Chapter 2

Terrestrial Bacteria from Agricultural Soils: Versatile Weapons against Aflatoxigenic Fungi

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Additional information is available at the end of the chapter

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1. Introduction

Invasion of food, feed and agricultural crops with mycotoxigenic fungi from the genera *Aspergillus*, *Fusarium* and *Penicillium* is an economic problem that is not yet under adequate control despite modern food production technologies and the wide range of preservation techniques available (Bennett & Klich, 2003). A small number of characterized fungi are as important as the genus *Aspergillus*, a taxonomic group which encompasses members with pathogenic, agricultural, industrial and pharmaceutical importance (Jamali et al., 2012). Nearly all fungi that produce aflatoxins, the most potent naturally occurring hepatocarcinogens, are members of the genus *Aspergillus* classified into the section *Flavi*. Among 22 closely related species in *Aspergillus* section *Flavi*, the members frequently encountered in agricultural products i.e. *Aspergillus flavus* and *A. parasiticus* are responsible for the majority of aflatoxin (AF) contamination events, with *A. flavus* being by far the most common (Varga et al., 2011). Aflatoxigenic fungi are common soil habitants all over the world and they frequently contaminate agricultural crops, such as peanuts, cottonseed, maize, and tree nuts (Bennett & Klich, 2003; Hedayati et al., 2007; Razzaghi-Abyaneh et al., 2006; Sepahvand et al., 2011). The fungal community structure composed of several players, species, strains, isolates and vegetative compatibility groups (VCGs), in the soil and on the crop determines the final AF concentration (Jamali et al., 2012; Razzaghi-Abyaneh et al., 2006). The life cycle of *A. flavus* in a pistachio orchard is shown in Fig. 1. AF contamination of agricultural crops is a major concern due to economical losses resulting from inferior crop quality reduced animal productivity and impacts on trade and public health. In a global context, AF contamination is an everlasting concern between the 35N and 35S latitude. Most of the countries in the belt of concern are developing countries and this makes the situation even worse because in those countries people frequently rely on highly susceptible crops for their daily nutrition and income. It has also been evident that AF more and more becomes a problem in countries that previously did not have to worry about AF contamination.

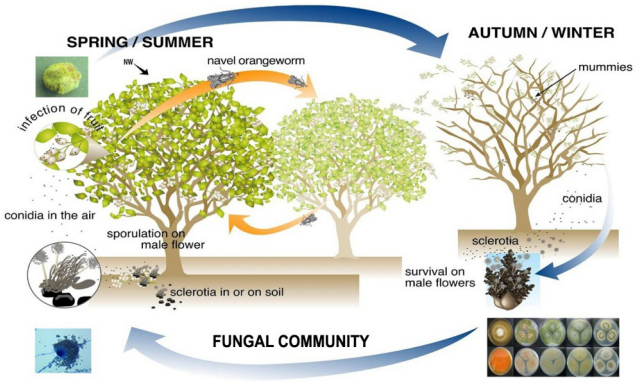

Figure 1. The life cycle of *A. flavus* is shown in a pistachio orchard. Infection of fruits with air-borne conidia occurs during Spring/Summer, while the fungus will survive by resistant structures named "sclerotia" during Autumn/Winter.

To ensure global safety on food and feed supplies, extensive researches have been carried out to effectively control and manage AF contamination of crops. The strategies for preventing AF contamination are generally divided into two categories including pre- and post-harvest controls (Kabak et al., 2006). Pre-harvest control strategies include appropriate field management practices (crop rotation, irrigation, soil cultivation, etc.), enhancing host resistance (transgenic or genetically modified crops), biological (application of antagonistic fungi and bacteria) and chemical control (fungicides, insecticides). Respect to biocontrol approaches, the rapid expansion in our knowledge about the role of microorganisms in inhibiting AF biosynthesis has enabled us to utilize them as potential AF biocontrol agents (Holmes et al., 2008; Raaijmakers et al., 2002). A large number of plants, mushrooms, bacteria, microalgae, fungi and actinomycetes have now been screened for the ability to inhibit toxigenic fungal growth and/or AF production (Alinezhad et al., 2011, Bagheri-Gavkosh et al., 2009; Ongena & Jacques, 2007; Razzaghi-Abyaneh & Shams-Ghahfarokhi, 2011; Razzaghi-Abyaneh et al., 2005, 2007, 2008, 2009, 2010, 2011). Substantial efforts have been carried out in identifying organisms inhibitory to AF biosynthesis through co-culture with aflatoxigenic fungi with the aim of finding potential biocontrol agents as well as novel inhibitory metabolites. The use of beneficial microorganisms is one of the most promising methods to the development of environmentally friendly alternatives to chemical pesticides in preventing the growth of aflatoxigenic fungi and subsequent AF contamination of susceptible crops. Among beneficial microorganisms, antagonistic bacteria are in the first line of investigation because of a much greater diversity than that of any other organism and possessing valuable pharmaceutically active molecules (Ongena & Jacques, 2007; Stein, 2005). Recent advances in analytical methods and enormous expanding of natural products libraries, cloning, and genetic engineering have provided a unique opportunity for isolation and structural elucidation of novel bioactive antifungal compounds from bacterial communities all over the world. It has been reported that, on average, two or three antibiotics derived from bacteria break into the market each year (Clark, 1996). Among an estimated number of 1.5 million bacterial species exists on our planet, only a little portion (less than 1%) has been identified yet of which a more little have tested for bioactive antifungal metabolites. Terrestrial bacteria are an interesting group of antagonistic microorganisms capable

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of efficiently inhibit toxigenic fungus growth and AF production. They mainly belong to the genera *Bacillus*, *Pseudomonas*, *Agrobacterium* and *Streptomyces* which have worldwide distribution (Holmes et al., 2008; Ongena & Jacques, 2007; Razzaghi-Abyaneh et al., 2011; Stein, 2005). Metabolites from *Bacillus subtilis* (Fengycins A and B, plipastatins A and B, iturin A, mycosubtilin, bacillomycin D), *Streptomyces* spp. (dioctatin A, aflastatin A, blasticidin A), and *Achromobacter xylosoxidans* [cyclo (L-leucyl-L-propyl)] are good examples of potent inhibitors of AF biosynthesis in laboratory conditions, crop model systems and also in the field (For review, see Razzaghi-Abyaneh et al., 2011). Since production of antifungal metabolites in bacteria is quite dependent to the strain and species, ongoing search on finding strange bacteria within the existing biodiversity to increase the chance of finding novel antifungals is currently done all over the world (Ranjbarian et al., 2011; Stein, 2005).

This chapter highlights comprehensive data on antagonistic bacteria isolated from agricultural soils of pistachio, peanuts and maize fields with an emphasis on their ability for inhibiting growth of aflatoxigenic fungi and AF production. We first describe how we can isolate and identify a large number of soil bacteria with antagonistic activity against toxigenic *A. parasiticus* by simple, efficient and low-cost screening methods. Next to be addressed will be a practical approach to isolation, purification and identification of antifungal metabolites from antagonistic bacteria by a combination of traditional and recent advanced technologies.

2. Biological control: a powerful management strategy

Biological control is defined as i) a method of managing pests by using natural enemies ii) an ecological method designed by man to lower a pest or parasite population to acceptable subclinical densities or iii) to keep parasite populations at a non-harmful level using natural living antagonists (Baker, 1987). The history of biological control dates back to an outstanding successful story, the biocontrol of the cottony-cushion scale (*Icerya purchasi*) on *Citrus* plant in California (Debach & Rosen, 1991). Biological control agents act against plant pathogens through different modes of action. Antagonistic interactions that can lead to biological control include antibiosis, competition and hyperparasitism (Bloom et al., 2003; Bull et al., 2002; Cook, 1993; Hoitink & Boehm, 1999). Competition occurs when two or more microorganisms require the same resources in excess of their supply. These resources can include space, nutrients, and oxygen. In a biological control system, the more efficient competitor, i.e., the biological control agent out-competes the less efficient one, i.e., the pathogen. Antibiosis occurs when antibiotics or toxic metabolites produced by one microorganism have direct inhibitory effect on another. Hyperparasitism or predation results from biotrophic or necrotrophic interactions that lead to parasitism of the plant pathogen by the biological control agent. Some microorganisms, particularly those in soil, can reduce damage from diseases by promoting plant growth or by inducing host resistance against a myriad of pathogens. Nowadays, atoxigenic *A. flavus* strains, biocompetitive bacteria and antagonistic yeasts has been effectively used to reduce AF contamination in field and laboratory conditions (Brown et al., 1991; Dorner et al., 1998, 1999; Hua et al., 1999; Palumbo et al., 2006). Commercial products from atoxigenic *A. flavus* under the names of AF36, AflaSafe and AflaGuard have been successfully used for biocontrol of aflatoxigenic fungi in maize, peanuts, cottonseed and pistachio fields in Southern US, Northern Mexico, Nigeria and West Africa (Atehnkeng et al., 2008; Donner et al., 2010).

3. Biocompetitive bacteria from agricultural soil

Regard to biocompetitive bacteria, *Bacillus subtilis* was first introduced as an inhibitor of growth and AF production of aflatoxigenic fungi by Kimura and Hirano (1988) and the effective compound, iturin A, had been patented for the control of AF in nuts and cereals (Kimura & Ono, 1988). Nowadays, ubiquitous inhabitants of agricultural soils i.e. the genera *Bacillus* and *Pseudomonas* are widely recognized as effective biocontrol agents of aflatoxigenic fungi. The broad host range, ability to form endospores and produce different biologically active compounds with a broad spectrum of activity made these bacteria as potentially useful biocontrol agents (Saharan & Nehra, 2011).

3.1. Soil sampling and bacterial isolation

One-hundred fifty soil samples were collected from pistachio, maize and peanut fields located in different regions of Damghan, Sari and Astaneh cities during June-July 2009. Sampling was done according to the latitude of each field. Each soil comprised from ten subsamples each of approximately 1000 mm^3 which were obtained using a sterile trowel at 10 m intervals. The subsamples were collected from the 50 mm top of the surface soil and then mixed thoroughly in a Nylon bag. The samples were air-dried in sterile Petri-dishes and stored at 4°C before use.

For bacteria isolation, 3 g of each soil sample was added to 10 ml of sterile normal saline solution (0.8 M), mixed vigorously by vortex for 2 min and centrifuge at 2500 rpm for 10 min. The amount of 10 µl aliquots of each sample supernatant was spread on to GY (Glucose 2%, Yeast extract 0.5%) agar and KB (King's B) agar plates and incubated for 3 days at 28°C. Discrete bacterial colonies were selected every 12 h and their purity was insured after transferring to master GY plate by tooth pick spot technique as shown in Fig. 2.

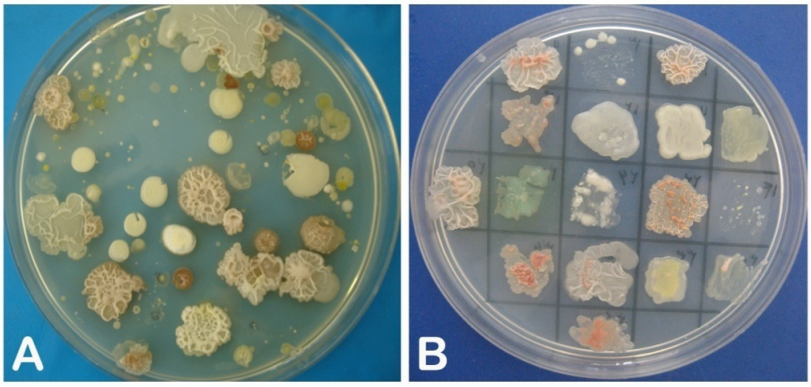

Figure 2. Various bacterial colonies appeared on GY agar after 3 days cultivation of soil suspensions (A). Separation and purification of colonies by using pick spot technique on GY agar master plates (B).

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3.2. Screening for antifungal activity by visual agar plate assay

For selecting bacteria that inhibit either fungal growth or AF production, a visual agar plate assay was used as described by Hua et al. (1999) with some modifications. A 5 µl aliquot of a conidial suspension (200 conidia/µl) of a norsolorinic acid (NA)-accumulating mutant of *Aspergillus parasiticus* NRRL 2999 was streaked on the center of a Potato dextrose agar (PDA) plate. A single streak of 10 µl aliquots of isolated bacteria grown overnight in 0.5X Tryptic soy agar (TSA; Difco, Becton Dickinson, Franklin Lakes, NJ) at 28°C was inoculated in peripheral lines in distance of 1.5 cm from central line by tooth pick. Screen plates were incubated for 3-5 days at 28°C and assessed visually for antifungal phenotypes (Fig. 3). Antifungal activity was assessed by comparing the zone of fungal growth inhibition in fungus co-cultured with bacteria as tests, in comparison with control plates which were inoculated only with the fungus. The effect of bacteria on AF production was assessed from the underside of the fungus where a decrease in the red pigment (NA) in the mycelium indicated inhibition of AF production by the bacterium (Fig. 3).

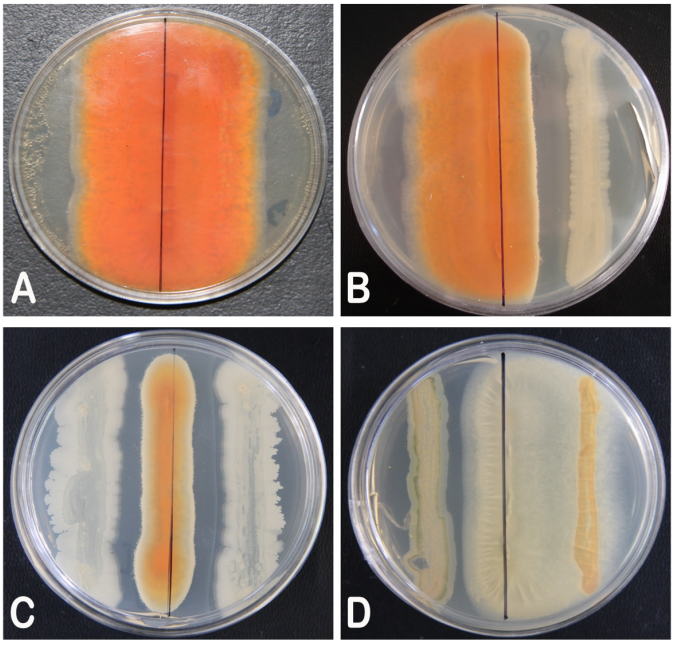

Figure 3. Visual agar plate assay shows screen identifying antagonistic bacteria with inhibitory activity against fungal (NA-accumulating mutant of *A. parasiticus* NRRL 2999) growth and/or NA accumulation (AF production):A) Control fungal culture against distilled water on both sides of GY agar.B) Control fungal culture against distilled water (left) and an antagonistic bacterium for fungal growth (right).C) Antagonistic bacteria for fungal growth with very weak inhibitory activity on NA accumulation on both sides.D) Antagonistic bacteria for both fungal growth and NA accumulation (left) and for only NA accumulation without affecting fungal growth (right).

Table 1 represents the results of antifungal phenotypes among soil bacteria isolated from pistachio, peanuts and maize fields. Different phenotypes were identified in all soils including NA and fungal growth inhibitors (type I), NA inhibitors (type II), growth inhibitors (type III) and finally non-inhibitors of NA and growth (type IV). The only exception were bacteria type II which was not isolated from peanuts field soils. In all fields, a pattern of type IV > type I > type III > type II were obtained regard to the number of antagonistic bacteria isolated. The phenotypes I and III are suitable candidates for biocontrol of AF-producing fungi in the field, while bacteria from type II are useful for elucidate AF biosynthesis pathway.

Fields of soil sampling	Total bacteria	Inhibitory bacteria	Inhibition of	
			NA	Fungal growth
Pistachio	290	37	+	+
		9	+	–
		22	–	+
		222	–	–
Maize	227	49	+	+
		6	+	–
		13	–	+
		159	–	–
Peanuts	87	19	+	+
		0	+	–
		16	–	+
		62	–	–

Table 1. Visual agar plate assay of antifungal phenotypes among soil bacteria isolated from pistachio, maize and peanuts field of Iran on PDA plates using a norsolorinic acid (NA) mutant of *A. parasiticus* NRRL 2999.

3.3. Identification of biocompetitive bacteria

The strongest antagonistic bacteria recognized from initial screening on PDA by visual agar plate assay were selected for identifying at genus and species level.

3.3.1. Biochemical identification

Selected bacteria were first determined to be either Gram-positive or Gram-negative using potassium hydroxide (Gregersen, 1978). Catalase and oxidase enzymatic activities were also determined (Barrow & Feltham, 1993). Gram-positive isolates were identified using GP2 MicroPlates (Biolog), whereas Gram-negative isolates were identified using GN2 MicroPlates (Biolog), according to the instructions of the manufacturer. Identification was based on the similarity index of carbon source utilization by each isolate relative to that of identified reference strains in the Biolog GP and GN databases.

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3.3.2. Molecular identification

Fig. 4 illustrates all the steps for molecular identification of antagonistic bacteria. Overnight bacterial cultures on LB medium at 30°C were streaked on TSA plates. Single colonies from cultures grown on 0.5X TSA at 28°C were suspended in 2.0 ml sterile distilled water. Bacterial cells were pelleted by centrifugation at 12,000 × g for 10 min. and resuspended in 0.1 ml sterile distilled water. Total DNA from bacteria was prepared from single colonies grown on TSA according to the QIAGEN instruction. The 16s rRNA gene fragment was amplified in PCR using 1 to 5 µl of each cell suspension as template and universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′AAGGAGGTGWTCCARCC-3′) (Lane, 1991). The PCRs were carried out using approximately 500 ng of total bacterial DNA, 10 µl of 10x PCR buffer, 8 µl of $MgCl_2$ (25 mM), 10 µl of deoxynucleoside triphosphates (dNTPs) (2 mM each), 3.3 µl of each primer (20 µM), 0.5 µl of *Taq* polymerase (5 U/µl), and enough Milli Q water so that the final volume of the mixture was 100 µl.

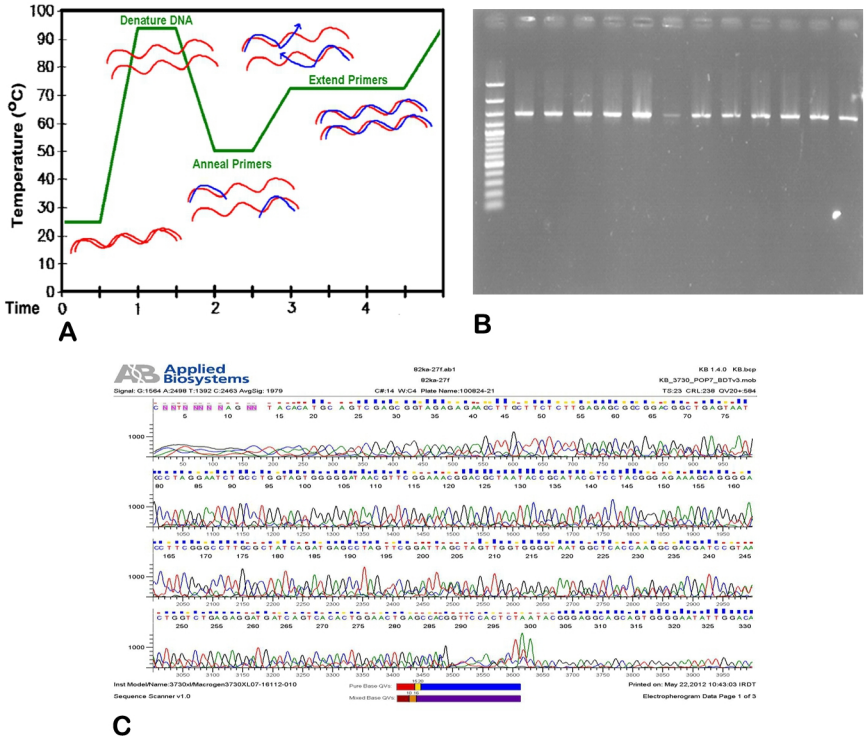

Figure 4. Molecular identification of antagonistic bacteria using PCR and DNA sequencing:A) PCR reaction temperature cycling; denaturing at 94°C, annealing at 55°C and extension at 72°C. Every cycle, DNA between primers is duplicated.B) An agarose gel stained with ethidium bromide shows PCR amplified bacterial DNAs (lines 2 to 13 from left). DNA molecular marker (100 bp DNA ladder) is shown in line 1 from left.C) Electroherogram data of purified DNA fragments of *Pseudomonas fluorescens* 82 which originated from sequence analysis by an ABI Prism Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The PCR mixtures were denatured at 95°C for 5 min, which was followed by 35 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 90s and then a final extension at 72°C for 5 min. Amplification was checked for purity by electrophoresis on a 1.0% agarose gel. The bands of interest were excised from the gel, and the DNA was purified using QIAquick PCR purification columns (Qiagen, Inc., Valencia, CA). Purified DNA fragments were sequenced using the same sets of primers that were used for amplification by an ABI Prism Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Bacteria were identified based on sequence similarities to homologous 16S rRNA gene fragments in the Ribosomal Database Project database (Cole et al., 2005) (accessed at http://rdp.cme.msu.edu/index.jsp).

3.4. Antagonistic activity against aflatoxigenic *A. parasiticus* NRRL 2999

Cell free culture supernatants of inhibitory bacteria were used in an antagonistic assay system. Table 2 represents the strongest antagonistic bacteria which were identified by a combination of biochemical and molecular methods in relation to their source of isolation.

Antagonistic bacteria	Strain number	Field	% of growth inhibition	% of AFB_1 inhibition	Surfactant production on blood agar
P. aeruginosa	320	Maize	63.9	95.3	+
	214	Maize	57.7	95.7	+
	155	Maize	48.9	78.2	–
	313	Peanuts	60.4	63.4	+
	257	Maize	64.5	85.3	+
	271	Maize	55.6	74.7	+
	293	Pistachio	55.7	73.6	+
	247	Maize	59.3	65.3	+
	287	Maize	59.0	87.6	+
	307	Peanuts	35.0	84.4	+
	168	Maize	62.6	96.9	+
	266	Maize	69.3	70.3	+
P. chlororaphis	236	Peanuts	15.3	65.9	–
P. fluorescens	82	Pistachio	72.7	91.1	–
B. subtilis	248	Maize	52.0	19.1	+
	298	Pistachio	70.6	18.7	+
	295	Pistachio	56.0	43.0	+
B. amyloliquefaciens	296	Maize	66.7	24.4	+

Table 2. Inhibitory effects of the strongest antagonistic bacteria selected from screening plates of visual agar plate assay on *A. parasiticus* NRRL 2999 growth and AF production in Potato dextrose broth. Control fungal culture had a growth rate of 51.17 mg and an AFB_1 amount of 697.78 ng/mg fungal dry weight.

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Identified bacteria (0.1 ml of bacterial inoculums containing Ca. 10^7 CFU/ml) were inoculated on 20 ml of PDB prepared in 100 ml capacity flasks and incubated for 48 h at 28°C in shaking condition (100 rpm). Cell free supernatant fluids were prepared by centrifuging the cultures at 23990×g for 15 min. The supernatant was supplemented with PDB to compensate for the consumption of nutrient by bacterial growth the pH of supernatant fluid was adjusted to that of the original medium. Supernatant fluids were sterilized by filtration through a 0.45 μm pore size nylon membrane. Five ml aliquots of sterilized bacterial supernatant were aseptically dispensed in 25 ml Erlenmeyer flasks and inoculated with 0.1 ml of a spore suspension of *A. parasiticus* NRRL 2999 containing Ca 10^7 conidia/ml. Cultures were incubated for 96 h at 28°C and analyzed for fungal growth and AF production. At the end of incubation period, fungal mycelia were separated from culture medium using filter paper. Mycelia dry weight was determined as an index of fungal growth by incubating a known weight of fungal biomass at 80°C for 3 h and then until a constant weight was obtained. AF was extracted from the culture medium using chloroform. The chloroformic extracts were concentrated by a rotary evaporator (EYELA N-1000, Japan) to dryness. Quantitation of AFB_1 was carried out using HPLC (KNAUER D-14163 UV-VIS system, Germany) (Razzaghi-Abyaneh et al., 2007). Fifty ml of each sample (chloroformic extract) were injected into the HPLC column (TSKgel ODS-80TS; 4.6 mm ID × 150 mm, TOSOH BIOSCIENCE, Japan) and eluted at a flow rate of 1 ml/min. by water-acetonitrile-methanol (60:25:15, v/v/v) as mobile phase. AFB_1 was measured at wavelength of 365 nm. The elution time of the samples was compared with AFB_1 standards and quantified on the basis of the ratio of the peak area of samples to those of the standards. As shown in Table 2, secretory metabolites of all tested antagonistic bacteria including *Pseudomonas aeruginosa* (12 isolates), *Bacillus subtilis* (3 isolates), and one isolate of each *Pseudomonas chlororaphis*, *P. fluorescens* and *Bacillus amyloliquefaciens* inhibited both *A. parasiticus* growth and AFB_1 production by different extents. Fungal growth was inhibited in the range of 15.3 to 72.7%, while AFB_1 synthesis was suppressed by 18.7 to 96.9%. The highest inhibition of fungal growth and AFB_1 production was related to *P. fluorescens* 82 and *P. aeruginosa* 168, respectively. In contrast to *Pseudomonas*, *Bacillus* species strongly inhibited fungal growth with a weak suppressive effect on AF production. All antagonistic bacteria except *P. aeruginosa* 155 from maize, *P. chlororaphis* 236 from peanuts and *P. fluorescens* 82 from pistachio were capable of producing surfactants as a part of their pathogenesis system (Table 2).

4. Purification of antifungal metabolites from soil bacteria: A practical approach

4.1. Culture conditions for metabolite production

As the first step for production of bioactive antifungals, different culture conditions including medium, incubation time and aeration should be optimized. In order to initial purification of inhibitory metabolites, the selected bacterium with strongest antifungal activity in initial screening was cultured on suitable liquid media such as GY (2% glucose, 0.5% yeast extract), SCD (2% bacto dextrose, 20% potato infusion), PDB (potato dextrose broth) or even KB (King´s B). The cultures were checked for optimal conditions of aeration (stationary cultures to shaking at different rpm from 100 to 250), incubation times (for at least 1 to maxi-

mum 7 days) and temperature (from 20 to 40°C). After culturing the bacterium at optimized condition, the whole culture as the main source of secretory metabolites was centrifuged at 8,000 x *g* for 30 min at room temperature. The cell free culture filtrate was then sterilized by filtration through a 0.22-µm-pore-size Millipore membrane (Millex-GV; Millipore) and kept at -20°C before use. The heat stability of the inhibitory metabolites can be examined by incubating the bacterial culture filtrate at 60, 80 and 100°C for 120 min or autoclaving at 121°C for 15 min. The acid and alkaline stabilities of the inhibitory metabolites can be checked by changing the pH of the culture medium to 1.5 and 11 by adding 1 M HCl or 1 M NaOH and incubating the solution at room temperature for 3 h.

4.2. Purification of antifungal metabolites

Consecutive steps of purification of bioactive metabolites from bacterial culture filtrate are summarized in Fig. 5. As the first step, the inhibitory bacterium should be cultured at optimized culture conditions from section 4.1. The next steps are Ion exchange column chromatography on Diaion HP20 resin, preparative thin layer chromatography on silica gel $60F_{254}$ and finally HPLC purification of bioactive metabolites.

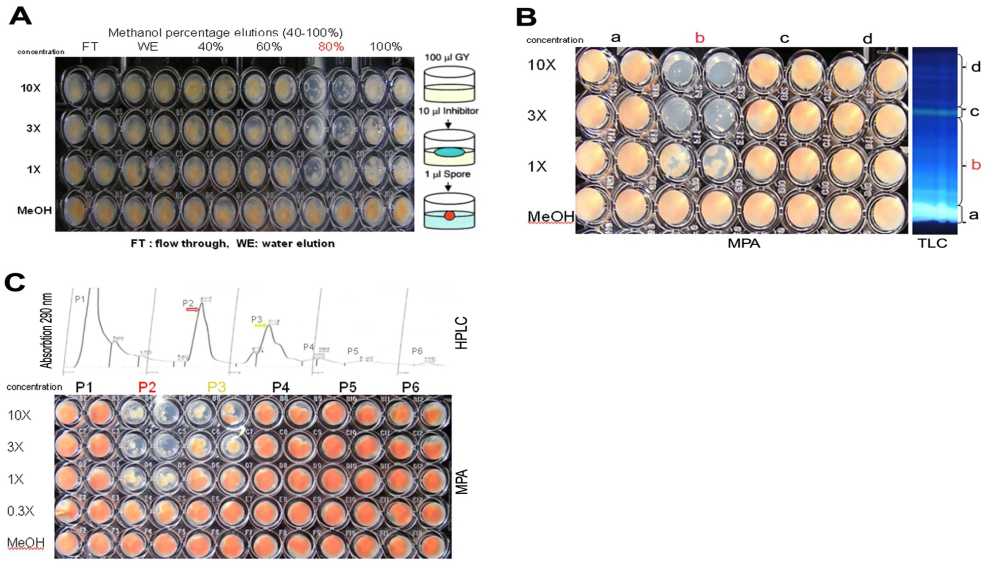

Figure 5. Sequential steps of purification of *A. parasiticus* growth inhibitory metabolites from bacterial culture filtrate:A) Stepwise elution of culture broth from a Diaion HP20 resin column using 40-100% aqueous MeOH. Fungal growth inhibition was reported for only 80% MeOH elution in microtiter agar plate assay (MPA).B) Further purification of fungal growth inhibitory metabolites from active Diaion HP20 column fraction (80% MeOH from step A) by thin layer chromatography (TLC). According to MPA result, section "b" was scrapped from TLC gel contained inhibitory compounds and thus, it was selected for further study.C) Final purification of inhibitory metabolites from section "b" of TLC in step B by normal-phase HPLC. Among 6 separated peaks shown (P1 to P6), two peaks i.e. P2 and P3 showed fungal growth inhibition in MPA.

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4.2.1. Metabolite production at pre-optimized culture conditions

The selected bacterium with strongest antifungal activity was cultured in 1000 ml capacity flasks contained 250 ml GY as selected medium from section 4.1. The cultures were incubated at pre-optimized conditions (28°C for 5 days with shaking at 120 rpm). The whole culture (2 liters totally) was then centrifuged at 8,000 × g at room temperature for 30 min. The supernatant was used for purification of the inhibitory metabolites.

4.2.2. Ion exchange column chromatography

A glass column (2.5 × 60.0 cm) was equilibrated with MeOH. Five hundred grams of Diaion HP20 resin was suspended in MeOH and then packed onto the glass column. After removing of MeOH, the column was equilibrated with distilled water. The culture broth of selected bacterium (500 ml) was loaded onto the column. The resin was washed with 3 liters of distilled water, and the substances bound to the resin were then stepwise eluted by using 2 liters each of 40, 60, 80, and 100% methanol (MeOH) in water. Each elution was concentrated to dryness with a rotary evaporator and dissolves in desirable amounts of 100% MeOH. The 80% MeOH fraction which showed the highest growth and/or AF inhibitory activity against NA-mutant of *A. parasiticus* NRRL 2999 in microtiter agar plate assay (MPA), was selected for further purification (Fig. 5A).

4.2.3. Preparative thin layer chromatography

The 80% MeOH fraction from section 4.2.2 (an approximate of 250 mg dry weight) was applied to Silica gel $60F_{254}$ TLC plate and then developed with a mixture of chloroform/methanol/water (65:25:4, v/v/v) as mobile phase. Total area developed on the TLC plate was divided into at least 5 regions under 365 nm UV light, and the silica gel was scraped separately from each region. The substances presented in the silica gel were extracted with tenfold amounts of 100% MeOH. Each fraction was concentrated to dryness, dissolves in a small amount of MeOH, and subjected to the MPA on 96-well microplates. The fraction "b" (75.6 mg dry weight) which contained the strongest inhibitory activity against fungal growth and/or AF production was selected for further purification (Fig. 5B).

4.2.4. High performance liquid chromatography (HPLC)

The fraction "b" from section 4.2.3 was finally purified by HPLC equipped with a Cosmosil 5C18-AR column (4.6 × 150 mm; 5 μm). After injecting the sample, the column was washed with MeOH/water (50:50, v/v) for 80 min. The flow rate was adjusted at 1.0 ml/min, and elution was monitored at 290 nm wavelength. The number of 6 separated peaks (P1 to P6) were collected from the ODS column as shown in Fig. 5. Based on the MPA results, two peaks i.e. P2 and P3 were able to inhibit fungal growth and pigment production by *A. parasiticus* NRRL 2999 (Fig. 5C). These peaks were selected for further characterization by LC-MS and MALDI-TOF.

4.3. Structural elucidation of antifungal metabolites

With a combination of Liquid chromatography-Mass spectrometry (LC-MS) and Matrix-assisted laser desorption/ionization (MALDI-TOF), we will be able to elucidate the chemical structure of a protein or peptide in a best way. LC-MS spectrum determines retention time and an approximate mass of a purified compound, while complementary MALDI-TOF enable us to explain chemical formula and precise mass of the compound as the final step of identification. LC-MS and MALDI-TOF spectra of purified antifungal are shown in Fig. 6.

4.3.1. Liquid chromatography-Mass spectrometry (LC-MS)

The LC-MS system usually consists of a LC-10Avp separation module equipped with a SPD-M10Avp photodiode array detector and LC-MS2010A single quadruple mass spectrometer with atmospheric pressure photo ionization (APPI) source. The probe can be operated in the positive/negative mode under the condition of defined probe voltage, temperature of 300°C, CDL temperature of 200°C, nabulization gas (N2) flow 2.5 1/min, and scan range 900-1600 m/z (sec/scan). The amount of 2 µl of each inhibitory peak purified from HPLC separation was injected to an Ascentis C18 column (150 mm × 2.1 mm, 5 µm) and washed with MeOH (65% aqueous solution) acidified with 0.1% acetic acid in a flow rate of 0.2 ml/min. The column temperature should be maintained at 40°C during the operation. Approximate mass and retention time of the compound were recorded at the end of analysis.

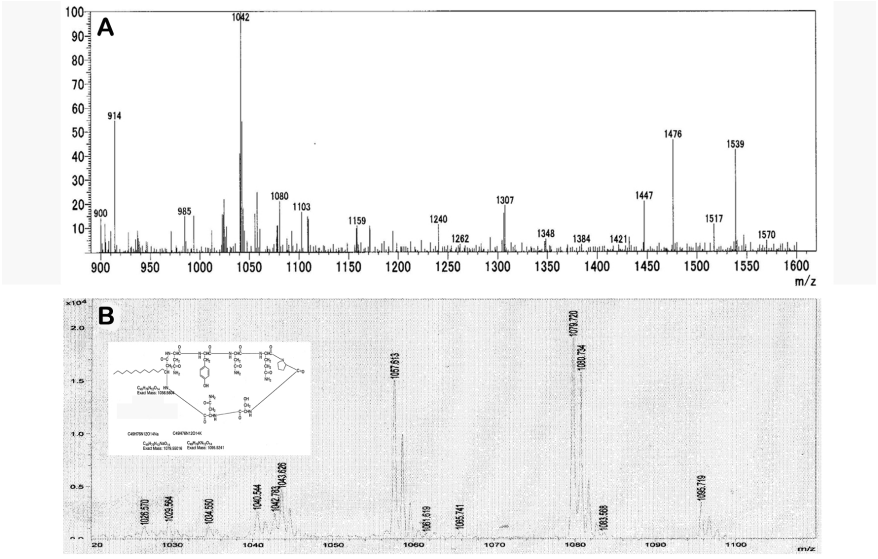

Figure 6. Liquid chromatography-Mass spectrometry (LC-MS) analysis of a HPLC purified inhibitory metabolite for *A. parasiticus* growth shows an approximate retention time of 17.0 min and a mass of 1042.0 m/z (A), while MALDI-TOF data indicates a structural formula of $C_{48}H_{76}N_{12}O_{14}$ and an exact mass of 1042.5447 m/z (B).

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4.3.2. MALDI-TOF

Matrix-assisted laser desorption ionization-time of flight spectrometer (MALDI-TOF) is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules (polymers, dendrimers and other macromolecules), which tend to be fragile and fragment when ionized by more conventional ionization methods. The MALDI-TOF is a two step process. First, desorption is triggered by a UV laser beam. Matrix material heavily absorbs UV laser light, leading to the ablation of upper layer of the matrix material. The second step is ionization which takes place in the hot plume. Aside from peptide mass fingerprinting and useful application in identifying of microorganisms such as bacteria and fungi, MALDI-TOF is used for the rapid identification of proteins isolated by using gel electrophoresis: SDS-PAGE, size exclusion chromatography, affinity chromatography, strong/weak ion exchange, isotope coded protein labeling (ICPL), and two-dimensional gel electrophoresis. MALDI-TOF analysis of inhibitory compounds with defined retention time and an approximate mass from LC-MS step reveals valuable data about chemical formula and exact mass and provides finally identification of the absolute configuration of the purified inhibitory bacterial metabolite (Fig. 6).

5. Concluding remarks and future prospective

AF contamination of food and feed remains a major risk for human and animal health all over the world. Despite the long history of our knowledge about AF, little has been documented on how we can virtually combat the global distress of AF contamination of crops and agricultural commodities. AF-producing fungi can infect grains from pre-harvest conditions in the field through to post-harvest stages in the stores. Several pre- and post-harvest strategies have being tested to reduce risk of AF contamination. One of the management strategies being developed is biological control using various antagonistic microorganisms such as fungi, bacteria, and actinomycetes by a competitive exclusion mechanism. Biological control in conjunction with other management practices has potential to dramatically reduce AF contamination. Natural population of *A. flavus* consists of toxigenic strains that produce considerable amount of AF and atoxigenic strains that lack the capacity to produce AF. Nowadays, introducing atoxigenic strains has been successfully used to compete and exclude toxigenic strains in the field thereby reducing AF production in contaminated crops. However, there are some important limitations from the type of vegetative compatibility groups which shows the progeny of the fungus for AF-producing ability to geographic limitations in selection of atoxigenic strains. Considerable tolerance of *B. subtilis* and *P. chlororaphis* to environmental stresses, their large capacity for producing diverse array of beneficial antifungal metabolites and their readily producing by current fermentation technology make them promising tools for biocontrol of aflatoxigenic fungi in practice. Bacterial population from the genera *Bacillus* and *Pseudomonas* identified in pistachio, maize and peanut fields in the present study with potent antagonistic activity against aflatoxigenic *Aspergillus parasiticus* can potentially be developed into new biocontrol agents for combating AF contamination of crops in the field. These bacteria must be evaluated for a set of selection criteria for further use in biocontrol field experiments. Inabili-

ty to produce toxic substances for biological systems and propensity to multiply, colonize and survive are the most important selection criteria to make sure that the selected antagonistic bacterial strains are safe and applicable when they introduced in to the environment. This endeavor shows biological control holds promise of offering a long-term solution for colonizing crops with aflatoxigenic fungi and thereby reducing AF contamination in the field.

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