CHAPTER 2

Chapter 2

Revealing Basic Physiology of *Klebsiella pneumoniae* Strain PB12

2.1. Introduction

Almost all natural aquatic and terrestrial environments are nutrient inadequate and, as a result, a major portion of the biosphere exists as oligotrophic (nutrient-depleted) habitats (Morita, 1997) The word "oligotroph" is a combination of the Greek adjective *oligos* meaning "few" and the adjective *trophikos* meaning "feeding". It is generally used to refer the environments (like deep oceanic sediments, caves, glacial and polar ice, deep subsurface soil, aquifers, ocean waters, and leached soils) which offer little to sustain life, organisms that survive in such environments, or the adaptations that support survival. In spite of the low-level of nutrients in oligotrophic waters, microbial numbers persist on the order of $0.5–5x10^5$ cells ml^{-1} (Whitman et al., 1998). Furthermore, of the three largest microbial habitats seawater, soil, and sediment/soil sub-surface the rates of cellular activity and turnover are highest in the open ocean (Whitman et al., 1998). In these oligotrophic environments, prokaryotes play a vital role in regulating the world's largest pool of organic carbon via accumulation, export, remineralization and transformation (Cole et al., 1988). Molecular investigations of microbial community have revealed a broad diversity of bacteria in oligotrophic environment, particularly members of the α- and γ-Proteobacteria, as well as members of the *Cytophaga–Flavobacterium–Bacteroides* group. The obscurity in isolating oligotrophs is well reported. The fundamental problem is that obligate oligotrophs are inherently sensitive to high nutrient concentation. As a result bacteria possessing the ability to adapt nutrient composition and generally grow on

media used in laboratory are mostly facultative oligotrophs. (Schut et al., 1997a). A number of aquatic facultative oligotrophs have been isolated including *Caulobacter*, *Hyphomicrobium* (Moaledj, 1978; Poindexter, 1981), *Cydoclastus oligotrophus* (Wang et al., 1996), and *S. alaskensis* (Schut et al., 1997b). Some of the factors which may restrict the ability of oligotrophs to adapt and its isolation include (1) intolerance to high concentrations of nutrients, (2) undesired growth substrates, (3) the lack of various growth factors or specific vitamins, (4) presence of inhibitory growth substrates or other additives, (5) inactivation by the close proximity to other cells (in colonies on agar plates), (6) susceptibility to the oxidative respiratory burst in the presence of fresh nutrients, and (7) the lethal effects of lytic phage.

In the oligotrophic ocean the limited availability of nutrients limits microbial growth which possesses a major impact on microbial physiology. Growth limitation relates largely to the availability of utilizable nutrients, such as carbon, nitrogen and phosphorus, however, the availability of trace metals, vitamins and a variety of physicochemical factors may also impact on the abundance of microorganisms. Although simple laboratory systems cannot confine the richness and complexity of natural ecosystems, such systems do offer a starting point for studying responses to controlled environmental changes and allow the rigorous experimental testing of hypothesis (Velicer & Lenski, 1999). The conventional and perhaps the most clear-cut method of microbial cultivation is the batch culture. Within this system at first all nutrients are in excess allowing the organism to grow at an optimal, unlimited rate. At a point where the concentration of one or more nutrients become limiting the exponential growth rate eventually ceases and the organism enters a starvation state.

The induction of stress resistance mechanisms is a defining characteristic of nonspore forming bacteria that are able to survive long periods of starvation. An ecologically relevant characteristics gained by microorganisms in aquatic environments is the ability to resist damaging effects of oxidative stress (e.g., hydrogen peroxide). This is possibly due to the common challenge to microorganisms by endogenous (due to ongoing metabolism) and exogenous oxidative stress in aquatic environment (Cooper & Zika, 1983). Reactive oxygen species (ROS) can cause damage to DNA, RNA, protein and lipids and as a consequence, cells have evolved a broad range of mechanisms to cope with this type of stress (reviewed in Storz and Imlay, 1999). Bacteria may require various oxidative stress induced proteins in order to defend the cellular machinery from endogenously derived oxidative stress (Dukan & Nystrom, 1999). In this context, defense against oxidative stress may also be a significant issue for slowly growing cells, since their capacity to replace damaged cellular components by new synthesis is limited by resource availability. To validate this, an attempt was made to check the survivability of PB12 in presence of a ROS producer, ZnO-PEI NPs (Chakraborti et al., 2014). Apart from ROS, Nitrous oxide (NO) has many of the properties of a prototypical signaling molecule. It is freely diffusible, transient, and highly reactive in biological systems. Some recent evidence suggests that NO and or its equivalents [S-nitrosothiols (SNO)] may also be involved in signaling in bacteria. Nitric-oxide synthases (NOSs) are widely distributed among prokaryotes and eukaryotes and have diverse functions in physiology. It was confirmed earlier that NOS in *B. subtilis* have the ability to synthesize NO from arginine (Adak et al., 2002), although it's physiological role remains obscure.

In contrast to our understanding of the physiological responses to starvation, little is known about the physiological changes that occur when nutrients are present, but at concentrations that result in sub maximal (being less than the maximum of which it is capable) rates of growth, such as those that are likely to occur in the ocean. Although starvation-induced cross protection is not observed, a marine oligotrophic ultramicrobacterium *Sphingomonas alaskensis* RB2256, maintains a high level of resistance to a variety of stress inducing agents (hydrogen peroxide, heat, ethanol and UV) regardless of whether it is growing or starved (Eguchi et al., 1996; Joux et al., 1999). For all the living organisms, cell size is a key characteristic. In bacteria, cell size plays, both directly and indirectly, an important role in fitness. For example, a bacteria susceptibility to predation by protists and host immune cells, such as neutrophils, depends on its cell size (Justice et al., 2008; Pernthaler, 2005). In addition, the cell size is relevant to mechanisms of antibiotic resistance and protection from bacterial phages (Miller et al., 2004; St-Pierre & Endy, 2008). It was noted earlier that there is a significant increase in antibiotic resistance to gentamicin in cells grown under osmotic stress (higher NaCl concentrations) (Crompton et al., 2014). The possible mechanism is mainly due to the down regulation of synthesis of cell-wall associated proteins under conditions of higher NaCl concentrations which could impede the passage of the antibiotic through the cell wall (McMahon et al., 2006; Frank & Patel, 2007). This increased resistance against antibiotics was often maintained after removal of the stress providing evidence of a stable phenotype and altered metabolism in response to the stress. Moreover, cell size is also closely related to cell proliferation via altering the surface area to volume ratio. Cell size influences the uptake of nutrients, the concentrations of cellular components and the

advancement of intracellular biochemical reactions (Young, 2006). Significantly, the initiation of chromosomal replication and the assembly of the division machinery also depend on cell size, resulting in homeostatic and recursive reproduction of an optimal cellular state (Donachie, 1968; Weart et al, 2007). These facts establish that the bacterial cell size is strongly coupled with its growth rate. During the last decade flow cytometry has been established as a major experimental technique in biology for enumeration of cells. Several reports on a wide variety of applications of this method to various parts of cell biology have been published. So far, however, the work has been limited almost exclusively to eukaryotic cells, while reports on prokaryotic cells have been very limited (Bailey et al., 1977). Previously, tedious physicochemical techniques (like dilution plating) have been used to unravel the growth kinetics of bacteria. Flow cytometry, however, seems ideally suited for the purpose provided sufficient sensitivity and appropriate staining procedures are fulfilled. In this chapter an attempt was made to explore the physiological trends choose by a facultative oligotrophic bacteria in order to reveal the strategy use to survive in nutrient deprived condition.

2.2. Materials and methods

2.2.1. Bacterial strain and growth

All experiments were done using the facultative oligotrophic strain *Klebsiella pneumoniae* PB12 (KF192506). Fresh inoculum was prepared by transferring a single colony of 24 h old culture of PB12 into 10 ml sterile LB (pH 7.0) in 100 ml Erlenmeyer flask. The inoculated medium was incubated at 30 °C for 4 h with agitation. The culture was harvested by centrifuging at 9587.5 x *g* for 5 min at 4 °C and washed twice with

sterile phosphate buffer saline (PBS) to remove traces of media if any. The washed pellet was finally suspended in 3 ml sterile PBS. Aliquots of approximately 10^4 cells were added to 10 ml of LB or diluted (0.01x or 10^{-2}) LB in 100 ml Erlenmeyer flask. The flask was kept at 30 °C (with shaking at 200 rpm) throughout the period of investigation. Survivability of PB12 cells in LB or diluted LB was assessed through dilution-plating of pure culture aliquots at different time intervals on fresh LB agar plates. Colony diameter of PB12 was determined along with the number of cells present on that particular colony was also enumerated using dilution-plating technique.

2.2.2. Scanning electron microscopic (SEM) studies

Overnight grown culture of PB12 cells (10^8 cells ml^{-1}) was washed and re-suspended in PBS as described previously (Chakraborti et al., 2014). Cells were fixed with 2% glutaraldehyde and dehydrated with series of ethanol treatments (10%, 30%, 50%, 70%, 90% and 100%) and examined by SEM (FEI Quanta-200 MK2) with an accelerating voltage of 20 kV. Multiple fields of visions were viewed at different magnifications.

2.2.3. Growth study using Flow cytometry or Fluorescence assisted cell sorting (FACS)

2.2.3.1. Instrumentation

Flow cytometry was performed with an FACS CALIBUR, Becton Dickinson, USA using CellQuest software. Sample excitation was done with an argon laser operating at 15 mW and 488 nm. Filter settings were 525 BP for FITC and 550 LP and 630 BP for

measurement of PI. Acquisition and data analysis were done with standard ELITE software, using the Immuno-4 program to determine the percentage of stained events.

2.2.3.2. Calibrations and discriminator

The flow cytometer was calibrated with Fluoresbrite plain microspheres (Polysciences, Inc., Warrington, PA), 0.72 µm in diameter, on forward scatter (FSC), side scatter (SSC), and FITC fluorescence. Fluorescence quantitation was done with fluorescein quantitation kits (Quantum 24 and Quantum 25, from Flow Cytometry Standards Corp., Research Triangle Park, NC). To determine the level of background noise, plain microspheres (Polysciences, Inc.), 0.79 µm in diameter, were used and were assumed to have no fluorescence. The mean fluorescence (logarithmic scale) was the fluorescence value corresponding to the calculated mean channel number (linear scale) of all events and was therefore not a true mean. Percentages of stained bacteria were determined with Immuno-4 software (Coulter). Sorting experiments were performed with gates on FSC >1,000 and on FSC <1,000 in combinations with gates on SSC or PI. Samples were injected through the instrument using the low pressure setting (12 pvrnin), which ensured the clear detection of individual bacterial cells. All media, buffers, and solutions were filtered through a 0.22-µm membrane filter (Nalgene Brand Products, Rochester, NY, USA) to remove microparticulates that would otherwise contribute to background noise during data acquisition. *K. pneumoniae PB12* cultured aerobically in LB broth (Difco, Detroit, Michigan, USA) at 30 °C overnight. A 1:100 dilution of this culture was made into fresh, sterile LB or 0.01x LB broths and incubated for another 8 h at 30 °C in a shaking waterbath (250 rpm) until the culture had entered early log-phase. Each sample was centrifuged at 8000 rpm for 5 min, the pellet re-suspended in 1 ml 0.1

M Tris-HC1 buffer, and then fixed by rapid injection into a 10 ml volume of ice-cold 70% ethanol. The fixed samples were kept at 4 °C until analysed. Following the fixation process, cells were prepared for flow cytometric analysis by using the following procedure. Cells were centrifuged and washed twice in 1 ml ice-cold 0.1 M Tris-HC1 buffer. FITC was added to give a final concentration of 20 mg l^{-1}. Cells were left to stain at room temperature for 15 min, then sedimented by centrifugation (as earlier), and resuspended in 1 ml 0.1 M Tris-HC1 buffer before flow cytometric analysis. Data was collected from 10,000 cells for each sample, at a flow rate of approximately 500 cells per second. All the photomultiplier amplifier gains were set in linear mode.

2.2.4. Determination of intracellular ROS

Measurements of intracellular ROS levels in PB12, grown in LB broth and 10^{-2} LB broth were made using 2', 7'-dichlorodihydrofluoroscein diacetate ($DCFH_2$-DA). Samples were incubated in the presence of 10 mM $DCFH_2$-DA in phosphate buffered saline (PBS) at 30 °C for 30 min then washed two times with PBS and centrifuged at 1200 rpm to remove the extracellular $DCFH_2$-DA. The trapped fluorescent dye (DCF) inside the cells used to evaluate and detect intracellular ROS. The fluorescence values at different conditions were monitored by excitation at 498 nm and emission 530 nm.

2.2.5. Determination of NO generation

NO generation was determined according to the method of Chakraborty et al. 2011. In brief, 100 µl of Griess reagent (containing 1 part of 1% sulfanilamide in 5% phosphoric acid, and 1 part of 0.1% of N-C-1napthyl ethylene diaminedihydrochloride) was added to 100 µl of sample, incubated at room temperature for 10 minutes, readings

were taken in a UV spectrophotometer at 550 nm and compared to a sodium nitrite standard curve (values ranging between 0.5 and 25 μM). The level of NO was expressed as μM/mg protein.

2.2.6. Determination of SOD activity

Cu,ZnSOD activity was assayed by the pyrogallol method (Marklund & Marklund 1974). The periplasmic fraction was obtained by a procedure described previously (Battistoni et al., 1996). The low expression level of Cu,ZnSOD and the presence of small amounts of cytoplasmic FeSOD and MnSOD in the periplasmic extracts prevent accurate measurements of Cu,ZnSOD. Therefore, to characterize our model we have determined the Cu, ZnSOD activity in periplasmic extracts before and after 15 min incubation with 2 mM diethyldithiocarbamate, a copper chelator which inactivates the Cu,ZnSOD enzyme without affecting the activity of MnSOD and FeSOD (Benov & Fridovich, 1994). Protein content was determined by the method of Lowry et al. β-Gal activity was measured by a previously described procedure (Sambrook et al., 1989). Cu,ZnSOD activity was determined by subtracting diethyldithiocarbamate resistant activity from the total SOD activity present in periplasmic extracts in the absence of such a copper-chelating agent. One unit is defined as the amount of Cu,ZnSOD necessary to achieve 50% inhibition of pyrogallol autoxidation.

2.2.7. Determination of catalase (CAT) activity

Catalase activity was measured in the cell lysate by the method decribed earlier (Luck, 1963). The final reaction volume of 3 ml contained 0.05 M Tris-buffer, 5 mM EDTA (pH 7.0), and 10 mM H_2O_2 (in 0.1 M potassium phosphate buffer, pH 7.0). About

50 µl aliquot of the lysates were added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. Catalase activity was calculated by using the molar extinction coefficient of 43.6 M^{-1} cm^{-1} for H_2O_2. The level of catalase was expressed in terms of mmol/min/mg protein.

2.2.8. Survivability of PB12 cells in presence of ZnO-PEI nanoparticles (ZnO-PEI NPs)

Fresh inoculum of *Klebsiella pneumoniae* PB12 was prepared by transferring a single colony of 24 h old culture into 10 ml sterile LB (pH 7) in 100 ml Erlenmeyer flask. The inoculated medium was incubated at 30 °C for 12 h with agitation (200 rpm). Survival experiments were performed by sub-culturing overnight grown culture in LB or 10^{-2} LB broths with agitation (200 rpm). After 8 h (for LB; nutrient rich medium) or 24 h (for 0.01x LB; nutrient-poor medium) of growth, cells were harvested by centrifuging at 10,000 × g for 10 min at 4 °C respectively. Pellets obtained were washed thrice with sterile phosphate buffer saline (PBS) to remove traces of media. Washed pellets obtained from LB or 0.01x LB was then re-suspended in PBS and the O.D was adjusted before any treatment with LD_{50} dosage (15 µg ml^{-1}) of ZnO-PEI NPs (Chakraborti et al., 2014) respectively for 30 min, serially diluted and plated on fresh LB agar plates. After overnight incubation at 30 °C, difference in percentage survival was then calculated from the cell count obtained before and after treatment with ZnO-PEI NPs.

2.2.9. Analysis of Raman Spectra of nanoparticles treated PB12 cells grown in diluted and undiluted Luria broth

A Renishaw RM1000 Raman spectrometer system (Gloucestershire, UK) equipped with equipped with a Leica DMLB microscope (Wetzlar, Germany) and a 785 nm near infrared diode laser source (maximum at 300 mW) was used in this study. Raman scattering signals were detected by a 578 x 385 pixels CCD array detector. Raman spectra were acquired from PB12 culture grown in LB (without ZnO-PEI NPs) or PB12 culture grown in 0.01x LB (without ZnO-PEI NPs) or PB12 culture grown in LB (treated with ZnO-PEI NPs) or PB12 culture grown in 0.01x LB (treated with ZnO-PEI NPs) using a 50x objective with a detection range from 700-1900 cm^{-1} in the extended mode. The measurement was conducted with a 10 s exposure time and c.10 mW laser power. Data analysis was performed using Delight version 3.2.1 (D-Squared Development Inc., LaGrande, OR, USA) software. Preprocessing algorithms such as smoothing and polynomial subtract were employed to analyse the data.

2.3. Results

2.3.1. Growth study

The growth of PB12 cells were observed in LB or diluted (0.01x) LB (without any supplementation) (Fig. 2.1a). The mean generation time of PB12 in LB or 0.01x LB was 25 min or 36 min respectively. The ability of PB12 to survive (without reduction in viable cell number since inoculation) and grow in a low nutrient medium establishes the oligotrophic nature of the strain. This means that, even at 10 times diluted LB, the nutrient concentration was high enough to support the growth of these cells; in other

words, the cells can carry out reproduction along with the macromolecular synthesis with whatever in their milieu over a given time period even at 10 times diluted LB. Colony diameter of PB12 grown on LB agar after 24 h was found to be 1.5 mm (Fig. 2.1b), whereas, colony diameter of PB12 on 0.01x LB after 24 h of incubation was 0.5 mm (Fig. 2.1c). After assessing the number of cells presents in this colony it was noted that colonies obtained on LB agar (d=1.5 mm) there are 1.8 x 10^8 cells present. Interestingly, 5.4 x 10^8 number of cells was obtained from the colony grown on diluted LB agar plates.

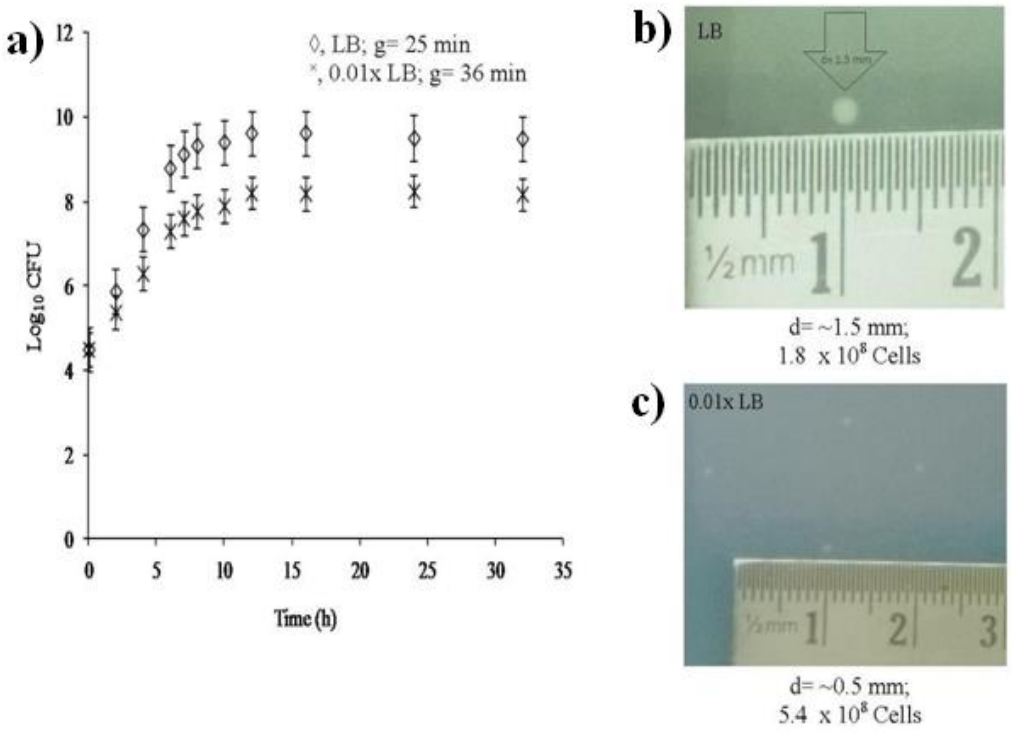


Fig.2.1. a) Viability and growth curve of *K. pneumoniae* PB12 in LB broth, ◊ (g= 25 min) and in 0.01x LB broth, × (g= 36 min); **b)** *K. pneumoniae* PB12 bacterial obtained on LB agar plate after 24 h of incubation (d= ~1.5 mm; CFU= 1.8 x 10^8 cells); **c)** *K. pneumoniae* PB12 bacterial obtained on 0.01x LB agar plate after 24 h of incubation (d=

~0.5 mm; CFU= 5.4 x 10^8 cells). The data represent the mean ± standard deviation of three independent experiments (n = 3).

2.3.2. SEM and FACS study

Scanning electron micrograph of PB12 cells grown on LB or 0.01x LB was found to be 1.55 ± 0.15 µm or 0.787 ± 0.18 µm respectively (Fig 2.2a & b). In an earlier studies, flow cytometry was successfully used to detect and quantify cell shape differences between the rod-shaped cells of *E. coli*: CS109 and the seriously deformed cells of *E. coli*: CS315-1K, a mutant lacking PBPs 4, 5, and 7 (Meberg et al., 2004). When PB12 cells grown in LB was compared with the cells grown in 0.01x LB in the absence of added fluorophore, the two populations could be distinguished from one another by examining the distribution of forward scattered light (Fig. 2.2c & d). The application of flow cytometry to the study of bacterial responses to antibiotics was done earlier (Gant et al., 1993). PB12 cells population grown in LB was skewed to the right on the *x* axis (forward- scattered light), indicating that the cells were possibly larger (Fig. 2.2c) than PB12 cells grown in 0.01x LB (which as skewed to the left on the *x* axis) (Fig. 2.2d).

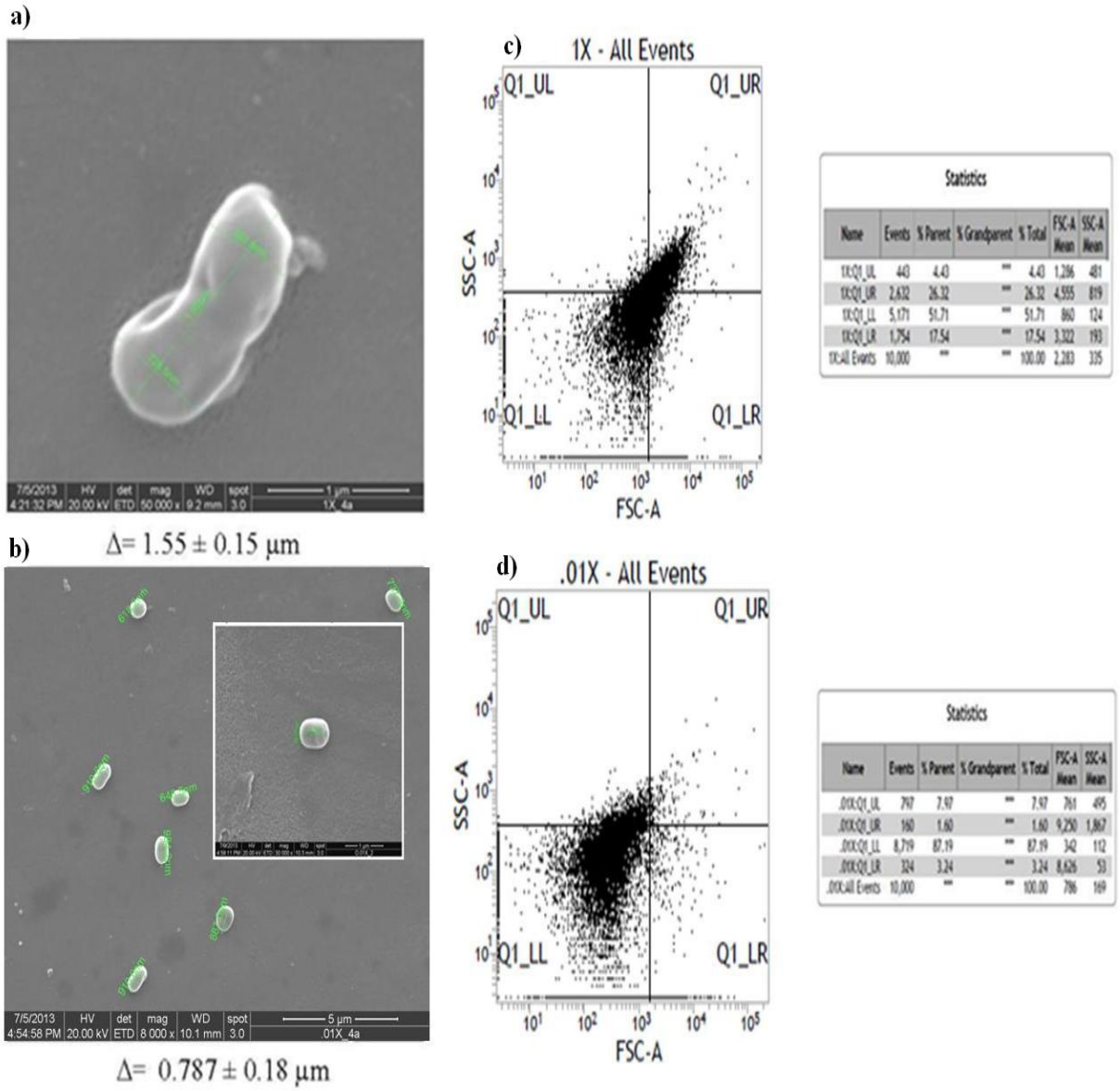
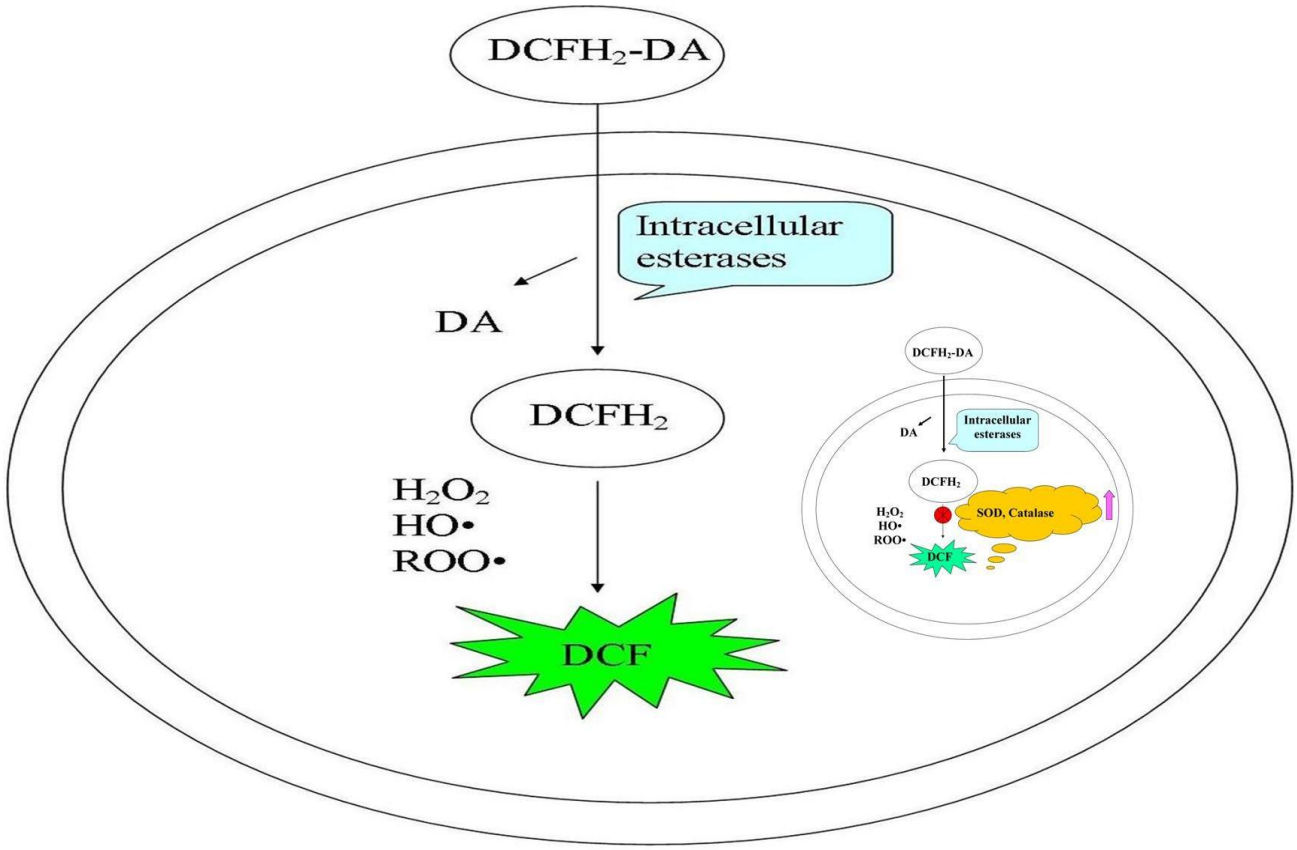


Fig.2.2. SEM micrograph of PB12 cell: **a)** grown in LB; **b)** grown in 0.01x LB; scatter plot of FSC vs SSC to visualize the distribution of PB12 cells based upon size; **c)** PB12 cells grown in LB; **d)** PB12 cells grown in 0.01x LB. The data represent the mean ± standard deviation of three independent experiments (n = 3).

2.3.3. Determination of intracellular ROS

Oxidative stress can cause several types of damage to the bacterial cell, including metabolic pathway disruptions and bacteriostatic and bactericidal effects (Berlett & Stadtman, 1997; Fridovich, 1998). Using an oxidant-sensing fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) intracellular ROS can be measured. $DCFH_2$-DA, a nonpolar dye, converted into the polar derivative $DCFH_2$ (nonfluorescent) by means of cellular esterase. After getting oxidized by intracellular ROS and other peroxides it switched to highly fluorescent DCF. Scheme 1 represents the basic principal of $DCFH_2$-DA dye. Fig. 2.3a and Fig. 2.3b indicates the intracellular ROS production in PB12 cells grown in LB and 0.01x LB respectively. Results showed that intracellular ROS production is more in LB grown cells compare to 0.01x LB grown cells. Oxygen derivatives like superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH) are usually generated as toxic by-products of aerobic metabolism in a cascade of monovalent reductions from molecular oxygen (Shimizu, 2014). Although these are not so reactive *per se*, O_2^- and H_2O_2 cause severe cell damage. H_2O_2 along with Fe^{2+} *via* the Fenton reaction produces OH, which reacts with any macromolecule such as protein, membrane constituents, and DNA (Greenberg et al., 1990; Liochev & Fridovich, 1994)



Scheme 2.1. The principal of the $DCFH_2$-DA dye. High intracellular level of SOD or Catalase enzyme prevents oxidation of $DCFH_2$ and the formation of fluorescent DCF product (figure inset).

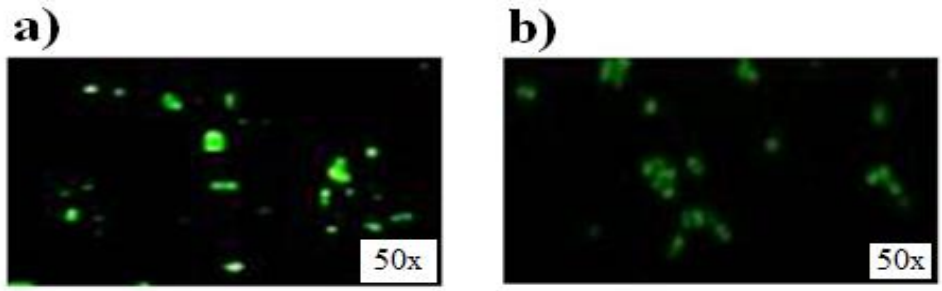


Fig. 2.3. Fluorescence micrographs of PB12 cells: **a)** grown in LB; **b)** grown in 0.01x LB.

2.3.4. Determination of NO generation

The bioactive gas nitric oxide (NO) has multiple biological functions in a very broad range of organisms. It is well-known that NO exerts both pro-oxidant and antioxidant effects, depending on the ambient redox status, the presence of other reactants, and the nature of the reaction (Wilson et al., 2008). It was noted that the PB12 cells grown in LB exhibited higher NO production than the cells grown in 0.01x LB (Fig. 2.4.).

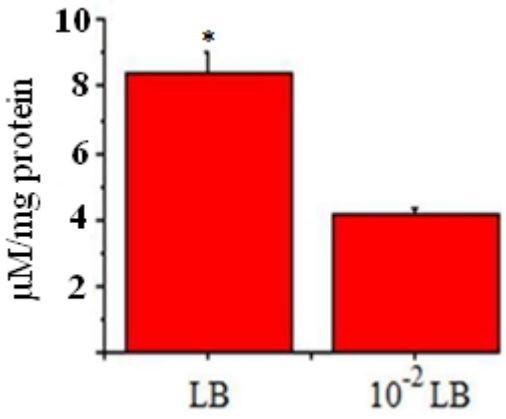


Fig. 2.4. Variations of NO production in *Klebsiella pneumoniae* PB12 cells grown in either LB or 0.01x LB (10^{-2}) broth.

2.3.5. Determination of Cu,ZnSOD and CAT activity

It now seems clear that starvation adaptation is important for cells to initiate long-term survival via developing resistance to oxidative stress. Clearly, oxidative stress is a condition likely to be professed by many bacteria, for example, in the form of reactive oxygen species. As SOD functions to detoxify superoxide, we sought to determine the

Cu,ZnSOD activity in *Klebsiella pneumoniae* PB12 grown in LB and 0.01x LB. Our present result focused in Fig. 2.5a, where Cu,ZnSOD activity has increased significantly ($P< 0.05$) in 0.01x LB grown PB 12 cells compared to LB grown PB 12 cells.

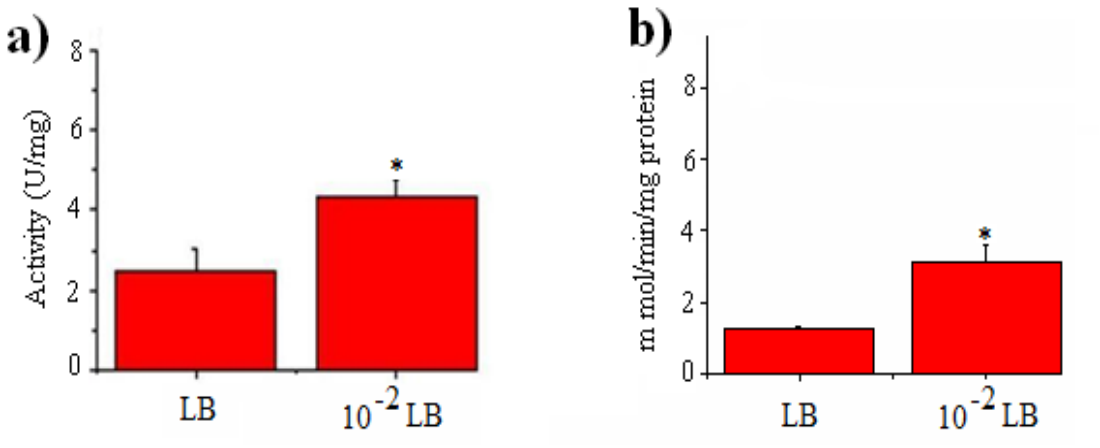


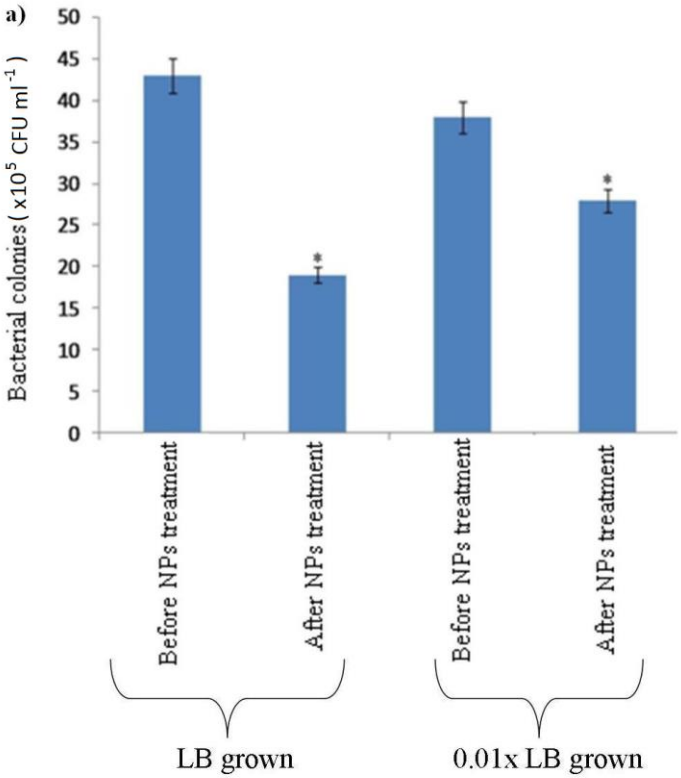
Fig. 2.5. a) Variations of Cu,ZnSOD activity in *Klebsiella pneumoniae* PB12 cells grown in LB or 0.01x (10^{-2}) LB; **b)** Variations of catalase activity *Klebsiella pneumoniae* PB12 cells grown in LB or 0.01x (10^{-2}) LB.

The superoxide radical is also generated by autoxidation of intracellular compounds. Its spontaneous dismutation in water, which yields H_2O_2 and also $^{-}O_2$, proceeds at a very high rate, yet in the cell the dismutation is additionally catalyzed by SOD. Superoxide free radicals are neutralized by the action of superoxide dismutase, generating hydrogen peroxide, which in turn is broken down by catalase. Here, catalase level has been found to be higher in 0.01x LB grown PB12 cells than LB grown cells (Fig. 2.5b). This rapid removal of O_2^- is very important for the cell because superoxide can give rise to very reactive ROS, such as H_2O_2, HO• or singlet oxygen; the peroxynitrite anion ($ONOO^-$) is formed when O_2^- reacts with NO. Despite its low

reactivity, O_2^- has been shown to inhibit antioxidant enzymes, such as catalase (Halliwell & Gutteridge 1986).

2.3.6. Survivability of PB12 cells in presence of ZnO-PEI nanoparticles (ZnO-PEI NPs) and its interpretation using Raman spectra

In one of the reports, it was established that ZnO-PEI NPs possess the potential to generate extracellular ROS and which is related to its antibacterial activity (Chakraborti et al., 2014). In this study, ZnO-PEI NPs was used as a model ROS generator to study its effect on PB12 cells grown in LB or 0.01x LB. Results showed that LD_{50} dosage of ZnO-PEI NPs cause only 35% reduction in bacterial growth of 0.01x LB grown cells whereas, approximately 50% reduction in bacterial growth was noted when LB grown cells were treated with same dosage of NPs ($p < 0.05$) (Fig. 2.6a). Moreover, Raman spectroscopy was used to derive more insight. Results showed that subtle differences were observed in the spectra of LB grown cells compared to 0.01x LB grown cells treated with same concentration of ZnO-PEI NPs (Fig. 2.6b). *Klebsiella pneumoniae* PB12 spectra showed distinctive absorption bands between 600 and 1800 cm^{-1}, that includes peak for proteins, lipids, carbohydrates and nucleic acids and these spectra are in good agreement with the previously published spectra (Chan et al., 2007). The main Raman peaks of PB12 cells showing bands around 853, 1005, 1252 and 1665 cm^{-1} were assigned to proteins; bands around 726, 783, 936, 1101, 1340 and 1577 cm^{-1} were assigned to nucleic acids; bands around 977 and 1453 cm^{-1} were assigned to lipids; and those around 1035 cm^{-1} was assigned to carbohydrates.


a)
Bacterial colonies (x10^5 CFU ml^{-1})
50
45
40
35
30
25
20
15
10
5
0
*
*
Before NPs treatment
After NPs treatment
Before NPs treatment
After NPs treatment
LB grown
0.01x LB grown

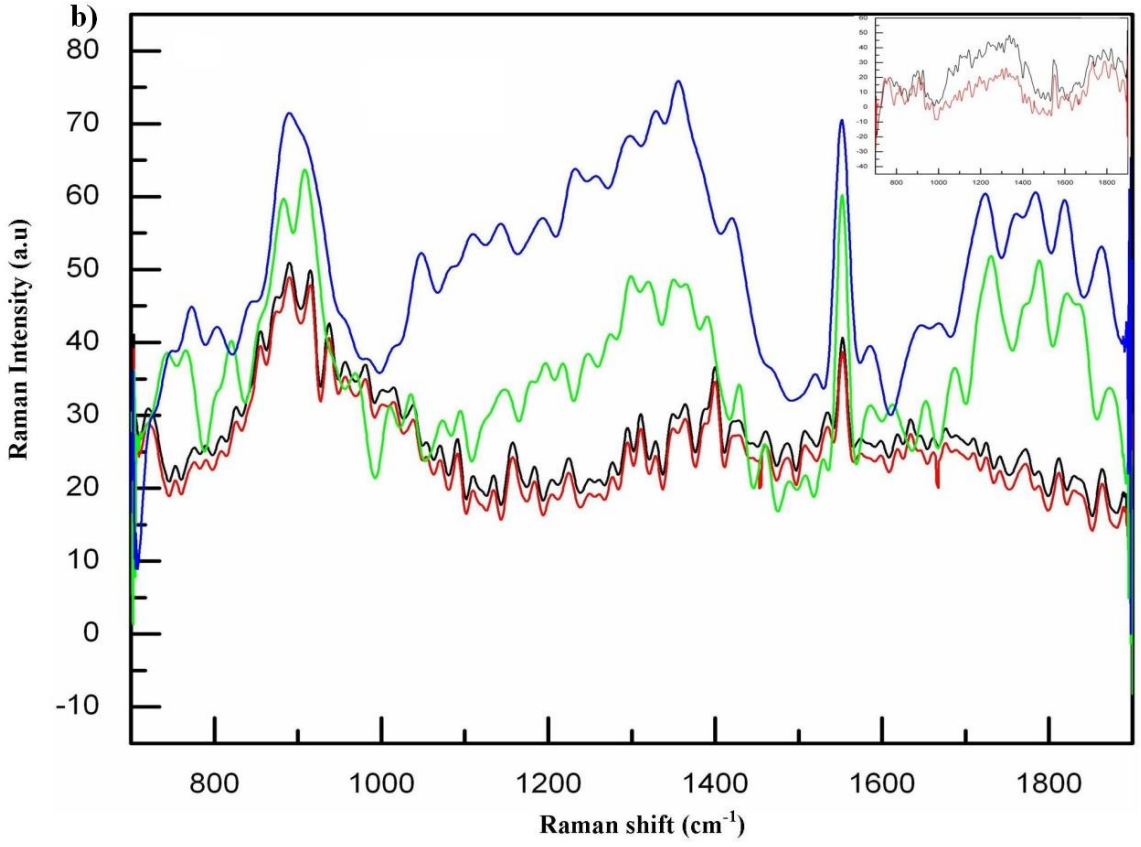

b)
Raman Intensity (a.u)
80
70
60
50
40
30
20
10
0
-10
800
1000
1200
1400
1600
1800
Raman shift (cm^{-1})

Fig.2.6. a) Histogram of number of bacterial colonies as function of NP treated and untreated PB12 cells grown in either LB or 0.01x LB broth; **b)** Raman spectra of PB12 cells grown in either presence or absence of ZnO-PEI suspension (red colour line, PB12 cells grown in 0.01x LB broth; black colour line, PB12 cells grown in LB; green colour line, ZnO-PEI NPs treated PB12 cells grown in 0.01x LB broth; ZnO-PEI NPs treated PB12 cells grown in LB broth). Measurements were taken from 700 to 1900 cm^{-1} at 10 s and c. 10 mW laser power.

2.4. Discussion

Bacterial stress can be caused by environmental modifications (physical/nutritional) that can have many consequences, such as retarded growth and cell death (Fridovich, 1998). Living organism always struggle to survive in response to variety of environmental perturbations. For this, living organisms sense environmental changes by detecting extracellular signals such as the concentrations of nutrients such as carbon, nitrogen, phosphate, sulfur, ion sources, or physical growth factors like pH, temperature, oxygen availability or stresses induced by oxygen, osmolarity, or solvent. These signals ultimately feed into the transcriptional regulatory systems, which affect the physiological and morphological changes to cope effectively for their survival (Seshasayee et al., 2006). Bacterial cells possess complex but efficient mechanisms to respond to the change in culture environment. This is mainly achieved by the so-called global regulators, where they generally act at transcriptional level. A two-component signal transduction system is considered to be the important means of detecting extracellular signals and transducing the signals into cytosol for metabolic regulation. These involve a phospho-relay from a transmembrane histidine protein kinase sensor to the target response regulator. In the case

of *E. coli*, 29 transcription factors (TFs) show such regulation with 28 histidine protein kinase (Kanehisa et al., 2006), where the genes encoding the two components are usually located within the same operon, enabling their coordinated expression. Coordinating growth with division is essential to ensure that cells are the appropriate size for a given environmental condition or developmental fate. This is true not only for multicellular plants and animals, but also for single-celled organisms that need to adapt quickly to rapid changes in environmental conditions. Like their eukaryotic counterparts, in the absence of environmental or internal pressure to increase size, exponentially growing bacteria cultured under a constant set of parameters exhibit little size variation between cells. Similar type of size variation was noted in PB12 cells when it allows growing in nutrient-poor condition. It is essential to note that cell size and shape are, not surprisingly, sensitive to changes in the morphogenesis of the bacterial cell wall because it was noted earlier that bacteria without such cell walls also have complicated morphologies (Miyata & Ogaki, 2006). It was found that bacterial morphology is determined by the coordinated operation of at least two mechanisms: one responsible for cell elongation and the other for division. By means of an actin homologue elongation is driven (e.g., MreB or its relatives) whereas, cell division is driven by the tubulin homologue, FtsZ. FtsZ assembles as a ring at the middle of the cell to localize and initiate cell division (Buddelmeijer & Beckwith, 2002), while MreB is required for cells to grow as rods instead of spheres and coordinates its activities with the FtsZ ring (Vats et al., 2009).

Bacteria in natural environments are relentlessly challenged by the need to familiarize it under varied nutrient availability and stress conditions. In response to such

changes, *Eschericia coli* (Jenkins et al. 1988), *Salmonella spp.* (Foster & Spector 1995), *Pseudomonas spp*. (Jørgensen et al. 1994), and *Vibrio spp.* (Östling et al. 1993) have been shown to elicit classical intracellular reorganization programmes. Naturally, these programmes are operative to make possible the development of cells for long-term survival as well as immediate recovery and consequently by a series of physiological and genetic alteration (Östling et al. 1993). In the present study, it was however attempted to understand how *Klebsiella pneumoniae* PB12 becomes accustomed to stasis, whether induced by lack of nutrients or as provoked by stress conditions. It was observed that Cu,ZnSOD production was higher in 0.01x LB grown PB12 cells compare to PB12 cells grown in LB broth. Literature reveals that periplasmic Cu,Zn-cofactored superoxide dismutase (SodC) protects Gram-negative bacteria from exogenous oxidative damage. Recently, a Cu,Zn-SOD has been found in *Escherichia coli* (Benov & Fridovich, 1994). The factors that may have contributed for the considerably increase in SodC is the selection of conditions under which bacteria are to be cultured. In fact it was found that the level of Cu,Zn-SOD activity was high when organisms are allowed to grown in 0.01x LB rather than in LB. In agreement with this observation, production of the *E. coli* putative Cu,Zn-SOD is strongly induced during aerobic growth (Benov & Fridovich, 1994). Moreover, Kroll et al. found that Cu,Zn-SOD activity can be considerably increased when organisms are grown in shaking liquid media rather than on nutrient agar plates. It was reported earlier that ZnO-PEI NPs possess the potential to generate ROS (Chakraborti et al., 2014). Viability assay in terms of decrease in CFU/ml was done in the presence of ZnO-PEI NPs. It was noted that 0.01x LB grown PB12 cells are more resistant towards NP challenge. This could be explained by the fact that 0.01x LB grown

PB12 cells showed high level of Cu,Zn-SOD and catalase activity compared to its other counterpart grown in undiluted LB. Moreover, the effect of NPs was also demonstrated using Raman spectroscopy which further supports our claim. It was reported earlier that *B. subtilis* utilizes endogenous and exogenous NO for rapid protection from oxidative damage (Gusarov & Nudler, 2005). It was well known that NO suppresses the Fenton reaction by transiently inhibiting cysteine reduction. Our hypothesis is: because the production of catalase and Cu,Zn-SOD is less in LB grown PB12 cells compare to 0.01x LB grown cells, the possible role of NO is to activate catalase vis-a-vis to detoxify excess H_2O_2. Similar type of explanation was also reported earlier (Gusarov & Nudler, 2005). These physiological studies conducted in this chapter may enlighten the survival strategy of a model facultative oligotrophic bacterium, PB12.