Some Biologically Active Substances from a Mycelial Biomass of Medicinal Caterpillar Fungus *Cordyceps sinensis* (Berk.) Sacc. (Ascomycetes)

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ABSTRACT: The accumulation of biomass, endo- and exopolysaccharides, lipids, phospholipids, fatty acids, proteins, and polyphenols as well as the antioxidant activity of *Cordyceps sinensis* submerged mycelium were studied. *C. sinensis* mycelial biomass contains up to 29% proteins, 15% endopolysaccharides, and over 7% lipids and is rich in phospholipids (up to 28% of total lipids) and unsaturated fatty acids ($C_{18:1}$ – up to 44%; $C_{18:2}$ – 53% of total fatty acids). Inoculum irradiation with light of different wavelengths affects mycelial biomass biosynthesis and exopolysaccharide production in *C. sinensis*.

KEY WORDS: *Cordyceps sinensis*, medicinal fungi, cultivation conditions, polysaccharides, proteins, lipids, phospholipids, polyphenols, antioxidant activity

I. INTRODUCTION

Cordyceps (Fr.) Link (Ascomycetes) is one of the genuses targeted for modern mycological, biotechnological, and pharmaceutical studies. Among the species of the genus *Cordyceps*, *C. sinensis* (Berk.) Sacc. is one of the most famous but poorly explored species because the fungus is only distributed in limited regions of East Eurasia.[1–3] *C. sinensis* is a medicinal species with a long and illustrious history. The Latin conjugation accurately describes the appearance of the club fungus, whose stroma extend from the mummified carcasses of insect larvae, usually caterpillar larva of the Himalayan Bat Moth *Thitarodes armoricanus*.

The fungus has been described in old Chinese medicine books from ancient times. *C. sinensis* has been known and used for many centuries in traditional oriental medicine. The range of therapeutic uses claimed for *C. sinensis* is indeed large. *Cordyceps* has been used to treat a wide range of conditions, including respiration; pulmonary diseases; renal, liver, and cardiovascular diseases; hyperlipidemia; and others. It is also regularly used in all types of immune disorders and as an adjunct in cancer therapy. Modern science has confirmed the efficacy of *Cordyceps* for most of the traditional uses.[4]

Major bioactive constituents of *C. sinensis* are cordycepin [3′-deoxyadenosine] and cordycepic acid [d-mannitol]. Many other nucleosides have been found in *C. sinensis* stroma, including uridine, several distinct structures of deoxyuridines, adenosine, 2′,3′dideoxyadenosine, hydroxyethyladenosine, cordycepin triphosphate, guanidine, deoxyguanidine. Other biologically active components found include various saccharides and polysaccharides of varied complexities, including cyclofurans, β-glucans, β-mannans, cross-linked β-mannan polymers, and complex polysaccharides

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consisting of both 5 and 6 carbon sugars joined together in branching chains comprising both α and β bonds. *C. sinensis* stroma contains a wide range of compounds considered nutritional. These are essential amino acids; vitamins B_1, B_2, B_{12}, E, and K; a wide range of sugars, proteins, and sterols; and a wide range of trace elements (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V, and Zr).[5]

Attempts have long been made to cultivate species of the genus *Cordyceps*, especially *C. sinensis*. By the mid-1980s, a majority of species of the genus *Cordyceps* available in the world's market were artificially cultivated. It has been shown that the analytical profile and medicinal properties of the mycelium are very similar to the wild fruiting body.[5]

There are two main methods used today in the cultivation of *C. sinensis*: the solid-substrate method and submerged fermentation. The latter method is the one primarily used in China. The majority of *Cordyceps* available on the market today is liquid cultured in this way.[5] This method is a very economical method for large-scale production. The ability to control the growth parameters results in a very consistent product, and there is very little variation in quality from batch to batch.

Fermentation technology is young. Mycological, biotechnological, and pharmaceutical aspects of *C. sinensis* obtained under submerged cultivation conditions are currently under considerable study. Although a considerable amount of information is available on various biologically active substances, nutrients, and mineral content in the *C. sinensis* fruiting body, examinations of submerged mycelium are usually limited by polysaccharides and cordycepin estimation. Apart from these biologically active compounds, the submerged mycelium of *C. sinensis* accumulates a number of other pharmaceutically and nutritionally valuable substances. These valuable products are also of great interest in *C. sinensis* biotechnology.

This research aims to study the influence of submerged cultivation conditions on the production of some biologically active substances by *C. sinensis*.

II. MATERIALS AND METHODS

A. Fungal Strain

The studied strain of *Cordyceps sinensis* 1600 was obtained from the Culture Collection of Higher Basidiomycetes Edible and Medicinal Mushrooms (KW) of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine.

B. Cultures and Growth Conditions

Mycelium of *C. sinensis* was grown in submerged conditions on glucose–peptone nutrient medium, g/L: glucose 30, peptone 2, KH_2PO_4 1, K_2HPO 1, $MgSO_4 \times 7H_2O$ 0,25, corn extract 2, deionized water 1 L. Effects of carbon sources on the synthesis of biomass and endopolysaccharides were studied on glucose–peptone nutrient medium in which glucose was replaced by maltose, starch, or sucrose. Effects of the temperatures on the accumulation of biomass and endopolysaccharides were studied at 20°C, 25°C, 30°C, and 35°C on glucose–peptone medium. The mycelium was grown in 250-mL Erlenmeyer flasks containing 50 mL of the cultivation medium.

The dynamics of the biomass, exopolysaccharides, proteins, lipids, endopolysaccharides, phospholipids, phenolic compounds, and antioxidant activity were investigated on the molasses-based medium in a fermenter (volume 10 L) at 20°C, with an intensity of aeration 1.0 L/L/min and an agitation speed of 100 rotations per minute (rpm).

C. Separation

Mycelium of *C. sinensis* was separated from the medium by centrifugation (9000 g for 20 minutes) and washed with distilled water.

D. Protein Determination

In the mycelium of the strain, protein content was estimated according to Kjeldal's method.[6] Dried mycelium (200 mg) was incinerated with con-

centrated sulfuric acid and then boiled with 30% hydrogen peroxide until the solution lost color. The nitrogen present is converted to ammonium sulfate. After the cooling of the solution, seignette salt and Nessler's reagent were added and the amount of ammonium was detected by photocolorimeter KFK-2MP (Russia).

E. Lipid and Fatty Acid Determination

Lipids from wet mycelium were extracted by the Folch method using a modification of the Bligh-Dyer modification.[7,8] To estimate the lipid content in the fungal mycelium, 10–20 g of crude mycelium was frozen with liquid nitrogen and ground in the mortar. The obtained homogenous mass was extracted using a mixture of chloroform:ethanol:water at a 1.0:2.0:0.8 ratio for 10–12 hours; then the mycelium was sieved on ash-free filtered paper, and the volume of the mixture was measured. To promote the separation of the chloroform phase, 2.5 mL of chloroform and water was added to each 9 mL of the extract and left for 4–6 hours. A lower chloroform fraction containing total lipids was collected and evaporated with a rotor evaporator, and the lipid amount was determined gravimetrically. The phospholipid content of the total lipids was determined by the photometric method after lipid incineration in 42% perchloric acid.[9] Fatty acid composition was analyzed on the chromatograph Chrom 5, with 15% polyethylene glycol succinate as the liquid (the temperature of the column was 160°C and the evaporation temperature was 210°C).[8,10]

F. Antioxidant Activity

The antioxidant activity of the ethanol extracts of mycelia was measured according to Nakatani and Kikuzaki[11] using the Kapich modification.[12] The antioxidant potential of fungal submerged mycelium was estimated via the ability of the extracts to inhibit the synthesis of the products reacting with thiobarbituric acid. The antioxidant activity value of the ionol-recognized antioxidant was assumed to be 100%.

G. Exo- and Endopolysaccharide Determination

Endopolysaccharides were determined according to Tang's method.[13] For the analysis of endopolysaccharides, the dried mycelia (100 mg) were extracted by using 1M NaOH at 60°C (1 hour), then the supernatant was assayed using the phenol–sulfuric acid method.

Exopolysaccharides were estimated in the cultural liquid and molasses-based media, also without mushroom mycelia.[14] Traces of exopolysaccharides were determined in the molasses-based medium. To extract the exopolysaccharides, the culture liquid was evaporated 2–3 times and mixed with an equal volume of ethanol. The mixture was allowed to react at 4°C until complete precipitation of the polysaccharides. The latter were separated by centrifugation, dialyzed, precipitated once again with ethanol, separated by centrifugation, and dried at 40°C.

H. Polyphenol Determination

To obtain extracts for the analysis of phenolic compounds, the submerged mycelium was ground, repeatedly frozen in liquid nitrogen, and extracted with 70% ethanol for 30 minutes in a boiling water bath with reverse refrigeration until a negative phenol assay was achieved. The final extract was centrifuged at 8000 rpm for 15 minutes. Total mono- and polyphenols were evaluated using the Folin-Denis reagent.[15]

I. Irradiation

Irradiation of mycelium grown by submerged cultivation was undertaken in a flat-bottomed flask. The argon ion laser (514.5 nm) and helium-neon laser (632.8 nm) (continuous wave [cw] and pulsed [discontinuous duty with a 1-ms impulse duration and a 2-ms repetition period]) modes of operation and the Ti:sapphire femtosecond laser (fs) (780 nm, 76 MHz repetition rate, 140 fs pulse duration), as well as light-emitting diodes, were used as sources of light. When choosing the light-treatment

regimen, the time of light exposure was selected to make the number of incident photons the same when irradiating mycelium with the light of different wavelengths. Nonirradiated (control) and irradiated mycelia were used for the inoculation of fermentation media.

J. Statistical Analyses

The data were analyzed by Excel statistical functions using a Microsoft Office XP software package. Values are presented as means ± standard error of the mean (SEM).

III. RESULTS AND DISCUSSION

At the initial stage of the research, we optimized the conditions of the *C. sinensis* submerged cultivation in order to attain high biomass and endopolysaccharide (one of the main biologically active compounds) production. The results obtained indicate that the composition of media, temperature, and the initial pH of media influence the synthesis of biomass and endopolysaccharides differently (Table 1; Figs. 1 and 2).

It was demonstrated that the use of sucrose as a carbon source contributed the most to biomass and endopolysaccharides accumulation, as compared with glucose, starch, or maltose (Table 1). Our data confirm those obtained by Kim and Yun.[16] One of the important factors affecting cell membrane functions, the utilization of different nutrients and fungi metabolic activity, is the pH value of the nutrient medium.[4,17,18] Cultivation of *C. sinensis* at different initial pH values showed that the highest biomass yield could be achieved at pH 7 (Fig. 1). Endopolysaccharide accumulation was greatest at initial pH 4–6 (Fig. 1). A similar pH effect on fungal growth and endopolysaccharide synthesis is known for other species of medicinal fungi.[14] The highest biomass and endopolysaccharide accumulation by *C. sinensis* was registered at 20°C–25°C. Increasing the temperature to 35°C resulted in a considerable inhibition of growth and biosynthesis of endopolysaccharides (Fig. 2).

It is known that the light factor is of great importance as a regulator of fungi morphogenesis and biological activity. It was demonstrated that the species may react differently to various spectral regions.[19] Our previous results proved the stimulation effect of light on the growth and biological activity of some culinary-medicinal species of Ascomycetes and Basidiomycetes.[20–23] The analysis of biomass and polysaccharide accumulation by *C. sinensis* after exposure to the blue light (450.0 nm) demonstrated the stimulation of biomass biosynthesis (Table 2). Neither the blue nor the red (632.8 nm) light influenced the accumulation of endopolysaccharides and both decreased slightly the yield of exopolysaccharides. These results may be explained by the existence of another photoreception mechanism for *C. sinensis*, which differed from the ones characterized for earlier investigated species.

The data obtained allowed the determination of the main parameters for the cultivation of *C. sinensis* in the fermentor. The molasses-based medium was used as a chipper alternative to the sucrose.

Our results indicated that the maximal biomass (16 g/L) yields on the 4th day of cultivation changed very little (Fig. 3). Exopolysaccharide accumulation

TABLE 1
Effect of Carbon Source on Biomass (g/L) and Endopolysaccharide Production (%) by *Cordyceps sinensis*

Carbon source	Biomass, g/L	Endopolysaccharides, %
Glucose	10.3 ± 0.3	12.3 ± 0.1
Maltose	8.9 ± 0.5	13.8 ± 0.2
Potato starch	11.1 ± 0.3	13.2 ± 0.2
Sucrose	11.4 ± 0.4	14.0 ± 0.3

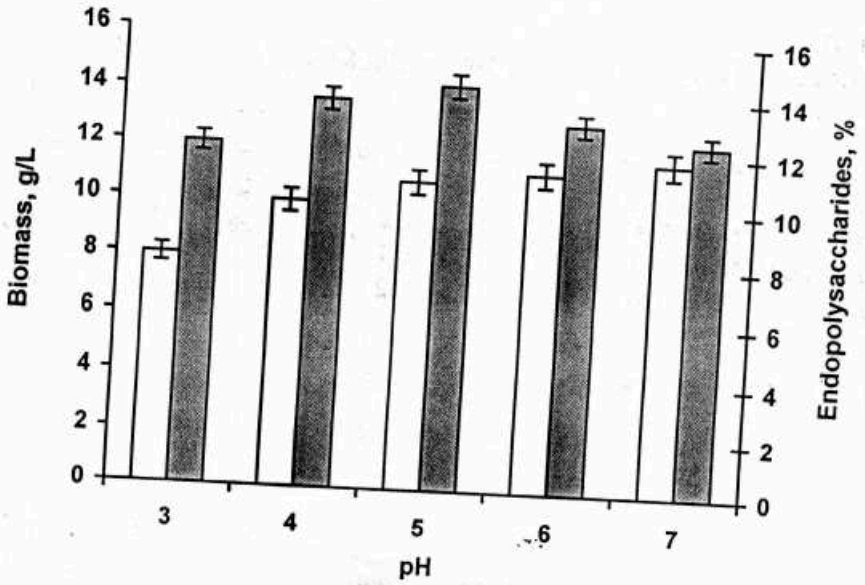


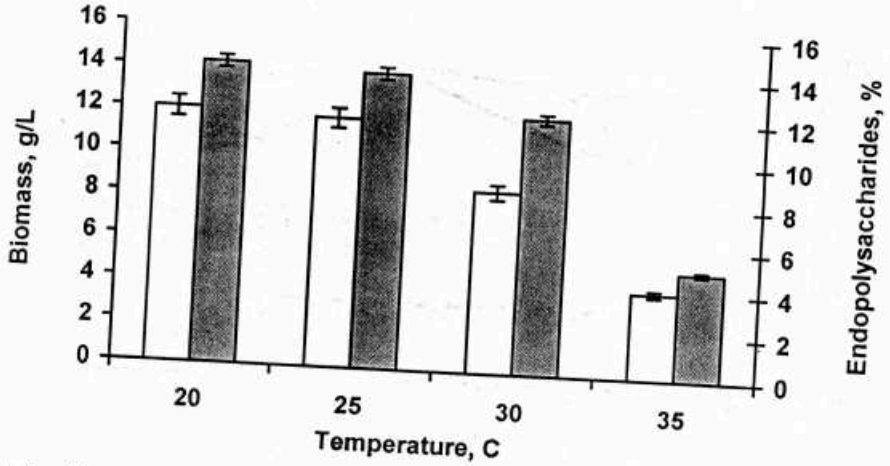
FIGURE 1. Effect of initial pH of glucose–peptone medium on (□) biomass (g/L) and (■) endopolysaccharides (%) production by *Cordyceps sinensis*.

in the medium took place almost in parallel to the mycelium growth but, as distinct from it, proceeded until the end of the fermentation process. Similar regularities were characterized for the growth of *Lentinus edodes* (Berk.) Singer in fermenter.[23]

It was shown that *C. sinensis* submerged mycelium contains up to 29% proteins, 15% endopolysaccharides, and over 7% lipids and is rich in phospholipids (up to 28% of total lipids) and unsaturated fatty acids ($C_{18:1}$ – up to 44%; $C_{18:2}$ – 53% of total fatty acids).

The protein and endopolysaccharide content in the submerged mycelial biomass of *C. sinensis* reached its maximum on day 3 of cultivation (Fig. 4). The content of the lipids continued to increase over the entire cultivation period. The ratio of phospholipids to total lipids increased only until day 3 of cultivation, whereupon it began to decrease (Fig. 4).

The fatty acid composition of *C. sinensis* lipids showed a predominance of unsaturated $C_{18:1}$ and $C_{18:2}$ fatty acids (Table 3). During cultivation, the



IGURE 2. Effect of cultivation temperature on biomass (g/L) and endopolysaccharide (%) production by *Cordyceps nensis* on glucose–peptone medium.

TABLE 2
Growth and Polysaccharide Synthesis by *Cordyceps sinensis* after Exposure to Light

Variant	Biomass, g/L	Endopolysaccharides, %	Exopolysaccharides, g/L
Control	10.0 ± 0.4	12.2 ± 0.2	5.5 ± 0.4
450.0 nm	11.5 ± 0.2	12.3 ± 0.1	3.3 ± 0.3
632.8 nm	9.4 ± 0.4	12.5 ± 0.2	3.8 ± 0.3

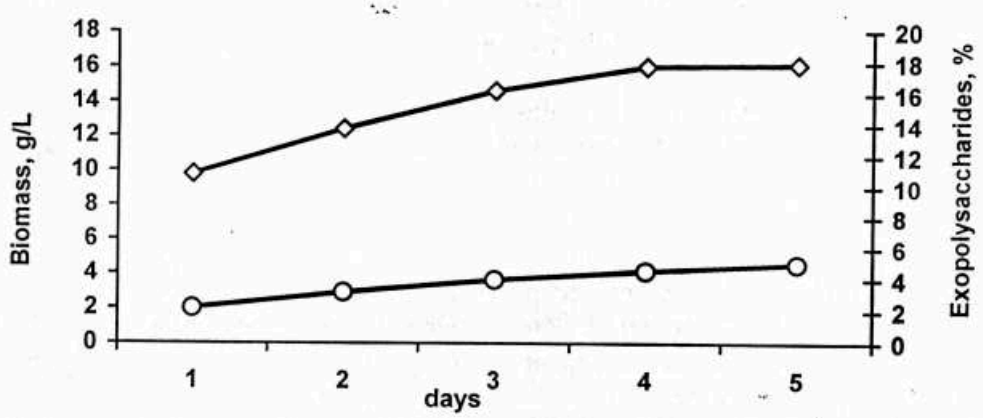


FIGURE 3. The growth of *Cordyceps sinensis* in fermenter on a molasses-based medium: (◊) biomass (g/L); (○) exopolysaccharides (g/L).

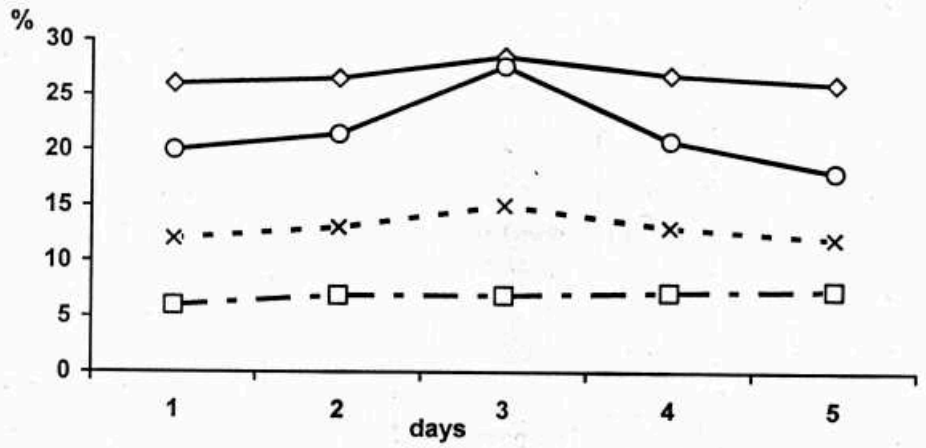


FIGURE 4. The growth of *Cordyceps sinensis* in fermentor on a molasses-based medium: (◊) proteins (%); (□) lipids (%); (×) endopolysaccharides (%); (○) phospholipids (% of total lipids).

TABLE 3
Fatty Acid Content (% of total fatty acids) of Lipids of *Cordyceps sinensis* Grown in a Fermentor on a Molasses-Based Medium

Constituent	Days			
	1	2	3	4
$C_{14:0}$	Trace	Trace	Trace	Trace
$C_{15:0}$	Trace	Trace	Trace	Trace
$C_{16:0}$	13.0	13.8	14.4	15.3
$C_{16:1}$	Trace	0.8	0.9	1.6
$C_{17:0}$	Trace	Trace	0.2	Trace
$C_{18:0}$	0.6	0.8	1.2	1.2
$C_{18:1}$	29.6	41.3	43.8	41.2
$C_{18:2}$	53.3	42.2	40.4	39.4
$C_{18:3}$	3.5	1.1	1.0	0.8
Unsaturated fatty acids	86.4	85.4	86.1	83.0
Saturated fatty acids	13.6	14.6	15.8	16.5
Ratio of unsaturated fatty acids to saturated fatty acids	6.4	5.9	5.5	5.0

content of linoleic acid ($C_{18:2}$) gradually decreased, whereas oleic acid ($C_{18:1}$) increased. Together with the increase of the content of saturated palmitic acid ($C_{16:0}$), these resulted in a decrease of the overall unsaturation of the *C. sinensis* lipids in the process of cultivation: the ratio of the sum of unsaturated to the sum of saturated fatty acids fell 1.3 times from the 1st to the 5th day of cultivation (Table 3). However, the ratio of unsaturated to saturated fatty acids of *C. sinensis* mycelium is 7–9 times higher than that of the earlier investigated species of medicinal Basidiomycetes.[23–27] The mycelium of *C. sinensis* differed from *Lentinus edodes* and *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. mycelia in the absence of heptadecenoic acid.[28]

Phenolic polymers are well-known natural antioxidants due to their conjugate chemical structure. A study of phenolic compound accumulation in *C. sinensis* submerged mycelium, together with a change in the mycelial alcohol extract antioxidant activity, showed that these two factors gradually increase during cultivation (Figs. 5A and 5B). Starting from the 2nd day of cultivation, the antioxidant activity changed slightly.

The results of the study show that *C. sinensis* submerged mycelial biomass contains a number of biologically active substances that could find application in pharmaceuticals, food, dietary supplements, and cosmeceuticals.

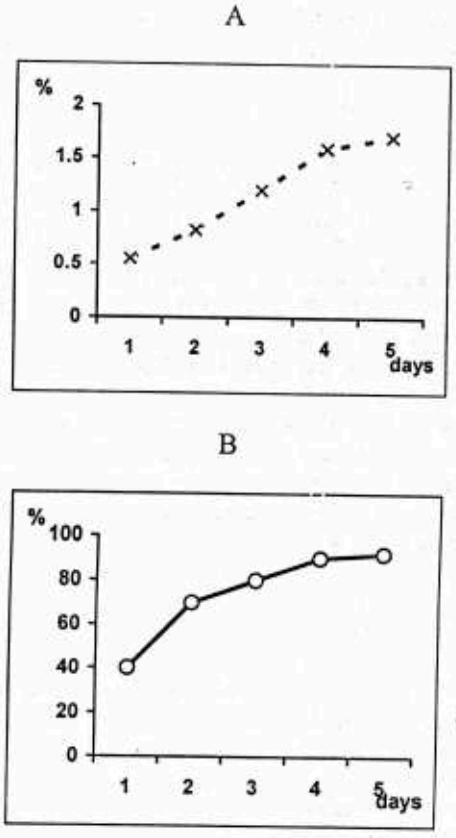


FIGURE 5. Dynamic of polyphenol accumulation (%) (A) and mycelium alcohol extract antioxidant activity (%) (B) during the growth of *Cordyceps sinensis* in fermentor on the molasses-based medium.

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