CHAPTER 4

The role of CatalaseA in DTT mediated thiol stress

4.1. Introduction

All the aerobic organisms posses the mechanism to cope up with potentially dangerous reactive oxygen species (ROS) like O_2^-, H_2O_2 and $^{\cdot}OH$, which are the by-products of incomplete reduction of molecular O_2 (Cadenas, 1989; Morgan et al., 1986; Halliwell, 1990). In bacteria, these oxidants can be either the products of their own metabolic redox reactions (Gonzalez-Flecha and Demple, 1995) or alternatively it can be from an external source such as host macrophage as in case of intracellular pathogenic bacteria such as *M. tuberculosis* (Chan et al., 1992; Hassett and Cohen, 1989). Both prokaryotic and eukaryotic cells are equipped with inducible defense systems that counteract oxidative damage. Extensive work has been done in *E. coli* and *Salmonella typhimurium,* on the mechanisms by which Gram negative organisms receive and respond to oxidative stress signals (Farr and Kogoma, 1991). However, there is very little information available on Gram positive bacteria in this regard.

The oxidative response of *Streptomyces coelicolor* has been studied to some extent. It produces two kinds of superoxide dismutases, whose levels are relatively high to effectively counteract the harmful effects of superoxide anion (Kim et al., 1998, a and b). It also produces multiple catalases (Kim et al., 1994) encoded by *catA*, *catB*, and *catC* genes (Cho and Roe, 1997; Cho et al., 2000; Hahn et al., 2000). *CatA* is the major vegetative catalase expressed during log phase and is inducible by H_2O_2. CatB is expressed during stationary phase and induced in response to osmotic stress. CatC is expressed transiently at late exponential to early stationary phase.

Although the oxidative stress is one of the most encountered stresses and its response is widely studied, induction of catalase by thiols due to reductive stress as observed in Chapter 3 cannot be ruled out. In eukaryotes thiols were reported to cause reductive stress (Ghyczy et al., 2003). In certain eukaryotic systems thiols were shown to induce the expression of catalase, Mn-superoxide dismutase (Morrison et al., 2005) and monooxygenase (Suh and Robertus, 2000). The

mechanism for this induction could be due to auto-oxidation of thiols to generate $O_2^{\cdot-}$. Secondly, they reduce disulphide bonds in proteins, accumulating unfolded proteins which in turn give a stress signal to the cell causing expression of stress responsive proteins. This is referred to as unfolded protein response (UPR) (Sidrauski et al., 1996).

Thiol containing compounds are generally good antioxidants and protect thiol groups from oxidation. Although, in the presence of metals and oxygen, DTT may induce free radicals and cause damage to proteins, lipids and DNA (Kachur et al., 1997). Through Fenton reaction, Fe^{3+} catalyzes the oxidation of DTT and is reduced to Fe^{2+} which can directly reduce the H_2O_2 to generate the free radical $^{\cdot}OH$. This reaction is the rate-limiting step, depending on the availability of reductants like reduced flavins, cysteine and other intracellular thiols and with ample supply of the above group of compounds under reductive stress may initiate a chain of free radical reactions (Netto and Stadtman, 1996). H_2O_2 as such is not much harmful but thiols and other reducing agents like flavins and cysteine have been shown to promote DNA damage by generating $^{\cdot}OH$ radicals from H_2O_2 through Fenton reaction (Woodmansee and Imlay, 2002; Park and Imlay, 2003).

4.2. Materials and Methods

4.2.1. Materials

P (protoplast) buffer: Sucrose (103 g), K_2SO_4 (0.25 g), $MgCl_2.6H_2O$ (2.02 g), trace element solution (2 ml), Distilled water to 800 ml. Dispense the above solution in 60 ml aliquots and autoclave. Before use, add to each flask in order 0.5% KH_2PO_4 (1 ml), 3.68% $CaCl_2.2H_2O$ (10 ml), 5.73% TES buffer, pH 7.2 (10 ml).

T (transformation) buffer: Mix the sterile solutions of 10.3% sucrose (25 ml), distilled water (75 ml), trace element solution (0.2 ml), 2.5% K_2SO_4 (1 ml). To 9.3

ml of this solution add 5 M $CaCl_2$ (0.2 ml), Tris-maleic acid buffer, pH 8.0 (0.5 ml). For use, add 3 parts by volume of this solution to 1 part by weight of PEG 1000, previously sterilized by autoclaving.

4.2.2. Catalase activity assay

For enzyme activity assays, *S. coelicolor* mycelia were collected from 1 ml culture by centrifugation and cells were lysed using 1 ml of lysis buffer (20 mM Tris, pH 7.5, 100 mM $MgCl_2$, 1 mg/ml lysozyme and 1 µM of PMSF). Cell debris was removed by centrifugation (12,000xg for 30 minutes) and supernatant was used for activity assays. Catalase activity was assayed by monitoring the degradation of H_2O_2 at 240 nm every 10 seconds for 2 minutes (Aebi, 1984). 1 ml of 10 mM H_2O_2 solution in 50mM potassium phosphate buffer, pH 7.0 was used as assay solution and 5-50 µl of cell lysate was added. Catalase specific activity was expressed as µmoles of H_2O_2 degraded per min per mg protein. Protein concentration was determined using Folin Lowry method (Lowry et al., 1951).

4.2.3. Superoxide dismutase activity (SOD) assay

For SOD activity assay, 100 to 200 µl supernatant was added in a quartz cuvette containing 750 to 850 µl of 0.2 mM potassium phosphate buffer, pH 8.0 followed by 50 µl of 0.5 mg/ml of pyrogallol. Auto-oxidation of pyrogallol was monitored by recording the absorbance at 420 nm every 10 sec for 3 minutes. Specific activity of SOD was estimated by units of enzyme required for the 50% inhibition of auto-oxidation of pyrogallol per mg protein (Marklund and Marklund, 1974).

4.2.4. Effect of *o*-phenanthroline on catalase induction

Cultures of *S. coelicolor* in mid-log phase were incubated with 1mM *o*-phenanthroline for 30minutes prior to DTT stress and catalase activity was assayed as earlier to estimate the level of induction. The direct effect of *o*-phenanthroline on catalase enzyme was estimated *in vitro* by adding 1mM *o*-phenanthroline to lysate prior to catalase assay.

4.2.5. Genomic DNA isolation from *Streptomyces coelicolor* (Hopwood et al., 1985)

Mycelium (1g/5 ml) was suspended in TE buffer, pH 8.0 and 2mg/ml lysozyme was added after washing with 10.3% sucrose. The culture was incubated at 30° C, triturated at every 15 min, until a drop of suspension on a microscopic slide is completely cleared by addition of a drop of 10% SDS followed by the addition of 1.2 ml of 0.5 M EDTA (i.e. to 0.1 M). The tubes were mixed gently and incubated at 30° C for 5 min. SDS was added to 1% final concentration and the centrifuge tubes were tilted immediately and incubated at 37 °C for up to 2 hours. Tris-saturated phenol (6 ml) was added and mixed thoroughly at RT (Room temperature) followed by addition of 6 ml of chloroform and mixed for 5 minutes at RT. Two phases were separated by centrifuging at 3000 rpm for 10 min. The aqueous phase was recovered carefully using a 1 ml cut tip and re-extracted with phenol: chloroform (1:1) as above followed by two extractions with equal volume of chloroform. DNA was precipitated by adding 1/10 volume of 5 M sodium acetate and equal volume of iso-propanol and washed with 70% ethanol; air dried and resuspended in TE.

4.2.6. Insertional inactivation of CatalaseA

In order to mutate *catA*, mutagenesis was performed through insertional inactivation of *catA* gene. The cloning was performed in the integration vector pSET152 (Figure 4.1). Plasmid pSET152 contains *att* site and the *int* gene encoding the enzyme integrase, which assist in the integration of the vector into the genome through *att* site. To develop catalaseA mutant it is necessary to prevent integration through the *att* site and allow integration through homologous recombination only (Figure 4.2).

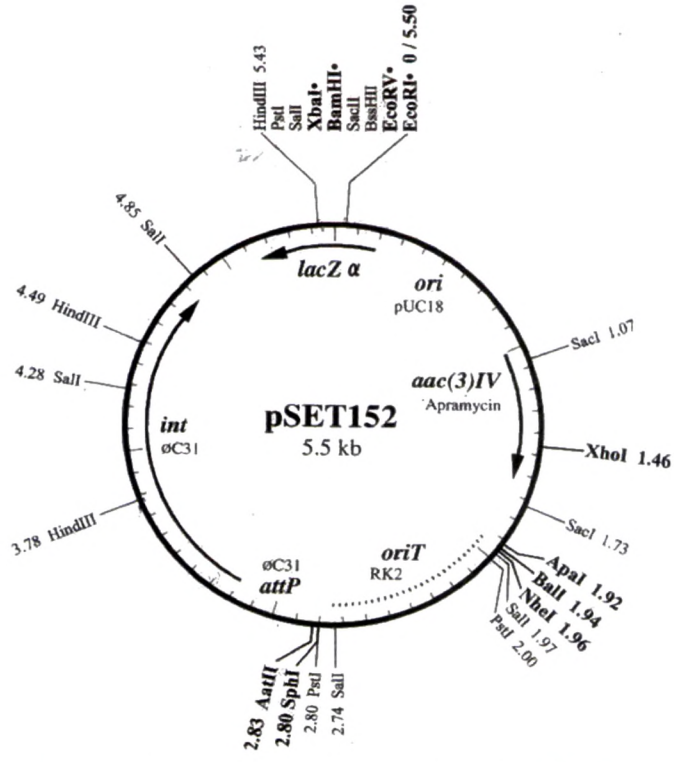


Figure 4.1: Restriction map of plasmid pSET152

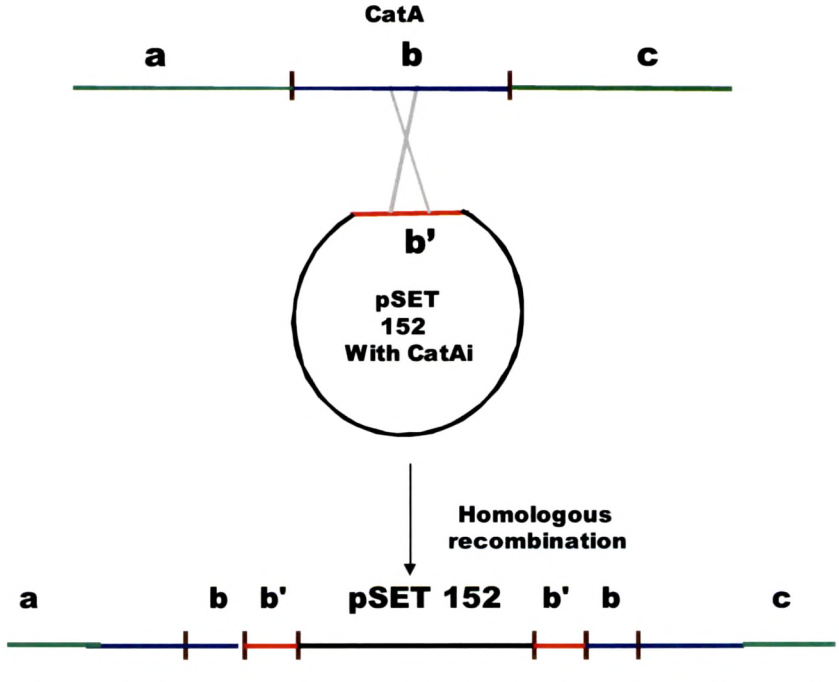


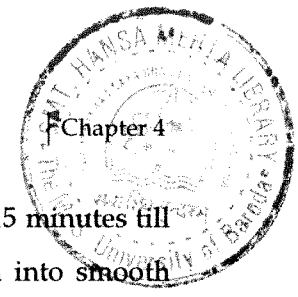
Figure 4.2: Schematic representation of strategy for insertional inactivation of catalaseA gene by homologous recombination.

Hence, *int* gene of the plasmid pSET152 was disrupted by digesting the plasmid with *Hind*III, since one of its target sites is located within the *int* gene (Figure 4.1). The largest fragment of the vector backbone (approximately 3.85 kb) was eluted from the agarose gel, purified and was self-ligated to generate pSET152 (Δ*Hind*III) plasmid, thus eliminating the *int* containing fragments. The internal region of *catA* gene (*catAi*) was amplified from the genomic DNA of *S. coelicolor* by PCR using forward primer 5′ CTG GGA TCC GGT CGG AAC 3′ and reverse primer 5′ GGT GGA TCC GGT AGT TCT CCG 3′ bearing the restriction sites *Hin*dIII and *Eco*RI at the 5′ end respectively to facilitate directional cloning. Plasmid pSET152 (Δ*Hin*dIII) and the PCR amplicon of *catAi* were digested with *Hin*dIII and *Eco*RI, purified and ligated to generate the recombinant plasmid, pSET(Δ*Hin*dIII)CatAi. The ligation mixture was transformed into *E. coli* strain DH5α by standard *E. coli* transformation protocol (Sambrook and Russel, 2001) and the transformants were selected on the Luria Agar plates containing 25 μM Apramycin. The recombinant plasmid was isolated from randomly selected transformant colonies and was confirmed by analyzing the restriction digestion pattern. The plasmid isolated from *E. coli* DH5α could not be used directly in *S. coelicolor* since it would be prone to degradation by methylation specific restriction enzymes present in the host (Zotchev et al., 1995). To overcome this, the confirmed recombinant plasmid was transformed in *dam*⁻ *dcm*⁻ strain of *E. coli* (ET12567), re-isolated and was then transformed in *S. coelicolor* using standard transformation protocol (Hopwood et al., 1985)

4.2.7. Transformation of plasmid DNA in *S. coelicolor* (Hopwood et al., 1985)

(i) Preparation of protoplasts

The spores of *S. coelicolor* were inoculated and grown in 10 ml YEME (Yeast extract malt extract) medium at 30° C on incubator shaker (170 rpm) for 36–40 h. The mycelium was used as inoculum (1-2%) for further experiments. The mycelium was grown for 21-24 h till the mid log phase. The culture was centrifuged and the mycelial pellet was washed twice with 10.3% sucrose solution. Mycelium was resuspended in 4 ml P buffer containing 4 mg lysozyme



(1 mg/ml) and triturated with glass pipette at an interval of every 15 minutes till the protoplastation occurred (confirmed by breaking of mycelium into smooth homogenous cells). After protoplastation is over the cells were resuspended gently in 4 ml P buffer and filtered through cotton assembly (Hopwood et al., 1985). The filtrate was centrifuged at 3000 rpm for 7 minutes and the pellet was washed twice with P buffer and resuspended in the remaining last drops of the supernatant.

(ii) Transformation of protoplasts

Protoplast (50 µl) was dispensed into different eppendorf tubes. Plasmid DNA pSET(Δ*Hin*dIII)CatAi (10 µl) was added to protoplasts and mixed by gentle tapping. Freshly reconstituted T buffer (200 µl) (0.25 g poly ethylene glycol (PEG)-1000 in 750 µl of T-buffer) was added immediately and mixed by pipetting up and down three times. The suspension was spread on R2YE plates with gentle spreading and allowed to dry in the laminar flow hood for 15 minutes. The plates were incubated at 30 °C. After incubation of 14-20 h the plates were overlaid with soft agar containing 25 µM apramycin. Resistant colonies were screened after 3 days of incubation.

4.2.8. Random mutagenesis of *S. coelicolor* by ethyl methanesulphonate (EMS)

The spores were germinated in 1ml 2 X YT medium for two hours and washed twice with one ml of 0.01 M potassium phosphate buffer, pH 7.0 and resuspended in 5 ml of the same buffer. The spore suspension was distributed into 1ml aliquots and treated with 0.185 mM EMS (20 µl/ml) for varied time periods (15 to 60 minutes). After EMS treatment the reaction was terminated by adding 0.5 ml 5% sodium thiosulphate. The spore suspensions subjected to mutagenesis were serially diluted and 100 µl of each dilution was plated on SMA (2% Soyabean 2% Mannitol 2%Agar) plates and incubated at 30 °C for 3-5 days and the colonies were counted and mutagenesis rate was calculated as compared to control. EMS treated spores were selected for the CatalaseA deficient mutant by overlaying H_2O_2 on the individual colonies and observing the O_2 bubbling

rate (Kim et al., 1994) as catalase acts on H_2O_2 to generate H_2O and O_2. Catalase deficient mutants will show slower bubbling rate as compared to wild type.

4.2.9. Growth curve of *S. coelicolor*

Since *S. coelicolor* shows mycelial growth, it is difficult to monitor growth rate like other bacteria either by taking OD_{600} or by serial dilution and colony count method. In order to perform the growth curve, the *S. coelicolor* spores were inoculated in 10 ml of YEME medium and incubated on shaker at 30 °C to get fine growth in stationary phase. The freshly germinated and grown culture was used for the growth curve as inoculum to 50 ml YEME flasks. 1 ml of culture was isolated after every hour and the mycelium was pelleted and washed with potassium phosphate buffer, pH 7.0 and resuspended in 0.5 ml of the same buffer and sonicated. Protein estimation was carried out for each sample in duplicates using Folin Lowry method (Lowry et al., 1951).

4.3. Results

4.3.1. Effect of DTT on catalase activity in *S. coelicolor*

From the analysis of catalase activity under DTT stress it was found that the activity was increasing in a dose dependent manner from 2 to 10 mM DTT (Figure 4.3), confirming the observation made in chapter 3 that the induced protein is CatalaseA. Further, the increase in catalase activity corresponded to the induced protein levels.

4.3.2. Effect of DTT on Superoxide Dismutase (SOD) activity in *S. coelicolor*

SOD is one of the important enzymes involved in protection against oxidative stress and is generally induced and works in concert with catalase in subsiding oxidative stress engendered by various chemical and physical agents. In general if DTT involves superoxide mediated stress response then there should be rise in SOD levels in *S. coelicolor* along with catalase. To establish the role of SOD in DTT stress, the SOD activity was measured at different time intervals (Figure 4.4).

From the graph it is clear that unlike catalase there is no significant change in SOD activity as compared to control. Hence, it can be inferred that superoxide level is not increasing in DTT mediated thiol stress.

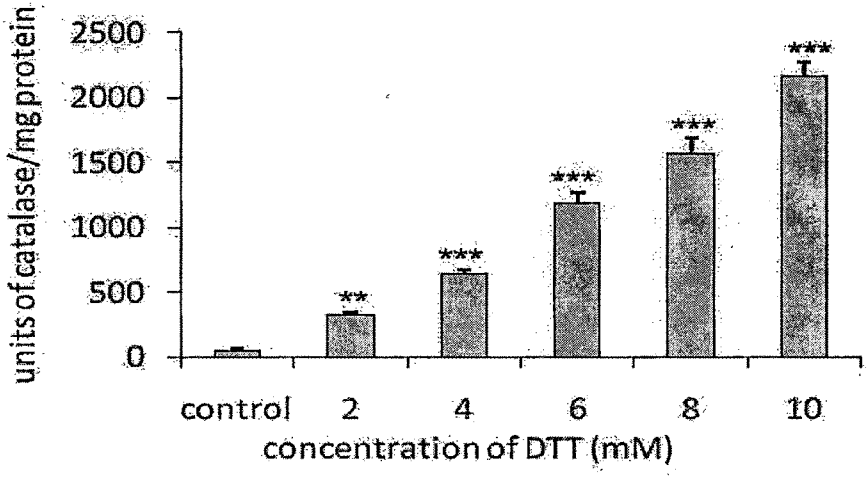


Figure 4.3: Effect of DTT on catalase activity in *S. coelicolor*: *S. coelicolor* cultures in midlog phase of growth were subjected to different concentrations DTT (2-10mM). The specific activity of catalase was observed after four hours of incubation at 30°C at different doses of DTT. Results are expressed as mean ± SEM of three observations. **p<0.001; ***p<0.0001.

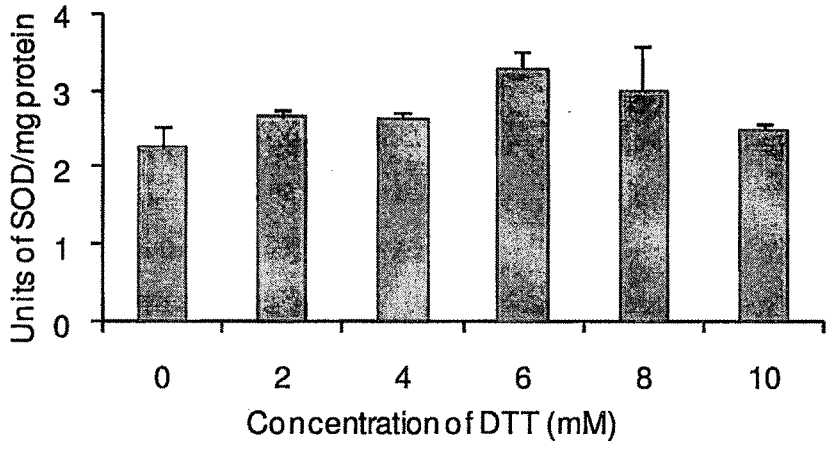


Figure 4.4: Effect of DTT on SOD activity in *S. coelicolor*: *S. coelicolor* cultures in midlog phase of growth were subjected to different concentrations DTT (2-10 mM). The specific activity of SOD was observed after four hours of incubation at 30 °C at different doses of DTT. Results are expressed as mean ± SEM of three observations.

4.3.3. Effect of Hydroxylamine (HA) on catalaseA induction

In order to determine the role of catalaseA under DTT stress, one of the ways is to inhibit the catalase activity and to see its consequences on the growth of *S. coelicolor*. The simplest approach is to inhibit the catalase activity using catalase inhibitor hydroxylamine (Blaschko, 1935; Sevag and Shelburne, 1942).

In order to check the effect of hydroxylamine on DTT stress, it is important to study the effect of HA on the growth and catalase activity in *S. coelicolor*. For that the growing *S. coelicolor* cultures were exposed to different concentrations of hydroxylamine (1-10 mM) for different time intervals of 0 to 4 hours followed by the measurement of growth and catalase activity (Figure 4.5 and 4.6). Since *S. coelicolor* shows mycellial phenotype, the growth was monitored in terms of the total protein content.

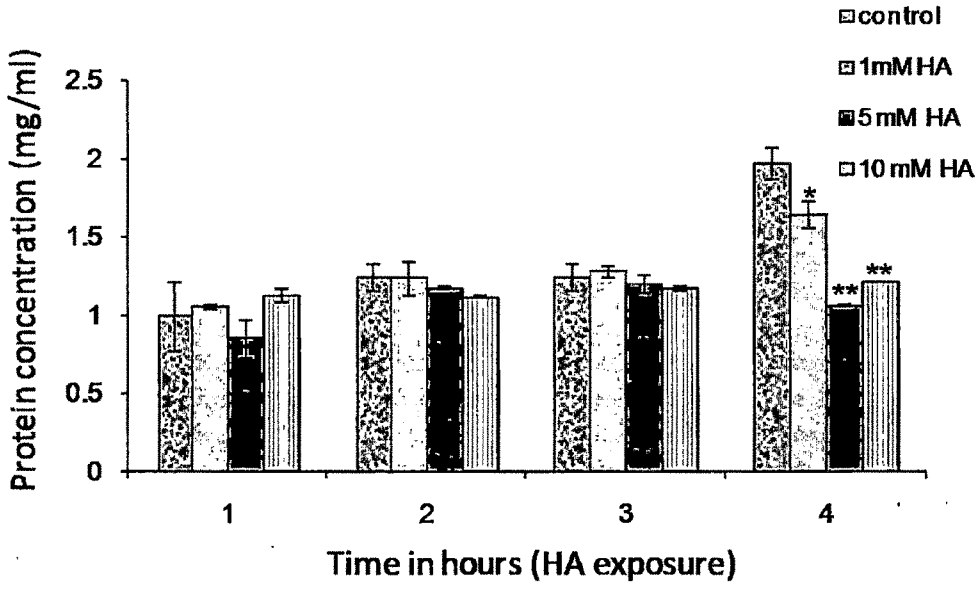


Figure 4.5: Effect of hydroxylamine on the growth of *S. coelicolor*. The midlog phase cultures of *S. coelicolor* were treated with 1-10 mM hydroxylamine and the growth was measured by protein estimation using Folin Lowry's method at different time intervals ranging from 1-4 hours. Results are expressed as mean ± SEM of three observations. * $p<0.05$; ** $p<0.001$

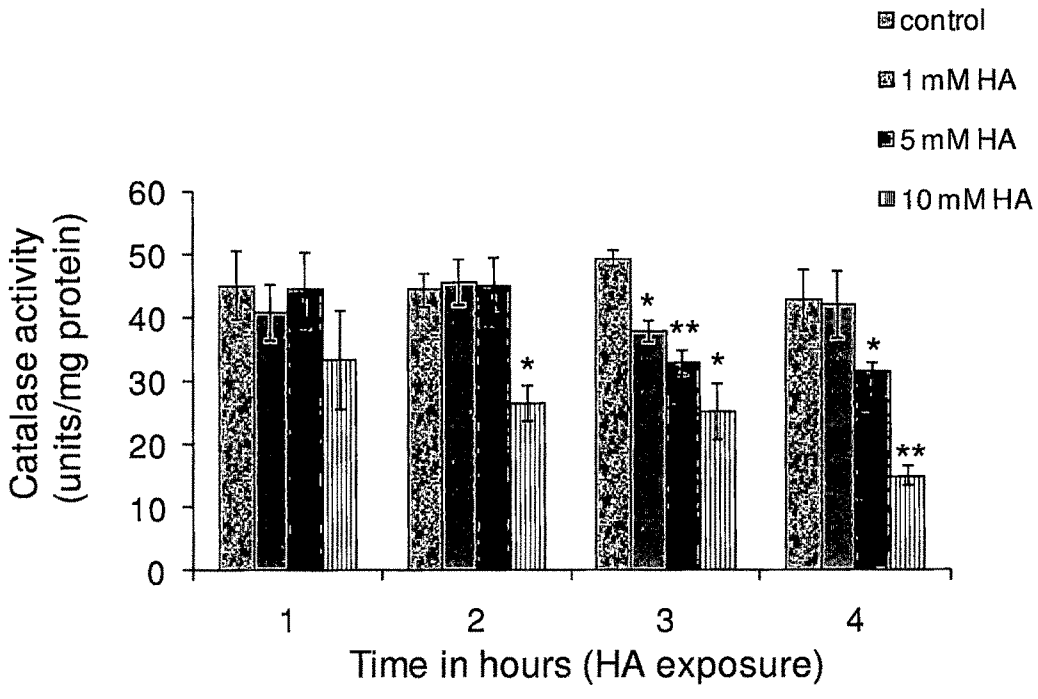


Figure 4.6: Effect of hydroxylamine on the catalase activity in *S. coelicolor*: The midlog phase grown *S. coelicolor* cultures were treated with 1-5 mM hydroxylamine and the catalase activity was measured at different time intervals ranging from 1-4 hours. Results are expressed as mean ± SEM of three observations. * $p<0.05$; ** $p<0.001$

Growth pattern in the presence of HA indicated that *S. coelicolor* showed dose dependent and time dependent suppression of the growth such that 1 mM HA significantly decreased the growth only after four hours, where as 5 mM and 10 mM of HA suppressed the growth after 3 hours of HA treatment. Catalase activity under HA stress followed the pattern similar to that of growth. At 10 mM of HA it showed decreased activity after 1 hour whereas in 1 mM and 5 mM of HA the activity decreased after 3 hours of HA exposure.

To study the importance of catalaseA on the survival of *S. coelicolor* under DTT stress, the *S. coelicolor* cultures were exposed simultaneously to HA and DTT and the catalase inhibition effects were monitored on the growth and catalase activity after 3 hours of treatment (Figure 4.7). Specific effect of catalase inhibition by HA

was studied by keeping DTT concentration constant at 10 mM and varying HA concentration from 1 to 5 mM. From Figure 4.7, it is evident that the catalase activity was reduced considerably in a dose dependent manner with HA after 3 hours, in addition the growth of *S. coelicolor* decreased in the same way. These results indicate the essential role of catalase in rescuing the bacterium from DTT mediated thiol stress.

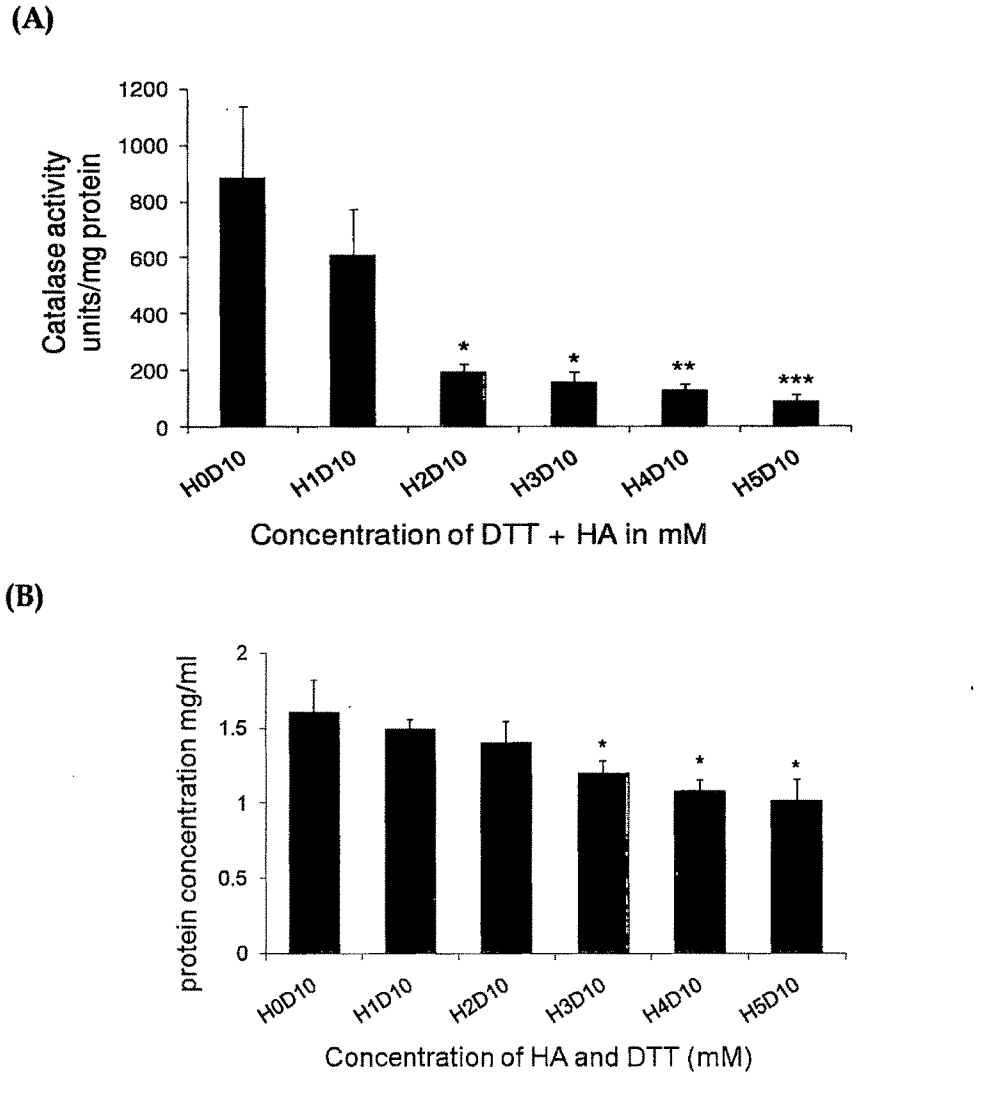


Figure 4.7: Combined effect of DTT and hydroxylamine on (A) Catalase activity (B) Growth of *S. coelicolor*. The growing *S. coelicolor* cultures were treated with different concentration of hydroxylamine (0-5 mM) along with fixed concentration of DTT (10 mM). Hydroxylamine was able to suppress significant catalase activity induced by DTT stress. Results are expressed as mean ± SEM of three observations. * $p<0.05$; ** $p<0.001$; ***$p<0.0001$

4.3.4. Generation of CatalaseA mutant by insertional inactivation (molecular approach)

As hydroxylamine is a nonspecific inhibitor and also it may have adverse effects on growth there are chances that it may interfere with the action of DTT by some alternate and unknown mechanisms. In order to rule out the possibility, it is more appropriate to get rid of catalase activity by generating catalaseA mutant of *S. coelicolor*. The most widely used method is to perform insertional inactivation of *catA* gene by homologous recombination. For this the clone was generated as per the section 4.2 described in materials and methods. The clone was confirmed by the release of 461 bp internal fragment of CatalaseA gene (CatAi) upon digestion with *Bam*HI (Figure 4.8).

Figure 4.8: *BamHI* restriction digestion pattern of *CatAi* cloned in pSET (Δ*Hin*dIII). Lane 1. 461 bp *CatAi* insert released (rounded up) from the vector; Lane 2. 100 bp ladder; Lane 3. λ *Bst*EII marker.

Homologous recombination was performed by transforming the pSET(ΔH)CatAi construct in *S. coelicolor*. Since the pSET(ΔH) is unable to self-replicate in the cell it has to be inserted into the genome. But in spite of high recombination frequency and the several attempts of repetitive transfer by conjugation and transformation, the recombinants/integrants could not be obtained suggesting that *catA* deletion mutant could be lethal thus proving that catalaseA activity is vital for *S. coelicolor*.

4.3.5. Generation of CatalaseA mutant by genetic approach

As insertional inactivation could not be achieved, we tried using an indirect method of genetic approach to get the catalaseA mutant. The *S. coelicolor* spores were randomly mutated using a chemical mutagen EMS. To get the mutants, EMS dose was kept constant and the time of exposure was normalized in order to achieve 90-99 % lethality (Table 4.1). From the Table 4.1 it is clear that 45 minutes with 0.185 mM of EMS exposure shows approximately 95% lethality.

Table 4.1. Relative viable count of *S. coelicolor* spores after EMS exposure for varied time intervals.

	Time of EMS exposure in minutes				
Serial dilution	0′	15′	30′	45′	60′
10^{-4}	MAT	MAT	MAT	MAT	~800
10^{-5}	>1000	~ 700	~ 400	~ 300	149
10^{-6}	284	112	49	5	-

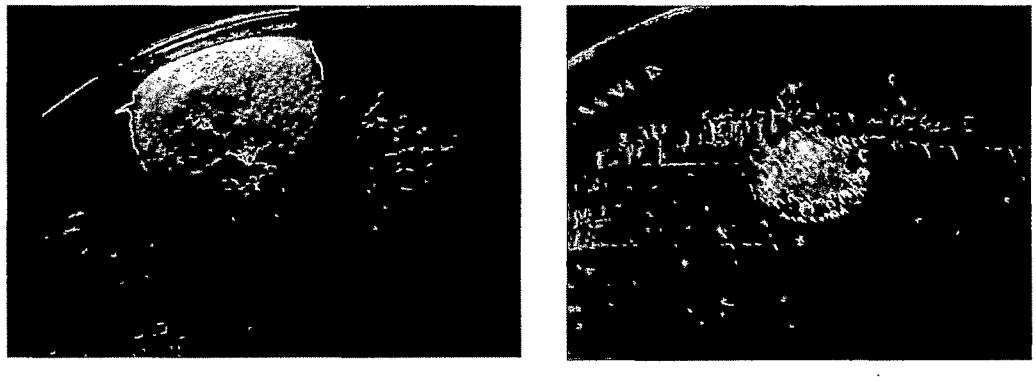
MAT represents the lawn growth with uncountable number of colonies.

The spores mutagenized at the standardized dose were screened for the catalase mutant by spraying 30% H_2O_2 over individual colonies and observing the oxygen evolution through formation of bubbles on the plate. The mutants with lower bubbling rates were isolated and screened for the catalase deficiency (Figure 4.9). One such mutant was found to possess very slow bubbling rate and identified as catalase deficient mutant.

4.3.6. Growth characteristics of CatalaseA mutant

Although the mutant showed decreased catalase activity by qualitative analysis (Figure 4.9), still residual catalase activity was present. As per the visual observation the mutant isolated above showed all the characteristic features of

the wild type *S. coelicolor* such as mycelial growth, germination, sporulation and pigment production. In order to check the effect on the growth rate, growth curve of the mutant was compared with that of wild type *S. coelicolor* (Figure 4.10) which showed that there was no significant change in the growth rate of mutant as compared to WT. This indicates that under normal conditions, the basal catalase activity present in the mutant is enough for the efficient survival of the organism.



Wild type Mutant

Figure 4.9: Comparative O_2 bubbling over wild type and mutant *S. coelicolor* colonies in response to H_2O_2 exposure. The wild type showed fast and vigorous bubbling compared to mutant indicating the latter to be as catalase deficient mutant. The bubbling rate was observed for 30-60 seconds immediately after H_2O_2 exposure.

4.3.7. Effect of DTT on catalase activity in the mutant

In order to establish the mutant as catalase mutant, the catalase activity of the mutant was measured during log phase (Figure 4.11). The catalase activity was reduced by more than 20 fold as compared to control indicating the mutant as catalase mutant and since the loss of catalase activity is during the log phase, it is specifically a catalaseA mutant, as it is the major catalase expressed during the log phase in *S. coelicolor* (Walker et al, 1995).

In the wild type *S. coelicolor*, as stated earlier there is more than 30 fold increase in the catalase activity under DTT stress (Figure 4.11). In contrast, the mutant

showed decrease in the catalase activity confirming the above hypothesis that the screened mutant is catalaseA mutant as it was observed in chapter 3 that catalaseA is one of the major proteins induced under DTT stress.

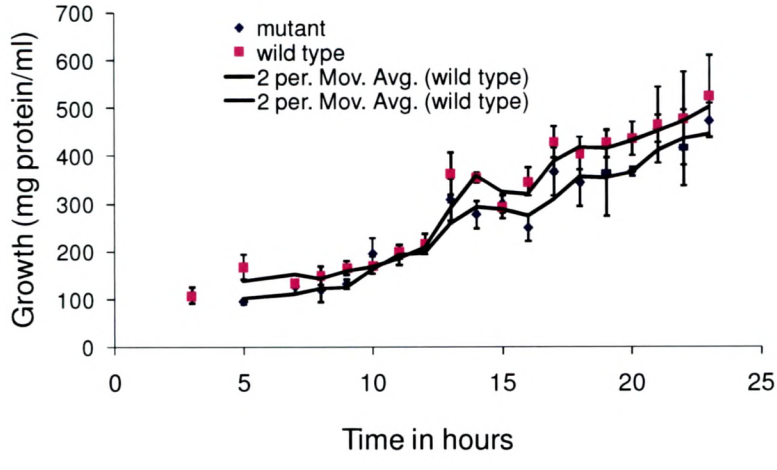


Figure 4.10: Comparative growth curve of wild type (WT) and catalase mutant of *S. coelicolor*. Growth was determined after 1% inoculation of freshly grown cultures of WT and mutant. The trendline shows 2% moving average of WT (bold line) and the mutant (dashed line). Results are expressed as mean ± SEM of three observations.

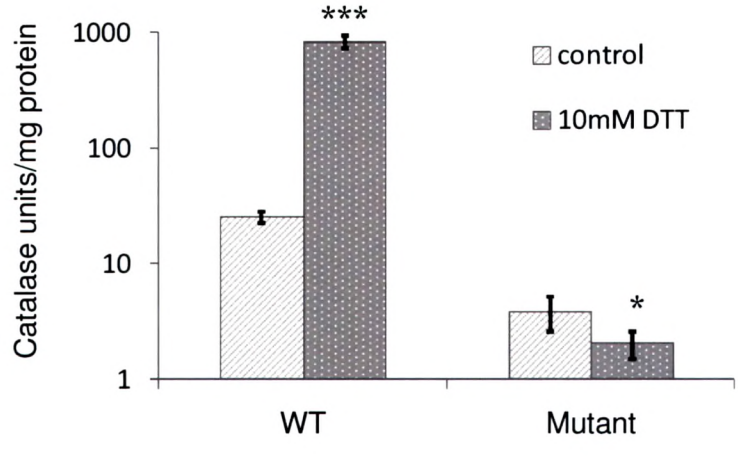


Figure 4.11: Influence of DTT on the catalase activity in the catalase mutant. Catalase activity was compared in wild type (WT) and catalase mutant (Mutant) in the presence and absence of DTT. The wild type showed increase, where as the mutant showed decrease in the activity. Because of large variation in the catalase activity, the Y axis is represented in the log scale. Results are expressed as mean ± SEM of three observations. **p<0.001; ***p<0.0001

4.3.8. Effect of DTT on the growth of catalaseA mutant

From the above results it is apparent that the mutant shows decline in catalaseA activity. At the same time the growth of the mutant is not affected, presumably the residual catalase activity present in the mutant is sufficient to take care of the survival of the organism and thus the mutant serves as a desirable model for understanding the significance of catalaseA in DTT stress. In order to study this phenomenon, effect of DTT on mutant physiology was observed. Results indicate that under normal conditions without any stress there is no change in the growth between WT and mutant but in the presence of DTT there is a significant reduction in the growth of the mutant strain as compared to wild type (Figure 4.12). This indicates that under DTT stress, induced catalaseA plays a protective role in S. *coelicolor* which is important for the protection against the stress and growth of the organism.

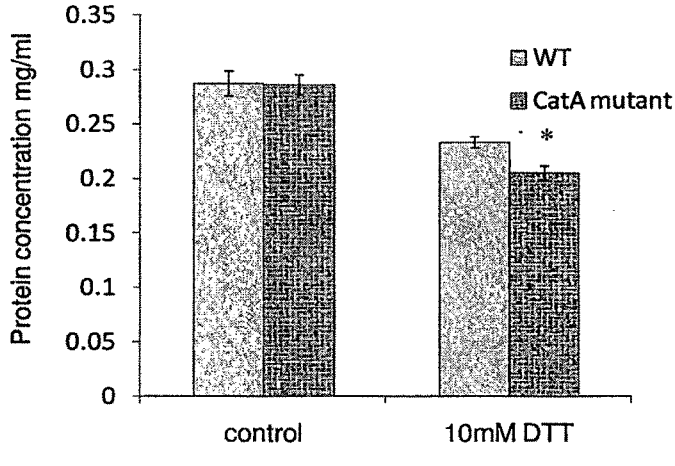


Figure 4.12: Effect of DTT on the growth of the catalaseA mutant. In control, there is no difference in the growth between WT and *catA* mutant. In the presence of 10mM DTT there is a significant reduction in the growth of *catA* mutant as compared to WT. Results are expressed as mean ± SEM of three observations. *$p<0.05$

4.3.9. Effect of *o*-phenanthroline on DTT stress

Earlier in chapter 2, it was established that the intracellular environment after DTT stress is reductive, hence the important question raised was, what must be the reason for the induction of catalaseA in *S. coelicolor*? One of the possible

reasons could be the amplification of Fenton reaction through continuous reduction of the limited pool of iron inside the cell. Besides, Fe ions are involved in major redox reactions. Therefore, it would be interesting to investigate the probable role of Fenton reaction and Fe in driving CatalaseA induction in DTT stress. One of the most important regulators of Fenton chemistry is the availability of free iron (Fe) itself. *O*-phenanthroline is a potent chelator of Fe which can reduce the available free iron required for continuous redox conversion of Fe^{2+} to Fe^{3+} and back in a cyclical fashion. This chelation lowers the level of reduced Fe^{2+} required for reduction of H_2O_2 to generate potentially dangerous hydroxyl ions ultimately blocking the Fenton reaction. Hence involvement of Fenton reaction was proved by scavenging Fe using *o*-phenanthroline and thereby dropping its availability. *S. coelicolor* cells were incubated with 1mM *o*-phenanthroline for 30minutes prior to DTT stress and the catalase activity was observed as earlier (Figure 4.13). It was observed that *o*-phenanthroline significantly interfered with the induction of catalaseA, resulting in suppression of catalase activity as compared to control. In fact the decrease in catalase activity was even lower than that of the basal activity in the control without DTT. This strongly indicated that Fenton reaction plays an important role in DTT mediated catalase induction.

CatalaseA contains Fe as a cofactor. Therefore it is possible that the *o*-phenanthroline may block the catalase activity directly at enzymatic level instead of altering the redox balance of the cell. In order to rule out this possibility, 1mM *o*-phenanthroline was added to the cell lysate (*in vitro*) prior to measurement of catalase activity so that if *o*-phenanthroline has direct effect on the catalase then it should show the same effect observed *in vivo* (Figure 4.13). From the *in vitro* results (Figure 4.14) it was found that there is no direct effect of *o*-phenanthroline on the catalase activity confirming our hypothesis that DTT drives the Fenton reaction leading to induction of catalaseA.

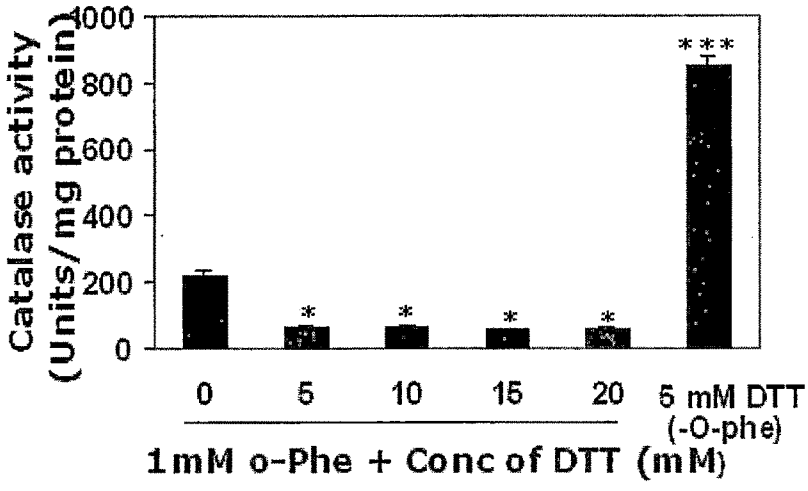


Figure 4.13: Effect of *o*-phenanthroline on DTT mediated catalaseA induction. First five columns represent the catalase activity in the presence of 1 mM *o*-phenanthroline and with varied concentration of DTT from 0-20 mM. The sixth column represents the catalase activity in the presence of 5mM DTT only. Results are expressed as mean ± SEM of three observations. * $p<0.01$; *** $p<0.0001$.

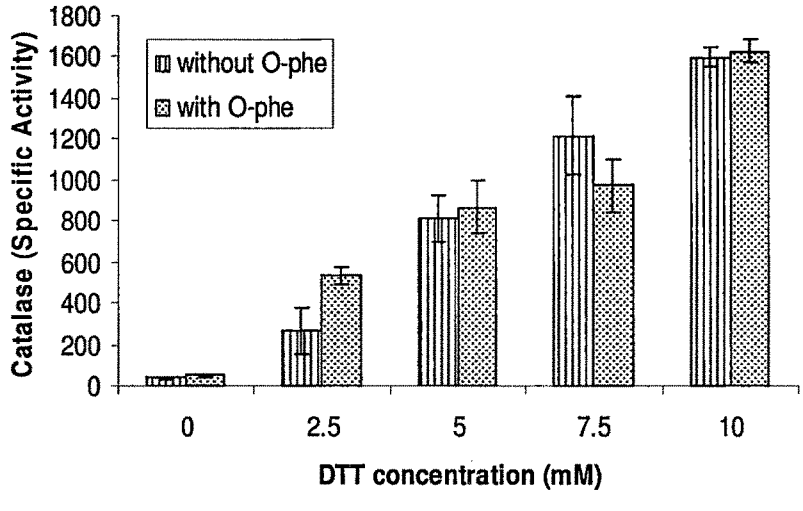


Figure 4.14: Effect of *o*-phenanthroline on catalase activity *in vitro*. DTT induced *S. coelicolor* cells were lysed and treated with 1 mM *o*-phenanthroline prior to catalase assay. Results are expressed as mean ± SEM of three observations.

4.4. Discussion

Catalase induction observed by proteomic analysis was further confirmed by enzymatic activity. It was apparent that induction in the catalase activity was elevated, which strongly suggests that the catalase must be playing a crucial role against DTT stress. In oxidative stress, two of the major enzymes involved are catalase and SOD. Catalase neutralizes H_2O_2 where as SOD acts upon superoxide ($O2^-$) to generate H_2O_2. In DTT stress, since there was no change in SOD activity, it implies that only H_2O_2 is the major ROS involved in DTT stress. In order to understand the role of catalase, down regulation of catalase by chemical inhibition with hydroxylamine as well as from the *catA* mutant generated by EMS mutagenesis showed diminished growth in the presence of DTT suggesting that catalaseA is playing a crucial role to defend against the DTT stress. Though, the mutant generated by random mutagenesis was having very low catalase activity, still the basal activity present was sufficient to grow under normal conditions but insufficient to sustain under DTT stress. Further, failure to get catalaseA deletion mutant suggests that the complete removal of catalase activity is lethal for *S. coelicolor*. This is supported by the fact that being a strict aerobe, *S. coelicolor* continuously generates ROS and in order to check the detrimental effects of ROS generation catalase is very important.

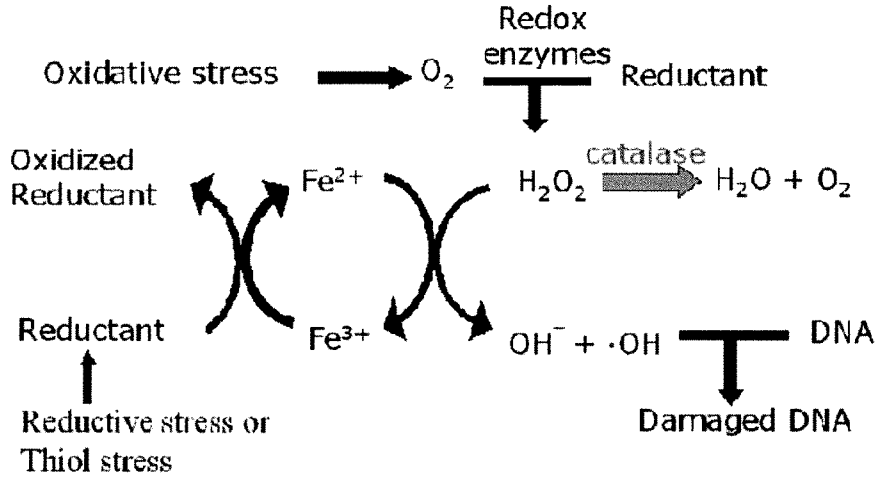


Figure 4.15: Mechanism of DNA damage under oxidative, reductive and or thiol stress. Removal of H_2O_2 by catalase averts DNA damage by hydroxyl free radicals.

CatalaseA suppression by *o*-phenanthroline implies that the induction of catalaseA under thiol stress was result of acceleration of Fenton reaction (Vekaria et al., 2007). Fenton reaction occurs in three steps (Equations 1, 2 and 3). Fe^{3+} ions react with a reductant forming Fe^{2+} ions and oxidized form of reductant. In turn Fe^{2+} ions react with hydrogen peroxide to generate hydroxide ions and hydroxyl radicals, converting back into Fe^{3+}. Hydroxyl radical reacts with both sugar and base residues of DNA causing detrimental effects.

Steps involved in the Fenton reaction, triggered by reduction of Fe by a reductant.

$$\text{reductant}^{red} + Fe^{3+} \rightarrow \text{reductant}^{ox} + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-} \text{ (the Fenton reaction)} \quad (2)$$

$$^{\cdot}OH + \text{DNA} \rightarrow H_2O + \text{DNA damage} \quad (3)$$

In order to prevent accumulation of hydrogen peroxide and subsequent formation of hydroxyl radicals organisms show induced expression of catalase, under oxidative as well as reductive stresses, as these two stresses represent two different halves of a cyclic reaction. In *E. coli* it was observed that NADH (Imlay and Linn, 1988) and reduced flavins in non-respiring bacteria caused oxidative DNA damage through Fenton reaction by transferring electrons to Fe^{3+} (Park and Imlay, 2003). It was also observed in *E.coli* that abnormally high level of cysteine can cause oxidative DNA damage by acting as adventitious reductant of Fe^{3+} (Trotter and Grant, 2002). Hence, ready availability of reductant in the form of free thiols rendered the organism excessively sensitive to hydrogen peroxide. Upsetting Fenton reaction at any step mentioned above made *E. coli* more resistant to hydrogen peroxide. Hence, it was concluded that even in *S. coelicolor* accelerated recycling of Fe^{3+}/ Fe^{2+} ions under thiol stress poses the threat of DNA damage and the bacterium tries to survive this challenge by catalaseA induction. The abolition of induction of catalase in *o*-phenanthroline under DTT stress confirms the hypothesis. This study indicates that catalase is a point of convergence for oxidative, reductive and/or thiol stresses (Figure 4.15).