Chapter 3

Quantification and analysis of the ethanol production by *Saccharomyces cerevisiae* under nitrosative stress

Introduction:

Excessive production of reactive nitrogen species including NO interferes with the structure as well as function of different macromolecules like DNA, proteins, enzymes, lipids etc. *in vivo* [1-3]. Protein modifications like protein tyrosine nitration (PTN) and *S*-nitrosylation are considered as the biomarkers of nitrosative stress [2, 3]. It has been reported that mitochondrial matrix proteins are the primary target of RNS [4]. Reports also suggest that the function of respiratory chain in *S. cerevisiae* may get hampered under nitrosative stress due to inactivation of several TCA cycle enzymes [5, 6]. Aconitase (catalyzes the reaction from citrate to isocitrate), one of the important enzyme of TCA cycle, has been reported to get affected under nitrosative insult [5, -8]. It is also a well-known marker of redox stress [9]. On the other hand, alcohol dehydrogenase (ADH), an important fermentative enzyme, may act as a Thus, higher activity of ADH may affect the metabolism via modulating fermentation (i.e. ethanol formation) [10, 11]. Hence, it was very important to investigate the status of ADH and aconitase under nitrosative stress.

Therefore, in this study the activity of ADH and aconitase along with the gene expression in the presence of sub-toxic dose of acidified sodium nitrite in *S. cerevisiae* were investigated under the specified experimental condition. To establish the phenomena as the effect of nitrosative stress, some of the key experiments were also repeated using *S*-nitrosoglutathione as a nitrosative stress agent.

Results:

To determine the status of ADH and aconitase under nitrosative stress, *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. Then, the cells were harvested, lysed and cell free-extract were prepared to investigate aconitase and ADH activity. The supernatants were used to quantify the ethanol and the concentration of reducing sugar as per the protocol of Zhange *et al.* (Mentioned in materials and methods). For the gene expression analysis, RNA was isolated from *S. cerevisiae* cells and cDNA was prepared for the experiments. All these parameters were compared with the control.

Effect of acidified sodium nitrite and *S*-nitrosoglutathione on the activity of aconitase:

Under the specified experimental condition, it was observed that the specific activity of aconitase was approximately dropped by 50% in the 0.5 mM ac. $NaNO_2$ treated cells as compared to the control **[Fig. 14]**.

Whereas, aconitase activity was not detected in the presence of 0.25 mM GSNO. These data clearly suggest that aconitase activity was suppressed in the presence of stress agent under the specified experimental condition **[Fig. 14]**.

Effect of acidified sodium nitrite on *ACO* genes expression:

As it was observed that aconitase activity was only present in acidified sodium nitrite treated sample, thus, the gene expression level of *ACO* genes were only determined in presence of ac. $NaNO_2$ and compared with the control. In the presence of 0.5 mM ac. $NaNO_2$, gene expression of *ACO1* was found to be increased by 1.2 fold **[Fig. 15A]** whereas gene expression of *ACO2* was dropped by 50% as compared to the control **[Fig. 15B]** which may be the cause of the reduction in the activity of aconitase.

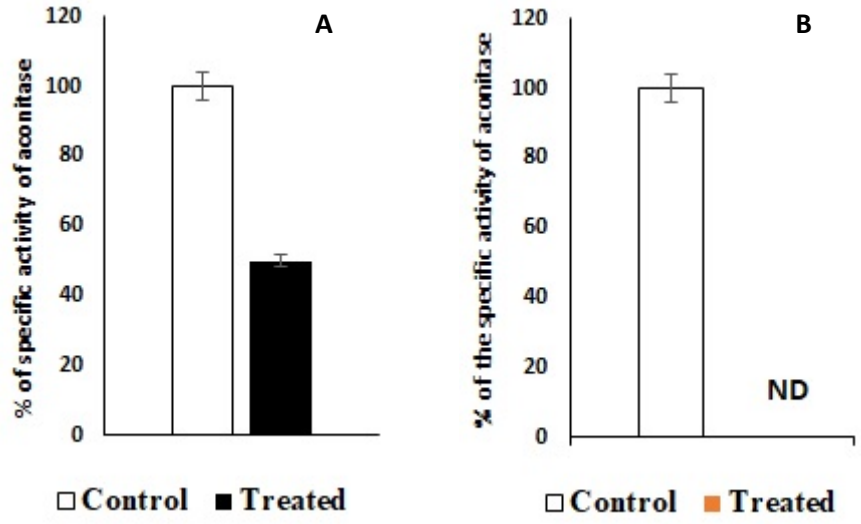

Fig. 14 (A) Effect of 0.5 mM acidified sodium nitrite and **(B)** Effect of 0.25 mM *S*-nitrosoglutathione on the specific activity of aconitase. Data is represented as the change in the percentage of specific activity as compared to the control. The enzyme assay was repeated for three times for each experimental set up and expressed as mean±SD. 100% specific activity equals to 7 mU/mg.

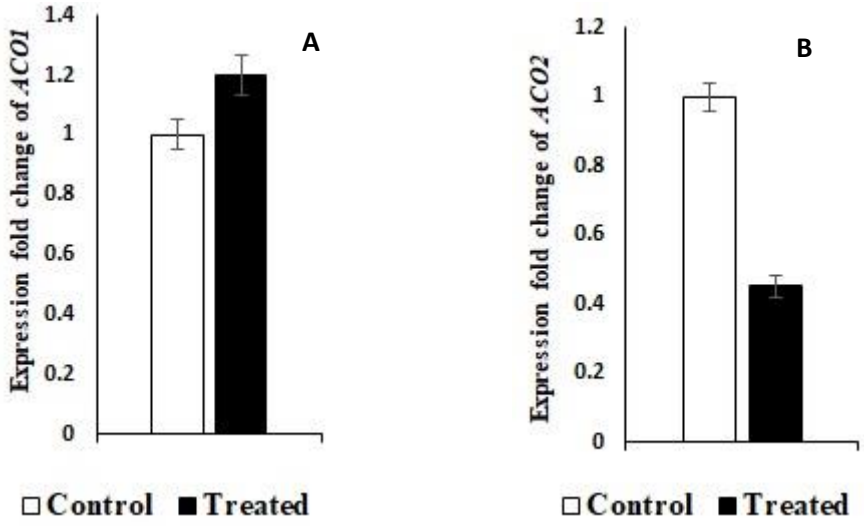

Fig. 15. (**A)** Effect of 0.5 mM acidified sodium nitrite on relative gene expression of *ACO1* and **(B)** relative gene expression of *ACO2*. The expression levels of *ACO1* and *ACO2* were normalized with that of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) in each set and expressed as relative fold change as compared to the control. Supporting information are mentioned in Table S1 and S2.

Effect of acidified sodium nitrite and on *S*-nitrosoglutathione ethanol production:

Initially, ethanol quantification was done by potassium dichromate method (Mentioned in materials and methods) and a sharp 1.3 fold increase in ethanol production was found in 0.5 mM ac. $NaNO_2$ treated cells as compared to control under the specified experimental condition. Later on, the kinetics of ethanol production was determined by a more clarified method of Zhang *et al.* (Mentioned in materials and methods). By performing this new method, the similar result was found i.e. when 0.5 mM ac. $NaNO_2$ was present, ethanol production increased significantly (~1.3 fold) in comparison to the control. The ethanol yield was increased by approximately 1.3 fold and consumption of sugar was also ~14% higher under the stress condition. The volumetric productivity was also increased by approximately 1.5 fold in the presence of 0.5 mM ac. $NaNO_2$. 66% of the theoretical ethanol yield was achieved in the presence of 0.5 mM ac. $NaNO_2$ **[Table 5]**.

In the presence of 0.25 mM GSNO, ~1.5 fold increase in ethanol production was discovered as compared to the control under the specified experimental condition. In this condition, ethanol yield was increased by approximately 1.3 fold and consumption of sugar was also 15% higher under the stress condition. The volumetric productivity was also increased by approximately 1.5 fold in the presence of 0.25 mM GSNO. 76% of the theoretical ethanol yield was achieved in the presence of 0.25 mM GSNO **[Table 5]**.

Effect of acidified sodium nitrite and *S*-nitrosoglutathione on the activity of ADH:

By performing spectrophotometric assay at 340 nm, it was found that alcohol dehydrogenase activity was increased by 1.3 fold in the presence of 0.5 mM ac. $NaNO_2$, as compared to the control **[Fig. 16A]**.

Similarly, alcohol dehydrogenase activity was increased by 3.5 fold in the presence of 0.25 mM GSNO as compared to the control **[Fig. 16B]**.

Table 5: Estimation of ethanol concentration, glucose consumption, ethanol yield, percentage of theoretical yield and volumetric productivity of 0.5 mM ac. $NaNO_2$ treated, 0.25 mM GSNO treated and untreated (control) samples of *S. cerevisiae*

Sample	Ethanol concentration (g/L)	Glucose consumed (g/L)	Ethanol yield (g/g of glucose)	% of theoretical yield	Volumetric Productivity (g/L/h)
Control	4.5±0.3	15±0.3	0.30	59	0.38
0.5 mM ac. $NaNO_2$ Treated	6±0.5	17±0.4	0.35	69	0.50
0.25 mM GSNO Treated	7±0.5	18±0.4	0.39	76	0.58

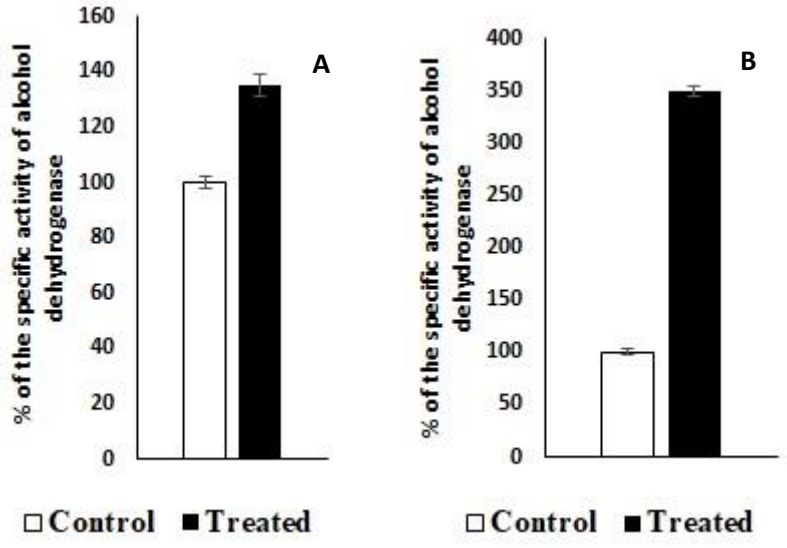

Fig. 16 (A) Effect of 0.5 mM acidified sodium nitrite and **(B)** Effect of 0.25 mM *S*-nitrosoglutathione on the specific activity of alcohol dehydrogenase. Data is represented as the change in the percentage of specific activity as compared to the control. The enzyme assay was repeated for three times for each experimental set up and expressed as mean±SD. 100% specific activity equals to 10 mU/mg.

In another set, CFE from *S. cerevisiae* was directly treated with 0.25 mM GSNO or 0.5 mM ac. $NaNO_2$ and ADH assay was performed. Interestingly, no change in ADH activity was observed in the treated CFE as compared to the untreated CFE **[Table 6]**, implying that GSNO and ac. $NaNO_2$ may not be involved in ADH protein modification. The inhibition assay of ADH was also studied using 0.1 mM 2,2,2-trifluoroethanol.

Table 6: Estimation of alcohol dehydrogenase activity of cell free extract (CFE) and treated CFE

Conditions	ADH activity (mU/mg)
CFE	4±NA
0.5 mM ac. $NaNO_2$ treated CFE	4±NA
0.25 mM GSNO treated CFE	4±NA
CFE + 2,2,2- trifluoroethanol	Not found
0.5 mM ac. $NaNO_2$ treated CFE + 2,2,2-trifluoroethanol	Not found
0.25 mM GSNO treated CFE + 2,2,2-trifluoroethanol	Not found

Effect of acidified sodium nitrite and *S*-nitrosoglutathione on *ADH* genes expression:

As ethanol production and ADH activity were significantly increased in the presence of 0.5 mM ac. $NaNO_2$ and 0.25 mM GSNO, hence, the gene expression level of *ADH1, ADH2* and *ADH3* genes under the same condition were investigated.

When 0.5 mM ac. $NaNO_2$ was present, the expression of *ADH1*, *ADH2* and *ADH3* genes were found to be increased by ~2.1 fold **[Fig. 17A]** ~2.4 fold **[Fig. 17B]** and ~3.5 fold **[Fig. 17C]** respectively as compared to the control.

Unlike 0.5 mM ac. $NaNO_2$, the expression level of *ADH1* **[Fig. 18A]** and *ADH2* **[Fig. 18B]**, were not significantly increased but the gene expression of *ADH3* **[Fig. 18C]** was increased by ~4 fold in the presence of 0.25 mM GSNO.

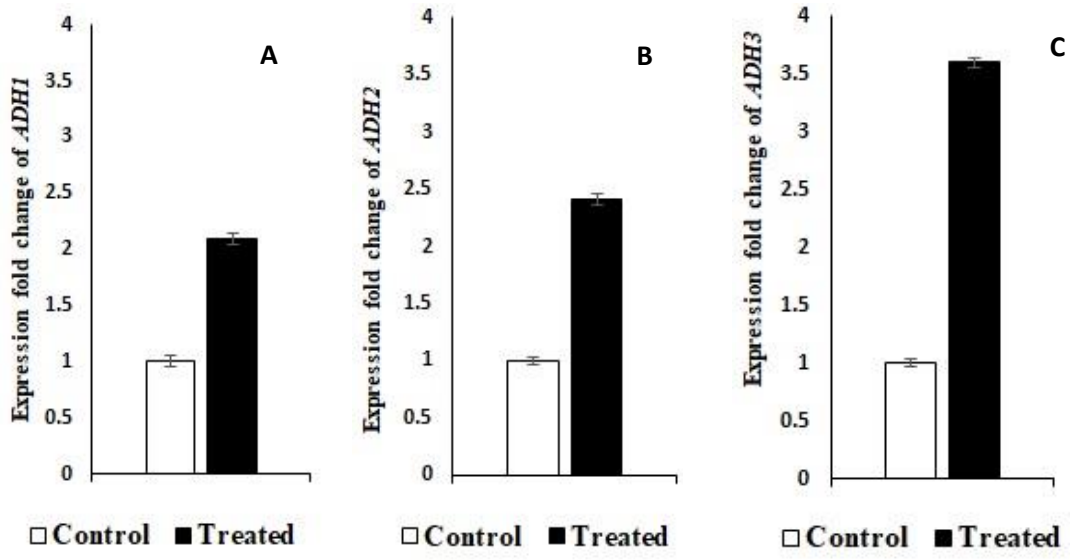

Fig. 17 (A) Effect of 0.5 mM acidified sodium nitrite on relative gene expression of *ADH1*, **(B)** relative gene expression of *ADH2*, **(C)** relative gene expression of *ADH3*. The expression level of *ADH1*, *ADH2* and *ADH3* genes were normalized with that of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) in each set and expressed as the change in relative fold change as compared to the control. Supporting information are mentioned in Table S3, S4 and S5.

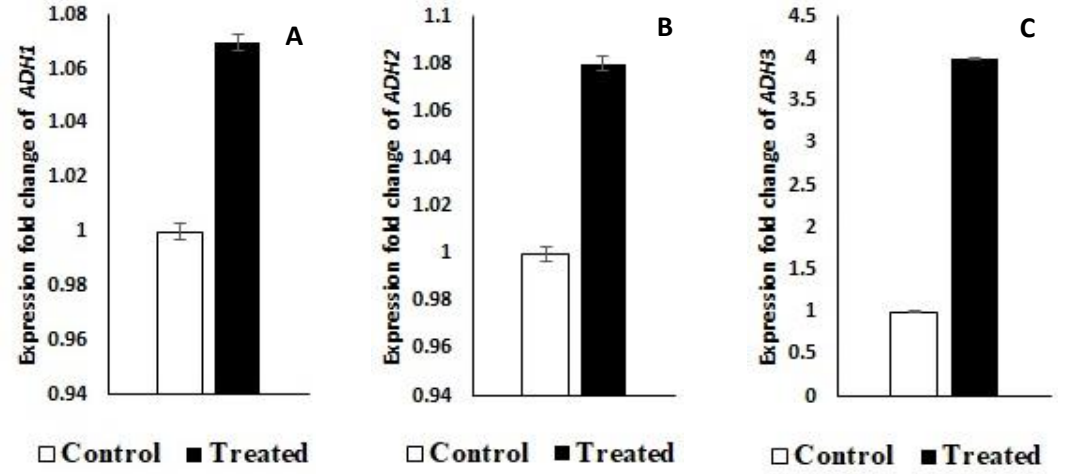

Fig. 18 (A) Effect of *S*-nitrosoglutathione on relative gene expression of *ADH1* **(B)** relative gene expression of *ADH2* and **(C)** relative gene expression of *ADH3*. The expression levels of *ADH* genes (*ADH1*, *ADH2* and *ADH3*) were normalized with that of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) in each set and expressed as relative fold change taking the normalized expression level in respective untreated control as unity. Supporting information are mentioned in Table S6, S7 and S8.

***In vitro* Protein tyrosine nitration (PTN) study and activity of pure aconitase and ADH in presence of acidified sodium nitrite:**

To see protein level modification in case of ac. $NaNO_2$ mediated nitrosative stress, formation of PTN, a key marker of redox stress [12, 13] was checked.

Depending on the concentration of ac. $NaNO_2$, PTN was assessed. By performing western blot analysis using 3-nitrotyrosine monoclonal antibody, PTN formation was observed in 0.3 and 0.5 mM ac. $NaNO_2$ treated aconitase but no impression of PTN formation was detected in untreated and 0.1 mM ac. $NaNO_2$ treated aconitase **[Fig. 19A]**. Here, 0.1 mM peroxynitrite treated aconitase was used as the positive control for this study. PTN study with pure ADH showed different result. The impression of PTN formation was only found in 0.1 mM peroxynitrite treated ADH. There was no impression of PTN formation in ac. $NaNO_2$ treated and untreated ADH **[Fig. 19B]**.

The specific activity of aconitase was also reduced with the treatment of higher concentration of acidified sodium nitrite. The reduction in the activity of aconitase was found to be the highest in 0.1 mM peroxynitrite treated sample **[Fig. 19A]**. The specific activity of ADH was found to be unaltered in acidified sodium nitrite treated samples as compared to untreated ADH but the specific activity of 0.1 mM peroxynitrite treated ADH was drastically decreased as compared to the untreated ADH **[Fig. 19B]**.

***In vitro S*-nitrosylation study and activity of pure aconitase and ADH in presence of acidified sodium nitrite:**

As GSNO is a nitrosylating agent thus *in vitro* formation of *S*-nitrosylation, an important biomarker of nitrosative stress, was checked in pure aconitase and ADH using *S*-nitrosylation western blot kit (Thermo-fisher). Here, strong signal of *S*-nitrosylation in 0.1 mM and 0.25 mM GSNO-treated aconitase was found **[Fig. 20]** but no impression of *S*-nitrosylation was recorded in GSNO-treated ADH samples (data not shown). In addition to it, the specific activity of GSNO-treated samples was drastically dropped. Whereas no significant decrease in activity was found in GSNO-treated ADH samples.

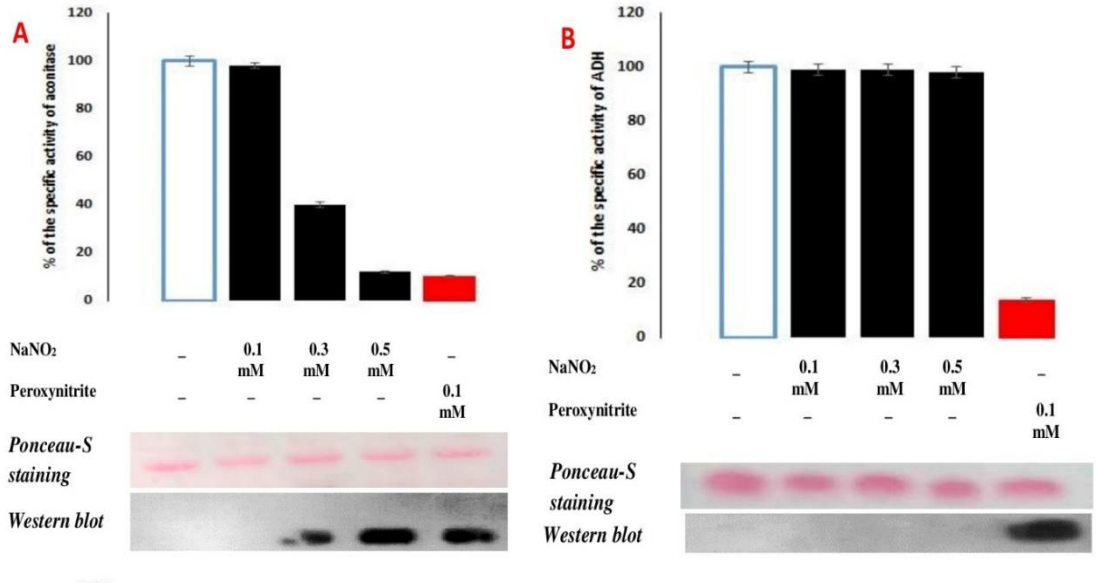

Fig. 19 Effect of different concentrations of acidified sodium nitrite (0.1, 0.3, 0.5 mM) and 0.1 mM peroxynitrite on the specific activity of pure proteins (aconitase and alcohol dehydrogenase) along with the protein tyrosine nitration (PTN) formation: **(A)** Western blotting for PTN and specific activity of aconitase. **(B)** Western blotting for PTN and specific activity of alcohol dehydrogenase. Data are expressed as the change in the percentage of specific activity as compared to the control. The assays were performed in triplicate and expressed as mean±SD. Western blot analysis for PTN was done by using anti 3-nitrotyrosine as the primary antibody and HRP conjugated goat anti-mouse IgG as the secondary antibody.

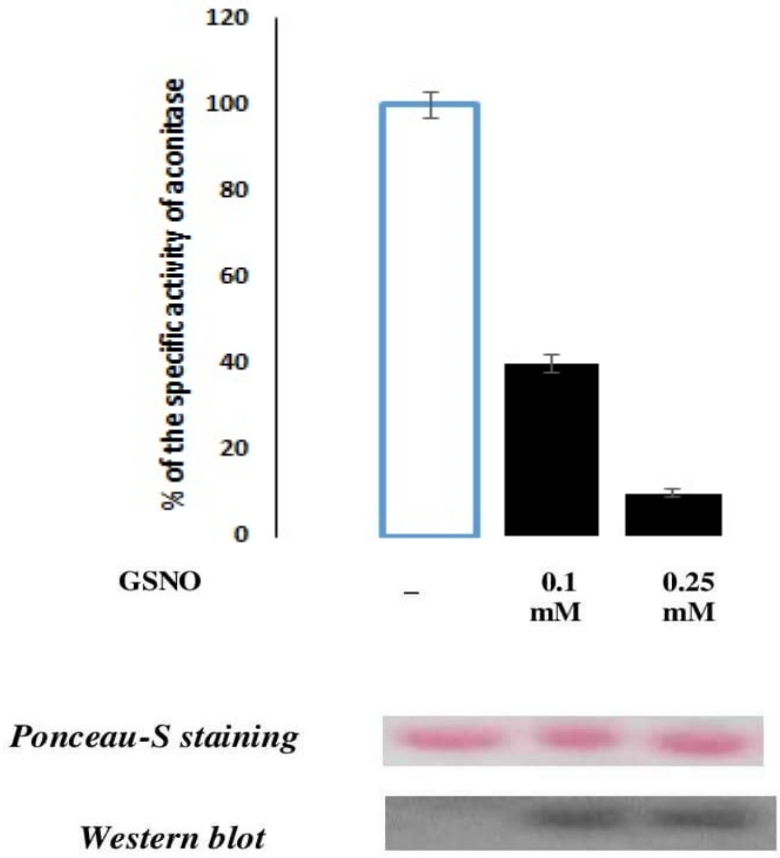

Fig. 20 Effect of different concentrations of *S*-nitrosoglutathione (0.1, 0.25 mM) on the specific activity of aconitase along with *S*-initrosylation formation: Data are expressed as the change in the percentage of specific activity as compared to the control. The assays were performed in triplicate and expressed as mean±SD. Western blot analysis for *S*-initrosylation was done by using anti-TMT as the primary antibody and HRP conjugated goat anti-mouse IgG as the secondary antibody.

Discussion:

Under the sub-toxic dose of GSNO or ac. $NaNO_2$, aconitase activity dropped significantly. As mentioned earlier, aconitase is a well-known redox stress marker [9]. This enzyme contains a Fe-S cluster in its active site. According to the evidences, oxidation of the active $[4Fe\text{-}4S]^{2+}$ can take place in presence of GSNO and paramagnetic cluster $[3Fe\text{-}4S]^{1+}$, is formed, resulting in the complete inactivation of the enzyme [14, 15]. *In vitro S*-nitrosylation formation in GSNO-treated aconitase samples also supports our findings. Whereas, western blot analysis with pure aconitase, revealed that PTN formation might be the cause of the reduced activity of aconitase under 0.5 mM ac. $NaNO_2$ stress. Tyrosine nitration generally contributes to the generation of additional negative charge to the protein and also adds comparatively bulky substituents to the protein which may lead to the alteration of local charge distribution as well as the configuration [16]. Thus, it can be deduced that 0.3 and 0.5 mM acidified sodium nitrite treatment induced PTN formation which triggered the alteration of configuration that might lead to the partial inhibition of aconitase. In addition to it, the gene expression study with 0.5 mM ac. $NaNO_2$, showed an interesting result. *ACO1* gene expression was found to be increased in presence of 0.5 mM acidified sodium nitrite. The major function of ACO1p is the conversion of citrate to isocitrate but this protein is also involved in different unrelated cellular processes, thus it acts as a moonlighting protein in yeast [17]. Among the different activities, maintaining of the mitochondrial DNA integrity is one of the important function of the ACO1p [17-19]. Hence, the higher expression of the *ACO1* indicated that the activity of ACO1p was required to maintain the mitochondrial DNA integrity in presence of 0.5 mM ac. $NaNO_2$ mediated nitrosative stress under the specified experimental condition. Unlike *ACO1*, the gene expression of *ACO2* was decreased by 50%, suggesting, 0.5 mM ac. $NaNO_2$ might affect the glucose metabolism via the TCA cycle. Altogether these results indicated that energy generation through the TCA cycle might be challenged under nitrosative stress. Because of the partial inhibition of aconitase, functioning of TCA cycle might be hampered. Thus, there was a possibility that cells might shift their metabolic flux towards formation of ethanol to increase reduced equivalent as the form of NADH inside the cell which may help to restore its cellular viability. Interestingly, increase in ethanol concentration was observed with higher glucose consumption under such condition that supports our hypothesis.

As ethanol production was increased significantly, hence, the activity of alcohol dehydrogenase (ADH) was estimated. Under nitrosative stress, the activity of ADH was increased significantly which supports the previous finding i.e. higher ethanol production. Further, the reason for this biochemical change was investigated. There were two possibilities of such modulations- either through higher expression of *ADH* genes (*ADH1*, *ADH2* and *ADH3*) or structural modification of ADH. To examine any structural modification, activity of ADH of GSNO or ac. $NaNO_2$ treated CFE was determined and compared with the activity of ADH of untreated CFE. Interestingly, CFE, treated with GSNO or ac. $NaNO_2$, showed no changes in ADH activity as compared to the ADH activity of untreated CFE. In addition, no impression of PTN was found in the presence of ac. $NaNO_2$, though Peroxynitrite, a potent nitrating agent, mediated inactivation of ADH was also reported earlier [20]. Again, no impression of *S*-nitrosylation formation was found in GSNO-treated ADH samples. Altogether these results suggest protein-level modification of ADH may not be possible in the presence of GSNO or ac. $NaNO_2$, probably due to the unavailability of suitable tyrosine and cysteine residue for nitration and *s*-nitrosylation respectively. Hence, next, the expression level of *ADH* genes (*ADH1*, *ADH2* and *ADH3*). were quantified.

In *S. cerevisiae*, ADH1 and ADH3 are mainly involved in ethanol production by using acetaldehyde as the substrate whereas ADH2 is involved in the reverse reaction i.e. production of acetaldehyde from ethanol [21]. Here, a significant increase in the expression level of *ADH3* in presence of 0.5 mM ac. $NaNO_2$ or 0.25 mM GSNO was found. A previous report showed higher ethanol production in *Dekkera bruxellensis* due to the overexpression of *ADH3* [21]. Thus, it can be concluded that ADH3 might have one of the most important role in ethanol production under nitrosative stress at least under the specified experimental condition. Interestingly, expression levels of *ADH1* and *ADH2* were only significantly increased in presence of 0.5 mM ac. $NaNO_2$. Unlike 0.5 mM ac. $NaNO_2$, almost no change in the expression of *ADH1* and *ADH2* were found in presence of 0.25 mM GSNO. The expression of *ADH2* was induced in presence of 0.5 mM ac. $NaNO_2$, which indicates that cells might be trying to utilize ethanol as a carbon source [22]. The activity of ADH2 might help to generate reducing equivalent in the form of NADH and maintain the redox status of the cell [23]. Overall, these results indicated probable metabolic reprogramming.

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