

Full Length Research Paper

Antifungal and antibacterial properties of surfactin isolated from *Bacillus subtilis* growing on molasses

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The aim of the study was to evaluate antifungal and antibacterial properties of surfactin isolated from *Bacillus subtilis* growing on molasses. Molasses replaced the traditional microbiological media to culture the *B. subtilis*. 10 phytopathogens and 30 *E. coli* strains were used in the study. The results demonstrated the ability of surfactin produced by *Bacillus sp.* growing on molasses to inhibit mycelial growth of the 4 fungi from 10 tested and all *E. coli* strains measured by agar plate inhibition assays. Fungi inhibited to the greatest degree as measured by the inhibition zones were *Botrytis cinerea* A 258 (~50% of inhibition), *Sclerotinia sclerotiorum* K 2291 (~50% of inhibition), *Colletotrichum gloeosporioides* A 259 (~40% of inhibition), *Phoma complanata* A 233 (~38% of inhibition), *Phoma exigua var. exigua* A 175 (~20% of inhibition). Among the *E. coli*, high inhibition growth was noted in 76% of the isolates. Application of natural products such as biosurfactant may be a new approach to biological control therefore reducing the need for synthetic chemical compounds.

Key words: Phytopathogens, *Escherichia coli*, *Bacillus subtilis*, surfactin, biocontrol, phytopathogens, biosurfactant.

INTRODUCTION

Gram-positive bacteria, including *Bacillus spp.*, produce a variety of antibacterial and antifungal metabolites, antibiotics such as zwittermicin-A and kanosamine and cyclic lipopeptides (LPs) from the surfactin, iturin and fengycin families (Emmert and Handelsman, 1999; Jacobsen et al., 2004; Ongena and Jacques, 2007). Cyclic lipopeptides have well potential in biotechnological applications because of their biosurfactant properties. Biosurfactants have numerous beneficial qualities including non-toxic, non-hazardous, biodegradable, environmental friendly, selective, effective under extreme

conditions, numerous industrial applications, and unique surface-active properties. In spite of these beneficial properties, their higher production cost compared to synthetic surfactants is a major drawback. Biosurfactants could potentially replace synthetic surfactants if cost of production was lowered substantially. One of the ways to reduce substrate cost for biotechnology at present is to use recycled agricultural wastes at the right balance and high concentrations of nutrients to support microbial growth and biosurfactants production.

So far, several renewable substrates including various

Table 1. Selected parameters of molasses as medium for *Bacillus* growth.

Parameter	Unit	Molasses
pH		7,65
COD	mgO ₂ /L	49075
BOD ₅	mgO ₂ /L	25060
Water content	%	17,75
Organic matter	%	82,15
Sucrose	%	51,25
Aminoacids	%	5,5
Total N	%	1,94

agricultural and industrial by-products and waste materials have been intensively studied for microorganism cultivation and biosurfactant production on the laboratory scale. These include olive oil mill effluent, waste frying oil, oil refinery wastes, soapstock, molasses, whey, starch wastes, cassava-flour processing effluent and distillery waste (Deleu and Paquot, 2004; Makkar and Cameotra, 2002). Value added products or benefits can improve the economics of such bioprocesses including microbial waste reduction. In our study, the antifungal and antibacterial properties of surfactin produced by *Bacillus subtilis* cultured on molasses were assessed.

MATERIALS AND METHODS

Microbial strains

The bacterial strain named T'-1 used in this study were isolated from 100-year-old oil refinery sludge in Czechowice-Dziedzice (Poland) as described by Berry et al. (2006). The isolate was identified on the previous study by the following method: 16S rRNA gene sequence analysis, fatty acids analysis (FAME) and BIOLOG™ system (Płaza et al., 2012). The fungal plant pathogens originated from the collection of the Department of Phytopathology and Mycology, University of Life Sciences in Lublin. They were previously isolated from various parts of caraway (*Sclerotinia sclerotiorum* K2291, *Phomopsis diachenii* K 657, *Septoria carvi* K 2082, *Colletotrichum dematium* K 425), angelica (*Botrytis cinerea* A 258, *Colletotrichum gloeosporioides* A 259, *Phoma complanata* A 233, *Phoma exigua* var. *exigua* A 175) and grapevines (*Phomopsis viticola* W 977, *Rhizoctonia solani* W 70). Their taxonomic position was based on macro- and microscopic features and pathogenicity tests (Machowicz-Stefaniak et al., 2011). Majority of these pathogens can infect a lot of plant species but some of them such as *Phomopsis viticola*, *Septoria carvi* and *Phomopsis diachenii* infect only one host or a few species within the same botanical family. Moreover, the studied strains represented both soil pathogens and above-ground plant pathogens. Bacteria *Escherichia coli* were isolated from the raw effluents from the wastewater treatment plant according to EN ISO 9308-1:2000.

Culture conditions for isolation of biosurfactant

Bacterial suspensions of *B. subtilis*, obtained from a nutrient agar slant incubated for 24 h at 30°C, in the liquid Standard Methods medium of the following composition (g x cm⁻³): peptone – 8, yeast extract – 2.5, glucose – 1 were adjusted to OD_{600nm} 0.65 (ca. 107-

108 CFU x dm⁻³). Then, three ml of the bacterial suspension was inoculated in 300 ml Erlenmeyer flasks containing 100 ml of the sterilized 10% (v/v) of molasses as nutrients sources. The characteristics of the molasses are presented in Table 1. The cultures were grown aerobically at 30°C for 96 h with constant shaking (110 rpm). After incubation, the cultures were centrifuged at 10000 g for 20 min. Then, the supernatants were used to isolate biosurfactant.

Isolation of surfactin

Biosurfactant was isolated as described Joshi et al. (2008). Biosurfactant was precipitated by adjusting the pH of the cell-free supernatant to 2.0 using concentrated HCl, then the acidified supernatant was stored overnight at 4°C. The precipitate was centrifuged at 9000 g for 15 min. Then, the biosurfactant pellet was suspended in the redistilled water and adjusted to pH 7.0 using 2 N NaOH. The surfactin concentration was expressed as described by Sheppard and Mulligan (1987).

In vitro antagonism experiment

Biosurfactant isolated from *Bacillus* sp. were tested for the ability to inhibit the growth of fungal plant pathogens in Petri dishes on potato dextrose agar (PDA) medium as described by Velmurugan et al. (2009) with some modifications. 200 µl of the surfactin solution were added drop by drop in wells made with a sterile cork borer at 1 cm diameter in gelified medium. Fungal growth inhibition was evaluated after plate incubation at 25°C for 3 or 10 days, depending on the pathogens growth. Sterilized molasses was used as the controls. Antifungal index (mycelial growth inhibition) was calculated as the follows:

$$\text{antifungal index (\%)} = \left(1 - \frac{A}{B}\right) * 100\%$$

where, A = diameter of fungal growth in the treatment samples
B = diameter of fungal growth in the controls

The ability to inhibit the growth of *E. coli* isolates by biosurfactant was performed in Petri dishes on standard method agar (SMA) medium. 200 µl of the surfactin were dispensed in wells made with a sterile cork borer at 1 cm diameter in the centre of gelified medium. The critical micelle concentration (CMC) value determined according to the method described by Sheppard and Mulligan (1987), was 0.127 mg/liter. The isolates from the solid medium were inoculated around the wells. Bacterial growth inhibition was evaluated after plate incubation at 37°C by 48 h. The experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Currently, the main drawback for the widespread use of biosurfactants is the disadvantageous economics of their production. Biosurfactants could potentially replace synthetic surfactants if cost of their production is lowered substantially. Achieving this goal requires finding alternative inexpensive substrates and highly efficient microorganisms for biosurfactant production. The choice of inexpensive raw materials is important to the overall economics of the process because they account for 50% of the final product cost. The best way to reduce substrate cost for biotechnology at present is to use wastes

Table 2. Effect of surfactin on tested phytopathogens.

Phytopathogen	Percentage (%) inhibition
<i>Botrytis cinerea</i> A 258	51,03 ± 2,04
<i>Rhizoctonia solani</i> W 70	16,67 ± 1,23
<i>Sclerotinia sclerotiorum</i> K 2291	52,42 ± 0,88
<i>Phomopsis diachenii</i> K 657	0,00
<i>Phomopsis viticola</i> W 977	7,14 ± 1,22
<i>Septoria carvi</i> K 2082	0,00
<i>Colletotrichum dematium</i> K 425	0,00
<i>Colletotrichum gloeosporioides</i> A 259	45,06 ± 2,45
<i>Phoma complanata</i> A 233	38,33 ± 0,97
<i>Phoma exigua</i> var. <i>exigua</i> A 175	20,27 ± 1,01

with the right balance of carbohydrates and lipids to support optimal bacterial growth and biosurfactants production, and which are either free and carry a cost credit for environmental benefit (Makkar and Cameotra, 2002). As known, millions of tons of hazardous and non-hazardous wastes are generated each year throughout the world. There is a great need for better management of these wastes via the concept: reduce, reuse, and recycle (Makkar and Cameotra, 2002).

The 16S rRNA gene sequencing could not clearly assign isolate T'-1 to any species of *Bacillus* as isolate showed > 99% similarity to two distinct species *B. subtilis* and *Bacillus amyloliquefaciens*. The metabolic profile of 94 biochemical tests as measured by the BIOLOG™ system, showed identification matches for *B. subtilis* ss spizenii with SIM value of 0.567 utilizing 68 substrates. However, FAME analysis showed that this strain was *B. subtilis*. The results identified that the surface active properties of free-cell supernatant were the best when bacteria grew on molasses. Replacing traditional microbiological media with agro-industrial wastes as substrates for biosurfactant production holds great potential. Moreover, this will reduce many management problems of processing industrial waste and cost of biosurfactant production.

Among the potential biosurfactant-producing microbes, *Bacillus* species are known to produce cyclic lipopeptides including surfactins, iturins, fengycins and lichenysins as the major classes of biosurfactants (Jacques, 2011; Perez-Garcia et al., 2011). Surfactin, one of the most effective surfactants produced by *Bacillus* strains showed a ST of 25 mN/m, an IT < 1.0 mN/m and CMC of 0.025 g x dm⁻³. Poliwoda et al. (2012) characterized the biosurfactant produced by T'-1 isolate cultured on molasses as surfactin. In Table 2 and Figure 1, antifungal activities of the surfactin are presented. Fungi inhibited to the greatest degree as measured by the inhibition zones were *Botrytis cinerea* A 258 (~50% of inhibition), *Sclerotinia sclerotiorum* K 2291 (~50% of inhibition), *Colletotrichum gloeosporioides* A 259 (~40% of inhibition), *Phoma complanata* A 233 (~38% of inhibition), *Phoma exigua* var. *exigua* A 175 (~20% of inhibition). In previous work,

the antifungal property of different surfactants – Rokamin K30, Rokamin SR5, Sulforokanol L270/1, SDS, Tergitol 15-S-7, Tween 80, natural surfactant – saponin and cell-free supernatant from *B. subtilis* growing on agro-industrial wastes were evaluated (Plaza et al., 2012). The results showed that surfactant like Rokamin K30, Rokamin SR5, Sulforokanol L270/1 inhibited mycelial growth two times more strongly than other chemical surfactants. Compared with the chemical surfactants, culture-free supernatant was more active than saponin, Tween 80 and sodium deodecyl sulphate (SDS).

On the other hand, among the *E. coli* isolates high inhibition caused by surfactin was noted (Table 3 and Figure 2). Ghribi et al. (2012) evaluated the antimicrobial activity of the biosurfactant produced by *B. subtilis* SPB1 growing on traditional microbiological medium against bacteria, fungi and yeast. The biosurfactant produced by *B. subtilis* SPB1 was characterized by an important antifungal activity, especially, against *Penicillium notatum*, *Penicillium italicum*, and *Aspergillus niger* and lower activity against *Rhizopus oryzae* and *Aspergillus oryzae*. This activity was clearly negative against *Alternaria alternata*, *Puccinia allii* and *Peronospora destructor*. Moreover, it was clear that this compound presented also an important antiyeast activity, mainly, against *Candida albicans*. The compound showed important antimicrobial activity against microorganisms with multidrug-resistant profiles. Its activity was very effective against *Staphylococcus aureus*, *Staphylococcus xylosus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*. The compound showed also higher activity against Gram-positive bacteria than against Gram-negative bacteria.

Bacteria of the genus *Bacillus* are known as producers of different number of secondary metabolites with the properties against bacteria, fungi and yeast growing on traditional microbiological media (Ongena et al., 2007; Arguelles-Arias et al., 2009; Perez-Garcia et al., 2011). Jacques (2011) described four main lipopeptides families from the *Bacillus* species: the surfactins, the iturins, the fengycins (or plipastatins) and the kurstakins. Joshi et al. (2008) described the antifungal compound produced by *B. subtilis* 20B cultured on LB medium. The growth

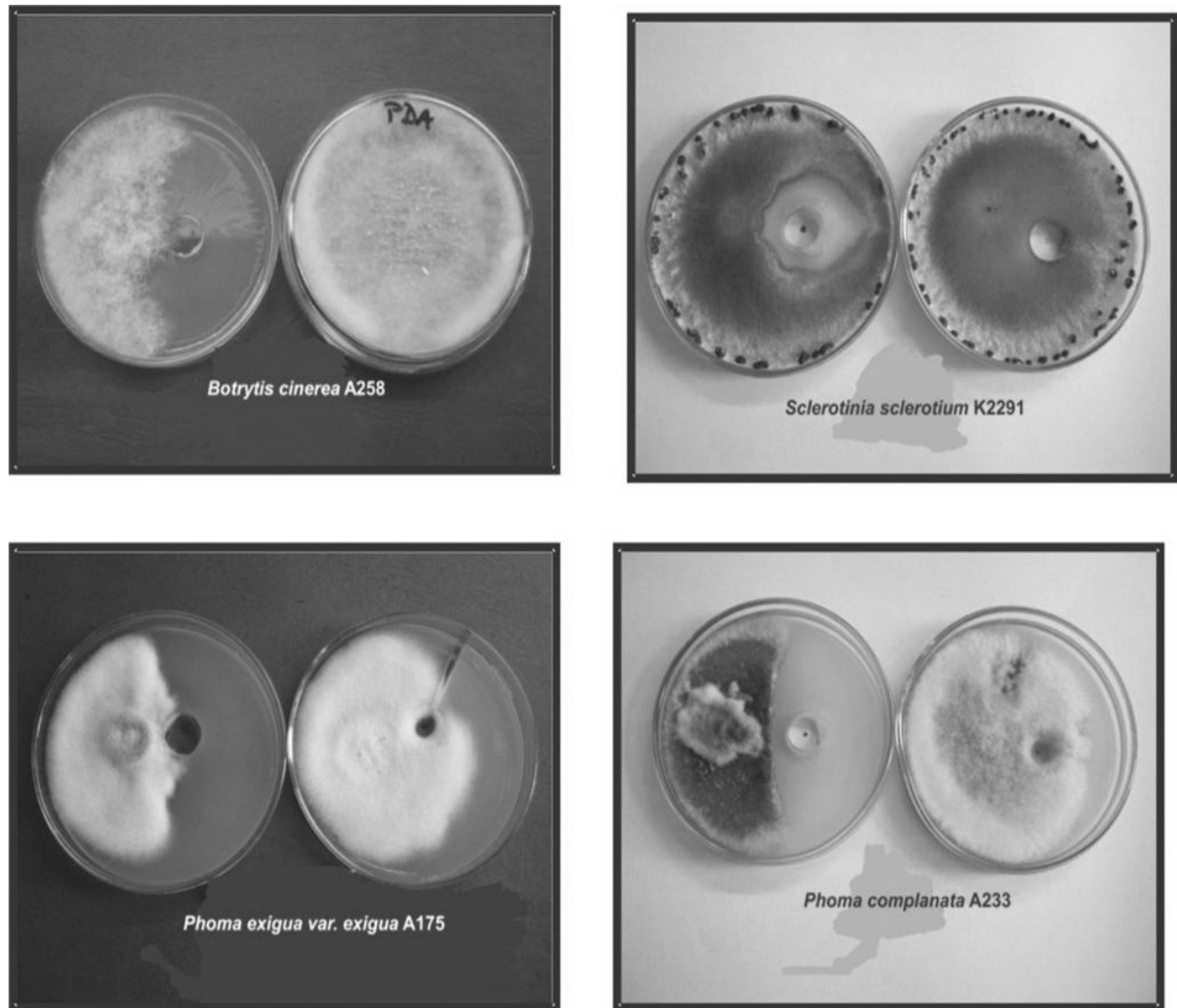


Figure 1. Growth inhibition of selected phytopathogens by surfactin (T⁻¹ isolate cultured on molasses). Left side, surfactin; right side, control.

inhibition of *Chrysosporium indicum*, *Alternaria burnsii*, *Fusarium oxysporium*, *Fusarium udum*, *Trichoderma herzanium* and *Rhizoctonia bataticola* was observed. Whereas, no inhibition of *Aspergillus niger* and *Penicillium chrysogenum* was observed. *B. subtilis* producing iturin A and surfactin was shown to be effective for the control of damping-off caused by *R. solani* in tomato plants (Asaka and Shoda, 1996). Ongena et al. (2005) describe the ability of *B. subtilis* strain GA1 to protect wounded apple fruits against grey mould disease caused by *Bacillus cinerea*. Treatment with endospores and vegetative cells from the strain GA1 was evaluated. The results confirm the potential of *B. subtilis* species for the control of post-harvest diseases for other fruits (Perez-Garcia et al., 2011).

The toxicity of iturins to fungi has been found to rely on their ability to permeate membranes (Jacques, 2011). Also, fengycins have fungitoxic activity but more speci-

fically against filamentous fungi. The fengycins action is less known compared to the other lipopeptides. Several studies present that lipopeptides are co-produced and active in synergistic way, for example surfactin with iturin, surfactin with fengycin and iturin with fengycin (Jacques, 2011; Ongena et al., 2007). Some of these species have more than antifungal properties, they have also antibacterial activity (Basurto-Cadena et al., 2012; Chen et al., 2012; Ghribi et al., 2012). Our results indicate that biosurfactant identified as surfactin produced by *Bacillus* strain cultured on molasses is capable of antifungal and antibacterial activities against a wide variety of phytopathogenic fungi and *E. coli* bacteria.

Conclusions

Most of biosurfactants applications depend on their specific properties. In addition, several biosurfactants have

Table 3. Growth inhibition of *E. coli* isolates by surfactin produced by *Bacillus* sp. cultured on molasses *E. coli* isolates.

<i>E. coli</i> isolate	Growth inhibition (after 48 h)
1	++
2	+++
3	+++
4	+++
5	+++
6	+++
7	Lack isolates
8	++
9	+++
10	+++
11	+++
12	+
13	++
14	+++
15	+++
16	++
17	+++
18	++
19	+++
20	+++
21	+++
22	+++
23	+++
24	+++
25	+
26	+++
27	+++
28	+++
29	+++
30	+++

“+++”, High inhibition of the bacterial growth; “++”, moderate inhibition; “+”, low inhibition.

been reported to have manifold biological activities like antibiotics, fungicides, insecticides, antiviral and anti-tumor agents or specific toxins and enzyme inhibitors. Developing knowledge of the biological properties is a key factor for introducing biosurfactants in high-added value products in different industries. Biosurfactants capable of suppressing pathogens and maintaining their population by competing against deleterious microorganisms could be successfully utilized as biopesticides for sustainable organic farming. The present research has importance in order to minimize the use of synthetic surfactants and fungicide, thus contributing to the environmental protection. The results here indicate that the *B. subtilis* growing on molasses produced surfactin which has antifungal and antibacterial activities. Despite possessing many commercially attractive properties and

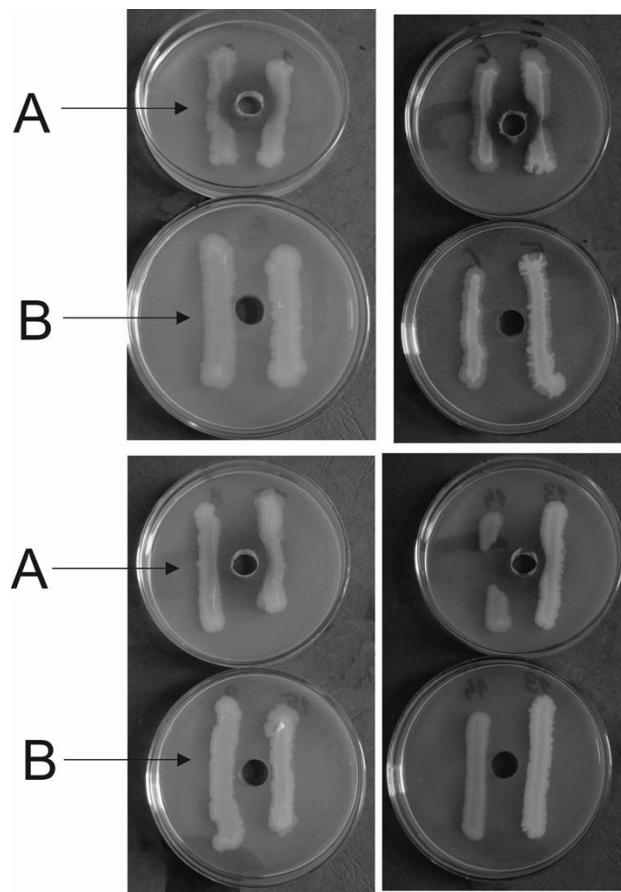


Figure 2. Growth inhibition of *E. coli* strains caused by surfactin produced by *Bacillus* T'-1 cultured on molasses; A - surfactin; B - control.

clear advantages compared with their synthetic counterparts, the production of microbial surfactants on a commercial scale up to now has not been realized because of their low yields and high production costs. One of the strategy is to use cheaper and waste substrates to lower the initial raw material costs involved in the biosurfactant production.

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