Chapter 1

Effect of NO and Reactive nitrogen species on the growth of wild type and flavohemoglobin deleted strain of *S. cerevisiae.*
1.1. Introduction

Flavohemoglobin is the main protective mechanism against NO in many microorganisms. Yeast flavohemoglobin encoded by the gene YHB1 and Yhb1 protein has been found to consume NO very efficiently [Crawford and Goldberg, 1998; Kim et al., 1999; Lewinska and Bartosz, 2006]. Yeast flavohemoglobin, a homologue of the mammalian cytohemoglobin, basically functions as an oxidoreductase. It is found to be important both under oxidative as well as nitrosative stress [Zhao et al., 1996; Buisson et al., 1998; Liu et al., 2000]. It mainly acts as nitric oxide dioxygenase and utilizes O\textsubscript{2} and NAD(P)H to convert NO to nitrate. *Saccharomyces cerevisiae* is an excellent model for studying the effect of NO and RNS on cellular growth as it can grow both under fermentative and respiratory proficient conditions. *S. cerevisiae* cells show fermentative growth in media containing glucose whereas respiratory proficient growth is observed when glycerol is used as sole carbon source. Previous studies showed that deletion of flavohemoglobin do not alter cell growth of *S. cerevisiae* which indicate that flavohemoglobin is not essentially required for the cell survival. However, distinct role of flavohemoglobin in *S. cerevisiae* has been mentioned under both oxic and anoxic conditions. It is found to be distributed in mitochondria and cytosol under anoxic condition indicating its important role to combat nitrosative stress. The extent of nitrosative stress by NO or RNS donors on any microorganism depends on several factors including the growth condition of the organism, its growth phase, concentration of NO or RNS donors and the duration of treatment. Previous studies showed that the effect of nitric oxide on *S. cerevisiae* was microbiostatic, not microbicidal [Bhattacharjee et al., 2010].

Before applying NO and RNS donors on *S. cerevisiae*, it is important to study the growth of wild type and flavohemoglobin deleted strains of *S. cerevisiae* under aerobic condition in different media e.g. complex and synthetic media containing glucose or glycerol as sole carbon source. To determine the effect of NO and RNS on *S. cerevisiae* growth, both wild type and flavohemoglobin deleted strain were used. In these studies, using both wild type and flavohemoglobin deleted ΔYhb1 stain of *S. cerevisiae*, the effects of Metal nitrosyl nitroprusside like Sodium Nitroprusside (SNP) which is a NO\textsuperscript{+} donating compound, nitrosothiols, nitrite and nitrate were analyzed in synthetic media containing graded concentrations of them.
1.2. Results

1.2.1. Flavohemoglobin deletion in *S. cerevisiae* does not affect cellular growth

To determine the effects of flavohemoglobin deletion on cell growth, *S. cerevisiae* cells were allowed to grow in both fermentative as well as respiratory proficient conditions. Cell growth was monitored for 24 hours by measuring the optical density at 600 nm every two-hour interval (as described in Materials and Methods) and plotted with respect to time to obtain the growth curve. Fig. 1a shows the growth curve of Y190 and ΔYhb1 strains of *S. cerevisiae* under fermentative growth condition in Yeast extract, Peptone, Dextrose (YPD) media. Fig. 1b represents the growth curve pattern of Y190 and ΔYhb1 strains of *S. cerevisiae* under respiratory proficient condition in Yeast extract, Peptone, Glycerol (YPG) media. The growth of *S. cerevisiae* cells was relatively slower in YPG media than in YPD media as glycerol was not a preferred carbon source for *S. cerevisiae* and fermentation was not possible under these conditions. Fig. 1c represents growth of Y190 and ΔYhb1 strains of *S. cerevisiae* in synthetic media (SC). Flavohemoglobin deleted ΔYhb1 strains of *S. cerevisiae* showed identical growth characteristics as compared to wild type strain in both fermentative as well as respiratory growth conditions.

1.2.2. Effects of Reactive Nitrogen Species (RNS) on the growth of wild type and flavohemoglobin deleted strain of *S. cerevisiae*

1.2.2.1. Effect of Sodium Nitroprusside (SNP) on growth

Sodium Nitroprusside is a NO donor compound which donate NO$^+$ (nitrosonium ion) (Fig. 2a). To check the effects of SNP on cell growth of *S. cerevisiae*, graded concentrations of SNP was added to synthetic media (SC Media) and cell growth was monitored for 24 hours by measuring the optical density at 600 nm every two-hour interval. Wild type strain of *S. cerevisiae* showed growth inhibition in SC medium containing SNP in a dose dependent manner (Fig. 2b). When cells were treated with graded concentrations (0, 1 and 3 mM) of sodium nitroprusside (SNP), ΔYhb1 strain was found to be more sensitive to SNP as compared to wild type Y190 strain of *S. cerevisiae*.
in presence of 1 mM SNP (Fig. 2c). No further significant decrease in growth was observed in ΔYhb1 strain of *S. cerevisiae* in presence of 3 mM SNP.

### 1.2.2.2. Effect of S-nitroso glutathione on growth

To study the effect of S-nitrosoglutathione (GSNO), wild type (Y190) *S. cerevisiae* cells were allowed to grow in presence of the graded concentrations of GSNO (Fig. 3a) (i.e. 0, 1, 3 and 5 mM) in SC media and cell growth was monitored for 24 hours by measuring the optical density at 600 nm every two-hour interval. There was a decrease in O.D. value in Y190 cells in presence of 1 mM GSNO, but the change was much less than that of SNP treated wild type cells of *S. cerevisiae* (Y190) (Fig. 3b). There was very little change in O.D. value in 3 mM and 5 mM GSNO treated cells of wild type strain of *S. cerevisiae* compared to 1 mM GSNO treatment. The ΔYhb1 strain showed dose dependent inhibition of growth upon GSNO addition (Fig. 3c).

### 1.2.2.3. Effects of nitrates and nitrites on the growth of *S. cerevisiae*

In order to check the effects of nitrite in growth of *S. cerevisiae* wild type and upon flavohemoglobin deletion, Y190 wild type and isogenic ΔYhb1 strain were grown in presence of 1 mM nitrite in SC media and cell growth was monitored for 24 hours by measuring the optical density at 600 nm every two-hour interval (Fig. 4a). Interestingly, ΔYhb1 strain of *S. cerevisiae* was much more tolerant towards 1mM nitrite compared to wild type strain (Y190). However, there was no effect of nitrate upon growth of *S. cerevisiae* wild type and ΔYhb1 mutant as there was no difference in growth in presence of 1mM nitrate in the media (Fig. 4b).
FIGURE 1.1 Growth curve of wild type Y190 and FlavoHb YHB1 deleted strains of *S. cerevisiae* in fermentative and respiratory media. The data represents mean ± SE, for n=3 experiments.
FIGURE 1.2 (A) Chemical formula of Sodium Nitroprusside (SNP). Growth curve of wild type Y190 and ΔYHB1 deleted strain in presence of Sodium nitrprusside (SNP, B and C). The data represents mean ± SE, for n=3 experiments.
FIGURE 1.3 (A) Chemical formula of S-nitroso glutathione (GSNO). Growth curve of wild type Y190 and \( \Delta Yhb1 \) deleted strain in presence of S-nitroso glutathione (GSNO, B and C) in synthetic complete media. The data represents mean ± SE, for n=3 experiments.
FIGURE 1.4 Growth curve of wild type Y190 and ΔYhb1 deleted strain in presence of 1mM Nitrite (A) and 1mM Nitrate (B) in synthetic media. The data represents mean ± SE, for n=3 experiments.
1.3. Discussion

Results obtained from the growth studies on wild type (Y190) and ΔYhb1 strains of *S. cerevisiae* in YPD, YPG and SC media under control conditions i.e. in absence of NO and reactive nitrogen species indicated that growth rate remained identical for both the strains if they were grown in identical media under similar experimental conditions. The lack of flavohemoglobin was apparently not detrimental to the cells grown under normal conditions in both respiratory proficient and in fermentative media.

Flavohemoglobin is known to combat nitrosative stress in *S. cerevisiae* and many other microorganisms [Liu et al., 2000]. The present study indicated that *S. cerevisiae* cells could tolerate various reactive nitrogen species donor compounds as for e.g. SNP, GSNO, NaNO2 and NaNO3 in the presence and in the absence of flavohemoglobin. However, *S. cerevisiae* cells showed more sensitivity towards SNP as well as GSNO in the absence of flavohemoglobin which indicated the presence of second line of defense in *S. cerevisiae* against nitrosative stress. These growth studies corroborated well with the previous studies conducted in respiratory proficient medium [Bhattacharjee et al., 2010]. Sodium nitroprusside (SNP), a NO+ donor, is much more toxic to *S. cerevisiae* than GSNO as SNP can inhibit cellular respiratory chain and mitochondrial function. The significant decrease in growth rate of wild type Y190 strain of *S. cerevisiae* following 3mM SNP treatment in SC media could be explained by the cyanide toxicity generated from SNP at higher concentrations. GSNO is an endogenous S-nitrosothiol (SNO) that plays a critical role in Nitric oxide (NO) signaling and is a source of bioavailable NO. Externally added GSNO had no significant effect on the growth of wild type Y190 strain of *S. cerevisiae* in SC media compared to ΔYhb1 strain which showed more sensitivity towards it.

It has been reported earlier that alcohol dehydrogenase III (ADH III encoded by SFA1 gene) of *S. cerevisiae* is actually GSNO reductase which converts GSNO to GSSG and NH3. It is important to note that GSNO is also a NO+ donor inside the cell. So it is conceivable that flavohemoglobin could play important role in the detoxification instead of having GSNO reductase. Thus it can be concluded that flavohemoglobin not only protect cells from NO donor compounds but also protect from RNS.
The effect of nitrite on wild type (Y190) as well as ΔYhb1 strain of *S. cerevisiae* was very much surprising. The higher sensitivity of wild type strain of *S. cerevisiae* towards nitrite could be explained by the fact that reduced efflux of nitrite from it. It has been reported that SSU1 is a sulphite exporter in *S. cerevisiae* [Park and Bakalinsky 2000] but in a recent observation, SSU1 was found to excrete nitrite and nitrate in the nitrate assimilatory yeast *Hansenula polymorpha* and also in *S. cerevisiae* [Cabrera *et al.*, 2014]. Another study showed that flavohemoglobin deletion caused over expression of the SSU1 gene at the transcriptional level under nitrosative stress in *S. cerevisiae* [Horan *et al.*, 2006]. Thus, it is possible that absence of flavohemoglobin induce *S. cerevisiae* cells to up regulate SSU1 for the clearance of nitrite from the cells which can be a precautionary measure under nitrosative stress.