SUPEROXIDE DISMUTASE (SOD) IS REGULARED BY ENVIRONMENTAL STRESS IN BACTERIAL SYSTEM

A

Thesis Submitted

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CERTIFICATE

This is to certify that the thesis entitled "**Superoxide dismutase (SOD) is regulated by environmental stress in bacterial system**." submitted by Ms.Anshum Tandon (3030101) in partial fulfillment of the requirements for the award of degree of Masters in Biotechnology to Thapar Institute of Engineering and Technology Patiala, is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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DECLARATION

I hereby declare that the work presented in the dissertation entitled "**Superoxide dismutase (SOD) is regulated by environmental stress in bacterial system**." Which is being submitted to the department of Biotechnology and Environmental sciences (DBTES), Thapar Institute Of Engineering and Technology (TIET), Patiala, in partial fulfillment of the requirements for the degree of MASTERS OF SCIENCES IN BIOTECHNOLOGY, as an authentic record of my dissertation work during a period of five months from January, 2005 to May, 2005, under the supervision of Dr. Sunil Khanna, Professor, Department of Biotechnology and Environmental Sciences, Thapar Institute Of Engineering and Technology, Patiala. The matter embodied in this dissertation has previously not formed the basis for any award of any degree or diploma.

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" We are limitless, for what we believe in our dreams."

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ABBREVIATIONS AND SYMBOLS

The abbreviation for chemicals and symbols follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature Biochem. J. (1996) 101, 1-7 or the instructions to authors by the Biochemical Journal (Biochem. J. (1973) 131, 1-20).

CHEMICALS

SDS	Sodium do-decyl sulphate.
Tris	Tris (hydroxymethyl) amino ethane.
Pnp	Para nitrophenol
Conc.	Concentration
NBT	Nitro blue tetrazolium.
Ppm	parts per million

SYMBOLS

°C	degree centigrade
cm	centimeter
G	gram
Hr	hour
μl	microlitre
μ	micro
μm	micrometer
μg	microgram
ml	milliliter
mg	milligram
M	molarity
Mm	millimolar
Min	minutes
N	normality
O.D	optical density
%	Percentage
xg	pellet obtained after centrifuging culture
Sec.	Seconds.

CONTENTS

INTRODUCTION

Oxygen, which has been present in only trace amounts for 2.5 billion years, begins to build-up in the atmosphere. Some oxygen is formed through photo-dissociation of water vapour. However most of the oxygen was probably produced as a by-product of photosynthetic autotrophs using light energy, to split water molecules and so build organic compounds. Primitive unicellular forms resembling modern blue-green algae (cyanobacteria) released oxygen, which accumulated in the atmosphere and was deposited in iron oxide beds on the floor of the ocean. Recent research has suggested that that non-oxygen producing bacteria species such as the purple and green bacteria are the most ancient photosynthetic bacteria. Another group of non-oxygen-producing bacteria, known as heliobacteria, evolved later and appear to have been the precursors of the forms that produce oxygen as a byproduct. Relatives of cyanobacteria appear to have given rise to chloroplasts in algae and green plants - the chloroplasts are the small bodies in plant cells that carry out photosynthesis in algae and other plants. This occurred through a process of engulfment where these primitive cyanobacteria were captured, engulfed and enslaving by other cells to become the solar driven carbohydrate factories within the cells. Chloroplasts contain their own RNA and are thought to have been derived from cells, which were once independent.

A similar process is thought to have involved the engulfing and enslavement of other bacteria to form mitochondria - the energy powerhouses of cells using oxidative phosphorylation to use oxygen and carbohydrate to release energy, carbon dioxide and water. These complex cells with organelles entrapped within them, and nuclei became the 'eukaryotes' - the next stages in evolution of unicellular organisms. In some ways these forms can be regarded as the first type of 'multi-cellular organism' - though they are not generally recognized as such. The organelles represent cells within cells. The final phase of development

of the modern atmosphere was the removal of the last remnants carbon dioxide (from 1-5% down to 0.04% today) and the build-up of oxygen to modern day proportions (from about 10% to 21% today). The oxygen that was produced was toxic to most forms present at the time and its build-up may have caused the first mass extinction on the planet opening up new habitats and opportunities for eukaryotes respiring oxygen. Anaerobic organism also exist today and must have followed a kind of evolutionary “adaptation” to the increasing atmospheric oxygen levels by restricting themselves to extreme environments where oxygen cannot penetrate in poisonous concentrations. Other organisms generated antioxidant defenses to tolerate oxygen and to use it for metabolic purpose, such as energy production gained by electron transport chains with oxygen a terminal acceptor.

1.1 Oxygen radicals

Electrons within atoms and molecules occupy regions of space known as "orbitals". Each orbital can hold a maximum of two electrons. A single electron alone in an orbital is said to be "unpaired" and a radical is defined as any species that contains one or more unpaired electrons. Such a definition embraces the atom of hydrogen (one unpaired electron) and the ions of such transition metals as iron, copper and manganese (cf. Holmberg,).

Oxygen radicals are produced during oxygen reduction to water in the intermediate steps:

$O_2 + 1e^- + H^+ AHO_2AH^+ + O_2^-$ (superoxide)

$HO_2 + 1e^- + H^+AH_2O_2$

$H_2O_2 + 1e^- + H^+AH_3O_2AH_2O + OH^-$ (hydroxyl radical)

$OH- + 1e^- + H^+AH_2O$

Small sugars, such as glycolaldehyde, glyceraldehydes, or dihydroxy acetone, autoxidize by a free pathway in which O_2^- serves as a chain propagator (Robertson *et.al.*, 1981; Mashino *et.al*, 1987). Enolization precedes autooxidation, and small sugars, unable to block the carbonyl by cyclization, are

consequently most readily autoxidized; in contrast, aldohexoses, which exist primarily as pyranoses, are relatively stable. Production of O_2^- and H_2O_2, during autoxidation probably explains the mutagenicity of small sugars (Garst *et.al.*,1982; Cohen *et.al* Greenwald : Elsevier), their abilities to inactivate the transsulfurase called rhodanese (Cannella C.,Berni R.,1983), and their abilities to cause the peroxidation of polyunsaturated fatty acids (Hicks *et.al*.,1988).

The relative resistance of aldohexoses towards autoxidation is abrogated when they react with amino compounds and is converted to fructosyl amines (Smith P.R., Thornalley S., 1992). This is the situation in glycated proteins, which do autoxidize with production of O_2^- and H_2O_2 (Sakurai T., Tsuchiya S.,1988). Oxidative damage, subsequent to glycation, has been reported for LDL (Sakurai T., Kimura S., Nakano M., Kimura H., 1991), collagen (Bailey A.J., Sims T.J., Avery N.C.,Miles C.A.,1993), the cytosolic Cu/Zn superoxide dismutases (SOD)(Ookawara *et.al*,1992), the extracellular C/Zn SOD (Adachi *et.al*.,1992), and serum albumin .

1.2 Superoxide

Superoxide ion is the one-electron reduction product of oxygen. Dissolved in organic solvents, it is an extremely reactive species, e.g. it can displace chlorine from such unreactive chlorinated hydrocarbons as carbon tetrachloride (CCl_4) (Sawyer *et.al.*, 1979). In aqueous solution O_2^- is poorly reactive, acting as a reducing agent (e.g. it will reduce cytochrome c or nitro-blue tetrazolium) and slowly undergoes the dismutation reaction, in which one molecule of superoxide reduces another one to form hydrogen peroxide (H_2O_2). The dismutation reaction occurs in stages; O_2^- must first combine with a proton to yield the hydroperoxyl radical, HO_2,

O_2^- + H+ ---> HO_2

$HO_2 + O_2^- + H^+$ ---> $H_2O_2 + O_2$

$O_2^- + O_2^- + 2H^+$ ---> $H_2O_2 + O_2$

At physiological pH the low concentration of H+ ions slows the rate of dismutation. Despite the low reactivity of O_2^- in aqueous solution, systems producing it do a great deal of damage *in vitro* (e.g. they fragment DNA and polysaccharides, kill bacteria and animal cells in culture) and *in vivo* (e.g. when O_2^- generating systems are injected into the footpads of rats, inflammation is produced, their instillation into the lungs of rats and rabbits produces oedema and cell death, and infusion of them into vascular beds produces endothelial cell damage and extensive leakage from the blood vessels) (Fridovich *et.al.*,1983: Halliwell *et.al*,1984). Depending on the circumstances, damage caused by O_2^- generating systems might be attributed to

(i) O_2^- itself, e.g. exposure of tissue fluids to O_2^- causes formation of a factor chemotactic for neutrophils that brings more of them into the area and hence can potentiate inflammation.
(ii) HO_2 radical, which is more reactive than O_2^- (Bielski BHJ, Shive GG,1979). Formation of HO_2 is favoured at pH values lower than "physiological", but the phagocytic vacuole operates at an acid pH and the pericellular pH of macrophages has been reported to be 6 or less (Etherington *et.al.* , 1979)
(iii) H_2O_2
(iv) hydroxyl radical
(v) singlet oxygen.

Singlet O_2 is an especially reactive form of oxygen capable of rapidly oxidising many molecules, including membrane lipids. Its formation in O_2^{--}generating

systems has often been proposed but clear-cut evidence for a damaging role of in such O_2 systems has not been obtained. One of the problems is that the "scavengers" of singlet O_2 frequently used react with other radical species as well (Halliwell *et.al.*, 1984)

Human and other animal neutrophils can kill some strains of bacteria under anaerobic conditions, when O_2^- cannot form. Obviously, the other mechanisms are important here. Many other bacterial strains are not killed in the absence of O_2, however, even though engulfment and vesicle fusion proceed normally. In chronic granulomatous disease (CGD), an inborn error of metabolism, the respiratory burst does not occur but other aspects of phagocytic action proceed normally. CGD was first described in humans because it was accompanied by severe and recurrent infections affecting lymph nodes, skin, lungs and liver (Tauber *et.al.*, 1983). The symptoms of CGD provide direct evidence for the production of O_2^- by human phagocytic cells *in vivo* and for its role in bacterial killing.

It follows therefore that if neutrophils become activated in the wrong place, or to excessive extents (as in the autoimmune diseases) then the oxygen radicals they release could do a lot of damage. It must be remembered, however, that phagocytic cells also produce hydrolytic enzymes (elastase, neutral proteases etc.), chemotactic factors, prostaglandins, leukotrienes and other chemicals, so that damage by activated phagocytes could be due to any one of these factors or to any combination of them. It cannot be attributed a priori to oxygen radicals.

1.3 Hydrogen Peroxide

O_2^- generating systems produce H_2O_2 by the dismutation reaction and a number of oxidase enzymes produce H_2O_2 directly, examples being glycollate oxidase and amino acid oxidases. Superoxide dismutase (SOD) enzymes remove O_2^- by greatly accelerating the dismutation reaction, so if we accept that O_2^- is formed *in*

vivo in humans then we must accept that H_2O_2 vapour is present in expired human breath (Williams *et.al.*, 1983), a likely source being H_2O_2 released from alveolar macrophages (Babior *et.al.*,1978) although a contribution from peroxide-producing oral bacteria (Carlsson *et.al.*,1983) cannot be ruled out. That H_2O_2 is formed *in vivo* in humans is further supported by the presence of enzymes specific for its removal, such as catalase and glutathione peroxidase. The latter enzyme requires selenium for its activity (Diplock *et.al.*,1981). H_2O_2 is probably more damaging than is O_2^- in *invitro* experiments in aqueous solution, but many cells seem to tolerate its presence and bacteria often produce H_2O_2. On the other hand, the toxicity of O_2^- generating systems to several animal cells in culture has been attributed to formation of. H_2O_2.

1.4 Hydroxyl radical

Hydroxyl radical is produced when water is exposed to high-energy ionising radiation and hence its properties have been well documented by radiation chemists (Bielski *et.al.*,1979: Willson *et.al.*,1978). Unlike the hydroxyl ion, the hydroxyl radical is fearsomely reactive, combining with most molecules found in vivo at near diffusion-controlled rates. Hence any OH produced *in vivo* will react at or close to its site of formation. The extent of the damage done would therefore depend on what the site of formation was (e.g. production of OH close to DNA could lead to strand breakage whereas production close to an enzyme molecule already present in excess in the cell, such as lactate dehydrogenase, might have no biological consequences).

Hydroxyl radical is produced whenever H_2O_2 comes into contact with copper (I) ions (Cu+) or iron (II) ions (Fe^{2+}). The substantial evidence suggests that metal complexes capable of causing hydroxyl radical formation are present *in vivo* in human cells. *In vivo* complexes of iron salts with phosphate esters such as ATP and GTP (Flitter *et.al.*, 1983: Floyd *et.al.*, , 1983) or with DNA are particularly important. (Floyd *et.al.*,1981).

Organisms take great care to ensure that as much iron or copper as possible is bound to transport proteins or functional proteins such as transferrin, caeruloplasmin or haemoglobin. Metals bound to these proteins are inactive or only weakly active in catalyzing OH production (Winterbourn *et.al.*, 1983). Since both H_2O_2 and metal complexes are present *in vivo* in humans, it is logical to assume that OH radicals can form. Direct evidence for this is difficult to obtain. Many methods exist for demonstrating the existence of OH *in vitro*, but *in vivo* any OH formed is likely to react so close to its site of formation that the use of these methods is impractical, although some new techniques (such as the ability of OH to convert dimethylsulphoxide into methane or its ability to hydroxylate aromatic rings in characteristic ways (Richmond *et.al.*, 1981) show promise for *in vivo* use. One can also attempt to infer the formation of OH radical in vivo by observing the damage done (as in rheumatoid arthritis). *In vitro*, phagocytic cells have been shown to produce OH radical (Charles *et.al.*, 1980) and the killing of bacteria can sometimes be prevented by reagents that react with this species .

It was mentioned in the previous section that the killing of animal cells in culture by O_2^- generating systems could sometimes be attributed to H_2O_2. It could, of course, be achieved by H_2O_2 itself; some enzymes are known to be inactivated by H_2O_2 although the best examples come from plant rather than animal systems (Charles *et.al.*,1980). There is another possibility, however, H_2O_2 generated externally crosses cell membranes easily and could penetrate inside the cell and cause OH to be formed. Externally added scavengers of OH would not prevent this since they could not reach the correct place. By contrast, O_2^- crosses cell membranes only slowly (Takahashi *et.al.*,1983) unless there is a specific channel for it (the only known example of this being the erythrocyte membrane, which has an "anion channel" through which O_2^- can move. Hydroxyl radical will never cross a membrane: it will react with whatever membrane component if meets first.

1.5 Superoxide Dismutase

The Superoxide dismutase (SOD), E.C.no.1.15.1.1 are metalloenzymes that catalyze the dismutation of superoxide to hydrogen peroxide and molecular oxygen. They have been found in nearly all organisms examined to date and play a major role in defense against oxidative stress. It plays an extremely important role in the protection of all aerobic life-systems, including man, against oxygen toxicity (and the free radicals derived from oxygen). There are three major classes of SODs in bacteria, which differ in their metal cofactors. The manganese containing (Mn SOD) and iron containing (Fe SOD) enzymes are cytoplasmic, while the copper-plus-zinc (Cu/Zn SOD) enzyme is periplasmic. In addition, a new class of nickel containing SOD has been recently discovered in *Streptomyces griseus* and *S. coelcolor* (Whittaker *et.al.*,1998) The Mn SODs and Fe SODs have very similar sequences and structures and are evolutionarily unrelated to Cu/Zn SODs. Usually FeSODs and MnSODs require specific metal for activity and can be distinguished on the basis of amino acid sequence and sensitivity to H_2O_2. However these criteria can be misleading and the purified protein must be analysed to correctly determine the metal at the active site. Small groups of Mn/FeSODs, termed cambialistic, are active with either manganese or iron incorporated into the same active site. They have been found in the anaerobic (aero tolerant) species *Propionibacterium shermanii, Bacteroides fragilis, Bacteroides thetaiotaaomicron, Streptococcus mutans*, and *Porphyromonas gingivalis* and in the aerobic methylotrphic *Methylomonas* strain J. The aerobic hypethemophilic *Aquifex pyrophilus* SOD is, presumably, also cambialistic.

1.6 Types of SOD

Superoxide Dismutase have been classified on the basis of metal cofactor into four classes: –

- Cu/Zn SOD
- Fe SOD
- Mn SOD
- Ni SOD

1.7 Cu/Zn SOD

1-3 Copper zinc Superoxide Dismutase, Cu/Zn SOD, is a dimeric protein with two identical subunits bonded noncovalently. Each subunit contains one copper(II) and one zinc(II) ion in the oxidized state of the metalloenzymes. During catalysis, copper is the redox partner of the superoxide radical. Cu/Zn SOD play a major role in antioxidant defence mechanisms; Cu/Zn SOD gene knock out mice exhibited more susceptibility to paraquat toxicity, but remained alive (Reaume *et.al*., 1996),while transgenic mice with over expressed Cu/Zn human SOD gene were protected from post-ischemic injuries. Cu/Zn SOD has been found in cytosol, mitochondria, and chloroplast of eukaryotes and in periplasm of some prokaryotes. The properties of these enzymes have been conserved throughout the evolution. The various classes found in fungi, plants, birds and mammals, are distinguishable from each other due to a few differences in amino acid composition and electron-paramagnetic resonance spectra (EPR), while structural differences have emerged between eukaryotic and prokaryotic Cu/Zn SOD.

Conserved structural and functional features of Cu/Zn SODs have been studied by comparison of the known 3- D structures and analysis of available amino acid sequences. Alignment of 38 SODs from various sources and the deduced

evolutionary tree indicated that cytosolic and extracellular enzymes followed independent evolutionary path. Cytosolic eukaryotic Cu/Zn superoxide dismutases share with prokaryotic enzymes are stable and flexible beta barrel fold, a conserved ligand stereo chemistry, the two metal ions-active site and a typical dimer assembly. However, recently it has been shown that in the prokaryotic SOD's the beta barrel elements is responsible for dimer interface, the strategy utilized for the electrostatic of the superoxide radical and the formation of the inter subunit disulfide bridge, are quite dissimilar from those which are highly conserved in their eukaryotic counterparts (Bourne *et al;* 1996).These observations tend to disprove the previous hypothesis of a lateral SOD gene transfer from eukaryotic to prokaryotic species. In homologous eukaryotic Cu/Zn SODs, local electric fields and electric flux in the proximity of the active site were found to be constant, suggesting that the spatial relationship of the charges and the protein surface have also been conserved during evolution. The only monomeric variant of Cu/Zn found in *E.coli* displays an anti parallel. Beta barrel structure in which disulfide bridge connections are modified, functional electrostatic residues are absent and the molecular surface region involved in the dimer formation is structurally altered displaying the net polar nature. These data suggest a modified control of substrate steering towards the catalytic centre.

1.8 Fe-SOD/Mn-SOD

Fe SOD is present in both aerobic and anaerobic bacteria, archaea bacteria and plants, whereas Mn SOD is present in bacteria, archaea bacteria, mitochondria and chloroplasts. In comparison with the Cu/Zn protein, the Mn SOD family has received less importance. Spectroscopic and magnetic studies revealed that the metals are tervalents and kinetic studies evidenced complexities in the catalytic mechanisms (Stallings *et al.*; 1983). Fe SOD and Mn SOD are closely related in sequence in structural homology suggesting a common ancestor. By contrast, they are folded differently with respect to the Cu/Zn family, considering that their encoding genes are unrelated. Recently a homo dimeric Fe SOD with amino acid

composition, EPR spectra and molecular weight (subunit of 22 Kda) similar to its aerobic counterparts has been isolated from the strictly anaerobic bacterium *Desulfovibrio gigas,* suggesting a role of this enzyme in combination with the catalase in the detoxification of oxygen byproducts in anaerobic environments (Dos Santos *et al*; 2000).

3D structures of Fe and Mn SOD have been reported for the enzyme from *E.coli, Bacillus stearothermophilus, Pseudomonas ovalis, Propionibacterium shermanii, Mycobacterium tuberculosis, Thermus thermophilus, Aquifex pyrophilus,* human mitochondria and the Archaea *Sulfolobus solfataricus and Sulfolobus acidocaldarius*. They are either homodimers or homotetramers (with subunit molecular weights of about 20 Kda). A high conservation has been found both in the primary and tertiary structures of the enzymes compared; residues essential for binding to metal cofactor near the subunit interface and residues forming the hydrophobic pocket around the active site are located in identical positions (Joshi *et.al.*,1993). The SODs from *B. circulans* and *A. aerogenes* were immuonologically distinct from each other, judged from an immuonoprecpitation test. The two SODs had high homologies with other bacterial Mn –SODs, especially the highest homology of 75.4% and 66.7%, respectively, with the *B. stearothermophilus* Mn- SOD (Lee *et.al.*, 1993).

Streptomyces coelicolor Mullar contains two superoxide dismutase, nickel-containing (NiSOD) and iron- and zinc-containing SOD (Fe/Zn SOD). The sodF gene encoding Fe/Zn SOD was isolated by using PCR primers corresponding to the N-terminal peptide sequence of purified Fe/Zn SOD and a C-terminal region conserved among known Fe/ZnSODs and Mn Sods. The deduced amino acids sequence exhibited highest similarity to Mn and Fe/Zn SODs from *Propionibacterium shermanii* and *Mycobacterium* spp. The transcription start site of the *sodF* gene was determined by primer extension. When the *sodF* gene was cloned in pIJ702 and introduced into *Streptomyces lividans* TK24, it produced at least 30 times more Fe/ZnSOD then the control cells. The *sodF* gene in

S.lividans TK24 was disrupted and found that the disruptant did not produce any Fe/ZnSOD enzymes activity but produced more Ni SOD. The expression of the cloned sodF gene in TK24 was repressed significantly by, consistent with the regulation pattern in nonoverproducing cells. This finding suggests that the cloned sodF gene contains the cis-acting elements necessary for Ni regulation. When the *sodF* mRNA in *S.coelicolor* muller cells was analyzed by S1 mapping of both 5' and 3' ends , it revealed that Ni caused a reduction in level of monocistronic *sodF* transcripts. Ni did not affect the stability of *sodF* mRNA. Indicating that it regulates transcription of *S.lividans* TK24 cells overproducing Fe/Zn SOD became more resistant to oxidants such as menadione and lawsone then the control cells, suggesting the protective role of Fe/Zn SOD. However, the *sodF* disruptant survival as well as wild-type strain in the presence of these oxidants, suggesting the complementing role of Ni SOD increased in the disruptant. (Kim *et.al*.,1998.)

1.9 Ni-SOD

A novel type of cytosolic SOD containing nickel as a cofactor has recently been reported in *Streptomyces* species. Some of these strains also contain Fe SOD. These enzymes are composed of four identical subunits of 13.4 kDa. The protein from *Streptomyces coelicolor* is processed *in vivo* (Kim *et al*; 1998) and in the repression of the gene encoding Fe SOD (Chung et.al, J Bacteriol 181,1999). The amino acid composition, N-terminal sequences and immunological properties demonstrated that they are distinct from the Mn, Fe or Cu/Zn and thus represents a new class of SOD. X-ray spectroscopic studies of the nickel site demonstrated that the coordination environment is completely different from other SODs being composed largely of S-donor ligands (Putative Cys and Met residues); kinetic investigations revealed that Ni SOD catalyses the redox chemistry of Superoxide with atleast the same efficiency as that of other SOD families.

1.10 Extracellular SOD

Extracellular SOD has been isolated first from mammalian sources. It is a tetrameric and glycosylated Cu/Zn SOD enzyme in humans, mice, and rats (Marklund *et.al.*, 1982). The amino acid sequence of the human enzyme has deduced from the gene and contains an 18 amino acid signal peptide, residues 96-193, with strong homology to the cytosolic Cu/Zn SOD, and residues 1-95 with no homology. Residues of the active site are conserved. Although, if this enzyme has been widely studied, its 3D structure is still unknown. The protein is involved in protection against oxygen free radicals. Transgenic mice secreting human EC-SOD in milk have been obtained and its use as powerful pharmaceutical to reduce hypertension has been postulated (Hansson *et.al* 1994). Extracellular SODs have also been found in non mammalian sources like *Schistosoma mansoni* (Hong *et.al* 1993), *Onchocerca volvulus* (James *et.al* 1994), where it appears to be a pathogenicity factor. Inactivation of secreted SOD with specific antibodies increases the susceptibility of the parasite to be killed by leukocytes (Beaman *et.al* 1985). A homodimeric Mn-SOD was also purified from culture supernatant of the Gram-Positive bacterium *Streptococcus pyrogenes* (Gerlach *et.al*,1998). The Archaeon *Sulfolobus solfataricus* also possesses an extracellular Fe-SOD, demonstrated to be involved in defence of cell bound enzymes against Superoxide radicals generated outside the *S. solfataricus* cytoplasm.

1.11 SOD – Structure and genetic analysis

Superoxide dismutases (SODs) catalyze the proton-dependent dismutation of two superoxide radical anions to molecular oxygen and hydrogen peroxide. The biological functions of SODs are very important because they prevent oxidative damage and inflammation, due to subsequent formation of oxygen intermediates derived from the superoxide, and are involved in anticancer and antiaging mechanisms. Cu/Zn SOD plays a protective role in the pathogenesis of selective

neuronal injury after brief ischemia and reduces the degree of necrotic and DNA fragmented neuronal death following global ischemia. The main function of SOD is to scavenge O2- radicals generated in various physiological processes, thus preventing the oxidation of biological molecules, either by the radicals themselves, or by their derivatives (Liochev and Fridovich, 2000). A number of environmental stresses can lead to enhanced production of O_2^- within plant tissues, and plants are believed to rely on the enzyme SOD to detoxify this reactive oxygen species.

Super oxide dismutase (SOD) is a key enzyme for protection of aerobic organisms against toxic radicals produced during oxidative process (McCord et al.,1997; Fridovich, 1999) since SOD has been discovered in the bacteria *Desulfovibrio desulfuricans* (Henry;1997)*, propionibacterium shermanii* (Meier *et.al* 1982, Synthesis of either Fe or Mn Superoxide dismutase with an apparently identical protein moiety by an anaerobic bacterium dependent on the metal supplied./J.Biol. chem., 257, 13977-13980) and *Bacteroids fragilis* (Gregory E.M. & Dapper C.H.,1983) as well as in primitive protozoa lacking mitochondria, such as *Entamoeba histoilytica* (Tannich *et.al.,* 1991), it is likely that other functions could be assigned to SOD, probably linked to the presence of metal ions, such as Mn, Fe or Cu/Zn; recently, a Ni SOD has been described(Kim et al, 1996). SODs are generally divided into two families based on sequence and structural homology: Cu/Zn SOD and Fe/Mn SOD, where enzymes of the latter family have either iron or manganese bound in the active site. To date, the structures of eight different Cu/Zn SODs and nine Mn or Fe SODs have been solved. Even though the three-dimensional structure of Cu/Zn SOD appears different from that of Mn or Fe SOD, it cannot be excluded that SOD could have evolved from an ancestral form, functioning in the cell as a metal carrier. Therefore, the study of structural properties of SOD from primitive organisms could provide clues to the understanding of structure-function relationships in this enzyme. Moreover, SODs from organisms adapted to

extreme growth conditions represent suitable models of proteins adapted to extreme environments.

The functional and structural aspects of SOD have been extensively reviewed (Bannister *et.al*, 1987; Touati *et.al*,,1988; Fridovich,1989; StallingsW.C.*et.al.*,1991) Fe and Mn-SOD catalyze the dismutation of super oxide in a two step reaction (Bull& Fee, J.A.,1985)

$O_2^- + M_3^-$ -- SOD → O2 +M_2^- -- SOD

$O_2^- + M_2^-$ -- SOD +$2H^-$ → $H_2O_2 + M_3^-$ -- SOD

Where M signifies the active site metal. Fe and Mn SOD are structurally similar with high degree of homology in the amino acid sequence.

The molecular environment around the metal is highly conserved in Fe and Mn-SOD structures, to the extent that what determines the metal specificity is as yet unclear. Some SODS are functional only if bound to the original metal, even through they are able to bind either Mn or Fe proposed that the inactivity of metal substituted SOD is due to in appropriate redox potentials in metal-substituted structures. There is also evidence of SODs functioning with either Mn or Fe (Gregory & Dapper, 1983; Meier *et al*, 1982, 1994). Vice versa, other Fe-SODs derived from Mn-SODs show very little activity at ph 8 while at ph 6 they display similar high activity with either Mn or Fe. In an attempt to elucidate the effects of metal replacement on the structure of the enzymes solved the crystal structure of *P. shermani* SOD, which works with either Fe or Mn bound in the active site. Recently, the Fe SOD from the thermophile bacterium *Aquifex pyrophilus* has been isolated and characterized and its three dimensional structure has been reported (Lim *et.al*, 1997b).

Intracellular parasitic protozoans of the genus *Leishmania* was found to be dependent on the elaboration of enzymatic and other mechanisms for evading toxic free- radical damage inflicted by their phagocytic macrophage host for their

survival. One such mechanism may involve superoxide dismutase, which detoxifies reactive Superoxide radicals produced by activated macrophages, but the role of this enzyme in parasite survival has not yet been demonstrated. A SOD gene from *L. tropica* was also cloned and generated SOD-deficient parasites by expressing the corresponding antisense RNA from an episomal vector. Such parasites have exhibited enhanced sensitivity to menadione and hydrogen peroxide in axenic culture, and a markedly reduced survival in mouse macrophages, indicating that SOD is a major determinant of intracellular survival of *Leishmania.* (Sanjay *et.al*, 2003)

The role of ruberythrin as a scavenger of oxygen radicals was suggested when studies on the food borne pathogen *Clostridium perfringes,* an obligate anaerobe, which showed growth under conditions of oxidative stress was carried out. In protein extracts, three major bands of SOD were obtained on gels after electrophoresis. The biggest band was purified by three chromatographic steps and an open reading frame of 196 amino acids (molecular weight 21,159Da) obtained, was sequenced with a strong homology to the *Desulfovibrio vulgaris* rubrerythin (*rbr)*. SOD activity was also observed in extracts of *E.coli strains* containing the recombinant *rbr* gene from *C.perfringes*. A biological function of ruberythrin as SOD was confirmed with the functional Complementation by the *rbr* gene of an *E.coli* mutant strain lacking SOD activity. (Yvan Lehman *et.al*, 1996)

A 2.7-kb fragment of a chromosomal DNA from *Clostridium perfringens* containing the Superoxide Dismutase- encoding gene, sod, previously, rubrerythrin from *C.perfringens* had been isolated and its gene (rbr) had been cloned (Lehmann *et.al*, 1996). Northern Blot experiments revealed a length of approximately 800 bases for each transcript of *rbr* and *sod* of C.perfringens. Thus, *rbr* and *sod* each represent a monocistronic operon. Neither *sod* nor *rbr* transcription was influenced by oxidative stress (Thomas *et.al*,, 1999). The nucleotide of the Mn-superoxide Dismutase gene of *Bacillus circulans* and the Fe

SOD gene of *Aerobacter aerogens* has also been sequenced by PCR. The two amino acids sequences deduced from the nucleotide sequences of PCR products had an identity of 66.1%. But, the SODs from *B.circulans* and *A. aerogenes* were immuonologically distinct (Lee *et.al*,,1993)

A single detectable Superoxide Dismutase was found in free-living growth conditions in *Sinorhizobium meliloti*, which was isolated from a genomic library by using a *sod* fragment amplified by PCR from degenerate primers as a probe. The *sodA* gene was found to be located in the chromosome and is transcribed monocistronically and encodes a 200 amino acid protein with a theoretical Mr of 22,430 and pI of 5.8. *S.meliloti* SOD complemented a deficient *E.coli* mutant, restoring aerobic growth of a *sodA sodB recA* strain. (Renata *et.al., 1999*).

1.12 Plant SOD

In plants, the role of SOD during environmental adversity, has received much attention since reactive oxygen species have been found to be produced during many stress conditions. Also, the possibilities for generating stress tolerant plant varieties by the genetic engineering of SOD are in progress. In plants, chloroplastic SOD is generally the most abundant SOD in green leaves, while in germinating seedlings and in etiolated material the cytoplasmic and mitochondrial SODs are prevalent (Foster *et.al*,,1990). This distribution reflects changes occurring in the subcellular sites of oxyradical formation i.e., during the greening process photosynthetic reactions become more dominant in cell metabolism, necessitating an increase in chloroplastic SOD. During subsequent growth to maturity, SOD activities appear to change little. However, observations suggests that expression of the different enzymes is to some extent determined by the availability of their metal cofactors (Del Rio *et.al*,1991)

Activities of all the SOD enzymes, along with other oxygen detoxifying enzymes such as catalase and glutathione reductase decreases as the plant senesces

(Dhindsa *et.al.*, 1981;Pauls *et.al.*, 1984). Processes that enhance the formation of oxyradicals and initiate lipid breakdown, such as lipoxygenase enzymes, are stimulated in senescing plant tissue (Lynch *et.al.*, 1984)), and the addition of hydrogen peroxide or hydrogen peroxide generating compounds to excised rice leaves promotes senescence (Parida *et.al.*,1978)). This suggests that free radicals play an important role in senescence and aging processes (Adelman *et.al.*,1988 ;:34Cerutti P.A., 985). To date, it has been shown that SOD activity is increased in cells in response to diverse environmental and xenobiotic stresses including paraquat, high light, water logging and drought. Apparently, each of the SOD isozymes are independently regulated according to the degree of oxidative stress experienced in the respected sub-cellular compartments, but how this is communicated at the molecular level is unknown.

In plants, the genes that regulate SOD expression may be isolated by methods requiring promoter analysis, gel shift assays, DNase I foot printing, and the screening of expression libraries with DNA sequence motifs known to be recognized by the factor of interest. Alternatively, plants mutants may be used to isolate regulatory genes by genetic approaches. Several plant mutants have been described that have mutations in regulatory genes controlling SOD expression e.g. *Conyza bonariensis*, (Shaaltiel *et.al.*, 1988) and *Lolium serene* (Harper *et.al.*,) , the lack of good genetic maps in these species makes them currently worthless for isolating the regulatory genes themselves. It has been suggested that some of the stress tolerant plant varieties analyzed with respect to SOD have acquired tolerance by increasing SOD Activity alone. Biochemical analysis of such mutants may suggests some role of SOD, a true evaluation of the effects of changing SOD activity alone in plants can be obtained only by genetic engineering. To obtain a complete concept, different SOD genes should be over expressed, because their enzyme products have different properties.

OBJECTIVE

- To study the effect of various stress causing agents on SOD activity in different bacterial isolates.

- Molecular characterization of various types of Superoxide dismutase.

MATERIALS AND METHODS

3.1 Preparation of inoculum

A loopful of thawed mother culture from glycerol stock was serially diluted by streaking on Luria agar (LA) plates to get isolated colonies. An isolated colony from the culture plate was use to inoculate 5ml sterile Luria Bertani (LB). Inoculums (1-2%)were further used to inoculate media. Each culture plate served to provide inoculums for a month. The purity of the culture was checked periodically.

3.2 Growth Patten Studies

The given bacterial culture 2619 *Pseudomonas aeruginosae* was grown on both LB (rich media) and BHB (basal media). Growth of the bacterium was measured by observing the optical density at 550nm. Studies were also carried out with pNP (50ppm), Lead (100ppm) and pyrene (50ppm) as stress in both LB and BHB to study their effect on the enzyme superoxide dismutase (SOD).

3.3 Superoxide dismutase activity

Superoxide dismutase activity was measured with an aliquot of the culture supernatant with potassium Phosphate buffer and reaction mixture. The reaction mixture consist of 50mM sodium/potassium phosphate buffer (pH 7.5), 58mM NBT, 9.9mM Methionine and 0.025% (v/v) Triton X-100. Finally, 2.45mM riboflavin was added to the reaction mixture & then exposed to strong light of 600lux for 8minutes.The color intensity was then determined spectrophotometrically at 560nm. For measuring Intracellular activity of the bacterial cell suspension, the suspension was sonicated at full blaste with 30sec pulse for 10min. The sonicated sample was centrifuged at 10,0000x g

for 15 min and the supernatant was used for enzyme activity. One unit of SOD was expressed as the quantity of enzyme required to inhibit the reduction of NBT by 50%.

3.4 Protein estimation

Protein was determined by biuret method (Itzhaki and Gill, 1964) using standard solution of bovine serum albumin (stock5mg/ml). The total volume of the protein sample was made upto 2ml and then 1ml Biuret reagent was added. After 10 mins, the absorbance was measured at 310nms against a blank in spectrophotometer (Hitachi., Japan)

3.5 Sample Preparation for Activity measurement

Cultures were inoculated in basal media with different stress causing compounds. The samples were withdrawn from each flask at various time intervals of 0,6,12,18,24,30,36,and 48 hrs, and were assayed for their SOD activity in both intracellular and extracellular fractions. The extracellular fraction was obtained as supernatant by centrifugating the culture at 10000x g for 10 mins. The intracellular fraction was prepared by sonicating the culture for three mins with a pulse of five seconds and then centrifugation at 10000x g for 10 mins was done. The supernatant was used for enzyme activity.

3.6 Chromosomal DNA Extraction

Cells were grown in luria broth media and harvested at mid log phase. Pellets were washed with TE (10:1,pH 8.0) and then 200μl of Rose solution was added. After incubation at 90°C for 20 min with intermitant shaking the sample was kept on ice for 5min.1ml of chloroform and isoamyl alcohol (24:1) was added at room temperature without disturbing. Cell debris and protein

form a separating layer between two solvent systems when centrifuged at 10,000x g for 10 min. The top layer were pipetted out and the supernatant containing DNA was treated with 50μl RNase (10mg/ml) and incubated for 30 min at 37°C. DNA was then precipitated with 2.5 volume of ethanol and incubated at -20°C for 30-60 min., which yielded pure chromosomal DNA. Evaporated ethanol properly and dissolved DNA in 50 μl TE (10:1,pH 8)

3.7 Agarose Gel Electrophoresis

Agarose (0.5gm) was added in 50ml of 1X TAE Buffer in a flask and boiled properly till agarose dissolved. Gel was cooled, poured in casting tray and allowed to solidify. Combs were removed and gel was kept into the gel apparatus containing 1X TAE buffer. Samples were loaded in wells using micropipette and lid was closed. Then volt, current and time was set and gel was allowed to run. After the gel was run, it was kept for staining in 100ml water containing 10μl Ethidium bromide for 20 mins. and visualized under UV light.

3.8 Polymerase chain reaction (PCR)

Two sets of primers were used to amplify the DNA obtained from different cultures – *P. aeruginosae*, bacterial isolates A15, A19 capable of pyrene degradation and metal tolerant G306, G314. The PCR program followed for both sets of primers as follows:

Primer set I

Forward Primer-23bp

F-CCTGCATTGCCATACGCCTACGA-3'

Reverse Primer-24bp

R-ATGACATCATTCCGACCACCATCC-3'

The Master mix was prepared using the following-

Components	Volume used (μl)
Sterile MQ H_2O	9
PCR buffer	2
$MgCl_2$	1.2
dNTPs	0.8
primer 3F	2
primer 4R	2
Taq DNA polymerase	2
DNA template	1

Program condition: -

Step	Temperature	Time	No. of cycles
Denaturation	94°C	5min	1
	94°C	30sec	
Annealing	61°C	30sec	30
Extension	72°C	1min	
Final Extension	72°C	7min	1
	4°C	Hold	

Primer set II

Forward Primer-39bp

F5'-GCGGGATCCAAGCTTAGGCGTGTTGGACCGTTTCCAAGA3'

Reverse Primer-36bp

R5'-ACGCGTCGACGAATTCCTTTCTTATTTAGCGGCTTG3'

The Mastermix was prepared using the following-

Components	Volume used (µl)
Sterile MQ H_2O	12.8
PCR buffer	2
$MgCl_2$	1.2
dNTPs	0.8
primer 5F	0.1
primer 6R	0.1
Taq DNA polymerase	2
DNA template	1

Program condition: -

Step	Temperature	Time	No. of cycles
Denaturation	95°C	3min	1
	95°C	30sec	
Annealing	37°C	60sec	30
Extension	72°C	60sec	
Final Extension	72°C	7min	1
	4°C	Hold	

RESULTS

4.1 Localization of superoxide dismutase enzyme activity

Pseudomonas aeruginosae was grown on Luria broth (LB) and superoxide dismutase (SOD) activity was determined at various time intervals in both extracellular and intracellular fractions. The growth of *Pseudomonas aeruginosae* reached the stationary phase at 14hrs and continued till 54hrs. Superoxide dismutase activity increased in both extracellular and intracellular as growth progressed and was optimum at 20hrs and then steadily declined. On comparing these activities, it was observed that higher activity was seen in case of extracellular fraction as compared to intracellular fraction (Figure 1).

4.2 Effect of p-nitrophenol on SOD activity

Pseudomonas aeruginosae used for the production of superoxide dismutase was grown in basal media (Bushnell Haas Broth, BHB) with and without p nitrophenol (pNP, 50ppm). The carbon source used in basal media was 1%(w/v) glucose. The optimum temperature used for growth of the cells was 37°C. The SOD activity and growth was monitored at different time intervals in both extracellular and intracellular fractions. The growth of *P. aeruginosae* in basal medium containing 50ppm pNP was maximum at 14hrs and then it remained almost stationary. The SOD activity in extracellular fraction increased with time till 6hr in medium without pNP, and 16 hr in medium with pNP (50ppm). Then a gradual decline in SOD activity was observed till 46 hrs. of growth. Further, it remained stable. The maximum SOD activity was achieved when bacterium entered in the stationary phase. In extracellular fractions, the SOD activity was found to be higher in media containing pNP (Figure 2), where as, in intracellular fraction almost the same SOD activity was exhibited in media with and without pNP. The maximum SOD activity was found between 12-18 hours of growth in basal media without pNP. While between 24-30 hours of incubation in media containing pNP.

Thus, an initial lag in intracellular fraction was observed in media containing pNP (Figure 3).

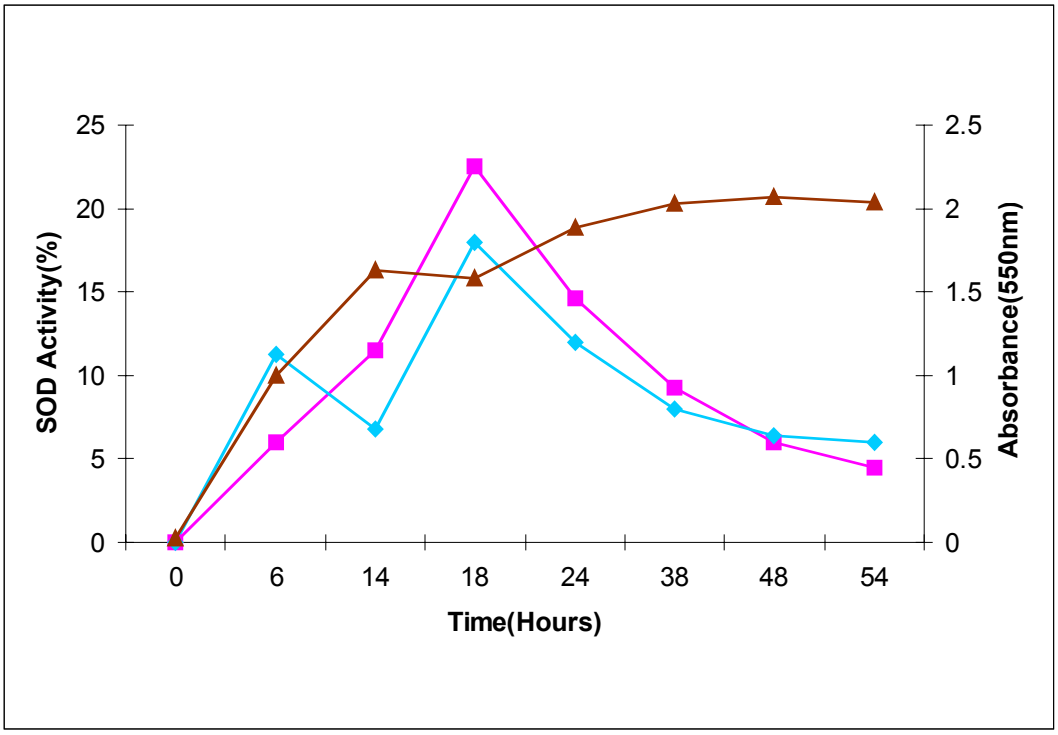


Figure 1: *Pseudomonas aeruginosae* was grown on Luria broth and growth was monitored at 550nm. Superoxide dismutase (SOD) activity was determined as described in section 3.3 in both extracellular and intracellular fraction.

Growth

SOD Activity (%) Extracellular

SOD Activity (%) Intracellular

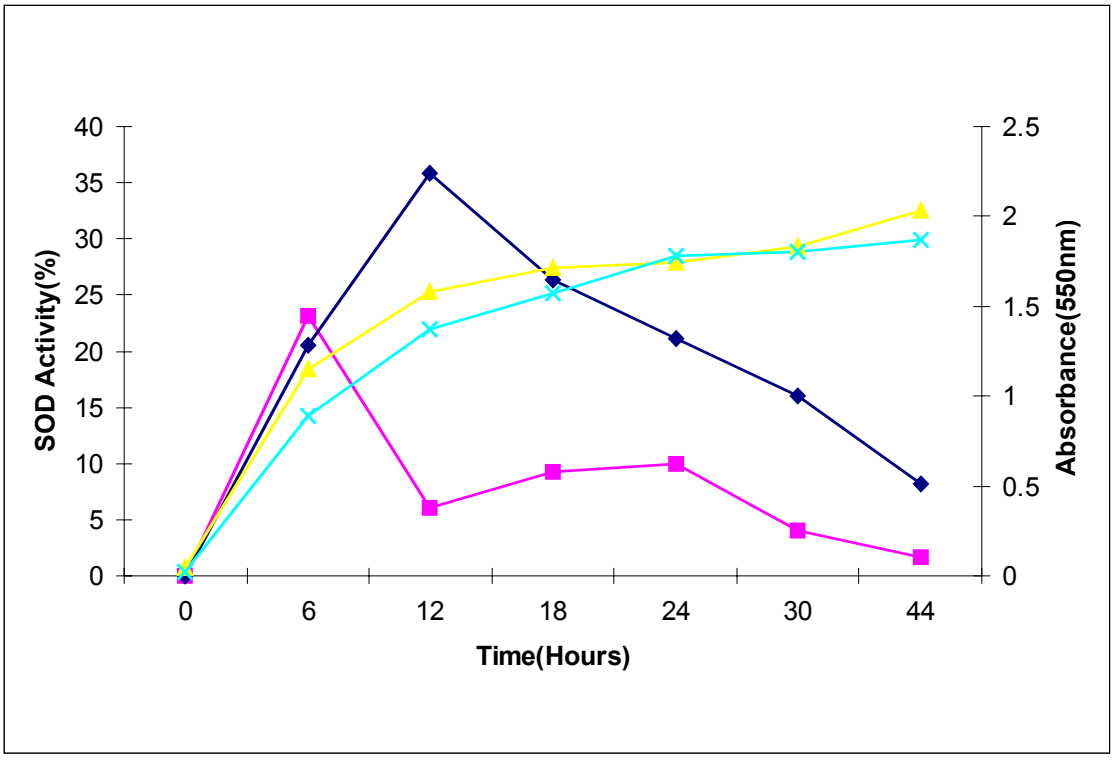


Figure 2: *Pseudomonas aeruginosae* was grown on Bushnell Hass Broth (BHB) with and without p nitrophenol, pNP (50ppm). Growth (OD 550nm) and SOD activity in extracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 50ppm pNP

SOD Activity (%)

SOD Activity (%) with 50ppm pNP

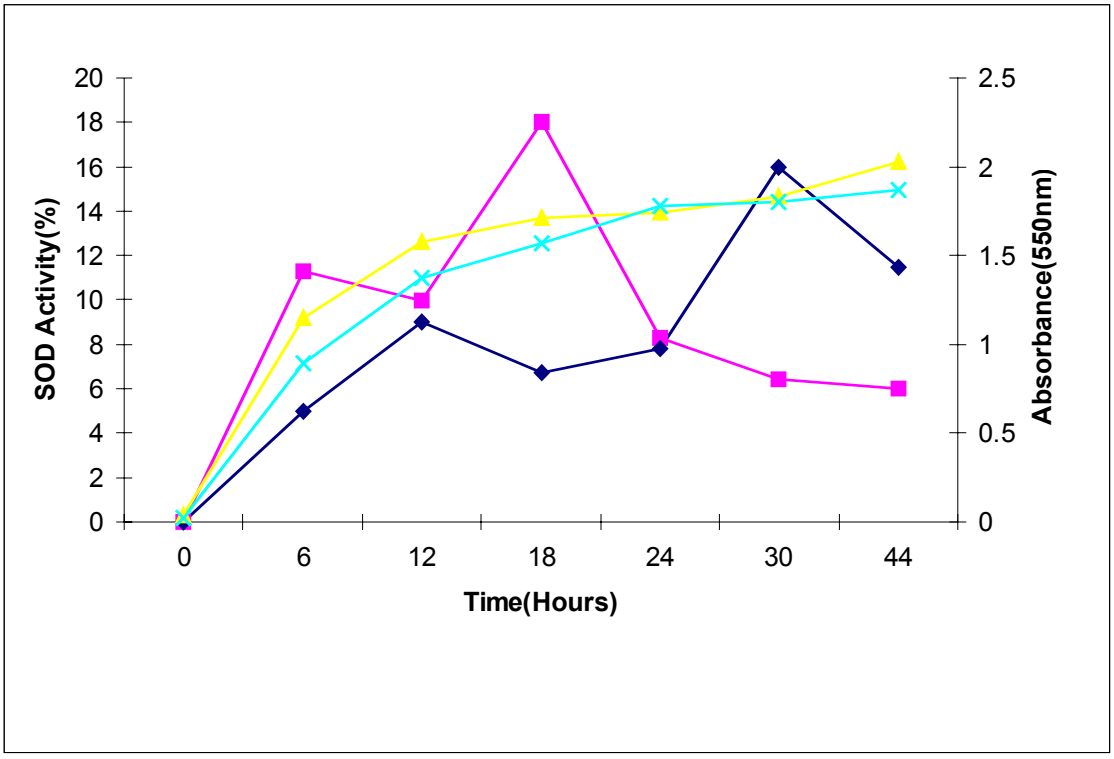


Figure 3: *Pseudomonas aeruginosae* was grown on Bushnell Hass Broth, BHB with and without p-nitrophenol (pNP, 50ppm). Growth (OD 550nm) and SOD activity in intracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 50ppm pNP

SOD Activity (%)

SOD Activity (%) with 50ppm pNP

4.3 Effect of pyrene on SOD activity

The effect of pyrene on the SOD activity of two bacterial isolate A15 and A19, capable of degrading 50ppm of pyrene as a sole carbon source was studied by its growth in BHB (with and without pyrene both containing 1% (w/v) glucose). The bacterial isolate A15 when grown in basal media containing pyrene (50ppm) exhibited exponential growth pattern for first 36 hrs and then remained stationary. whereas, when isolate A15 was grown in basal medium without pyrene, stationary phase was achieved within 24 hrs of growth and biphasic SOD activity was exhibited in extracellular fraction. In this, SOD activity increased exponentially within the first 6 hrs and then declined and again increased up till 24 hrs of growth. Then a gradual decline in SOD activity was observed till 54hrs of growth. In extracellular fraction of both bacterial isolate A15 and A19, rapid enhancement in SOD activity was observed in medium containing pyrene 50ppm) as compared to medium without pyrene (Figure 4 and Figure 6) and maximum SOD activity was obtained in 18 hr in both isolates, whereas, only bacterial isolate A19 exhibited higher SOD activity in medium containing pyrene in intracellular fraction (Figure7). Isolate A15 showed gradual rise in SOD activity till 36hrs in medium without pyrene (Figure 5).

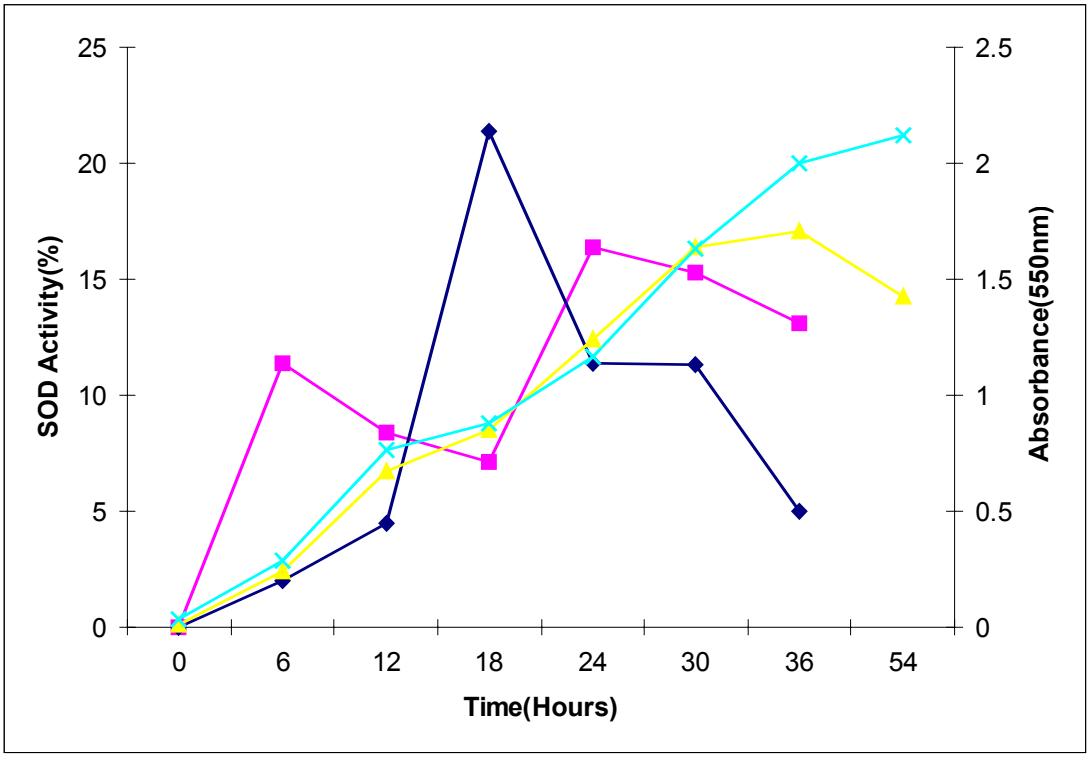


Figure 4: Bacterial isolate A15 capable of degrading pyrene as a sole carbon source was grown on BHB (1% (w/v) glucose) with and without pyrene (50ppm). Growth (OD 550nm) and SOD activity in extracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 50ppm pyrene

SOD Activity (%)

SOD Activity (%) with 50ppm pyrene

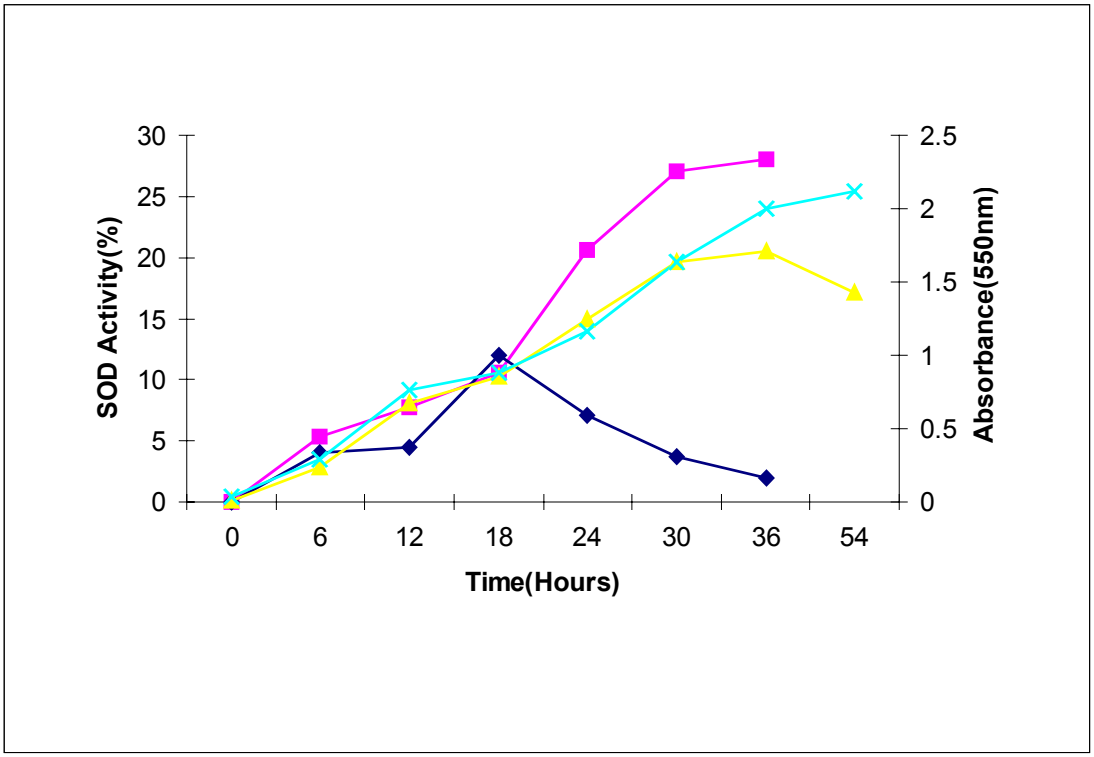


Figure 5: Bacterial isolate A15 capable of degrading pyrene as a sole carbon source was grown on BHB (1% (w/v) glucose) with and without pyrene (50ppm). Growth (550nm) and SOD activity in intracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

---▲--- Growth

---✕--- Growth with 50ppm pyrene

---■--- SOD Activity (%)

---◆--- SOD Activity (%) with 50ppm pyrene

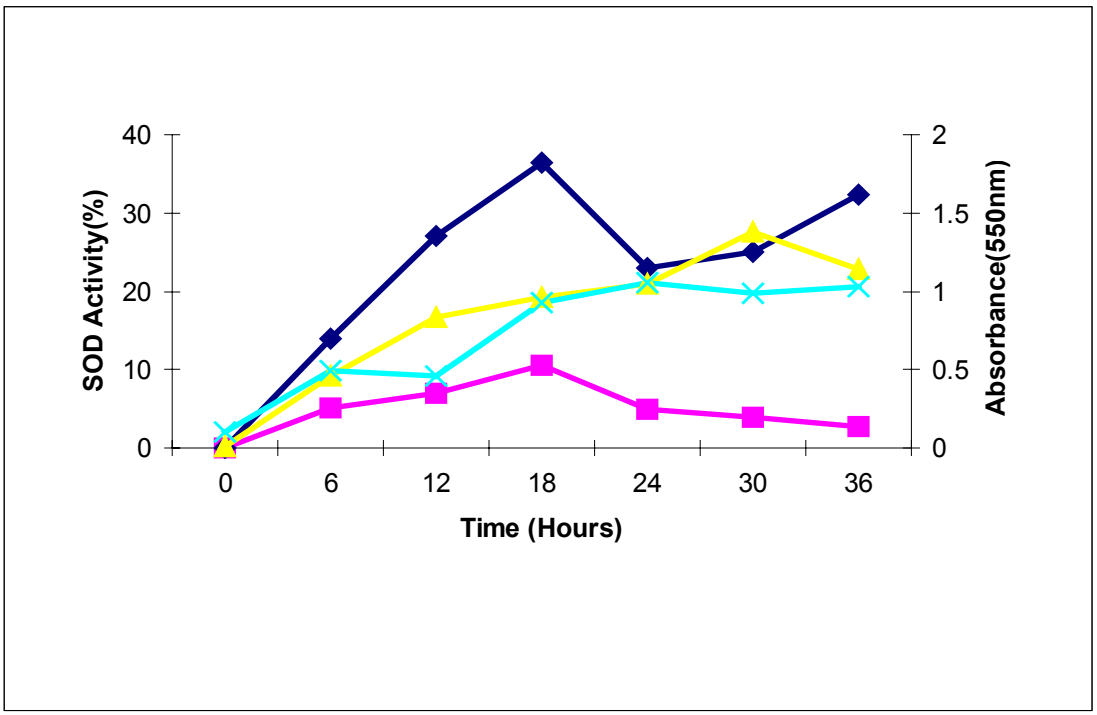


Figure 6: Bacterial isolate A19 capable of degrading pyrene as a sole carbon source was grown on BHB (1% (w/v) glucose) with and without pyrene (50ppm). Growth (550nm) and SOD activity in extracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 50ppm pyrene

SOD Activity (%)

SOD Activity (%) with 50ppm pyrene

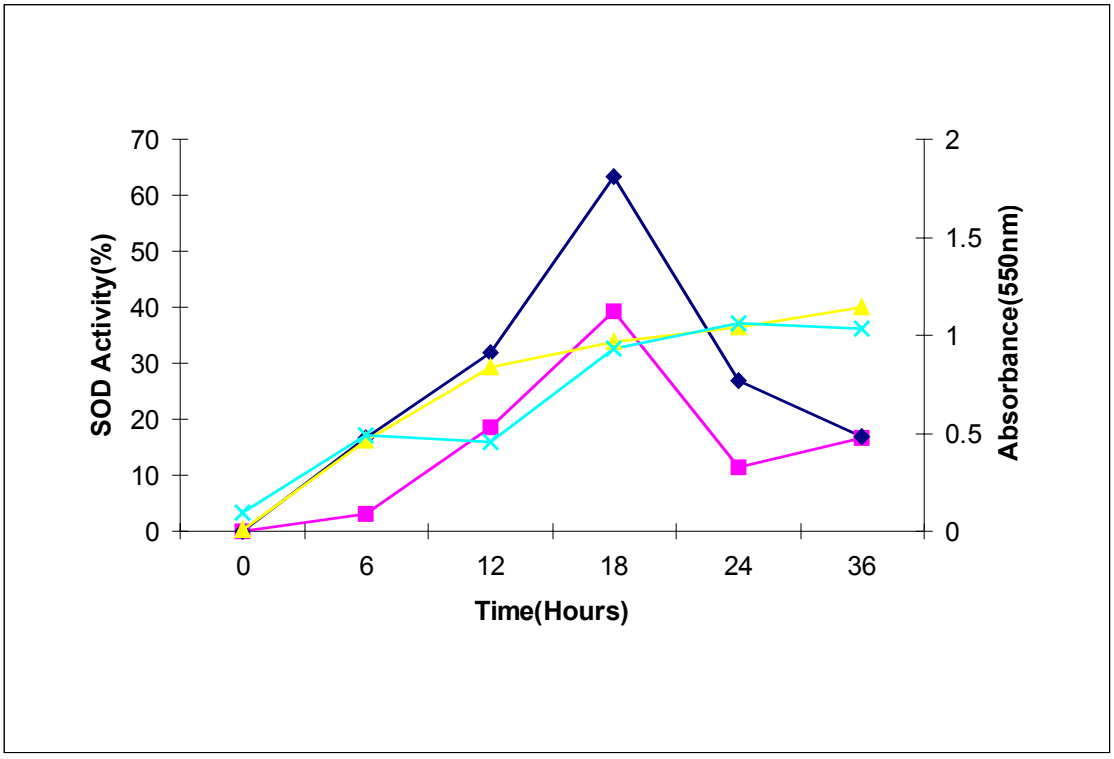


Figure 7: Bacterial isolate A19 capable of degrading pyrene as a sole carbon source was grown on BHB (1% (w/v) glucose) with and without pyrene (50ppm). Growth (550nm) and SOD activity in intracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 50ppm pyrene

SOD Activity (%)

SOD Activity (%) with 50ppm pyrene

4.4 Effect of lead metal on SOD activity

The effect of lead (Pb) on SOD activity was studied in two bacterial isolate G314 and G306, tolerant to 100ppm lead. The studies were carried out in BHB with and without lead (100ppm), containing 1% (w/v) glucose. Bacterial isolate G314 exhibited very less growth in basal medium containing Pb as compared to basal medium without Pb. But SOD activity increased to almost double in both extracellular and intracellular fractions in cells grown with lead. When isolate G306 was grown in basal medium with Pb, again the growth was comparatively lower than in the medium without Pb, but, was higher when compared to growth of isolate G314. In isolate G306, SOD activity showed rapid rise till 34 hr in case of extracellular fraction. In intracellular fraction SOD activity increased with time till 10 hr. Then a gradual decline in SOD activity was observed till 34 hrs of growth. The SOD activity in extracellular fraction showed higher SOD activity in medium containing lead (100ppm) in both isolates G314 and G306 (Figure8 and 11) whereas, only G314 exhibited higher SOD activity in intracellular fraction in medium with lead (100ppm) as compared to intracellular fraction of G306 (Figure 9 and Figure11).

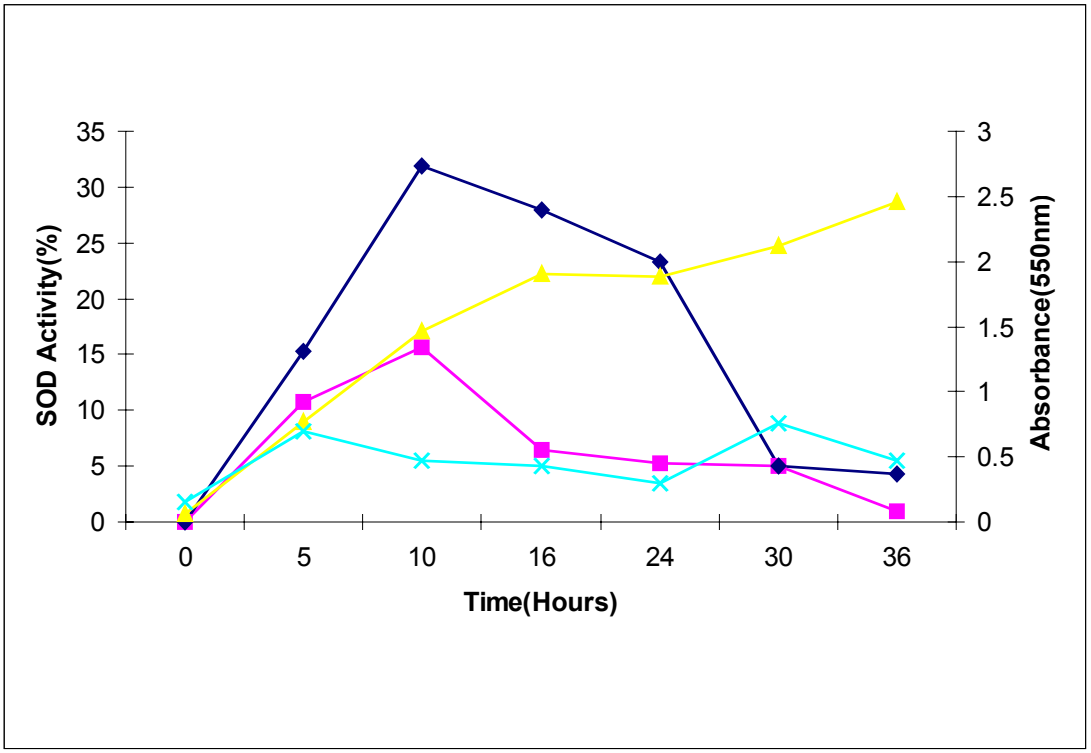


Figure 8: Bacterial isolate G314 tolerant to metal, lead (Pb) was grown on BHB (1% (w/v) glucose) with and without Pb (100ppm). Growth (OD 550nm) and SOD activity in extracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 100ppm lead

SOD Activity (%)

SOD Activity (%) with 100ppm lead

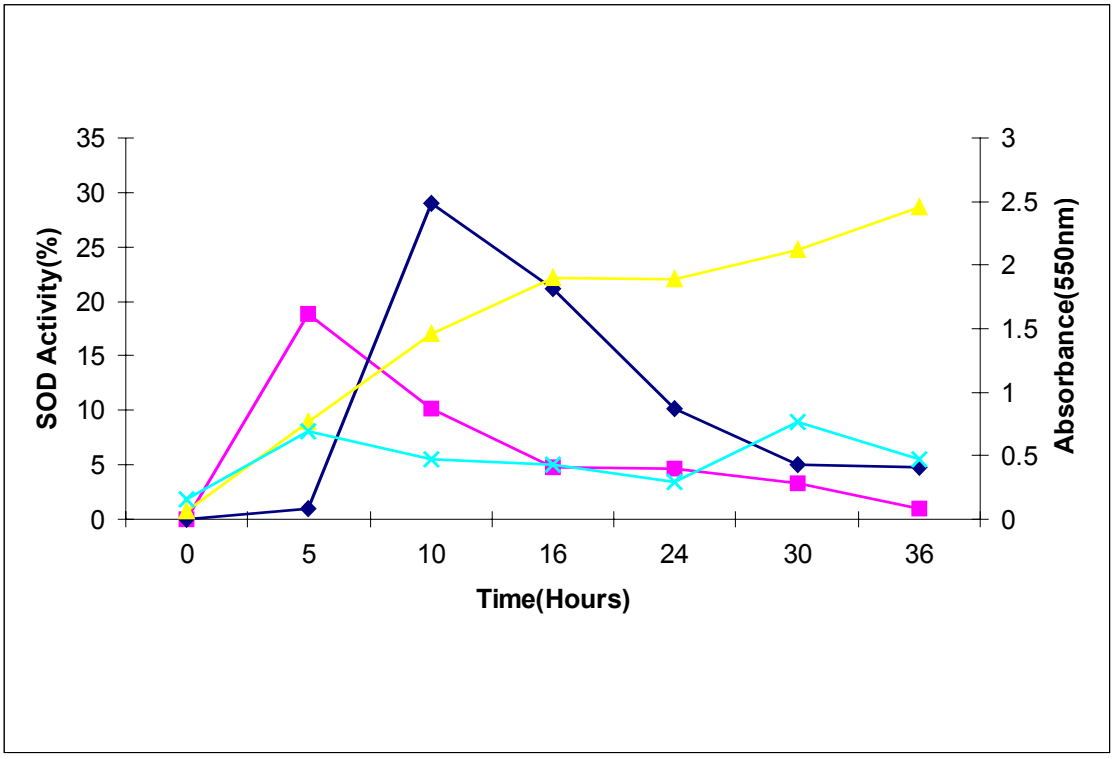


Figure 9: Bacterial isolate G314 tolerant to metal, lead (Pb) was grown on BHB (1% (w/v)glucose) with and without Pb (100ppm). Growth (OD 550nm) and SOD activity in intracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 100ppm lead

SOD Activity (%)

SOD Activity (%) with 100ppm lead

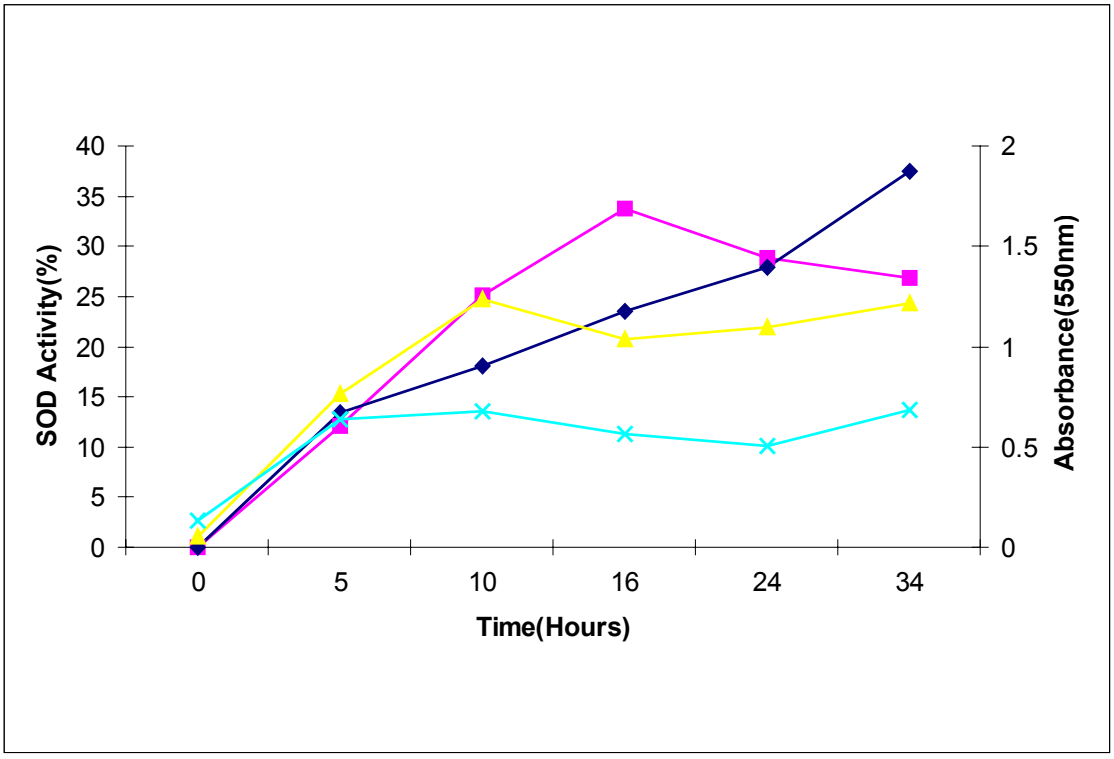


Figure 10: Bacterial isolate G306 tolerant to metal, lead (Pb) was grown on BHB (1% (w/v) glucose) with and without Pb (100ppm). Growth (OD 550nm) and SOD activity in extracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 100ppm lead

SOD Activity (%)

SOD Activity (%) with 100ppm lead

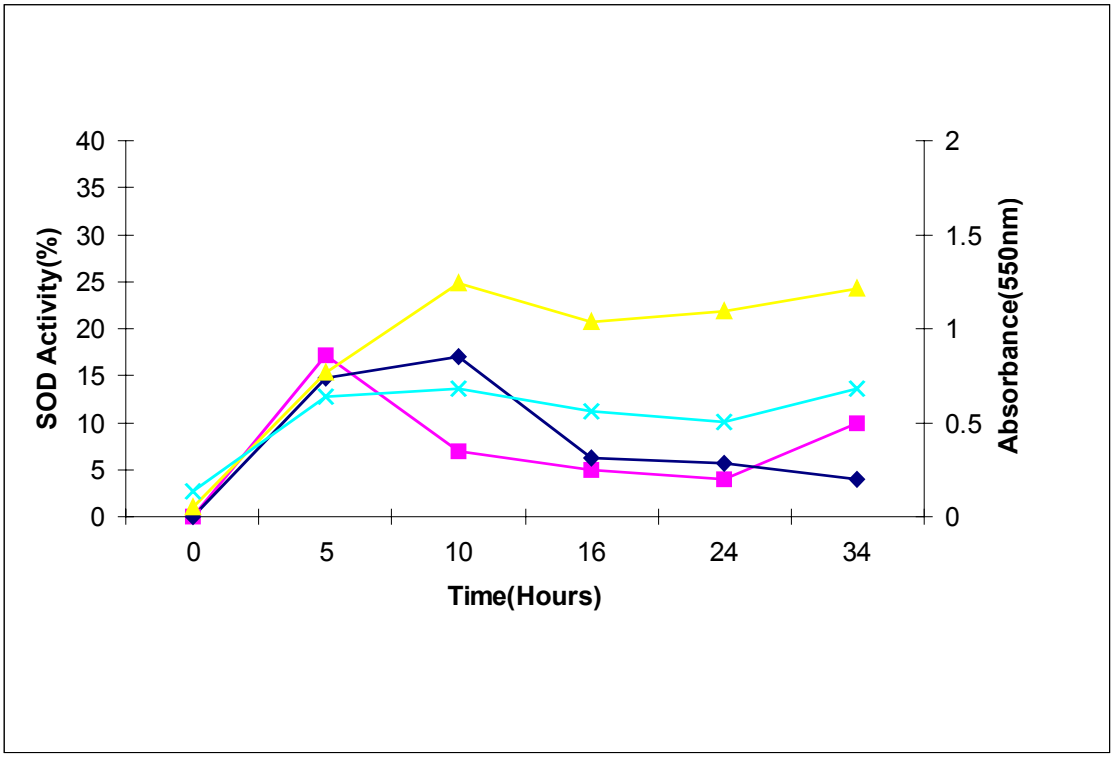


Figure 11: Bacterial isolate G306 tolerant to metal, lead (Pb) was grown on BHB (1% (w/v) glucose) with and without Pb (100ppm). Growth (OD 550nm) and SOD activity in intracellular fraction was measured at different time intervals as described in section 3.2 and 3.3 respectively.

Growth

Growth with 100ppm lead

SOD Activity (%)

SOD Activity (%) with 100ppm lead

4.5 Molecular characterization studies

Molecular characterization was carried out using different sets of primers. These primers were found to amplify SOD gene in different bacterial systems. Two sets of SOD primer I obtained were derived from *Pseudomoas aeruginosae* origin, which on amplification gives 430bp and 320bp (Mn SOD) product respectively. The other set of primer II were from Streptomyces *spp*. from which, a single DNA fragment corresponding to the expected 480-bp amplification product (Mn SOD) was expected.

Primer sequences : -

Primer I

Forward Primer-23bp

F-CCTGCATTGCCATACGCCTACGA-3'

Reverse Primer-24bp

R-ATGACATCATTCCGACCACCATCC-3'

(S. Heim *et.al*., 2003)

Primer II

Forward Primer-39bp

F5'-GCGGGATCCAAGCTTAGGCGTGTTGGACCGTTTCCAAGA-3'

Reverse Primer-36bp

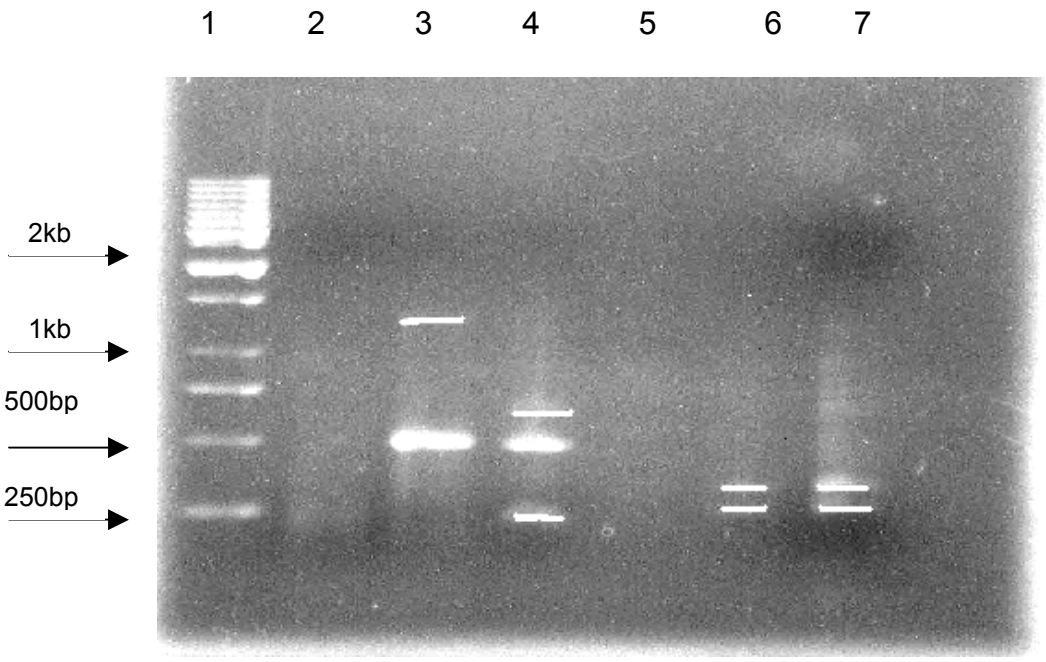
R5'-ACGCGTCGACGAATTCCTTTCTTATTTAGCGGCTTG-3'

(Claire Poyart *et.al*., Jan. 2000)

PCR amplification (primer I)

The PCR amplification using the primer I were carried out for all bacterial isolates and were subjected to agrose gel electrophoresis , which resulted in following pattern as in Figure 12. Out of six bacterial isolates (A4, A15, A19, G306, G314, *P. aeruginosae*) , four isolates showed amplification. Isolate A15 gave two bands of approximately 1.5kb and 480bp, whereas, isolate A19 resulted into three bands of approximately 600bp, 480bp and 250bp. Isolate G314 and P.

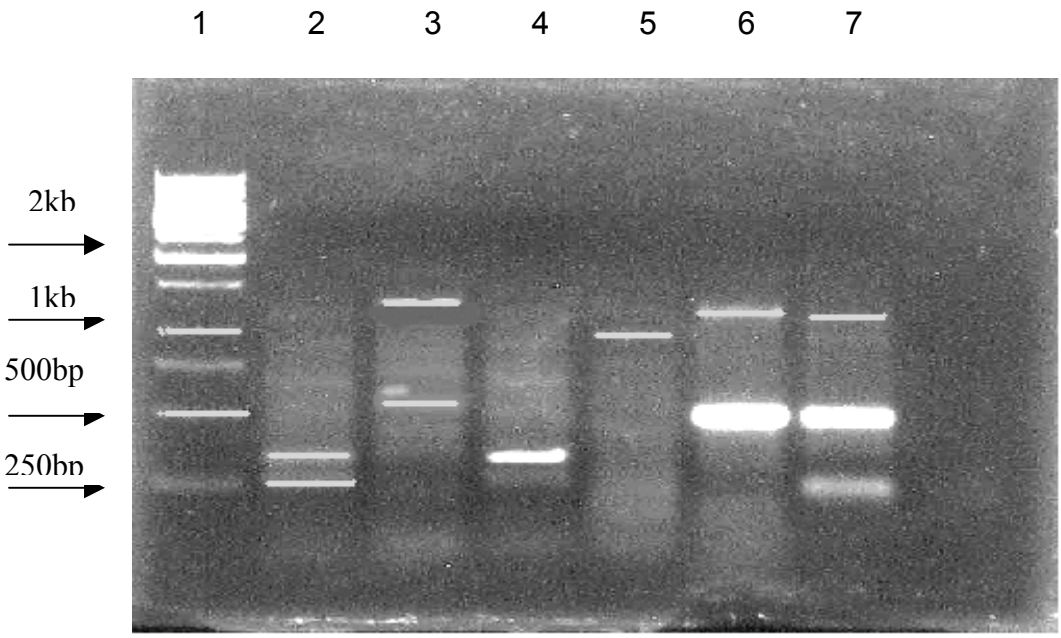
aeruginosae gave similar band pattern with bands of 430bp and 320bp as expected.



Lane 1 : 1Kb ladder Lane 2 : Isolate A4 Lane 3 : Isolate A15
Lane 4 : Isolate A19 Lane 5 : Isolate G306 Lane 6 : Isolate G314
Lane 7 : *P.aeruginosae*

PCR amplification (primer II)

The PCR amplification with primer set II resulted into a different pattern, but in this all six isolates gave amplification product. The band pattern obtained resulted into two bands each of isolate A4 (approx. 250bp& 350bp),A15 (approx. 500bp & 1.2Kb), G314 (approx. 480bp & 1Kb) and *P. aeruginosae* (approx. 480bp & 1Kb) ; and single band for isolate A19 (approx. 300bp) and G306 (approx. 800bp).



Lane 1 : 1Kb ladder Lane 2 : Isolate A4 Lane 3 : Isolate A15

Lane 4 : Isolate A19 Lane 5 : Isolate G306 Lane 6 : Isolate G314

Lane 7 : *P.aeruginosae*

DISCUSSION

Ever since the introduction of molecular oxygen into our atmosphere, by O_2 evolving photosynthetic organisms, about two billion years ago, reactive oxygen intermediates (ROI) have been the unwelcome companions of aerobic metabolism. In contrast to O_2, these partially reduced or activated derivatives of oxygen (O2, O_2^-, H_2O_2, and OH^-) are highly reactive and toxic, and can lead to the oxidative destruction of cells. Both environmental chemistry and metabolic biochemical reactions can constantly generate in vivo free radicals and other oxygen-derived species that can cause severe damage to almost all biomolecules, especially to DNA, proteins, and lipids. The superoxide anion has been shown to be the most readily generated and spread radicle among organism and it is a common intermediate of oxidative stress processes in the cells. The antioxidant defense system of superoxide dismutases (SOD) scavenges and minimizes the formation of this radical, and thus plays a major role in reducing cumulative oxidative damage in different cell compartments both in aerobic and anaerobic cells. In the cell, the cytosol SODs are constitutively present and induced by many oxidative agents able to raise the superoxide concentrations. Presence of SODs, however, in extracellular cell-associated locations demonstrates how valuable they are in maintaining the integrity of cells against oxidative stress generated by the cell environment, particularly upon increased oxygenation. Because SODs have recently been found in Archae, which are prokaryotes, sometimes living in extreme environments, even in anaerobic ones, these enzymes can be considered essential: they may have allowed the evolution of aerobic respiration starting from an ancient from of oxygen insensitive life.

In aerobic organisms molecular oxygen in unreactive but is capable of giving rise to lethal excited state free radicals and derivatives. Utilization of oxygen proceeds most readily via a complete stepwise four-electron reduction to water

during which partially reduced reactive intermediates is generated. In higher plants this energy is readily obtained from light quantas via such transfer of molecules. All these activated oxygen species are extremely reactive and cytotoxic to all organism. Reactive oxygen species can result from various environment stimuli such as UV light radiation, herbicide to various other stresses, which are known to induce free radical formation.

Through selective pressure and evolution numerous defense mechanism both enzymatic and non-enzymatic have emerged to protect cells from oxidative injury. One of the most important enzymatic defense is superoxide dismutase that scavenges the hydroxyl anion with mixed action with catalase. They convert potentially dangerous superoxide radical to water and oxygen averting cellular damage.

The previous study in lab suggests the need to observe various microbes, which are capable of growing on a variety of difficult to degrade chemical molecules. Thus, research was carried out to study defence mechanism developed similar to oxidative stress in microbes exposed to recalcitrant chemicals. In present studies, it was found that the SOD protein was constitutive in all the five isolates (P.aeruginosae, A15, A19, G306, G314) studied as was also observed by Bradley et.al.,1992) in fungi. Growth pattern studies when carried out in basal media with and without stress It was observed that there was rapid enhancement in SOD activity on minimal media with stress conditions, particularly in extracellular fraction. This indicates that in the presence of pNP, pyrene or metal lead the cells generated oxy-intermediates and this leads to the increase in SOD activity. During the course of studies on superoxide dismutase in *P. aeruginosae,* 50% increase in SOD activity was observed in extracellular fraction, whereas no noticeable enhancement was observed in intracellular fraction. An initial lag period in SOD activity was observed during pNP stress.

The addition of pyrene elicits the enhanced activity of the enzyme superoxide dismutase in both the bacterial isolates A15 and A19, capable of degrading pyrene as sole carbon source. The isolate A19 showed four fold enhancement in extracellular SOD activity as compared to two fold increase in isolate A15. The isolate A19 exhibited maximum SOD activity (70%) in all the five isolates studied. This shows that the response to pyrene addition might involve an enhanced metabolism under these conditions, which had definitely led to rapid production of oxyanions and thus increased in levels of SOD to scavange those oxyanions.

Similar studies, when carried out in metal (lead) tolerant bacterial isolate G306 and G314 exhibited increased SOD activity in both extracellular and intracellular fractions. Almost, two fold increased in SOD activity was observed in isolate G314, whereas SOD activity levels was exhibiting continuous rise during in isolate G306 the course of studies.

At this point, a direct relationship between growth on complex substrates and superoxide dismutase inductions could not be established. Our observation that presence of various stress causing agents in the environment elicits the enhanced activity of the enzyme whose functions is to scavange oxygen free radicals demonstrates as association between environmental and oxidative stress. Similar correlations between SOD activity enhancement and elevation of temperature have been observed in *E.coli*. (Privalle and Fridovich, 1987) and mammalian cells (Loven *et.al.,* 1985). Further, molecular characterization studies were carried out using two sets of primer, which were from different origin. When PCR amplification was carried out using primer set I (*Pseudomonas aeruginosae* origin) isolates A15, A19, G314 and *P. aeruginosae* could be amplified except A4 and G306. Pyrene degrading isolates A15 and A19 showed slightly a similar band pattern, a single band of approximately of 480bp, whereas isolate G314 and *P.aeruginosae* exhibited similar band amplification of 430bp and 320bp. Isolate A15 gave two bands of approximately 1.5kb and 480bp, whereas, isolate

A19 resulted into three bands of approximately 600bp, 480bp and 250bp. The primer set II (*Streptomyces spp.* origin) also showed amplification of SOD gene in all isolates. In this, all gave different band pattern but, isolate G314 and P. aeruginosae exhibited quite a similar band pattern, two bands of 1kb and 480bp. Thus, it can be concluded that isolate G314 and *P. aeruginosae*. The two bands each in isolate A4 of approx. 250bp& 350bp and of approx. 500bp & 1.2Kb were obtained in A15 respectively. Thus further, identification of the isolates needs to be carried out and their phylogenetic history known. Also, studies should be carried out to ascertain if the expression of the various forms of SOD is governed by the type of environmental stress which the microbes is exposed to or their exists different types of SOD in the same microbe.

SUMMARY

1. SOD activity was observed in both the extracellular and intracellular fractions with and without stress and SOD enzyme was a constitutive protein.
2. *Pseudomonas aeruginosae* exhibited 50% increase in SOD activity in extracellular fraction when grown in medium containing 50ppm p nitrophenol.
3. Bacterial isolates A15 and A19, capable of growing solely on pyrene, showed enhanced SOD activity on addition of 50ppm pyrene. Isolate A19 showed four fold enhancement in extracellular SOD activity as compared to two fold increase in isolate A15.
4. Isolate A19 exhibited maximum SOD activity (70%) among all the five isolates studied.
5. Lead tolerant bacterial isolate G314 showed a two fold increase in SOD activity, while continuous rise in SOD activity was seen in isolate G306 during growth when both were grown in the presence of 100 ppm of lead.
6. The PCR amplification using the primer I (*Pseudomonas spp*.) showed amplification in four isolates. The band pattern obtained resulted into two bands of approximately 1.5kb and 480bp in A15, whereas, isolate A19 resulted into three bands of approximately 600bp, 480bp and 250bp. Isolate G314 and *P. aeruginosae* gave similar band pattern with bands of 430bp and 320bp.
7. The PCR amplification with primer set II (*Streptomyces spp*.) resulted into two bands of 250bp & 350bp in A4, 500bp & 1.2kb in A15, and 480bp & 1kb in G314 and P. aeruginosae respectively. A single band was obtained in case of isolate A19 (300bp) and G306 (800bp).

APPENDIX

Standard solutions

All the solutions and buffers were prepared in distilled water at room temperature and stored it 4°C.

Bovine serum albumin (1mg/ml)

BSA	50mg
Distilled water	50ml

Para nitro phenol (50 ppm)

PNP	0.001gm
Distilled water	10ml

Tris-HCl Buffer (pH=7.0—7.5)

Desired amount of tris was weighed and half amount of distilled water (of final volume) was adde Then pH was adjusted with dilute HCl and final volume was made up with distilled water.

Potassium phosphate Buffer

KH_2PO_4 (0.2mM)	16ml
K_2HPO_4 (0.2mM)	84ml
Distilled water	100ml

Biuret Reagent

NaOH	30%
CuSO4.5H2O	0.21%

Dissolved in ice bath and cupric sulphate added slowly with continuous stirring. Made up the final volume to 100ml. Reagent discarded if a blackish or reddish brown precipitate persisted.

Nitroblue tetrazolium (58mM)

NBT	14.5mg
Sterile Distilled water	10ml

Note : Store in dark coloured brown bottle.

Riboflavin (2.4mM)

Riboflavin	150mg
Sterile DW	100ml

Note : Store in dark coloured brown bottle at 4°C.

Methionine (9.9mM)

Methionine	150mg
Sterile DW	5ml

TE (Tris-EDTA) Buffer

Tris-Cl (pH=8)	10mM
EDTA (pH=8)	1mM

1X TAE (Tris Acetate EDTA) Buffer

Tris acetate	0.04M
EDTA	0.001M
pH = 8.0	

Reaction mixture (SOD assay)

Phosphate buffer	27.0 ml
NBT	1.0 ml
Methionine	1.5 ml
Triton X 100 (1%)	0.75 ml

5X TBE (Tris borate EDTA)

Tris Base	54gm
Boric acid	27.5gm
EDTA (0.05M)	20ml

pH= 8.0

Final volume is made upto 1Litre.

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