Differential catalase activity and tolerance to hydrogen peroxide in the filamentous cyanobacteria *Nostoc punctiforme* ATCC 29113 and *Anabaena* sp. PCC 7120

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Abstract

Photoautotrophic cyanobacteria often confront hydrogen peroxide (H2O2), a reactive oxygen species potentially toxic to cells when present in sufficiently high concentrations. In this study, H2O2 tolerance ability of filamentous cyanobacteria Nostoc punctiforme ATCC 29133 (Nostoc 29133) and Anabaena sp. PCC 7120 (Anabaena 7120) was investigated. Nostoc 29133 was better able to tolerate H2O2-induced inhibition of chlorophyll a and photosystem II performance, as compared to Anabaena 7120. The intracellular hydroperoxide level (indicator of oxidative status) also did not exhibit as much a rise in Nostoc 29133, as it did in Anabaena 7120 after H2O2 treatment. Accordingly, Nostoc 29133 showed higher intrinsic constitutive catalase activity than Anabaena 7120 indicating that the superior tolerance of Nostoc 29133 stems from its higher ability to decompose H2O2. It is suggested that difference in H2O2 tolerance between closely related filamentous cyanobacteria, as is borne out by this study, may be taken into account for judicious selection and effective use of strains in biotechnology.

Introduction

Hydrogen peroxide (H_2O_2) is one of several reactive oxygen species (ROS) produced as a by-product of photosynthesis and/or respiratory processes in aerobic organisms (Latifi et al. 2009; Imlay 2013). The oxygen evolving photosynthetic cyanobacteria, major drivers of global carbon and nitrogen cycle and potential sources of biofuels and commodity chemicals, produce H_2O_2 mainly through superoxide dismutase (SOD) catalyzed disproportionation of superoxide radical ($O_2^{\bullet-}$), a by-product of photosynthetic electron transport activity (Banerjee et al. 2013; Singh et al. 2014; Kitchener and Grunden 2018). Incomplete oxidation of H_2O at the donor side of photosystem II (PSII) also generates H_2O_2 in cyanobacteria (Pospíšil 2009). A wide variety of naturally occurring stressors such as high light, ultraviolet rays, salinity, herbicides, heavy metals, high and low temperature etc. further increase the intracellular concentration of H_2O_2 in cyanobacteria (Latifi et al. 2009; Chauvat and Chauvat 2015; Mironov et al. 2019). Besides, cyanobacteria may also encounter H_2O_2 sourced from metabolic activities of other organisms or from photo-oxidation of chromophoric dissolved organic matter in their natural environments (Diaz and Plummer 2018; Zinser 2018).

While low-concentration H_2O_2 may function as a second messenger in cell signal transduction pathways, sufficiently high concentrations of H_2O_2 cause oxidative stress leading to loss of membrane integrity, destruction of light-harvesting pigments, impairment of PSII reaction center protein D1 and photosynthetic activity, and ultimately cell death in cyanobacteria (Drábková et al. 2007 a, b; Nishiyama et al. 2011; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). It has been suggested that such adverse effects are most often not directly caused by H_2O_2, but rather by the ROS hydroxyl radical ($HO^{\bullet}$) formed from interaction of H_2O_2 with free intracellular ferrous iron via Fenton chemistry (Imlay 2013). Hence, cyanobacteria must promptly neutralize H_2O_2 to avoid formation of cell lethal $HO^{\bullet}$.

Cyanobacteria possess various antioxidative enzymes such as thiol-specific peroxiredoxins (Prxs) and catalases to neutralize H_2O_2 (Latifi et al. 2009; Banerjee et al. 2013). Whereas, Prxs reduce low concentrations of H_2O_2 (K_M in µM range), catalases efficiently decompose high concentrations of H_2O_2 (K_M in mM range) (Tichy and Vermaas 1999). Comparative genome sequence analysis in cyanobacteria has revealed that unlike Prxs, the distribution of catalases is not uniform; a large number of them (nearly 50 %) lack catalase-encoding gene (Bernroitner et al. 2009). Accordingly, several studies also revealed higher sensitivity to H_2O_2 in some cyanobacteria which lack catalase, such as the unicellular fresh water *Microcystis aeruginosa* and marine-dwelling *Prochlorococcus* (Morris et al. 2011; Mikula et al. 2012). Conversely, the ones containing catalase (heme-dependent KatG) like unicellular, non-diazotrophic cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 have been shown to tolerate high concentrations of H_2O_2 (Tichy and Vermaas 1999; Perelman et al. 2003). On the other hand, many KatG-lacking filamentous diazotrophic cyanobacteria, particularly the species of *Anabaena*, which possess at least one catalase with manganese as cofactor (Mn-catalase), are reported to lack catalase activity, irrespective of whether they were grown in the presence or absence of H_2O_2 (Bagchi et al. 1991; Bernroitner et al. 2009; Banerjee et al. 2012; Chakravarty et al. 2016; Ballal et al. 2020). Correspondingly, *Anabaena* sp. PCC 7120 despite possessing two Mn-catalases, was more sensitive to H_2O_2, as compared to *Synechocystis* sp. PCC 6803 (Pascual et al. 2010). A report also suggests that owing to higher basal level catalase activity *Synechococcus* sp. PCC 7942 is more tolerant to H_2O_2 than *Synechocystis* sp. PCC 6803 (Gupta and Ballal 2015). It is presently unclear whether variation in H_2O_2 tolerance also exists among filamentous cyanobacteria. Such information may be important, as this may lead to identification of stress resilient strains to be used in biotechnological applications (Kitchener and Grunden 2018).

In this study, H_2O_2 tolerance ability of *Nostocpunctiforme* ATCC 29133 (hereafter *Nostoc* 29133) was evaluated with respect to the reference strain *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120) to determine possible differences, if any, between these two taxonomically closely related filamentous cyanobacteria. However, unlike *Anabaena* 7120, *Nostoc* 29133 has a symbiotic origin and isolated from the cycad *Macrozamia* (Meeks et al. 2001; Campbell et al. 2008). *Nostoc* 29133 has previously been shown to adapt to a variety of stresses, including UVA radiation, herbicide methyl viologen and heavy metals (Soule et al. 2009; Moirangthem et al. 2014; Hudek et al. 2017). This cyanobacterium is also considered a potential candidate for production of biofuels and many other high-value compounds (Moraes et al. 2017). The findings presented in this study show that *Nostoc* 29133 is relatively more tolerant to H_2O_2 than *Anabaena* 7120 due to its higher intrinsic constitutive H_2O_2 decomposition activity. This suggests that differences in H_2O_2 tolerance may exist even between closely related filamentous cyanobacteria. Further, the unique H_2O_2 stress-tolerant property of *Nostoc* 29133 is likely to add to its biotechnological value.

Materials And Methods

Cyanobacterial strains and culture conditions

The batch cultures of *Nostoc* 29133 (*Nostoc punctiforme* strain ATCC 29133-S, also known as UCD 153, Campbell et al. 2008) and *Anabaena* 7120 (*Anabaena* sp. PCC strain 7120) were grown at 25 °C in BG11-liquid medium, pH 7.5, containing 17.6 mM sodium nitrate buffered with equimolar 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) from stock cultures maintained on slants containing BG11-solid medium with 1.5 % agar (Rippka et al. 1979). The cultures were continuously illuminated with cool fluorescent light (photon fluence rate of 20-23 μmol m^{-2} s^{-1}) during growth period, as described earlier (Moirangthem et al. 2014).

Determination of H_2O_2 tolerance

For H_2O_2 tolerance assay, the actively growing cultures, 100 ml each of *Nostoc* 29133 and *Anabaena* 7120, were pooled separately and washed twice with fresh BG11-medium. Such cultures were inoculated separately in 30 ml of fresh BG11-medium at equal chlorophyll *a* (Chl *a*) concentration. The H_2O_2 stock (30 % v/v; Merck, India) was diluted to 10 mM concentration with sterile Milli-Q water and subsequently different volumes of H_2O_2 were added to the cyanobacterial cultures at the start of the experiment to obtain concentrations ranging from 0-0.5 mM H_2O_2, and incubated as described above. Growth was monitored periodically by measuring the concentration of Chl *a* spectrophotometrically (Cary 60, Agilent, USA) in methanolic extracts of cyanobacterial cultures using absorbance value at 663 nm × 13.43, where 13.43 represents extinction coefficient of Chl *a* (Mackinney 1941; Moirangthem et al. 2014).

Pulse amplitude modulated (PAM) fluorometry

The maximal efficiency of PSII photochemistry (F_v/F_m), which is a common and quick indicator of photosynthetic performance of cells, was measured in H_2O_2 treated and untreated cultures of both the cyanobacterial species after 15 min of dark adaptation using a Dual-PAM-100 fluorometer (Waltz, Effeltrich, Germany), as described earlier (Moirangthem et al., 2014). The F_v/F_m values were derived from $F_v = (F_m-F_0)$, where F_v represents the variable fluorescence signal, F_0 minimal fluorescence signal of dark adapted cells, and F_m the maximal fluorescence signal after application of a saturating light pulse (Schreiber et al. 1995).

Determination of total intracellular hydroperoxide

The total hydroperoxide levels was measured in cyanobacterial cells after incubation in the presence of different concentrations of H_2O_2 (0-0.5 mM) for one day by a ferrous oxidation/xylenol orange (FOX) assay method as described earlier (DeLong et al. 2002; Moirangthem et al. 2014; Wolff 1994).

Determination of catalase activity

The cultures of *Nostoc* 29133 and *Anabaena* 7120 were exposed to increasing concentrations of H_2O_2 for a day. Such cells were centrifuged at 2300×*g* for 5 min, and the cell pellets obtained were washed twice with 36 mM potassium phosphate buffer (pH 7.4). The cell-free extracts were obtained by sonication of

the cell pellets in the same buffer added with 1 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF), as described earlier (Moirangthem et al. 2014). The total protein in cell extracts was measured according to Bradford (1976). 50 µg of total protein was added to 50 mM phosphate buffer (pH 7) and 10 mM H_2O_2 in a final volume of 1 ml. Catalase activity was determined by measuring the disappearance of H_2O_2 at 240 nm using an extinction coefficient of 43.6 M^{-1} cm^{-1} (Beers and Sizer 1952).

Statistical analysis

All experiments were performed at least three times and the results are presented as mean ± standard deviation. The significant differences (*P* values less than 0.05) were analyzed by Student's two-sample *t*-test (Microsoft word version 10) between control and H_2O_2 treated cyanobacterial cultures.

Results And Discussion

Nostoc 29133 exhibits higher tolerance to H_2O_2 than *Anabaena* 7120

Cyanobacteria are often exposed to H_2O_2 generated in surrounding environments (Diaz and Plummer 2018; Zinser 2018). Being a small and neutral molecule, extracellular H_2O_2 can readily cross cell membranes and enter into cells. At high concentrations, H_2O_2 can be highly damaging to cellular growth and metabolism, especially if there is free ferrous iron available in the cell since $HO^{\bullet}$ radicals will be formed by Fenton chemistry (Latifi et al. 2009; Banerjee et al. 2013; Imlay 2013). In order to reveal the potential differences in H_2O_2 tolerance of filamentous *Nostoc* 29133 and *Anabaena* 7120, the active cultures of both cyanobacterial strains were subjected to increasing concentrations of exogenously added H_2O_2 (0.1, 0.25, and 0.5 mM), and Chl *a* content of cultures was measured for 6 days. This range of H_2O_2 concentration was chosen based on studies in *Aphanizomonen ovalisporum*, a filamentous cyanobacterium, which fails to survive in the presence of 0.5 mM H_2O_2 (Kaplan-Levy et al. 2015). When incubated with 0.1 mM H_2O_2, Chl *a* content of *Nostoc* 29133 (Fig. 1a) and *Anabaena* 7120 (Fig. 1b) was inhibited more or less to similar extent compared to their respective controls. However, a distinct difference between the two strains was observed with increased doses of H_2O_2. Whereas, 0.25 and 0.5 mM H_2O_2 inhibited Chl *a* content of *Nostoc* 29133 by 12 to 20 % compared with untreated control (considered 100 %), similar treatments led to complete inhibition in *Anabaena* 7120. These results suggest that tolerance to H_2O_2 is higher in *Nostoc* 29133 compared to in *Anabaena* 7120, despite the two being taxonomically closely related to each other (order *Nostocales*) (Rippka et al. 1979). Differences in H_2O_2 tolerance among cyanobacteria have been described earlier, but such comparisons have been mostly between different taxonomical groups, for example, comparisons of *Anabaena* 7120 and *Synechocystis* sp. PCC 6803 (Pascual et al. 2010), and of *Cylindrospermopsis* and *Planktothrix* (Yang et al. 2018). Therefore, H_2O_2 tolerance seems to be a species dependent feature in cyanobacteria, and may be related to differential accumulation of H_2O_2 and/or $HO^{\bullet}$ (Drábková et al. 2007 a, b; Yang et al. 2018).

This possibility was investigated by measuring the intracellular hydroperoxide concentrations in *Nostoc* 29133 and *Anabaena* 7120.

Nostoc 29133 displays lower intracellular hydroperoxide levels than *Anabaena* 7120

Total intracellular hydroperoxide levels (includes lipid hydroperoxides) was determined in the cultures of *Nostoc* 29133 and *Anabaena* 7120 exposed to H_2O_2 for 1 day by FOX assay (Wolff 1994; DeLong et al. 2002). As shown in Fig. 2, incubation with 0.1 mM H_2O_2 barely affected the hydroperoxide levels in either strain relative to their respective controls. However, exposure to higher concentrations of H_2O_2 led to differential increase in hydroperoxide levels; *Nostoc* 29133 displayed considerably lower levels relative to *Anabaena* 7120. It is highly likely that relatively lower accumulation of hydroperoxides within cells of *Nostoc* 29133 protected it from H_2O_2 lethality, as opposed to that in *Anabaena* 7120. To investigate if the ability to keep a low intracellular hydroperoxide concentration is also shown in a lower damage to the metabolism, the inhibition of PSII activity was measured in *Nostoc* 29133 and *Anabaena* 7120.

Nostoc 29133 exhibits lower inhibition of PSII performance than *Anabaena* 7120

PSII is a major target of H_2O_2 in many cyanobacteria (Drábková et al. 2007 a, b; Nishiyama et al. 2011; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). The maximum photochemical efficiency of PSII (F_v/F_m), an indicator of PSII electron transport capacity, is a quick and sensitive parameter to assess PSII performance in cyanobacteria (Schreiber et al. 1995). Thus, F_v/F_m was measured in whole cells of *Nostoc* 29133 and *Anabaena* 7120 following 1 day treatment with increasing concentrations of H_2O_2 (Fig. 3). The H_2O_2 treated cultures (0.1 and 0.25 mM) of *Nostoc* 29133 did not show any appreciable change in F_v/F_m values compared to control cultures, though F_v/F_m was inhibited to a smaller extent in 0.5 mM treated cultures. In *Anabaena* 7120, treatment with 0.1 mM H_2O_2 resulted in a mild inhibition of F_v/F_m compared with control, however, higher concentrations completely inhibited F_v/F_m. These results suggest lower inhibition of PSII performance in *Nostoc* 29133 than in *Anabaena* 7120, which may be an effect of tighter regulation of intracellular hydroperoxide levels in the former thus limiting free H_2O_2 to inhibit the PSII.

Nostoc 29133 possesses higher catalase activity than *Anabaena* 7120

To probe the underlying reason for differential accumulation of hydroperoxides in *Nostoc* 29133 and *Anabaena* 7120, catalase activity was evaluated in the cell extracts of 1 day old H_2O_2-treated and untreated cultures. The antioxidative enzymes such as catalases and Prxs are known to participate in scavenging H_2O_2 in cyanobacteria (Tichy and Vermaas 1999; Perelman et al. 2003; Bernroitner et al. 2009; Latifi et al. 2009; Banerjee et al. 2013). However, unlike catalases, Prxs are susceptible to H_2O_2-mediated overoxidation and inactivation (Pascual et al. 2010). As shown in Fig. 4, catalase activity was nearly 20-fold higher in control cultures of *Nostoc* 29133 than that in control cultures of *Anabaena* 7120. While a 1.2-fold increase in catalase activity was observed in *Nostoc* 29133 after 0.25 mM H_2O_2

treatment, an approximately 3-fold increase in this activity was observed in *Anabaena* 7120, compared to their respective controls. However, such an increase in catalase activity was clearly not enough to prevent death by H_2O_2 in *Anabaena* 7120 (Fig. 1b). As opposed to *Anabaena* 7120, a high intrinsic constitutive catalase activity seems to contribute to prompt and efficient decomposition of H_2O_2 resulting in lower intracellular hydroperoxide levels and higher tolerance to H_2O_2 in *Nostoc* 29133. This correlation of H_2O_2 tolerance and intrinsic catalase activity has also been demonstrated earlier in a comparative study between *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 (Gupta and Ballal 2015). It should be stressed that several cyanobacteria, including the filamentous forms, lack catalase activity and are sensitive to H_2O_2 (Bagchi et al. 1991; Bernroitner et al. 2009; Morris et al. 2011; Banerjee et al. 2012; Mikula et al. 2012; Yang et al. 2018; Piel et al. 2019). In this context, *Nostoc* 29133 may be unique, as it possesses catalase activity and a superior ability to tolerate H_2O_2. The findings presented here should pave the way for further in-depth characterization of catalases in this filamentous cyanobacterium and their roles in adaptation to H_2O_2 and other abiotic stress conditions, which presumably generate oxidative stress.

Conclusion

This study highlights differences in H_2O_2 tolerance between two closely related filamentous cyanobacteria *Nostoc* 29133 and *Anabaena* 7120. *Nostoc* 29133 exhibited lower inhibition of chlorophyll *a* and PSII performance, as compared to *Anabaena* 7120 in response to exogenous H_2O_2. The higher tolerance of *Nostoc* 29133 to H_2O_2 was accompanied by a tighter control of intracellular hydroperoxide level supported by higher intrinsic constitutive catalase activity, in contrast to that in *Anabaena* 7120. H_2O_2 stress tolerant photoautotroph like *Nostoc* 29133 is likely to be an important biotechnological resource, and may be exploited as a potential source of valuable antioxidant catalase.

Declarations

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Figures

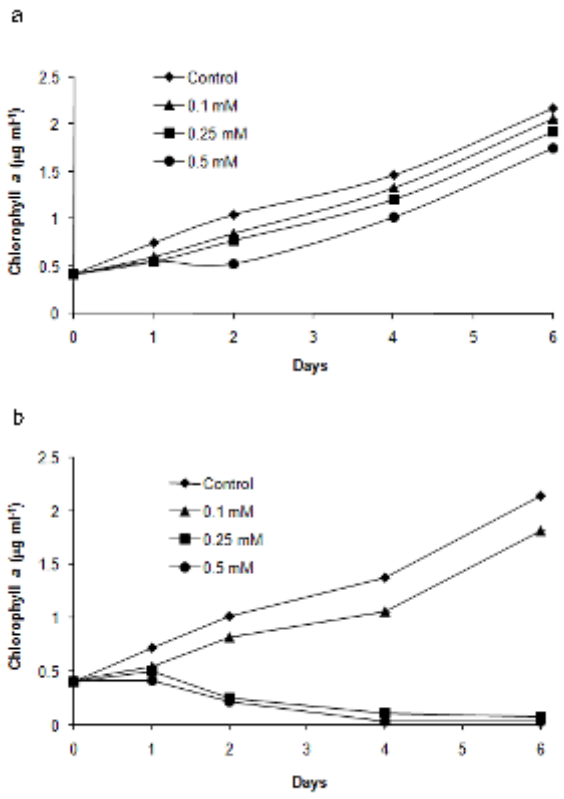

Figure 1

Growth response of Nostoc 29133 and Anabaena 7120 to H2O2. The actively growing Nostoc 29133 (a) and Anabaena 7120 (b) cultures equivalent to 0.5 µg ml-1 of Chl a were seeded in fresh BG11-medium

containing H2O2 (0-0.5 mM). The Chl a content of cultures was measured at periodic intervals for 6 days. The bars indicate standard deviation of three independent experiments and are smaller than the symbols

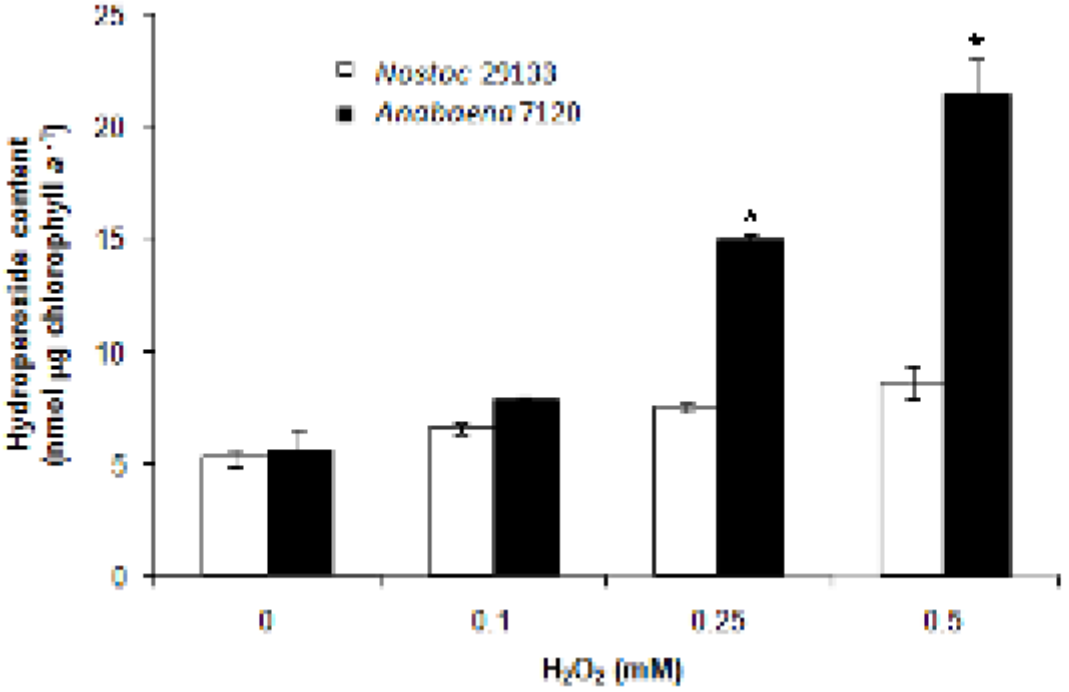

Figure 2

Effect of H2O2 on total hydroperoxide level in Nostoc 29133 and Anabaena 7120. The cyanobacterial cultures treated or untreated with H2O2 for 1 day in fresh BG11-medium were used to measure the total intracellular hydroperoxide level by ferrous oxidation-xylenol orange method. The bars indicate standard deviation of three independent experiments. Asterisk (*, $P < 0.05$) on the bars represent significant difference as compared to respective control value

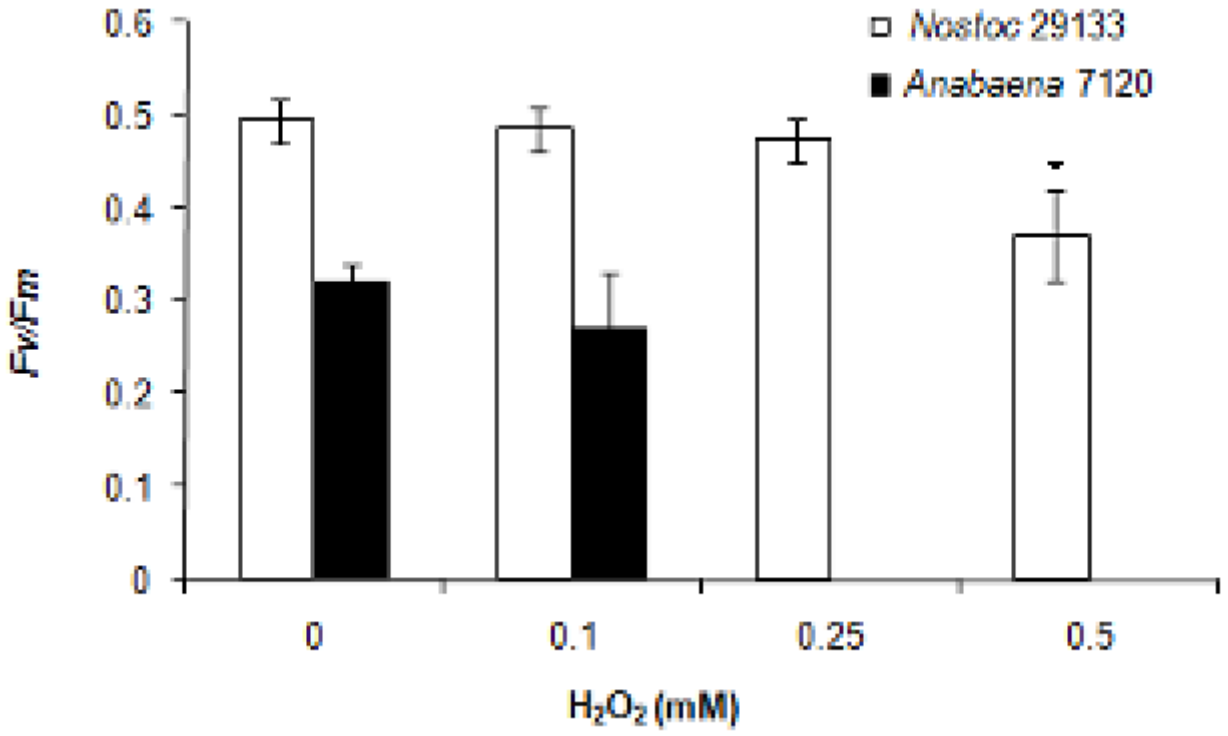

Figure 3

Effect of H2O2 on maximum photochemical efficiency (Fv/Fm) of PSII in Nostoc 29133 and Anabaena 7120. The cyanobacterial cultures untreated or treated with different concentrations of H2O2 for 1 day in BG11-medium were harvested, washed and resuspended in the same medium. Such cultures (Chl a concentration 10 µg ml-1) were dark adapted for 15 min followed by Fv/Fm measurement. The bars indicate standard deviation of three independent experiments. Asterisk (*, $P < 0.05$) on the bar represent significant difference as compared to respective control value

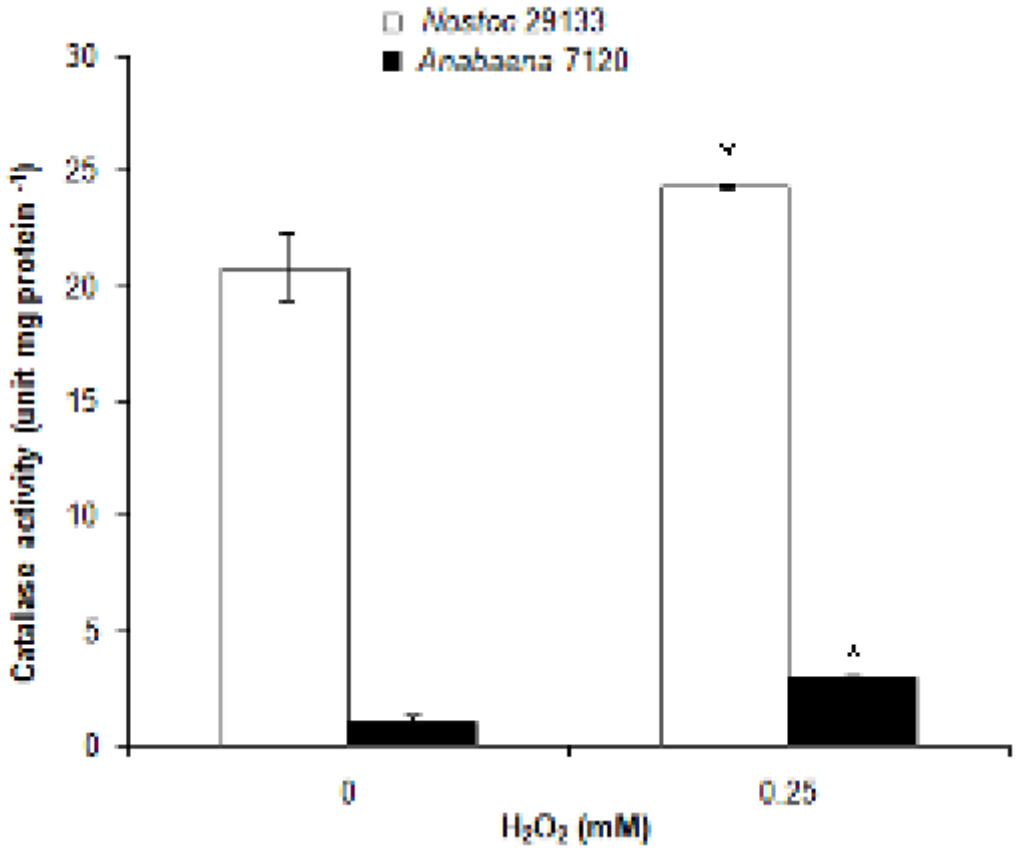

Figure 4

Catalase activity assay in Nostoc 29133 and Anabaena 7120. The cyanobacterial cultures treated or untreated with H2O2 for 1 day in fresh BG11-medium were harvested, washed and resuspended in 36 mM phosphate buffer (pH 7.4) and broken by ultrasonication. The cell extracts equivalent to 50 µg total protein was used for measuring catalase activity. The bars indicate standard deviation of three independent experiments. Asterisk (*, $P < 0.05$) on the bars represent significant difference as compared to respective control value