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ORIGINAL PAPER

Further improvement in ganoderic acid production in static liquid culture of *Ganoderma lucidum* by integrating nitrogen limitation and calcium ion addition

Huan-Jun Li[1] · De-Huai Zhang[1] · Li-Liang Han[1] · Xuya Yu[1] · Peng Zhao[1] · Tao Li[1] · Jian-Jiang Zhong[2] · Jun-Wei Xu[1]

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Abstract To further improve the ganoderic acid (GA) production, a novel integrated strategy by combining nitrogen limitation and calcium ion addition was developed. The effects of the integrated combination on the content of GA-T (one powerful anticancer compound), their intermediates (squalene and lanosterol) and on the transcription levels of GA biosynthetic genes in *G. lucidum* fermentation were investigated. The maximum GA-T content with the integrated strategy were 1.87 mg/ 100 mg dry cell weight, which was 2.1–4.2 fold higher than that obtained with either calcium ion addition or nitrogen limitation alone, and it is also the highest record as ever reported in submerged fermentation of *G. lucidum*. The squalene content was increased by 3.9- and 2.2-fold in this case compared with either individual strategy alone. Moreover, the transcription levels of the GA biosynthetic genes encoding 3-hydroxy-3-methyglutaryl coenzyme A reductase and lanosterol synthase were also up-regulated by 3.3–7.5 and 1.3–2.3 fold, respectively.

Keywords Ganoderic acid production · *Ganoderma lucidum* · Bioprocessing strategy · Medicinal mushroom fermentation · Bioactive secondary metabolites

Introduction

Medicinal mushrooms are viewed as a rich source of biologically active and therapeutically useful agents. *Ganoderma lucidum* has long been used for treating and preventing various diseases in East Asia due to its beneficial effects [1–3]. Ganoderic acids (GAs) are the major constituents in *G. lucidum* and they play an important role in its biological effects [4, 5]. Among the GAs, ganoderic acid T (GA-T) is considered as one of most promising antitumor and anti-metastasis candidates based on extensive *in vitro* and *in vivo* pharmacological studies [6–8].

The biosynthesis of GAs is proceed via the mevalonate/isoprenoid (MVA) pathway. This pathway includes the conversion of farnesyl diphosphate to squalene, 2, 3-oxidosqualene, and lanosterol. Lanosterol is the common cyclic intermediate of triterpenes in *G. lucidum*. The final steps following lanosterol formation are yet unclear, but most likely include a series of oxidation, reduction, and acylation reactions [4]. Some genes involved in the MVA pathway have been isolated and characterized in *G. lucidum*. The 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMGR), squalene synthase (SQS), and lanosterol synthase (LS) are key enzymes in the GA biosynthesis [9].

Due to the important pharmacological function of GA-T and other GAs, the demand for them has increased in recent years [3, 10]. Various cultivation methods have been developed to increase GA production in *G. lucidum*. The submerged fermentation of mushroom is considered as a promising approach for the efficient production of major

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✉ Jun-Wei Xu
xjuwei@163.com; jwxu@kmust.edu.cn

[1] Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, China

[2] State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic and Developmental Sciences, and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dong-chuan Road, Shanghai 200240, China

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metabolites because it is fast, cost effective, and easy to control the product quality [5, 11]. Many attempts such as optimization of fermentation conditions [12–14], addition of inducers [15–20] and development of new bioprocessing strategies [21, 22] as well as overexpression of key GA biosynthetic genes [23–25] have been made to increase GA production in the submerged culture of *G. lucidum*. For example, increased GA-T production, compared with traditional shaking culture, was demonstrated in static liquid culture of *G. lucidum* [26]. However, to meet the demand for GA-T for large-scale pharmacological testing and clinical trials, further increase in GA-T production is expected to reduce the product cost.

Our previous work demonstrated that nitrogen limitation is an efficient strategy for increasing the production of GA-T, and the nitrogen-regulated transcription factor AreA may be involved in regulation of GA biosynthesis [27]. A calcium ion addition strategy was also developed to enhance GA-T production, and the results showed that Crz1 (calcineurin-responsive zinc finger), a transcription factor, is pivotal for regulating GA biosynthetic genes at the transcription level [28]. Bioinformation analysis found that both Crz1 and AreA binding sites exist in the promoter sequences of *hmgr*, *sqs* and *ls* (Supplementary data), implying the GA biosynthesis may be synergistically regulated by both nitrogen status of the cells and calcineurin signal transduction pathway. It would be interesting to investigate whether the integration strategy with simultaneous nitrogen limitation and calcium ion addition will lead to more GA-T accumulation in static liquid culture of *G. lucidum*.

As introduced above, therefore this work developed an integrated fermentation strategy by combining nitrogen limitation and calcium ion addition to enhance the GA production in static liquid culture of *G. lucidum*. The influence of the integrated strategy on GA biosynthesis was carefully investigated by quantitatively analyzing GA content, intermediate accumulation, and transcription levels of key biosynthetic genes.

Materials and methods

Strain, prelature and culture conditions

G. lucidum CGMCC 5.616 was purchased from the China General Microbiological Fermentation Center and grown in potato dextrose agar slants at 30 °C. The preculture of *G. lucidum* was conducted by the method of Xu et al. [26]. The mycelia were isolated by centrifugation (10 min, 10,000*g*), and washed 3 times with sterile water, and resuspended in fermentation medium [27]. The fermentation medium consisted of the following components (g/L): glucose 20, KH_2PO_4 1, K_2HPO_4 2, NaCl 0.2, $MgSO_4$ 0.5, vitamin B1 0.05, $CaCl_2$, and L-glutamine. The concentrations of $CaCl_2$ and L-glutamine varied according to the experiment requirement. The resuspended mycelia were moved to plate for liquid static culture process [26].

For comparison of different cultivation strategies, the control experiment was conducted in broth containing an initial glutamine concentration of 60 mM. In the nitrogen limitation experiment, cells were transferred to broth containing an initial glutamine concentration of 3 mM glutamine [27]. In the Ca^{2+} addition experiment, 10 mM $CaCl_2$ was added on day 2 to *G. lucidum* broth containing an initial glutamine concentration of 60 mM according to the previous work [28]. In the integrated strategy fermentation, 10 mM $CaCl_2$ was added on day 2 to *G. lucidum* broth containing an initial glutamine concentration of 3 mM.

Sampling, analysis of biomass, medium sugar, GA-T, total crude GAs, squalene and lanosterol

Biomass and residual sugar concentration were measured by gravimetric method and 3, 5 dinitrosalicylic acid (DNS) method, respectively [27]. Total crude GAs, individual GAs, squalene and lanosterol were extracted and determined according to the method described earlier [23, 25].

Total RNA extraction and cDNA synthesis

Total RNA was extracted using TriZol Reagent (Invitrogen) according to the manufacture's instruction. RNA concentration was determined using a Nanodrop 2000 (Thermo). After extraction, the total RNA was treated with RNAase-free DNAase I (Fermentas) to remove genomic DNA contamination. 1 μg of total RNA was reverse-transcribed using the Superscript RNAase H- First-strand synthesis kit (Invitrogen) following the manufacturer's recommendations.

Real-time quantitative PCR (qRT-PCR) amplification

The transcription levels of the HMGR gene, SQS gene and LS gene were determined by qRT-PCR using the procedure reported previously [23]. Transcription levels were normalized against *G. lucidum* 18S rRNA gene (an internal control), which was constantly expressed under all experimental conditions. For each gene, the reference samples (60 mM glutamine) were set at 1.0, and the results of other samples were expressed as the fold changes over the reference sample.

Statistical analysis

Data are the average of three independent sample measurements. Statistical analysis was performed with Student's *t* test. The results were considered significant for *p* values <0.05 in a two-tailed analysis.

Results and discussion

Effect of the integrated strategy of nitrogen limitation and calcium ion addition on GA production

Figure 1 shows the time profiles of cell growth and sugar consumption in cultures with different strategies. The maximum biomass was 12, 11.2, 8.0 and 8.8 g/L in the control culture, with calcium ion addition, with nitrogen limitation, and with coupling of calcium ion addition and nitrogen limitation, respectively. Compared with the control, the integrated strategy reduced to some extent final dry cell weight due to low nitrogen availability, which was agreed with our previous report [27].

To examine the effect of the integrated strategy on GA biosynthesis by *G. lucidum* in detail, the time profiles of the contents of both GA-T and total crude GAs under various culture conditions were compared in Fig. 2a. The content of GA-T with the integrated strategy increased rapidly until day 8 then declined slightly on day 12, still maintaining a higher level than those under other culture conditions. The maximum GA-T content of 1.87 mg/ 100 mg DW was obtained under the integrated strategy, which was 8.9-, 4.2-, and 2.1-fold of that obtained in the control, the calcium ion addition and the nitrogen limitation conditions, respectively. These results are well agreement with previous reports that the production of GA-T is increased under nitrogen limitation or Ca^{2+} induction conditions. The maximum GA-T content in this study was 1.55 and 1.20 times higher than that reported for a liquid static culture of *G. lucidum* under nitrogen limitation and elicitation with Ca^{2+}, respectively [27, 28]. The GA-T content obtained here was the highest as ever reported in submerged cultures of *G. lucidum*. The results indicated that the integrated strategy was more effective in promoting GA-T biosynthesis than other approaches. The content of total crude GAs showed a similar trend as the GA-T, and the maximum level with the integrated strategy was 2.0-, 1.5-, and 1.1-fold that obtained with the other three conditions, respectively (Fig. 2b). The combination of nitrogen limitation and calcium ion addition resulted in a synergistic enhancement of accumulations of both GA-T and total GAs. This may be due to the up-regulation of some GA biosynthetic genes in *G. lucidum* under a series of signal transduction cascades being more strongly acted by the integration strategy than other conditions [27, 28].

Effect of the integrated strategy on accumulations of squalene and lanosterol

As squalene and lanosterol are important intermediates in the GA biosynthetic pathway, we analyzed the contents of squalene and lanosterol in different culture conditions to understand whether the integrated strategy affected the intermediate accumulation or not. Figure 3a shows that the maximum squalene content obtained with the integrated strategy was 48.7 μg/g DW on the day 8, which was 5.2-, 3.9-, and 2.2-fold that under the control, the calcium ion addition and the nitrogen limitation conditions, respectively. Although the squalene content decreased to 15.6 μg/g DW at day 16, it still maintained at a higher level than the other cases. The amount of lanosterol with the integrated strategy increased to a maximum of 27.5 μg/g DW on day 8, but decreased to 20 μg/g DW at the end of fermentation (Fig. 3b). The maximum content of lanosterol with the integrated strategy was 1.4-fold and 1.2-fold higher than those obtained with either the calcium ion addition or the nitrogen limitation strategy. As shown in Fig. 3a, the content of squalene was higher in the nitrogen limitation condition than that in the control. In *Schizochytrium* sp., it was reported that ammonium sulfate

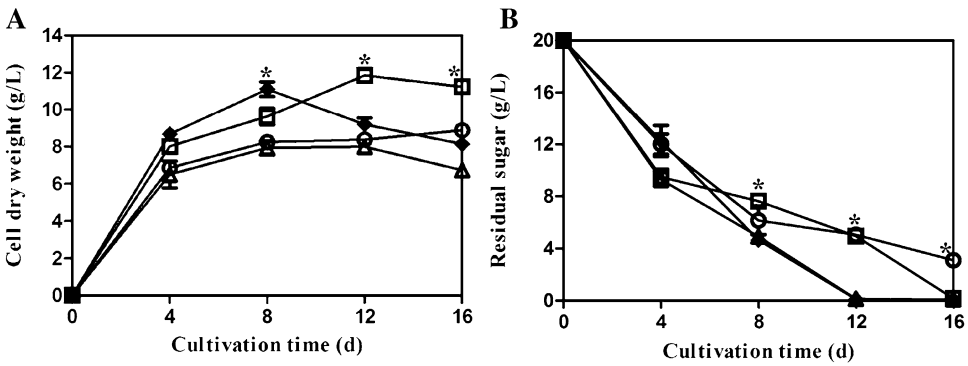

Fig. 1 Time profiles of biomass (**a**) and residual sugar (**b**) in static liquid culture of *G. lucidum* (*unfilled square*), with calcium ion addition (*filled diamond*), with nitrogen limitation (*unfilled triangle*), and with coupling of calcium ion addition and nitrogen limitation (*unfilled circle*), respectively. *d* days

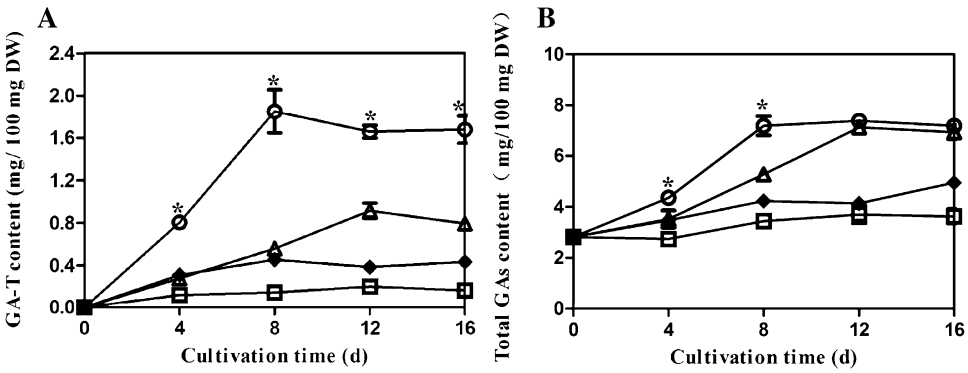

Fig. 2 Time profiles of GA-T content (**a**) and total crude GA content (**b**) in static liquid culture of *G. lucidum*, with calcium ion addition, with nitrogen limitation, and with coupling of calcium ion addition and nitrogen limitation, respectively. *d* days. The symbols are the same as those in Fig. 1

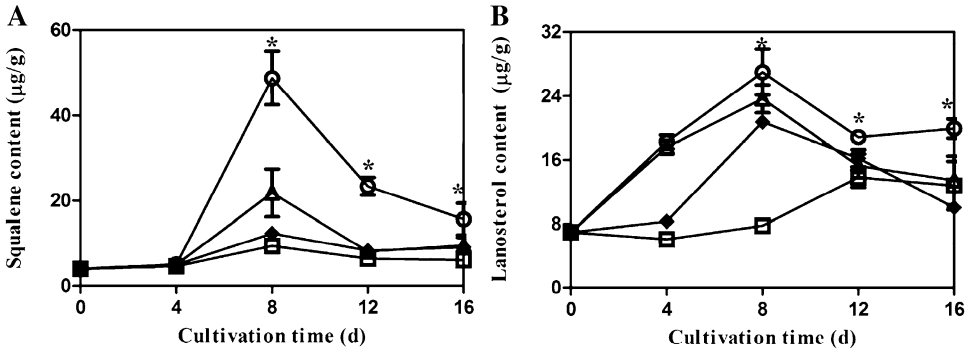

Fig. 3 Time profiles of squalene content (**a**) and lanosterol content (**b**) in static liquid culture of *G. lucidum*, with calcium ion addition, with nitrogen limitation, and with coupling of calcium ion addition and nitrogen limitation, respectively. d, days. The symbols are the same as those in Fig. 1

limitation can efficiently enhance squalene biosynthesis [29]. The accumulations of squalene and lanosterol were found to increase under Ca^{2+} induction, which is in line with another report showing that the increased cellular calcium ion levels induce up-regulation of some genes involved in MVA pathway [30]. The results also showed that more intermediates accumulated throughout the cultivation under the integrate strategy than the other conditions. This observation coincided with previous reports that more accumulation of intermediates is beneficial to the GA production [15, 16, 23, 25]. Our results suggested that increased GA-T accumulation may be related to the increased accumulations of its precursors such as squalene and lanosterol.

Gene expression response to different culture strategies

To understand the possible molecular mechanisms responsible for the enhanced GA-T production under the integrated strategy, the transcription levels of three biosynthetic genes (*hmgr*, *sqs* and *ls*) of GAs were analyzed by qRT-PCR in different culture conditions. As shown in Fig. 4, the transcription level of *hmgr* under the integrated strategy on day 4 was about 7.5 and 3.3 times higher than those under the nitrogen limitation strategy and the calcium ion addition strategy, respectively. In the case of *sqs*, the transcription level on day 4 under the integrated strategy was induced by 1.8-fold and 1.6-fold compared with those under the nitrogen limitation strategy and the calcium ion addition strategy, respectively. For *ls*, the transcription level on day 4 under the integrated strategy was 2.3-fold and 1.3-fold higher than those in the nitrogen limitation strategy and the calcium ion addition strategy, respectively. The transcription levels of three biosynthetic genes were much higher under the integrated strategy on day 4 than those obtained in other culture conditions. Among the three genes monitored, *hmgr* exhibited a higher transcription level compared to *sqs* and *ls* under the integrated strategy. Our results indicated that the calcium ion addition and the nitrogen limitation strategies may exert a combined effect on transcription of GA biosynthetic genes especially *hmgr*. It is known that HMGR is a key enzyme in the MVA pathway that leads to the biosynthesis of various precursors used for the synthesis of terpenes [31]. Those results were in accordance with our previous report that the overexpression of *hmgr* in *G. lucidum* results in a remarkable increase of GA production [23]. Bioinformation analysis showed that both calcineurin-responsive zinc finger (Crz) and AreA/NIT2 binding sites exist in the promoter sequences of *hmgr*, *sqs* and *ls* (Supplementary data). The accumulation of GA-T began to increase sharply from day 4 under the integrated strategy, which was consistent with the increased expressions of *hmgr*, *sqs* and *ls* on day 4. The above results suggested that the higher transcription levels of GA biosynthetic genes in the early-

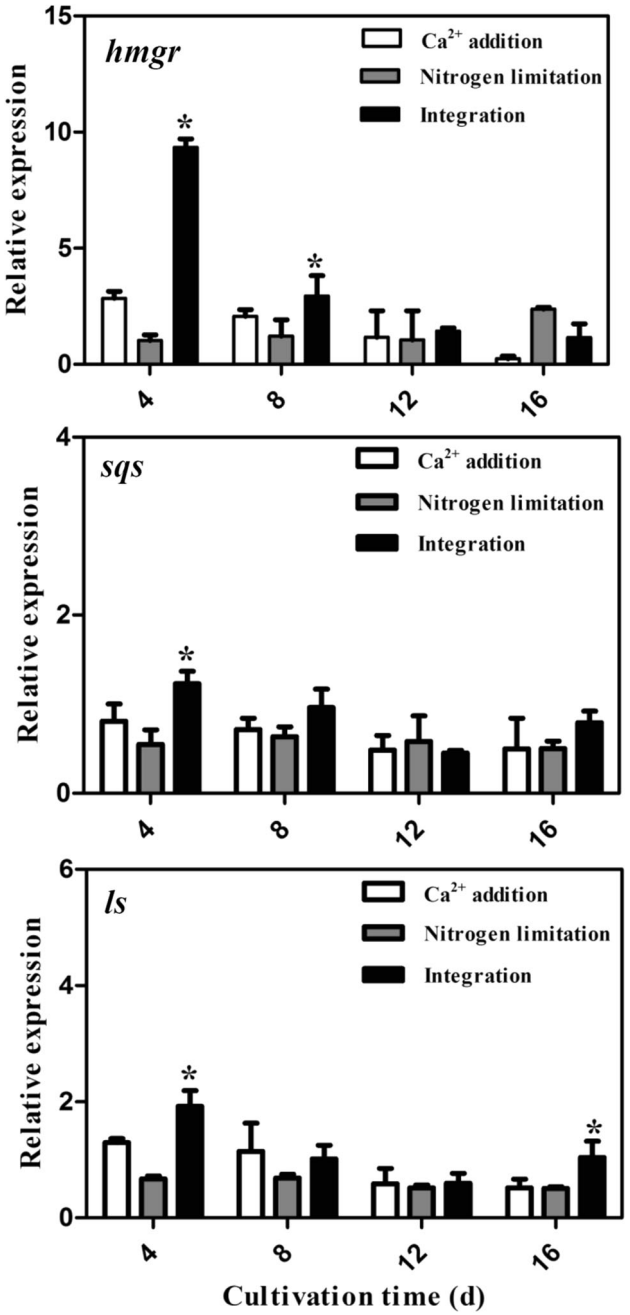

Fig. 4 Transcription levels of three biosynthetic genes in static liquid culture of *G. lucidum* with calcium ion addition, with nitrogen limitation, and with coupling of nitrogen limitation and calcium ion addition. Expression of the samples from the control experiment is set at 1.0, and the results under other culture conditions are expressed as the fold changes over the reference sample. *d* days

stage culture may result in higher production of GA-T. The similar phenomenon was also observed in the enhanced GA production by phenobarbital induction and acetic acid induction [15, 16]. Previous studies also reported that there is a positive correlation between biosynthetic gene expression and the content of triterpenes in *Arabidopsis* [32], *Eleutherococcus senlicosus* [33] and *G. lucidum* [18, 26], to which this work also agreed well. Those information may be useful to other related studies for enhancing triterpene production by genetic or bioprocessing approaches in future.

Conclusions

This work demonstrated a novel integrated strategy for efficient production of antitumor GA-T and total GAs in static liquid cultures of *G. lucidum* by simultaneously adopting calcium ion addition and nitrogen limitation strategy. The integrated strategy could induce the GA biosynthesis through up-regulation of the biosynthetic gene transcription and increased supply of precursors. The work will be helpful to develop a more efficient fermentation process for hyperproduction of the antitumor secondary metabolites.

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