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Extraction Technology, Structural Characteristics and Antibacterial Activity of Melanin from a Strain of *Lachnum*

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Based on the single factor experiment, Box-Behnken experiment was designed to obtain the optimum extraction conditions of *Lachnum* YM-346 melanin (LM346): NaOH 1.00 μg·mL^{-1}, ultrasonic time 58.02 min and ultrasonic temperature 64.61°C. Under these conditions, the yield of melanin was 3.19 % higher than that by alkali extraction and acid precipitation. Infrared spectroscopy showed that LM346 had characteristic absorption peaks at 1654.213 cm^{-1} and 3324.19 cm^{-1} , which represented the C=O and O-H stretching vibration of indole ring, ^{1}HNMR revealed that LM346 had absorption peaks at 7.0-7.5 ppm and 2.5-5.0 ppm as a result of indole CH=C and NH, and pyrolysis/GC/MS indicated that there were a large amount of pyrrole, benzene and their derivatives in the pyrolysis products, suggesting that LM346 was an indole type melanin. The antibacterial activity of this melanin against the test bacteria was higher than that of tetracycline.

Key words: Melanin from *Lachnum*; Box-Behnken design; Ultrasonic-assisted extraction; Indole-type; Antibacterial activity.

Melanin is a category of phenolic or indole biomacromolecule with complex structures[1], and can be divided into eumelanins, phaeomelanin and allomelanin[2]. At present, the method of alkali extraction and acid precipitation is frequently used to extract melanin[3-5], whereas there are also reports of using ultrasonic-assisted extraction to increase the yield of melanin[6]. Melanin has many biological activities and can be used as light protectant, antioxidant, chelator, liver-protectant and antimicrobial. Research shows that *Monodictys castaneae* SVJM139 pigment has antimicrobial activity, and has been used in therapy of wound infection and skin disease induced by pathogenic bacteria[7].

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Currently, infrared spectroscopy (IR), mass spectrometry (MS), nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) are often used to examine the structure of melanin[8,9], and pyrolysis/GC/MS or H_2O_2 hydrolysis/GC/MS is widely used to analyze the structure type of melanin[10-13]. We have found that melanin from *Lachnum singerianum* is a kind of pheomelanin-like pigment[9].

This study is intended to extract the intracellular melanin in *Lachnum* YM-346 by method of ultrasonic-assisted alkali extraction and acid precipitation, and reveal its antibacterial activity and structural characteristics.

MATERIALS AND METHODS

Materials

Lachnum YM-346 was isolated and preserved in the Laboratory of Microbial Resources and Application of Hefei University of Technology.

Fermentation

Lachnum YM-346 was inoculated in a 500 mL triangular flask contained 300 mL culture medium, and was incubated at 26 °C for 15 d in shaking(150 r·min^{-1}). Fermentation medium was as follows: potato extract 20%; glucose 20.0 g·L^{-1}; yeast extract 5.0 g·L^{-1}; tyrosine 19.06 mg·L^{-1}; magnesium sulfate 2.03 mmol·L^{-1} and pH 7.5.

Extraction technology of LM346

Method of Ye et al. with minor modification was used to extract LM346[14]. The fermentation broth was drawn and filtrated to obtain the mycelium, which was dried at 50-55 °C and added with NaOH solution with the solid-liquid ratio of 2.5 % (g·mL^{-1}). After being put in the ultrasonic cell disruptor for some time, the sample was centrifuged at 5000 r·min^{-1} for 10 min. The supernatant was obtained and diluted 20 times, and the absorbance at 500nm value was determined. The greater the absorbance value is, the higher the yield is. The single factor experiment was conducted to examine the effects of the concentration of NaOH solution, ultrasonic power, ultrasonic time, ultrasonic temperature on the yield of melanin by changing only one parameter at a time, leaving all others unchanged. The NaOH concentrations were 0.5, 1.0, 1.5, 2.0 and 2.5 mol·L^{-1}, the ultrasonic powers were 40, 60, 80, 100 and 120 W, the ultrasonic temperatures were 40, 50, 60, 70, 80 and 90°C, and the ultrasonic times were 30, 40, 50, 60 and 70 min, respectively.

According to results of the single factor experiment, appropriate NaOH concentration, ultrasonic time and ultrasonic temperature were selected for Box-Behnken experiment and the experimental results were verified. The experiment was repeated three times, and 1000 times the average of absorbance values was used. Using multiple regression analysis with Design Expert 7.0 software, the Box-Behnken experimental results were fitted into the quadratic polynomial model $Y=\beta_0+\alpha_1 A+\beta_1 B+\gamma_1 C+\alpha_1\beta_1 AB+\alpha_1\gamma_1 AC+\beta_1\gamma_1 BC+\alpha_{11}A^{2+2}{}_{11}B^{2+3}{}_{11}C^2$, and *F*-test was employed for analysis of variance to evaluate the significance of the quadratic polynomial model and its regression coefficient.

Structural characteristics of LM346

Method of Wang *et al*. with minor modification was used for the determination[15]. LM346 solution (50 mg·L^{-1}) of 1mL was obtained, and its absorbance value at its maximum absorption wavelength was measured. The color value $E^{1\%}_{1cm}$ = AB/M, where A is the absorbance value, B is the dilution times, and M is the mass of melanin.

Method of Ye *et al*. was used for analysis[14]. TU-1810PC UV-Vis spectrophotometer (Shanghai, China) was used to determine the maximum absorption wavelength of LM346 between 190-800 nm.

Method of Ye *et al*. was used for the analysis[14]. LM346 of 2 mg was evenly mixed with 400 mg dry KBr, and pressed into tablets. FT-IR spectra were measured on a 6700 FT-IR spectrometer between 3500-500 cm^{-1}.

AVANCE AV 400 spectrometer (Bruker, Switzerland) was used for ^{1}HNMR analysis of LM346. Injection conditions: solvent: D_2O/NaOD; temperature: 298 K; delay time: 1.000 s; sampling time: 1.0224741 s; observation spectrum width: 8012.82 Hz; repetitions: 8; observation frequency: 500.1300236 MHz; FT size: 16384.

The method of Gómez-Marín et al. with minor modification was used[13]. LM346 of 30 mg was accurately weighed, added with 100 mg NaOH powder and 30 mg $Na_2S_2O_4$, put in a crucible and evenly mixed by dripping a small amount of water, followed by reaction at 300°C for 10 min and cooling with running water. The pH value of the sample was adjusted to around 1.0 with 7 mL 6 mol·L^{-1} HCl. After centrifugation at 5000 r·min^{-1} for 10min, the supernatant was obtained and extracted 3 times using 15 mL absolute ether. All the organic phases were combined and heated in water bath to 70°C to remove the ether, and the residue was dissolved with acetonitrile for later use. Acetonitrile solution of 0.5 mL was obtained, added with 0.5 mL N,O-Bis (trimethylsilyl) trifluoroacetamide and sealed for reaction at 125°C for 30 min. The sample was analyzed by GC/MS.

7890A-5975C gas chromatograph-mass spectrometer (Agilent 19091S-433: 492.70546, America) was used for the analysis. GC-MS conditions were as follows: HP-5 silica capillary column (30 m × 250 µm × 0.25 µm) adopted; Helium used as the carrier gas; The flow speed 1 mL·min^{-1}; Injection volume 1 µL; Split ratio 40:1; Initial column temperature 50°C (2 min), increased to 300°C at the rate of 5°C·min^{-1} (8 min) by a temperature program; Ion source temperature 230°C; ionization mode EI (70 eV).

Antibacterial activity of LM346

The method of Duan et al. with minor modification was used[16]. *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Listeria monocytogenes* were used for the test. Suspensions of the test bacteria (10^6 cfu·mL^{-1}) of 1mL were spread into the bacteria agar plates. Three sterile oxford cups were placed at the middle of each plate with equal distance from each other, added with 0.3 mL LM346 solutions sterilized by micropore filter with the concentrations of 0.5, 1.0 and 2.0 mg·mL^{-1}, respectively, and incubated at 37 °C for 48 h. The diameters of the inhibition zones were measured. The sterile water was used as the control, and tetracycline (0.1 mg·mL^{-1}) was used as the positive control. All tests were repeated three times.

The method of Visalakchi & Muthumary with minor modification was used[7]. LM346 solutions (0.5 mL) of different mass concentrations (0.02-0.20 mg·mL-1) were added into tubes containing 10 mL bacteria culture medium, added with 0.1 mL Staphylococcus aureus suspension and fully shaken to enable the bacterial concentration in each tube to be 106 cfu·mL-1, and incubated at 37 °C for 48 h. Observe the bacteria growth, the culture medium turning turbid indicated the growth of bacteria. The sterile water was used as the control.

RESULTS AND DISCUSSION

Extraction technology of LM346

Single factor experiment showed that the optimum ultrasonic-assisted extraction conditions of LM346 were as follows: NaOH concentration: 1.0 mol·L^{-1}, ultrasonic time: 60 min, ultrasonic temperature: 70°C, and ultrasonic power: 80 W.

The appropriate ultrasonic time, NaOH concentration and ultrasonic temperature were selected based on the single factor experiment to conduct the Box-Behnken experiment following Table 1, and Design-expert 7.0 was used to analyze the data, as shown in Table 2. The ultrasonic temperature had the greatest effect on the absorbance (i.e. the yield), followed by the NaOH concentration, and the ultrasonic time. The regression equation is:

$$Y=518.40+3.88A+12.00B-31.13C-23.75AB-48.00AC-18.25BC-125.20A^2-277.45B^2-69.20C^2 \quad ...(1)$$

Variance analysis of the regression coefficients of the quadratic polynomial model and the experimental results (Table 2) showed that F_{model} was 293.41 and the probability P_{model} was less than 0.0001, indicating that Eq. (1) was significant; that the lack of fit probability P_{lose}(0.696) was greater than 0.05, suggesting that no lack of fit factor existed; that the determination coefficient R^2 was

Table 1. Box–Behnken design and observed responses

No.	*A* Ultrasonic time/min	*B* C_{NaOH} / (mol·L^{-1})	*C* Ultrasonic temperature /(°C)	*Y* Absorbance
1	80	1	80	241
2	60	0.5	60	1733
3	80	1.5	70	116
4	60	1	70	504
5	60	1	70	528
6	40	1	80	339
7	60	1.5	80	134
8	40	1.5	70	146
9	60	0.5	80	153
10	60	1	70	501
11	60	1	70	534
12	60	1.5	60	227
13	80	0.5	70	133
14	40	0.5	70	68
15	80	1	60	405
16	40	1	60	311
17	60	1	70	525

0.9974, implying that Eq. (1) could well reflect the true relation between the selected parameters and the response values. In addition, the coefficient of variation (CV) was 4.47%, and less than 5%, indicating that Eq. (1) had a good reproducibility[17], and R^2_{Pred}(0.9854) was agreed well with R^2_{Adj}(0.9940). Based on Eq. (1), two factors were chosen as variables, and another factor was regarded as the center point to make Fig. 1. With the increase of the ultrasonic time, the absorbance

Table 2. Estimated regression coefûcients for the quadratic polynomial model and the analysis of variance for the experimental results

Source	Coefficient Estimate	Standard Error	df	Sum of Squares	*F*-Value	*P*-Value
Intercept				Model		
	518.4	5.93	1	4.64E+05	293.41	< 0.0001
A	3.88	4.69	1	120.13	0.68	0.4356
B	12	4.69	1	1152	6.56	0.0375
C	-31.13	4.69	1	7750.13	44.11	0.0003
AB	-23.75	6.63	1	2256.25	12.84	0.0089
AC	-48	6.63	1	9216	52.45	0.0002
BC	-18.25	6.63	1	1332.25	7.58	0.0284
A^2	-125.2	6.46	1	66000.17	375.63	< 0.0001
B^2	-277.45	6.46	1	3.24E+05	1844.66	< 0.0001
C^2	-69.2	6.46	1	20162.69	114.75	< 0.0001
Lack of Fit			3	340.75	0.51	0.696
Error			4	889.2		
R^2	0.9974	Adj-R^2	0.9940	Pred-R^2	0.9854	
C.V.%	4.47	*PRESS*	6841.37			

Table 3. Compounds identified in the pyrolysis-GC-MS analysis of LM346

Compounds[a]	Abbreviation[b]	% total peak heights
Acetic acid	C	11.08
Benzene	A	3.39
Toluene	A_1	1.34
Pyrrole	B	27.69
Pyrrole, 2-methyl-	B_1	2.19
Pyrrole, 3-methyl-	B_2	1.94
Pyrrole, 2,3-dimethyl-	B_3	1.04
Benzene ethyl-	A_2	1.87
Benzene,1,4-dimethyl-	A_3	1.35
Styrene	A_4	3.26
compounds N.I	n.i	6.47
Phenol	H	14.31
Benzenenitrile	F	6.55
Phenol, 2-methyl-	H_1	3.96
Phenol, 4-methyl-	H_2	2.62
1,2-Benzenediol	H_3	3.23
Benzoacetonitrile,4-methoxy-	F_1	4.14
Indole	E	1.39
Isoindole-1,.3-dione	E_1	2.18

[a]Compounds— products identified by Py-GC-MS analysis.
[b]Abbreviation of selected group of compounds; n.i., Non identiûed compound; A-Benzene type; B—Pyrrole type; C-Acetic acid type; E—Indole type; F-Benzenenitrile type; H-Phenol type.

Table 4. Antimicrobial activity of LM346

Bacteria	Inhibition zone(mm) at different concentration (mg·mL^{-1})			Control	
	0.5	1.0	2.0	Sterile water	Tetracycline(0.1)
Staphylococcus aureus	18.0±0.28^{a}	19.2±0.28^{a}	21.7±0.31^{a}	-	17.3±0.23
Escherichia coli	9.3±0.26^{b}	11.4±0.30^{b}	13.0±0.33^{b}	-	10.6±0.18
Salmonella typhi	11.8±0.25^{b}	12.6±0.28^{b}	14.3±0.32^{b}	-	13.9±0.22
Listeria monocytogenes	17.9±0.27^{a}	19.1±0.30^{a}	20.1±0.32^{a}		12.4±0.21

Results are reported as means standard deviation. Means bearing different letters in a row are significantly different $P<0.05$. — means no inhibition zone.

increased first and then decreased, similarly to the NaOH concentration and the ultrasonic temperature (Fig. 1).

The interaction terms between the ultrasonic time and the NaOH concentration and between the ultrasonic time and ultrasonic temperature had extremely significant effect on the absorbance ($P_{AB}<0.01, P_{AC}<0.01$), and the interaction term between the NaOH concentration and the ultrasonic temperature had significant effect on the absorbance ($P_{BC}<0.05$) (Table 2). Besides, Fig. 1 showed that Eq. (1) had a maximum value. Using Eq. (1), 1000 times the maximum absorbance value was calculated to be 440.279, with the ultrasonic time, NaOH concentration and ultrasonic temperature being 58.02 min, 1.00 mol/L and 64.61 °C, respectively. Under the above conditions, verification test was performed to obtain 1000 times the absorbance value of LM346 solution to be 453±4.6, with the yield of 13.66 % (dry weight basis), increasing by 3.19 % compared to that by alkali extraction and acid precipitation and increasing by 4.81 % compared to that of *Lachnum singerianum* YM-292 melanin by alkali extraction and acid precipitation[9].

Structural characteristics of LM346

Low color value is an important factor to inhibit the industrial production of pigment by liquid fermentation[18]. The color values ($E^{1\%}_{1cm}$) of LM346 was 221.0, higher than that of melanin isolated from Osmanthus fragrans' seeds ($E^{1\%}_{1cm}$lmax = 60.24) [15].

LM346 had maximum absorption peaks at 211 nm, which was similar to the maximum absorption wavelengths of *Lachnum singerianum* melanin and *Lachnum*YM-223 melanin[16,19]. The UV–VIS spectrum showed an exponential decay without distinguishing characteristics in the region between 202-800 nm.

Infrared spectroscopy had been used in study of the chemical structure of melanin in Taihe Silkies, *Monascus* red pigment and *Laetiporus sulphureus* melanin, etc.[8,20,21].In the infrared spectrum of LM346 (Fig. 2), there were indole O-H stretching vibration (3324.19 cm^{-1}), C-H stretching vibrations (2934.131 cm^{-1} and 2819.638 cm^{-1}), quinone C=O stretching vibration (1654.213 cm^{-1}) and C-N stretching vibrations (1390.71 cm^{-1} and 1236.02 cm^{-1}), indicating that LM346 contains

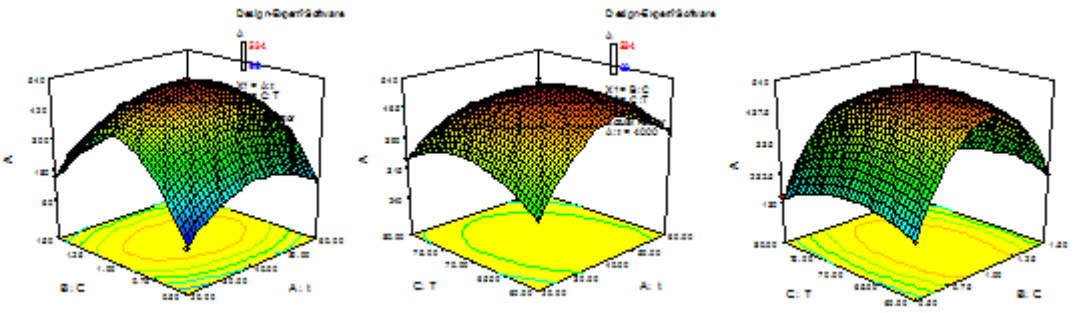

Fig. 1. Response surface curve for Selenium in cells showing the interaction between ultrasonic time (t), concentration of NaOH Solution (C) and ultrasonic temperature (T)

substituted indole quinone structure and is a indole type melanin.

^{1}H-NMR spectrum of LM346 exhibited a series of broad peaks, which reflected the complicated structure of the melanin biomacromolecule (Fig. 3). The chemical shifts of ^{1}H-NMR spectrum indicated that this melanin might contain the following groups: indole CH=C (7.0-7.5 ppm), indole NH (2.5-5.0 ppm), CH_3 connected to the indole ring (2.2-2.5 ppm), -CH_2-CH_3 of the Aliphatic alkyl fragments (1.0-2.0 ppm) etc.[22], which further proved that the basic structure of LM346 is indole quinone.

Table 3 gave the pyrolysis characteristic products and their percentages of LM346. According to the chemical composition, the main pyrolysis products can be divided into following groups: A—benzene (A, A_1, A_2, A_3 and A_4), B—pyrrole (B, B_1, B_2 and B_3), C—acetic acid (C), E—indole (E and E_1), F—benzenenitrile (F and F_1) and H—phenol (H, H_1, H_2 and H_3).

The pyrolysis products of LM346 contained high quantities of pyrrole (27.69 %), benzene and their derivatives, among which the total content of pyrrole, 2-methylpyrrole, 3-methylpyrrole and 2, 3-dimethylpyrrole reached 33.9

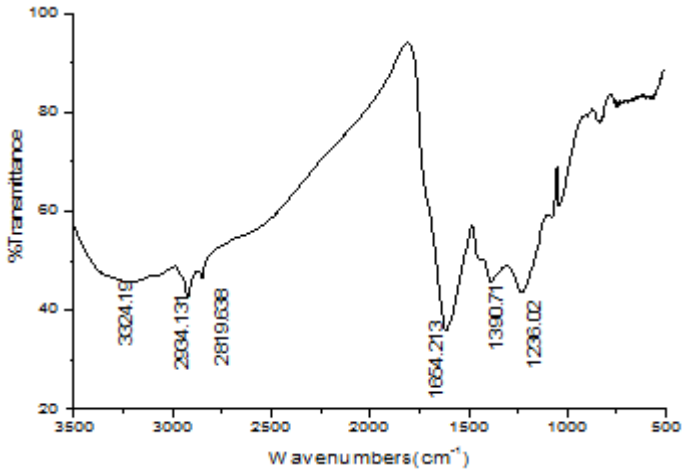

Fig. 2. Infrared spectrum of LM346

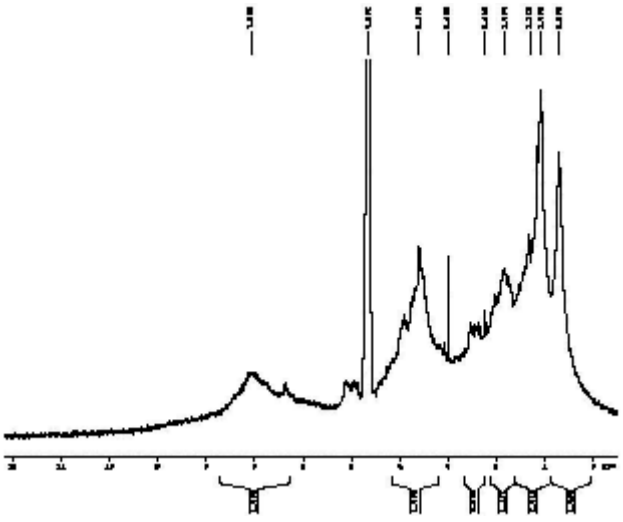

Fig. 3. ^{1}H NMR of LM346

%, the total content of benzene, methylbenzene, ethylbenzene, 1, 4-dimethylbenzene and styrene accounted for 11.21 %, which were similar to those of the degradation products of synthesized DOPA-melanin[11]. Thermal degradation of indolequinone units during pyrolysis might generate pyrrole, benzene and their alkyl derivatives[23]. In the pyrolysis products were also phenols (phenol, 4-methylphenol, catechol) and acids (acetic acid), which might be produced the degradation of hydroxyindole and indolequinone units, or might be from uncyclic aminoacid-type units [11]. In addition, the degradation products also contained unidentified compounds (7.09 %), which might be some complicated pyrrole or indole derivatives. The presence of pyrrole, benzene and their derivatives and the absence of sulfur compound in the pyrolysis products of LM346 further demonstrated that this melanin is an indole melanin.

Pigments of some *streptomyces* and *eumycetes* can effectively inhibit the human pathogens[7,24]. LM346 has antibacterial activity of different degrees against the four test bacterium, and the diameter of the inhibitory zone increased with the increase of its concentration. The antibacterial activity against the *Staphylococcus aureus* was highest (Table 4). The inhibitory zone diameters of 2 $mg \cdot mL^{-1}$ LM346 against *Staphylococcus aureus, Listeria monocytogenes, Salmonella typhi* and *Escherichia* coli were 21.7 ± 0.31 mm, 20.1 ± 0.32 mm, 14.3 ± 0.32 mm and 13.0 ± 0.33 mm, respectively, all greater than those of 0.1 $mg \cdot mL^{-1}$ tetracycline against them. The MIC of LM346 against *Staphylococcus aureus* was 80 $\mu g \cdot mL^{-1}$. These results indicated that LM346 can be used as antibiotic medicine.

CONCLUSION

LM346 was extracted by ultrasonic-assisted alkali extraction and acid precipitation, and the yield increased by 3.19 % (dry weight basis) compared to that by alkali extraction and acid precipitation. Infrared spectroscopy, ^{1}H-NMR and pyrolysis/GC/MS indicated that LM346 is an indole type melanin. LM346 had antibacterial effects of different degrees on *Staphylococcus aureus, Listeria monocytogenes, Salmonella typhi* and *Escherichia coli*.

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