Research Article

pISSN: 2345-5357
eISSN: 2423-4214

APPLIED FOOD BIOTECHNOLOGY, 2024, 11 (1):E7
Journal homepage: www.journals.sbmu.ac.ir/afb

OPEN ACCESS

Ganoderic Acid Production via Aerial Co-cultivation of *Ganoderma lucidum* with *Bacillus subtilis* and *Aspergillus niger* Using Bubble Column Bioreactor

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Abstract

Background and Objective: *Ganoderma lucidum*, with its medicinal characteristics, is one of the most beneficial fungi in traditional Asian medicine. This fungus low efficiency of ganoderic acid production has limited its use as a valuable secondary metabolite. Environmental stresses and elicitors such as microbial volatile organic compounds in co-cultures can increase ganoderic acid production. To investigate effects of variables of co-culture time and volume on *Ganoderma lucidum* growth and ganoderic acid production, *Bacillus subtilis* and *Aspergillus niger* were aerially co-cultured with *Ganoderma lucidum*.

Material and Methods: To investigate fungus growth and production of ganoderic acid using bubble column bioreactor, effects of independent variables of temperature, initial inoculation, length-to-diameter ratio (L: D) and aeration were investigated using Taguchi method. Then, effects of co-culture of *Ganoderma lucidum* with *Bacillus subtilis* and *Aspergillus niger* under optimum conditions were investigated.

Results and Conclusion: Optimizing effects of co-culture time and volume variables led to 2.9-fold increases in production of ganoderic acid, compared to the control sample. Optimization of biomass production in the bioreactor showed that biomass production increased significantly by increasing the initial inoculation percentage and temperature. These two variables significantly affected ganoderic acid production and its optimum production point was 10% of initial inoculation, temperature of 25.6 °C, L: D of 4:8 and aeration rate of 0.64 vvm. Gas holdup investigation for air-water and air-fermentation media systems showed that the presence of suspended solids and aeration rate affected gas holdup. Microbial volatile organic compounds in co-culture of microorganisms can increase ganoderic acid production by *Ganoderma lucidum*.

Conflict of interest: The authors declare no conflict of interest.

Article Information

Article history:
- Received 27 Oct 2023
- Revised 5 Dec 2023
- Accepted 25 Dec 2023

Keywords:
- *Ganoderma lucidum*
- Ganoderic acid
- Aerial co-cultivation
- Bubble column bioreactor
- Taguchi method

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How to cite this article?

Kianirad S, Shakiba D, Hatamian-Zarmi A, Mokhtari-Hosseini Z-B, Ebrahimi Hosseinzadeh B. Ganoderic Acid Production Via Aerial Co-cultivation of Ganoderma lucidum with Bacillus subtilis and Aspergillus niger Using Bubble Column Bioreactor. Appl Food Biotechnol. 2024; 11 (1): e7. http://dx.doi.org/10.22037/afb.v11i1.43684

1. Introduction

Mushrooms have been used in traditional medicine and nutrition for centuries due to their various medicinal characteristics and their popularity continues to grow in the modern era as their potential health benefits are revealed. Mushrooms contain a wide range of bioactive compounds, including polysaccharides, terpenoids, phenolic compounds, vitamins and proteins, which have been shown to possess antioxidant, anti-inflammatory, immunomodulatory,anti-cancer, probiotic, antimicrobial and anti-diabetic characteristics [1,2]. Studies show that extracts from most of these fungi are used as dietary supplements and medications in traditional Chinese medicine and numerous of their compounds are currently produced on industrial scales [3,4]. *Ganoderma* (*G.*) *lucidum* is one of the medicinal fungi that has been interested by the researchers and scientists. This fungus is known in Asian countries as the fungus of immort-

ality and its use has begun since nearly two thousand years ago [5].

The nutritional profile of *G. lucidum* shows that the fungus includes significant quantities of water-soluble proteins, dietary fibers and major minerals. Moreover, *G. lucidum* is an affluent source of multiple vitamins, including riboflavin and niacin [6]. Overall, this superfood with high nutritional benefits is used worldwide. The *G. lucidum* can treat migraines, hypertension, arthritis, gastritis, diabetes, lupus erythematosus, hepatitis and cardiovascular diseases [7]. Therefore, it can be addressed as a curative functional food. These characteristics are linked to the secondary metabolites of *G. lucidum*, including polysa-ccharides, phenolic compounds and terpenoids [8]. Of these compounds, polysaccharides and triterpenes have mostly been studied [9]. Triterpenes are a large group of organic compounds with an aromatic structure [10,11]. Ganoderic acid (GA) and ganoderic alcohols belong to this group. Naturally, GA has received further attentions due to its similarity to lanoxin and oxygen structure [12]. Studies show that GAs can inhibit cholesterol synthesis, tumor growth and hepatitis B virus antiviral activity. Recently, activity of GA against the human immunodeficiency virus has been reported [13-17].

Low production of GA has limited its use as a valuable secondary metabolite. Since the fruiting body of *G. lucidum* needs nearly six months to produce [11], submerged culture of this fungus is used to increase the production. The *G. lucidum* cell growth and production efficiency of its metabolite can be affected by physicochemical factors of the culture media and extraction methods [18]. Esmaelifar et al. detected that *Agaricus (A.) bisporus* extract, maltose and vitamin B_1 significantly increased the antioxidant activity of *G. lucidum* polysaccharides. They showed that the cultivation of *G. lucidum* in bioreactors increased the cell growth [19]. In 2011, production conditions of GA in bioreactors were studied regarding factors such as pH, oxygen pressure and shear stress [20]. Heydarian et al. reported the highest GA production in the initial glucose concentration of 35 g. l^{-1} and presence of KH_2PO_4 and vitamin B_1 [21]. Moreover, other methods have been reported to increase GA production efficiency. Further investigating various extraction methods, Zhen et al. achieved high yields of GA using *in situ* effervescence reaction-assisted mechanochemical extraction method by grinding *G. lucidum* with sodium bicarbonate and citric acid [22]. Sun et al. chose a various route to enhance the GA production using high spore-producing strain of *G. lucidum* and protoplast of dikaryotic CGMCC 5.0026 strain. Promotion of sporulation led to 1.7-3.42 times increases in GA-T, GA-Mk and GA-Me, compared to the original strain [23]. Heat stress is an alternative option to induce GA production. By the upregulation of two spermidine biosynthesis genes under heat stress, GA production increased significantly compared to the wild-type control. It was concluded that heat stress promoted the conversion of spermidine from putrescine, which included inhibitory roles in GA biosynthesis [24].

Microbial volatile organic compounds (mVOCs) are other affectors that stimulate growth and production of secondary metabolites. Some of these compounds are produced only in co-cultures and by exposure of microorganisms to competitive conditions [25]. Therefore, studies of indirect and aerial connections between the microorganisms in aerial co-cultures are critical. Despite the great importance of mVOCs and their roles in nature, limited studies have been carried out in this field. In previous studies, the current authors investigated effects of aerial co-cultures on the growth and metabolite production of *G. lucidum*. Kalantari et al. showed that aerial co-cultivation of *G. lucidum* with *Bacillus (B.) subtilis* and *Pseudomonas aeruginosa* led to decreases in growth of the mycelia of this fungus. Furthermore, quantity of GA increased more than 2.8-fold. Studies have shown that this effect is linked to changes in cell membrane permeability, cell wall composition and structure and DNA damage [26]. It has been shown that aerial co-cultivation of *G. lucidum* with *Pleurotus ostreatus* for six days increases the exopolysaccharide specific yield by at least 2.2-fold [27]. Hatamian-Zarmi et al. increased biomass and exopolysaccharide production of Shiitake mushrooms under the effects of mVOCs of *Aspergillus niger* and *Schizophyllum commune* in aerial co-culture [28]. Regarding the current authors' studies on investigating factors affecting production of GA and optimizing production of this metabolite, effects of aerial solid state/liquid state co-culture of *G. lucidum* with *A. niger* and *B. subtilis* microorganisms on biomass growth and GA production were investigated in the present study. Furthermore, production conditions of GA in bubble column bioreactor were optimized and co-culture of the microorganisms in the bioreactor was investigated.

In this study, factors such as co-culture time and volume, initial inoculation percentage and temperature, various L: Ds and aeration rate were optimized and then effects of environmental stress and stimuli on *G. lucidum* biomass and GA production were investigated.

2. Materials and Methods

2-1- Microorganisms and chemicals

The *G. lucidum* was provided by the microbial bank of the Biotechnology Laboratory, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran, with CCGMC 5.616 strain number. Moreover, *A. niger* (UTMC 5018) and *B. subtilis* (UTMC 1416) were provided by the Persian Type Culture Collection and the Microbial Technology and Product Research Center, University of Tehran, Tehran, Iran. Nutrient agar, nutrient broth, potato

dextrose agar (PDA) and potato dextrose broth culture media and other chemicals were purchased from Merck, Germany.

2-2- Preparation of solid co-culture media of Ganoderma lucidum and growth study

The aim of this study was to compare quantity of the culture volume on various times of co-culture (Days 0, 2 and 4). Hence, 15 ml of PDA media for the cultivation of *A. niger* and *G. lucidum* were poured into 100 ml flasks and placed horizontally for a few hours to solidify the agar. Then, the first 2 cm of all flasks were marked as the starting line. The NA medium was used for the cultivation of *B. subtilis* and the previous steps were repeated. After preparing the fungal culture media, 5-mm discs were separated from a PDA plate containing *G. lucidum* and placed at the end of the flask before the starting line. Then, all inoculated flasks were incubated at 30 °C and regular checking was carried out. As soon as each fungus reached the starting line, it was transferred to the refrigerator. After the growth of microorganisms up to the mark line, all the flasks reached the starting line and were transferred to the refrigerator; thus, all flasks were synchronized. To co-cultivate with *B. subtilis*, the microorganism was cultured in a separate flask. Then, the *G. lucidum* flask was air-tightly attached to the *B. subtilis* flask on Days 0, 2 and 4 of incubation. Connection was sealed with parafilm to prevent air from entering or leaking out of the system. System was then transferred into an incubator set at 30 °C. The incubation period typically included nearly a week. After growth was complete, growth length was investigated for one of the flasks using ruler. To co-culture *G. lucidum* with *A. niger*, the previous steps were repeated.

2-3- Preparation of liquid culture media for the co-culture of Ganoderma lucidum

To optimize quantity of GA produced, appropriate values of co-culture time and volatile flask volume variables were assessed through the co-culture of *G. lucidum* with *A. niger* and *B. subtilis* in various conditions. To prepare the liquid culture, 40 ml of potato dextrose broth media were prepared in 100 ml flasks for *G. lucidum* and *A. niger* and 40 ml of NB media were prepared for *B. subtilis*. Then, 1 ml of inoculum prepared for *G. lucidum* was added to the fungus flask and incubated at 30 °C and 180 rpm (Jaltjahiz, Iran). To aerial co-culture, on days 6, 8 and 10, liquid cultures of *B. subtilis* and *A. niger* were connected to *G. lucidum* (Fig 1, A, B). On Day 12, biomass and GA extraction was carried out based on Fang et al. [29] protocol. Due to the bacterial and fungal growth cycle differences, *B. subtilis* was cultured in fresh flask 3 d after culture and replaced the previous flask. Liquid culture was carried out to assess effects of 2 and 3 times the volume of the volatile flask (*B. subtilis*) and each flask of *G. lucidum* was co-cultured with two or three flasks of *B. subtilis* to assess its effects on GA production.

2-4- Biomass separation and ganoderic acid extraction

After separating biomass from the supernatant using filter papers, biomass was dried via freeze-drying. For extracting GA, 3 ml of absolute ethanol were added to 100 mg of the dried biomass for 1 w (this step was repeated twice). Biomass was centrifuged (AWEL MF 20-R, France) at 5000 g for 15 min. and the precipitate was dissolved in a few milliliters of water. Then, an aqueous solution was extracted with 2 ml of chloroform; GA was extracted from chloroform with sodium hydrogen carbonate solution (5%). The solution pH was regulated by adding hydrochloric acid (pH<3). The final extraction was carried out using chloroform and quantity of GA was assessed by dissolving it in chloroform using optical spectroscopy.

2-5- Optimization of ganoderic acid production in bubble column bioreactor using Taguchi method

Briefly, *G. lucidum* was cultured in a bubble column bioreactor to study growth and production of GA. Taguchi method was used to investigate effects of independent variables such as temperature, initial inoculation size, L:D and aeration rate on GA production (Fig 1, C).

2-6- Co-culture in bioreactor

After reporting optimal conditions of GA production, co-cultures of *G. lucidum* and *B. subtilis* were carried out to assess the co-culture effects. Therefore, *B. subtilis* was cultured and the exhaust air was connected to a bioreactor containing *G. lucidum* using silicone tube. Every 24 h, 100 ml of NB media were added to the bioreactor for the continuous culture of bacteria. Unlike the flask, culture period included 5 d in the bioreactor. Hence, co-culture was carried out from Day 1.

2-7- Assessment of gas holdup in bioreactor

Since the bioreactor was made of glass, the liquid level could be seen. Thus, the liquid level could be assessed in aerated and non-aerated states. Gas holdup could be calculated using equation 1 [30].

$$\varepsilon = (H_B - H_R)/H_B \quad \text{Eq. 1}$$

Where, H_R was height of the non-aerated liquid and H_B was height of the aerated liquid. However, the method accuracy decreased during the formation of foam and this method could not be used in metal and non-transparent reactors. The gas holdup included a significant dependence on the gas aeration rate, which could be calculated by Eq 2 [31].

$$\varepsilon = \alpha v_g^{\ \beta} \quad \text{Eq. 2}$$

In general, α and β are constants included various values depending on the type of sparger and flow regime [31]. With increases of gas velocity, dependence of gas holdup on gas velocity decreased. This dependence occurred in non-Newtonian and Newtonian fluids such as biological suspensions and fermentation with solid raw materials.

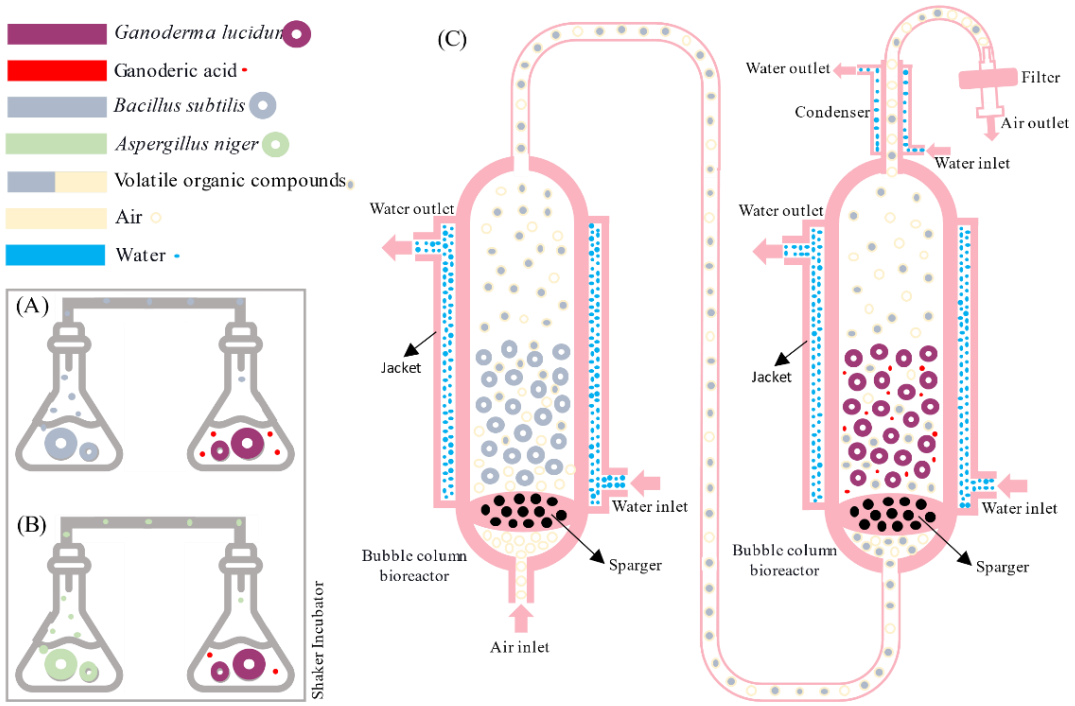

Figure 1. (A) Co-culture of *Ganoderma lucidum* and *Bacillus subtilis* in flasks; (B) co-culture of *Ganoderma lucidum* and *Aspergillus niger* in flasks; and (C) co-culture of *Ganoderma lucidum* and *Bacillus subtilis* in bubble column bioreactor.

For homogeneous flows, β values were in the range of 0.1-1.2 [30]. This study assessed gas holdup in water-air and culture media-air systems at four aeration levels of 0.5, 1, 1.5 and 2 vvm and four L: D levels of 1:6, 3:2, 4:8 and 5:1.

3. Results and Discussion

3-1- Assessment of affecting variables on Ganoderma lucidum growth and ganoderic acid production

One factor at a time approach was used to investigate affecting variables of co-culture time of *G. lucidum* with *B. subtilis* and *A. Niger* and volume of the volatile flask on *G. lucidum* growth and GA production.

3-1-1- Investigation of affecting variables on Ganoderma lucidum growth

To show effects of volume on *G. lucidum* growth rate in co-culture system, two-fold volume of the volatile flask (co-culturing of *G. lucidum* with two flasks of *A. niger* and *B. subtilis*) was investigated (Tables 1 and 2). Results showed that the co-culture with *A. niger* and *B. subtilis* led to decreases in the growth of *G. lucidum*. On Day 1, limited growth rates were seen in co-culturing samples, indicating higher effects of the microbial volatile organic compounds (mVOCs) on the fungi. Moreover, less growth rates were observed in co-cultured samples on Day 4 due to the insignificant effects of mVOCs. These results verified effects of mVOCs on the fungal growth.

Furthermore, assessment of the extracted GA from *G. lucidum* in co-cultures with *B. subtilis* in various volumes showed that the GA content in two-fold volumes was 2.31 times greater than that of the control.

3-2- Optimization of Ganoderma lucidum growth and ganoderic acid production in bioreactor

Out of the affecting factors on GA production, inoculum size (A), temperature (B), L: D (C) and aeration rate (D) were chosen as the major factors for the optimization using Taguchi method. These factors were optimized at three levels with the L9 array of Taguchi (Table 4). Dry weight of the biomass and produced GA were assessed as responses. Results were analyzed using Design Expert 11 software. The highest quantity of GA was produced in the L5 array with 10% inoculum size, temperature of 25 °C, L: D of 4:8 and aeration rate of 10 VVM. Therefore, 0.813 mg GA per 100 mg of biomass and 2.8 g biomass per litter were produced.

3-2-1- Optimization of Ganoderma lucidum growth in bioreactor

Results of optimization of *G. lucidum* growth indicated that temperature, L: D, aeration rate and multiplication of the initial inoculum size by temperature (AB) included significant effects on dry biomass weight (p<0.05) (Fig. 2). Moreover, R^2 of 99% suggested appro-priate overlapping of the experimental data and predicted numbers of the presented model for dry biomass weight.

Dry weight biomass = 22.066 - 4.153A - 0.599B - 0.733213C + 1.60D + 0.144AB + 0.065AC

3-2-2- Optimization of GA production in bioreactor

Results showed that initial inoculum size, temperature, aeration rate, multiplication of inoculum size by temperature (AB) and multiplication of the initial inoculum by the length-to-radius ratio (AC) significantly affected the GA production (p<0.05) (Fig. 3). Moreover, R^2 of 99% indicated appropriate overlapping of the experimental data and predicted numbers based on the presented numbers for the produced GA.

Total GA = -2.603 + 0.369A + 0.123B - 0.146C - 0.071D - 0.015AB + 0.027AC

3-3- Assessment of co-culture of Ganoderma lucidum and Bacillus subtilis in bioreactor

After achieving the optimized point in GA production, co-culture of *G. lucidum* and *B. subtilis* was carried out in bioreactor (Fig. 4). The total produced GA included 0.536 mg in 100 mg of dry biomass weight, which was lesser than the optimized state. Differences might be due to the aeration rate differences in the bioreactor and flasks. As previously stated, oxygenation rate was one of the affecting parameters in GA production; thus, continuous aeration in the bioreactor might explain these differences.

3-4- Gas holdup

3-4-1- Investigating effects of aeration intensity on gas holdup

Gas holdup was investigated in various aerations and volumes. Figure 5-A shows gas holdup increased for all L:Ds with an increasing aeration rate. This was due to the increases in the gas volume of the bioreactor, production of smaller bubbles and increases in retention time of the smaller bubbles through rapid circulation of the liquid in high aeration Results showed that when the quantity of aeration increased from 0.5 to 2 vvm, gas holdup increased nearly 3-4 times.

3-4-2- Investigating effects of fermentation time on gas holdup

Changes of gas holdup in the bioreactor occurred based on the intensity of aeration in distilled water (DW) and culture media at Days 0 and 5 of fermentation with various L: D. A5t all L: Ds and apparent gas velocities, the gas holdup was greater in DW than fermentation media (Figure 5-B). Increases in gas holdup could be seen at zero fermentation time due to dissolved solid particles in the fermentation media, which caused gas holdup. Gas holdup completely decreased in the fermentation media on Day 5.

Table 1. Effects of co-cultivation with *B. subtilis* and *A. niger* on the growth of *Ganoderma lucidum*

Co-culture of *Ganoderma lucidum*	Co-culture on day 0 (mm)			Co-culture on day 2 (mm)			Co-culture on day 4 (mm)	
	Day 2	Day 4	Day 6	Starting line	Day 4	Day 6	Starting line	Day 6
Aspergillus niger	5.5±0.5	24.0±0.7	34.0±2.0	5.0±0.4	19.0±1.6	35.0±2.8	22.5±2.3	40.0±3.8
Bacillus subtilis	10.5±0.8	28.0±1.2	38.5±1.5	7.0±0.7	19.5±2.0	40.0±3.7	24.0±1.5	42.5±2.6
Control	12.0±1.0	38.0±1.5	44.5±3.0	4.5±0.5	26.5±2.4	50.0±5.5	28.0±3.4	50.0±3.0

Table 2. *Ganoderma lucidum* growth in solid co-culture system with two-fold volumes.

Co-culture of *G. lucidum*	Co-culture on day 0 (mm)			Co-culture on day 2 (mm)			Co-culture on day 4 (mm)	
	Day 2	Day 4	Day 6	Starting line	Day 4	Day 6	Starting line	Day 6
A. niger	7.0±1.0	9.0±0.8	10.0±1.5	10.0±0.5	12.0±3.0	13.0±1.2	30.0±4.0	36.0±2.2
B. subtilis	7.0±0.5	13.0±1.5	18.0±3.0	9.0±1.6	17.0±2.7	29.0±4.5	32.0±1.4	41.0±3.4
Control	8.0±1.5	22.0±2.5	46.0±4.0	10.0±1.0	25.0±3.5	50.0±6.4	29.0±3.6	50.0±5.0

A. niger= Aspergillus niger, B. subtilis= Bacillus subtilis, G. lucidum= Ganoderma lucidum

Table 3. The effect of co-cultivation time and volatile substance producing agent on GA production (mg in 100 mg biomass).

Co-culture of *G. lucidum*	Day 6	Day 8	Day 10
Aspergillus niger	0.24±0.01	0.44±0.03	0.32±0.02
Bacillus subtilis	0.41±0.05	0.72±0.07	0.29±0.01
Control	0.48±0.03	0.38±0.01	0.43±0.03

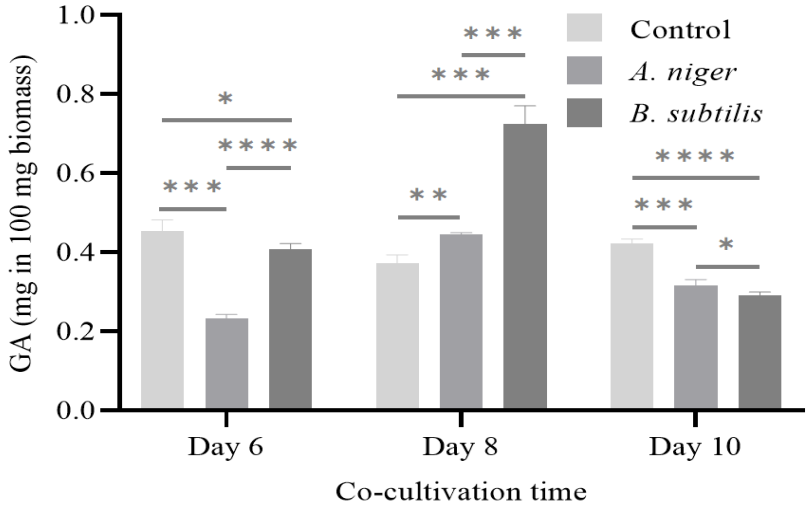

Figure 2. The effect of co-cultivation time and volatile substance producing agent on GA production (mg in 100 mg biomass)

Table 4. The results of design of experiment with Taguchi method.

Run	Variables				Results			
	A (%)	B (°C)	C	D (VVM)	Biomass (g. l^{-1})		GA (mg per 100 mg biomass)	
					Predicted	Actual	Predicted	Actual
1	5.00	30	1:6	1.0	6.03	5.94	0.525	0.5225
2	5.00	25	3:2	1.5	5.56	5.47	0.245	0.2429
3	1.00	25	1:6	0.5	6.23	6.27	0.237	0.2374
4	1.00	28	3:2	1.0	4.56	4.64	0.332	0.3352
5	10.00	25	4:8	1.0	2.84	2.88	0.813	0.8139
6	1.00	30	4:8	1.5	3.42	3.46	0.325	0.3252
7	5.00	28	4:8	0.5	3.68	3.59	0.437	0.4347
8	10.00	30	3:2	0.5	6.39	6.43	0.485	0.4861
9	10.00	28	1:6	1.5	6.43	6.47	0.277	0.2780

A: inoculum (%), B: temperature (˚C), C: L:D, and D: aeration rate (vvm).

Over time of fermentation and mushroom growth, viscosity of the media increased and caused formation of larger bubbles, which decreased gas holdup. The process of changes in a gas holdup of L: D = 4:8 can be seen in Figure 5-B. Moreover, dependence of gas holdup on aeration speed at L: D = 4:8 is shown in Table 5.

The β values in all three water systems, fermentation media on Day 0 and fermentation media on Day 5 were in the range of homogeneous flow. Results showed significant differences between the gas holdup of various aeration rates (p<0.05).

Numerous studies have been carried out on mVOCs of the microorganism growth. Studies have shown that bacteria affect the cell cycle of fungi by affecting growth of mycelia and inducing formation of fruiting bodies. This effect is highly species dependent [22]. Volatile compounds produced by fungal and bacterial strains are mostly alcohols, aldehydes, esters, ketones, aromatic compounds, acids, furans, phenols and nitrogen compounds. These compounds, which are produced only in simultaneous cultures, can include various biotechnological uses in pharmaceutical, food and cosmetic industries [32]. In this study, it was observed that releases of volatile organic compounds of *A. niger* and *B. subtilis* included inhibitory effects on *G. lucidum* growth. Effects of mVOCs on growth decrease and changes in the hypha were seen in other studies [9, 18]. Morphologic studies have demonstrated potential inhibitions in fungal growth by the microbial volatile organic compounds with generation of hyphae, changes in membrane permeability, changes in composition and structure of the cell wall and damages to the DNA can inhibit the fungi growth [11,15,16]. Studies have shown that oxygen limitation in *G. lucidum* media culture leads to growth decreases and enhancements in GA production in this fungus [26,33]. Effects of high carbon dioxide concentration on inhibiting *Pleurotus* growth was investigated by Zadražil et al. The best concentration of carbon dioxide for the mycelium growth of the fungal species was reported as 16-22%. Furthermore, 36% concentration of carbon dioxide inhibited the growth of fungal species [34].

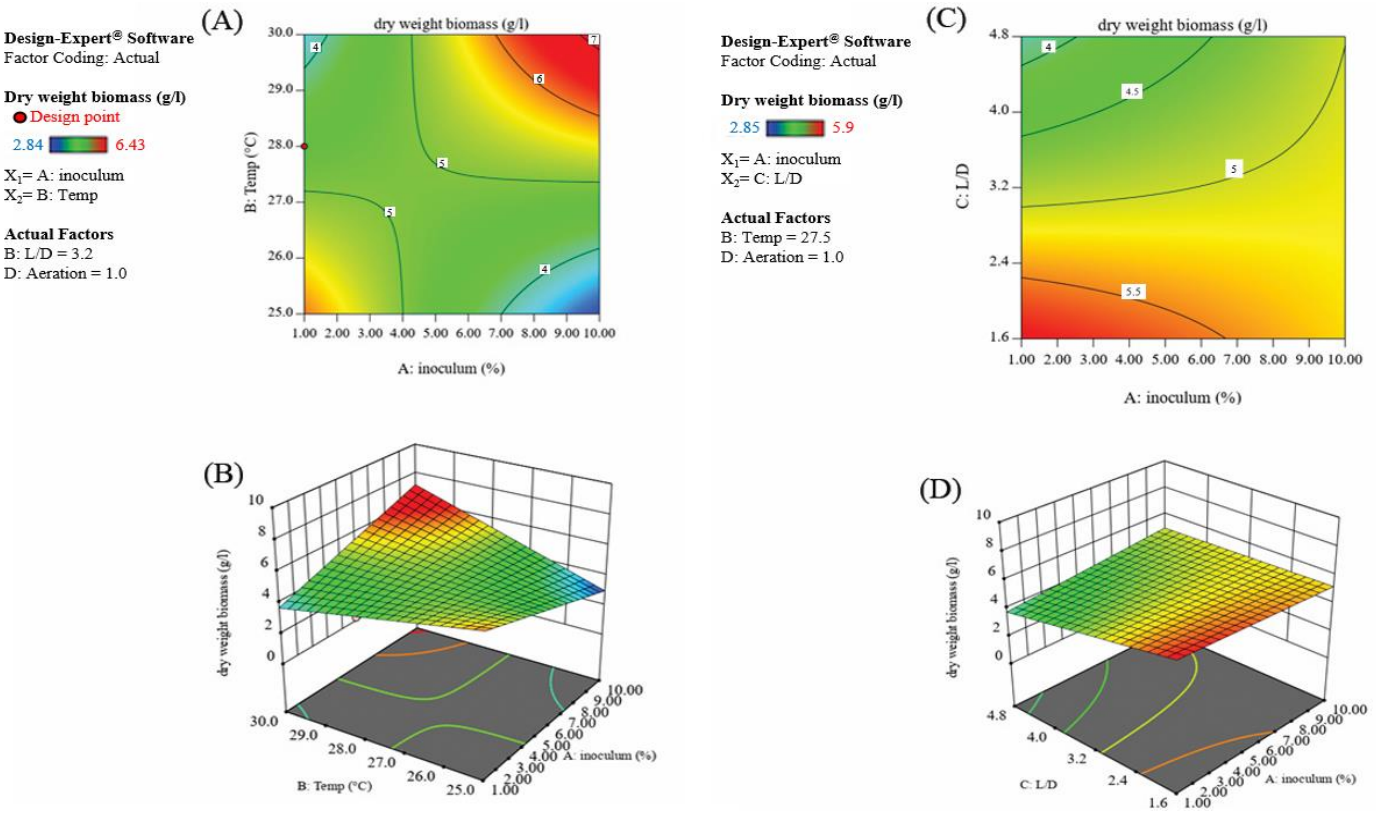

Figure 3. 2D (A) and 3D plots of inoculum size (%) and temperature interactions versus biomass dry weight, and 2D (C) and 3D (D) plots of inoculum size (%) and L:D interactions versus biomass dry weight.

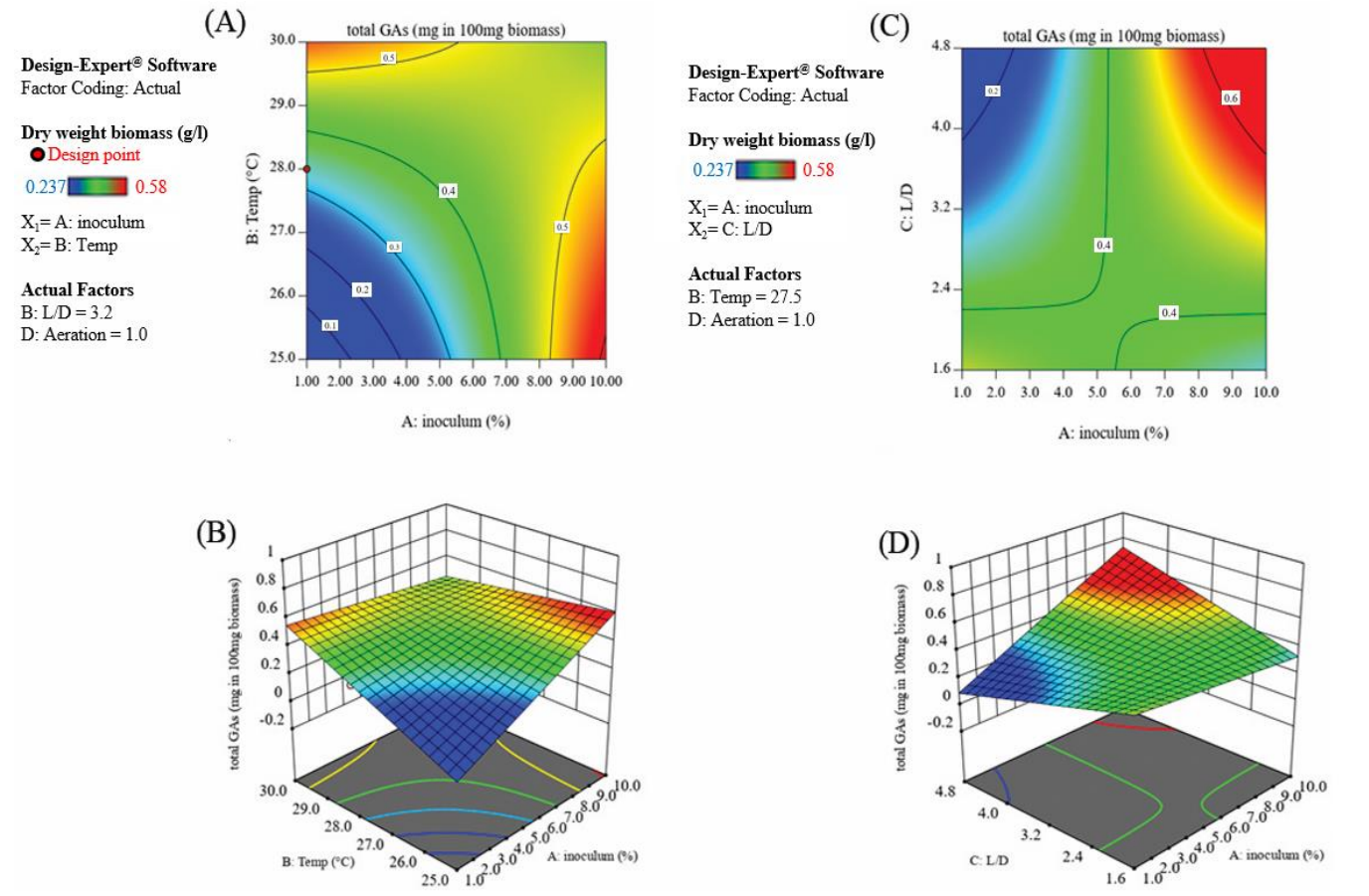

Figure 4. 2D (A) and 3D plots of inoculum size (%) and temperature interactions versus GA production, and 2D (C) and 3D (D) plots of inoculum size (%) and L:D interactions versus GA production.

Figure 5. (A) Culture of *G. lucidum* in bubble column bioreactor, (B) co-culture of *G. lucidum* and *B. subtilis* in bubble column bioreactor.

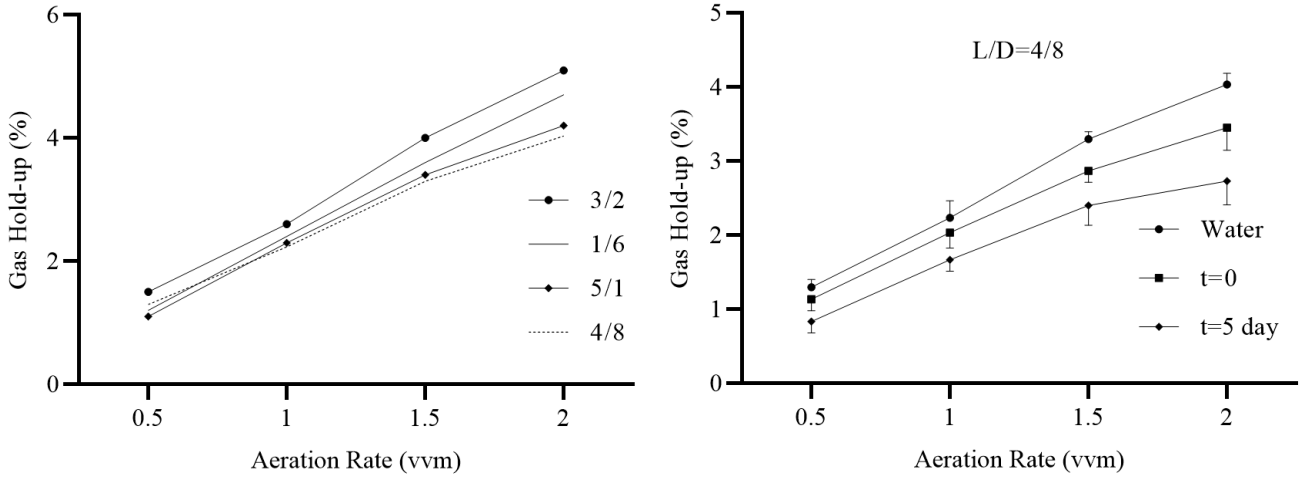

Figure 6. (A) Gas holdup changes in the distilled water-air system at different L:Ds in terms of vvm, (B) Gas holdup changes in the distilled water-air system and culture medium-air system at zero time and fermentation medium-air after 5 days at L:D=4:8 in terms of vvm

Numerous parameters play roles in increasing the GA content. In one study, it was reported that time was the primary factor in increasing GA content and the highest yield was achieved when the ultrasonic-assisted extraction was carried out at 64.2–70 ◦C for 1.2 h [35]. Addition of various substrates during the fermentation process was another method of enhancing the GA yield [36]. Yan et al. studied roles of two various sterilized oleic acids on GA production. Addition of high-temperature oleic acid (121 ◦C, 30 min) to the media after 32 h resulted in 1.44, 3.55 and 0.38-fold increases of GA R, S and T productions, respectively. Addition of filter-sterilized oleic acid on Day 5 enhanced the GA R, S and T productions up to 3.11, 5.19 and 1.44 times higher than those in the control group, respectively [37]. In another study, addition of graphene-based nanomaterials [graphene oxide (GO)] decreased graphene oxide [(rGO) and (rGO/Fe_3O_4)] as elicitors were investigated on Day 5 of fermentation. While GO and rGO included inhibitory effects on the GA production rate, elicitors improved the GA content, compared to the control group. Supplementation with 50 mg rGO resulted in the maximum GA content [38].

As observed in this study, co-culture with *A. niger* included further effects on the growth decreases of *G. lucidum*, compared to co-culture with *B. subtilis*. It could be concluded that the higher biomass production and faster growth of *A. niger*, comparing to *G. lucidum*) resulted in further oxygen consumption by this fungus and hence production of a higher concentration of carbon dioxide, which decreased *G. lucidum* growth. In addition, lower *G. lucidum* growth and higher GA production in the two-fold volume might be due to the further oxygen consumption and generation of high carbon dioxide concentrations by *B. subtilis* and *A. niger*; similar to the previous studies. Studies have shown that oxygen limitation or carbon dioxide accumulation might lessen the toxic effects of mVOCs [34, 39]. It could be assumed that decreases in GA growth in the three-fold volume might be due to the carbon dioxide accumulation, oxygen decrease and decrease in the microbial volatile organic compounds. Lin et al. investigated effects of oxygen content on biomass and GA production. They observed that 3-hydroxy-3-methyl-glutaryl-CoA reductase, squalene synthase and lanosterol synthase in the GA production pathway were affected by the oxygen pressure. At a high oxygen pressure, produced H_2O_2 affected the ganoderic production [40,41]. During the liquid culture of *G. lucidum* in bioreactor, oxygen supplement and shear stress significantly affected the cell growth, morphology and GA production [40,41]. For biomass and extracellular polysaccharide production, optimized temperature and aeration rate included 27 ◦C and 1 vvm, respectively.

4. Conclusion

In this study, effects of two parameters of co-culture time and volumes were investigated on the co-culture of *G. lucidum* with *B. subtilis* and *A. niger* using one factor-at-a-time approach. It was indicated that the co-culture of *B. subtilis* on Day 8 led to 1.5 times higher growth, compared to the control sample. Moreover, co-culture of *B. subtilis* with *G. lucidum* in 2-fold volume resulted in 2.3-fold increases in GA production. Assessment of *G. lucidum* growth in solid culture on higher times and volumes of co-culturing exhibited further inhibitions, which were the signs of microbial volatile compound effects on *G. lucidum* growth rate. Major parameters in microbial co-culture include microbial volatile organic compounds, oxygen, CO_2, culture media composition and the microbial strains. Changes in each of these parameters affect the microorganism growth rate. To optimize the fungal growth in bioreactor, the initial inoculum size, temperature, length-to-diameter ratio, and aeration rate were chosen for Taguchi analysis. Optimized condition for GA production included 10% initial inoculum size, temperature of 25.6 ◦C, L: D of 4:8, and aeration rate of 0.64 vvm. Gas holdup for air-water and fermentation media-air systems was investigated in various aeration intensities. Gas holdup increased with increasing aeration rate for all L:Ds. Furthermore, gas holdup in DW was higher than that in fermentation environments due to solid particles in the fermentation media.

5. Acknowledgements

The authors thank all the staff, who helped them in the Biotechnology Laboratory of the Faculty of New Sciences and Technologies, University of Tehran.

7. Conflict of Interest

The authors declare no conflict of interest.

8. Conflict of Interest

The authors report no conflicts of interest.

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Research Article
pISSN: 2345-5357
eISSN: 2423-4214
OPEN ACCESS

APPLIED FOOD BIOTECHNOLOGY, 2024, 11 (1):E7
Journal homepage: www.journals.sbmu.ac.ir/afb

تولید گانودریک اسید از طریق کشت هوایی توام *گانودرما لوسیدوم* با *باسیلوس سوبتیلیس* و *آسپرژیلوس نایجر* با استفاده از بیوراکتور ستون حبابی

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چکیده

سابقه و هدف: قارچ *گانودرما لوسیدوم* با خواص دارویی خود یکی از قارچ‌های مفید در طب سنتی آسیایی است. راندمان کم این قارچ‌ها در تولید گانودریک اسید ، کاربرد آن را به عنوان یک متابولیت ثانویه با ارزش محدود کرده است. تنش‌های محیطی و محرک‌هایی مانند ترکیبات آلی فرار میکروبی در کشت‌های توام تولید گانودریک اسید را افزایش می‌دهند. برای بررسی اثرات متغیرهای زمان و حجم کشت توام بر رشد *گانودرما لوسیدوم* و تولید گانودریک اسید ، *باسیلوس سوبتیلیس* و *آسپرژیلوس نایجر* به صورت توام با *گانودرما لوسیدوم* کشت هوایی شدند.

مواد و روش ها: برای بررسی رشد قارچ و تولید گانودریک اسید با استفاده از بیوراکتور ستون حبابی، اثر متغیرهای مستقل دما، تلقیح اولیه، نسبت طول به قطر (L:D) و هوادهی با استفاده از روش تاگوچی بررسی شد. سپس کشت توام قارچ *گانودرما لوسیدوم* با *باسیلوس سوبتیلیس* و *آسپرژیلوس نایجر* در شرایط بهینه بررسی شد.

یافته‌ها و نتیجه‌گیری: بهینه‌سازی متغیرهای زمان و حجم همزمان کشت منجر به افزایش ۲/۹ برابری تولید اسید گانودریک در مقایسه با نمونه شاهد شد. بهینه‌سازی تولید زیست‌توده در بیوراکتور نشان داد که با افزایش درصد و دمای تلقیح اولیه، تولید زیست‌توده به طور قابل توجهی افزایش یافت. این دو متغیر به طور معنی‌داری بر تولید اسید گانودریک اثر داشته و شرایط بهینه با ۱۰ درصد تلقیح اولیه، دمای ۲۵/۶°C، ۴:۸ L:D و سرعت هوادهی ۰/۶۴ vvm بود. بررسی احتباس گاز برای سامانه‌های محیط کشت تخمیر هوا-آب و هوا نشان داد که وجود مواد جامد معلق و سرعت هوادهی بر احتباس گاز تأثیر می‌گذارد. ترکیبات آلی فرار میکروبی در کشت مشترک میکروارگانیسم‌ها می_توانند تولید گانودریک اسید توسط *گانودرما لوسیدوم* را افزایش دهند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۲۷ اکتبر ۲۰۲۳
داوری ۵ دسامبر ۲۰۲۳
پذیرش۲۵ دسامبر ۲۰۲۳

واژگان کلیدی

- *گانودرما لوسیدوم*
- گانودریک اسید
- کشت هوایی توام
- بیوراکتور ستون حبابی
- روش تاگوچی

***نویسندگان مسئول**

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