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Regular paper

Biological activity of surfactins — a case of a biosurfactant produced by *Bacillus subtilis* PCM 1949*

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Biosurfactants are microbial surface active compounds which, contrary to synthetic surfactants, are natural in origin, biodegradable and less toxic to a human organism. For that reason, there is a great research potential in studies aimed at their purification, finding potential ways of their utilization and decreasing their production costs. This paper demonstrates the process of isolating and purifying a surfactin synthesized by *Bacillus subtilis* PCM 1949. Surfactin samples were prepared by a classical organic solvent extraction and were studied using mass spectrometry (MS). Analysis of the susceptibility profile of microorganisms utilized in the diffusion-plate tests demonstrated that their sensitivity to this surfactin is differentiated and depends on the microorganism species. In our studies, we found that the selected strains of bacteria and fungi were insensitive to this surfactin at a wide range of concentrations.

Key words: surfactin, *Bacillus subtilis*, biosurfactants, biological activity

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INTRODUCTION

Surfactin was discovered by Arima and coworkers from the culture broth of *Bacillus subtilis* (Arima *et al*., 1968). This compound is a cyclic lipoheptapeptide, containing seven residues of D- and L-amino acids and one residue of a β-hydroxy fatty acid, closed by lactone formation (Shaligram & Singhal, 2010) (Fig. 1).

The ability to biosynthesize surfactin is a property of bacteria from the *Bacillus* genus. Surfactin is formed by non-ribosomal peptide synthetases (NRPSs), which govern all necessary steps in this process. Surfactin possesses an antibiotic activity (Stein, 2005), and its amphipathic properties cause it to be stable under both, hydrophobic and hydrophilic conditions. It has the ability to reduce surface tension of water from 72 to 27 mN/m at a concentration as low as 0.005% (Mnif & Ghribi, 2015).

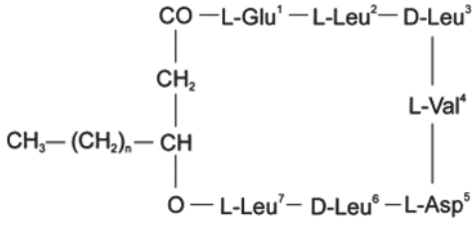

Figure 1. Primary structure of surfactin (n = 9–11).

In addition to its properties as a detergent, this biosurfactant exhibits strong antibacterial and antifungal properties (Nagórska *et al*., 2007).

Bacterial cells can attach to surfaces and after cell division and proliferation, form aggregates commonly referred to as biofilms. Gudiña *et al.* described that biosurfactants, surfactin among them, can affect the adhesion of microorganisms, because they partition the interfaces of fluid phases with distinct polarities and hydrogen bonding (Gudiña *et al*., 2013). Surfactin inhibits the adhesion of pathogenic organisms to solid surfaces or infection sites. In vinyl urethral catheters, it eliminates biofilm formation by *Salmonella, Escherichia* and *Proteus* genus, (Singh & Cameotra, 2004; Seydlová & Svobodová, 2008; Krasowska, 2010). These properties can be potentially utilized in medicine, in prevention of nosocomial infections. In recent years, it has become known that surfactin induces apoptosis in breast cancer cells through a ROS/JNK-mediated mitochondrial/caspase pathway (Cao *et al*., 2010). In this case, the mechanism of apoptosis results in inducing the reactive oxygen species (ROS) formation which leads to a homeostasis disorder in one of the metabolic pathways (Duarte *et al*., 2014).

Researchers from Germany studied the effect of surfactin on different viruses as well, and discovered its activity against herpes viruses, retroviruses and other enveloped viruses carrying RNA and DNA (Vollenbroich *et al*., 1997). The antiviral activity of surfactin is primarily due to the physicochemical interaction between the membrane active surfactant and the virus lipid envelope. This phenomenon induces the outflow of the virus's genetic material, and at appropriate concentrations of surfactin, probably the complete destruction of the capsid (Kracht *et al*., 1999).

The aim of this study was to determine the influence of surfactin produced by *Bacillus subtilis* PCM 1949 on given microorganisms. Moreover, the optimal conditions for this biosurfactant production process were determined and the steps of its incubation, isolation and purification were developed.

MATERIALS AND METHODS

Studied bacterial strains and fungi species. *Bacillus subtilis* PCM 1949, *Serratia marcescens* PCM 501, *Escherichia coli* PCM 384, *Pseudomonas fluorescens* PCM 2123, *Penicil-*

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Abbreviations: TLC, thin layer chromatography; R_f, retardation factor; MS, mass spectrometry; NRPSs, non-ribosomal peptide synthetases; ROS, reactive oxygen species

Table 1. Study of the examined bacterial strains' sensitivity to surfactin

Surfactin concentration [mM]	Bacterial strains		
	Serratia marcescens PCM 501	*Escherichia coli* PCM 384	*Pseudomonas fluorescens* PCM 2123
	Width of the inhibition zone (mm) ± 1 (mm)		
30	–	12	–
25	–	11	–
20	–	10	–
15	–	–	–
10	–	–	–
5	–	–	–
2.5	–	–	–

lium purpurogenum ATCC 66107, *Penicillium citrinum* ATCC 36382, *Beauveria bassiana* ATCC 22567, *Beauveria felina* DSM 4678, *Cladosporium* sp. LOCK E125, *Fusarium culmorum* LOCK E136 and *Fusarium oxysporum* LOCK E90 were derived from Polish Collection of Microorganisms, deposited at the Institute of Immunology and Experimental Therapy in Wroclaw. *Bacillus subtilis* PCM 1949 was used for the surfactin production and the other strains were employed to study the biological activity of this biosurfactant.

Surfactin production, isolation and identification. For crude surfactin production from *Bacillus subtilis* PCM 1949, 500 ml of mineral medium containing mineral salts, glucose as a carbon source and $NaNO_3$ as a nitrogen source, were inoculated and incubated for 14 days at 37°C (Abdel-Mawgoud *et al*., 2008). The *Bacillus* culture was centrifuged at 4000 rpm and 25°C for 20 min, acidified using 1 M HCl (Sigma-Aldrich, USA) and stored in a refrigerator for 24 hours. After this time, the culture was centrifuged again at 4000 rpm and 4°C for 10 min. The supernatant was decanted, and the precipitate of surfactin was frozen and subsequently subjected to a freeze-drying process, in order to use it for further study. Each 0.1 g of obtained surfactin powder was then extracted with 25 ml of ethyl acetate (Sigma-Aldrich, USA), and the solvent was evaporated with a rotary wash at 90 rpm and 20°C for 15 min (Chen & Juang, 2008). The identification of the compound obtained after extraction was achieved by mass spectrometry. Moreover, the recovered surfactin powder was dissolved in the CH_3OH (Sigma-Aldrich, USA) and analyzed using the TLC method in the $CHCl_3$/ CH_3OH /NH_4OH (80/25/4, v/v/v) developing system. The secondary amino groups were visualized with ninhydrin and R_f factor value was calculated.

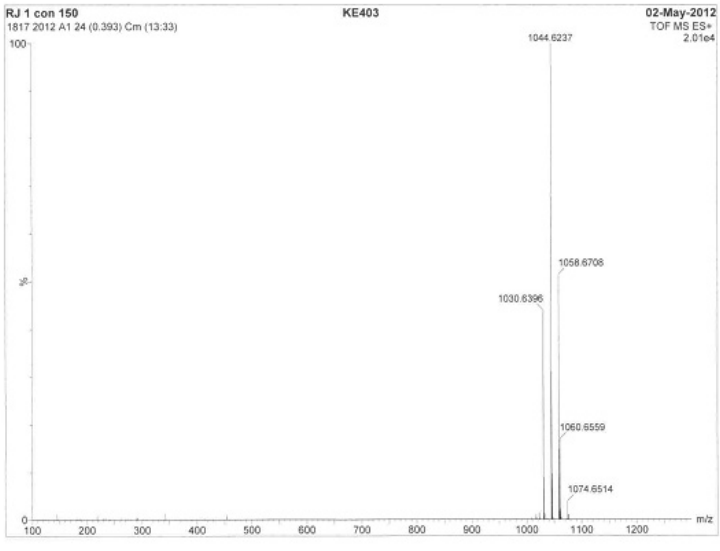

Figure 2. Mass spectrum of surfactin.

Surfactin biological activity studies. The biological activity studies of surfactin were a crucial part of our work, and for this purpose, cultures of *Serratia marcescens* PCM 501, *Escherichia coli* PCM 384, *Pseudomonas fluorescens* PCM 2123, *Penicillium purpurogenum* ATCC 66107, *Penicillium citrinum* ATCC 36382, *Beauveria bassiana* ATCC 2256, *Beauveria felina* DSM 4678, *Cladosporium* sp. LOCK E125, *Fusarium culmorum* LOCK E136 and *Fusarium oxysporum* LOCK E90 were prepared. In order to observe the sensitivity of the bacteria to our antibiotic, Petri dishes with 10 ml of Mueller-Hinton agar (Thermo Scientific, USA) were used. Carpet cultures with 24 hour broth cultures of selected bacterial strains were cultivated. For this purpose, 0.1 ml of the culture of each microorganism was diluted to 1×10^{-2} and then transferred to the surface of a Petri dish with Mueller-Hinton agar. Simultaneously, the isolated fungi species were plated on the Petri dishes with Sabouraud medium with chloramphenicol (Thermo Scientific, USA), and incubated for 7 days at 26°C. With the obtained fungal culture, inocula, suspended in 10 ml Triton X 100 (Thermo Scientific, USA), were applied to the plate with Sabouraud medium in 0.2 ml volume. On each plate, 1 filter paper disk with 10 µl of surfactin in phosphate buffer pH 7.4 was applied. The concentration of surfactin was in the range from 2.5 to 50 mM. The plates were incubated for 24 or 72 hours at different temperatures, suitable for the particular microorganism. Next, we observed the growth of microorganisms and on this basis investigated whether the strain was sensitive to surfactin or not.

RESULTS

Mass spectrum analysis of surfactin revealed several isomers of this biosurfactant. We obtained a signal from the protonated surfactin form with a mass of 1030.6 m/z, a sodium adduct with a mass

Table 2. Study of the examined fungi species' sensitivity to surfactin

Surfactin concentration [mM]	Fungi species						
	Beauveria bassiana ATCC 22567	*Cladosporium* sp. LOCK E125	*Beauveria felina* DSM 4678	*Fusarium culmorum* LOCK E136	*Fusarium oxysporum* LOCK E90	*Penicillium purpurogenum* ATCC 66107	*Penicillium citrinum* ATCC 36382
	Width of the inhibition zone (mm) ± 1 (mm)						
50	–	–	–	20	–	–	–
40	–	–	–	19	–	–	–
30	–	–	–	17	–	–	–
20	–	–	–	15	–	–	–
10	–	–	–	12	10*	–	–
5	–	–	–	–	–	–	–
2.5	–	–	–	–	–	–	–

*Indicates *Fusarium oxysporum* LOCK E90 artifact at a 10 mM surfactin concentration.

of 1044.6 m/z and also a potassium adduct – 1060.7 m/z (Fig. 2). These values are close to the results reported in the literature (Kowall *et al.*, 1998).

The influence of surfactin at different concentrations on the growth of several bacterial strains and fungi species was observed (Table 1, Table 2).

The results presented in Table 1 demonstrate that only *Escherichia coli* PCM 384 was sensitive to surfactin at the concentration range from 20 mM to 30 mM.

The results presented in Table 2 demonstrate that *Fusarium culmorum* LOCK E136 was sensitive to surfactin at the concentration range from 10 mM to 50 mM.

DISCUSSION

Mass spectrum analysis determined that the examined compound contained surfactin in several isoforms. This fact confirms findings described in the literature which indicate that surfactin is found in several isoforms which differ in the structure of leucine, valine and isoleucine arrangement, mainly at positions 2 and 7 (Kowall *et al.*, 1998).

The influence of the available antibiotics and chemotherapeutic compounds on the growth of microorganisms that cause many diseases, infections and allergies, requires an accurate assessment. Some pharmaceuticals have a cytotoxicity that is too high to utilize them as therapeutic agents. Therefore, while developing the effective antifungal or antibacterial treatment for a given species, the proper selection of the active agent, the dose, schedule of administration and also the form of drug applications should all be taken into account.

The microbial susceptibility profile to surfactin was evaluated in our work (Table 3).

The results of our studies on the biological activity of surfactin are surprising. We have demonstrated that all examined bacterial strains, with the exception of *Escherichia coli* PCM 384 have a high resistance to surfactin.

The antibacterial and antifungal activity of the studied biosurfactant is varied. It is noticeable, that the susceptibility profile to our compound seems to depend on the species of the microorganism. *Fusarium culmorum* LOCK E136 is particularly interesting in this case, due to its sensitivity to surfactin at the concentration range from 10 mM to 50 mM.

Based on the results from the Table 1 and Table 2, it may be seen that with an increased concentration of surfactin, the width of the inhibition zone of microorganism's growth was increased. In accordance with this trend, the widest growth inhibition zone of *Fusarium culmorum* LOCK E136 (20 mm ± 1 mm) was observed at the highest surfactin concentration used (50 mM) (Table 2). Interestingly enough, results obtained for *Fusarium oxysporum* LOCK E90 were considered to be an artifact. The morphology of the mycelium may have changed due to the utilized solvent. Thus, in one of the plates, at a relatively low concentration of 10 mM surfactin, clear

Table 3. Study of the microbial susceptibility profile to surfactin

Microbial species	Family	Resistance to surfactin	Sensitivity to surfactin
Serratia marcescens PCM 501	Enterobacteriacae	+	–
Escherichia coli PCM 384	Enterobacteriacae	–	+
Pseudomonas fluorescens PCM 2123	Pseudomonaceae	+	–
Penicillium purpurogenum ATCC 66107	Trichocomaceae	+	–
Penicillium citrinum ATCC 36382	Trichocomaceae	+	–
Beauveria bassiana ATCC 22567	Cordycipitaceae	+	–
Beauveria felina DSM 4678	Cordycipitaceae	+	–
Cladosporium sp. LOCK E125	Davidiellaceae	+	–
Fusarium culmorum LOCK E136	Nectriaceae	–	+
Fusarium oxysporum LOCK E90	Nectriaceae	–	+

distinctive crescent-shapes appeared. This area could be mistakenly recognized as a growth inhibition zone, but it is not. In our study, *F. oxysporum* LOCK E90 was classified as resistant to surfactin at the whole range of concentrations.

The studies conducted here presented an insensitivity to surfactin among most of the strains employed in the experiments. Some of these microorganisms are pathogens (*F. culmorum*) or opportunistic microorganisms (*E. coli, P. fluorescens*), which can be the source of contamination in production lines and can be the cause of various diseases in people with impaired immunity. The lack of correlation of *in vitro* and clinical results present in the literature may be accounted for by the fact that natural bacterial biofilms and fungal mycelia are much more difficult to treat with antibiotics, chemotherapeutic agents and disinfectants compared with a suspension of fungal spores and bacterial cells utilized in standard tests and occur quite rarely *in vivo*.

Although there are many known compounds with antimicrobial and antitumor activity, there is a constant search for new substances produced for e.g. by microorganisms.

Due to their biological activity, lipopeptide biosurfactants produced by *Bacillus subtilis* present a great potential for biotechnological and the biopharmaceutical applications. In spite of this immense potential, their use is still limited, possibly due to their high production costs, insufficient knowledge of their molecular action mechanism and the fact that their toxicity to the cells is unclear.

For this purpose, the future research projects dedicated to these compounds should focus on studying their chemical structure and discovering new properties, useful in many industrial fields, including biomedical ones.

Conflict of interest

The authors declare no conflict of interest.

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