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A comparative study on the production of exopolysaccharides between two entomopathogenic fungi *Cordyceps militaris* and *Cordyceps sinensis* in submerged mycelial cultures

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ABSTRACT

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Aims: The present study comparatively investigates the optimal culture conditions for the production of exopolysaccharides (EPS) and cordycepin during submerged mycelial culture of two entomopathogenic fungi *Cordyceps militaris* and *Cordyceps sinensis*.

Methods and Results: Fermentations were performed in flasks and in 5-l stirred-tank fermenters. In the case of *C. militaris*, the highest mycelial biomass (22·9 g l^{-1}) and EPS production (5 g l^{-1}) were achieved in a medium of 40 g l^{-1} sucrose, 5 g l^{-1} corn steep powder at 30°C, and an initial pH 8·0. The optimum culture conditions for *C. sinensis* was shown to be (in g l^{-1}) 20 sucrose, 25 corn steep powder, 0·78 $CaCl_2$, 1·73 $MgSO_4 \cdot 7H_2O$ at 20°C, and an initial pH 4·0, where the maximum mycelial biomass and EPS were 20·9 and 4·1 g l^{-1} respectively. Cordycepin, another bioactive metabolite, was excreted at low levels during the early fermentation period (maximum 38·8 mg l^{-1} in *C. militaris*; 18·2 mg l^{-1} in *C. sinensis*).

Conclusions: The two fungi showed different nutritional and environmental requirements in their submerged cultures. Overall, the concentrations of mycelial biomass, EPS and cordycepin achieved in submerged culture of *C. militaris* were higher than those of *C. sinensis*.

Significance and Impact of the Study: *C. militaris* and *C. sinensis* are representative insect-born fungi which have been longstanding and widely used as traditional medicines in eastern Asia. Comparative studies between two fungi are currently not available and this is the first report on the optimum medium composition for submerged culture of *C. sinensis*.

Keywords: cordycepin, *Cordyceps militaris*, *Cordyceps sinensis*, entomopathogenic fungi, exopolysaccharide, medium optimization, submerged culture.

INTRODUCTION

The entomopathogenic fungi of the genus *Cordyceps*, whose members are known to be exclusively endoparasitic to insects, are an interesting research subject and have received considerable attention, particularly from eastern Asian countries.

Many types of the polysaccharides extracted from the fruit bodies of *Cordyceps* species have demonstrated various bioactive properties including, but not limited to, immunomodulating, anti-tumour and hypoglycaemic activities (Kiho *et al.* 1993; Kiho and Ukai 1995; Kuo *et al.* 1996; Song *et al.* 1998; Yang *et al.* 2000; Kim *et al.* 2001; Koh *et al.* 2003a,b; Li *et al.* 2003).

Cordycepin, a bioactive metabolite contained in the fruit bodies of these fungi, also exibihits various biological activities (Trigg *et al.* 1971; Sugar and McCaffery 1998; Zhou *et al.* 2002; Li *et al.* 2003; Yun *et al.* 2003). Unfortunately, *Cordyceps* are very difficult to collect because of their very small size and restricted area of growth. Thus the

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usage of cordycepin has been limited. Recently, mass production of these fungi, through artificial cultivation, has been successfully established and they are currently produced on a large scale. Although most investigators have sought to cultivate these fungi on a solid media, it may be more advantageous to do so in submerged media (Choi *et al.* 1999; Lee *et al.* 2001; Sung *et al.* 2002a,b).

Submerged cultures of entomopathogenic fungi give rise to higher productions of mycelial biomass and exopolysaccharide (EPS) in a more compact space, within a shorter period of time, and with less chance of contamination when compared with those cultivated on solid media (Xu *et al.* 2002; Kim *et al.* 2003a,b). Our group has previously demonstrated the optimization of culture parameters for different species of *Cordyceps militaris*, such as temperature, initial pH, carbon and nitrogen levels, and mineral source (Park *et al.* 2001, 2002b,c,d). However, there is no report available regarding the submerged cultivation of *Cordyceps sinensis*, even though this fungus is widespread in many countries and contains various beneficial functionalities in their fruit bodies.

To date, the submerged culture conditions for only three *Cordyceps* species have been reported, including *C. militaris*, *Cordyceps pruinosa* and *Cordyceps jiangxiensis*, even though over 40 species are known (Park *et al.* 2001, 2002a,b; Xu *et al.* 2002; Kim *et al.* 2003a,b; Xiao *et al.* 2004a,b). The objectives of the present study were to comparatively investigate the optimal submerged culture conditions of two entomopathogenic fungi, *C. militaris* and *C. sinensis*, for the production of mycelial biomass and EPS, and to partially characterize their fermentation products.

MATERIALS AND METHODS

Micro-organism

The strains of *C. militaris* NG1 and *C. sinensis* were kindly provided by NonGong Mushroom Co. (Kyungbuk, Korea) and Prof J.M. Sung of Kangwon National University (ChunCheon, Korea) respectively. The stock culture was maintained on potato dextrose agar (PDA) slants. Slants were incubated at 25°C for 7 days and then stored at 4°C for use as subcultures every month.

Inoculum preparation and culture conditions

Two *Cordyceps* species were initially grown on PDA medium (2·4% potato dextrose broth and 2% agar) in a Petri dish and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized house-developed cutter. Unless otherwise specified, the liquid seed cultures were prepared in a 250-ml flask containing 50 ml of YM medium (in g l^{-1}: 10 glucose, 3 yeast extract, 3 malt extract and 5 meat peptone) at 25°C on a rotary shaker incubator at 150 rev min^{-1} for 4 days. The main flask culture experiments were performed in 250-ml flasks containing 50 ml of YM medium after inoculating with 4% (v/v) of the seed culture. In the case of *C. militaris*, the fermentation culture were carried out in a 5-l stirred-tank fermenter (KF-250, KoBioTech, Seoul, Korea) under the following conditions: temperature, 30°C; aeration rate, 2 vvm; agitation speed, 150 rev min^{-1}; initial pH, 8·0; working volume, 3 l. In the case of *C. sinensis*, fermentations were conducted under the following conditions: temperature, 20°C; aeration rate, 2 vvm; agitation speed, 150 rev min^{-1}; initial pH, 4·0; working volume, 3 l.

Optimization experiments

To determine the optimal temperature and initial pH for mycelial biomass and EPS productions, the two fungi were individually cultivated at various pH levels ranging from pHs 3 to 9 and temperatures ranging from 15 to 35°C. To formulate suitable medium compositions for both mycelial growth and EPS production in flask culture of two fungi, 11 important carbon sources and nine nitrogen sources were each provided at concentrations of 10 and 5 g l^{-1} respectively (Tables 1 and 2). All experiments were carried out in duplicate and average values were calculated.

Analytical methods

The culture broth was harvested by centrifugation (9000 *g* for 20 min) and the supernatant was filtered through

Table 1 Effect of carbon sources on mycelial growth and exopolysaccharide (EPS) production by *Cordyceps militaris* and *C. sinensis* in shake flask culture*

Carbon sources (10 g l^{-1})	*C. militaris*† (g l^{-1})		*C. sinensis*† (g l^{-1})	
	Mycelial biomass	EPS	Mycelial biomass	EPS
Cellobiose	8·67 ± 0·19	1·11 ± 0·11	7·24 ± 0·27	0·62 ± 0·10
Dextrose	7·86 ± 1·10	0·90 ± 0·20	6·18 ± 0·22	0·52 ± 0·05
Fructose	8·33 ± 0·15	1·06 ± 0·21	6·83 ± 0·63	0·66 ± 0·04
Glucose	8·96 ± 0·14	1·02 ± 0·06	8·59 ± 0·37	0·61 ± 0·01
Lactose	7·97 ± 0·21	0·88 ± 0·08	5·17 ± 0·07	0·58 ± 0·03
Maltose	8·71 ± 0·01	1·20 ± 0·03	8·52 ± 0·04	0·69 ± 0·07
Mannitol	8·80 ± 0·23	0·80 ± 0·43	7·46 ± 0·76	0·65 ± 0·02
Sorbitol	8·08 ± 0·24	0·76 ± 0·17	7·86 ± 0·17	0·54 ± 0·06
Starch	9·02 ± 0·21	1·04 ± 0·24	8·48 ± 1·64	0·64 ± 0·07
Sucrose	9·56 ± 0·13	1·25 ± 0·14	9·05 ± 0·51	0·68 ± 0·08
Xylose	6·06 ± 0·30	0·50 ± 0·10	7·84 ± 0·16	0·53 ± 0·05

*Fermentations were carried out in shake flasks for 4 days at 25°C with initial pH 5·0.

†Values are mean ± SD of duplicate experiments.

Table 2 Effect of nitrogen sources on mycelial growth and exopolysaccharide (EPS) production by *Cordyceps militaris* and *Cordyceps sinensis* in shake flask culture*

Nitrogen sources (5 g l^{-1})	*C. militaris*† (g l^{-1})		*C. sinensis*† (g l^{-1})	
	Mycelial biomass	EPS	Mycelial biomass	EPS
Ammonium chloride	0·42 ± 0·02	0·48 ± 0·07	0·97 ± 0·05	0·13 ± 0·01
Ammonium nitrate	0·56 ± 0·02	0·56 ± 0·19	0·87 ± 0·03	0·09 ± 0·16
Ammonium phosphate	2·20 ± 0·04	1·53 ± 0·47	0·86 ± 0·02	0·18 ± 0·05
Ammonium sulfate	0·54 ± 0·06	0·48 ± 0·03	0·89 ± 0·19	0·10 ± 0·08
Corn steep powder	16·93 ± 0·05	2·34 ± 0·27	9·13 ± 0·17	1·12 ± 0·06
Malt extract	6·95 ± 0·07	0·98 ± 0·12	2·47 ± 0·03	0·29 ± 0·14
Meat peptone	11·88 ± 1·72	1·58 ± 0·43	8·93 ± 0·01	1·03 ± 0·04
Polypeptone	7·90 ± 0·08	0·77 ± 0·02	6·57 ± 0·16	0·36 ± 0·1
Yeast extract	16·79 ± 0·29	1·80 ± 0·10	5·85 ± 0·11	0·62 ± 0·02

*Fermentations were carried out in shake flasks for 4 days at 25°C with initial pH 5·0.
†Values are mean ± SD of duplicate experiments.

Whatman filter paper No. 2 (Whatman International Ltd, Maidstone, UK). The resulting culture filtrate was mixed with four volumes of ethanol, stirred vigorously and left overnight at 4°C. The precipitated EPS was centrifuged at 9000 *g* for 20 min and the supernatant was discarded. The EPS precipitate was lyophilized and weighed to obtain the concentration of EPS. The mycelial biomass was measured after repeated washing of the mycelial pellets with distilled water and drying overnight at 90°C. The filtrate from the membrane filtration (Whatman filter paper No. 2) was analysed for residual sugar concentration by HPLC (Shimadzu Co., Osaka, Japan) using an Aminex HPLC-42C column (0·78 × 30 cm; Bio-Rad Laboratories, Hercules, CA, USA) equipped with a refractive index detector.

Fractionation and compositional analysis of the EPS

The ethanol precipitate of the EPS was dissolved in 0·2 mol l^{-1} NaCl solution creating an EPS concentration of 5 g l^{-1}, and the EPS solution was then loaded onto a Sepharose CL-6B column (2·4 × 100 cm; Sigma Chemical Co., St Louis, MO, USA). The column was eluted with the same solution at a flow rate of 0·6 ml min^{-1}. The carbohydrate moiety of the EPS was measured by monitoring the absorbance at 480 nm (Dubois *et al.* 1956), while the protein moiety was monitored at 280 nm. The EPS fractions were pooled and lyophilized for further analysis. The total sugar content of the EPS was determined by the phenol sulfuric acid method, with glucose as the standard. The sugar composition was analysed by gas chromatography (model Star 3600CX; Varian Co., Lexington, MA, USA) with a fused silica capillary column (Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector. The total protein was determined by the Bradford method, with bovine serum albumin as the standard.

Quantification of cordycepin

Culture broths were harvested by centrifugation (9000 *g* for 20 min), and then the supernatant was filtered through 0·45-μm filter membranes prior to injection into the capillary electrophoresis (CE) system (Li *et al.* 2001). Analyses were carried out with a CE instrument (P/ACE 5500 system; Beckman, Fullerton, CA, USA) in an uncoated silica capillary (27 cm in height, 20 cm inlet to detector) × 50 μm i.d. capillary column at a constant voltage of 10 kV and a temperature of 25°C. All samples were pressure-injected for 5 s. Before injection, the capillary column was rinsed with 0·1 mol l^{-1} HCl, 0·1 mol l^{-1} NaOH and deionized water for 2 min, and then washed again with 25 mmol l^{-1} borate buffer for 2 min. The experimental detection wavelength was set at 258 nm with a photodiode array detector. A standard curve for the quantification of cordycepin was prepared prior to sample analysis

RESULTS

Effect of temperature and initial pH

In the case of *C. militaris*, maximum EPS concentrations were obtained at 30°C, whereas maximum mycelial biomass were found at 20°C (Fig. 1). Both EPS yield and mycelial growth were found to be noticeably decreased when incubated in temperatures above 30°C. In the case of *C. sinensis*, maximum productions of the EPS and mycelial biomass were both achieved at 20°C (Fig. 1). Fig. 2 shows the effect of initial pH on both EPS production and mycelial growth, suggesting that initial pH significantly affected fermentation results. For *C. militaris*, maximum EPS concentrations were obtained in cultures grown at an initial pH 8·0, while maximum mycelial biomass was achieved at pH 5. In contrast, *C. sinensis* demonstrated optimal mycelial growth and EPS production at pH 4·0 (Fig. 2).

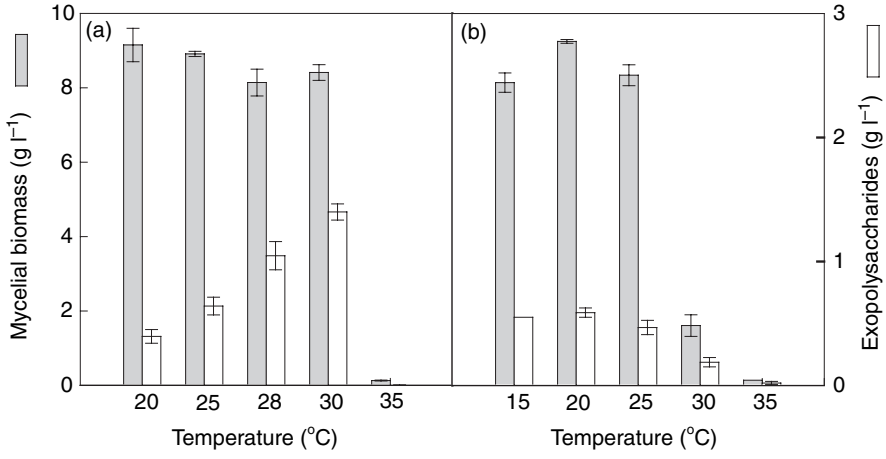

Fig. 1 Effect of temperature on mycelial growth and exopolysaccharides in shake flask cultures of *Cordyceps militaris* (a) and *Cordyceps sinensis* (b). All experimental data are mean ± SD of duplicate experiments

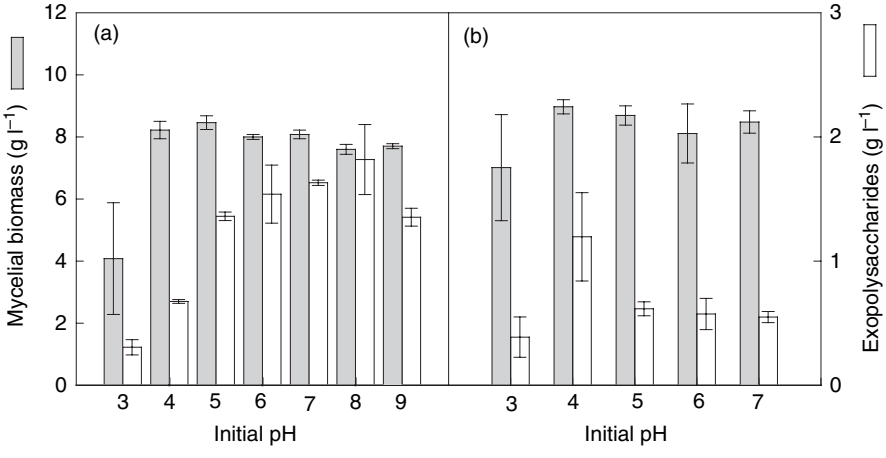

Fig. 2 Effect of initial pH on mycelial growth and exopolysaccharides in shake flask cultures of *Cordyceps militaris* (a) and *Cordyceps sinensis* (b). All experimental data are mean ± SD of duplicate experiments

Effect of medium composition

Of the carbon sources tested, the two fungi yielded the highest mycelial growth and EPS production when cultivated in media prepared using sucrose, though at differing concentrations. *Cordyceps militaris* demonstrated maximal mycelial growth and EPS production in medium with 40 g l^{-1} sucrose (Fig. 3). *Cordyceps sinensis* demonstrated optimal concentrations that were lower than those required by *C. militaris* (15 g l^{-1} for mycelial growth and 20 g l^{-1} for EPS production). Table 2 shows the effect of nitrogen source on both mycelial growth and EPS production, where the highest mycelial biomass and EPS production were achieved in the medium containing 5 g l^{-1} corn steep powder for *C. militaris*. In comparison with organic nitrogen sources, inorganic nitrogen sources gave rise to relatively lower mycelial biomass and EPS production. It is interesting to note that higher concentration of the nitrogen source (25 g l^{-1}) was required by *C. sinensis* than was used for *C. militaris* (5 g l^{-1}) (Fig. 4), which is opposite to the results for the carbon source requirements, as previously shown in Fig. 3. The influence of bioelements on mycelial growth and EPS production were examined by supplementing various mineral sources (7 mmol l^{-1}) into the medium (Table 3). Although no significant effect was observed in the case of *C. militaris*, *C. sinensis* demonstrated enhanced production of mycelial biomass and EPS when cultured in media containing both calcium and magnesium.

Fermentation results

Fig. 5a shows typical time profiles of mycelial biomass and EPS production by *C. militaris* in a 5-l stirred-tank fermenter under optimized culture conditions (sucrose 40 g l^{-1}, corn steep powder 5 g l^{-1}, 30°C and pH 8·0). The EPS production reached a maximum concentration of 5·05 g l^{-1} at day 16, where maximum mycelial biomass indicated 22·9 g l^{-1} (Fig. 5a). In the case of *C. sinensis*, under optimal culture condition (in g l^{-1}: sucrose 20, corn steep powder 25, $CaCl_2$ 0·78, $MgSO_4 \cdot 7H_2O$ 1·73, 20°C and pH 4·0), the maximum concentrations of EPS and mycelial

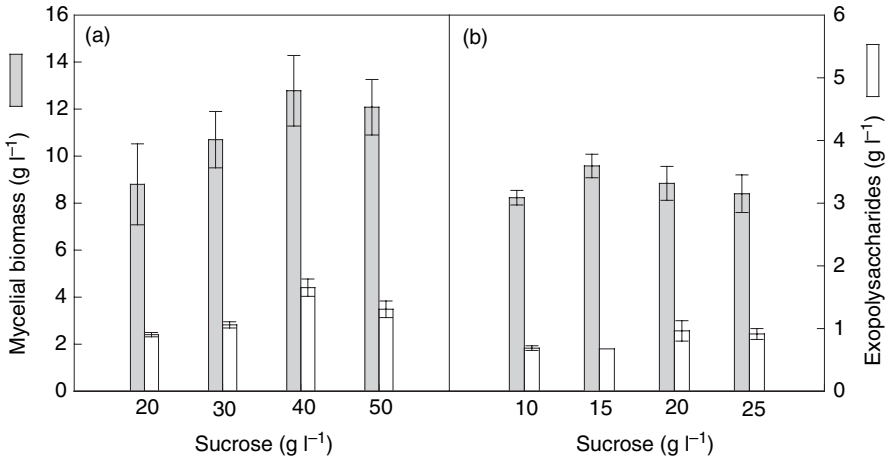

Fig. 3 Effect of carbon source on mycelial growth and exopolysaccharides in shake flask cultures of *Cordyceps militaris* (a) and *Cordyceps sinensis* (b). All experimental data are mean ± SD of duplicate experiments

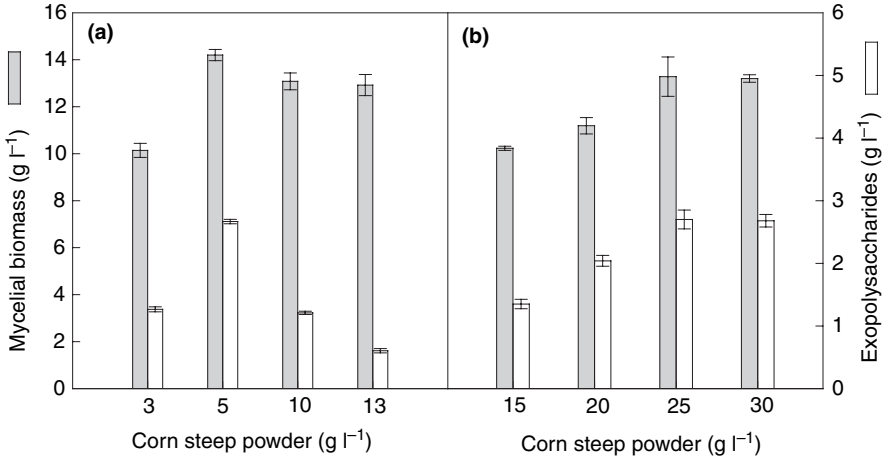

Fig. 4 Effect of nitrogen source on mycelial growth and exopolysaccharides in shake flask cultures of *Cordyceps militaris* (a) and *Cordyceps sinensis* (b). All experimental data are mean ± SD of duplicate experiments

Table 3 Effect of mineral sources on mycelial growth and exopolysaccharide (EPS) production by *Cordyceps militaris* and *Cordyceps sinensis* in shake flask*

Mineral sources (7 mmol l^{-1})	*C. militaris* (g l^{-1})		*C. sinensis* (g l^{-1})	
	Mycelial biomass	EPS	Mycelial biomass	EPS
Control†	12·80 ± 1·14	1·70 ± 0·21	8·34 ± 0·28	0·48 ± 0·06
$CaCl_2$	11·44 ± 0·54	1·63 ± 0·10	9·55 ± 0·07	0·78 ± 0·08
$FeSO_4{\cdot}7H_2O$	9·09 ± 0·21	1·49 ± 0·02	8·86 ± 0·06	0·63 ± 0·13
KH_2PO_4	7·60 ± 0·00	1·95 ± 0·06	8·72 ± 0·06	0·91 ± 0·21
K_2HPO_4	11·62 ± 1·86	1·65 ± 0·25	8·40 ± 0·02	1·01 ± 0·10
$MgSO_4{\cdot}7H_2O$	10·24 ± 0·65	1·51 ± 0·12	8·61 ± 0·17	1·08 ± 0·01
$CaCl_2$ + $MgSO_4{\cdot}7H_2O$	ND‡	ND	11·24 ± 0·24	1·25 ± 0·02

*Fermentations were carried out in shake flask for 4 days at 25°C with initial pH 5·0. Values are mean ± SD of duplicate experiments.
†No supplementation of mineral ions.
‡ND, not determined.

biomass were 20·94 and 4·15 g l^{-1}, respectively, at day 16 (Fig. 5b).

Fractionation and compositional analysis of the EPS

In the course of gel filtration chromatography on Sepharose CL-6B, five fractions of EPS (F1–F5 in Fig. 6a) were obtained from the culture filtrate when cultivated at basal conditions for *C. militaris*. In contrast, the crude EPS obtained from the mycelial culture broth of optimized condition yielded three fractions when passed through the same column (F1–F3 in Fig. 6b). Fig. 7 show the elution profiles of crude EPS produced by *C. sinensis* under the basal condition (YM medium) and optimized condition. Three fractions of EPS (F1–F3 in Fig. 7a) were coeluted, with F2 being the major fraction under basal condition, while two fractions (F1 and F2 in Fig. 7b) were obtained from the optimal medium. The chromatographic and compositional

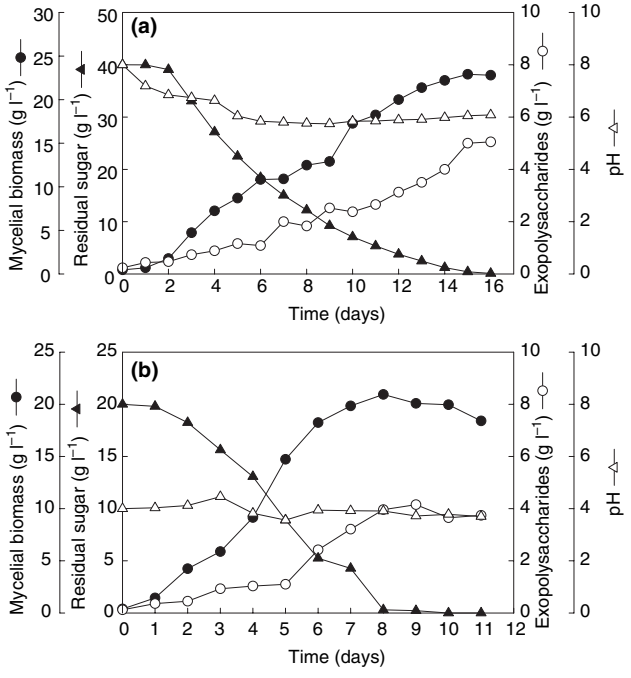

Fig. 5 Time profiles of mycelial biomass and exopolysaccharide production during submerged culture of *Cordyceps militaris* (a) and *Cordyceps sinensis* (b) in a 5-l stirred-tank fermenter under the optimal conditions

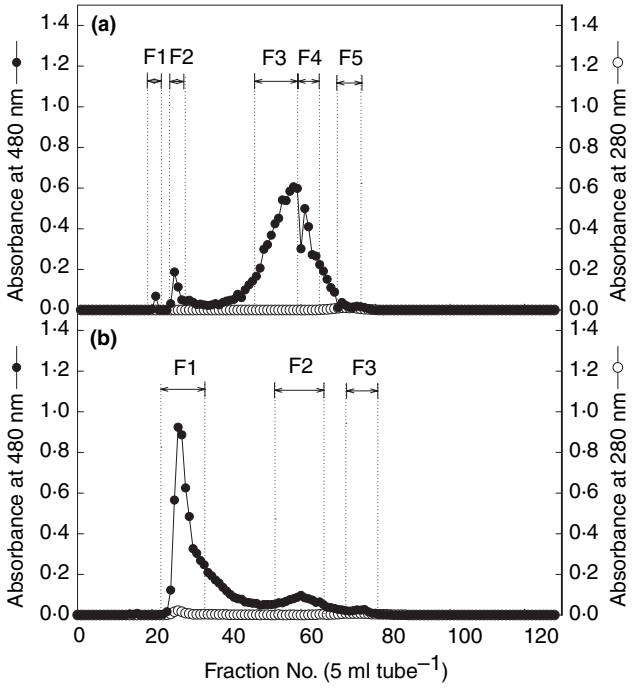

Fig. 6 Elution profiles of the exopolysaccharides (EPS) produced by submerged culture of *Cordyceps militaris* in Sepharose CL-6B chromatography. EPS fractions produced from basal conditions (a) and optimal conditions (b). Eluates were analysed by measuring the absorbance at 480 nm for carbohydrate and at 280 nm for protein

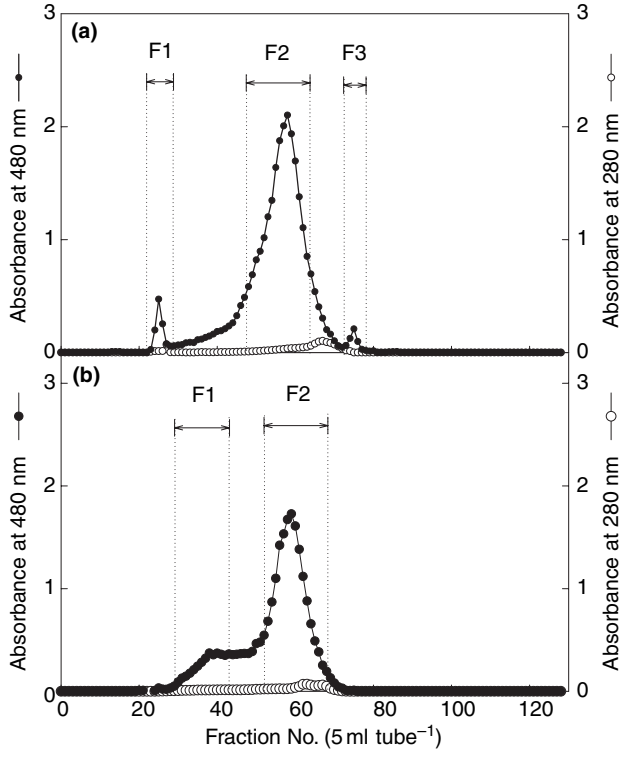

Fig. 7 Elution profiles of the exopolysaccharides (EPS) produced by submerged culture of *Cordyceps sinensis* in Sepharose CL-6B chromatography. EPS fractions produced from basal conditions (a) and optimal conditions (b). Eluates were analysed by measuring the absorbance at 480 nm for carbohydrate and at 280 nm for protein

analyses evidenced that all fractions of the EPS are polysaccharides without protein moieties (also see Table 4). The EPS from *C. militaris* was obviously glucan, whereas the EPS from *C. sinensis* were galactomannans. The most abundant fractions (e.g. F3 in Fig. 6a, F1 in Fig. 6b, and F2 in Fig. 7) were pooled as purified EPS and their chemical compositions were illustrated in Table 4.

Measurement of cordycepin by CE

The cordycepin concentration excreted to the fermentation broth during submerged cultures of the two fungi were determined by using CE. Initially, the standard curve was established as dependence of peak area on the cordycepin concentrations, as shown in the inset of Fig. 8. The linear calibration equations for cordycepin quantification was $y = 587{\cdot}4743x$ ($R^2 = 0{\cdot}9984$). Peaks were identified in two ways: (i) by comparing the migration times of the unknown peaks with those of the standard cordycepin eluted with the same conditions, and (ii) by spiking *C. militaris* and *C. sinensis* sample with standard solutions of cordycepin. Fig. 8 shows the time profiles of cordycepin concentration

Table 4 Variances in sugar composition of the exopolysaccharides (EPS) produced from submerged mycelial culture of two entomopathogenic fungi *Cordyceps militaris* and *Cordyceps sinensis* under basal and optimal medium

	Compositions (%)*					
	C. militaris			*C. sinensis*		
Sugar	Basal medium	Optimized medium	Purified EPS	Basal medium	Optimized medium	Purified EPS
Ribose	ND†	0·58	0·67	0·14	ND	ND
Arabinose	3·20	3·91	1·88	1·93	5·89	4·61
Xylose	1·83	2·91	0·79	0·66	3·60	2·08
Mannose	8·45	2·29	1·92	54·14	38·50	42·19
Galactose	5·58	0·85	1·28	30·11	41·64	43·37
Glucose	80·93	89·46	93·46	13·02	10·37	7·75

*Crude exopolysaccharides produced from basal and optimum medium respectively. The most abundant fractions were pooled as purified EPS (F3 and F1 in Fig. 6; F2 in Fig. 7).
†ND, not detected.

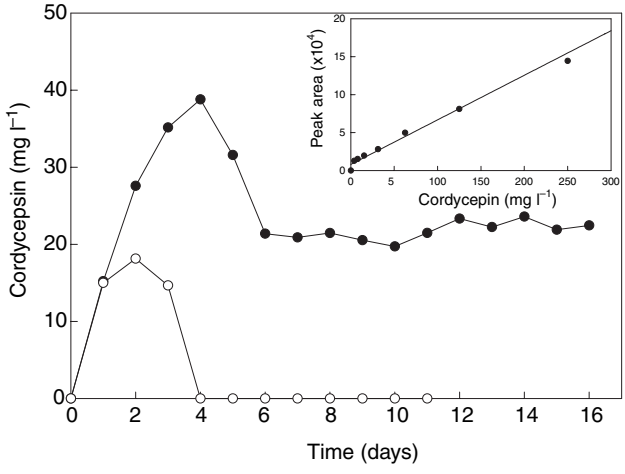

Fig. 8 Time profiles of cordycepin production during submerged culture of *Cordyceps militaris* (●) and *Cordyceps sinensis* (○) in a 5-l stirred-tank fermenter. Standard curve was established as a dependence of peak area on the cordycepin concentrations (see inset)

during submerged culture of *C. militaris* and *C. sinensis* in a 5-l stirred tank fermenter. The maximum concentrations of cordycepin from *C. militaris* and *C. sinensis* were 38·80 and 18·19 mg l^{-1} respectively.

DISCUSSION

Many types of bioactive metabolites from the fruiting body of *Cordyceps* were found to have a variety of biological activities. However, extracellular metabolites are entirely limited to cordycepin and only three reports describing extracellular polymeric substances (e.g. EPS) are currently available (Yamada 1984; Song *et al.* 1998; Yang *et al.* 2000). The authors suggested that the extracellular polymeric substances have strong biological activities including hypolipidaemic, anti-tumour, and anti-complementary activity as detected from fruiting bodies of *Cordyceps*. In this regard, much attention should be given to the use of submerged culture of these fungi for the production of mycelial biomass and its bioactive components.

Nutritional requirements of higher fungi play critical role in EPS production in submerged cultures. It has been found that medium constituents strongly affect chemical composition, structure and productivity of EPS (Wagner *et al.* 2004; Hsieh *et al.* 2005). In the present study, it was also found that the optimal submerged culture conditions for maximum mycelial growth and EPS production depend strongly on the species and strains used (Park *et al.* 2001, 2002a,b,c; Xu *et al.* 2002; Kim *et al.* 2003a,b).

It is generally understood that mycelia of many different mushrooms and entomopathogenic fungi will grow, to some extent, on a wide range of carbon sources (Yang *et al.* 2003). However, the carbon source yielding maximum growth differs from species to species. Both *Cordyceps* species investigated in this study utilized sucrose as the preferred carbon source. This may make it possible to use a cheaper alternative sources of sucrose, such as raw sugar or molasses, for mass production of mycelial biomass and EPS by large-scale submerged cultivations. As medium cost is one of the critical factors affecting the economy of the industrial fermentation process, further study using less expensive ingredients should be considered.

It has been suggested that using a combination of two carbon sources in the submerged cultivation of higher fungi could result in higher mycelial growth and EPS production compared with those containing a single carbon source (Hwang *et al.* 2003a,b; Xiao *et al.* 2004b). Comparing the carbon source requirement of the *Cordyceps* species in this study with previous studies, different sugars at different concentrations were required for mycelial growth and EPS production in their submerged cultures. For example, *C. pruinosa* utilized 25 g l^{-1} sucrose as the suitable carbon source, whereas *C. jiangxensis* required 20 g l^{-1} maltose for both mycelial growth and EPS production. In contrast, the *C. militaris* species showed significantly different carbon source requirement. For instance, *C. militaris* C738 required a high concentration of sucrose (60 g l^{-1}), while two different strains of *C. militaris* required 40 g l^{-1} sucrose for mycelial growth and EPS production (Park *et al.* 2001; Kim *et al.* 2003b). Bearing in mind that mycelial biomass is also a useful product containing several bioactive polysaccharides, production yield of mycelial biomass should be simultaneously considered in the processes of submerged culture of mushrooms and entomopathogenic fungi.

Accordingly, while each higher fungus frequently requires different carbon source for maximum mycelial biomass and EPS production, use of the same carbon source is recommended from a practical point of view.

The omission of nitrogen in the medium greatly affects fungal growth and metabolite production. Nitrogen source may be supplied to media in the form of ammonia, nitrate, or as organic compounds, such as amino acids or proteins. It has been reported that the optimal nitrogen source of mycelial biomass and EPS production in several entomopathogenic fungi was corn steep powder (Bae *et al.* 2000; Park *et al.* 2001; Hwang *et al.* 2003a). In comparison with organic nitrogen sources, inorganic nitrogen sources often yield relatively lower mycelial biomass and EPS production than organic sources in liquid cultures of higher fungi. Based on our previous results, corn steep powder is considered to be the most useful nitrogen source in submerged cultivation of many higher fungi for EPS production (Park *et al.* 2001; Kim *et al.* 2002a, 2003a; Xu *et al.* 2002; Hwang *et al.* 2003a,b). However, Xiao *et al.* (2004b) demonstrated that soybean steep powder and tryptone were more efficient for mycelial growth and EPS production, respectively, in a submerged culture of *C. jiangxiensis*. It should be mentioned here that some of the enhancements obtained from using the organic sources of nitrogen may indeed not only reflect the form that the nitrogen is in, but also the fact that other non-nitrogen components could play a role in the improvements.

It has been reported that mineral ions play a pivotal role in fungal growth and in their secondary metabolite formations. Chardonnet *et al.* (1999) found that external Ca^{2+} can play an indirect role in fungal growth by altering internal Ca^{2+}, which controls the cytoplasmic Ca^{2+} gradient, and the activity of fungal enzymes involved in cell wall expansion. The direct effect of Ca^{2+} on the fungal cell wall may also be a significant factor in cell membrane permeability interactions. In contrast, Papagianni (2004) found that Ca^{2+} accumulation seemed to inhibit the synthesis of fungal biopolymers, possibly through an effect on enzymes such as β-glucan synthesis. For higher $CaCl_2$ concentrations, the calcium ion content of the cell wall increased, resulting in reduced protein and neutral sugar contents. The effects of these agents appear to be mediated by a gradient of cytoplasmic free Ca^{2+}, which is obligatorily present and involved in active growth. Mg^{2+} is also essential to all fungi. It is a cofactor in enzymatic reactions, stabilizes the plasma membrane, and its uptake is ATP dependent. The positive action of Ca^{2+} and Mg^{2+} on mycelial biomass and EPS production was obvious in the present submerged cultures (Xiao *et al.* 2004b). In this study, a combination of Ca^{2+} and Mg^{2+} ions gave rise to enhanced mycelial growth and EPS production in *C. sinensis*, but not in *C. militaris*. The reason for this difference is not yet clear.

Many investigators claim that the morphology of fungal mycelia under different initial pH values is a critical factor in biomass accumulation and metabolite formation (Wang and McNeil 1995; Shu and Lung 2004). The medium pH may affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis (Gerlach *et al.* 1998; Shu and Lung 2004). In the case of *C. sinensis*, the optimal pH value and temperature for both mycelial biomass and EPS production were pH 4·0 and 20°C respectively. This is comparable with many kinds of mushrooms with relatively low temperature optima (e.g. 20–25°C) in their submerged cultures (Bae *et al.* 2000; Park *et al.* 2001). It has been reported that several kinds of higher fungi have more acidic pH optima for mycelial biomass and EPS accumulation during their submerged cultures (Lee *et al.* 1989; Hwang *et al.* 2003b; Kim *et al.* 2003a; Shu and Lung 2004).

The EPS produced in this study were proved to be glucans (from *C. militaris*) and galactomannans (from *C. sinensis*). A variety of different types of polysaccharides in many species of *Cordyceps* have been reported in the literature, most of which are polysaccharides extracted from the fruit body or the mycelia. Kim *et al.* (1994) reported that the polysaccharides from ascocarps of *C. militaris* were composed of glucose (78·6%), galactose (19·1%), and arabinose (2·2%). Koh *et al.* (2002) investigated the general composition of hot water extract from cultured mycelia of *C. sinensis* and found it to be 83·9% carbohydrate and 11·8% protein, where the carbohydrates were mainly composed of glucose, mannose, galactose and arabinose.

Yu *et al.* (2001) isolated proteoglycans from the stromata of *C. militaris*, which consisted of mainly glucose (88·6%). Galactomannans or galactoglucomannans have been fractionated from the fruit bodies of many different species of *Cordyceps* (Kiho *et al.* 1993, 1996, 1999). Recently, Koh *et al.* (2001, 2003a,b) isolated proteoglycans from the hot water fraction of the dried mycelia of fermented *C. sinensis*, composed mainly of glucose and mannose, with a small amount of galactose and arabinose.

As for the extracellular polysaccharides from mycelial cultures of *Cordyceps*, different constituents have been demonstrated according to species and culture conditions. For instance, Song *et al.* (1998) reported that the exopolymer produced from the culture broth of *C. militaris* consisted mainly of galactose (87·2%), with small amount of glucose (9·3%) and arabinose (3·5%). Extracellular β-glucans were produced by submerged mycelial culture of *Cordyceps ophioglossoides*. (Yamada 1984; Ohmori *et al.* 1988).

Taken together, it is apparent that the chemical compositions in fruit bodies of *Cordyceps* fungi are strongly dependent upon many factors, such as harvest time, storage duration of the fruit bodies, and preparation methods of the

polysaccharides. However, submerged culture of *Cordyceps* can be a more promising choice for producing the bioactive polysaccharides without causing significant change in their chemical compositions.

Cordycepin is a nucleoside analogue 3′-deoxyadenosine with a broad spectrum of biological activities. It was first extracted from *C. militaris* by Cunningham *et al.* (1950) from the supernatant of the fermentation broth in *C. militaris* and was later found to be present in small amounts in *C. sinensis*. Cordycepin is an example of a compound that is primarily extracellular in nature. Many studies have been carried out on cultured *Cordyceps* mycelium looking for the presence of cordycepin. The authors have shown that cordycepin is generally present in solid/substrate-grown *Cordyceps*, but not in liquid-cultured *Cordyceps*. The presence or absence of cordycepin is dependent upon many factors, including the method of mycelial culture.

Many of the studies dealing with cordycepin analysis have applied TLC and HPLC techniques (Huang *et al.* 2003; Li *et al.* 2004; Ma *et al.* 2004; Velikinac *et al.* 2004). However, TLC is only used for qualitative and semi-quantitative analysis. As for HPLC, reconditioning of the column requires a great amount of time and a large volume of organic solvent. Moreover, the columns are expensive and require frequent changing to lessen the risk of elution loss. Capillary zone electrophoresis is the most widely used mode of CE under native conditions and it is also the first fully automated electrophoresis method (Li *et al.* 2001; D'Acunto *et al.* 2002; Ling *et al.* 2002; Jung *et al.* 2004). In this study, cordycepin concentrations produced by both *Cordyceps* species were unexpectedly low (<40 mg l^{-1}), which are similar to the levels appearing in the literature (Cunningham *et al.* 1951; Frederiksen *et al.* 1965; Hsu *et al.* 2002). Mao and Zhong (2004) demonstrated that enhanced production of cordycepin (maximum 345 mg l^{-1}) was obtained by medium optimization for cordycepin, not for EPS, by submerged cultivation of *C. militaris*. They reported that cells grew best in a galactose medium but cordycepin production was optimal in a glucose medium. They also reported that a two-stage dissolved oxygen (DO) control was efficient for cordycepin production. DO was controlled at 60% from the beginning of cultivation, and then shifted to a lower control level of 30% when cordycepin productivity started to decrease (Mao and Zhong 2004). The low cordycepin concentrations achieved in this study were probably because of lack of medium optimization, as they were focused on maximizing mycelial growth and EPS production, rather than cordycepin production.

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