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RESEARCH ARTICLE

Purification and identification of *Bacillus subtilis* SPB1 lipopeptide biosurfactant exhibiting antifungal activity against *Rhizoctonia bataticola* and *Rhizoctonia solani*

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Abstract This study reports the potential of a soil bacterium, *Bacillus subtilis* strain SPB1, to produce lipopeptide biosurfactants. Firstly, the crude lipopeptide mixture was tested for its inhibitory activity against phytopathogenic fungi. A minimal inhibitory concentration (MIC), an inhibitory concentration at 50 % (IC50 %), and an inhibitory concentration at 90 % (IC90 %) values were determined to be 0.04, 0.012, and 0.02 mg/ml, respectively, for *Rhizoctonia bataticola* with a fungistatic mode of action. For *Rhizoctonia solani*, a MIC, an IC50 %, and IC90 % values were determined to be 4, 0.25, and 3.3 mg/ml, respectively, with a fungicidal mode of action. For both of the fungi, a loss of sclerotial integrity, granulation and fragmentation of hyphal mycelia, followed by hyphal shriveling and cell lysis were observed with the treatment with SPB1 biosurfactant fraction. After extraction, separation, and purification, different lipopeptide compounds were identified in the culture filtrate of strain SPB1. Mass spectroscopic analysis confirmed the presence of different lipopeptide compounds consisting of surfactin isoforms with molecular weights of 1007, 1021, and 1035 Da; iturin isoforms with molecular weights of 1028, 1042, and 1056 Da; and fengycin isoforms with molecular weights of 1432 and 1446 Da. Two new clusters of lipopeptide isoforms with molecular weights of 1410 and 1424 Da and 973 and 987 Da, respectively, were also detected. This study reported the ability of a *B. subtilis* strain to co-produce lipopeptide isoforms with potential use as antifungal compounds.

Keywords Lipopeptide · Mass spectroscopy · Surfactin, iturin, and fengycin · *Rhizoctonia* sp. · Antifungal

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Introduction

Attacks by fungus can be disastrous to crops despite the preventive measures adopted to keep it in control. Management of fungus rot is generally a difficult challenge, and once initiated, epidemics are difficult to contain. Extensive use of chemicals to control plant diseases has disturbed the ecological balance of microbes inhabiting soil, leading to development of resistant strains of pathogens, groundwater contamination, and obvious health risks to humans. One of the biggest ecological challenges being faced by the microbiologists and plant pathologists in the future is the development of environmental-friendly alternatives to the currently used chemical pesticides for combating a variety of crop diseases. As a consequence of the recent demand for eco-friendly disease management, investigation of the antifungal activity of microbial derived products has become of major interest. As suggested by Fatima et al. (2009), biological control of plant diseases is gaining attention due to increased pollution

concerns because of pesticides use for crop protection and development of pathogen resistance. The use of environmental-friendly microorganisms has proved useful in plant growth promotion and disease control in modern agriculture (Fatima et al. 2009). They are known to suppress soil-borne plant pathogens through the production of secondary metabolites including antibiotics and therefore improve the productivity of several crops. As reported by Okigbo (2005), *Bacillus* sp. and its related genera are reported for production of wide range of cyclic lipopeptides active against various fungal species.

Lipopeptides are among of the most popular and interesting class of microbial surfactants. They include mainly surfactin, fengycin, iturin, and lichenysin compounds which are amphiphilic membrane active peptide antibiotics with potent antimicrobial, antiadherent, antiinflammatory, immune modulator, anticancer, antifibrin clot formation, antiviral, antimycoplasma, and hypocholesterolemic activities for a large spectrum of application in medical and pharmaceutical fields (Mnif and Ghribi 2015). They are also of great interest in agricultural as biocontrol and insecticidal agents; bioremediation for their contaminant biodegradation and metal sequestering role; and in food processing industries for their emulsifying, foaming, and dispersing properties (Mnif and Ghribi 2015; Ongena and Jacques 2008).

Surfactin is the most known, interesting, and studied compound among the whole. It has been characterized for the first time in 1968 by Arima et al. (1968) with a primary structure described as a macrolide consisting of a heptapeptide sequence L-Glu(1)-L-Leu(2)-D-Leu(3)-L-Val(4)-L-Asp(5)-D-Leu(6)-L-Leu(7) linked to a ß-hydroxy fatty acid with 13, 14, or 15 carbon atoms (Arima et al. 1968). After that, surfactin production and structure elucidation have been reported in many studies (Ben Ayed et al. 2014; Kowall et al. 1998; Lin et al. 1994). In fact, a vast natural diversity occurs, giving rise to homologues or isomers differing from each other by the length (12 to 16 atoms of carbon) and the ramification of the fatty acid chain, and to isoforms, characterized by some differences in the peptidic sequence (Dufour et al. 2005; Leclère et al. 2005; Price et al. 2007; Pecci et al. 2010). Rather than being genetically determined, these variations depend on the specific *Bacillus* strain and environmental conditions (Kowall et al. 1998). Also, they can be related to alterations in nutritional culture conditions, especially feeding with some specific amino acid residues (Coronel-León et al. 2015). Loiseau et al. (2015) reported the co-production of two surfactin isoforms by *B. subtilis*. However, previous works noticed that surfactin can possess six isoforms (Kowall et al. 1998; Leclère et al. 2005). Bacon et al. (2012) reported the production of seven isomers with fatty acid chain ranging from C11 to C17, while Abdel-Mawgoud et al. (2008) reported the production of nine different isoforms. Among the produced lipopeptides, surfactin is the most recognized family. It can be produced mainly by *B. subtilis* species (Abdel-Mawgoud et al. 2008; Huang et al. 1993; Kowall et al. 1998; Liu et al. 2015; Sang et al. 2005; Willenbacher et al. 2014; Zeriouh et al. 2013), *B. pumilis* species (Morikawa et al. 1992; Seydlová and Svobodová 2008), *B. licheniformis* species (Li et al. 2008, 2010; Lin et al. 1994; Tendulkar et al. 2007), *B. amyloliquefaciens* species (Buensanteai et al. 2008; Horowitz and Griffin 1991; Sun et al. 2006), and *B. mojavensis* species (Ben Ayed et al. 2014). Similarly, *B. subtilis* species can produce other lipopeptide isoforms belonging to fengycin (Guo et al. 2013; Yánez-Mendizábal et al. 2012; Pathak et al. 2012; Tang et al. 2014; Yánez- Rebib et al. 2012), bacillomycin (Gong et al. 2014; Luo et al. 2014a), and iturin families (Jin et al. 2014; Yun-feng et al. 2012).

Lipopeptides are largely produced by microorganisms belonging to the *Bacillus* genus. In this work, we aimed to purify and identify lipopeptide compounds produced by *B. subtilis* strain SPB1, showing potent antifungal activities against phytopathogenic fungi. Lately, we have reported that *B. subtilis* strain SPB1 could produce lipopeptide biosurfactants with highly emulsification activity (Ghribi et al. 2012a). It was demonstrated as enhancer of hydrophobic compound bioavailability and biodegradability and could be widely applied in bioremediation technology (Mnif et al. 2013a, 2015a). Moreover, previous studies proved that SPB1 biosurfactant could be of a great interest in the bread-making industry (Mnif et al. 2012, 2013b). Here, we report the purification and identification of the biosurfactant produced by *B. subtilis* strain SPB1 along with potential antifungal activity against phytopathogenic fungi. Recently, we reported the effective use of the crude lipopeptide preparation of *B. subtilis* strain SPB1 as a natural fungicide for the control of *Fusarium solani* infestation in tomato and potato tubers (Mnif et al. 2015b).

Materials and methods

Microorganism

B. subtilis strain SPB1 (HQ392822), a biosurfactant producing bacterium, was isolated in our laboratory from a Tunisian soil contaminated by hydrocarbons. It was selected on the basis of the high hemolytic and emulsification activities of its biosurfactant and which exhibited also a broad spectrum of action, including insecticidal activity against lepidopteran larvae (Ghribi et al. 2011, 2012a, 2012c) and antimicrobial activity against microorganisms with multidrug-resistant profiles (Ghribi et al. 2012b). It was identified as *B. subtilis* by morphological, biochemical, and 16S rDNA sequence analysis (Ghribi et al. 2012b).

Lipopeptide biosurfactant extraction and purification

Culture conditions and a crude lipopeptide preparation were carried out as described by Mnif et al. (2015b). This serves as a crude lipopeptide to study the antifungal activity. For identification study, lipopeptide extraction and purification were performed as suggested by Coronel-León et al. (2015). The obtained crude lipopeptide was subjected to three extractions with an ethyl acetate-methanol mixture (2:1, *v*/*v*). The organic phases were combined, passed over anhydrous sodium sulfate, concentrated in a rotary vacuum evaporator (Büchi, Switzerland), and weighed. Lipopeptide compounds were chromatographed on a silica gel column. Elution was carried out with chloroform/methanol/ammonium hydroxide (65:35:5); the fractions were collected in vials. The process was monitored by thin layer chromatography with the same solvent of elution. They were revealed by ninhydrin specific for amino acid moiety and phosphomolybdic acid specific for fatty acid moiety. Fractions showing the presence of both amino acid and fatty acid parts were analyzed by tandem mass spectrometry (4800 Plus MALDI TOF/TOF, AB SCIEX, CA, USA).

Mass spectrometry

The molecular weight of the components of the surfactants was determined by negative- and positive-ion mode electrospray ionization (ESI) analyses (LC/MSD-TOF, Agilent Technologies, CA, USA) (Coronel-León et al. 2015). The capillary voltage were 4 and 3.5 kV for the positive and negative modes, respectively, with nitrogen as the nebulizing and drying gas. Tandem mass spectrometry (4800 Plus MALDI TOF/TOF, AB SCIEX, CA, USA) was used in the experiment. The full mass spectrum was acquired in the reflector positive-ion mode for the lipopeptide, using dihydroxybenzoic acid (DHB) as matrix.

Phytopathogen fungus

R. bataticola and *R. solani* were kindly provided by Dr. Mohamed Ali Triki (Olive Tree Institute of Tunisia). They were maintained at 4 °C in potato dextrose agar plates and at −20 °C in tryptone salt medium (tryptone 1 g, NaCl 8.5 g, Tween 20 1 %, glycerol 15 %, and distilled water 1 l).

Study of the antifungal activity of SPB1 biosurfactant

In vitro antifungal activity of SPB1 biosurfactant was checked initially by the disk diffusion method. After that, we studied the inhibition of radial growth of *R. bataticola* and *R. solani*. Both studies were performed as described by Mnif et al. (2015b). The mycelial growth inhibition was calculated according to the present formula:

$\text{MGI}(\%) = ((dc - dt)/dc) \times 100$, where dc and dt represent the mycelial growth diameter in control and treated Petri plates, respectively.

The minimal inhibitory concentration (MIC) defined as the smallest concentration that inhibits the fungal growth totally and the 50 % inhibitory concentration of the lipopeptide (IC50) values were determined.

The fungistatic–fungicidal nature of the SPB1 lipopeptide biosurfactant was tested by controlling revival of growth of the inhibited mycelia disk following its transfer to nontreated PDA (Mnif et al. 2015b). A fungicidal effect was where there was no growth, whereas a fungistatic effect was where temporary inhibition of fungal growth occurred. The agar disks of *R. bataticola* and *R. solani*, which failed to grow, were either transferred onto agar media without SPB1 biosurfactant. Petri plates were incubated for 5 days. The experiments were conducted in triplicates.

Results and discussion

Study of the antifungal activity of the *B. subtilis* SPB1 crude biosurfactant

Study of the antifungal activity of SPB1 biosurfactant against R. bataticola

We examined the growth of *R. bataticola* during 3 days of incubation in presence of different biosurfactant concentrations. Growth patterns were observed in the presence of five increasing concentrations (0.02, 0.04, 0.06, 0.08, and 0.1 %) in comparison to a negative control without biosurfactant addition. As presented in Fig. 1, a total inhibition of almost 100 % was observed for concentrations equal and superior to 0.04 mg/ml. A very few growth was detected in the presence of 0.02 mg/ml of SPB1 biosurfactant, and the percentage of inhibition recorded is statistically significant from negative control and the other studied doses. Mean values were statistically significant according to Duncan test at p values <0.05. In fact, a MIC, an IC50, and IC90 % values were determined to be 0.04, 0.012, and 0.02 mg/ml, respectively. Hence, *R. bataticola* was shown very sensitive towards SPB1 biosurfactant.

In order to assess the nature of the antifungal potency of the lipopeptide fraction of *B. subtilis* strain SPB1 towards *R. bataticola*, an agar with mycelia of the studied fungi showing a total inhibition of growth (in the presence of 0.04 mg/ml biosurfactant) was placed on the center of a new Petri dish without biosurfactant addition and incubated at 25 °C for 5 days. Obtained result showed that the mycelium of *R. bataticola* was able to regain growth. So, we can assume that SPB1 biosurfactant had a fungistatic mode of action towards *R. bataticola*. Microscopic observations demonstrate

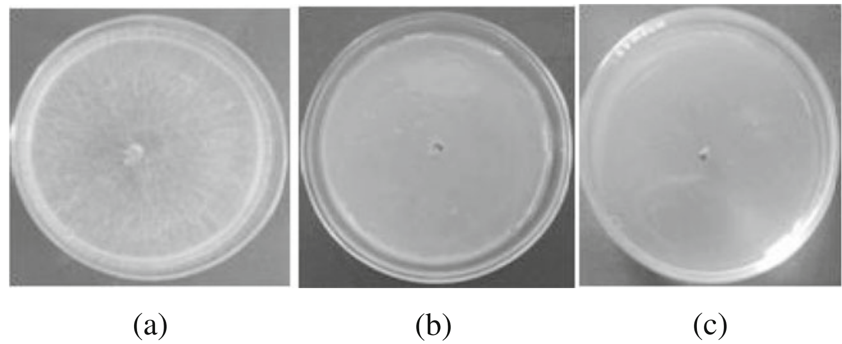

Fig. 1 Antifungal activity of SPB1 biosurfactant towards *R. bataticola*. Effect of different concentration on the antifungal potency, negative control (**a**), in the presence of 0.02 mg/ml biosurfactant (**b**), and in the presence of 0.04 mg/ml biosurfactant (**c**)

that SPB1 biosurfactant reduced highly growth of *R. bataticola* and altered hypha and sclerotium morphologies. In fact, as presented in Fig. 2, bloomed sclerotia with teared mycelium were observed when *R. bataticola* was treated with 0.04 mg/ml SPB1 biosurfactant. Growing concentration accentuates the degree of alteration. In fact, a loss of sclerotial integrity, granulation and fragmentation of hyphal mycelia, followed by hyphal shriveling and cell lysis were observed when using 0.04 mg/ml SPB1 biosurfactant.

R. bataticola is a diverse omnipresent soil and seed-borne necrotrophic fungal pathogen. It has a global distribution and can infect more than 500 plant species including monocot and dicot plant hosts (Sharma et al. 2012). As reported by Sundravadana et al. (2011) and Sharma et al. (2012), high levels of pathogenic and genetic variations in *R. bataticola* (RB) from different parts of the world were described. This pathogen causes different types of diseases, viz., seedling blight, root rot, charcoal rot, wilt, stalk rot, stem blight, fruit rot, seedling decay, and leaf blight in crop plants, and can therefore cause up to 60 % yield loss in crop production (Sundravadana et al. 2011). In contrast to many pathogens favored by change to moisture conditions (Garrett et al. 2006), *R. bataticola* may become more problematic in agricultural areas, where climate change results in longer drought periods and higher temperatures when the crop is exposed to moisture stress conditions (Sharma et al. 2012). So, an urgent need for the control of these three disastrous fungi was developed.

Study of antifungal activity of SPB1 biosurfactant towards R. solani

Growth of *R. solani* was observed during 3 days of incubation in the presence of different biosurfactant concentrations. Growth patterns observed in the presence of increasing concentration of SPB1 biosurfactant ranging from 0.1 to 4 mg/ml show a weak inhibition of the mycelia growth of *R. solani* in comparison to a negative control without biosurfactant addition as observed in Fig. 3. In fact, mycelium growth of *R. solani* in the presence of 0.5 and 2 mg/ml were about 21.22 and 12.56 mm, respectively. At 4 mg/ml of biosurfactant, a total inhibition of the growth of the respective fungi was recorded. Hence, a MIC, IC50, and IC90 % values were determined to be 4, 0.25, and 3.3 mg/ml, respectively. An agar with mycelia of the *R. solani* fungi showing a total inhibition of growth (in the presence of 4 mg/ml biosurfactant) was placed on the center of a novel Petri dish without biosurfactant addition and incubated at 25 °C for 5 days. Result show that the respective fungus was not able to regain growth. With this, we can assume that SPB1 lipopeptide extract had a fungicidal activity towards *R. solani*.

Microscopic observations of the inhibited mycelium of *R. solani* by different concentrations of lipopeptide preparation demonstrate that SPB1 biosurfactant was able to lyses hyphae of the respective fungi (Fig. 4). In fact, the negative control shows normal articulate hyphae. Hyphal fragmentation and cell wall lysis indicated the fungicidal nature of the metabolite.

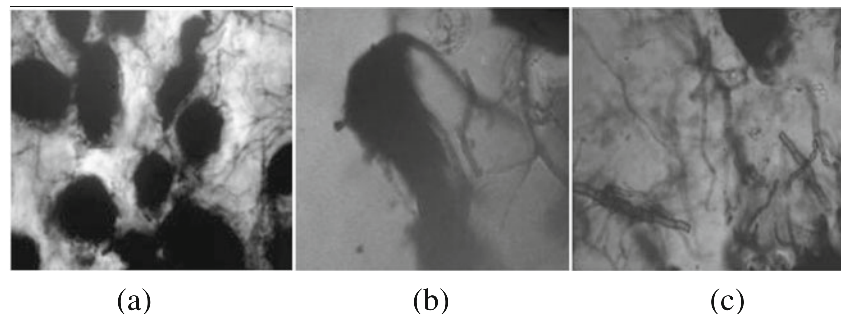

Fig. 2 Effect of SPB1 lipopeptide on *R. bataticola* mycelial growth. Representative microscopic pictures (10×25 magnification) of hypha and sclerotium of *R. bataticola* grown in medium without lipopeptide (**a**, control) and with lipopeptide at concentrations of 0.02 mg/ml (**b**) and 0.04 mg/ml (**c**)

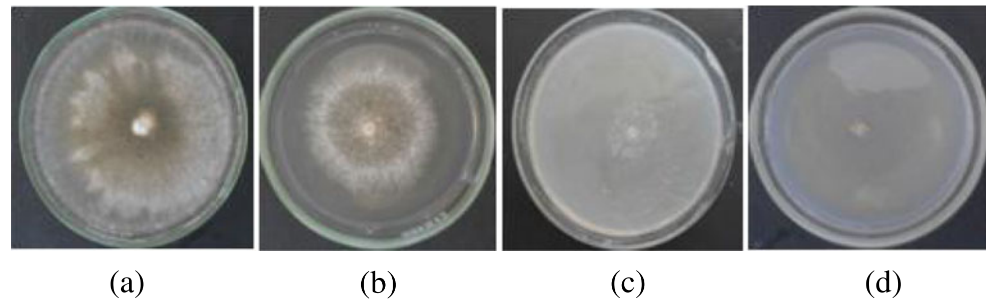

Fig. 3 Antifungal activity of SPB1 biosurfactant towards *R. solani*. Effect of different concentrations on the antifungal potency, negative control (**a**) and in the presence of 0.5 mg/ml biosurfactant (**b**), 2 mg/ml biosurfactant (**c**), and 4 mg/ml biosurfactant (**d**)

According to literature reviews and studies, fungus *R. solani*, which causes black scurf of potato tubers and makes its quality worsen, belong to commonly appearing potato pathogens (Kurzawinska and Mazur 2008). Sclerotia of the mentioned fungi occurring on sets can be the source of infection for plants and descendant tubers (Kurzawinska and Mazur 2008). However, many biocontrol strategies were developed to combat these phytopathogenic fungi. Many literature reviews described the use of biopreparation and chemical compounds to inhibit the growth of *R. solani* (Kurzawinska and Mazur 2008; Walters et al. 2004; Erper et al. 2011). Other studies reported the application of microbial isolates such as bacteria and fungi as biosystems to control the invasion of these fungi (Asaka and Shoda 1996; Montealegre et al. 2010; Naeimi et al. 2010). Here, we evaluated the efficiency of the biosurfactant fraction to control *R. solani* growth.

Present studies reported the efficiency of SPB1 lipopeptide preparation to inhibit *R. bataticola* and *R. solani* growth. Results are similar to those published by Senthilkumar et al. (2009), reporting the loss of sclerotial integrity, the granulation and fragmentation of hyphal mycelia, and cell lysis of the pathogenic fungi *R. bataticola* when treated by antifungal metabolite produced by *Paenibacillus* sp. Similar report describe the inhibition of *R. solani* sclerotium production when treated with many *Trichoderma* isolates (Naeimi et al. 2010). Therefore, iturin A produced by *B. subtilis* strain BS-99-H had inhibition potency towards *Pestalotiopsis eugeniae* presented by a swelling and a deformation of fungus hyphae leading to a fungicidal effect (Lin et al. 2010).

Identification of lipopeptide biosurfactants produced by *B. subtilis* strain SPB1

The key components of the use of emulsifiers are essentially the cost of their production and the ease of their recovery. Several techniques were developed to extract and purify lipopeptides. As described in the methodology part, lipopeptide fractions were extracted by a mixture of ethyl acetate/methanol (2:1) followed by an elution through silica gel (60) column chromatography.

Mass spectrum analyses of the purified fractions are presented in Fig. 5a, b. They showed five well-resolved clusters of peaks, the first at *m*/*z* values between 996 and 1010 Da (family A), the second at *m*/*z* values between 1030 and 1058 Da (family B), the third at *m*/*z* values between 1051 and 1079 Da (family C), the fourth at *m*/*z* values between

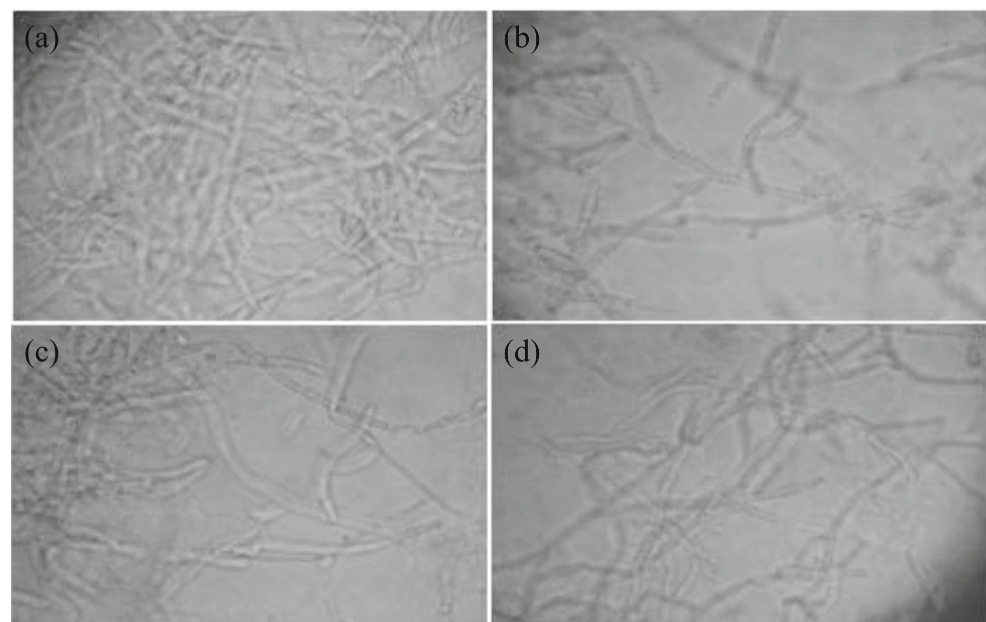

Fig. 4 Effect of SPB1 lipopeptide on *R. solani* mycelium growth. Representative microscopic pictures (10×40 magnification) of hypha *R. solani* grown in medium without lipopeptide (**a**, control) and with lipopeptide at different concentrations (**b**, **c**, and **d**)

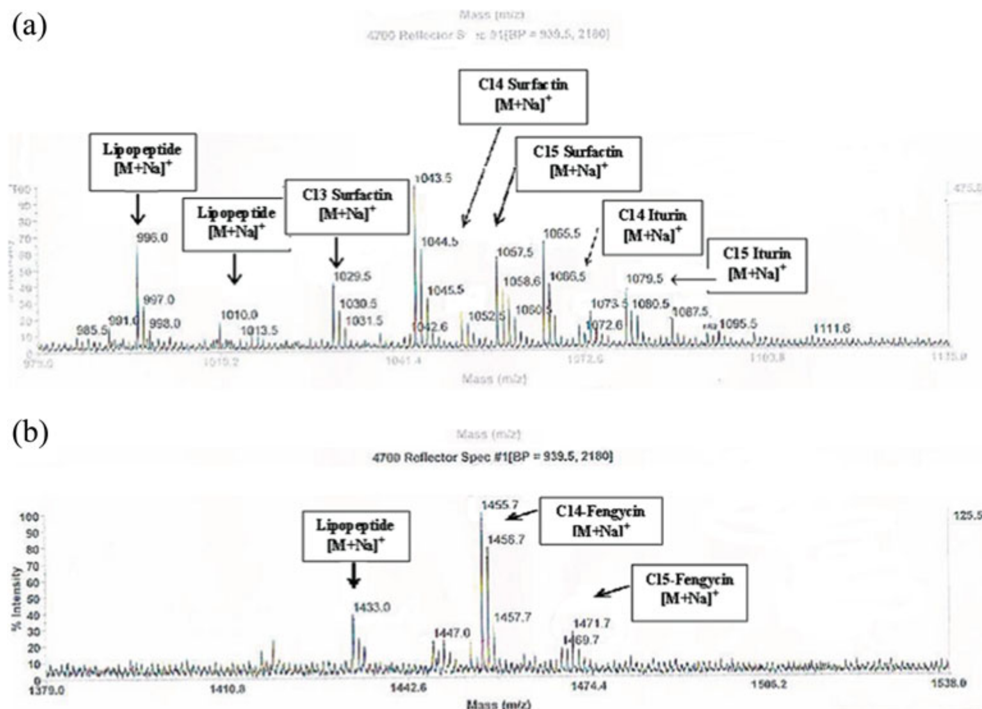

Fig. 5 **a** Mass spectroscopy (LC/MSD-TOF) spectra of molecular mass of SPB1 lipopeptide biosurfactants. Spectra of lipopeptides (**a**), surfactin (**b**), and iturin (**c**) produced by *B. subtilis* SPB1. **b** Mass spectroscopy (LC/MSD-TOF) spectra of molecular mass of SPB1 lipopeptide biosurfactants. Spectra of lipopeptides (**d**) and fengycin (**e**) produced by *B. subtilis* SPB1

1433 and 1447 Da (family D), and the fifth at *m*/*z* values between 1455 and 1469 Da (family E). By comparing the mass with the mass numbers reported for the lipopeptide complexes from other *Bacillus* strains, each group of peaks could be attributed to different lipopeptide isoforms. Each isoform group can belong to the same family and have probably the same amino acid sequence with difference in the length of the fatty acid chain. Family B corresponds to the surfactin family with molecular weights of 1007, 1021, and 1035 Da (Kowall et al. 1998; Price et al. 2007; Chen et al. 2008; Pecci et al. 2010; Pyoung et al. 2010; Luo et al. 2014a, b; Ben Ayed et al. 2014); family C corresponds to the iturin family with molecular weights of 1028, 1042, and 1056 Da (Price et al. 2007; Chen et al. 2008; Pyoung et al. 2010); and family E corresponds to the fengycin family with molecular weights of 1432 and 1446 Da (Vater et al. 2002; Pathak et al. 2012; Guo et al. 2013; Luo et al. 2014b; Tang et al. 2014; Ben Ayed et al. 2014) (Table 1). However, the other two families, A and D, corresponded to two new clusters of lipopeptide families with molecular weights of 1410 and 1424 Da and 973 and 987 Da, respectively (Table 1).

Structurally, lipopeptides are amphiphilic anionic cyclic compounds that contain peptidic moieties linked to a ß-fatty acid tails (Mnif and Ghribi 2015). They consist of short linear chains or cyclic structures of amino acids, linked to a fatty acid via ester or amide bonds or both (Mnif and Ghribi 2015). A lactone bridge between the β-hydroxyl function of the acid and the carboxy-terminal function of the peptide confers a cyclic structure to this molecule (Mnif and Ghribi 2015). Lipopeptides constitute a diverse group of metabolites produced by various bacterial and fungal genera (Mnif and Ghribi 2015). Moreover, there is considerable structural diversity as a consequence of differences in the nature of the fatty acid component, as well as in the type, number, and configuration of the amino acids in the peptide chain (Fracchia et al. 2012). Cyclic lipopeptides belonging to surfactin, fengycin, iturin, and lichenysin families represent the four major classes of lipopeptide biosurfactant isoforms produced by *Bacillus* strains.

In the context of biocontrol of plant diseases, the three families of *Bacillus* lipopeptides—surfactins, iturins, and fengycins—were at first mostly studied for their appreciable antibacterial or antifungal properties (Ongena and Jacques 2008). Regarding many literature reviews and studies, microbial-derived lipopeptides were described as potential inhibitors of phytopathogen growth. As suggested by Raaijmakers et al. (2010), the main natural functions of lipopeptides from *Bacillus* species are believed to the control of other microorganisms, motility, and attachment to surfaces, although they may also have a signaling function to coordinated growth and differentiation. Canova et al. (2010) reported the suppression of *R. solani* by *Paenibacillus* sp. (IIRAC30) derived surfactin series. Moreover, lipopeptide biosurfactant remains a very interesting alternative to control *R. solani* invasion. To know, iturin A produced by *B. amyloliquefaciens* (Yu et al. 2002) and cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. (Nielsen et al. 2002) were discussed to suppress *R. solani*. Mycelial growth of *Fusarium moniliforme* and *F. graminearum* and *Sclerotinia sclerotiorum* were effectively inhibited in vitro by *B. subtilis*

Table 1 Different lipopeptides identified by mass spectrometry

Family	Mass peak (*m*/*z*)	Nature of the lipopeptide isomers	References
Family A	996	Lipopeptide $[M + Na]^+$	
	974	Lipopeptide $[M + H]^+$	
	973	Lipopeptide	
	1010	Lipopeptide $[M + Na]^+$	
	988	Lipopeptide $[M + H]^+$	
	987	Lipopeptide	
Family B	1030	C13-surfactin $[M + Na]^+$	Kowall et al. 1998; Price et al. 2007; Chen et al. 2008; Pecci et al. 2010; Pyoung et al. 2010; Ben Ayed et al. 2014; Luo et al. 2014a, b
	1008	C13-surfactin $[M + H]^+$	
	1007	C13-surfactin	
	1044	C14-surfactin $[M + Na]^+$	
	1022	C14-surfactin $[M + H]^+$	
	1021	C14-surfactin	
	1058	C15-surfactin $[M + Na]^+$	
	1036	C15-surfactin $[M + H]^+$	
	1035	C15-surfactin	
Family C	1051	C13-iturin $[M + Na]^+$	Price et al. 2007 ; Chen et al. 2008 ; Pyoung et al. 2010
	1029	C13-iturin $[M + H]^+$	
	1028	C13-iturin	
	1065	C14-iturin $[M + Na]^+$	
	1043	C14-iturin $[M + H]^+$	
	1042	C14-iturin	
	1079	C15-iturin $[M + Na]^+$	
	1057	C15-iturin $[M + H]^+$	
	1056	C15-iturin	
Family D	1433	Lipopeptide $[M + Na]^+$	
	1411	Lipopeptide $[M + H]^+$	
	1410	Lipopeptide	
	1447	Lipopeptide $[M + Na]^+$	
	1425	Lipopeptide $[M + H]^+$	
	1424	Lipopeptide	
Family E	1455	C14-fengycin $[M + Na]^+$	Vater et al. 2002; Guo et al. 2013; Ben Ayed et al. 2014; Luo et al. 2014b; Tang et al. 2014
	1433	C14-fengycin $[M + H]^+$	
	1432	C14-fengycin	
	1469	C15-fengycin $[M + Na]^+$	
	1447	C15-fengycin $[M + H]^+$	
	1446	C15-fengycin	

B-FS01 derived fengycin (Hu et al. 2007) and bacillomycin and fengycin derived from *Bacillus* spp. (Ramarathnam et al. 2007), respectively. Also, iturin A produced by *B. subtilis* BS-99-H inhibits germination of *P. eugeniae* spores (Lin et al. 2010). In fact, fengycin-type lipopeptides were as suppressing agent of *Fusarium* wilt and foot rot (Rebib et al. 2012; Cao et al. 2012).

In the past decades, research on lipopeptides has been fueled by their antimicrobial, antitumor, immunosuppressant, and surfactant activities (Raaijmakers et al. 2010). Owing to their microbial origin, lipopeptide compounds are low or non-toxic, biodegradable, demonstrate excellent surface activity, possess high specificity, show effectiveness under extreme conditions, and can be reused through regeneration too as compared to synthetic surfactants (Lima et al. 2011; Sachdev and Cameotra 2013). Consequently, they are widely used in many fields such as environment, chemical, food and cosmetic industries, medical and pharmaceutical fields, and in agricultural field (Sachdev and Cameotra 2013).

In this study, we report the potential involvement of different lipopeptide isoforms in the biocontrol activity of *B. subtilis* SPB1 towards phytopathogenic fungi. In fact, cyclic lipopeptides (CLPs) produced by *B. subtilis* strains have been shown to protect host plants from a number of pathogens

(Falardeau et al. 2013). Our findings are similar to those published by Li et al. (2014) and Waewthongrak et al. (2014) reporting the co-production of surfactin, iturin, and fengycin isoforms involved in the biocontrol of *Plasmodiophora brassicae* and *F. solani* and *Penicilium digitatum*, respectively. Also, Liu et al. (2014) reported that the involvement of three isoforms, surfactin, iturin, and fengycin, affected spore germination and membrane permeability of spores from four fungal plant pathogens (*Alternaria solani*, *Fusarium sambucinum*, *Rhizopus stolonifer*, and *Verticillium dahliae*). Similarly, Ben Slimene et al. (2012) showed the production of iturins, surfactins, and fengycins with long-chain fatty acids and other not yet identified compounds by spores of *B. subtilis*, an antagonist L194 strain against *Phoma medicaginis* pathogenic fungi. In a study published by Luo et al. (2014b), *B. subtilis* 916 co-produce not only the three families of well-known lipopeptides, surfactin, bacillomycin L (iturin family), and fengycin but also produce a new family of lipopeptide called locillomycin active against *F. oxysporum*. However, Cao et al. (2012) reported the involvement of *B. subtilis* SQR 9-derived fengycin and bacillomycin in the inhibition of mycelial growth and spore germination of *F. oxysporum*. Waewthongrak et al. (2014) suggested that fengycin and surfactin act as elicitors of defense-related gene expression in "Valencia" fruit following infection by *P. digitatum*.

According to literature reviews and studies, many essential oils were described as potent inhibitors of in vitro *R. bataticola* growth (Sharma et al. 2012; Beg and Ahmad 2002; Kundu et al. 2013). However, biocontrol using microbial-derived compounds remains the best alternative. Regarding previous studies, in vitro growth of *R. bataticola* was inhibited by antifungal metabolite secreted by the endophytic bacterium belonging to *Bacillus* and *Paenibacillus* strains (Senthilkumar et al. 2009) and *Pseudomonas fluorescens* (Sujatha and Ammani 2013).

Conclusion

The production of lipopeptides by *B. subtilis* SPB1 was confirmed by spectrometric analysis. Results demonstrated the ability of the strain to produce a mixture of lipopeptide isoforms. After extraction and purification, SPB1 biosurfactant was shown to be composed of different lipopeptide isoforms belonging to surfactin, iturin, and fengycin families in addition to two new un-identified lipopeptide clusters. The lipopeptide mixture exhibited strong antifungal activity against *R. solani* and *R. bataticola*. In vitro antifungal assay determined a minimal inhibitory concentration of 0.04 mg/ml with a fungistatic mode of action towards *R. bataticola* and 4 mg/ml with a fungicidal mode of action for *R. solani*. In conclusion, the present work shows that the SPB1 strain constitutes a promising biocontrol agent against plant diseases induced by phytopathogenic fungi. Its mode of action seems to involve synergism between various secreted lipopeptide antibiotics, some of which remain to be characterized.

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Compliance with ethical standards

Conflict of interest No conflict of interest is declared.

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