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Full Length Article

Improvement of Antagonistic Activity of *Bacillus megaterium* MHT6 against *Fusarium moniliforme* using He-Ne Laser Irradiation

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Abstract

Corn ear rot has been the most important disease of the world. It is mainly caused by *Fusarium moniliforme* which produce a number of mycotoxins including Fumonisin B1 (FB1). Aiming at supplying a potentially effective biological control agent against *F. moniliforme, Bacillus megaterium* MHT6 isolated from China, was tested for its antagonistic activity against *F. moniliforme*. Moreover, both antifungal activity and the active compound of strain MHT6 were tested and identified. In order to improve its antifungal activity, a random mutagenesis generated using He-Ne laser irradiation was applied on *B. megaterium* MHT6. After the mutation, one hundred bacterial colonies were obtained and their effects against *F. moniliforme* were assessed in a plate assay. Eight colonies (8%) exhibited higher inhibition activity as compared to the wild type MHT6. Among them, the mutant MHT 88 which showed the strongest antagonistic ability was selected out. Through bacterial motility assays, the mutant MHT88 showed stronger swarming motility than wild type MHT6. According to FPLC and MALDI-TOF-MS, the production of two main antagonistic lipopeptides such as surfactin and fengycin increased in mutant MHT88. Especially the surfactin, which can't be detected in the wild strain MHT6, was obviously observed in mutant. Moreover, the cell-free supernatant of the mutant MHT88 showed stronger capability in degrading FB1 compared to MHT6. This study provided a promising biological agent to inhibit *F. moniliforme* and suppress corn ear rot disease. © 2015 Friends Science Publishers

Keywords: Biological control; *Bacillus megaterium*; *Fusarium moniliforme*; Surfactin; Fengycin; Fumonisin B1 (FB1)

Introduction

Corn is an important cultivated plant in China and around the world. Corn ear rot caused by *Fusarium moniliforme* is a destructive disease for the decreasing yield of corn (Bezuidenhout *et al.*, 1988; Lazzaro *et al.*, 2015). Moreover, the fungus produces a number of mycotoxins including Fumonisin B1(FB1) during the process of growth and invasion of grains (Lizárraga-Sánchez *et al.*, 2015). The FB1, as one of the mainly produced mycotoxins by *F. moniliforme*, has not only caused the significant economic losses but also been associated with high incidences of oesophageal and liver cancer in several areas in China (Chen *et al*., 2015).

In nature, the FB1-producing mold *F. moniliforme* share the same habitat with other microorganisms (Lizárraga-Sánchez *et al*., 2015), such as *Bacillus substilis*, *B. megaterium*, *B. pumilus* etc., which can inhibit FB1 production. This inhibition may result from many factors including competition for space and nutrients and the antitoxin metabolites in general (De Melo *et al*., 2015).

At present, corn ear rot is usually controlled by treating ears with fungicide (De Curtis *et al*., 2011). However, due to the development of resistance of pathogenic fungus and the hazardous effects on public health and environment, many countries have restricted the use of chemical fungicides and pesticides (Rahman, 2013; Cai *et al*., 2015). Therefore, considerable attention has been paid to applying biological control methods as a suitable alternative to the use of chemical fungicides (Feliziani *et al*., 2013).

As potential biological agent, the bacterium *B. substilis* shows great promise in alleviating problems with plant pathogens (Romero *et al*., 2007; McCormick, 2013). *B. substilis* has been found to produce antibiotic compounds such as lipopeptides including fengycin, iturin and surfactin families (Alvarez *et al.*, 2012). Especially the lipopeptides of fengycin have displayed strong antifungal activity against a wide range of plant pathogens fungus (Pérez-García *et al.*, 2011; Yánez-Mendizábal *et al.*, 2012; Falardeau *et al*., 2013). Previous research has reported the fengycin family of polypeptides produced by *Bacillus* sp. could inhibit the

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FB1-producing fungi (Bacon *et al*., 2012; McCormick, 2013; Yuan *et al*., 2013). It can inhibit growth of *F. moniliforme* and reduce the FB1 production by cutting the transcription of gene FUM8, which is the key gene for FB1 production (Falardeau *et al.*, 2013). Zhang *et al.* (2014) indicated that stimulative effects of low intensity He-Ne laser irradiation could enhance the cell-cycle progression. He-Ne laser irradiation, as a new mutagenesis technology, with the advantage on high mutation rate and mutant capacity, has attracted considerable interest in microorganism mutation breeding.

B. megaterium, can produce antimicrobial peptides, showing antagonistic activity against much more pathogens. The objective of these studies was to test the antagonistic activity of *B. megaterium* MHT6 against *F. moniliforme* and identify its main antifungal active compound. In order to improve its antagonistic activity, the strain was irradiated by He-Ne Laser irradiation. After testing the mutants' antagonistic activity, motility and the ability on degrading FB1, the mutant MHT88 showed a large potential in the biological control against *F. moniliform*e.

Materials and Methods

Pathogenic Fungus and Antagonistic Bacteria

The pathogenic fungus *F. moniliform*e L8, isolated from naturally infected corn ear before, was conserved in microbiological laboratory, Northwest University, China. The pathogen fungus was maintained on potato dextrose agar (PDA) and incubated at 25–28°C. *B. megaterium MHT6*, with the antagonistic activity against many pathogens, has been isolated and conserved in microbiological laboratory, Northwest University, China.

Antagonistic Activity of *B. megaterium* MHT6 cell against *F. moniliforme* L8

The antagonistic ability of MHT6 cell *against F. moniliform*e L8 was tested by using the dual-culture method (Oldenburg *et al*., 1996; Karandashov *et al*., 2000). Under sterile condition, strain MHT6 was inoculated onto the plates at a distance of 2.5 cm from the center, and a 5 mm diameter fungal plug was inserted in 9 cm PDA center of each plate. After 7 days incubation at 30°C, the antagonistic properties were quantified by measuring the radius of pathogen colony towards the antagonist colony. Each colony was performed in triplicate. Taking the plate without inoculating antagonistic isolates as control, the diameter of inhibition zone was measured and data were processed through statistical analysis by ANOVA using SPSS19.0 software (SPSS, Inc., USA).

The antagonistic ability of MHT6 fermentation broth *against F. moniliform*e L8 was tested by culture filtrate test using oxford cup method (Yoshida *et al*., 2001; Xu *et al*., 2013). The MHT6 was inoculated in 250mL nutrient broth at 37°C for 72 h. After centrifuging the culture at 10,000 g for 10 min, supernatant was collected and filtered through a 0.22 μm cellulose nitrate filters under sterile conditions. The filtrates were poured into sterilized PDA in 20% (v/v) concentration; 20 mL of the mixed agar was poured into Petri plate and solidify. A 5 mm diameter of fungal plug was inoculated in the center of each plate and incubated for 7 days at 30°C. Using PDA without filtrate as control, the diameter of the mycelial growth of *F. moniliforme* was measured. Each colony was performed in triplicate. Data analysis was performed by ANOVA using SPASS19.0 software (SPSS, Inc., USA), the means for each treatment were separated at P=0.05.

Purification and Identification of Antifungal Active Compound in MHT6

Thermo stability and other properties of the antifungal compound: 1.5 mL of crude supernatant was displaced in Eppendorf-sterile-tubes. The tubes were processed following three treatments: (a) Maintained at room temperature for 20 min; (b) heating in water bath at 50ºC for 20 min; (c) 120ºC heating 20 min. 100 μL of each treatment was separately tested against *F. moniliforme* L8 for inhibitory activity. Meanwhile, we also detected the tolerance of MHT6's antifungal compound on proteinase K, strongly acidic (pH < 5) and alkaline (pH > 11) conditions.

Purification of antifungal active compound in MHT6 by FPLC: Cell-free supernatants of MHT6 were mainly extracted following the method described by (Chitarra *et al*., 2003). Generally speaking, strain was grown in NB medium for 3 days at 30°C with shaking at 150 rpm. Cell-free supernatants were obtained by centrifugation at 10,000 g for 20 min at 4°C. After adjusting the pH to 2 with 6 M hydrochloric acid (HCl), the supernatant was kept overnight at 4°C and centrifuged at 10,000 g for 20 min at 4°C. The precipitate of the cell-free supernatant was collected and dissolved in 50% methanol. The presence of antifungal compounds in the extraction prepared from strain MHT6 was analyzed by FPLC as described previously (Smyth *et al*., 2014).

A 10 μL aliquot of the extract fraction was loaded onto a SOURCE™ 5RPC ST 4.6/150 column and separated by FPLC with an AKTA Purifier (GE Healthcare, Uppsala, Sweden). The products were eluted by solvent A and solvent B with a linear gradient of 20–100% acetonitrile and 0.065% trifluoroacetic acid (TFA), flow rate was 1 mL/min, elution time was over 60 min. Solvent A was 20% acetonitrile in 0.065% TFA (V/V), solvent B was 80% acetonitrile containing 0.05% TFA (V/V). The main peak clusters were collected manually. Finally, each peak cluster was concentrated under vacuum using a rotary evaporator. The concentrated compounds were used for the antagonism tests against *F. moniliforme* L8 *in vitro* as described above.

Analysis of major antifungal active compound of MHT6 by MALDI-TOF-MS: The major antifungal active

compounds were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in the Fourth Military Medical University, China. MALDI-TOF mass spectra were recorded using a Bruker Reflex. MALDI-TOF instrument (Bruker Daltonik, German) containing a 337 nm nitrogen laser for desorption and ionization. For mass spectrometric analysis of crude lipopeptides, 1 to 2 μL samples of fractions were mixed with an equal volume of matrix medium [a saturated solution of α-cyano-4-hydroxycinnamic acid in 70% aqueous acetonitrile containing 0.1% (vol/vol) TFA]. Positive-ion detection and the reflector mode were used. The acceleration and reflector voltages were 16 and 20 kV. Post source decay mass spectra were obtained using the same samples. The identity of the pure homolog was obtained by comparing the *MS.* (deduced from the m/z ratio of [M+H]+ions or [M+Na]+ions detected in electro spray ionization mass spectrometry) with data reported in the literature (Athukorala *et al.,* 2009; Pathak and Keharia, 2013; Yang *et al.,* 2015).

Screening for Mutant

He-Ne laser radiation: Culture activation: The antagonistic strain MHT6 was incubated for 30 h. The culture was washed with 5 mL of sterilized saline water. The rinse solution was placed into a conical flask with glass pearls and then shaken on a Cyclotron oscillator for 30 min. The OD600 value was adjusted to 0.986 by using a photoelectric Turbid meter.

He-Ne laser irradiation: Under sterile conditions, 2 mL of the suspension was placed into test tubes. After adjusting the He–Ne laser (λ=632 nm, 2.5 mm of light spot diameter, 9 mW of output power, and 25 cm of irradiation distance), the suspension was irradiated for15 min using the He–Ne laser generator of Northwest University Photo electricity Factory. The suspension without irradiation was used as a negative control. Each irradiating time was repeated three times. The experiments were performed in triplicate.

Mutant strain cultivation: After irradiation, the suspension was serially diluted (10^{-5}, 10^{-6} and 10^{-7}). Then, 0.2 mL of each diluted suspension was spread onto NA plates. Each diluted suspension was spread on three plates. After incubation at 37°C for 20 h, the mutants were counted by the viable colonies, and their colonial morphology was observed. Finally, the survival rate and the positive mutation rate were calculated.

Antagonistic activity of mutants' cell against *F. moniliform*e L8: *Antagonistic activity of* 100 selected mutants cells against *F. moniliform*e L8 was tested and evaluated by using dual-culture method (Oldenburg *et al.*, 1996; Karandashov *et al.*, 2000). The diameter of inhibition zone was measured. Each colony was performed in triplicate. Data analysis was performed by ANOVA using SPASS19.0 software (SPSS, Inc., USA) at P=0.05

Antagonistic activity of mutants' fermentation broth against *F. moniliform*e L8: Antifungal activities of cell-free supernatants in eight selected mutants (M36, M56, M88, M68, M78, M80, M86, M67) were tested by culture filtrate test using oxford cup method (Yoshida *et al.,* 2001; Xu *et al*., 2013). The diameter of inhibition zone was measured. Each colony was performed in triplicate. Data analysis was performed by ANOVA using SPASS19.0 software (SPSS, Inc., USA) at P=0.05

Bacterial Motility Assays

According to method described by (Bindel Connelly *et al.*, 2004), swimming and swarming motilities of wild strain and mutants were analyzed. Both mutants and the wild strain MHT6 were respectively inoculated on LB plates containing 0.3% and 0.7% agar for 18 h of incubating at 37°C.

Purification and Identification of Antifungal Active Compound in Mutant MHT88

The method of purification and identification of antifungal active compound in mutant MHT88 was as same as the method of MHT6 which described above.

Effect of Strain MHT6 and Mutant MHT88 on Degradation of Fumonisin B1

Cell free supernatants of MHT6 and mutant MHT88 were prepared by centrifuging at 10,000 g for 15 min after growing for 72 h in LB broth at 37°C. The degradation experiment was performed in 2 mL Eppendorf-tubes in a final volume of 1.5 mL of cell free supernatants. A stock solution of 50 ppm of standard FB1 (Sigma Aldrich) was used to supplement cultures to a final concentration of 1.5 ppm. In the control, FB1 was added to LB broth. The mixtures were incubated in the dark at 37°C without shaking for 72 h. The residual FB1 was estimated by measuring optical density at 365 nm in UV spectrophotometer. The experiment was conducted two times in a completely randomized design with three replicates for each treatment. Data analysis was performed by ANOVA utilizing the SPSS 19.0 software at P=0.05. The optical density (OD) values were converted to ppm by plotting the standard curve of different concentrations of FB1 (0.1–10 ppm) against OD365 (Fig. 2).

Biocontrol of Corn Ear Rot Disease in Field Experiment

In corn tasseling stage, *B. megaterium* MHT6 and its mutant MHT88 were grown in LB medium at 37°C for 24 h, then the cells were harvested by centrifugation at 10,000 g, 30 min. After adjusting the bacterial concentration to 10^9 CFU/mL by sterile distilled water, the two strains' both concentrations was respectively diluted to 1% and 0.2%. The bacterial broth of MHT6 and MHT88 with different concentration was separately sprayed onto the corn ears,

taking the distilled water as control. The spray was done for 5 times, with the frequency of 7 days per one time. Control efficacy was calculated as following equation:

Control efficacy = (Infection rate of control-Infection rate of treatment)/Infection rate of control×100%.

Results

Antagonistic Ability of the Cell and Fermentation Broth in *B. megaterium* MHT6 against *F. moniliforme* L8

By dual culture test and culture-filtrate test, antagonistic ability of the cell and fermentation broth in MHT6 against *F. moniliforme* L8 was tested, and the results are shown in Table 1. From the Table, we can see the *B. megaterium* MHT6 could inhibit the growth of *F. moniliforme* L8. Meanwhile, we also found the strain cell showed stronger antagonistic activity than its cell-free supernatant.

Screening of Bacterial Mutants

Following the application of He-Ne laser irradiation, 100 colonies were selected. After testing and evaluating the inhibition effect of 100 selected mutants against *F. moniliform*e L8 by using dual-culture method (Oldenburg *et al*., 1996; Karandashov *et al*., 2000), the diameter of inhibition zone for every mutants was measured. The data were analyzed through ANOVA using SPSS19.0 software (SPSS, Inc., USA) at the 5% level of probability.

As it was shown in Table 1, there are eight mutants (M36, M56, M67, M68, M78, M80, M86, M88) with significant increase in antagonistic activity against *F. moniliforme*, which were selected for culture-filtrate test (Yoshida *et al.*, 2001).

After the statistical analysis (Table 1), the mutants' cells still showed stronger antagonistic activity than their extracellular metabolites (De Melo *et al.*, 2015). Meanwhile, all the mutants showed significant increase in the antagonistic activity compared with wild type MHT 6, which indicated that He-Ne laser irradiation, as an effective mutagenesis method, could increase the fungal inhibition of potential biocontrol agents. Considering the comprehensive evaluation on antagonistic capability, we selected mutant MHT88 for further studies.

Motility Assays of MHT6 and MHT88

In order to test the bacterial motility, the mutant MHT88 and MHT6 were respectively tested on 0.3% and 0.7% agar plates containing LB medium (Bindel Connelly *et al.*, 2004). As it was shown in the results the mutant MHT88 is capable of spreading from the site of inoculation. Bacteria facilitate their growth and survival by forming cooperative and multicellular communities, which enable them to improve swarming motility and colonization competence (Zeriouh *et al.*, 2014).

Table 1: Effect of cell and cell-free supernatants of MHT6 and its mutant MHT88 on inhibiting *F. moniliforme* L8

	Inhibition zone (mm)	
Treatment	Cell	Cell-free supernatant
MHT6	7 ± 0.010^{e}	5.67 ± 0.35^{e}
MHT36	9.37 ± 0.25^{d}	6.58 ± 0.45^{cde}
MHT56	9.67 ± 0.06^{d}	7.65 ± 0.39^{cd}
MHT67	12.36 ± 0.11^{c}	9.88 ± 0.05^{c}
MHT68	12.58 ± 0.46^{bc}	10.38 ± 0.43^{bc}
MHT78	13.68 ± 0.25^{b}	11.32 ± 0.10^{b}
MHT80	13.88 ± 0.08^{b}	12.06 ± 0.35^{ab}
MHT86	14.00 ± 0.20^{ab}	13.14 ± 0.23^{a}
MHT88	15.36 ± 0.36^{a}	13.68 ± 0.24^{a}

Data represent the means ± standard deviation from three replicates.
The experiment was conducted three replicates;
Values followed by the same letter within a column are not significantly different at P=0.05

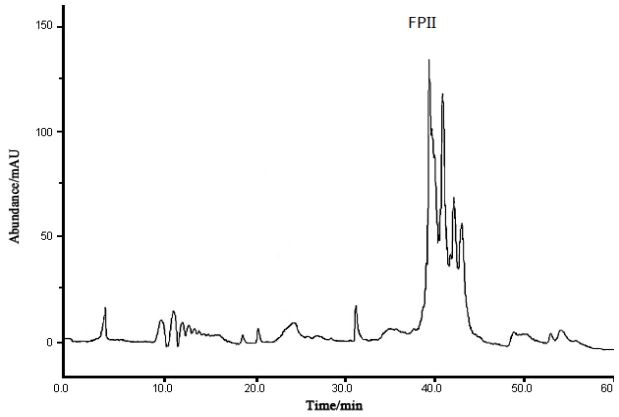

Fig. 1a: FPLC analysis of antifungal production fromMHT6

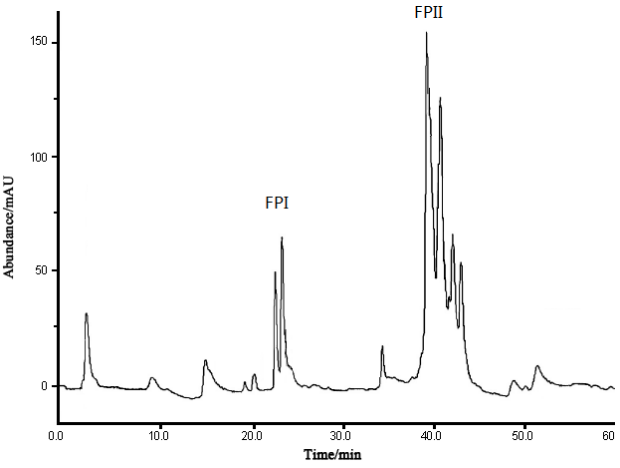

Fig. 1b: FPLC analysis of antifungal production fromMHT88

Besides a genetic control (swr operon) swarming could also be modulated by lipopeptides production (Calvio *et al.*, 2005). Surfactin mutants lost the ability in swarming and spreading on surfaces (Kinsinger *et al*., 2003). Luo reported

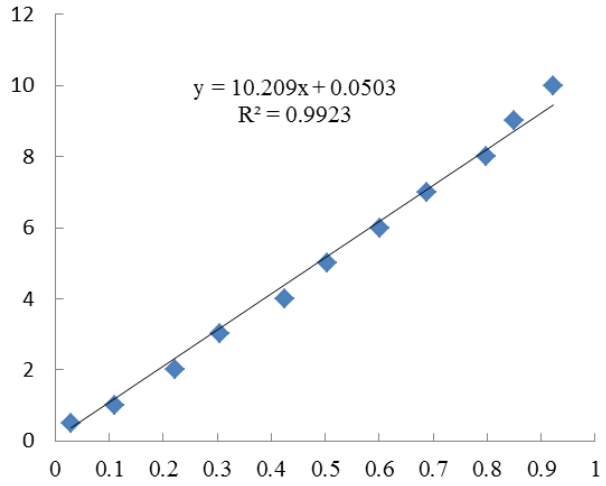

Fig. 2: Standard curve of different concentration of FB 1 against OD_{365}

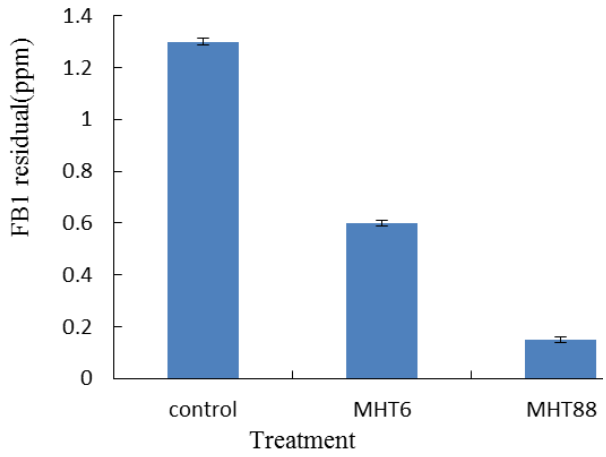

Fig. 3: FB1residual (ppm) in the liquid medium in the presence of supernatant of MHT6 and MHT88 after 72h of incubation at 37 °C. Error bar represent standard deviations of the means from three replications .Control is LB broth containing FB1 without bacterial supernatants

that Bacillomycin L and surfactin contribute synergistically to the phenotypic features of *B. substilis* 916 and the biocontrol of rice sheath blight induced by *Rhizoctonia solani*. Overproduction of the antifungal lipopeptides mycosubtilin and surfactin was directly related to an enhanced spreading on a swarming medium (Luo *et al*., 2015). These lipopeptides are able to reduce surface tension and permit surface spreading. Furthermore, they could be useful in the surface colonization. We supposed that the increased spreading of the mutant MHT88 on the semi-solid agar medium was probably due to increased lipopeptides expression such as the surfactin, which could facilitate faster medium colonization than the wild type in a dual culture assay. This speculation could be identified in the following studies.

Table 2: The surface spreading ability of MHT6 and MHT88 on semi-solid agar plate with 0.3% and 0.7% agar

Treatment	Diameters of bacterial colony (cm)	
	MHT6	MHT88
0.3%	0.37±0.015	2.70±0.025
0.7%	0.10±0.006	2.49±0.036

Table 3: Thermo stability of the antifungal compound separated from supernatant of MHT6 and MHT88

Treatment	Inhibition zone of antibiotic metabolite(mm)	
	MHT6	MHT88
Room temperature (20±2°C), 20 min	4.88±0.17	13.68±0.45
Water bath (50±2°C), 20min	4.88±0.25	13.68±0.13
Autoclaving (120±1°C, 1 atm), 20 min	4.88±0.12	13.88±0.24

Table 4: The antagonistic effect of factions separated from MHT6 and MHT88 against *F. moniliform*e L8

Inhibition zone(mm)		
Treatment	FP-I	FP-II
MHT6	-	4.66±0.06
MHT88	0.2±0.22	7.78±0.36
Control	0	0

The experiment was conducted three replicates; the culture with 50%Methanol was taken as control

Purification and Identification of Antifungal Active Compound of MHT6 and MHT88

Thermo stability and other properties of the antifungal compound: By testing the thermal stability of antifungal compound, the result was shown in Table 3. The inhibition zone of each treatment was same, which indicated three kinds of thermal treatments did not affect the antagonistic activity of the compound in MHT6 and MHT88. Therefore, the antibiotic compound production should be thermo resistant.

Antifungal activity of fraction separated by FPLC: We prepared the lipopeptides extract from the cell-free supernatant of strain MHT6 and MHT88, and found that the extract showed a different inhibitory effect toward the growth of *F. moniliforme* L8. To identify the main antifungal active compound of MHT 88, the extract was respectively separated by FPLC, which yielded two main fractions (FP-I and FP-II) (Fig. 1b).Whereas, there is only one fraction (FPII) separated from MHT6 (Fig. 1a). Meanwhile, we can see the peak of FPII in MHT88 is higher than that in MHT6.

The fractions obtained from the MHT6 and MHT88 were separately tested for their inhibitory activity by oxford cup method (Xu *et al*., 2013). The results given in Table 4 showed that fraction FP-II showed significant inhibitory activity against the growth of *F. moniliforme* L8. It indicated that the FP-II is the major antifungal active compound of *B. megaterium.* Meanwhile, we also found the inhibition zones formed by fractions from MHT88 are all larger than that from MHT6. The Fig. 1 and Table 4 revealed that after the mutation, the mutant's metabolite showed stronger antagonistic activity than wild type MHT6.

Table 5: Mass spectrometry analysis antifungal production produced by cell-free supernatant of MHT6 and MHT88

Strain	Fraction	Mon isotopic experimental masses(m/z)	Mon isotopic theoretical masses(m/z)	Intensity	Structure assignment
MHT6	FPII	1449.77	1449.79	++	Fenycin C15, $[M+H]^+$
		1491.80	1491.83	++	Fenycin C18, $[M+H]^+$
MHT88	FPI	1030.64	1030.4	++	Surfactin C13, $[M+Na]^+$
		1058.88	1058.88	++	Surfactin C15, $[M+Na]^+$
	FPII	1449.74	144974	+++	Fenycin C15, $[M+Na]^+$
		1463.75	1463.78	++	Fenycin C16, $[M+H]^+$
		1477.78	1477.82	++	Fenycin C17, $[M+H]^+$
		1491.82	1491.83	++	Fenycin C18, $[M+H]^+$

(+) represents low; (++) represents moderate; (+++) represents high

Identification of antifungal fraction by MALDI-TOF-MS: In order to characterize the antifungal active compound, we conducted MALDI-TOF-MS analysis to determine the molecular mass of the compound separated from MHT6 and MHT88. Comparative analysis of mass spectra revealed quantitative and qualitative variability in m/z ion masses corresponding to every fraction (Table 5). The antifungal production produced by MHT6 was mainly fenycin (C15 and C18). The antifungal production produced by MHT88 was Surfactin (C13, C15) and fenycin (C15, C16, C17 and C18). Combined with the result of FPLC and MALDI-TOF-MS, we can conclude that after mutation, the surfactin gene expressed increasing the expression of fengycin gene too.

Degradation of FB1 in Medium by Mutant MHT88 and Wild Strain MHT6

According to previous report (Benedetti *et al.,* 2006), Fumonisin B1 (FB1) could be degraded by a *Bacillus* sp. strain. In this study, we tested and compared the FB1 degradation effect of the cell-free supernatants obtained from mutant and wild type. Estimation of residual FB1 was accompanied by measuring OD365 in UV spectrophotometer in the presence of supernatant fluids of strain MHT6 and the mutants MHT88 after 72 h of incubation at 37°C. The optical density (OD) values were converted to ppm by plotting the standard curve of different concentrations of FB1 (0.1–10 ppm) against OD365 (Fig. 2)

The results showed that mutant MHT 88 could considerably reduce FB 1 in the liquid medium after 72 h as compared to wild strain MHT 6 (Fig. 3). As was shown in the previous study, fengycin could degrade the FB 1 and reduce the FB 1 production by cutting the transcription of gene FUM 8, which is the key gene for FB 1 production (Falardeau *et al,.* 2013). Therefore, the result that mutant MHT 88 showed stronger ability in degrading FB 1 demonstrate the expression of fengycin enhanced after the mutation, which was in accordance with the results of FPLC and MALDI-TOF-MS.

The Biocontrol Effect of MHT 6 and MHT 88 on Corn Ear Rot Disease in Corn Field Experiment

At last, we examined the control effect of MHT 6 and MHT 88 in corn ear rot disease. The biocontrol efficiencies of wild-type MHT 6 strain and the mutant strain MHT 88 were compared in corn field by assessing the control effect after spraying the biological agent onto the corn ear. The distilled water was taken as control, As was shown in the Table 5, the biological agent MHT 88 showed the significant biological effect compared to its wild strain MHT 6 (Table 6).

Table 6: Control effect of MHT6 and MHT88 on corn ear rot disease against *F. moniliforme*

Treatment	Concentration%	Control effect%
MHT88	100	90.4a
	1	84.6b
	0.2	80.5c
MHT6	100	79.1bc
	1	73.8cd
	0.2	67.4d
CK	-	-

Data are means of three replicates; Mean within a column followed by different letters are significantly different at p=0.05 according to DMRT

Discussion

Bacillus megaterium MHT 6, with ability of inhibiting the growth of *F. moniliforme* L8, could be its potential biological control agents. By dual culture test and culture-filtrate test, the antagonistic activity of the strain cell is stronger than its extracellular metabolites, this result suggests that other interactions such as special competition and nutrition competition exist between the antagonistic bacteria and the pathogens (Hodge and Fitter, 2013). In order to enhance the antagonistic ability of the MHT6's extracellular metabolites, a random mutagenesis using He-Ne laser irradiation was applied.

To enhance the antagonistic capability of this biological control agent, strain MHT6 was mutated using He–Ne laser irradiation. As a new efficient mutation breeding technique, He–Ne laser irradiation has energy density, good monochromaticity and satisfactory directionality, which could induce of phr gene expression in *E. coli* strain KY706/pPL-1 (Kohli *et al*., 2001). Moreover, it also could increase gene transcription and translation (Vacca *et al.,* 1994). These characteristics explain why He–Ne laser irradiation is widely used in microbial breeding (Gao *et al.,* 2014).

Following the application of He-Ne laser irradiation, both the cell and the fermentation broth of mutants showed significant enhancement compared their wild type MHT6, it

means that with the appropriate excitation and emission settings such as He-Ne laser induced or change the gene expression which activating the metabolite production (Makkonen *et al.*, 2013).

To successfully suppress soil-borne pathogens, the biological agents need to effectively colonize on the plants. Poor root colonization by biocontrol agent may result in low biocontrol ability. The surfactin deficient mutant derived from the wild strain significantly decreased colonization ability on the plant and then reduced the biocontrol ability against pathogen infection (Zeriouh *et al.*, 2014). The result of strain motility assays indicates the mutant MHT88 exhibited higher motility than wild strain MHT6. Combined with the MALDI-TOF-MS profile of fraction FP-I in strain MHT6, we can conclude that the strain MHT88 could express surfactin. When comparing the population density on the corn ear of wild strain MHT6 and mutant strain (date not shown), we noticed that mutant exhibited high capability of population to the wild type-strain. This result implied the by the mutation, the mutant MHT88 conclude surfactin to compete with pathogens for colonization, which improved their biocontrol ability.

From this research, the antifungal compound FPII isolated from MHT6 and MHT88 by FPLC and identified as fengycin lipopeptides by MALDI-TOF-MS. After testing antifungal activity of fraction FPII and FPI (Table 4), we can find the FPII, which identified as fengycin showed significant inhibition against *F. moniliforme* L8. The results showed fengycin might play a major role in inhibiting the growth of *F. moniliforme*. This hypothesis should be confirmed in the further study by constructing a fengycin-deficient mutant and comparing the antagonistic ability between wild type and mutant.

After comparing the fumonisin-degrading effect of wild type MHT6 and MHT88, we can see the mutant MHT88 showed stronger activity in degrading FB1. Previous research identified degradation of FB1 by microorganisms is likely composed of two essential steps: hydrolysis to hydrolyzed fumonisin B1 (HFB1) and deamination of the hydrolysis product (Heinl *et al.*, 2011). Benedetti et al. (Benedetti *et al.*, 2006) demonstrated that different bacterial isolates of maize ecosystem have the ability to degrade FB1, among of which, *Sphingomonas sp*. MTA144 (Meca *et al.*, 2013), could encode aminotransferase FBI, showing strong fumonisin degrading activity (Hartinger *et al.*, 2011). Hu *et al.* reported that fengycin could inhibit the transcription of some key genes involved in the production of FB1 (Hu *et al.*, 2009). Combined with the result in this study, we can conclude that the increase of fengycin expression could be the main cause of its enhancement of FB1-degrading ability.

Conclusion

The enhancement of mutant's antifungal activity against *F. moniliforme* L8 is probably because of the increase in fengycin production. The improvement of mutant's motility, which is mainly due to the expression of surfactant, could enable mutant to colonize surfaces. Moreover, the mutant MHT 88 could cause greater FB1 reduction than wild type MHT6. In the biological control of corn field experiment, different concentration of fermentation broth of MHT 88 showed significant effect on disease control, which indicated the mutant MHT 88 could be served as a promising biocontrol agent for corn protection.

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