EFFECT OF PLANT OILS ON MYCELIAL BIOMASS PRODUCTION, BIOSYNTHESIS AND ANTIOXIDANTS OF EXOPOLYSACCHARIDE BY *OPHIOCORDYCEPS SINENSIS*

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ABSTRACT

Ophiocordyceps sinensis (syn. *Cordyceps sinensis*) is a well-known entomopathogenic fungus with many significant bioactivities such as antioxidants, immunomodulatory and antitumor, etc. In Vietnam, its mycelial biomass has been cultured artificially in a liquid medium and has been studied application since 2013. Many previous researches demonstrated that exopolysaccharides (EPS) was secreted by the fungus with many integral bioactive activities. Thus, the aim of this study is to enhance the biosynthesis potential of EPS of *O. sinensis* fungus by implementing sunflower oils and coconut oils at concentrations from 1% to 5% (v/v), and olive oil at concentrations between 1% and 10% in culture medium. The results showed that the mycelial biomass and EPS production of *O. sinensis* fungus increased considerably compared to non-oil medium. The EPS yields for olive oil (5%), sunflower oil (3%) and coconut oil (4%) were 5.94 g/L, 2.56 g/L and 2.43 g/L, respectively. Moreover, the ABTS (2,2'- azino - bis (3 – ethylbenzothiazoline – 6 - sulphonic acid) radical scavenging activity of EPS extracted from the olive oil-containing medium of 5% rose significantly compared to the control EPS. As a result, the data has demonstrated that the 5% olive oil-containing medium had the ability to boost EPS production of *O. sinensis* fungus and improve in vitro antioxidant potential of EPS. Thus, it creates a scientific basis to explore the EPS source effectively from *O. sinensis* fungus in the future.

Keywords: Ophiocordyceps sinensis, exopolysaccharide, antioxidants, mycelial biomass, ABTS (2,2'- azino - bis (3 – ethylbenzothiazoline – 6 - sulphonic acid)).

1. INTRODUCTION

Ophiocordyceps sinensis is known as Dong Chong Xia Cao in the Traditional Chinese Medicine (TCM). It parasitizes larvae of moths belonging to the *Lepidoptera*, especially *Hepialus* and *Thitarodes* [1]. Previous studies have illustrated that the fungus has many vital bioactivities such as boosting immune system, reducing cholesterol, antitumor, antioxidants and decreasing blood sugar levels, etc. In recent years, *O. sinensis* is at risk of extinction because of overexploitation as well as global climate change [2]. Thus, the feasible and sustainable solution to maintain this medicinal mushroom is the fermentation technology of the *O. sinensis* mycelium.

Currently, the implementation of plant oils in the culture of medicinal mushrooms is being considered by scientists. The plant oils stimulate the growth of fungi and EPS production during the fermentation process [3]. Dong and Yao (2010) conducted to survey and assess the antioxidants of EPS extracted from *Cordyceps sinensis* by six *in vitro* tests, including inhibited linoleic acid peroxidation, DPPH, superoxide anion, $OH^{\bullet}$ radical scavenging potential, reducing capacity and complex formation with iron. The results indicated that EPS was highly effective in inhibiting linoleic acid peroxidation, scavenging more than 80% of DPPH radicals and reducing the ability of complex formation with iron [2].

In addition, the plant oil is known as an anti-foaming agent during fermentation. Hence, it stimulates the growth of fungi and secondary metabolism for several medicinal fungi [3, 4]. Yang *et al* (2000) carried out to culture the *Ganoderma lucidum* on a medium within fatty acids and plant oils. The data showed that oleic acid and palmitic acid stimulated considerably EPS and mycelial biomass production. Furthermore, at 0.15 g/100 mL of olive oil, the yield of biomass production increased from 0.2 g/mL to 0.46 g/mL. Otherwise, linoleic acid inhibited the growth of the fungus and EPS production at 0.1 g/100 mL [5]. However, the information on stimulating the growth of the *O. sinensis* and EPS biosynthesis by plant oils is still extremely limited. Therefore, in this study, a survey of the effects of olive oil, sunflower oil, and coconut oil on mycelial biomass production and EPS biosynthesis of *O. sinensis* mushroom as well as the antioxidant capacity of the EPS was conducted.

2. MATERIALS AND METHODS

2.1. Material

Ophicordyceps sinenis strain was supplied by Dr. Truong Binh Nguyen (Dalat University, Da Lat, Lam Dong, Vietnam). It was maintained on PDA medium at 4 °C.

2.2. Methods

2.2.1. Surveying the effects of plant oils on mycelial biomass and EPS production of Ophiocordyceps sinensis

Preparing 10 L of the liquid medium containing: 2 kg potato, 500 g saccharose, 60 g peptone, 40 g yeast extract, 5 g KH_2PO_4, 5 g K_2HPO_4, 5 g $CaCl_2$, 2 g $MgSO_4$, 1% (v/v) Tween 80 and plant oils (1 – 5% coconut oils; 1 – 5% sunflower oils and 1 - 10% olive oils). Then, it was autoclaved at 121 °C for 30 minutes. The medium was added two Erlenmeyer flask containing 400 mL of the inoculum into the medium and poured out each plastic container (500mL capacity) with 200 mL and incubated at 22 °C for 30 days.

C is the symbol of the control without Tween 80 and plant oils

Tw is the symbol of the medium within 1% Tween 80

C1 - C5 are the symbols of the coconut oil-containing media of 1 - 5%

S1 - S5 are the symbols of the sunflower oil-containing media of 1 - 5%

O1 – O10 are the symbols of the olive oil-containing media of 1 - 10%

2.2.2. Harvesting the mycelial biomass

The biomass was washed with n-hexane 20% to remove oils and dried at 55 °C to constant mass. The dry weight of the biomass (g/L) was evaluated [6].

2.2.3. Extraction of exopolysaccharide

The culture broth was isolated and treated with n-hexane to remove oils. It was then concentrated by a rotary vacuum evaporator. The EPS was isolated by precipitating with ethanol 96° in the ratio 1:4 (v/v) at 4 °C for 24 hours and centrifuging (6000 rpm, 20 min).

Finally, the sample was lyophilized and stored at 4 °C. The polysaccharide content of EPS was determined by the phenol-sulfuric acid method [7].

2.2.4. ABTS radical scavenging assay

The ABTS (2,2'- azino - bis (3 – ethylbenzothiazoline – 6 - sulphonic acid) radical scavenging assay was carried out according to Roberto *et al* (1999) with several minor modifications [8]. The reaction consisted of three stages. Stage 1, formation of $ABTS^{\bullet+}$ radicals: mixed ABTS 7 mM and $K_2S_2O_8$ 2.45 mM with a ratio 1:1 (v/v), then incubated in the dark for 12 – 16 hours at room temperature. Stage 2, diluted $ABTS^{\cdot+}$ with PBS buffer pH 7.4 to an absorbance of $OD_{734nm} = 0.70 \pm 0.02$. Stage 3, reaction mixture: added 3000 µL $ABTS^{\cdot+}$ into 100 µL sample, incubated in the dark for 30 min, the absorbance was measured at OD 734 nm. Vitamin C was used as standard.

The $ABTS^{+}$ scavenging percentage of the samples was calculated by using the following equation:

$$S\% = \frac{A_0 - A_1}{A_0} x100$$

where A_0 is the absorbance of the blank control and A_1 is the absorbance of the samples.

2.2.5. Data analysis

Data were evaluated for statistical significance with Student's T-Test followed by GraphPad Prism Statistic. The numbers were repeated at least 3 times and were expressed as Mean ± Standard deviation.

3. RESULTS AND DISCUSSION

3.1. Effects of plant oils on mycelial biomass production

The biomass yields of *O. sinensis* in various plant oil-containing media including coconut oils (1 - 5%), sunflower oils (1 - 5%) and olive oils (1 - 10%) were represented in the Figure 1.

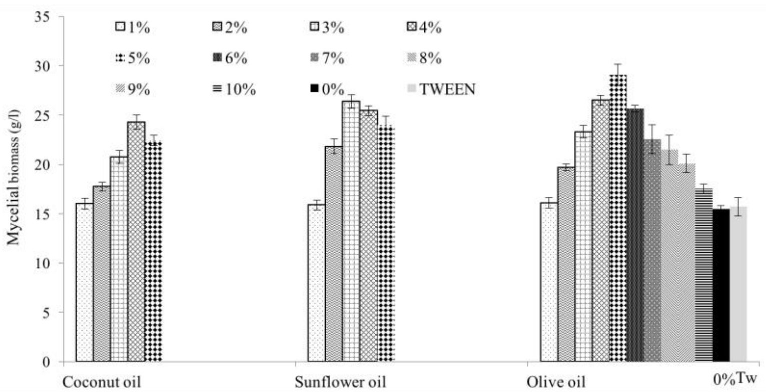

Figure 1. The biomass yields of *O. sinensis* fungus in different plant oil-containing media

The data shows that Tween 80 did not affect the biomass production of the fungus. The yield of biomass for Tw was 15.7 ± 0.71 g/L, which was also similar to control (about 15.5 ± 0.53 g/L). By contrast, regarding coconut oils (1 – 5%), the biomass yields were higher than that of control (Figure 1). Specifically, the dry weight was the highest at 4% of coconut oil (about 24.3 g/L). Bolla *et al* (2011) also demonstrated that although coconut oil stimulated the biomass production of the fungus [3]. The coconut oil has a low level in oleic acid and high in saturated fatty acids, so it can partially restrict for stimulating the growth of fungi.

Similarly, sunflower oil also improved the biomass production of *O. sinensis* from 15.9 g/L to 26.4 g/L. In specific, at 3% sunflower oil, the yield was the highest 26.4 g/L and was about 37% higher than that of control. The content of linoleic acid in sunflower oil accounted for about 65 – 70% [7, 9, 10]. It was a major ingredient related to stimulating the biomass production of fungi because it could enhance nutrient uptake in the culture medium [4, 10]. However, this mechanism remains unclear.

Noticeably, olive oil-containing medium was the best for the growth of the fungus. The biomass yield was the highest about 29.06 g/L at 5% of olive oil, which was almost twice as much as control. The study of Park *et al* (2002) showed that the biomass yield of *Cordyceps militaris* fungus rose significantly when adding olive oil in the culture medium [11]. More 70% of the olive oil content was oleic acid [4, 7, 12]. Several previous studies revealed that oleic acid stimulated the growth of the fungi in a liquid medium [9, 10].

Therefore, the results showed that the coconut oil was an inadequate nutrient source for the growth of the *O. sinensis* fungus because the main constituents of the oil were saturated fatty acids [3]. Meanwhile, sunflower oil and olive oil stimulated the fungal growth, which was similar to the report of Hsieh *et al* (2008) for culturing *G. frondosa* fungus [4]. The rate of oleic acid in both these oils occupied a vast majority of about 70% which was easily used by the fungi. In particular, at 5% of olive oil, the biomass yield of *O. sinensis* was the highest.

3.2. Effects of plant oils on EPS biosynthesis

After harvesting the biomass, EPS crudes were isolated from different plant oil-containing media including coconut oils (1 - 5%), sunflower oils (1 - 5%) and olive oils (1 -10%) as shown in the Figure 2. It was a type of extracellular polysaccharide with many vital bioactivities that was secreted culture medium during the grown of fungi.

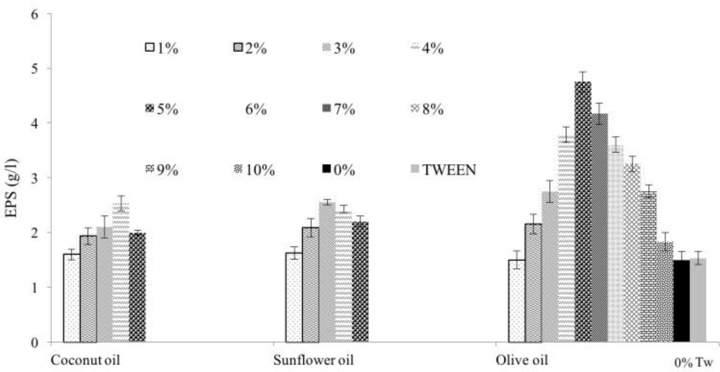

Figure 2. The EPS biosynthesis of *O. sinensis* in different plant oil-containing media

The EPS content of the control reached to 1.59 ± 0.12 g/L. The figure for the medium within 1% Tween 80 was similar (about 1.57 ± 0.08 g/L). Thus, Tween 80 (1%) was not effect on EPS biosynthesis of *O. sinensis* fungus. However, the figure for plant oil-contaning media considerably increased. Of those, in a coconut oil-containing medium of 4%, the EPS content rose from 1.5 g/L to 2.4 g/L. Similarly, regarding sunflower oil, the amount of EPS was directly proportional increase to the concentration of 1 – 3% and dropped at more than 3%. Particularly, at the concentration of 3%, the EPS was secreted the most (about 2.56 g/L), which was 1.5 times higher than that of the control. Likewise, the EPS biosynthesis of *O. sinensis* fungus considerably grew when supplementing olive oil in the culture medium.

The number of EPS increased from two to four times compared to the control. The data was the same to the study of Hsieh *et al* (2008) when they cultured *Grifola frondosa* in sunflower oil and olive oil-containing media [4].

In conclusion, the results have demonstrated that plant oils not only stimulated the growth of *O. sinensis* fungus, but also enhanced the EPS biosynthesis of the fungus. Thus, to improve the mycelial biomass production and EPS biosynthesis of *O. sinensis* fungus, it was suggested that the olive oil-containing medium of 5% was the most suitable medium.

3.3. The polysaccharide content of EPS crudes

The polysaccharide content of EPS crudes was determined by phenol-sulfuric acid as shown in the Table 1. In general, the rate of polysaccharide of EPS crudes made up a slight majority of about 45 – 70%.

Table 1. The polysaccharide content of EPS crudes

Sample	Polysaccharide content (%)	Sample	Polysaccharide content (%)
C	55.09 ± 0.38		
Tw	48.97 ± 0.40		
C1	45.32 ± 0.71	O1	51.65 ± 1.05
C2	48.69 ± 0.54	O2	57.98 ± 0.62
C3	56.85 ± 0.49	O3	60.53 ± 0.16
C4	55.97 ± 0.98	O4	62.21 ± 0.55
C5	53.61 ± 0.72	O5	64.31 ± 0.95
S1	55.70 ± 1.54	O6	69.65 ± 1.32
S2	59.44 ± 1.27	O7	61.98 ± 0.65
S3	65.43 ± 0.50	O8	60.53 ± 0.17
S4	66.76 ± 0.72	O9	63.21 ± 0.59
S5	61.05 ± 0.53	O10	60.78 ± 0.23

The polysaccharide content of EPS extracted from the 1% Tween 80 medium was lower than that of the control, being 48.97 % and 55.09% respectively. This indicated that Tween 80 (1%) slightly impact to decrease the polysaccharide content of EPS. The figure for coconut oil-containing medium of 1 – 2% concentration was the same. By contrast, in terms of sunflower oil and olive oil, the polysaccharide content of EPS crudes gradually increased from 55.09% to 69.65% (except 1% of olive oil). Noticeably, the EPS crudes of the olive oil (6%) had the highest percentage of polysaccharide (about 69.65%). At an olive oil-containing medium of 5%, polysaccharide content of EPS was 63.21 %.

Consequently, the results revealed that the plant oils seemed to modify the polysaccharide content of EPS crudes, which could affect their bioactivities.

3.4. $ABTS^+$ radical scavenging activity of EPS crudes

The $ABTS^+$ (2,2'- azino - bis (3 – ethylbenzothiazoline – 6 - sulphonic acid) radical scavenging method was one of the effective and simple antioxidant assays. The antioxidant potential of samples was evaluated by an IC_{50} value. It was the value at which the sample reduced 50% of free radical concentration.

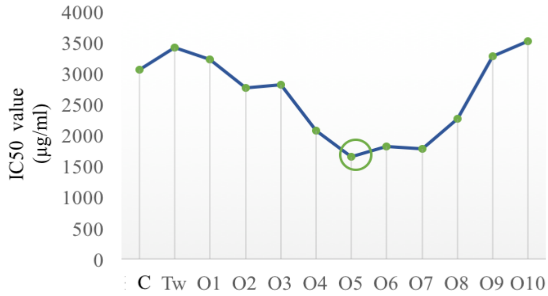

Figure 3. The ABTS$^+$ radical scavenging activity of EPS crudes of olive oil media

The ABTS$^+$ radical scavenging activity of the EPS crudes of the olive oil media was screened at the concentration range of 0 – 6000 µg/mL (Figure 3). Overall, the IC_{50} values of all the samples were determined from about 1600 to 3500 µg/mL. At the olive oil-containing media of 1 - 5%, the IC_{50} value of the EPS samples decreased to 1655.63 µg/mL (O5 medium), from 3200 µg/mL (O1 medium). In contrast, the figure for olive oil-containing media of 5 - 10% rose from about 1800 to 3500 µg/mL. This indicated that EPS of the olive oil (5%) was the highest ABTS$^+$ radical scavenging potential, which doubled compared to the control.

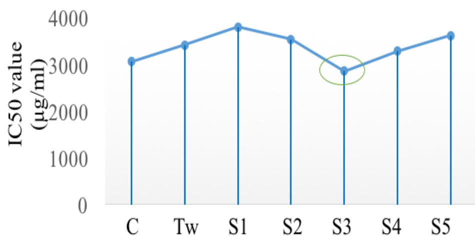

Figure 4. The ABTS$^+$ radical scavenging activity of EPS crudes of sunflower oil media

Similarly, the ABTS$^+$ radical scavenging activity of the EPS crudes extracted from the sunflower oil-containing media was also screened at the concentration range of 0 - 6000µg/mL (Figure 4). The IC_{50} value of the EPS crudes were higher than that of the control, excepted the EPS sample extracted from S3 medium. Similarly, as for the coconut oil-containing media, this activity of EPS crudes steadily decreased compared to the control (Figure 5).

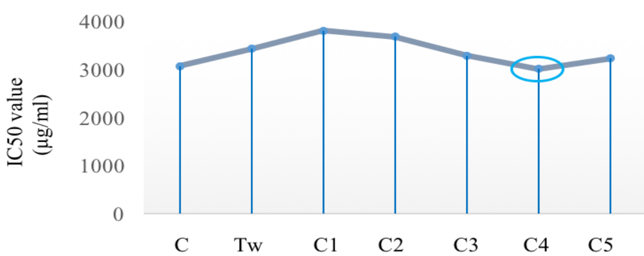

Figure 5. The ABTS$^+$ radical scavenging activity of EPS crudes of coconut oil media

Therefore, the results demonstrated that the sunflower oil and coconut oil inhibited the ABTS$^+$ radical scavenging activity of the EPS crudes, while the olive oil (especially at a concentration of 5%) improved this potential of the EPS crudes. Yan *et al* (2013) reported that EPS fractions isolated from the mycelial biomass and the culture medium that had also the ability to reduce OH^- and ABTS$^+$ radicals significantly [13].

4. CONCLUSION

The study has indicated that the plant oils were an important ingredient for culturing *O. sinensis* fungus. The olive oil and sunflower oil effectively affected the growth of the fungus and EPS biosynthesis. Noticeably, the olive oil (5%) was the best medium for the *O. sinensis* fungus. It stimulated to increase the biomass production and EPS biosynthesis as well as the polysaccharide content of EPS considerably. Furthermore, the $ABTS^+$ radical scavenging potential of EPS also strongly improved with an IC_{50} value of 1655.63 µg/mL, which doubled compared to the control (with 3067.71 µg/mL). Meanwhile, the coconut oil and sunflower oil inhibited the $ABTS^+$ radical scavenging activity of EPS. In conclusion, it is suggested that using the olive oil (5%) to culture *O. sinensis* fungus is an integral strategy because it not only stimulates the mycelial biomass production of the fungus and the EPS biosynthesis but also improves the *in vitro* antioxidant activities of EPS. By doing this, the medicinal mushroom will be effectively exploited in the future.

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TÓM TẮT

ẢNH HƯỞNG CỦA DẦU THỰC VẬT TỚI SỰ SẢN XUẤT SINH KHỐI, SINH TỔNG HỢP VÀ HOẠT TÍNH KHÁNG OXY HOÁ CỦA EXOPOLYSACCHARIDE TỪ NẤM *OPHICORDYCEPS SINENSIS*

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Ophiocordyceps sinensis (đồng danh *Cordyceps sinensis*) được biết đến là một loài nấm ký sinh côn trùng với nhiều hoạt tính sinh học quan trọng như kháng oxy hóa, đáp ứng miễn dịch và kháng khối u… Tại Việt Nam, sinh khối sợi nấm *O. sinensis* đã được nuôi cấy nhân tạo trong môi trường lỏng và đã nghiên cứu ứng dụng từ năm 2013. Một số nghiên cứu trước đây chứng minh nấm tiết ra một lượng lớn exopolysaccharide (EPS) giàu hoạt tính sinh học trong môi trường nuôi cấy. Mục đích của nghiên cứu này là nâng cao khả năng sinh tổng hợp EPS của nấm *O. sinensis* bằng cách bổ sung dầu hướng dương và dầu dừa ở nồng độ từ 1- 5% (v/v), và dầu ô liu ở nồng độ 1 - 10% (v/v) trong môi trường nuôi cấy. Sản xuất sinh khối sợi nấm và EPS của nấm *O. sinensis* tăng lên đáng kể so với môi trường không bổ sung dầu thực vật. Hiệu suất thu nhận EPS của các môi trường chứa 5% ô liu, 3% dầu hướng dương và 4% dầu dừa lần lượt là 5,95 g/L, 2,56 g/L và 2,43 g/L. Bên cạnh đó, hoạt tính bắt gốc tự do ABTS (2,2'- azino - bis (3 – ethylbenzothiazoline – 6 - sulphonic acid) của EPS tách chiết từ môi trường chứa 5% olive tăng đáng kể so với EPS đối chứng. Như vậy, dữ liệu đã chứng minh dầu thực vật có khả năng kích thích sản xuất EPS của nấm *O. sinensis* và nâng cao hoạt tính kháng oxy hóa *in vitro* của EPS. Kết quả nghiên cứu tạo cơ sở khoa học để khai thác hiệu quá nguồn EPS từ nấm *O. sinensis* trong tương lai.

Từ khóa: Ophiocordyceps sinensis, exopolysaccharide, nuôi cấy lỏng, ABTS (2,2'- azino - bis (3 – ethylbenzothiazoline – 6 - sulphonic acid))