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Production, structure elucidation and anticancer properties of sophorolipid from *Wickerhamiella domercqiae*

Chen Jing, Song Xin**, Zhang Hui, Qu Yinbo*

State Key Lab of Microbial Technology, Shandong University, Jinan 250100, China

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Abstract

A yeast strain Y_{2A} producing a large amount of biosurfactants was isolated from oil-containing wastewater and identified as *Wickerhamiella domercqiae* by BIOLOG analysis and routine yeast identification method. The crude biosurfactants produced from Y_{2A} were obtained by extract with ethyl acetate and proved to be a mixture of glycolipids by thin layer chromatography (TLC). The main product was separated and purified by HPLC and then characterized as sophorolipid by nuclear magnetic resonance (NMR) and mass spectroscopy (MS). This is the first report on sophorolipids produced from *W. domercqiae*. Subsequently, the cytotoxic effects of the sophorolipid on cancer cells of H7402, A549, HL60 and K562 were investigated by MTT assay. The results showed a dose-dependent inhibition ratio on cell viability according to the drug concentration $\leq$62.5 μg/ml. These findings suggested that the sophorolipid produced by *W. domercqiae* have anticancer activity.

Keywords: Sophorolipid; *Wickerhamiella domercqiae*; Human cancer cell; Anticancer activity

1. Introduction

Sophorolipids (SLs), a kind of extracellular biosurfactant, which were produced by yeasts have been known for a long time [1]. Each of them is comprised of one sophorose molecule, hydrophilic part, linked to one hydroxyl fatty acid, lipophilic part, by one or two crosslines [2]. They are distinguished from sophorose acetylated grade, hydroxyl fatty acid chain length, and hydroxyl group position in the fatty acid [3].

Sophorolipids as biosurfactant show good surfactant properties so that they can be used in many ways. Some researches show that sophorolipid of lactonic form is able to inhibit the growth of some microorganisms [4]. Others show that sophorolipids can be used in cosmetic as a high value skin moisturizer, also in the petroleum industry and in food areas as emulsifiers [5]. However, sophorolipids are reported to be secreted by yeasts of *Candida* sp., such as *Candida bogoriensis*, *C. apicola*, *C. bombicola*, etc. [1–10]. Furthermore, little is known about the pharmacological roles of sophorolipids as anticancer drugs [6,7].

The aim of this study, was to isolate new yeast strains producing sophorolipids of different structures and to explore new applications of sophorolipids as pharmaceuticals.

2. Materials and methods

2.1. Yeast strain

Wickerhamiella domercqiae Y_{2A} was isolated from an oil-containing wastewater sample by enrichment culture techniques and measurements of the oil film-collapsing activity and surface tension of culture broth.

2.2. Media and cultivation

2.2.1. Enrichment medium

2% (v/v) rapeseed oil, 0.2% (w/v) yeast extract, 0.4% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) $Na_2HPO_4{\cdot}12H_2O$ and 0.05% (w/v) $MgSO_4{\cdot}7H_2O$. The sample (50 ml in 300 ml flask) was incubated on a rotary shaker for 48 h at 30 °C and 160 rpm.

2.2.2. Solid medium (w/v)

2% glucose, 1% yeast extract, 2% peptone and 2% agar. After enrichment cultivation, the culture was spread onto agar plates and incubated at 30 °C. The isolated sole colonies were transferred to slants. The composition of seed medium was the same as the solid medium without agar. The seed medium (50 ml in 300 ml flask) was incubated on a rotary shaker for 24 h at 30 °C and 160 rpm. Seed culture broth (2.5 ml) was transferred to the fermentation medium.

* Corresponding author. Tel.: +86 531 88365234; fax: +86 531 88565610.
** Co-corresponding author. Tel.: +86 531 88361379; fax: +86 531 88565610.
E-mail addresses: songx@sdu.edu.cn (Song X.), quyinbo@sdu.edu.cn (Qu YB).

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2.2.3. Fermentation medium

Five percent (w/v) glucose, 2% (v/v) rapeseed oil, 0.2% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) $Na_2HPO_4{\cdot}12H_2O$ and 0.05% (w/v) $MgSO_4{\cdot}7H_2O$, pH 6.5. The fermentation medium (50 ml in 300 ml flask) was cultivated for 96 h at 30 °C and 160 rpm.

2.3. Oil film-collapsing activity

The oil film-collapsing activity was assayed according to the method in reference [11].

2.4. Surface tension measurement

The surface tension of culture broth was measured using the ring method by a Tensiometer (Chengde, Hebei, China).

2.5. TLC

Two milliliters of culture broth was extracted with 2 ml ethyl acetate and the extract was applied to TLC for determination of glycolipids. Stationary phase: silica gel GF254. Developing system [12]: $CHCl_3/CH_3OH/H_2O = 65/15/2$ (v/v/v). Visualizing reagents: α-naphthol/sulphuric acid. Fuchsia-colored sugar positive spots could be observed after the plate was dried at 105 °C for 5 min.

2.6. Separation and preparation

Culture broth was extracted with ethyl acetate of equal volume, and the organic phase was vacuum-dried at 50 °C to remove the ethyl acetate. The residue was washed with hexane to remove the remaining rapeseed oil. After vaporizing the residual hexane at 50 °C under vacuum, the crude glycolipids were obtained and applied to further separation by HPLC (Agilent, USA), which was performed using a Kromasil C18, 5 μm (250 mm × 4.6 mm) column. The flow rate was 1 ml/min and the composition of the acetonitrile–water fluid phase was programmed from 20 to 90% in 55 min. The injection volume was 5 μl and the eluent was monitored with UV detector at 207 nm. The main product occurring at retention time 38.15 min was collected. Preparative HPLC was performed using a SHIMADZU LC column. The flow rate was 8 ml/min, and the injection volume was 1000 μl. The composition of fluid phase was programmed as the same of analytical HPLC.

2.7. Structure elucidation

^1H and ^{13}C NMR spectra of the purified glycolipid were recorded in $CDCI_3$ on a Bruker AV600 NMR spectrometer. Two-dimensional NMR spectra, ^1H–^1H correlation spectroscopy (COSY), the ^1H–^1H total correlation spectroscopy (TOCSY), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) were performed at 600 MHz by standard procedures. Chemical shifts were expressed in parts per million down field from an internal standard of tetra methylsilane (TMS). MS analysis for the purified glycolipid was performed on an API4000 mass spectrometer. Mass spectra were analyzed from 400 to 1000 *m/z*, and negative ions were detected.

2.8. Identification of the yeast strain

The yeast strain was identified using BIOLOG system, Biolog Universal Yeast Agar, Biolog Yeast Microplate and routine methods of yeast identification [13,14].

2.9. Preparation of drug

Purified sophorolipid was dissolved in ethanol to ensure that the final concentration of ethanol in the culture medium was below 0.05% (v/v), then applied to cells. Ethanol at a concentration below 0.1% (v/v) could not affect the viability of the cells.

2.10. Cell culture

H7402 (liver cancer line), A549 (lung cancer line), HL60 and K562 (leukemia lines), four human cancer cells were obtained from Shandong Academy of Medical Sciences of China. The cells were cultured in flat flasks with RPMI1640 medium (Gibco, USA) supplemented with 10% calf serum (Hangzhou Sijiqing, China), 100 units/ml penicillin and 100 μg/ml streptomycin sulfate (AMRESCO, USA) in an incubator with an atmosphere of 5% CO_2 at 37 °C. The cells were routinely at 1.0×10^5 cells/ml into 96-well plates.

2.11. MTT assay

Fifty microliters of the cell suspension of 1.0×10^5 cells/ml was seeded into wells of a 96-well plate. For H7402 and A549 cells, 24 h after planting, the experiments were performed. After the treated cells were incubated for either 24 or 48 h, cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenytetrazolium; Sigma, USA) assay [15]. In brief, 10 μl MTT was added to the wells, the cells were cultured for 4 h at 37 °C in an incubator with an atmosphere of 5% CO_2. Then 100 μl of 10% SDS was added to the wells and the cells were cultured overnight. The formation of formazan was measured at 570 nm using microplate spectrophotometer (Spectra Max 190, USA).

2.12. Data analysis

The results are expressed as the mean ± S.D., and accompanied by the number of experiments performed independently. Statistical analysis was done by *t*-test.

3. Results

3.1. Isolation and identification of yeasts producing biosurfactants

More than 200 strains were isolated from oil and wastewater samples. These yeast strains were cultured in fermentation media, 11 strains showing large halos (more than 3 cm diameter) were isolated by measuring the oil film-collapsing activity of the culture broth. By surface tension measure and TLC analysis, we found two strains (Y_{58} and Y_{2A}) have the ability of producing glycolipids. The surface tensions of their culture broth were 38.4 and 37.3 mN/m, respectively. It can be observed from Fig. 1 that more than six glycolipids were distinguishable. However, the strain of Y_{58} lost the ability after several transfers. So Y_{2A} was finally selected as a potential producer of glycolipids for this study.

3.2. Identification of strain Y_{2A}

By using BIOLOG system and conventional methods for yeast identification, strain Y_{2A} was identified as *W. domercqiae*, the probability and similarity values for Y_{2A} and *W. domercqiae* were 100% and 0.63, respectively. Cell morphology, growth properties and colony appearance of strain Y_{2A} were identical to those of *W. domercqiae* (data not shown).

3.3. Purification of glycolipids-type biosurfactant

Analytical HPLC was used to separate the crude glycolipids. The results are shown in Fig. 2. It can be seen that the crude glycolipids are mixture containing three main products occurring