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Study of Alterations of Bacterial Membrane Proteins involved in β-Lactam Sensitivity

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Abstract

The selective nature of organisms to prevailing stress– β-lactam antibiotics is obviously concerned with the architecture of outer membrane. *Bacillus subtilis* Gram-positive bacteria advocates variation in achieving cellular morphogenetic dimensions. In the present study more protonated species of β-lactam i.e. Ceftriaxone proved to be more effective in contrast to cefazolin as far as sensitivity of *Bacillus subtilis* is concerned. The MIC_{50} for ceftriaxone (CT) was observed to be 1.5 ppm in contrast to first generation β-lactam antibiotic i.e. cefazolin with MIC_{50} 18 ppm concentration. The relative sensitivity of bacterial cells to both the cephalosporins was more positively inclined to third generation species. The role of modified cephalosporin was observed under various physiological situations under laboratory conditions such as pH, Temp, chelating agent and Mg^{2+} ion concentrations. The sensitivity of *Bacillus subtilis* to β-lactam was more potentiated at acidic pH than at alkaline pH. At alkaline pH only 16% inhibition of growth of *Bacillus subtilis* in Dye's minimal media at 37 ± 1^0C was seen in contrast to 60% inhibition of growth in the presence of CZ. The growth was enhanced / potentiated in the presence of chelating agent – EDTA at 0.25 mM concentration in combination with MIC_{50} of CT and CZ 84% inhibition of growth was recorded in the presence of CT (MIC_{50}) in contrast to 82% inhibition in the presence of CZ (MIC_{50}). The growth inhibition with 0.25 mM EDTA alone was also seen to be 82%.

Keywords: β-lactam, EDTA, MIC_{50}, Ceftriaxone sodium, Cefazolin sodium

1. INTRODUCTION

Antibiotic resistant gram-positive bacteria have become an increasing problem in the last two decades. Despite advances in antimicrobial therapy the incidence of severe infections caused by multiple drug resistant bacteria has been increasing over the past 20 years. Whereas resist ant gram negative bacteria were a major problem in the 1970's, the past decade has seen an increase in the number of problems associated with multidrug resistant gram positive bacteria, including methicillin resistant S*taphylococcus aureus* (MRSA), methicillin – resistant coagulase-negative *Staphylococci* (MRCONS), penicillin-resistant S*treptococcus pneumoniae*, and vancomycin-resistent enterococci (Baquero.F., 1997 and Moellering. R.C., 1998).

β-lactams are the antibiotic compounds most widely used against hospital and community acquired infections. However, resistance has emerged in both gram positive and gram-negative bacteria, limiting their therapeutic efficacy. The choice of appropriate treatment depends on analysis of susceptibility data that indicates a specific mechanism of resistance. Correct interpretation of susceptibility tests permits a rational approach to the resistance problem and selection of alternatives for treatment. The laboratory must first be able to identify accurately microorganism to the species level and then test a minimum of relevant antimicrobials. β-lactam resistance in enterobacteriaceae is mainly due to the production of plasmid or chromosomal encoded β-lactamases. In Gram-negative non-fermenting bacteria, impermeability and efflux are relatively more important to the treatment selected. In Gram-positive proteins (PBPs), production of new PBPs or synthesis of β-lactamases (Arias and Panesso, 2003).

Resistant to β-lactam containing antimicrobial agents continues to increase frequently due to the presence of β-lactamases in gram-negative bacteria. Over the past twenty-five years broad-spectrum enzymes such as TEM and SHV variants and the metabolic β-lactamases have become more prolific. As a result of the ability of plasmids to continue to acquire additional resistance determinants, many of the β-lactamases producing gram-negative pathogens have become multi-drug resistant. In combination with decreased permeability, the organism can become virtually

untreatable with current therapies. The major groups of β-lactamases that pose β-lactamages, the plasmid mediated cephalosporinages; the inhibitor resistant TEM or SHV derived β-lactamases and the carbopenem hydrolyzing β-lactamases. These enzymes that can be transferred on mobile elements are the most serious of the newer β-lactamases and include enzymes in each of the four groups of cephalosporins (Bush K., 1999).

The sequence for the genome of *Bacillus subtilis* was completed in 1997 and was the first published sequenced living bacterium. The genome is 4.2 mega base pairs long with 4,100-protein coding regions. Bacillus and plant growth promoting rhizobacterium are known to synthesis antifungal peptides. This ability has lead to subtilis in biocontrol, Bacillus *subtilis* has been shown to increase crop yields, although it has not been shown because it enhances plant growth, or inhibits diseases growth. Bacilli are rod shaped, gram positive, sporulating, aerobes or anaerobes. Most bacilli are saprophytes each bacterium creates spores, which are resistant to heat, cold, radiations, desiccation, and disinfectants. Bacilli exhibit an array of physiologic abilities to live in a wide range of habitats, including many extreme desert sands, hot springs, and arctic soils. Species in the generation can be thermophillic, psychorophillic, acidophilic alkaliphilic, halophilic and capable of growing at pH values, temperature concentrations where few other organisms can survive *Bacillus subtilis*, inhibit the rizosphere, which is the interface between root and the surrounding soil. The plant roots and associated biofilm can have a significant effect often on the surrounding soil, creating a unique environment. It has recently been shown that *Bacillus subtilis* engages in cannibalism. They use cannibalism as the easy tool to fight extreme cases. For survival in harsh environments, bacilli can form spores, but it is very costly to them easier way, is for the bacteria to produce antibiotics that destroy neighboring bacilli, so that their content helps in allowing for the survival of the few of the bacteria.

The Bacteria have evolved variety of unique mechanisms by which they can immobilize proteins on their surface. Such mechanisms are directly related to the involvement of either phosphoglycerides (PG) showing attachment with protein by covalent linkage or other proteins involved in formation of non-covalent binding of protein to PG or other polymers for eg. Techoic acids. These covalently, linked protein of gram positive species are known to be involved in confirming survival of infectious particles with in an infected host (Schneewind et. al., 1995).

Penicillin binding proteins PBPs helps in catalyzing the cell wall assembly (Ghuysen. 1991). These proteins polymerize PG on the external surface of the cell membrane *Bacillus subtilis* genome reveals presence of three classes of PBPs (Murray et. al., 1998). Details genomic analysis of *Bacillus subtilis* shows presence of six low Mol.wt PBP encoding genes, dacA, dacB, dacC, dacF, PbPE and PbPX (Popham et. al., 1999).

However, class 'A' PBPs are involved in promotion of polymerization of glycan and transpeptidation HMW PBPs are known to be functional enzymes that have been characterized on the basis of sequence similarity. These proteins continue to cleave β-lactam rings thereby reducing the binding of proteins. In contrast HMW PBPs of class 'B' are not fully explored till date. Subsequent to this 5-15 nm thick S-layer is found which covers the total cell surface and provides a selective advantage of serving as protective coat (Sleytr et. al., 1993; Messner and Sleytr., 1992; Hovnalle et. al., 1988; and Sleytr and Messner., 1983). It has been reported that 15% of total protein of bacterial cell contributes to S-layer protein as confirmed in case of growth analysis (Sleytr et. al., 1993). Under stress conditions the loss of survival of cells is associated either with damage of outer cell wall or by lysis of apart of population. Presently it is confirmed that several compounds are being leaked out in intracellular spaces it response to adaptation to extreme environmental conditions (Tsiomenko and Tuimetova., 1995; Luphashin et. al., 1991 and Melechove., 1985; 1983). There are several species of gram positive bacteria which are known to colonize mucosal surface of animals and humans without causing any disease such organisms constantly interact with the host immune system which is involved in prevention of occurrence of disease. Proteins of the outer surface of gram-positive bacteria display their functional domains to the ambient media. The number of appearance of newly synthesized proteins as triggered by stress conditions supports the view that extracellular molecular proteins are involved positively in protective behavior under unfavorable situations (Roshchina and Pectrov., 1997). In case of gram-positive bacteria up to 20% polypeptides synthesized are known to be located on outer membrane. The cellular couplings mechanisms directly involve in primary as well secondary transport system are dependent upon such functional proteins. The involvement of proteins in translocation is a challenging task to explore, as most of the functional carriers under stress conditions are chemically proteins. Therefore, susceptibility of gram-positive bacteria – *Bacillus subtilis* to antibiotic stressed might be involve in alteration of outer membrane as well as cellular proteins.

2. Method

The present study was conducted at the department of Microbiology, Barkatullah University, Madhya Pradesh, India. Institutional ethical board approved the study and the informed consent was obtained from all the subjects before the commencement.

2.1Organism and Culture Conditions

Bacillus subtilis NCIM 2063 strain used for the present study is a non pathogenic Gram- positive rod, and obligate aerobe (Plate 01: a; b). It is known to form protective end spore thereby providing tolerance to extreme environmental conditions. It was obtained from National Chemical Laboratory (NCL) Pune, India. The bacterial culture was routinely maintained on presterilized NAM at 04^0C. The culture was routinely monitored by single colony isolation method on Nutrient Agar plates for confirmation of purity of culture.

Chemical as well as reagents used in the present experiments were of reagent grade. However, antibiotics preferred for the experimental purpose for eg. Ceftriaxone (CT) sodium and Cefazolin (CZ) sodium were procured from Lupin Laboratories Ltd. M.P., India under the common generic names. During experimentation *Bacillus subtilis* cultures were grown in Erlenmeyer flask containing presterilised growth medium. Incubation was done at 37^0C ± 0.1 in thermostatically controlled orbital shaker (Lab India, Model, 3521) under aerobic condition with plate rotation of 180 revolutions per minute.

2.2 Inoculum Preparation

Fresh colonies were picked up and suspended in 1 ml of the Dye's minimal medium. The population size of the inocula was estimated regularly by the optical density method as at 540 nm to avoid sporulation.

2.3 Chemicals & Protein Assay

All the chemicals were of reagent grade obtained from Loba Cheme. Ltd., India and Himedia Labs. Ltd., India. Lysozyme ATP (Adenosine triphosphate, EDTA), Ascorbic acid, Ammonium molybdate, TCA (trichloroacetic acid), Folin ciocalteous phenol reagent were obtained from Merck India. Ltd., India. Triphenyl tetrazolium chloride and sodium succinate were purchased from Loba Chemicals, India. Cytochrom C (Sigma type III, horse heart), phenazine methosulphate and N-N, N-N, tetramethyl –P – Phenyldiamine (TMPD) were purchased from Himedia Laboratories Pvt. Ltd. India. The molecular weight marker proteins for eg. Bovine serum albumin (67kDa), Ovalalbumin (45 kDa), Chymotrypsinogen (25 kDa), Cytochrome (12.5 kDa) and Insulin (5.7 kDa). were procured from Sigma Chemical, USA. Protein concentration in the supernatant (released) was estimated by Lowry's (1951) and ultraviolet absorption method (1951) (Kalckar., 1947; Warburg and Christian., 1941).

2.4 Preparation of Membrane Vesicles

Bacillus subtilis cells ere harvested and converted to membrane vesicles by employing Konings and Kaback (1973) procedure with slight modifications (Singh and Bragg., 1976). *Bacillus subtilis* culture was harvested at mid exponential phase of growth out washed twice with 0.3 M (pH 8.0). Tris HCl buffer for 10 minutes by repeated centrifugation at 10,000 rpm (C-30, refrigerated centrifuge, Remi, India). The washed cells were then suspended in 30 mM Tris – HCl containing 20% (w/v) sucrose at 22^0C. This was followed by the addition of aqueous solution of EDTA (1mM) and Lysozyme (5 $mg.g^{-1}$ of cells). The suspension was then stirred gently. After 30 minutes of incubation at 22^0C, the suspension was diluted 10 folds with $MgCl_2$ (0.2 mM) containing small amount of deoxyribonuclease). Now the resulting lysate was rapidly stirred and the decrease in turbidity was followed for 20-40 mins at 420 nm, until the turbidity reached a constant minimum value. The lysate was then centrifuged at 15,000 xg for 30 minutes to recover whole cells and lysed spheroplast membrane in the pellet. The resulting pellet was then resuspended in Tris-HCl buffer (30mM, pH 8.0). This was followed by low speed centrifugation (3000xg), separating the whole cells from the membrane vesicles. The supernatant containing membrane vesicles were carefully removed and then passed through Millipore membrane filter (0.45 μ) to remove any soluble protein. The membrane vesicles so obtained were then used for transport assay studies.

2.5 ATPase Assay

ATPase activity was estimated in 3.0 ml of 100 mM Tris-HCl buffer (pH 6.8), 5mM ATP (disodium salt) *and 5mM $MgCl_2$ by the method of Davis and Bragg (1972) with slight modifications. The reactions were* stopped by addition of 1.5 ml of 10% (v/v) trichloracetic acid (TCA) after 30 mins. of incubation at 37^0C. The solutions were then

centrifuged at 10,000xg for 10 mins. Aliquots of TCA supernatant were than assayed for inorganic phosphate (Pi) by the method of Ames (1955). Assay of enzyme activity was performed by adding 2.5 ml of a reagent containing 1% (w/v) ascorbic acid, 10% (w/v) TCA, 0.31 gms of ammonium molybdate and 1.8 ml concentrated H_2SO_4. After incubation at 45^0C for 20 mins the absorbance was read at 660 nm with Shimadzu 1610 UV-VIS Spectrophotometer. The calibration curve of Pi was prepared with KH_2PO_4 solution, the Pi released during hydrolysis catalyzed by ATPase was measured by development of blue column protein content of membrane vesicles as well as bacterial cell was measured by Lowery's et al., (1951) method.

2.6 Assay of protein profile using HPLC

Confirmation of stress proteins as evident by the presence of β-lactams , CT and CZ was done by the HPLC (Shimadzu model LC-10AT) for the study of molecular weight determination of proteins found in the sample Bovine serum albumin (67 kDa), Ovalalbumin (45 kDa), Chymotrypsinogen (25 kDa), Cytochrome (12.5 kDa) and Insulin (0.57 kDa) was used in pure form. The migration of protein in the column was observed when 25 µl of protein samples were injected. The proteins show different rate of movement due to their different molecular size for every sample 4000 psi pressure was maintained and flow rate was adjusted to 1.0 ml min-1 the solid phase was protein-PAK column (Shimadzu) and mobile phase was 0.1 M phosphate buffer (pH 6.8). The proteins were measured at absorbance of 280 nm after carrying out elution process for 30 minutes. Protein –PAK column (7.8 mmx30 cm, total permeation volume of 12 ml) provide rapid separation, purification and characterization of proteins using gel filtration. These columns are packed with a rigid hydrophobic porous silica gel and are manufactured with exclusive bonding processes that improve column stability and minimize non-specific adsorption. HPLC technique offers advantages of speed and specificity and uses equipment that is versatile in its operation. Column is the essential feature of HPLC. The column allows high resolution at speedy flow rate. HPLC offers advantages in speed and ease of sample recovery. Therefore, it offers greater advantage over electrophoresis and open column chromatography. The column Chromatographic analysis helps in separation and identification of biological samples. Allowing the sample to move in a column containing partitioning material and eluting the mixture by pumping the solvent through the column do this.

2.7 Effect of various factors

The concentration used for CT and CZ was 1.5 ppm and 18 ppm respectively. The membrane vesicles of *Bacillus subtilis* thus prepared are treated in buffer solution and samples are separated by centrifugation at 10,000 rpm for 10 minutes (Remi, C-24 refrigerated centrifuge). The detection of protein was done at 0, 30 minutes time intervals. Using HPLC at 280nm pH, temperature and EDTA are used as stress conditions similar to growth studies in the presence and absence of β-lactam antibiotics (CT and CZ). Total protein content of *Bacillus subtilis* was measured and the membrane vesicles are also being considered for detection of membrane protein. Similar to detection of stress proteins the data is supported by estimating cellular enzymes in the presence of varying physiological condition of pH, Temp, and EDTA etc.

3. Results

Similar to growth inhibition studies as observed with reference to β-lactam antibiotics under various stress conditions the cellular metabolism was also seen to be affected. Prokaryotic cells including *Bacillus subtilis* test organism shows differed cellular metabolism in the influence of several factors. Once the cell enters in propagation of growth all the coordinated pathways are being driven by chemical energy either present or being synthesized simultaneously. The status of intermediary metabolic reactions is governed directly by various vital reactions of the cells governed by membrane potential. The membrane potential indirectly is regulated and maintained by the translocation of H^+ ions across the transducing membranes. The direct involvement of activated transducers can be indirectly accessed by monitoring cellular energy status directly or indirectly (Serrano., 1998 and Goffeau and Slayman., 1981). In the present investigation the level of inhibition of cellular activity of *B.subtilis* is monitored similar to growth performance study, to generate direct involvement of energy status, in growth of *B.subtilis* in Dye's minimal media. Such observations will help in elucidation of molecular mechanism of drug resistance / sensitivity in case of gram-positive bacteria. In control conditions of optimum pH of 7.0 ± 0.5 the response of Bacteria to β-lactam stress was modified as it seen in growth observation studies.

As compare to growth of *Bacillus subtilis* the pattern of ATPase enzyme activity was seen as illustrated in Figure 2. As compare to acidic pH the enzyme activity at alkaline pH was less however at neutral pH 630

μgPi.mg.protein^{-1} .min^{-1} ATPase activity was seen. The pattern of inhibition of ATPase enzyme activity in *B.subtilis* at pH 7.0 in the presence of CT and CZ at their MIC_{50} concentrations (1.5 ppm and 18 ppm) observed to be consistent with the trend followed in Figure 1. As compared to alkaline pH of 8.0 the enzyme activity was more at pH 5.0 under similar condition of antibiotic concentrations. Not much variation at alkaline pH with respect to CT and CZ β-lactam is seen which might be due to alterations in energy budget of the cells, when subjected to β-lactam antibiotics. Therefore, from the present observation it can be concluded that at alkaline pH β-lactam are less sensitive than acidic pH. The transmembrane proton gradient by H^+-ATPase which is involved as an energy transmitter for processes such as synthesis of energy molecule ATP, transport of nutrients, maintenance of K^+ balance and ultimate regulation of internal pH (Zilberstein et. al., 1984). Apart from this the proton pumps are involved in the control of various dimensions of cellular metabolism. In case of extreme cellular stress such as pH, the alkaline pH tends to be more supportive n contrast to acidic pH as under unfavorable conditions extracellular pH leads to diminished rate of energy generation thereby affecting the rate of ATPase activity. However when *B.subtilis* cells were subjected to acidic pH of 5.0 a slight increase in H^+ ATPase activity appears to be due to increased ADP and AMP pools. In such conditions the energy supply by ATP molecule is used for pumping out of protons, which is required to maintain the stable pH. The ATPase activity observation in case of *B.subtilis* at neutral pH 7.0 was observed to be slightly more pronounced as than in case of presence of β-lactam antibiotics at all pH condition. The reduced ATPase activity as seen in Fig. 2 could be correlated with the observed influence of β-lactams in response to growth studies as represented in Fig. 2.

On analyzing the protein release during the course of treatment of *B.subtilis* cells with CT and CZ at 25^0C the pattern of protein concentration with respect to pH conditions reflects remarkable variation (Fig. 3, 4). As reviewed by observed data the concentration of protein in all the pH conditions were found to be the pH conditions were found to be in the same pattern at pH 5.0, pH 7.0 and pH 8.0. However, the protein concentrations in the supernatant after harvesting the *B.subtilis* cells was maximum in the influence of CZ and In case of CT, in membrane vesicles derived from growing cells of *B.subtilis* 0.7 mg^{-1}.ml^{-1} was released at pH 8.0 where as only 0.9 mg^{-1}.ml^{-1} protein release was measured after 30 minutes of incubation from membrane vesicles. The quantity of protein released from intact cells of *B.subtilis* when subjected to both the antibiotics at MIC_{50} concentrations followed the same pattern of membrane vesicles (Fig. 3). Presence of more protein content in the external medium after treatment with β-lactam reflects the probable cause of enhanced sensitivity of *Bacillus subtilis* cells. Further characterization of speciation of protein thus liberated under antibiotic stress would be explained in subsequent observations, with High performance chromatography technique.

When cells were grown in the presence of 1 mM Mg^{2+} ion concentration in combination with chelator 0.25 mM the pattern of protein as observed by HPLC studies. No much effect on pattern of inhibition of growth by chelation (EDTA, 0.25 mM) contrary to, that the protein released from the intact cells when *Bacillus subtilis* was grown in the presence of EDTA (0.25 mM) protein leakage in the exterior of the cell was potentiated by CZ. Therefore, from the present study it could be inferred that the toxicity of CT as observed greater than CZ was not due to washing of membrane proteins but alteration of intake (uptake) pattern regulated by cellular transport mechanism. Similar to EDTA, Mg^{2+} ion alone was found to enhance protein release even at 5mM concentration up to 15 mM in the presence of CZ not in the absence, of β-lactam. However, the absence of Mg^{2+} ions in the growth medium caused similar pattern of protein release into the exterior of the cells. The observation pattern thus confirms that Mg^{2+} does not pay any role in making availability of rather structured, as well as functional protein of *B.subtilis* cells (Fig. 5). The resolution analysis of proteins in the presence of 1.0 mM Mg^{2+} and 0.25 mM EDTA shows appearance of protein peak after 20 mins of sample run in the column showing presence of different molecular configuration of protein greater than 67 kDa when referred with standard curve.

The Gram-positive bacteria, *Bacillus subtilis* when subjected to antibiotic stress the profile of protein as studied in the presence of Ceftriaxone (CT) and Cefazolin (CZ) β-lactam antibiotic stress reflects variation in protein profile by HPLC studies. Similarly *Listeria monocytogenes* Gram-positive bacteria cells has been reported to have acid shock with HCL had significantly greater heat resistance when compared with non-acid-shocked cells (Farber and Pagotto; 1992). Studies with Gram-negative *Escherichia coli* have shown that a shift to a lower pH induces the synthesis of at least four heat shock proteins (Lee et al. 2003).It has been shown that acid induced death is the direct result of lowered pH_i (Foster and Hall 1991). Severe acidic pH creates a situation whereby protons leak across the membrane faster than housekeeping pH homeostasis (the ability of an organism to maintain its cytoplasmic pH at a

value close to neutrality, despite fluctuations in the external pH) systems can remove them (O'Driscoll et al. 1997). The result is an intracellular acidification to levels that damage or disrupt key biochemical processes (Bearson et al., 1998). Weak acids in their unprotonated form can diffuse into the cell and dissociate thereby lowering the intracellular pH (pH_{in}) and resulting in the inhibition of various essential metabolic and anabolic processes. In response to encounters with acids, microorganisms have evolved complex inducible acid survival strategies (Abee and Wouters 1999).

The gradual susceptibility of *Bacillus subtilis* to CT and MIC_{50} (1.5 ppm) in contrast to CZ MIC_{50} (18 ppm) appears to be differential selectivity of bacterial membrane to both the β-lactams even at neutral pH / the sensitivity to drugs was more pronounced at alkaline pH. The integrity of membrane proteins in the influence of CT reflects valuable amount of release of protein (Fig. 6), after 30 mins of run shows reason for susceptibility to β-lactam. In the present study the availability of proteins under stress was seen to be modified under various physiological conditions. The appearance of drug resistance in therapeutic purposes under treatment conditions might be responsible for induction of variable protein species under clinical procedures. The appearance of proteins in the medium cannot be confirmed by present investigation that they were genetically induced by β-lactam antibiotics.

4. Discussion

The sensitivity of *Bacillus subtilis* to β-lactam was more potentiated at acidic pH than at alkaline pH. At alkaline pH only 16% inhibition of growth of *Bacillus subtilis* in Dye's minimal media at 37 ± 1^0C was seen in contrast to 60% inhibition of growth in the presence of CZ. The growth was enhanced / potentiated in the presence of chelating agent – EDTA at 0.25 mM concentration in combination with MIC_{50} of CT and CZ 84% inhibition of growth was recorded in the presence of CT (MIC_{50}) in contrast to 82% inhibition in the presence of CZ (MIC_{50}). The growth inhibition with 0.25 mM EDTA alone was also seen to be 82%. It could be concluded from the present observation that EDTA alone has negative role in bacterial survival. The chelation of ions by EDTA is known to disrupt the orientation and integrity of cell wall thereby causing leakage of cellular substances thus resulting into retardation of *Bacillus subtilis* cell growth. Mg^{2+} at a concentration of 15mM with 0.75mM EDTA was highly effective in all the condition of control as well as with CT and CZ antibiotics. However ATPase activity in the presence of 15mM Mg^{2+} ion + EDTA (0.75mM) was seen to be 56, 18 and 22 $\mu gPi.mg.protein^{-1}.min^{-1}$ in the absence of antibiotics and in the presence of CT and CZ respectively. As far as effect of temperature is observed at 25^0C and 50^0C in relation to drug sensitivity the level of inhibition in absence of antibiotic and in their presence was more pronounced at 25^0C and 50^0C. This might be because of conformational change of *Bacillus subtilis* due to temperature stress. However, at routine temperature of 37^0C no inhibition of growth was recorded in control conditions whereas, both the cephalosporins selected for the present study were found to have similar level of inhibition of growth of *Bacillus subtilis* i.e. 62% and 60% the presence of CT and CZ respectively.

5. Conclusion

The selective nature of organisms to prevailing stress– β-lactam antibiotics is obviously concerned with the architecture of outer membrane. *Bacillus subtilis* Gram-positive bacteria advocates variation in achieving cellular morphogenetic dimensions. It is known to disrupt the barrier of stress applicable in the growth condition by undergoing morphological as well as physiological modifications. The induction of spore formation is an established fact with *Bacillus subtilis*, which offers obstructions in laboratory appearance as well as positive diagnosis due to this property. The therapeutic application of cephalosporins therefore faces a challenging situation to successful, susceptibility for drug treatment and application.

Modifications of compositional structure by strengthening ionic density with reference to cephalosporin administration in clinical trials have become a routine task for therapy. In the present study more protonated species of β-lactam i.e. Ceftriaxone proved to be more effective in contrast to cefazolin as far as sensitivity of *Bacillus subtilis* is concerned. The MIC_{50} for ceftriaxone (CT) was observed to be 1.5 ppm in contrast to first generation β-lactam antibiotic i.e. cefazolin with MIC_{50} 18 ppm concentration. The relative sensitivity of bacterial cells to both the cephalosporins was more positively inclined to third generation species. The role of modified cephalosporin was observed under various physiological situations under laboratory conditions such as pH, Temp, chelating agent and Mg^{2+} ion concentrations. The sensitivity of *Bacillus subtilis* to β-lactam was more potentiated at acidic pH than at alkaline pH. At alkaline pH only 16% inhibition of growth of *Bacillus subtilis* in Dye's minimal media at 37 ± 1^0C was seen in contrast to 60% inhibition of growth in the presence of CZ. The growth

was enhanced / potentiated in the presence of chelating agent – EDTA at 0.25 mM concentration in combination with MIC_{50} of CT and CZ 84% inhibition of growth was recorded in the presence of CT (MIC_{50}) in contrast to 82% inhibition in the presence of CZ (MIC_{50}). The growth inhibition with 0.25 mM EDTA alone was also seen to be 82%. It could be concluded from the present observation that EDTA alone has negative role in bacterial survival. The chelation of ions by EDTA is known to disrupt the orientation and integrity of cell wall thereby causing leakage of cellular substances thus resulting into retardation of *Bacillus subtilis* cell growth. Mg^{2+} at a concentration of 15mM with 0.75mM EDTA was highly effective in all the condition of control as well as with CT and CZ antibiotics. However ATPase activity in the presence of 15mM Mg^{2+} ion + EDTA (0.75mM) was seen to be 56, 18 and 22 $\mu gPi.mg.protein^{-1}.min^{-1}$ in the absence of antibiotics and in the presence of CT and CZ respectively. As far as effect of temperature is observed at 25^0C and 50^0C in relation to drug sensitivity the level of inhibition in absence of antibiotic and in their presence was more pronounced at 25^0C and 50^0C. This might be because of conformational change of *Bacillus subtilis* due to temperature stress. However, at routine temperature of 37^0C no inhibition of growth was recorded in control conditions whereas, both the cephalosporins selected for the present study were found to have similar level of inhibition of growth of *Bacillus subtilis* i.e. 62% and 60% the presence of CT and CZ respectively.

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Figures

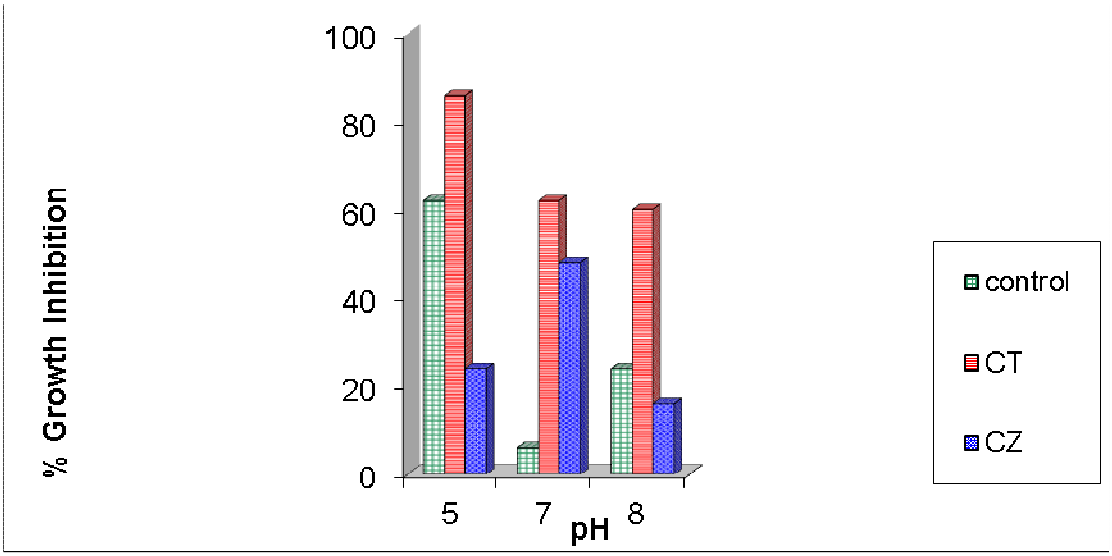

Figure 1: Percentage growth inhibition of *Bacillus subtilis* at pH (5, 7, and 8) in presence and absence of CT and CZ

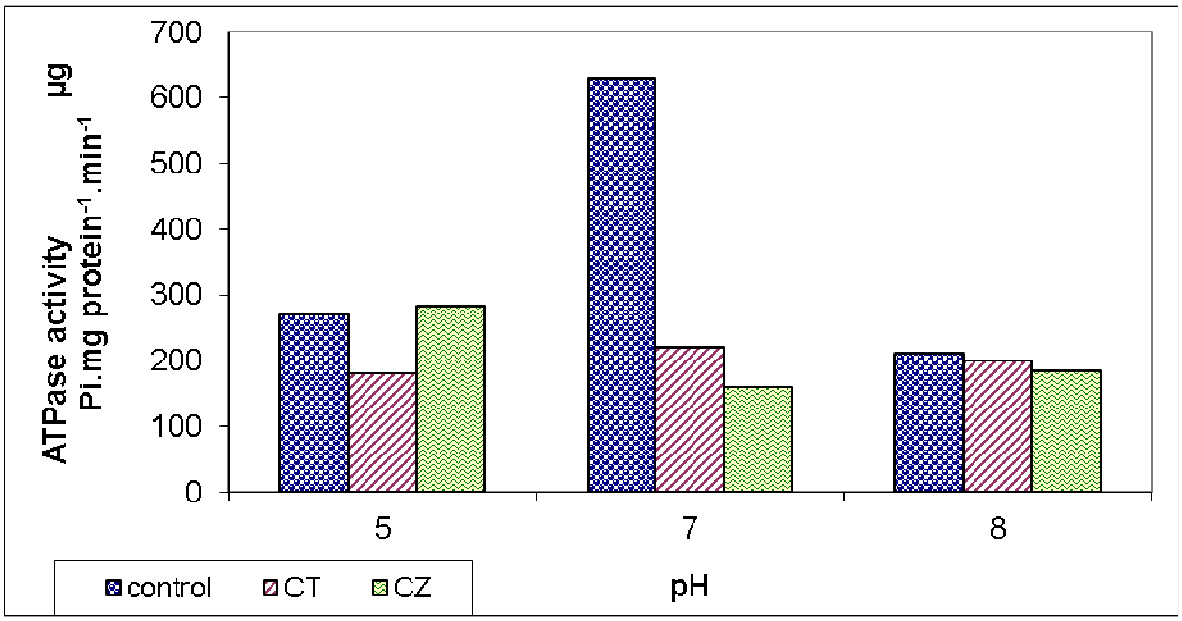

Figure 2: Effect of varying pH conditions (5, 7, and 8) on ATPase activity of *B.subtilis* in presence and absence of CT and CZ at 37± 1^0C.

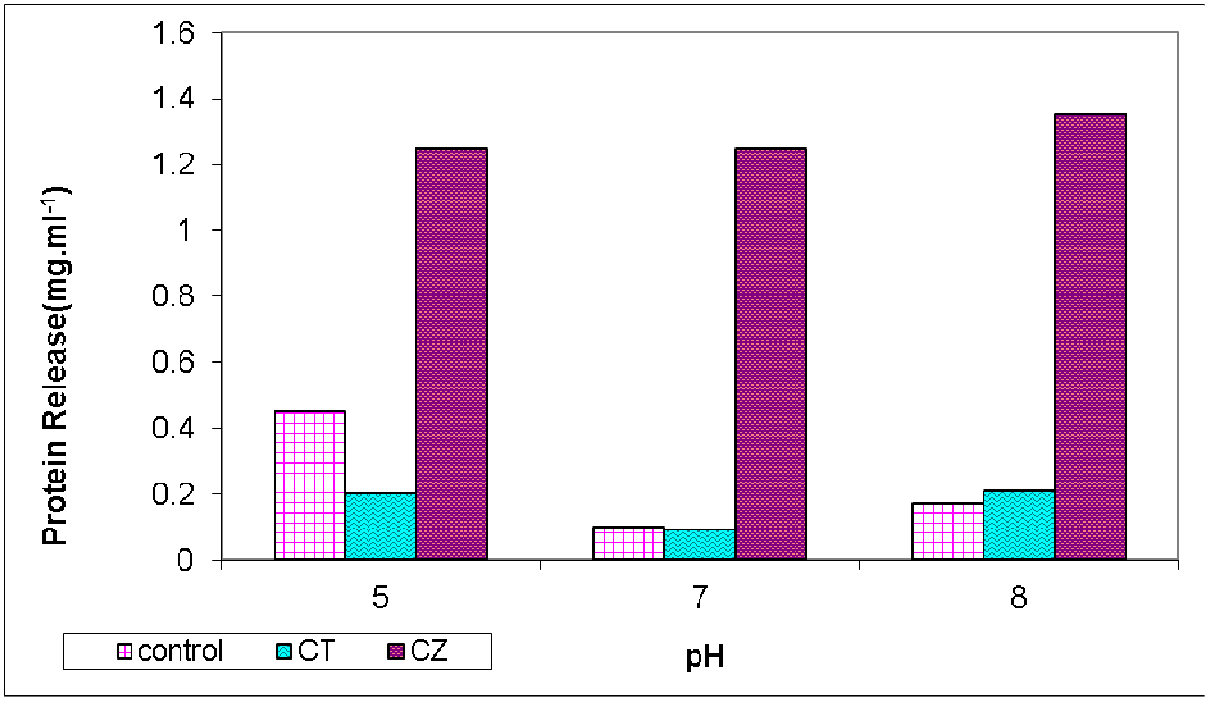

Figure 3: Effect of varying pH conditions (5, 7, and 8) on protein release in supernatant of intact cells of *B.subtilis* in presence and absence of CT and CZ after 30 mins of incubation.

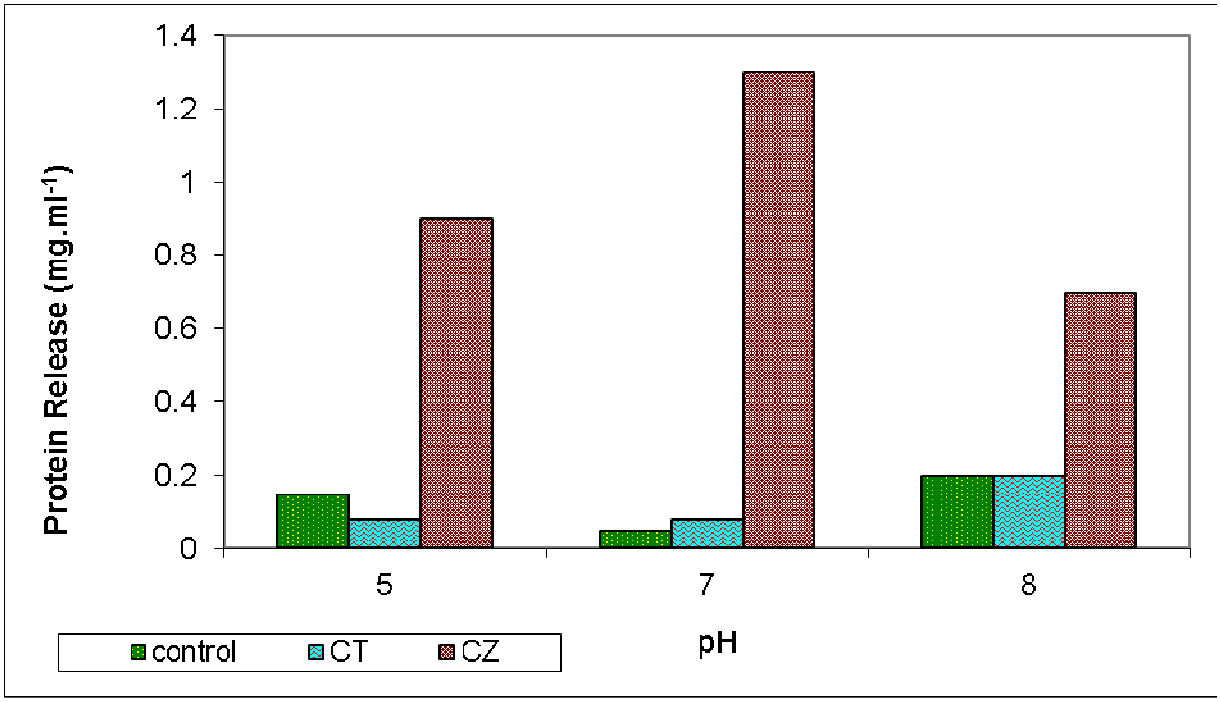

Figure 4: Effect of varying pH conditions (5, 7, and 8) on protein release of membrane vesicles of *B.subtilis* suspended in Tris-HCL buffer in presence and absence of CT and CZ after 30 mins of incubation.

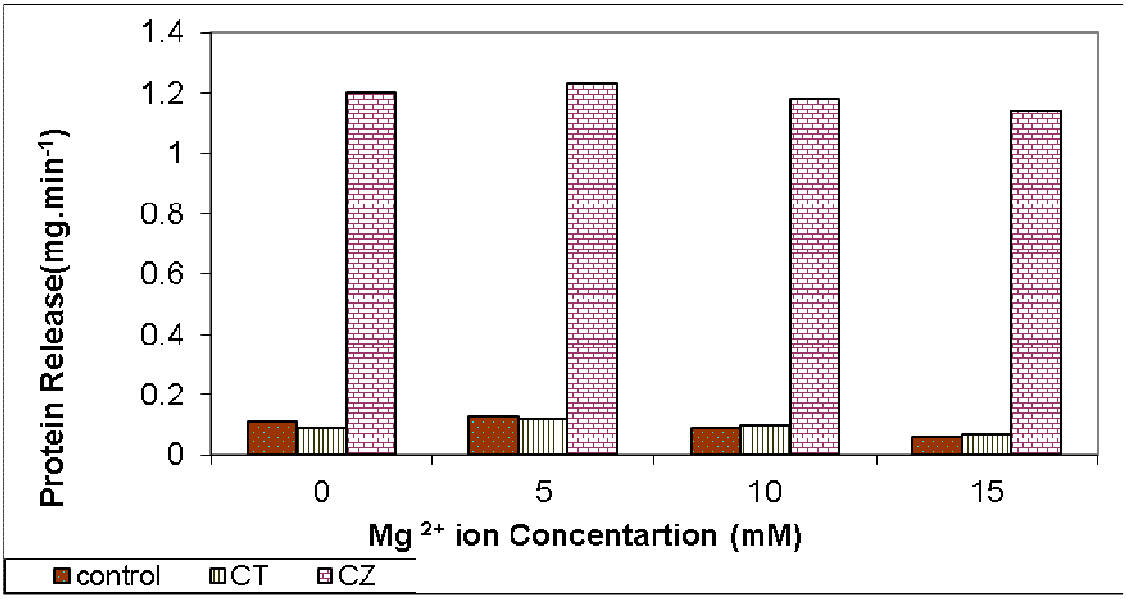

Figure 5: The Protein released in supernatant of intact cells of *B.subtils* when grown in varying concentrations of Mg^{2+}ion (1, 5, 10, 15mM) in presence and absence of CT and CZ after 30 mins of incubation.

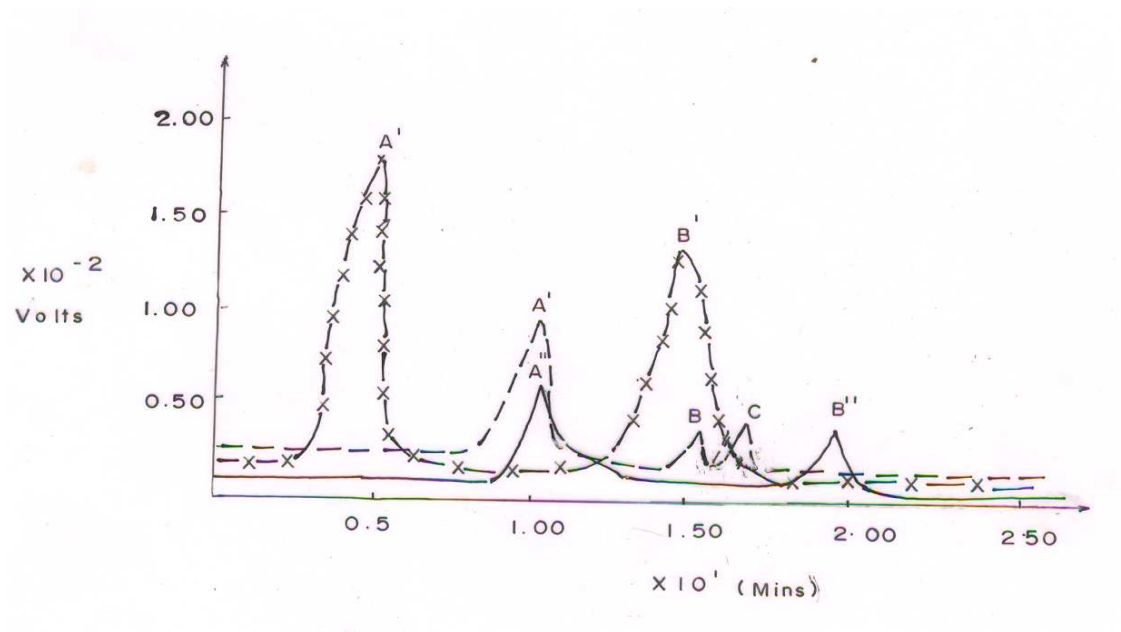

Figure 6: Molecular weight determination of protein released in supernatant of intact cells of *B.subtilis* in the presence of antibiotic stress at pH 7.0 as monitored by HPLC. (----------Without antibiotic,-x-x- presence of CT _____and presence of CZ)