ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Anti-Oxidant enzyme levels and quantification of Reactive oxygen species in *Mycobacterium aurum*

RadhikaPothi

Faculty of Health and Medical sciences, University of Surrey, Guildford, Surrey GU27XH, United Kingdom.

Manuscript Info *Abstract*

Manuscript History:

Received: 13 July 2013
Final Accepted: 24 July 2013
Published Online: August 2013

Key words:
Mycobacterium aurum, Chlorination, Antioxidants, Growth phases

Chlorination is one of the safest disinfection methods for drinking water but there are few waterborne microbes which are highly tolerant for chlorine treatment. Mycobacteria are found distributed in water channels in most of the countries. They are well known for their characteristic cell wall rich in mycolic acid. High lipid content predetermines their resistance towards chemicals, antibiotics and environmental stress. Apart from thick lipid rich cell wall the genus has other defensive mechanisms like antioxidant enzyme production like superoxide dismutase and catalase which prevents the organism from oxidative stress mainly caused by chlorination. This study investigates the level of antioxidant enzyme levels in correlation with quantified reactive oxygen species and cell survival across growth phases of *Mycobacterium aurum*which is one among known GC rich actinomyceteand a surrogate strain for mycobacterium tuberculosis.

Introduction

Reactive oxygen species (ROS) are involved in the cell growth, differentiation, progression, and death. Low concentrations of ROS may be beneficial or even indispensable in processes such as intracellular signalling anddefence during environmentally unfavourable conditions. But higher levels of antioxidants can have deleterious effect on the host cell survival. As a safeguard against the accumulation of ROS, several non-enzymatic and enzymatic antioxidant activities exist (Jose et al., 1999) Therefore, when oxidative stress arises as a consequence of unfavourable conditions, a defence system promotes the regulation and expression of these enzymes especially in Mycobacterium aurum(Helmann, 2003). Mycobacteria have been reported in treated and distributed drinking water. The isolation of mycobacteria from both environmental and treated drinking water samples was first reported in the early 1900s. However, it has been in the last three or four decades these environmental mycobacteria have been recognized as pathogens of human disease (Cloete, 2003). It is a pathogen infecting both humans and animals. Mycobacterium species are chlorine resistant organisms and common inhabitants of drinking water systems (Carson et al., 1978, 1988). Quantification of reactive oxygen species has been extensively studied Helbling and VanBriesen (2007). Antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) have been postulated to protect against biological oxidative damage by scavenging oxygen free radicals M2.

Catalase and superoxide dismutase (SOD) prevents cellular damage by converting ROS to oxygen and water [Kumar et al., 2011]. It has been shown that several biochemical and physiological changes were involved when microbial cells were exposed to salt, pH, and temperature stress. However, there is a lack of information on the behaviour of the antioxidant enzymes (catalase and SOD) against these stresses which induce production of ROS. Therefore in this study we induced ROS in *M.aurum* using hydrogen peroxide and hypochlorous acid to study the antioxidant enzyme levels in different phases of cell growth. Also by determining the effect of ROS on cell survival along with superoxide dismutase and catalase enzyme levels paved way for further research into the insights of Mycobacterium's tolerance to chlorine treatments as chlorination is considered one of the safest disinfection methods of drinking water around the world (Lisle et al., 1998; Ryu and Beuchat, 2004).

Materials and Methods

All the glassware used were washed with detergents and rinsed with hot water and then with deionized water. They were allowed to air dry, then capped with aluminum foil and dried overnight in the oven at 65º C; these steps were taken to avoid the interferences of organic material from the environment (Helbring and VanBriesen, 2007).

Cell Culture Preparation and Quantification

Organisms selected was *Mycobacterium aurum* (ATCC 23366), an organism that is neither gram positive nor gram negativeand that is considered to be resistant to treatment with disinfectants. All the cultures were purchased from ATCC. They were grown in batch cultures in liquid growth media,*M.aurum* in Mdidlebrook 7H10 agar along with Middlebrook OADC Enrichment (Difco) and glycerol (Acros Organics). When needed, the cells were separated from the media and resuspended in Sorensen's Phosphate buffer.

Cell concentration changes were assayed by light scatter (optical density,OD) at 610 nm or by colony counts. For the OD method, standard curves for*M.aurum* was developed, and used to assess the concentration changes.*M.aurum*was plated and quantified by spread plate method on MB 7H10 with MB OADC Enrichment and glycerol; incubation at 37^{o}C for 15 days. All measurements were conducted at least in duplicate for each experiment. Each set of experimental conditions was repeated in triplicate. Means and standard deviations from the repeated experiments are reported. In all experiments, replicates agreed within about 7%. Prior to each experiment, cell cultures were aseptically transferred to sterile plastic tubes and centrifuged at 6000g for 5min. The liquid growth media was separated from the pellet and discarded. Cells were re-suspended in a volume of 0.1M chlorine demand-free Sorensen's phosphate buffer at a pH of 7.4 equal to the volume of the discarded supernatant. Sorensen's phosphate buffer was prepared by combining a 0.2M sodium phosphate monobasic stock solution with a 0.2M sodium phosphate dibasic anhydrous stock solution and deionized water at a volumetric ratio of 57:243:600. Re-suspended cells were centrifuged, separated, and re-suspended in phosphate buffer three additional times in order to completely separate the cells from their growth media, to avoid interaction of the oxidant and the media (Helbling and VanBriesen, 2007).To ensure the purity of microbial cultures, Ziehl Nielson staining was used for *M.aurum.*

Stock Solutions

HOCl was freshly prepared by dissolving equal amount of distilled water and sodium hypochlorite as 1 mole of sodium hypochlorite reacts with water to give 1 mole of HOCl.

H_2O + NaOCl$\rightleftharpoons$NaOH + HOCl In the context of chlorination, the term `free chlorine' refers to a group of three species, namely, dissolved Cl_2, HOCl (hypochlorous acid), and OCl^- (hypochlorite)(Snoeyink and Jenkins, 1980). When chlorine is dissolved in water, equilibrium exists between these three species, and their ratio depends on the temperature and pH (Dukan and Touati, 1996). The equilibrium reaction is as follows:

$Cl_2 + H_2O \rightleftharpoons HOCl + H^+ \rightleftharpoons Cl^-$

The equilibrium constant, Keq, for the above reaction is 4.5 x 10-4 at 25^{o}C (Snoeyink and Jenkins, 1980). The HOCl formed can further dissociate as follows:

$HOCl \longrightarrow H+ + OCl^-$

With an equilibrium constant of 10-7.54. HOCl is the predominant species over most of the pH range of typical drinking waters (pH: 5 – 8), and thus we have focused on the effects of HOCl in this work. To maintain our solutions with HOCl dominance, they were buffered at pH 7.pH measured before and after each experiment confirmed a narrow range of values was maintained (7.3-7.4).

Various concentrations of H_2O_2 were prepared from 8.8 M stock solution. Hydrogen peroxide and hypochlorous acid solutions from 0-7.82mg/L and 0-8.4mg/L respectively, were prepared for test cases (0, 0.03, 0.07, 0.1, 0.14, 0.18, 0.23 mM). 0.2 M Sterile sodium thio-sulfate (Na_2SO_3) solution was prepared by dissolving 5.69 g of $Na_2S_2O_3.5H_2O$ in 100 ml of distilled water. As these oxidants decay over time in storage and are light sensitive they were stored in dark bottles under cold storage (4ºC).

Treatment of cultures with HOCl and H_2O_2

Cell solutions prepared as described above were placed in flasks with magnetic stir-bars. Inoculum concentration was measured by OD and plate counting as described above. Oxidants prepared as described above were added to the cell suspensions and incubated for 5 min at a shaker speed of 200 rpm maintained at 37ºC. The residual free chlorine was quenched with an equal volume of 0.2 M sterile sodium thio-sulfate (Na_2SO_3) solution. To remove the excess sodium thio-sulfate, cells were centrifuged and re-suspended in fresh media.

ROS (superoxide and hydroxyl radical) Measurements

Superoxide Radical Quantification

Superoxide radicals were measured using hydroethidium (HE) fluorescence due to its specificity for this ROS (Barbacanne et al., 2000). When HE is oxidized by superoxide it originates ethidium (E+), a fluorescent compound (λexcitation = 520 nm; λemission = 610 nm). 10 µM of HE was added to the bacterial culture and incubated for 15 minutes. The culture was centrifuged and the cells were collected and washed with phosphate buffer saline (PBS) thrice. Finally, the cells were suspended in PBS and the fluorescence was taken with excitation and emission wavelength of 520 nm and 610 nm, respectively. Concentration of superoxide was found from a calibration curve which was made using potassium superoxide from Sigma-Aldrich as standard (Valentine et al.,1984).To make the calibration curve, known amount (mmoles) of KO_2 were added with 20 µl (1 mM stock in methanol) of hydroethidine (HE) dye. Sufficient amount of dimethylsulfoxide (DMSO) solvent was added to make the final volume to 2ml (Final concentration of HE becomes 10 µM). Similarly 20 µl of HE in 2 ml final volume of DMSO (without KO_2) was taken as blank. Fluorescence readings were taken immediately after making the above mixture. To get the standard curve fluorescent readings were plotted against known concentrations of KO_2. The detailed procedure of solubilization of KO_2 using crown ether is described by Valentine et al., 1984.

Hydroxyl radical measurement

Hydroxyl was measured using 3'-(p-aminophenyl) fluorescein (APF). APF is a non-fluorescent molecule until it is reacted with either hydroxyl radicals, resulting in cleavage of the aminophenyl ring from the fluorescein ring system, which is highly fluorescent (Cohn et al., 2008). APF was used directly from the purchased vial without further treatment. Similar to superoxide measurement 10 µM of APF was added to the cultures followed by the incubation for 30 minutes in dark for 37ºC and the fluorescent was taken with the excitation and emission wavelength of 490 and 520 nm respectively.

Enzyme Assays

Superoxide Dismutase (SOD) Assay

The method described by Paoletti and Mocali (1990) was used for the assay of SOD activity with modification in assay volume. The assay volume (2.030 ml) contained 1.6 ml 100 mMtriethanolamine-diethanolamine-HCl buffer (pH 7.4), 80 µl of 7.5 mM NADPH, 50 µl of 100 mM EDTA- 50 mM $MnCl_2$ solution (pH 7.0), 100 µl of the cell extract was kept at room temperature for 5 min to stabilize. Then 200 µl of 10 mMmercaptoethanol was added and mixed well. The decrease in absorbance at 340 nm was monitored for 20 min over a 5-min interval at 25°C in a spectrophotometer (Bio-rad: Smartspec 3000). The enzyme activity was calculated using a calibration curve made from standard SOD (Sigma-Aldrich).

Superoxide dismutase

SOD activities were determined according to Paoletti et al. (1986) and Paoletti and Mocali (1990) with slight modifications.This method is 10 to 40 times as sensitive as other commonly used assay procedures. The assay mixtures (200µl) consisted of 75 mMtriethanolamine, 75 mMdiethanolamine, pH 7.5; 1.25 mM manganese chloride, 2.5 mM EDTA, 0.3 mM NADH, 1.0 mMmercaptoethanol, and varying amounts of enzyme preparation. The unit of activity is defined as the amount of enzyme causing 50 percent inhibition of the rate of the superoxide-driven NADH oxidation (McCord and Fridovich, 1969). Accordingly, each determination consisted of three assays run at different enzyme concentrations, and the amount of enzyme preparation (µg fresh-weight equivalents) required for 50% inhibition was computed by semi log linear regression (Paoletti et al., 1986; Paoletti and Mocali, 1990). Boiled enzyme preparations caused a marginal inhibition of NADH oxidation (<10%) in the upper range of the enzyme concentrations, possibly resulting in a slight overestimation of some of the enzyme activities. The cause of this activity is not known. It may reflect a non-enzymatic dismutation of the superoxide radical facilitated by some component(s) of the enzyme preparations (Gunter et al., 2002)

Catalase assay

Catalase activity was determined in cell free extracts using a standard colorimetric assay (Kubal and D'Souza 2004) by monitoring the rate of H_2O_2 degradation. One unit of catalase activity is defined as amount of enzyme required to break down 1 nmol of H_2O_2 in 1 min at 25^{o}C. The SOD activity was assayed using a standard method (Paoletti et al. 1986; Paoletti and Mocali, 1990). One unit of SOD activity is defined as the amount of protein which produced 50% inhibition of the rate of NADH oxidation observed in the control.

Results

Anti-oxidant enzyme levels

A common strategy to investigate the effects of ROS is to examine the intracellular enzymes that help to maintain ROS levels, namely, catalase and superoxide dismutase (SOD). Standard curves were plotted for catalase enzyme using various concentration of hydrogen peroxide Fig (1) (2 panel a and b).

Figure 1.Catalase standard curve.

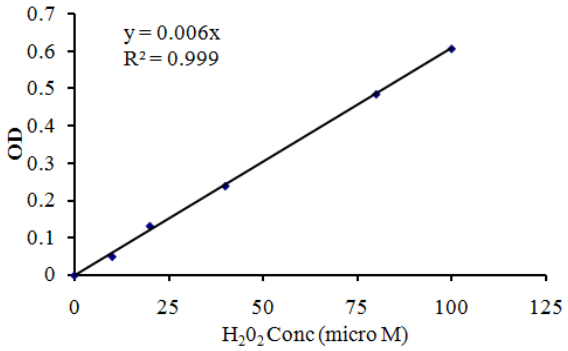
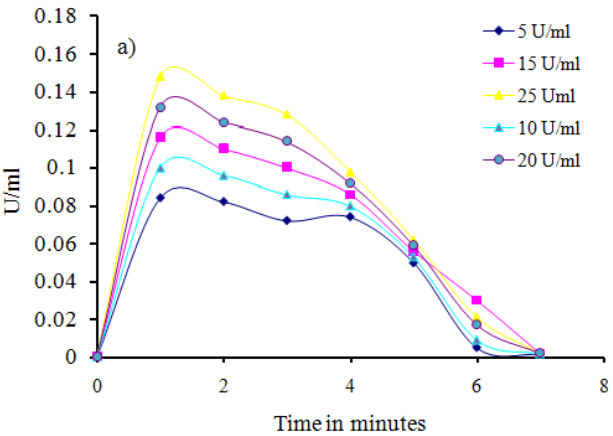
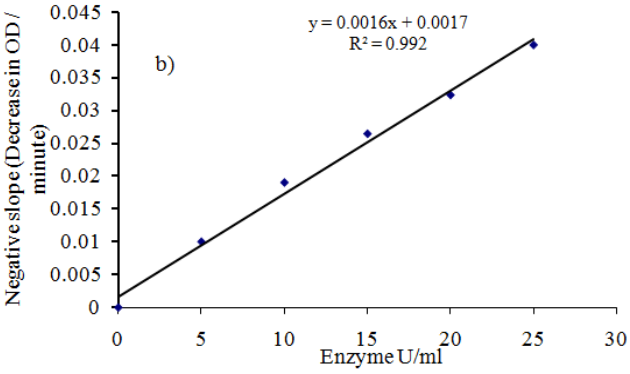


Figure 2. Panel a) SOD Standard curve. Use the heat inactivated enzyme for the control reaction (Blank). Incubate the above mentioned reaction mixture for 5 min at room temperature (25ºC) to equilibrate the oxidation of NADH due to NADH oxidase activity possibly occurring in the cell lysate.Add 100 µL of Beta Mercapto -ethanol which generates superoxide radical in the reaction mixture.Monitor the time course decrease in absorbance at 340 nm due to NADH oxidation for 5 min. Panel b) the slope of the linear portion of the curves so obtained is used for the calculations of the rate of NADH oxidation.

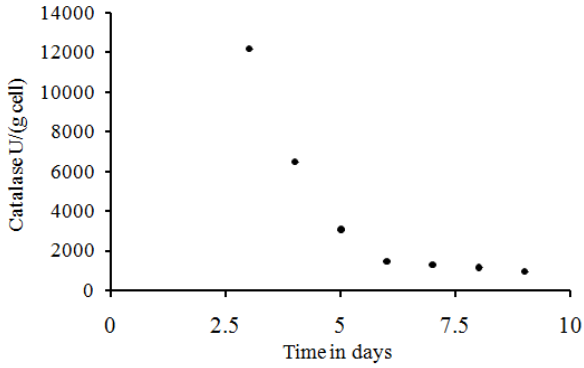


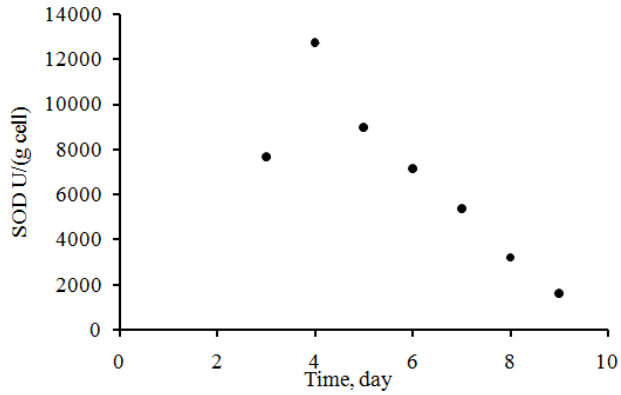


Role of Catalase and SOD

The specific intracellular catalase levels, presented in Figure 3 panel b, show that the level in the early-log phase, 12208 U (g cell)-1, is 8.4-fold higher than the value in the mid-log phase (1461 U (g cell)-1) and 12.7-fold higher than that in the early-stationary phase (964 U (g cell)-1). Thus, specific intracellular levels of catalase, the enzyme that acts against hydroxyl radicals was highest in the log-phase, which could possibly explain the robustness of log-phase cells compared to the other phases.

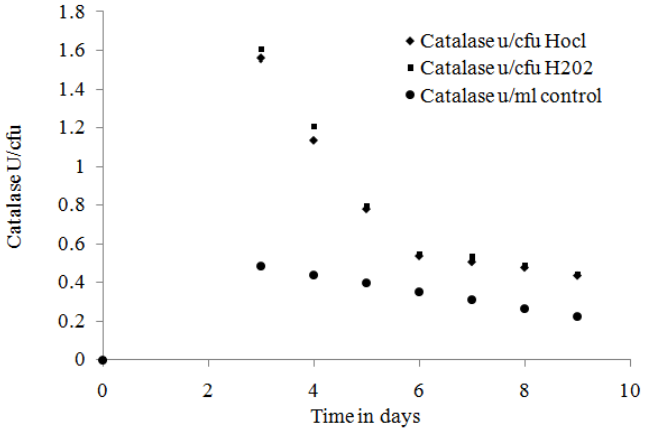
Figure 3. a) Specific intracellular catalase levels across different growth phases of *M.aurum*. b) Specific intracellular superoxide dismutase levels across different growth phases of *M.aurum*.





The specific intracellular SOD levels, presented in Figure 3 panel b are interesting – they are comparable between the early– and mid – log phases (7653 and 7128 U (g cell)-1, respectively), but it is 4.8-fold lower (1598 U (g cell)-1) in the early-stationary phase. Thus, the specific intracellular levels of SOD that acts against superoxide was lowest in the early-stationary phase, which along with the low levels of catalase, explains the sensitivity of M. aurum in the early stationary phase to chlorination. To further analyse and validate the antioxidant enzyme activity in the cells which helps their survival during oxidative stress, superoxide and hydroxyl levels were measured in relation to catalase and superoxide dismutase.

Figure 4. Catalase enzyme levels under normal conditions and during oxidative stress (treatment with H_20_2 and HOCl across early log, exponential and early stationary phase in *M.aurum*)



Initially catalase enzyme level was measured up to 9 days across all three growth phases (early log, exponential and early stationary phase) with both HOCl and H_20_2 treatment (Fig 6). Under normal conditions maximum catalase level was 0.6U/cfu during early log and exponential phase. Enzyme level stayed constant until early stationary phase with not many differences. While during exposure to oxidative stress (HOCl and H_20_2 treatment) enzyme level was detected to be similar with a maximum of ~1.6U/cfu during early log and exponential phase. This clearly indicates that catalase does have a role in oxidative stress management in *mycobacterium aurum*

Figure 5. Specific intracellular superoxide level nmol/cfuvs Time in days in control and oxidative stressed cells with HOCl and H_20_2 from which catalase enzyme levels were measured

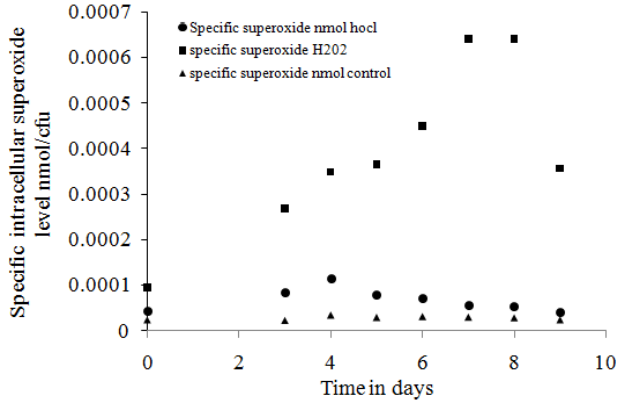
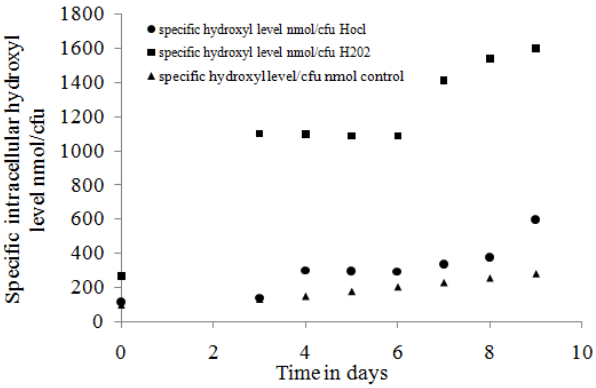


Figure 6. Specific intracellular Hydroxyl level nmol/cfuvs Time in days in control and oxidative stressed cells with HOCl and H_20_2 from which catalase enzyme levels were measured



To correlate the catalase enzyme level with superoxide and hydroxyl production in the same culture, reactive oxygen species was quantified (Fig 5, 6). Superoxide level was found 2 folds higher in H_20_2 treated culture when compared to superoxide radicals in control and HOCl treated culture. Irrespective of the enzyme levels early stationary phase showed more superoxide production compared to early log and exponential when catalase enzyme level was found high (Fig 6) which confirms catalase role in managing oxidative stress.As explained above superoxide dismutase enzyme was measured across growth phases of *M.aurum* (Fig 8). Enzyme level was higher during H_20_2 treatment and nearly similar effect was found during HOCl treatment. Control culture had 1 fold lower enzyme production compared to oxidative stressed culture enzyme levels.

Figure 7. Superoxide dismutase enzyme levels under normal conditions and during oxidative stress (treatment with H_20_2 and HOCl across early log, exponential and early stationary phase in *M.aurum*)

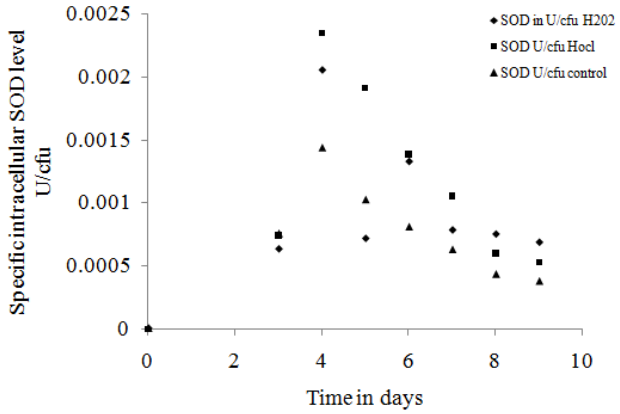


Figure 8. Specific intracellular superoxide level nmol/cfuvs Time in days in control and oxidative stressed cells with HOCl and H_20_2 from which superoxide dismutase enzyme levels were measured

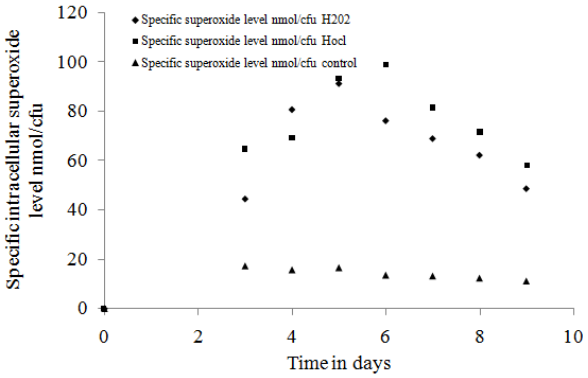
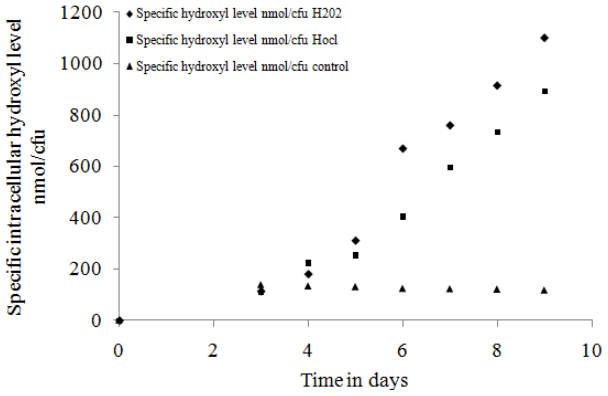


Figure 9. Specific intracellular hydroxyl levelnmol/cfuvs Time in days in control and oxidative stressed cells with HOCl and H_20_2 from which superoxide dismutase enzyme levels were measured.



When intracellular superoxide and hydroxyl radicals were measured (Fig 8, 9)to relate how superoxide dismutase enzyme influence oxidative stress management, superoxide level was found to be higher in HOCl treated culture when compared to H_20_2 treated cultures of up to 100 nmol/cfu and 85 nmol/cfu. Hydroxyl levels were found to be more linear during both H_20_2 and HOCl treatment with a maximum reaching upto 1200 nmol/cfu which was quite high compared to specific intracellular superoxide level in the cultures. Level of superoxide dismutase did not correlate well with intracellular ROS production in *M.aurum.*

Discussion

It is certainly interesting to reveal the changes occurring during stress and to simultaneously monitor alterations in different growth phases of an organism. (Covert et al., 1999) In a recent study investigating antioxidant enzyme levels and reactive oxygen production, it was demonstrated that 73% of the variance in growth phases can be explained by antioxidant enzyme levels like superoxide dismutase and catalase abundance (Lu et al., 2007). Conversely, this finding suggests that about one quarter of the variance of ROS levels may be additionally controlled other factors like carbon metabolism (Eila et al., 2007), extrapolysaccharide production (Eila et al., 2004), iron utilisation (Elizabeth et al., 2006) and cell wall composition especially in mycobacteria as they have amycolic acid rich thick cell wall which acts as a defensive agent against any antibiotic attack (Falkinham et al., 1984). As the cellular phenotype is ultimately governed by the protein(s) in other words might be enzymes expressed from mRNAs, expression profiling data would be better corresponding to the amount of cellular proteins (Du Moulin et al., 1998). With that as an effective research, in this study the correlation between antioxidant enzyme levels (superoxide dismutase and catalase) along different growth phases (early log, exponential and early stationary phase) of *M.aurum* was studied under normal conditions and when exposed to hydrogen peroxide and hypochlorous acid at the right pH.It was interesting to find that SOD that acts against superoxide had no effects on the reactive oxygen production in the cultures rather catalase provided a valid proof that it controls the ROS production during the exponential phase. While early stationary phase cultures were still found to be resistant or in other words had more superoxide and hydroxyl radicals in spite of antioxidant enzyme levels. Above results also suggest that apart from the antioxidant enzyme levels there are other parameters which help the organism survive during the oxidative stress and that needs more investigation.

Conclusion

The specific intracellular levels of SOD that acts against superoxide was lowest in the early-stationary phase, which along with the low levels of catalase, explains the sensitivity *of M. aurum* in the early stationary phase to chlorination. Specific intracellular levels of catalase, the enzyme that acts against hydroxyl radicals was highest in the log-phase, which could possibly explain the robustness of log-phase cells compared to the other phases.

Acknowledgment

Indian Institute of Technology Madras and Indo US forum for funding.

References

1. B.s Kubal and S.F.D'Souza (2004). Immobilization of catalase by cross-linking of

permeabilized yeast cells in a protein support, Bhabha atomic research centre newsletter.162,Issue no.273.

2. Barbacanne MA, Souchard JP, Darblade B, Iliou JP, Nepveu F, Pipy B, Bayard F, Arnal JF (2000): Detection of superoxide anion released extracellularly by endothelial cells using cytochrome c reduction, ESR, fluorescence and lucigenin-enhanced chemiluminescence techniques. Free RadicBiol Med 29: 388-396.

3. Carson LA, Cusick LB, Bland LA, Favero MS (1988). Efficacy of chemical dosing methods for isolationgnontuberculous mycobacteria from water supplies of dialysis centers. Applied Environmental Microbiology 54: 1756-1760.

4. Cloete, TE (2003). Resistance Mechanisms of Bacteria to Antimicrobial Compounds. International Biodeterioration& Biodegradation, 51 (4): 277-282.

5. Cohn C, Mueller S, Wimmer E, Leifer N, Greenbaum S, Strongin DR, Schoonen M. 2006: Pyrite-induced hydroxyl radical formation and its effect on nucleic acids. Geochemical Transactions 7.

6. Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma, Jr. 1999 Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol. 65:2492–2496.

7. Du Moulin, G. C., K. D. Stottmeier, P. A. Pelletier, A. Y. Tsang, and J. Hedley- Whyte. 1988. Concentration of Mycobacterium avium by hospital hot water systems. JAMA 260:1599–1601

8. EilaTorvinen, Markku J. Lehtola, Pertti J. Martikainen, and Ilkka T. Miettinen1 2007 Survival of Mycobacterium avium in Drinking Water Biofilms as Affected by Water Flow Velocity, Availability of Phosphorus, and Temperature Applied and environmental microbiology p. 6201

9. EilaTorvinen,SiniSuomalainen, Markku J. Lehtola, Ilkka T. Miettinen, OutiZacheus, Lars Paulin, Marja-LeenaKatila, and Pertti J. Martikainen 2004 Mycobacteria in Water and Loose Deposits of Drinking Water Distribution Systems in Finland Finland Applied and environmental microbiology p. 1973

10. Elizabeth D. Hilborn., Terry C. Covert, Mitchell A. Yakrus, Stephanie I. Harris,Sandra F. Donnelly, Eugene W. Rice, Sean Toney, Stephanie A. Bailey, and Gerard N. Stelma, Jr.2006 Persistence of Nontuberculous Mycobacteria in a Drinking Water System after Addition of Filtration Treatment Applied and environmental microbiology, p-5864

11. Falkinham, J. O., III, K. L. George, B. C. Parker, and H. Gruft. 1984. In vitro susceptibility of human and environmental isolates of Mycobacterium avium, M. intracellulare, and M. scrofulaceum to heavy-metal salts and oxyanions. Antimicrob. Agents Chemother. 25:137–139.

12. Gunter F. Weirich, Anita M. Collins, Virginia P. Williams (2002). Antioxidant enzymes in the honey bee, Apismellifera, Apidologie 33,3-14.

13. Helbling DE, VanBriesen JM (2007) Free chlorine demand and cell survival of microbial suspensions. Water Research, 41: 4424-4434.

14. Helmann JD, Winston MF, Gaballa A, Kobel PA, Morshedi MM, Fawcett P, et al (2003). The global transcriptional response of Bacillus subtilis to peroxide stress is oordinated by three transcription factors. J Bacteriol 185: 243–53.

15. Jose M mates, Cristina perez. Gomez and Ignacio nunez De castro (1999). Antioxidants enzymes and human diseases. Clinical biochemistry, Vol.32, No.8, 595-603.

16. Lisle JT, Broadaway SC, Prescott AM, Pyle BH, Fricker C, Mcfeters GA (1998). Effects of Starvation on Physiological Activity and Chlorine Disinfection Resistance in Escherichia coli O157: H7. Applied and Environmental Microbiology 64 (12): 4658-4662.

17. Lu.P et al., 2007. Absolute protein expression profiling eatimates the relative contributions of transcriptional and translational regulation. Nature Biotechnology. Vol 25:117-124.

18. Luo Y, Han Z, Chin SM, Linn S (1994). Three chemically distinct types of oxidants formed by iron-mediated Fenton reactions in the presence of DNA. ProcNatlAcadSci USA 91:12438–42.

19. McCord J M &Fridovich I 1968. The reduction of cytochrome c by milk xanthine oxidase. J. Biol. Chem. 243:5753-60.

20. Paoletti F., Aldinucci D., Mocali A., Caparrini A. (1986) A sensitive spectrophotometric method for the determination of superoxide

dismutase activity in tissue extracts, Anal. Biochem. 154, 536–541.

21. Paoletti F., Mocali A. (1990) Determination of superoxide dismutase activity by purely chemical system based on NAD(P)H oxidation, Methods Enzymol. 186, 209–220.

22. Ryu JH, Beuchat LR (2004). Factors Affecting Production of Extracellular Carbohydrate Complexes by Escherichia Coli O157: H7. International Journal of Food Microbiology 95: 189-204.

23. Carson LA, Petersen JJ, Favero MS, Aguero SM (1978). Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants. Applied Environmental Microbiology 36: 839-846.

24. SawanKumar ,Gayathiri T. Kalyanasundaram, Sathyanarayana N. Gummadi (2011). Differential Response of the Catalase, Superoxide Dismutase and Glycerol-3-phosphate Dehydrogenase to Different Environmental Stresses in Debaryomycesnepalensis NCYC 3413, 62:382-387.

25. Snoeyink,V.L and Jenkins,D (1980). Water chemistry, John wiley sons Inc.
Valentine,H.T; Tritton,L.M;Furnival,G.M (1984). Subsampling trees for biomass, volume or mineral content. Forest science. 30(3):673-681.

****