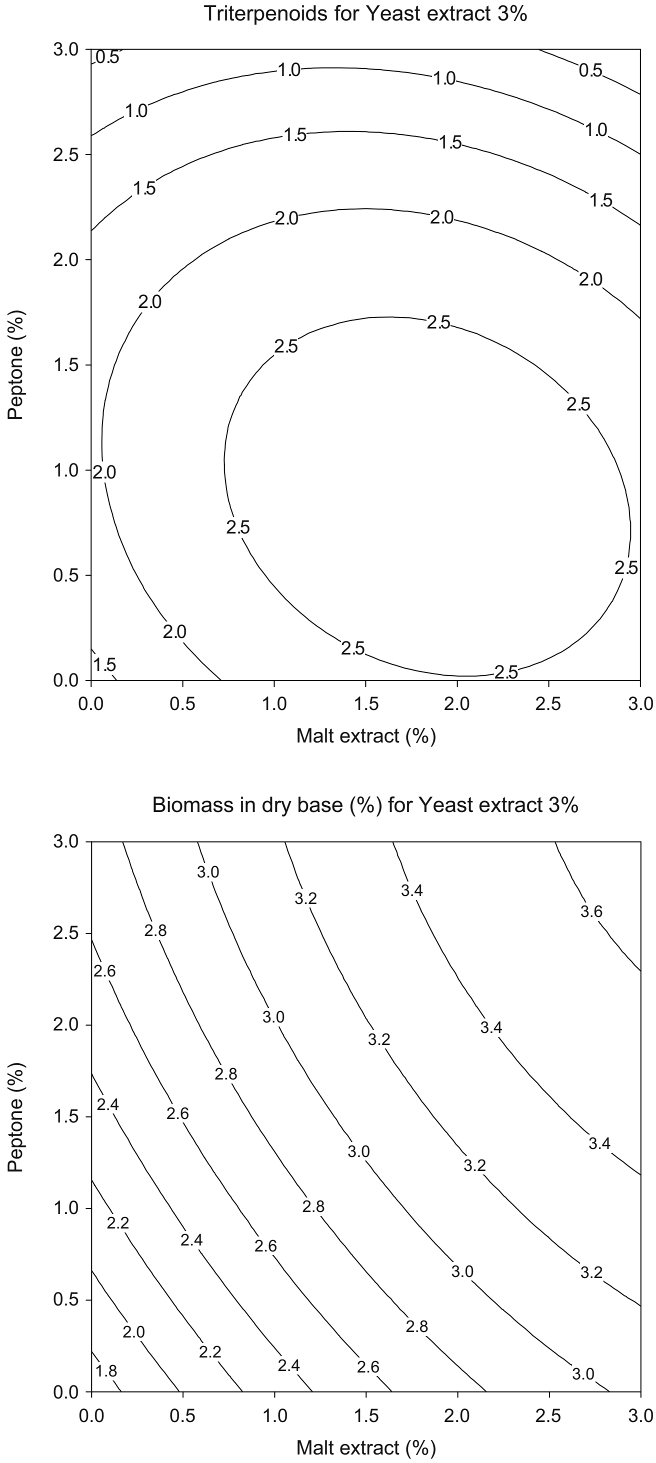


Fig. 3 Contour plot for the production of triterpenoids and biomass at 0~3% concentration of malt extract and peptone (yeast extract=3%)

Design of experiments

The optimum medium composition was optimized using Box–Behnken design (Box and Behnken 1960). The crucial factors involved in the study and their concentrations are given in Tables 1 and 2. A total of 15 runs were carried out simultaneously, with runs 13–15 as three replications. The central point of the design arrangement was decided according to the C/N ratio and the path of steepest ascent. The composition of basal medium was: malt extract 1.5%, peptone 1.5%, and yeast extract 1.5%. Control conditions were: malt extract 2%, peptone 1.3%, and yeast extract free.

Response surface methodology

Data analysis was carried out using RSREG (Response Surface Regression) in Statistical Analysis System (SAS, Cary, NC, USA). A second order model was employed to fit the data individually for the responses Y_1 (biomass) or Y_2 (triterpenoids) by the general model (Giovanni 1983) with three factors: X_1 (malt extract), X_2 (peptone), and X_3 (yeast extract), with each factor coded to be in the levels of −1, 0, and +1.

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2$$

The coded points for this experimental design are given in Tables 1 and 2. The model was evaluated in terms of statistically significant coefficients, R^2 and P-value.

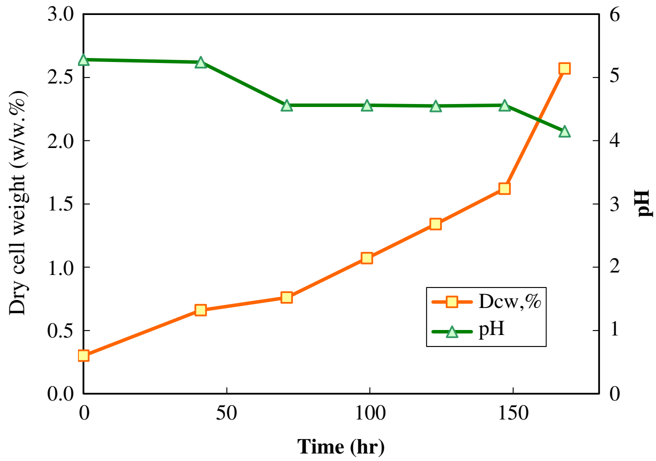


Fig. 5 Time course of *A. cinnamomea* strain AC0623 cultivation in a 100-l fermentor. *DCW* Dry cell weight

Results

Effect of carbon sources in batch flask culture

Glucose, sucrose, and fructose were compared on their effects of biomass yield and triterpenoid content in 500 ml Hinton shake flask culture with initial pH 5.5. The results were shown in Table 3. Glucose in a concentration of 3% seemed to be the most suitable carbon source for mycelium growth of *A. cinnamomea* AC0623 because the dried biomass and triterpenoids reached 1.52% and 0.35 mg/ml, respectively. The lower final pH of glucose lets it take advantages in submerged culture than sucrose or fructose as carbon source because contamination can also be inhibited.

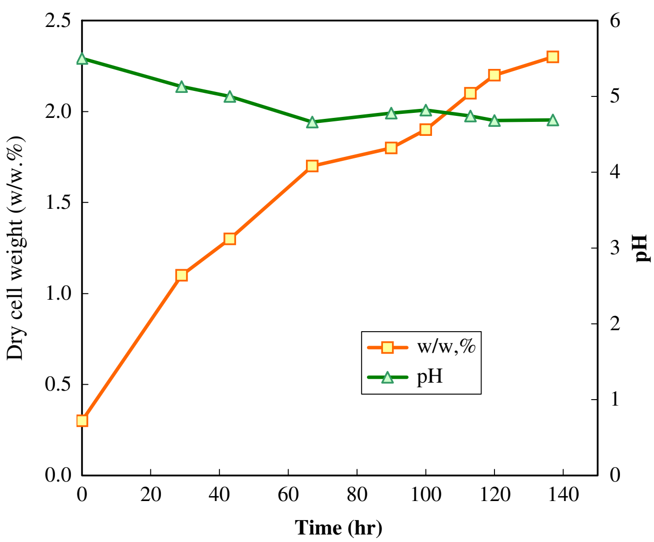


Fig. 4 Time course of *A. cinnamomea* strain AC0623 cultivation in a 5-l fermentor. *DCW* Dry cell weight

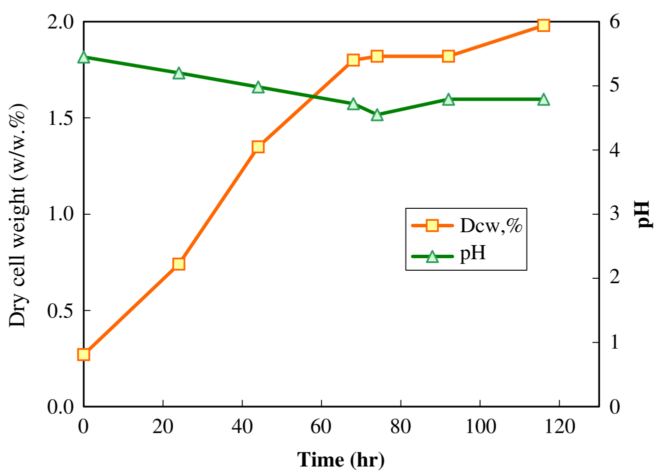


Fig. 6 Time course of *A. cinnamomea* strain AC0623 cultivation in a 700-l fermentor. *DCW* Dry cell weight

Table 5 Effect of culture conditions on the production of biomass and triterpenoids by *Antrodia cinnamomea* AC0623 in shake flask

Items	DCW (%, w/w)[a]	Triterpenoids (mg/g)
Control	1.18	16.7
Yeast extract 3%	3.10	26.0
RSM condition	3.20	31.8

[a]*DCW* Dry cell weight of biomass

Regression equation, R^2 value of model

The following equations, where the factors take their coded value, were obtained from regression analysis for the biomass and triterpenoid production, respectively:

$$\begin{aligned} Biomass(\%) &= 2.25 + 0.3213\ X_1 + 0.5463\ X_2 + 0.8550\ X_3 \\ &\quad - 0.1725\ X_1^2 - 0.1225\ X_2^2 - 0.0350\ X_3^2 \\ &\quad - 0.1000\ X_1X_2 + 0.2525\ X_1X_3 - 0.1375\ X_2X_3 \end{aligned}$$

$$\begin{aligned} Triterpenoids\ (\%) &= 2.80 + 0.06\ X_1 - 0.3013\ X_2 + 0.2113\ X_3 \\ &\quad - 0.6038\ X_1^2 - 1.0213\ X_2^2 - 0.3713\ X_3^2 \\ &\quad - 0.3000\ X_1X_2 + 0.0850\ X_1X_3 - 0.4825\ X_2X_3 \end{aligned}$$

The percentages of variability in the responses accounted for by the factors (R^2 value) for the models are given in Table 4. The R^2 values of biomass and triterpenoid production were 98.74 and 83.72%, respectively. High confidence was obtained because the R^2 value had reached 80–90%. Also, the statistics' P-value for the overall regression is significant at the 5% level, which further supports that the model is adequate in approximating the response surface of the experimental design.

Optimum conditions based on RSM

Figure 1 shows three-dimensional (3-D) response surface plots of the effect of malt extract and peptone concentration on the production of triterpenoids. The results indicated that the optimal basal medium for the highest triterpenoid production was yeast extract 3%, peptone 1%, and malt extract 2.5%. Figure 2 shows 3-D response surface plots of the effect of malt extract and peptone concentration on the production of biomass. The results indicated that the optimal basal medium for the highest biomass production was composed of yeast extract 3%, peptone 3%, and malt extract 3%. Because production of both biomass and triterpenoids may increase at the same time, any decision on the optimum conditions should consider the relationship between these two elements. By multiplying the data of Figs. 1 and 2, when the yeast extract concentration reached 3%, the production of both biomass and triterpenoids increased. Figure 3 shows the optimum conditions (yeast extract 3%, peptone 1%, and malt extract 2.5%) would be expected from contour plot. The biomass and triterpenoid contents would be 3.1% and 26 mg/g, respectively, under optimum conditions.

Jar fermentor cultures

The cultured media of 5-, 100-, and 700-l fermentors are shown as following: glucose 3%, bacto-peptone 1%, malt extract 2.5%, $MgSO_4 \cdot 7H_2O$ 0.03%, KH_2PO_4 0.03%, and K_2HPO_4 0.03%, initial pH 5.5 which are based on the optimum conditions from shaking flask cultures. Figures 4, 5, and 6 showed the time course of *A. cinnamomea* strain AC0623 cultivation in 5-, 100-, and 700-l fermentors. Comparison of the biomass and triterpenoid production among different scale of cultivation is shown in Table 6. Because the scale of fermentor is increased, the triterpenoid yield of 700-l fermentor may reach to 63 mg/g.

Discussion

The nutritional medium requirement for biomass and triterpenoid production by *A. cinnamomea* AC0623 strain was optimized. Box–Behnken was applied to optimize biomass and triterpenoid production. Glucose is superior compared to sucrose or fructose as carbon source in the culture medium (Table 1). The design of center point is based on the C/N ratio and path of steepest ascent. Firstly, we fixed glucose 3% and malt extract 1.5%, and combined them with nitrogen source peptone and yeast extract; each concentration was increased from 0.5 to 2.5%. The results showed that peptone 1.5% and yeast extract 1.5% were better than any other culture media. The larger the pellet, the lesser amount of biomass was obtained in Hinton flask or jar fermentor cultivation. Because the oxygen transfer from outside to inner cell was difficult, the cell growth rate will then be inhibited. According to response surface methodology, the optimum concentrations of nitrogen source were determined. As shown in Fig. 1, as the yeast extract concentration increased from 0 to 3%, the curve of triterpenoid production gradually increased. When the malt extract was kept around 1.5%, the production of triterpenoids gradually increased along with peptone concentration from 0 to 1.5%, and gradually decreased along with peptone concentration from 1.5 to 3.0%. As shown in Fig. 2, as the yeast extract concentration increased from 0

Table 6 Comparison of the final biomass yield and triterpenoid content of *Antrodia cinnamomea* AC0623 cultivated in fermentors

Fermentor scale	DCW (%, w/w)[a]	Triterpenoids (mg/g)
5–l	2.3	30
100–l	2.6	32
700–l	2.0	63

[a]*DCW* Dry cell weight of biomass

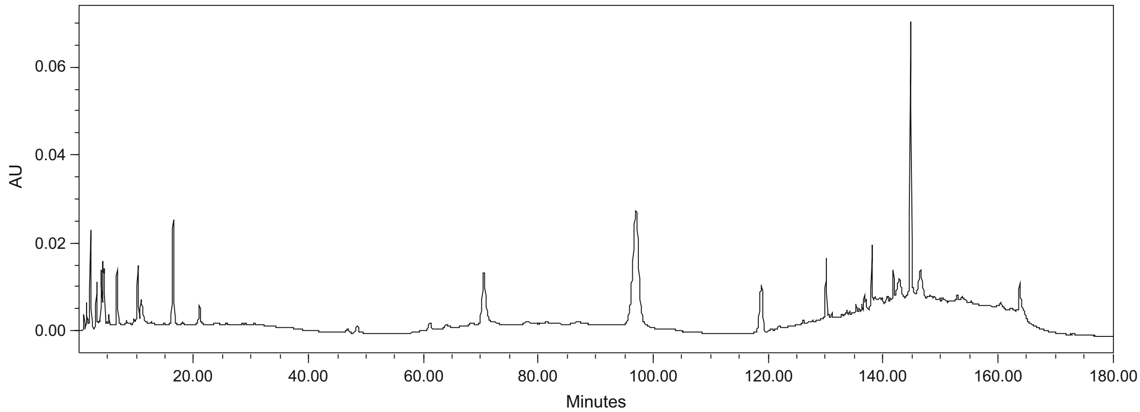


Fig. 7 Triterpenoid chromatogram of *A. cinnamomea* AC0623 strain

to 3%, the curve gradually increased, i.e., the production of biomass gradually increased. As production is optimum at the malt extract which was kept around 1.5%, the production of biomass gradually increased along with peptone concentration from 0 to 3.0%. The maximum value of biomass is about 3.7% (w/w). Obviously, the effect of factors on triterpenoid production was higher than on biomass production. If the biomass reached the highest point, it may result in the decrease of triterpenoid production.

Based on the above findings, RSM was used to verify the experimental results on biomass and triterpenoid production. As shown in Table 5, the results and control group indicated that the production of biomass could be increased from dry cell weight 1.18% (w/w) to 3.20% (w/w), and triterpenoids content could be increased from 16.7 to 31.8 mg/g. Orthogonal effect should be take into account in the experimental design. On the other hand, it was found that the biomass of the fermentor culture was not better than those of flask cultures. Generally speaking, the agitation speed of 100- or 700-l was lower than 5-l during fermentation process. This could be attributed to a higher shear stress present in fermentor cultures because higher agitation speed might break down the mycelium, which had a negative effect on cell growth of fungi (Table 6). At present, few papers have precisely mentioned about the quantitative analysis of triterpenoids. Strictly speaking, it is necessary for us to use high-performance liquid chromatography analysis and to sum the total area of each peak from triterpenoid chromatogram. Figure 7 shows the triterpenoid chromatogram of *A. cinnamomea* strain AC0623.

Medium composition and culture conditions may affect the growth of fungi and alter metabolite production. To obtain bioactive compound, the mycelium production by submerged culture was investigated. Response surface methodology was used to optimize medium composition for the increment of biomass and triterpenoid production. The results indicate that when a submerged culture in shaking flasks was operated at 28°C, at initial pH 5.5, and at rotation speed 105 rpm, the bioimass and triterpenoids content could be increased to dry cell weight 3.20% (w/w) and 31.8 mg/g, respectively. The experiments were further scaled up to 100- and 700-l fermentors. Higher content of triterpenoids (63 mg/g) was obtained in 700-l fermentation by means of the control of cultural conditions and the modification of medium composition based on the RSM. On the other hand, the 2.0%(w/w) dry cell weight of biomass is also suitable for commercial production.

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