Metabolic networks to combat zinc toxicity in *Pseudomonas fluorescens*

By
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Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science (MSc) in Chemical Sciences

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Abstract:

Heavy metals play an important role in organisms, but in elevated concentrations all metals become toxic due to their interference with normal biological processes. The aim of this study was to determine how *Pseudomonas fluorescens* reprograms its metabolic networks to overcome metal toxicity stemming from zinc (Zn), an environmental pollutant. Under Zn stress, this microbe survived despite the fact that two important pathways, the tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation were ineffective. *P. fluorescens* upregulated the enzymes citrate lyase (CL), phosphoenolpyruvate carboxylase (PEPC) and pyruvate phosphate dikinase (PPDK), a strategy which enabled the metabolism of citrate, the sole source of carbon, into pyruvate and ATP. Levels of pyruvate were found to be high in the spent fluid and soluble CFE of Zn-stressed cells. Phosphoenolpyruvate (PEP) provided an important route to ATP via substrate-level phosphorylation. Antioxidant systems such as catalase and superoxide dismutase (SOD) were stimulated by Zn toxicity. The increased activity of NADPH-producing enzymes such as isocitrate dehydrogenase (ICDH), malic enzyme (ME), and glucose 6-phosphate dehydrogenase (G6PDH) was necessary to create a reductive environment. This metabolic shift is critical to counter the oxidative stress triggered by zinc toxicity and to generate ATP in an O₂-independent manner. This metabolic engineering evoked by Zn may be tailored in bioremediation and metal chelation technologies.
Acknowledgments:

I would like to send my appreciation and respects to my supervisor Dr. V. Appanna and my co-supervisor Dr. M. Chahma for their insight and guidance throughout my research.

I would like to thank my committee members Dr. A. Omri and my external examiner for their assistance with my thesis.

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Finally, I would like to thank my mum, dad, mother in law, sibling and my husband, Hassan for their love and encouragement to continue my degree.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-KG</td>
<td>α-Ketoglutarate</td>
</tr>
<tr>
<td>α-KGDH</td>
<td>α-Ketoglutarate dehydrogenase</td>
</tr>
<tr>
<td>ACN</td>
<td>Aconitase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGODH</td>
<td>Acetylating glyoxylate dehydrogenase</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATTC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BN PAGE</td>
<td>Blue native polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>CFE</td>
<td>Cell free extract</td>
</tr>
<tr>
<td>Cl</td>
<td>Chloride</td>
</tr>
<tr>
<td>CL</td>
<td>Citrate lyase</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>Complex I</td>
<td>1st protein complex of Electron Transport Chain</td>
</tr>
<tr>
<td>Cyt C</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenol</td>
</tr>
<tr>
<td>DDH₂O</td>
<td>Deionized distilled water</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrobenzoic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide (oxidized form)</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ICDH</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>ICL</td>
<td>Isocitrate lyase</td>
</tr>
<tr>
<td>INT</td>
<td>Iodonitrotetrazolium</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>ME</td>
<td>Malic enzyme</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MS</td>
<td>Malate synthase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide (oxidized form)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidized form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPC</td>
<td>Phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PEPS</td>
<td>Phosphoenolpyruvate synthase</td>
</tr>
<tr>
<td>Pᵢ</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PPᵢ</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate Kinase</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine methosulphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyfluoride</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SLP</td>
<td>Substrate level phosphorylation</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive species</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>The Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N’, N’– tetramethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>Vol</td>
<td>Volume</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZnBP</td>
<td>Zinc binding protein</td>
</tr>
<tr>
<td>ZFP</td>
<td>Zinc finger protein</td>
</tr>
<tr>
<td>ZnO</td>
<td>Zinc oxide</td>
</tr>
<tr>
<td>ZnNPs</td>
<td>Zinc oxide nanoparticles</td>
</tr>
</tbody>
</table>
1. Introduction:

1.1: Zinc occurrence, physical and chemical properties:

Zinc (Zn) is the 25th most abundant element in the Earth’s crust; it is widely distributed in nature, and ranges between 0.0005% and 0.02% of the Earth's crust (Ochu, J. O., 2012). It has been estimated that the world production of zinc is approximately 7.1 million metric tons (Alan, N., 2008). Zn is found in its metallic chemical element as a bluish-white, shiny metal. It is a divalent metal and is the first element in group 12 of the periodic table. Also, as a strong Lewis acid, Zn can accept a pair of electrons, which makes Zn an effective catalytic agent in many enzymatic reactions and facilitates its coordination to proteins through the thiol group of cysteine or the imidazole of histidine (Stefanidou, M., 2006). Thus, Zn has evolved to be an essential micronutrient in all form of life including humans, bacteria and plants (Broadley, M. R., 2007).

1.1.1: Zinc as a micronutrient:

Zn is an important mineral required by the human body; it can be found in various foods such as meat (particularly in the liver), sea foods as well as cereals (peanuts and other dairy products) (Kaji, M., 2006). A rich amount of Zn can be found in animal products and some diets that provide large quantities of Zn are found (Table 1).
Table 1: Zn containing foods (adapted from Hotz, C., 2004)

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Zn content mg/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver, kidney (beef, poultry)</td>
<td>4.2-6.1</td>
</tr>
<tr>
<td>Meat (beef, pork)</td>
<td>2.9-4.7</td>
</tr>
<tr>
<td>Seafood (fish)</td>
<td>0.5-5.2</td>
</tr>
<tr>
<td>Eggs (chicken, duck)</td>
<td>1.1-1.4</td>
</tr>
<tr>
<td>Dairy (milk, cheese)</td>
<td>0.4-3.1</td>
</tr>
<tr>
<td>Beans, lentils (soy, chickpea)</td>
<td>1.0-2.0</td>
</tr>
<tr>
<td>Whole-grain cereal (wheat, maize)</td>
<td>0.5-3.2</td>
</tr>
<tr>
<td>Vegetables</td>
<td>0.1-0.8</td>
</tr>
<tr>
<td>Fruits</td>
<td>0.0-0.2</td>
</tr>
</tbody>
</table>

Zn is an essential nutrient for growth in infants and young children as well as adults (Rosado, J.L., 2003). The recommended dietary allowances (RDA) of Zn in the United States are shown in (Table 2), which is organized by age and sex.

Table 2: The daily RDA of Zn (adapted from Kaji, M., 2006).

<table>
<thead>
<tr>
<th>Age</th>
<th>Zn mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal infants from birth to 12 months of age</td>
<td>5</td>
</tr>
<tr>
<td>Children 1 to 10 years of age</td>
<td>10</td>
</tr>
<tr>
<td>Males older than 11 years of age</td>
<td>15</td>
</tr>
<tr>
<td>Females older than 11 years of age</td>
<td>12</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>15</td>
</tr>
<tr>
<td>Lactating women</td>
<td></td>
</tr>
<tr>
<td>First 6 months after delivery</td>
<td>19</td>
</tr>
<tr>
<td>Second 6 months after delivery</td>
<td>16</td>
</tr>
</tbody>
</table>
It has been reported that the estimated amount of Zn needed by an infant is about 20 μg/g of tissue (Kaji, M., 2006). Zn concentration increases during development. However, the recommended daily doses of Zn can change based on an individual’s health. Individual requirements of Zn depend on numerous factors including stress, medication, illness and the type of diet a person is consuming. Zn requirements should be assessed based on these factors in order to obtain healthy amounts of Zn in the body (Watts, D. L., 1988).

There are some similarities between mammals and plants in their uptake and transport of trace elements (Shanker, A. K., 2008). Zn is one of the important micronutrients required by plants. It is a component of several proteins in plants. In higher plants, Zn is absorbed as a divalent ion through divalent ion channels (Graham, R. D., 2003). Zn is an essential trace element for various enzyme activities and the growth and development of heterotrophic bacteria (Bong, C. W., 2010).

1.1.2: Essentiality of zinc and zinc-containing enzymes:

Zn is one of the most essential micronutrients required by most organisms, including humans, animals, plants and microbes. It plays a pivotal role in many biological processes as a catalytic, structural and regulatory ion as depicted in (Table 3) (Stefanidou, M., 2006). It is an important component for various enzymatic reactions and it is essential in transcription, cell signaling and regulation of pH (Lemire, J., 2008). It has been reported that more than 300 enzymes in different species of microorganisms, plants, and animals require Zn to function effectively (Chirwa, M., 2012). Moreover, Zn is also critical in a variety of functional proteins. Consequently, numerous Zn-containing enzymes and proteins participate in the metabolism of proteins, nucleic acids, carbohydrates and lipids (Vallee, B., 1993).
Table 3: Biological importance of Zn (adapted from Stefanidou, M., 2006)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-</td>
<td>Zn is essential for cell proliferation and differentiation.</td>
</tr>
<tr>
<td>2-</td>
<td>Zn is found in many enzymes such as carbonic anhydrase and alcohol dehydrogenase.</td>
</tr>
<tr>
<td>3-</td>
<td>Zn is important in proteins such as zinc finger proteins.</td>
</tr>
<tr>
<td>4-</td>
<td>Zn is essential as a transcription factor and for cellular signaling.</td>
</tr>
<tr>
<td>5-</td>
<td>Zn protects biological structures from damage.</td>
</tr>
<tr>
<td>6-</td>
<td>Zn corrects the immune function.</td>
</tr>
</tbody>
</table>

The major roles of Zn occurring directly in nature are either catalytic or structural (Lee, Y. M., 2008). Moreover, it is important in the homeostasis of cell processes such as DNA synthesis, normal growth, brain development, behavioral response, reproduction, membrane stability, bone formation and wound healing. Zn is associated with proteins via three amino acid ligands and one molecule of water (Auld, DS., 2001). The nitrogen in histidine is used most frequently along with glutamate, oxygen in aspartate and sulfur in cysteine. Zn is also found in its cocatalytic site in multi-Zn enzymes with two or more Zn or other metals such as Mg atoms (Stefanidou, M., 2006). Structural Zn atoms are known in which Zn is fully bound to four histidines and occurs on enzymes such as alcohol dehydrogenase, aspartate transcarbamoylase and protein kinase C (Figure 1) (Vallee, B., 1993).
1.1.3: Carbonic anhydrase:

Carbonic anhydrase (CA) is an excellent example of catalytic Zn, which mediates the conversion of carbon dioxide (CO$_2$) and a proton (H$^+$) to bicarbonate or carbonic acid. CA is the first Zn metalloenzyme that has been fully investigated (Escudero-Almanza, DJ., 2012). CA plays several physiological roles such as enhancing CO$_2$ exchange in the erythrocytes, kidneys and lungs, leading to acid-base balance and promoting HCO$_3^-$ secretion (Wang, Y., 1998). It is essential in eukaryotic cells for respiration, photosynthesis, calcification and pH homeostasis. Moreover, CA has been found in many organisms, including mammals as well as plants. There
are many isoforms of carbonic anhydrase such as α, β, γ. All mammalian isoforms contain an α class of this enzyme, while bacteria and the archaea domain belong to β and γ classes. The CA family consists of 13 isozymes in mammals (Smith, K. S, 2000). In this enzyme, the Zn is coordinated with the imidazole rings of three histidine residues: His 94, His 96, and His 119 as shown in (Figure 2) (McCall, K. A., 2000).

**Figure 2:** The ribbon diagram of the structure of human carbonic anhydrase (taken from McCall, K. A., 2000).

Figure 3 shows the summary of the conversion of CO$_2$ to bicarbonate catalyzed by carbonic anhydrase (CA). First, Zn can facilitate the release of proton from water molecules, which contributes to hydroxide formation (Escudero-Almanza, DJ., 2012). Then, Zn binds the hydroxide and attacks the CO$_2$ to form bicarbonate.
Figure 3: The mechanism of carbonic anhydrase II (McCall, K. A., 2000).

CA has been found in the red blood cells and its function is to transport CO₂. The latter, which comes from the respiring tissues, enters the red blood cells by diffusion and is hydrated to form H⁺ and HCO₃⁻. The intracellular hemoglobin buffers H⁺, while HCO₃⁻ needs to be transported back to the plasma for Cl⁻ exchange. When the HCO₃⁻ is formed, the CO₂ is transported to the blood (Henry, R. B, 1984).

In prokaryotes, CA has two pivotal functions: transporting CO₂ and HCO₃⁻ and providing
enzymatic reactions from CO₂ and HCO₃⁻. In aquatic photosynthetic organisms such as *Chlamydomonas reinhardtii*, CA plays essential roles in the CO₂ concentrating mechanism, which can help to adapt to CO₂ starvation in the environment. The prokaryotic CA is involved in carboxylation and decarboxylation processes, which fulfill important physiological functions (Supuran, C. T, 2007). Furthermore, prokaryotic CA has been found in numerous bacteria including *Neisseria sicca, N. lactamica and N. Mucosa* (Nafi, B. M., 1990). The overexpression of CA in *Pseudomonas fluorescens* has been shown to confer calcium (Ca) resistance. In this instance, the enhanced production of HCO₃⁻ enables the precipitation of toxic levels of Ca as calcite (CaCO₃) (Anderson, S., 1992). It has been reported that prokaryotic CA has medical implications because of its presence in many pathogenic species, where it is necessary for microbial virulence (Smith, K. S., 2000). Moreover, CA is an important component in plants located in chloroplasts and cytoplasm in C₃ plants and is essential in photosynthesis (Waraich, E. A., 2011).

1.2: **Zinc as a transcription factor:**

Gene expression regulates various vital biological processes such as development, differentiation and diseases (Sera, T., 2009). The transcription factors are the main modulators of gene expression and bind the promoter regions of genes in order to facilitate the transcription process. Zn finger proteins (ZFPs) that bind DNA are coordinated with histidine and cysteine residues in the Cys₂ His₂ type motif (Figure 4). Zn finger DNA bindings have many repeats involving 30 amino acids. A variety of motifs have been uncovered. For instance, there are more than 700 ZFPs with DNA-binding motifs in humans (Sera, T., 2009).
C (cysteine; yellow) and H (histidine; blue) bind the Zn ion and the linker sequence is in green. The two hydrophobic residues are shown in red, while the structure of black residues is not essential and indicates those responsible for contacting DNA during sequence-specific binding (Knight, R. D., 2001).

Engineered ZFPs provide a novel approach to regulate many cellular processes and to target specific genes. Many beneficial aspects exist for using ZFPs to bind RNA and to interact between proteins; however, the major role of ZFPs is the recognition of DNA. Also, Zn finger motifs have been utilized as scaffolds, which can stretch known DNA interactions with amino acid residues. Various Zn finger domains joined together will extend the DNA sequence by adding 3-4 bp to the recognized sequence with each new attached Zn finger domain. Since the targeted DNA sequence does not need to be symmetric, ZFPs associated with DNA-binding proteins have more benefits than other DNA binding motifs derived from natural transcription
factors, which can bind the DNA as a dimer. This characteristic makes ZFP a suitable option for binding to a DNA sequence (Gommans, W. M., 2005). To control gene expression, it is important to understand the gene function and to promote drugs to treat several diseases. Designed Zn finger protein transcription factors (ZFP TFs) offer an attractive tool for controlling of gene expression. ZFP TFs has been used to activate or repress multiple endogenous gene targets. For these proteins or gene regulation techniques to be successful in drug discovery or direct agents in the clinic, the action with the genome has to be precise (Tan, S., 2003).

Various designed (ZFPs) exist in many fields, including functional genomics, transgenic animals and plants, somatic cell genetics, drug discovery, therapeutics, and genome-editing genome corrections (Rebar, E., 2004). Functional genomics is a powerful tool due to its potential to inactivate a single promoter, which yields ZFP-repressor as beneficial tools to examine gene functions. ZFP TFs afford a good technique for construction of food plants with Zn useful agricultural traits. ZFP activators have diverse applications for enhancing the expression of pharmacological proteins. They also offer powerful tools for constructing cell lines for drug screening. ZFPs have been used for treating a variety of genetic diseases such as ZFP-nucleases, which provide a promising strategy for genome correction (Rebar, E., 2004).

### 1.2.1: Zinc in signaling:

Zn is important for a number of proteins, including enzymes that belong to cellular signaling pathways and transcription factors. A Zn signal has been observed in monocytes when treated with lipopolysaccharide and these signals regulate inflammatory signaling (Haase, H., 2009). It has also been indicated that Zn acts as a neurotransmitter. In neurons, exocytotic stimuli increases released Zn into the surrounding milieu and it is taken up into the cytoplasm via gated Zn channels in adjacent cells. Released Zn migrates to neighboring cells such as postsynaptic neurons and glial cells and functions as modulators and mediators between cell-to-cell signaling.
Therefore, it acts as an autocrine, paracrine, transcellular, transmembrane signaling factor and a neurotransmitter. Zn functions as a signaling molecule because Zn mimics the action of hormones, growth factors and cytokines (Yamasaki, S., 2007).

The role of Zn as neurotransmitter is different from the idea that Zn acts as a second messengers in cells because neurotransmitters carry the information between cells, whereas the secondary messengers act intracellularly. The first secondary messenger discovered is cyclic adenosine monophosphate (cAMP). To date, a few of secondary messengers have been identified, like Ca\(^{2+}\), cyclic guanylic acid (cGMP), NO, lipid mediators, G-proteins, protein kinases and protein phosphates (Murakami, M., 2008).

Zn has impacts on toll like receptor 4 (TLR 4) as shown in (Figure 5), inducing cytokine secretions in monocytes. Increased free Zn is a physiological signal containing both TLR 4 signal transductions, and elevated concentration of Zn leading to TLR4 signal inhibition (Beyersmann, D., 2001). After stimulation with LPS, the intracellular Zn signal will rapidly be observed. It is an essential in activation of mitogen-activated protein kinases (MAPKs) by TLR 4. Zn-activated MAPK is involved in most cell types. It has been suggested that the mechanisms inhibit interleukin-1 receptor related kinase (IRAK) and up-regulates A20, which removes the activation of the tumor necrosis factor receptor-associated factor 6 (TRAF6) or an inhibition of cyclic nucleotide phosphodiesterases (PDE). Cyclic nucleotide phosphodiesterase (PDE) leads to increasing cGMP and activation of protein kinase A (PKA), which inhibits Raf and thereby TLR-mediated NFkB activation (Haase, H., 2009).
1.2.2: Zinc in metabolism:

Zn ions are an integral part of homeostatic mechanisms that regulate several metabolic activities in cells and tissues. It has been reported that increasing intracellular Zn can influence carbohydrate metabolism. In hepatocytes, Zn can stimulate muscle glycolysis, inhibits the synthesis of glycogen and alters cellular energy metabolism (Rofe, A. M., 2000). It has been shown that Zn-binding protein (ZnBP) in vitro is around 11.5 KDa and interacts with essential enzymes of carbohydrate metabolism such as the liver enzymes aldolase, phosphofructokinase, hexokinase-1, glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase,
glyceraldehyde-3-phosphate dehydrogenase and fructose-1, 6-bisphosphatase. Studies show that the effect of Zn ions on carbohydrate metabolism of hepatocytes has become indirect evidence of the involvement of ZnBP in the regulation of glycolysis and gluconeogenesis (Brand, I. A., 1996). Also, there are many Zn containing enzymes found in glycolysis shown in (Figure 6) such as aldolase and enolase (Pelicano, H et al., 2006).

**Figure 6:** Glycolysis with Zn-containing enzymes. HK, hexokinase; PGI, phosphoglucone isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase(Pelicano, H et al., 2006).
Zn is an important plant nutrient because of its involvement in a variety of critical aspects of metabolism. Plant roots require Zn as a divalent ion, and Zn is then distributed via the whole plant in a complex series of processes (Sagardoy, R., 2009). In prokaryotes, Zn plays a role in various enzymatic activities. It has been known that Zn-containing proteins called metalloproteases are found in numerous bacteria such as *Bacillus, Streptomyces and Pseudomonas* species (Hu, Y., 2012). The best example of protease produced by *Bacillus* is thermolysin. The protease contains a single polypeptide chain associated with thiol or disulfide group located in the active site and four calcium ions to stabilize the protein. Zn metalloproteases have effective therapeutic applications. *Pseudomonas aeruginosa*, which is an opportunistic pathogen causing a diverse array of fatal infections has various extracellular products such as metalloproteases, elastase and alkaline protease (Finlayson, E.A., 2011). Elastase is a Zn-containing metalloprotease that functions to degrade numerous essential substances including, elastin, laminin, fibrin, human collagen and immunoglobulins (Hase, C. C., 1993). Additionally, anthrax lethal factor is also a Zn-dependent endopeptidase and constituent of the anthrax toxin secreted by *Bacillus anthracis* (Nguyen, T. L., 2007).

Zn has a critical role in the function and structure of proteins from prokaryotes. It has been identified that *Escherichia coli* has numerous Zn-binding proteins including acetate kinase (AckA) that needs metal for expression of catalytic function as shown in (Table 4) (Katayama, A., 2002).
### Table 4: Zn-binding proteins in *Escherichia coli* (taken from Katayama, A., 2002)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ada</td>
<td>Transcription factor for ada operon</td>
</tr>
<tr>
<td>Cdd</td>
<td>Cytidine deaminase</td>
</tr>
<tr>
<td>Def</td>
<td>Peptide deformylase (PDF)</td>
</tr>
<tr>
<td>DnaG</td>
<td>Primase for DNA replication</td>
</tr>
<tr>
<td>DnaJ</td>
<td>Molecular chaperone</td>
</tr>
<tr>
<td>Fba</td>
<td>Fructose 1,6-bisphatase</td>
</tr>
<tr>
<td>Fpg</td>
<td>Formamidepyrimidine-DNA glycosylase</td>
</tr>
<tr>
<td>FtsH</td>
<td>ATP-dependent protease</td>
</tr>
<tr>
<td>Fur</td>
<td>Ferric uptake regulation protein</td>
</tr>
<tr>
<td>HemB</td>
<td>5-Aminolevulinic acid dehydrogenase</td>
</tr>
<tr>
<td>LpdA</td>
<td>Lipoamide dehydrogenase</td>
</tr>
<tr>
<td>MetE</td>
<td>Cobalamin-indepd. Methionine synthase</td>
</tr>
<tr>
<td>MetH</td>
<td>Cobalamin-depd. Methionine synthase</td>
</tr>
<tr>
<td>PhoA</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Ppa</td>
<td>Inorganic pyrophosphatase</td>
</tr>
<tr>
<td>Pta</td>
<td>phosphotransacetylase</td>
</tr>
<tr>
<td>RpoA</td>
<td>RNA polymerase α subunit</td>
</tr>
<tr>
<td>RpoC</td>
<td>RNA polymerase βi subunit</td>
</tr>
<tr>
<td>RpoB</td>
<td>RNA polymerase β subunit</td>
</tr>
</tbody>
</table>
1.2.3: Zinc deficiency:

Zn is essential in a variety of biological processes. Therefore, a deficiency of this metal has several impacts on the immune and nervous system *in vivo*. It has been reported that immune function decreases after Zn depletion. For example, Zn deficiency in mice will affect the reduction of natural killer cell-mediated cytotoxic activity and host defense against pathogens and tumors (Yamasaki, S., 2007). In humans, Zn contributes to various health problems. Zn deficiency in animals leads to many manifestations such as growth failure, hair loss, epidermal hyper keratinized and testicular atrophy (Prasad, A. S., 1998).

Zn deficiency leads to many diseases such as gastrointestinal disorder, renal disease, sickle cell anemia, AIDS, and some types of cancers (Stefanidou, M., 2006). Most human organs are affected by severe Zn deficiency including the epidermal, gastrointestinal, central nervous, immune skeletal and reproductive systems. Zn depletion is the major contributor for the syndrome of “adolescent nutritional dwarfism” that has been discovered in mid-Eastern countries (Hambidge, M., 2000).

Zn deficiency has been shown to lead to prostate cancers because Zn is an important component in several transcription factors, antioxidant defense enzymes and DNA repair proteins. Zn also helps protect the DNA from damage and protects sulfhydryl groups from oxidation. A high level of Zn protects the prostate from oxidative stress by reducing the generation of free radicals and increasing the antioxidant defense. Zn depletion may affect the cell cycle and apoptosis by controlling of cell signaling pathways. For example, Zn depletion reduces the circulation level of insulin as growth factor-1 and leads to intracellular receptor tyrosine kinase disruption, which has an impact on cell proliferation and survival. Moreover, Zn deficiency is known to impair the DNA repair functions because it has a pivotal function in DNA repair proteins. Zn supplementation has a beneficial role in decreasing the susceptibility to
prostate cancer and reduces the risk associated with prostate cancer (Ho, E., 2009).

Zn is an important nutrient in plants. Zn deficiency in tomatoes contributes to impair stem elongation and reduced protein and starch synthesis. Also, severe Zn deprivation leads (Figure 7) to root apex necrosis or causes reddish-brown or bronze tints in leaves. There are many regions concerned with Zn-deficient in plants including soil in India, Turkey, China, and Western Australia (Broadley, M. R., 2006). Zn deficiency is the most widespread disorder in rice that causes stunted growth, leaf bronzing and seedling mortality (Rose, M. T., 2012). Also, Zn deprivation is common in wheat, contributing to severe depression in wheat production. Furthermore, Zn-deficient plants release a variety of organic components from roots including sugars, amino acids and phenolic compounds, which create a suitable environment for infection.

Zn is a pivotal trace element due to its participation in various biological processes; however, under elevated concentration, Zn is toxic. Zn should be tightly controlled. Microorganisms are essential in the soil plant system because of their importance in recycling of nutrients. For instance, Arbuscular mycorrhiza (AM) fungi help acquire nutrients via nitrogen fixation (Ramezanian, A., 2010).
Figure 7: Zn deficiency symptoms in plants (taken from Broadley, M. R., 2006).

1.3: The occurrence of zinc in the environment and the environmental problem of zinc:

Heavy metals can be released into the environment by natural and anthropogenic activities, which concentrate these heavy metals in soils and sediments. It has been shown that the concentration of heavy metals in sediments found in natural water bodies is much higher than the overlaying waters. Also, in soils, heavy metals may be found in phosphate minerals, and hydrous oxides of aluminum, iron and manganese. In order for heavy metals to be available and enter the cells, they have to be in their soluble forms (Bondarenko, O., 2008). The major factor that solubilizes the metals from mineral surfaces is pH. Under normal pH conditions, most metal hydroxide minerals have very low solubility because the hydroxide ion is associated with pH. When the pH decreases, the soluble form of metal hydroxide minerals increases. Consequently,
the dissolved metals become more available and may incorporate with biological processes because the ionic form of the metal is toxic, especially in aquatic systems (Salomons, W., 1995).

Zn, as an example of a heavy metal, is promoted in the environment due to anthropogenic sources, such as mining and smelting (Figure 8). Zn mining and smelting activities are the major sources of metal pollutants in the environment. It has been reported that there are high amounts of released Zn in the environment. Under certain conditions, some Zn released from mining might transfer and accumulate in an area that could have effects on plants, animals and humans. Zn mining industries are also the major contributor to water pollution. Polluted soils have decreased nutrients and reduced microbial diversity, which has a negative impact on plant growth. Consequently, these polluted soils, generated by Zn distribution and heavy metals released from Zn mining such as cadmium (Cd), and lead, become toxic and are potential risks to human and environmental health (Zhang, X., 2011).

The amount of Zn in the soil increases from the disposal of the wastes of metal manufacturing industries. An industrial process such as galvanization is one factor that leads to human exposure to Zn by inhalation of Zn-containing smoke. Most bombs contain Zn oxide or Zn chloride, which has led to several cases of Zn containing fume inhalation (Plum, L.M., 2010). Automobiles are also another contributor of soil pollutions because they contain gas and various oils that have led to soil contaminations (Kadi, M. W., 2009). Moreover, Zn can be increased by emissions arising from transport systems, fuel combustion, vehicular or road/track construction material and cargo leakage particles, which leave metal deposit into the soil via dry or wet deposition (Liu, H., 2009).

In addition, increased manufacturing and the use of nanoparticles contribute to a large release of engineered nanoparticles into the environment. ZnO nanoparticles (ZnO NPs) have
been used in diverse applications such as semiconductors, catalysts, paints and many other consumer products such as sunscreen to block UV radiation. Thus, increasing the production and the use of ZnO has led to exposure to humans and other organisms. Nanoparticles released into the environment through wastewater treatment promote the exposure to the ecosystem as well as to aquatic organisms. It has been shown that ZnO NPs have a higher toxicity than Zn\(^{2+}\) ion alone; however, these particles dissolve quickly and the Zn ion is the major source of toxicity (Reed, R. B., 2012).

Zn can be combined with other elements, such as oxygen, sulfur, and chloride to form Zn compounds, which are found in waste site as Zn chloride, Zn sulfide, and Zn sulfate. Zn compounds have numerous uses in the industry. Zn sulfide and Zn oxide are used to make white paints, ceramics, textiles, fertilizers, pigments, batteries and many other products. Zn chloride is known to be a key contributor to the smoke generated by smoke pumps. Zn compounds have many applications in the pharmaceutical industry such as in vitamins, diaper rash and antidandruff shampoo (Liu, H., 2009). Zn oxide (ZnO) has been an essential industrial material and has been used in various applications. As well, ZnO is used in the rubber industry and photocopy paper as a photoconductive ingredient. In recent years, ZnO has been investigated for many usages in transistors, solar cells and memory devices.

Zn phosphate (Zn\(_3\) (PO\(_4\))\(_2\)) is another Zn compound that has numerous uses including in corrosion-resistant paints for metal structure and as filler in the manufacture of vulcanizates to promote heat resistance. It has been reported that Zn phosphate is used as an anti-galling agent in the coupling of drill strings for industrial oil and gas (Moezzi, A., 2012). Zn sulfide (ZnS) on the other hand has several applications as important materials in photonics research, and it has been utilized in the fabrication of light-emitting diodes, electroluminescent devices, ultraviolet sensors
and solar cells (Zhao, L., 2004). Hence, Zn compounds are widely distributed in nature due to their involvement in numerous aspects of our daily life.

Zn can interact with Cd, which induces oxidative damage and affects the soil environment as well as the ecosystem. Cd is the most toxic heavy metal and it is not involved in any physiological functions. While Zn is an essential trace element for all forms of life including human and bacterial species, it can be toxic in elevated concentration. As Cd and Zn belong to the group IIB of the periodic table, they tend to have similar physico-chemical properties. In a Zn smelter, Zn pollution of the soil could increase Cd levels and the presence of one chemical in the soil affects the availability and mobility of others (Van Gestel, CA. M., 1997). Due to their common occurrence in nature, they may compete with one another for similar ligands. Cd can induce oxidative damage and interfere with important metals such as Zn. Cd can displace Zn in biological systems because of its higher affinity for S-ligands and N-donors. Indeed, Zn benefits against Cd-induced oxidative stress because increased Zn will decrease the accumulation of Cd, which helps to reduce the toxicity. The interactions between Cd and Zn are found not only in organisms, but also in soil, where metals can compete for the sorption site and this can lead to the interaction of metals. Because the metals interact at a high exposure level, they become bioavailable in the soil (Qiu, H., 2011). Moreover, Cd is known to substitute for Zn in metalloenzymes and has a very strong affinity for biological structures, including proteins, enzymes and nucleic acids which contain sulphydryl groups. When Cd displaces Zn, Cd interferes with Zn’s absorption. The impacts of Cd are a disruption of Zn-dependent metabolic processes such as the cellular production of DNA, RNA, and proteins that could modify Zn homeostasis and contribute to serious risks in human’s organs and can cause chronic or acute diseases (Brzóska, M. M., 2001). Zn is one of the 129 priority pollutants as listed by the
Environmental Protection Agency (EPA) because millions of tons of Zn waste is discarded and can contribute to numerous hazards in the environment. Excessive Zn is known to lead to iron and copper deficiencies, nausea, fever, headache, tiredness and abdominal pain (Alan, N., 2008).

**Figure 8:** Occurrence of heavy metals in the environment (Aktar, W., 2011).

**1.3.1: Toxicity of zinc:**

Zn production can be found in many regions, such as China, Australia, India, United States and Canada. China is one of the top countries in Zn production and in 2001, it produced 950 000 tons of Zn found near smelting area sites. Heavy metal contamination has increased in these areas in recent years (Wang, C., 2009). It has also been reported in China in 1985 that the mine-tailing dam of Chenzhou lead/ Zn mine collapsed due to heavy rains. In that phenomenon, a strip of frame about 400m large on both sides of the river channel was covered by a 15 cm
thick layer of black sludge. The toxic sludge contaminated the soil surface that led to the uptake in crops (Liu, H., 2005). Also, due to increases in both industry and population in most cities in China, pollution has become the major concern in the country (Wang, Q. Y., 2001).

1.3.2: Effect of excess zinc in plants:

Excess heavy metals in the soil are a major factor behind many problems in plants, microorganisms, animals and humans (Liu, H., 2009). Zn is the second important transition metal after iron and it is involved in many biological processes in most organisms. Excess Zn negatively affects plants via inhibition of seed germination, plant growth, root development and leaf chlorosis. In the cell, excess Zn can modify mitotic activity and affect membrane integrity and permeability, thus killing the cells. At the cellular level, excess Zn can also modify gene expression. It has been found that various genes in biological processes, such as lignin biosynthesis and several gene-encoding proteins are associated with defenses to combat oxidative stress. Excess Zn can lead to the induction of oxidative stress (Wang, Q., 2009).

High Zn concentrations disrupt the absorption of other nutrient elements. Consequently, the imbalance of nutrients may increase heavy metal toxicity in plants (Wang, C., 2009). Soil contaminated by heavy metals affects plant uptake, physiological processes and ecological organisms. This can lead to potential risks to human health through the consumption of food from polluted soil (Wang, Q., 2001).

Zn toxicity is also found in soil contaminated by mining and smelting activities. The Zn toxicity symptoms occur in the leaves of some crops. Crops react differently to Zn toxicity depending on soil pH. In acidic soils, graminaceous species are less susceptible to Zn toxicity than most dicots (Broadley, M. R., 2007). It has also been shown that the crops found in acidic soil suffer from Zn toxicity and species that have high concentration of Zn uptake such as spinach and beet are more susceptible to excess Zn (Sagardoy, R., 2009). Excess accumulation of
Zn in plants is the major factor in physiological constraints contributing to decreased vigor and plant growth, interfering with various activities of vital enzymes for normal metabolism. Although Zn is an important trace element, it can be phytotoxic at elevated concentration by reducing crops when plant leaves reach around 300-1000 μg Zn g\(^{-1}\) dry mass (Souza, J. F., 2007).

The average Zn concentration needed for the plants to grow is about 25-150 mg Kg\(^{-1}\). However, when Zn reaches to 400-500 mg Kg\(^{-1}\), it inhibits the growth of plants (Sagardoy, R., 2009). Excess Zn also inhibits CO\(_2\) assimilation because of structural and functional disturbances in the photosynthesis. Excess Zn induces Fe or Mg deficiency, which leads to increased chlorosis and necrosis in young leaves and could also lead to a decrease in the accumulation of photosynthetic pigments (Vassilev, A., 2011). For example, Zn toxicity in \textit{P. vulgaris} leads to the inhibition of photosystems II and I, which decreases photosynthesis. Also, in \textit{Spinacia oleracea} (spinach), excess Zn can reduce ATP synthesis and the activity of chloroplasts (Reichman, S. M., 2002). Excess Zn induces the generation of reactive oxygen species and substitutes for other metals in the active sites of protein. Zn toxicity can decrease tissue water content and change the P and Mg concentration in plant tissues (Sagardoy, R., 2009). Under Zn stress conditions, Zn sensitive grasses induce P deficiency symptoms, which has an impact on P absorption by effects on P metabolism (Reichman, S. M., 2002).

Excess Zn stimulates the production of many enzymes in \textit{P. vulgaris}. It also leads to inhibition of physiological activity such as inhibition of chloroplast NADPH production. A high concentration of Zn in \textit{O.sativa} contributes to inducing peroxidase, auxin oxidase and ascorbic acid oxidase, whereas the activity of catalase, α-amylase, ATPase and phytase are inhibited. Zn toxicity also results in a decrease of the accumulation of the amino acid in \textit{panax quinquefolium} (American ginseng) roots. However, the toxic concentration of Zn in \textit{D. cespitosa} roots is shown
to cause the accumulation of asparagine and proline. Under conditions of Zn toxicity, Zn inhibits stem cell elongation. It has been shown that excess Zn in *H. annuus* cells has led to mitotic abnormalities (Reichman, S. M., 2002).

A variety of metals such as Zn, Cu and Mn are important for the function of living organisms as a trace element for many biological processes. They are essential for the structure of proteins and pigments, in the redox process, regulation of the osmotic pressure, and maintenance of the ionic balance and enzyme component of the cells. Although some metals like Zn are important micronutrients in most bacteria, at elevated concentrations Zn inhibits the growth of most bacteria. Heavy metals could modify the conformational structures of nucleic acids and proteins and also may inactivate some enzymes, which slows growth and destroys cell membranes (Bong, C. W., 2010). Bacteria facilitate the transport of the metals from root to shoot. *B. cepacia* promotes Zn accumulation in shoots, while at low concentration of Zn, it accumulates in roots. Recent studies have shown that bacteria enhanced the plant’s potential for Zn phytoremediation because they protect the plant against the metal’s inhibitory effects (Li, W. C., 2007).

**1.3.3: Effect of excess zinc in aquatic systems and microorganisms:**

Heavy metal contamination of aquatic eco-organisms is a serious pollution issue. Most heavy metals have harmful effects on all organisms when they are in high concentration. There are many ways to release heavy metals in an aquatic environment, including natural or anthropogenic sources. The accumulation of heavy metals contaminates aquatic environments. Metals are known to contaminate aquatic eco-systems and be hazardous to aquatic life because of their high toxicity, tendency to bioaccumulate and their availability via anthropogenic sources. Consequently, that will contribute to potential risks for all organisms (Aktar, W., 2011). Fish are one of the important aquatic organisms. Heavy metals can be accumulated in fish, which can
become a major contributor to metal pollution resulting in harmful impacts on human health (Aktar, W., 2011).

The free metal ion is the most toxic form due to their binding to certain ligands (Atchison, G. J., 1987). It has been reported that at high concentrations, Zn could inhibit Ca regulation in fish. Zn in fish acts as both an essential micronutrient and as a toxicant. One example of the indirect impact of Ca absorption is the impairing of reproduction in rainbow trout exposed to 50 μg/L Zn. The inhibition of Ca uptake leads to imbalances in the uptake and loss of Ca, which decreases the plasma Ca concentration called hypocalcemia. Since Zn and Ca share common uptake pathways, they seem to compete for the same ion channel. Therefore, elevated levels of Zn affect Ca uptake (Santore, R. C., 2002). The exposure to high amounts of Zn can disrupt acid-base regulation. This might be because of the inhibition of carbonic anhydrase (CA) activity, which has effects on CO₂ excretion. Other possible reasons for the toxicological effect of Zn are its impacts on Ca regulation. The exposure to high concentration of Zn increases an adverse respiratory response in fish due to an acute inflammatory reaction, which could destroy the bronchial surface (Santore, R. C., 2002). It has been shown that the lifetime for rainbow trout in a solution of Zn increased 2.35 times when the temperature decreased from 22-12 °C (Reed, P., 1980).

Moreover, low dissolved oxygen concentration has been shown in many polluted rivers to increase the toxicity to fish. It has been shown that the concentration of Zn required to kill 50 percent of fish was 1.4 times higher at an oxygen concentration of 8.9 mg/L than it was at 3.8 mg/L. Zn is most toxic at a pH of 6 and hardness of 50 mg/L. At a pH of 8 or higher, the solubility of Zn is decreased because when the pH increases, the solubility decreases. Zn is toxic to fish at pH of 8 or higher because the pH undergoes a local decrease near the fish gills and the
insoluble form changes to a soluble form. The toxic concentration of Zn causes many modifications to the morphology and physiology of fish. High amounts of Zn also destroy the gills and damage the heavy secretions of mucus in sticklebacks. Acute toxic concentrations of Zn also cause severe cytological damage to the gills (Reed, P., 1980).

1.4: Zinc Toxicity in humans:

Zn is an important trace element, but at high concentrations it is toxic due to its interference with various biological processes. Excess Zn could cause various dysfunctions, which can result in impairment of growth and reproduction. Symptoms of Zn toxicity include vomiting, diarrhea, bloody urine, icterus (yellow mucus membrane), liver failure, kidney failure and anemia (Fosmire, G., 1990). The general poisoning impacts of heavy metals happen because of their interference with normal metabolic processes. This is especially true, when heavy metals are ingested in the acid medium of the stomach, and change to their stable oxidation states such as Zn\(^{2+}\), Cd\(^{2+}\), and Hg\(^{2+}\). They then combine with biological molecules such as proteins and enzymes to form strong bonds as shown in (Figure 9) (Duruibe, J. O., 2007).
Figure 9: Interactions of cysteine or methionine with heavy metals (Duruibe, J. O., 2007).

Zn leads to many effects, including neurological disorders, disruption of Fe homeostasis and perturbation of cholesterol metabolism (Lemire, J., 2008). Zn is abundant in the brain with about 200 ng/mg protein. The largest amount of Zn, around 80% or more, is found interacting tightly with proteins. The second pool of Zn is about 10% and occurs in the vesicles of specific neurons. In the third pool, Zn exists in free form or ionic form in the cytoplasm. At elevated concentration, Zn is neurotoxic and leads to various brain injuries, including stroke, epilepsy, mechanical trauma and Alzheimer’s disease. Zn can kill neurons by impeding ATP synthesis and impairing mitochondrial function. Zn is non-toxic, but Zn concentrations can be increased during injurious stimuli.

Zn also affects many energy pathways such as glycolysis, tricarboxylic acid cycle and the electron transport chain, which leads to depletion in energy production. Zn inhibits some glycolytic enzymes such as glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and
phosphofructokinase. Zn inhibition to GAPDH could be injurious to cells. In neurons, elevated Zn concentration results in the building up of glycolytic metabolites such as dihydroxyacetone phosphate and fructose 1,6-bisphosphate. Depletion of downstream 1,3-biphosphoglycerate and pyruvate and reduction of ATP levels in the cells are also results of Zn toxicity. Zn also inhibits the TCA cycle and the electron transport chain in the mitochondria. It has been reported that Zn inhibits rat liver mitochondrial respiration via complex I (Dineley, K. E., 2003).

This soft acid metal can interact strongly with enzymatic imadizole and sulfhydryl groups. Mitochondrial ACN is inhibited due to the ability of Zn to disrupt the Fe-S cluster, which is needed for catalysis. Also, other enzymes dependent on Fe-S clusters might be inhibited, such as SDH and Cyt C Ox. This inhibition will diminish the production of ATP and will result in electron leakage and ROS production in the mitochondria (Lemire, J., 2008). Also, Zn impedes the α-ketoglurate dehydrogenase complex C (KGDHC) of the TCA cycle shown in (Figure 10) (Dineley, K. E., 2003). KGDHC inhibition happens when Zn interacts with thiol groups in lipoic acid residues on the E$^-2$- subunit of the enzyme, which makes it inactive. This divalent metal also interacts with thiol groups of the antioxidant glutathione (Lemire, J., 2008).

Elevated Zn concentration leads to ROS accumulation and lipid peroxidation in neurons (Dineley, K. E., 2003). Zn inhibition of the TCA cycle is the major contributor to ROS production. Another reason is that high amounts of Zn in neurons activates the generation of superoxide by the enzyme NADPH oxidase. The disruption of the mitochondria leads to mitochondrial permeability transition (MPT), which means the opening of very large and non-selective pores. These pores allow the facilitation of transport and the opening is the main step to start apoptotic and necrotic cell death. The consequences of initiating MPT include mitochondrial-swelling, efflux of mitochondrial Ca stores and the release of many molecules
from mitochondria such as glutathione, cytochrome C and apoptosis-inducing factor (AIF). It has recently been shown that Zn induced MPT in brain mitochondria at a concentration of 10 nM causing swelling as well as releasing cytochrome C and apoptosis inducing factor (Dineley, K. E., 2003).

![Diagram of Zn inhibition targets](image)

**Figure 10:** The targets of Zn inhibition (adapted from Dineley, K. E., 2003). This figure represents the bioenergetic targets for Zn inhibition. Zn may impede cellular energy production via the inhibition of glycolysis, TCA cycle and mitochondrial electron chain. The elevated Zn reduced ATP levels and increases ROS production, which contribute to MPT.

### 1.4.1: Adaptation to elevated zinc concentrations:

Metallothioneins (MTs) are small cysteine-rich proteins present in eukaryotes including humans and plants. The family has four isoforms. MTs I and II occur in all types of cells, while MT III is expressed in neurons. MT IV is present in neurons and in epithelial tissues. MTs play essential roles in regulating Zn uptake, distribution, storage and release. It is known that MTs
bind Zn more tightly than other proteins. In human hepatocytes Zn-bound MTs represents 5-10% of total Zn. MTs are potent scavengers of heavy metals such as Zn and can detoxify harmful heavy metals and ROS produced by various stressors. MTs are able to capture hydroxyl radicals, which are partly responsible for ROS toxicity. MTs protect biological structures and DNA from oxidative damage by distributing Zn. Zn metabolism is modified in Alzheimer’s disease, and it has been found that MT-3 was reduced in extracts from Alzheimer’s disease brain. Therefore, MTs help to protect neurons from oxidative stress as well as modulate neurotransmission during inflammation. The liver is the main site of Zn regulation through induction of ZnMT (Stefanidou, M., 2006).

The first MT known in plants was the wheat Ec (for early cys-labelled) protein. Recently, more than 50 MT-like sequences have been found in a variety of plants. They are different from mammalian and fungal MTs. MTs play roles in bacteria, including SmtA from Synechoccus, which can sequester and detoxify four Zn ions per molecule of metallothionein. Also, it has been noted that other bacterial MTs can bind multiple Zn ions such as Bmt from Anabena, Pseudomonas aeruginosa and Pseudomonas Putida (Blindauer, C. A., 2002). Histidine has been shown to be an effective chelator of Zn. Supplementation with histidine impacts Zn uptake in many physiological assays, including absorption by intestinal preparations from fish, crustaceans and mammals and Zn uptake by cells such as erythrocytes. It has recently been found that the ability of amino acids in a medium to protect astrocytes from the toxicity of Zn was more effective with histidine than with cysteine, glutamine and threonine. (Murphy, J. T., 2011).

Because of the potential toxicity of metals, organisms have to possess various strategies to tightly regulate the distribution of metal ions. There are principal mechanisms for the detoxification of heavy metals such as efflux, sequestration and chelation. For example, the
Arabidopsis halleri orthology of HMA4 is important for hypertolerance of Zn. The loss of the major facilitator superfamily Z1F1 leads to Zn hypersensitivity in Arabidopsis (Arabidopsis thaliane) (Tennsteadt, P., 2009). In higher plants, the tripeptide glutathione is essential for distribution of reduced sulfur and also pivotal in plant defense mechanisms against environmental stress, including heavy metal stress, xenobiotic stress, oxidative stress and pathogen attack. It has also been noted that glutathione is an important factor in Zn-phytochelator synthesis that are present in the roots of maize seedlings (Tukendorf, A., 1996).

Also, it has been reported that the main components of metal balance are transport, chelation and sequestration processes. These are tightly regulated to ensure the effective delivery and distribution at the cellular and organism level, resulting in metal tolerance. The metal ion uptake via transporters is coordinated by chelators and chaperones, which are involved in metal trafficking. Essential metals are transported to the cytosol and then to the organelle. The uptake to the organelle is catalyzed by a metal ion pump, which can interact with a chaperone. Detoxification of the excess metal is achieved through sequestration in vacuoles (Clemens, S., 2001).

Phytochelators (PCs) are small, metal-binding peptides of the general structure (gGluCys)nGly, n=2-11 (Pawlik, B.S., 2003). They have been found in most plants and certain fungi. Phytochelators are enzymatically synthesized by a PC synthase, which is induced rapidly after exposure to various metals. It has been shown that in Arabidopsis Cad1 mutant, PC-Cd is an important complex to detoxify Cd in the plant (Clemens, S., 2001). PC precursor and GSH act as antioxidants, and they have an essential role in plant adaptation. Some plant species grow under elevated heavy metal concentration in the soil (Baccio, D. D., 2005). It is assumed that thiol peptide like GSH and phytochelation play a role for defense against Zn in algae. Studies
show that both Zn-tolerant ecotype of *S. tenue* (T) and Zn sensitive ecotype of *S. tenue* (S) produce similar amounts of phytochelators in response to short-term exposure to Zn (Pawlik, B. S., 2003). It has been shown that the overexpression of the γ-glutamyl cysteine synthetase gene from *E. coli* in *Brassica juncea*, leading to an increase in the biosynthesis of glutathione and PCs results in tolerance to Cd.

Plants have a wide range of potential strategies to cope with environmental stress such as heavy metal toxicity. For example, some ecotype species enhance oxidative defense to counter heavy metals. In *mycorrhizas*, the role to detoxify heavy metal is taken on by the cell wall and through extracellular exudates. Tolerance also could be mediated by the plasma membrane, either by reducing the uptake of heavy metals or by promoting the efflux pumping of metals from the cytosol. Figure 11 summarizes the cellular mechanisms for metal detoxification in higher plants (Hall, J. L., 2002).
Figure 11: Summary of the cellular mechanism for metal detoxification (Hall, J. L., 2002). The sequestration of metal movement to roots by mycorrhizas. 2. Binding to cell wall and root exudates. 3. Reducing influx across plasma membrane. 4. Active efflux into apoplast. 5. Chelation in the cytosol by several ligands. 6. Repairing and protecting the plasma membrane against the stress conditions. 7. Transporting by PC-Cd complex in to the vacuole. 8. Transporting and accumulating of metals in the vacuole (taken from Hall, J. L., 2002).

Although the root of the cell wall is directly in contact with metals in soil, the uptake onto the cell wall must be limited. It has been shown that the heavy metal-tolerant Silene vaularis ssp. Humilis accumulates a wide range of heavy metals in epidermal cell walls by binding to protein or as silicates. Root exudates have various roles including the production of metal chelators. One example has identified the role of root secretion in adapting to metal stress via sequestration by organic acids such as oxalic acid, which is essential for detoxification of Al and which accumulates non-toxic Al-oxalate in the leaves (Hall, J. L., 2002). Also, the enhanced
accumulation of the cell wall is one effective detoxifying strategy in *Pseudomonas* (Mago, R., 1994).

Other mechanisms for regulating intracellular metal levels at the plasma membrane involve the active efflux of metal ions. In bacteria, efflux pumping is an important basis for the most toxic ion resistance mechanism and includes transporters such as p-type ATPases or cation/H⁺ antiporters (Hall, J. L., 2002). Efflux pumping systems play a role in detoxifying a variety of metals such as Cd, Zn, Co and Ni and efflux transporters are pivotal for metal ion homeostasis in animals. For example, plasma membrane Zn transporter (ZnT-1) is isolated from rat kidney. Cell transformed with ZnT-1 mutant lacking the first membrane-spanning domain showed Zn sensitivity. Also, Zn efflux involves some forms of secondary active transport. It has been shown that for bacterial and mammalian mechanism, the CPx-ATPases and CDF family including ZT Zn efflux transporters of humans and rodents are the most important strategies for metal efflux adaptation (Hall, J. L., 2002).

One promising alternative biochemical mechanism is a treatment of metal-containing wastewaters with sulfate reducing bacteria (SRB). The important application of SRB to wastewater treatment is dependent on their ability to reduce sulfate to sulfide, which then reacts with most metals to form insoluble sulfides (Radhika, V. S., 2006). Consequently, H₂S-producing organisms can adapt to heavy metals. In yeast, metal adaptation has been associated with H₂S production. *Saccharomyces cerevisiae* tolerant to copper and mercury produce more H₂S than their non-tolerant strains. It has also been reported that in some conditions sulfide-producing organisms can protect sensitive organisms from the toxic impact of metals. For example, in an experimental study, *D. desulfuricans* was grown in a mixed culture with a metal sensitive strain of *Pseudomonas aeruginosa*, which can adapt to a greater concentration in mixed
culture than a pure culture. That is because of the H₂S produced by the sulfate reducer protected the pseudomonad. This study has promoted the potent of biotechnological usages of bacterial sulphate for removing the toxic metal from the aqueous waste (withe, C., 1996). Figure 12 depicts the bacterial mechanisms to detoxify Zn toxicity by using different chelators. However, how Zn affects metabolism and how metabolic reconfiguration plays a role in Zn detoxification are not fully understood.

**Figure 12:** Zn detoxification in bacteria.
1.5: Thesis objective:

A living organism can evoke three possible strategies during extreme environmental conditions. The organism may adapt, become dormant or die. Our laboratory has studied several ways how organisms adapt to abnormal situations. The soil microbe, *Pseudomonas fluorescens*, is an effective model system to study how a variety of environmental stressors affect living organisms due to its adaptation to extreme environment. This microbe can also thrive in mineral media with several carbon sources and can be tailored for biotechnological applications. Zn is an environmental pollutant of concern as elevated levels of this divalent metal may have a serious impact on the biosphere and on human health. Although microorganisms are known to devise intricate strategies to fend the toxic impact of Zn, little is known about the metabolic networks that participate in the survival of these organisms. In cultured hepatocytes (Lemire, J., 2008) it has been demonstrated that a metabolic shift in Zn-stressed cells leads to the pooling of α-KG and enhances NADPH production, which enable the cell to quell the toxicity. The role of α-keto acids in combating Al and Ga toxicity has also been demonstrated in *P. fluorescens* (Lemire, J., 2010).

In the current study, this soil microbe will be grown in millimolar amounts of Zn and the production of energy independent of oxidative phosphorylation will be studied. The metabolic networks involved in the utilization of citrate, the sole carbon source, and in the production of the antioxidant NADPH will investigated. Also, as oxidative phosphorylation is perturbed by Zn, the influence of substrate level phosphorylation (SLP) will be assessed in order to delineate the energy budget in these Zn-stressed bacteria. Enzymes which are involved in citrate catabolism and the generation of energy via SLP, such as citrate lyase (CL) and pyruvate phosphate dikinase (PPDK) will be investigated.
The role of such antioxidants as pyruvate and α KG in nullifying Zn toxicity will also be examined. This work will provide a holistic metabolic approach to the detoxification of Zn by *P. fluorescens* and help to elaborate biotechnological processes that may be involved in the decontamination of Zn in the environment. Indeed, bioremediation is a cost effective alternative to conventional treatments, and holds a lot of promise in treating metal contaminants (Kumar, A., 2010).
2. Materials and methods:

2.1: List of Reagents and Equipment:

Products purchased from Sigma Chemical Company (St. Louis, Missouri)

2-Thiobarbituric acid,
Dithio-bis(2-nitrobenzoic acid)(DTNB)
6-amino caproic acid
Adenosine 5’ triphosphate (ATP)
Adenosine 5’ diphosphate (ADP)
Adenosine 5’ monophosphate (AMP)
Ammonium chloride (NH₄Cl)
BisTris
Citric acid monohydrate
Coenzyme A (sodium salt)
Coomassie G 250
Cytochrome C
Dithiothreitol (DTT)
Glucose-6-phosphate dehydrogenase EC. 1.1.1.49 (from porcine heart)
Glycerol
Glycine
Glyoxylic acid (monohydrate)
Hydrogen peroxide (30% (w/v))
Iodonitrotetrazolium chloride
Isocitrate dehydrogenase EC 1.1.1.42 (from porcine heart)
Malic dehydrogenase EC. 1.1.1.40 (from porcine heart)

2-mercaptoethanol

n-Dodecyl β-D-maltoside

Nicotinamide adenine dinucleotide (oxidized form)

Nicotinamide adenine dinucleotide (reduced form)

Nicotinamide adenine dinucleotide phosphate (oxidized form)

Nicotinamide adenine dinucleotide phosphate (reduced form)

Phenylmethysulphonylfluoride (PMSF)

Phosphoenolpyruvate (PEP)

Potassium phosphate monobasic (KH$_2$PO$_4$)

Pyruvic acid (sodium salt crystalline)

Sodium phosphate dibasic (Na$_2$HPO$_4$)

Sodium dodecyl sulphate (SDS)

Sucrose

Tricarballylic acid

Tricine

Tris (hydroxymethyl) aminomethane (Tris) HCl and Tris base (Trizma Base)

**Reagents purchased from ICN Biochemical (Cleveland Ohio)**

α-Ketoglutaric acid

dinitrophenylhydrazine

D,L-isocitric acid trisodium salt

Oxalic acid dehydrate
Reagents purchased from other companies:

Acrylamide; Bio-Rad Laboratories (Mississauga, Ontario)

Alliance 2487 Dual Wavelength Absorbance Detector; Waters Ltd (Mississauga, Ontario)

Alliance 2695 separation module, High performance liquid chromatographer Water Ltd (Mississauga, Ontario)

Bacto-Agar; DFCO laboratories (Detroit, Michigan)

Bis-acrylamide; Bio-Rad laboratories (Mississauga, Ontario)

Bradford reagent; Bio-Rad laboratories (Mississauga, Ontario)

Centrifuge Model J2-MI; Beckman Instruments (Mississauga, Ontario)

Fumaric acid; Fisher Scientific (Unionville, Ontario)

Gyratory waterbath shaker model G-76; New Brunswick Scientific (Edison, New Jersey)

Hydrochloric acid (HCl); CanLab (Toronto, Ontario)

Magnesium chloride tetrahydrate; BDH Laboratory Chemicals Division (Toronto, Ontario)

Malic acid; BDH Laboratory Chemicals Division (Toronto, Ontario)

N,N,N’,N’-Tetramethylenediamine (TEMED); Bio-Rad Laboratories (Mississauga, Ontario)

*Pseudomonas fluorescens* ATCC 13525; American Type Culture Collection (Rockville, Maryland)

Sodium hydroxide (NaOH); Fisher Scientific (Unionville, Ontario)

Spectrophotometer model Ultrospec 3000; Amersham Pharmacia Biotech (Baie d’Urfe, Quebec)

Succinic acid; BDH Laboratory Chemicals Division (Toronto, Ontario)

Sulphuric acid (H$_2$SO$_4$); CanLab (Toronto, Ontario)
2.1.1: Organism and culture conditions:

The bacterial strain *Pseudomonas fluorescens* 13525 was obtained from the American Type Culture Collection (ATCC). The microbe was cultured on phosphate medium containing citric acid in 2% agar. The sterile agar test tubes were maintained at 4°C. Distilled and deionized H₂O (ddH₂O) was utilized in all experiments.

2.1.2: Agar preparation:

In 250 mL of double distilled water NaHPO₄ (2.4 g); KH₂PO₄ (1.2 g); NH₄Cl (0.4 g); MgSO₄.7H₂O (0.08 g); citric acid monohydrate (1.6 g) and 400 µL of trace elements was added. The trace element solution consisted of FeCl₃.6H₂O (2 µM); MgCl₂.4H₂O (1 µM); Zn(NO₃)₂.6H₂O (0.05µM); CaCl₂ (1 µM); CoSO₄.7H₂O (0.25 µM); CuCl₂.2H₂O (0.1 µM); NaMoO₄.2H₂O (0.1 µM). The pH of the trace element solution was adjusted to 2.75 with dilute HCl to prevent precipitation of the metals and the solution was stored at 4°C. The pH of the agar media was adjusted to 6.8 with dilute NaOH and the final volume was brought to 400 mL with ddH₂O. The solution was gently warmed and Bactoagar® (6.6 g) was added and stirred until completely dissolved. Approximately 7 to 10 mL was placed in test tubes and capped for slant preparation. Following autoclave sterilization for 20 min at 17lbs/in², 121°C, the test tubes were placed lying down to solidify at room temperature. Slants were stored in the refrigerator at 4°C.

2.1.3: Pre-culture media:

The media used for the growth of the pre-culture contained Na₂HPO₄ (6.0 g), KH₂PO₄ (3.0 g), NH₄Cl (0.8 g), MgSO₄.7H₂O (0.2 g), citric acid monohydrate (4.0 g) and trace element solution (1.0 mL) per litre of ddH₂O. The pH was increased to 6.8 with dilute NaOH and the media was separated into 100 mL aliquots in 250mL Erlenmeyer flasks. The flasks were capped with foam plugs and autoclaved for 20 min at 17lbs/in², 121°C. The pre-culture media was inoculated with a loop of *Pseudomonas fluorescens* stored on agar slants. Stationary phase of
growth was attained following 24 h of incubation at 26°C in a gyratory water bath shaker model
G76 (New Brunswick Scientific) at 140 rpm.

2.2: Control and stress media:

Control media did not contain Zn. Studies were performed using normal phosphate (NP)
conditions. The media was prepared with the following reagents: Na₂PO₄ (0.06 g), KH₂PO₄ (0.03
g), NH₄Cl (0.8 g), MgSO₄.7H₂O (0.2 g), citric acid monohydrate (4.0 g) and trace element
solution (1 mL) per litter. The pH was adjusted to 6.8 with dilute NaOH. The media was
separated in 200 mL amounts in 500 mL Erlenmeyer flasks, stopped with foam plugs and
autoclaved for 20 min at 121°C. The media was inoculated with 1 mL of the pre-culture bacteria
and the cultures were incubated at 26°C in the water bath for 24 h.

Media supplemented with Zn at various concentrations was prepared in the same manner
as the control media with the following change: 4 g of citric acid monohydrate and the metal
were first allowed to complex in approximately 50 mL deionized water for 30 min prior to being
added to the remainder of the media. Studies involved the use of Zn-chloride (ZnCl₂) complexed
to citric acid. Studies were performed utilizing the following concentrations of ZnCl₂: 0.1 mM, 1
mM, 5 mM and 10 mM. Cultures were collected after 38 h of growth as this corresponded to
the same growth phase.

2.2.1: Growth profile and spent fluid analysis:

At various time of growth cells from the control and Zn-stressed cultures were harvested.
10 mL of the media were obtained and centrifuged at 10,000 x g for 20 min to separate the cells.
The pellets then were solubilised by heat in 1 mL of 1 N NaOH and the Bradford assay was
performed to measure protein concentration in the each culture at various points of growth.
For regulation experiments, to assure that Zn was contributing to metabolic shifts, Zn culture which was grown to stationary phase was transferred into control medium and vice versa. Following 8 h of incubation, the cells were collected and cell free extract (CFE) was isolated for enzymatic activity and HPLC studies.

2.2.2: Harvest of *P. fluorescens*:

*Pseudomonas fluorescens* was collected by the method outlined (Figure 13). First, the growth medium was centrifuged at 10,000 x g for 15 min at 4°C. 10 mL of supernatant was taken for spent fluid (SF) analyses. The supernatant was removed and 0.85% NaCl was used to resuspend the bacterial pellet. The bacteria were then recentrifuged at 10,000 x g for 15 min. The Bradford protein assay was utilized to assess cellular growth with BSA as a standard. After bacterial harvesting by the method outlined above, cells were resuspended in cell storage buffer (CSB) containing 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM PMSF and 1 mM DTT at pH 7.6. The cells were then sonicated using a Brunswick sonicator 3 times for 15 Sec while the cells were stored on ice.
Figure 13: Collection of bacterial cells *P. fluorescens* cultures were centrifuged at 10,000 x g for 15 min at 4°C. The supernatant (spent fluid) was then collected for metabolite analysis. Pellets were washed with 0.85 % NaCl and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was discarded and the pellets were resuspended in CSB.

After harvesting, the bacterial cells were re-suspended in cell storage buffer (CSB) containing 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM PMSF, 1 mM DTT at pH 7.3. The cells were sonicated (on ice) using Brunswick sonicator, power level 4 at 15 Sec for 3 times. Then, the cell free extracts (CFE) were centrifuged at 180,000 x g for 3 h. The membrane pellets were re-suspended in CSB and stored at 4°C for use as outlined (Figure 14).
Figure 14: Separation of soluble and membrane CFE. This figure outlines the separation of soluble and CFE. The total CFE was centrifuged at 180,000x g for 3 h at 4°C. The membrane fraction (pellet) was re-suspended in CSB. The soluble fraction was stored at 4°C.

2.2.3: High performance liquid chromatography (HPLC) studies:

The relative levels of numerous TCA cycle metabolites from spent fluid and soluble CFE from control and Zn-stressed cells were discerned by high performance liquid chromatography (HPLC). (2 mg/mL) Protein equivalent of the soluble fraction was heated for 10 min. The samples were diluted tenfold using Milli-Q water and filtered into screw top sampling vials (Fisher) using pasteur pipettes packed with 1mm of cotton. The samples were then run in mobile phase containing 20 mM KH$_2$PO$_4$, pH 2.9 prepared in Milli-Q water. The samples were loaded into the HPLC (Waters Alliance 2695 separation module) and Empower software was used in the automatic injection protocol for the HPLC. TCA cycle metabolites and nucleotides were detected by using a Waters model 2487 UV-Vis dual wavelength detector operation at 210 nm for organic acids and 254 nm for nucleotides. Peaks were identified by comparing to known standards and
spiking the samples with standard solutions. To verify the metabolic shift of citrate, 2 mg/mL protein equivalent of soluble CFE was incubated for 30 min at 26°C in reaction buffer (25 mM Tris, 5 mM MgCl₂, pH 7.0) containing 2 mM citrate.

Also, to study the disparate citrate metabolism in control and Zn cultures, 2 mg/mL protein equivalent of soluble CFE was incubated for 30 h at 26°C in reaction buffer (25 mM Tris, 5 mM MgCl₂, pH 7.0) containing 2 mM citrate with 0.5 mM AMP. HPLC profile aided to identify the metabolic changes that led to the production of pyruvate and ATP via SLP. 2 mg/mL Protein equivalent of soluble CFE was incubated for 30 min at 26°C in reaction buffer (25 mM Tris, 5 mM MgCl₂, pH 7.0) containing 2 mM oxaloacetate with 0.5 mM of Pᵢ and 1 mM AMP. Various reaction products were monitored.

2.3: Oxidized protein measurement:

The soluble protein fractions isolated from control and Zn cultures were utilized to assess the level of oxidized proteins. One mg of soluble protein was added to 1 mL of 2% 2,4-dinitrophenylhydrazine (DNPH) and left to react for 60 min. Then, 200 uL of 50% trichloroacetic acid (TCA) was added to each sample. The protein was then spun down at 14,000 rpm for 10 min. The supernatant was discarded and washed three times with a solution of 10% TCA. The pellets were then washed three times with a solution of ethylacetate:ethanol in a 1:1 ratio. The final precipitate was dissolved in 1.0 ml of 6 M guanidine and the absorbance was measured at 370 nm. The extinction coefficient (ε) was equal to 21.5 nmol⁻¹ L⁻¹ cm⁻¹ (Frank, J., 2000).

2.3.1: Oxidized lipid measurement:

Thiobarbituric acid (TBA) can react with aldehyde equivalents resulting in oxidized lipids. The amount of thiobarbituric acid reactive species (TBARS) was measured in the membrane fraction of control and Zn cultures. Four mg of membrane protein equivalent was
heated with 15% trichloroacetic acid (TCA), 0.375% TBA/0.25 N HCl in a final volume of 1.0 mL for 15 min. Following the development of a pinkish color, samples were centrifuged at 10,000 x g for 10 min. The absorbance of the supernatant was measured at 532 nm. The extinction coefficient (ε) was equal to 1.56 \times 10^5 M^{-1} cm^{-1} (Aydin, S., 2005).

2.3.2: Sulfhydryl measurement:

Free and total sulfhydryl was measured. For free sulfhydryl, 50 µL of the sample and 2X25 µL TCA were spun down for 15 min at 1500 rpm supernatant and room temperature and the supernatants were pooled. Fifty µL of supernatant was added to 200 µL of Tris-HCL (pH 8.9) and 20 µL 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB). The absorbance was measured after 15 min at 412 nm. For total sulfhydryl measurement, 20 µL of the sample was added to 75 µL of Tris-HCl (pH 8.2), 25 µL DTNB and 400 µL methanol. Samples were then spun down at 3000 g for 5 min at room temperature. The absorbance was then measured at 412 nm (Mhoinicka, M., 1999).

2.3.3: Fe-S cluster measurement:

Iron-sulfur (Fe-S) clusters were monitored using the soluble fraction of control and Zn-stressed cells by UV/Vis scanning spectroscopy. Briefly, 3.0 mg/mL of soluble CFE protein was measured by scanning between 395 and 420 nm. Fe-S-containing proteins were observed as a band in the 415-425 nm range (Middaugh, J., 2005).

2.4: Spectrophotometric monitoring of enzyme activity in CFE:

The CFE from *P. fluorescens* were isolated as mentioned earlier. The protein concentration of each fraction was measured by Bradford assay.

2.4.1: Measuring catalase activity:

The activity of catalase was measured with following reagent, p-anisidine, and the absorbance was monitored at 458 nm. 200 µg of protein equivalent from control and Zn stress
were incubated with 15 mM H$_2$O$_2$. 10 mM p-Anisidine was added directly in final volume of 1.0 mL and the absorbance was measured after 3 h (Igamberdiev, V., 1995).

2.4.2: Measuring superoxide dismutase (SOD) activity:

The activity of SOD was measured with INT, having an oxidized absorbance of 485 nm. Two hundred µg protein equivalent of CFE from control and Zn-cultures were incubated with 5 mM menadione. Fifteen µL of INT was added for final volume of 1.0 mL and the absorbance was measured after 3 h (Poinas, A., 2002).
2.4.3: Electrophoresis:

Table 5: Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) buffers:

<table>
<thead>
<tr>
<th>Blue Cathode Buffer (1L)</th>
<th>Colorless Cathode Buffer (1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.96 g tricine (50 mM)</td>
<td>8.96 g Tricine (50 mM)</td>
</tr>
<tr>
<td>3.13 g BisTris (15 mM)</td>
<td>3.138 g BisTris (15mM)</td>
</tr>
<tr>
<td>0.2 g Coomassie blue G 250</td>
<td>pH 7.0 at 4 °C</td>
</tr>
<tr>
<td></td>
<td>pH 7.0 at 4 °C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3x Gel Buffer (50 mL)</th>
<th>Anode Buffer (1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.84 g aminocaproic acid (1.5M)</td>
<td>10.45 g BisTris (50mM)</td>
</tr>
<tr>
<td>1.567 g BisTris (150 mM)</td>
<td>pH7.0 at 4°C</td>
</tr>
<tr>
<td>pH 7.0 at 4 °C</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Coomassie Blue Staining Solution</th>
<th>Destaining Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% methanol</td>
<td>50% methanol</td>
</tr>
<tr>
<td>10% acetic acid</td>
<td>10% acetic acid</td>
</tr>
<tr>
<td>0.2% Brilliant Blue R 250</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: 4-16% protocols for BN-PAGE:

<table>
<thead>
<tr>
<th></th>
<th>4% (μL)</th>
<th>16% (μL)</th>
<th>Stacking Gel (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acryl-Bis mix (49.5 %T, 1.5% C)</td>
<td>234</td>
<td>937</td>
<td>273</td>
</tr>
<tr>
<td>3x buffer</td>
<td>967</td>
<td>967</td>
<td>1136</td>
</tr>
<tr>
<td>ddH2O</td>
<td>1699</td>
<td>223</td>
<td>2000</td>
</tr>
<tr>
<td>75% glycerol</td>
<td>-</td>
<td>773</td>
<td>-</td>
</tr>
<tr>
<td>10% ammonium persulfate (APS)</td>
<td>9.7</td>
<td>7.6</td>
<td>30</td>
</tr>
<tr>
<td>Tetramethylethelialinedia mine (TEMED)</td>
<td>1.0</td>
<td>0.8</td>
<td>5</td>
</tr>
</tbody>
</table>

2.5: In-gel activity staining for soluble enzymes:

The soluble CFE from *P. fluorescens* grown in control and Zn medium at various growth times and conditions was prepared for gel electrophoresis by diluting the fractions with 3 X BN buffer and water to a final concentration of 4 mg/mL protein equivalent and 1 X BN buffer. To each sample lane 60 μg of protein was loaded and electrophoresed under native conditions. Following BN-PAGE, the gels were incubated in reaction buffer (25 mM Tris-HCl, 5 mM
MgCl\textsubscript{2}, pH 7.4) for 15 min. The gels were then placed in the appropriate buffer (reaction buffer with the desired substrates, cofactors and enzymes for coupled reactions) to detect enzymatic activity. The activity in-gel was visualized using phenazine methosulfate (PMS) or 2,6-dichloroindophenol (DCPIP) and iodonitrotetrazolium chloride (INT). Enzymatic reactions that use NAD\textsuperscript{+} or NADP\textsuperscript{+}, which are then converted to NADH and NADPH respectively, were easily detected as PMS reduces INT and the latter appears as a pink formazan precipitate at the location of the enzyme in-gel. Reverse reactions (NAD(P)H $\rightarrow$ NAD(P)) were detected using DCPIP rather than PMS as shown in (Figure 15). Under such conditions, it is critical to avoid light exposure, so all reactions are performed in the dark (Singh, R., 2005) and (Auger, C., 2011).
2.5.1: NADPH producing enzymes: ICDH, ME, G6PDH:

INT and PMS were used to visualize these enzymes. The tetrazolium is readily reduced by NADH or NADPH in the presence of PMS to form insoluble formazan that will localize
directly at the site of enzymatic activity on the gel. The gels were placed in reaction buffer plus 0.2 mg/mL PMS, 0.4 mg/mL INT, 0.5 mM NADP⁺ and the following substrate depending on the respective enzyme to be detected: 5 mM isocitrate for isocitrate dehydrogenase (ICDH)-NADP⁺ activity, 5 mM malate for malic enzyme (ME) activity and 5 mM glucose-6-phosphate for glucose-6-phosphate dehydrogenase (G6PDH) activity (Beriault, R., 2005). The total volume of the reaction buffer was 1.5 ml per lane. After visualization of a pink precipitate at the site of enzyme catalysis, the gel was placed in destaining solution (50% methanol, 10% acetic acid). This stopped the reaction and served to remove the Coomassie G 250 from the gel leaving a clear gel with a pink band at the site of enzymatic activity.

2.5.2: Detection of CL activity by BN-PAGE:

The in-gel activity of citrate lyase (CL) was visualized by using a reaction mixture containing 5 mM citrate, 0.5 mM NADH, 5 units malate dehydrogenase (MDH), 0.4 mg/mL INT and 0.2 mg/mL DCPIP. The total volume was 1.5 mL/lane. The band was observed after 10 min (Auger, C., 2011).

2.5.3: Detection of PEPC activity by BN-PAGE:

The in-gel activity of phosphoenolpyruvate carboxylase (PEPC) was visualized using a reaction mixture containing 5 mM phosphoenolpyruvate (PEP), 5 mM HCO₃⁻, 5 units MDH, 0.4 mg/mL INT and 0.2 mg/mL DCPIP. The total volume was 1.5 mL/lane. The band was observed after 10 min.

2.6: Detection of PEPCK activity by BN PAGE:

The in-gel activity of phosphoenolpyruvate carboxykinase (PEPCK) was visualized using a reaction mixture containing 10 mM PEP, 1 mM adenosine diphosphate (ADP), 10 mM HCO₃⁻, 0.5 mM NADH, 10 units MDH, 0.4 mg/mL INT and 0.2 mg/mL DCPIP. Total volume per lane was 1.5 mL and the band was observed after 10 min of incubation (Lemire, J., 2008).
2.6.1: Detection of PPDK activity by BN-PAGE:

The in-gel activity of pyruvate phosphate dikinase (PPDK) was visualized using a reaction mixture containing 5 mM PEP, 0.5 mM adenosine monophosphate (AMP), 0.5 mM sodium pyrophosphate, 0.5 mM NADH, 10 units of lactate dehydrogenase (LDH), 0.2 mg/mL of DCPIP and 0.4 mg/mL of INT. Total volume per lane was 1.5 mL and the band was observed after 10 min of incubation.

2.6.2: Detection of PEPS activity by BN PAGE:

The in-gel activity of phosphoenolpyruvate synthase (PEPS) was visualized using the reaction mixture containing 5 mM PEP, 0.5 mM AMP, 0.5 mM inorganic phosphate, 0.5 mM NADH, 10 units of lactate dehydrogenase (LDH), 0.2 mg/mL of DCPIP and 0.5 mg/mL of INT. Total volume per lane was 1.5 mL and the band was observed after 10 min of incubation (Auger, C., 2012).

2.6.3: Detection of PK activity by BN-PAGE:

The in-gel activity of pyruvate kinase (PK) was visualized using a reaction mixture containing 5 mM PEP, 1 mM ADP, 0.5 mM NADH, 5 units LDH, 0.4 mg/mL INT and 0.2 mg/ml DCPIP. Total volume per lane was 1.5 mL and the band was observed after 10 min of incubation.

2.7: Detection of AK activity by BN-PAGE:

The in-gel activity of adenylate kinase (AK) was visualized using a reaction mixture containing 5 mM ADP, 5 mM glucose, 0.5 mM NADP, 5 units of hexokinase, 5 units of G6PDH, 0.4 mg/mL INT and 0.2 mg/mL PMS. Total volume per lane was 1.5 mL and the band was observed after 40 min of incubation.
2.7.1: Detection of ICL activity by BN-PAGE:

The in-gel activity of isocitrate lyase (ICL) was visualized using a reaction mixture containing 5 mM isocitrate, 0.5 mM NAD\(^+\), 10 units LDH, 0.4 mg/mL INT and 0.2 mg/mL PMS. The total volume was 1.5 ml/lane. The band was observed after 3 h (Hamel, R., 2001).

2.7.2: Detection of AGODH activity by BN PAGE:

The in-gel activity of acetylation glyoxylate dehydrogenase (AGODH) was visualized using a reaction mixture containing 5 mM glyoxylate, 0.5 mM NADP, 0.66 mM COA, 0.4 mg/mL INT and 0.2 mg/mL PMS. The total volume was 1.5 ml/lane.

2.7.3: Detection of glutathione reductase (GR):

The in-gel activity of glutathione reductase (GR) was visualized using a reaction mixture containing 5 mM GSSG, 0.5 mM NADP\(^+\), 0.4 mg/mL INT and 0.2 mg/mL DCPIP. The total volume was 1.5 mL/lane. The band was observed after 30 min.

2.8: In-gel activity staining for membrane enzymes:

Following the isolation of membranes as described above, the proteins were solubilized using n-dodecyl-beta-D-maltoside. Samples were prepared by diluting the membrane fraction, 3 X BN buffer and 10% maltoside with ddH\(_2\)O to provide a final concentration of 4 mg/mL protein equivalent in 1 X BN and 1% maltoside. The samples were incubated in an ice bath for 30 min prior to storage at -20°C to allow the maltoside to exert its effect.

2.8.1: Detection of complex I activity by BN-PAGE:

For the detection of complex I, the gel was incubated in a volume of 1.5 mL/lane consisting of reaction buffer containing 5 mM KCN, 5 mM NADH and 0.4 mg/mL INT. the detection of ETC components requires KCN to stop the transfer of electrons to other components of chain, allowing the enzyme to transfer the electron to reduce INT and initiate formazan precipitation. The detection of the complex was evident after approximately 5 min.
2.8.2: Detection of complex IV activity in BN-PAGE:

For the detection of complex IV, the gel was incubated in a volume of 1.5 mL/lane consisting of reaction buffer containing 5 mg/ml diaminobenzidine, 562.5 mg/mL sucrose and 10 mg/mL cytochrome C. The detection of complex IV was evident after approximately 10 min (Lemire, J., 2008).

2.8.3: Detection of αKGDH activity by BN-PAGE:

For the detection of α- ketoglutarate dehydrogenase (αKGDH), the gel was incubated in a volume of 1.5 mL/lane consisting of reaction buffer, 5 mM α-ketoglutarate, 0.5 mM NAD⁺, 0.2 mg/mL of PMS and 0.4 mg/mL INT. The detection of α-KGDH was observed after 20 min (Mailloux, R. J., 2009).

2.9: Detection of MDH activity by BN-PAGE:

For the detection of malate dehydrogenase (MDH), the gel was incubated in a volume of 1.5 mL/lane consisting of reaction buffer, 5 mM malate, 0.5 mM NAD⁺, 0.2 mg/ml of PMS and 0.4 mg/mL INT. The activity of MDH was observed after 20 min (Singh, R., 2005).

2.9.1: Detection of FUM activity by BN-PAGE:

The detection of fumarase (FUM) was possible with the addition of MDH from porcine heart (160 units). The gel was incubated in a volume of 1.5 mL/lane consisting of reaction buffer, 5 mM fumarate, 0.5 mM NAD⁺, MDH, 0.2 mg/mL PMS and 0.4 mg/mL INT.

2.9.2: 2D SDS-PAGE and protein expression:

The activity bands for PPDK from control and various concentration of Zn: 0.1 mM, 1 mM, 5 mM and 10 mM were denatured by soaking the band in a solution of 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol for 30 min. The bands were then rinsed for 10 s with SDS-PAGE electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine and 1% (w/v) SDS, pH. 8.3) and placed in the wells of an SDS gel. Electrophoresis was performed at 80 V throughout the
stacking gel until the running front entered the separating gel. The voltage was increased to 150 V until the blue marker reached the bottom of the separating gel. Then, the gels were destained with 10% acetic acid/50% methanol overnight. Protein expression was then determined with the Bio-Rad silver staining kit (Singh, R., 2005).

2.9.3: SDS buffers:

Table 7: SDS buffers:

<table>
<thead>
<tr>
<th>5X electrophoresis buffer (1L)</th>
<th>30% acrylamide stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.1 g tris-HCl</td>
<td>29.2 g acrylamide</td>
</tr>
<tr>
<td>72.0 g glycine</td>
<td>0.8 g bisacrylamide</td>
</tr>
<tr>
<td>5.0%(w/v)SDS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4X Tris/SDS pH 6.8</th>
<th>4X Tris/SDS pH 8.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M tris</td>
<td>1.5 M tris</td>
</tr>
<tr>
<td>0.4 % (w/v) SDS</td>
<td>0.4 % (w/v) SDS</td>
</tr>
<tr>
<td>pH adjust to 6.8 with 6 N HCl</td>
<td>pH adjust to 8.8 with 6 N HCl</td>
</tr>
</tbody>
</table>

Table 8: 10% SDS solution setup:

<table>
<thead>
<tr>
<th></th>
<th>Running gel (µL)</th>
<th>Stacking gel (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>2500</td>
<td>650</td>
</tr>
<tr>
<td>4X Tris/SDS (pH 8.8)</td>
<td>1875</td>
<td>-</td>
</tr>
<tr>
<td>4X Tris/SDS (pH 6.8)</td>
<td>-</td>
<td>1250</td>
</tr>
<tr>
<td>ddH2O</td>
<td>3125</td>
<td>3050</td>
</tr>
<tr>
<td>10% APS</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>TEMED</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

2.10: Statistical analyses:

All experiments were performed in triplicate and in biological duplicate. The student t test was used to determine the significance of differences in enzyme activity and metabolite levels in control and Zn- stressed cells. For n=3, the means were considered to be significantly different and p is said to be (p≤0.05).
Figure 16: Summary for experimental work.
3. Results:

3.1: Growth profile of *P. fluorescens* exposed to Zn:

When *Pseudomonas fluorescens* was exposed to Zn at various concentrations (0.1 mM, 1 mM, 5 mM, and 10 mM), the rate of growth to the stationary phase was slower than the control. Additionally, the biomass decreased as the concentration of Zn increased as shown (Figure 17).

![Figure 17](image)

*Figure 17:* The growth curve of *P. fluorescens* in control and Zn in media; ■ control, ● 0.1 mM Zn, ○ 1 mM Zn, ▲ 5 mM Zn, □ 10 mM Zn (Appanna, V., 1995).

The growth pattern of control cultures did not display a distinct lag phase and it reached the stationary phase in approximately 30 h. In the presence of Zn, *P. fluorescens* required more time to reach the stationary phase than it did in the control culture, as shown (Figure 17). The delay in time indicates that the microbe was adapting to the Zn-stressed condition. Also, the
decrease in cellular yield was dependent on the concentration of Zn. It decreased dramatically at 10 mM Zn, owing to the toxic effects engendered at this concentration.

At the stationary phase of growth most of the Zn was found in the spent fluid and just a little was associated with cell; citrate was rapidly consumed in both control and Zn-stressed cultures (Appanna, V., 1995). Therefore, it was necessary to analyze the spent fluid to see what metabolites may be chelating Zn in order to exude this toxic moiety.

**3.1.1: HPLC analysis of the spent fluid:**

HPLC studies were performed on spent media. The metabolite profiles were obtained. In the presence of 5 mM Zn, there was higher amount of oxalate and pyruvate compared to the control (Figure 18).
Figure 18: Metabolite accumulation in the spent fluid. A: control, B: Zn (these chromatograms represent 1 biological of replicate of 3).

3.1.2: HPLC analysis of the soluble CFE:

After the spent fluids of control and Zn stressed cultures were analyzed, the nature of metabolites from soluble CFE was assessed. HPLC analysis was used to probe the metabolic shift associated with the adaptation to Zn in *P. fluorescens*. The stressed culture showed the accumulation of pyruvate and an increase in ATP levels to the control. Phosphoenolpyruvate (PEP) levels were also higher in the bacteria subjected to Zn (Figure 19).
Figure 19: HPLC analysis of soluble CFE from control and Zn soluble CFE. ■: control. □: Zn-stressed cells. n=3, ± SD. ** denotes that the difference between control and Zn statistically significant p ≤ 0.005 (metabolites were detected at 210 nm).

3.1.3: The effect of Zn on cellular components:

3.2: Sulphydryl measurements:

As Zn can coordinate with sulphydryl groups in various proteins containing cysteine and methionine, elevated concentration of Zn may oxidize the sulphydryl moieties rendering them inactive. It is critical to measure the free and total sulphydryl groups in control and Zn-stressed cells. The levels of these moieties were lower in the stressed cells (Figure 20).
Figure 20: Sulfhydryl group measurements. ■ control and □ Zn-stressed *P. fluorescens*. A: Free sulfhydryl group. The difference between control and Zn is not considered to be statistically significant. \( p = 0.43, n = 3 \). B: Total sulfhydryl. \( p \leq 0.05, n= 3 \). *: denotes statistically significance.

As Zn appeared to reduce free sulfhydryl and contribute to an increase in the oxidative environment, the activity of glutathione reductase (GR) was measured. GR cleaves the
glutathione disulfide to glutathione. This enzyme was found to be downregulated under Zn stress (Figure 21).

3.2.1: BN PAGE of glutathione reductase:

![BN PAGE of glutathione reductase](image)

**Figure 21**: The in-gel activity of glutathione reductase; control = untreated cells; Zn = cells treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments

3.2.2: Fe-S measurement in cellular fractions:

Spectrophotometric analysis was applied to examine the integrity of Fe-S clusters in the soluble CFE. The absorption pattern of the Fe-S clusters around 400 nm was monitored by UV/Vis wavelength scanning in control and Zn-stressed cultures (Figure 22).

Figure 22 demonstrates the Fe-S cluster measurement of the control and Zn respectively. As seen, the Fe-S cluster was reduced in Zn-stressed cells, which might lead to exposure of oxidative stress via the release of free Fe into the milieu.
Figure 22: The UV-Vis spectra of soluble CFE. A: control, B: Zn stressed cells. The arrow is indicative of the Fe-S cluster.
The effects of oxidative stress on cellular components:

3.2.3: Oxidized lipids:

A TBARS (thiobarbituric acid reactive species) assay was performed by using the membrane fraction of control and Zn-stressed cells. It was shown that the bacterial concentration of TBARS was less in Zn compared to the control cells (Table 9).

Table 9: Measurement of oxidized lipids in control and Zn-stressed *P. fluorescens*. Mean± SD, n=3, *: denotes a statistically significant difference compared to control (p ≤ 0.05).

<table>
<thead>
<tr>
<th>Malondialdehyde equivalent (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control: 0.050 ± 0.00125*</td>
</tr>
<tr>
<td>Zn: 0.030 ± 0.00152*</td>
</tr>
</tbody>
</table>

3.3: Oxidized proteins:

DNPH was utilized to visualize oxidized proteins in soluble CFE from control and Zn-stressed cells. The soluble CFE obtained at the same growth phase was analyzed. More oxidized proteins were recorded in the stressed cultures (Figure 23).

Figure 23: Oxidized protein in control and Zn-stressed *P. fluorescens*; ■ control, □ Zn-stressed. n=2, ± SD. The difference between control and Zn is not considered statistically significant; p=0.225.
As the metabolites were disparate in the two cultures, it became important to investigate the various metabolic networks such as the TCA cycle and oxidative phosphorylation. To accomplish that, BN-PAGE was prepared to measure enzyme activities. Also, as oxidative stress is triggered by Zn toxicity, it was indeed critical to measure the oxidative stress defenses such as catalase and SOD. Table 10 provides a list of different enzymes examined.

**Table 10:** The activity of various enzymes in control and Zn-stressed *P. fluorescens.*↑: increase, ↓: decrease, -: no discernible bands.

<table>
<thead>
<tr>
<th><strong>ROS Defense enzymes</strong></th>
<th>control</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Superoxide dismutase(SOD)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td><strong>NADPH-producing enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase-NAD⁺(ICDH-NAD⁺)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Malic enzyme (ME)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Glucose 6 phosphate dehydrogenase (G6PDH)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td><strong>TCA cycle enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase-NAD⁺(ICDH-NAD⁺)</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>α-Ketoglutarate dehydrogenase (αKGDH)</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Malate dehydrogenase (MDH)</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Fumarase (FUM)</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Oxidative phosphorylation enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex I</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Complex IV</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Citrate metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate lyase (CL)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxylase (PEPC)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase (PEPCK)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Pyruvate phosphate dkinase (PPDK)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Phosphoenolpyruvate synthase (PEPS)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Pyruvate kinase (PK)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Adenylate kinase (AK)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td><strong>Glyoxylate shunt</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocitrate lyase (ICL)</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Acetylating glyoxylate dehydrogenase (AGODH)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glyoxylate dehydrogenase (GDH)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.3.1: The activation of antioxidant enzymes catalase and SOD:

Catalase and SOD are two enzymes that can detoxify ROS. The former catalyzes the decomposition of hydrogen peroxide to water and oxygen while the latter catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. These enzymes were probed by spectrophotometric analysis in *P. fluorescens* cells from control and Zn-stressed cultures. As shown (Table 11), both catalase and SOD were increased in Zn-stressed cells to deal with oxidative stress.

**Table 11**: Catalase and SOD activities; control soluble CFE=100%, n = 3 ± SD, p ≤ 0.05, *: denotes that the difference is statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>Catalase</th>
<th>SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn soluble CFE</td>
<td>325.8 % ± 0.00615*</td>
<td>283.4 % ± 0.072148*</td>
</tr>
</tbody>
</table>

3.3.2: Oxidative phosphorylation and TCA cycle enzymes:

As the tricarboxylic acid (TCA) cycle and oxidative phosphorylation are essential for aerobic energy production, it is important to decipher the state of these metabolic networks. BN-PAGE in gel-activity staining was utilized to monitor two enzymes in the ETC: complex I and complex IV. These enzymes were found to have negligible activity in membrane CFE from Zn-stressed cells compared to controls (Figure 24). Also, due to changes in metabolite levels observed in Zn-stressed *P. fluorescens* evoked by Zn, the activity of enzymes in the TCA cycle was measured. All NAD dependent enzymes: ICDH, KGDH and MDH had diminished activity in Zn stressed cells (Figure 25). These changes were clearly revealing a repatterning of metabolic modules in *P. fluorescens* subjected to Zn.
Figure 24: In-gel activity of oxidative phosphorylation enzymes. A: Complex I. B: Complex IV; control = untreated cells; Zn = cells treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments.

Figure 25: BN PAGE of TCA cycle enzymes in *P. fluorescens*. In-gel activity staining; control = untreated cells; Zn = cells treated with 5 mM Zn (cells were obtained at same growth phase). A: ICDH-NAD+, B: α-KGDH, C: MDH, D: FUM. Data are representative of 3 independent experiments.

3.3.3: NADPH producing enzymes:

NADPH provides the important reducing power to cope with oxidative stress and is pivotal for the organism’s survival. Thus, the analysis of NADPH producing enzymes was important to understand how the organism deals with oxidative stress. The data indicate that most NADPH-producing enzymes were increased in Zn-stressed cells (Figure 26).
Figure 26: In-gel activity staining of NADPH producing enzymes. A: ICDH-NADP+, B: ME, C: G6PDH; control = untreated cells; Zn = cells were treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments.

As ICDH-NADP$^+$ activity was sharply increased, the influence of varying concentrations of Zn on this enzyme was evaluated. Soluble cell free extract obtained at the same growth phase from cells grown in different concentrations of Zn were probed. There was an increase in activity of ICDH-NADP$^+$ with increased exposure to Zn shown (Figure 27: A). However, at 10 mM Zn, a decrease was observed. To prove that indeed Zn was the cause of the upregulation of ICDH-NADP$^+$ activity, cells grown in Zn were incubated in control media and control cells were subjected to Zn media respectively for 8 h shown (Figure 27: B). While a decrease in activity observed with the former, an increase in activity was evident in control cells exposed to Zn. This indicates that Zn was indeed the cause of activity of ICDH-NADP$^+$. Similar observations have been observed in our laboratory with other oxidative stress (Singh, R., 2008).
Figure 27: In gel-activity staining of ICDH-NADP+. A: ICDH-NADP+ with Zn concentration 0.1 mM, 1 mM, 5 mM and 10 mM, E: regulation experiment (Control media were incubated in Zn media followed by 8 h and Zn culture incubated in control media followed by 8 h).

3.4: Alternative metabolic pathway:

3.4.1: Citrate metabolism:

The inability of this soil organism to metabolize citrate via the TCA cycle under Zn stress prompted it to form an alternative citrate degradation pathway via CL, which cleaves the citrate to produce oxaloacetate and acetate without the participation of ATP and Coenzyme A (Auger, C., 2011). Also, to confirm the citrate was consumed in the Zn-stressed cells, the HPLC profiles of control and Zn-stressed cells incubated 2 mM of citrate were examined. Citrate was rapidly consumed in the Zn-stressed cells with the concomitant production of pyruvate. Numerous disparate unidentified peaks were also evident, thus suggesting that the metabolic networks operative in the Zn-stressed cells were different from the control cells. While no discernible CL activity band was evident in the control cells, in the Zn-stressed cells a sharp band was obtained (Figure 28).
Figure 28: Citrate degradation. Panel I: The in-gel activity of citrate lyase; control = untreated cells; Zn = cells were treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments. Panel II: HPLC result of A: control and B: Zn-stressed cultures given 2 mM of citrate after 30 min of incubation (these chromatograms represent 1 biological of replicate of 3).
3.4.2: Oxaloacetate metabolism:

As oxaloacetate levels were increased because of the upregulation of CL and downregulation of MDH in the Zn-stressed cells, it became important to know how oxaloacetate was being metabolized. PEPC and PEPCK are two enzymes that convert the oxaloacetate to high energy PEP. Although no discernible bands for PEPC and PEPCK were in control cells, both enzymes were found higher in activity in the Zn-stressed cells as shown (Figure 29). Also, the effects of various concentration of Zn on these enzymes were investigated. Soluble CFEs obtained at the same growth phase from cells grown in different concentration of Zn were probed in gel activity. There was an increase in the activity of PEPC with more concentration. However, at 10 mM Zn, a decrease was observed. To verify that Zn is the main cause of the increase in the activity of PEPC, cells grown in Zn were incubated in control media and control cells were also incubated to Zn for 8 h. It has found that an increase activity in the control and a decrease activity in Zn cells. This indicates that Zn was the reason of increased activity of PEPC.
3.4.3: **Pyruvate metabolism:**

Pyruvate was higher in Zn-stressed cells than it was in the control cells, in both spent fluid and the soluble CFE. The upregulation of PEP may trigger the production of pyruvate. PK, PEPS and PPDK are enzymes mediating the conversion of high energy PEP to pyruvate and ATP. These enzymes were upregulated under Zn stress as shown (Figure 30).
**Figure 30**: Pyruvate producing enzymes. A: PPDK, B: PK, C: PEPS; control = untreated cells; Zn = cell treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments.

As PPDK was upregulated under Zn-stress, it was critical to monitor this enzyme with variety of Zn concentrations. Soluble CFEs obtained at the same growth phase from cells grown in diverse concentration of Zn showed an increase of the activity of PPDK with increased Zn. However, there was a decrease at 10 mM. To prove that Zn was the cause of the upregulation of this enzyme, cells grown in Zn was incubated in control media and control cells was also incubated in Zn media for 8 h. The latter revealed an increase in activity while the former underwent a reduction in PPDK activity. This points to the fact that Zn was the main cause of upregulation the activity of PPDK. Furthermore, soluble CFE of 5 mM Zn was obtained at different time intervals and there were increase of PPDK activity with increased the time of growth. That indicates that at 38 h was the perfect time for *P. fluorescens* to adapt to Zn (Figure 31).
Figure 31: in-gel staining of PPDK activity. A: PPDK in control and increasing Zn concentrations (0.1 mM, 1 mM, 5 mM, and 10 mM). B: Time profile of PPDK in Zn-stressed cells (20 h, 26 h, 34 h, 38 h). C: control and Zn regulation experiment (control media were put in Zn media followed by 8 h and Zn culture were put in control media followed by 8 h). B: PK, C: PEPS; control = untreated cells; Zn = cell treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments).

2D SDS-PAGE followed by silver staining was utilized to determine the relative levels of PPDK expression in Zn-treated cells. The enzyme appeared as multiple bands in the SDS gel, which means this enzyme contains of multiple subunits. Only the prominent subunit of PPDK is shown (Figure 32).
Figure 32: 2D SDS PAGE for PPDK. Bands from BN-PAGE in-gel activity assays were excised and loaded into a SDS gel. control and increasing Zn concentrations (0.1 mM, 1 mM, 5mM and 10 mM); control=untreated cells, Zn= cells treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments. This arrow indicates the PPDK band.

To verify the fate of citrate is providing ATP via substrate level phosphorylation (SLP), the soluble CFE was given citrate and AMP and the reaction was monitored by HPLC (Figure 33).
Figure 33: HPLC analysis of CFE incubated with citrate. A: control, B: Zn. 2 mM citrate with AMP for 30 min. (these chromatograms represent 1 biological of replicate of 3).

In order to confirm that ATP production was performed via SLP and as CL, PEPC, PEPCK, PEPS, PK and PPDK enzymes are upregulated, it is important to monitor the metabolite by HPLC. Soluble CFE from control and Zn stress was incubated in a reaction mixture containing, 2 mM oxaloacetate, 1 mM AMP and 0.5 P_i. There were significant increases in PEP, oxaloacetate, pyruvate and ATP under Zn stressed cells as shown (Figure 34).
Figure 34: ATP production by SLP. ■: control. ■: Zn-stressed cells. n=3, ± SD. * denotes that the difference between control and Zn is statistically significant p ≤ 0.005. (Metabolites were detected at 210nm).

3.5: Adenylate kinase activity:

Adenylate kinase catalyzes the interconversion of 2 ADP to ATP and AMP. Due to the increase in AMP-dependent enzyme activities such as PEPS and PPDK, we monitored adenylate kinase (AK) activity in Zn-stressed bacteria as shown (Figure 35).

Figure 35: In-gel activity of adenylate kinase; control= untreated cells; Zn = cells treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments.
3.5.1: Oxalate production:

As oxalate was abundant in the spent fluid, it was necessary to understand how this organic acid was being produced. ICL activity was measured in control and Zn-stressed cells and it was upregulated in the presence of Zn, as shown in (Figure 36). The increase in this enzymatic activity led us to look at enzymes that metabolize the production of glyoxylate. Acetylating glyoxylate dehydrogenase is capable of producing oxalate. However, no activity was detectable (Figure 36: D). Figure 37 depicts all the summary of the results obtained.

![Figure 36: Oxalate production; control = untreated cells; Zn = cells treated with 5 mM Zn (cells were obtained at same growth phase). Data are representative of 3 independent experiments. A: ICL activity in BN PAGE, B: ICL in different concentration Zn 0.1 mM, 1 mM, 5 mM, 10 mM C: control and Zn regulation (control media were put in Zn media followed by 8 h and Zn culture put in control media followed by 8 h), D: acetylating glyoxylate dehydrogenase.](image)
Figure 37: The summary of results.
4. Discussion:

The preceding results point to the disruption of the Fe-S cluster, which is associated with many enzymes in the TCA cycle and oxidative phosphorylation, thus affecting the energy production in Zn-stressed *Pseudomonas fluorescens*. Although most organisms succumb to the toxic influence of Zn, some plants and bacteria are known to have evolved intricate defense strategies to counter this divalent metal (Hall, J. L., 2002). These data reveal the metabolic networks that create a reducing environment in *P. fluorescens* exposed to Zn stress.

The decrease of Fe-S clusters, sulfhydryl groups, and increased in oxidized proteins are indicative of Zn’s ability to disrupt the TCA cycle and oxidative phosphorylation and promote an oxidative environment. The soil microbe utilizes an alternative machinery to cope with the Zn toxicity by upregulating such enzymes as CL, PEPCK, PEPC, PPDK, PEPS and PK to create ATP via substrate level phosphorylation. Enhancing the production of NADPH-generating enzymes such as ICDH, G6PDH and ME, the organism produces enough of this moiety to quell Zn-induced oxidative stress. The upregulation of the activity of antioxidant enzymes such as catalase and superoxide dismutase allows *P. fluorescens* to scavenge ROS to protect against Zn toxicity.

4.1: Zinc is toxic to the microbe:

Although Zn is a micronutrient and plays important roles in the growth of microbes, at high concentrations Zn is toxic due to its ability in creating an oxidative environment. For instance, ZnCl₂ at 0.5 mM inhibits the growth of *E. coli* (Babich, R., 1978). There are many microbial strategies for uptake of metals including adsorption, binding to cellular surfaces and the accumulation of the metal ions within the cytoplasm. Moreover, some microbes bind to the metal by the cell wall (Muhammad, N., 1998). However, these disparate uptake routes may have different toxic influences.
4.1.1: Growth of *P. fluorescens* under Zinc toxicity:

It appears the microorganism adapting to Zn as Zn is known to perturb sulfhydryl groups. The data indicate that Zn has increased oxidized proteins, as shown (Figure 23), thus increasing oxidative stress within the cell and affecting protein function. However, Zn has lowered lipid peroxidation within the cell. Zn can inhibit lipid peroxidation initiated by Fe by binding the membrane polar head groups of phospholipids and preventing oxidation (Zago, M. P., 2000). Thus, it is not unlikely that in this instance, the divalent metal may be aiding in the oxidation of lipids.

It has been established that Fe-S clusters are a target of Zn stressed cells. It is also shown by UV spectrophotometric analysis (Figure 22) that the Fe-S cluster was decreased compared to control. This is because Zn can displace free iron, leading to oxidative stress within the cell. Zn can also interact with thiol groups of antioxidants such as glutathione, contributing to the depletion of reduced GSH which promotes oxidative stress (Lemire, J., 2008). Figure 21 shows the activity of glutathione reductase (GR), which was downregulated in Zn-stressed cells. Zn can work as pro-oxidant and inhibit several of antioxidant enzymes such as GR in oral bacteria including, *Streptococcus mutans* (Phuong, N. T., 2006). In eukaryotes, Zn also inhibits GR, leading to the promotion of cell death in the synaptic vesicles of neurons. High concentrations of Zn in astrocytes increase oxidative stress while decreasing antioxidant production. Thus, Zn could increase the production of oxidized glutathione (GSSG) by inactivating GR. This inhibition would impair the clearance of H₂O₂ in the cells (Bishop, G. M., 2007). Therefore, it is important to measure the total and free sulphydryl in control and Zn- stressed conditions. As seen, there is a decrease of sulphydryl groups in Zn-treated *P. fluorescens* compared to control. This indicates that Zn could increase the oxidation of free sulphydryl groups. Therefore, Zn affects to proteins, Fe-S, sulphydryl group and GR activity where shown in (Figure 38).
To deal with the onslaught of Zn, the antioxidant defense mechanisms should be promoted to quell the toxic ROS radicals generated in *P. fluorescens*. Due to the modulation of the TCA cycle that occurs in Zn-stressed conditions, *P. fluorescens* shifts its metabolic networks by limiting the pro-oxidant NADH and increasing NADPH producing enzymes such as ICDH, ME, and G6PDH (Figure 26: A, B, C). NADPH is an important reducing power to cope with oxidative stress and is pivotal for the organism’s survival. (Beriault, R., 2007). Also, ICDH-NADP⁺ activity was increased with Zn concentration, which indicates that the cells attempt to counter the oxidative stress by enhancing the NADPH production. Therefore, increasing the amount of NADPH is necessary to clear ROS from the cells. Also, increased activity of ICDH is essential for producing the α-KG, which is known to be an effective antioxidant (Lemire, J., 2008). Furthermore, the accumulation of this keto acid acts as an essential tool against oxidative stress. It has been reported in previous work that *P. fluorescens* induced the antioxidant enzymes catalase and SOD in order to nullify oxidative stress when exposed to Ga (Lemire, J., 2010).

**Figure 38:** Summary of the toxic effects of Zn in *P. fluorescens*. 
Catalase is an essential enzyme involved in the degradation of H$_2$O$_2$ and was approximately three fold higher in Zn stressed cells compared to control (Table 11). Also, SOD, which dismutates the superoxide into hydrogen peroxide and oxygen, is also increased under Zn stress to quell the toxicity of free radicals. The organism is therefore adapting to curtail Zn stress. These enzymes are known to be upregulated during oxidative stress.

As Fe-S clusters are disrupted under Zn-stressed conditions, it is critical to know how the TCA cycle performs its function in the presence of this metal. The TCA cycle contains several enzymatic steps where electron transfer proceeds through Fe-S clusters (Middaugh, J., 2005). Therefore, several enzymes of these pathways were disrupted. In addition, the ETC, which is the site of oxidative phosphorylation, contains several Fe-S clusters and was also perturbed in Zn-stressed cells. Therefore, decreased activities have been observed in complex I and complex IV and fumarase (Figure 24, Figure 25). These enzymes are the prime targets during oxidative stress (Lemire, 2010).

Moreover, most TCA cycle enzymes, especially NAD$^+$ dependent enzymes such as ICDH, α KGDH, and MDH are downregulated in the stressed condition compared to the control as shown (Figure 25). Figure 39 also represents the effects of Zn to important enzymes in TCA cycle and oxidative phosphorylation. This downregulation of enzymes is important to decrease NADH production, which a potential source of ROS through oxidative phosphorylation. Also, inhibiting these enzymes is necessary for the accumulation of α-keto acids, such as α-KG and pyruvate, which act as antioxidants. This modulation evoked by Zn in stressed cells is pivotal to counter ROS stress and in generating an alternative energy-producing pathway.
4.1.2: Metabolic adaptation in *P. fluorescens* under Zinc:

The TCA cycle and oxidative phosphorylation are reliant on Fe. Aconitase, fumarase, complex I, and complex IV all need Fe to fulfill their functions (Lemire, J., 2010). The perturbation of Fe-S will affect these enzymes, thus crippling the central metabolism under Zn stress. To circumvent this problem, *P. fluorescens* elaborates a different version of the TCA cycle. This microbe stimulates the production of ICL. This enzyme cleaves isocitrate into glyoxylate and succinate and it is a key enzyme, leading to many anaplerotic reactions and allows the use of a diverse array of nutrients such as acetate and fatty acids (Appanna, V., 2003).

As shown (Figure 36: A), ICL activity is initially upregulated under Zn stress. That might indicate that the bacterium is using a semi-functional TCA cycle with ICL. Also, ICL activity was increased in control in regulation experiment, thus pointing to an intimate relationship between ICL activity and Zn. Furthermore, this metabolic adaptation leads to the generation of glyoxylate, which is important for the production of the two organic acids oxalate and malate. It
has been reported that oxalate is increased during Al stress to allow for sequestration of Al and detoxification of this trivalent metal as shown in (Figure 40) (Hamel, R., 2001).

**Figure 40:** The modulation of the TCA cycle under Al stress (adapted from Hamel, R., 2001). This scheme shows the modulation of TCA cycle enzymes and the production of oxalate generated by Al stress. ICL: isocitrate lyase, ICDH: isocitrate dehydrogenase, KGDH: ketoglutarate dehydrogenase, GOE: glyoxylate oxidizing enzyme. Red enzyme: decrease, green enzyme: increase.

The data in this study indicate a higher amount of oxalate in the spent fluid of Zn-stressed cells compared to the control which might indicate that this microbe is using oxalate as a detoxifier of Zn, but this still needs further investigation. As shown (Figure 36: D) that no activity was observed in acetylating glyoxylate dehydrogenase, which oxidizes glyoxylate to oxalate. The spontaneous oxidation of glyoxylate under intracellular situations or the presence of other enzyme like lactate dehydrogenase (LDH) may lead to enhanced oxalate.

In Zn-stressed cells, *P. fluorescens* appeared to degrade citrate via TCA cycle ineffectively. Thus, in order for this microbe to survive it has to adapt to this condition by
shifting the metabolic machinery towards such enzymes as CL, PEPC, PEPCK, PPDK, PEPS and PK to enhance the production of the antioxidant pyruvate (Mailloux, R. J., 2008) and ATP. CL, which cleaves citrate into oxaloacetate and acetate without the participation of ATP and coenzyme A was upregulated. Therefore, CL is an essential enzyme in the metabolic reprogramming evoked by Zn stress. As shown (Figure 29) that PEPC and PEPCK were upregulated, as they help to deal with oxaloacetate generated by CL. While PEPC mediates the conversion of oxaloacetate in the presence of inorganic phosphate to PEP, PEPCK converts the oxaloacetate into PEP in the presence of ATP or GTP. Both of these enzymes produce high energy PEP that is more abundant in Zn-stressed cells as shown by HPLC analysis of the soluble CFE (Figure 19).

Also, PK, PEPS, and PPDK were upregulated for aiding in the production of pyruvate and ATP. PK mediates the conversion of PEP and ADP into pyruvate and ATP, while PEPS uses AMP and inorganic phosphate (P$_i$) rather than ADP. PPDK theoretically is more efficient because this enzyme utilizes AMP with pyrophosphate (PP$_i$). As pyrophosphate-dependent metabolism can produce 2 ATP per 1 oxaloacetate, PPDK renders glycolysis more efficient (Auger, C., 2012). Also, this enzyme increased with increasing Zn concentration.

Indeed, HPLC analysis shows that there is a significant increase in pyruvate levels in spent fluid and soluble CFE in Zn-stressed cells compared to the control (Figure 18, 19). This increase in α-keto acids such as pyruvate allows for the accumulation of an effective antioxidant agent to counter the toxicity generated by Zn. The pooling of pyruvate is assured by the upregulation of CL, PEPC and PPDK (Figure 41). It has been shown that these enzymes come out in the same spot in the gel reaction, which might indicate a metabolon that would render the process of ATP and pyruvate production very effective. These stipulations require further studies.
Metabolon means that certain enzyme come together to perform the specific function. In addition, it has shown that pyruvate was increased in soluble CFE treated with 2 mM of citrate, which could be a contributor of the alternative TCA cycle. The citrate consumption was increased under Zn stress, which allowed the soil microbe to degrade the citrate, the sole of carbon. Further, when this microbe was treated with citrate and AMP, the pyruvate was high in Zn. Also, the data in (Figure 34) represent the increase of PEP, oxaloacetate, pyruvate and ATP when soluble CFEs in control and stress given 2 mM of oxaloacetate, P_i and AMP. That confirms that the pyruvate and ATP were produced from this pathway. As oxidative phosphorylation was affected in Zn-stressed conditions, and the organism was surviving, it became critical to understand how this soil microbe was fulfilling its energy needs.

Figure 41: The metabolic reconfiguration under Zn toxicity. CL: citrate lyase, PEPC: phosphoenolpyruvate carboxylase, PEPCK: phosphoenolpyruvate kinase, PK: pyruvate kinase, PEPS: phosphoenolpyruvate synthase, PPDK: pyruvate phosphatase dikinase.
As oxidative phosphorylation is shut down and the AMP-dependent enzymes are up-regulated under Zn stress, it is tempting to explore one enzyme that can maintain the AMP production namely adenylate kinase (AK). The latter is a phosphotransferase enzyme that mediates the conversion of 2 ADP to produce AMP and ATP and is found in eukaryotic and prokaryotic cells (Markaryan, A., 2001). AK has numerous functions in genetics, development and maintenance of energy (Dzeja, P., 2009). It has been reported that the pathogenic strains of *Pseudomonas aeruginosa* can secrete AK to kill the macrophages through the activation of P2Z receptor and enhance the cell death that is found in cystic fibrosis patients (Markaryan, A., 2001).

AK can regenerate AMP for PPDK and PEPS and generate ATP from 2 ADP molecules. PPDK working with AK is more efficient because AK can produce 2 ATP from 2 ADP, while PK produces only 1 ATP from ADP (Figure 42). Therefore, increasing the AK in Zn-stressed bacteria might be an effective way to maintain ATP production (Chastain, C. J., 2011).

![Figure 42: AK and ATP production. AK: adenylate kinase, PPDK: pyruvate phosphate dikinase.](image)

Figure 42: AK and ATP production. AK: adenylate kinase, PPDK: pyruvate phosphate dikinase.
5. Conclusion:

The perturbation of the TCA cycle and oxidative phosphorylation under Zn stress led to the discovery of novel stratagems for the adaptation to Zn toxicity in *P. fluorescens*. This soil microbe reengineers the TCA cycle, leading to the production of pyruvate, an antioxidant, in order to cope with oxidative stress stemming from Zn. The resulting pyruvate production occurs due to the participation of the enzymes CL, PEPC, PEPCK, PK, PEPS and PPDK. This shift allows for the production of ATP and survival as oxidative phosphorylation is ineffective under Zn stress. NADPH production is enhanced and NADH synthesis is curtailed in order to nullify the toxic impact of Zn. Also, *P. fluorescens* increases the antioxidants such catalase and SOD to cope the oxidative stress generated by Zn (Figure 43, Figure 44).

This work adds two important concepts to the understanding of adaptation in *P. fluorescens* exposed to Zn. The role of metabolic machinery in alternative fashion to fulfill the energy needs and production of pyruvate, an antioxidant was elaborated. Although there are various ways for detoxifying metal stress, this work expand our knowledge of how this bacteria could scavenge the metal toxicity to survive. To further build upon the findings of this work, the role of chelators like oxalate, malate and histidine needs to be explored.
Figure 43: Summary of the adaptation in *P. fluorescens* evoked by Zn stress.
Figure 44: Metabolic shift triggered by Zn toxicity. This shift towards Fe-limited metabolism is evident in *P. fluorescens* and allows for a reductive environment to neutralize Zn-stressed conditions.

5.1: Future works:

To better understand the chelation of Zn in *P. fluorescens*, we have to measure some metabolites and find a better direct assay to monitor oxalate in the CFE. Also, malate, owing to its ability to bind zinc, could be another metabolite to investigate. As ICL was upregulated in Zn-stressed cells and acetylating glyoxylate dehydrogenase displayed no activity, we expect that malate synthase could be mediating the conversion of glyoxylate into malate (Figure 45). The latter could be converted to pyruvate, an antioxidant, which is increased (Figure 36) where we find the accumulation of pyruvate in spent fluid and soluble CFE under Zn stress. Furthermore,
when we give soluble CFE of control and Zn-stressed cells glyoxylate we find more pyruvate in the latter, which might indicate that they come from this enzyme. However, the use of oxalate as a chelator still needs to be confirmed. Histidine is also a potent chelator of Zn and may protect against its toxicity (Murphy, J. T et al., 2011). Therefore, the production of this amino acid should be explored.

**Figure 45:** The route for the production of pyruvate. Pyruvate is known to be an antioxidant. ME: malic enzyme; MS: malate synthase; ICL: isocitrate lyase.
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