

## On the reliability of fungal materials used in studies on *Ophiocordyceps sinensis*

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**Abstract** *Ophiocordyceps sinensis* ( $\equiv$  *Cordyceps sinensis*) is one of the best known traditional Chinese medicines, with great benefits to human health and huge economic value. The reliability of fungal materials used in studies of the species is particularly important because contradictory results have been found in various studies in the past decades. Examination of fungal materials specified in reports on *O. sinensis* showed great variation in both sources and culture conditions of living strains. To test the reliability of the materials used, experiments were carried out to study the effect of culture conditions on the growth of living strains of *O. sinensis* by using six reliable strains representing the major production regions of the fungus on the Tibetan Plateau. The results showed that *O. sinensis* is a slow-growing fungus at comparatively low temperature, and that temperature and growth period are crucial factors which can be verified by experiment. Analyses of fungal materials used in 152 papers on *O. sinensis* from PubMed since 1998 showed that 41 papers lacked detailed information on the fungal materials; 26 used natural products, 11 used artificially cultivated fruit bodies, and 80 used fermentation products from living strains. Of the latter category (using fermentation products), 64 of the papers were found to use unreliable (45) or uncertain (19) strains for fermentation products based on the temperature and growth period for *O. sinensis* strains verified in this study. Apart from the natural products of *O. sinensis*, which require scientific identification, a total of at least 116 papers (over three-quarters) used unreliable, uncertain or

unspecified materials, including so-called cultivated fruit bodies which were apparently from other species. The reliability of materials or living strains used in studies on *O. sinensis* is discussed in this paper, and suggestions are made for use of reliable fungal materials in further studies of this fungus.

**Keywords** Fungal materials · Reliability · Growing temperature · *Ophiocordyceps sinensis*

### Introduction

*Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora [ $\equiv$  *Cordyceps sinensis* (Berk.) Sacc.], an entomogenous fungus on moth larvae, is one of the best known traditional Chinese medicines. It has been used as a tonic in China for hundreds of years and has been officially classified as a drug in the Chinese Pharmacopeia [1], having the effects of invigorating the lung and nourishing the kidney. The bioactive properties of the fungus have also been confirmed by pharmacological studies [2–4]. It has been used to treat a wide range of conditions, including respiratory, renal, liver, and cardiovascular diseases, hyposexuality, and hyperlipidemia [5–7].

Natural production of *O. sinensis* is limited because the fungus is endemic to the Tibetan Plateau from an altitude above 3,000 m up to the snow line [8–10]. The fungus was used exclusively in the Emperor's palace and traded for its weight in gold in ancient China. On the market today, the fungus has become available to ordinary people, but its price may reach twice that of gold. Owing to market demand and overexploitation, the fungus is now a species in danger [11]. Much effort has been devoted to cultivation of fruiting bodies and production of mycelia by

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fermentation of *O. sinensis*. As fruit-body cultivation has not been developed successfully, production of mycelium by submerged culture is considered to be rewarding and popular. Many strains have been isolated from natural *O. sinensis* [8, 12], and some of them have been manufactured in large quantity by fermentation technology [5, 12].

There has been increasing interest in secondary metabolites of higher fungi for discovering new drugs or lead compounds in recent years [13, 14]. As one of the important natural drugs in China, *O. sinensis* has also been bioassayed for its pharmacological activities. However, some contradictions have been found in the results; for example, oral administration of mycelia of *O. sinensis* resulted in improvement of steady-state energy status in mouse liver and enhancement of endurance in mice swimming test [15, 16], yet oral treatment with *O. sinensis* mycelia did not show positive effects on aerobic capacity or endurance exercise performance [17], and no enhancement of muscle tissue oxygen saturation was suggested [18]. Conflicting results in literature regarding the pharmacological functions of *O. sinensis* to alter apoptotic homeostasis have also been reported and analyzed [19]. These discrepancies not only hamper progress in in-depth investigation of this fungus but also reduce consumer confidence in its products.

Examination of fungal materials specified in reports on *O. sinensis* showed great variation in sources and culture conditions of living strains. One of the possible reasons for contradictions in the reported results is different materials used in various studies. One hundred and fifty-two papers on the study of *O. sinensis* from PubMed since 1998 were analyzed, focusing on the sources of materials and the culture conditions for living strains. The materials used in studies included natural products, artificially cultivated fruit bodies, and fermentation products. Among the literature analyzed, there were 41 papers lacking detailed information on fungal materials; 26 used natural products, 11 used cultivated fruit bodies, and 80 used fermentation products from living strains. For living strains, various culture conditions were adopted, e.g., liquid culture at 30°C for 4 days with pH 6.8 and constant stirring [20], 150 rpm at 26°C for 48–72 h [21], 150 rpm at 25°C, pH 5 with airflow rate of 1.0 vvm for 7 days [22], 150 rpm at 25°C for 3 days [23], 200 rpm at 22°C for 5 days with 10% inoculum and pH 5.5 [24], 100 rpm at 18°C for 40 days with pH 5.5–6.0 [25, 26], and so on. Growing temperatures from 18°C to 30°C were found, and growth periods from 48 h to 40 days. Thus inconsistencies have obviously occurred in the culture conditions for living strains. It is obvious that the fungal materials used in studies on *O. sinensis* were not the same, and the contradictory results reported may stem largely from these different materials

used. As more work on *O. sinensis* is published, the reliability of fungal materials becomes increasingly important. The fungal material, including living material, used in the reports should be verified as to whether they were in fact *O. sinensis*. Some ready-made materials cannot be verified through examination of the papers, but the culture conditions for living strains can be compared.

Based on the analysis of fungal materials of *O. sinensis* used in the literature, differences in culture conditions for living strains were found in media (ingredient, pH, solid or liquid), shaking speed or airflow, dissolved oxygen, inoculum volume, growth period, and temperature. In the present work, experiments were carried out to verify culture conditions of the fungus. Among several hundred strains isolated by this research group from the production region of the species on the Tibetan Plateau, six were selected to represent the major production areas. The strains used were further determined by means of both morphological and molecular methods to ensure correct identification of the fungus. Based on the experimental results, the authenticity of materials used in previous studies on *O. sinensis* was analyzed. The reliability of the materials or living strains used in studies is discussed, and suggestions are made for use of reliable fungal materials in further studies of this fungus.

## Materials and methods

### Fungal strains

Fungal strains used in this study were isolated from *O. sinensis* collected from the main production regions, namely Sichuan, Qinghai, Tibet, and Yunnan, and maintained on potato dextrose agar (PDA) supplemented with 5% (mass/volume ratio) wheat bran and 0.5% peptone [25] at 4°C as stock. Details of the strains used in this study are listed in Table 1.

To ensure the correctness of strains of *O. sinensis*, the identity of the strains was determined by DNA sequence analysis, through which the internal transcribed spacer (ITS) of nuclear ribosomal DNA was amplified and the sequences were compared with a dataset generated in this laboratory containing ITS sequences from dried specimens and living strains of *O. sinensis* obtained from various regions of the Tibetan Plateau, in addition to the morphological characters observed in culture.

All strains were subjected to DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing, following the method of Jiang and Yao [27]. Each fragment was sequenced in both directions for confirmation, and the two strands of sequences were assembled using the Seqscape programme (ABI Prism® SeqScape

**Table 1** Growth rate of *Ophiocordyceps sinensis* strains from various regions at different temperatures on solid medium

Strain no.	Origin	Altitude	Collecting date	GenBank accession number	Mycelial growth rate at different temperatures							
					4 ± 1 <sup>a</sup>	12 ± 1	15 ± 1	18 ± 1	21 ± 1	25 ± 1	28 ± 1	LSD <sup>b</sup>
285	Baima Snow Mountain, Yunnan	4,000 m	4 June, 2001	EU570927	0.161 ± 0.006 <sup>c</sup>	0.340 ± 0.034	0.421 ± 0.001	0.442 ± 0.001	0.333 ± 0.016	0	0	0.028
762	Kangding County, Sichuan	4,220 m	9 June, 2000	EU570923	0.195 ± 0.019	0.470 ± 0.026	0.530 ± 0.173	0.518 ± 0.013	0.440 ± 0.001	0	0	0.020
880	Xiaojin County, Sichuan	4,150 m	30 April, 2004	EU570944	0.240 ± 0.001	0.380 ± 0.020	0.530 ± 0.010	0.558 ± 0.063	0.493 ± 0.023	0	0	0.051
961	Naqu County, Tibet	4,700 m	10 June, 2004	EU570945	0.165 ± 0.043	0.379 ± 0.001	0.502 ± 0.022	0.487 ± 0.012	0.330 ± 0.006	0	0	0.026
964	Jiacha County, Tibet	4,000 m	10 June, 2004	EU570945 <sup>d</sup>	0.107 ± 0.007	0.295 ± 0.016	0.486 ± 0.025	0.500 ± 0.014	0.333 ± 0.022	0	0	0.028
977	Menyuan County, Qinghai	3,470 m	28 May, 2004	EU570939 <sup>e</sup>	0.110 ± 0.010	0.300 ± 0.020	0.460 ± 0.020	0.480 ± 0.020	0.380 ± 0.020	0	0	0.028
LSD					0.036	0.040	0.033	0.051	0.028	0	0	

<sup>a</sup> Temperature (°C)

<sup>b</sup> LSD is the least significant difference at *P* = 0.05

<sup>c</sup> Average growth rate (mm/days) as mean ± standard deviation (SD) of triple determinations

<sup>d</sup> The ITS sequence of 964 is identical to that of 961

<sup>e</sup> The ITS sequence of 977 is identical to that of the field collection, submitted to GenBank by this laboratory, from which the living strain was isolated

Software<sup>TM</sup>, version 1.1), and by careful manual examination to eliminate base-calling errors. The sequences obtained in this study were aligned and compared with a dataset generated by this laboratory, containing over 100 ITS sequences of dried specimens and living strains of *O. sinensis* from almost all the major production areas of the fungus. Phylogenetic analyses were performed using both PAUP4.0b10 for Macintosh [28] and MrBayes v.3.1.2 [29] to determine the species identity of the strains.

All strains in Table 1 were investigated on solid media to determine the effect of growth temperature. Strain 762 (CGMCC 2793), for which the nutritional requirements of mycelial growth of the fungus has been studied before [25], was used in the other experiments.

#### Inoculum preparation and basic culture conditions

Before inoculation, the strains were incubated in a Petri dish on the same medium as for stock at 18°C for 60 days. For liquid culture, seed cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml liquid medium, inoculated with a 5-mm agar disc from the 60-day culture. The flasks were rotated at 100 rpm and 18°C for 15 days.

Unless otherwise specified, cultures were performed under the following conditions: temperature 18°C, initial pH 5.6, medium volume 50 ml in a 250-ml flask inoculated with 10% by volume seed culture and agitated at 100 rpm in dark for 40 days. Mycelia were harvested by centrifugation at 8,000g for 15 min to separate them from liquid medium. After repeated washing of the mycelial pellets with distilled water and drying at 70°C to constant weight, the mycelial biomass was determined. All experiments were performed in triplicate.

#### Effect of culture temperature

A 5-mm agar disc with mycelia of *O. sinensis* was punched with a sterilized cutter from the inoculum preparation and transferred to a fresh Petri dish with the same medium. The inoculated dishes were sealed with parafilm and incubated at 4°C, 12°C, 15°C, 18°C, 21°C, 25°C, and 28°C. Colony growth was measured weekly for 8 weeks. Cultures with no growth at higher temperature, such as 25°C and 28°C, were moved to 18°C after 10, 20, and 30 days subsequently and re-examined after 30 days of incubation to determine whether they resumed growth at 18°C. Growth rate was determined by measuring colony diameter.

Liquid cultures prepared as described for basic culture conditions above were incubated statically for 60 days at the same temperature range described above, except 28°C, to determine the effect of temperature on mycelial growth.

### Effect of initial pH and light

The effect of initial pH on mycelial growth was estimated by setting different initial pH values. Before sterilization, medium pH was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 using 1 N HCl or 1 N NaOH; corresponding culture pH of 4.3, 4.8, 5.5, 6.0, 6.7, 7.2, 7.6, and 8.2 was obtained after autoclaving. The pH value was determined using a Sartorius Basic pH meter PB-10 (Germany).

The influence of light on mycelial growth was investigated by growing fungal cultures under photoperiods of 12 h light/12 h dark and 24 h dark with 10,000 lux in an incubator (HPG-280B, Harbin, China). Flasks under the 24 h dark regime were covered with aluminum foil.

### Effect of dissolved oxygen and inoculum volume

The response of *O. sinensis* to levels of dissolved oxygen was investigated by shaking flasks [30, 31]. Different levels of oxygen concentration were simulated by filling 50, 100, 150 or 200 ml medium into a 250-ml Erlenmeyer flask and 100 ml into a 500-ml Erlenmeyer flask. Different volumes of seed culture at 2%, 5%, 10%, 15% or 20% (volume ratio) were inoculated into a 250-ml flask containing 50 ml medium to study the effect of inoculum volume on mycelial growth.

### Growth curve of *O. sinensis* in liquid and on solid culture

Thirty Erlenmeyer flasks were prepared for determination of the growth curve of *O. sinensis* in liquid culture. The optimal conditions (temperature 18°C, initial pH 6.0, medium volume 50 ml in a 250-ml flask, inoculated with 10% volume of seed culture, agitated at 100 rpm in dark) were used. Mycelia were harvested from three flasks every 5 days, and mycelial dry weight measured. The final pH value in the culture was read at time of harvesting. After sampling, the fermentation supernatant was stored at -20°C and then thawed for analyses of residual sugar and ammoniacal nitrogen concentration. Residual sugar level was assayed by phenol-sulfuric acid method [32]. Ammoniacal nitrogen concentration was estimated using formaldehyde titration assays [33].

For solid culture, dishes with inoculum were sealed with parafilm and incubated at 4°C, 12°C, 15°C, 18°C, 21°C, and 23°C. The culture lasted 100 days, and colony growth was measured every 10 days.

### Statistical analysis

Data obtained from the experiments were analyzed by one-way analysis of variance (ANOVA). Tests of significant

differences were determined by Duncan's multiple-range tests or least significance difference (LSD) at  $P = 0.05$  using SPSS 11.0 (SPSS Inc.).

## Results

### Strain identification

In addition to the morphological characters in culture, the ITS sequence was amplified from cultures of the strains for molecular identification. The complete ITS sequences of the strains were 535–543 bp long and have been submitted to GenBank (for accession numbers see Table 1). Sequence similarity among these sequences was found to be 97.4–100%. Phylogenetic analyses confirmed the species identity of the strains to be *O. sinensis*.

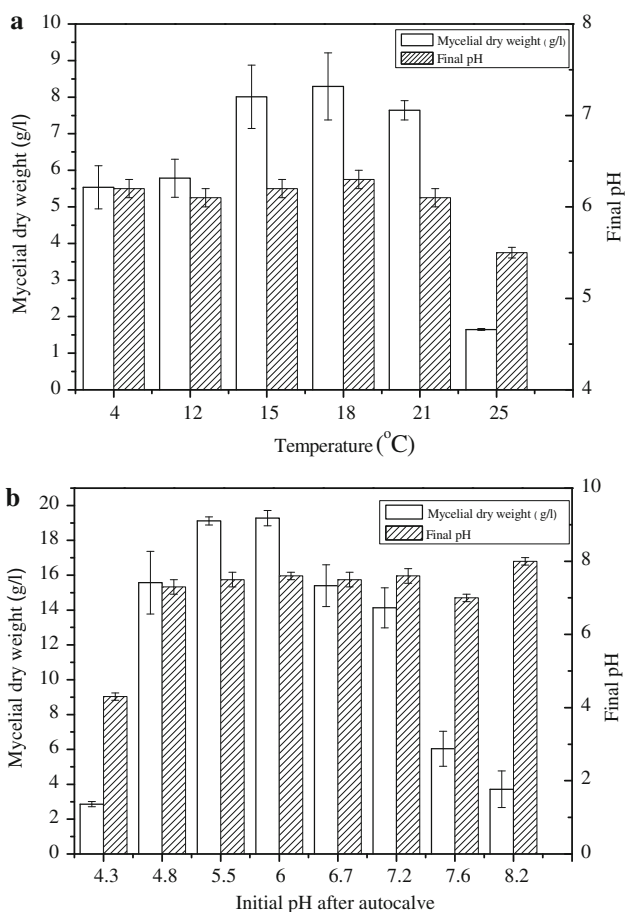
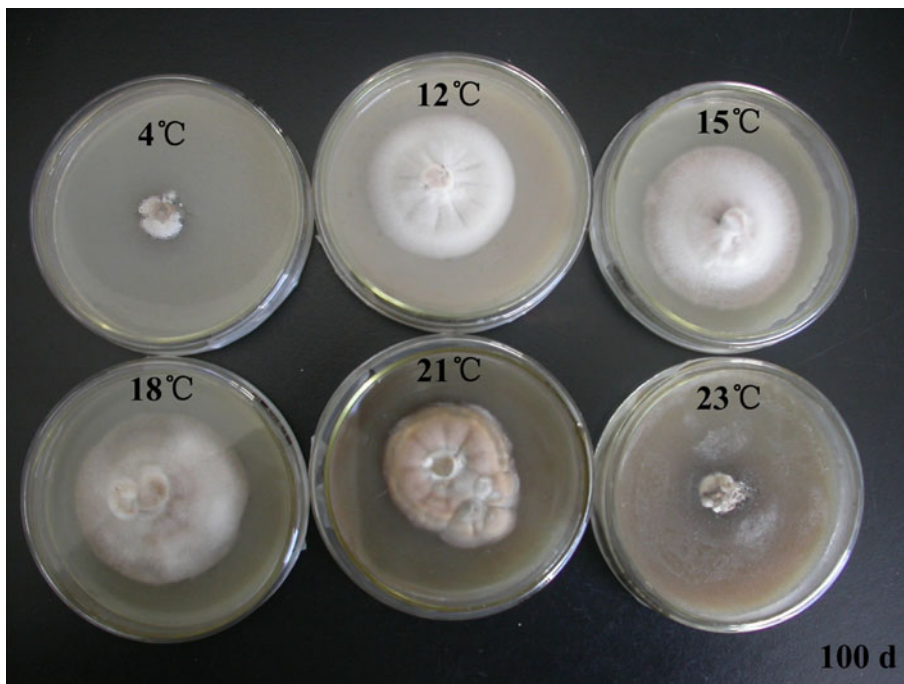
### Temperature

Mycelial growth of all six strains followed a similar trend in response to temperature (Table 1). Rate of mycelial growth increased with temperature from 4°C up to 18°C and then decreased rapidly as temperature increased further. There was only weak growth at 23°C even after culture for 100 days (Fig. 1). The color of colonies also varied, from white, sandy beige, to dark brown, as temperature increased (Fig. 1).

Least significance difference (LSD) at  $P = 0.05$  was used for comparison of means of each treatment. The optimum growth temperature was 15°C and 18°C, with no significant difference between these two temperatures for all six strains ( $P = 0.05$ ). Significant difference in growth rate was found among the strains at the same temperature. In general, strains 762 and 880 grew faster than the others at every tested temperature, with no significant difference in maximum mean growth rate between these two strains. For all tested strains, no growth was observed at 25°C or 28°C after 10, 20 or 30 days. After moving to 18°C for another 30 days incubation, those moved from 25°C at 10 and 20 days and from 28°C at 10 days resumed growth and reached 20–30 mm diameter in 56 days, but the others failed to regrow and were apparently dead.

The growth rate of strain 762 at different temperatures in liquid culture was similar to that found on the solid medium as determined by mycelial dry weight (Fig. 2). This strain could grow at temperatures ranging from 4°C to 21°C, with maximum biomass at 18°C. However, there was no significant difference in growth rate between 15°C and 18°C. There was no growth at 25°C. Final pH increased more or less from the initial pH 5.6 at each temperature, except at 25°C, where no change of pH value was observed.

**Fig. 1** Colony of *Ophiocordyceps sinensis* on solid medium at different temperatures after 100 days



**Fig. 2** Effect of temperature and initial pH on mycelial growth of *Ophiocordyceps sinensis* in liquid culture. Strain 762 (CGMCC 2793) was used, and growth lasted for 40 days

#### Initial pH and light

Mycelia of *O. sinensis* grew well at initial pH values between 4.8 and 7.2, but mycelial dry weight dropped sharply beyond this range (Fig. 2). At pH 4.3 and 8.2, very weak growth was observed. Maximum biomass was found at initial pH 6.0; however, there was no significant difference in biomass between pH 6.0 and 5.5. The final pH of the supernatant after harvest of mycelia was observed to be 7.0–7.8 in the media of different initial pH with good growth of mycelia, except 4.3 and 8.2, where there was little change of the value (Fig. 2).

The mycelial dry weight for 24 h dark and 12 h light/12 h dark treatments was  $18.43 \pm 1.15 \text{ g l}^{-1}$  and  $13.16 \pm 1.63 \text{ g l}^{-1}$ , respectively, with the same final pH value of 7.5. However, there was no significant difference between these two treatments.

#### Dissolved oxygen and inoculum volume

Mycelia of *O. sinensis* could grow in each medium volume, but mycelial dry weight increased with decreasing medium volume from 200 to 50 ml in a 250-ml flask (Table 2). The maximum mycelial dry weight was obtained with medium volume of 50 ml in a 250-ml flask, but there was no significant difference among medium volumes from 50 to 150 ml in a 250-ml flask and 100 ml in a 500-ml flask.

The mycelial yield from different inoculum volumes is also presented in Table 3. Mycelial dry weight increased

**Table 2** Effect of medium volume on mycelial growth of *Ophiocordyceps sinensis*

Medium volume/flask capacity (ml/ml)	Mycelial dry weight ( $\text{g l}^{-1}$ )*	Final pH
50/250	22.673 $\pm$ 1.92 5 a	7.1
100/250	18.360 $\pm$ 2.885 a	7.0
150/250	14.710 $\pm$ 3.210 ab	6.9
200/250	10.013 $\pm$ 0.258 b	6.2
100/500	21.345 $\pm$ 0.940 a	7.0

\* Values are mean  $\pm$  SD of triple determinations, and values followed by the same letter are not significantly different by Duncan's multiple-range test ( $P = 0.05$ )

**Table 3** Effect of inoculum volume on mycelial growth of *Ophiocordyceps sinensis*

Inoculum volume (% $v v^{-1}$ )	Mycelial dry weight ( $\text{g l}^{-1}$ )*	Final pH
2	17.053 $\pm$ 2.802 b	6.6
5	20.080 $\pm$ 1.802 b	7.1
10	24.540 $\pm$ 0.953 a	7.2
15	26.800 $\pm$ 1.921 a	7.2
20	25.680 $\pm$ 0.764 a	7.2

\* Values are mean  $\pm$  SD of triple determinations, and values followed by the same letter are not significantly different by Duncan's multiple-range test ( $P = 0.05$ )

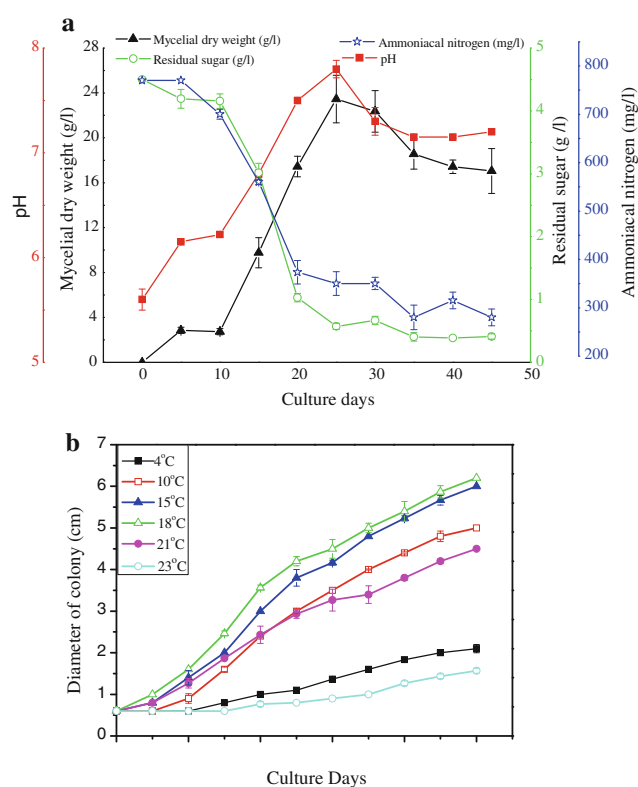
with inoculum volume from 2–15% and decreased slightly at 20%. However, there was no significant difference among inoculum volumes of 10%, 15%, and 20%.

The growth curve of *O. sinensis* in liquid culture under optimized conditions

To study the growth period and kinetics of mycelial growth, a typical time course of mycelial growth of *O. sinensis* was conducted in Erlenmeyer flasks under the optimal conditions as determined by the above experiments.

In submerged cultures, the lag phase lasted for 10 days, followed by the exponential phase for about 15 days (Fig. 3A), with maximum mycelial dry weight of 23.45  $\text{g l}^{-1}$  at day 25. The stationary phase lasted only 2 days and soon turned to death phase. The initial pH value of the medium increased during the lag and exponential phases to 7.8, and then decreased slightly by the end of incubation. The kinetics of residual sugar and of ammoniacal nitrogen showed similar time courses during the entire culture period. They decreased sharply at the exponential growth phase and then declined slowly until the batch was terminated (Fig. 3a).

In solid culture at 12°C, 15°C, 18°C, and 21°C, the lag phase also lasted 10 days, followed by the exponential



**Fig. 3** Growth curve of *Ophiocordyceps sinensis*: **a** in liquid culture under optimal conditions (temperature 18°C, initial pH 6.0, medium volume 50 ml in a 250-ml flask, inoculated with 10% volume of seed culture, agitated at 100 rpm in dark), and **b** on solid culture at different temperatures. Strain 762 (CGMCC 2793) was used, and growth lasted 100 days

phase, as determined by colony diameter (Fig. 3b). The diameter increased with time during 100 days. However, there was only very weak growth at 23°C during 100 days.

## Discussion

The increasing interest in the pharmaceutical effects of *O. sinensis* [6, 7] has stimulated many publications in recent years, but contradictory results have been reported from time to time. The reliability of the fungal materials used in studies of this fungus has become a very important issue because contradictions in the results were probably caused by different materials, especially different living strains for fermented products, as judged from information provided in papers analyzed in this study. As living strains were used to prepare the fungal material in more than half of the papers analyzed, investigation on culture conditions for the growth of living strains of *O. sinensis* was conducted using reliable strains in the present work, based on previous results of nutritional requirements for mycelial growth of the fungus in submerged cultures [25], to

identify factors that can verify the reliability of fungal strains used in the literature. Among all the tested factors, temperature and growth period, which can be easily verified by experiment, were crucial to mycelial growth of *O. sinensis* strains, although all culture conditions, except light, showed significant effects on growth.

The optimal temperature for mycelial growth of *Cordyceps* (Fr.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora and *Ophiocordyceps* (Petch) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora depends on the species and strains involved [34–36]. The growing temperature range for all six tested reliable strains of *O. sinensis* from various regions of the Tibetan Plateau and identified by morphological, physiological, and molecular characters was observed to be 4–21°C with an optimum between 15°C and 18°C, both on solid medium and in liquid culture. It was also demonstrated on solid medium that the fungus grew poorly above 21°C and stopped growth at 25°C or above. The optimal temperature for mycelial growth of *O. sinensis* is apparently lower than that for other species in the same or related genera, such as 20°C for *C. militaris* [35, 37], 26°C for *O. jiangxiensis* JXPJ0109 [36], and 28°C for *Paecilomyces tenuipes* C240 (an anamorph of *Cordyceps takaomontana* Yakush and Kumaz) [38]. Nevertheless, the strains of *O. sinensis* can withstand higher temperature for certain periods, e.g., 20 days at 25°C and 10 days at 28°C, although they will eventually die if kept at higher temperature for longer, e.g., 30 days at 25°C or 20 days at 28°C, as revealed by the results above.

The optimal initial medium pH for mycelial growth of *O. sinensis* was determined in this study to be 5.5–6.0, similar to that of other related fungi, e.g., *C. militaris* [35] and *O. jiangxiensis* JXPJ0109 [36], and the range of initial pH was from 4.8 to 7.2, similar to that of most fungi. Apparently, there is no special requirement on initial pH for growth of *O. sinensis*, although a very low optimal initial pH 4.0 for *O. sinensis* was reported by Kim and Yun [34], where the identity of the fungus is doubtful judged from the growing temperature of 25°C and growth period of 7 days.

The effects of light on ascospore germination, conidiophore production, and stomatal development in *O. sinensis* have been studied previously [39–41], but no report has been found so far on its mycelial growth in liquid culture. Similar to *Aspergillus parasiticus* Speare [42], no significant effect of light on mycelial growth of *O. sinensis* was observed in this study. The mycelial dry weight of *O. sinensis* was found to increase with the reduction of the medium volume in a flask, which suggests that oxygen has affected the growth of mycelia as the lower volume of medium in a flask is considered to be favorable for oxygen transport [43]. However, the results of this study showed that *O. sinensis* could grow at all the medium volumes tested, suggesting that the level of dissolved oxygen is not crucial for growth of this fungus.

The relationship between inoculum volume and mycelial growth has been investigated in some species of *Cordyceps* and *Ophiocordyceps*, e.g., 2–8% inoculum volume for mycelial growth of *C. militaris* with little or no obvious effect on liquid cultures [35] and optimized inoculum volume of 2.5–7.5% from a test range of 2.5–10% for mycelial growth of *O. jiangxiensis* JXPJ 0109 [36]. The optimal inoculum volume of 10–20% for mycelial growth of *O. sinensis* observed in the present study is higher than reported for other related species, possibly owing to the long lag phase and slow growth rate of the fungus. Nevertheless, mycelia of *O. sinensis* were found to grow in every inoculum volume tested, although inoculum size may affect the growth rate of the fungus.

The growth curve observed in this study showed that the growth period of *O. sinensis* was comparatively long even under the optimal conditions, both in liquid and on solid cultures. The growth curve in liquid culture was close to sigmoid, with lag, exponential, stationary, and death phases. The lag phase (about 10 days) was relatively longer than that of other species reported in the same or related genera, e.g., 2 days for *C. militaris* [35] and 1 day for *O. jiangxiensis* JXPJ 0109 [36]. Fermentation can be terminated at 25–30 days if production of mycelia is the main purpose. The growth curve on solid culture also suggested that mycelia of *O. sinensis* grew very slowly, reaching only 60 mm in 100 days.

It is evident that *O. sinensis* is a slow-growing fungus at comparatively low temperature, a characteristic feature of typical growing conditions on the Tibetan Plateau, which can be applied in comparative analyses to test the fungal strains used in various studies on the species. In many papers on *O. sinensis*, however, the growing temperature was over 21°C and the growth period was shorter than 1 week, e.g., liquid cultures at 25°C for 7 days used for a study on effects of hot-water extract on the activation of macrophages and the intestinal immune system [4], flasks incubated at 25°C for 3 days for the optimal medium to produce polysaccharides [23], and fermentation at 22°C for 5 days for comparison of chemical composition and bioactive ingredients with natural *O. sinensis* [24]. There are many more such examples scattered throughout the literature; however, the fungal strains used in those reports were apparently not *O. sinensis* in the light of the experiment results of the present work. Among the 80 papers with descriptions of culture conditions for living strains of *O. sinensis*, 64 (80%) were found to use unreliable (45) or uncertain (19) strains for fermentation products based on the temperature and growth period for the species as verified in this study. The alpine habitats of *O. sinensis* on the Tibetan Plateau make collection and isolation of the fungus very difficult. The psychrophilic and slow growth characters of the species further increase the difficulty of

obtaining the correct fungal strain. In fact, 22 fungal names, involving 13 genera, were found to be associated with isolates from *O. sinensis*, and many of them have no close link to the species, although they may have been considered as the anamorphic (asexual) stage of the fungus [8, 12]. Possibly owing to differences in disciplines, researchers in the fields of pharmacology and drug discovery may not be aware of the complexity in determination of fungal material related to *O. sinensis*. This may be the reason why unreliable and uncertain strains of the fungus have been used in many papers over many years.

Of the 152 papers on *O. sinensis* analyzed, 11 indicated that artificially cultivated fruit bodies were used as the fungal materials. Although it has been claimed that stromata or fruit bodies of this species have been successfully cultivated artificially [44–47], the production of these cannot be repeated. The so-called cultivated fruit bodies used in those papers could not be *O. sinensis*, but rather some other species. It is striking that information on the fungal material used was lacking in more than one-quarter (41) of papers analyzed. The results reported in those papers cannot be referenced, because the reliability of the fungal material cannot be assessed. In 26 of the papers where natural products were used, the species identity of the products requires further verification. Many counterfeit products of the fungus have been found on the market due to its great economic value. Gypsum, wheat flour, soybean flour, and corn flour put into molds for compression as *O. sinensis* and the rhizome of *Stachys geobombycis* C.Y. Wu have been sold as the fungus by unscrupulous merchants [48]. Apart from the natural products of *O. sinensis* which require scientific identification, a total of at least 116 (over three-quarters) of the 152 papers analyzed used unreliable, uncertain or unspecified materials. Reliability and reproducibility of the experiments depend upon the authentication of source materials and, indeed, any study should be based on reliable materials. Caution regarding the identity of fungal material used in scientific research and in commercial applications should be emphasized to ensure correct results.

Results based on unreliable materials cannot be of any use in the discovery of new drugs from natural products and will not advance scientific research. It is important to employ the correct material or living strain of fungi in the relevant work. Materials or strains should be authenticated by professionals through available scientific methods to ensure the reliability of materials in the first step of research work. It is also important to give explicit information on the origin of materials and identity determination, also including the Latin name of biological species, culture conditions for fermented product, and voucher materials for further confirmation in studies on *O. sinensis* or on any other medicinal fungi.

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