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Electronic Supplementary Information

Characterization of biosurfactant lipopeptide and its performance evaluation for oil-spill remediation

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1. materials

Lipopeptide (cell-free foamate of *Bacillus subtilis* HSO12126, deal with acid precipitation, drying); XinJiang crude oil (obtained from Xinjiang oilfield, density of 0.8706 g/cm^3, viscosity of 25.43 mPa·s at 50 °C); Zebrafish, green microalgae (purchased from the local aquafarm); Activated sludge (collected from the aerobic tank). Other materials were all analytical reagents. Unless otherwise specified, all of the aqueous solutions used in this study were prepared with deionized water.

2. Methods

2.1 Determination of effectiveness and stability

A stock solution of oil in dichloromethane (DCM) was prepared by adding 500 mg of oil into 25 mL DCM. In order to generate a six-point calibration curve, a series of specific volume of stock standard solution was adjusted to a final volume of 10 mL separately with DCM. Then the absorbance of oil solutions relative to a DCM blank was measured at 650 nm. The absorbance vs. oil concentration curve was established.

50 mL artificial seawater was added into 60 mL Pear shape separatory funnel, 250 mg oil was dispensed directly onto the surface of the artificial seawater with 1 mL Syringe. Then 5 mg lipopeptide was dispensed at the center of the oil slick in a separatory funnel with dropper, resulting in a lipopeptide-to-oil ratio of 1:50. The funnel was placed on a horizontal oscillator for 10 mins at 240 rpm and then remained stationary for 30 s. Thereafter, 20 mL of the sample was put in a 25 mL test tube for subsequent centrifugal extraction. The extract was adjusted to a final volume of 20 mL for the spectrophotometric analysis at 650 nm. The effectiveness was calculated based on the ratio of oil dispersed in the water column according to the calibration curve to the total oil which depended on the total oil added to the funnel.

The stability of the lipopeptide was investigated under different environmental conditions. The effectiveness was measured at various NaCl concentrations (0, 1%, 2%, 3%, 4%*w/v*), temperatures (15, 20, 25 ◦C) and pH values (5, 7, 9, 11). Furthermore, the lipopeptide dispersity was affected by DORs (1:1250, 1:500, 1:250, 1:50, 1:10). All analyses were performed in triplicate.

2.2 Determination of lipopeptide toxicity to aquatic organisms

Tap water artificially aerated was used as culture liquid. The zebrafish must be raised over 7 days, and it was not allowed to proceed to test if the mortality rate exceeds 10%. The fish must be starved 24 h before the tests. Water parameters in all experiments were as follows: pH 7.5±0.5; temperature 23±2 ◦C. As for experiment, aquariums of 20 cm*20 cm*20 cm were filled with 3 L water with various lipopeptide concentrations. The concentrations, arranged in a logarithmic series, were selected based on range-finding-tests (one replicate per treatment) which were performed under the same conditions as the final tests (two replicates per treatment). 10 zebrafish were put into each aquarium. Behaviors and mortality were monitored and recorded every four hours for 24 h posttreatment, with tests coinciding with control survival <90% excluded. The median lethal concentration (LC_{50}) of lipopeptides at 24 h was calculated from a regression equation (Y=probit values, transrormed from percent mortality; X = concentration).

In order to be in the exponential growth phase when used to inoculate the test solutions, *Pseudokirchneriella subcapitata* was precultured 1-3 days under the same test conditions as the subsequent test. The experiment was performed in 100-mL conical flasks with 50 mL media prepared. The initial cell densities were at 10^5 cells/mL for both control and test groups. All flasks were incubated at 23 ± 2 ◦C and exposed to fluorescent light whose intensity was 4500 lux in the wavelength range of 400-700 nm. Test flasks were shaken manually twice a day. A preliminary range-finding test was carried out to confirm concentrations covering several orders of magnitude of difference in the final definitive test. At the day of experiment, five concentrations of lipopeptide were made and tested. Flasks containing solution without tested lipopeptide were used as control. Both control and test flasks were inoculated for three days (72 h). Every 24 h the algal density was quantified with the hemocytometer. Triplicate measurements were made on each sample. The pH value of all samples was measured with pH meters at the beginning and at the end of experiments. The concentration causing 50% growth inhibition of algae was determined and used as toxic

end point and was expressed as EC_{50}.

2.3 Determination of biodegradability

The concentration of activated sludge was adjusted to 16 g/L. Before the formal experiments, 5 mL activated sludge was added into nutrient salt media which the total concentration of surfactant was 30 mg/L. The flaks were cultivated at 25±3 ◦C and 200 rpm for three days. 5 mL culture solution was taken out to repeat the steps above to gain the final injection. As for formal experiments, 5 mL injection was added into 500-mL conical flasks containing 250 mL nutrient salt medium with different surfactant concentrations, and the flasks were incubated at the same conditions with pre-experiment. At day 0, 1, 3, 5 and 7, 20 mL cultured solution was collected into 50-mL graduated tube, which was shaken twice per second for 30 s and then remained stationary for 30 s. The volume of the foam was calculated because it was considered to be a liner relationship with the concentration of surfactant. The biodegradation (D, %) was calculated according to the following equation:

$$D = \frac{V_0 - V_x}{V_0} \times 100\% \quad (1)$$

Where V_0 and V_X were the volume of the foam at the beginning and at the sampling date of the incubation period respectively.

2.4 Determination of bioremediation

In a word, 250-mL conical flasks were filled with 100 mL of artificial seawater, 5 mL of microbial inoculation, 0.5 g of Xinjiang crude oil, and 0.05 g of lipopeptide or commercial dispersant or none. Control flasks contained seawater and oil. All flasks were placed in an orbital shaker rotating at 200 rpm and kept at 25±5 ◦C for 0, 1, 2, 7 days. The *n*-hexane was used as the solvent to extract oil to 20 mL after incubation. After that, 5 μL (dissolved in 100 μl hexane) of chlorohexadecane was added as a surrogate standard. Then the extraction was analyzed by GC–MS.