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Original Article

IN VITRO EVALUATION OF ANTIFUNGAL POTENTIAL AND ELECTRON MICROSCOPIC STUDIES OF *BACILLUS AMYLOLIQUEFACIENS AGAINST ASPERGILLUS SPECIES*

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ABSTRACT

Objective: Bacteria are able to synthesize a wide range of metabolites with fungicidal properties. The present study focused on the *in vitro* evaluation of antifungal potential of *amyloliquefaciens DSM-1067 against Aspergillus* spp. and its electron microscopic studies.

Methods: An *invitro* evaluation of antifungal activity of bacterial secreted and cellular proteins was determined by microbroth dilution, disc diffusion and spore germination inhibition assays (SGIA). The cytotoxicity of these bacterial proteins was determined by hemolytic assay, and the effect of *Bacillus amyloliquefaciens DSM 1067 lysate* proteins on *Aspergillus fumigatus* was visualized by scanning electron microscopy (SEM).

Results: *Bacillus amyloliquefaciens DSM-1067* lysates showed the highest activity by inhibiting the growth of *A. fumigatus*, *A. flavus* completely at a concentration of 31.25 µg/ml. *In vitro* toxicity experiments resulted that the lysate of *B. amyloliquefaciens* DSM-1067was non-toxic against human erythrocytes even at high concentrations. These findings thus emphasize its usefulness in the development of new antifungal therapies. SEM analysis demonstrated the *in vitro* inhibition of *A. fumigatus* growth by *B. amyloliquefaciens* DSM-1067cytosolic proteins leading to wrinkled hyphae, irregular branching patterns. It also showed disruption of conidiophores development. These cytological effects of *B. amyloliquefaciens* DSM-1067on the hyphal growth of *A. fumigatus* can explain its potent anti-*Aspergillus* activity.

Conclusion: The present investigation revealed that *B. amyloliquefaciens* DSM-1067 lysate protein can act as a potential candidate for exploration in the development of effective and non-toxic treatments against *Aspergillus* induced diseases. Its effect on the development of conidiophores and hyphal growth are studied in the present study.

Keywords: Anti-*Aspergillus, Bacillus amyloliquefaciens,* Toxicity, *Aspergillus* species, Scanning electron microscopy.

INTRODUCTION

The incidence of systemic mycoses caused by filamentous fungi has risen significantly in the last decades mainly due to the increased use of immunosuppressive therapy [1]. Exposure to *Aspergillus*species may cause allergic reactions in hyper-sensitized hosts and/or invasive infections in highly immunosuppressed individuals [2]. Among the human pathogenic species of *Aspergillus*: *A. fumigatus* is the primary causative agent of induced infections, followed by *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* [3]. Reports have shown that the organ transplant patients suffering from invasive aspergillosis (IA) are predominant and a serious matter of concern [4, 5]. Also limitations of currently available antifungal agents are associated with high toxicity, complex drug interactions and limited efficacy showing mortality rates up to 60-90% [6]. The employment of natural bio resources for treating invasive fungal infections has been focused in the recent decades [7-9]. It has been reported that the *Serratiamarcescens* exhibited strong *in vitro* antifungal activity against pathogenic fungi including *Aspergillus* species [10]. In another report *B. amyloliquefaciens* produces a "broad-spectrum" antifungal proteins, among them baciamin was the one which showed potent antifungal activity [11]. Several strains of *B. subtilis* and *B. amyloliquefacien s*have been reported to produce lipopeptides displaying strong antifungal activity with low toxicity, high biodegradability [12]. The *B. amyloliquefaciens* strain "TF-28" was reported as a particular endophytic bio control strain which showed an effective inhibitory effect on *F. moniliforme* [13]. There is a series of bacteria which are rarely reported for having significant properties and clinical utility (including treatment of IA) with no mechanism of action. In view of this, the present investigation was undertaken to explore the anti-*Aspergillus* properties of bacterial isolates for their future use to develop novel antifungal therapies.

MATERIALS AND METHODS

Bacterial strains

Bacillus amylo liquefaciens DSM-1067(MTCC 2248), *Serratia marcescens* (MTCC 9527), *Streptomyces noursei* (MTCC 6210), *Streptococcus mutans* (MTCC 890), *E. coli* (MTCC 1671), *Staphylococcus aureus* (MTCC 3160), *Bacillus cereus* (MTCC 10085), *Bacillus subtilis (*MTCC1133), *Bacillus subtilis* (MTCC1483), *Streptomyces spp.*. (MTCC 4066), *Micrococcus luteus* (MTCC 8132) were purchased from an Institute of Microbial Technology, Chandigarh, India. These strains were cultured in Luria Broth (LB) for 3 days at 37 °C in an incubator shaker. The cells were counted by the turbidometry method and used for performing various experiments.

Pathogens

Clinical isolates of *Aspergillus spp.* obtained from Indian Agriculture Research Institute, New Delhi, India were employed in the current study. Three pathogenic species of *Aspergillus spp.* namely *A. fumigatus*, *A. flavus* and *A. niger*were cultured in laboratory on Sabouraud dextrose agar plates. The plates were inoculated with stock cultures of *A. fumigatus*, *A. flavus* and *A. niger* and incubated in a BOD incubator (Hicon, India) at 37 °C. The spores were harvested from 96 h cultures and suspended homogeneously in phosphate buffer saline (PBS), pH 7.4 in a test tube. A homogeneous spore suspension was obtained by incubating the tube at 37 °C for 60 min with intermittent shaking. The spores in the suspension were counted and their number was adjusted to 1 x 10^8 spores/ml before performing the experiments.

Preparation of bacterial supernatant and lysate

The 72 h log phase cultures of bacterial strains were centrifuged at 3256 g for 30 min in Sigma refrigerated centrifuge. The supernatant was collected in a flask for testing the antifungal activity and pellet was washed with PBS thrice and suspended in sonication buffer (50 mmol/l Tris-HCl, 50 mmol/l Ethylenediaminetetraacetic acid, 5 mmol/l 5 mmol/l Dithiothreitol, 1 mmol/l Phenyl methane sulfonyl fluoride). The cell suspension was sonicated by following the program: 20 s bursts at 200 W and 10 s cool periods (2 min) using a sonicator. The sonicated material was centrifuged at 16350 g for 30 min using Sigma refrigerated centrifuge. The supernatant was collected and used as lysate. The lysate was dialysed against distilled water at 4 °C for 24 h with several changes of water and lyophilized.

Protein concentration of bacterial supernatant and lysate was determined by BCA method [14].

Antifungal activity

The antifungal activity of bacterial components was analysed by micro broth dilution assay (MDA), disc diffusion assay (DDA) and spore germination inhibition assay (SGIA) as described earlier [15]. Each experiment was repeated at least three times.

Microbroth dilution assay

The test was performed in 96-well culture plates (Tarsons, Kolkata, India). Autoclaved Sabouraud dextrose broth (90 µl) was added to the wells of culture plates. Various concentrations of bacterial proteins in the range of 1000.0 to3.9 µg/ml) were prepared in the wells by two-fold dilution method and these wells were inoculated with 10 µl of spore suspension containing 1 x 10^6 spores. The plates were incubated at 37 °C and examined macroscopically after 48 h for the growth of *Aspergillus* mycelia. Appropriate control wells treated with Amphotericin B or without any treatment were included in the study. A protein was considered to be active if the wells appeared clear without any visible growth of *Aspergillus* and the results were expressed as minimum inhibitory concentration (MIC).

Disc diffusion assay

The disc diffusion test was performed in radiation sterilized Petri plates of 10.0 cm diameter. Different concentrations ranging from 50.00 to 3.20 µg of proteins/disc of the bacterial extracts were impregnated on the sterilized discs (5.0 mm in diameter) of What man filter paper No. 1. These discs were placed on the surface of the agar plates, which were already inoculated with *Aspergillus* spores. The plates were incubated at 37 °C and examined after 48 h for zone of inhibition, if any, around the discs. The diameter of zone of inhibition was recorded. The concentration, which developed the zone of inhibition of at least 6.0 mm diameter, was considered as MIC. Amphotericin B was used in assay as a standard control drug. An additional control disc without any sample but impregnated with equivalent amount of solvent was also used in the assay.

Spore germination inhibition assay

The *Aspergillus* species were grown on Sabouraud dextrose agar plates and their homogenous spore suspension was prepared in the Sabouraud dextrose broth. Various concentrations (1000 to 3.9 µg/ml) of the bacterial proteins in 90 µl of culture medium were prepared in 96-well flat bottom micro culture plates (Tarsons, Kolkata, India) by double dilution method.

The wells were prepared in duplicates for each concentration. The wells were inoculated with 10 µl of spore suspension containing 100±5 spores. Appropriate control wells treated with Amphotericin B or without any treatment were included in the study. The plates were incubated at 37 °C for 16 h and then examined for spore germination under the compound microscope (Labomed, USA). The number of germinated and non-germinated spores was counted and the percent spore germination inhibition (PSGI) was calculated using following formula:

PSGI = (100-No. of spores germinated in drug treated well/No. of spores germinated in control well) x 100

The activity of the preparations was represented as the MIC_{90} which inhibits the germination of spores in the range of 90-100%.

Haemolytic assay

The basic method of Latoud et al., 1986 [16] with slight modifications was employed to determine the hemolytic effect of bacterial proteins on mammalian cells. Human erythrocytes, collected from apparently healthy individuals, were washed three times with PBS by centrifugation at 1500 revolutions per min (rpm) for 10 min. A 2% erythrocyte suspension was incubated at 37 ºC for 1 h with different concentrations of protein lysate ranging from 1000.0 to 15.60 µg/ml. After incubation, cells were pelleted at 5000 rpm for 10 min in refrigerated centrifuge (Sigma, Germany). The supernatant was collected and the Absorbance at 450 was determined using a spectrophotometer (UV Vis Spect Lambda Bio 20; Perkin Elmer). In negative control sets, only buffer was used for background lysis, whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes. For each sample, the percentage of maximum haemolytic activity was determined.

Scanning electron microscopy

The *A. fumigatus* was cultured in 100 ml of L-asparagine broth using a spore suspension of 1 x 10^6spores/ml for 24 h in a BOD incubator shaker at 37 °C, 100 rpm and treatment with MIC_{50} of *B. amyloliquefaciens* DSM-1067 protein to examine its effect on fungus morphology and spore germination along with control untreated cultures. Fungal mass recovered from culture and used for SEM studies. The cells were fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.2) at 48 °C for 2h. All specimens were washed four times with phosphate buffer and post-fixed with phosphate buffered 2% osmium tetroxide at 48 °C for 4 h. After washing overnight with the same buffer, the specimens were dehydrated in graded ethanol and finally freeze-dried in t-butyl alcohol. The specimens were coated with gold film (approximately 10 nm). Samples were examined using a Carl Zeiss EV040 Gemini field emission scanning microscope with an acceleration voltage of 20 kv using the Everhart Thornley SE detector and the in-lens SE detector in a 50:50 ratio.

RESULTS

The secreted and cellular proteins (lysate) of *B. amyloliquefaciens* DSM-1067, *S. marcescens, S. noursei, S. mutans, E. coli,S. aureus, B. cereus, B. subtilis, Streptomyces spp.* and *M. Luteus* were prepared.

Table 1: Antifungal activity of secreted proteins of bacterial strains evaluated by microbroth dilution assay against pathogenic fungi (*A. fumigatus, A. flavus and A. niger*)

Bacteria evaluated for antifungal activity		MIC_{90} of Bacterial secreted proteins (µg/ml)		
MTCC No.	**Bacterial species**	***A. fumigatus***	***A. flavus***	***A. niger***
1482	*Bacillus amyloliquefaciens*	125	125	250
9527	*Serratia marcescens*	125	250	250
1577	*Escherichia coli*	62.5	62.5	125
6210	*Streptomyces noursei*	125	125	125
2248	*Bacillus amyloliquefaciens DSM-1067*	125	125	250
3160	*Staphylococcus aureus*	31.2	31.2	62.5
10085	*Bacillus cereus*	15.6	15.6	31.5
8601	*Bacillus subtilis*	-	-	250
8142	*Bacillus subtilis*	-	-	-
890	*Streptococcus mutans*	250	250	-
1671	*Escherichia coli*	125	250	-
8132	*Micrococcus luteus*	250	250	500
4066	*Streptomyces spp.*	250	125	250
1302	*Escherichia coli*	62.5	62.5	250
8848	*Bacillus amyloliquefaciens*	62.5	62.5	125
10439	*Bacillus amyloliquefaciens*	125	125	250
Drug [Total =16]	Amphotericin B	1.25	1.25	2.50

*****Protein extracts of 16 bacterial isolates. MIC: minimum inhibitory concentration, MIC reported are the mean value of triplicates.

Table 2: Antifungal activity of lysate proteins of different bacterial strains evaluated by microbroth dilution assay against pathogenic fungi (*A. fumigatus, A. flavus and A. niger*)

Bacteria evaluated for antifungal activity		MIC_{90} of Bacterial secreted proteins(µg/ml)		
MTCC No.	**Bacterial Species**	***A. fumigatus***	***A. flavus***	***A. niger***
1482	*Bacillus amyloliquefaciens*	62.50	62.50	31.20
9527	*Serratia marcescens*	62.50	62.50	125
1577	*Escherichia coli*	62.50	62.50	250
6210	*Streptomyces noursei*	62.50	62.50	31.25
2248	*Bacillus amyloliquefaciens DSM-1067*	31.2	31.25	62.50
3160	*Staphylococcus aureus*	125	125	62.50
10085	*Bacillus cereus*	15.6	15.6	62.50
8601	*Bacillus subtilis*	-	-	-
8142	*Bacillus subtilis*	-	-	-
890	*Streptococcus mutans*	250	250	500
1671	*Escherichia coli*	500	250	-
8132	*Micrococcus luteus*	250	250	500
4066	*Streptomyces spp.*	500	250	125
1302	*Escherichia coli*	250	-	-
8848	*Bacillus amyloliquefaciens*	250	125	125
10439	*Bacillus amyloliquefaciens*	125	125	250
Drug [Total =16]	Amphotericin B	1.25	1.25	2.50

*Protein extracts of 16 bacterial isolates. MIC: minimum inhibitory concentration. MIC reported are the mean value of triplicates.

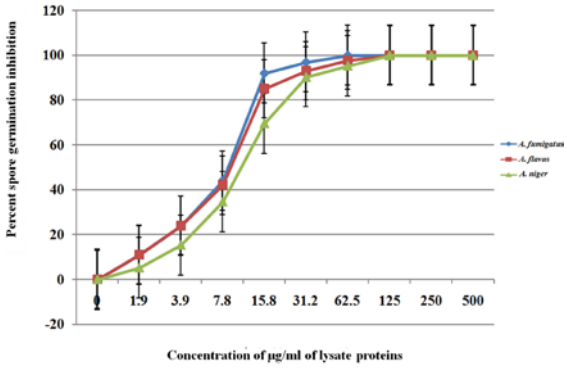


Fig. 1: Graph showing the growth inhibition of Aspergillus species by lysate proteins of Bacillus amyloliquefaciens DSM-1067, demonstrated through percent spore germination assay

Aspergillusfumigatus (A. fumigatus), Aspergillusflavus (A. flavus) and Aspergillusniger (A. niger)

The antifungal activity of secreted and cellular proteins of these bacterial strains was evaluated by MDA in comparison of positive standard drug Amphotericin B. The lysates were showed mild to moderate activity against *Aspergillus* species as represented in table 1 and 2.

The secreted proteins of *B. amyloliquefaciens, S. marcescens, S. noursei, E. coli, S. aureus* and *B. cereus* depicted significant antifungal activity against *Aspergillus* isolates having a MIC in the range between 125 µg/ml to 15.6 µg/ml. Out of them *B. cereus* showing the highest activity against *A. fumigatus*. Similarly cellular lysate proteins of *B. amyloliquefaciens* DSM-1067 *and S. marcescens* showed the MIC at31.25 µg/ml and 62.50 µg/ml having the highest activity against *A. fumigatus* and *A. flavus* in MDA and PSGI (fig. 1) and showing MIC at 62.50 µg/ml and125 µg/ml against *A. niger.*

The DDA experimental results in table 3 and 4 demonstrated the lowest concentration of *B. Amyloliquefaciens* DSM-1067 lysate proteins was 6.20 µg/disc which showed significant activity against of *A. fumigatus*, while it was 12.5 µg/disc in case of *S. marcescens.*

Table 3: Antifungal activity of lysate proteins *of Bacillus amyloliquefaciens DSM-1067* and ***Serratia marscecens*** **against *Aspergillus spp.* demonstrated by disc diffusion method**

Zone of inhibition (mm)						
A. fumigatus			***A. flavus***		***A. niger***	
mean±SD		**CV**	**mean±SD**	**CV**	**mean±SD**	**CV**
Bacillus amyloliquefaciens DSM-1067						
Lysate proteins concentrations (µg/disc.)						
50	11.0±0.10	0.010	11.00±0.10	0.010	10.80±0.3	2.60
25	10.90±0.20	0.060	10.90±0.10	0.020	10.03±0.15	0.023
12	9.03±0.05	0.003	9.03±0.05	0.003	6.50±0.10	0.010
6.20	6.30±0.26	0.070	6.16±0.15	0.020	0.0	0.0
3.10	0.0	0.0	0.0	0.0	0.0	0.0
AmpB 2.5	8.30±0.05	0.003	8.40±0.15	0.020	8.03±0.15	0.023
Serratia marcescens						
50	10.50±0.10	0.010	10.70±0.05	0.003	8.90±0.10	0.010
25	9.20±0.20	0.040	9.10±0.10	0.010	6.50±0.05	0.0
12	6.10±0.15	0.020	6.06±0.05	0.003	0.0	0.0
6.20	0.0	0.0	0.0	0.0	0.0	0.0
3.10	0.0	0.0	0.0	0.0	0.0	0.0

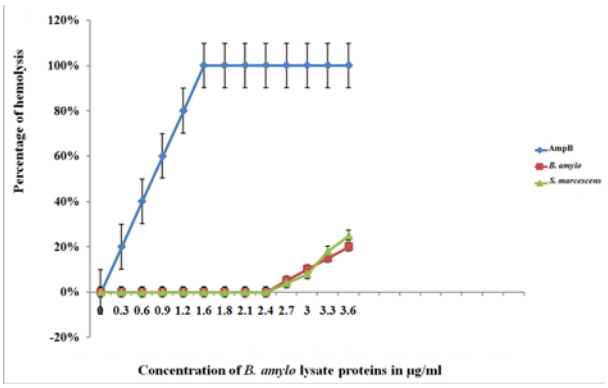
*CV: Coefficient of variance, Amp B: Amphotericin B

Table 4: Minimum inhibitory concentrations (MICs) of lysate proteins of *Bacillus amyloliquefaciens DSM-1067* and *Serratia marcescens* against *Aspergillus spp.*, evaluated by various antifungal susceptibility tests

Pathogen	* MIC of *Bacillus amyloliquefaciens DSM-1067* (µg/ml)			MIC of *Serratia marcescens* (µg/ml)		
	MDA	PSGI	DDA	MDA	PSGI	DDA
A. fumigatus	31.25	31.25	6.20	62.50	62.50	12.50
AmpB	(1.25)	(1.25)	(0.75)			
A. flavus	31.25	31.25	6.20	62.50	62.50	12.50
AmpB	(1.25)	(1.25)	(0.75)			
A. niger	62.50	62.50	12.50	125.00	125.00	25.00
AmpB	(2.50)	(2.50)	(0.75)			

*MIC reported are the mean value of triplicates, Amp B: Amphotericin B (Standard drug)

The toxicity studies of the lysate proteins of all bacterial strains were evaluated against human erythrocytes. Among them *B. amyloliquefaciens DSM-1067*and *S. marcescens* lysates were found to be nontoxic up to the concentration of 250.0 µg/ml (i.e. cell lysis less than 5%) which were significant as compared to standard drug Amphotericin B i.e. 100% lysis at a concentration of 37.5 µg/ml (fig. 2).



***Amphotericin B (AmpB) *B. amyloliquefaciens DSM-1067* (*B. amylo*) and *S. marcescens*(*S. marceses*)**
Fig. 2: Graph showing hemolytic activity of bacterial lysate proteins in comparison to standard drug using human erythrocytes

The SEM analysis revealed that the control sample (without any treatment) showed typical conidial morphology of *A. fumigatus* where fungal hyphae were long-single stranded, stretched, elongated and highly dense with interconnected conidiophores (fig. 3a). Whereas severe defects on fungal hyphal growth, structure and conidial morphology were examined through SEM analysis when *A. fumigatus*conidia (1 x 10^6spores/ml) were treated with cellular proteins of *B. Amyloliquefaciens* DSM-1067 *(*MIC_{50}) as shown in fig. 3b.

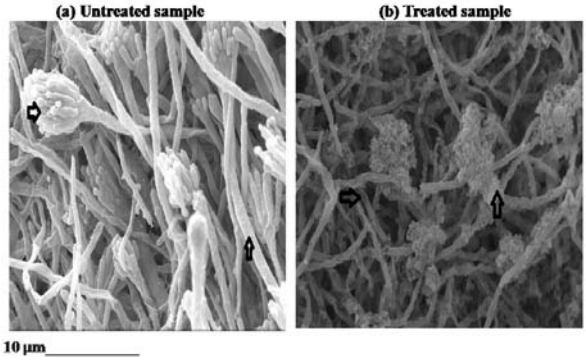


Fig. 3: Scanning electron microscopy images of *Aspergillus fumigatus* untreated sample

(a) hyphae are long, straight with smooth surfaces and branched conidiophores, whereas in bacterial lysate protein treated sample of *Aspergillus fumigatus* (b) hyphae are short, irregular along with shrinking of hyphal walls and damage conidiophores as well as no conidia were observed (arrows) in treated samples

DISCUSSION

In the present study crude lysate proteins of *B. amyloliquefaciens DSM-1067*and *S. marcescens* showed significant antifungal activity against pathogenic species of *Aspergillus*. Although the MIC of active fractions is in the range of 30-60 µg/ml which is less as compared to standard drugs, but they are 10-25 fold less toxic than standard to compensate the higher doses. The MIC of *B. amyloliquefaciens* DSM-1067 as compared to the *S. marcescens* is lower, determined by MDA, DDA and PSGI and also less toxic. The products of *B. amyloliquefaciens* have been reported to be used as a biocontrol agent with antifungal activity [17]. Such properties of *B. amyloliquefaciens* DSM-1067 may be due to biologically active proteins, as indicated in present investigation. Moderate type of activity was observed in case of *S. noursei*. The lysate exhibited better activity than the supernatant (supernatant MIC 125µg/ml and lysate MIC 62.50 µg/ml). The antifungal properties of *Streptomyces* supernatant containing secretory proteins are reported with an appreciable level of activity in supernatant; however, observers did not evaluate the anti-*Aspergillus* properties of *Streptomyces* lysates [12]. The lysates and supernatants of *S. mutans, Micrococcus luteus and B. subitis* were found to be less active against *A. fumigatus.* The *S. aureus* supernatant exhibited better activity than the lysate and *B. cereus* having significant activity of cellular lysate against *Aspergilli*, but its cytotoxicity was quite high.

The SEM studies revealed the effect of *B. Amyloliquefaciens DSM-1067* treatment which demonstrated inhibition of *A. fumigatus* growth, affected hyphal morphology with shrunken hyphal walls, disruption of both hyphal tips and conidiophores and ultimately led to the destruction of whole hyphae. Findings of the present study revealed that the *B. amyloliquefaciens* DSM-1067 had significant activity against *Aspergilli* and it found to be good/eco-friendly bacterium. It can further be used as probiotic "which are having potent candidate with therapeutic value" against *Aspergilli* and its nontoxic behavior may discover potential bioactive molecules for treating *Aspergillus*-induced diseases.

CONCLUSION

A group bacterial strain was screened for anti-*Aspergillus* activity against pathogenic clinical isolates of *Aspergillus* species. The lysate protein of *B. amyloliquefaciens* DSM-1067 showed the highest anti-*Aspergillus* activity having MIC (31.2 µg/ml) and its morphological changes of hyphae lateral walls, disruption of the hyphal tips and branches, abrupt conidiophores germination as well as demolishing of whole hyphal structure. Less toxicity against human cells shows that it may be used as the source of bioactive protein(s) which might have be utilized as therapeutic agents against aspergillosis because various strains of *Bacillus* are already used as the potential source of probiotics.

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ABBREVIATION

DDA: Disc diffusion assay, MDA: Microbroth dilution assay, MIC: Minimum inhibitory concentration, PBS: Phosphate buffer saline, PSGI: Percent spore germination inhibition, RPM: Revolutions per minute, SEM: Scanning electron microscopy, SGIA: Spore germination inhibition assay

CONFLICT OF INTERESTS

Declared None

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