Arch Microbiol (2017) 199:939–944
DOI 10.1007/s00203-017-1359-0

SHORT COMMUNICATION

Molecular analysis and biochemical characteristics of degenerated strains of *Cordyceps militaris*

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Received: 30 January 2017 / Revised: 2 March 2017 / Accepted: 7 March 2017 / Published online: 20 March 2017

Abstract *Cordyceps militaris* has commercially been cultivated, but its degenerated subcultures have gradually resulted in the reduced production. In this study, the biological characteristics and DNA change of degenerated strains of *C. militaris* were analyzed in detail. The results showed that the degenerated strains exhibited the lower growth rate, and the deficiency in fruit body formation and pigment production. The degradation of strains was not attributable to DNA changes identified by RAPD and SRAP. Compared to normal strains, the biochemical indexes of degradation strains and normal strains showed that the carotenoid content of degradation strains was significantly lower, the activities of cellulase and amylase of degradation strains were slight lower, and the EPS content was lower, but the IPS was higher. All these results suggested that the degradation of *C. militaris* may be caused by the inhibition or in harmony of metabolite synthesis involved in the metabolic regulation, which should be further verified.

Keywords Biochemical characteristics · *Cordyceps militaris* · Molecular identification · Strain degeneration

Abbreviations

C. versicolor	*Cordyceps militaris*
C. sinensi	*Cordyceps sinensi*
EPS	Extracellular polysaccharide
IPS	Intracellular polysaccharide
RAPD	Random amplified polymorphic DNA
SRAP	Sequence-related amplified polymorphism

Communicated by Erko Stackebrandt.

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Introduction

Cordyceps militaris, an important medicinal and nutritious edible fungus, is closely related to *Cordyceps sinensi*. They are in the lineage of Clavicipitaceae, *Cordyceps* (Sung et al. 2007). By comparison, *C. militaris* has a wider geological distribution than that of *C. sinensi*, mainly including France, Germany, England, Canada, and USA. *Cordyceps militaris* can be artificially cultivated for its robust adaptability to different climate manifested by its wide distribution, production quantity, and ease in cultivation. For quite a long history, *C. militaris* and *C. sinensi* have been consumed as famous and precious component in the traditional Chinese medicine and as cooking material for luxurious cuisines (Das et al. 2010). The majority of pharmacologically active ingredients (cordycepin, adenosine and polysaccharides) extracted from *C. militaris* and *C. sinensi* are very nearly the same (Kang et al. 2015; Reis et al. 2013; Tuli et al. 2014). Therefore, *C. militaris* is the best substituent when the supply cannot meet the increasing consumption demand of *C. sinensi* in global market. However, the degeneration phenomena of *C. militaris* strain hindered the industrialized cultivation and the industrialization development. Therefore, the strain regeneration of *C. militaris* has attracted considerable research attention, especially the detection at early period.

Currently, there have been a few studies on the degeneration of *C. militaris* strain mainly caused by the high-frequency mutation of gene, low isozyme expression (Li

et al. 2003), dehydrogenase activity (Lin et al. 2010), and nucleus phase change (Wang et al. 2010). In general, the degenerated strains of *C. militaris* have some special characteristics like less primordium, spore quantity change, and abnormal fruit body after cultivation. Although these studies had provided some valuable information on the degeneration of *C. militaris* strain, a little is known about the other characteristics of degenerated strain, especially on the mechanism of degeneration. In the present study, the mechanism of degeneration will be explored on DNA and metabolic levels by molecular markers (RAPD and SRAP) and biochemical determination. The data obtained in this work will contribute to a better method for the detection of degenerated strains at early period and reduce the risk of production failure of *C. militaris* on large-scale cultivation.

Experimental procedures

Microbial strains and culture media

Strains cm-3, cm-5, cm-6, and cm-7 are normal, and cm-6D2 and cm-6D3 are degenerated strains originated from cm-6, which cannot form fruit body. The strain F_{12} is a culture of 12 subculture generation from the original strain cm-6. Liquid medium was prepared with the corresponding raw materials (potato 200 g; glucose 20 g; peptone 3 g; yeast extract 3 g; KH_2PO_4 1 g; $MgSO_4$ 0.5 g; VB_1 0.1 g; L^{-1}). Solid medium was made on the basis of liquid medium by adding 20 g/L agar. Due to the sensitivity of vitamin to heat inactivation during a typical autoclave process, it was directly added to the medium at a proper stage of medium preparation. Solid cultivation medium consisted of oatmeal and liquid medium at a ratio of 1:1 (w/v).

Verification of degenerated strains

The mycelial spawn from the liquid culture of strains cm-6, cm-6D2, cm-6D3, and F_{12} was inoculated onto a cultivation medium. Every bottle was packed with 24 g of cultivation substrate. The characteristics of fruit bodies were determined and recorded when the harvest of matured fruit bodies was complete.

Molecular operations

All primers or primer pairs used in this study were listed in Table 1. PCR amplification was carried out with the corresponding primer or primer pair to finish RAPD and SRAP analyses (Li et al. 2007). Thermal cycles were implemented with the following program: the first five cycles were run at 94 °C (5 min), 94 °C (1 min), 35 °C (1 min), and 72 °C (1 min) for predenaturing, denaturing, annealing, and extension, respectively. Then, the annealing temperature was raised to 50 °C for another 35 cycles. After the completion of the 35 cycles, the reaction mixture was incubated for 10 min at 72 °C. After the PCR amplification, the samples were subjected to analysis with the conventional agarose electrophoresis. The results of electrophoresis were recorded with imaging system.

Determination of biochemical characteristics of *C. militaris* strains

(1) Mycelial characteristics: The mycelial blocks (5×5 mm) from strains cm-3, cm-5, cm-7, cm-6, cm-6D2, cm-6D3, and F12 were placed on solid plates, incubated in dark for the mycelial overgrowth, and then continued to culture for 48 h in light condition. The characteristics of mycelia were observed and recorded. (2) Analysis of intracellular, extracellular polysaccharides, carotenoid, cellulase, and amylase: The hyphal (intracellular and

Table 1 Oligoes used in this study

Used in	(Forward) primer	Sequence (5′→3′)	Reverse primer	Sequence (5′→3′)
RAPD	A12	TCGGCGATAG		
	A16	AGCCAGCGAA		
	C7	GTCCCGAGCA		
	B18	CCACAGCAGT		
	S22	TGCCGAGCTG		
	S62	GTGAGGCGTC		
SRAP	Me1	TGAGTCCAAACCGGATA	Em3	GACTGCGTACGAATTGAC
	Me2	TGAGTCCAAACCGGAGC	Em6	GACTGCGTACGAATTGCA
	Me3	TGAGTCCAAACCGGAAT	Em8	GACTGCGTACGAATTAGC
	Me5	TGAGTCCAAACCGGAAG		
	Me6	TGAGTCCAAACCGGTAG	Em13	GACTGCGTACGAATTGGT

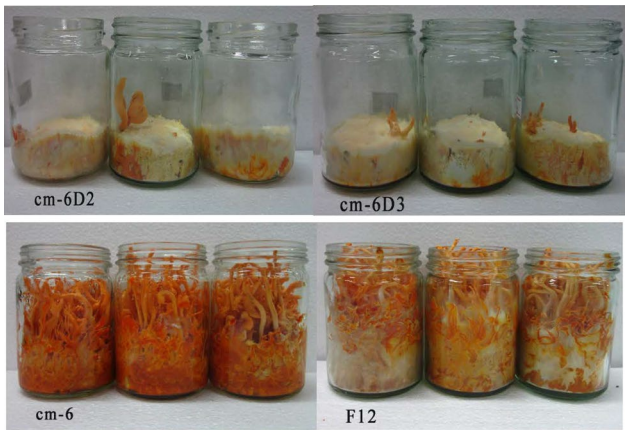

Fig. 1 Cultivation of degenerated and normal strains

extracellular) polysaccharide was extracted with the method reported (Wang and Li 2009), and quantified by phenol–sulfuric acid colorimetric method. The produced carotenoid was determined with the method reported (Fu 2005). The cellulase activity was determined with the filter paper assay (Meddeb-Mouelhi et al. 2014). One unit (U) of cellulase was defined as the amount of enzyme that could catalyze the generation of 1 ug glucose in 1 min (Ferrari et al. 2014). The amylase activity was assayed with the iodine–starch colorimetric method reported (Visvanathan et al. 2016). One unit (U) of amylase was defined as the amount of enzyme that releases 1 µmol of reducing sugar as maltose per minute under the assay condition and is expressed as U/mL of substrate in submerged fermentation. All experiments were carried out in triplicate to ensure the reproducibility and the mean of three independent experiments was presented.

Results and discussion

Verification of degenerated strains

The growth rate of degenerated strains (cm-6D2 and cm-6D3) was normal, and mycelia were thin with a tint of yellow. Mycelia of original strain (cm-6) were dense with a golden color, and mycelia of F_{12} were dense with a slight yellow color. These results suggested that the degenerated strains completely lost the ability to differentiate primordium and produce fruiting bodies, and, therefore, were recognized as completely degenerated strains (Fig. 1). However, the degenerated strains were not identified and explained explicitly the difference from original strain in Li's research (Li et al. 2003). According to the former research (Wang et al. 2010), the genetic variation of *C. militaris* from Britain, China, Japan, Korea, and Norway was extremely small and did not correlate with geographical

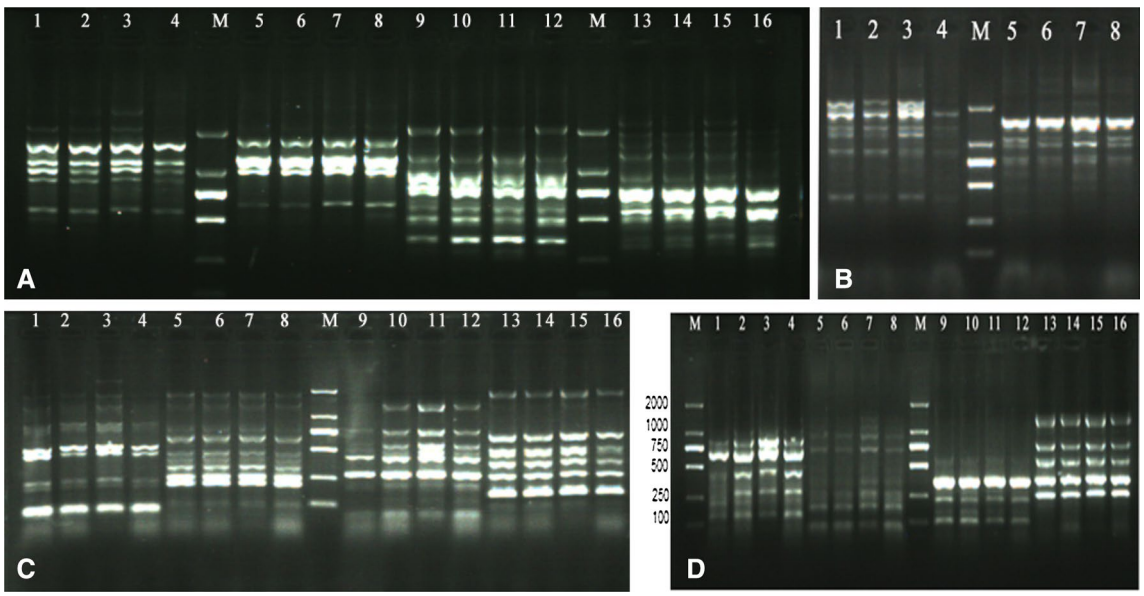

Fig. 2 RAPD and SRAP analyses. **a** RAPD analysis with primers B18, C7, S22 and S62; *Lanes 1, 5, 9, and 13* cm-6; *Lanes 2, 6, 10, and 14* cm-6D2; *Lanes 3, 7, 11, and 15* cm-6D3; *Lanes 4, 8, 12, and 16* F_{12}. **b** RAPD analysis with primers A12 and A16; *Lanes 1 and 5* cm-6; *Lanes 2 and 6* cm-6D2; *Lanes 3 and 7* cm-6D3; *Lanes 4 and 8* F12. **c**: SRAP analysis with primers Me2-em6, Me5-em6, Me6-em3, and Me6-em6; *Lanes 1, 5, 9, and 13* cm-6; *Lanes 2, 6, 10, and 14* cm-6D2; *Lanes 3, 7, 11, and 15* cm-6D3; *Lanes 4, 8, 12, and 16* F_{12}. **d**: SRAP analysis with primers Me3-em13, Me3-em8, Me6-em8, and Me1-em3; *Lanes 1, 5, 9, and 13* cm-6; *Lanes 2, 6, 10, and 14* cm-6D2; *Lanes 3, 7, 11, and 15* cm-6D3; *Lanes 4, 8, 12, and 16* F_{12}

origins. Mass production does not affect the genetic stability of *C. militaris*.

RAPD and SRAP analyses

Amplification of RAPD marker was performed with primers B18, C7, S22, S62, A12, and A16 (Fig. 2a, b). The results indicated that cm-6, cm-6D2, cm-6D3, and F_{12} were almost exactly the same and no genetic difference existed, which demonstrated that these four strains still belonged to the same strain. As shown in Fig. 2c and d, the amplification of SRAP markers was carried out with primers me2-em6, me5-em6, me6-em3, me6-em6, me3-em13, me3-em8, me6-em8, and me1-em3, respectively. These amplified bands were distinct with great amount, indicating that the phylogenetic relationship among different strains was close and the strains still belonged to the same strain. Compared to the phenotypic results, these results also demonstrated that there was no genetic variation of *C. militaris* during the degeneration process of these strains, which is contrary to other research (Li et al. 2003). This discrepancy may be attributed to the difference of strains used in experiments.

Biological characteristics

(1) Characteristics of mycelia from different strains: As shown in Fig. 3, mycelia formed by strains cm-6D2 and cm-6D3 were creamy white with slow discoloration and a light bottom. Mycelia of strain cm-6 were orange with a deep bottom and secreted more yellow pigment. Mycelia of strain F_{12} had a bread-shape middle uplift and fluffy cross-linked with a deeper color on the back. Mycelia of strains cm-3, cm-5, and cm-7 were orange with a deep golden bottom color. (2) Determination of polysaccharides

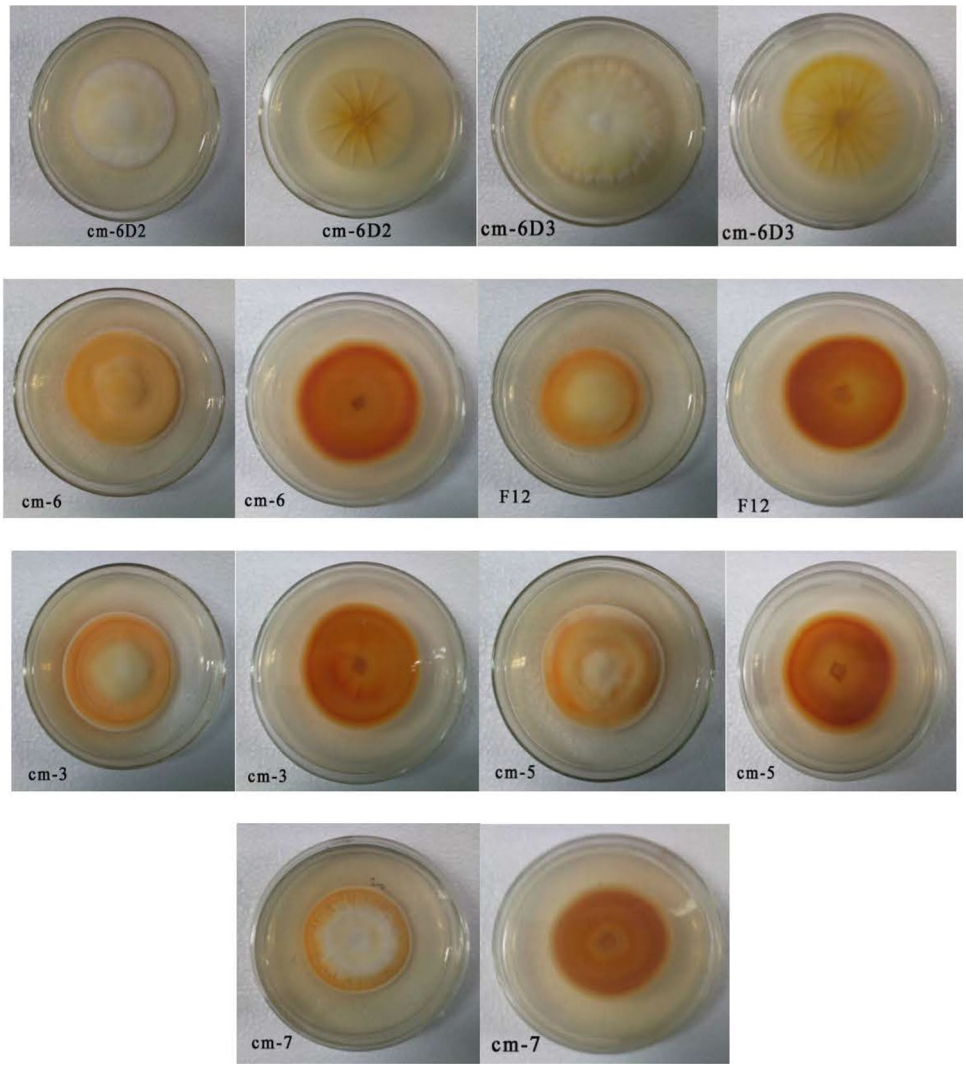

Fig. 3 Mycelial morphology of degenerated and normal strains

produced by different strains: As shown in Fig. 4a, the intracellular and extracellular polysaccharides were analyzed in detail. The results showed that, compared to normal strains (16.93 mg/g), the concentration of intracellular polysaccharides produced by degenerate strains was higher and can reach 25.63 mg/g by cm-6D3 strain. The production of intracellular polysaccharides by strains cm-5 and cm-7 was almost the same as cm-6. On the contrary, compared to degenerate strains, the concentrations of extracellular polysaccharides by strains cm-3, cm-5, cm-6, and cm-7 were relatively higher, while the production by strains cm-6D2, cm-6D3 and F_{12} were all lower than that of normal strains. (3) Determination of carotenoids produced by different strains: Mycelia of *C. militaris* turned yellow after illumination and could synthesize a certain amount of carotenoids. The production of carotenoids produced by different strains was shown in Fig. 4b. Carotenoid production by degenerated strains cm-6D2 and cm-6D3 was 2.63 and 6.89 μg/g, respectively. However, the production of carotenoids by strains F_{12}, cm-6, cm-3, cm-5, and cm-7 was much higher, specifically reaching to 23.64, 38.68, 20.24, 18.73, and 21.27 μg/g. These data indicated that the ability to produce carotenoids by degenerate strains is lower than normal strains. The content of carotenoid in strain F_{12} was also lower than the original strain cm-6. This decreased carotenoid production by degenerate strains could be used as an effective assessment indicator in evaluating the degeneration of strain. (4) Determination of cellulase activity: As shown in Fig. 4c, the cellulase activities of all strains (cm-6, cm-6D2, cm-6D3, and F12) were relatively low, specifically reaching to 0.788, 0.695, 0.659, and 0.602 U/mL, respectively. The cellulase activity of strain cm-6 was higher than that of degenerated strains and F_{12}, and no obvious correlation was observed between the degenerated strains and normal strains. All cellulase activities were all low, indicating that the ability to decompose cellulose by *C. militaris* was weak. Therefore, it was difficult to use cellulose as a main carbon source, which is

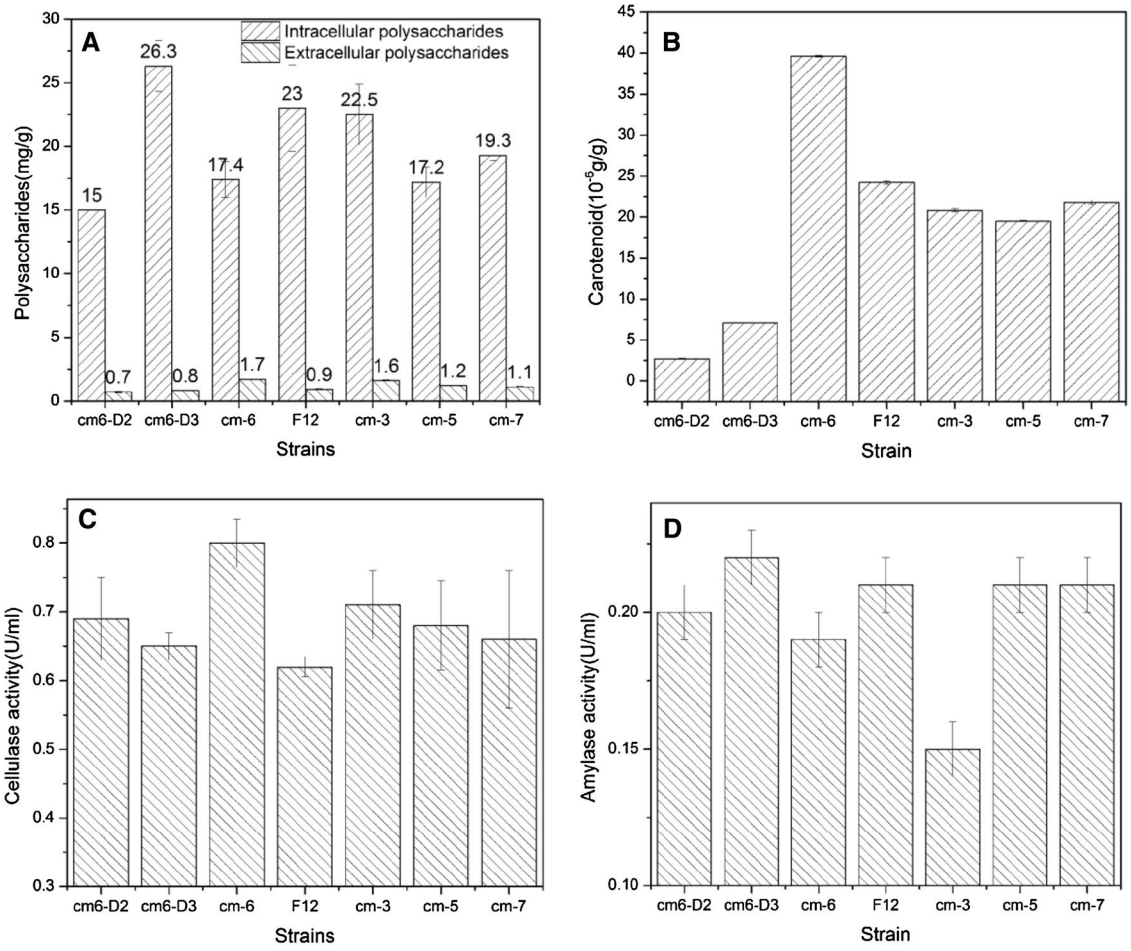

Fig. 4 Comparison of some phenotypes between normal strains and degenerated strains of *C. militaris*. The accumulation of polysaccharides (**a**), carotenoid (**b**), the capability in producing cellulase (**c**), and amylase (**d**) was compared for normal strains (cm-6, cm-3, cm-5, and cm-7) and degenerated strains (cm6-D2, cm6-D3). Data were presented as the mean of three independent assays

consistent with the finding that it was hard to cultivate *C. militaris* using vinasse as substrate (Morrell-Falvey et al. 2015). (5) Determination of amylase activity: Analysis of amylase activity in degenerated strains and normal strains showed that the amylase levels in cm-6D2, cm-6D3 and F_{12} were close and were all higher than that of cm-6, suggesting that the degenerated strain can secrete high levels of amylase (Fig. 4d).

Interestingly, it was found that the ability to synthesize carotenoids and secrete polysaccharides was reduced and the activity of cellulase and amylase also decreased slightly. According to the current work and some reports (Li et al. 2003; Lin et al. 2011), all results indicated that there were significant differences in esterase, dehydrogenase, and other metabolites between degenerated and normal strains, suggesting that the metabolic function of degenerated strains decreased. The degeneration of *C. militaris* strains might be due to the uncoordinated synthesis of metabolites or inhibition of metabolites in metabolic regulation, but the conclusion remains to be verified. The data obtained showed that the difference in metabolites played an important role in causing the degeneration of strains. Cellulose activity, amylase activity, carotenoid, and extracellular polysaccharide levels could be used as reference indexes to evaluate the degeneration of strains. Of course, a possible alternative to studying the degeneration phenomenon of *C. militaris* is metabolic analysis including metabolic regulation mechanism and metabolites secretion, which will become a new focus on studying the degeneration of *C. militaris* at the metabolic regulation level.

Concluding remarks

The degradation of *C. militaris* strains generally resulted in the lower growth rate, the deficiency in fruit body formation, and the reduction in pigment production. This degradation was not attributable to the DNA changes and may be caused by the inhibition or in harmony of metabolite synthesis of metabolic regulation, because many kinds of metabolites (carotenoid, cellulase, amylase, EPS, and IPS) changed in varying degrees.

Acknowledgements This work was financially supported by the Natural Science Foundation of China (Grant No. 31201669), the Natural Science Foundation of Fujian Province for Distinguished Young Scholars (Grant No. 2014J06010), the major platform for the development of edible fungus industry technology in Fujian Province (Grant No. 2014N2001), and the Major Extension Program of Agriculture Technologies from The Ministry of Finance and Agriculture of China (Grant No. KNJ-153011-3).

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