

Full Length Research Paper

Optimization of mycelium biomass and exopolysaccharides production by *Hirsutella* sp. in submerged fermentation and evaluation of exopolysaccharides antibacterial activity

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Hirsutella is a hyphomycete that has been used as biopesticide. Many compounds with antimycobacterial activity have been reported. However, there were fewer reports about fermentation condition for the *Hirsutella* and activity of its exopolysaccharides. In the present investigation, the fermentation condition and medium for mycelia growth and exopolysaccharides production were optimized and the antibacterial activity of exopolysaccharides (EPS) from *Hirsutella* sp. was studied. The optimized conditions and medium were as follows: initial pH 5.5, potato extract 20% (w/v), sucrose 2.5%, peptone 0.5%, K₂HPO₄ 0.2%, MgSO₄ 0.05% and fermentation for 4 days. The highest production of EPS and mycelium biomass yield reached 2.17 and 10.06 g/l, respectively. The content of sugar and protein of EPS were 92.7 and 5.2%, respectively. The monosaccharide component of EPS was mannose, galactose and glucose with a molar ratio of 4.0:8.2:1.0. Its molecular weight was 23 kDa. The antibacterial effect of EPS was observed that they were most effective against gram-positive bacterium, especially *Bacillus subtilis* and *Micrococcus tetragenus*. The MIC of exopolysaccharides to *B. subtilis* and *M. tetragenus* was 1 and 2.5 mg/ml, respectively. These results are helpful to expand the application of *Hirsutella* sp. as a biological pesticide.

Key words: *Hirsutella* sp., exopolysaccharides, mycelium biomass, antibacterial activity.

INTRODUCTION

The hyphomycete genus *Hirsutella* Pat. includes about 94 species (Mycobank, 2007), some of them are pathogenic to insects, mites and other invertebrates. Thus, they have been regarded as biological control of plant-parasitic nematodes. For example, *Hirsutella rhossiliensis* and *Hirsutella minnesotensis* were used to control the soybean cyst nematode (Chen and Liu, 2005; Zhang et al., 2008). And *Hirsutella thompsonii* was also treated as a potential microbial control agent (Kanga and James, 2002; Kumar and Singh, 2008). Meanwhile, many active compounds have been found from *Hirsutella*, such as the

insecticidal protein hirsutellin A, antimycobacterial cyclohexadepsipeptide Hirsutellide A, and antimycobacterial alkaloids Hirsutellones A - E (Liu et al., 1995; Vongvanich et al., 2002; Isaka et al., 2005).

In the previous survey, *Hirsutella sinensis* was identified as the anamorph of *Cordyceps sinensis* (Chen et al., 2001; Liu et al., 2001). It is known that *Cordyceps* have been regarded as popular and effective folk medicines for a long time. The disease curing activities are owed to the active components in the fungi including amino acids, vitamins, polysaccharides, cordycepin and adenosine (Jiao et al., 1990). Furthermore, polysaccharides have been demonstrated in numerous biological activities such as immunostimulating, antioxidative, antitumor and antimicrobial activities and they are used in world wide research (Kiho et al., 1993; Kou et al., 1996; Kuo et al.,

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1996; Song et al., 1998; Yang et al., 2000; Yu et al., 2001).

Many fungi that can produce polysaccharides responded to environmental factors directly and to some extent, the nutritional conditions determined the degree of exopolysaccharide formation (Yang and Liau, 1998; Park et al., 2001; Kim et al., 2002). Unfortunately, the influence factors on the exopolysaccharide production by *Hirsutella* have not been previously examined.

Meanwhile, there was little report about the antibacterial activity of exopolysaccharide (EPS) produced by *Hirsutella* sp. In the present study, the optimal submerged fermentation medium and cultural condition for mycelia growth and EPS production by *Hirsutella* sp. in shake flasks were investigated. Moreover, the EPS produced by *Hirsutella* sp. was extracted from the broth and the antibacterial activity was tested.

MATERIALS AND METHODS

Microorganism

A strain of *Hirsutella* sp. parasitized on *Antheraea pernyi* Geurin-Meneville was collected in Baekdu Mountain, China. It was isolated from the fruiting body and was identified by Prof. X.L.Jiang (Ocean University of China). It was deposited in China Center for Type Culture Collection and was numbered CCTCC M207152. The stock culture was maintained on potato dextrose agar (PDA) slants, which were incubated at 24 °C for 15 d and then stored at 4 °C.

Inoculation preparation and culture condition

After *Hirsutella* sp. was initially grown on PDA medium in a Petri dish for 7 d, 5 ml conidia suspension (concentration 2×10^7 conidia/ml) was inoculated into 250 ml flasks containing 100 ml of seed culture medium and incubated on a rotary shaker at 180 r/min and 24 °C for 7 d. The seed culture medium and the basal fermentation medium was consisted of potato extract 20% (w/v), sucrose 2%, yeast extract 0.1%, K_2HPO_4 0.15% and $MgSO_4$ 0.05% at initial pH 6.0. The fermentation experiments were performed in 250 ml flasks containing 100 ml of the medium after inoculating with 5% (v/v) of the seed culture. Incubation was conducted at 24 °C on a rotary shaker at 180r/min.

Mycelia dry weight

The amount of mycelia dry weight in freely suspended cultures was determined by filtering mycelia through tared filter paper (Whatman No. 1) and dried to a constant weight at 70 °C overnight.

Determination of the initial pH in the medium and incubation times

The effect of the initial media pH was investigated in the basal medium (potato extract 20% (w/v), sucrose 2%, yeast extract 0.1%, K_2HPO_4 0.15% and $MgSO_4$ 0.05%) at different values of initial pH (4.0 -7.0) in shake-flask cultures for 5 d. The initial pH condition was adjusted after sterilization of the media. The effect of incubation times on mycelium biomass and EPS production was studied in the basal medium from 1 to 7 d.

Selection of carbon and nitrogen sources for fermentation in shake flasks

The effect of carbon sources on mycelium biomass and EPS production was investigated by a set of experiments using four kinds of carbon sources separately at the concentration of 25 g/l based on the basal medium, including glucose, sucrose, maltose and soluble starch. The effect of nitrogen sources on biomass and EPS production was investigated using seven kinds of different nitrogen sources separately at the concentration of 5 g/l based on the basal medium, including peptone, yeast extract, soy powder, silkworm pupa powder, NH_4NO_3 , $(NH_4)_2SO_4$ and $NaNO_3$. Concentrations of other components in the medium were fixed at original levels. Fermentation was carried out under optimized initial pH and incubation time.

Optimization the culture medium by orthogonal matrix method

L_9 (3^4) orthogonal array was used for the optimization of biomass and EPS production. Four factors on three different levels were involved in the design, including sucrose, peptone, K_2HPO_4 and $MgSO_4$. Fermentation was carried out under optimized initial pH and incubation time. Data were analyzed using MINITAB 13.30 (Minitab Inc., State College, PA., USA). EPS production was considered as the primary response for optimization and biomass was taken into account as a secondary response. All experiments including fermentation and assay were performed in triplicate.

Preparation of the crude EPS

The cultivated broth of *Hirsutella* sp. was centrifuged at 4,500r/min to separate the mycelia from the liquid. The supernatant was pooled followed by vacuum-concentrated at 50 °C till giving a final volume of about 100 ml. The supernatant was precipitated by adding two times of 95% ethanol (v/v) to the volume and stirred vigorously. After the resulting mixture was left at 4 °C overnight, it was separated by centrifugation, washed exhaustively with 95% ethanol, dissolved in deionized water and dialyzed using cellulose sacks (Sigma). Then the non-dialyzed portion was lyophilized and the crude polysaccharides were extracted by the Sevag method to remove the dissociative protein (Matthaei et al., 1962).

Determination of the chemical characterization of the EPS

Total sugar content in EPS was determined by the phenol-sulfuric acid method using D-glucose as the standard (Dubois et al., 1956). Protein contents were also determined, by the Bradford method using a Bio-Rad Kit, with bovine serum albumin as standard.

The EPS was hydrolyzed in 3 ml 2 mol/l trifluoroacetic acid (TFA) (100 °C, 8h). The monosaccharide was acetylated (Borchardt and Piper, 1970) and determined by gas chromatography (GC) as alditol acetates, using a DB225 capillary column (30 cm × 25 mm), temperature 220 °C and a flame ionization detector. Inositol was used as an internal standard.

Molecular weight estimation of the EPS

The molecular weight of EPS was determined by gel permeation chromatography (GPC) on a SB-804 HQ column. The sample was dissolved in distilled water at a concentration of 5 mg/ml and 20 ml of this solution was injected. The column was operated at 40 °C and eluted with distilled water at a flow rate of 0.6 ml/min. The column was calibrated by various standard dextrans.

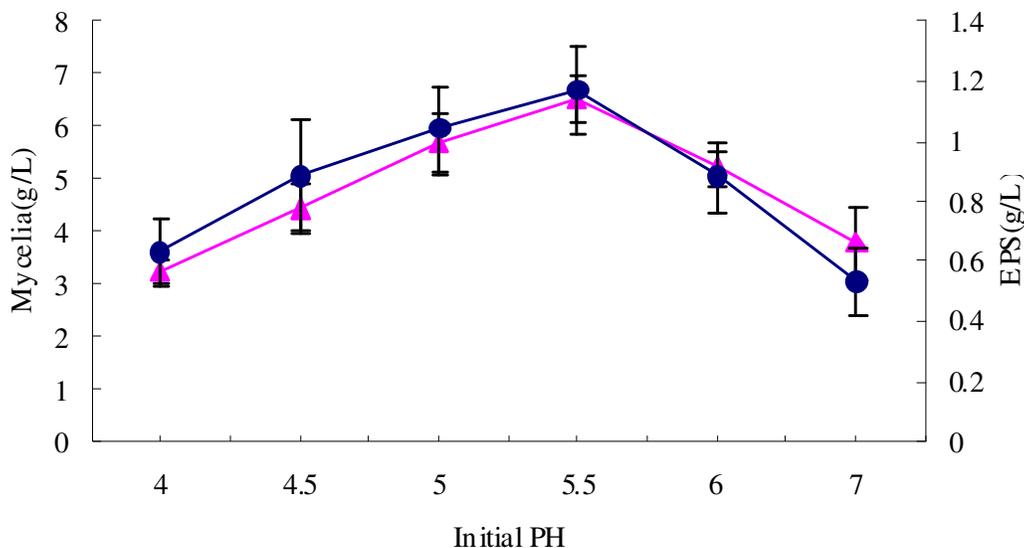


Figure 1. Effect of initial pH on the mycelial growth and exopolysaccharide production by *Hirsutella* sp. in shake-flask culture: (●) the concentration of exopolysaccharide and (▲) mycelial dry weight.

Determination of antibacterial activity of the EPS

Bacterial strains used for determining antibacterial activity were *Pseudomonas putida*, *Escherichia coli*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *Salmonella* sp., *Shigella flexneri*, *Aeromonas* sp., *Micrococcus tetragenus*, *Staphylococcus aureus*, *Micrococcus lysodeikticus* and *Bacillus subtilis*, which were obtained from the microbiology laboratory in Ocean University of China. Antibacterial activity was evaluated by the filter paper disc method (Nath et al., 2008). The polysaccharides were dissolved in a minimum amount of deionized water. A suspension of an overnight cultural of each test organism containing 1.0×10^3 cells/ml was added to the medium. Whatmann filter paper discs (diameter 6.5 mm) were sterilized by dry heat at 140 °C for 1 h and saturated with the solution of EPS (concentration: 10,20,50 mg/ml). These discs were air-dried at room temperature to remove any residual solvent, which might interfere with the determination. These discs were then placed on the surface of a sterilized agar nutrient medium that was inoculated with test microorganism (by using a sterile swab) and air-dried to remove the surface moisture. The thickness of the agar medium was kept equal in all Petri dishes. Before incubation, Petri dishes were placed for 1 h in a cold room (5 °C) to allow diffusion of the compound from the disc into the agar plate. Thereafter, the discs were incubated at 37 ± 1 °C for 20 - 24 h. The zone of inhibition of growth was measured, which indicates the inhibitory activity of the compounds on the growth of the microorganism.

Determination of the minimum inhibitory concentrations (MIC) to bacteria

MIC was determined by methods of Chen and Tu (1994). Firstly, EPS (50 mg/ml) was dissolved a minimum amount of deionized water (1% final concentration) and diluted by nutrient broth culture medium in 96-well cell culture cluster. The dilution concentration of EPS in every well was 10, 5, 2.5, 1, 0.5 and 0.25 mg/ml, respectively. Then bacterial culture solution (*P. putida*, *V. anguillarum*, *M. tetragenus*, *S. aureus* and *B. subtilis*), with contents of 10^7 colony-forming units/ml, was added into each well. After the samples were incubated at 37 °C for 20 - 22 h, the MIC of bacteria comprised the

concentration of the blank well.

Statistical analysis

Unless otherwise stated, data were expressed as mean \pm SD and analyzed statistically by ANOVA method.

RESULTS AND DISCUSSION

Optimization of the fermentation condition and medium

Effect of initial pH

The pH of the culture medium is a vital factor that governs mycelial growth and exopolysaccharide production. The optimal pH for the mycelial growth and exopolysaccharide production was 5.5; at higher values of pH the mycelial biomass and exopolysaccharide production declined sharply (Figure 1). The highest mycelium biomass and exopolysaccharide production were 6.53 and 1.04 g/l, respectively. Likewise, other researchers have a reported optimum pH of 5.5 for other exopolysaccharide-synthesizing fungi, such as *Pleurotus pulmonarius* (Nour et al., 2004).

Time course of cultivation

Figure 2 displays the kinetics of mycelial growth and exopolysaccharide accumulation by *Hirsutella* sp. in the basal medium of shake-flask cultures. We observed a rapid increase in the exopolysaccharide concentration within the first 3 days, during which time the cells entered

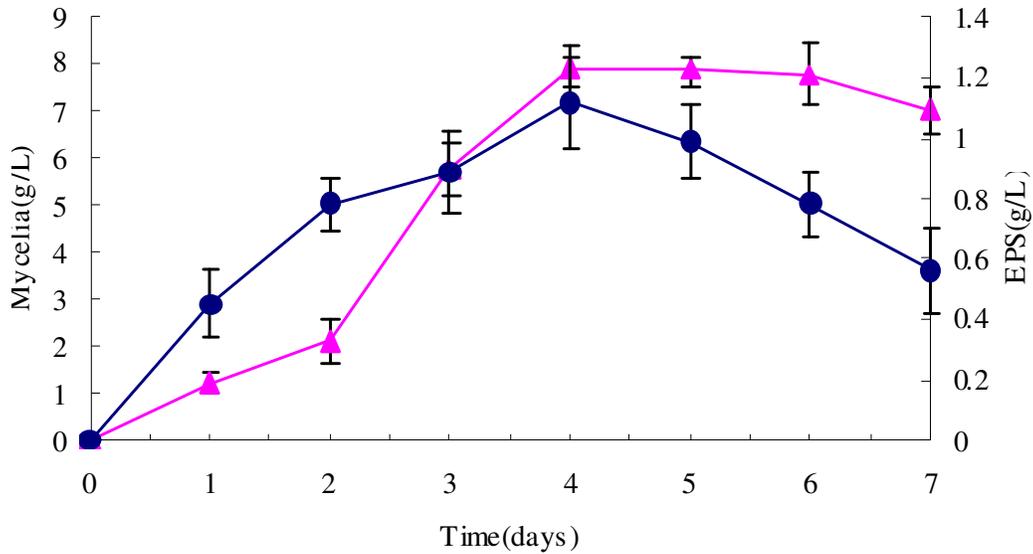


Figure 2. Typical time profiles of the mycelial growth and exopolysaccharide production by *Hirsutella* sp. in shake-flask culture under the basal medium: (▲) mycelial dry weight and (●) the concentration of exopolysaccharide.

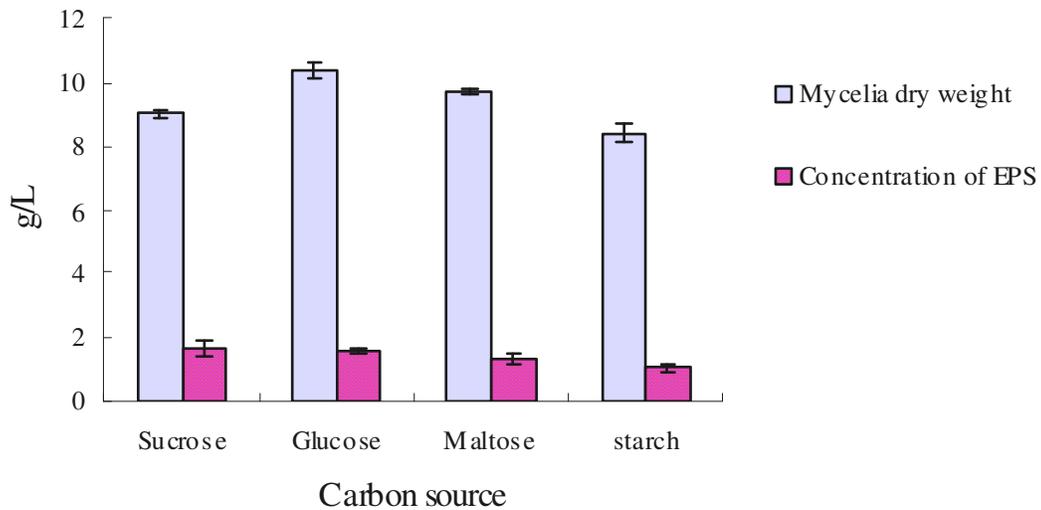


Figure 3A. Effect of carbon sources on the mycelia growth and the EPS produced by *Hirsutella* sp. in 250 ml flasks containing 100 ml of medium. All points represent the means \pm SD of triplicates.

an exponential growth phase. The mycelial biomass and exopolysaccharide concentrations were the highest at day 4 and the corresponding maximum values were 7.94 and 1.12 g/l, respectively; after day 7, these values decreased. Thus, we performed all of the following fermentations for 4 days, optimizing the conditions in the flask culture.

Effects of carbon and nitrogen sources

It was reported that sucrose was the most suitable carbon

source for mycelium growth production in *Cordyceps militaris* (Park et al., 2001; Kim et al., 2003), while glucose was selected for *Phellinus gilvus* (Hwang et al., 2003; Xu et al., 2003). Maltose was also known as an efficient carbon source for EPS production by mushrooms (Bae et al., 2000; Wu et al., 2003). Starch was chosen as carbon source for *Pleurotus tuber-regium* production; it also can get maximum yield of mycelium after 10 days fermentation (Jung et al., 1997). According to Figure 3A, the concentration of EPS was 1.62 and 1.54 g/l, respectively, when sucrose and glucose were used as a carbon source. They were no significant difference. Thus, we

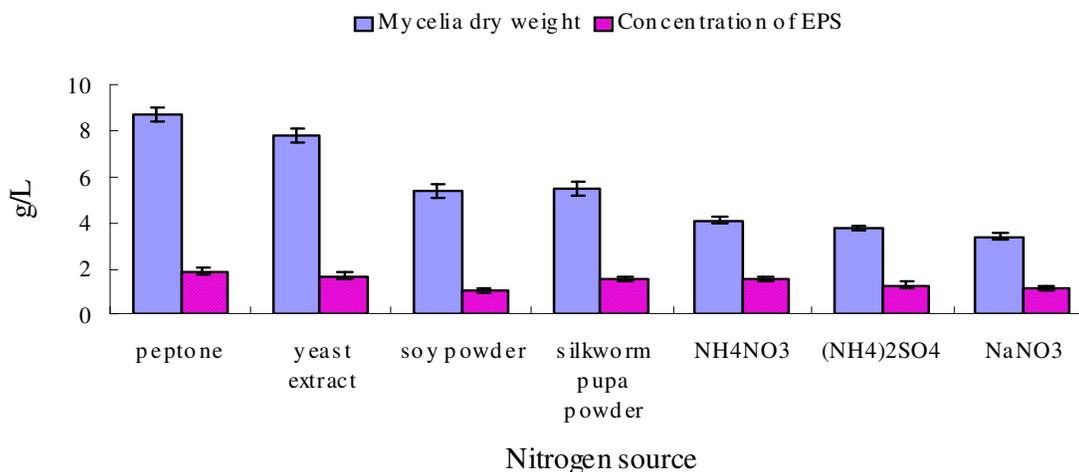


Figure 3B. Effect of nitrogen sources on the mycelia growth and the EPS produced by *Hirsutella* sp. in 250 ml flasks containing 100 ml of medium. All points represent the means \pm SD of triplicates

Table 1. Result of L9 (3^4) orthogonal design to the mycelium growth and EPS of *Hirsutella* sp. in shake flasks.

No.	A Sucrose	B Peptone	C K ₂ HPO ₄	D MgSO ₄	Mycelia dry weight (g/l)	Exopolysaccharide (g/l)
1	2%	0.3%	0.1%	0.05%	6.38 \pm 0.32	1.18 \pm 0.27
2	2%	0.5%	0.15%	0.1%	6.46 \pm 0.46	1.65 \pm 0.38
3	2%	0.7%	0.2%	0.15%	7.34 \pm 0.29	1.43 \pm 0.23
4	2.5%	0.3%	0.15%	0.15%	6.58 \pm 0.41	1.31 \pm 0.22
5	2.5%	0.5%	0.2%	0.05%	10.06 \pm 0.23	2.17 \pm 0.37
6	2.5%	0.7%	0.1%	0.1%	9.1 \pm 0.31	1.57 \pm 0.38
7	3%	0.3%	0.2%	0.1%	8.65 \pm 0.42	1.46 \pm 0.31
8	3%	0.5%	0.1%	0.15%	7.93 \pm 0.15	1.78 \pm 0.21
9	3%	0.7%	0.15%	0.05%	7.31 \pm 0.26	1.56 \pm 0.35

Values are mean \pm SD of triple determinations.

chose sucrose as the carbon source based on the economic consideration for the large scale fermentation. The effects of nitrogen sources on the production of mycelium biomass and EPS were studied in the fermentation medium containing various nitrogen sources. As illustrated in Figure 3B, the highest levels of mycelium biomass and EPS were obtained when peptone was used as the sole nitrogen source. The dry weight of the biomass and the concentration of EPS were 8.7 and 1.88 g/l, respectively. Additionally, it was found in the study that organic nitrogen source are more suitable for the biomass production. It was reported that organic nitrogen sources were absorbed by the cells easier than the inorganic ones (Jung et al., 1997; Kang et al., 1997).

Orthogonal matrix method

The carbon sources, nitrogen sources and mineral salts

were regarded as correlated factors of the culture medium, especially the carbon and nitrogen sources. To study the relationships between medium components and optimize their concentration for mycelium growth and EPS production, the orthogonal matrix L9 (3^4) method was applied. In 9 experiments of L9 (3^4) orthogonal projects, we selected and varied three concentration levels as shown in Table 1. The concentration was chosen after single factor experiment on different concentration of carbon and nitrogen sources, the results were not showed here. The fermentation medium used in these experiments included 20% potato extracts. The results are included in Table 2. According to the magnitude of R, the decreasing order of effects of the factors on mycelium growth was determined as K₂HPO₄ > sucrose > peptone > MgSO₄. The effects of the factors on the concentration of EPS was determined as peptone > sucrose > K₂HPO₄ > MgSO₄. According to the magnitude of K, to obtain a high mycelium growth and a high production of

Table 2. Analysis of media on mycelia biomass and exopolysaccharide production of *Hirsutella* sp. in shake flask culture with L9 (3⁴) orthogonal test.

Parameter	Mycelium dry weight(g/l)				Exopolysaccharide(g/l)			
	A	B	C	D	A	B	C	D
K1	6.73	7.2	7.8	7.92	1.42	1.32	1.51	1.64
K2	8.58	8.15	6.78	8.07	1.68	1.87	1.51	1.56
K3	7.96	7.92	8.68	7.28	1.60	1.52	1.69	1.51
R1	1.85	0.95	1.9	0.79	0.26	0.55	0.18	0.13

Symbols A, B, C and D represent factors of sucrose, peptone, K₂HPO₄ and MgSO₄, respectively.

K_i = \sum mycelia biomass or exopolysaccharide in thrice experiment at Xi/3. Values are means \pm SD of triple determinations.

R_j = maximum K_i - minimum K_i. Values are means \pm SD of triple determinations.

Table 3. Antibacterial activity (diameters of inhibition zone; mm) of exopolysaccharide from *Hirsutella* sp.

Microorganism	Concentration of polysaccharide (mg/ml)		
	10	20	50
<i>Pseudomonas putida</i>	5.12 \pm 0.87	12.03 \pm 1.63	18.43 \pm 2.33
<i>Escherichia coli</i>	—	—	6.14 \pm 1.45
<i>Vibrio parahaemolyticus</i>	—	5.07 \pm 1.65	8.18 \pm 2.04
<i>Vibrio anguillarum</i>	3.04 \pm 1.02	10.41 \pm 1.97	15.05 \pm 1.87
<i>Vibrio alginolyticus</i>	—	7.14 \pm 2.22	12.87 \pm 1.45
<i>Salmonella</i>	—	—	5.25 \pm 1.36
<i>Shigella flexneri</i>	—	—	8.42 \pm 1.78
<i>Aeromonas</i>	—	5.02 \pm 0.96	9.19 \pm 2.33
<i>Micrococcus tetragenus</i>	7.02 \pm 2.43	16.26 \pm 1.73	30.06 \pm 1.95
<i>Staphylococcus aureus</i>	4.25 \pm 1.46	14.21 \pm 2.05	24.98 \pm 1.28
<i>Micrococcus lysodeikticus</i>	—	7.05 \pm 1.35	17.01 \pm 1.89
<i>Bacillus subtilis</i>	6.16 \pm 1.57	15.23 \pm 1.45	35.32 \pm 2.63

The data presented are means \pm SD of three determinations.

EPS, the optimal concentration was sucrose 2.5%, peptone 0.5%, K₂HPO₄ 0.2%, MgSO₄ 0.05%. Consequently, the maximal mycelium yield reached 10.06 g/l and the maximal EPS production was 2.17 g/l.

Preliminary study on crude EPS

Analysis of the crude EPS extracts from *Hirsutella* sp.

The *Hirsutella* sp. was cultivated and the broth was treated according to the methods mentioned above to get the crude exopolysaccharide. The crude exopolysaccharide extract from *Hirsutella* sp. was obtained with yields of 0.17% (lyophilized weight). The result of chemical analysis of the crude polysaccharides and its extract are summarized as following: white powder, good water-solubility, content of sugar and protein in the extract solution being in turn 92.7, 5.2%. The monosaccharide component of EPS was mannose, galactose and glucose

with a molar ratio of 4.0:8.2:1.0. The molecular weight of EPS was 23 kDa.

Antibacterial activity of the EPS *in vitro*

Antibacterial activity of the purification polysaccharides *in vitro* is showed in Table 3. Among all microorganisms test, the exopolysaccharides was found to be most effective against gram-positive bacterium, especially *B. subtilis* and *M. tetragenus* with a zone of inhibition 30.06 and 35.32 mm, respectively, at a concentration of 50 mg/ml. Moreover, with the increasing concentration, the EPS showed significant antibacterial activity against all selected microorganisms by a dose-dependent manner. As elucidated in Table 3, the activity was lowest in the inhibition of gram-negative bacterium, especially *E. coli*, *Salmonella* and *Shigella flexneri* at a low concentration whose inhibition zones were not observed. Even though for all that, the EPS also showed the effect towards *P.*

putida, *V. anguillarum*, *M. tetragenus*, *S. aureus* and *B. subtilis* at low concentration. In addition, the MIC of EPS to *P. putida*, *V. anguillarum*, *M. tetragenus*, *S. aureus* and *B. subtilis* was 5, 10, 2.5, 5 and 1 mg/ml, respectively. The antimicrobial activity of polysaccharides depends on a variety of factors, including its type (e.g., plain or derivative), molecular weight, composition and even the chelating activities (Rabea et al., 2003). Different theories have been put forward to explain polysaccharides' antimicrobial mode of action, especially molecular weight. Raafat reported that molecules of chitosan larger than 10 kDa were responsible for its antimicrobial activity (Raafat et al., 2008). Our results indicated that the EPS had a significant antimicrobial activity. Its molecular weight was 23 kDa. Therefore, future work will clarify the molecular relevance to its antimicrobial activity.

Conclusion

In the research described here, we determined that the concentrations of the components of the medium, the initial value of pH and the choices of the carbon sources, nitrogen sources are the factors that mostly affect the production of exopolysaccharides by *Hirsutella* sp. When cultivated in the optimal fermentation medium potato extract 20%, sucrose 2.5%, peptone 0.5%, K₂HPO₄ 0.2%, MgSO₄ 0.05% at pH 5.5 for 4 days, as concluded in this paper, the maximal mycelium yield reached 10.06 g/l and the maximal EPS production was 2.17 g/l. Further more, the result indicated that the exopolysaccharides produced by *Hirsutella* sp. showed antibacterial activity against gram-positive bacterium, especially *M. tetragenus* and *B. subtilis*. This could provide more data for application of *Hirsutella* sp. as a biological pesticide.

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