

EVALUATION OF A LIPOPEPTIDE BIOSURFACTANT FROM *BACILLUS NATTO* TK-1 AS A POTENTIAL SOURCE OF ANTI-ADHESIVE, ANTIMICROBIAL AND ANTITUMOR ACTIVITIES

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ABSTRACT

A lipopeptide biosurfactant produced by *Bacillus natto* TK-1 has a strong surface activity. The biosurfactant was found to be an anti-adhesive agent against several bacterial strains, and also showed a broad spectrum of antimicrobial activity. The biosurfactant induced a significant reduction in tumor cells viability in a dose-dependent manner.

Key words: Biosurfactant, anti-adhesive, antimicrobial activity, antitumor

Microbial compounds which exhibit pronounced surface activity are classified as biosurfactants. Biosurfactants including a structurally diverse group are produced by several types of microorganisms such as bacteria, yeasts and fungi as membrane components or secondary metabolites (9,19,25). They attracted attention as hydrocarbon dissolution agents for the first time in the late 1960s (1). In recent years, the interest in biosurfactants has been remarkably increasing due to many advantages compared with chemical surfactants, including lower toxicity, biodegradability, ecological acceptability, complex molecules and specific activity at extreme conditions (temperature, pH, salinity) (32,45).

Among the many classes of biosurfactants, lipopeptides represent a class of microbial surfactant with remarkable surface properties and biological activities, such as surplus crude oil recovery, food-processing, de-emulsification, antimicrobial and antitumor, antiviral, antiadhesive activities and so on (3,4,17,18,33,41). Surfactin, produced by various *Bacillus subtilis* strains, is one of the most powerful and effective lipopeptide-type biosurfactant so far known, which is consisted of a seven amino acids linked *via* a lactone bond to a β -hydroxy fatty acid with 13, 14 or 15 carbon atoms (5,15). Apart from many characteristic functional activities of biosurfactants, surfactin can also inhibits fibrin clot formation, induces formation of ion channels in lipid bilayer membranes and

inhibits cyclic adenosine monophosphate (cAMP) (1,4, 24). In addition, a good number of authors have documented that the antiviral action of surfactin is primarily due to a physiochemical interaction between the membrane active surfactant and the virus lipid membrane (37).

Atomic force microscope (AFM) is a powerful imaging tool which has been frequently employed to image a wide variety of samples under a wide variety of conditions. In the past years, AFM has created remarkable interest in applying it to the study of biological structures. Images have appeared in the literature showing DNA, single proteins, structures such as gap junctions, and living cells (22,27,28,29).

Natto is a traditional Japanese food. Recently, its medical benefits are widely recognized in Japan resulting in its increased popularity. Some of its beneficial effects are prevention of heart attacks, strokes, cancer, osteoporosis, obesity and intestinal disease caused by pathogens (10,14). *Bacillus natto*, an edible strain, identified as *B. subtilis*, is claimed to be the organism mainly responsible for natto fermentation. Moreover, ingestion of *Bacillus natto* cells is safe for humans under normal host condition (40).

Bacillus natto TK-1, which produces an effective biosurfactant, was provided by TUST Collection Center (Tianjin University of Science and Technology, Tianjin, China). The biosurfactant was determined to be a lipopeptide consisting of

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a fatty acid tail linked to a peptide moiety consisting of 7 amino acid residues (6).

For preparation of the inoculum, *Bacillus natto* TK-1 was grown in a nutrient broth medium at 37°C, 170 rpm for 16 h. The fermentation medium used for biosurfactant production was composed of 5.0 g/L sucrose, 20.0 g/L peptone, 0.5 g/L yeast extract, 0.02 g/L MgSO₄•7H₂O, 1.4 g/L Na₂HPO₄•12H₂O, and 0.4 g/L KH₂PO₄, which was inoculated by 1.8 ml of the seed medium into a 500-ml flask with 60 ml fermentation medium. The bacterial cells were then incubated under the same condition as the seed cultures for 24 h.

Ten microorganisms were used as test organisms in this study. Five bacterial strains were obtained from ATCC (American Type and Collection Center), including *Micrococcus luteus* (ATCC 28001), *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 27325), *Bacillus cereus* (ATCC 7004), *Staphylococcus aureus* (ATCC 10780). Five fungal strains, *Botrytis cinerea*, *Fusarium moniliforme*, *Penicillium candidum*, *Gibberella zeae* and *Colletotrichum truncatum* were provided by TUST Collection Center (Tianjin University of Science and Technology, Tianjin, China). Bacterial strains were cultivated at 37°C and maintained on LB broth. Cultures of fungi were grown on potato dextrose agar (PDA) at 28°C.

Human chronic myelogenous leukemia cells (K562), human hepatic carcinoma cells (BEL-7402), buffalo rat liver cells (BRL) and human embryonic kidney cells (HEK293) were obtained from Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China) and cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 kU/L penicillin, 100 mg/L streptomycin and 1% glutamine. All cells were cultured at 37°C in a humidified incubator containing 5% CO₂. Cells in logarithmic growth phase were used for further experiments.

The preparation of biosurfactant was described previously by Cao *et al.* (6). Briefly, the bacterial cells were removed by centrifugation at 10 000 rpm at 4°C for 10 min. The supernatant was subjected to acid precipitation by adding 6 M HCl to pH 2.0 and putting the precipitate to form at 4°C overnight. The precipitate was collected by centrifugation and resuspended by a little distilled water adjusted to pH 7.0 to make most of precipitate dissolved and finally lyophilized. The brown lyophilized material was then extracted three times with methanol. After evaporation, the crude biosurfactant was isolated by thin-layer chromatography (TLC) on a silica gel plate with a developing solvent consisting of chloroform-methanol-water (65:25:4 [vol/vol/vol]). Then the purified single point was scratched off from the TLC plate to test whether the point contained the biosurfactant by oil spreading (26). The crude biosurfactant was used for further studies.

The surface tensions of various concentrations of biosurfactant were determined by the Ring method using a SIGMA70 system unit tensiometer (KSV Instruments, Trumbull, CT). Measurements were performed at 20°C. The whole

experiment was repeated at least three times. The critical micelle concentration (CMC) was determined as the intersection of the linear part of this curve of surface tension vs. the concentration. Furthermore, the surface activity of biosurfactant was also measured by an oil spreading test (26). Briefly, 30 ml distilled water at pH 7.0 was added to a petri dish (90 mm diameter) and then 1.0 ml paraffin oil to the surface of water. When the water was covered by the oil completely, 5 µl biosurfactant with various concentrations was put on the center of the oil membrane. The size of the resultant oil-displaced circle area reflects the surface activity of biosurfactant. Each sample was repeated in triplicate and the control plate was evaluated with buffer (PBS).

The anti-adhesive activity of the crude biosurfactant against the bacterial strains was quantified according to a previously reported adhesion assay (23). Briefly, each well of a sterile 96-well microtiter plate was filled with 200 µl biosurfactant of several concentrations (ranging from 2.5 to 40 g/L) and incubated for 24 h at 4°C, and then washed twice with PBS. Control wells contained PBS only. A 200 µl aliquot of a washed bacterial suspension was added and incubated in the wells for 4 h at 4°C. Unattached organisms were removed by washing the wells three times with PBS. The adherent microorganisms were fixed with 200 µl of 99% methanol per well, and after 10 min the plates were emptied and left to dry. Then the plates were stained with 1% crystal violet solution at room temperature for 5 min. Excess stain was rinsed off by placing the plate under running tap water. Subsequently the plates were air dried, the dye bound to the adherent microorganisms was resolubilized with 200 µl of 33% (v/v) glacial acetic acid per well. The absorbance of the dissolved dye, corresponding to the number of adherent microorganisms, was measured in a microplate reader at 595 nm. The percentage reduction in adherence was calculated using the following equation:

$$\% \text{reduction in adherence} = [(A_{\text{control}}) - (A_{\text{sample}})] / A_{\text{control}} \times 100$$

The disc diffusion method (42) was used to evaluate the antimicrobial activity of biosurfactant. Briefly, the microorganisms were prepared by mixing 1 ml of inoculum (adjusted to 2-5×10⁷ CFU/ml) with 15 ml respective medium (LB for bacteria and PDA for fungi) at 45°C and allowed to set in a petri dish (90 mm diameter). Wells were then made (8 mm diameter) with a sterile cork borer. 30 µl of the test solutions and control were added to each disc, respectively. Petri dishes were incubated at 37°C during 24 h for bacteria strains and at 28°C during 36 h for fungi strains. At the end of the incubation period, the presence of clear inhibition zones around the discs was measured. The experiments were carried out in triplicate.

Then, we further observed the morphological changes of *S. typhimurium* in response to biosurfactant treatment by AFM. The AFM analysis was performed as described by Li *et al.* (2,22). Briefly, *S. typhimurium* in LB were collected by centrifugation (10 000 rpm, 10 min) and resuspended in deionized distilled water. Two samples were prepared: one was untreated,

the other was treated with biosurfactant (3.2 g/L for both organisms). After the samples were incubated at 37°C for 50 min, all organisms were collected by centrifugation (10 000 rpm, 10 min) and resuspended in deionized distilled water again. A 6 µl drop of bacteria suspension was applied onto a glass slide. All organisms morphology was observed with AFM (JSPM-5200, Nanoscope, Japan) in tapping mode. A single-crystal silicon cantilever was used and the AFM data were collected at a typical line scan rate of 1.0 Hz.

Cell viability was determined by a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (31). Briefly, exponentially growing cells (1×10^5 /ml) were added to each well of 96-well tissue culture plate with a total volume of 100 µl per well. The biosurfactant at various concentrations was added to each well. After cultured with biosurfactant for 48 h, MTT (5 mg/ml in PBS) was added and incubated for 4 h. The formazan precipitate was dissolved in 150 µl DMSO and the absorbance at 570 nm was measured by an ELISA reader (Multiskan EX, Labsystems). The 50% inhibitory concentration (IC_{50}) of biosurfactant in cells was calculated by MTT assay. In addition, 5-fluorouracil (5-FU) was used as a standard drug.

The chromatin condensation of BEL-7402 cells was analyzed by fluorescence microscopy. Briefly, BEL-7402 cells were treated with biosurfactant (20 mg/L) for 48 h with a combination of the fluorescent DNA-binding dyes Hoechst 33258 (Sigma). Cells were harvested and washed two times with PBS (pH 7.2), and then were labeled by 10 mg/L Hoechst 33258 for 5 min. The cells were then observed under a fluorescence microscope (Olympus, Japan) using an ultraviolet (UV) filter pack.

After isolation of the biosurfactant by acidic precipitation followed by solvent extraction, the purification was followed by TLC. The surface-active substances migrated at the position of R_f 0.58-0.60 as a single point on the TLC. The relationship between surface tension and concentration of the crude biosurfactant was determined by the Ring method. CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. The CMC of the crude biosurfactant was estimated to be around 512 mg/L and the minimum surface tension of biosurfactant was 30.1 mN/m (Fig. 1a). Although the isolated biosurfactant was not as effective as many of biosurfactants described in the literature, for example, surfactin produced by *B. subtilis* ATCC 21332 with a CMC of 250 mg/L and the minimum surface tension 27.9 mN/m (8), it should be noted that the biosurfactant studied here was not as much purified as the ones described in the literature. In addition, the area of the oil-displaced circle was in good proportion to the concentration of the biosurfactant (Fig. 1b). The higher diameters represent the higher concentration of biosurfactant.

In recent years, the remarkable increase in drug-resistant bacteria makes a search for novel means and drugs of fighting

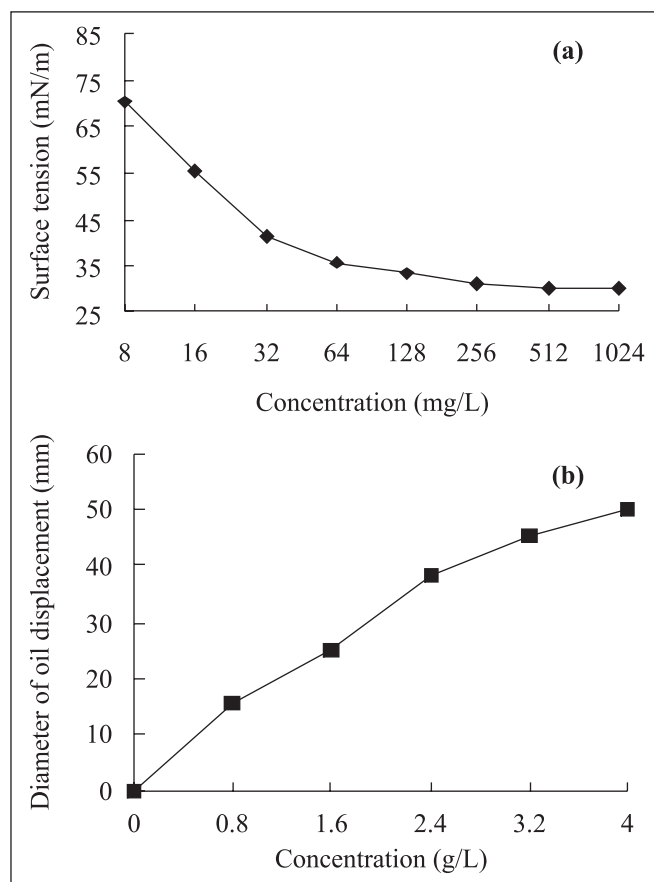


Figure 1. (a) Surface tensions of biosurfactant at various concentrations. Surface tension was measured by the Ring method at 20°C; (b) Relationship between diameter of oil displacement and various concentrations of biosurfactant. Each sample was repeated in triplicate and the control plate was evaluated with PBS.

bacterial infections imperative. Attempts to reduce or inhibit microbial adherence is a viable means to control infection, since such adherence is one of the initial stages of the infectious process. Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites (34). A biosurfactant of *P. fluorescens* was found to inhibit the adhesion of *Listeria monocytogenes* LO28 to polytetrafluoroethylene and stainless steel surfaces (30). There are reports of inhibition of biofilm formed by uropathogens and yeast on silicone rubber by biosurfactants produced by *Lactobacillus acidophilus* (43). *Lactobacillus fermentum* RC-14 releases surface-active components that can inhibit adhesion of uropathogenic bacteria, including *Enterococcus faecalis* (11). The mechanism of anti-adhesive properties of many drugs appears to be alteration of cell surface hydrophobicity.

To further test anti-adhesive activity of the biosurfactant, we measured the adherence of a variety of bacteria to 96- well microtiter plates *in vitro*. As shown in Table 1, the biosurfactant was found to significantly inhibit the adhesion of *S. typhimurium*, *E.coli* and *S. aureus*. The highest anti-adhesive activity was exhibited against *S. typhimurium* with inhibition percentage till 51.9%. Results obtained from all treated bacteria indicated that the anti-adhesive effect depended on the dose applied and the bacteria tested. The anti-adhesive activity against *M. luteus* will occur when the concentration of biosurfactant is higher than 6.4 g/L.

Zones of inhibition diameter produced by different concentrations of biosurfactant were measured using disc diffusion method. As shown in Table 2 and Fig. 2, biosurfactant exhibited interesting antibacterial and antifungal activities. The antimicrobial activity increased with increasing concentration

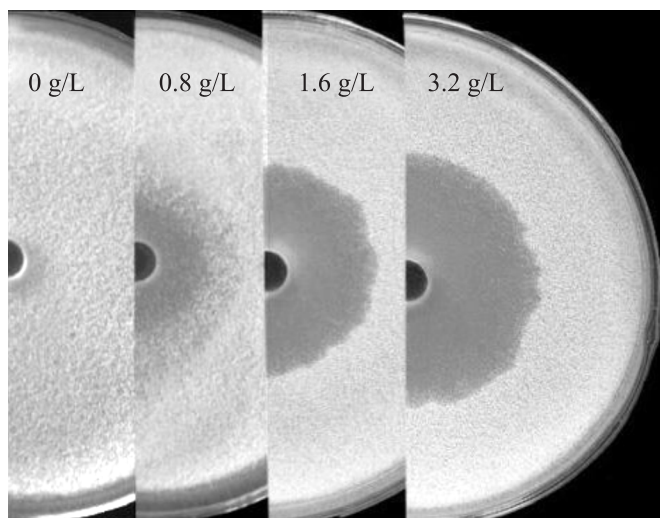


Figure 2. Antimicrobial activity of biosurfactant at various concentrations (0-3.2 g/L) against *B. cinerea*.

Table 2. Antibacterial and antifungal activities of the crude biosurfactant at different concentrations (0.8-3.2 g/L) using disc diffusion method.

Microorganisms	Zone of inhibition diameter (mm)		
	0.8 g/L	1.6 g/L	3.2 g/L
Bacteria			
<i>Micrococcus luteus</i>	8.1	13.4	18.0
<i>Salmonella typhimurium</i>	7.4	10.3	12.0
<i>Staphylococcus aureus</i>	7.7	9.0	11.4
<i>Bacillus cereus</i>	—	—	—
<i>Escherichia coli</i>	—	—	—
Fungi			
<i>Botrytis cinerea</i>	38.2	41.3	48.8
<i>Fusarium moniliforme</i>	16.5	20.1	28.4
<i>Colletotrichum truncatum</i>	6.7	7.3	11.4
<i>Penicillium candidum</i>	6.1	8.4	10.8
<i>Gibberella zeae</i>	—	—	—

‘—’ = no inhibition.

of biosurfactant. It has a strong activity against *B. cinerea*, *F. moniliforme*, *M. luteus* and *S. typhimurium*. Recently, several lipopeptide biosurfactants produced by *B. subtilis* (21,36,44) and *B. licheniformis* (9,13,46) have been shown to have antimicrobial activity. Vater *et al.* (41) had described the antifungal properties of surfactin. Hiradate *et al.* (12), Yu *et al.* (47) and Cho *et al.* (7), reported about another lipopeptide, iturin, produced by *Bacillus strains* to suppress phytopathogenic fungi. In our study, the lipopeptide biosurfactant from *Bacillus natto* TK-1 exhibited both antibacterial and antifungal activities.

Previous studies showed that various interesting biological properties of lipopeptide biosurfactants were presumed to be the result of interactions with the membranes of target cells

Table 1. The anti-adhesive activity of the crude biosurfactant at different concentrations (0.8-12.8 g/L) against a variety of bacteria to 96- well microtiter plates *in vitro*.

Bacteria	Mean % reduction in adherence				
	0.8 g/L	1.6 g/L	3.2 g/L	6.4 g/L	12.8 g/L
<i>Salmonella typhimurium</i>	5.3	9.7	18.5	28.4	51.9
<i>Escherichia coli</i>	4.1	9.0	19.8	30.6	44.3
<i>Staphylococcus aureus</i>	4.9	7.5	16.5	20.7	30.2
<i>Micorococcus luteus</i>	—	—	—	5.9	11.2
<i>Bacillus cereus</i>	—	—	—	—	—

‘—’ = no reduction in adherence.

(37). By Scanning electron and optical microscopies, Thimon *et al.* (39) discovered iturin produced by *B. subtilis*, which affects the morphology and membrane structure of yeast cells. Studies by Kim *et al.* (20) also proved that fengycin from *B. thuringiensis* CMB26 was capable of affecting the cell surface of the phytopathogenic fungus *C. gloeosporioides*, an of the bacteria *E. coli* O157 and *P. rapae crucivora*. In our study, the bactericidal effects upon *S. typhimurium* were examined using AFM. It can be observed from the images that before treatment of biosurfactant the *S. typhimurium* showed normal smooth surfaces. Meanwhile, clustering of cells was observed. In contrast, cells treated with biosurfactant for 50 min exhibited dramatic changes in the structure of the cell walls. The outer membrane of the bacteria was extremely rough and some membrane residues were found around the cells. Cells were scattered and few clustering were seen (data not shown).

In recent years, there are few reports about lipopeptide biosurfactants with effect on tumor cells. As part of our program to evaluate the cytotoxic potential of the biosurfactant, K562 and BEL-7402 cells were treated with a series of biosurfactant concentrations for 48 h, and then cell viability was evaluated by MTT assay. The results in Fig. 3a showed that biosurfactant was able to suppress the viability of K562 and BEL-7402 cells in a dose-dependent manner. The IC_{50} for K562 and BEL-7402 cells at 48 h were 19.1 mg/L and 30.2 mg/L, respectively. In addition, the effects of biosurfactant on the survival rate of normal cell lines were also examined, including BRL and HEK293. The IC_{50} for BRL and HEK293 cells at 48 h were 105.4 mg/L and 93.8 mg/L, respectively (Fig. 3b). Compared with K562 and BEL-7402 cells, biosurfactant at the same concentration had lower toxicity to the normal cell lines *in vitro*.

Direct observation of the cells under the fluorescent microscope tends to suggest that biosurfactant induces apoptosis of BEL-7402 cells. The nuclear morphology was analyzed by Hoechst 33258 staining. Cytoplasm and chromatin condensation, were detected in BEL-7402 cells at 48 h of treatment with 20 mg/L biosurfactant (data not shown). Apoptosis is a tightly regulated form of cell death, also called the programmed cell death (35). It has a central role in the pathogenesis of human disease when the genes controlling the apoptotic process are suppressed, overexpressed or altered by mutation (38). Disordered apoptosis is implicated in a variety of human diseases. Research into apoptosis is proceeding at a fast pace and this has led to the possibility of new therapeutic approaches to some tumor diseases (16). Our study demonstrated that the biosurfactant induced morphological changes. BEL-7402 cells treated with biosurfactant were characterized by chromatin condensation and cell shrinkage in the early stage, and then the nucleus and cytoplasm fragment, forming membrane-bound apoptotic bodies which can be engulfed by phagocytes.

In the present study, the biosurfactant produced by *Bacillus natto* TK-1 has a strong surface activity. From the results of

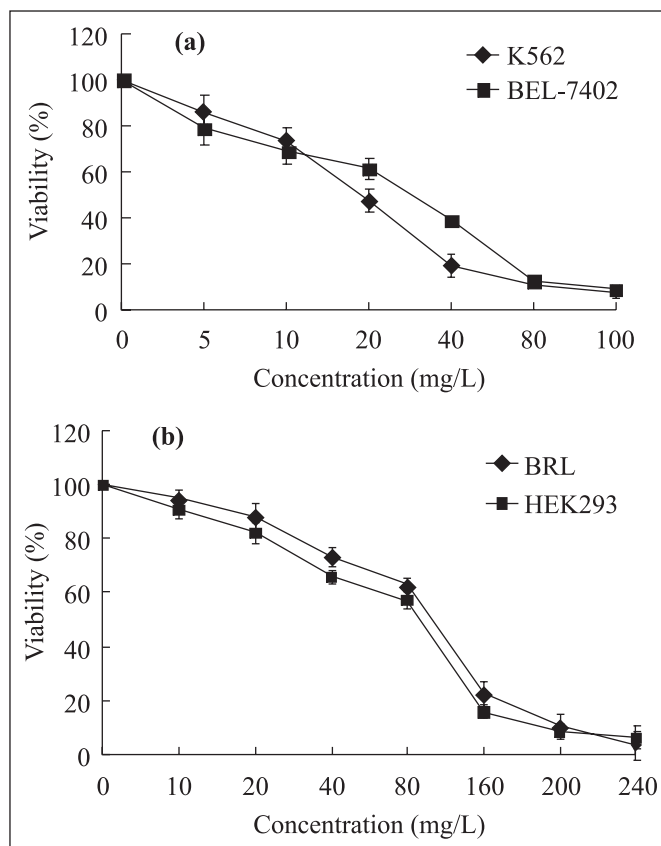


Figure 3. Effects of biosurfactant on the viability of tumor and normal cells. K562 and BEL-7402 cells (a), and BRL and HEK293 (b) were treated with biosurfactant at the indicated concentrations for 48 h. The viability was determined by MTT assay. Each value represents mean \pm S.D. of three independent experiments.

anti-adhesive, antimicrobial and antitumor activities assay, biosurfactant exhibits attractive therapeutic and biotechnological properties, which represents a promising candidate as a new drug. The information of the morphological changes of bacteria provided by AFM indicated that the disruption of the outer membrane of *S. typhimurium* cells was the possible mechanism of the action of the lipopeptide biosurfactant. In addition, by fluorescence examination, biosurfactant induced chromatin condensation of BEL-7402 cells and showed common features of apoptosis.

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RESUMO

Avaliação de um lipopeptídeo biosurfactante de *Bacillus natto* TK-1 com fonte potencial de atividade antiadesiva, antimicrobiana e antitumoral

Um lipopeptídeo biosurfactante produzido por *Bacillus natto* TK-1 apresenta intensa atividade de superfície. Verificou-se que o biosurfactante apresentou atividade antiadesiva contra várias cepas bacterianas, e também atividade antimicrobiana de amplo espectro. O biosurfactante causou uma redução significativa na viabilidade de células tumorais, de forma dose-dependente.

Palavras-chave: biosurfactante, atividade antiadesiva, atividade antimicrobiana, atividade antitumoral

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