Chapter 2

Characterization of physiochemical properties of *Saccharomyces cerevisiae* under nitrosative stress

Introduction:

Redox homeostasis is one of the most important factor to maintain the cellular integrity. ROS and RNS including NO interfere with redox homeostasis, resulting in oxidative or nitrosative stress. Hence, alteration of redox homeostasis is a key marker of oxidative or nitrosative stress [1]. Thiol is one of the most important component to maintain the redox homeostasis *in vivo* [2]. The most abundant thiol that is present in almost all form of life, is glutathione [3]. It is a tripeptide containing glycine, glutamate and cysteine residue. Generally, two forms of glutathione exist in the cellular environment i.e. reduced glutathione (GSH) and oxidized glutathione (GSSG). Intracellularly, GSH is synthesized via the activity of two enzymes: γ-glutamylcysteine ligase (GCL) and GSH synthetase (GS). At first glutamate reacts with the cysteine by the action of GCL to form dipeptide γ-glutamylcysteine and then glycine reacts with the dipeptide by the action of GS and GSH is formed [4]. It acts as the redox buffer of the cell due to the generation of a huge amount of reducing equivalent [5]. GSH can be oxidized to GSSG by the action of GSH peroxidase (GPx). GPx uses GSH as the substrate to detoxify the effect of H_2O_2, lipid peroxides etc. that can interfere with the redox homeostasis. Again, GSSG can be reduced to GSH by the activity of NADPH dependent glutathione reductase (GR) [3, 6]. Thus, the activities of GPx and GR are very crucial for maintaining the redox status. In addition to it, intracellular thiol status is determined as the ratio of reduced to oxidized forms, i.e., GSH/GSSG. Change in the ratio of GSH/GSSG is considered as an important hallmark of nitrosative stress [7]. GSH has a protective role against NO mediated stress. The intracellular GSH can bind efficiently with NO which in turn reduces the activity of NO mediated destruction. Hence, it is very important to determine the GSH/GSSG ratio to investigate the redox status *in vivo* [8].

Saccharomyces cerevisiae also counteracts the stress by inducing different enzymes known as stress responsive enzymes e.g. catalase, Glutathione reductase (GR), superoxide dismutase (SOD), GSNOR etc. which in turn can also maintain the redox homeostasis. Thus it became imperative to determine the activities in these enzymes in the presence of ac. $NaNO_2$ and GSNO. In addition to it, *in vivo* generation of ROS and RNS is associated with the alteration of redox homeostasis. Thus, it became very important to investigate and quantify the amount of ROS and RNS in the context of this study.

Results:

To examine the effect of ac. $NaNO_2$ and GSNO at their respective sub-toxic doses (0.5 mM for ac. $NaNO_2$ and 0.25 mM for GSNO) on the cellular glutathione status, *S. cerevisiae* cells were first grown in YPD medium and then treated with either 0.5 mM ac. $NaNO_2$ or 0.25 mM GSNO. Following the treatment, cells were harvested, lysed and different parameters were determined using cell-free extract. The change in the glutathione status, GR and catalase activity were compared with the control. Treated and control cells were also checked for ROS and RNS including NO generation by performing FACS and Confocal microscopy.

Effect of acidified sodium nitrite on the redox homeostasis of *S. cerevisiae*:

To characterize the glutathione status of treated and untreated *S. cerevisiae* cells, reduced glutathione concentration (GSH), oxidized glutathione concentration (GSSG), total thiol concentration (GSH+GSSG) and GSH/GSSG ratio were measured. It was found that total glutathione concentration was not significantly altered in the presence of 0.5 mM ac. $NaNO_2$ as compared to the control. Whereas the concentration of GSSG was found to be decreased by ~2.3 fold and the concentration of GSH was increased by ~1.8 fold in the presence of 0.5 mM ac. $NaNO_2$ as compared to the control. overall, a sharp increase in GSH/GSSG ratio (4.2 fold higher) was found in the 0.5 mM ac. $NaNO_2$ treated cells as compared to the control under the specified experimental condition **[Table 3]**. In addition to it, GR activity was also found to be increased by 4 fold in the presence of 0.5 mM ac. $NaNO_2$ in comparison to the control **[Table 3]**. It was also observed that the activity of catalase was increased by approximately 2.4 fold in the 0.5 mM ac. $NaNO_2$ treated sample as compared to the control **[Table 3]**. Altogether these findings suggested that the redox homeostasis of the cells were significantly altered in the presence of 0.5 mM ac. $NaNO_2$ and the cells were trying to thwart it out.

To study the alteration of redox homeostasis, it was very important to investigate the *in vivo* generation and accumulation of ROS and RNS including NO [16]. Thus, confocal microscopy **[Fig. 9&10]** and FACS **[Fig. 11]** were performed. It was observed that the ROS was generated in both the treated and control cells with no significant change. On the contradictory, the generation of RNS was only observed in

the 0.5 mM ac. $NaNO_2$ treated cells (79%), clearly suggesting that the changes observed in the treated cells were solely due to the generation of RNS including NO.

Table 3: Estimation of total glutathione (GSH+GSSG), GSH/GSSG and activity of glutathione reductase (GR), and catalase in both treated and untreated (control) samples of *S. cerevisiae*

Sample	(GSH+GSSG) nmol/mg of protein	GSH nmol/mg of protein	GSSG nmol/mg of protein	GSH/GSSG	GR activity (mU/mg protein)	Catalase activity (mU/mg protein)
Control	77.6±2.4	33.9±1	43.7±2	0.78	4.3±NA	4.1±NA
0.5 mM ac.$NaNO_2$ treated	78.27±1.9	59.9±1.1	18.27±1.7	3.28	17.2±1.5	10±0.6

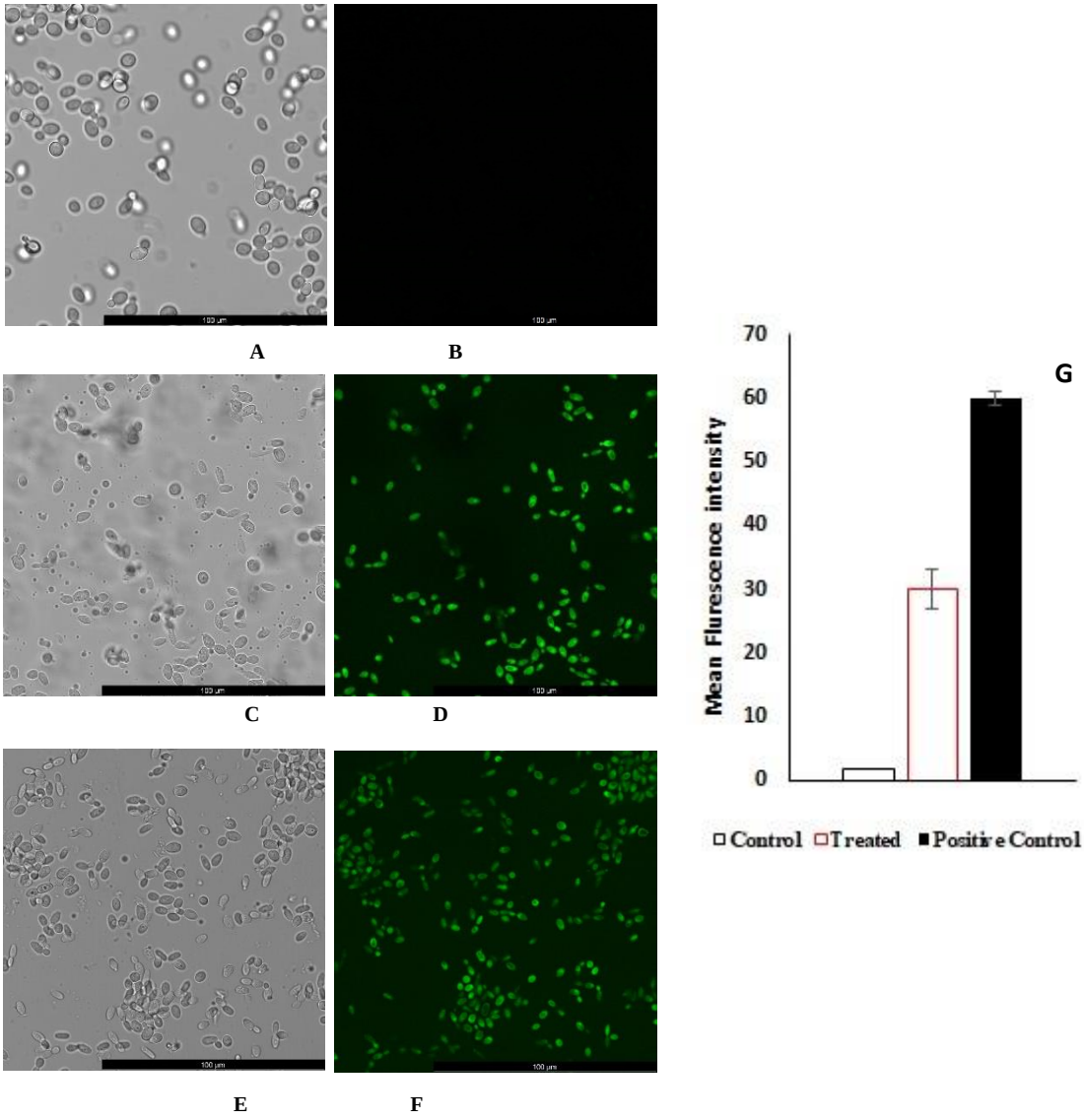

Fig. 9 Effect of acidified sodium nitrite on Reactive nitrogen species (RNS) including nitric oxide (**NO) generation:** The presence of NO was visualized as green colour using DAF-FM (excitation at 495 nm and emission at 515 nm). Phase contrast and corresponding fluorescence images of *S. cerevisiae* control (A and B), 0.5 mM acidified sodium nitrite treated (C and D) and positive control [peroxynitrite treated] (E and F). Micrographs were recorded at 45X. Bar=100 µm. The mean fluorescent intensity (G) was determined by using Leica LAS X software and represented as Mean±SD.

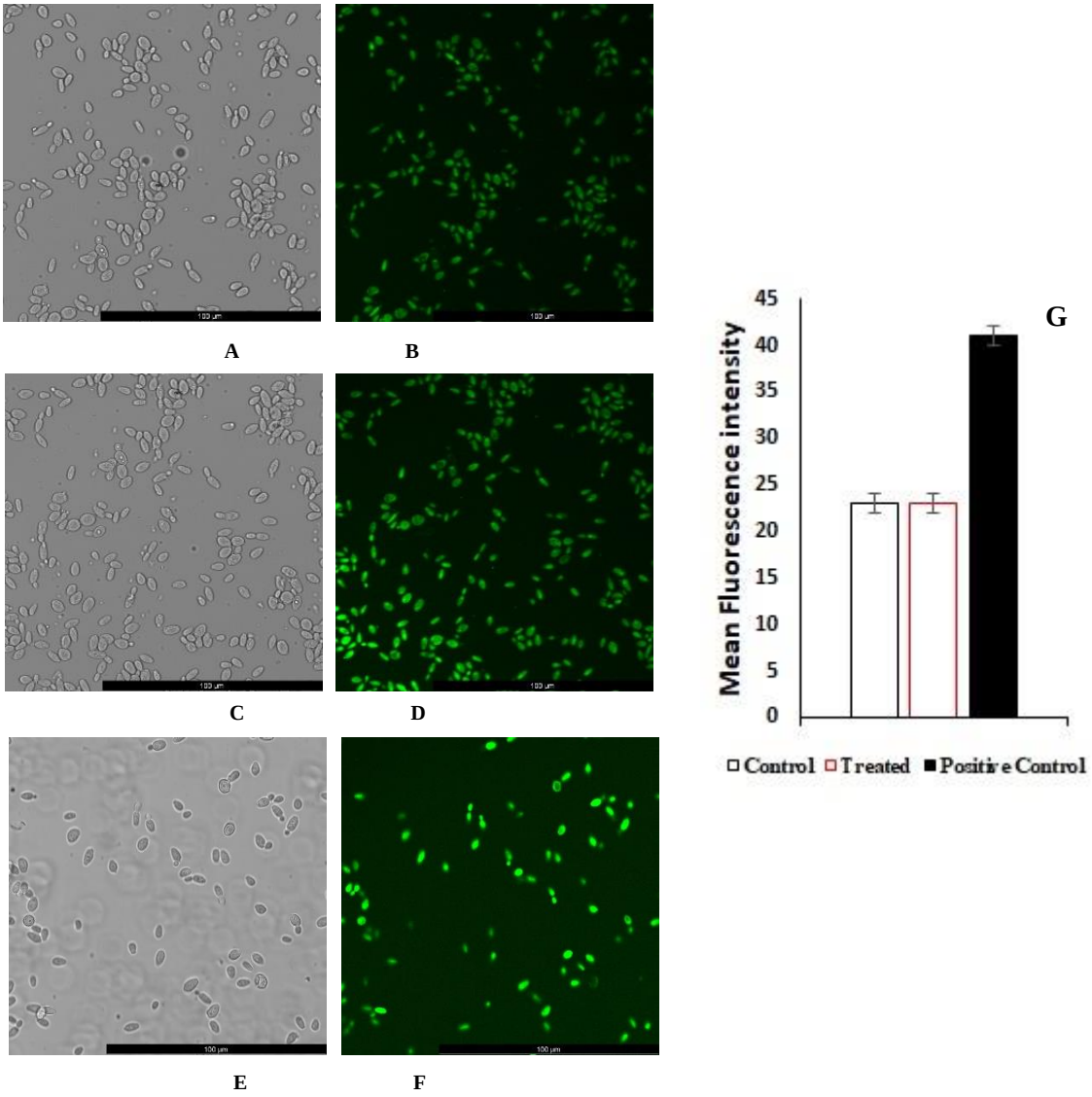

Fig. 10 Effect of acidified sodium nitrite on reactive oxygen species (ROS) generation: The presence of ROS was visualized as green colour using H_2DCFDA (excitation at 495 nm and emission at 515 nm). Phase contrast and corresponding fluorescence images of *S. cerevisiae* control or untreated (A and B), 0.5 mM acidified sodium nitrite treated (C and D) and positive control [H_2O_2 treated] (E and F). Micrographs were recorded at 45X. Bar=100 µm. The mean fluorescent intensity (G) was determined by using Leica LAS X software and represented as Mean±SD.

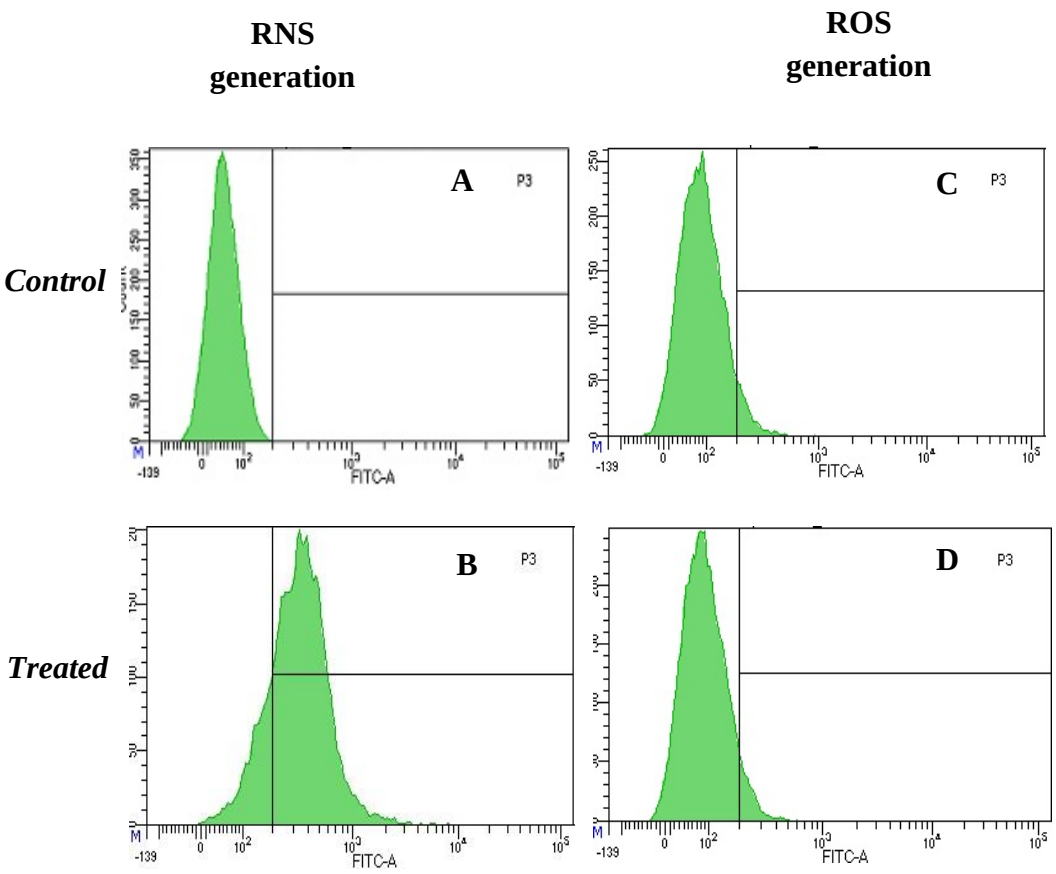

Fig. 11 Effect of 0.5 mM acidified sodium nitrite on reactive nitrogen species and reactive oxygen species generation: FACS analysis for the reactive nitrogen species (A, B) and reactive oxygen species (C, D). FACS analysis was done by using FACS Diva software. Excitation and emission were set at 495 nm and 515 nm respectively (for both the reactive nitrogen and oxygen species)

Effect of *S*-nitrosoglutathione on the redox homeostasis of *S. cerevisiae*:

To investigate the alteration in redox homeostasis *in vivo* in the presence of 0.25 mM GSNO, GSSG/GSH ratio, GR, GSNOR, and catalase activity, were assessed. Under the specified experimental condition, it was observed that the concentration of oxidized glutathione (GSSG) was decreased by 2.4 fold and reduced glutathione (GSH) was increased by 1.6 fold in the 0.25 mM GSNO treated cells as compared to the control. This resulted in a 3.9 fold increase in the GSH/GSSG ratio in treated cells in comparison to the control **[Table 4]**. A sharp 3.3 fold increase in GR activity was observed in the treated cells **[Table 4]**. Furthermore, treated cells also showed 4.3 fold higher activity of GSNOR (GSNO reductase) as compared to control, indicating that cells were expressing these enzymes to counteract the deleterious effect of GSNO **[Table 4]**. The activity of catalase was also found to be increased by 2.6 fold in presence of 0.25 mM GSNO as compared to control **[Table 4]**, implying that any ROS produced during the process was detoxified.

The presence of reactive species (ROS and RNS including NO) were detected and quantified under specified experimental conditions using confocal microscopy and mean fluorescence intensity. RNS was only detected in GSNO-treated cells **[Fig. 12]**. Whereas ROS was found in both the treated and untreated samples, there was no significant difference in ROS generation **[Fig. 13]**. Hence, it can be assumed that the effect observed under the specified experimental condition is solely due to the generation of RNS including NO by 0.25 mM GSNO.

Table 4: Estimation of total glutathione (GSH+GSSG), GSH/GSSG and activity of glutathione reductase (GR), catalase and *S*-nitrosoglutathione reductase (GSNOR) in both treated and untreated (control) samples of *S. cerevisiae*

Sample	(GSH+GSSG) nmol/mg of protein	GSH nmol/mg of protein	GSSG nmol/mg of protein	GSH/ GSSG	GR Activity (mU/mg protein)	Catalase Activity (mU/mg protein)	GSNOR Activity (mU/mg protein)
Control	81.92±2	34.2±0.5	47.72±1.2	0.7	3.95±0.5	3.88±0.4	1±0.02
0.25 mM GSNO treated	75.4±2	55.3±0.4	20.1±1.1	2.75	13.82±0.7	9.97±0.5	4±0.46

*Data has been rounded off to the nearest whole number for the publication

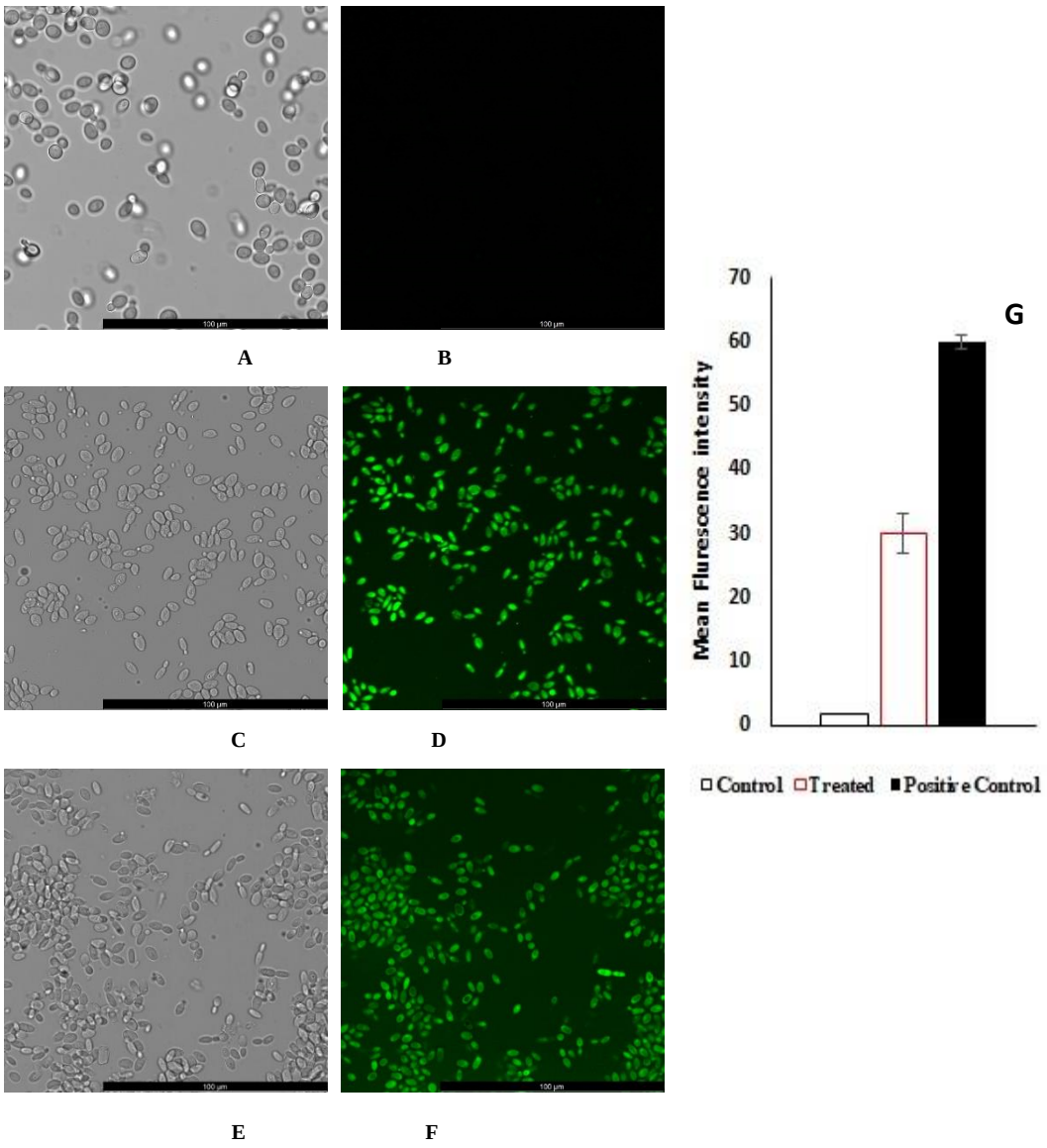

Fig. 12 Effect of *S*-nitrosoglutathione on Reactive nitrogen species (RNS) including nitric oxide (**NO) generation:** The presence of NO was visualized as green colour using DAF-FM (excitation at 495 nm and emission at 515 nm). Phase contrast and corresponding fluorescence images of *S. cerevisiae* control (A and B), 0.25 mM GSNO treated (C and D) and and positive control [peroxynitrite treated] (E and F) Micrographs were recorded at 45X. Bar=100 µm. The mean fluorescent (G) was determined by using Leica LAS X software and represented as Mean±SD.

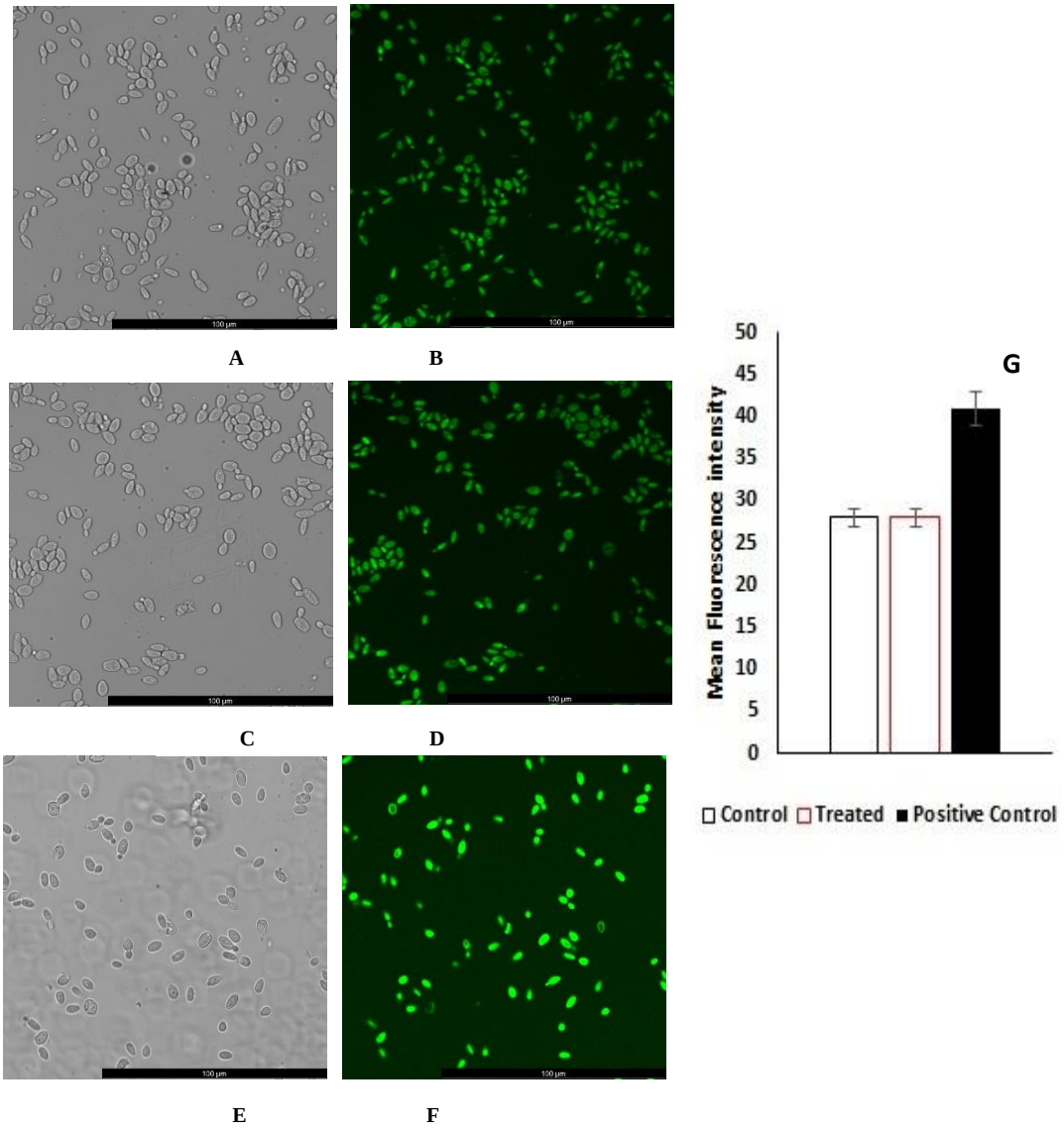

Fig. 13 Effect of *S*-nitrosoglutathione on reactive oxygen species (ROS) generation: The presence of ROS was visualized as green colour using H_2DCFDA (excitation at 495 nm and emission at 515 nm). Phase contrast and corresponding fluorescence images of *S. cerevisiae* control or untreated (A and B), 0.25 mM GSNO treated (C and D) and positive control [H_2O_2 treated] (E and F). Micrographs were recorded at 45X. Bar=100 µm. The mean fluorescent intensity (G) was determined by using Leica LAS X software and represented as Mean±SD.

Discussion:

Under sub-toxic dose of GSNO and ac. $NaNO_2$, some significant changes in physicochemical properties as well as redox homeostasis of *S. cerevisiae* were found in comparison to control, indicating that the cells were trying to overcome the stress for survival. GSH is regarded as a stress response component that plays an important role in the inhibition of NO activity, metal toxicity and so on [10, 12, 13]. When the GSH level decreases, NO activity induces DNA damage as well as protein modifications such as protein tyrosine nitration, *S*-nitrosylation [14, 15]. This study showed an increase in GSH/GSSG ratio under GSNO and ac. $NaNO_2$ stress, suggesting that the treated cells were trying to increase the reduced equivalent inside the cell in the form of GSH [16]. GSH acts as the redox buffer under the stress conditions and maintains the redox homeostasis as per the requirement of the cell [17]. Higher activity of GR under sub toxic dose of GSNO and ac. $NaNO_2$ also supported the finding. Thus, it can be concluded that the higher activity of GR under stress condition might contribute to the higher GSH/GSSG ratio. In addition to this, activity of catalase was also found to be increased in treated cells that might be involved to detoxify any reactive species that were generated by the action of ac. $NaNO_2$ and GSNO [10, 18-20]. Though it is well-known for its oxidative stress response activity, but the activity of catalase may also get stimulated under nitrosative stress to overcome the hostile situation [20].

GSNOR activity was induced in GSNO treated cells. GSNOR activity was very important to reduce GSNO. It cleaves GSNO into GSSG and NH_3. Hence, the possible outcome under GSNO stress is increase in the concentration of GSSG due to the action of GSNOR and subsequent reduction of GSSG to GSH by GR. This implies that an elevated level of reduced equivalents was required to maintain redox homeostasis *in vivo*.

Results from FACS and confocal microscopy confirmed that there was no such significant difference in ROS generation in the acidified sodium nitrite treated cells as compared to the control whereas formation of was observed only in the treated cells. Therefore, it can be concluded that the observed phenomena were only due to acidified sodium nitrite mediated nitrosative stress.

Similarly, generation of RNS was also found in GSNO treated cells. There was no such significant difference in ROS generation in GSNO treated cells as compared to the control. Therefore, it can be concluded that the observed phenomena were only due to GSNO mediated nitrosative stress.

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