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Biological activities of crude exopolysaccharides produced by the bacteria isolated from plasmodia of slime molds

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Abstract

Slime molds are an unusual group of fungus-like protozoans, and one of their trophic stages (the plasmodium) is often found to be associated with certain unique bacteria not normally found in their regular habitats. Four different strains of bacteria were previously isolated from plasmodia (generated from fruiting bodies under laboratory conditions) of the slime molds *Physarella oblonga* and *Fuligo septica*. The bacteria were genetically classified as members of the genera *Enterobacter*, *Stenotrophomonas*, and *Alcaligenes*. The crude exopolysaccharides (EPSs) from the four species of bacteria were isolated and evaluated for their antioxidant and anti-proliferative activities. Mild *in vitro* antioxidant activities were recorded for all the samples. However, at a concentration of 1 mg/mL, the crude EPSs from *Enterobacter* sp. (C1) and *Stenotrophomonas* sp. 1 (C2) showed significant anti-proliferative activities against HeLa cervical cancer cells, with the antiproliferative rates of 41.31% and 36.19%, respectively. Remarkably, they displayed negligible antiproliferative activities toward bovine aortic endothelial cells. These data suggested that further investigations, including purification of the EPS samples and evaluation of their cytotoxicity against HeLa cells, should be carried out.

Keywords: Antiproliferative activity, Symbiotic bacteria, Exopolysaccharides, *Fuligo septica*, *Physarella oblonga*

1. Introduction

The slime molds (called myxomycetes by biologists) derive their common name from the fact that one the trophic stages in their life cycle (the plasmodium) often leaves behind slime tracks (exopolysaccharides: EPSs) on solid surfaces when moving from one place to another. Despite the amount of material in the slime tracks being appreciable, the bioactivity of this material has not been intensively studied. Slime molds possess characteristics of both fungi (forming fruiting bodies) and protozoans (motile, phagocytic and lacking cell walls); thus, they are an unusual group of microorganisms and have been found to possess a large number of novel bioactive compounds [1].

Previous studies have isolated a significant number of novel compounds from slime molds, and these have various biological properties such as antimicrobial and anticancer activities [2]. These biological activities suggest that slime molds have a great potential for further applications in the medical and pharmaceutical fields. Therefore, it is essential to optimize their growth under laboratory conditions [3]. One of the biotic factors governing the growth of the plasmodia of slime molds relates to the bacteria with which they are associated. Technically, if the plasmodia are in favorable conditions, they will feed on the associated bacteria or their metabolites and overgrow the bacteria. However, if the conditions favor bacterial growth, then the plasmodia would be inhibited. Although there have been some studies of bioactivities of myxomycete plasmodia [1,4], there is still very little understandings of the bacteria associated with plasmodia. Previous studies regarding this aspect include those of Cohen and Venkataramani [5,6]. In those studies, the bacteria associated with plasmodia of some species of

myxomycetes, including *Physarum polycephalum, Stemonitis herbatica, Badhamia* sp., and *Physarum nicagauense* were isolated and identified. Cohen demonstrated that most bacteria associated with the plasmodia of *P. polycephalum, Badhamia* sp., and *B. utricularis* are Gram-negative and most are motile. They were superficially at least referable to those two great heterogeneous groups of common soil and water bacteria, which are *Achromobacter* and *Flavobacterium*. Venkataramani [6] claimed that the plasmodia of *P. anicaroauense* were associated with two kinds of Gram-negative, rod-shaped bacteria. Biochemical tests were conducted on those bacteria and they were found to be *Proteus* sp. and *Klebsiella* sp. However, none of these studies attempted to investigate bioactive compounds derived from the associated bacteria, including exopolysaccharides (EPSs) produced by the bacteria.

EPSs are high-molecular-weight polymers that are composed of sugar residues and secreted by microorganisms into the surrounding environment [7]. Microbial EPSs have gained a great deal of interest due to their potential biological activities [8]. EPSs extracted from bacteria and fungi have been revealed to possess inhibitory activities against Gram-positive and Gram-negative bacteria [1]. EPSs of bacteria and other microorganisms have been demonstrated to assert antimicrobial activities on several pathogenic bacteria, including *Staphyloccoccus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis*, and *Salmonella typhi* [9]. Moreover, EPSs also possess abilities to inhibit cancer cell lines such as breast cancer (MCF-7 cell), cervical cancer (Hela cell) and T-cell leukemia cancer (Jurkat cell) [10-12].

Until recently, the bacteria associated with slime molds have not been intensively studied. In fact, none of the investigation relating to the production of EPS from the bacteria associated with the plasmodia of slime molds has been internationally published. As these bacteria are unique, they may produce some types of unusual EPSs as well. Therefore, the research described herein was carried out to evaluate the bioactivities of the crude EPSs produced by the bacteria isolated from the plasmodia of *F. septica* and *P. oblonga*. The bacteria were previously identified by 16S r RNA sequencing as *Enterobacter* sp. (C1), *Stenotrophomonas* sp. 1 (C2), *Stenotrophomonas* sp. 2 (C4), and *Alcaligenes* sp. (C5).

2. Materials

Enterobacter sp. (C1), *Stenotrophomonas* sp. 1 (C2), *Stenotrophomonas* sp. 2 (C4), and *Alcaligenes* sp. (C5) were isolated from the plasmodia of two slime molds, which are *Fuligo septica* and *Physarella oblonga*. Fruiting bodies of these slime molds were collected from an orchid farm (Tam Diep city, Ninh Binh province, Vietnam) and the specimens are deposited at the Applied Microbiology Laboratory (International University-VNU HCM, Ho Chi Minh city, Vietnam). The plasmodial cultures were generated from the collected fruiting bodies.

3. Methods

3.1 EPS production

A loop of each bacterium was transferred from the stock culture slant into an Erlenmeyer flask containing 100 mL of Luria-Bertani (LB) broth. The flask culture was shaken at 120 rpm at room temperature for a day and the culture was subsequently used as an inoculum.

An inoculum amount of 10% was added to a nutrient broth medium. One liter of nutrient broth contained 10 g sugar, 10 g yeast extract, 3 g K_2HPO_4, 1 g KH_2PO_4 and 0.5g $MgSO_4$; pH was adjusted to 5.5. Then the cultures were shaken at 120 rpm at room temperature (27-30 ℃) for 3 days and terminated for EPS isolation [13].

3.2 Crude EPS isolation

Crude EPS was extracted using the procedure described by Li et al. [9] with some modifications. The culture was centrifuged at 8500 rpm for 15 min. The pellet was discarded and a 3-fold volume of cold absolute ethanol was added to the supernatant. The mixture was kept at 4 ℃ for 24 h. After that, the mixture was centrifuged at 8500 rpm for 15 min at 4 ℃. The supernatant was discarded; the crude EPS was collected and dried at 60 ℃ until the weight was constant.

3.3 DPPH radical scavenging activity of the bacterial crude EPSs

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity of the extracted EPSs was determined using the method as described in Molyneux et al. [14] and Hassan et al. [15]. A DPPH solution with a concentration of 0.2 mM was prepared by adding 7.88 mg of DPPH to 100 mL of ethanol. The dried EPS was dissolved in sterile distilled water and diluted to obtain various concentrations of between 2, 5 to 10 mg/mL. A volume of 100 μL of the EPS solution with a certain concentration was added to each well in a 96-well plate. After that, 100 μL of 0.2 mM DPPH solution was added to each well. A DPPH solution without the EPS sample

was used as the control, and ascorbic acid was used as the standard. The 96-well plate was covered with aluminum foil and left at room temperature for 15 min. The absorbance was measured at 517nm against blank containing sample with ethanol. Antioxidant activity was calculated by using the below equation (1).

$$\%\ Radical\ scavenging\ activity = \frac{absorbance\ of\ blank - absorbance\ of\ sample}{absorbance\ of\ blank}\ x\ 100 \quad (1)$$

3.4 Antiproliferative activities of the bacterial crude EPSs

The antiproliferative properties of crude EPSs on tested cell lines was evaluated by a MTT (3-[4,5-Dimethylthiazol-2-Yl]-2,5-Diphenyltetrazolium Bromide)-based assay [16]. The cancer cell lines used in current study included cervical carcinoma HeLa, and liver carcinoma HepG2 cells. Moreover, inhibition of polysaccharides against non-transformed cell line (bovine aortic endothelial cells-BAEC) was also determined.

Ten mg of crude EPS was dissolved in PBS (phosphate buffer saline, 0.01 M, pH 7.4) to obtain an EPS solution with a concentration of 10 mg/mL. The tested cells in 180 µL of Dulbecco's modified eagle medium (DMEM) were seeded in a 96-well microplate at a concentration of $5x10^3$ cells per well and cultivated at 5% CO_2, 37 ℃ for 24 h. The EPS solution was added to each well to reach a concentration of 1mg/mL and incubated for another 24 h. After that, a washing with 100 µL PBS was applied to each well. Then 50 µL of the DMEM medium that contains 2 mg/mL MTT was added to each well and incubated for 4 more hours. Afterwards, 200 µL of dimethyl sulfoxide (DMSO) was used to dissolve MTT formazan after 4 h and measured at 540 nm. PBS was used as the negative control. Cell inhibition (%) was calculated according the below equation (2).

$$Cell\ inhibition\ (\%) = \frac{OD_{control} - OD_{sample}}{OD_{control}} x100\% \quad (2)$$

OD_{sample} and $OD_{control}$ represent the absorbance of the test sample and the negative control, respectively.

3.5 Data analysis

All the experiments were conducted in duplicate except for the antiproliferative activity test, which was carried out in triplicate. Data were analyzed using excel and the results were exhibited as mean ± standard deviation. Statistical analysis was performed with the use of one-way ANOVA. Differences were considered as significant if $p<0.05$.

4. Results

4.1 EPS production of the bacteria isolated from Fuligo septica and Physarella oblonga plasmodia

EPSs were collected from the cultures as previously described. The amounts of dried EPS are shown in Figure 1. Among the four species of bacteria, *Enterobacter* sp. (C1) produced the highest amount (10.03 g/L). However, statistical analysis showed no significant difference in the amounts of EPS produced by the four species of bacteria ($p>0.05$)

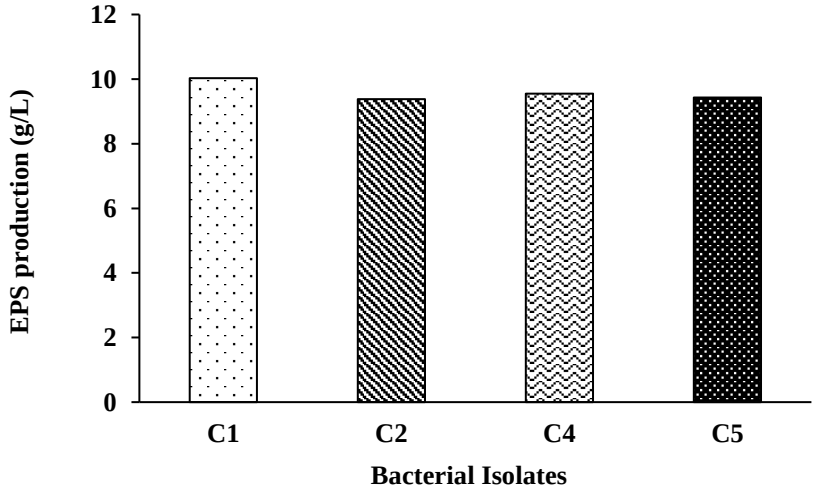

Figure 1 EPS production of different species of bacteria in nutrient broth medium after three days of incubation C1: *Enterobacter* sp.; C2: *Stenotrophomonas* sp. 1; C4: *Stenotrophomonas* sp. 2; C5: *Alcaligenes* sp.

4.2 Antioxidant activity of the bacterial crude EPSs

At an EPS concentration of 2 mg/mL, none of the samples could scavenge DPPH as no color change was observed (data not shown). However, within the range of 5 to 20 mg/mL, all the samples showed the color change from purple to yellow, which indicated that the EPS samples from the other four species had abilities to scavenge DPPH.

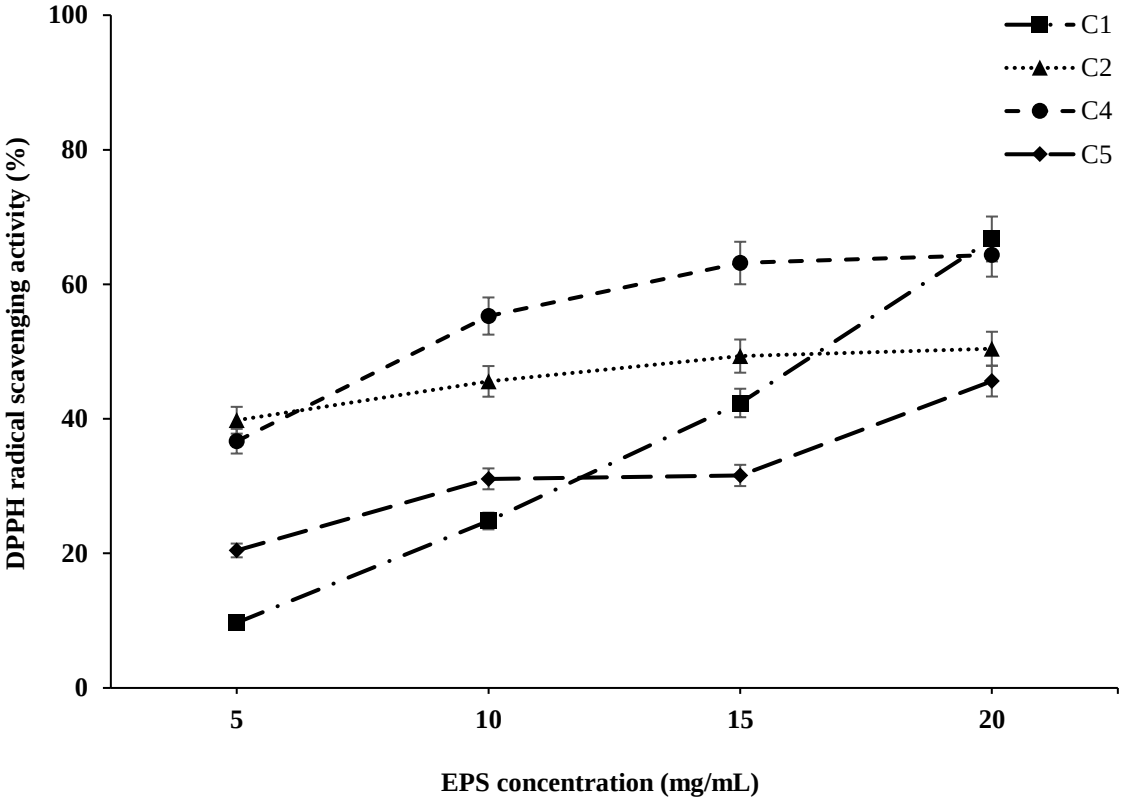

Figure 2 Antioxidant activities of the bacterial crude EPSs at four different concentrations C1: *Enterobacter* sp.; C2: *Stenotrophomonas* sp. 1; C4: *Stenotrophomonas* sp. 2; C5: *Alcaligenes* sp.

In the range of 5 to 15 mg/mL, the EPSs from *Stenotrophomonas* sp. 1 (C2) and *Stenotrophomonas* sp. 2 (C4) expressed higher antioxidant activities compared with other samples (Figure 2). However, at a concentration of 20 mg/mL, the EPS from *Enterobacter* sp. (C1) showed the highest antioxidant activity, which was slightly higher than that of the *Stenotrophomonas* sp. 2 (C4) but significantly higher than the others (p<0.05).

4.3 Antiproliferative activity of the bacterial crude EPSs by MTT assay

The data in Figure 3 indicate that all the EPS samples exhibited inhibitory activities against the cervical cancer HeLa and liver cancer HepG2 cells at a concentration of 1 mg/mL. Data were statically analyzed and there were significant differences between samples (p<0.05). Remarkably, the EPS produced by strains of *Enterobacter* sp. (C1) and *Stenotrophomonas* sp. 1 (C2) could inhibit the proliferation of HeLa cell populations to 41.31% and 36.19%, respectively (Figure 3A).

The anti-proliferative activities of the bacterial EPS samples against HeLa cells were stronger than that towards HepG2 in most cases. Only the EPS from *Alcaligenes* sp. (C5) possessed a slightly higher inhibition against HepG2 (22.47%) than HeLa (20.88%).

Another noteworthy point was that all the bacterial crude EPS samples displayed remarkably low antiproliferative activities (smaller than 5%) toward the bovine non-transformed cells (BAEC) (Figure 3C).

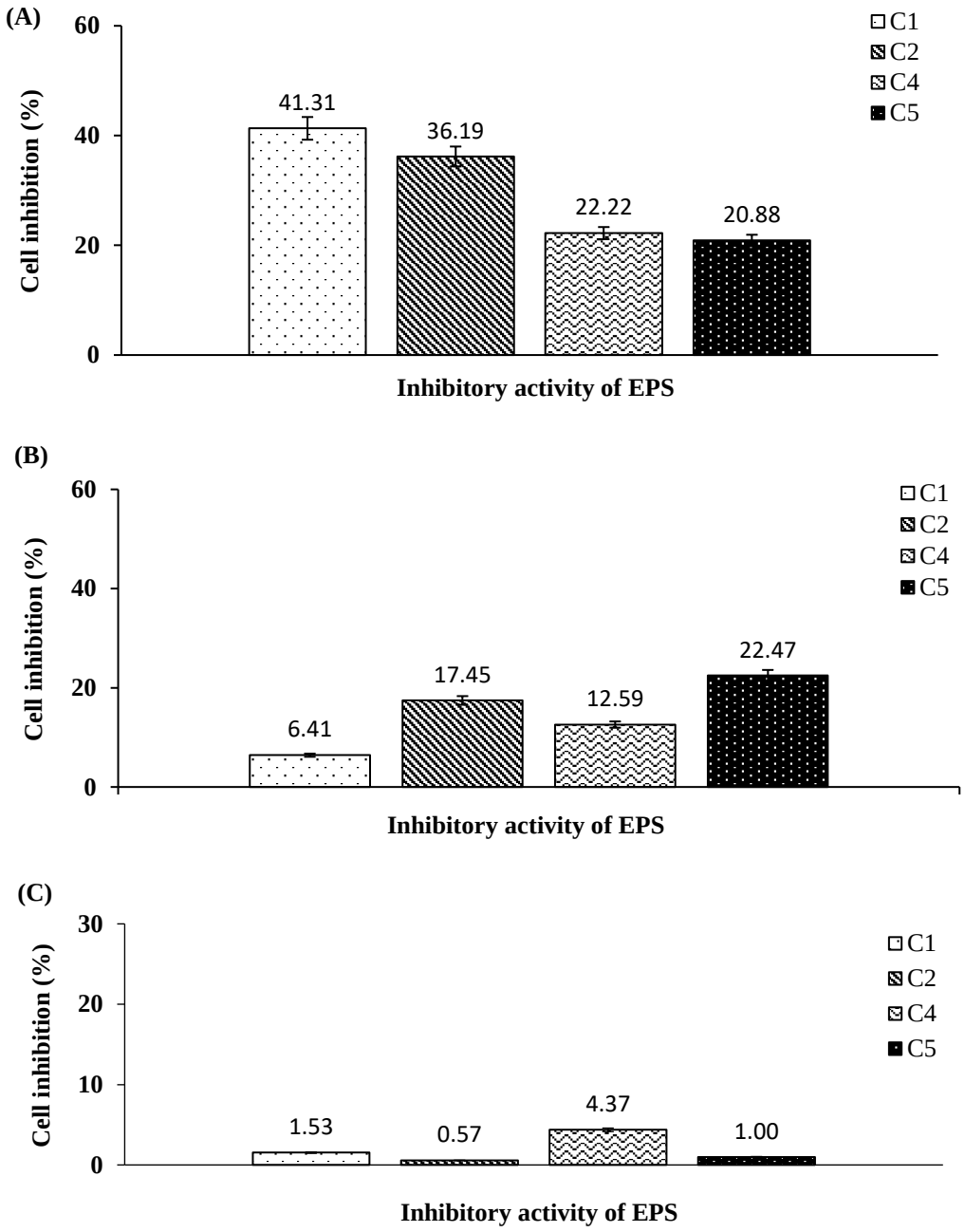

Figure 3 Antiproliferative activities of the bacterial crude EPS samples at the concentration of 1 mg/mL against (A) cervical carcinoma HeLa; (B) liver carcinoma HepG2 and (C) non-transform BAEC cells. C1: *Enterobacter* sp.; C2: *Stenotrophomonas* sp. 1; C4: *Stenotrophomonas* sp. 2; C5: *Alcaligenes* sp.

5. Discussion

The yields of EPS achieved from the four bacterial isolates in this current study were similar (ranging from 9.38 to 10.03 g/L; $p>0.05$). These amounts are significantly lower than that of a marine *Cyanothece* sp., for which the EPS was collected from a 11-day old culture using a similar precipitation method, and the EPS productivity was 22.34 g/L [17]. This is probably just due to the nature of the bacteria; different species of bacteria have different capacities of EPS production. In addition, the medium composition and the age of the culture would influence the amounts of EPSs excreted. But it should be noted here that in addition with the main contents of polysaccharides; the crude EPSs would also contain proteins, polypeptides, and trace amount of metal ions [18-20].

In terms of bioactivities, generally the crude EPS samples were found to possess mild antioxidant activities since the samples showed scavenging activities toward DPPH at relatively high concentrations (5 to 20 mg/mL),

whereas ascorbic acid completely scavenged DPPH at the concentration of 100 µg/mL (data not shown). However, notably, the samples, especially the samples from *Enterobacter* sp. and *Stenotrophomonas* sp. 1, expressed significant antiproliferative activities against HeLa cells. Mild activities on HepG2 of the crude EPSs from *Stenotrophomonas* sp. 1 and *Alcaligenes* sp. also were observed. Surprisingly, these samples displayed significantly low cytotoxicity against non-transformed cells (the antiproliferative activities were smaller than 5%). These data suggest that they would have the potential for further anticancer drug research as they could selectively inhibit the growth of cancer cells with little negative effects on normal cells. The cytotoxicity activities of bacterial EPSs can be different toward different cell lines and can also vary from one species to another. Deproteinated EPSs isolated from *Enterobacter cloacae* showed mild antiproliferative activity towards HeLa cervical cancer cells. At the concentration of 1 mg/mL, the inhibition rate was 19.49% [21]. In another study, EPS from *Pseudomonas fluorescens* at a concentration of 537 µg/mL was found to reduce the viability of HepG2 to 50% [23]. In addition, EPSs from lactic bacteria have been intensively investigated and they have been found to exhibit the highest antiproliferative activities (17-28%) on HT-29 cells at a concentration of 5-50 µg/mL, while displaying no effect on the viability of intestine cells [16,22]. Exopolysaccharides play an important role in bacterial, fungal, and algal defense systems [24]. In recent years, EPSs have demonstrated a considerable potential as anti-tumor drugs. The results of the current study suggest that further research on EPSs produced by the bacteria associated with slime molds is warranted in order to re-evaluate their cytotoxicity toward at least cervical cancer cells (HeLa cell).

6. Conclusions

The results of this present study suggest that the crude EPSs isolated from the bacteria associated with *P. oblonga* and *F. septica* plasmodia display mild antioxidant activities, but they possess quite high antiproliferative activities towards cancer cells. The crude EPS of *Enterobacter* sp. could inhibit the proliferation of HeLa cancer cells with the rate of 41.31% at the concentration of 1 mg/mL, whereas they showed negligible inhibition toward normal cells at the same concentration. Purification of the EPS samples for further investigation would be recommended.

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