Chapter 4

Quantification and analysis of different key enzymes involved in glucose metabolism including ethanol fermentation under nitrosative stress

Introduction:

Saccharomyces cerevisiae cells have multiple and diversified mechanisms to regulate metabolic enzymes for adjusting metabolism under the different perturbations like genetic and environmental stresses, broadly termed as 'metabolic reprogramming' [1]. Several factors like transcriptional regulation, alteration in protein concentration, enzymatic activity, post translational modification, allosteric regulation etc. are mainly involved in metabolic reprogramming [1-4]. Complex interplay of genes expression under different internal and external stimuli or stress also contributes to the process [4].

In yeast cells, carbon metabolism is mainly facilitated via fermentation and TCA cycle. During fermentation, glucose first converts to pyruvate and then get reduced to ethanol, leading to the generation of energy and important intermediates which may act as growth factors. Though *S. cerevisiae* is a Crabtree positive (can generate energy via fermentation in presence of oxygen) yeast but TCA cycle is also very important for this organism to generate ATP, utilize non-fermented sugars, production of the precursors for different biosynthetic pathways and so on [1-3]. Under nitrosative stress, mainly TCA cycle enzymes are severely affected due to the protein modifications like protein tyrosine nitration, *s*-nitrosylation etc., thus, generation of energy under such condition may be hampered [5-8]. In contrast to this, different studies showed that cell viability of *S. cerevisiae* cells was not affected in the presence of sub-toxic dose or lower concentration of RNS [9-11]. But definitive studies regarding the characterization of glucose metabolism along with the metabolic reprogramming under nitrosative stress in *S. cerevisiae*, is not yet well-established.

Hence, in this study, activity of some key enzymes involved in different pathways (TCA cycle, glyoxylate pathway, PDH bypass pathway) of carbon metabolism were investigated under the specified experimental condition to delineate the glucose metabolism in the presence of acidified sodium nitrite. These data were also validated using a bioinformatics tool. This study may prove to be helpful to characterize the metabolic response of *S. cerevisiae* under acidified sodium nitrite mediated nitrosative stress.

Results:

To characterize the glucose metabolism under nitrosative stress, *S. cerevisiae* cells were first grown in YPD medium and then treated with either 0.5 mM ac. $NaNO_2$. Then, the cells were harvested, lysed and cell free-extract were prepared to investigate the activity of different key enzymes by performing enzymatic assays. All these results were compared with the control.

Effect of acidified sodium nitrite on citrate concentration and citrate synthase:

To study the citrate metabolism in the presence of 0.5 mM acidified sodium nitrite, the concentration of citrate and citrate synthase (CS) were assayed. CS is an important enzyme of the TCA cycle which catalyzes an irreversible reaction to form citrate from oxaloacetic acid (OAA) and acetyl-CoA [12]. Here, the concentration of citrate was found to be decreased by approximately 50% (both intracellular and extracellular), indicating that the synthesis of citrate might have decreased under stress condition **[Fig. 21A]**. Hence, the activity of CS was assayed. Here, the specific activity of CS was seen to be decreased by approximately 50% under the stress condition as compared to the control **[Fig. 21B]**, suggesting, the citrate metabolism as well as the TCA cycle might be hampered in the presence of 0.5 mM acidified sodium nitrite.

Effect of acidified sodium nitrite on key enzymes of glucose metabolism:

As the specific activity of CS was significantly affected in presence of 0.5 mM acidified sodium nitrite, thus next the utilization of pyruvate was checked via TCA cycle by assaying two important enzymes pyruvate dehydrogenase (PDH) [which catalyzes the conversion from pyruvate to acetyl-CoA] [12] and an anaplerotic enzyme pyruvate carboxylase (PC) [which catalyzes the formation of OAA from pyruvate] [12]. Interestingly, the specific activity of PDH and PC were observed to be decreased by approximately 50% and 15% respectively in the presence of 0.5 mM acidified sodium nitrite in comparison to the control **[Fig. 22A & B]**. Next, the fate of OAA in the TCA cycle was investigated. Therefore, the specific activity of malate dehydrogenase (MDH) which catalyzes the reversible conversion of OAA to malate, was assessed. Interestingly, it was found that the specific activity of MDH sharply increased by approximately 1.3 fold under the stress condition in comparison to the control **[Fig. 22C]**, indicating, TCA cycle was amortized under the stress condition but the higher

activity of MDH revealed that the conversion of oxaloacetic acid to malate might increase under 0.5 mM acidified sodium nitrite mediated nitrosative stress. On the other hand, ethanol production was also found to be increased under the same condition. These two phenomena jointly indicated towards a possibility of shifting of metabolic flux towards pyruvate under nitrosative stress. Hence, to check that, the activity of MDH (decarboxylating), that catalyzes the conversion of malate to pyruvate, was assayed. Here, ~1.3 fold increase in specific activity of MDH (decarboxylating) was observed in 0.5 mM ac. $NaNO_2$ treated cells **[Fig. 22D]**. Furthermore, specific activity of pyruvate decarboxylase (PDC) that catalyzes the conversion from pyruvate to acetaldehyde, was also determined. Here, a sharp 3.2 fold increase in the specific activity of PDC was found in the treated cells **[Fig. 22E]**, suggesting shifting of metabolic flux towards fermentation in presence of 0.5 mM ac. $NaNO_2$. In addition to it, a sharp decrease in the specific activity of isocitrate dehydrogenase (~50%) was observed in the treated cells. **[Fig. 22F]**. Isocitrate dehydrogenase (ICDH) is an important rate-limiting enzyme of TCA cycle [12]. Thus, it can be deduced from the obtained data that TCA cycle might be affected in presence of 0.5 mM ac. $NaNO_2$. Thus, next the activity of aldehyde dehydrogenase (ALDH), an important enzyme for the PDH-bypass pathway, was assessed [13]. Here, the activity of ALDH was found to be decreased by approximately 64% in the treated cell as compared to control **[Fig. 22G]**. Further, the activity of malate synthase (MS), an important enzyme of glyoxylate shunt (an anaplerotic variant of TCA cycle) [14], was also assessed and it was observed that the activity of malate synthase was decreased by approximately 40% under the stress condition **[Fig. 22H]**.

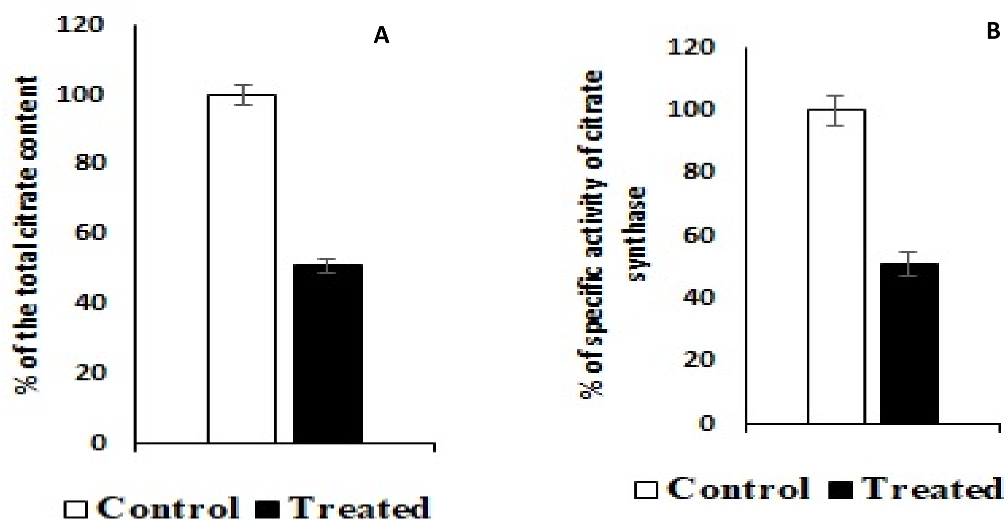

Fig. 21 Effect of 0.5 mM acidified sodium nitrite on **(A)** the total citrate content (Extracellular and Intracellular), and **(B)** specific activity of citrate synthase. Data are expressed as the change in the percentage of specific activity as compared to the control. Assays were performed in triplicate and expressed as the mean±SD. Supporting information regarding citrate content are mentioned in Table S9.

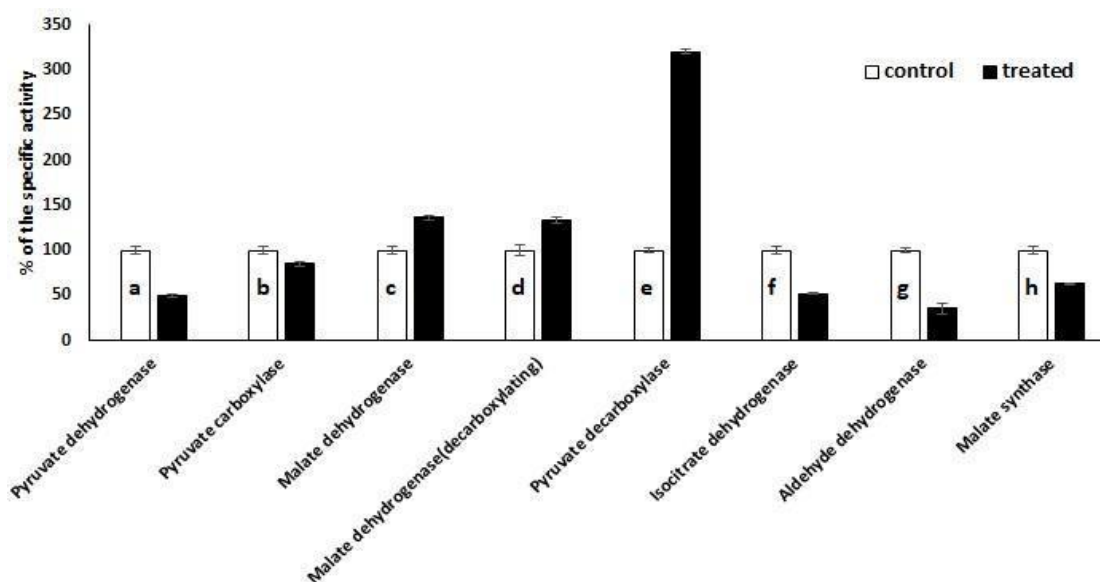

Fig. 22 Effect of 0.5 mM acidified sodium nitrite on the specific activity of **(A)** Pyruvate dehydrogenase **(B)** Pyruvate carboxylase, **(C)** Malate dehydrogenase, **(D)** Malate dehydrogenase (decarboxylating), **(E)** Pyruvate decarboxylase, **(F)** Isocitrate dehydrogenase, **(G)** Aldehyde dehydrogenase, **(H)** Malate synthase. Data are expressed as the change in the percentage of specific activity as compared to the control. Assays were done in triplicate and represented as mean±SD.

Network and functional annotation studies with the altered protein activities:

Network and functional annotation studies were performed to validate our findings that were obtained from different enzymatic assays. Under nitrosative stress, the enzymes with altered activity, were subjected for the analysis. It was found that malate dehydrogenase and pyruvate decarboxylase predominantly participated in the activated enzyme network with the highest number of connections **[Fig. 23A]**. Due to the higher activity of these two enzymes, it was predicted that yeast cellular system might be involved primarily in biological processes such as pyruvate metabolic process, malate metabolic process, and gluconeogenesis **[Table 7]** whereas the highest connectivity was found in citrate synthase, isocitrate lyase, pyruvate dehydrogenase and aconitase in the network generated by the enzymes with decreased activity under nitrosative stress **[Fig. 23B]**. In connection with the downregulated enzymes, TCA cycle, glyoxylate cycle, glutamate biosynthetic process, and acetate biosynthetic process were predicted to be negatively affected under the stress condition. From the point of view of cellular component, the enzymes at mitochondrial matrix or Mitochondrion, were predicted to be the most abundant. In addition to this, the activity of malate dehydrogenase activity and alcohol dehydrogenase (NAD) were predicted as the most enriched molecular functions in the treated yeast cells. On the other hand, molecular functions with the aldehyde dehydrogenase activity, transferase activity, transferring acyl groups, acyl groups converted into alkyl, and lyase activity, were predicted to be downregulated due to the decreased activity of these enzymes **[Table 7]**.

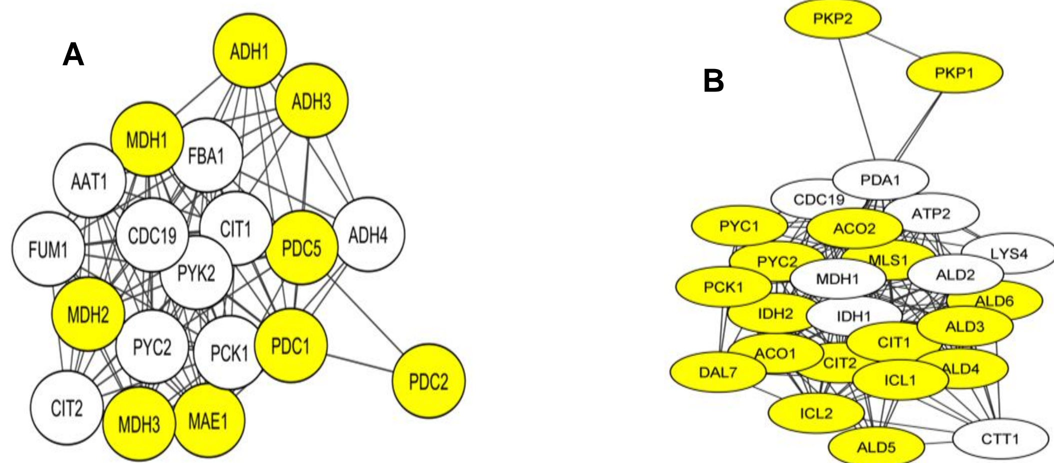

Fig. 23 Network representation of enzymes in the presence of 0.5 mM acidified sodium nitrite. **(A)** Network representation of enzymes with increased activities and **(B)** Network representation of enzymes with decreased activities. Highlighted colour denotes the enzymes with experimentally validated activities.

Table 7. Functional enrichment by activation/ deactivation of enzymes

	Term	% of genes	P-Value	Benjamini adjusted P-Value
Enrichment by activated enzymes due to stress				
Biological Process	pyruvate metabolic process	31.6	3.8E-10	8.7E-9
	malate metabolic process	26.3	3.8E-10	8.7E-9
	Fermentation	10.5	9.7E-3	4.0E-2
Cellular Components	mitochondrial matrix	31.6	3.1E-5	4.1E-4
	cytosol	42.1	2.1E-2	9.0E-2
Molecular Function	malate dehydrogenase activity	15.8	3.8E-5	6.1E-4
	alcohol dehydrogenase (NAD) activity	15.8	1.9E-4	2.3E-3
	Pyruvate kinase activity	26.3	3.6E-4	3.5E-3
Enrichment by deactivated enzymes due to stress				
Biological Process	tricarboxylic acid cycle	38.5	2.8E-15	1.5E-13
	glyoxylate cycle	19.2	6.6E-8	1.2E-6
	acetate biosynthetic process	11.5	1.2E-4	9.1E-4
Cellular Components	Peroxisomal matrix	42.3	4.3E-11	9.0E-10
	mitochondrial nucleoid	15.4	1.3E-4	9.2E-4
Molecular Function	aldehyde dehydrogenase activity	19.2	1.7E-7	9.3E-6
	transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer	15.4	9.8E-6	1.3E-4
	lyase activity	23.1	1.1E-4	9.7E-4

Discussion:

Under the sub-toxic dose of acidified sodium nitrite, the activity of some of the TCA cycle enzymes were found to be decreased significantly. Among the enzymes of the TCA cycle, CS is very important for mitochondrial functioning. Hence, the reduction

in the specific activity of CS in presence of 0.5 mM acidified sodium nitrite, indicated that the mitochondrion functioning was highly affected under nitrosative stress [15]. In addition to it, reduction in the activity of ICDH (catalyzes the conversion from isocitrate to α-ketoglutarate) [12], and PDH (catalyzes the conversion from pyruvate to acetyl CoA) [12], pointed towards depletion in citrate metabolism under nitrosative stress. Previous reports suggest that ICDH and PDH activity can be affected under redox stress i.e. oxidative and nitrosative stress [16, 17]. Formation of acetyl CoA is the vital factor for the shifting of glucose metabolic flux towards respiration [12]. The formation of acetyl-CoA can also occurr by a PDH-independent alternative pathway known as PDH-bypass pathway where the activity of PDC, ALDH are required among other enzymes [13]. Reduction in the activity of ALDH (oxidizes acetaldehyde to acetate [13]) might affect the acetyl-CoA production. In addition, lower availability of acetyl-CoA might also affect the activity of MS under stress condition. MS is an important enzyme of glyoxylate cycle, an anaplerotic variant of TCA cycle present in *S. cerevisiae* [14]. Hence, reduced activity of MS might also affect the glyoxylate cycle. Acetyl-CoA can also act as the positive allosteric modulator of PC, important anaplerotic enzyme [18]. It replenishes the intermediates of TCA cycle by catalyzing the reaction from pyruvate to oxaloacetic acid [12]. Thus, lower production of acetyl-CoA in presence of 0.5 mM acidified sodium nitrite, might interfere with the activity of PC [12, 18]. Hence, it can be concluded that the requirement for the replenishment of the intermediates of TCA cycle might be affected in presence of 0.5 mM acidified sodium nitrite, suggesting reduction in TCA cycle under nitrosative stress.

The elevated activity of MDH and MDH (decarboxylating) in presence of 0.5 mM acidified sodium nitrite suggested the possibility that OAA, formed by the activity of PC, might have been rerouted to pyruvate via malate formation. Hence, it can be understood that the flow of glucose metabolic flux towards the TCA cycle was reduced. Previous report also suggests that malate can cross the mitochondrial membrane but OAA cannot [12]. Thus, it can be understood that MDH activity was upregulated to form malate from OAA and subsequent conversion of malate to pyruvate by the activity of MDH (decarboxylating) under nitrosative stress. Though the affinity of MDH (decarboxylating) is very low (K_m= **50 mM),** but the activity of this enzyme can be induced in *S. cerevisiae* under adverse conditions like starvation [19]. This enzyme is also involved in accumulating the intracellular flux of NADPH [20], an important factor

of stress response [21], suggesting the role of MDH (decarboxylating) as a stress response enzyme. On the other hand, MDH can also participate in the generation of cytosolic NADH, an important factor of antioxidant system and energy metabolism [12, 22]. Therefore, it can be understood that in presence of 0.5 mM acidified sodium nitrite, when TCA cycle was heavily affected, higher activity of the MDH and MDH (decarboxylating) contributed for the generation of energy intermediates which in turn shifted the glucose metabolic flux towards fermentation. It has also been reported earlier that the activity of MDH (decarboxylating) can be upregulated during alcoholic fermentation in *S. cerevisiae* [23]. Report also suggest that the activity of MDH (decarboxylating) can be strongly induced at the time of switching of the metabolic flux from respiration to fermentation in *S. cerevisiae* [24]. In addition to it, the higher activity of PDC and ADH under stress condition also suggested the upregulation of ethanol fermentation. Thus, a metabolic reprogramming via shifting of metabolic flux from respiration to fermentation might have taken place in *S. cerevisiae* under acidified sodium nitrite mediated nitrosative stress. A model of metabolic reprogramming in *S. cerevisiae* in the presence of 0.5 mM acidified sodium nitrite mediated nitrosative stress, is proposed in **Fig. 24**.

To validate our findings, the wet lab data were also subjected for functional enrichment analyses and fermentation was predicted as one of the most activated biological processes under the experimental condition, clearly corroborating with the findings. In addition, malate metabolic process and pyruvate metabolic process were also predicted to be upregulated biological process whereas TCA cycle, glyoxylate shunt were predicted to be downregulated under such condition, clearly indicating towards higher ethanol production in *S. cerevisiae* under 0.5 mM acidified sodium nitrite mediated nitrosative stress. This metabolic reprogramming might not only be very important for the energy generation but it seemed like a part of the nitrosative stress response strategies. This reprogrammed glucose metabolism might be coupled with the cellular antioxidant machinery to overcome the stress condition. Hence, the cell viability was not significantly altered in 0.5 mM acidified sodium nitrite treated cells as compared to the control.

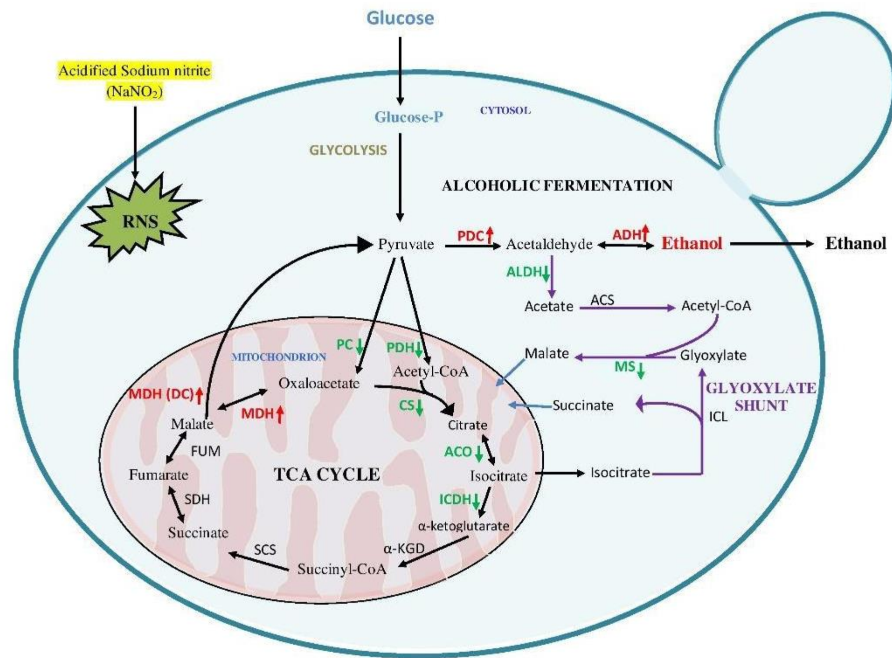

Fig. 24 Proposed switching of glucose metabolism in the presence of 0.5 mM acidified sodium nitrite. green arrows represent upregulated enzymes and red arrows represent downregulated enzymes in the presence of 0.5 mM acidified sodium nitrite. In this condition, energy generation through TCA cycle was compromised due to the lower activity of pyruvate dehydrogenase (PDH), citrate synthase (CS), aconitase (ACO), isocitrate dehydrogenase (ICDH), pyruvate carboxylase (PC) but the glucose metabolic flux was rerouted via higher activity of malate dehydrogenase (MDH) and malate dehydrogenase (decarboxylating) [MDH(DC)] towards pyruvate which was further metabolized via the fermentative pathway with the help of higher activity of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) which resulted in higher production of ethanol. In addition to it, activity of malate synthase (MS) and aldehyde dehydrogenase (ALDH) were also reduced that might affect the glyoxylate shunt (an anaplerotic variant of TCA cycle) and PDH-bypass pathway (an alternative route of acetyl-CoA synthesis without the activity of PDH).

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