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ORIGINAL ARTICLE

In vitro antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom

Li Bin · Li Wei · Chen Xiaohong · Jiang Mei · Dong Mingsheng

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Abstract A melanin derived from *Auricularia auricula* was characterized based on ultraviolet-visible (UV), infrared (IR), electron paramagnetic resonance (EPR) spectra and chemical tests. The antibiofilm activity of the isolated *A. auricula* melanin against *Escherichia coli* K-12, *Pseudomonas aeruginosa* PAO1 and *P. fluorescens* P-3 was examined using crystal violet and LIVE/DEAD BacLight staining as well as confocal laser scanning microscopy (CLSM). The results showed that *A. auricula* melanin could inhibit significantly biofilm formation of *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3, while no inhibitory effects on growth were found. CLSM analysis showed that *A. auricula* melanin treatment resulted in thinner and looser biofilm compared with the control. The current study reveals for the first time the antibiofilm activity of melanin derived from *A. auricula.*

Keywords *Auricularia auricula* · Melanin · Antibiofilm · *Escherichia coli* · *Pseudomonas aeruginosa* · *Pseudomonas fluorescens*

Introduction

Biofilms are film-like structures formed by aggregates of bacterial cells on biotic and abiotic surfaces. These naturally existing biofilms are major threat to human beings (Wang et al. 2007), with 80% of bacterial infections being caused by biofilms (Rasmussen and Givskov 2006). Bacteria occurring in biofilms are between 10- and 1,000-fold more resistant to antibiotics, leading to serious clinical problems, particularly regarding avoidance of host immune systems (Brooun et al. 2000). Biofilms are a major cause of nosocomial infections. They cause persistent infections by forming biofilms on the surface of in vivo medical devices such as contact lens, artificial joints, and synthetic valves (Wang et al. 2007). Bacterial attachment to surfaces and subsequent biofilm formation are important steps in the establishment of chronic infections and persistence in host tissues (Costerton et al. 1999). Biofilms can delay wound healing significantly (Percival and Cutting 2009). In the food industry, biofilms can be a source of recalcitrant contaminations, causing food spoilage, and are possible sources of public health problems such as outbreaks of food-borne pathogens. Biofilms are difficult to eradicate due to their resistant phenotype (Camilli and Bassler 2006; Domka et al. 2007). However, the mechanisms by which bacteria growing in biofilms attain this resistance are still unknown. Moreover, the resistance to antimicrobial agents of biofilm-embedded bacteria make it important to search for novel agents that can effectively kill these bacteria. Novel strategies are therefore required to deal with biofilm-mediated infections.

Auricularia auricula, commonly known as 'tree-ear', is a species of edible mushroom found worldwide. From ancient times, the mushroom has been used widely in Chinese cuisine, and is known for its pharmaceutical effects in folk medicine. *Auricularia auricula* has been reported to have many biological activities, including antitumor (Misaki et al. 1981; Mizuno et al. 1995), hypocholesterolemia (Cheung 1996), hypoglycemic (Takeujchi et al. 2004), antioxidant (Finkel and Holbrook 2000; Acharya et al. 2004) and anticoagulant (Yoon et al. 2003) activity. These potent medicinal functions are mediated mostly by non-starch polysaccharide components, especially beta-glucans (Zhang et al. 1995).

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Nowadays, there is considerable interest in the exploitation of this fungus. Most recently, Zhu et al. (2011) reported that the extracted pigments could effectively inhibit the production of violacein, a quorum-sensing (QS)-regulated behavior in *Chromobacterium violaceum* CV026.

Here, the pigment from *A. auricula* fruiting bodies was characterized, and the ability of this pigment to inhibit biofilm formation of *Escherichia coli* K-12, *Pseudomonas aeruginosa* PAO1 and *P. fluorescens* P-3 was investigated by monitoring biofilms using confocal laser scanning microscopy (CLSM).

Materials and methods

Bacterial strains and growth conditions

Pseudomonas aeruginosa PAO1 and *P. fluorescens* P-3 were procured from Fisheries Research Institute, Shanghai, China. *Escherichia coli* K-12 was supplied by Nanjing Center for Disease Control and Prevention, Jiangsu province, China. The three bacteria were used to determine biofilm formation and were cultured routinely in Luria-Bertani (LB) broth (0.5% yeast extract, 1% tryptone, 0.5% NaCl) at 37°C for the exponential growth phase experiments.

Extraction and purification of *A. auricula* pigment

The fruiting bodies of *A. auricula* produced in Liaoning province, China were purchased from a local supermarket. Taxonomic identification of fruiting bodies was confirmed by a senior mycologist (Xin Zhentao, Institutes of Edible Mushroom, Shanghai Academy of Agricultural Sciences), based on its clearly ear-like shaped fruit bodies and its cylindrical basidia with three transverse septa (internal cross-walls dividing the hyphae). Analysis of internal transcribed spacer (ITS) regions was used to determine species identification of fruiting bodies of *A. auricula*. After milling of dried fruiting bodies to a fine powder, 10 g dried powder was extracted using 300 ml 3 M HCl for 1 h at 60°C. The melanin precipitates were recovered by centrifugation at 5,000 rpm for 20 min and sonicated with 300 ml 1.5 M NaOH for 1 h. The supernatant was acidified with 2 M HCl to pH 2, then incubated for 12 h at 80°C. The precipitate obtained was purified by acid hydrolysis using 6 M HCl at 100°C for 2 h to remove carbohydrates and proteins, and treated with chloroform to wash away lipids. The precipitate was then dried at 30°C, re-dissolved in 2 M NaOH and centrifuged at 5,000 rpm for 20 min. The supernatant was precipitated by the addition of 2 M HCl, washed with distilled water and lyophilized, and then stored at −20°C for further experiments (Selvakumar et al. 2008).

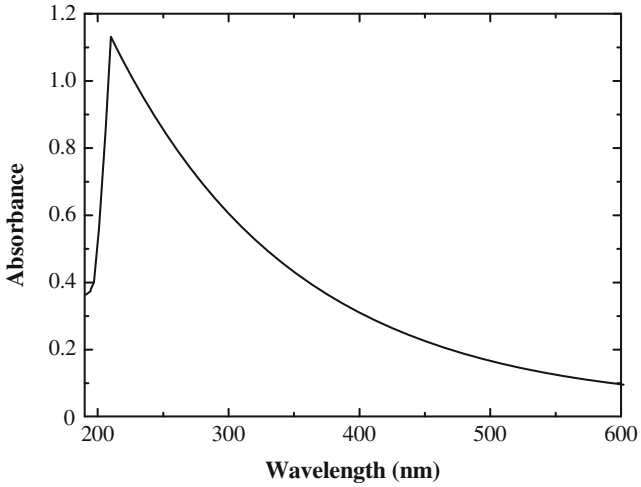

Fig. 1 UV absorption spectrum of *Auricularia auricula* pigment

Characterization of *A. auricula* pigment

The physical and chemical characteristics of *A. auricula* pigment were measured according to standard procedures (Paim et al. 1990), including solubility in water and common organic solvents (ethanol, hexane, acetone, benzene and chloroform), oxidized bleaching by $KMnO_4$, $K_2Cr_2O_7$, NaOCl and H_2O_2, and reaction with $FeSO_4$. The melanin obtained from *A. auricula* was dissolved in 3 ml borate buffer (pH 8) and its ultraviolet-visible (UV) absorption spectrum was recorded by a U-1800 HITACHI spectrophotometer (Hitachi, Japan) (Ravishankar et al. 1995). The infrared (IR) spectrum was recorded with a Thermo IR 200 Fourier transform infrared spectrometer (Thermo, Barrington, IL) in the frequency range 4,000–500 cm^{-1}. KBr samples were obtained from a uniformly prepared mixture contained 2 mg *A. auricula* pigment sample and 150 mg spectrometric grade KBr (Ellis and Griffiths 1974; Ravishankar et al. 1995). The

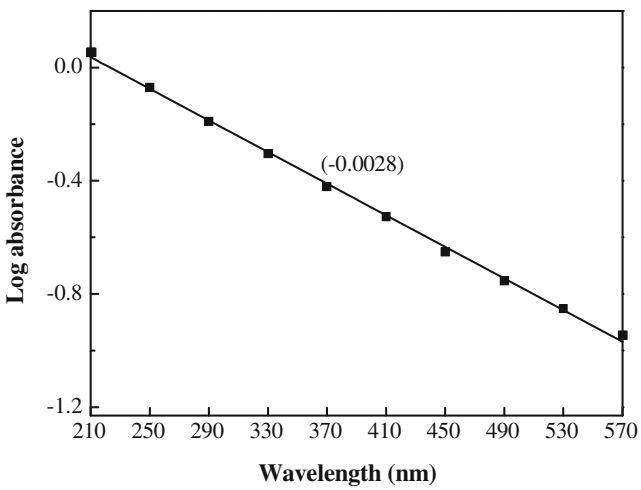

Fig. 2 A plot of log optical density against wavelength

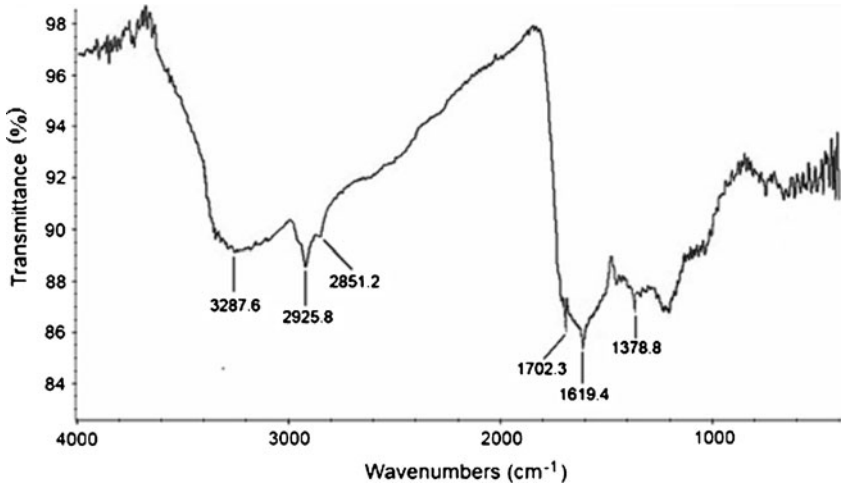

Fig. 3 Infrared (IR) spectrum of *A. auricula* pigment

electron paramagnetic resonance (EPR) spectrum of the pigment was taken in a Bruker EMX- 10/12 EPR Spectrophotometer (Bruker, Germany).

Determination of minimal inhibitory concentration

Minimal inhibitory concentration (MIC) was determined by a microtiter broth dilution method as recommended by the Clinical and Laboratory Standards Institute (Khan et al. 2009). The MIC was defined as the lowest antibiotic concentration that yielded no visible growth. Cell suspensions (100 μl) were inoculated into the wells of 96-well microtitre plates in the presence of *A. auricula* pigment with different final concentrations. The inoculated microplates were incubated at 37°C for 24 h before being read.

Biofilm formation inhibition assay

The quantification of biofilm formation using minimal biofilm eradication concentration assay system devices (MBEC, Innovotech, Edmonton, Canada) was performed according to the manufacturer's instructions and as described previously (Girennavar et al. 2008). The MBEC device is similar to a 96-well plate. The lid of the MBEC device contains 96 pegs on which biofilms formed. The planktonic cells were growing in the 96 wells. Overnight cultures were inoculated (1: 100) in LB media for *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3. Aliquots of these diluted cultures were added to a 96-well plate. To assess the impact of *A. auricula* pigment on biofilm formation, *A. auricula* pigment was dissolved in appropriate concentrations of dimethylsulfoxide (DMSO), sterilized by filtration through a 0.22 μm membrane filter and added to these culture suspensions. *Auricularia auricula* melanin was omitted in the control. The lid of the MBEC device (containing the pegs on which biofilms formed) was inserted into the plate. The plate was incubated at 37°C for 24 h without shaking. After incubation, suspension cultures in wells were spread on LB plates to confirm any antibacterial activity of the samples, and the peg lid was washed three times with sterile distilled water. The biofilms on the peg lid were stained with 125 μl 0.3% crystal violet per well for 20 min. The excess dye was removed by washing the peg lid three times with sterile distilled water. Dye associated with the attached biofilm was dissolved with 200 μl 95% ethanol. Aliquots of the solubilized dye from each well (125 μl) were transferred to a separated well in an optically clear flat-bottom 96-well plate and the optical density (OD) was measured at 590 nm. Each data point was averaged from eight replicate wells and the standard deviation (SD) was calculated.

Confocal laser scanning microscopy

The biofilms were monitored under a CLSM (Leica TCS SP2; Leica Microsystems, Heidelberg, Germany) as described previously (Berney et al. 2007; Rasmussen

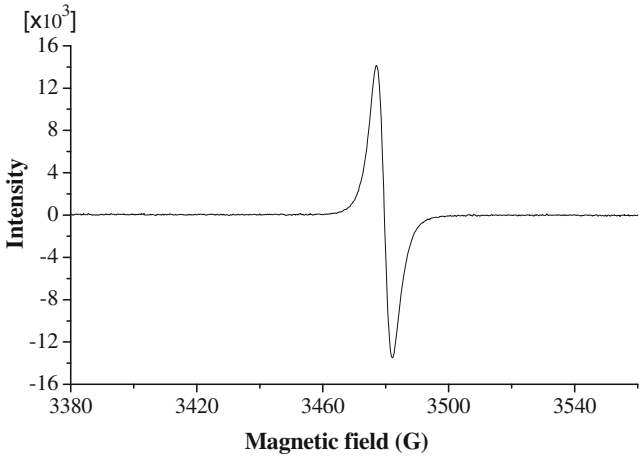

Fig. 4 Electron paramagnetic resonance (EPR) spectrum analysis of *A. auricula* pigment

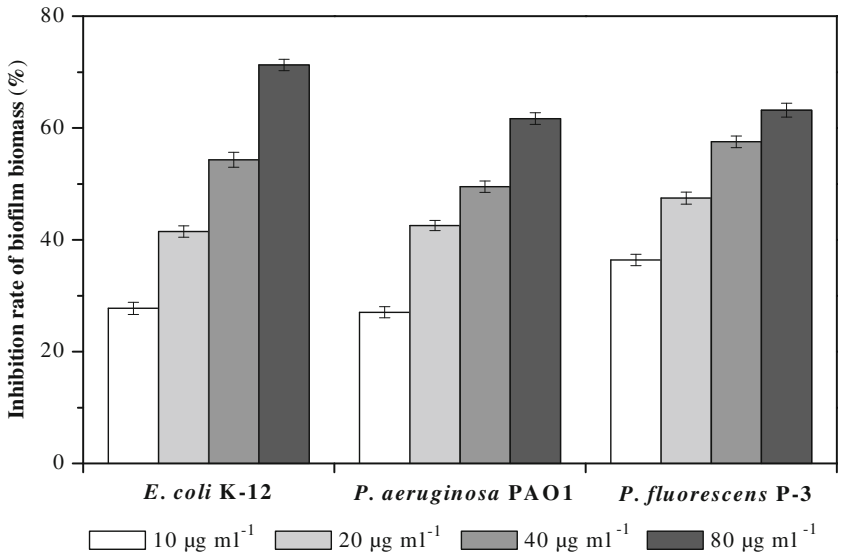

Fig. 5 Effect of *A. auricula* melanin on biofilm formation by *Escherichia coli* K-12, *Pseudomonas aeruginosa* PAO1 and *P. fluorescens* P-3

et al. 2005). Overnight cultures of strains incubated in LB medium were diluted to a final density of 1.0×10^6 CFU ml^{-1} with fresh medium. CLSM was performed on biofilms formed on plastic coverslips (0.2 mm thick and 13 mm in diameter; Nunc, Roskilde, Denmark) by dispensing 1 ml cell suspensions into the wells of 24-well microtitre plates. Plates were incubated statically at 37°C for 48 h. The biofilms were exposed to the *A. auricula* pigment (80 μg ml^{-1}) for a further 24 h. Coverslips with cells grown in the medium with DMSO (1%) were employed as control. At the end of incubation, the coverslips were gently washed with sterile distilled water and stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's introductions. Stained coverslips were washed gently twice with sterile distilled water and observed with CLSM. The Leica confocal software was used for analysis of biofilm images, which allowed for collection of z-stacks three-dimensional reconstruction. Images were acquired from random positions of biofilms formed on the upper side of the coverslips. The thicknesses of biofilms were also determined directly from the confocal stack images.

Statistical analysis

The results were expressed as means ± SD. Analysis of variance (ANOVA) was conducted and differences between variables were tested for significance by one-way ANOVA with post hoc Tukey test using the SPSS V16.0 program. Differences at $P<0.05$ were considered statistically significant. Correlation analysis was carried out using EXCEL program.

Results

Physico-chemical characterization of *A. auricula* pigment

The *A. auricula* melanin exhibited the same, previously reported, physical and chemical properties as natural melanin (Bilinska 1996; Ellis and Griffiths 1974; Paim et al. 1990). It was insoluble in water, ethanol, hexane, acetone, benzene and chloroform, and dissolved only in alkali, precipitated in alkaline $FeCl_3$ and below pH 3. *A. auricula* melanin was bleached in H_2O_2, $KMnO_4$, $K_2Cr_2O_7$ and NaOCl, and produced a blue color with $FeSO_4$/ferricyanide.

Table 1 Effect of *Auricularia auricula* pigment on the growth of *Escherichia coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3. Data are expressed as means ± SD

A. auricula pigment concentration (μg ml^{-1})	*E. coli* K-12 (Log $_{10}$ CFU ml^{-1})	*P. aeruginosa* PAO1 (Log $_{10}$ CFU ml^{-1})	*P. fluorescens* P-3 (Log $_{10}$ CFU ml^{-1})
0 (Control)	8.235±0.013 [a]	9.725±0.002 [b]	7.068±0.002 [c]
DMSO (1%)	8.216±0.003[a]	9.722±0.005[b]	7.066±0.002 [c]
5	8.236±0.006 [a]	9.723±0.002 [b]	7.068±0.003 [c]
10	8.240±0.003 [a]	9.719±0.003 [b]	7.055±0.002 [c]
20	8.238±0.001 [a]	9.724±0.004 [b]	7.067±0.003 [c]
40	8.235±0.002 [a]	9.722±0.004 [b]	7.075±0.004 [c]
80	8.242±0.002 [a]	9.726±0.003 [b]	7.069±0.002 [c]

[a]Different letters indicate significant differences ($P<0.05$) for each concentration among the different pathogenic bacteria

The nature of the melanin was investigated further by UV, IR and EPR. In the UV spectrum, the absorbance of melanin first increased and then progressively decreased as the wavelength increased, in agreement with the results of other (Bell and Wheeler 1986). The absorption spectrum showed characteristic absorption peaks in the UV regions at 210 nm (Fig. 1), due to the presence of many complex conjugated structures in the melanin molecule (Cockell and Knowland 1999). The decrease in absorption with increasing wavelength is almost linear in the case of melanins. Hence, the slopes of linear plots are often used to identify melanins (Chet et al. 1967; Ellis and Griffiths 1974; Ravishankar et al. 1995). Daniel (1938) showed that the log of optical density of a melanin solution when plotted against wavelength produces a linear curve with negative slopes from −0.0015 to −0.0030 (Ellis and Griffiths 1974; Suryanarayanan et al. 2004). Such characteristic straight lines with negative slopes have been obtained for some terrestrial and marine fungi (Chet et al. 1967; Ellis and Griffiths 1974; Ravishankar et al. 1995). The melanin of *Phyllosticta capitalensis* gave a straight line with a negative slope of −0.0015 (Suryanarayanan et al. 2004). The pigment of *A. auricula* also gave a straight line with a negative slope of −0.0028 (Fig. 2).

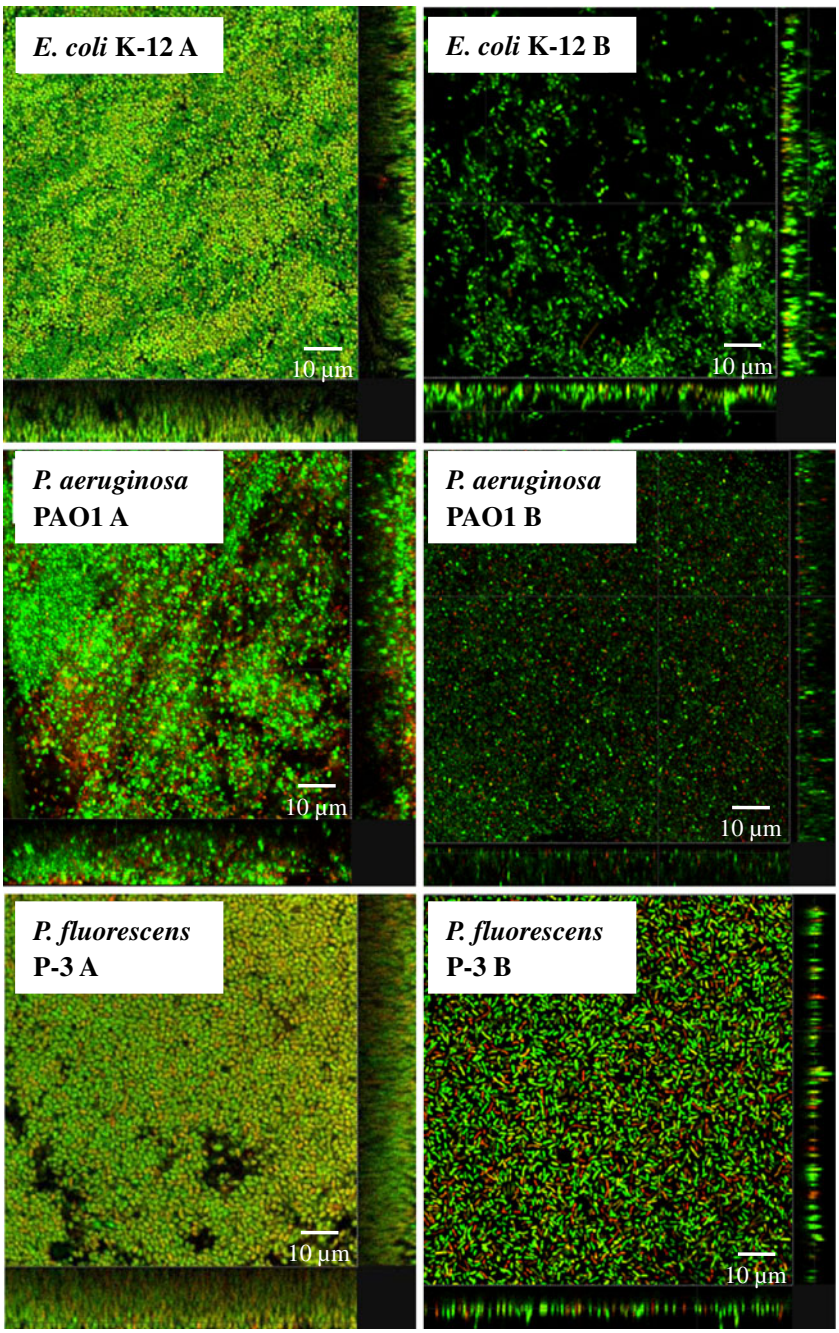

Fig. 6 Confocal laser scanning microscopy (CLSM) images of biofilms formed by *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3. **a** DMSO (1%), **b** *A. auricula* melanin (80 µg ml^{-1})

IR spectra have been employed to study the structural peculiarities of melanin pigments (Bonner and Duncan 1962). The IR spectrum of the isolated melanin was in accordance with the results of Suryanarayanan et al. (2004), who found typical absorption bands of hydroxyl group at 3,287.6 cm^{-1}, of N–H stretch at 2,925.8 cm^{-1} and of carboxylic groups at 1,702.3 cm^{-1} (Fig. 3).

Characteristic EPR behavior is another diagnostic feature of melanin according to the organic free radicals present (Enochs et al. 1993). The peak at 2.0042 (G value) of the EPR spectrum of *A. auricula* melanin (Fig. 4) indicated the presence of free radicals, as also reported by Enochs et al. (1993). Though these spectra were performed in an attempt to obtain structural information on melanin, it is still difficult to know the exact structure of melanin polymers (Selvakumar et al. 2008), which needs further investigation in the future.

Effects of *A, auricula* pigment on biofilm formation

The *A. auricula* melanin MIC values were 160 μg ml^{-1}, 640 μg ml^{-1} and 320 μg ml^{-1} for *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3, respectively. Figure 5 shows the effects of *A. auricula* melanin on the formation of *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 biofilms. *Auricularia auricula* melanin exhibited concentration-dependent inhibitory activity, which showed a reduction in biofilm biomass with the increase in concentration. As shown in Fig. 5, *A. auricula* melanin at a sub-MIC of 80 μg ml^{-1} inhibited biofilm formation of *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 up to 71.3, 61.7 and 63.2%, respectively. Interestingly, *A. auricula* melanin inhibited biofilm formation of the three strains without inhibiting growth of their planktonic cells (Table 1), thus proving that biofilm inhibition by *A. auricula* melanin is not due to an antibacterial effect.

Image analysis of bacterial biofilms by CLSM

The effects on *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 biofilms of *A. auricula* melanin were also studied using CLSM. CLSM z-section analyses showed that the three strains formed thick and compact biofilms when grown in the absence of *A. auricula* melanin. In contrast, *A. auricula* melanin at a sub-MIC of 80 μg ml^{-1} resulted in thinner and looser cell aggregations on surfaces instead of normal biofilm architecture (Figs. 6, 7). Besides the difference in biofilm thickness, *A. auricula* melanin also influenced the surface area covered. The CLSM top-view images of *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 biofilms showed that the biofilms of control groups (without *A. auricula* melanin) covered the entire surface of the coverslips. In the presence of *Auricularia auricula* melanin at a concentration of 80 μg ml^{-1}, CLSM assessments of the three bacteria revealed an obvious decrease in the surface coverage of coverslips, and the density of bacteria was decreased significantly (Fig. 6).

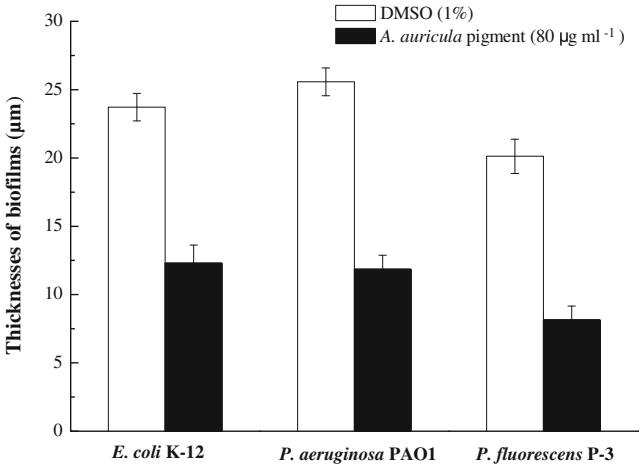

Fig. 7 Influence of the thickness of biofilms of *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 of *A. auricula* melanin

Discussion

In this study, a pigment from *A. auricula*, which has been used as a delicious food in China for many years, was isolated and characterized. It was shown that *A. auricula* melanin inhibits QS-regulated biofilm formation in *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 without interfering with their growth. A wide spectrum of QS-regulated behaviors, such as biofilm formation, has been reported in several bacteria (Waters et al. 2008). In our research, *A. auricula* melanin was found to inhibit biofilm formation at a range below MIC. The inhibitory effect was regulated by QS. A significant number of reports have appeared on the persistence of some foodborne pathogens on food contact surfaces and biofilms, leading to serious economic and health problems. Pathogen outbreaks associated with biofilms have been related to the presence of *E. coli* and *Pseudomonas* spp. (Hentzer et al. 2002; Herzberg et al. 2006). Effective control of these pathogenic bacteria and dental plaque is the key to the prevention and treatment of these diseases. Research may contribute to the prevention of bacterial diseases without raising further concerns about antibiotic resistance.

Melanin is a common substance produced by animals, plants and micro-organisms. Melanins are irregular, dark-brown polymers that are used frequently in medicine, pharmacology and cosmetics preparations (Casadevall et al. 2000; Dastager et al. 2006; Jacobson 2000; Langfelder et al. 2003). As shown in previous studies, melanin has a number of healthful functions, such as antioxidation (Tu et al. 2009), free

radical-scavenging (Wu et al. 2008), anti-HIV (Manning et al. 2003) and immunomodulatory (Sava et al. 2001) activity. However, there is little information available in literature investigating the antibiofilm activity of melanin from *A. auricula*. In this study, a pigment from *A. auricula* was characterized as melanin based on UV, IR, EPR spectra and chemical tests. Biofilm formation in *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 was regulated by QS. In this paper, *A. auricula* melanin inhibited QS-regulated biofilm formation in *E. coli* K-12, *P. aeruginosa* PAO1 and *P. fluorescens* P-3 without interfering with the growth of these organisms. Biosynthesis of melanin with tyrosinase transforms the tyrosine into L-DOPA (3, 4-dihydroxy phenyl-L-alanine), which is further converted into dopachrome and auto-oxidized to indol-5,6-quinone. The latter is polymerized spontaneously into DOPA-melanin, which is a dark-brown pigment (Mencher and Heim 1962). Many heterocyclic compounds, e.g., dopaquinone, leucodopachrome and cysteinyldopas, participate in the whole pigment synthesis process. These intermediate compounds all contain carboxyl and amino groups that easily form a carbonyl group with heterocycle by intra- or inter-molecular dehydration. After dehydration, the conformation of the generating compound is similar to N-acyl homoserine lactone without the side chain. Presumably, these inhibitory compounds could inhibit acyl homoserine lactone (AHL)-regulated behaviors by binding competitively to the AHL receptor protein. This may explain why natural melanin from *A. auricula* can inhibit QS systems in bacteria. Because *A. auricula* has been used safely for a long time, the isolated melanin has a great advantage over the use of toxic compounds for humans. These findings suggest that melanin from *A. auricula* might have potential for application in clinical medicine and the food industry.

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