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SHORT COMMUNICATION

Stimulatory effect of ethanol on libertellenone H biosynthesis by Arctic fungus *Eutypella* sp. D-1

Chu Shen[1] · Ning Xu[1] · Yanyun Gao[1] · Xiaoyue Sun[1] · Ying Yin[1] · Menghao Cai[1] · Xiangshan Zhou[1] · Yuanxing Zhang[1,2]

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Abstract Libertellenone H (**1**) was a promising antitumor diterpenoid isolated from Arctic fungus *Eutypella* sp. D-1, however, its production was very limited. In this study, we investigated the effects of ethanol on cell growth and libertellenone H production. The mycelium in ethanol-feeding cultures was fragmented and dispersed, and the titer of libertellenone H was remarkably increased to 4.88 mg l^{-1} in an optimal feeding manner, which was 16.4-fold higher than the control group. To provide an insight into the cell response to ethanol, genes critical to the libertellenone H biosynthesis were successfully cloned and their transcription levels were determined. The results suggested that the gene transcription levels of 3-hydroxy-3-methyl glutaric acyl coenzyme A reductase and geranylgeranyl diphosphate synthase were up-regulated by ethanol stimulation. The results from this study were helpful for further understanding of the ethanol function on diterpenes biosynthesis as well as developing more effective strategies for over-production of these desired secondary metabolites.

Keywords Arctic fungus · Libertellenone H · Ethanol feeding · 3-Hydroxy-3-methyl glutaric acyl coenzyme A reductase · Geranylgeranyl diphosphate synthase

✉ Ying Yin
yy1047@126.com

✉ Menghao Cai
cmh022199@ecust.edu.cn

[1] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

[2] Shanghai Collaborative Innovation Center for Biomanufacturing Technology, 130 Meilong Road, Shanghai 200237, China

Introduction

In recent years, extreme environments, such as the deep sea, hypersaline locations, deserts, and polar regions, have attracted attention as new resources for novel bioactive compounds in drug discovery [1]. Many novel and bioactive secondary metabolites have been successfully discovered from polar regions, which are characterized by low temperature, low fresh water availability, frequent freeze–thaw cycles, osmotic stress, desiccation, low nutrients, and strong UV radiation [2].

As shown in Fig. 1, libertellenone H (**1**) was a structurally novel diterpenoid isolated from Arctic fungus *Eutypella* sp. D-1. Compared with its homolog libertellenone A (**2**), B (**3**), and C (**4**), libertellenone H showed significant inhibition in breast cancer cell line Mcf-7. Libertellenone B had only mild inhibitory effect, and libertellenone A, C did not show inhibition at all [3]. Thus, libertellenone H was a promising antitumor drug candidate. However, the production of libertellenone H was very limited, so that subsequent pharmacological and pharmacodynamic studies were severely restrained. Therefore, different strategies were necessary to improve the biosynthesis level of libertellenone H.

During the last few decades, a large variety of environmental stimulants including light, ultrasonication, temperature, and many chemicals have been developed to improve the production of secondary metabolites [4]. Among these factors, the stimulating role of alcohols, especially ethanol, has been studied by many investigators. In the culture of *Streptomyces clavuligerus* NP1, the presence of ethanol or methanol made the mycelium fragmented and dispersed, and markedly promoted the production of cephalosporin(s) [5]. Ethanol or methanol also increased the permeability of the cell membrane and the metabolic activity of the enzymes related to citrate synthesis [6, 7]. Besides, ethanol triggered

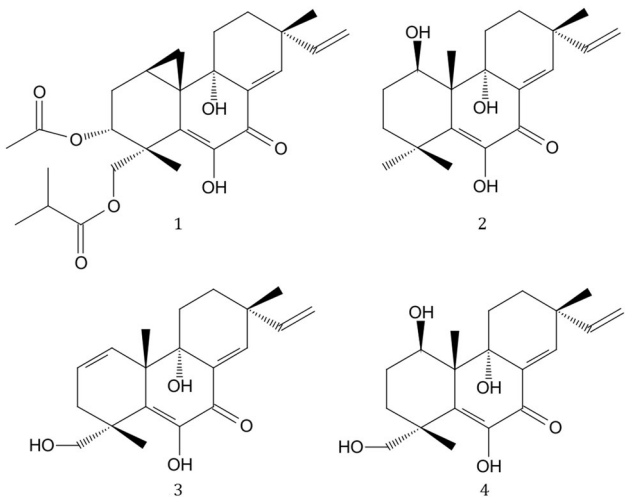

Fig. 1 Chemical structure of libertellenone H (1), A (2), B (3), and C (4)

various responses, including the increase of reactive oxygen species (ROS) and transcriptional levels of validamycin A biosynthetic genes, which finally enhanced antibiotic production [8]. In general, ethanol, as a kind of cultural stimulants, has been widely applied in citric acid, antibiotics, and other secondary metabolites biosynthesis. However, few groups reported the effects of ethanol on diterpenes biosynthesis as well as its inner mechanism.

In this study, we investigated the stimulatory effect of ethanol on libertellenone H biosynthesis. As ethanol might be toxic to the cells and could affect their viability and metabolic activity, different feeding concentrations and feeding times of ethanol were tested to find an optimal condition for ethanol supplement in *Eutypella* sp. D-1 culture. Then the effects of ethanol on mycelial morphology, cell growth, and libertellenone H production were measured in the optimal feeding manner. On the other hand, to discover the mechanism of ethanol effect on libertellenone H production, gene transcription levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and geranylgeranyl pyrophosphate synthase (GGPPS), which were two critical enzymes involved in the diterpenoid biosynthetic pathway, were examined. The characterization of ethanol-induced gene expression will explain libertellenone H accumulation, and it might also help to design new strategies for desired terpenoid compound hyperproduction.

Materials and methods

Microorganism

Eutypella sp. D-1 (CCTCC M 2013144) was kindly provided by the Second Military Medical University, isolated from the soil of London Island of Kongsfjorden of Ny-Ålesund District (altitude of 100 m) of Arctic [3]. Mycelia of *Eutypella* sp. D-1 were stored in 30 % (w/v) glycerol at −20 °C.

Fermentation conditions

Medium I contained 125 g glucose l^{-1}, 3.3 g $NaNO_3$ l^{-1}, 0.07 g $K_2HPO_4{\cdot}3H_2O$ l^{-1}, 0.4 g $MgSO_4{\cdot}7H_2O$ l^{-1}, 0.625 g KCl l^{-1}, 0.7 g yeast extract l^{-1}, 0.003125 g $CoCl_2{\cdot}6H_2O$ l^{-1}, 0.01875 g $FeSO_4$ l^{-1}, 0.0065 g $CaCl_2$ l^{-1}, 15 g L-ornithine hydrochloride l^{-1} and was adjusted to pH 5.8. Medium II contained 51.4 g sucrose l^{-1}, 3.3 g $NaNO_3$ l^{-1}, 0.07 g $K_2HPO_4{\cdot}3H_2O$ l^{-1}, 0.4 g $MgSO_4{\cdot}7H_2O$ l^{-1}, 0.625 g KCl l^{-1}, 0.7 g yeast extract l^{-1}, 0.003125 g $CoCl_2{\cdot}6H_2O$ l^{-1}, 0.01875 g $FeSO_4$ l^{-1}, 0.0065 g $CaCl_2$ l^{-1}, 15 g L-ornithine hydrochloride l^{-1} and was adjusted to pH 5.8.

The strain was first cultured on potato dextrose agar (PDA) medium at 28 °C for 7 days. To obtain inoculums, a rounded agar block (10 mm diameter) was picked by a puncher from agar plate, divided into four pieces, and then inoculated into 100 ml medium I in 500 ml shake flask. The first stage seed was incubated at 28 °C and 180 rpm for 3.5 days on a rotary platform shaker. To get the second stage seed, 5 ml of first stage seed was inoculated into 100 ml medium I in 500 ml shake flask and cultivated for 2 days under the same conditions. Then all fermentation cultures were performed in 250 ml flasks with 50 ml of medium II, which was inoculated with 2.5 ml second stage seed. The flasks were incubated at 20 °C and 180 rpm on a rotary platform shaker for 9 days. Three duplicates were carried out for each condition.

Ethanol feeding experiments

To investigate the effects of the ethanol amount on fermentation process, ethanol was equally fed three times (72, 96, and 120 h) with final concentrations of 1, 2, 4, and 6 %, respectively. Afterward, the effects of ethanol feeding time on cell growth and libertellenone H synthesis were also investigated. In this experiment, method A means equally feeding ethanol three times at 24, 48, and 72 h; method B means equally feeding ethanol three times at 72, 96, and 120 h; method C means equally feeding ethanol three times at 120, 144, and 168 h; method D means equally feeding ethanol three times at 168, 192, and 216 h; method E means equally feeding ethanol every day. The final ethanol concentration was 4 % in every method.

Analytical methods

The off-line pH of the broth was detected by an FE20 pH meter (Mettler-Toledo). The total biomass of *Eutypella* sp. D-1 was determined by dry cell weight (DCW) [9].

Before the analysis of residual sugar, broth was centrifuged at 13,000*g* for 5 min and the supernatant was obtained. The total residual sugar was determined by standard anthrone–sulfuric acid method [10].

For the analysis of libertellenone H titer, 10 ml broth, 20 ml ethyl acetate and 30 glass beads were added into a 250 ml shake flask, and then incubated at 20 °C and 180 rpm on a rotary platform shaker for 36 h. Afterward, the mixture was centrifuged at 5000*g* for 10 min. The upper-liquor was collected and evaporated under reduced pressure. The obtained crude gum was dissolved with 1.5 ml methanol and centrifuged at 13,000*g* for 10 min. At last, the extract was microfiltrated and prepared for further HPLC analysis.

The reverse phase HPLC system of Agilent 1200 with a C18 column (Kromasil^{TM}, Sweden, 250 mm × 4.6 mm, 5 μm 100 Å-spherical silica) was used to analyze the concentration of libertellenone H. The mobile phase was solutions of 0.1 % acetic acid (solvent A) and acetonitrile (solvent B) and a linear gradient was performed from 5 to 60 % solvent B in 10 min followed by 60 % solvent B in 20 min. The flow rate, UV absorption, and operating temperature were set as 1.0 ml min^{-1}, 332 nm, and 25 °C, respectively. The concentration of libertellenone H in fermentation broth was calculated according to the corresponding purified standard.

Gene cloning and assay of gene expression by quantitative real-time PCR (qRT-PCR)

The mycelia of *Eutypella* sp. D-1 in fermentation medium were filtered and collected. Genomic DNA was extracted from mycelia using plant genomic DNA kit (Tiangen Biotech Co., Ltd., China). Degenerate PCR and tail PCR [11] were applied to obtain the target gene (*hmgr* and *ggpps*) sequences.

Total RNA was extracted by RNAsimple Total RNA Kit (Tiangen Biotechnology Co., Ltd, China). The residual genomic DNA was removed by RQ1 RNase-Free DNase (Promega, America). RNA samples were reverse-transcribed with RevertAid^{TM} M-MuLV system (Fermentas, Canada). Reverse transcription was achieved using PrimeScript^{TM} RT Reagent Kit (TAKARA, Japan).

The transcriptional level of *hmgr*, *ggpps,* and *β-tubulin* was determined by qRT-PCR on an Applied Biosystems 7500 (America) with FastStart Universal SYBR Green Master (ROX) (Roche, Switzerland). Primers were designed by Primer 5.0. *Hmgr*: forward primer, 5′-CCTTGGAGATCATG-TTCATAC-3′; reverse primer, 5′-CTGGCTGGTACGAATCTT-3′; *ggpps*: forward primer, 5′-CCT-TGTTCTCGGTGTACTT-3′; reverse primer, 5′-CTGGCTGGTACGAATCTT-3′; *β-tubulin*: forward primer, 5′-AACAAGTATGTTCCTCGT-3′; reverse primer, 5′-CAAAGACGAAGTTGTCGG-3′. The PCR conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 30 s at 72 °C. According to the study of Livak and Schmittgen [12], the $2_T^{-\Delta\Delta C}$ method was applied to post-qRT-PCR calculations to analyze relative gene expression. *β-tubulin* was set as endogenous control and the final data for the gene expression were normalized with it.

Statistical analysis

The significances of the differences between treatments were determined by *P* values using one-way analysis of variance (ANOVA). It was defined as significant when $P < 0.05$. All data were analyzed by Origin 8.0 (OriginLab Corporation, USA).

Results and discussion

Ethanol feeding optimization

The effects of different ethanol concentrations on fermentation were determined first. As shown in Fig. 2a, in general, the presence of ethanol inhibited the cell growth. However, libertellenone H titer and yield coefficient of libertellenone H titer to DCW ($Y_{T/X}$) in ethanol feeding cultures were increased compared with the control. The maximum libertellenone H titer and $Y_{T/X}$ was obtained when 4 % ethanol was fed ($P < 0.05$). Before reaching 4 %, libertellenone H titer increased slowly with ethanol concentration ($P < 0.05$). This suggests that the positive effect of ethanol is dose-dependent and actually the similar phenomenon was also observed in other reports [6, 8]. On the other hand, when ethanol concentration reached 6 %, libertellenone H titer remarkably decreased, accompanied by a severe cell growth defect (45.6 % decline in DCW, $P < 0.05$). This might be due to strong disturbances on fungal metabolism and mycelial morphology caused by ethanol. Similar findings were also reported by Moyer and Aftab Nadeem [6, 13]. At higher concentrations, some alcohols may greatly inhibit cell growth, which in turn impacts product synthesis. According to the results, the optimal feeding concentration of ethanol is 4 %.

As the appropriate timing for feeding alcohols to the cell culture is considered to be one of the most important factors in secondary metabolism [8], the cultures feeding with 4 % ethanol at different time points were also investigated for cell growth and product synthesis. As shown in Fig. 2b, ethanol fed at different time points had diverse effects on cell growth and libertellenone H synthesis. In general, ethanol fed on different time points restrained cell growth at various degree compared with the control. The final pH

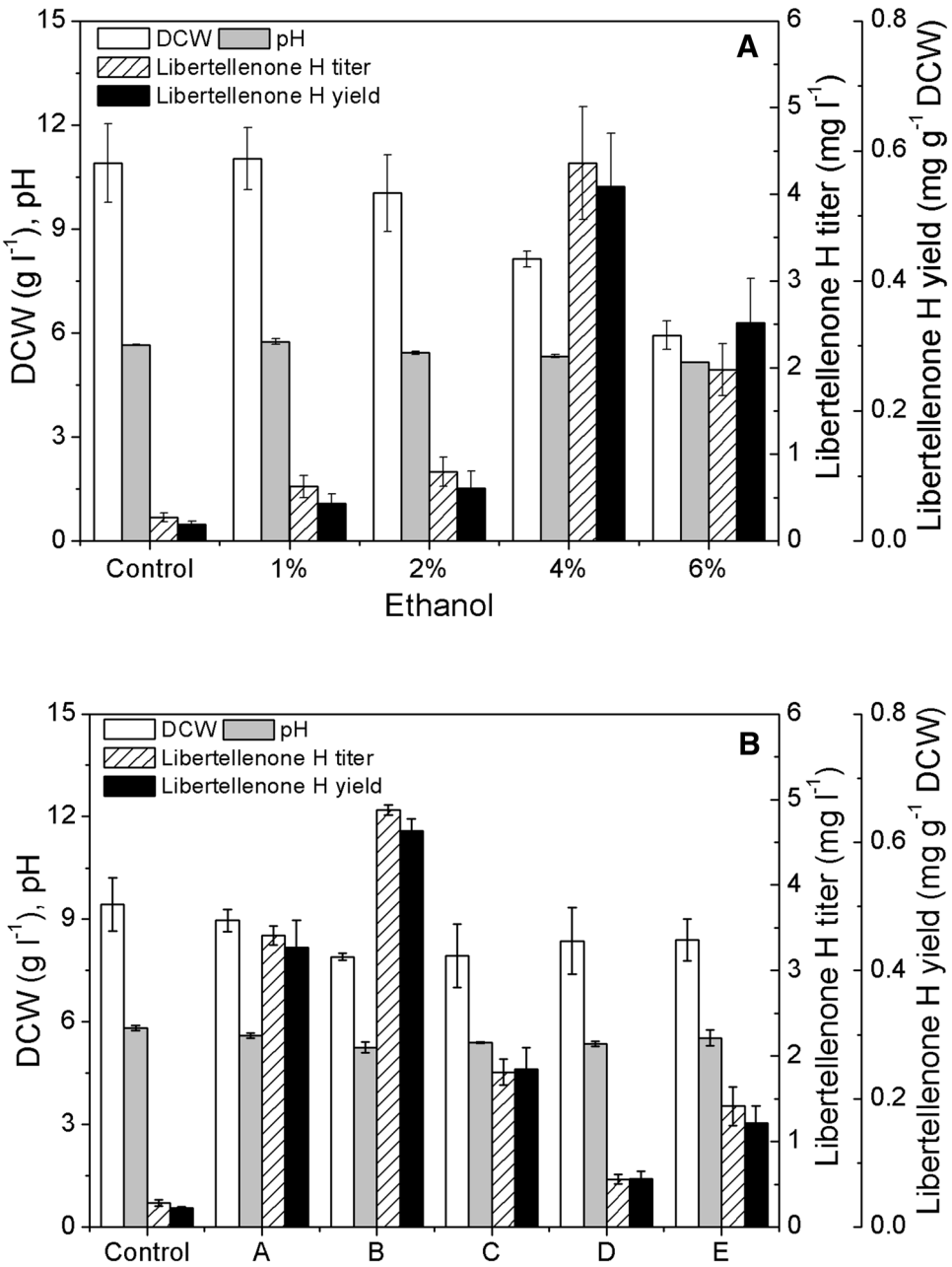

Fig. 2 Effects of ethanol amount and feeding time on cell growth and libertellenone H titer of *Eutypella* sp. D-1. **a** Different concentrations of ethanol were fed equally three times at 72, 96, and 120 h. **b** Different feeding time of ethanol. Method A means equally feeding ethanol three times at 24, 48, and 72 h; method B means equally feeding ethanol three times at 72, 96, and 120 h; method C means equally feeding ethanol three times at 120, 144, and 168 h; method D means equally feeding ethanol three times at 168, 192, and 216 h; method E means equally feeding ethanol every day. The final feeding concentration of ethanol was 4 %

in ethanol feeding cultures was slightly lower than control ($P < 0.05$, except in method E). The highest titer (4.88 mg libertellenone H l^{-1}) and $Y_{T/X}$ (0.618 mg libertellenone H g^{-1} DCW) was achieved by method B (equally feeding ethanol three times at 72, 96, and 120 h), which was 16.4-fold and 19.8-fold higher than the control ($P < 0.05$), respectively. In this study, feeding ethanol too early or too late, both reduced the stimulating effect of ethanol on libertellenone H production. It seems that, feeding ethanol too early might disturb the normal growth of mycelium, which is not beneficial for libertellenone H biosynthesis. On the other hand, although feeding at the middle or late phase of the fermentation could overcome the negative effects of ethanol on cell growth, it failed to stimulate libertellenone H biosynthesis. It is likely that the genes related to product synthesis might be transcribed and translated already at early phases, therefore ethanol feeding after that cannot do much. This study shows similar results to another study reported in submerged culture of *Streptomyces hygroscopicus* 5008, in which ethanol feeding in the later phase of growth resulted in less enhancement of validamycin A production [8]. However, feeding 0.2 % ethanol at different times did not affect cell mass or carotenoid accumulation in *Phaffia rhodozyma* [14], which

might be due to the low concentration of ethanol applied in their study. Furthermore, it has been reported that the end of the exponential growth phase is another good time point for elicitors to induce secondary metabolite synthesis [15, 16]. Therefore, the optimum feeding time is probably correlated with different kinds of elicitors and strains.

Consequently, the optimal ethanol feeding condition in this study was determined as equally feeding ethanol three times at 72, 96, and 120 h with a final concentration of 4 % in the medium. Under this condition, libertellenone H titer increased by 16.4-fold. Afterward, this optimal ethanol feeding condition was applied for further investigation.

Time course of cell growth, pH, sugar utilization, and product biosynthesis with ethanol feeding

In the fermentation process, ethanol obviously changed the mycelial morphology compared with the control. The mycelia were long, dense, and twisted with each other to form large mycelial clumps with large hairy region in control media (Fig. 3a). However, in ethanol-supplemented cultures, most hyphae were fragmented and dispersed due to the toxicity of ethanol and a small amount of hypha gathered together to form only small clumps (Fig. 3b). It has been reported that fungal morphology was closely related with production of secondary metabolites in submerged cultures of filamentous fungi [17–19]. So it is possible that the higher production with alcohol supplements was at least partially due to a specific morphological change.

Time course of cell growth, pH, and sugar utilization with ethanol feeding are shown in Fig. 4a. The biomass in ethanol feeding group was lower than the control. And ethanol feeding also led to a slightly lower pH from day 3 to day 9. As for the sugar utilization profiles, ethanol could increase sugar utilization from day 3 to day 9, and might result in more organic acid accumulation, which is always in accordance with pH value reduction.

The effect of ethanol on product biosynthesis is shown in Fig. 4b. In ethanol-supplemented cultures, libertellenone H titer increased slowly during ethanol feeding period, but rapidly after feeding was stopped, and reached the maximum on day 9. Meanwhile, the titers of libertellenone A, B, and C, which were the homologs of libertellenone H (Fig. 1), also increased greatly and even accumulated largely after day 9 (Fig. 4b). They are all typical diterpenoids and synthesized by mevalonate pathway. As a result, we speculated that ethanol feeding induced more metabolic flux flow to the mevalonate pathway and thus, enhanced the biosynthesis of diterpenoid compounds such as libertellenone A, B, C, and H in this fungus. As libertellenone A, B, C, and H are homologs, they share some early steps of biosynthesis, but have distinct later steps since they have different side chain groups. In particular, libertellenone H shows the largest uniqueness, which has acetyl, isobutyryl, and three-membered ring in its structure. This may be the reason for the difference in the accumulation time of libertellenone A, B, C, and H. Next, we will focus on the transformation relationships between libertellenone A, B, C, and H. The biosynthesis of libertellenone H may be further enhanced by the inhibition of the synthesis of libertellenone A, B, and C.

In view of the positive effect of ethanol, many researchers have further studied the mechanism of the effects of lower alcohols on product biosynthesis. In the cultures of yeast *P. rhodozyma*, the accumulation of carotenoid by ethanol feeding was accompanied by elevated expression of HMG-CoA reductase and might relate with activation of oxidative metabolism [14]. As trace metals, manganese, iron, and zinc are critical to cell growth, it has

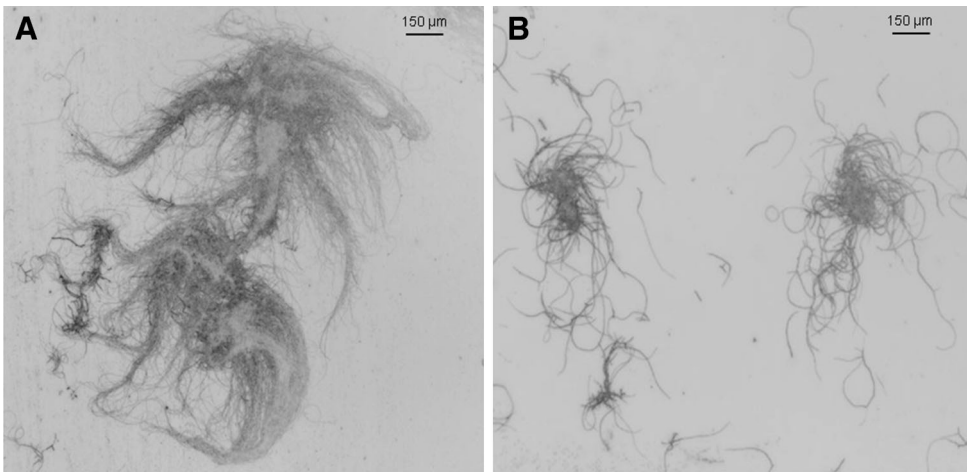

Fig. 3 Effects of ethanol feeding on *Eutypella* sp. D-1 morphology (×2.5). **a** Morphology of control cells without ethanol feeding. **b** Morphology of cells with ethanol feeding

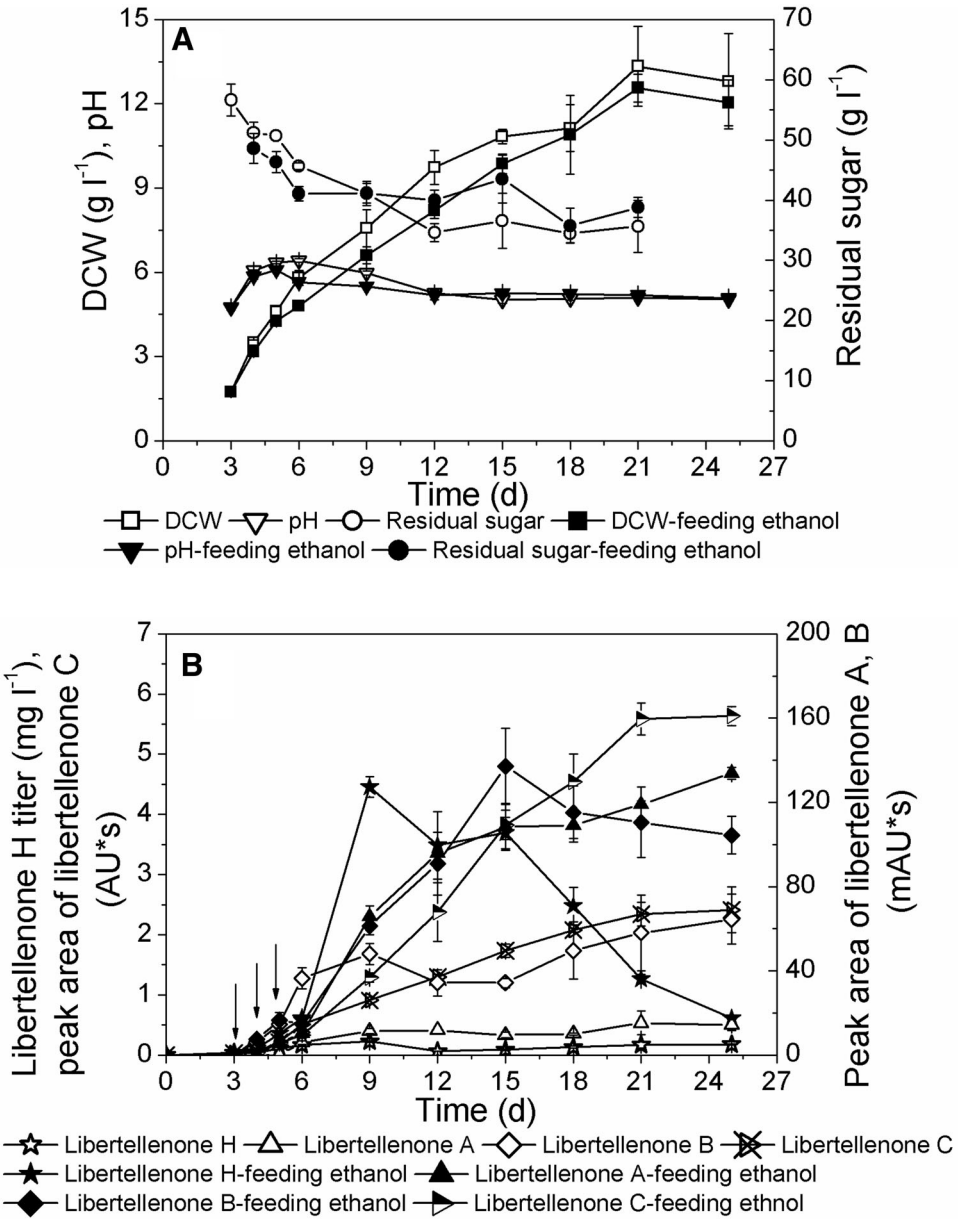

Fig. 4 Comparis-on of time profiles of DCW, pH, residual sugar, libertellenone H, A, B, and C between ethanol feeding and control. **a** DCW, pH and, residual sugar. **b** Libertellenone H, A, B, and C. *Arrows* indicates the ethanol feeding time

been proven that alcohols remarkably enhanced production by increasing their tolerance in fungus [20]. Additionally, in the fermentation of *S. hygroscopicus* 5008, the mechanism of the effects of ethanol on validamycin A production could be explained by the change of intracellular ROS signals, the transcription level of key genes and activities of key enzymes in carbon metabolism [8].

However, to the best of our knowledge, the specific mechanism of the effects of ethanol on diterpenoid is still unclear. In this study, the mechanism of ethanol stimulation on the biosynthesis of libertellenone H was further analyzed on the transcription level of key genes related to diterpenoid synthesis.

Effects of ethanol on the transcriptional level of libertellenone H biosynthesis-related genes

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR), the rate-limiting enzyme of mevalonate pathway is one of the most highly regulated enzymes in nature. It catalyzes the conversion of HMG-CoA to mevalonic acid [21]. Although Gu et al. [14] showed some evidence that increased carotenoid production by ethanol was accompanied by HMGR induction and possible activation of oxidative metabolism, the role of HMGR in libertellenone H biosynthesis under ethanol treatment still needs to be determined. Therefore, the expression profile of *hmgr* gene in *Eutypella* sp. D-1

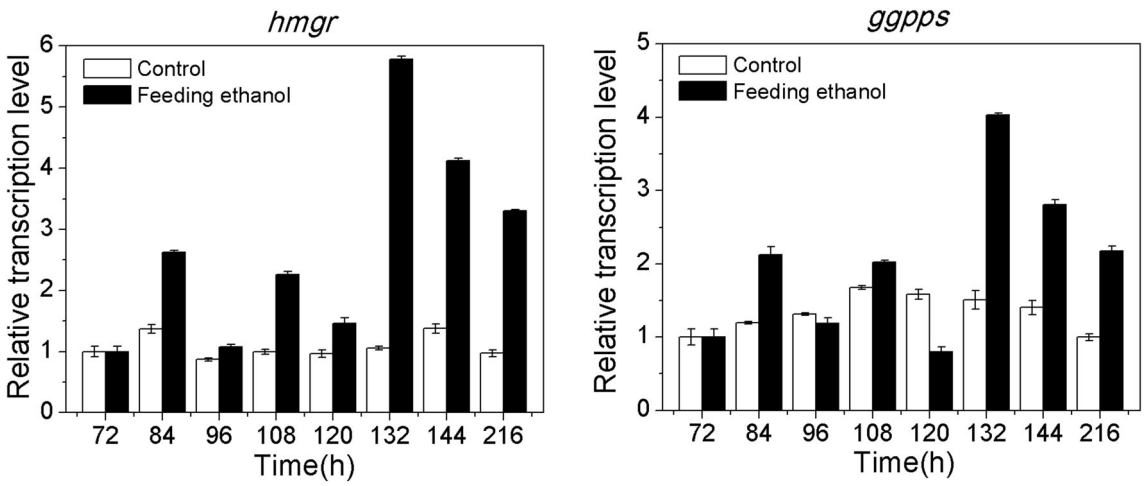

Fig. 5 Transcriptional level of biosynthetic genes *hmgr* and *ggpps* with (*dark*) or without (*blank*) ethanol. Untreated cells collected on the third day were defined as reference samples with expression level 1.0, and results were expressed as the fold increase of mRNA level over the reference sample

was investigated. Moreover, since geranylgeranyl pyrophosphate (GGPP) is one of the branching points in the mevalonate pathway [22], geranylgeranyl pyrophosphate synthase (GGPPS) is also a key enzyme in diterpenoid synthesis. Several reports showed that elicitors such as jasmonic acid and methyl jasmonate induced gene transcriptions and/or enzyme activities of GGPPS in the taxoid biosynthetic pathway [9, 23]. However, few groups concerned the effect of ethanol on GGPPS transcriptions. Thus, we also investigated the expression profile of *ggpps* gene according to ethanol feeding.

First, the sequences of ORF and flanking regions of HMGR and GGPPS were obtained by degenerate PCR and Tail PCR. The HMGR ORF was 3610 bp (GenBank accession No. KT320871). The GGPPS ORF was 1224 bp (GenBank Accession No. KT320870). Then the transcription levels of *hmgr* and *ggpps* with and without ethanol in the medium were determined by qRT-PCR. As shown in Fig. 5, the transcription levels of both *hmgr* and *ggpps* were significantly increased at time points 84, 108, and 132 h (12 h after every ethanol treatment). As for *hmgr* transcription, it reached 1.9-fold at 84 h and 2.3-fold at 108 h compared with control ($P < 0.05$). Afterward, significant transcriptional change of *hmgr* was observed. It reached 5.5-fold over control ($P < 0.05$) at 132 h, and then decreased progressively when ethanol feeding was stopped. The response of the *ggpps* gene expression levels along those time points by ethanol induction showed a similar trend with *hmgr*. The expression level of *ggpps* reached 2.7-fold over control ($P < 0.05$) at 132 h and also declined after that. The relative inconspicuous increase in transcription level of *hmgr* and *ggpps* after the first and second ethanol feeding might be explained by the following reasons. First, the amount of ethanol in culture medium was still relatively low after the first and second feedings and the obvious stimulation could be obtained after the third feeding. Second, the environment around mycelium changed greatly when ethanol feeding started, since ethanol was toxic to the cells. It is likely that cells needed some time to recover from this environmental change, which led to the relative low transcription level at the beginning. Although transcription levels of *hmgr and ggpps* decreased when ethanol feeding was stopped from 132 to 216 h, the libertellenone H titer was largely accumulated (Fig. 4b). This indicates that gene up-regulation occurs earlier than libertellenone H accumulation under ethanol treatment. The result was in consistent with the rapid accumulation of validamycin A in the stationary phase while the up-regulation of structural genes happened earlier (at 24 h after ethanol treatment) [8].

Although more regulatory and functional details of the two metabolic genes involved in the secondary metabolism in *Eutypella* sp. D-1 remain to be elucidated, our results indicate that overexpression of the positive key metabolic genes might facilitate more secondary metabolic flux into libertellenon H biosynthesis.

Conclusions

Our study provides experimental evidences for the positive effect of ethanol on libertellenone H production by *Eutypella* sp. D-1. With ethanol treatment, libertellenone H biosynthesis was significantly elevated (16.4 folds), as well as its homologs libertellenone A, B, and C. The transcriptional analyses of key genes *hmgr* and *ggpps*

preliminarily reveal the mechanism of ethanol effects on libertellenone H and its homologs. The information obtained from mycelium morphology, physiological data, and gene transcription will help to further understand the role of the ethanol on diterpenes biosynthesis. It also provides some guidance to develop more effective strategies for over-production of other desired secondary metabolites.

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