Impact of Rifampin Induction on the Fermentation Production of Ganoderic Acids by Medicinal Mushroom *Ganoderma lucidum*

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Abstract

Backgrounds and Objectives: Ganoderic acids are the most valuable secondary metabolites in *Ganoderma lucidum* traditional medicinal mushrooms, which have shown antitumor properties in many studies. However, application of ganoderic acids is limited due to low yield production. Recently, it was shown that static liquid culture could be a proven technology for producing ganoderic acids in *Ganoderma lucidum*, and that applying elicitors could be a potential strategy to improve their production.

Materials and Methods: In this work, the effect of rifampin, a cytochromes P450 inducer, on production of ganoderic acids was studied, and Response Surface Methodology was applied to optimize the elicitor induction. Then total ganoderic acid in the harvested mycelia was extracted and its absorbency was measured.

Results and Conclusion: The results showed an increase in the concentration of ganoderic acid in all samples. Moreover, optimum concentration and induction time of rifampin were obtained. The proposed model predicted the maximum ganoderic acid production as 18.6 mg g^{-1} in which the optimal concentration and time induction obtained were 100 μM and day 9, respectively. This work demonstrated a useful method for the enhanced production of ganoderic acids by *Ganoderma lucidum*.

1. Introduction

Ganoderma lucidum is a medicinal mushroom that has been used as a Chinese traditional folk remedy for centuries [1]. Today a variety of commercial *G. lucidum* products are available, such as powders, dietary supplements, tea and coffee [2]. In addition, most compounds of this fungus have medicinal properties. Therefore, increasing the production of these compounds can be useful [3,4]. More than 150 compounds have been separated from *Ganoderma spp*. [5,6]. Ganoderic acids (GAs) extracted from *G.*

lucidum have been reported to be responsible for many of the pharmaceutical activities of this fungus.

However, *G. lucidum* usually takes several months to form its fruiting body under culture conditions. It is also difficult to control the quality of the produced fruiting body [7]. Thus, submerged fermentation of *G. lucidum* is observed as a replacement for efficient production of GAs [8,9]. However, in spite of the improvements made in recent years, yields of GAs in *G. lucidum* mycelia and the fermentation through my-

celia are still low; this presents a major hurdle in the commercialization of the production of GAs [10,11]. Static liquid culture of *G. lucidum* is a proven technology for production of GAs [12]. Induction using an elicitor is an effective method of enhancing the production of secondary metabolites [13].

Recently, Liang et al reported the enhanced production of GAs under induction by phenobarbital in the submerged culture of *G. lucidum* [14]. GA biosynthesis involves a series of reactions including hydroxylation, oxidation, and demethylation, most of which are presumed to be catalyzed by P450-type enzymes [14].

Rifampin strongly induced cytochrome P-450 3A and 2C dependent enzyme [15]. Given the importance of P450 in the regulation of biosynthesis of secondary metabolites such as GAs; it is reasonable to consider the addition of a P450 inducer as a potential strategy to enhance the production of GAs [16]. Rifampin is a typical P450 inducer that can increase transcription of several plant cytochrome P450 enzymes [17,19].

In this work, rifampin was added during the static liquid culture step of a two-stage cultivation of *G. lucidum* to improve the production of GAs. After screening, the induction process was optimized using a response surface methodology (RSM) in order to predict the levels of the key factors for a maximized production of GAs.

2. Materials and Methods
2.1. *G. lucidum*

G. lucidum CCGMC 5.616, provided by the Industrial Fungi Biotechnology Research Department of Ferdowsi University of Mashhad, was maintained on potato dextrose agar plates, and stored at 4°C.

2.2. Medium and culture conditions

The pre-culture was prepared in Yeast extract-Peptone-Glucose *medium* and incubated for 5 days at 30°C and 150 rpm. Then 1 ml of it was inoculated into a 100 ml flask containing 30 ml medium. The flask was incubated on a rotary shaker for 4 days at 30°C and 150 rpm. After this initial shake flask culture period, it was converted into static liquid culture [20].

2.3. Addition of elicitor

Rifampin was purchased from Hakim Pharmaceutical Company (Tehran, Iran). It was dissolved in dimethyl sulphoxide (DMSO) [21] and sterilized by filtering through 0.22 μm polyvinylidene difluoride syringe before being added to the cultivation medium at three different final concentrations (50,500, and 1000 μM) on day 9 after culture. Each 100-ml flask contained 30 ml broth. The DMSO concentration in the broth was 0.05 %vv^{-1} following the addition of rifampin. DMSO was also added to some cultures at the same concentration but without rifampin induction as a control. After 14 days, the mycelia floating in each flask were harvested, washed and centrifuged at 12500 ×g for 5 min with distilled water; then they

were dried at 50°C under a rotary vacuum, followed by measuring their weight. Cell dry weight measured using gravimetric method.

2.4. Experimental design for RSM

After confirming the effect of rifampin on increasing the production of GAs, RSM was carried out in order to find the optimum conditions for the extraction of GAs. Central composite design was used in order to optimize the dependent variables (i.e. CDW and GAs) as responses while the independent variables chosen were rifampin concentration and induction time (Table 1).

Therefore, to investigate the effect of rifampin, 13 experiments were designed using Minitab software (ver. 16.2.4.4). The statistical analysis of the model was performed in the form of Analysis of Variance. The quadratic models were represented as response surface graphs, which give the infinite number of combinations of the two factors selected keeping the other constant. The optimization of the process was aimed at finding the optimum values of independent variables (rifampin concentration and induction time), which would give maximum GA production. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on GA production. The optimum values of the selected variables were obtained both by solving the regression equation and also by analyzing the response surface contour plots. ($p \leq 0.05$) Finally, the model was used to predict the optimum value of the factors, which gives maximum GAs.

Table 1. Variable levels for optimization of GA production

Variable	Level (-1)	Level (0)	Level (1)
Concentration of rifampin (μM)	10	100	190
Time (day)	6	9	12

2.5. GA measurement

Total GAs in the harvested mycelia was extracted as previously described [20]. The dried mycelia (100 mg) were extracted by 50 %vv^{-1} ethanol (3 ml) for 1 week (twice). After removal of mycelia by centrifugation, the supernatants were dried at 50°C under vacuum. The residues were suspended in water followed by extraction with chloroform. The GA existing in the chloroform extract was further extracted with 5 %wv^{-1} NaHCO$_3$. After adding 2N HCl to adjust the pH of the NaHCO$_3$ phase to be lower than 3.0, the GAs in the NaHCO$_3$ phase were again extracted with chloroform. After removal of chloroform by evaporation at 40°C, the GAs were dissolved in absolute ethanol, and their absorbency was measured at 245 nm in a spectrophotometer using thymol as standard.

3. Results and Discussion
3.1. Effect of rifampin induction on the cell growth and production of Gas

The concentration and induction time of rifampin certainly affected the yield of CDW and GA. In the first step, tests for investigating rifampin induction on GA production were carried out. *G. lucidum* cells were treated with 50, 500 and 1000 μM rifampin on the day 9. The CDW and yield of GAs measured on the day 14 are summarized in Table 2. *G. lucidum* cell growth was inhibited slightly by DMSO and more severely by rifampin. On the other hand, the CDW and yield of GAs showed very little change compared to the control when DMSO was added. Induction with 50 μM rifampin had strong effect, while 1000 μM rifampin increased the yield of GAs slightly. Increasing the concentration to 1000 μM decreased the yield slightly compared to 50 μM. The results indicated that the optimal rifampin dosage for induction was around 50 μM.

Table 2. The effect of rifampin concentration on cell dry weight and yield of GAs on the day 9 of static liquid culture

Culture conditions	CDW (g l^{-1})	Gas (mg g^{-1} CDW)
Control	12.2	8.6
DMSO	10.4	7.6
Rifampin (50 μM)	10.1	16.4
Rifampin (500 μM)	9.3	15.6
Rifampin (1000 μM)	7.8	10.1

All experiments were done in triplicate, and the SD of each data was <10%.

3.1. Optimization of concentration and induction time of rifampin

The experiments were designed to evaluate the impact of concentration and induction time of rifampin. The CDW and yields of total GAs measured are summarized in Table 3. The results suggest that these variables significantly affect the production yield.

The total GAs showed different stimulatory responses to rifampin induction at different growth stages. Many parameters can influence the performance of GA production and CDW rate by rifampin. Figure 1 illustrates the contour and surface plots of CDW response versus the variation of the two significant parameters. The equations revealed that elicitor concentration had a strong negative linear effect, and induction time had a positive linear effect on CDW ($p \leq 0.05$). In the all samples treated with rifampin, CDW was decreased compared to the control.

However, these two variables had no linear effect on the Gas' extraction rate; the middle variables also indicated positive effects on the extraction rate ($p \leq 0.05$) (Figure 2). Higher yields of total GAs were observed when the cells were exposed to 100 μM rifampin on the day 9 (dark green regions in Figure 2).

Thus, day 9 was the optimal induction time. Induction of 190 μM rifampin had little effect, while 100 μM rifampin increased the yield of GAs. Increasing the concentration to 190 μM decreased the GA yield slightly compared to decreasing the concentration to 10 μM. Figure 2 shows that the effect of induction time on GAs production is higher than that of concentration. The polynomial model for CDW rate and GAs was regressed by mainly considering the significant terms:

$$CDW = 10.2153 - 0.6166C + 0.6333T$$

$$GAs = +17.54000 - 0.42678C - 0.89246T - 1.77000C^2 - 2.47000T^2$$

The significance of each coefficient was determined using the F-test and p-value in Table 4. The corresponding variables would be more significant if the absolute F-value becomes greater and the p-value becomes smaller. According to Table 4, p-value is less than 0.05 for quadratic term of rifampin concentration (A^2) and time induction (B^2). As a result, optimum GAs production is a function of the mentioned terms. The maximum production of GAs under the optimum conditions was predicted as 18.6 mg g^{-1}. In order to proving the authenticity of optimal conditions, it was repeated again. Concentration and induction time of rifampin were obtained as 91 μM and day 9, respectively. The coefficient of determination (R^2) of the predicted model was 0.8877 for CDW and 0.9135 for GAs, suggesting a good correlation; the predicted model seemed to reasonably represent the observed values.

Thus, the response was sufficiently explained by the model, and concentration and induction time were effective parameters for having higher regression coefficients.

Table 5 shows comparison between the present study and other studies. Accordingly, Liang et al. for the first time used phenobarbital as a inducer and observed that addition of 100 μM phenobarbital on day 9 was found to be optimal resulting in a maximal amount of total GAs (41.4 mg/g cell dry weight) and 64% increase in the level of GAs in the treated cells. Phenobarbital and rifampin are typical P450 inducers that can increase transcription of several different cytochrome P450 isoenzymes. Rifampin affects on CYP2C19 and CYP3A4 isoenzymes, and phenobarbital affects on CYP2B1 and CYP2C isoenzymes [16]. Because of the differences in the affected isoenzymes of these two elicitors, difference in GA production seems to be reasonable. However, the maximum percentage of increase in GA production was obtained by induction of 10 mM Mn^{2+} on the day 4. Mn^{2+} plays important role in the cellular physiology and metabolism of various organisms. Addition of Mn^{2+} enhances GA production in the liquid culture of *G. lucidum* through inducing calcineurin signal pathway [22].

Zhang et al. evaluated the effect of cellulase as an elicitor on GA production by two-stage cultivation of *G. lucidum*, using lactose as a carbon source. Loading of 5 mg l^{-1} cellulase on the day 3 resulted in the maximal GA titer of 1608 mg l^{-1}.

Because of the differences in growth conditions, medium and cultivation time, GAs productivity are different. However, compared to the control, cellulase increased GA production by 21% [23].

Table 3. Full factorial central composite design for evaluation of the two variables of time and rifampin concentration on production of GA and biomass

Experiment number	Variables		Responses	
	Concentration of rifampin (μM)	Time (day)	CDW (g l^{-1})	GAs (mg g^{-1} CDW)
1	10	6	10.4	12.2
2	10	12	11.6	11.6
3	190	6	9.2	11.2
4	190	12	10.8	10.6
5	100	6	9.8	16.6
6	100	12	10.8	12.4
7	10	9	10.6	16.4
8	190	9	8.9	15.4
9	100	9	10.1	18.6
10	100	9	9.8	17.1
11	100	9	10.3	16.4
12	100	9	10.1	17.6
13	100	9	10.4	18

Table 4. Regression coefficients for determination of variable significance on CDW and GAs and their significance for the response surface model

| Independent factor	Regression coefficient		F-value		P-value	
	CDW	GAs	CDW	GAs	CDW	GAs
Constant	10.0586	17.7414	78.374	14.780	0.001	0.0010
Con	-0.6167	-0.500	23.88	0.200	0.002	0.3000
Time	0.6333	-0.900	25.19	4.000	0.002	0.0870
Con*Con	-0.1052	-2.344	0.32	12.090	0.510	0.0100
Time*Time	0.4448	-3.74483	5.72	23.370	0.048	0.0010
Con*Time	0.1000	-1.273	0.42	0.200	0.538	0.9960

R^2 (CDW)= 0.8877
R^2 (GAs)= 0.9135

Table 5. Comparison between the present and other studies that have increased production of total GA yield

Elicitor	Concentration and time induction	Statistical analysis	GA content (mg g^{-1} CDW)	GAs productivity (mg l^{-1}) compared by their controls	Percentage increase of total GAs compared by their controls	Ref.
Rifampin	Conc : 100 μM Time :day 9	RSM	18.6	186 Cont: 104.9	77%	Present study
Mn^{2+}	Conc :10mM Time : day 4	-	55	990 Cont: 450	124%	[22]
Cellulase	Conc :5 mg l^{-1} Time : day 3	-	14.1	1608 Cont: 1334.5	21%	[23]
Phenobarbital	Conc :100 μM Time: day 9	-	41.4	738 Cont: 450	64%	[14]

Insect Extract (Catharsius molossus)	Conc :200 mg l^{-1} Time : day 0	-	20.8	313.7 Cont:231.7	35%	[24]
Fungal Extract (Penicillium citrinum)	Conc :60 mg l^{-1} Time :day 8	-	23	315.5 Cont: 264	19%	[25]
Methyl jasmonate	Conc :254 μM Time :day 6	-	40.5	-	45%	[26]

a

b
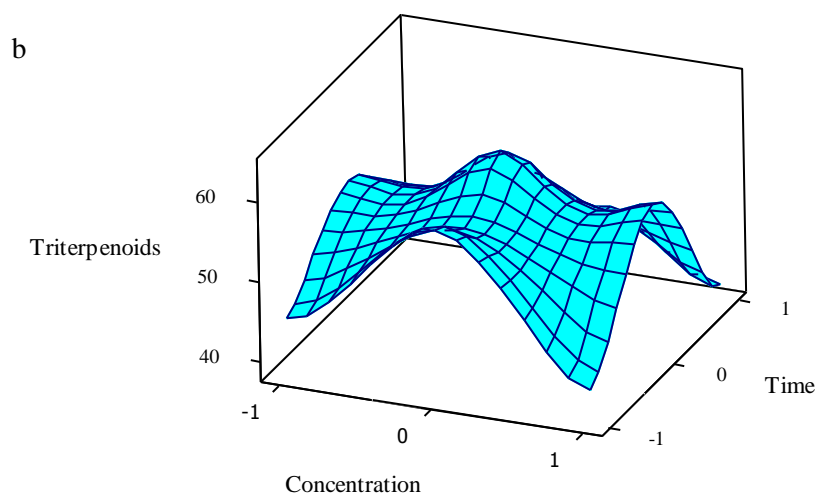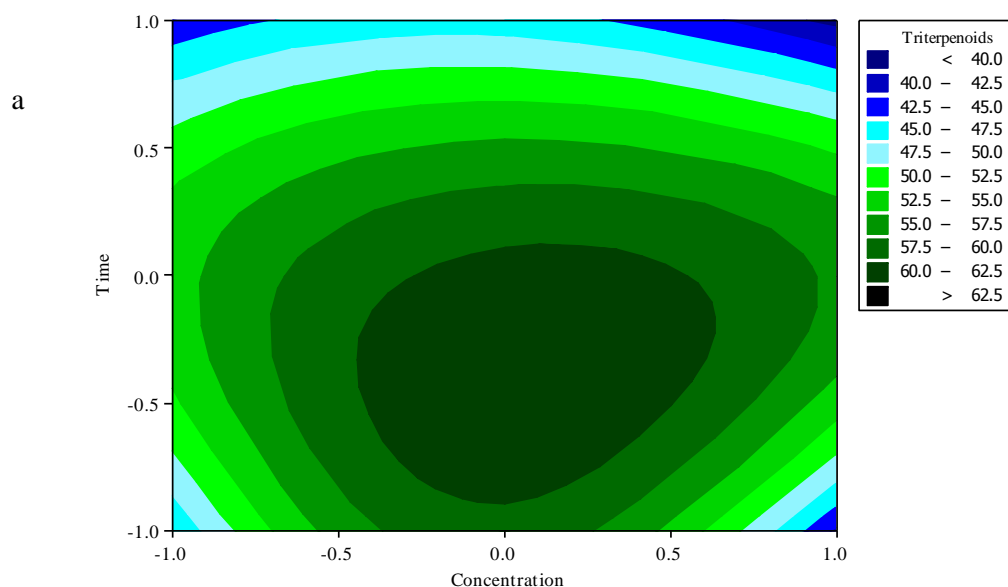
Figure 1. Contour (A) and surface (B) plots for response (CDW) with respect to concentration and time (legend; CDW: Cell dry weight).
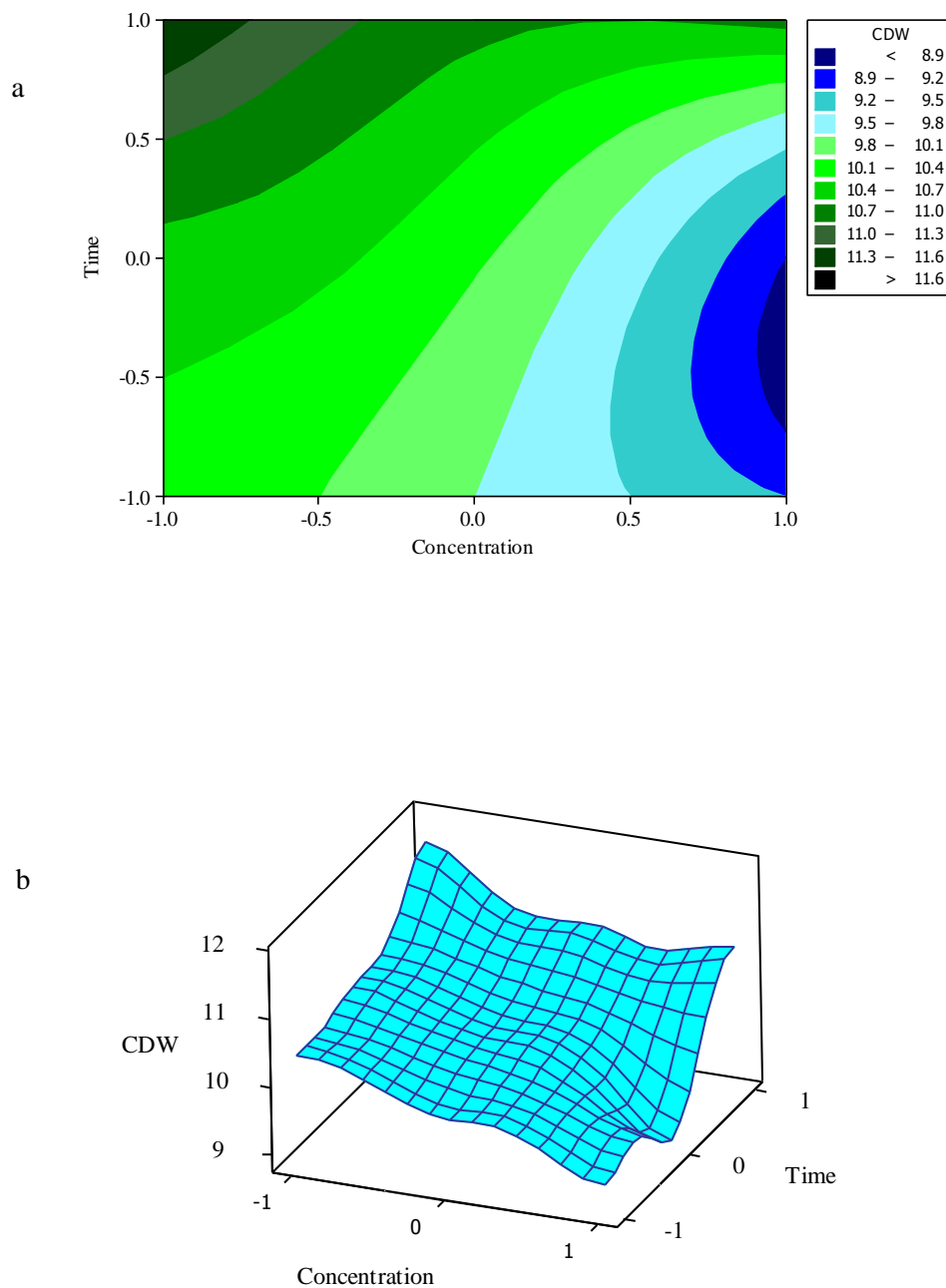
Figure 2. Contour (a) and surface (b) plots for response (GAs) with respect to concentration and time (legend; CDW: Cell dry weight).

In addition, the use of bio-extracts of insects and fungi could increase GA production by 35% and 19%, respectively [24,25]. According to the results, the ether extract of *Catharsius molossus* at 200 mg l^{-1} concentration led to a significant increase in GA concentration from 231.7 to 313.7 mg l^{-1} [24] while the maximum GA production (315.5 mg l^{-1}) was obtained via elicitation of polysaccharide elicitor from *Penicillium citrinum* [25].

Ren et al. focused on methyl jasmonate induction that influenced GA biosynthesis and determined that addition of 254 μM methyl jasmonate solubilized in Tween-20 to the culture on day 6 could increase GAs production of 4.52 mg g^{-1} dry weight. The resulting GA yield was 45.3% higher than the untreated control sample [26]. This work has presented experimental data on the use of rifampin induction as elicitor. At the end of study, 84% increase in GA production was observed and the rifampin induction was optimized using RSM".

It provides effective strategy for enhancing the production of total GAs in *G. lucidum* in response to treatment with a strong P450 inducer.

4. Conclusions

The concentration and induction time of elicitor are critical to the manipulation of secondary metabolite accumulation [27,28]. In this work, the addition of rifampin at the day 9 inhibited cell growth and greatly reduced the production of GAs (Table 1). RSM was effectively used to determine the optimum concentration and induction time of rifampin. Based on the profiles of RSM, induction of 100 μM rifampin on the day 9 retained higher amount of GAs whereas higher CDW was obtained in the lowest concentration and the final day. It can provide a simple and effective strategy for enhancing the production of total GAs by *G. lucidum* fermentation.

5. Acknowledgment

6. Conflict of interest

The authors have declared that there is no conflict of interest.

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