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Short communication

Antifungal and aflatoxin-reducing activity of extracellular compounds produced by soil *Bacillus* strains with potential application in agriculture



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ABSTRACT

Toxigenic *Aspergillus flavus* and *A. parasiticus* fungal strains can contaminate a wide variety of food crops with the subsequent production of aflatoxins (AFs) resulting in severe economic losses and public health issues. Biological control is a promising approach to manage AFs contamination in pre- and post-harvested crops. In the present study, the effect of soil-borne *Bacillus* spp. strains on aflatoxigenic *A. parasiticus* growth and AFs production was evaluated and the culture supernatant of the most effective strain was evaluated for the presence of antifungal lipopeptides. Six *Bacillus* spp. strains were able to reduce *A. parasiticus* growth rate significantly ($p < 0.05$). *Bacillus* spp. RC1A was able to inhibit fungal growth almost completely, reducing growth rate to 0.16 mm/h and increasing Lag phase duration (31.72 h) ($p < 0.0001$). RC1A could also reduce AFB₁ concentration produced by *A. parasiticus* ($p < 0.0001$). Organic solvent extraction and chromatographic analysis of RC1A culture supernatant showed the presence of bands corresponding to three of the main groups of lipopeptides (surfactin, iturin A and fengycin) at the expected retention factor (Rf) values; they were also confirmed by MALDI-MS analysis. These fractions were able to inhibit *A. parasiticus* growth and AFB₁ production to non-detectable levels when tested separately in liquid culture media. The further study of the antifungal compounds produced by these strains will determine their potential use to manage AFs contamination in crops and feeds.

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1. Introduction

Aspergillus section *Flavi* (especially *A. flavus* and *A. parasiticus*) can contaminate a wide variety of food crops including maize, cottonseed, peanuts, and tree nuts. The subsequent production of aflatoxins (AFs) in susceptible crops results in severe losses for

growers every year. Aflatoxins, especially aflatoxin B₁ (AFB₁), are extremely toxic secondary metabolites with carcinogenic, mutagenic and teratogenic effects (IARC, 2002; Zhang, Shi, Hu, Cheng, & Wang, 2008).

Many strategies have been investigated to manage AFs contamination in crops. Biological control appears to be the most promising approach to control AFs in both pre- and post-harvested crops. Bacterial strains with the ability to produce antifungal substances have been tested mainly to control post-harvest fungal contamination. Many *Bacillus* strains (especially *B. subtilis*, *B. amyloliquefaciens* and *B. circulans*) are known to suppress fungal growth *in vitro* due to the production of antifungal antibiotics especially the

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non-ribosomally synthesized cyclic lipopeptides of the surfactin, iturin and fengycin families (Afsharmanesh, Ahmadzadehb, Javan-Nikkhab, & Behboudib, 2014; Caldeira, Santos Arteiro, Coelho, & Roseiro, 2011; Cho et al., 2009, 2003; Das, Mukherjee, & Sen, 2008; Gong et al., 2015; Ji et al., 2013). Lipopeptides are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antifungal activities which can be used as biopesticides for plant and post-harvest protection. Moreover, lipopeptides are easily biodegradable in soils, constituting a healthier and environmentally friendly alternative to synthetic fungicides (Caldeira et al., 2011). Although the use of synthetic fungicides is the most effective treatment to control fungal disease, there is an urgent need to find equally effective but safer means of controlling post-harvest fungal pathogens, mainly due to the toxicity of the synthetic fungicide residues exert on human health and the environment (Droby, 2006).

In the present study, the effect of soil-borne *Bacillus* spp. strains on aflatoxigenic *A. parasiticus* growth and AFs production was evaluated and the culture supernatant of the most effective strain was evaluated for the presence of lipopeptides.

2. Materials and methods

2.1. Strains

Thirteen *Bacillus* strains were obtained from lake sediment and soil samples according to methodology described by Sosa-Pech et al. (2012). Strains were cultivated in nutrient agar (NA) plates and morphological traits (colony morphology, color, size, shape, elevation, margins) Gram stain and catalase test were recorded after 24 h incubation at 28 °C. Spore formation was observed in malachite green stained slides of 72 h cultures. Strains showing *Bacillus* genus' characteristics (Gram-positive, rod-shaped, spore forming bacilli) were maintained in NA slants and three colonies of each strain were inoculated in microtubes containing 0.2% glycerol and stored at –80 °C for future studies. *Bacillus* species were identified using a MALDI-TOF mass spectrometer (Bruker Daltonics MALDI Biotyper) using single colonies of 24 h cultures of each strain. Three strains were identified as *B. mojavensis*, two as *B. subtilis*, five as *B. cereus*, one as *B. megaterium* and two as *B. mycooides*.

2.2. Influence of *Bacillus* strains on *Aspergillus parasiticus* growth and aflatoxin production

Malt extract agar (MEA) dishes amended with two ml of 24 h *Bacillus* cultures (10^8 cells/ml) and MEA control dishes were inoculated with a central spot of a *A. parasiticus* NRRL 2999 conidial suspension in 0.2% soft agar (10^3 cells/ml) and incubated at 25 °C until the colony on the control plates reached the edge of the dish. The diameter of growing fungal colonies was measured in two directions at 90° from each other to obtain the mean diameter for each colony. The growth rate (mm/h) was calculated by linear regression of colony diameter against time during the linear phase of growth for each set of conditions tested. The linear section of the graph was extrapolated to a zero increase in diameter (i.e. 5.0 mm diameter) and the intercept on the time axis was defined as the Lag phase (hours in which the colony reaches 5.0 mm of diameter). Three agar plugs were taken from each plate and AFs accumulated in the culture media were extracted with chloroform according to Geisen (1996) with some modifications and the extracts were analyzed for AFB₁ by HPLC according to Trucksess, Stack, Nesheim, Albert, and Romer (1994). Briefly, AFs were extracted from agar plugs with 1 ml chloroform by centrifugation at 8000 rpm. The organic layer (800 µl) containing the toxin was collected, filtered

and evaporated to dryness under N₂ stream. Samples were redissolved in 400 µl mobile phase and 200 µl aliquots were derivatized with 700 µl trifluoroacetic acid: acetic acid: water (20:10: 70, v/v) solution. Fifty µl aliquots were injected in a Waters Alliance 2695 system coupled to a fluorescence detector (Waters 2487). Chromatographic separations were performed on stainless steel, C18 reversed phase column (Luna Phenomenex, 150 × 4.6 mm id., 5 µm particle size). Water (4 v/v): methanol (1 v/v): acetonitrile (1 v/v) was used as mobile phase at a flow rate of 1.5 ml min⁻¹ and the limit of detection (LOD) was 0.5 ng/ml. The fluorescence of AFB₁ derivatives was recorded at excitation and emission wavelengths of 360 and 460 nm, respectively. A calibration curve was constructed by injecting AFB₁ standards of 5; 30 and 50 ng/ml and quantification of the toxin levels in samples were calculated by comparison of peak areas. The AFB₁ standards solutions were prepared according to AOAC (1995). The experiment was repeated three times (three replicates per strain) and the results were obtained comparing average values ± standard error (SE).

2.3. Extraction of the antifungal compounds

Bacillus mojavensis RC1A was selected and the cell-free culture supernatant (CFCS) was obtained by centrifugation (8000 rpm, 15 min at 4 °C) and filtration (0.22 µm cellulose nitrate filters) of a 24 h culture in nutrient broth. The extraction of the antifungal compounds was performed according to Ji et al. (2013). Briefly, the CFCS was mixed with equal volume of hexane, chloroform, ethyl acetate, and n-butanol, successively. Each fraction was collected, concentrated and dissolved with methanol. The crude extract from the butanol layer was dried to remove methanol *in vacuo* and separated by silica gel column chromatography (70–230 mesh; Merck, Darmstadt, Germany) with chloroform:methanol (20:1), chloroform:methanol (10:1), chloroform:methanol (5:1) and chloroform:methanol (2:1) as mobile phase. All fractions were collected and a preparative thin layer chromatography was made to separate compounds in the extracts (especially lipopeptides) and an aliquot of each was stored at –20 °C for antifungal activity testing. The plates were developed in chloroform:methanol:H₂O (65:25:4, v/v). The chromatograms were air-dried and compounds revealed under UV light (265 and 360 nm) by spraying with water. The different fractions were separated by scraping the silica from the TLC plates, compounds were extracted with chloroform:methanol (2:1, v/v) and the extracts were tested for antifungal activity against *A. parasiticus* NRRL 2999. An aliquot of each extract was taken to confirm lipopeptides presence by HPLC in further studies. Lipopeptides were also extracted from CFCS using n-butanol by an alternative methodology described by Afsharmanesh et al. (2014). After separation and complete evaporation of the butanol layer, the remaining residue was dissolved in methanol. This methanolic fraction was separated by thin layer chromatography (TLC) on normal-phase HPTLC silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany) and developed with chloroform:methanol:water (65:25:4, v/v). Lipopeptides were visualized by spraying distilled water onto the TLC sheets and their corresponding retention factor (R_f) values were estimated (Razafindralambo et al., 1993) and compared with characteristic R_f values of lipopeptides obtained in other studies (Afsharmanesh et al., 2014).

2.4. Antifungal activity

The antifungal activity against *A. parasiticus* NRRL 2999 of all the fractions obtained was tested as described by Palumbo, Baker, and Mahoney (2006). Sterile 48-well plates containing 1 ml yeast extract saccharose (YES) broth per well were added 50 µl of the

different extracts (two replicates each). Two wells containing only YES broth and two containing YES +50 μ l chloroform:methanol (2:1 v/v) were included as controls. Two wells containing YES +50 μ l pure CFCS and two wells containing YES +50 μ l of a 72 h culture of RC1A in nutrient broth were also included. Twenty μ l of an *A. parasiticus* conidial suspension (10^5 cells/ml) were inoculated in each well and plates were incubated at 28 °C for 7 d without shaking. Fungal growth was visually recorded daily. After incubation, fungal mycelium was recovered by filtration onto Whatman no. 4 paper filters and dried overnight. Fungal dry weights were measured for each well. The extent of the extracts' inhibition of *A. parasiticus* growth was determined relative to control cultures of *A. parasiticus* in YES broth without antifungal extracts. After removing the mycelium, YES broth was removed from each well and extracted with equal volume of chloroform, the chloroform layer was separated and analyzed for AFs by HPLC according to Trucksess et al. (1994). The experiment was repeated two times (two replicates per extract) and the results were obtained comparing average values \pm standard error (SE).

2.5. Confirmation of lipopeptides production by MALDI-TOF

The production of lipopeptides by *B. mojavensis* RC1A and *B. subtilis* RC6A was confirmed by analyzing the presence of these compounds in the CFCS and butanolic extract of culture supernatants of the two strains that showed antifungal activity. Samples were analyzed by ultraviolet matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) performed on the Bruker Ultraflex Daltonics Time-of-Flight/Time-of-Flight (TOF/TOF) mass spectrometer (Leipzig, Germany). Mass spectra were acquired in linear positive ion modes. From *B. mojavensis* RC1A sample solutions were prepared as follows: (i) From solvent free butanolic extracts (dried material), methanol solution was prepared and (ii) from solvent free butanolic extracts (dried material), aqueous solution (pH = 8) were prepared. Besides, CFCS from *B. mojavensis* RC1A and from *B. subtilis* R6CA and culture nutrient media (as base line control) were used without any modification. External mass calibration was made using β -cyclodextrin aqueous solution (1 mg/

mL) (MW, $[M+Na]^+ = 1157.35730$ and $[M+K]^+ = 1173.33010$) with 9H-pyrido[3,4b]indole (nor-harmane, nHo) as matrix in positive and negative ion mode. The matrix signal, as $[M+H]^+$ or $[M-H]^-$ was used as an additional standard for calibration in the corresponding ion mode. Sample solutions were spotted on a MTP 384 target plate polished steel from Bruker Daltonics (Leipzig, Germany). For MALDI-MS matrix solutions were prepared by dissolving nHo in acetonitrile/water (1:1, v/v) solution. For MALDI-MS experiments dry droplet sample preparation or sandwich method was used according to Nonami, Fukui, and Erra-Balsells (1997), loading successively 0.5 μ l of matrix solution, analyte solution and matrix solution after drying each layer at normal atmosphere and room temperature. The matrix to analyte ratio was 3:1 (v/v) and the matrix and analyte solution loading sequence was: i) matrix, ii) analyte, iii) matrix, iv) matrix. Desorption/ionization was obtained by using the frequency-tripled Nd:YAG laser (355 nm). Experiments were performed using firstly the full range setting for laser firing position in order to select the optimal position for data collection, and secondly fixing the laser firing position in the sample sweet spots. The laser power was adjusted to obtain high signal-to-noise ratio (S/N) while ensuring minimal fragmentation of the parent ions and each mass spectrum was generated by averaging 100 lasers pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively. Best results, shown in Fig. 3, Figs. S1 and S2 and Table 3, were obtained in positive ion mode with methanolic solutions and with the corresponding CFCS.

2.6. Statistical analyses

Aflatoxin B₁ concentration in YES medium in each *A. parasiticus* + *Bacillus* co-cultures was compared with control plates and with the other co-cultures using ANOVA ($p < 0.05$). Fisher's LSD test ($p < 0.05$). Growth rate and Lag phase values of co-cultures were compared with each other and with controls using ANOVA ($P < 0.0001$) and Fisher's LSD test ($P < 0.0001$). In the *A. parasiticus* NRRL 2999 growth inhibition and AFB₁ production by *Bacillus mojavensis* RC1A extracts, mycelium weight was compared

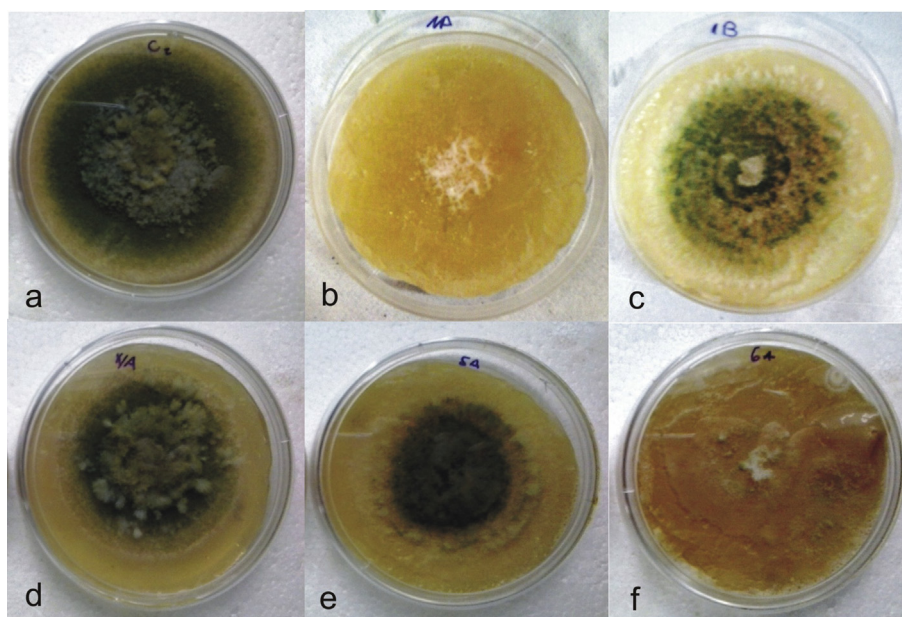


Fig. 1. Inhibition of *Aspergillus parasiticus* NRRL 2999 growth by *Bacillus* strains isolated from soil. a) Control, b) *B. mojavensis* RC1A, c) *B. subtilis* RC1B, d) *B. mycooides* RC4A, e) *B. cereus* RC5A, f) *B. subtilis* RC6A.

using ANOVA ($p < 0.05$) and Scott & Knott Test and AFB₁ production was compared using ANOVA ($p < 0.05$) and LSD Fisher Test. InfoStat software was used for all statistical analyses.

3. Results

3.1. Influence of *Bacillus* strains on *Aspergillus parasiticus* growth and aflatoxin production

Six out of *Bacillus* 13 strains were able to reduce *A. parasiticus* growth rate significantly ($p < 0.05$) compared to controls (0.53 mm/h). *Bacillus mojavensis* RC1A was able to inhibit fungal growth almost completely, reducing growth rate to 0.16 mm/h and increasing Lag phase duration significantly (31.72 h) ($p < 0.0001$). *Bacillus subtilis* RC6A did also affect Lag phase duration (37.99 h) delaying fungal growth significantly ($p < 0.0001$) (Fig. 1, Table 1). Only *B. mojavensis* RC1A was able to significantly reduce ($p < 0.0001$) the concentration of AFB₁ produced by *A. parasiticus* and accumulated in the culture media (Table 1).

3.2. Antifungal activity of compounds produced by *Bacillus mojavensis* RC1A

No visible fungal growth was observed in wells containing

pure *Bacillus mojavensis* RC1A culture or CFCS and *A. parasiticus* from day 1 to day 7. The same result was observed in wells containing three of the chloroformic phase extracts (Cl3 Rf = 0.47; Cl4 Rf = 0.65; Cl6 Rf = 0.91), two of the hexane phase (He3 Rf = 0.39; He6 Rf = 0.76) and one of the methanolic phase (Me2 Rf = 0.31) obtained after the extraction described by Ji et al. (2013) (Fig. 2). Also, no visible *A. parasiticus* growth was observed in wells containing three of the compounds separated by TLC from the chloroform:methanol fractions obtained in the column chromatography of the methanolic extract from the butanolic fraction for the characterization of antifungal lipopeptides (compound 1 from chloroform:methanol (20:1, v/v) fraction (Me 20:1–1), Rf = 0.82; compound 1 from chloroform:methanol (10:1, v/v) fraction (Me 10:1–1), Rf = 0.81 and compound 4 from chloroform:methanol (2:1, v/v) fraction Me 2:1–4), Rf = 0.82) (Table 2). Eighteen of the extracts and compounds tested were able to inhibit *A. parasiticus* growth significantly (ANOVA $P < 0.001$; Scott & Knott $P < 0.05$) when mycelia were weighed. Twelve of the compounds and extracts tested that inhibited visible fungal growth, also reduced AFB₁ production significantly ($P < 0.05$), 58% of these to non-detectable levels ($P < 0.05$) (Table 2). According to their Rfs, some of these compounds could be identified as surfactin (Rf-0.7–0.8), fengycin (Rf-0.1–0.2) and iturin A (Rf-0.4) (Afsharmanesh et al., 2014).

Table 1

Influence of *Bacillus* strains on *Aspergillus parasiticus* NRRL 2999 growth rate, Lag phase and aflatoxin B₁ (AFB₁) production and accumulation *in vitro*. Different letters within the same column indicate statistically significant differences. AFB₁ concentration in YES medium in each *A. parasiticus* + *Bacillus* co-cultures was compared with control plates and with the other co-cultures ($P < 0.05$). Growth rate and Lag phase values of co-cultures were compared with each other and with controls using ANOVA ($P < 0.0001$) and Fisher's LSD test ($P < 0.0001$) (InfoStat software).

Isolate	AFB ₁ (µg/g) Media ±SD*	Growth rate (mm/h)		Lag phase (h)	
		Media**	SE	Media**	SE
<i>B. mojavensis</i> RC1A	3,15 ^d ± 1,33	0,16 ^h	0,02	31,72 ^{ab}	2,06
<i>B. subtilis</i> RC1B	144,66 ^{bc} ± 59,02	0,37 ^f	0,02	23,80 ^{bc}	2,06
<i>B. cereus</i> RC1C	7,715,10 ^a ± 827,16	0,51 ^{bc}	0,02	15,59 ^c	2,06
<i>B. mojavensis</i> RC3A	283,72 ^{bc} ± 10,47	0,36 ^f	0,02	18,34 ^c	2,06
<i>B. mojavensis</i> RC3B	212,16 ^{bc} ± 57,05	0,53 ^{bc}	0,02	11,79 ^c	2,06
<i>B. megaterium</i> RC3C	191,13 ^{bc} ± 59,55	0,63 ^a	0,02	15,05 ^c	2,06
<i>B. cereus</i> RC3E	7,090,50 ^a ± 244,18	0,54 ^b	0,02	13,39 ^c	2,06
<i>B. cereus</i> RC3F	1,064,52 ^b ± 1,270,44	0,49 ^{bed}	0,02	19,14 ^{bc}	2,06
<i>B. mycooides</i> RC4A	77,06 ^{bc} ± 3,00	0,41 ^{ef}	0,02	18,41 ^c	2,06
<i>B. cereus</i> RC5A	175,82 ^{bc} ± 6,47	0,43 ^{de}	0,02	22,04 ^{bc}	2,06
<i>B. subtilis</i> RC6A	130,65 ^{bc} ± 10,89	0,24 ^g	0,02	37,99 ^a	2,06
<i>B. cereus</i> RC6B	61,22 ^{bc} ± 8,48	0,48 ^{cd}	0,02	14,01 ^c	2,06
<i>B. mycooides</i> RC6C	76,40 ^{bc} ± 1,86	0,50 ^{bc}	0,02	19,09 ^{bc}	2,06
Control	123,77 ^{bc} ± 41,66	0,53 ^{bc}	0,02	15,43 ^c	2,06

SD: Standard deviation; SE: standard error.

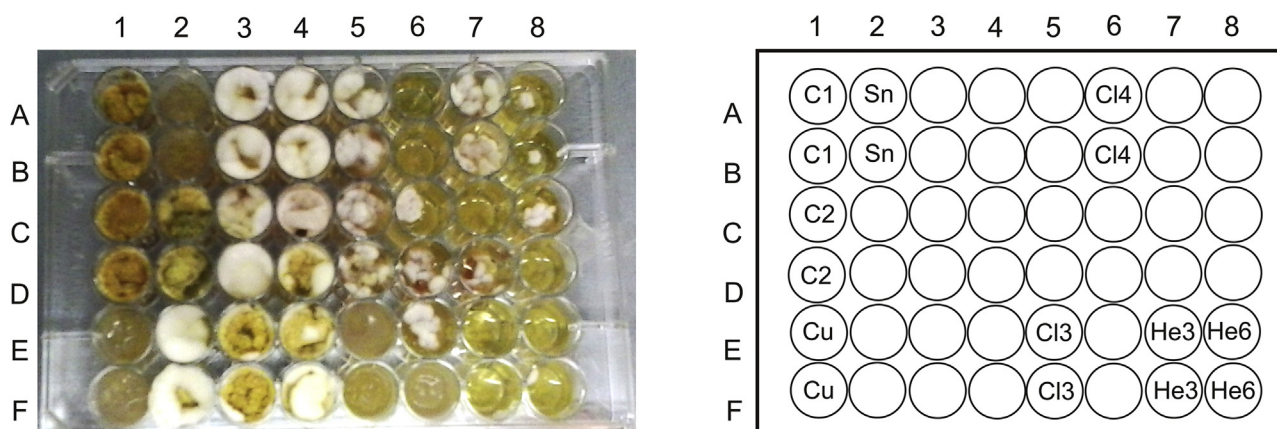


Fig. 2. Antifungal activity of *Bacillus mojavensis* RC1A culture (Cu), cell free culture supernatant (Sn) and different compounds obtained by organic solvent extraction and TLC separation. Compounds indicated as Cl3, Cl4 He3 and He6 were able to inhibit fungal growth completely and reduce aflatoxin B₁ to non-detectable levels.

3.3. Confirmation of lipopeptides production by MALDI-TOF

MALDI-TOF mass spectra analysis revealed signals compatible with surfactin, iturin, and fengycin homologues in both, *B. mojavensis* RC1A and *B. subtilis* RC6A CFCS as well as in butanolic extract (Table 3). As shown in Fig. 3 and Figures. S1 and S2 (see Supplementary material) signals observed are clearly located in two different *m/z* regions: those observed in *m/z* 960–1200 (Fig. 3a) and the second group located in the region *m/z* 1400–1650. Accordingly to data in the literature (Price, Rooney, Swezey, Perry, & Cohan, 2007; Pathak, Keharia, Gupta, Thakur, & Balaram, 2012; Vater et al., 2002; Torres, Petroselli, Daz, Erra-Balsells, & Audisio, 2015; Yang, Wei, & Mu, 2006) in the first region signals can be assigned mainly to surfactins and iturins, and in the second *m/z* region to fengycins, observed their molecular ions as $[M+H]^+$, $[M+Na]^+$ and/or $[M+K]^+$ species. In some samples (and not in all

duplicates) polymyxins were detected as very low intensity signals (*m/z* 1145 and *m/z* 1202; Table 3). Signals related with kurstakins (*m/z* range 901–970) and bacitracin (*m/z* range 1400–1420) were not detected in any condition for any of the analyzed samples (Fig. 3, Figs. S1 and S2). The best relative intensity ratio between signals of the first group and signals in the second group were observed when CFCS from *B. mojavensis* RC1A (Fig. 3a and b) and CFCS from *B. subtilis* RC6A (Figs. S2a and S2b) were analyzed.

4. Discussion

In the present study, soil-borne *Bacillus* strains able to inhibit *A. parasiticus* growth and reduce AFB₁ formation and accumulation were detected. Similar results were obtained by Munimbazi and Bullerman (1998) who reported 35 and 56% of inhibition in *A. parasiticus* mycelial growth when exposed to CFCS of two

Table 2
Inhibition of *Aspergillus parasiticus* NRRL 2999 growth and aflatoxin B₁ (AFB₁) production by different extracts and fractions containing antifungal compounds from *Bacillus mojavensis* RC1A after organic solvent (Cl: chloroform, Me: methanol, He: hexane, Bu: butanol) extraction (complete extracts) and after column chromatography and TLC (indicated by abbreviation of the solvent and number of the compound). Aqueous fraction (Aq), pure RC1A 24 h culture and cell free culture supernatant (CFCS) were also included in the test. Culture medium (Control 1) and culture medium + 50 µl chloroform:methanol (2:1 v/v) (Control 2, the solvent used for the extracts) were included as controls. Visible fungal growth was qualitatively evaluated considering as positive (+) when the visible growth on the surface of the media was reduced at least to two third parts compared to the controls.

Extract	Visible fungal growth inhibition	Average mycelium weight (g) ± SE*	AFB ₁ (µg/ml)**	TLC Rf
Cl3	+	0.00 ± 0.01 ^a	0.00 ± 0.36 ^a	0.47
Pure CFCS	+	0.00 ± 0.01 ^a	0.00 ± 0.36 ^a	–
Cl4	+	0.00 ± 0.01 ^a	0.00 ± 0.36 ^a	0.65
Pure culture	+	0.00 ± 0.01 ^a	0.00 ± 0.36 ^a	–
Me 2	+	0.00 ± 0.01 ^a	0.00 ± 0.36 ^a	0.31
He 3	+	0.00 ± 0.01 ^a	0.00 ± 0.36 ^a	0.27
Cl6	+	0.01 ± 0.01 ^a	0.69 ± 0.36 ^{a,b,c,d,e,f}	0.91
He6	+	0.01 ± 0.01 ^a	1.54 ± 0.36 ^{c,d,e,f,g,h}	0.76
Me 20:1–1	+	0.01 ± 0.01 ^a	0.00 ± 0.36 ^a	0.82
Me 2:1–4	+	0.01 ± 0.01 ^b	1.64 ± 0.36 ^{d,e,f,g,h}	0.84
Me 5:1–1	+	0.02 ± 0.01 ^b	0.64 ± 0.36 ^{a,b,c,d}	0.79
He5	+	0.02 ± 0.01 ^b	1.67 ± 0.36 ^{f,g,h}	0.67
He4	+	0.02 ± 0.01 ^b	1.95 ± 0.36 ^{g,h}	0.53
Me 10:1–1	+	0.02 ± 0.01 ^b	0.59 ± 0.36 ^{a,b,c}	0.81
Complete Cl	+	0.02 ± 0.01 ^b	1.81 ± 0.36 ^{g,h}	–
Me 5:1–2	+	0.02 ± 0.01 ^b	0.52 ± 0.36 ^{a,b}	0.83
He2	+	0.02 ± 0.01 ^b	1.39 ± 0.36 ^{b,c,d,e,f,g,h}	0.27
Me 2:1–2	+	0.02 ± 0.01 ^b	0.65 ± 0.36 ^{a,b,c,d,e}	0.68
Complete Aq	–	0.03 ± 0.01 ^c	1.70 ± 0.36 ^{g,h}	–
He1	+	0.03 ± 0.01 ^c	2.02 ± 0.36 ^{g,h}	0.20
Complete He	–	0.03 ± 0.01 ^c	1.88 ± 0.36 ^{g,h}	–
Me 20:1–2	+	0.03 ± 0.01 ^c	1.27 ± 0.36 ^{b,c,d,e,f,g,h}	0.90
Complete Me 2:1	–	0.03 ± 0.01 ^c	1.83 ± 0.36 ^{g,h}	–
Cl2	–	0.03 ± 0.01 ^c	2.24 ± 0.36 ^h	0.26
Me 2:1–3	–	0.03 ± 0.01 ^c	1.6 ± 0.36 ^{f,g,h}	0.73
Cl5	+	0.03 ± 0.01 ^c	1.87 ± 0.36 ^{g,h}	0.77
Complete Bu	–	0.04 ± 0.01 ^c	1.70 ± 0.36 ^{g,h}	–
Complete Me 5:1	–	0.04 ± 0.01 ^c	1.89 ± 0.36 ^{g,h}	–
Me 2:1–5	–	0.04 ± 0.01 ^c	1.71 ± 0.36 ^{g,h}	0.92
Bu3	+	0.04 ± 0.01 ^c	1.19 ± 0.36 ^{b,c,d,e,f,g}	0.90
Cl1	–	0.04 ± 0.01 ^c	2.01 ± 0.36 ^{g,h}	0.16
Me 10:1–2	+	0.04 ± 0.01 ^c	1.81 ± 0.36 ^{g,h}	0.93
Complete Me 20:1	–	0.04 ± 0.01 ^c	1.66 ± 0.36 ^{e,f,g,h}	–
Me 2:1–1	+	0.04 ± 0.01 ^c	1.98 ± 0.36 ^{g,h}	0.62
Me 5:1–3	–	0.04 ± 0.01 ^c	1.90 ± 0.36 ^{g,h}	0.91
Aq1	+	0.04 ± 0.01 ^c	1.29 ± 0.36 ^{b,c,d,e,f,g,h}	0.25
Bu2	+	0.04 ± 0.01 ^c	1.63 ± 0.36 ^{d,e,f,g,h}	0.72
He7	–	0.05 ± 0.01 ^c	1.65 ± 0.36 ^{d,e,f,g,h}	0.87
Bu1	+	0.05 ± 0.01 ^c	1.76 ± 0.36 ^{g,h}	0.48
Me1	+	0.05 ± 0.01 ^c	1.79 ± 0.36 ^{g,h}	0.25
Complete Me 10:1	–	0.06 ± 0.01 ^c	2.05 ± 0.36 ^{g,h}	–
Control 1	–	0.03 ± 0.01 ^c	2.03 ± 0.36 ^{g,h}	–
Control 2	–	0.03 ± 0.01 ^c	1.88 ± 0.36 ^{g,h}	–

SE: Standard error.

TLC: Thin layer chromatography.

Rf: Retention factor.

*Different letters within the same column indicate statistically significant difference ($p < 0.05$). ANOVA and Scott & Knott Test. InfoStat.

**Different letters within the same column indicate statistically significant difference ($p < 0.05$). ANOVA and LSD Fisher Test. InfoStat.

Table 3

Main peaks detected by MALDI mass spectrometry analysis of the lipopeptides present in the CFCS and butanolic extract of CFCS produced by different strains of *Bacillus* spp. Assignments are based on reference data: Price et al. (2007); Pathak et al. (2012); Vater et al. (2002); Torres et al. (2015); Yang et al. (2006).

m/z exp	Lipopeptide	Species	<i>B. mojavensis</i> RC1A		<i>B. subtilis</i> RC6A
			Butanolic extract	CFCS	CFCS
986	surfactin	C10 [M+Na] ⁺	+	+	+
1000	surfactin	C11 [M+Na] ⁺	+	+	+
1014	surfactin	C12 [M+Na] ⁺	+	+	+
1030	surfactin	C13 [M+Na] ⁺	+	+	+
1036	surfactin	C15 [M+H] ⁺	+	+	+
1044	surfactin	C14 [M+Na] ⁺	+	+	+
1052	iturin	C13 [M+Na] ⁺	+	+	+
1058	surfactin	C15[M+Na] ⁺	+	+	+
1066	iturin	C14[M+Na] ⁺	+	–	–
1074	surfactin	C15 [M+K] ⁺	+	+	+
1081	iturin	C14 [M+K] ⁺	+	+	+
1088	surfactin	C16 [M+K] ⁺	+	+	+
1098	iturin	C18[M+H] ⁺	+	+	+
1102	surfactin	C17 [M+K] ⁺	+	+	+
1109	iturin	C16 [M+K] ⁺	+	–	–
1113	iturin	C19[M+H] ⁺	–	+	+
1120	iturin	C18[M+Na] ⁺	+	+	+
1134	iturin	C19[M+Na] ⁺	+	+	+
1145	polimyxins D1	[M+H] ⁺	–	+	–
1159	not assigned		+	+	+
1182	polimyxins D1	[M+K] ⁺	+	+	+
1197	not assigned		–	–	+
1436	fengycin	Ala-6-C14[M+H] ⁺	–	–	+
1449	fengycin	Ala-6-C15[M+H] ⁺	+	–	+
1462	fengycin	Ala-6-C16[M+H] ⁺	+	+	+
1471	fengycin	Ala-6-C15[M+Na] ⁺	+	–	+
1478	fengycin	Ala-6-C17[M+H] ⁺	–	+	+
1485	fengycin	Ala-6-C16[M+Na] ⁺	+	+	+
1492	fengycin	Val-6-C16[M+H] ⁺	+	+	+
1499	fengycin	Ala-6-C17[M+Na] ⁺	+	+	+
1506	fengycin	Val-6-C17[M+H] ⁺	+	+	+
1513	fengycin	Val-6-C16[M+Na] ⁺	–	+	+
1516	fengycin	Ala-6-C18[M+ Na] ⁺	+	+	+
1529	fengycin	Val-6-C17[M+ Na] ⁺	+	+	+
1544	fengycin	Val-6-C18[M+ Na] ⁺	–	+	+
1558	fengycin	Val-6-C19[M+ Na] ⁺	+	–	–
1572	not assigned		+	+	–
1586	not assigned		–	+	+
1600	not assigned		+	+	+
1613	not assigned		–	+	+
1629	not assigned		–	+	+

B. pumilus strains. Similarly, Kong, Shan, Liu, Wang, and Yu (2010) and Afsharmanesh et al. (2014) reported inhibition of *A. flavus* growth in presence of *B. megaterium* and *B. subtilis* CFCSs and washed cells. In our study, all species identified were members of *B. subtilis* group and *B. cereus* group. The importance of inhibiting fungal growth in the field and post-harvest relies in the fact that mycotoxin production and accumulation in agricultural products could be avoided, delayed or reduced. The thin layer chromatography analysis of butanolic extract of *B. mojavensis* RC1A (member of *B. subtilis* group) was compared with TLCs of lipopeptides produced by *B. subtilis* strains UMAF6614 (surfactin, bacillomycin D and fengycin) and UMAF6639 (surfactin, iturin A and fengycin) taking their Rf values obtained by Afsharmanesh et al. (2014) and Romero et al. (2007) as references. TLC analysis showed the presence of bands corresponding to three of the main groups of lipopeptides at the expected retention factor (Rf) values. These fractions were able to inhibit fungal growth *in vitro* when isolated from the CFCS.

The results obtained with the MALDI-TOF-MS technique demonstrated that *B. mojavensis* RC1A produces four families of lipopeptides with different homologous compounds: fengycins, surfactins, iturins and bacillomycins. All four groups of lipopeptides were also confirmed in CFCS and butanolic extract of *B. subtilis* RC6A, another strain that reduced *A. parasiticus* growth

significantly. Co-production of surfactin, iturins and fengycin families have been reported in *B. subtilis* and *B. amyloliquefaciens* strains. Ben Ayed et al. (2015) reported the production of fengycins and surfactins in *B. mojavensis* A21, a strain isolated from marine water. The co-production of lipopeptides from different families by *B. mojavensis* RC1A and *B. subtilis* RC6A is an interesting characteristic that could support their potential applications in different biotechnological fields (Razafindralambo et al., 1997). Moreover, structural diversity of lipopeptides observed by MALDI-MS, may offer several potential applications. Different isoforms and homologues exhibit different properties and activities, which depend in particular on the chain length (Ben Ayed et al., 2015). Surfactins are probably the most powerful biosurfactants described, and despite their moderate antifungal activity, they show a strong synergistic effect in combination with iturin A (Maget-Dana, Thimon, Peypoux, & Ptak, 1992). Surfactins have also shown several pharmacological activities including, antimicrobial, antiviral, antitumoral and antifibrinolytic (Vollenbroich, Pauli, Özel, & Vater, 1997). They act in a synergistic manner with fengycin which may improve their activities (Razafindralambo et al., 1997). Fengycins, quite abundant in the present SFCS samples, have demonstrated specific antifungal activity against filamentous fungi inhibiting phospholipase A2 (Nishikiori, Naganawa, Muraoka, Aoyagi, & Umezawa, 1986).

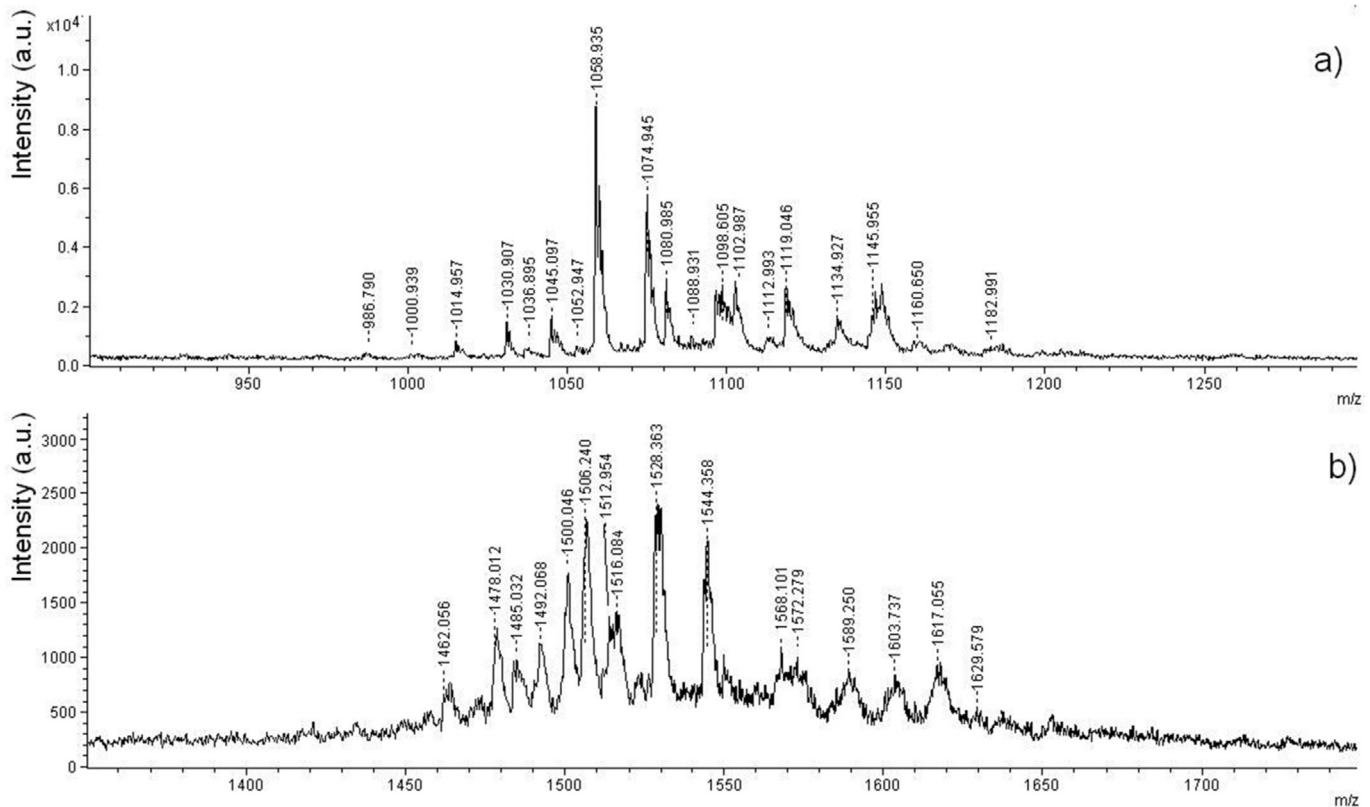


Fig. 3. MALDI mass spectra of CFCS from *B. mojavensis* RC1A: (a) *m/z* range, 900–1300; (b) *m/z* range, 1300–1800. Positive ion mode. Matrix: nHo.

5. Conclusions

Bacillus strains isolated from soil and lake sediment from our region have demonstrated to produce compounds capable to inhibit growth of *A. parasiticus* (a major fungal contaminant in stored crops, food and feed) and to diminish the production of AFB₁, potent carcinogenic and mutagenic mycotoxin associated with cancer in humans and responsible for worldwide substantial economic losses and public health issues. The further study of these strains and the compounds they produce in order to determine their absence of toxicity will decide their potential application in biological-based products to control fungal and mycotoxins contamination in food and feedstuffs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.foodcont.2017.10.020>.

References

- Afsharmanesh, H., Ahmadzadehb, M., Javan-Nikkhabh, M., & Behboudib, K. (2014). Improvement in biocontrol activity of *Bacillus subtilis* UTB1 against *Aspergillus flavus* using gamma-irradiation. *Crop Protection*, 60, 83–92.
- AOAC International. (1995). *Official methods of analysis* (16th Edition, Vol. 1). Arlington: AOAC International, VA.
- Ben Ayed, H., Nasri, R., Jemil, N., Ben Amor, I., Gargouri, J., Hmidet, N., & Nasri, M. (2015). Acute and sub-chronic oral toxicity profiles of lipopeptides from *Bacillus mojavensis* A21 and evaluation of their in vitro anticoagulant activity. *Chemico-Biological Interactions*, 236, 1–6.
- Caldeira, A. T., Santos Arteiro, J. M., Coelho, A. V., & Roseiro, J. C. (2011). Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCM1 1051. *Process Biochemistry*. <https://doi.org/10.1016/j.procbio.2011.05.016>.
- Cho, S. J., Lee, S. K., Cha, B. J., Kim, Y. H., & Shin, K. S. (2003). Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. *FEMS Microbiology Letters*, 223, 47–51.
- Cho, K. M., Math, R. K., Hong, S. Y., Asraful Islam, S. M., Mandanna, D. K., Cho, J. J., et al. (2009). Iturin produced by *Bacillus pumilus* HY1 from Korean soybean sauce (kanjang) inhibits growth of aflatoxin producing fungi. *Food Control*, 20, 402–406.
- Das, P., Mukherjee, S., & Sen, R. (2008). Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans*. *Journal of Applied Microbiology*, 104, 1675–1684.
- Droby, S. (2006). Improving quality and safety of fresh fruits and vegetables after harvest by the use of biocontrol agents and natural materials. *ISHS Acta Horticulturae*, 709, 45–51.
- Geisen, R. (1996). Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Journal of Official Applied Microbiology*, 19, 388–392.
- Gong, A. D., Li, H. P., Yuan, Q. S., Song, X. S., Yao, W., He, W. J., et al. (2015). Antagonistic mechanism of iturin a and plipastatin a from *Bacillus amyloliquefaciens* S76–3 from wheat spikes against *Fusarium graminearum*. *PLoS ONE*, 10(2), e0116871. <https://doi.org/10.1371/journal.pone.0116871>.
- International Agency for Research on Cancer (IARC). (2002). IARC monographs on the evaluation of carcinogenic risks to humans: Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. *IARC Monographs*, 82, 171.
- Ji, S. H., Paul, N. C., Deng, J. X., Kim, Y. S., Yun, B. S., & Yu, S. H. (2013). Biocontrol activity of *Bacillus amyloliquefaciens* CNU114001 against fungal plant diseases. *Mycobiology*, 41(4), 234–242.
- Kong, Q., Shan, S., Liu, Q., Wang, X., & Yu, F. (2010). Biocontrol of *Aspergillus flavus* on

- peanut kernels by use of a strain of marine *Bacillus megaterium*. *International Journal of Food Microbiology*, 139, 31–35.
- Maget-Dana, R., Thimon, L., Peypoux, F., & Ptak, M. (1992). Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. *Biochimie*, 74, 1047–1051.
- Munimbazi, C., & Bullerman, L. B. (1998). Inhibition of aflatoxin production of *Aspergillus parasiticus* NRRL 2999 by *Bacillus pumilus*. *Mycopathologia*, 140, 163–169.
- Nishikiori, T., Naganawa, H., Muraoka, Y., Aoyagi, T., & Umezawa, H. (1986). Plipastatins, new inhibitors of phospholipase A2 produced by *Bacillus cereus* BMG302- f F67 III. Structural elucidation of plipastatins. *Journal of Antibiotics (Tokyo)*, 39, 755–761.
- Nonami, H., Fukui, S., & Erra-Balsells, R. (1997). β -carboline alkaloids as matrices for matrix-assisted ultraviolet laser desorption time-of flight mass spectrometry of proteins and sulfated oligosaccharides: A comparative study using phenylcarbonyl compounds, carbazoles and classical matrices. *J. Mass Spectrom*, 32, 287–296.
- Palumbo, J. D., Baker, J. L., & Mahoney, N. E. (2006). Isolation of bacterial antagonists of *Aspergillus flavus* from almonds. *Microbial Ecology*, 52, 45–52.
- Pathak, K. V., Keharia, H., Gupta, K., Thakur, S. S., & Balaran, P. (2012). Lipopeptides from the banyan endophyte, *Bacillus subtilis* K1: Mass spectrometric characterization of a library of fengycins. *J. Am. Soc. Spectrom*, 23, 1716–1728.
- Price, N. P., Rooney, A. P., Swezey, J. L., Perry, E., & Cohan, F. M. (2007). Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations. *FEMS Microbiol Lett*, 271, 83–89.
- Razafindralambo, H., Paquot, M., Hbid, C., Jacques, P., Destain, J., & Thonart, P. (1993). Purification of antifungal lipopeptides by reversed-phase high performance liquid chromatography. *Journal of Chromatography*, 639, 81–85.
- Razafindralambo, H., Popineau, Y., Deleu, M., Hbid, C., Jacques, P., Thonart, P., et al. (1997). Surface-active properties of surfactin/iturin A mixtures produced by *Bacillus subtilis*. *Langmuir*, 13, 6026–6031.
- Romero, D., de Vicente, A., Olmos, J. L., Dávila, J. C., & Pérez-García, A. (2007). Effect of lipopeptides of antagonistic strains of *Bacillus subtilis* on the morphology and ultrastructure of the cucurbit fungal pathogen *Podosphaera fusca*. *Journal of Applied Microbiology*, 103, 969–976.
- Sosa-Pech, M., Ruiz-Sánchez, E., Mejía-Bautista, M., Reyes-Ramírez, A., Cristóbal-Alejo, J., Valencia-Boatín, A., et al. (2012). *In vitro* antagonistic activity of *Bacilli* class isolates from the Yucatán peninsula against four phytopathogenic fungi. *Universidad Y Ciencia Trópico Húmedo*, 28(3), 279–284.
- Torres, M. J., Petroselli, G., Daz, M., Erra-Balsells, R., & Audisio, M. C. (2015). *Bacillus subtilis* subsp. *subtilis* CBMDC3f with antimicrobial activity against Gram-positive foodborne pathogenic bacteria: UV-MALDI-TOF MS analysis of its bioactive compounds. *World J Microbiol Biotechnol*, 31, 929–940.
- Trucksess, M. W., Stack, M. E., Nesheim, S., Albert, R. H., & Romer, T. R. (1994). Multifunctional column coupled with 625 liquid chromatography for determination of aflatoxins B1, B2, G1, and G2 in corn, almonds, Brazil nuts, peanuts, and pistachio nuts: Collaborative study. *Journal of the AOAC International*, 77, 1512–1521.
- Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N., & Cameotra, S. S. (2002). Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. *Appl Environ Microbiol*, 68, 6210–6219.
- Vollenbroich, D., Pauli, G., Özel, M., & Vater, J. (1997). Antimycoplasmal properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. *Applied and Environmental Microbiology*, 63, 44–49.
- Yang, S. Z., Wei, D. Z., & Mu, B. Z. (2006). Determination of the amino acid sequence in a cyclic lipopeptide using MS with DHT mechanism. *Journal of Biochemical and Biophysical Methods*, 68(1), 69–74.
- Zhang, T., Shi, Z. Q., Hu, L. B., Cheng, L. G., & Wang, F. (2008). Antifungal compound from *Bacillus subtilis* B-FS06 inhibiting the growth of *Aspergillus flavus*. *World Journal of Microbiology and Biotechnology*, 24, 783–788.