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RESEARCH PAPER

Oxygen Limitation Improves Ganoderic Acid Biosynthesis in Submerged Cultivation of *Ganoderma lucidum*

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Abstract The effects of oxygen limitation on the production of ganoderic acid (GA), a secondary metabolite with anti-tumor activity, and on transcription levels of triterpene biosynthesis genes were investigated in liquid cultures of *Ganoderma lucidum*. A low oxygen supply level was beneficial to total GA biosynthesis, but negative to the cell growth. The higher GA production was obtained under micro-aerobic conditions (*i.e.* initial overall k_La values at 0.02 and 0.13/h). The maximum GA production of 272.3 ± 11.5 mg/L was obtained at an initial overall k_La of 0.13/h, which was 1.7-fold that at a normal cultivation condition (an initial k_La of 5.51/h). For four major individual GAs, the production level of GA-Mk, -T, -S, and -Me in the hypoxia-induced cells was increased by 50, 87, 62, and 111%, compared with that of the control, respectively. Meanwhile, the transcriptions of four key genes encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, mevalonate-5-pyrophosphate decarboxylase, squalene synthase and squalene epoxidase in the triterpene biosynthetic pathway were up-regulated under the hypoxia condition (at an initial k_La of 0.13/h). Reactive oxygen species was generated in response to hypoxia, which seemed to be involved in the regulation of GA biosynthesis. The information obtained provides an insight into the role of oxygen limitation in the GA biosynthesis, and will be helpful for optimizing the fermentation process on a large scale.

Keywords: ganoderic acid, traditional medicinal mushroom, gene expression, submerged culture, oxygen limitation

1. Introduction

Ganoderma lucidum (Fr.) Krast (Polyporaceae) is a traditional medicinal mushroom, and has been widely used as a tonic in promoting longevity and health in Mainland China and some other Asian regions, for several thousand years. Ganoderic acids (GAs) as oxygenated triterpenes are the main active ingredients, besides polysaccharides produced by this higher fungus [1,2]. Over one hundred GAs have been isolated from the fruiting bodies, spores and cultured mycelia of *G. lucidum* [1,2], and have received wide interest, due to their interesting pharmacological functions, such as anti-tumor [3-5] and anti-HIV-1 activities [6].

Many researches aimed to obtain high GA content and high production of total crude GAs, by studying various cultivation conditions in *G. lucidum* fermentation [7-12]. For example, our group has carried out a lot of work on improving GA production in submerged cultivation, and in a two-stage cultivation process combining liquid fermentation and static culture [7-9]. Ren *et al.* added methyl jasmonate to the culture of G. *lucidum*, and the GA yield was 45.3% higher than the untreated control sample [10]. Tang *et al.* developed a multiple Cu^{2+} addition strategy to enhance the total GA accumulation [11]. From another aspect, recently a lot of individual GAs were isolated and identified, together with their function study, and we have tried to optimize

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fermentation conditions for individual GA production. For example, Liang *et al.* added 100 μM phenobarbital on day 5 after the transition of shaking culture, to the static liquid culture of *G. lucidum*, resulting in increased levels of ganoderic acid-Mk, -T, -S, and –Me in the treated cells by 47, 28, 36, and 64%, respectively [9]. Zhao *et al.* reported that the levels of four kinds of individual GAs were several-fold increase, when the cells were under nitrogen limitation condition [12].

Oxygen concentration is crucial to microbial cultures [13], and it has been reported to affect the cell growth and metabolite formation in the fermentation of many microorganisms, such as *Bacillus brevis* and *Antrodia cinnamomea* [14,15]. The supply of oxygen based on the initial volumetric oxygen transfer coefficient (k_La) is generally used as a controlling strategy in bioprocesses [16-18]. In some cases, high product concentrations were obtained under an oxygen limitation condition, which was regarded as a growth-limiting nutrient [19,20]. Oxygen deprivation (hypoxia) is accompanied by reactive oxygen species (ROS) formation, and is characterized by a set of metabolic changes collectively called 'oxidative stress response' [21]. In the fermentation of *G. lucidum*, Tang and Zhong reported that a higher dissolved oxygen tension (DOT) (25% of DOT) was beneficial to the cell growth, while 10% of DOT was favorable to the GA biosynthesis [22]. In the latter case under oxygen limitation, a higher GA content was obtained, compared to that at 25% of DOT. Based on such a finding, a DOT shift strategy successfully resulted in a significant enhancement of overall GA production (GA content multiplied by cell concentration) [23].

However, there has been a lack of understanding of the mechanism of GA biosynthesis induction caused by low oxygen level in the fermentation of *G. lucidum*. In this study, the impact of oxygen supply levels on GA biosynthesis was investigated at various values of initial k_La in shake flasks. Furthermore, a gradual transition from normoxia to hypoxia condition was designed, to investigate the effects of oxygen deficiency on GA biosynthesis in submerged fermentation. The response of ROS and the transcription levels of GA biosynthetic genes, including 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), mevalonate-5-pyrophosphate decarboxylase (MVD), squalene synthase (SQS), squalene epoxidase (SE) and lanosterol synthase (LS), were also detected in the oxygen deficiency condition.

2. Materials and Methods

2.1. Maintenance and preculture of *G. lucidum*

The strain of *G. lucidum* CCGMC 5.616 was maintained on potato-agar-dextrose slants. The slant was inoculated with mycelia, and incubated at 28°C for 7 days, then stored at 4°C for about 2 weeks. The preculture was conducted by the method of Fang and Zhong [8].

2.2. Cultivation procedure

The details of culture medium and conditions were as described by Fang and Zhong [8]. Oxygen limitation was made by sealing a shake flask with hydrophobic fluoropore membrane (ø0.6 cm) (Shanghai Jiafeng Gardening Co., Ltd., Shanghai, China), or using a sterile rubber stopper, adjusting the ventilation mouth size of flask with adhesive plaster, instead of 8-layer cotton gauze, which was used as a control.

2.3. Determination of initial overall k_La value

The initial overall k_La value in flask was estimated with the non-steady state method [24], by using a dissolved oxygen probe (East China University of Science and Technology, Shanghai, China). A 250-mL shake flask with dissolved oxygen (DO)-sensor port near the bottom was used to determine the initial k_La value. The process followed was as reported by Xu and Zhong [16].

To reveal the effect of oxygen limitation on the cell growth and GA production, micro-aerobic conditions were accomplished by drilling holes with a needle (0.82 mm diameter) in the hydrophobic fluoropore membrane sealed on the ventilation mouth of a conical flask. The initial overall k_La value of the flask containing 50 mL medium with 1 hole, 4 holes, no adhesive plaster and 8-layer cotton gauze at 120 rpm and 30°C was determined to be 0.02, 0.13, 0.33, and 5.51/h, respectively.

2.4. Sampling, determination of cell dry weight (DW), and residual medium sugar

For sampling, three flasks were taken each time. The dry cells were obtained by centrifuging a sample at 31,475 × *g* for 15 min, and the precipitated cells were washed twice with distilled water, dried at 50°C for sufficient time to a constant weight, and then the DW was measured with gravimetric method. Residual sugar concentration was analyzed by phenol-sulfuric acid method [25].

2.5. Assay of total and individual ganoderic acids (GAs)

Total crude GAs were extracted and measured by spectrophotometer (at 245 nm), using thymol as standard [8]. For determining individual GAs, high-performance liquid chromatography (HPLC) was used on an Agilent 1200 series (5 μm Agilent Zorbax SB-C18 column, 250 × 4.6 mm). Details of the HPLC analysis method were described elsewhere [26]. The purities of the individual GAs (GA-Mk, GA-T, GA-S, and GA-Me) were higher than 98%, based on HPLC results.

2.6. ROS measurement

Intracellular ROS generation was measured by a fluorometric assay with 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Applygen Technologies Inc.). For assessing ROS generation, *G. lucidum* cells were incubated with DCFH diacetate (final concentration of 10 μM) for 30 min, before oxygen limitation started. One-ml aliquots of medium taken at various intervals were centrifuged at 10,000 × *g* for 5 min to remove media, resuspended in 1 ml PBS buffer, and monitored by a "Fluorescence Multi-well Plate Reader", with excitation and emission wavelengths of 485 and 535 nm, respectively. Intensities are reported as percentages of initial values.

2.7. Dissolved oxygen concentration detection

The dissolved oxygen (DO) concentration in medium was monitored every minute, with a DO electrode connected to a multifunction analysis meter (East China University of Science and Technology, Shanghai, China). The respiration rate is given by the derivative calculated between two successive measurements (μmol/h/g/ DW).

2.8. RNA isolation and quantitative real-time PCR (qRT-PCR)

Aliquots of 0.1 g mycelia were collected by filtration from the culture media, and frozen in liquid nitrogen. The total RNA was extracted with 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) following Invitrogen's procedure, and treated with RNase-free DNaseI (MBI Fermentas, Canada), and then reverse-transcribed to cDNA with ReverTra Ace-α-cDNA Synthesis Kit (Toyobo, Japan) for RT-PCR, following the vendor's instructions. Afterwards, transcript levels of 18S rRNA, *hmgr*, *mvd*, *sqs*, *se* and *ls* were determined by quantitative real-time PCR, using SYBR Green I on the Mastercycler® ep realplex 2S detection system (Eppendorf, Germany). Primers were designed using Primer Express software (Applied Biosystems). The sequences of the primer for amplification of *hmgr*, *sqs*, and *ls* were those described by Xu *et al.* [26]. For *mvd* and *se*, the following primer sets were used: *mvd*-forward, 5'-TCGGACTCG CTTGCGGTAGA-3' and *mvd*-reverse, 5'-CGTGCTTGATAC GGTGCTG-3'; *se*-forward, 5'-AGGGAGAACCCGAAG CATT-3' and *se*-reverse, 5'-CGTCCACAGCGTCG CAT AAC-3'. PCR reactions were carried out with the MaximaTM SYBR Green qPCR Master Mix (MBI Fermentas, Canada), according to the manufacturers protocol. After an initial denaturation step at 95°C for 10 min, amplification occurred in three steps: 15 sec of denaturation at 95°C, 30 sec of annealing at 60°C, and 30 sec of extension at 72°C, for a total of 40 cycles. Identical thermal cycling conditions were used for all targets. The gene for 18S rRNA was used as the reference gene, because its expression was found to be stable under our experimental conditions. The expression level of the different genes was normalized with respect to the *G. lucidum* 18S rRNA expression level. For each gene, the reference sample (without oxygen-limited sample) was defined as the expression level 1.0, and results were expressed as the fold of mRNA level over the reference sample. Post qRT-PCR calculations to analyze relative gene expression were performed according to the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen [27].

2.9. Statistical analysis

All data of shake flasks were the average of three independent samples. The error bars indicate the standard deviation (SD) from the mean of triplicates. Data were analyzed with Student's t-test. The difference between treatments was considered significant, when $P < 0.05$ in a two-tail analysis.

3. Results and Discussion

3.1. Effect of hypoxia on GA biosynthesis

Fig. 1 shows time profiles of the total crude GA accumulation (in mg/100 mg DW of biomass) during a conventional submerged cultivation of *G. lucidum* at a severe oxygen limitation, by sealing the flask with a sterile rubber stopper from day 0, 1, and 3. No matter which day the oxygen limitation was imposed, the total crude GA content increased remarkably, in comparison with the control (normal shaking flask covered with 8 layers of cotton gauze). The highest total crude GA content was 3.43, 3.11, and 3.31 mg/100 mg

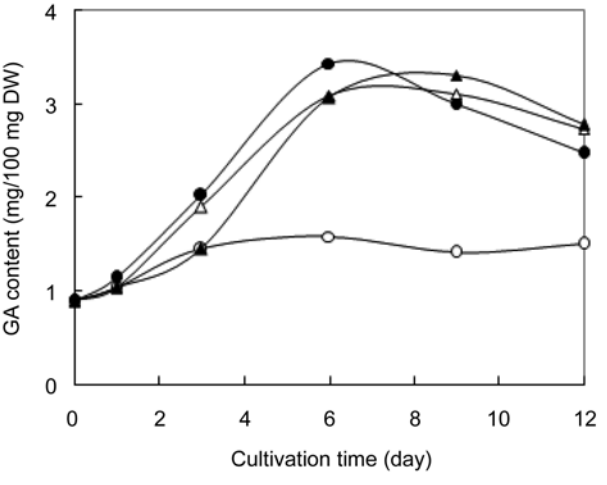

Fig. 1. Effect of oxygen limitation on ganoderic acid (GA) content during the submerged fermentation of *G. lucidum* in shake flasks. Symbols: artificial oxygen limitation from day 0 (dark circle), day 1 (open triangle), day 3 (dark triangle), and control (without artificial oxygen limitation, open circle).

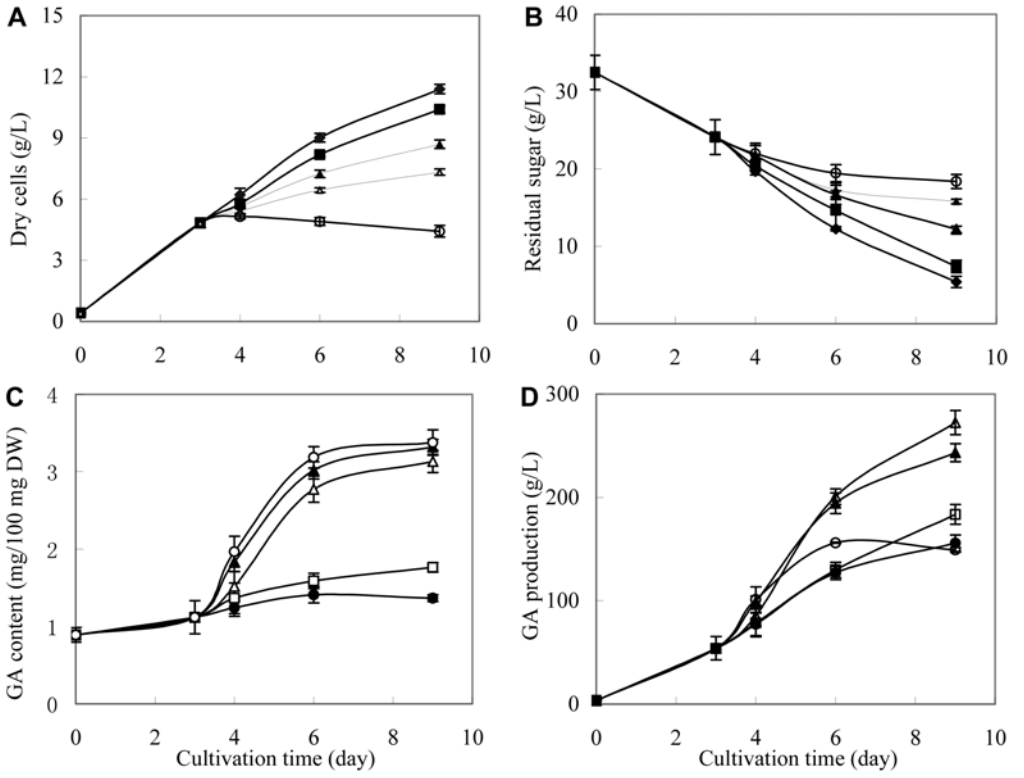

Fig. 2. Time courses of (A) cell growth, (B) sugar consumption, (C) total crude GA content, and (D) total crude GA production of the cells at different initial overall k_La from day 3 of the submerged culture of *G. lucidum*. Symbols for initial overall k_La values: 0.02 (dark triangle), 0.13 (open triangle), 0.33 (open square), and 5.51/h (dark circle). Sealed submerged culture from day 3 (open circle) was also done. The error bars in the figure indicated the standard deviations from three independent samples.

DW, respectively, with oxygen limitation at day 0, 1, and 3, which was 2.17-, 1.96-, and 2.09-fold of the control. The results suggested that the GA biosynthesis was significantly affected by oxygen limitation. In order to investigate how much oxygen supply was beneficial to the biosynthesis of GA, an oxygen supply strategy, by controlling the initial overall k_La value, was established in the further experiments.

3.2. Effects of oxygen supply on cell growth and GA production

Figs. 2A and 2B indicate that the increase of initial k_La value from 0.02 to 5.51/h led to faster sugar consumption and higher cell growth rate from day 3. At an initial k_La value of 0.02, 0.13, 0.33, and 5.51/h, as well as in the sealed culture, the sugar consumption rate calculated as [(Final residual sugar concentration-Initial residual sugar concentration)/(culture time)], was 1.40, 2.00, 2.80, 3.13, and 0.96 g/L/day on day 6, respectively. The final cell concentration (based on dry weight) increased from 7.32 ± 0.15 g/L at an initial k_La value of 0.02/h, to 11.4 g/L at that of 5.51/h, and the average cell growth rate [(Final cell density-Initial cell density)/(culture time)] was 0.42 g/L/ day and 1.09 at an initial k_La value of 0.02 and 5.51/h, respectively. The consumption of sugar corresponded well with the cell growth and initial k_La value, and faster sugar consumption and cell growth were observed at a higher initial k_La value. Within the range of initial k_La investigated, an increase of oxygen supply was beneficial to the growth of *G. lucidum*. Oxygen supply was thus considered as a key factor influencing the growth of *G. lucidum* in the liquid cultivation.

Regarding the production of GA (Figs. 2C and 2D), a rapid increase of total GA content and production was observed as the initial k_La decreased to 0.02 and 0.13/h, and the GA content level had no obvious difference from that under sealed culture. Further increase in initial k_La values led to a sharp drop in the accumulation of GA, in both its content, and total production. The maximum GA content (3.32 ± 0.10, 3.13 ± 0.14 mg/100 mg DW) under low initial overall k_La of 0.02 and 0.13/h reached 1.35 and 1.22 folds higher, than that under normal condition (k_La at 5.51/h). Interestingly, the GA content obtained under an initial k_La value of 0.33/h had no significant increase ($P < 0.05$). The results suggested that the relatively low oxygen supply level was beneficial to GA biosynthesis. On the other hand, the maximum GA production at initial k_La values of 0.02, 0.13, 0.33, and 5.51/h was obtained as 243.3 ± 8.7, 272.3 ± 11.5, 183.5 ± 9.6, and 155.7 ± 8.0 mg/L at day 9,

respectively. Their corresponding productivity (from day 3) was 31.9, 36.4, 21.6, and 16.9 mg/L/day. It should be mentioned that the highest production and productivity obtained at an initial k_La value of 0.13/h were 1.7 and 2.2-fold those at an initial k_La of 5.51/h, respectively. However, in the case of absolute oxygen limitation by sealing flask surface, its highest GA production was 156.1 ± 9.9 mg/L at day 6, and after that, a slight decrease was observed, due to the decrease of its biomass, although its GA content increased a bit. From the above results, it is clear that for *G. lucidum*, as an aerobic fungus, starvation of oxygen caused its poor cell growth and low GA production, but a certain degree of oxygen limitation (under low initial k_La values) was beneficial to the GA biosynthesis, while cell growth was fairly good. A change of metabolic flux towards metabolite production under oxygen limitation has also been reported by other groups [20,28].

Until now, although over one hundred triterpenoids (including GA derivatives) have been reported, in our work, only the individual ganoderic acids GA-Mk, GA-T, GA-S, and GA-Me were identified in the mycelia of *G. lucidum*. The content of 4 individual GAs observed at day 6 is shown in Table 1. The content of GA-Mk, GA-T, GA-S, and GA-Me was much higher under lower oxygen supply than the control. In the case of initial k_La value at 0.13/h, the individual GA-Mk, -T, -S, and -Me levels were 98.4 ± 6.4, 99.5 ± 7.7, 113.1 ± 7.1, and 51.4 ± 3.1 μg/g DW, respectively. They were 50, 87, 62, and 111% increase, compared with the control. The highest levels were GA-T and S, indicating that oxygen limitation not only enhanced GA biosynthesis, but also changed the distribution (heterogeneity) of individual GAs in the submerged culture of *G. lucidum*. It was interesting that the powerful anti-tumor GA-T (3,29) and GA-S (30) had quite high content under oxygen limitation. A similar phenomenon was also observed in a process of liquid static culture of *G. lucidum* under various oxygen levels in the gaseous phase, as we reported previously; the different ratio of GA-S, GA-T and GA-Me was obtained at various oxygen levels (31). Tang and Zhong reported that an initial k_La value within the range of 16.4 ~ 96.0/h had a significant effect on the cell growth, cellular morphology and metabolites biosynthesis. The maximum GA content in the culture was obtained at a high initial k_La value (96.0/h), because an increase of initial k_La led to a bigger size of mycelia aggregates, caused by limitation of oxygen within mycelia pellets [22]. Our results showed a requirement of a low initial k_La for higher GA biosynthesis, and those results confirmed that oxygen limitation could stimulate GA biosynthesis, in submerged cultivation of *G. lucidum*.

Although a higher GA content was obtained at an oxygen-limited level, a much higher cell density was achieved under adequate oxygen supply; therefore a higher total production of GAs was reached in the latter case. For large-scale production, an optimal strategy with shift of oxygen supply may be carried out, which requires further study.

3.3. Oxidative burst and gene expression responding to oxygen limitation

ROS generation was observed in several groups as an integral part of the response to oxygen deprivation [21]. Rathore *et al.* reported that hypoxia may distinctively activate NADPH oxidase (Nox), to increase intracellular ROS concentration and Ca^{2+} through mitochondrial ROS-PKCε signaling axis, in pulmonary artery smooth muscle cells [32]. In living cells, researchers have hypothesized that ROS might act as an upstream signaling molecule, which regulated differentiation, metabolic regulation and apoptosis [33]. In this work, the oxygen limitation led to the deficiency of oxygen supply (hypoxia). How *G. lucidum* cells responded to hypoxia in defense reactions was investigated here. As shown in Fig. 3, the cells rapidly

Table 1. Effects of oxygen supply on cell dry weight, content of total crude GAs, and content of individual GAs on day 6 of liquid culture[a]

Treatment	Biomass (g/L)	Content of total crude GAs (mg/100 mg DW)	Individual GAs (μg/g DW)			
			Mk	T	S	Me
I	9.0 ± 0.2	1.4 ± 0.1	63.5 ± 4.8	51.2 ± 4.1	69.8 ± 5.5	24.4 ± 2.1
II	8.2 ± 0.1	1.6 ± 0.1	66.6 ± 5.4	55.8 ± 4.4	80.5 ± 5.0*	26.4 ± 2.8
III	7.2 ± 0.3*	2.8 ± 0.3*	98.4 ± 6.4*	99.5 ± 7.7*	113.1 ± 7.1*	51.4 ± 3.1*
IV	6.4 ± 0.2*	3.0 ± 0.2*	95.5 ± 7.9*	116.2 ± 4.0*	121.6 ± 9.9*	52.9 ± 3.2*
V	4.9 ± 0.2*	3.2 ± 0.2*	95.1 ± 6.7*	110.9 ± 5.4*	127.3 ± 8.7*	49.8 ± 3.1*
VI	4.5 ± 0.3	1.7 ± 0.2**	50.5 ± 3.5**	62.9 ± 5.9**	70.0 ± 2.6**	27.5 ± 1.3**

I: the control, with 8-layer cotton gauze, initial k_La value of 5.51/h.
II, III, IV: initial k_La value of 0.331, 0.128, and 0.021/h.
V: sealed submerged culture from day 3.
VI: with 10 μM DPI addition in non-aerated submerged culture from day 3.
[a]Standard deviation was calculated from three independent samples.
*$p < 0.05$ for II, III, IV,V compared to I.
**$p < 0.05$ for VI compared to V.

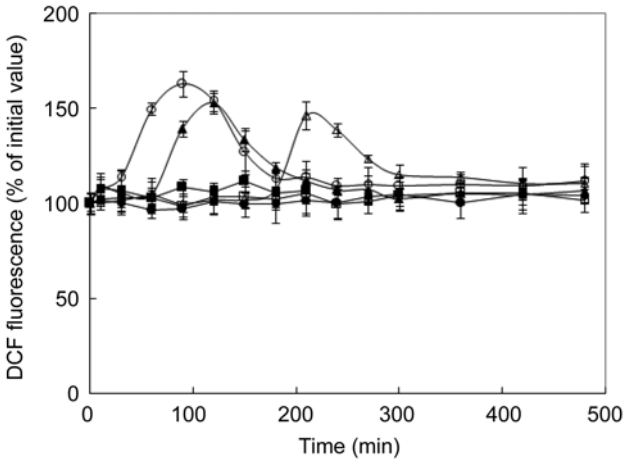

Fig. 3. Intracellular ROS generation in *G. lucidum* cells. The oxygen supply was controlled with initial overall k_La value at 0.02 (dark triangle), 0.13 (open triangle), 0.33 (open square), and 5.51/h (dark circle) from day 3 of the submerged culture of *G. lucidum*. Sealed submerged culture from day 3 (open circle), and with 10 μM DPI addition in sealed submerged culture from day 3 (dark square) were also done. Each data point represents the mean ± SD from three independent samples.

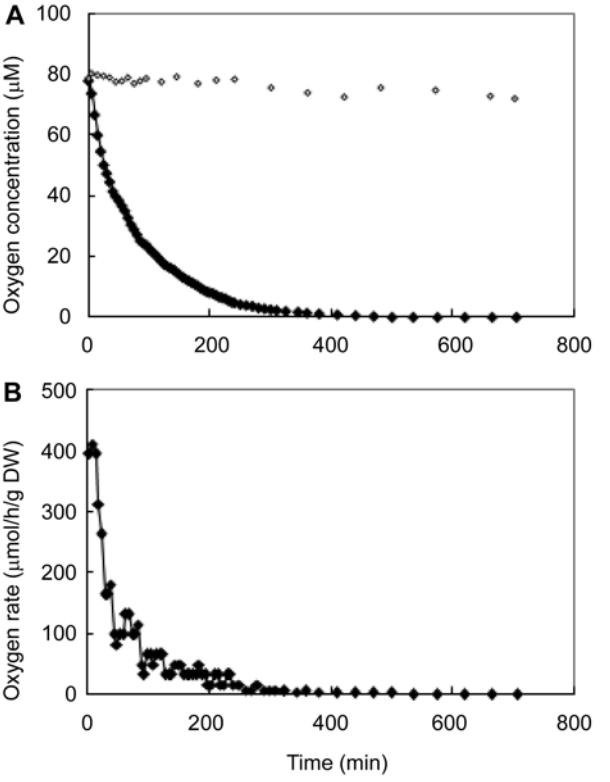

Fig. 4. Oxygen concentration in (A) the cultured medium, and (B) oxygen consumption by submerged culture of *G. lucidum* under oxygen deficiency condition. The oxygen supply was controlled with an initial overall k_La value of 5.51/h (open diamond), and sealed submerged culture (dark diamond) from day 3 was also done.

generated ROS after alteration to hypoxia, and ROS production was considerably increased at 90, 120, and 210 min under severe oxygen limitation conditions (including sealed submerged culture, and those at an initial overall k_La of 0.02 and 0.13/h). Under sealed oxygen limitation condition, the DO concentration decreased from 80 to 0 μM. The oxygen consumption of *G. lucidum* was rapidly decreased in 45 min after the beginning of the experiment. The cells had a low oxygen uptake rate, the cell growth rate decreased, and cell death occurred later, so the biomass was reduced at the end of fermentation (Fig. 1A). Under initial k_La values of 0.02 and 0.13/h, the DO was almost zero, well below the critical DO level of about 35 μM; thus the cells were considered to be hypoxia, too. The oxygen supply was not enough for cells to uptake, and cells also had low growth rate and low sugar consumption rate. The appearance time of the ROS peak change means the successive change of cellular responses to hypoxia conditions suffered under corresponding severe oxygen limitation conditions. The ROS peak appeared earlier, under more severe oxygen limitation condition, in a sequence of sealed, initial k_La 0.02/h, then initial k_La 0.13/h. In contrast, the ROS level of the control and at an initial overall k_La of 0.33 was not obviously changed, and always lower than that of low oxygen supplied samples over the period studied (480 min). It was concluded that the oxygen supply level at initial overall k_La of 0.33/h satisfied the basic requirement for cell growth.

Oxygen consumption process in the nutrient solution was studied with a severe oxygen limitation from day 3 (the biomass was 4.8 g/L). When the airtight cover was first sealed, oxygen concentration in the medium decreased gradually in the nutrient solution (Fig. 4A). Seven hours later, the oxygen concentrations were below 1 μM. This experimental setup led to a gradual development of an aerobiosis as described by Morard *et al.* [33]; a similar tendency was found in the oxygen-supplied cultivation at an initial overall k_La value of 0.02 and 0.13/h. The oxygen level of the control and that at an initial overall k_La of 0.33 were relatively steady, and at a higher level of 80 μM over the period (data not shown). The oxygen uptake rate of *G. lucidum* (Fig. 4B) was obtained with the difference between two successive measures over a 5-min time course (Fig. 4A). In this experimental condition, the oxygen uptake rate rapidly decreased in 45 min after the beginning of the experiment, and the critical oxygen level reached about 35 μM (Fig. 2B). Thereafter, the cells were considered to be hypoxia [34]. The oxidative burst (ROS production) occurred when the cells encountered hypoxia conditions.

It is known that GAs as triterpenes are synthesized through the mevalonate pathway [35,36], which includes

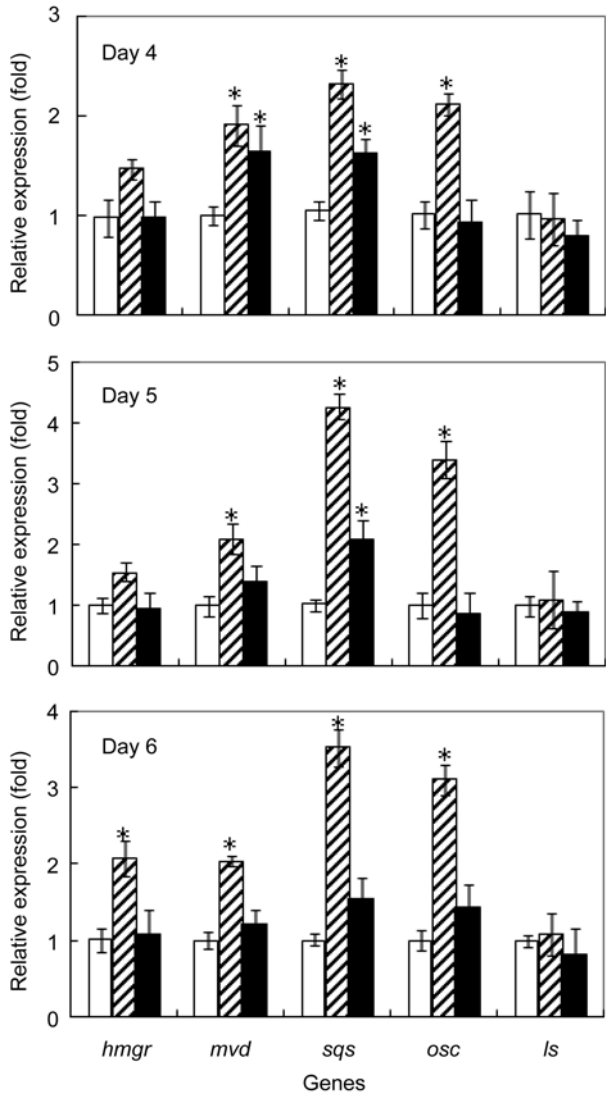

Fig. 5. Biosynthetic gene expressions in *G. lucidum*. Symbols for oxygen supply conditions: initial k_La value of 5.51/h (as control, blank bar), sealed submerged culture from day 3 (hatched bar), with DPI addition in non-aerated submerged culture from day 3 (dark bar). The error bars in the figure indicated the standard deviations from three independent samples. Differences were considered statically significant at $P < 0.05$.

steps catalyzed by HMGR, MVD, SQS, SE and LS. However, it is unclear how the genes encoding these enzymes would respond to hypoxia. The mRNA expression levels of the five corresponding genes, *i.e. hmgr, mvd, sqs, se* and *ls* under oxygen limitation or not were examined, using qRT-PCR. As shown in Fig. 5, the transcription level of *hmgr* in oxygen limitation condition was not obviously increased in the two days cultivation ($P < 0.05$), but it was 1.1 times higher than the control on day 3. For *mvd*, the gene transcription level kept about 2 times that of the control in the entire cultivation process. In the case of *sqs* and *se*, the highest transcription levels in hypoxia culture were about 4.4 and 3.5 times higher than that of the control on the second day. The expression level of *ls* was not changed over the period as studied. Liang *et al.* reported that the increased gene expressions of *hmgr*, *sqs*, and *ls* on days 6 and 7 following phenobarbital induction on day 5 correlated with the enhanced accumulation of GAs in phenobarbital-treated cells [9]. HMGR catalyzes the first step specific to isoprenoid biosynthesis [37]. SQS is generally considered a crucial enzyme at the point of pathway crossroad controlling the carbon flux into the terpenoid synthesis [38]. SE plays a central role in triterpene biosynthesis, because it catalyzes a key reaction of the triterpene biosynthetic pathway, and generates a number of triterpenoid carbon skeletons [10]. LS catalyzes the cyclization of 2,3-oxidosqualene to form lanosterol, which is the lanostane ring skeleton of GAs [39]. In the *G. lucidum* system, among the five genes monitored in this work, *sqs* and *se* showed a continual increase in expression from day 4 to day 6, and relatively higher transcription levels, compared to the other three. This suggests that SQS and SE may be more critical than HMGR, MVD and LS to GA biosynthesis in *G. lucidum*. Up-regulation of the four biosynthetic genes occurred in oxygen limitation-cultured cells, which parallels the increase in the content of total crude GA and four individual GAs. These trends suggest that the increase in the GAs contents in the oxygen limited cells may be due to increased transcription of biosynthetic genes, as observed for GAs biosynthesis affected by phenobarbital induction or nitrogen deficiency [9,12].

In order to determine whether ROS was involved in the enhanced biosynthesis of GA by hypoxia, 10 μM diphenylene iodonium (DPI) (Sigma, USA), a NADPH oxidase inhibitor, was added at 30 min before oxygen limitation. As shown in Fig. 3, the addition of DPI obviously inhibited the formation of ROS. The cell growth and GAs biosynthesis were inhibited simultaneously (Table 1), suggesting that the generation of ROS was induced by hypoxia, and interestingly, it was involved in the low oxygen-induced GA biosynthesis. Whether hypoxia-induced ROS regulated the expression of genes in the GA biosynthesis pathway was further investigated. As shown in Figs. 5A, 5B, and 5C, when DPI was added to the culture medium, the mRNA transcription levels of genes were reduced, except that samples of *mvd* gene on the first day and *sqs* gene in the initial two days were still on a higher level. All of the genes expression decreased to the same level of control on day 3. The results suggested that the hypoxia-induced ROS might activate the *hmgr*, *mvd*, *sqs* and *se* genes transcription in *G. lucidum* cells, and lead to high yield GA accumulation. We found a high oxygen concentration in gaseous phase in the liquid static culture of *G. lucidum*

stimulated extracelluar H_2O_2 generation, and affected GA metabolism, *via* induction of its biosynthetic genes expression [40]. The above facts imply that ROS was induced in hypoxia and hyperoxia, which were extreme conditions for the cells.

4. Conclusion

The production of total crude GAs and individual GAs in the submerged cultivation of *G. lucidum* under oxygen-limiting was higher than that in normoxia condition. Oxygen limitation induced ROS generation in *G. lucidum* cells, which played an important role in hypoxia-induced GA biosynthesis, through transcriptional induction of its biosynthetic genes. The findings may help to further understand the mechanism of oxygen regulation of GA biosynthesis. In this work, we have just analyzed a couple of known genes directly involved in the pathway of GA biosynthesis. We suppose that a global analysis of transcriptome and metabolome profiles would lead to more useful information. It is also possible that some other genes may regulate the biosynthesis as a global regulator, or as an activator/inhibitor to precursors/GAs biosynthesis. Thus, further investigation is required, to have deep understanding of the mechanism of GA biosynthesis regulation.

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