CHAPTER 2

Standardization of conditions for thiol stress and establishing reductive nature of thiol stress

2.1. Introduction

Thiols play important role in the redox regulation. Most of the studies on effects of redox imbalance are about the oxidative stress in which the cellular thiols generally get oxidised (Leichert and Jakob, 2004; Leichert et al., 2008; Bardwell, 2005). As discussed earlier most of the intracellular protein cysteines are maintained in their reduced form, under oxidative stress some of the regulatory proteins get oxidized and can act as redox switches. Reductive stress, on the other hand is anticipated to have opposite effect on the organism there by preventing disulphide bond formation especially in regulatory proteins mentioned in chapter 1, in addition to membrane bound and extracellular proteins.

DTT is a strong reducing agent that is often used to promote reductive environment and rarely for induction of reductive stress. It can cross membranes and prevent disulfide bond formation. Reductive effects of DTT are well studied in the eukaryote yeast, where it can block folding and transport of many proteins especially in endoplasmic reticulum (ER) with proteins such as carboxypeptidase Y, which contains five disulphide bonds (Simons et al., 1995). DTT treatment leads to accumulation of misfolded proteins which induces Ire1 signaling leading to unfolded protein response (UPR) (Cox et al., 1993; Kohno et al., 1993). Microarray analysis shows that DTT induces many proteins which help to counteract misfolding of proteins and UPR response. Besides, it alters the expression of many genes unrelated to ER and UPR indicating that these gene products are likely to protect the organism against reductive stress (Gasch et al., 2000). Reductive stress is the shift in the balance of some important biological couples like NAD^+/NADH, $NADP^+$/NADPH and GSSG/GSH towards more reducing side. These are the couples linked thermodynamically and their redox status directly reflects the redox environment of the cell (Schafer and Buettner, 2001).

Bacteria unlike eukaryotes do not show compartmentalization. In *E. coli*, the oxidative protein folding and disulphide bond formation occur in the periplasmic space. Studies related to unfolded protein response were carried out in

periplasmic space. Contrary to this Gram positive bacteria do not contain periplasmic space. The primary objective of this study was to standardize conditions for thiol stress, as thiol stress leads to reduction of disulphide bonds in proteins, thereby inducing the unfolded protein response and subsequently identify the proteins which show differential expression.

2.2. Materials and Methods

2.2.1. Chemicals and Biochemicals

Fine chemicals like DTT, acrylamide, DTNB [5,5'-Dithio-bis(2-nitrobenzoic acid)], MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), alcohol dehydrogenase were purchased from Sigma Chemical Co., St. louis, USA; other chemicals like potassium sulphate, Glucose, Sucrose, Maltose were purchased from Qualigens, India. Sodium dodecyl sulphate was from S.D. fine chem. Ltd, India. Chemicals used for SDS-PAGE, 2D-PAGE solvents like acetonitrile, chloroform, ethyl acetate, methanol, HPLC grade water was from either Merck Research labroratories, USA or E. Merck (India) Ltd., Mumbai, India. Agarose, Methanol, isopropanol, proline, lysozyme were obtained from SRL India. Culture media including Luria broth, Luria agar, peptone, tryptone, yeast extract, bactopeptone, tryptone Soya broth, Malt extract, cas-amino acids, agar agar were obtained from Hi-media, India. All other chemicals used in this study were also of analytical grade and obtained from local sources.

2.2.2. Microbial Strains

All the microbial strains used in the study were either procured from culture collection or were obtained as gift from various authentic sources. The microbial strains used in the present work are listed in Table 2.1.

Table 2.1 List of bacterial strains used in this study

Strains	Genotype/phenotype	Source/reference
Streptomyces coelicolor A(3)2	Produces antibiotic actinorhodin	MTCC
Streptomyces lividans TK24	Str-6, SLP2$^-$, SLP3$^-$	MTCC
Streptomyces griseus	Producer of type II polyketide – Chromomycin A3	Dr.Rosazza, University of Iowa, USA
Escherichia coli (DH5a)	deoR, endAl, gyrA96, hsdR17 (rk – mk+), recAl, supE44, thi -1, Δ (lac ZyA – arg F), U169, Φ80dlacZΔM15.	Hanahan, 1985, Lab Collection
Escherichia coli S 17.1	recA –, mob+ , thi, pro, hsdR-, hsdM+/RP4-2- Tc::Mu-Km::Tn7	Lab Collection

2.2.3. Growth conditions and standardization of DTT concentration necessary for inducing stress in *S. coelicolor*

Streptomyces coelicolor cultures were grown to log phase in YEME media at 30 °C where as *E. coli* was grown in LB media and exposed to 2-10 mM DTT for one to four hours. The mycelia were harvested by centrifugation and washed twice with sonication buffer (20 mM Tris, pH 7.5, 100 mM $MgCl_2$, 0.1% Triton X-100). The pellets were harvested after centrifugation and incubated at 4°C for 30 min after resuspending in sonication buffer containing 1 mg/ml lysozyme, 1 mM phenylmethyl sulfonyl fluoride (PMSF). The mycelia were sonicated thrice for 30 seconds in continuous mode. The lysates were centrifuged at 10,000 g for 10 min and the supernatant was collected. The protein concentration of the supernatant was estimated using Folin Lowry method.

2.2.4. Total thiol estimation by 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB)

In order to check the intracellular redox status of *S. coelicolor* under DTT stress, the total thiol content was estimated. Total intracellular thiol content was estimated using the method given by Sedlak and Lindsay (1968) with slight modifications. *S. coelicolor* culture was suspended in 0.1M sodium phosphate buffer, pH 7.2 containing 0.1% triton X-100 followed by sonication (3 continuous pulses of 30 seconds). 1 ml of culture was taken to set the reagent blank. 30 µl of DTNB solution (40 mg/ml DTNB in 1% sodium citrate) was added and OD was measured at 412 nm. The thiol content was expressed as the equivalent of thiol content of DTT from the standard curve of DTT.

2.3. Results

2.3.1. Effect of DTT on the growth of *S. coelicolor*

Being a reducing agent, DTT can alter the redox balance of the cell and hence it may affect the overall growth of the organism. In order to check this effect, the growth was measured at different concentrations of DTT. *S. coelicolor* shows mycelial growth which makes it difficult to monitor the growth by means of OD measurement or colony count method. So, to overcome this limitation, the effect on growth was monitored by measuring the total protein content of the cultures after four hours of DTT treatment (Figure 2.1). From the graph it is clear that there is a dose dependent reduction in the growth of *S. coelicolor* in the presence of DTT. After four hours of stress, the protein amount was less in DTT treated sample as compared to untreated control. But the total protein was still increasing in a time dependent manner with respect to 0hr control/DTT treated sample. Hence, decrease in protein content in DTT treated sample is due to growth inhibition.

2.3.2. Effect of DTT on the intracellular thiol content in *S. coelicolor*

The best indicator of redox status of the cell is GSH/GSSG ratio. But being actinomycetes, *S. coelicolor* does not contain glutathione; instead it contains mycothiol as a intracellular thiol buffer. With the intention of probing the redox

status of the cell in the presence of DTT, total thiol content, that is protein bound and free thiol content were monitored by DTNB method. From figure 2.2 it is clear that the total thiol content was increased in the presence of DTT, which indicates the cellular environment is still reducing and it is shifted more towards the reducing side.

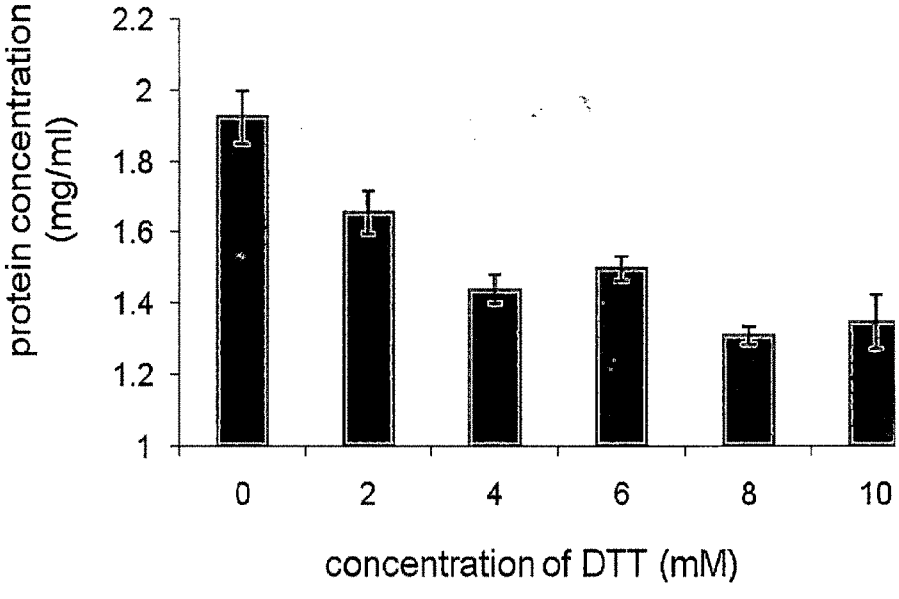

Figure 2.1: Effect of DTT on the growth of *S. coelicolor* in log phase measured by estimating protein content

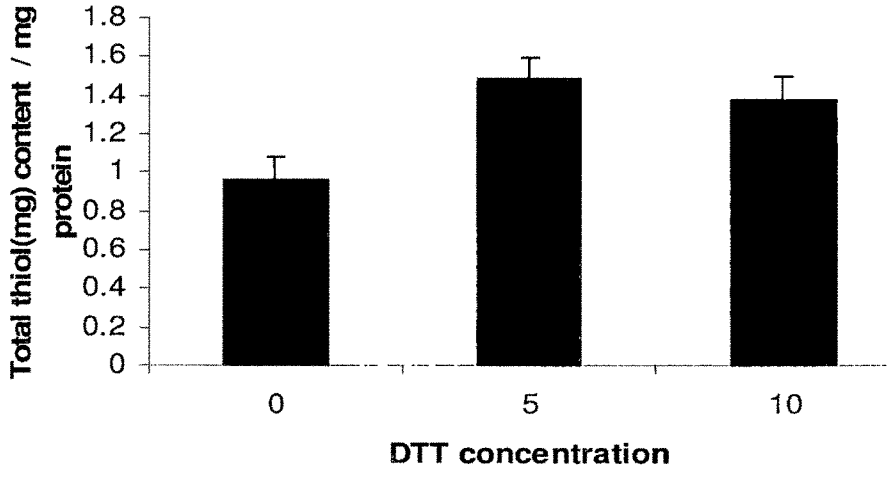

Figure 2.2: Effect of DTT on the total thiol content of the cell.

2.3.3. Effect of DTT on protein expression profile of *S. coelicolor* (observed in SDS-PAGE)

In order to check the generalized effects on the cellular expression, the DTT treated cells were checked for the alterations in the protein profile. As seen in the Figure 2.3 there was a prominent induction of a protein band of approximately 55 kDa in the presence of DTT, in a time and dose dependent manner. The protein was suspected to play an important role in the regulation against DTT stress in *S. coelicolor*.

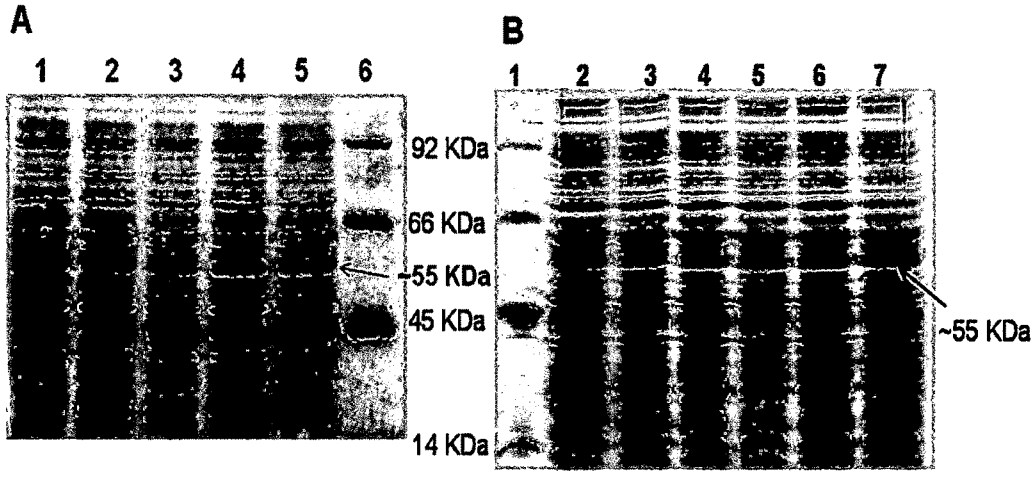

Figure 2.3: Effect of DTT on the protein expression profile in *S. coelicolor*: (A) Effect of DTT on the protein expression profile in time dependent manner. Lane A1, control (0 hour DTT treatment). Lane A2–A5, (1 hour, 2 hour, 3 hour and 4 hour post 10mM DTT treatment respectively). Lane A6, Protein molecular weight marker. **(B) Effect of DTT on the protein expression profile in dose dependent manner.** Lane B1, Protein molecular weight marker. Lane B2, control, without DTT treatment. Lane B2 – B7, different concentrations of DTT (2 mM, 4mM, 6mM, 8mM and 10mM respectively) Arrows indicate ~55 kDa protein band, which is over expressed in the presence of DTT in a time and dose dependent manner.

2.3.4.) Effect of DTT on protein expression profiles of *S. lividans, S. griseus* and *E. coli* (observed in SDS-PAGE)

To verify if similar effect of DTT stress is there in other organisms or it is specific to *Streptomyces coelicolor* only, we performed the induction experiment with other

species of *Streptomyces*, *S. griseus* and *S. lividans* and also in an unrelated Gram negative species, namely *E. coli* after following growth kinetics and taking cultures in similar stages of growth by observing OD_{600}. *E. coli* was studied just to compare if there is any induction in any of the protein band or not. It was found that a similar induction band was seen in *S. griseus* and *S. lividans* (Figure 2.4 A & B), but was absent in *E. coli* (Figure 2.4 C).

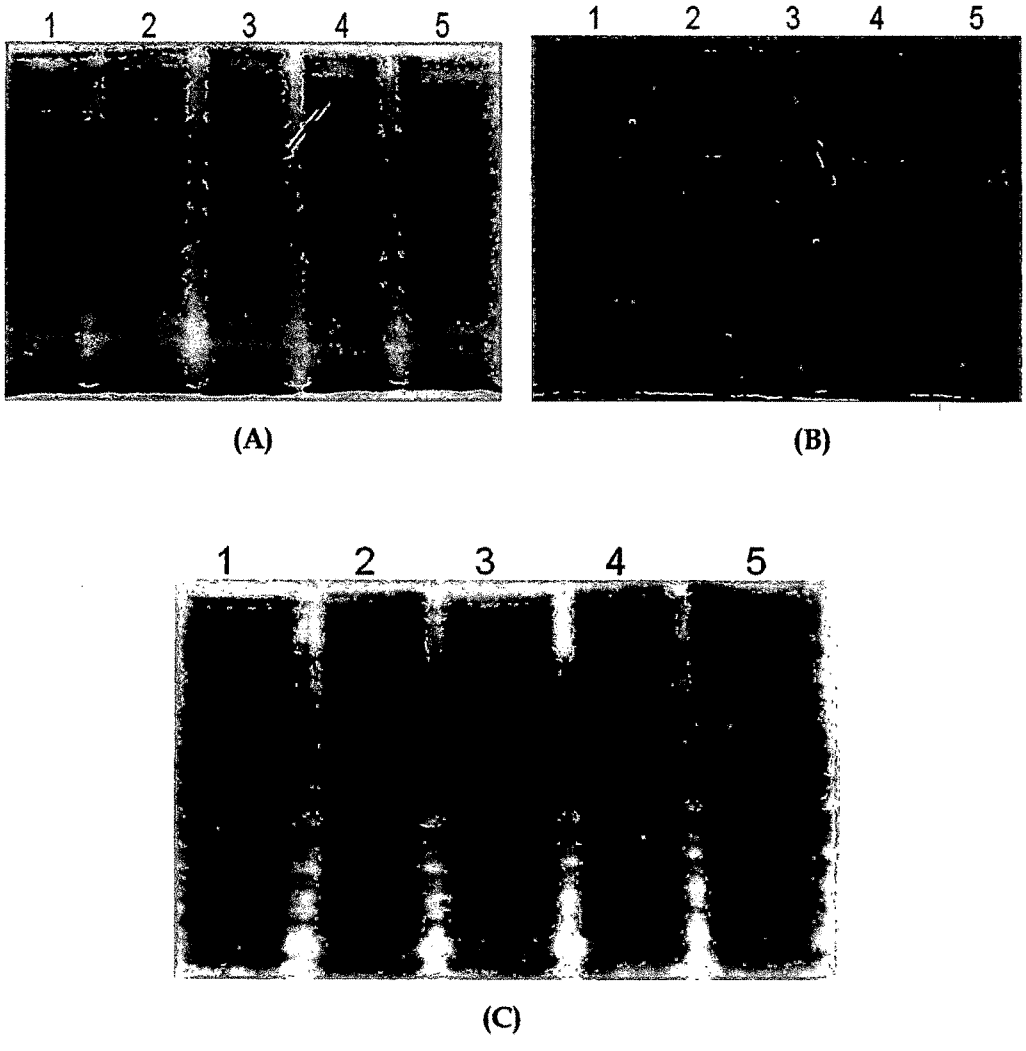

Figure 2.4: Effect of DTT on other bacterial species (A) *S. lividans*, (B) *S. griseus* and (C) *E. coli*. In all the three figures Lane 1 shows protein profile of control, without DTT treatment. Lane 3 – 5 show protein profiles obtained after treatment of the bacteria with different concentrations of DTT (1 mM, 2 mM, 5 mM and 10 mM). Arrows in A and B indicates dose dependent over expression of the band of ~55 kDa protein in the presence of DTT.

2.4. Discussion

From the above studies it was observed that in the presence of DTT, the stress experienced by the cells is of reductive type. As mentioned earlier the intracellular environment is more reducing, but still there are some of the components which may be maintained in their oxidized state. Results on the thiol status indicate that there is an increase in the total thiol content so cellular status is switching more towards the reductive side. From the *S. coelicolor* SDS-PAGE pattern it was seen that in the presence of DTT, there is a remarkable induction of a protein band of approximately 55 kDa in dose dependent manner. Protein band with approximately same molecular weight was observed to be induced in other *Streptomyces* species, where as there was no striking difference in any protein band in Gram negative bacterium, *E. coli*.