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Research Article

A novel *Ganoderma lucidum* G0119 fermentation strategy for enhanced triterpenes production by statistical process optimization and addition of oleic acid

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A novel enhanced triterpenes fermentation production process by *Ganoderma lucidum* G0119 with the addition of oleic acid in the medium has been developed and optimized. All of the six exogenous additives tested were found to exhibit stimulatory effect on mycelial growth and triterpenes biosynthesis by *G. lucidum*. The results show that oleic acid addition had significant role in promoting triterpenes production. The optimal concentration and time of oleic acid addition were determined to be 30 mL/L and 0 h, respectively. Furthermore, three significant factors influencing triterpenes production were identified as glucose, magnesium sulfate and temperature using the Plackett–Burman design. The optimized conditions by central composite design were 27.83 g/L glucose, 1.32 g/L magnesium sulfate, 26.2°C temperature. The triterpenes fermentation yield with the optimized medium based on actual confirmatory experimental data in 6 L fermentor was 1.076 g/L versus the statistical model predicted value of 1.080 g/L. Our innovatively developed triterpenes fermentation production technology and process has been proven to produce high triterpenes productivity and yield conceivably useful for industrial production.

Keywords: Central composite design / *Ganoderma lucidum* / Oleic acid / Optimization / Triterpenes

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1 Introduction

Ganoderma lucidum (Fr.) Krast (Polyporaceae) is a famous traditional Chinese medicinal mushroom. Polysaccharides and triterpenes are the two of its major bioactive components [1]. Interestingly, recent studies show that triterpenes have new biological activities including anti-tumor and anti-HIV-1 [2, 3]. Latest report demonstrates that *G. lucidum* is capable of reducing obesity in mice by influencing the content of microbiota in the gut [4].

Normally, *G. lucidum* is available in the form of mature fruiting bodies and spores under soil culture condition. However, the production of fruiting bodies and spores requires a long cultivation time of about six months [5]. Consequently, the triterpenes yield is rather low in soil cultivation, the submerged fermentation is considered to be the better alternative for efficient production of triterpenes [6–8]. It has been a major concern that

the triterpenes in the submerged fermentation of *G. lucidum* is quite low as recent studies identified many physiologically active *G. lucidum* metabolites for potential nutraceutical applications.

Compared with the soil culture methods, the liquid submerged fermentation of triterpenes is more attractive due to inexpensive materials, mild reaction conditions, and environmental compatibility. The production of mycelia and triterpenes by some mushroom species in submerged cultures has prospered in the Orient in recent years. In order to enhance the production efficiency, the control of environmental conditions or the modification of media composition would be vital.

Many previous studies were centered on the influence of various factors in the shake flask culture of *G. lucidum* mycelium [9]. We recently identified that secondary metabolite production by *G. lucidum* is affected by several factors [10]. To accelerate mycelial growth and metabolite production by *G. lucidum*, the effects of environmental conditions [11], inoculation density [12], medium composition [4], pH [1, 13], oxygen supply [7], pH-shift and dissolve oxygen tension-shift integrated fed-batch fermentation [14], two-stage culture process [6], etc. have been studied. Designing an appropriate fermentation condition, together with developing productive strains are crucial for the

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Abbreviations: CCD, central composite design; P-B, Plackett–Burman; RSM, response surface methodology

Table 1. Reported effects of various process parameters on triterpenes production in fermentation with various strains of *Ganoderma lucidum*

<i>G. lucidum</i> strain	Fermentation condition	Triterpenes content (mg/100 mg)	Triterpenes yield (g/L)	Reference
Not stated	Inoculum density	1.86	0.267	[12]
CCGMC 5.616	Initial pH	1.20	0.208	[1]
CCGMC 5.616	Nitrogen source and initial glucose	1.27	0.212	[6]
CGMCC 5.616	Two-stage culture	3.19	0.582	[8]
HG	Environmental condition	2.63	0.346	[16]
CGMCC 5.616	Addition of phenobarbital	4.14	0.803	[17]
CGMCC 5.616	pH-shift and DOT-shift integrated	3.53	0.798	[18]
CGMCC 5.616	pH-shift and DOT-shift integrated	3.70	0.755	[14]
CGMCC 5.616	Pulse feeding of lactose	2.05	0.367	[7]
CGMCC 5.616	Oxygen supply	3.36	0.450	[19]
CGMCC 5.616	K_La and $A_S^{a)}$	4.96	0.976	[20]
CGMCC 5.616	Cupric ion addition	3.00	0.348	[21]
SB97	Complex media	2.31	0.496	[22]
CGMCC 5.616	Three-stage light irradiation	3.10	0.466	[23]
CGMCC 5.616	Oxygen concentration	4.47	1.050	[24]
CGMCC 5.616	Nitrogen limitation	5.20	0.564	[25]
CGMCC 5.616	Fungal elicitors	2.83	0.120	[26]
G0119	Oleic acid addition	7.12	1.076	This work

^{a)}Initial volumetric oxygen transfer coefficient and area of liquid surface per liquid volume.

improvement of the microbial fermentation processes [15]. For triterpenes production, numerous attempts have been made to enhance the triterpenes content and yield (Table 1).

In this report, the effects of six exogenous additives on mycelial growth and triterpenes production were investigated separately for the fermentation. Our results indicate that oleic acid can promote mycelial growth as well as enhance triterpenes production significantly. Oleic acid plays a key role in process in *G. lucidum* mycelial fermentation. However, oleic acid stimulation of triterpenes production in *G. lucidum* has never been reported. In this study, *G. lucidum* G0119 was employed to investigate its response to oleic acid improve triterpenes fermentation. To our knowledge, this is the first report on enhancing triterpenes fermentation production by addition of exogenous oleic acid in *G. lucidum*. The effects of oleic acid on mycelial growth and triterpenes accumulation were investigated. The enhancement of triterpenes fermentation production by oleic acid was optimized by experiments using the Plackett–Burman (P-B) design and Central Composite Design (CCD). The P-B design has been employed to determine the significant factors triterpenes synthesis. The CCD was used to identify the optimum levels of the significant variables for enhancement of triterpenes production by *G. lucidum* G0119.

2 Materials and methods

2.1 Strain and slant culture

G. lucidum G0119 (Hunong Lingzhi No.1 variety) preserved by the Agricultural Culture Collection of China (Edible Fungi Branch) was used in this study. It was maintained on potato dextrose agar (PDA) slants which were inoculated and incubated at 26°C for 10 days, then stored at 4°C for about 1 month.

2.2 Media and culture conditions

The seed medium contained: potato 200 g, glucose 20 g, distilled water 1000 mL, pH 5.5. For the seed culture, three loops of agar culture in pea size from a slant culture were inoculated into a 250 mL flask with 100 mL seed medium, and cultivated for 10 days in a reciprocal shaker at 150 rpm and 26°C. The seed culture (10%, v/v) was then inoculated into the fermentation medium.

The fermentation medium contained (g/L): glucose 30, yeast extract 3, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 2, pH 5.5. For liquid submerged fermentation, the seed culture was inoculated at 10% (v/v) into a 250 mL flask with 100 mL seed medium. Culture conditions of temperature, agitation rate, and growth period were fixed at 26°C, 150 rpm, and 192 h (8 days), respectively. All experiments were performed in triplicate.

The fermentation in 6 L fermentor: the fermentation medium contained (g/L): glucose 30, yeast extract 3, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 2, pH 5.5. 10% (v/v) of seed culture was inoculated into a 6 L fermentor with a working volume of 4 L. The pH was controlled automatically at 5.5. Aeration rate was 5 L/min and agitation speed was controlled at 100 rpm. Fermentation time was 192 h.

2.3 Exogenous additives addition

There have been reports that fatty acids, oils and surfactants promoted the production of fungal metabolites [5]. It was also reported that the production of microbial exopolysaccharide was stimulated with some fatty acids [5]. Six exogenous additives (oleic acid, soybean oil, hexane, dodecane, acetic ester and Tween 80) were separately added to the fermentation medium at 20 mL/L concentrations in flasks fermentation for 192 h.

2.4 Oleic acid addition

Liquid submerged fermentation process for triterpenes production lasted 192 h in flasks. Oleic acid was used and the additive concentration and additive time were shown as follows:

Oleic acid with various additive methods (sterilized at the 121°C for 30 min or filtration-sterilized with 0.22 μm microfiltration membrane) were added to the medium in flasks.

Oleic acid at various concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mL/L) were added to the medium in flasks.

Oleic acid at various additive times (0, 24, 48, 72, 96 and 120 h) was added to the medium in flasks.

2.5 Plackett–Burman design

In this study, the P-B design [27] was applied to determine the most significant factor affecting triterpenes production. A total of nine variables including oleic acid, glucose, yeast extract, monopotassium phosphate, magnesium sulfate, pH value, temperature, shaker speed and inoculum volume, and two dummy or unassigned variables were studied in 12 experiments for 192 h fermentation time. Two dummy variables, with levels which do not change in the design, were introduced to estimate the population standard deviation. The experimental designs with the name, symbol code, and actual level of the variables are shown in Tables 2 and 3.

Table 2. Range of various factors studied in the P-B design

Variable	Variable code	Unit	Low level (−1)	Low level (+1)
Oleic acid	X_1	mL/L	20	40
Glucose	X_2	g/L	25	35
Yeast extract	X_3	g/L	2.0	4.0
Monopotassium phosphate	X_4	g/L	1.5	3.0
Magnesium sulfate	X_5	g/L	1.5	3.0
pH value	X_6	None	4.6	5.6
Temperature	X_7	°C	25	32
Shaker speed	X_8	r/min	100	200
Inoculum volume	X_9	mL/L	50	150

Table 3. P-B design matrix for screening of fermentation parameters for triterpenes production by *G. lucidum* G0119

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	Triterpenes concentration (g/L)
1	+1 ^{a)}	−1	−1	−1	+1	−1	+1	+1	−1	0.785 ± 0.036 ^{b)}
2	+1	+1	+1	−1	−1	−1	+1	−1	+1	0.569 ± 0.022
3	+1	−1	+1	+1	+1	−1	−1	−1	+1	0.463 ± 0.020
4	+1	+1	−1	+1	+1	+1	−1	−1	−1	0.335 ± 0.013
5	−1	−1	+1	−1	+1	+1	−1	+1	+1	0.410 ± 0.017
6	−1	+1	−1	+1	+1	−1	+1	+1	+1	0.382 ± 0.011
7	+1	+1	−1	−1	−1	+1	−1	+1	+1	0.443 ± 0.018
8	−1	−1	−1	+1	−1	+1	+1	−1	+1	0.889 ± 0.037
9	+1	−1	+1	+1	−1	+1	+1	+1	−1	0.899 ± 0.039
10	−1	+1	+1	+1	−1	−1	−1	+1	−1	0.360 ± 0.010
11	−1	+1	+1	−1	+1	+1	+1	−1	−1	0.488 ± 0.018
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.845 ± 0.033

^{a)} +1 and −1 represent the high and low levels, respectively.

^{b)} Each experimental data point represents the mean ± standard from three independent samples ($n = 3$).

2.6 Response surface methodology (RSM) design

RSM is a frequently used technique for building models and determining the optimal process conditions. The relationship of the significant variables identified by the P-B experiment was expressed in a second order equation.

The CCD for three factors using five coded levels was employed (Table 4). The extreme level of axial points was chosen to be 1.68 (Table 4) in order to make this design rotatable.

2.7 Analytical methods

Determination of mycelial biomass: The mycelial biomass of *G. lucidum* was determined by weighing the dry mycelial samples collected at various times which were centrifuged for 15 min at 10 000×g. The resulting pellet was washed repeatedly with distilled water and dried at 60°C until a constant weight was achieved.

Determination of glucose: For glucose concentration determination, 20 μL culture samples were centrifuged for 20 min at 10 000×g after removing the sediment and were diluted to 2 mL with distilled water, added with 1.5 mL 3, 5-dinitrosalicylic acid reagent [28]. Samples were diluted to 25 mL and then determined with ELISA (model Synergy HT; BioTek Instruments, Inc., USA) at 520 nm.

Table 4. Independent variables and experimental design levels for CCD

Independent variables	Unit	Coded symbols	Level				
			1.68	1	0	−1	−1.68
Glucose	g/L	Gx ₁	46.82	40.00	30.00	20.00	13.18
Magnesium sulfate	g/L	Mx ₂	3.68	3.00	2.00	1.00	0.32
Temperature	°C	Tx ₃	34.39	32.00	28.50	25.00	22.61

Determination of total triterpenes: The method of Feng et al. [10] was used. 100 μ L sample was diluted to 0.2 mL with 5% (w/v) vanillic aldehyde, added with 0.5 mL perchloric acid reagent. After 20 min at 60°C water bath, the sample was diluted to 5 mL with acetic acid and the resulting absorbance at 550 nm was determined with an ELISA (model Synergy HT; BioTek Instruments, Inc., USA) using ursolic acid as standard.

2.8 Statistical analysis

Data analyses were carried out using Design Expert 8.0.6 (Stat-Ease Inc., Minneapolis, USA), and analyses of variance as well as RSM were conducted by ANOVA and RSM procedure. Mean values were considered significantly different when $P \leq 0.05$. All experiments were repeated in triplicates and the average of results was used for analysis.

3 Results and discussion

3.1 Effects of various exogenous additives on triterpenes fermentation

The mycelial growth, glucose consumption rate and triterpenes production were investigated using six different exogenous additives: oleic acid, soybean oil, hexane, dodecane, acetic ester and Tween 80. As shown in Fig. 1, the maximum mycelial dry weight (12.15 g/L), glucose concentration (13.70 g/L) and triterpenes production (0.776 g/L) were observed when oleic acid served as the major exogenous additive. The exogenous additives in the medium improved the mycelial dry weight, glucose consumption rate and triterpenes production. Addition of oleic acid into the medium was found to be the most effective. Addition of dodecane into the medium, the mycelial dry weight, glucose consumption rate and triterpenes production were 9.02, 18.50 and 0.095 g/L, respectively, as compared to the control of 10.85, 13.20 and 0.127 g/L, respectively. Addition of soybean oil into the medium, the mycelial dry weight, glucose consumption rate and triterpenes production were 10.36, 11.30 and 0.511 g/L, respectively.

3.2 Determination of the optimal sterilization method for oleic acid additive for triterpenes fermentation

As shown in Fig. 2, the mycelial dry weight, glucose consumption rate and triterpenes production exhibited significant differences

under the influence of various additives. Under the condition of high temperature sterilization method, the mycelial dry weight, glucose consumption rate and triterpenes were 9.65, 16.70 and 0.362 g/L, respectively. When the oleic acid was filter-sterilized with 0.22 μ m microfilter membrane, the mycelial dry weight, glucose consumption rate and triterpenes was 11.87, 13.60 and 0.786 g/L, respectively. Under this condition, the mycelial dry weight and triterpenes yield were 29.98% and 8.47 times higher than the control (without the addition of oleic acid), respectively. For the triterpenes fermentation with the filter-sterilized oleic acid additive, the triterpenes yield was higher than the control and that with heat-sterilized oleic acid additive. It is apparent that filter-sterilization of oleic acid additive was better than heat-sterilized oleic acid additive for triterpenes fermentation production. It is conceivable that heat-sterilization of the oleic acid additive diminish its effect in boosting up the triterpenes fermentation yield.

3.3 Optimization of the concentration of the filter-sterilized oleic acid additive for triterpenes fermentation enhancement

To determine the optimal concentration of filter-sterilized oleic acid addition to the triterpene fermentation medium, various levels of oleic acid at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mL/L were tested. The mycelial dry weight, glucose consumption rate and triterpenes production at the end of the triterpenes fermentations with the above level of oleic acid additive are presented in Fig. 3. The optimal level of oleic acid was found to be 30 mL/L. The mycelial dry weight and triterpenes production increased up to the 30 mL/L optimal oleic acid level. Both were lower with oleic acid was higher than 30 mL/L. However, glucose consumption rate continued to increase with higher than 30 mL/L oleic acid. It is conceivable that higher than 30 mL/L oleic acid in the triterpenes fermentation might lead to some damaging effect on the cell membrane. Consequently, 30 mL/L filter-sterilized oleic acid was experimentally proven to be the optimal level for the improvement of triterpenes fermentation production by *G. lucidum* G0119.

3.4 Determination of the optimal time for the addition of filter-sterilized oleic acid for enhanced triterpenes fermentation

The filter-sterilized oleic acid (30 mL/L) was added to the culture broth at 0, 24, 48, 72, 96 and 120 h. The mycelial dry

Figure 1. Effects of various exogenous additives on (A) mycelial growth, (B) glucose consumption rate and (C) triterpenes production during the fermentation process. Each value was presented as a mean $\pm$ standard deviation ($n = 3$).

Figure 2. Comparison of heat sterilization and filter-sterilization of oleic acid on mycelial growth, glucose consumption rate and triterpenes production during the fermentation process oleic acid additive. Each value was presented as a mean $\pm$ standard deviation ($n = 3$).

weight, glucose consumption rate and triterpenes production at various addition times are shown in Fig. 4. The above oleic acid addition times showed no significant difference on promoting mycelial growth and enhancing triterpenes production. By changing addition time, mycelial dry weight and triterpenes

production showed similar results. Accordingly, 30 mL/L oleic acid has been chosen to be added at 0 h for better operational efficiency. The resulting maximum mycelial dry weight (13.42 g/L) and triterpenes production (0.855 g/L) have been achieved.

Figure 3. Effects of the addition of various concentrations of filter-sterilized oleic acid at the start of fermentation on mycelial growth, glucose consumption rate and triterpenes production during the 192-h fermentation process. Each value was presented as a mean $\pm$ standard deviation ($n = 3$).

Figure 4. Effects of oleic acid addition time on the mycelial growth, glucose consumption rate and triterpenes production during the fermentation process. Each value was presented as a mean $\pm$ standard deviation ($n = 3$).

3.5 Determination of the significant factors for triterpenes production

Table 5 shows the P-B experimental design and analysis. The standard deviations, calculated using dummy variables, were not equal to 0 (Table 5), suggesting the existence of interactions amongst factors in the fermentation. The P values less than 0.05 are indicative of significant factors at the probability level of 95%. The p -value of 0.0050 for glucose indicates the most significant positive effect on triterpenes production. In addition, increasing the level of temperature and magnesium sulfate enhanced the triterpenes production significantly. The pH value, oleic acid, monopotassium phosphate, shaker speed, and inoculum volume did not significantly affect triterpenes production within the levels tested. Glucose is the main source of

glucose-6-phosphate and pyruvate which is transferred to acetyl coenzyme A in the metabolic pathway. Acetyl coenzyme A is the important precursor of triterpenes compounds [29]. Two other significant factors for mycelial growth and triterpenes production are temperature and magnesium sulfate which are the other two significant factors. These factors were optimized in the next step.

3.6 Optimization of triterpenes fermentation parameters with RSM

The relationship of the three aforementioned significant factors was investigated using RSM. Triterpenes concentration was the response variable. CCD was performed, and the levels of glucose

Table 5. Analyses of various variables on triterpenes production from the P-B design experiments

Variable	Estimated coefficient	Standard deviation	F value	<i>p</i> -value (Prob> <i>F</i>)
X ₁ -Oleic acid	0.0196	0.0203	0.9340	0.4358
X ₂ -Glucose	−0.3573	0.0254	197.8255	0.0050
X ₃ -Yeast extract	−0.0613	0.0152	16.1545	0.0567
X ₄ -Monopotassium phosphate	−0.0234	0.0135	2.9706	0.2269
X ₅ -Magnesium sulfate	−0.1268	0.0135	87.5371	0.0112
X ₆ -pH value	0.0034	0.0071	0.2329	0.6770
X ₇ -Temperature	0.0963	0.0102	89.7494	0.0110
X ₈ -Shaker speed	−0.0258	0.0102	6.4630	0.1261
X ₉ -Inoculum volume	−0.0462	0.0102	20.6881	0.0451

Table 6. CCD variables and the resulting triterpenes yields

Test	Variables			Triterpenes yield (g/L)
	Glucose (Gx ₁) (g/L)	Magnesium sulfate (Mx ₂) (g/L)	Temperature (Tx ₃) (°C)	
1	30.00	3.68	28.50	0.788 ± 0.040 ^a
2	30.00	2.00	28.50	1.031 ± 0.042
3	30.00	0.32	28.50	0.932 ± 0.031
4	20.00	3.00	25.00	0.744 ± 0.029
5	40.00	3.00	25.00	0.545 ± 0.021
6	46.82	2.00	28.50	0.139 ± 0.028
7	30.00	2.00	28.50	0.965 ± 0.033
8	40.00	3.00	32.00	0.256 ± 0.029
9	30.00	2.00	28.50	1.006 ± 0.037
10	30.00	2.00	28.50	0.974 ± 0.036
11	13.18	2.00	28.50	0.582 ± 0.032
12	20.00	3.00	32.00	0.366 ± 0.032
13	30.00	2.00	28.50	0.951 ± 0.039
14	20.00	1.00	32.00	0.421 ± 0.039
15	40.00	1.00	25.00	0.748 ± 0.011
16	30.00	2.00	22.61	0.871 ± 0.010
17	20.00	1.00	25.00	0.871 ± 0.025
18	40.00	1.00	32.00	0.281 ± 0.038
19	30.00	2.00	34.39	0.160 ± 0.034
20	30.00	2.00	28.50	0.987 ± 0.037

Each experimental data point represents the mean ± standard from three independent samples (*n* = 3).

(Gx₁), magnesium sulfate (Mx₂), and temperature (Tx₃) were optimized. In accordance with the experimental data of CCD (Table 6), sum of squares, mean squares, *F* value and *p*-value for the RSM model of triterpenes production are presented in Table 7. The response expressed in terms of variables Gx₁, Mx₂ and Tx₃ (coded value) is as follows:

$$\begin{aligned}
 Y = & 0.99 - 0.096Gx_1 - 0.048Ex_2 - 0.20Tx_3 \\
 & - 0.0055Gx_1Ex_2 + 0.089Gx_1Tx_3 + 0.031Ex_2Tx_3 \\
 & - 0.23Gx_1^2 - 0.050Ex_2^2 - 0.17Tx_3^2
 \end{aligned}$$

where *Y* is the dependent variable, the arithmetic mean response of the 20 runs is 0.99. The estimated coefficients for glucose (Gx₁), magnesium sulfate (Mx₂), and temperature (Tx₃) are −0.096, −0.048, and −0.20, respectively. The main effects (Gx₁, Mx₂ and Tx₃) represent the average result of changing one factor

at a time from a low to high value. The interactive terms (Gx₁ Mx₂, Gx₁ Tx₃ and Mx₂ Tx₃) show how the response changes when three factors are changed simultaneously. The polynomial terms (Gx₁², Mx₂², and Tx₃²) are included to investigate non-linearity. The regression model has been evaluated for model significance and the lack-of-fit. Analysis of variance (ANOVA) has been used for these evaluations. As shown in Table 7, both linear and quadratic terms of the equation are significant, indicating a nonlinear relationship between the influence variables and triterpenes concentration. The coefficient of determination for the regression equation is *R*² = 0.9887, suggesting that the model is a good fit to the experimental data. The adjusted *R*² indicates that the three influence factors identified in the P-B experiment fitted 97.86% of the variance in the response variable. In addition, the lack of fit Prob>*F* was 0.0861. Consequently, the model has practical significance.

Table 7. ANOVA of quadratic regression equation for the response surface

Source	Sum of squares	Mean square	F value	p-value
Model	1.7959	0.1995	97.43	< 0.0001*
Gx ₁	0.1266	0.1266	61.81	< 0.0001*
Mx ₂	0.0313	0.0313	15.28	0.0029
Tx ₃	0.5656	0.5656	276.19	< 0.0001*
Gx ₁ Mx ₂	0.0002	0.0002	0.12	0.7359
Gx ₁ Tx ₃	0.0006	0.0006	0.31	0.5906
Mx ₂ Tx ₃	0.0078	0.0078	3.81	0.0795
Gx ₁ Gx ₁	0.7382	0.7382	360.46	< 0.0001*
Mx ₂ Mx ₂	0.0356	0.0356	17.37	0.0019*
Tx ₃ Tx ₃	0.4233	0.4233	206.66	< 0.0001*
Residual	0.0205	0.0020		
Lack of fit	0.0162	0.0032	3.76	0.0861
Pure error	0.0043	0.0009		

*Values of “Prob > F” less than 0.0500 indicate model terms are significant.

R² = 0.9887; Adj R² = 0.9786.

Table 8. Confirmatory experiments for the optimal triterpenes fermentation production medium

Medium	Glucose (g/L)	Magnesium sulfate (g/L)	Temperature (°C)
Original	30.00	3.00	26.0
Center	30.00	2.00	28.5
Best	27.83	1.32	26.2

Table 9. Fermentation parameters with various media in 6 L fermentor

Parameters	Medium		
	Original	Center	Best
Initial glucose concentration (g/L)	29.07 ± 0.80 ^{a)}	29.04 ± 0.85	28.8 ± 0.90
Final glucose concentration (g/L)	11.06 ± 0.42	10.24 ± 0.48	8.56 ± 0.43
Fermentation time (h)	192	192	192
Maximum mycelial dry weight (g/L)	13.584 ± 0.550	14.932 ± 0.502	15.122 ± 0.561
Triterpenes yield (g/L)	0.846 ± 0.044	0.976 ± 0.048	1.076 ± 0.052
Glucose consumption rate (g/(L·h))	0.094 ± 0.003	0.098 ± 0.002	0.105 ± 0.003
Mycelial yield on glucose (g/g)	0.754 ± 0.026	0.794 ± 0.028	0.747 ± 0.032
Triterpenes yield on glucose (g/g)	0.047 ± 0.002	0.052 ± 0.002	0.053 ± 0.003
Mycelial productivity (g/(L·h))	0.071 ± 0.001	0.078 ± 0.002	0.079 ± 0.002
Triterpenes productivity (× 10 ⁻² g/(L·h))	4.408 ± 0.115	5.083 ± 0.108	5.604 ± 0.125

^{a)} Each experimental data point represents the mean ± standard from three independent samples (n = 3).

3.7 Confirmation by fermentation experiments in shake flasks and fermentors

To examine the validity of the empirical model, confirmatory experiments with the original medium, center medium and best optimal medium composition were carried out to verify the predicted value of triterpenes production in 6 L fermentor (Table 8). The changes of key variables (initial glucose concentration, final glucose concentration, fermentation time, maximum mycelial dry weight, triterpenes concentration, glucose consumption rate, mycelial yield on glucose, triterpenes yield on

glucose, mycelial productivity, triterpenes productivity) during the fermentation are shown in Table 9. The average triterpenes production yield from the confirmatory experiments is 1.076 g/L which is just 3.72% lower than the predicted value of 1.080 g/L. Our novel optimized medium achieved 2.47% higher triterpenes fermentation production yield of 1.054 g/L which was the highest previously published [24] (Table 1). In addition, our current triterpenes productivity of 0.056 g/(L·h) is 1.54 times higher than the best reported in the published literature of 0.036 g/(L·h) [20].

Figure 5. Profiles of pH and dissolved oxygen levels in liquid submerged fermentation of triterpenes by *G. lucidum* G0119 under the best optimized condition.

The statistically optimized results fit very well with the actual fermentation results. As shown in Fig. 5, addition of oleic acid led to relatively high dissolved oxygen level, which resulted in the increase of triterpenes production and faster glucose utilization. When the dissolved oxygen level was reduced, a concomitant increase in triterpenes production occurred. Furthermore, comparing with the control, the pH variation profile was similar to that with the addition of oleic acid.

As summarized in Table 1, previously published triterpenes production yield by liquid submerged fermentation were mostly relatively low. Only two reports showed similar triterpenes concentration to our current results. Zhang and Zhong [24] reported the previously highest triterpenes production yield of 1.050 g/L, but the fermentation time was 12 days with two-stage fermentation in shake flasks. Earlier, Tang and Zhong [20] obtained the highest triterpenes production of 0.976 g/L under culture condition with initial volumetric oxygen transfer coefficient and area of liquid surface per liquid volume. However, it took 24 days of fermentation time and two-stage fermentation in 7.5 L three-layer static bioreactor. We have developed and optimized a simple and effective strategy with addition of 30 mL/L oleic acid in liquid submerged fermentation with the fermentation time less than 192 h (8 days). Using this new process, the production of triterpenes was achieved by combination of high productivity and high yield. It is conceivable that such significantly improved fermentation production of triterpenes strategy could be implemented at the large industrial production scale. It is quite possible that presence of oleic acid might improve the permeability of the cell membranes conceivably leading to improve precursor substrates uptake and triterpenes secretion.

4 Concluding remarks

In this study, oleic acid was identified as the key exogenous additive. P-B design was applied to investigate ten fermentation variables to estimate the most important factors affecting triterpenes fermentation production. The optimal fermentation conditions determined by the CCD were 27.83 g/L glucose, 3 g/L yeast extract, 2 g/L KH_2PO_4 , 1.32 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 5.5, and 30 mL/L of filter-sterilized oleic acid has been identified and proven to be for triterpenes fermentation with concomitant

high productivity and yield conceivably feasible for the industrial scale.

Practical application

In this study, *Ganoderma lucidum* G0119 was used to investigate the improvement of triterpenes fermentation by statistical process optimization and addition of oleic acid. The actual triterpenes fermentation yield with the optimized medium based on confirmatory experimental data in 6 L fermentor was 1.076 g/L versus the statistical model predicted value of 1.080 g/L. A simple and efficient enhancement of triterpenes fermentation by the addition of filter-sterilized oleic acid in the fermentation medium was developed for liquid submerged fermentation. The resulting fermentation time was 192 h. Using this strategy, the significantly improved production of triterpenes was achieved with high yield and high productivity. It is conceivable that such significantly improved fermentation of triterpenes strategy could be implemented at the large industrial production scale.

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