Inhibition of fungal growth and mycotoxins formation of selected toxigenic fungi by different bacterial strains

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ABSTRACT

Four bacterial strains (*Bacillus amyloliquefaciens* BICS 08b, *B. subtilis* AUMC 63, *Lactococcus lactis* BICS 081b and *Pseudomonas fluorescence* BICS 0149b) were tested for their ability to inhibit both growth and mycotoxins production by different 11 toxigenic fungi; five local isolates from different Egyptian food sources and six standard strains obtained from CBS, Central Bureau voor Schimmelcultures, Holland. *Pseudomonas fluorescence* BICS 0149b completely inhibited growth of nine fungi and completely inhibited toxin production by the 11 tested fungi. *Bacillus subtilis* AUMC 63 reduced fungal growth of the 11 fungal isolates under study by 40-100%, and reduced their toxins formation by 20- 100%. *Lactococcus lactis* BICS 081b highly reduced both fungal growth and mycotoxins production by 76-100% and 60-100%, respectively. *Bacillus amyloliquefaciens* BICS 08b completely inhibited toxins formation by almost fungi while it was not highly affected their growth. From this result, some bacterial strains may be successfully used as a biological control agent of food contamination with molds and mycotoxins

Key words: toxigenic fungi, mycotoxins, antagonistic bacteria.

INTRODUCTION

Fungi are able to grow on different kinds of food: cereals, meat, milk, fruit, vegetables, nuts, fats and their products. The fungal growth may result in several kinds of food-spoilage, off-flavors, toxins, discoloration, rotting and formation of pathogenic or allergenic propagules (**Chelkowski, 1991; Tipples, 1995; Filtenborg *et al*., 1996).** If the contaminating fungi are pathogens could also cause human illness. Toxigenic fungi, on the other hand, under favorable conditions could produce mycotoxin in foods.

Mycotoxin is produced during the growth of fungi and can be found out or in the hyphae and spores of these organisms (**Zucchi and Melo, 2009; Köppen *et al*., 2010**). If ingested, mycotoxins may cause acute or chronic disease episodes, termed mycotoxicosis (**Köppen *et al*., 2010**; **Medeiros *et al*., 2012**). Mycotoxin long-term exposure has also been related to several mycotoxicosis, such as carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic neurotoxic and immunosuppressive (**Richard, 2007; Medeiros *et al*., 2012**).

Several strategies have been applied to eliminate mold growth and mycotoxins production or to remove/ destroy the preformed toxins from grains and foodstuffs (**Bullerman *et al*., 1984; Angelo, 2000**). The ideal decontamination procedure should be easy to use, inexpensive and should not lead to the formation of compounds that are still toxic or can alter the nutritional and palatability properties of the food. Chemical antifungal agents have long been used to control fungi in foods (**Bullerman *et al*., 1984; Bullerman, 2000**). In some situations, the prolonged use or overuse of these chemicals has led to the development of fungal resistance. Also, the levels of chemicals used in the food industry are fungistatic and do not kill or completely inhibit growth of an indefinite time **(Bullerman, 2000**). However, increasing consumer concerns about chemicals in foods has led to interest in novel preservation methods. Currently the global trend is turned to safer and eco-friendly alternative approaches (**Mari *et al*., 2007; Sharma *et al*., 2009**). It has been reported that antagonistic microorganisms or their antimicrobial metabolites have some potential as natural bio preservatives to control undesirable fungi.

Several bacterial species such as *Bacillus subtilis*, *Lactobacilli* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp. have shown the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus* spp. in laboratory experiments. **Palumbo *et al*. (2006)** reported that a number of *Bacillus*, *Pseudomonas*, *Ralstonia* and *Burkholderia* strains isolated from California almond samples could completely inhibit *A. flavus* growth. Several strains of *B. subtilis* and *P. solanacearum* isolated from the non-rhizophere of maize soil were also able to inhibit aflatoxin accumulation (**Nesci *et al*., 2005**). **Nanis (2012) and Chelkowski (1989)** demonstrated that some lactic acid bacteria have promising ability as natural food-grade bio control agents of mold growth and mycotoxin production.

The present investigation was aimed to evaluate the potential of four bacterial strains (*Bacillus amyloliquefaciens* BICS 08b, *B. subtilis* AUMC 63, *Lactococcus lactis* BICS 081b and *Pseudomonas fluorescence* BICS 0149b) for bio-control of the fungal growth and toxin production by 11 toxigenic fungi.

MATERIALS AND METHODS

Selection of toxigenic fungi

A total of 11 toxigenic fungal isolates were selected for studying the biocontrol activities of four bacterial strains on their growth and toxins formation. The toxigenic selected fungal isolates were 5 isolates from different food sources in Sohag Governorate, Egypt and recorded as highly toxin producers (local isolates), these isolates were *Aspergillus flavus* 30 (Aflatoxin B_1, B_2, G_1 and G_2 producer), *A. ochraceus* 76 (Ochratoxins A, B), *Aspergillus nidulans* 69 (Sterigmatocystin), *Penicillium digitatum* 131 (Patulin) and *Alternaria alternata* 5 (Alternariol). Other six highly toxigenic fungal isolates were purchased from CBS (Centraal Bureau voor Schimmelcultures), Fungal Biodiversity Center of Holland and used as a standard isolates. These isolates were *Aspergillus parasiticus* CBS 571.65 (Aflatoxin B_1, B_2, G_1 and G_2), *A. ochraceus* CBS 589.68 (Ochratoxin A), *Penicillium griseofulvum* CBS 589.68 (Patulin), *P. scabrosum* CBS 530.97 (Fumagillin), *Fusarium equiseti* CBS 406.86 (Zearalenone) and *Phaeosphaeria nodorum* CBS 438.87 (Alternariol).

Collection of antagonistic bacteria

Three bacterial strains were obtained from the Identification and Biological Control Unit of Agriculture Research Center (ARC), Cairo, Egypt. These isolates were *Bacillus amyloliquefaciens* BICS 08b, *Lactococcus lactis* BICS 081b and *Pseudomonas fluorescence* BICS 0149b. Other one bacterial strain obtained from Assuit University Mycological Center (AUMC); this isolate was *Bacillus subtilis* AUMC 63.

The effect of antagonistic bacterial strains on growth of toxigenic fungal isolates

For testing antimicrobial activity of bacteria, potato- dextrose agar (PDA) medium was used as follow: 2ml suspension of antagonistic bacteria were surface plated aseptically into each of 55 sterile Petri-dishes for each bacterial strain and 15 -20 ml of PDA medium cooled to just above the solidifying temperature were added to each dish. The dishes were rotated by hand in abroad swirling motion so that the bacteria were dispersed in the agar medium. The 11 toxigenic fungi were, individually, inoculated at the center of each dish (5 dishes for each fungal isolate under study from each bacterial culture). Growth of each fungus was measured after 7 days of incubation at 28 ± 2°C. Fungal growth without bacterial inoculum was used as control (**Gheorghe *et al.*, 2008**).

Testing the effect of bacterial strains on mycotoxins formation

In order to determine the effect of bacterial strains on mycotoxin formation, the previous four bacterial strains were tested using potato - dextrose liquid medium. Erlenmeyer flasks of 250 ml capacity were used. Each flask contained 50 ml medium. The flasks were sterilized at 121°C for 20 minutes and inoculated after cooling with 2 ml of the toxigenic fungal inoculum suspension + 2ml of the antagonistic bacterial inoculum suspension. The cultures were incubated at 28 ± 2°C as static cultivation for 10 days. At the end of incubation period, the content of each flask (medium + toxigenic fungi + antagonistic bacteria) were homogenized for five minutes in a high speed blender (16000 rpm) with 100 ml chloroform. The extraction procedure was repeated three times. The combined chloroform extracts were washed with equal volume of distilled water, dried over anhydrous sodium sulphate, filtered then concentrated to near dryness. The antagonistic effect of bacterial strains on toxins formation was determined for each toxigenic

fungal isolates under study as previously described by **Gheorghe *et al*. (2008)** with some modification and compared it with the control. Mycotoxin levels were detected using thin layer chromatography (**Scott *et al*., 1970; Gimeno, 1979; El-kady and Moubasher, 1982**).

RESULTS AND DISCUSSION

The antifungal activity of four bacterial strains on the growth and toxins production by the 11 toxigenic fungal strains was examined and the results recorded in **Tables (1- 4**). The results revealed that *Pseudomonas fluorescence* BICS 0149b completely inhibited the growth of nine toxigenic fungal isolates and reduced the growth of *A. parasiticus* CBS 571.65 and *A. flavus* 30 by 82% and 68%, respectively. Furthermore, bacterial strain completely inhibited toxin production by all the 11 tested fungal strains. **Moshtaq *et al*. (2010)** revealed that *Pseudomonas fluorescence* showed highest antifungal activity against *Penicillium italicum* (94%) and *Pseudomonas malophilia* reduced colony diameter of *Aspergillus flavus*, *Penicillium italicum* and *Penicillium simplicissmum* up to 88-98%.

Bacillus amyloliquefaciens BICS 08b completely inhibited the growth of standard *Phaeosphaeria nodorum* CBS 438.87 and reduced the growth of the other five standard toxigenic fungi by 13- 60%. Also, this bacterial strain inhibited the growth of the five local toxigenic strains by 27- 68%. This bacterial strain completely inhibited the production of aflatoxin, alternariol, fumagillin, sterigmatocystin and zearalenone, On the other hand. ochratoxin A was reduced by 60% and patulin production was inhibited by 40- 80% by this bacterial strain. **Arrebola *et al*. (2010)** reported that *B. amyloliquefaciens* is an efficient antagonist to inhibit the mycelial growth of *B. cinerea*, *P. expansum* and *R. stolonifer*. **Bluma and Etcheverry (2006)** reported a significantly reduction in aflatoxin B_1 when *A. flavus* grew with *B. amyloliquefaciens*. *B. amyloliquefaciens* was shown to effectively reduce *F. verticillioides* propagules and fumonisin content in maize kernels at harvest when applied as seed coating (**Pereira *et al*., 2007**).

Bacillus subtilis AUMC 63 completely inhibited the growth of *F. equiseti* CBS 406.86, *P. digitatum* 131 and *Phaeosphaeria nodorum* CBS 438.87 and reduced their toxins by 80 or 100%. The growth of the other eight toxigenic fungi was reduced by 40- 94%, while their toxins formation was reduced by 20- 80%. *Bacillus subtilis* is known to produce a number of antifungal compounds inhibit the growth of a large number of fungi, such as *Aspergillus*, *Penicillium* and *Fusarium* species (**Zuber *et al*., 1993; Munimbazi and Bullerman, 1998**). **Samuel *et al*. (2011)** indicated that two *Bacillus* strains can potentially be utilized to detoxify zearalenone in food and feeds. *Bacillus subtilis* showed promise for reducing mycotoxin contamination by *F. verticillioides* (**Bacon *et al*., 2001**). Several studies suggest the use of bacteria such as *Streptococcus*, *Lactobacillus* and *Bacillus* as sources of carboxypeptidase A, the main cause of ochratoxin A degradation (**Fuchs *et al*., 2008**).

Lactococcus lactis BICS 081b completely inhibited the growth and toxins formation of the standard toxigenic fungal strains except *P. griseofulvum* CBS 315.63 (their growth was inhibited by 91% and their toxin was reduced by 80%) Also, this bacterial strain completely inhibited the growth and toxin formation of the local strain of *P. digitatum* 131 in addition to inhibition the growth of the other four local toxigenic strains by 76- 94% and reduced their toxins formation by 60- 100%. **Florianowicz (2001)** reported the ability of *Lactococcus cremoris* to control

mycotoxinogenic mould growth**. Fuchs *et al.* (2008)** indicated that some lactic acid bacteria were able to reduce rot caused by *Penicillium expansum* by 10-50%. **El- Nezami *et al.* (1998) and Peltonen *et al.* (2001)** reported that *L. rhamnosus* strains removed 54.6 and 80% of aflatoxin B_1, respectively. **Haskard *et al.* (2001)** reported that viable cells of *L. lactis* and *L. casei* remove 59 and 21.8% of aflatoxin B_1, respectively. **Gomah and Zohri (2014)** indicated the ability of two species of lactic acid bacteria to reduce both growth and mycotoxins production by three species of *Fusarium*.

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Table (1): The inhibitory effect (%) of some bacterial strains on growth of some standard toxigenic fungal strains (growth diameter measured by cm after 7days of incubation on PDA medium at 28 ºC).

Table (2): The inhibitory effect (%) of some bacterial strains on growth of some local toxigenic fungal strains (growth diameter

Toxigenic fungal strains / **Bacterial strains**	***A. parasiticus* CBS 571.65 (aflatoxin producer)**		***A. ochraceus* CBS 589.68 (ochratoxin producer)**		***F. equiseti* CBS 406.86 (zearalenone producer)**		***P. griseofulvum* CBS 315.63 (patulin producer)**		***P.scabrosum* CBS 530.97 (fumagillin producer)**		***Phaeosphaeria nodorum* CBS 438.87 (alternariol producer)**	
	Fungal growth (cm)	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**
Control	**5.5**	**0**	**4.0**	**0**	**6.0**	**0**	**2.3**	**0**	**0.5**	**0**	**2.3**	**0**
***Bacillus amyloliquefaciens* BICS 08b**	3.5	36	2.5	37	2.6	57	2.0	13	0.2	60	0	100
***Bacillus subtilis* AUMC 63**	0.9	83	2	50	0	100	1.0	56	0.1	80	0	100
***Lactococcus lactis* BICS 081b**	0	100	0	100	0	100	0.2	91	0	100	0	100
***Pseudomonas fluorescence* BICS 0149b**	1.0	82	0	100	0	100	0	100	0	100	0	100

measured by cm after 7days of incubation on PDA medium at 28 ºC.

Toxigenic fungal Isolates / **Bacterial isolates**	***Alternaria alternata* 5 (alternariol producer)**		***A. flavus* 30 (aflatoxin producer)**		***A. nidulans* 69 (sterigmatocystin producer)**		***A. ochraceus* 76 (ochratoxin producer)**		***P. digitatum* 131 (patulin producer)**	
	Fungal growth (cm)	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**	**Fungal growth (cm)**	**Inhibition %**
Control	**5.0**	**0**	**5.5**	**0**	**5.0**	**0**	**3.5**	**0**	**2.5**	**0**
***Bacillus amyloliquefaciens* BICS 08b**	1.9	62	4.0	27	3.0	40	2.4	31	0.8	68
***Bacillus subtilis* AUMC 63**	0.2	96	2.0	63	3.0	40	0.2	94	0	100
***Lactococcus lactis* BICS 081b**	0.7	86	0.4	92	1.2	76	0.2	94	0	100
***Pseudomonas fluorescence* BICS 0149b**	0	100	1.6	68	0	100	0	100	0	100

Table (3): The inhibitory effect (%) of some bacterial strains on mycotoxins formation by standard toxigenic fungal strains grown on potato dextrose broth at 28 ºC for 10 days.

Toxigenic fungal strains / Bacterial strains	*A. parasiticus* CBS 571.65 (aflatoxin producer)		*A. ochraceus* CBS 589.68 (ochratoxin producer)		*F. equeseti* CBS 406.86 (zearalenone producer)		*P. griseofulvum* CBS 315.63 (patulin producer)		*P.scabrosum* CBS 530.97 (fumagillin producer)		*Phaeosphaeria nodorum* CBS 438.87 (alternariol producer)	
	Toxin level	Inhibition of toxin production %	Toxin level	Inhibition of toxin production %	Toxin level	Inhibition of toxin production %	Toxin level	Inhibition of toxin production %	Toxin level	Inhibition of toxin production %	Toxin level	Inhibition of toxin production %
Control	**+5**	**0**	**+5**	**0**	**+5**	**0**	**+5**	**0**	**+5**	**0**	**+5**	**0**
***Bacillus amyloliquefaciens* BICS 08b**	0	100	+2	60	0	100	+3	40	0	100	0	100
***Bacillus subtilis* AUMC 63**	+1	80	+3	40	0	100	+2	60	+1	80	+1	80
***Lactococcus lactis* BICS 081b**	0	100	+1	80	0	100	+1	80	0	100	0	100
***Pseudomonas fluorescence* BICS 0149b**	0	100	0	100	0	100	0	100	0	100	0	100

Table (4): The inhibitory effect (%) of some bacterial strains on mycotoxins formation by local toxigenic fungal strains grown on potato- dextrose broth at 28 ºC for 10 da

Toxigenic fungal strains / Bacterial strains	*Alternaria alternata* 5 (alternariol producer)		*A. flavus* 30 (aflatoxin producer)		*A. nidulans* 69 (sterigmatocystin producer)		*A. ochraceus* 76 (ochratoxin producer)		*P. digitatum*131 (patulin producer)	
	Toxin level	**Inhibition of toxin production %**	**Toxin level**	**Inhibition of toxin production %**	**Toxin level**	**Inhibition of toxin production %**	**Toxin level**	**Inhibition of toxin production %**	**Toxin level**	**Inhibition of toxin production %**
Control	**+5**	**0**	**+5**	**0**	**+5**	**0**	**+5**	**0**	**+5**	**0**
***Bacillus amyloliquefaciens* BICS 08b**	0	100	0	100	0	100	+2	60	+1	80
***Bacillus subtilis* AUMC 63**	+1	80	+3	40	+4	20	+2	60	+1	80
***Lactococcus lactis* BICS 081b**	0	100	+1	80	+2	60	0	100	0	100
***Pseudomonas fluorescence* BICS 0149b**	0	100	0	100	0	100	0	100	0	100