Unraveling the mode of antifungal action of Bacillus subtilis and Bacillus amyloliquefaciens as potential biocontrol agents against aflatoxigenic Aspergillus parasiticus

Unraveling the mechanism of antifungal action of *Bacillus subtilis* and *Bacillus amyloliquefaciens* against aflatoxigenic *Aspergillus parasiticus*

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Abstract

Aspergillus parasiticus is the known fungal contaminant, plant pathogen and aflatoxin producer on foodstuffs and agricultural commodities. In the present study, the antifungal effects of culture filtrates of two soil-isolated strains of *Bacillus* were studied with special reference to the possible mode of actions against *Aspergillus parasiticus* NRRL2999. The growth suppression and IC_{50} were determined by microbioassay technique. Changes in membrane ergosterol content, mitochondrial dehydrogenase activity and chitin and β-1,3-glucan content of the fungal cell wall were evaluated as the different possible targets of bacterial antagonists in the presence of different doses of culture filtrates. According to the results, both bacteria showed good antifungal (up to 92%) and also proteolytic activities (up to 10.5 ± 0.4 mm clear zones on gelatin agar). Bacterial antagonists decreased ergosterol content of the fungal cell membrane (9.0-80%) in a dose-dependent manner. They also affected dehydrogenase activity of fungal cell mitochondria. Interestingly, at 2500 µl, the slight resume of fungal growth and cell wall compositions, referred as paradoxical effect, was exhibited. Finally, electron microscopic photographs (SEM and TEM) showed drastic changes in structure and forms of the treated fungal cells including hyphae folding, wrinkling, cell depletion and vacuolization. As commercial antifungals only target one organelle of the fungal cells and may cause fungal resistance after a while, applying bacterial culture filtrates targeting multiple sites in the fungal cells could be new insight into reduces fungal resistances.

Keywords: *Aspergillus parasiticus*, *Bacillus* species, Antifungal activity, Biological control, Mode of action, Aflatoxins

Introduction

Food and agricultural products are always exposed to fungal contamination in the field or during storage. *Aspergillus*, *Penicillium* and *Fusarium* are the main field fungi. They act as the plant pathogens cause many economic losses to food and agricultural industries. They could also produce hazardous toxins that cause chronic diseases in human and animals (Schuster et al. 2002; Rasooli and Razzaghi-Abyaneh, 2004; Razzaghi-Abyaneh 2013) .The strains of the genus *Aspergillus* are of the major contaminants of foodstuffs. A. *parasiticus* could produce both B and G groups of aflatoxins on food crops especially, nuts. Animals that feed by these foodstuffs may exhibit various health problems including weakness, higher susceptibility to environmental stresses, and loss in fertility and increase in mortality. Aflatoxin could transfer to human by consumption of animal products and consequently cause cancer and death (Allameh, et al. 2005). There are also reports about human infection (Goncalves et al. 2012; Goncalves et al. 2013)

Due to increasing resistance to common antifungal agents, considerable attention has been focused on discovering new resources of antifungals that could affect various targets in fungal cells. Control of pathogenic fungi by antagonistic microorganisms, is an emerging and sustainable strategy (Ongena et al. 2004; Siahmoshteh et al. 2016). The antagonist should have special characteristics to consider being biological control agent such as genetic stability, simple nutritional requirements, survival in adverse environmental conditions, and growth on cheap substrates in fermenters and lack of toxigenicity to humans (Holmes et al. 2008; Razzaghi-Abyaneh et al. 2011).

Terrestrial bacteria especially, the strains of the genus *Bacillus* are the trustworthy group of antagonistic microorganisms (Zhao et al. 2012; Ahmed, et al. 2014) . *Bacillus* strains are capable of producing wide range of antagonistic compounds with strong antifungal activity (Ongena and

Jaques, 2008). These metabolites may act individually and/or interactively on critical cellular processes and inhibit the growth and toxin production of fungi (Razzagh-Abyaneh et al. 2013).

Commercial antifungals each target specific organelle of the fungal cells while culture filtrate of antagonistic microorganisms may contain various antifungal metabolites and targets different parts of a cell to reduce resistance to treatments. Knowing about all possible antagonistic mechanism will increase the efficacy of the biocontrol agent. In the present study, the inhibitory effect of culture filtrates of two indigenous bacteria from the genus *Bacillus* against growth of *A. parasiticus* as a pathogenic fungus with the ability to produce both B and G group of aflatoxin, has been evaluated. Then, the effect of culture filtrates as the sources of versatile antifungal metabolites on different organelles of *A. parasiticus* cells including chitin, β-1,3- glucan, ergosterol content and mitochondrial enzyme activity was investigated. Proteolytic activity and proteinaceous features of antifungal compounds were also studied. Electronic microscopes (TEM and SEM) were also used to show any changes in fungus hypha and its internal structure. To our knowledge, there are not any reports on the underlying mechanism of antifungal actions of *Bacillus* strains against *A. parasiticus*. Therefore, further research on this subject seems to be necessary.

Materials and methods

Microbial strains

Two bacterial isolates from the genus *Bacillus* with known antifungal ability identified molecularly as *B. subtilis* (UTB2) and *B. amyloliquefaciens* (UTB3) were chosen for this study (Siahmoshteh et al. 2017). Bacterial strains were cultured in TSB medium for 72 hours and the

supernatant was separated by centrifuging 14000 rpm for 0 minutes and then sterilized by 0.22µ filters. Different doses of culture filtrate of each strain used as the antagonist against *Aspergillus parasiticus* NRRL2999. The fungus spore suspension was prepared by scraping the surface of 7 day-old SDA slant and immersing the spores in 0.1 % Tween 80 solution.

Fungal growth inhibition

The effect of culture filtrates of the bacteria on *A. parasiticus* growth was measured using microbioassay technique (Razzaghi-Abyaneh et al. 2007) in 6-well microplates (Jetbiofil, China). Briefly, 50µl of 10^7 spores/ml of fungus was added to each well. Glucose Yeast Extract Broth (GYB) used as the culture medium. Different doses of bacterial culture filtrate harvested at 72 h incubation (2500, 1500, 750, 500, 250), was added to each well. The control well had no culture filtrate. After 96 h incubation at 28 °C, fungal biomass of each well was gathered and washed thoroughly with distilled water. The biomass of each well was dried at 80 °C to reach a constant weight. The dry weight of the treated samples was compared with the controls and the difference was reported as the fungal growth inhibition. The same method was used to determine the IC_{50} of culture filtrate of each bacterial strain.

Effect of proteinase K on the antifungal activity of *Bacillus* species

After proving the antagonistic activity of bacterial CFC, they were treated by 0.1 mg/ml proteinase K (each mg 15-30 unit) to indicate the proteinaceous nature of antifungal metabolites. Samples were incubated at 37°C for 1 hour (Nagpure et al. 2014). The inhibitory effect of the treated samples on the growth of *A. parasiticus* was determined using well diffusion method by

spreading 10^5 spore/ml of the fungus on the plate surface. The lack of inhibition zone around the wells is the indicator of proteinaceous nature of bacterial culture filtrate.

Ergosterol Assay

Ergosterol content of the plasma membrane of *A. parasiticus* was determined according to (Ahmad et al. 2010) with some modifications. Briefly, certain amount of biomass from microbioassay technique was dried at 60 °C for 3 h. Three milliliters of 25% alcoholic potassium hydroxide solution was poured on each sample and vortex mixed for 1 min. The samples were incubated at 80 °C for 1 h. After reaching to room temperature, the extraction occurred by adding 1 ml distilled water and 3 ml n-hexane to the sample tubes. The hexane layer was separated and transferred to -18 °C freezer for 18 h. Each sample diluted with fivefold in 96% ethanol. Scanning was operated between 200 and 300 nm using UV/Vis spectrophotometer (PerkinElmer EZ 301, USA). Ergosterol content was calculated as a percentage of the net weight of the mycelial mass by the following equations: percent ergosterol + percent 24(28)

DHE = [(A282/290×F)]/pellet weight, percent 24(28) DHE= [(A230/518×F)]/pellet weight and percent ergosterol = [percent ergosterol + percent 24(28) DHE]—percent 24(28) DHE, where F is the dilution factor, and 290 and 518 are the E values (in percent per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.

Protease Activity of culture filtrate

The protein content of culture filtrate was determined by the Bradford method (Bradford, 1965). Bovine serum albumin was used as the standard protein. Protease activity of culture filtrate was proved by well diffusion method using gelatin as the substrate. In brief, an agar medium

containing 2% of gelatin powder was prepared and autoclaved. One hundred µl of each bacterial culture filtrate was poured in to wells with 8mm diameter. Proteinase K (Sigma, U.S) was also used as positive control in two different concentrations, 10 and 50mg/ml (each mg, 15-30 unit). The plates were incubated in 37°C for an overnight. The clear zones around the wells indicate the protease activity of each culture filtrate.

Determination of mitochondrial dehydrogenase activity

Dehydrogenase activity of fungal mitochondria after treatment with bacterial culture filtrate was determined by MTT method. The test was carried out in 96-well microplate according to lopes *et al*., 2013 with modifications (Lopes et al. 2013). Briefly, 100 µl of 10^5 spore/ml was added to each well containing 100 µl of two-fold serial dilution of culture filtrate in TSB. After 24h of incubation, 20 µl of 5mg/ml MTT in phosphate buffer (PBS) was added to the cells. After 30 minutes at 35° C, the plate was centrifuged at 500 g for 5 minutes. The insoluble purple-color formazan was solubilized in 200 µl of DMSO.

Chitin and glucan assay of the fungal cell wall

Biomass of *A. parasiticus* in contact with different doses of bacterial culture filtrate, were harvested after four days of incubation at 28 °C. The mycelia were inactivated by incubating at 60 °C for 1 hour. The biomass of each treatment was disrupted by glass beads in a microsmash (TOMY, Japan). The pellets were washed 5 times with 1M NaCl solution to solubilize the remaining plasma debris; the cell walls were boiled for 10 minutes in a buffer (500mM Tris-HCl, SDS, β-mercaptoethanol and EDTA). The pellets were freeze-dried and then hydrolyzed by boiling in 3 M fluoroacetic acid for 3 h. The acid evaporation was occurred at 65°C. The chitin

content was determined according to Kapteyn et al. (2000). The glucan of each hydrolyzed sample was determined according to (Fortwendel et al. 2009) by fluorimetric method.

Electron microscopic analysis (SEM and TEM)

A small specimen of fungal mycelium which was treated by IC_{50} concentration of bacterial culture filtrate on microbioassay experiment was prepared for electron microscopic observations. For both SEM and TEM, the samples were firstly fixed in 3% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2). After at least 3three hours, the samples were washed with phosphate buffer and passed through 2% molten agar. Then, the specimens immersed in 1% aqueous solution of osmium tetroxide in phosphate buffer, pH 7.2. After one hour, the samples were exposed to dehydration by a graded water–acetone series (10% steps for 30–90% each of 60min, 100% for 180 min and finally 100% overnight). For SEM, the specimens were covered by a thin layer of gold (13 nm) to increase the electrical conduction and the samples were observed under SU-3500 SEM, Hitachi (Japan). Thin sections of dehydrated samples were prepared by Leica Ultracut UCP on 100-mesh grids to observe by TEM (EM10C-100 KV, Zeiss).

Data analysis

All data were subjected to the analysis of variance (one-way ANOVA) using an SPSS version 23.0 program for Windows (http://www.spss.com/). Differences with $P<0.05$ were considered significant.

Results

Fungal growth inhibition

As indicated in Table 1 both bacterial strains were able to inhibit the growth of *A. parasiticus* NRRL2999 in a dose-dependent manner. UTB2 suppressed the fungal growth in the range of 4 to 90 % and UTB3 in the range of 5 to 92 %. Surprisingly, at 2500 µl the inhibitory effect of culture filtrate of UTB2 has been decreased to 85%. This phenomenon is termed "paradoxical effect". It is stated that in a certain range of higher concentrations paradoxically showed resume in growth (Loiko and Wagener, 2016).

Effect of proteinase K on the antifungal activity of *Bacillus* species

Well diffusion method was used to indicate the proteinaceous nature of the antifungal metabolites present in culture filtrates by inhibiting the growth of *A. parasiticus* on agar plate. By measuring the inhibition zone, no significant difference was observed between proteinase-treated and non-treated culture filtrate of both strains. Such results could offer that the inhibitory components which present in both bacterial culture filtrates are not necessarily of protein. Otherwise, they would be hydrolyzed by the enzyme and lose their inhibitory effect. The pH of both culture filtrates was 8.4±0.03. This pH value is in the range of optimum proteinase activity (7.5-9.0) (www.sigmaaldrich.com). According to Cawoy *et al.* (2015), the main inhibitory components of *Bacillus* strains are Iturins and Fengycins. The other secretory inhibitory components are surfactins. This family of components is also of LPs.

Effect of culture filtrates on ergosterol content of membrane

Ergosterol is an important component of the fungal cell membrane which is a bioregulator and responsible for the membrane flexibility. The ergosterol content was determined at 250, 500,

750, 1500, 2500 µl of bacterial CFC. Both bacterial culture filtrates reduce ergosterol content of *A. parasiticus* cell membrane (Table 2). It shows that cell ergosterol could be considered to be a target for the antifungal compounds of the bacterial CFC.

Protein content and protease activity of culture filtrate

Protein content of bacterial CFC was determined by Bradford method. Culture filtrates contains 3.72 ± 0.46 and 1.48±0.11 µg of protein for UTB2 and UTB3, respectively. After a night of incubation at 37° C, the presence of clear zone around the wells of culture filtrate on gelatin agar indicates the hydrolysis of gelatin by protolithic enzymes. Clear zone of 10 ± 0.1, 10.5 ± 0.4, 7.5 ± 0.1 mm was observed for UTB2, UTB3 and Proteinase K 50 mg/ml. No clear zone was observed around the wells containing 20 mg/ml of proteinase K. According to results of this test, culture filtrate of both bacterial strains showed good proteolytic activity but without significant difference with each other.

Determination of mitochondrial dehydrogenases activity

Mitochondrial dehydrogenases (MD) activity of *A. parasiticus* after exposing to bacterial culture filtrate was determined by through colorimetric assay using MTT as substrate in 96-well plates. The efficacy of culture filtrates on activities of MD is shown in Figure 1. Inhibitory effect of CFC on activities of MD is also dose-dependent i.e different amounts of CFC caused different degrees of inhibition on dehydrogenases activity as the activity inhibition in low doses of CFC is not statistically different ($p < 0.05$) with control sample. In the highest dose of CFC (200 µ) more than 60% of enzyme deactivation was recorded. In each treatment, CFC of UTB2 showed higher impact on enzyme activity.

Effect of culture filtrates on fungal cell wall components

The effect of different doses of culture filtrates of UTB2 and UTB3 on cell wall compositions of *A. parasiticus* was evaluated by determining the amount of chitin and β-1,3 -glucan. None of the samples significantly affected the chitin composition of the cell wall of *A. parasiticus* ($p < 0.05$). The glucan content of the cell wall of the fungus was significantly decreased by increasing the amount of CFC. But applying 2500 µl CFC of UTB2 had no statistically significant effect on glucan ratio. It is suggested that at high concentrations of antifungals, the synthesis of β-1,3-glucan could be restored (Loiko & Wagener, 2016).

Electron microscopic analyses (SEM and TEM)

The morphological changes of *A. parasiticus* cells after 4 days of exposure to 2500 µl of bacterial culture filtrates were examined by electronic microscopes, SEM and TEM. The effects of CFC on the morphological structure of fungal hyphae observed by SEM are shown on Figure 2. In control sample (Fig. 2.a and b) the hypha maintain their normal and intact shape while in the samples treated by both culture filtrates drastic changes in the appearance of hypha were occurred. Massive collapse, depression, folding and wrinkling are observable in the photographs (Fig. 2.c, d, e and f).

TEM photographs of control samples showed normal hypha and intact outer cell wall. High electron density in control sample indicates a normal cytoplasm containing all organelles (Fig.3, a and b). In reverse, complete cytoplasm and internal organelles depletion, detachment of cell membrane and vacuolation are observed in cells treated by bacterial culture filtrates (Fig. 3, b, c, d and e). Slightly higher thickness of the wall in comparison to control by using culture filtrate of UTB2 is also observable (Fig. 3,b and c).

Discussion

Bacillus subtilis and *B. amyloliquefaciens* are of the most known bacterial antagonist. They are able to produce different antifungal metabolites. Some strains are also used as commercial biocontrol agents in the field and on food (Ongena et al. 2005; Vitullo et al. 2012; Siahmoshteh et al. 2017). Bacterial strains of the present study were able to produce antifungal substances in TSB medium. This media is easily accessible which consider an advantage in mass production and commercialization of the antagonist. Both bacterial culture filtrates showed growth inhibition in a dose-dependent manner. But applying the highest dose of the CFC of UTB2, showed a fungal growth resume. This phenomenon which is called paradoxical effect is a known phenomenon in using antifungal agents against pathogenic fungi especially from the genus *Candida* and *Aspergillus* (Hall, Myles, Pratt, & Washington, 1988 ;Kurtz, et al., 1994; Plummer, et al., 2007 ;Verwer, van Duijn, Tavakol, Bakker-Woudenberg, & van de Sande, 2011 ;Hadrich, et al., 2014 ;Rueda, Cuenca-Estrella, & Zaragoza, 2014; Loiko and Wagener, 2016). Paradoxical effect was firstly described by Hall et al (1988) in applying cilofungin against *Candida albicans* and *Candida tropicalis*. High doses of antifungals cause reverse effect and reactivate or increase the expression of some genes. It is a kind of defense mechanism for the fungus. It usually affect the expression of the cell wall genes, chitin synthase and β-1,3-glucan synthase. The results of this study also shows reume in β-1,3-glucan content. The same results obtained for using high doses of echinocandins. These antifungal agents target the biosynthesis of β-1,3-glucan. Surprisingly, to appear paradoxical effect, expression of the β-1,3-glucan synthase Fks1 is essential (Loiko and Wagener. 2016). Secreted antifungal metabolites of the strains of genus *Bacillus* (iturins and Fengycins) are structurally the same as echinocandins i.e both are of the

cLPs. This may decipher the results of cell wall components of this study while applying high doses of culture filtrates. To indicate the proteinaceous nature of antifungal metabolites of culture filtrates, they were treated by proteinase K. By measuring the inhibition zone, no significant difference was observed between proteinase-treated and non-treated culture filtrate of both strains. Such results could offer that the inhibitory components which present in both bacterial culture filtrates are not necessarily of protein. Otherwise, they would be hydrolyzed by the enzyme and lose their inhibitory effect.

Both strains showed high proteolytic activity. *Bacillus* strains are well-known for production of proteases especially, alkaline protease (Takami et al. 1989; Degering et al. 2010). These proteases are characterized by their activity and stability under high alkaline conditions. As the pH of the both culture filtrates of this study are above 8, the proteolytic activity is related to alkaline proteases which are secreted into culture medium.

As the enzymes are proteinaceous in features, proteolytic activity may lead to inactivation of broad spectrum of enzymes which are the key components of the viable cells. This could partly explain the antifungal activity of bacterial culture filtrate against our pathogenic fungus.

Mitochondria are organelles which play important roles in viable cells of higher organisms by their enzymes activities. MD (lactate dehydrogenase, malate dehydrogenase, succinate dehydrogenase) are key enzymes in respiratory chains of the cells which produce ATP. In our study, activities of MDs are partly inhibited by both bacterial CFC, which may interfere with the citric acid cycle and inhibit the synthesis of ATP in the mitochondria of *A. parasiticus*.

Cell membrane ergosterol is the target of some common antifungal drugs from azoles and polyenes. Azoles could inhibit the biosynthesis of ergosterol by inhibiting 14a-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, 14a-methylated sterols. The

precursors include lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol. This causes the loss of integrity in plasma membrane. The antifungal mechanisms of polyenes are not fully understood but they are able to bind to sterol compounds, especially, ergosterol. This caused the formation of the channels in the cell membrane (Valiante et al. 2015).

The results of the present study showed the effect of culture filtrates of our strains on both internal and external structure of *A. parasiticus* cells. The results were confirmed by SEM and TEM. A considerable morphological alteration of the hyphal internal and cell wall structure was observed applying high doses of CFC against *A. parasiticus*. The photographs show collapse and squashed due to the lack of cytoplasm. In fact, the antifungal compounds change the cell permeability which results in an imbalance in intracellular osmotic pressure, subsequent disruption of internal organelles, leakage of cytoplasm, and finally cell death (Tolouee et al. 2010; Li et al. 2012). TEM observation also indicated that the antagonistic metabolites penetrated into the cytoplasm and damaged the organelles. A thicker cell wall was observable around the cells of *A. parasiticus* treated by the highest dose of CFC of UTB2. Glucans are the outer layer compounds of the cell wall. Thicker outer layer of the cell wall at applying high dose of antagonist could confirm the resume of β-1,3-glucan synthesis. The TEM observations of *A. flavus* cell wall reported by Kurtz et al (1994) are in harmony with our result. They reported a thick cell wall with an extensive outer layer for *A. flavus* when the fungus treaed by lipopeptide antifungal agent.

Different classes of antifungals used to suppress fungal growth. These drugs usually target specific organelles of the fungus. Chitin and β-glucan are the main constituents of fungal cell walls which were not found in human cells. Therefore the antifungal drugs that target these organelles are efficient in treating patients. But some fungal strains show resistance to these

drugs. For this reason, there is an increasing attention to discover a novel eco-friendly source of antifungals to substitute the current drugs (Tian et al. 2012).

Altogether, results of the present study indicate both *B. subtilis* and *B. amyloliquefaciens* could target mitochondrial dehydrogenase, cell membrane, ergosterol content, cell wall and produce proteolytic compounds. Culture filtrates of these bacteria as the rich sources of antifungal metabolites with the capability of targeting different organelles could be proposed as the replacement for commercial antifungal agents but much more investigations are needed to make it practical.

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Figure legends:

Fig. 1 Inhibitory effect of different doses of culture filtrates (CFC) of UTB2 and UTB3 on mitochondrial dehydrogenases activites.

Fig. 2 effects of Bacterial culture filtrates on the morphological structure of *A. parasiticus* NRRL2999 hyphae observed by SEM. **a,b** Untreated hyphae of *A. parasiticus*. **c,d** The morphology of *A. parasiticus* hyphae after treatment with culture filtrate of UTB2 at different

magnitudes. **e,f** The morphology of *A. parasiticus* hyphae after treatment with culture filtrate of UTB3 at different magnitudes.

Fig. 3 The internal morphology of *A. parasiticus* hyphae observed by TEM. **a,b** The internal morphology of untreated control hyphae of *A. parasiticus*. **c,d** The internal morphology of *A. parasiticus* hyphae after treatment with culture filtrate of UTB2 at different magnitudes. **e,f** The internal morphology of *A. parasiticus* hyphae after treatment with culture filtrate of UTB3 at different magnitudes.

Table 1

Table 1 Inhibition of *A. parasiticus* growth by different doses of bacterial cell culture filtrate (CFC)

	CFC (µl)	Fungal growth (mg)	
		Mean ± SD	Inhibition (%)
UTB2	0	27.5 ± 1.1	0
	250	26.3 ± 0.6	4
	500	16.3 ± 2.8	41
	750	12.0 ± 2.0	77.1
	1500	2.8 ± 0.4	90.0
	2500	4.1 ± 0.3	85
UTB3	0	27.5 ± 1.1	0
	250	26.1 ± 0.8	5
	500	16.9 ± 2.0	38
	750	6.3 ± 1.2	56.2
	1500	5.0 ± 1.0	82.1
	2500	2.2 ± 0.7	92

Table 2

Table 2 Ergosterol content of cell membrane of *A. parasiticus* after exposure to bacterial cell culture filtrate (CFC)

CFC dose (µl)	Ergosterol inhibition (%)	
	Bacillus species	
	UTB2	UTB3
250	11.0 ± 3.5[d]	9.1 ± 1.4[d]
500	24.6 ± 4.8[c]	22.6 ± 5.0[c]
750	45.5 ± 5.9[b]	39.7 ± 4.5[b]
1500	80.7 ± 7.1[a]	75.0 ± 5.4[a]

Figure 1

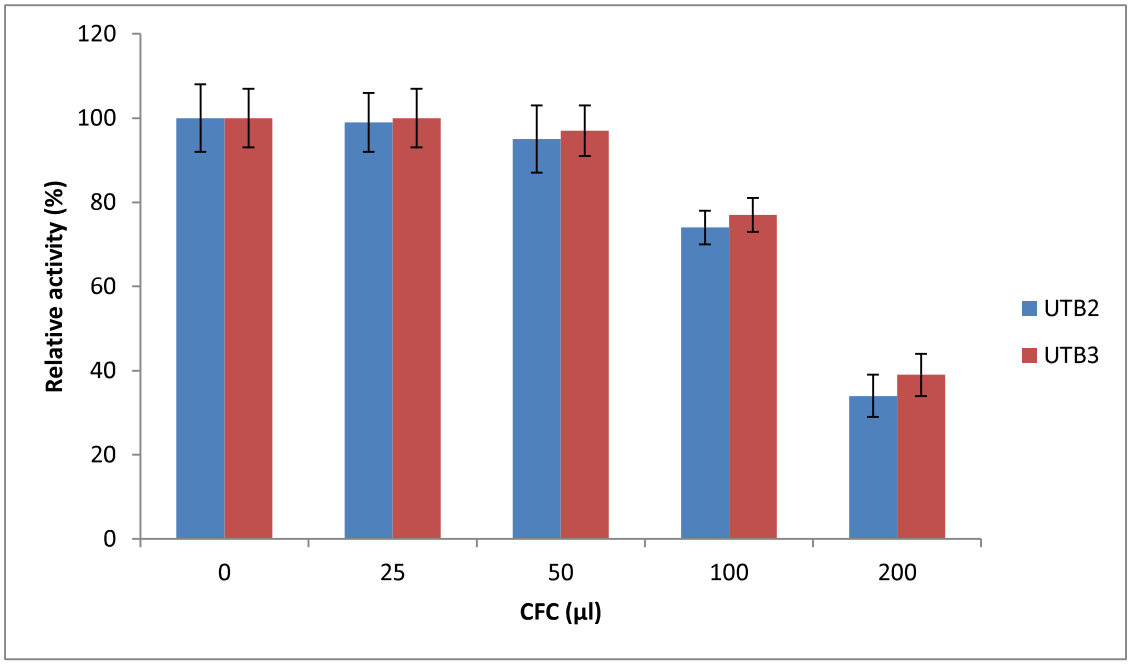
120
100
80
60
40
20
0
Relative activity (%)
0
25
50
100
200
CFC (µl)
UTB2
UTB3

Figure 2

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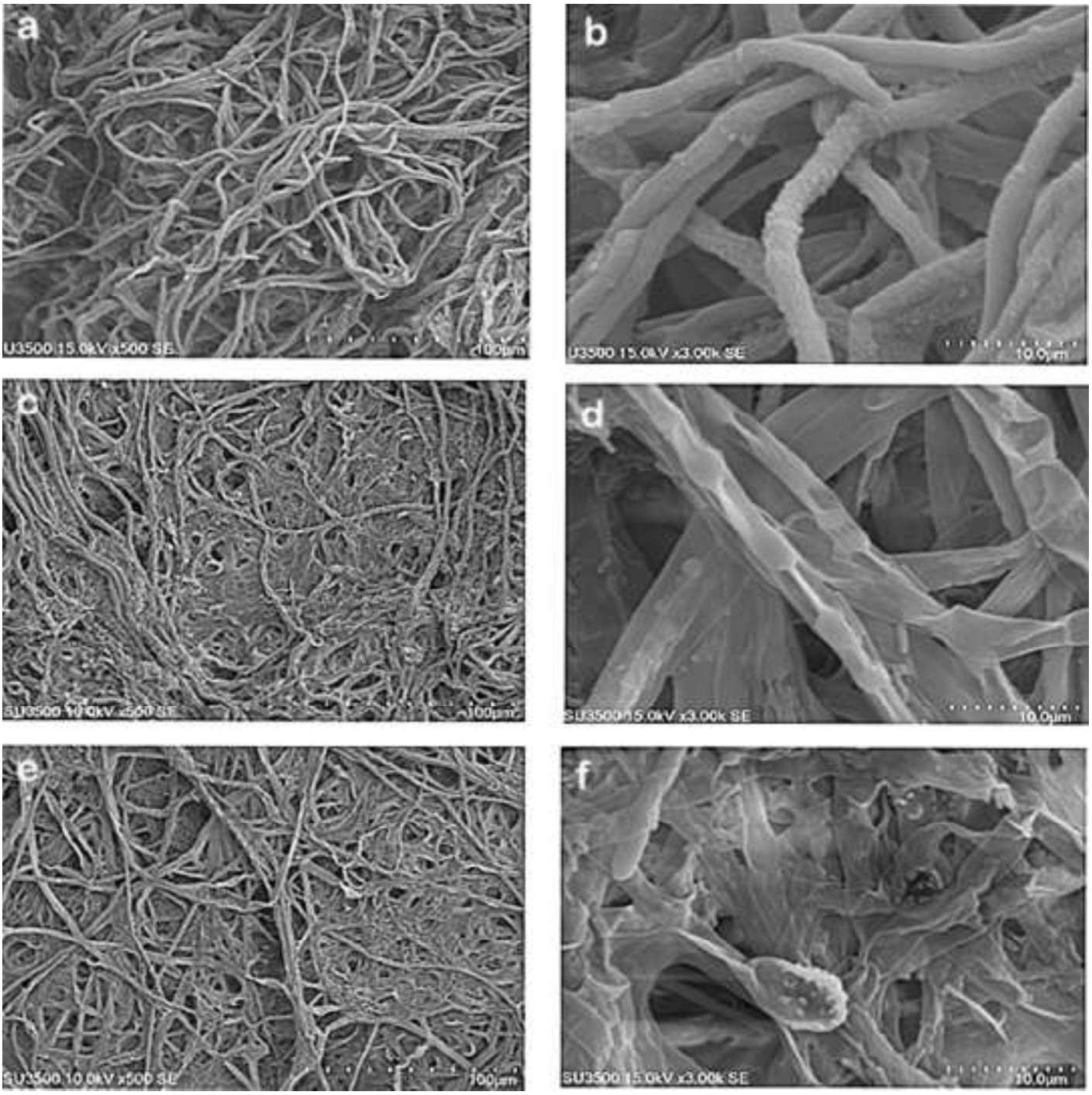

Figure 3

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