

Role of Membrane Architecture in Development of Sensitivity to Cephalosporin Group of Antibiotics

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Abstract

Due to efficient adaptation process the spread of resistance among microbial pathogens is always a major problem to therapy. The resistance towards an antibiotic can be either biochemical or genetic. The molecular orientation of bacterial cell wall is responsible in determining drug resistance. The role of cephalosporins for e.g.: ceftriaxone & ceftazidime was studied in relation to its toxicity to *B. subtilis* (Gram positive) & *E. coli* (Gram negative). In the presence of ceftazidime an abnormally high level of protein release was observed when intact cells as well as membrane vesicles. In the presence of ceftazidime protein release was observed to be enhanced in contrast to ceftriaxone. The protein export in case of intact bacterial cells was higher than membrane vesicles suggesting the involvement of membrane proteins in drug sensitivity/resistance. The extent of protein released was also found to be modulated when both the cells were subjected to temperature treatment. However, maximum protein export was seen when gram positive & gram-negative cells were subjected to EDTA concentrations. In contrast availability of Mg^{2+} ions in the medium resulted in slight fall in protein release indicating stabilization of membrane vesicles as well as bacterial cell wall, which might have resulted in lowered protein export due to involvement of transport system. From this study it can be concluded that outer membrane orientation determines the therapeutic value of ceftazidime & ceftriaxone.

Keywords: Ceftriaxone sodium, Ceftazidime sodium, Ethylene diamine tetra acetate, Proton motive force, Minimum Inhibitory Concentration

1. Introduction

Man has always lived in an environment that abounds with living organisms. Micro-organisms can be defined as living creatures so small that individuals cannot be seen without the aid of microscope. Bacteria are the simplest organisms to which the term cell can be applied. They are the smallest form of organic life that is visible under a compound microscope. Because of their ubiquitous nature, a number of studies have been carried out on these organisms.

Gram-positive and gram-negative bacteria are basically classified on the basis of their cell wall architecture. In bacterial cells the main cellular components has been divided into cytosole, cytoplasmic membrane and cell wall. The cytosole surrounded by cytoplasmic membrane and outer envelope is known as cell wall (Giesbrecht et. al., 1976). The cell wall of Gram-positive bacteria is a peptidoglycan having additional molecules of teichoic acid, trichromic acid, polyphosphates and carbohydrates. (Hancock., 1997; Salton., 1994). The peptidoglycan layer of bacterial cell consist of disaccharide N-acetyl Muramic acid (β -1-4)-N-acetylglucosamine (MurNAC-GlcNAc) (Ghuysen and Strominger. 1963a; 1963b). The length of the Glycine molecule varies from 5-30 subunits depending upon the bacterial species. (Henz et.al., 1993; Snowden and Perken; 1990; and Glauner., et .al., 1987). In majority of bacterial cell D-lactyl moiety of each muramic is amide linked to short peptide component of peptidoglycan. (Tipper et.al., 1967; Munoz et .al., 1966 and Ghuysen et.al., 1965). The wall peptides are cross linked with other peptides that are attached to a neighbouring glycan strand (Tipper and Burman., 1969; Tipper and Storming., 1968), thereby generating a three dimensional molecular network that surrounds the cell and provides desired exoskeletal function.

Gram- positive and gram- negative bacteria are basically classified on the basis of their cell wall architecture. The envelopes of different microorganisms differ considerably. A gram negative bacterium has more complex structure as compared to its counterpart, i.e., gram positive bacteria. In bacterial cells the main

cellular components has been divided into cytosole, cytoplasmic membrane and cell wall. The cytosole surrounded by cytoplasmic membrane and outer envelope is known as cell wall (Giesbrecht et. al., 1976). The cell wall surrounds the inner cytoplasmic membrane, maintaining its shape and protecting mechanically fragile cytoplasmic membrane from rupture due to high internal osmotic pressure generated by the cytoplasm.

Many antibiotics exert their action either in or outside the cytoplasm membrane, and only those, which have targets within the cytoplasm, must find means of penetration of the cell interior. Whereas, the fungal cell envelope is a complex structure in which the extension zone (hyphen tip) is bound by a wall made up of chitin or cellulose micro-fibrils embedded in the matrix of proteins. Glycans and glycoproteins may also be incorporated. The inner layer of the secondary wall is made up of chitin, overlaid by a proteinaceous layer, outside which is a glycoprotein reticulum, again overlaid by a layer of mixed α and β -glucans (Gooday and Trinci., 1980). The fungal membrane contains high proportions of carbohydrates in addition to phospholipids and sterols such as cholesterol and ergo sterol. The relative proportions vary according to the growth phase, which in turn affects the response of the organism to antimycotic drugs.

In some bacteria during the life cycle cell can lose these outer walls and deficient forms thus generated are referred to as L-forms or L-Phase variants (L for Lister Institute, where they were discovered). L-forms can be induced artificially by treatment with lysozyme or penicillin that disrupts the cell wall integrity. When gram-positive cell is exposed to either of these two chemicals cell wall synthesis is completely hampered / disrupted and become protoplast. However when a gram-positive cell is exposed to such chemicals it loses its peptidoglycan, but retains its outer membrane and spheroplast is formed.

Since bacterial cells are prokaryotic in their cellular organization therefore the cell membrane provides a site for various functions such as energy reaction, nutrient processing and biosynthesis. Apart from this, the major action of cell membrane is to regulate transport of nutrients, as well as, waste from external environment to cell interior. The cytoplasmic membrane of bacterial cell is selectively permeable with special carrier mechanism for passage to various molecules. The glycocalyx and cell wall act as a barrier for the passage of large molecules but they are not the primary transport apparatus. The cell membrane is also involved in secretion of discharge of metabolic product into extra cellular environment. In addition, bacterial membranes are important site for a number of metabolic activities. In these cells, most of the enzymes of respiration and other energy processing activities are located on the membrane. Enzyme system located on the cell membrane also synthesize structural macromolecules to be incorporated into the cell envelop. These membranes are also responsible for secretion of enzymes and toxins into the environment.

The cytoplasmic membrane lies directly outside the cytoplasm. It acts as a selective permeable barrier between the cytoplasm and the cell environment and is also the site at which may important and in primarily comprises of phospholipids and proteins. The membrane proteins are enzymes and carrier proteins, the latter mediating the specific transport of nutrients and ions. The membranes proteins constitute about 20% of the total bacterial proteins, which are mainly lipophilic on their surface. The electron transport system, the cytochromes, quinones, iron-sulphur proteins and flavin adenine dinucleotides are embedded in the cytoplasmic membrane, which acts membrane (transmembrane proteins) or they may be exposed on only one face. The cytoplasmic membrane of *Bacillus subtilis* contains predominantly derivative of ethanolamine in the inner layer and glycerol in the outer layer. During metabolism, protons are extruded to the exterior of the bacterial cell, the net result being acidification of the cell exterior, which also becomes positively charged relative to the cell interior. This combined potential, the concentration or osmotic effect of the proton and its electropositivity, is the electrochemical potential of the proton ($\Delta\mu_{H^+}$) which can be quantified and expressed in terms of electrical units (mV). It is the potential of the proton motive force (ΔpH) which derives ancillary activities (Russell and Chopra., 1990). Energy transduction can occur (1) in membranes or (2) in non-membranous components of the cell. In these two processes two different forms of convertible energy currencies are used. These are $\Delta\mu_{H^+}$ and ATP in (1) and (2) respectively.

According to Fig. 1, the transmembrane differences in the electrochemical potential of H^+ ions ($\Delta\mu_{H^+}$ or protonic potential) holds a central position in the energy transduction pattern occurring in 'protonic' membranes. $\Delta\mu_{H^+}$ consists of electrical, chemical and concentrational (osmotic) components, that is, the electric potential difference ($\Delta\psi$) and the pH difference (ΔpH , Skuhachev. 1980).

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.

2.1 Organism 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°C. 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°C ± 0.1 in thermostatically controlled orbital shaker (Lab India, Model, 3521) under aerobic condition with plate rotation of 180 revolutions per minute. The pH of the media was adjusted by pre addition of 1 ML⁻¹ HCL or 1 ML⁻¹ NAOH solution. Glucose (0.5% w/v) was added to presterilised growth medium as carbon source; however carbohydrates such as Sucrose, Fructose etc. are also preferred in other experimentation.

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 Determination of MIC

The minimum inhibitory concentrations (MIC) of the β-lactam were determined by the optical density method. The MIC was defined as the lowest antibiotic concentration inhibiting 50% of the visible bacterial growth under optimal conditions.

2.4 Growth studies

Erleneyer flasks (250 ml) were prepared to contain 50 ml of the sterile media. These flasks were inoculated with 0.25 ml aliquotes taken from an overnight growth culture. (0.341~O.D.) in the same media. Incubation was done at 37± 1 ° C in the thermostatically controlled orbital shaker (Labline, Model no. 3215) under aerobic conditions with a platform rotation of 180 rev. min⁻¹ Growth recorded at 550 nm using Shimadzu (1610) spectrophotometer. Aliquotes were aseptically removed from the flasks at hourly intervals and placed in a 1 cm path length cuvette of the spectrophotometer. Growth inhibition studies of *B.subtilis* was also carried out by subjecting the bacterium to adverse condition of growth such as varying temperature, pH, EDTA, varying Mg²⁺ ion concentrations. The behaviour of the bacterium was determined both in the presence and absence of antibiotic stress condition, measured spectrophotometrically (Shimadzu – 1610) at 540 nm against a medium blank.

3. Results

The Growth response of *Bacillus subtilis* under varying physiological conditions show different growth pattern. The capability of structural transformation under stress conditions in the form of endospore makes the observation somewhat critical. In order to avoid transition between sporulation and vegetative growth the inoculum size was considered as important factor in determining the MIC50 for the CT and CZ antibiotics. To maintain proper inoculum cell were harvested in early log phase inspite of late log phase. The MIC50 in case of CT observed was 1.5 ppm in contrast to CZ, which shows MIC50 at 18 ppm. When *Bacillus subtilis* cell were inoculated in presterilised Dyes medium they showed differential growth tendency in the presence and absence of CT and CZ (Fig. 2). In the presence of antibiotic stress the log phase, which was seen to be 7 hrs under controlled conditions, was extended followed by stationary phase. Prominent log phase was observed when cell were grown in the absence of CZ, along with observations of optical density in relation to increasing cell density protein content of the culture at similar time intervals was made and presented as illustrated in (Fig. 2).

In all the cases CT was found to be more effective than CZ at MIC50. The increased sensitivity of *Bacillus subtilis* in case of CT might be due to high sodium concentration as structural component, which was lacking in CZ. The sodium content of CT and CZ appears to be 83.0 mg.g⁻¹ and 48.3 mg.g⁻¹, respectively. The increased sensitivity of bacteria in the presence of CT can be reflected as facilitation of β-lactam antibiotics across the bacterial membrane.

It is already known that in evolutionary pattern of transport system presently Na⁺ ion offers greater advantage over H⁺/OH⁻ antiport system. As far as variation in protein concentration is concerned the cellular protein content was measured by Lowery's method and plotted with respect to time (Fig. 2). The protein content was seen at late log phase as 45.2 and 40.3 μg/ml in contrast to control conditions in the presence of CT and CZ respectively. The growth of microorganisms when subjected to varying temperature condition undergoes

modifying metabolic processes. It has been seen that the synthesis of fatty acids is directly influenced by temperature as observed in case of Bacilli (Fujii and Fulco., 1977). In spite of those bacterial cells are seen to repair the initial heat injury leading to lysis of bacterial cells (Condon et al., 1996). The inhibitory effect of heat stress are seen to result from the damage of proteins and the permeabilisation of membranes, particularly the cytoplasmic membrane, H⁺ gradient and lowering in the intracellular pH (Magger and Ferreira., 1993; Benschoter and Ingram., 1986 and Weitzel et. al., 1987). *Bacillus subtilis* showed highly reduced growth rate i.e. inhibition up to 92% under controlled conditions at 250C in the absence of antibiotics (Fig. 3). CT and CZ induced inhibition to bacterial cell, which was found to be 87.48% and 88.24% respectively at 250 C temperatures. In contrast at higher temperature of 500 C, 88.28%, 81.62% and 90.26% inhibition of growth was recorded. 100% survival of *Bacillus subtilis* was considered at 370C. However, 42% and 44% growth inhibition in case of CT and CZ was recorded respectively.

Furthermore the growth as well as sensitivity of *Bacillus subtilis* was observed to be effected in the presence of various pH range (Fig. 4). At pH 5.0 β -lactam untreated *Bacillus subtilis* showed 62% reduction in contrast to 86% and 84% inhibition of growth in presence of CT and CZ respectively.

At alkaline pH 8.0 the growth inhibition was found to be 52% and 12% respectively. The percent inhibition was more pronounced in case of CT as compared to CZ at both pH conditions.

The integrity of bacterial membranes lies in the availability of ionic strength of the medium. Chelating agents are known to modify property of membrane thus altering movement of molecules from outside to cell interior, variation in Mg²⁺ ions. Concentration is known to affect the permeability of bacterial cell membrane. When cells are grown in the presence of chelating agent in laboratory as well as natural conditions the property of exchange of material across cell membrane gets altered. A chemical change in the cell surface occurs concomitantly, since most of the lipopolysaccharide (LPS) is released during treatment (Leive. 1968). When *Bacillus subtilis* is grown in the varying concentration of EDTA – a chelating agent growth performance was found to be gradually reducing as no growth was observed beyond a concentration of 0.08mM (Fig. 5). In the absence of EDTA as well as β -lactam antibiotics the growth of bacteria at 370C was found to be 100%. In the presence of CT and CZ the percentage survival of *Bacillus subtilis* was reduced in the presence of CT in contrast to CZ with increase in concentration on EDTA i.e. 0.5mM the survival was highly reduced in the combination of CT and CZ. Increasing concentration of EDTA in combination with drugs was found to promote drug sensitivity. The appearance of susceptibility to β -lactams as mediated by chelating agent (EDTA) under laboratory conditions supports the significance of hydrophobic interactions between phospholipids and the lipid ‘A’ moiety of LPS that undoubtedly contributes to the integrity and stability of bacterial membranes. The decyclation of phospholipids in the outer membrane weakens these hydrophobic interactions, perhaps to permit sufficiently, the release of LPS and leads to increased sensitivity to the β -lactam antibiotics. The permeability change induced due to EDTA treatment has been shown to be non-specific, resulting in increased permeability to several micro and macromolecules (Leive, 1968).

4. Discussion

The growing culture of bacterial cells are known to pump H⁺ ions into the bacterium where negatively charged groups are located resulting in a weak competition between H⁺ and cations. The Mg²⁺ ion content of the medium binds with the outer cell surface and induces cell wall biosynthesis either directly or indirectly. However, translation and transcription in cells, is also given by the Mg²⁺ ion, as it is a structural component necessary for maintaining structural integrity of ribosomal subunit (Tissieres., 1959). In total cellular metabolism shows deviation from normal routine when proteins are not made available. In the present study Mg²⁺ ions alone were found to modulate survival of *Bacillus subtilis* in Dye’s minimal medium at 370C (Fig. 6). Mg²⁺ ions with increasing concentrations upto 10mM were found to be effective. The observation indicates facilitation of growth in combination with Mg²⁺ ions in the presence of CT and CZ. Therefore it can be established that Mg²⁺ ion when present in combination with therapeutic drugs modify the sensitivity to the bacterium. The treatment of β -lactam can be potentiated in combination of Mg²⁺ ions to make more success while treatment. The fact that structure of gram - positive bacterial walls has a high affinity for metal ions as they are negatively charged at normal pH conditions (Urratia – Meera et al., 1992). Mg²⁺ ions in combination with EDTA have been found to have protective behaviour towards treatment of β -lactams (Fig. 7). The growth performance of *Bacillus subtilis* in combination with EDTA + Mg²⁺ ions in the presence of β -lactams shows reduced inhibition of 0.25mM EDTA + Mg²⁺ ions.

5. Conclusion

Bacillus subtilis survives in the environmental condition exposed to various prevailing stress condition for eg. Salt, pH, Temperature etc. Therefore a complex behaviour of organism is seen under stress conditions (Volker,

1994). In the present study observations reflects that the therapeutic significance of β -lactam antibiotics viz. CT and CZ is influenced by laboratory condition might be true for pharmaceutical applications. Alkaline pH was sensitive than acidic pH of 5.0. In case of CT 86% and 52% inhibition of growth of *Bacillus subtilis* was found at pH 5.0 and 8.0 in contrast to only 12% inhibition at pH 8.0 in case of CZ. As compared to CZ, CT was found to have lower MIC₅₀ with *Bacillus subtilis* although both are β -lactam groups only differing in 1 level of generation as reflected by structured modifications. Higher temperature conditions for e.g. 500C showed more sensitivity that optimum temperature of 370C. At lower temperatures of 250C the normal growth of *Bacillus subtilis* was reduced in contrast to optimum temperature. Drug sensitivity in case of EDTA- a chelating agent was found to be pronounced when *Bacillus subtilis* was grown in Dye's medium. However, Mg²⁺ ions when applied in combination with EDTA protected the chelating behaviour of EDTA thus making cell more sensitive to treatment. The Mg²⁺ alone was found to potentiate the effect of CT and CZ in combination when added to the growth media. The study reflects the capability of *Bacillus subtilis* in determining the coexistence of cells under various stress conditions. Although application of β -lactams starting from first generation (CZ) to appearance of third generation (CT) shows present day need of control of several infections caused by gram positive and gram-negative counterparts. In the current observation establishment of phenomenon with respect to non-pathogenic isolate give a model for interpretation of further observations.

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Figures

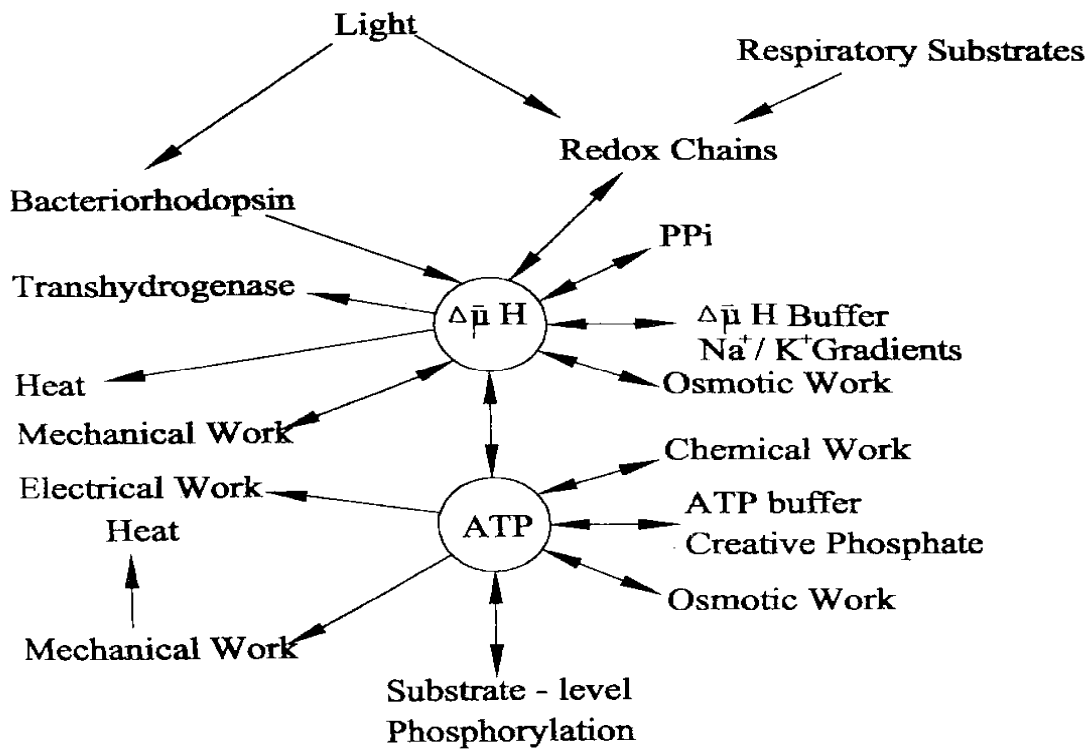


Figure 1: Transmembrane differences in the electrochemical potential of H⁺ ions ($\Delta\bar{\mu}H$ or protonic potential)

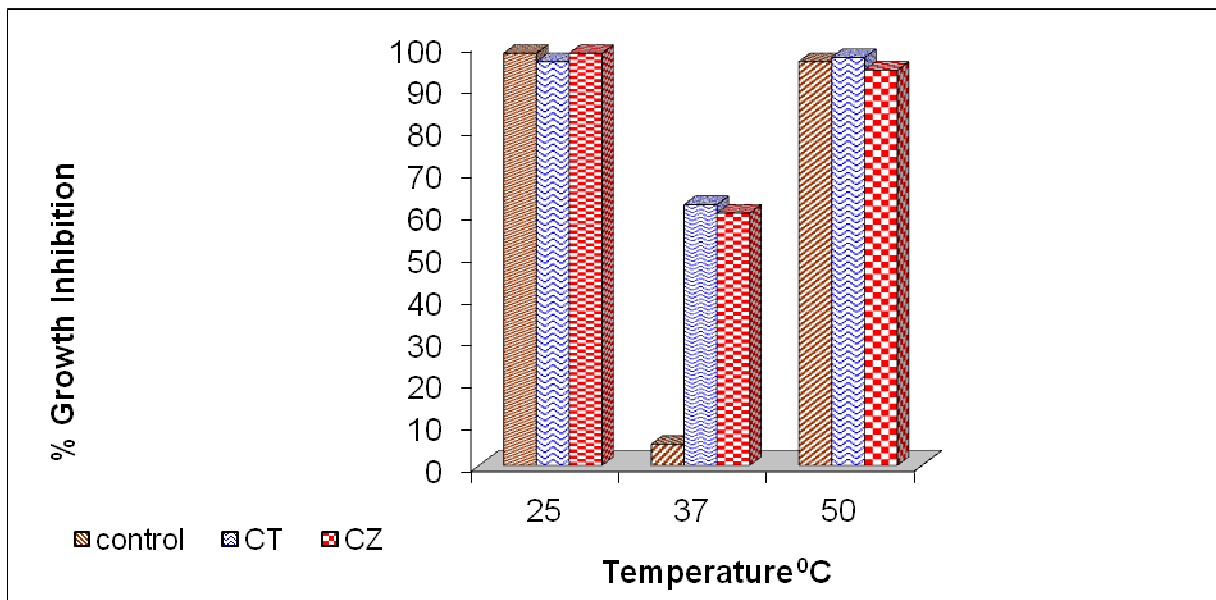


Figure 2: Percentage growth inhibitions of *Bacillus subtilis* at varying temp. (25, 37, 50 °C) in presence and absence of CT and CZ at pH 7.0

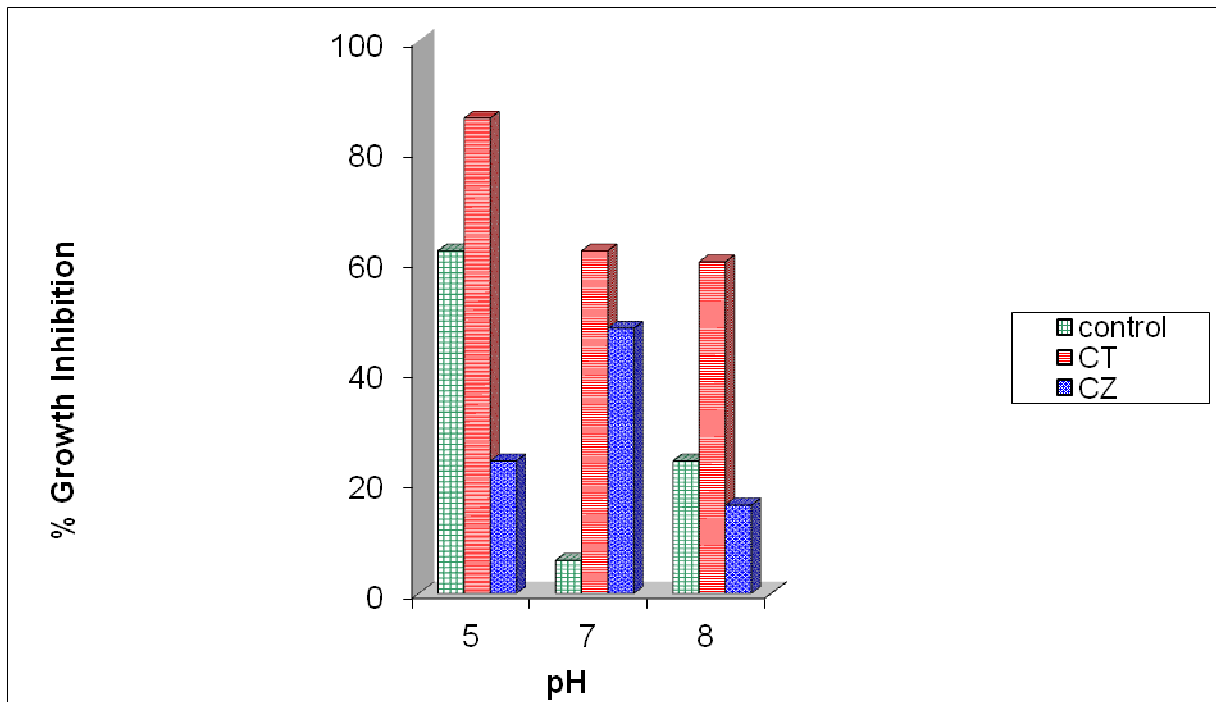


Figure 3: Percentage growth inhibition of *Bacillus subtilis* at pH (5, 7, and 8) in presence and absence of CT and CZ.

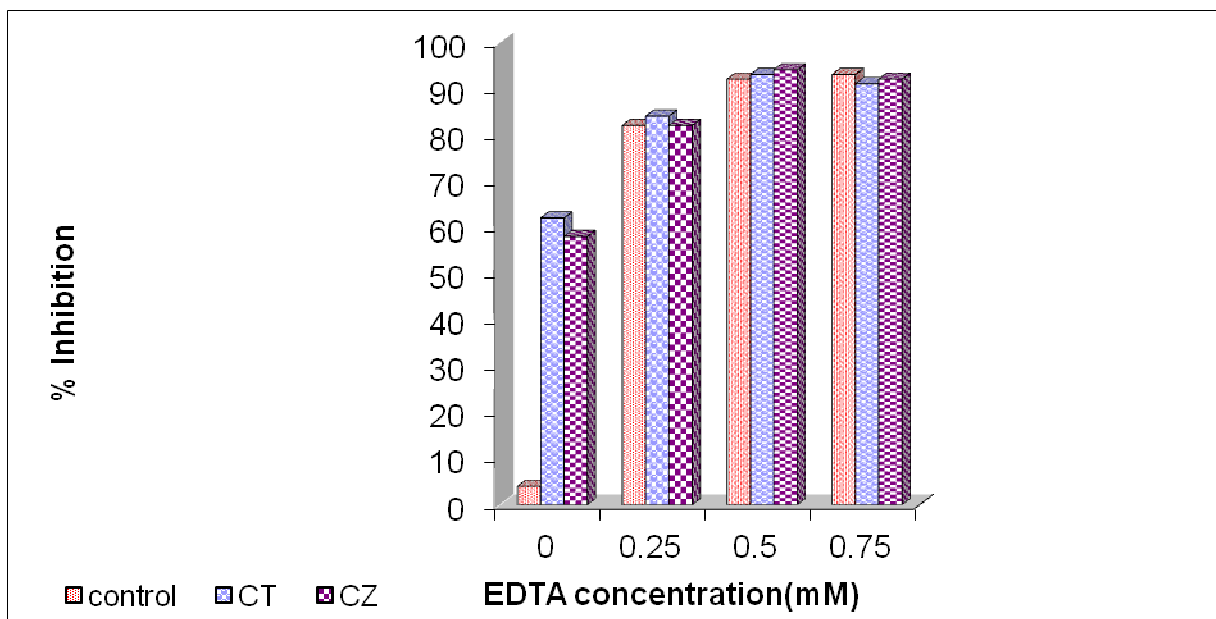


Figure 4: Percentage growth inhibition of *Bacillus subtilis* at varying EDTA concentration (0.25, 0.5, and 0.75) in presence and absence of CT and CZ at pH 7.0

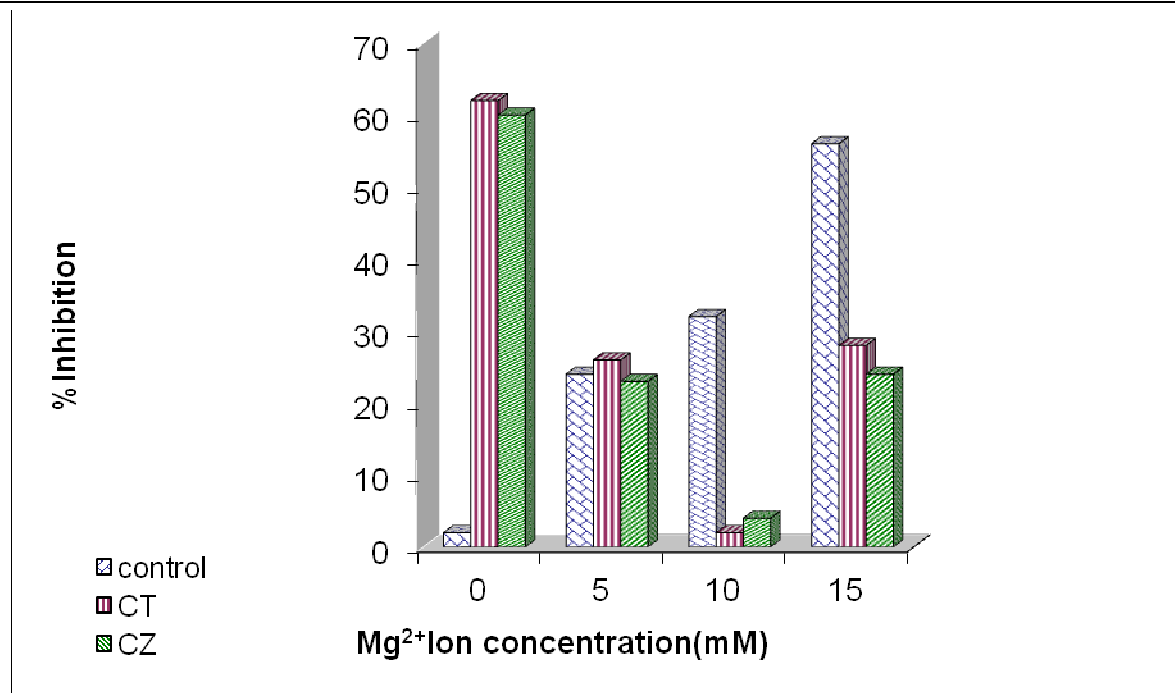


Figure 5: Percentage growth inhibition of Bacillus subtilis at varying Mg²⁺ concentration (0, 5, 10, 15 mM) in presence and absence of CT and CZ at pH 7.0.

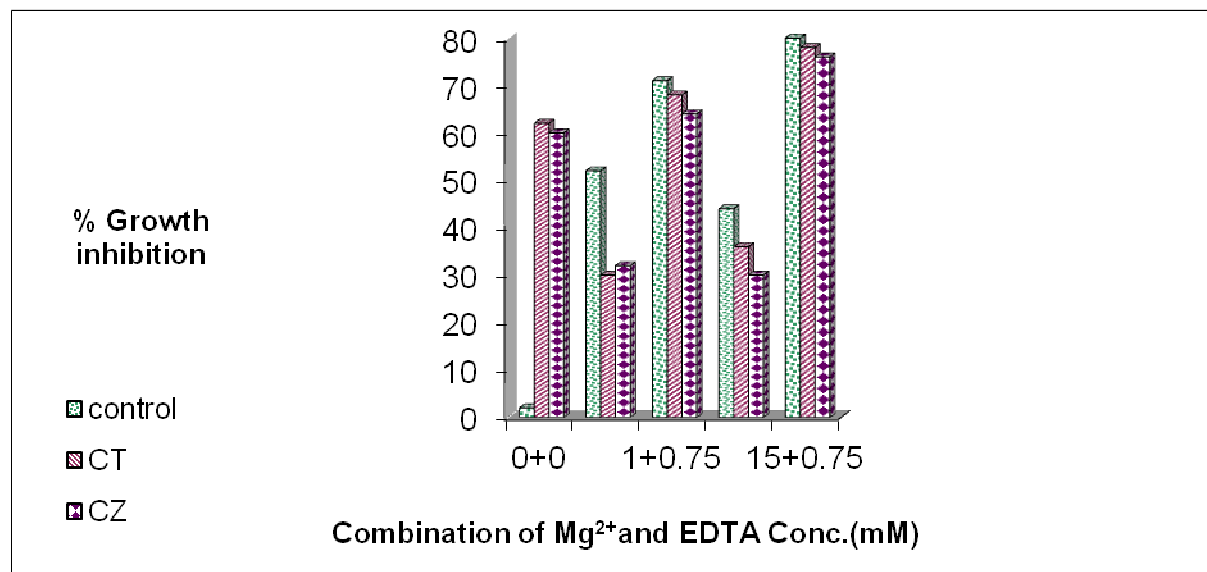


Figure 6: Percentage growth inhibition of Bacillus subtilis at varying combination of Mg²⁺ and EDTA concentration (0+0, 1+0.25, 1+0.75, 15+.025, 15+0.75 mM) in presence and absence of CT and CZ at pH 7.0.

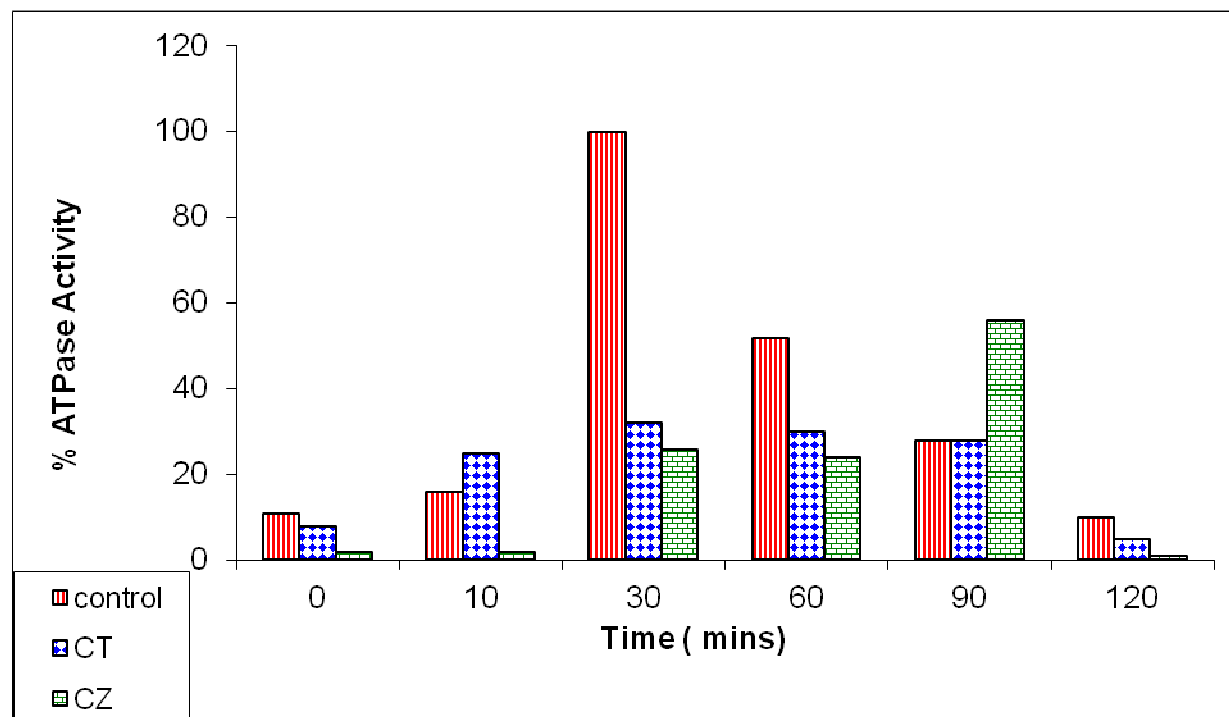


Figure 7: The percentage ATPase activity of *B.subtilis* in the presence and absence of CT and CZ (MIC₅₀, 1.5 ppm and 18ppm respectively) at 37±1°C, pH 7.0 100% ATPase activity = 660µg Pi.mg protein⁻¹.min⁻¹

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