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Morphological and Ultrastructural Studies for the Biological Action of Penicillic Acid on Some Bacterial Species

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Abstract: Nine species of bacteria were tested for sensitivity against Penicillic Acid (PA) using a disc diffusion assay. Among the tested bacterial species, *Salmonella typhi* appeared to be the most sensitive bacteria. *Bacillus cereus* showed the less response to the inhibitory effect of PA. The response of treated cells of *S. typhi* and *B. cereus* to PA was detected using light and electron microscopic examination. PA induced the formation of flattened and filamentous cells due to increase in lengths and widths of cells. This was more clear in treated cells of *S. typhi* than those of *B. cereus*. The cell wall and cytoplasmic membrane of *S. typhi* appeared to be damaged and a part of cytoplasmic material may be released through cracks. Moreover, deformation in the submicroscopic structure of *S. typhi* cells that treated with PA have been observed and some cells showed an irregularity in shape. Examination of the ultrathin sections of the treated *B. cereus* cells with PA, indicated that mature stainable sporangial formation in most cells and developmental stages of sporulation are obtained at a faster rate compared with those of growing vegetative control cells. Studying the antibacterial activities of PA were extended to show bacteria that resistant to some conventional antibiotics, were being sensitive to the saturated antibiotic discs with PA.

Key words: Penicillic acid, ultrastructure, *Salmonella typhi*, *Bacillus cereus*

INTRODUCTION

Penicillic acid (PA, 3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid) is a secondary metabolite produced by a variety of *Aspergillus* and *Penicillium* species (Smith and Moss, 1985; Jiménez *et al.*, 1991; Bresler *et al.*, 1995; Kang *et al.*, 2007). PA is a carcinogenic mycotoxin, displaying a variety of biological activities including antiviral and antitumor (Suzuki *et al.*, 1971), antidiuretic (Murnaghan, 1946), antifungal (Kang and Kim, 2004; Yamji *et al.*, 2005) and phytotoxic properties (Ahmad and Egbal, 2002; Martínez-Luis *et al.*, 2005; Yamji *et al.*, 2005). PA is also cytotoxic (He *et al.*, 2004), hepatotoxic (Chan *et al.*, 1980) and nephrotoxic (Austwick, 1983). Ueno *et al.* (1995) showed that PA can cause induction of DNA fragmentation. The potential risk of PA for human health was suggested when PA had been isolated from agricultural products such as poultry feed, blue eye diseased corn, commercial corn, dried beans and tobacco products (Kurtzman and Ciegler, 1970; Pero *et al.*, 1972; Thrope and Johnson, 1974) and from mold-fermented sausage (Ciegler *et al.*, 1972).

PA was proved to have antibacterial activity. It was found to exhibit significant antibiotic activity against both Gram-positive and Gram-negative bacteria (Oxford, 1942; Oxford *et al.*, 1942; Geiger and Conn, 1945; Wirth *et al.*, 1956).

PA was also reported to induce morphological effects on bacteria by induction of the enlargement of cell volume and the formation of elongated filamentous cells (Auffray *et al.*, 1984).

The efficiency of conventional antibiotics in preventing bacterial proliferation is the source of their success, but at the same time is also the cause of their failure, where in many cases, the imposed selective pressure by the use of conventional antibiotics lead to increase the expression of degrading

enzymes and development of drug-efflux systems which operate with increasing efficiency and therefore actively reduce the internal concentration of the antibiotics (Rasmussen *et al.*, 2005). Anwar *et al.* (1990) showed that bacteria in the biofilm mode exhibit a higher tolerance to antimicrobial treatments. The prospect of the 21st century as a post-antibiotic era highlights, the importance of novel strategies to control bacterial diseases (Camara *et al.*, 2002). Quorum Sensing (QS) is a cell-to-cell communication phenomenon whereby bacterial cells are able to indirectly monitor their own population density and regulate their behaviour in a multicellular fashion as the density changes (Williams, 2002). Rasmussen *et al.* (2005) found that patulin and PA produced by *Penicillium* species, act as Quorum Sensing Inhibitor (QSI) compounds.

The main objectives of this research were to study the sensitivity of clinical pathogenic bacteria to the activity of PA; to show the morphological and ultrastructural effects of PA on Gram-positive and Gram-negative bacteria and to make a simple trial to test the capability of PA to function as QSI.

MATERIALS AND METHODS

Microorganisms

Bacillus cereus, *B. subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staph. citreus* were kindly obtained from the National Research Centre (NCR), El-Dokki, Giza, Egypt. *E. coli* and *Salmonella typhi* group D were kindly obtained from the Microbiology Department, Faculty of Medicine, Zagazig University, Egypt. *Penicillium hirsutum* was isolated from paddy rice grains, selected from 9 toxigenic isolates as a high producer of PA and was identified to the species level according to Frisvad and Samson (2004).

Culture Media

Nutrient agar and nutrient broth were used for maintaining and growing of the bacterial species under the experimental conditions. Czapek-Dox's agar (Thom and Raper, 1945) was used for isolation, purification, maintaining and identification of the fungal isolate. Raulin-Thom broth (Bentley and Keil, 1962; Sekiguchi *et al.*, 1987) was used for production and optimization of PA.

Isolation and Determination of PA

PA was isolated from culture filtrate according to Keromnes and Thouvenot (1985) with some modifications, culture filtrate of the fungal isolate was extracted two times with equal volumes of ethyl acetate, shaken for about 30 min and allowed to stand for 30 min. The combined solvent fractions were dried with anhydrous Na₂SO₄ and then evaporated till a dark brown material was obtained (Bentley and Keil, 1962; Martinez-Luis *et al.*, 2005). This material was dissolved in 20 mL ethyl acetate and then passed through column of silica gel (C18). PA was recovered as white needles by the addition of 20 mL fold excess of petroleum ether, then determined qualitatively after spotting on TLC plates with standard PA (Sigma Chemical Co., Louis St., USA) by the method described by Ciegler and Kurtzman (1970) and Aziz and Moussa (2002) and quantitatively determined spectrophotometrically according to Bentley and Keil (1962).

Antibacterial Assay of PA

The isolated PA was dissolved in known volumes of sterilized distilled water to give the desired concentrations at 200, 300, 400, 500, 600, 700, 800 and 900 µg mL⁻¹. These concentrations were applied to small filter paper discs (11 mm in diameter) dropwise using a micropipette. The discs were air dried and put gently on the surface of the tested Petri dishes containing 25 mL nutrient agar inoculated with 0.1 mL of bacterial cell suspension of the tested bacterial species (approximately, 10⁷ cells mL⁻¹) (triplicate plates were made for each bacterial species). The plates were then pre

incubated at 4°C for 2 h to allow uniform diffusion into the agar. After that, the plates were incubated for 18-24 h at 35°C and the resulted inhibition zones around the discs were then measured.

Light Microscopic Examination

PA was dissolved in a known volume of sterilized distilled water and added to sterilized nutrient broth to give the final concentration of PA solution at 800 µg mL⁻¹. Control treatments were made by adding sterilized distilled water only to the broth. The media with and without PA were then inoculated with 0.1 mL bacterial cell suspension (10⁷ cells mL⁻¹) of *Salmonella typhi* or *Bacillus cereus*. Incubation was carried out for 18 h at 35°C, after that the control and sample cultures were centrifuged at 3000 rpm for 15 min, washed with distilled water and the precipitated cells were stained with modified Gram's method as described by Silvertown and Anderson (1961). The induced morphological changes by PA were examined, analyzed and photographed using a computerized microscope (Motic images, 2000, version 1.3, www.motic.com, Micro-Optic. Industrial Group Co. Ltd. Japan).

Ultrastructure of PA Treated Cells of *S. typhi* and *B. cereus*

The procedure of preparation of ultrathin slices for transmission electron microscope was proceeded in the Central Laboratory of Electron Microscope (Ain Shams University, Cairo, Egypt). Bacterial cells were collected by centrifugation, fixed in 2.5% glutaraldehyde (Gupta and Berridge, 1966) for three hours, post-fixed in 1% osmium tetroxide (Palade, 1952), dehydrated in a serial dilution of ethyl alcohol, passed in three changes of acetone : ethanol (1:2, 1:1 and 2:0) and embedded in resin capsule (Luft, 1961). The capsules containing the samples were cut on ultrathin microtome by diamond knives into ultrathin sections (70 nm). They were carried on copper grids. Ultrathin slices were then contrasted with uranyl acetate (Sptempack and Ward, 1964), followed by lead citrate (Reynold, 1963) for 30 min. The grids were examined under JEOL 1200 Ex II transmission electron microscope and the electron micrographs were printed on Ilford cards.

Screening for the Efficiency of Antibiotics in Preventing Bacterial Growth

The biological effect of twelve types of antibiotics discs (Pasteur Lab., Egypt, 6 mm diameter), namely; nalidixic acid, chloramphenicol, tobramycin, ampicillin, erythromycin, neomycin, tetracycline, amoxicillin, bacitracin, ofloxacin, vancomycin and penicillin was tested on the growth of the tested bacterial species. The inhibiting and non-inhibiting effects of antibiotics on bacterial growth were recorded after incubation for 24 h at 35°C.

Combined Effect of Antibiotics and PA on the Tolerant Bacterial Cells

PA solution (800 µg mL⁻¹) was applied to penicillin discs dropwise using a micropipette and tested against all the experimental bacterial species except *Str. faecalis*. The combined effect of both PA and bacitracin was also tested against *K. pneumoniae* under the mentioned incubation conditions.

The results were analyzed by ANOVA (at 0.05 and 0.01 levels).

RESULTS AND DISCUSSION

A total of nine different species of bacteria was tested for sensitivity against PA (Table 1). *S. typhi*, *Staph. aureus*, *Staph. citreus* and *Str. faecalis* showed their response to the inhibitory effect of PA at the tested concentrations (200 to 900 µg mL⁻¹). A weak inhibitory effect of PA against *B. cereus* and *P. aeruginosa* was observed and the sensitivity of these bacteria to the mycotoxin appeared at the high concentrations (600 to 900 µg mL⁻¹) reaching their maximum at 900 µg mL⁻¹ (15 and 16 mm, respectively). It has been found that *B. subtilis* was more sensitive to PA than *B. cereus*. On the other hand, PA has no any inhibitory effect against *E. coli* and *K. pneumoniae*.

Table 1: Relation between concentrations of PA ($\mu\text{g mL}^{-1}$) and the inhibition zones of the tested bacteria

Concentrations of PA ($\mu\text{g mL}^{-1}$)	Diameter of inhibition zones (mm)							
	200	300	400	500	600	700	800	900
<i>B. cereus</i>	-	-	-	-	14.0	14.5	14.5	15.0
<i>B. subtilis</i>	-	12.0	13.0	15.5	16.5	17.0	17.0	18.0
<i>E. coli</i>	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	-	-	15.0	15.0	15.5	16.0
<i>S. typhi</i>	15.0	15.5	16.5	19.5	19.5	21.0	22.5	23.0
<i>Staph. aureus</i>	13.0	15.0	15.0	15.0	15.0	15.0	17.0	19.0
<i>Staph. citreus</i>	15.0	15.5	16.5	17.0	17.0	17.5	18.0	19.5
<i>Str. faecalis</i>	13.0	13.5	15.5	17.0	17.0	17.0	17.0	19.0

Table 2: Morphogenetic effect of PA ($800 \mu\text{g mL}^{-1}$) on the length and width of *S. typhi* and *B. cereus*

Morphology	<i>S. typhi</i>		<i>B. cereus</i>	
	Control cells	Treated cells	Control cells	Treated cells
Average length (μm)	0.896	1.45	2.33	3.43
Average width (μm)	0.240	0.331	0.669	0.867

The results of these studies indicate that *S. typhi* (Gram-negative-bacteria) considered to be the most sensitive bacteria to the inhibitory influence of PA and *B. cereus* (Gram-positive-bacteria) showed a very weak response to the inhibitory influence of PA, would, therefore be the two bacterial species of choice for making further studies on the effect of PA on bacteria throughout the subsequent experiments of the present work. Likely, Madhyastha *et al.* (1994) found that PA had inhibitory effect against *B. brevis*, *B. cereus* var. *mycoides*, *B. cereus*, *B. megaterium*, *B. subtilis* and *B. thuringiensis* and similarly they showed that PA did not have any inhibitory effect against *E. coli*. Olivigni and Bulleman (1978) found that *B. subtilis* was more sensitive to PA compared to *B. cereus* var. *mycoides*, *B. megaterium*, *E. coli* and *Staph. aureus*. PA exhibited significant antibiotic activity against both gram-positive and Gram-negative bacteria (Geiger and Conn, 1945; Wirth *et al.*, 1956). Oxford (1942) reported that PA was more active against members of *coli*-typhoid *Salmonella* group of Gram-negative organisms. Namikoshi *et al.* (2003) reported that PA had inhibitory effect against the marine bacteria, *Ruegeria atlantica* TUF-D. Rasmussen *et al.* (2005) found that $147 \mu\text{g mL}^{-1}$ of PA had no any effect on the growth of *P. aeruginosa*.

The results in Table 2 indicate that PA induced increases in lengths and widths of both *S. typhi* and *B. cereus* cells compared with the control values, but *S. typhi* is more sensitive to the effect of PA than those of *B. cereus*. Moreover, it was seemed that PA induces the formation of elongated cells and this was more clear in the treated cells of *S. typhi* than those of *B. cereus*. Similar results were reported by Auffary *et al.* (1984), who showed that PA and other mycotoxins induced the formation of elongated filament cells in *B. thuringiensis* subsp. *thuringiensis* and these filamentous cells appeared to lack septa. Boutibonnes (1979) used the concentration $2000 \mu\text{g mL}^{-1}$ of PA to make inhibition for *B. thuringiensis* (Berliner) growth. Meanwhile, he showed that PA at this concentration induced enlargement of the cell volume during determination of the length of the treated cells.

Many previous authors have been concerned with various physiological effects of PA in bacteria but they did not show the mode of action of PA on bacteria through an ultrastructural study. In a normal cell of *S. typhi* (Fig. 1a and b), the outer membrane has a typical wavy appearance and the cytoplasmic membrane is continuous close contact with the cell wall. In contrast, the cell wall and the cytoplasmic membrane of treated cells are more denser, linear (Fig. 2a and b) and appears to be damaged from either both sides or the end of the cell (Fig. 2a). Moreover, a part of the cytoplasmic material may be released through formation of cracks (Fig. 2c) and this may explain the antibacterial activity of PA against Gram-negative-bacteria as owing to increased permeability of the cell wall and cytoplasmic membrane. Similarly, the antibacterial activity of polymyxin group against Gram-negative-

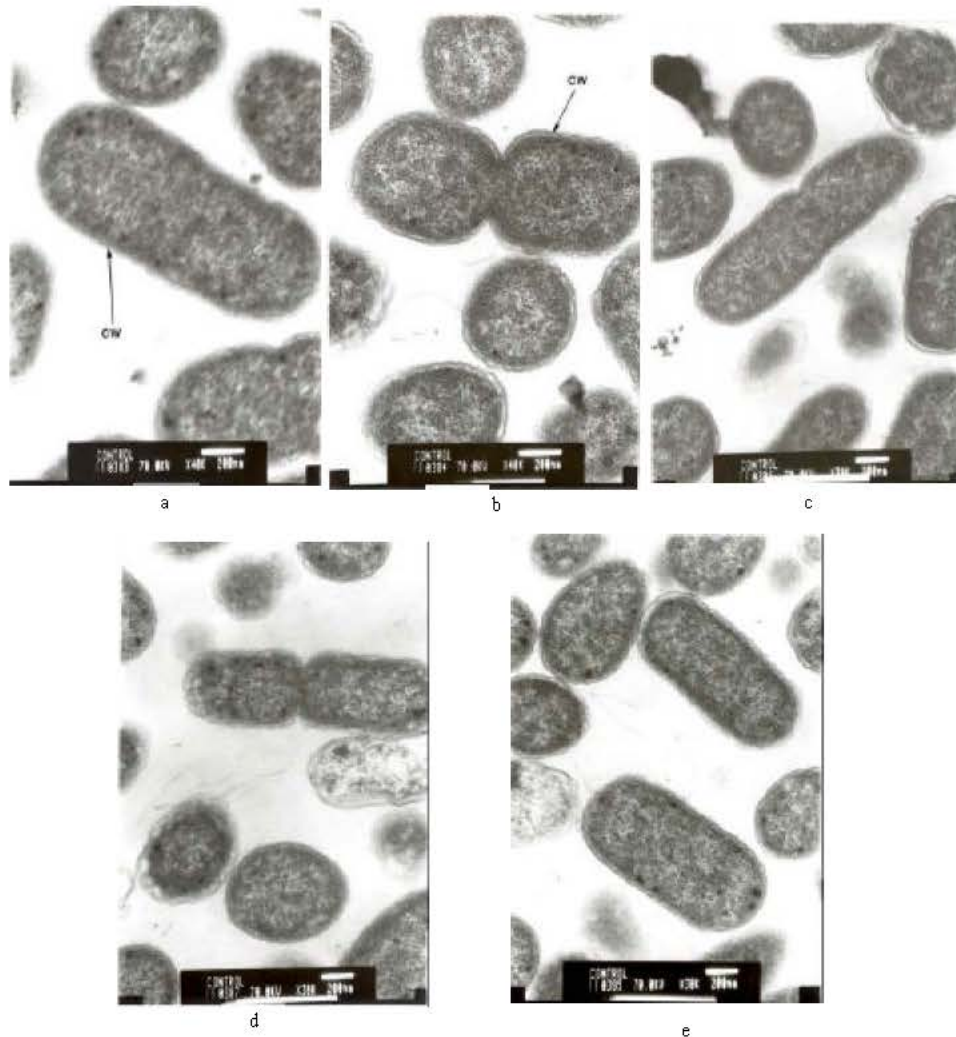


Fig. 1: Transmission electron micrographs of ultrathin sections of control *S. typhi* cells. Cw: Cell Wall. Bars = 200 nm

bacteria was due to increased permeability of the cell envelope consisting of the cell wall and cytoplasmic membrane and the resultant leakage of the cell contents (Newton, 1956; Koike *et al.*, 1969). The usual submicroscopic structure of the cells (Fig. 1a and c) is no longer observed clearly in the treated cells. The differences between DNA-rich and ribosomes rich cytoplasmic regions seem to be deformed (Fig. 2a and b). Ueno and Kubota (1976) reported that PA, a carcinogenic mycotoxin, has the ability to modify bacterial DNA. Umeda *et al.* (1977) showed that PA induces single and double stranded DNA breaks. The images of treated cells with PA revealed that cells were more flattened and filamentous (Fig. 2a and c) and some cells showed an irregularity in shape (Fig. 2d), compared with the untreated cells (Fig. 1a-c). Boutibonnes (1979) found that PA induced enlargement of cell volume of *B. thuringiensis* (Berliner) and this cell abnormality induction resembles those obtained with mitomycin C. Auffray *et al.* (1984) showed that PA induced the formation of elongated filaments cells in *B. thuringiensis* subsp. *thuringiensis*.

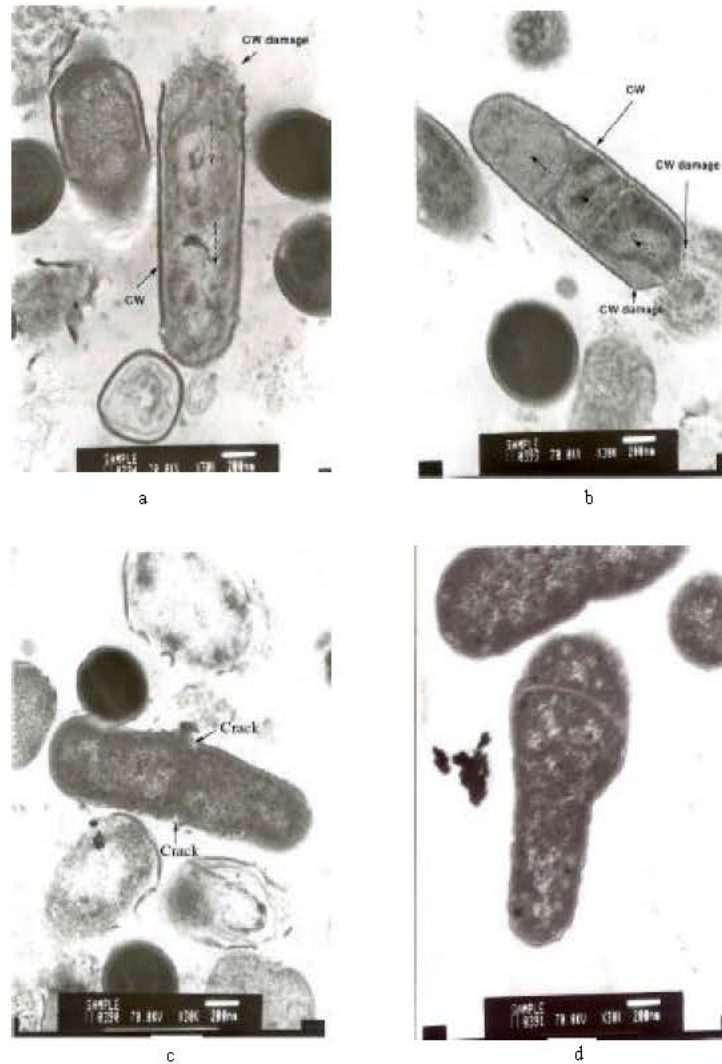


Fig. 2: Transmission electron micrographs of ultrathin sections of treated *S. typhi* cells with PA ($800 \mu\text{g mL}^{-1}$). CW, Cell Wall ; cut arrows in 2a and 2b marks deformation in the DNA-rich and ribosomes-rich cytoplasmic regions. Bars = 200 nm

In electron micrographs of thin sectioned of normal *B. cereus* cells, the nuclear material appears in the form of an axially disposed filament (Fig. 3a and b). The development of a sporal mesosome occurs at this time which subsequently becomes associated with the segregation of that portion of the axial filament which will be enclosed by the spore septum. The large circular electron -transparent area represents a granule of poly- β -hydroxy-butyrate (PHB) and the smaller, diffuse, transparent areas (gl, Fig. 3d) scattered throughout the cytoplasm are glycogen particles (Ellar and Lundgren, 1966). It was noticed that most of the untreated *B. cereus* cells grow vegetatively after 18 h of incubation (Fig. 3b-d). Few cells exhibit stage 4 of sporulation (Fig. 3e and f) at which the cortex development was manifested by the electron-dense layer (Ellar and Lundgren, 1966). By examination of the ultrasections of *B. cereus* treated with PA, it was found that mature stainable sporangia formed in all cells and all the treated cells exhibit stages 4 and 6 of sporulation (Fig. 4a-e) and this may be attributed to the presence of PA in the growth medium that induced accelerated rate in sporulation of

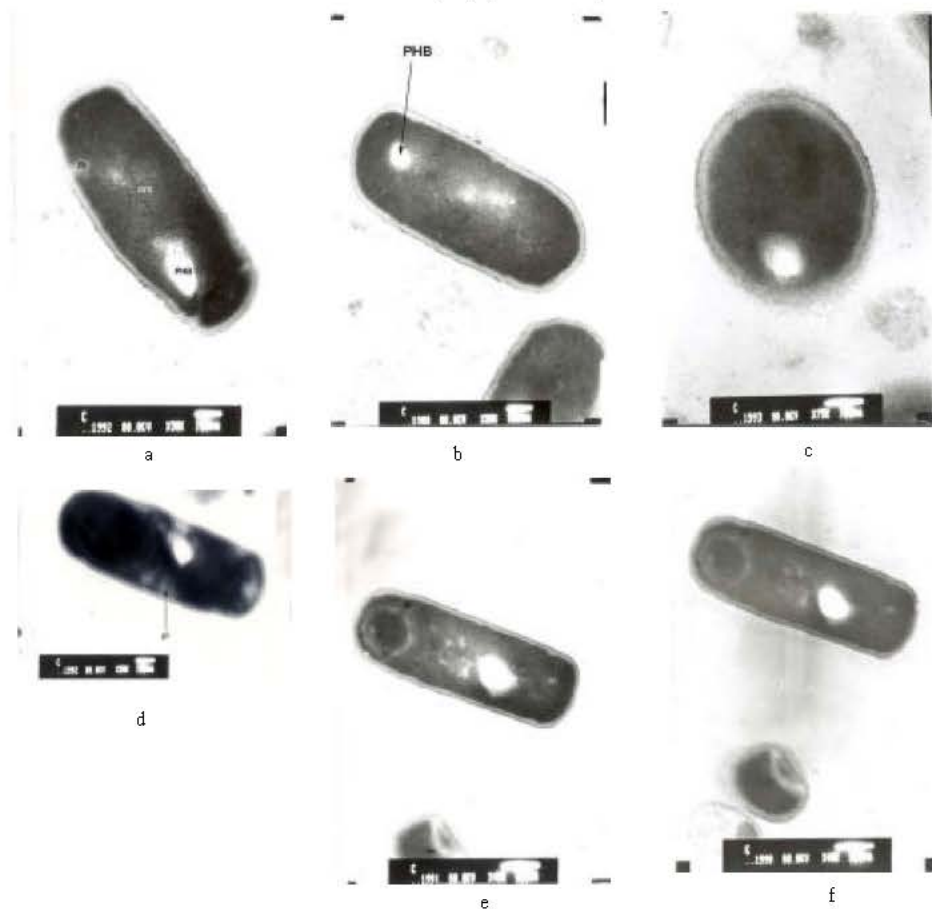


Fig. 3: Transmission electron micrographs of ultrathin sections of control *B. cereus* cells. PHB, poly- β -hydroxy-butyrate inclusion; gl, smaller electron-opaque areas believed to contain glycogen; m, mesosome ; nm, nuclear material. Bars = 200 nm

B. cereus cells. Ellar and Lundgren (1966) reported that addition of a mixture of Spore Wall Peptide Components (SPWPC) to growth medium, shortens the period of vegetative growth of *B. cereus* and obtains the developmental stages of sporulation in a faster rate. The SPWPC mixture ($200 \mu\text{g mL}^{-1}$) composed of L-aspartic acid, α - ϵ -diaminopimelic acid, D-glucosaminic acid and L-alanine. An irregularity was also observed in the shape of the spore (Fig. 4d and e) grown in PA solution, compared with the natural shape of spore which is a holosphere as showed by Ellar and Lundgren (1966). It is clear that *B. cereus* cells were not affected greatly by PA as *S. typhi* cells, where the cell wall and cytoplasmic membrane of *B. cereus* were not affected by PA except in some few cells (Fig. 4a). Geiger and Conn (1945) reported that the antibiotic activities of PA is due to its reaction with the sulfhydryl groups of bacterial enzyme systems or with sulfhydryl-containing metabolites essential to bacteria. The toxic effects of PA have been considered to be caused by its reaction with, for example enzymes and it has been shown to react with several amino acids to form less toxic products (Ciegler *et al.*, 1972). Kimmel and Tipton (2005) showed that PA is an irreversible inactivator of GDP-mannose dehydrogenase (which catalyzes the committed step in alginate biosynthesis) from *P. aeruginosa*.

By screening of the capability of different types of antibiotics discs for inhibiting of the growth of the tested bacterial species (Table 3), it was found that penicillin had no any inhibitory effect against

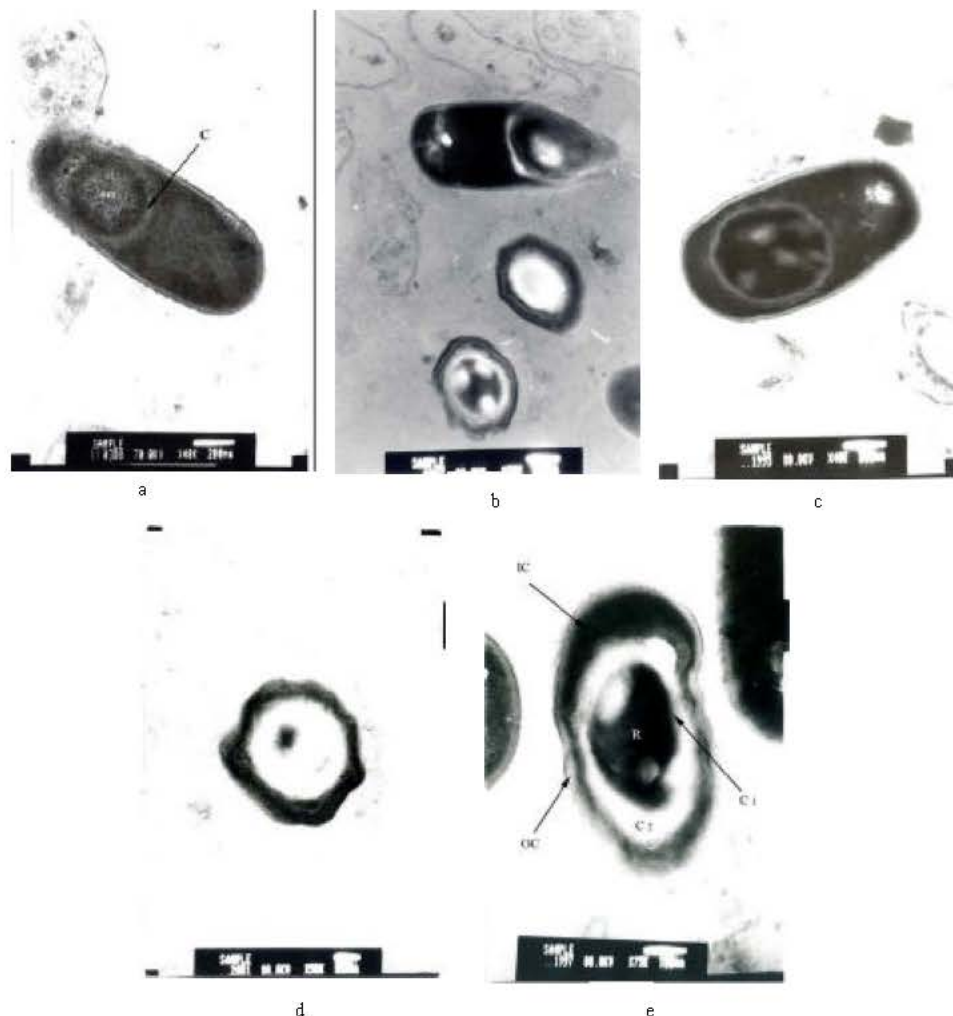


Fig. 4: Transmission electron micrographs of ultrathin sections of treated *B. cereus* cells with PA ($800 \mu\text{g mL}^{-1}$). nm, nuclear material; C, Cortex; C₁, inner dense layer of cortex; C₂, less dense cortical layer; OC, outer coat; IC, inner coat; R, ribosomal aggregate. Bars = 200 nm

the tested bacterial species except *Str. faecalis* which was the lonely tested species that was sensitive to penicillin inhibitory effect. The results also indicated that *K. pneumoniae* showed no response to the inhibitory effect of bacitracin, though this antibiotic had an inhibitory effect against the other tested bacterial species. Table 4 reveals that addition of PA to the penicillin discs had an inhibitory effect on the growth of *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi*, *Staph. aureus* and *Staph. citreus*. It was found that *S. typhi* showed the greatest sensitivity and *B. cereus* showed the lowest sensitivity to the combined effect of PA and penicillin. The results also show that *K. pneumoniae* that was resistant to bacitracin discs, was being sensitive to bacitracin discs saturated with PA. Even though PA is a mycotoxin, this experiment demonstrates that fungi produce QSI. On going future work, we prospect to find non-toxic QSI produced by fungi. This agree with the previous study (Rasmussen *et al.*, 2005) confirming that PA is QSI. It has previously been shown that *P. aeruginosa* biofilm cells are highly tolerant to antibiotic treatments (Anwar *et al.*, 1990). Hentzer *et al.* (2003) showed that biofilms treated with the QSI compound furanone C-30 became

Table 3: Diameter of inhibition zones (mm) of different bacterial species resulted from using a disc diffusion assay of different types of antibiotics

Antibiotic	Diameter of inhibition zones (mm)											
Bacterial species	A 1	A 2	B	C	E	N ₁	N ₂	O	P	T ₁	T ₂	V
<i>B. cereus</i>	21.0	15.0	15.0	25.0	29.0	27.0	16.0	28.0	-	25.0	18.0	17.0
<i>B. subtilis</i>	15.0	21.0	10.0	28.0	24.0	17.0	15.0	26.0	-	24.0	19.0	17.0
<i>E. coli</i>	15.0	13.0	11.0	32.0	25.0	25.0	15.0	30.0	-	25.0	17.0	20.0
<i>K. pneumoniae</i>	21.0	18.0	-	26.0	25.0	20.0	14.0	27.0	-	22.0	17.0	24.0
<i>P. aeruginosa</i>	17.0	24.0	12.0	32.0	28.0	26.0	18.0	28.0	-	21.0	20.0	20.0
<i>S. typhi</i>	27.0	25.0	10.0	35.0	32.0	31.0	20.0	36.0	-	30.0	20.0	24.0
<i>Staph. aureus</i>	15.0	15.0	10.0	30.0	20.0	22.0	18.0	27.0	-	20.0	17.0	22.0
<i>Staph. citreus</i>	25.0	16.0	11.0	31.0	26.0	27.0	16.0	29.0	-	27.0	18.0	21.0
<i>Str. faecalis</i>	46.0	4.02	20.0	36.0	36.0	34.0	21.0	41.0	26.0	30.0	23.0	30.0

A 1 = Ampicillin, A 2 = Amoxycillin, B = Bacitracin, C = Chloramphenicol, E = Erythromycin, N₁ = Nalidixic acid, N₂ = Neomycin, O = Ofloxacin, P = Penicillin, T₁ = Tetracycline, T₂ = Tobramycin, V = Vancomycin and - = no inhibition zone

Table 4: Combined effect of penicillin or bacitracin with PA on the tolerant species (diameter, mm)

Bacterial species	Diameter of inhibition zones (mm)	
	P+penicillic acid	B+penicillic acid
<i>B. cereus</i>	9.0	
<i>B. subtilis</i>	23.0	
<i>E. coli</i>	19.0	
<i>K. pneumoniae</i>	19.5	19.0
<i>P. aeruginosa</i>	17.0	
<i>S. typhi</i>	28.0	
<i>Staph. aureus</i>	18.5	
<i>Staph. citreus</i>	22.5	

susceptible to both SDS and tobramycin. Rasmussen *et al.* (2005) found that when *P. aeruginosa* biofilm treated with tobramycin, only a few cells were dead, where as almost all the cells in the biofilm treated with both tobramycin and either patulin or PA, were dead.

The results of Table 1 were analyzed statistically by ANOVA by comparison of the bacterial species at the mean values of different PA concentrations as a whole. At 0.05 level, significant values were obtained in comparison of *B. cereus* with all bacterial species except *P. aeruginosa*. Significant values were also obtained in comparison of *S. typhi* with *B. cereus*, *B. subtilis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. At 0.01 level, a high significance was obtained in comparison of *B. cereus* with all bacterial species except *P. aeruginosa*. A high significance was also obtained in comparison of *S. typhi* with *B. cereus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*.

For average lengths: at 0.05 level, significance values and at 0.01 level, high significant values were obtained in comparison of the control lengths of both *S. typhi* and *B. cereus* cells with those of treated. For average widths: at 0.05 level, insignificant values were obtained in comparison of the control widths of both *S. typhi* and *B. cereus* cells with those of treated.

At 0.05 level, a significance was obtained in comparison of *B. cereus* or *S. typhi* with all bacterial species. At 0.01 level, a high significance was obtained in comparison of *B. cereus* with all bacterial species also, high significance was obtained in comparison of *S. typhi* with all bacterial species except *B. subtilis*.

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