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Impact of different fermentation characteristics on the production of mycelial biomass, extra-cellular polysaccharides, intra-cellular polysaccharides, and on the antioxidant activities of *Cordyceps militaris* (L.) Fr. (strains AG-1, PSJ-1)

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Abstract: The mycelial biomass, antioxidant activity and production of extra- and intra-cellular polysaccharides production [EPS, IPS] of *Cordyceps militaris* strains AG-1, PSJ-1 were evaluated under different submerged liquid culture (SLC) conditions**.** At 24 ^{0}C mycelial biomass and polysaccharide production of AG-1, PSJ-1 was optimal using PVC media and static culture conditions; (AG-1: 21.85 ± 1.00; PSJ-1: 18.20 ± 1.84 g l^{-1}), and oven drying at 40 °C (AG-1: 25.95 ± 0.84, PSJ-1: 23.55 ± 0.69 mg g^{-1}) compared with hot water extraction (AG-1: 7.07 ± 0.15, PSJ-1: 7.39 ± 0.61 mg g^{-1}). Maximum biomass, EPS and IPS production were observed when the initial pH was 6.7: AG-1: 12.92 ± 0.33, 209.70 ± 1.56, 32.62 ± 0.87; PSJ-1: 9.03 ± 0.24 g l^{-1} , 198.16 ± 0.85 mg g^{-1}, 30.63 ± 1.96 mg g^{-1}, respectively. The use 3.5 % coconut oil improved biomass, EPS, IPS production, which were 8.27 ± 0.09, 8.01 ± 0.01 g l^{-1} ; 1208.00 ± 8.60, 1110.40 ± 7.20 mg g^{-1}; 32.43 ± 0.49, 29.74 ± 0.44, for AG-1 and PSJ-1, respectively. Both culture condition and oven drying methods had significant effects on H_2O_2 and radical scavenging activity, $ABTS^{+}$ radical activity, lipid peroxidation, and also had effects on total flavonoid and, total phenolic contents. The use of crude submerged liquid culture and oven drying on strains AG-1, PSJ-1 led to extracts with potent antioxidant activity, suggesting the therapeutic use of polysaccharides from strains AG-1, PSJ-1.

Key words: antioxidant activities; Cordyceps militaris; extra-cellular polysaccharides and intra-cellular polysaccharides; oven drying; pH

Vpliv različnih postopkov fermentacije na produkcijo biomase micelija, ekstra in itracelularnih polisaharidov in na antioksidacijsko aktivnost kokonovega glavatca (*Cordyceps militaris* (L.) Fr., seva AG-1, PSJ-1)

Izvleček: Biomasa micelija, antioksidacijska aktivnost in produkcija zunaj- in znotraj celičnih polisaharidov [EPS, IPS] so bile ovrednotene pri kokonovem glavatcu (*Cordyceps militaris* (L.) Fr., seva AG-1, PSJ-1) v razmerah različnih submerznih tekočih kultur (SLC) **.** Pri 24 ^{0}C sta bili biomasa micelija in produkcija polisaharidov pri obeh sevih optimalni v gojišču PVC in stabilnih ramerah; (AG-1: 21,85 ± 1,00; PSJ-1: 18,20 ± 1,84 g l^{-1}), po sušenju v pečici na 40 °C (AG-1: 25,95 ± 0,84, PSJ-1: 23,55 ± 0,69 mg g^{-1}) v primerjavi z ekstrakcijo z vročo vodo (AG-1: 7,07 ± 0,15, PSJ-1: 7,39 ± 0,61 mg g^{-1}). Največja biomasa in produkcija obeh tipov polisaharidov (EPS in IPS) sta bili doseženi pri začetnem pH 6,7: AG-1: 12,92 ± 0,33; 209,70 ± 1,56; 32,62 ± 0,87; PSJ-1: 9,03 ± 0,24 g l^{-1}; 198,16 ± 0,85 mg g^{-1}; 30,63 ± 1,96 mg g^{-1}. Uporaba kokosovega olja (3,5 %) je povečala biomaso in produkcijo polisaharidov v obeh sevih in sicer: 8,27 ± 0,09; 8,01 ± 0,01 g l^{-1}; 1208,00 ± 8,60: 1110,40 ± 7,20 mg g^{-1}; 32,43 ± 0,49; 29,74 ± 0,44. Oba načina gojenja in metode sušenja v pečici so imeli značilen vpliv na aktivnost nevtralizacije H_2O_2 in prostih radikalov, aktivnost $ABTS^{+}$ radikala in peroksidacijo lipidov. Imeli so tudi vpliv na vsebnost celokupnih flavonoidov in fenolov. Uporaba surove submerzne tekoče culture in sušenje obeh sevov v pečici sta dali izvlečke s potencialno antioksidacijsko aktivnostjo, kar nakazuje terapevtsko uporabo polisaharidov iz obeh sevov kokonovega glavatca.

Ključne besede: antioksidacijska aktivnost; Cordyceps militaris; zunaj- in znotraj celični polisaharidi; sušenje v pečici; pH

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1 INTRODUCTION

Cordyceps militaris (L.) Fr. (Ascomycota: Hypocreales) is an entomopathogenic fungus that is used in traditional Asia medicine; it is a common parasite of lepidopteran larvae (Shih et al., 2007). The medicinal properties of *Cordyceps militaris* result from its ability to produce bioactive compounds including cordycepin. At present, liquid fermentation is used for biomass production. However, optimization is required to maximize production of bioactive compounds. The efficacy of extracts production depends mainly on the strain used, nutrient sources in the culture medium, and cultivation parameters (Dong et al., 2012). During the fermentation, *C. militaris* grows in liquid suspension, which is thought to be the best way to produce complex organic compounds including cordycepin (Mao et al., 2005). Vegetable oils can enhance growth rates of mycelium in liquid culture (Schisler & Volkoff, 1977) and reports suggest that oils and fatty acids promote production of fungal metabolites (Kojima et al., 1972).

In recent years, considerable research attention had focused on natural compounds with hypoglycemic activity. Polysaccharides extracted from various medicinal fungi species have shown hypoglycemic activity (Kiho et al., 1997). Although many researchers have tried to optimize submerged liquids culture conditions to promote extra–cellular polysaccharides (EPS) production by fungi, the nutritional requirements and medium conditions for submerged cultures are poorly understood, yet the method remains popular (Park et al., 2001). For *C. militaris* in particular, submerged liquid culture has rarely been studied (Nielsen et al., 1995).

Currently, antioxidants extracted from field-collected *C. militaris* fruiting bodies, are investigated extensively for their ability to protect organisms and cells from oxidative damage due to aging and cell degeneration (Cazzi et al., 1997). Traditionally, field-collected *C. militaris* is used widely in both food and pharmaceutical preparations (Isildak et al., 2004). Field-collected and cultivated *C. militaris* is becoming increasingly popular as a functional food (due to antioxidant activity) as well as for its medicinal properties (Elmastas et al., 2007).

In this study, we determined the optimal cultivation conditions (pH and type of vegetable oils used) for production of *C. militaris* mycelial biomass. Moreover, we quantified the effect of cultivation conditions on biomass production of biologically active ingredients, extra and intra-cellular polysaccharide production**,** antioxidant activities, total phenol content, flavonoid content. We also characterized the biological activity of methanol extracts of mycelial and filtrates obtained using the static cultures method.

2 MATERIALS AND METHODS

2.1 FUNGAL STRAINS

Two field-collected strains of *Codiceps militaris* AG-1, and strain PSJ-1 were obtained from the Plant Physiology and Value Added Microorganisms Laboratory, Department of Plant Industry, National Pingtung University of Science and Technology (NPUST), Taiwan. Mycelia from each of the two strains were cultivated and maintained in the collection on mannitol yolk polymyxin selective agar (MYPS) (Dang et al., 2018) at 24 °C.

2.2 MEDIA

Five submerged liquid culture media were used to evaluate the growth and production of secondary metabolites: (1) MYPS media: 4 g l^{-1} malt extract powder; 4 g l^{-1} yeast extract; 6 g l^{-1} peptone; 10 g l^{-1} sucrose; 0.3 g l^{-1} Vitamin B1, and 1000 ml distilled water; (2) PVC media: 30 g l^{-1} glucose; 10 g l^{-1} corn powder; 1 g l^{-1} KH_2PO_4; 0.6 g l^{-1} K_2HPO_4; 0.7 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.25 g l^{-1} $FeSO_4 \cdot 7H_2O$; 0.5 g l^{-1} vitamin B1, 6 g l^{-1} peptone, and 1000 ml distilled water. (3) PD culture medium: 200 g l^{-1} potatoes; 20 g l^{-1} dextrose; 30 g l^{-1} sucrose; 0.5 g l^{-1} Vitamin B1, and 1000 ml distilled water, (4) Malt-extract medium (ME): 5g l^{-1} malt extract powder; 5g l^{-1} peptone; 20g l^{-1} glucose; 0.3 g l^{-1} Vitamin B1, and 1000 ml distilled water (Atlas, 1993); (5) Czapek-Dox medium (CD): 30 g l^{-1} Sucrose; 2 g l^{-1} $NaNO_3$, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.5 g l^{-1} KCl; 0.3 g l^{-1} vitamin B1 and 1000 ml distilled water (Stevens, 1981). Each cultivation attempt was conducted in five (5) repetitions per medium.

2.3 DETERMINING THE EFFECT OF DIFFERENT MEDIA ON BIOMASS OF *C. militaris* AND QUANTITY OF HOT WATER EXTRACTED

Discs (6 mm diameter cut from culture grown on MYPS plates) of mycelia from the two strains of *C. militaris* were inoculated into the liquid culture medium. Five discs were used for 250 ml of liquid media in each 500 ml Erlenmeyer flask. Flasks were incubated at 24 °C in a temperature controlled rotary shaker (Orbital Shaker Model SK-302AB, Sun Kuan Instruments Co., Kaoshung, Taiwan) for 18 days. The shaking function was set 'static' for the first 5 days and then to 'shake' (93 rpm) for the following 13 days) (Park et al., 2002). After the incubation period mycelia were harvested from each flask by filtration through a pre-weighed filter paper (Whatman No. 1, Whatman Ltd.Toyo Roshi Kaisha, Ltd. Japan), weighed

(wet weight) and then vacuum-dried (dry weight per unit volume of media). The filtrate was concentrated under reduced pressure (rotary evaporator) at 4 °C. Dried filtrates and mycelia were ground into fine powders (20 mesh) in a mill grinder before a hot water extraction (Jo et al., 2010).

2.4 EFFECT OF DIFFERENT PH ON BIOMASS AND ON YIELD OF EXTRAC-CELLULAR AND INTRA-CELLULAR POLYSACCHARIDES AFTER SUBMERGED CULTURE OF *C. militaris*

The effect of pH was done in PVC cultivation medium with pH adjusted to 4.5, 5.5, 6.7, 7.5, or 8.0. 1N HCl or 1N NaOH were used for adjusting the pH. Strains AG-1, PSJ-1 of *C. militaris* in were then grown at each pH in submerged culture; under static conditions for 18 days at 24 °C. After 18 days the biomass (dry weight) was determined and the extra and intra-cellular polysaccharides extracted and weighed. Each treatment was conducted 5 repetitions.

2.4.1 Extra-cellular polysaccharides (epd) and intracellular polysaccharide (ips)

Extra-cellular polysaccharides (EPS) were extracted from the culture filtrate from each flask using standard methods with minor modifications (Fang & Zhong, 2002). Specifically, mycelial biomass in the medium was centrifuged at 10,000 g for 20 min. The supernatant obtained was mixed with three volumes of pure ethanol and incubated at 4 °C for 24 hours. The resulting precipitate (EPS) was then separated by centrifugation at 8000 g for 10 min, washed with ultrapure water and lyophilized prior to weighing. For intra-cellular polysaccharides (IPS), mycelial biomass was subjected to extraction with boiling water for one hour and the mixture was filtered through Whatman No. *1* filter paper. The filtrate was allowed to precipitate with four volumes of 95 % (v/v) ethanol following incubation overnight at 4 °C. The resulting precipitate (IPS) was separated by centrifugation at 8000 g for 10 min, washed with ultrapure water and lyophilized before determining dry mass (Sharma et al., 2015).

2.4.2 Extract of oils on biomass and ips yield after submerged culture of *C. militaris*

The effects of adding different types of oils to the culture medium on biomass and production of extra and intra-cellular polysaccharides during submerged culture was done in PVC medium only. The following oils were used: sunflower oil (HALA Taisun Col Ltd., Taiwan), olive oil (Olitalia sunflower oil, Italia), coconut oil (coconut oil virgin, Viet Delta Industry Co., Ltd, Vietnam), suet volum % (Pingtung City Supermarket, Taiwan), and peanut oil (HACCP, Taiwan) at concentrations of 1.5 %, 2.5 %, and 3.5 %. One type of oil was used per setup. Fermentation was done at 24 °C, at an initial pH of 6.7, in static cultivations conditions for 18 days. Biomass (dry weight) and extra-cellular and intra-cellular polysaccharide production were measured as described previously. Each oil type and % treatment was conducted in five repetitions.

2.5 ANTIOXIDANT ACTIVITIES OF MYCELIAL EXTRACTS OF *C. militaris* GROWN UNDER DIFFERENT SUBMERGED CULTURE CONDITION

The antioxidant activity of mycelial extracts of *C. militaris* was in a submerged culture of three different media (MYPS, PVC, PD) under of three protocoles: (1) shake (shaking regimes for 18 days at 24 °C); (2) static (for 18 days at 24 °C); (3) static + shake (static for the first 5 days and then 'shake' at 93 rpm for the following 13 days). After cultivation mycelia were oven dried, methanol extracts were then evaluated for antioxidant activity *In vitro*. We specifically quantified the total phenolic content and total flavonoid content. For each test, each treatment was conducted in five repetitions.

2.5.1 Mycelial drying method (oven drying)

Fresh mycelia were dried using the oven drying (OD) method (40 °C for 72 h at RH = 65 %). Oven drying was done in hot air oven (Rotek Instruments, B & C Industries, Cochin, India). (Mediani et al., 2013). The samples were ground to powder with a grinder (Yuqi, DM-6, Taiwan), and stored at -20 °C prior to methanol extraction.

2.5.2 Hot water extract

Approximately 2 g from each dryed and grinded sample was extracted with 200 ml of boiling distilled water for 2 h. The extract was filtered through filter paper (Advantec No. 1, Japan) while the residue was re-extracted twice under same conditions. The filtrates obtained from the three separate extractions

were combined, concentrated and lyophilized. The lyophilized extracts were weighed and stored at 4 °C prior to use.

2.5.3 Methanol crude extraction from oven-dried mycelia.

Approximately 2 g from each dryed and grinded (DM-6, Taiwan) sample was extracted with 200 ml of 95 % methanol at 75 ^{0}C after 2 h using a bath/circulator, and filtered through muslin cloth. The extracts were filtered through Whatman No. 2, Whatman Ltd. Toyo Roshi Kaisha, Ltd. Japan) filter paper. The residues were then extracted with an additional 100 ml of methanol. This was then filtered again through 100 mm of filter paper. Extractions from each sample were done twice and the combined filtrates were concentrated, under conditions of reduced pressure, in a rotary evapotator at 40 ^{0}C until dry. The dried extracts obtained were re-dissolved in methanol to a concentration of 100 mg ml^{-1} and were stored at 4 °C prior to the analyses of antioxidant attributes. For downstream experiments the dried methanol, filtered, evaporated under reduced pressure and vacuum-dried at 40 ^{0}C to get the viscous residue needed for estimation of antioxidant activities.

2.5.4 Scavenging of hydroxyl radicals

The hydroxyl radical scavenging activity of *C. militaris* methanol extract was measured according to the method of (Halliwell et al., 1992). Stock solutions of EDTA (1 mM) were prepared in DMSO and $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) in distilled deionized water. For each extract the scavenging activity was determined at different concentrations: 0.5, 1, 2.5, 5, 7.5, and 10 mg ml^{-1} in methanol dissolved in distilled water, 330 µl of phosphate buffer (50 mM, pH 7.5) and 100 µl of ascorbic acid. For each extract/ concentration 1000 µl were added to a solution made up of 100 µl of EDTA, 10 µl of $FeCl_3$, 100 µl of H_2O_2, 360 µl of deoxyribose and incubated at 37 °C for 1 h. After this time 1 ml of the incubated mixture was mixed with 1 ml of 10 % TCA and 1 ml of 0.5 % TBA (in 0.025 M NaOH containing 0.025 % butylated hydroxyl anisole) and the development of pink chromogen was measured spectrophotometrically at 532 nm. The hydroxyl radical scavenging activity of each extract was reported as percentage inhibition of deoxyribose degradation and was calculated according to the formula (1):

$$Scavenged\ (\%) = \frac{(A0 - A1) \times 100}{A0}$$

Where A0 was the control absorbance and A1 was the absorbance of the solution containing either the extract or absorbance of the solution containing either the extract or the standard absorbance.

2.5.5 Scavenging of ABTS$^{\cdot+}$, a radical cation.

The scavenging activity of the extracts was estimated using the ABTS$^{\cdot+}$ decolorization method (Arumagam et al., 2006; Re et al., 1999). A stock solution for evaluation of antioxidant activity was produced my mixing 5 ml of 7 mM ABTS and 88 µl of 140 mM $K_2S_2O_8$; this solution was allowed to complete radical generation for 12–16 h in darkness at room temperature. The stock solution was diluted with ethanol and PBS (pH 7.4) to give an absorbance of 0.75 at 734 nm. The scavenging activity of each strains of *C. militaris* methanol extract was determined at concentrations: 0.5, 1, 2.5, 5, 7.5, and 10 mg ml^{-1}. For each extract/ concentration combination 1 ml of extract was added to 1 ml of diluted stock solution and, 5 min after the initial mixing, the absorbance was measured at 734 nm, using ethanol as the blank solution. All measurements were performed in triplicate. The total antioxidant activity (TAA) percentage was calculated using the formula (2):

$$TAA\ \% = (Ac - \frac{As}{Ac}) \times 100$$

Where A = absorbance of stock solution and As = absorbance of the extract.

2.5.6 Antioxidant activity in relation to lipid peroxidantion

The antioxidant activity of *C. militaris* two strains methanol extracts was determined using a 1,3-diethyl-2-thiobarbituric acid (DETBA) method (Furuta et al., 1997). Extracts were evaluated at different concentrations (0.5, 1, 2.5, 5, 7.5, and 10 mg ml^{-1} in methanol); for each sample was added to 50 ml of linoleic acid emulsion (2 mg ml^{-1} in 95 % ethanol) and a positive control butylated hydroxytoluene (BHT) (0.1 mg ml^{-1}) were used in this study. The mixture was incubated at 80 °C for 60 min; it was then cooled in an ice bath and mixed with 200 ml of 8 % sodium dodecyl sulfate (SDS), 400 ml of deionized water, and 3.2 ml of 12.5 mM DETBA (Aldrich Chemical Co., WI, USA) in sodium phosphate buffer (pH 3.0).

After thoroughly mixing it was incubated at 95 °C

for 15 min and then cooled in an ice bath. Ethyl acetate (4.0 ml) was then added to the mixture, the mixture was centrifuged at 1000 g at 20 °C for 15 min. The fluorescence of the ethyl acetate layer was then measured in a spectrofluorometer with excitation set at 515 nm and emission set at 555 nm. Each value was expressed as the mean of triplicate measurements +/- standard deviation. The percentage of lipid peroxidation was determined against a blank with no sample added (100 %). The antioxidant activity was expressed as the inhibition of lipid peroxidation using the formula (3):

$$\textit{Antioxidant activity}\ (\%) = (1\text{- Percentage of lipid peroxidation}) \times 100$$

2.5.7 IC50 VALUES IN AN ANTIOXIDANT ACTIVITY.

The results of antioxidant activity, hydroxyl radicals, scavenging activity of ABTS+ radical cation, lipid peroxidation, respectively were normalized and expressed as IC50 (mg extract/ml). A lower IC_{50} value (mg extract/ml) corresponds to the higher antioxidant activity of *C. militaris* mycelial (obtained by submerged cultivation) extract.

2.6 QUANTIFYING ANTIOXIDANTS

2.6.1 Total phenolic content (tpc) of the extracts.

Total phenolic contents of each methanol extract was determined using gallic acid as a standard (Chan et al., 2009). A sample of 0.2 ml from each extract (10 mg ml^{-1} in methanol) was mixed with 2 ml of Folin-Ciocalteu's phenol reagent, 2N (Sigma). The mixture was incubated at room temperature for 5 minutes. Then 1.8 ml of 20 % sodium carbonate (Na_2CO_3, Nihon Shiyaku) was added, and the mixture centrifuged at 3000 g for 10 min. The mixture was then incubated for 90 min at room temperature. The absorbance of each reaction mixture was measured at 735 nm using a spectrophotometer (Hitachi U-2800, Japan). Gallic acid was used as a standard to create a calibration curve. The TPC was obtained by interpolation from linear regression analysis in mg gallic acid equivalents (mg GAE)/g of dry extract. All tests are performed in triplicate. Two strains mycelial concentration of the sample solution was determined based on a standard curve regression equation (4):

$A = 0.8533C + 0.0211, r^2 = 0.997$

(Where A is the absorbance and C is the concentration). Then, the extraction rate of TPC in the *C. militaris* sample was calculated.

2.6.2 Total flavonoid content (tfc) of the extracts.

The TFC was determined according to the method of Jia et al. (1999). A sample of 0.2 ml from each extract (100 mg ml^{-1} in methanol) was mixed with 1.5 ml of distilled water. To this 0.1 ml of 10 % aluminium nitrate [Al(NO3)3] and 0.1 ml of 1 M potassium acetate (CH3COOK) was added. After 40 min at room temperature, absorbance was measured at 415 nm. Quercetin was used as a standard for a calibration curve. Flavonoid contents was determined by interpolation from linear regression analysis in mg quercetin equivalents (QE)/g dry extract. All tests were done in triplicate. Two strains mycelial concentration in the sample solution was determined based on a standard curve regression equation (5):

$A = 3.2173A + 0.0618, r^2 = 0.997$

(Where A is the absorbance and C is the concentration). From, this extraction rate of TFC in *C. militaris* samples was calculated.

2.7 EXPERIMENTAL DESIGN AND DATA ANALYSIS

All experiments were done at the Department of Plant Industry, NPUST, Taiwan. One-way analysis of variance (ANOVA) was done followed Duncan's multiple range tests for means comparisons ($p \leq 0.05$). All analysis was done in SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA). For each test, each value is expressed as mean ± SE and with 5 replicates (n = 5).

3 RESULTS AND DISCUSSION

3.1 EFFECT OF CULTIVATION MEDIA ON *C. militaris* BIOMASS PRODUCTION

Mycelial wet mass, dry mass and extra-cellular polysaccharide content of both strains were all the greatest in cultures grown in PVC media compared with the other media evaluated (Table 1 and Fig. 1). Overall, significantly more mycelial biomass was produced by strain AG-1 than by strain PSJ-1 in submerged culture. Overall, the cultivation methods used for both AG-1, PSJ-1 in this study had low or similar quantities of extracts as obtained in other set-ups. For example, studies on *C. militaris* strain BCC2816 reported 36 mg of extract from mycelium grown in 5 l of potato dextrose broth medium at 25 °C (Rukachaisirikul et al., 2004.), while 19.1 g l^{-1} were collected from mycelia produced in 57 ml medium at temperature 28 °C, and pH of 6.2 (Chunyan et

Table 1: Effect of different liquids media and mycelial biomass on static culture fresh mass, oven dries mass, crude hot water extract of strains AG-1 and, PSJ-1 after 18 days of cultivation. Valuea are expressed as mean± standard deviation (n = 5). Means within the same column followed by the same letters are not significantly different from each other at $p \leq 0.05$ according to Duncan's multiple range tests.

	Mycelial Biomass					
	Static culture fresh mass ($g\ l^{-1}$)		Oven dried mass (40 ^{0}C) ($mg\ l^{-1}$)		Boiling distilled water crude extract ($mg\ l^{-1}$)	
Media	AG-1	PSJ-1	AG-1	PSJ-1	AG-1	PSJ-1
MYPS	17.88 ± 0.87^{ab}	15.03 ± 0.71^{ab}	23.73 ± 1.02^{ab}	22.74 ± 0.75^{ac}	7.53 ± 0.32^{a}	6.87 ± 0.69^{ab}
PD	10.71 ± 1.13^{cc}	8.92 ± 1.28^{ab}	22.90 ± 0.06^{ac}	24.02 ± 0.04^{aa}	6.10 ± 0.09^{bc}	5.91 ± 0.44^{ac}
PVC	21.85 ± 1.00^{aa}	18.20 ± 1.84^{aa}	25.95 ± 0.84^{aa}	23.55 ± 0.69^{ab}	7.07 ± 0.15^{ab}	7.39 ± 0.61^{aa}
ME	14.45 ± 1.37^{bb}	13.59 ± 0.78^{ac}	18.76 ± 0.85^{bc}	16.96 ± 0.56^{b}	5.26 ± 0.50^{be}	5.05 ± 0.43^{bc}
CD	10.17 ± 0.85^{cd}	8.63 ± 0.77^{bb}	21.49 ± 0.68^{ab}	18.31 ± 0.75^{c}	5.99 ± 0.04^{bd}	2.80 ± 0.51^{c}

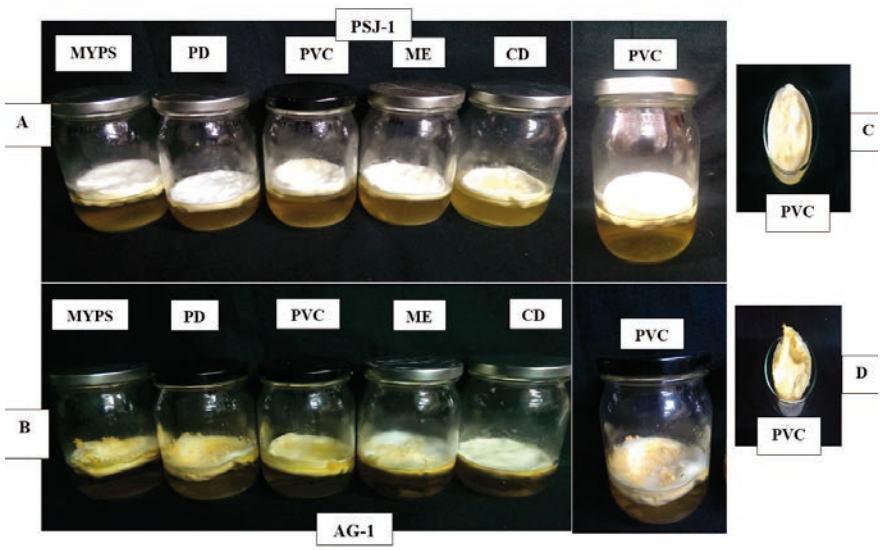

Figure 1: Effect of difficult submerged culture, fresh mycelial weight extract cordycepin production, MBDW: Mycelial Biomass dry weight (g/L), EPS: exopolysaccharide, IPS: Intracellular polysaccharides (mg/g DW). PVC media. of *C. militaris* cultured at 18 days. A: PSJ-1 strain and B: AG-1 strain. C: fresh mycelial of PSG-1 on PVC liquid media; D: fresh mycelial and fruiting body of AG-1 on PVC liquid media.

Table 2: Effect of initial pH and between strains AG-1, PSJ-1 strains on the mycelial biomass, extra-cellular and intra-cellular polysaccharide production after 18 days in static culture in PVC media at 24 0C. Data show the mean±standard deviation (n = 5). Means within the same column followed by the same letters are not significantly different to each other according to Duncan›s multiple range tests ($p \leq 0.05$)

	Mycelial biomass (dry mass; $g\ l^{-1}$)		Extra- cellular polysaccharides ($mg\ l^{-1}$)		Intra-cellular polysaccharides ($mg\ l^{-1}$)	
pH	AG-1	PSJ-1	AG-1	PSJ-1	AG-1	PSJ-1
5	4.63 ± 0.03^{c}	3.95 ± 0.06^{b}	38.9 ± 0.36^{d}	32.06 ± 0.55^{d}	13.31 ± 1.10^{ad}	12.20 ± 0.84^{ad}
5.5	5.71 ± 0.05^{ac}	4.88 ± 0.09^{b}	45.85 ± 0.87^{cd}	35.06 ± 1.18^{d}	15.84 ± 0.79^{cd}	14.69 ± 0.95^{cd}
6	8.61 ± 0.71^{bc}	7.88 ± 0.34^{ab}	168.56 ± 0.94^{ab}	168.16 ± 0.67^{ab}	23.68 ± 0.19^{bc}	22.62 ± 0.12^{bc}
6.7	12.92 ± 0.3^{b}	9.03 ± 0.24^{a}	209.70 ± 1.56^{a}	198.16 ± 0.85^{a}	32.62 ± 0.87^{b}	30.63 ± 1.96^{b}
7.5	6.70 ± 0.69^{ac}	7.93 ± 0.52^{a}	69.50 ± 4.05^{bc}	57.39 ± 2.18^{bc}	9.94 ± 0.86^{ade}	8.66 ± 0.73^{ad}
8	5.28 ± 0.43^{c}	5.07 ± 0.43^{ba}	39.73 ± 2.87^{d}	35.16 ± 1.73^{cd}	8.44 ± 0.74^{ae}	7.83 ± 0.62^{d}

Table 3: Effect of different type of oils and between strains AG-1, PSJ-1 strains on mycelial biomass, extra-cellualar and intra-cellular polysaccharide production of C. militaris after 18 days of cultivation in static culture in PVC media (initial pH 6.5) at 24 °C. Data show the means of five independent experiments±standard deviation (SD). Each value is expressed as mean±standard deviation (n = 5). Within columns the mean values with different capital letters within a row are significantly different ($p \leq 0.05$) to each other according to Duncan's multiple range tests)

		Mycelial dry biomass ($g\ l^{-1}$)		Extra-cellular polysaccharide ($mg\ l^{-1}$))		Intracellular polysaccharide ($mg\ g\ DM^{-1}$)	
Type of oils		AG-1	PSJ-1	AG-1	PSJ-1	AG-1	PSJ-1
Sunflower oil	1.5 %	3.49 ± 0.04^{cdm}	3.22 ± 0.01^{ik}	254.80 ± 12.69^{ag}	223.00 ± 5.84^{gh}	6.62 ± 0.20^{ci}	6.48 ± 0.05^{bh}
	2.5 %	4.17 ± 0.08^{ik}	3.96 ± 0.03^{fg}	291.00 ± 3.74^{af}	245.20 ± 2.49^{g}	6.75 ± 0.07^{ci}	6.36 ± 0.03^{bh}
	3.5 %	4.67 ± 0.03^{hi}	4.21 ± 0.06^{ef}	348.80 ± 2.40^{de}	316.20 ± 3.15^{de}	14.56 ± 0.28^{ade}	12.85 ± 0.21^{ef}
Oliver oil	1.5 %	3.32 ± 0.07^{d}	3.12 ± 0.08^{m}	113.38 ± 2.94^{hi}	107.10 ± 1.00^{ik}	6.86 ± 0.05^{ci}	6.26 ± 0.03^{bh}
	2.5 %	3.68 ± 0.03^{cm}	3.47 ± 0.07^{hi}	139.80 ± 1.71^{hi}	125.00 ± 1.52^{I}	8.80 ± 0.08^{bch}	7.45 ± 0.04^{bgh}
	3.5 %	3.90 ± 0.02^{ck}	3.72 ± 0.03^{gh}	154.20 ± 2.22^{gh}	127.60 ± 1.36^{hI}	5.59 ± 0.10^{ci}	5.21 ± 0.19^{b}
Coconut oil	1.5 %	5.87 ± 0.02^{bg}	5.55 ± 0.07^{cd}	434.00 ± 1.73^{cd}	406.60 ± 2.87^{bc}	17.98 ± 0.14^{ad}	16.54 ± 0.61^{e}
	2.5 %	6.67 ± 0.07^{af}	6.14 ± 0.02^{c}	701.20 ± 7.75^{bc}	683.60 ± 4.95^{ab}	18.17 ± 1.17^{de}	17.01 ± 0.97^{de}
	3.5 %	8.27 ± 0.09^{E}	8.01 ± 0.0^{a}	1208.00 ± 2.30^{b}	1110.40 ± 3.16^{a}	23.61 ± 1.31^{cd}	20.39 ± 1.55^{cd}
Suet	1.5 %	5.33 ± 0.10^{h}	5.14 ± 0.05^{de}	276.40 ± 3.17^{afg}	246.00 ± 1.64^{fg}	7.97 ± 0.90^{chi}	7.00 ± 0.92^{bh}
	2.5 %	6.50 ± 0.13^{afg}	6.26 ± 0.04^{bc}	309.00 ± 2.40^{ef}	294.20 ± 2.03^{ef}	10.45 ± 1.18^{defg}	9.58 ± 0.64^{agh}
	3.5 %	6.18 ± 0.05^{abg}	6.05 ± 0.02^{c}	411.60 ± 3.75^{de}	389.00 ± 3.03^{cd}	12.18 ± 1.04^{abf}	11.10 ± 0.66^{afg}
Peanut oil	1.5 %	5.19 ± 0.04^{hi}	5.06 ± 0.02^{df}	55.48 ± 0.57^{m}	53.53 ± 0.35^{m}	9.95 ± 0.12^{bhi}	9.37 ± 0.07^{agh}
	2.5 %	6.92 ± 0.02^{f}	6.78 ± 0.01^{ab}	60.40 ± 0.16^{ik}	59.53 ± 0.20^{m}	6.90 ± 0.05^{ci}	6.72 ± 0.05^{bgh}
	3.5 %	8.46 ± 0.14^{ef}	8.22 ± 0.05^{a}	103.60 ± 2.23^{i}	100.48 ± 1.70^{km}	46.17 ± 0.81^{d}	43.51 ± 0.21^{c}

al., 2009). Our results are in agreement with (Ing-Lung Shih et al., 2007), whose studies showed that metabolites and cell growth of 15.5 $g\ l^{-1}$ (shaking) after 36 days, and 14.0 $g\ l^{-1}$ (static) after 30 days.

3.2 EFFECT OF PH ON THE MYCELIAL *C. militaris* BIOMASS AND EXTRA- AND INTRA-CELLULAR POLYSACCHARIDES PRODUCTION

Initial pH affected the growth of mycelial, as well as the production of extra and intra-cellular polysaccharides, for both AG-1, PSJ-1 strains when cultivated using the submerged culture method (Table 2). Submerged liquids culture dry mass, EPS, and IPS were the highest when the initial pH ranged from 6.0 to 6.7, and were the lowest at the highest and the lowest pHs evaluated (5 and 8). Previous reports have suggested that low pH may increase quantities of extracellular polysaccharides in continuous production of many types of Basidiomycetes and Ascomycetes, but this may have been due to oil supplementation (Hsieh et al., 2005; Park et al., 2020; Kim et al., 2001). The optimal pH for cordycepin production by different strains of *C. militaris* has been reported to be in the range of 4-7 (Kang et al., 2014; Zhong et al., 2011). Other studies reported that *C. militaris* strain BCC2816 achieved the highest dry mycelial mass (19.1 $g\ l^{-1}$), and optimal cordycepin yield (1.8 $mg\ l^{-1}$) at 28 °C, pH 6.2 (Chunyan et al., 2009). In general, the effects of pH in static culture of strains AG-1, PSJ-1 in the present study were lower compared with other studies. For instance, *C. militaris* strain 3936 produced cordycepin in the pH range of 4.5 to 7.0, with the highest levels at pH 5.5 (213 $mg\ l^{-1}$), which was also consistent with earlier reports (Leung et al., 2007).

3.3 EFFECT OF DIFFERENT OILS ON BIOMASS AND ON EPS AND IPS YIELD AFTER SUBMERGED CULTURE OF TWO *C. militaris* STRAINS

Addition of oil sources led to significant increased levels of mycelial biomass, EPS and IPS (Table. 3). The greatest mycelial dry mass was achieved in coconut oil (Table. 3). Mycelial biomass, EPS and IPS production were higher when media were supplemented with sunflower oil, olive oil, coconut oil, suet, and peanut oil; for all oils growth increased as toe % oil increased. The results are in agreement with (Hsieh et al., 2006), who showed that mycelial growth was increased when media were supplemented with > 1 % olive oil; this was also associated with greater IPS production. Previous reports have also shown that production of extra-cellular polysac-

Table 4: Hydroxyl radical scavenging ability at different concentrations of *C. militaris* two strains grown using nine submerged culture methods that incorporated different media and shaking regimes. The concentration causing 50 % inhibition (IC50) is recorded. Mean values with different capital letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* two strains followed by the same letters are not significantly different at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were oven dried (n = 5).

		Sample concentration (mg ml^{-1})							
Submerged culture method	Strains	0.50	1.00	2.50	5.00	7.50	10.00	IC50 (µg ml^{-1})	R^2
MYPS (shake)	AG-1	19.16 ± 0.39^{b}	28.69 ± 1.03^{ac}	41.46 ± 1.04^{a}	56.56 ± 1.01^{c}	66.32 ± 0.61^{ad}	80.26 ± 0.62^{ad}	2.30	R^2 = 0.9966
	PSJ-1	8.94 ± 0.86^{ad}	8.71 ± 0.39^{a}	45.58 ± 0.34^{b}	61.21 ± 0.60^{b}	69.66 ± 0.83^{df}	77.00 ± 0.41^{acd}	2.01	R^2 = 0.9191
PVC (shake)	AG-1	4.99 ± 0.63^{e}	40.77 ± 1.20^{b}	15.68 ± 0.42^{c}	69.57 ± 1.51^{B}	76.77 ± 0.61^{b}	81.42 ± 1.01^{a}	2.49	R^2 = 0.7894
	PSJ-1	10.86 ± 1.01^{ad}	25.44 ± 1.73^{c}	34.96 ± 0.64^{ac}	57.03 ± 0.60^{bc}	76.54 ± 0.62^{c}	78.16 ± 0.62^{ac}	2.33	R^2 = 0.9697
PD (shake)	AG-1	10.10 ± 0.39^{d}	21.49 ± 0.83^{d}	35.89 ± 0.83^{b}	47.74 ± 1.07^{d}	76.07 ± 0.61^{b}	78.63 ± 0.22^{bd}	2.16	R^2 = 0.9694
	PSJ-1	8.25 ± 1.39^{abcd}	38.91 ± 0.63^{b}	12.19 ± 1.23^{e}	47.97 ± 0.59^{ae}	68.41 ± 1.01^{bdf}	76.54 ± 0.24^{ad}	1.84	R^2 = 0.7819
MYPS (static)	AG-1	19.16 ± 1.19^{b}	29.15 ± 0.82^{ac}	40.53 ± 0.23^{a}	51.92 ± 0.70^{acd}	76.54 ± 0.85^{b}	82.58 ± 0.41^{ac}	2.34	R^2 = 0.9752
	PSJ-1	26.36 ± 1.26^{c}	10.80 ± 0.79^{ae}	34.50 ± 1.19^{ac}	49.13 ± 0.41^{ad}	73.52 ± 0.41^{ac}	79.79 ± 0.41^{ac}	2.19	R^2 = 0.8607
PVC (static)	AG-1	10.34 ± 0.63^{cd}	31.01 ± 1.04^{bc}	43.32 ± 0.62^{a}	56.79 ± 1.06^{c}	71.89 ± 0.61^{c}	84.67 ± 0.40^{c}	3.57	R^2 = 0.9941
	PSJ-1	8.48 ± 0.26^{abcd}	17.77 ± 0.42^{d}	31.48 ± 0.61^{ad}	45.64 ± 0.83^{e}	69.80 ± 0.83^{adf}	82.58 ± 0.41^{b}	3.01	R^2 = 0.9825
PD (static)	AG-1	13.82 ± 0.59^{bc}	26.60 ± 1.27^{ace}	36.59 ± 0.39^{b}	55.40 ± 1.20^{c}	68.18 ± 1.01^{cd}	78.40 ± 0.39 ^{bd}	3.04	R^2 = 0.0772
	PSJ-1	5.46 ± 0.59^{bd}	28.92 ± 0.82^{bc}	33.10 ± 0.39^{a}	50.52 ± 0.41^{ad}	62.83 ± 0.62^{e}	75.84 ± 0.62^{d}	2.79	R^2 = 0.9803
MYPS (static + shake)	AG-1	6.39 ± 0.27^{ae}	23.11 ± 0.64^{de}	34.50 ± 1.44^{b}	54.47 ± 0.84^{ac}	72.59 ± 0.62^{bc}	77.70 ± 0.41^{be}	2.46	R^2 = 0.9842
	PSJ-1	4.76 ± 0.60^{b}	13.36 ± 0.23^{de}	29.15 ± 0.62^{d}	56.56 ± 0.61^{c}	71.43 ± 0.70^{abc}	78.40 ± 0.41^{ac}	2.40	R^2 = 0.9714
PVC (static + shake)	AG-1	9.87 ± 0.24^{d}	25.20 ± 0.23^{ade}	32.87 ± 0.60^{b}	52.15 ± 0.60^{ac}	64.46 ± 0.41^{a}	80.72 ± 0.23^{ad}	2.91	R^2 = 0.9922
	PSJ-1	11.50 ± 0.42^{cd}	26.13 ± 1.19^{c}	37.51 ± 0.63^{bc}	56.33 ± 1.02^{c}	65.85 ± 0.80^{bef}	73.29 ± 0.47^{de}	2.65	R^2 = 0.9847
PD (static + shake)	AG-1	9.64 ± 1.00^{ad}	28.46 ± 1.00^{ac}	36.35 ± 0.61^{b}	50.29 ± 0.61^{ad}	59.58 ± 0.80^{e}	77.24 ± 0.84^{b}	2.55	R^2 = 0.9881
	PSJ-1	7.32 ± 0.38^{abd}	19.40 ± 0.47^{d}	33.33 ± 0.23^{b}	52.85 ± 0.46^{cd}	67.02 ± 0.82^{bdF}	78.63 ± 0.22^{ac}	2.40	R^2 = 0.9948
Control	BHT	88.65 ± 0.30^{a}	88.87 ± 0.04^{a}	89.01 ± 0.06^{a}	89.56 ± 0.25^{a}	89.72 ± 0.14^{a}	90.16 ± 0.06^{a}		

Table 5: Scavenging activity of ABTS.+ radical cation and inhibition concentration at 50 % (IC50) values of mycelial extracts of *C. militaris* two strains grown using nine submerged culture methods that incorporated different media and shaking regimes. Mean values with different upper-case letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* two strains followed by the same lower-case letters are not significantly different to each other at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were oven dried n = 5).

		Sample concentration (mg ml^{-1})							
Submerged culture method	Strains	0.50	1.00	2.50	5.00	7.50	10.00	IC50 (µg ml^{-1})	R^2
MYPS (shake)	AG-1	5.92 ± 1.15^{ee}	16.26 ± 1.08^{be}	40.01 ± 2.14^{ac}	13.75 ± 1.60^{d}	65.98 ± 1.47^{b}	66.26 ± 0.81^{a}	3.46	$R^2 = 0.7283$
	PSJ-1	16.26 ± 1.84^{acd}	41.68 ± 1.14^{b}	45.03 ± 0.68^{ac}	35.54 ± 1.41^{cd}	62.63 ± 0.68^{ab}	63.46 ± 1.83^{c}	3.06	$R^2 = 0.7949$
PVC (shake)	AG-1	8.16 ± 1.57^{ab}	23.80 ± 2.09^{ab}	34.14 ± 1.46^{a}	45.31 ± 1.20^{b}	57.04 ± 1.67^{a}	73.53 ± 1.58^{cb}	4.56	$R^2 = 0.9944$
	PSJ-1	8.72 ± 1.39^{bd}	22.97 ± 2.14^{c}	39.45 ± 1.67^{ad}	48.10 ± 1.81^{ad}	61.51 ± 0.93^{ab}	66.26 ± 0.64^{ad}	3.10	$R^2 = 0.9927$
PD (shake)	AG-1	7.32 ± 1.46^{b}	22.96 ± 1.12^{ab}	34.42 ± 1.19^{a}	58.72 ± 1.57^{ab}	63.75 ± 1.09^{ab}	66.54 ± 0.93^{a}	2.58	$R^2 = 0.95$
	PSJ-1	4.53 ± 1.14^{b}	22.97 ± 2.37^{c}	41.96 ± 1.20^{acd}	50.90 ± 1.01^{abd}	57.04 ± 1.30^{ab}	65.98 ± 2.40^{ac}	2.87	$R^2 = 0.0935$
MYPS (static)	AG-1	10.95 ± 1.30^{ab}	22.40 ± 1.86^{b}	52.29 ± 0.93^{b}	37.77 ± 1.03^{bc}	62.85 ± 1.99^{ab}	76.32 ± 1.20^{b}	4.09	$R^2 = 0.8763$
	PSJ-1	16.82 ± 1.19^{ac}	31.62 ± 1.93a	44.47 ± 1.20^{ac}	58.44 ± 0.77^{b}	65.98 ± 1.60^{b}	71.29 ± 0.90^{ab}	3.22	$R^2 = 0.9724$
PVC (static)	AG-1	17.38 ± 1.11^{a}	36.37 ± 1.19^{a}	43.08 ± 0.93^{bc}	51.18 ± 1.74^{ab}	65.42 ± 1.67^{b}	76.58 ± 0.60^{b}	4.57	$R^2 = 0.9821$
	PSJ-1	9.28 ± 1.40^{abcd}	24.64 ± 1.74b^{bc}	35.81 ± 1.65^{d}	45.87 ± 1.47^{d}	65.98 ± 1.47^{b}	73.52 ± 1.82^{b}	4.03	$R^2 = 0.9896$
PD (static)	AG-1	8.16 ± 0.84^{ab}	18.21 ± 3.74^{bd}	34.14 ± 1.46^{a}	46.71 ± 1.65^{a}	61.51 ± 1.41^{ab}	72.13 ± 1.09^{ab}	3.45	$R^2 = 0.9969$
	PSJ-1	12.63 ± 0.83^{acd}	25.20 ± 1.19^{c}	48.94 ± 1.37^{bc}	34.98 ± 2.42^{c}	64.31 ± 1.56^{b}	71.29 ± 0.81^{ab}	2.79	$R^2 = 0.8668$
MYPS (static + shake)	AG-1	6.21 ± 0.80^{bd}	40.84 ± 1.65^{a}	14.86 ± 1.35^{d}	20.17 ± 0.73^{cd}	63.19 ± 0.84^{ab}	72.96 ± 1.11^{cb}	3.87	$R^2 = 0.6346$
	PSJ-1	15.43 ± 2.88^{acd}	26.88 ± 1.19^{ac}	41.12 ± 0.87^{ac}	53.97 ± 1.29^{abd}	66.26 ± 1.34^{b}	69.61 ± 0.81^{abc}	3.22	$R^2 = 0.9706$
Liquid (static + shake)	AG-1	6.21 ± 0.32^{bd}	20.73 ± 1.01^{cb}	38.33 ± 1.13^{ac}	59.56 ± 0.73^{a}	67.94 ± 1.67^{b}	74.64 ± 2.23^{ab}	4.49	$R^2 = 0.9706$
	PSJ-1	9.56 ± 1.13^{abcd}	34.98 ± 1.46^{abc}	60.12 ± 2.61^{b}	48.94 ± 1.59^{ad}	66.26 ± 1.43^{b}	70.11 ± 1.02^{abc}	3.65	$R^2 = 0.811$
PD (static + shake)	AG-1	10.39 ± 0.56^{b}	37.21 ± 1.07^{a}	42.80 ± 0.73^{c}	49.78 ± 2.12^{b}	63.19 ± 0.93^{ab}	72.96 ± 0.17^{cb}	3.50	$R^2 = 0.9449$
	PSJ-1	17.10 ± 1.29^{c}	31.62 ± 2.04^{ac}	45.87 ± 1.47^{ac}	57.32 ± 2.20^{b}	66.26 ± 1.57^{b}	71.85 ± 1.25^{ab}	3.42	$R^2 = 0.976$
Control	BHT	76.00 ± 1.73^{a}	76.00 ± 1.15^{a}	77.33 ± 0.88^{a}	78.33 ± 1.20^{a}	78.67 ± 0.88^{a}	79.33 ± 0.67^{A}		

charides and mycelial growth of the *C. militaris* significantly increased (nearly tripling) following the addition af vegetable oils to the medium (Park et al., 2002).

3.4 SCAVENGING OF HYDROXYL RADICALS

In our study, we found that all mycelial extracts were able to scavenge hydroxyl radicals but that the scavenging activity was affected by concentration (Table 4). At 10 mg ml^{-1}, both AG-1, PSJ-1 strains had the greatest hydroxyl radical scavenging activities compared with other concentrations; values ranged from: 77.70 ± 0.41 to 84.67 ± 0.40 % AG-1 and 73.29 ± 0.47 to 79.79 ± 0.41 % for PSJ-1. In terms of grown of the strain in different shaking regimes, IC_{50} values ranged from AG-1: 2.16-3.57 mg ml^{-1}, PSJ-1: 1.84-3.01 mg ml^{-1}. The results are similar to previous studies, where 76 % of the highest scavenging capacity of hydroxyl radicals was achieved at a concentration of 3.67 g l^{-1} (sample/water). (Shen & Shen, 2001). Also in previous studies, boiling water extracts of cultured and natural *C. sinensis* (Berk.) Sacc. mycelia achieved lower radical scavenging activities (IC_{50}: 0.96 ± 0.06 mg l^{-1} and 1.03 ± 0.03 mg ml^{-1} at concentration of 0.25–2.0 mg ml^{-1}, respectively) (Dong & Yaoa, 2007). In contrast, other studies achieved greater hydroxyl radical scavending ability using hot water extractions of mycelium (37.1 ± 3.8 % to 74.2 ± 4.7 %). (Yuxiang et al., 2006).

3.5 SCAVENGING ACTIVITY OF THE CATION RADICAL $ABTS^{\cdot+}$.

$ABTS^{\cdot+}$ scavenging activity for mycelial extracts from strains AG-1, PSJ-1 increased as concentrations increased (Table 5), and was greatest at the 10 mg ml^{-1} concentration, ranging from 66.54 ± 0.93 to 77.15 ± 0.81 for AG-1 and from 65.98 ± 2.40 to 76.58 ± 0.60 for PSJ-1. The $ABTS^{\cdot+}$ radical scavenging activity was achieved by a mycelial extract from strain AG-1, grown on PVC without shaking (static); overall IC_{50} values for AG-1 ranged from 2.58-4.57 mg ml^{-1}. For PSJ-1 IC_{50} values obtained in the following treatments: MYPS shake (3.06 mg ml^{-1}), PVC shake (3.10 mg ml^{-1}), MYPS static (3.22 mg ml^{-1}), PD static (2.79 mg ml^{-1}), MYPS static+shake (3.22 mg ml^{-1}), PVC static+shake (3.65 mg l^{-1}); and PD static+shake (3.42 mg ml^{-1}), as shown in Table 5. In the studies of others, using different extraction and fermentation methods of *Cordyceps militaris* SN-18 also exhibited excellent $ABTS^{\cdot+}$ radical scavenging activity with an EC_{50} of: 5.25 ± 0.03 mg ml^{-1} at 4 mg ml^{-1} (Yu Xiao et al., 2014). According to Sapan et al. (2015), EPS and IPS extracts from mycelia of *C. cicadae* exhibited strong $ABTS^{\cdot+}$ radical scavenging activity with EC_{50}: 6.38 ± 0.12 mg ml^{-1} (EPS) and 5.23 ± 0.25 mg ml^{-1} (IPS), at a concentration of 8.0 mg ml^{-1}.

3.6 INHIBITION OF LIPID PEROXIDATION

Lipid peroxidation by extracts from strains AG-1, PSJ-1 increased with increasing concentration. A concentration of 10 mg ml^{-1} of strains AG-1, PSJ-1 resulted in mycelial extracts that exhibited the highest antioxidant activity compared with the other concentration, and the results were comparable to butylated hydroxyl toluene used as a positive control (BHT) (Table 6). At 10 mg ml^{-1} there was no significant difference in the inhibition ambition of strains AG-1, PSJ-1 ($p < 0.05$). Lipid peroxidation inhibition activity of the mycelia extract from strain AG-1 grown on the PD medium under 'shake' conditions was significantly stronger than the other extracts with the lowest IC_{50} values (1.88 mg ml^{-1}) (Table 6). Based on estimated IC_{50} from the inhibition assays of various *Cordyceps* strain, concentrations ranged from 0.08 to 5 mg ml^{-1}. These values are in line with the doses of *Cordyceps* prescribed medicinally, which ranges from 1-10 g per dose, (Zhu et al. 1998). For example, using a concentration of 15.0 mg ml^{-1} of *C. militaris* extract, inhibition of lipid peroxidation reached nearly 50 %, at 4.0 mg ml^{-1} (Chun-Lun Wang et al., 2015).

3.7 TOTAL PHENOLIC CONTENT (TPC).

In this study the TPC of mycelial extract from strain AG-1 ranged from 1.75 ± 0.07 to 3.74 ± 0.18 mg g^{-1} (Table 7) and were highest when grown in PVC medium under static conditions (3.74 ± 0.18 mg g^{-1} extract). For AG-1, the TPC was significantly higher in treatments PVC static, MYPS static, PD static, PVC static+shake, MYPS static+shake, PD static+shake, PVC shake, MYPS shake, and PD shake than PD static treatment. For strain PSJ-1 the TPC of mycelial extracts were lower than those from strain AG-1, with values that range from 1.94 ± 0.21 to 3.23 ± 0.10 mg g^{-1}. The results of AG-1, PSJ-1 were similar to some previous reports: 3.91 ± 0.16 mg GAE/g extract (Chun Lun Wang et al., 2015); 3.9 ± 0.2 mg GAE/g extract (Lee et al., 2013). This confirms that antioxidant potential is positively correlated with TPC (Kaur & Kapoor, 2002). The TPC in our study was much higher, and thus incomparable to the results of previous researchers. In one study TPC obtained from methanolic extracts from natural and cultured *C. sinensis* were: 17.07 ± 0.38 mg GAE/g extract while in another they varied from 12.02-12.14 mg GAE/g (Junqiao Wang et al., 2015). Thus, the TPC of extracts from AG-1, PSJ-1

Table 6: Inhibition of lipid peroxidation and inhibition concentration at 50 % (IC50) values of mycelial extracts of *C. militaris* two strains grown using nine submerged culture method that incorporated different media and shaking regimes. Mean values with different upper-case letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* two strains followed by the same lower-case letters are not significantly different to each other at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were oven dried (n = 5).

		Sample concentration (mg ml^{-1})							
Submerged culture method	Strains	0.50	1.0	2.50	5.00	7.50	10.00	IC50 (µg ml^{-1})	R^2
MYPS (shake)	AG-1	8.33 ± 1.45^{abc}	14.67 ± 1.76^{cd}	28.00 ± 1.15^{b}	37.67 ± 1.86^{cd}	58.00 ± 1.53^{ab}	66.33 ± 1.20^{ad}	1.79	$R^2 = 0.9827$
	PSJ-1	11.67 ± 1.20^{acd}	22.00 ± 1.15^{a}	35.00 ± 1.00^{ac}	47.33 ± 1.20^{b}	57.33 ± 1.33^{ad}	65.33 ± 1.20^{abc}	1.69	$R^2 = 0.9509$
PVC (shake)	AG-1	13.33 ± 0.88^{ac}	30.67 ± 1.20^{abc}	40.00 ± 1.15^{a}	50.33 ± 0.88^{ac}	61.00 ± 1.53^{b}	70.67 ± 0.67^{ac}	2.02	$R^2 = 0.944$
	PSJ-1	18.00 ± 1.15^{b}	35.33 ± 0.88^{c}	21.67 ± 1.20^{e}	47.67 ± 1.45^{b}	61.00 ± 1.15^{ab}	68.00 ± 0.58^{ab}	1.74	$R^2 = 0.9468$
PD (shake)	AG-1	10.33 ± 0.88^{abc}	37.67 ± 1.45^{b}	37.00 ± 1.00^{a}	57.00 ± 0.58^{b}	60.00 ± 0.58^{b}	62.00 ± 0.58^{d}	1.88	$R^2 = 0.8298$
	PSJ-1	8.33 ± 0.88^{d}	14.00 ± 0.58^{b}	25.33 ± 1.20^{bde}	36.33 ± 2.33^{a}	46.00 ± 1.53^{cd}	58.67 ± 0.67^{c}	1.51	$R^2 = 0.9898$
MYPS (static)	AG-1	12.00 ± 1.15^{ac}	5.33 ± 0.33^{e}	28.00 ± 1.00^{b}	48.00 ± 1.00^{c}	57.33 ± 0.88^{ab}	68.00 ± 1.15^{ac}	2.06	$R^2 = 0.9787$
	PSJ-1	12.33 ± 0.67^{acd}	21.33 ± 0.67^{ae}	39.00 ± 1.00^{ac}	41.33 ± 0.33^{ab}	58.00 ± 1.00^{abd}	66.00 ± 1.53^{abc}	1.67	$R^2 = 0.9319$
PVC (static)	AG-1	14.67 ± 0.88^{c}	28.33 ± 1.67^{ac}	37.00 ± 1.00^{Aa}	49.67 ± 0.33^{ac}	60.00 ± 1.15^{b}	77.67 ± 1.45^{b}	2.14	$R^2 = 0.983$
	PSJ-1	12.33 ± 0.33^{acd}	27.33 ± 1.45^{ad}	40.33 ± 1.45^{c}	47.00 ± 1.00^{b}	58.33 ± 1.20^{abd}	70.33 ± 0.33^{b}	1.83	$R^2 = 0.94$
PD (static)	AG-1	5.80 ± 0.53^{b}	25.40 ± 0.80^{c}	39.67 ± 0.33^{a}	54.33 ± 0.67^{ab}	61.33 ± 0.67^{b}	69.00 ±1.00^{ac}	1.95	$R^2 = 0.8806$
	PSJ-1	9.33 ± 0.67^{cd}	19.00 ± 0.58^{abe}	29.33 ± 0.67^{abd}	42.00 ± 1.15^{ab}	63.33 ± 0.88^{b}	68.67 ± 0.67^{ab}	1.85	$R^2 = 0.9742$
MYPS (static + shake)	AG-1	7.67 ± 1.76^{ab}	33.67 ± 0.33^{ab}	16.33 ± 0.88^{bc}	23.00 ± 1.00^{e}	58.00 ± 0.58^{ab}	68.67 ± 0.88^{ac}	1.84	$R^2 = 0.9232$
	PSJ-1	13.00 ± 1.53^{abc}	21.67 ± 0.88^{ae}	36.00 ± 0.58^{ac}	46.67 ± 1.33^{b}	53.33 ± 0.88^{d}	66.00 ± 2.08^{abc}	1.73	$R^2 = 0.945$
Liquid (static + shake)	AG-1	11.00 ± 0.58^{abc}	27.67 ± 1.33^{c}	41.00 ± 0.58^{a}	53.93 ± 1.03^{ab}	60.67 ± 0.88^{b}	71.33 ± 1.76^{bc}	2.02	$R^2 = 0.8872$
	PSJ-1	12.33 ± 0.33^{acd}	22.00 ± 1.00^{ae}	34.33 ± 0.33^{acd}	44.33 ± 1.45^{ab}	56.33 ± 0.88^{ad}	69.00 ± 3.21^{ab}	1.93	$R^2 = 0.9633$
PD (static + shake)	AG-1	13.00 ± 1.00^{ac}	6.00 ± 0.58^{de}	31.00 ± 0.58^{ab}	50.00 ± 1.15^{ac}	54.00 ± 0.58^{a}	65.33 ± 0.67^{ad}	1.92	$R^2 = 0.9519$
	PSJ-1	14.67 ± 0.33^{ab}	24.33 ± 1.45^{ad}	35.67 ± 1.76^{ac}	42.33 ± 0.33^{ab}	53.33 ± 1.33^{ad}	62.33 ± 0.88^{ac}	1.67	$R^2 = 0.961$
Control	BHT	78.27 ± 0.58^{a}	78.67 ± 0.88^{a}	78.67 ± 0.33^{a}	79.67 ± 0.33^{a}	82 ± 0.58^{a}	83.00 ± 0.58^{a}		

Table 7: The total phenolic contents and total flavonoids contents of mycelial extracts of *C. militaris* grown using nine submerged culture method that incorporated different media and shaking regimes. Mean values with different upper-case letters within a row are significantly different to each other ($p < 0.05$). Means within the same column of *C. militaris* strains AG-1, PSJ-1 followed by the same lower-case letters are not significantly different to each other at $p \leq 0.05$ according to Duncan's multiple range tests. Extracts were all oven dried (40 °C) (n = 5).

	Total phenolic contents (mg ml^{-1})		Total flavonoids content (mg ml^{-1})	
Submerged culture method	AG-1	PSJ-1	AG-1	PSJ-1
MYPS (shake)	1.94 ± 0.10^{bd}	2.02 ± 0.17^{b}	4.91 ± 0.08^{b}	4.86 ± 0.13^{b}
PVC (shake)	3.04 ± 0.41^{ab}	2.37 ± 0.08^{b}	5.55 ± 0.14^{ba}	5.48 ± 0.31^{b}
PD (shake)	1.75 ± 0.07^{d}	1.94 ± 0.21^{b}	4.93 ± 0.28^{ab}	4.80 ± 0.11^{b}
MYPS (static)	2.76 ± 0.10^{abc}	2.37 ± 0.21^{ab}	5.43 ± 0.15^{ba}	5.28 ± 0.11^{b}
PVC (static)	3.74 ± 0.18^{a}	3.23 ± 0.10^{a}	6.01 ± 0.22^{a}	5.70 ± 0.21^{b}
PD (static)	2.72 ± 0.16^{bc}	2.25 ± 0.10^{b}	5.33 ± 0.17^{ab}	5.22 ± 0.18^{b}
MYPS (static + shake)	2.10 ± 0.18^{cd}	1.98 ± 0.20^{b}	4.88 ± 0.22^{b}	4.90 ± 0.13^{b}
PVC (static + shake)	2.33 ± 0.07^{bcd}	2.25 ± 0.14^{b}	5.48 ± 0.28^{ab}	5.05 ± 0.10^{b}
PD (static + shake)	2.02 ± 0.27^{bd}	1.94 ± 0.17^{b}	4.86 ± 0.07^{b}	4.70 ± 0.07^{b}

in this study depended not only on the use of different submerged culture method but also on the mycelial drying methods used.

3.8 TOTAL FLAVONOID CONTENT (TFC).

The TFC of mycelial extracts from AG-1 ranged from 4.86 ± 0.07 to 6.01 ± 0.22 mg g^{-1} (Table 7). Extracts from mycelial grown in PVC medium and static conditions also gave the highest TFC for AG-1 (6.01 ± 0.22 mg g^{-1} extract). For AG-1 TFC was significantly greater in mycelial growm in the PVC static, MYPS static, PD static, PVC static+shake, MYPS static+shake, PD static+shake, PVC shake, MYPS shake, and PD shake than in mycelial grown in the PD static treatment. TFC of PSJ-1 mycelial were lower than those of strain AG-1, and rangerd from 4.70 ± 0.07 to 5.70 ± 0.21 mg g^{-1} (Table 7). In our study TFC was significantly dependent on the strain used and on the nine different submerged culture strategies. Previous work on strains AG-1, PSJ-1 have already shown that TFC is less dependent on drying method than the indicated TFC of *C. militaris* waster medium was 4.26 ± 0.05 mg of QE g^{-1} (distilled water) (Chun Lun Wang et al., 2015), and that while the TPC from the growing medium of *C. militaris* was 3.91 ± 0.16 mg GAE g^{-1} the TFC was higher at 4.26 ± 0.05 mg of QE g^{-1} (Chun Lun Wang et al., 2015). However, other studies have shown that the TFC of mycelial of *C. militaris* grown on fermented of unfermented wheat were: 7.08 ± 0.46 mg l^{-1}; ethanol 80 %: 7.36 ± 0.37 mg l^{-1} (ethanol extracts); and 6.07 ± 0.19 mg l^{-1} (water extract) (Yu Xiao et al., 2014).

4 CONCLUSIONS

The impact of various submerged culture strategies (shaking regime, drying methods, oil supplements) on mycelium growth, EPS and IPS production, and antioxidant activity of *C. militaris* strains AG-1, PSJ-1 in submerged culture are presented. Submerged culture method drying methods and oils played an important role in mycelial biomass growth, EPS production, IPS production, and antioxidant activity of extracts. Submerged culture methods for optimal production of mycelial extracts with high levels of polysaccharides was achieved. The optimal initial pH for mycelial growth and extra-cellular polysaccharide production was 6.7. Of the five different types of oil sources tested, coconut oil, suet, and sunflower oil, all improved the dry mass of mycelia produced and also the production of both EPS and IPS. Specifically, maximum mycelium growth and extra-cellular polysaccharide concentrations were achieved in the PVC media containing 3.5 % coconut oil. Results from various original H_2O_2 radical scavenging activity, $ABTS^{\cdot+}$ radical activity, and lipid peroxidation assays revealed that extracts of mycelial from AG-1, PSJ-1 had significant antioxidant activity, and could represent a potential source of antioxidants of great importance for the treatment of disease. The strong antioxidant activity was related to the total flavonoid content (TFC), and total phenolic content (TPC). Future work on the identification, isolation and structural characteristics of the active components will be the target of our follow-up studies, as these compounds have the potential for use as phyto-therapeutic agents.

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