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Adaptational Changes in Cellular Morphology of *Bacillus subtilis* strain KPA in Response to certain Antimicrobials

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Abstract:
Objective- To determine the impact of certain antimicrobials stress on the cell morphology of a novel bacterial isolate.
Materials and Methods-In this regard, isolation and molecular characterization of the isolate from poultry farm faeces soil sample was done by serial dilution followed by morphological characteristics and biochemical tests of pure isolated culture. Further, the identification of bacterium as *Bacillus subtilis* strain KPA was confirmed by subjecting its amplicon (483 bp) to 16S rRNA gene sequence analysis and pairwise alignment through BLAST tool. A definite volume of antimicrobial agents such as *Allium sativum*, ampicillin and Mercuric chloride at their sub- MIC values was added to the lag phase culture of strain KPA. The bacteria grown in presence of these antimicrobials were observed using Gram staining technique at different magnifications of light microscope after 48 h of incubation.
Results- A drastic change in the cell morphology of stationary phase strain KPA was observed. *Allium sativum* and ampicillin were responsible for increase in the length of bacterial cell. Interestingly, cell aggregation was also noticed upon addition of *A.sativum* and ampicillin.On the other hand, cells treated with Mercuric chloride were able to change their morphology from rod shape to more or less round shape.
Conclusion-The stress response is a metabolic program activated in response to unfavorable conditions. These morphological changes clearly represent an adaptive response of strain KPA towards the stress.
Key words- Antimicrobials, *Bacillus subtilis*, Gram staining, light microscope, sub- MIC.

Introduction

Soil microorganisms are often exposed to drastic changes in environmental conditions. They have adaptive defence mechanisms, which allow them to survive and function in unfavourable conditions. Adaptive mechanism works in response to the stress conditions. The biological purpose of the stress response is to protect cells against lethal environmental factors and to repair damage due to stress conditions. The stress response is manifested as a change in the metabolic activity of the cell, resulting from the repressions of synthesis of most of the proteins formed in the cell under normal physiological conditions and induction of the synthesis of a specific group of proteins enabling the cell to function in the new conditions. [1] Bacteria are often exposed to changes in the environmental conditions. One of the physiological changes in the bacterial cell due to unfavourable environmental factors is their morphological changes. Bacterial cellular responses to different stress evoking physical and chemical stimuli have attracted current interest in Microbiology. A range of bacteria, including *E. coli*, *Bacillus* sp. and *Pseudomonas* sp.[2, 3, 4] have been reported to elicit an array of survival strategies in response to such changes. Stress responses among microbial population against an array of

environmental physico-chemical stimuli including temperature, salt, oxidative stress etc. are well known.[5, 6, 7] Natural plant products, antibiotics and toxic heavy metals provide stress to the bacteria below their inhibitory concentration values, resulting in the change of their cellular morphology. Several antimicrobial agents were isolated from plant including secondary metabolites such as essential oil, coumarins, flavonoids etc. The secondary metabolites of the medicinal plants have antibacterial, antiviral, antifungal etc. activities. These active secondary metabolites at low concentration are causative agents for providing mild stress conditions to the bacterium, resulting in the change of morphology and other metabolic activities of the cell. Natural antibiotics are secondary metabolites of the microorganisms living in natural environment. The microorganism undergoes several morphological changes such as filamentation, spheroplast formation and cell lysis in the presence of antibiotics.[8] The changes vary with the bacterial strain, the concentration of antibiotic, the length of exposure to the antibiotic and the inoculum size.[9] Many bacteria adapt to environmental stress through morphological changes, especially exposure to toxic heavy metals. Heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity.[10] Morphological alterations were induced when the bacterial cells were incubated with sub-inhibitory concentrations of some heavy metals. Bacteria utilize different strategies to adapt to varying environmental situations including exposures to high concentrations of heavy metals. One of the strategies that bacteria adapt against the stress conditions is the change in morphology. Such changes were observed in phototrophic bacteria on exposure to metalloid oxyanions [11]and in *Pseudomonas putida* and *Enterobacter* sp, in presence of toxic organic compounds.[12] As cellular morphological changes of bacteria in response to stress including high temperature, nutrients unavailability, oxidative stress, low and high pH, salt concentrations etc. have been studied but still there is less reports on the morphological studies of bacteria especially *Bacillus* sp. in presence of medicinal plants, antibiotics and heavy metals as antimicrobial agents. In view of this the present study was investigated to isolate novel strain of *Bacillus* sp. and to understand their morphological changes through light microscope at different magnifications under mild stress condition of *Allium sativum* (Medicinal plant), ampicillin (Antibiotic) and Mercuric chloride (Heavy metal).

Materials and Methods

Sample collection, Isolation and Screening

Poultry faeces sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India). Faeces sample was brought to the laboratory in aseptic condition. A serial dilution of the sample (1 gram of faeces soil) was made using sterile saline until a dilution of $10^{-6}$was obtained. 100 µl of this dilution was spread over nutrient agar petriplates and incubated at 37ºC for 24 hours. Pure culture was isolated and subcultured in the same medium at 37ºC. The culture was streaked and kept in incubator at 37°C for 24 hours and was preserved in slants at 4±2°C.

Morphological and Biochemical tests

Purified isolate was characterized by Biochemical analysis using Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test, Urease test, Oxidase test and Amylase test (according to the Bergey's Manual of Systemic Bacteriology). Gram staining, Endospore staining and Motility test were performed under Morphological tests.

Isolation of Genomic DNA

Two ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. One ml of UniFlex™ Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol: chloroformwas added and mixed well. The samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex™ Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporates. The pellet was resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at -20ºC.

Amplification of 16S rRNA genes by PCR, Sequencing and Alignment

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5` AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/.

Antimicrobials of interest

Allium sativum (Spice), ampicillin (Antibiotic) and Mercuric chloride (Heavy metal) were used to provide stress to the novel bacterial strain.

Sample preparation

Allium sativum (Garlic) was purchased from local market of Nungambakkam, Tamil Nadu (India). The garlic bulbs without the outer skins were grinded in a sterilized mortar and pestle. The fine garlic mesh was centrifuged at 6000 rpm for 10 minutes. The supernatant was filter sterilized by using a 0.2 µm syringe filter to produce sterile supernatant. Heavy metal salt solution was prepared by mixing Mercuric chloride ($HgCl_2$) in sterilized distilled water at the concentration of 25 mg/L. Ampicillin (10 µg) was prepared by mixing appropriate volume of DMSO.

MIC and sub- MIC determination of antimicrobials

MIC and sub- MIC values of *Allium sativum* juice, Mercuric chloride and ampicillin were determined by Microdilution method.[13] Serial dilutions of *Allium sativum* juice, Mercuric chloride and ampicillin were prepared from 100% to 1% concentration. Serial dilutions of antimicrobials were added to bacterial culture in Microtitre plate. The plate was incubated at 37ºC for overnight. The bacterial growth was detected by the addition of 3-(4, 5-dimetylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solutions (10 mg/ml) in the wells. The highest dilution of antimicrobials inhibiting the bacterial growth was considered as MIC value. Half of the value of MIC was considered as sub- MIC value.

Inoculum preparation

Fifty millilitre of inoculum medium containing nutrient broth (pH- 7.0) was transferred to 250 ml of conical flask and cotton plugged. The flask was sterilized at 121ºC, 15 lb pressure for 15 minutes. A loopfull of bacteria was inoculated aseptically into the cooled medium and kept for incubation overnight at 37ºC in a rotatory shaker.

Shake flask fermentation

The fermentation was carried out in conical flasks (volume capacity 250 ml), each flask containing 50 ml of Nutrient Broth. One flask was kept as control (no addition of antimicrobial agents). Rest of the flasks was labelled as *Allium sativum*, ampicillin and Mercuric chloride. The flasks were sterilized at 121ºC for 15 minutes. Each flask was inoculated with 500 µl of overnight bacterial inoculum. The flasks were kept in the rotatory shaker at 37ºC for 2 h (**Fig. 1-** lag phase of novel strain). The flasks were taken out and each flask except control was inoculated with appropriate volume of antimicrobials (from sub-MIC value) as labelled on the flasks. All the flasks were again kept in rotatory shaker for 48 h of incubation.

Cell morphology

Morphological features of the isolated bacteria were identified by using Gram staining technique under light microscopy made from control cultures as well as from the cultures grown at definite concentration of antimicrobial agents. An aliquot of 20 µl of each bacterial suspension was withdrawn by 48 h intervals and the shape and organization of cells were observed under light microscope at 10X, 40X and 100X magnification.

Results

Isolation and identification of new strain of bacteria

The isolated bacterial strain was identified as *Bacillus* sp. based on morphological and biochemical characteristic. Genomic DNA of the isolate was visualized under UV. The amplicon of 483 bp was observed using PCR amplification. In the present study, 16S rRNA gene sequencing of the isolate was investigated. The isolate was identified as *Bacillus subtilis* strain KPA by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. The comparison showed that the similarity of 16S rRNA gene sequences was 99%. The identities of strain KPA were determined by comparing them with the available sequences of the strains and with high scored rRNA sequences in BLAST search. The novel isolated sequence was deposited in Genbank (Accession number-KC918878), maintained by NCBI, USA.

MIC and sub-MIC determination of *Allium sativum,* ampicillin and Mercuric chloride

The effects of antimicrobial on bacterial cell growth were studied to determine the minimal inhibition concentration (MIC) value which could inhibit the bacterial growth. In this experiment, the bacterial viability was evaluated by using MTT solution as an indicator. The results from Table 1indicated that the MIC values of *A. sativum*, ampicillin and Mercuric chloride on *B. subtilis* strain KPA was 10%, 10% and 20% respectively. The sub-MIC values (which is 0.5 x MIC) for each treatments was further calculated as 5%, 5% and 10% for *A. sativum*, ampicillin and Mercuric chloride respectively.

Impairment of cell morphology due to stress

The changes in cell morphology of strain KPA under mild stress condition of *Allium sativum*, ampicillin and Mercuric chloride were observedusing Gram staining technique under light microscope at different magnifications. Light microscopic examination of stressed cells confirmed the heterogeneous nature of the cellular suspension when compared with non-treated stationary phase cells. The morphology of the cell of non-treated stationary phase cells at different magnifications (Fig.-2a, 3a, 4a) are quite similar to those of Gram (+) rod shaped *Bacillus subtilis* in general. Strain KPA grown in presence of *Allium sativum* were observed at 10X magnification and were found to be disrupted after 48 h of incubation. The cells were clearer and were observed in the form of aggregates at 40X magnification. On the other hand the length of the cell was found to be increased and cell density was found to be decreased when observed at 100X magnification (Fig.- 2b, 3b, 4b). The effect of ampicillin on strain KPA was observed as forming aggregates at 10X magnification. The length of the cells was observed to be increased at 40X and 100X magnifications (Fig.- 2c, 3c, 4c). The effect of Mercuric chloride on strain KPA was totally different from other two antimicrobials tested here. The cell size was observed to be shortened at 10X magnification. The cells were found to be separated apart when observed at 40X magnification. A drastic change in the morphology of strain KPA was viewed when it was observed at 100X magnification. The cells had lost their rod shaped morphology and were changed to more or less round shape (Fig.- 2d, 3d, 4d).

Table-1: Shows MIC and sub- MIC values for antimicrobials against strain KPA

Antimicrobials	MIC value (%)	Sub- MIC value (%)
Allium sativum	10	5
Ampicillin	10	5
Mercuric chloride	20	10

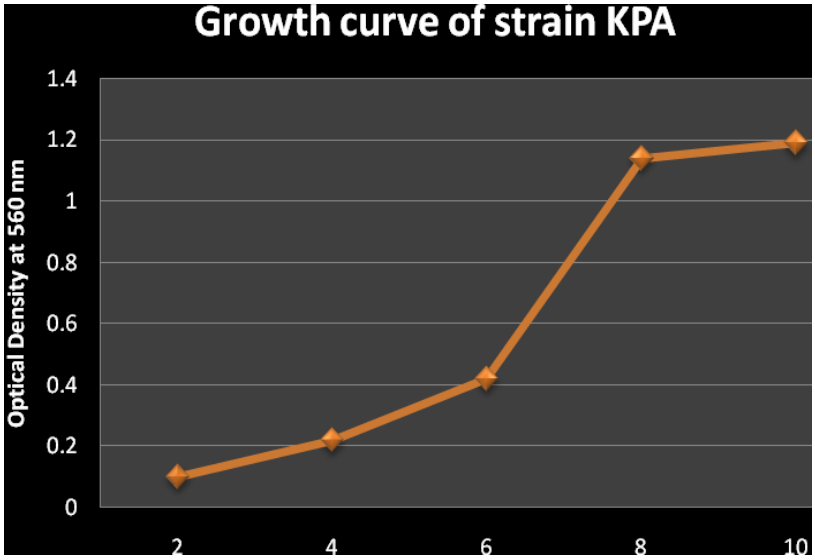

Fig- 1: Growth curve of strain KPA

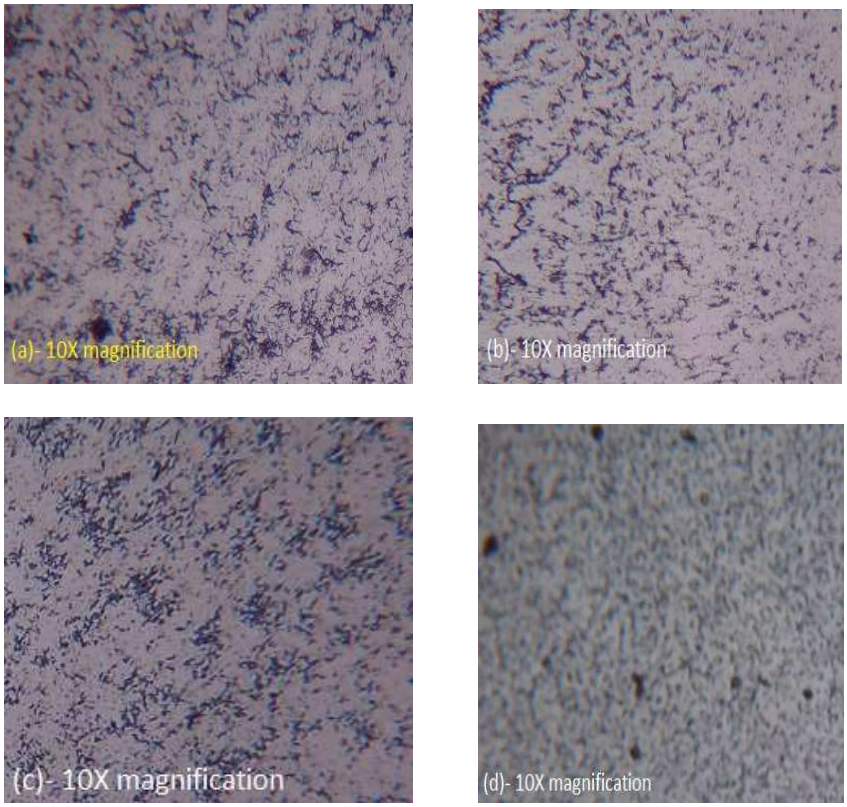

Fig.- 2: Shows cell morphology changes at 10X magnification. (a) Represents Gram staining of non-treated strain KPA, (b) represents Gram staining of bacteria grown in presence of *Allium sativum*, (c) represents Gram staining of bacteria grown in presence of ampicillin and (d) represents Gram staining of bacteria grown in presence of Mercuric chloride.

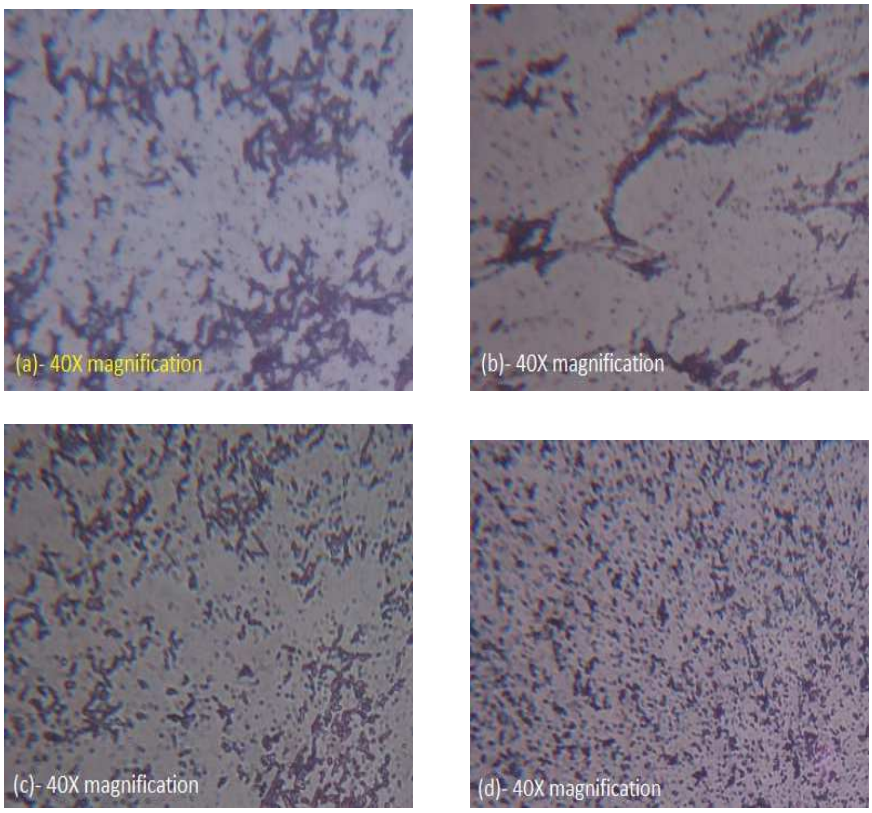

Fig.- 3: Shows cell morphology changes at 40X magnification. (a) Represents Gram staining of non-treated strain KPA, (b) represents Gram staining of bacteria grown in presence of *Allium sativum*, (c) represents Gram staining of bacteria grown in presenceof ampicillin and (d) represents Gram staining of bacteria grown in presence of Mercuric chloride.

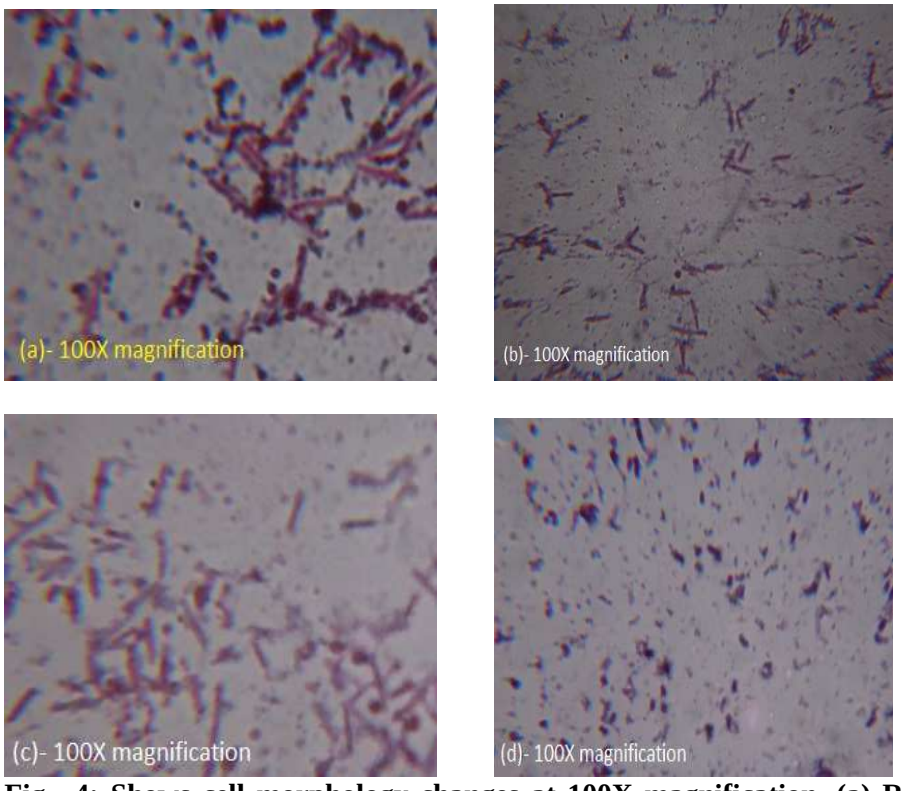

Fig.- 4: Shows cell morphology changes at 100X magnification. (a) Represents Gram staining of non-treated strain KPA, (b) represents Gram staining of bacteria grown in presence of *Allium sativum*, (c) represents Gram staining of bacteria grown in presence of ampicillin and (d) represents Gram staining of bacteria grown in presence of Mercuric chloride.

Discussion

Morphological variations of microorganisms in a novel environment are the most visible indicator of organismal adaptation.The changes in morphology as an adaptive response to adverse environmental conditions have already been reported with several bacterial species.[14,15] In the present investigation the morphology of different antimicrobial agents treated cells of *B. subtilis* strain KPA at different magnifications of light microscope provided strong evidence that the antimicrobial agents below their inhibitory concentration are stressful for the bacterial populations, characterized by changes in the shape and size of the bacteria. Our study reveals that strain KPA have evolved an adaptive response to *Allium sativum*, ampicillin and Mercuric chloride stress and have changed their shape. The apparent shape of a bacterium is determined by the geometry of its growing cell wall.[16, 17] Recently, a number of prokaryotic cytoskeletal proteins, such as FtsZ, MreB and crescentin, have been shown to be important for shaping the bacterial cell.[18, 19] These proteins regulate visible morphological changes that require cell wall growth and remodeling. Other proteins, such as PBP2 [20], RodA [21,22], and RodZ [23] can also affect the morphology of bacteria. These proteins colocalize with MreB to form a helical complex and are indispensable for the proper assembly of the MreB helix. Depletion of these proteins can also disrupt the MreB helix and lead to altered cell shapes. Low *et al*. [24] reported that Garlic (*Allium sativum*) and its bioactive components have the ability to change the morphology of *Candida albicans* by suppressing hyphae production. Hyphal production is an essential virulence determinant of *C. albicans* for invasive infections, therefore garlic and itsconstituents can be effective not only against colonizing *C. albicans* strains present in mucosal infections, but also virulent strains causing systemic or invasive candidiasis. In the present investigation *Allium sativum* was also able to change the morphology of strain KPA by increasing the length of the cell. This may be due to the reason that after 48 h of incubation (stationary phase), the cells were metabolically inactive and the cell size is inversely proportional to metabolic rate of the bacterial cell. *Allium sativum* at sub-MIC value triggers several morphological and biochemical changes enabling the strain KPA to escape the deleterious effects of this spice. Bacteria in the stationary phase are characterized by a number of adaptations that enable them to utilize available sources of energy and carbon sparingly. Most often this is achieved by means of restricted energy expenditures for growth and reproduction, a reduced metabolism rate, and activation of autolysis processes that ensure the supply of new food substrates. Cell structures such as cell envelope, the nucleoid, and the ribosomes become modified in order to increase their stability. As a result of these changes, the cells become resistant to harmful physical and chemical agents (Swiecilo and Zych-Wezyk, 2013). In this study the length of bacterial cell was increased under the stress of ampicillin. Our study favors the finding of Oli *et al*.[25]who demonstrated that cell morphology of antibiotic treated cells of *E. faecalis* under SEM were found to be increased. Morphological alterations were induced when the bacterial cells were incubated with sub-inhibitory concentrations of some heavy metals (e.g., Cd, Cu, Ni and Zn). Loosely packed coccobacillus type normal cells formed characteristics chains of coccidal lenticular shape with constrictions at the junctions between them in presence of Cd or Ni. Cu induced transformation of cells to becoming round-shaped, while Zn turned the cells filamentous and aggregated.[26] The cell wall of *B. subtilis*, as one type of Gram positive bacteria, has been well characterized and it has metal sequestering properties.[27] Cells length were decreased by (33.4%) for *B. subtilis* than cells cultivated in metal free medium. In the present investigation cell morphology of *Bacillus subtilis* strain KPA, under the mild stress condition of Mercuric chloride was changed from rod shape to more or less round shape and the cell size was also reduced. Our study strongly favors the finding of Chakravarty *et al*.(2007) who demonstrated that bacterial cells in presence of heavy metals were mostly aggregated and elongated form. The rod shape was lost and the strain was observed as a mixed population of spherical and elongated cells in packed aggregation as well as in individual form.

Conclusion

Our investigation clearly demonstrated the impact of certain antimicrobials such as *Allium sativum*, ampicillin and Mercuric chloride on cellular morphology of *Bacillus subtilis* strain KPA. The length and the shape of the bacterial cell were found to be changed when the bacterium was treated with antimicrobial agents at their sub minimum inhibitory concentration. To sum up, the reported results shed new light on the behavior of Gram positive bacteria under mild stress conditions. These antibacterial agents trigger several morphological and biochemical changes enabling strain KPA to escape the deleterious effects of certain antimicrobials. Further study is necessary to observe the drastic changes in the morphology of strain KPA through Scanning electron microscope (SEM). Another investigation should also be continued to know about the genes of strain KPA which are affected by these certain antimicrobials at their sub-MIC values.

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