Investigation Into The Growth Phase Dependent Sensitivity Of Mycobacterium Aurum To Chlorine And Quantification Of Reactive Oxygen Species

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Abstract: Chlorination is one of the best known disinfectant methods for treating drinking water. *Mycobacterium aurum* and *Escherichia coli* are common waterborne organisms and as mycobacterial species are much resistant to reactive oxygen species, the initial effect H2O2 and HOCl was quantified in *Escherichia coli* and *Mycobacterium aurum* with the concentrations ranging from 0 to 0.16mM.The influence of reactive oxygen radicals with all the three growth phases like early log (3rd day in *M.aurum* and 3rd hour in *E.coli*), mid log (9th day and 9th hour) and early stationary phase (12th day and 12th hour) was checked and a correlation between the intracellular superoxide and hydroxyl with normalized change in CFU was expressed. The organism's susceptibility to H2O2 and HOCl was observed to be dependent on its growth phase. The possible reasons for their difference in resistance were analysed.

Keywords: *Mycobacterium aurum, Escherichia coli*, Chlorination, Reactive oxygen species.

1 Introduction

Chlorination is the predominant disinfectant method used for treating drinking water due to its low cost and ease of application [1]. Nevertheless, the mechanism of disinfection by chlorine is not well understood. Although the mechanisms involved in microbial inactivation by chlorine are not fully understood, it is believed that reaction with microbial membranes increases cell permeability, which results in leakage of macromolecules and cell death [2], [3]. More recent studies have shown that membrane damage is not the main mechanism of inactivation for cells exposed to oxidative stresses; subtler effects such as an uncoupling of the electron chain or enzyme inactivation may be dominant (Virto et al, 2005). On the other hand, Mycobacteria, the group of well-known human pathogens that are also found in treated drinking waters, are chlorine resistant [5], [6]. Chlorine resistant organisms are of particular interest in water safety because they persist and remain infective. Further, the naturally occurring microbial flora in water distribution systems persist despite the presence of the chlorine residual [7], [8], [9]. Resistance to free chlorine has been attributed to unique variations in the cellular structure and protein composition of different organisms [10]. In addition to physical attributes that aid in resistance to free chlorine, microorganisms may defend against oxidative stresses by inherent or adapted resistance mechanisms that produce extracellular polymeric substances (EPS). These substances are known to react with the oxidative disinfectant, which probably effectively reduces the concentration of disinfectant at the cell wall or cell membrane where cell damage can occur [11], [12]. Reactive oxygen species (ROS), which include predominantly superoxide (O2*-), hydroxyl (OH-) and hydrogen peroxide (H_2O_2), are fundamental molecules that mediate cellular processes [13], and also have been shown to have a regulatory role in the cell – they participate in cell signaling through covalent modifications of target proteins [14], [15], [16]. Exposure of bacterial cells to HOCl, the predominant chlorine species at the pH of interest in aqueous systems, is known to induce hydroxyl radicals (OH-) in them [17]. The OH- radicals are the most damaging of the reactive oxygen species (ROS) in the cell, and have very short half-lives [5]. The superoxide radicals (O2*-), on the other hand, are believed to participate to a larger extent in cell signaling due to their large half-lives of existence [15], [16], [18]. Further, the direct quantification of ROS reported in the literature is rather limited, with most studies focusing on the qualitative description of ROS based on related enzyme characterizations, or identification of ROS. In this work, we have directly quantified the ROS species of interest and related it to the chlorination effects. When chlorine is dissolved in water, equilibrium exists between dissolved Cl_2, HOCl (hypochlorous acid), and OCl- (hypochlorite) [19], and their ratio depends on the temperature and pH [17]. The equilibrium constant, Keq, for the above reaction is 4.5 x 10-4 at 25 ^{o}C [20]. The HOCl formed can further dissociate to H+ and OCl- with an equilibrium constant of 10-7.54. HOCl is the predominant species over most of the pH range of typical drinking waters (5 to 8), and thus we have used freshly prepared HOCl in our studies; direct use of HOCl helps in better quantification of the reactive species inducing agent. Since the chlorine sensitivity studies are normally performed with early stationary phase cultures as reported in the literature [21], the early stationary phase (12th day *M. aurum* and 12th hour *E. coli)* cultures were treated with different concentrations of HOCl, and the extent of killing was examined. The sensitivity of Mycobacterium cells to chlorine is suspected to be dependent on the culture growth phase [22], and hence we studied chlorine sensitivity in the mid-

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log and early-log phases.

2 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 [1].

Cell Culture Preparation and Quantification

Organisms selected were *E.coli* (ATCC 11775), a gram negative inhabitant of water widely used as indicator organism and a test for chlorine treatment and *Mycobacterium aurum* (ATCC 23366), an organism that is neither gram positive nor gram negative. And 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 like *M.aurum* in Mdidlebrook 7H10 agar along with Middlebrook OADC Enrichment (Difco) and glycerol (Acros Organics); and E.coli in TSB (Difco) agar (Fisher scientific). 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 each organism were developed, and used to assess the concentration changes Both *M.aurum* and *E.coli* were plated and quantified by spread plate method on MB 7H10 with MB OADC Enrichment and glycerol; and NB media with TSB (Difco) agar (Fisher Scientific) plates, incubation at 37oC for 15 days and 24 hrs respectively. 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. Resuspended 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 [1]. To ensure the purity of microbial cultures they were checked continuosly by staining methods. Gram staining and Ziehl Nielson staining were used for *E.coli* and *M.aurum* respectively.

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.

$H_2O + NaOCl \longrightarrow NaOH + HOCl$

In the context of chlorination, the term `free chlorine' refers to a group of three species, namely, dissolved Cl2, HOCl (hypochlorous acid), and OCl- (hypochlorite) [23]. When chlorine is dissolved in water, equilibrium exists between these three species, and their ratio depends on the temperature and pH [17]. The equilibrium reaction is as follows:

$Cl2 + H_2O \longrightarrow HOCl + H+ + Cl-$

The equilibrium constant, Keq, for the above reaction is 4.5 x 10-4 at 25 ^oC [23]. 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. Innoculum 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 [24]. 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 [24].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

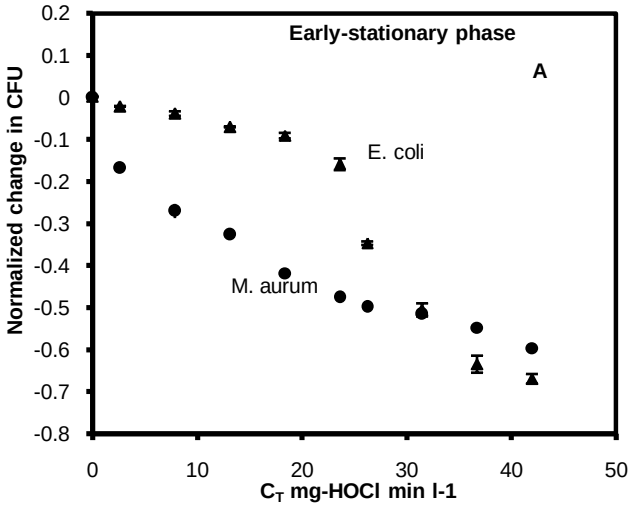
concentration of HE becomes 10 µM). Similarly 20 µl of HE in 2 ml final volume of DMSO (without KO_2) was taken as blank. Fluorescence readings were taken immediately after making the above mixture. To get the standard curve fluorescent readings were plotted against known concentrations of KO_2. The detailed procedure of solubilization of KO_2 using crown ether is described by [25].

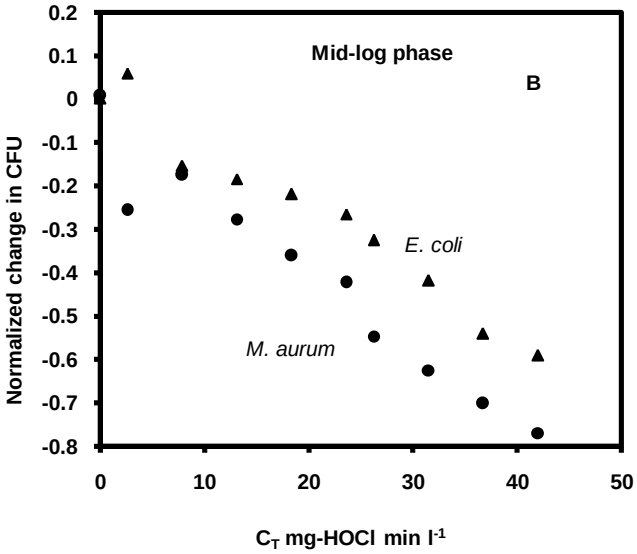
Hydroxyl radical measurement

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 [16]. 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. Further, all the glassware used were washed with detergents and rinsed with hot water and also with deionized water. They were allowed to air dry, and then dried overnight in oven capped with aluminium foil. These steps were taken to avoid the interferences of organic material from the environment [1]. Lack of microbial contamination was also ensured during the experiments. For example, the acid-fast stain test was used to ensure integrity of the *Mycobacterium aurum* cultures.

Results and Discussion

M. aurum is known to be resistant to oxidative disinfectants like hypochlorous acid but at higher concentration of H_2O_2 and HOCl M. aurum is getting killed, this clearly showed a growth phase dependent sensitivity of mycobacterium to disinfectants demanding further study in *M. aurum* with higher concentration of disinfectants at different growth phases and more focus on the reasons for growth phase dependency. The pH values measured before and after the HOCl treatment showed that during the exposure to HOCl, the pH was in the range of 7.3 – 7.4 for all the experiments. The narrow range in pH was achieved through the use of Sorensen's buffer, as detailed in the Materials and Methods. The extent of killing is expressed as a normalized change in cfu upon treatment, i.e. (cfu after treatment – cfu before treatment)/(cfu before treatment). A decrease in the normalized change in cfu indicates cell killing, and an increase indicates growth activation. It is known [1], [26] that the effect of killing agents such as chlorine depends on both the exposure concentration as well as the exposure time. Thus, the product of exposure concentration and time, CT, has been used as the more meaningful parameter to describe killing results. A plot of the normalized change in cfu as a function of CT is given in Figure 1A, in the range of CT values found in the literature. As expected for both organisms, the sensitivity to chlorine increased with CT, but, interestingly, for most values of CT that were used, the *M. aurum* was more sensitive to chlorine compared to *E. coli* when cells in the early-stationary phase were studied. The sensitivity of Mycobacterium cells to chlorine is suspected to be dependent on the culture growth phase [19], [26] and hence we studied chlorine sensitivity in the mid-log and early-log phases. The results are shown in Figures 1B and 1C for the mid-log (9th day *M. aurum*; 9th hour, *E. coli*) and early log (3rd day *M. aurum*; 3rd hour, *E. coli*) cultures, respectively. In the mid-log phase, *M. aurum* was still more sensitive to HOCl compared to E. coli in the range of CT values studied, but the difference between their sensitivities at CT values less than 25, as indicated by the difference in values of normalized change in CFU, was less compared to the early-stationary phase. Nevertheless, in the early-log phase, the behavior was opposite: *M. aurum* was less sensitive to chlorine, compared to *E. coli*.





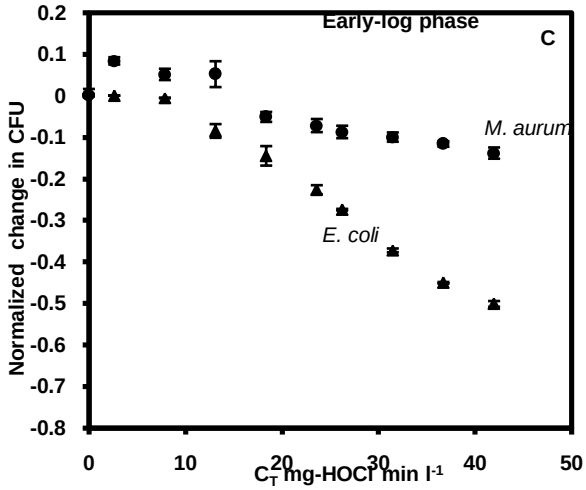


Figure 1. Growth phase dependency of *M.aurum* and *E.coli* at different phases of growth like, A) Concentration of HOCl vs Normalized change in CFU at early stationary phase (12th day for *M.aurum* and 12th hour for *E.coli*) B) Concentration of HOCl vs Normalized change in CFU at Mid-log phase (9th day for *M.aurum* and 9th hour for *E.coli*) and C) Concentration of HOCl vs Normalized change in CFU at early log phase (3rd day for *M.aurum* and 3rd hour for *E.coli*).

Further, the sensitivity of *E. coli* to chlorine did not significantly vary with the growth phase compared to *M. aurum*, although there were growth-phase dependent minor variations. Thus, we further studied the growth phase dependent sensitivity of *M. aurum* to chlorine.

Specific intracellular superoxide levels

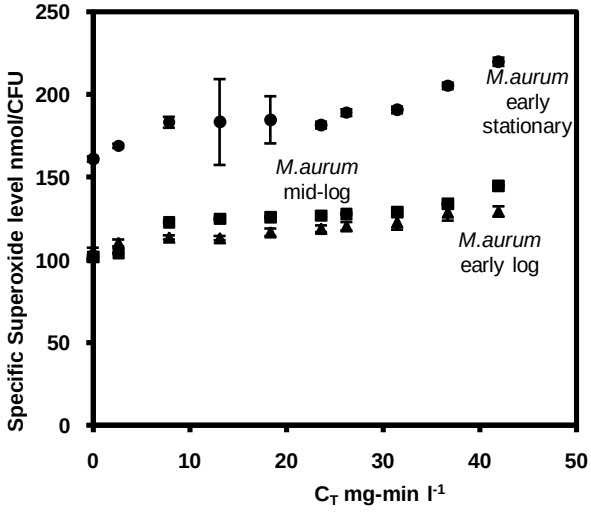


Figure 2. Graph showing the concentration of H_20_2 vs specific superoxide level nmol/CFU in all the three growth phases (early stationary, mid-log and early log phase) in *M.aurum*. Compared to Mid-log phase (9th day) early stationary phase (12th day) culture showed higher levels of specific intracellular superoxide production upto 225nmol/CFU while the levels of siS was almost same 170nmol/CFU and 160nmol/CFU in both mid-log (9th day) and early log phase (3rd day) cultures of *M.aurum* respectively.

Although the intracellular antioxidant enzyme levels like catalase and superoxide peroxidase can provide an idea of the ROS activity, a direct quantification of the ROS would help toward a better understanding. Intracellular superoxide is known to act as a regulatory molecule of cellular metabolism [27], and hence we quantified the specific intracellular superoxide levels (siS) during chlorination. The results given in Figure 4 shows that the siS increased with CT. Also, the siS in the early stationary phase was about 50% higher than the mid-log phase values at all CT values studied; the values in the mid-log and the early-log phases were comparable. Nevertheless, the siS did not seem to correlate well with the extent of *M. aurum* killing when all three growth phases are considered, as shown in Figure 5. Thus, although the siS do vary with the growth phase, the difference in siS does not seem to explain the growth-phase dependent differences in chlorine sensitivity.

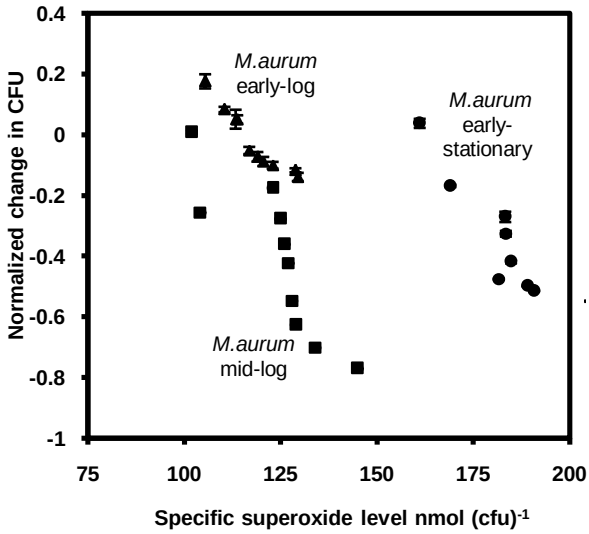


Figure3. Specific superoxide level nmol/CFU vs Normalized change in CFU in all three growth phases (Early log, Early stationary and Mid-log). Early log and mid log were more susceptible to ROS when compared to early stationary phase culture.

Early log phase and Mid log phase cultures are much susceptible for superoxide killing compared to early stationary phase which demands further explanation and research in the factors which influence the growth phase killing like antioxidant enzyme levels like catalase and superoxide dismutase. In mycobacterial species extra cellular polysaccharide production can also add to the protection layer against the reactive oxygen species.

Specific intracellular hydroxyl levels

HOCl is known to predominantly induce intracellular hydroxyl [13]. Thus, we quantified the specific intracellular hydroxyl (siH) levels during the chlorination process, and related it to the cell killing effect of chlorination. The variation in SiH levels with CT for *M. aurum* is presented in Figure 5A.

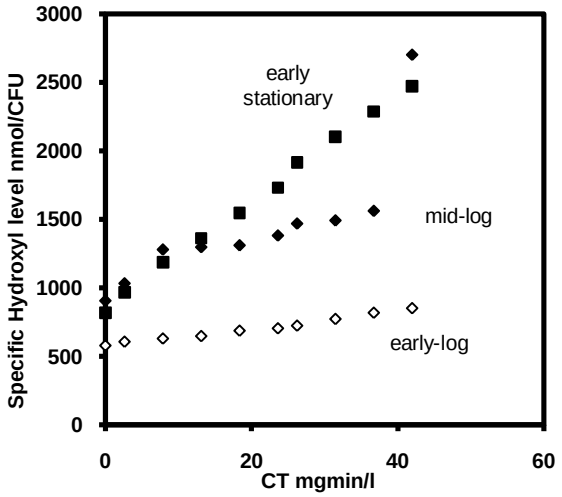


Figure4. Graph showing the concentration of HOCl vs specific hydroxyl level nmol/CFU in all the three growth phases (early stationary, mid-log and early log phase) in *M.aurum*. early stationary phase culture showed significant level of hydroxyl production of 2400nmol/CFU when compared to Mid log phase with 1500 nmol/CFU andEarly log phase with 600 nmol/CFU.

The results presented in Figure 5 show that in all the three growth phases that were studied, the *M. aurum* killing, as represented by the normalized change in CFU) is well correlated with the siH induced by chlorination. Although the cell killing was found in all three growth phases it gradually decreased with the increase in hydroxyl levels.

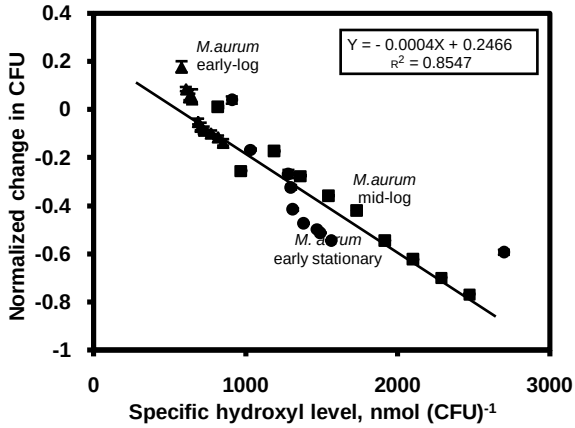


Figure 5. Specific hydroxyl level nmol/CFU vs Normalized change in CFU in all three growth phases (Early log, Early stationary and Mid-log). In all the three growth phases that were studied, the *M. aurum* killing, as represented by the normalized change in CFU) is well correlated with the siH induced by chlorination.

Correlation of *M. aurum* killing extent with siH across growth phases and inducing agents

The correlation of cell killing extent by chlorination with siH was studied when another ROS inducing agent, namely hydrogen peroxide (H_2O_2), was used. Unlike the results of superoxide levels and growth phase dependent cell killing, specific hydroxyl level showed a sequencial correlation with the normalize change in cell viablility. Correlation between specific intracellular superoxide and hydroxyl level nmol/CFU and normalized change in CFU in all three growth phases like Early log phase (3rd day), Mid log phase (9th day) and Early stationary phase (12th day) of *M.aurum* showed a linear decrease in normalized change in CFU as the superoxide and hydroxyl levels increased. The results given in Figure 6 show a good correlation, with the same slope. This strengthens our hypothesis that siH is a fundamental parameter that mediates cell killing in all the three growth phases (Early log, Mid log and early stationary phase) although mycobacterium is resistant to reactive oxygen radicals.

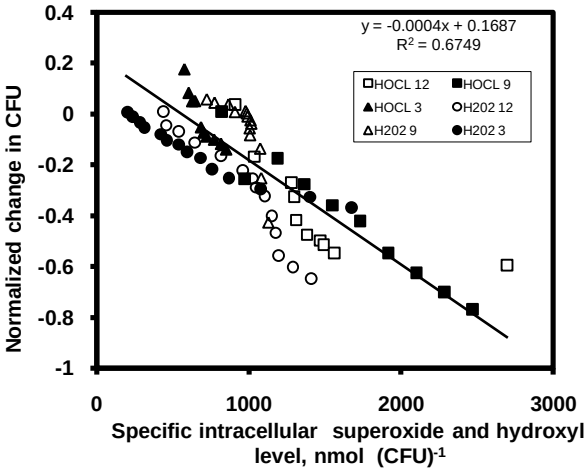


Figure6. Graph showing correlation between specific intracellular superoxide and hydroxyl level nmol/CFU and normalized change in CFU in all three growth phases like Early log phase (3rd day), Mid log phase (9th day) and Early stationary phase (12th day) of *M.aurum*. There is a linear decrease in normalized change in CFU as the superoxide and hydroxyl levels increased.

Correlation of killing extent with siH across growth phases and inducing agents and organisms

When the correlation in the previous section is extended to another organism, *E. coli*, the correlation still seems valid.

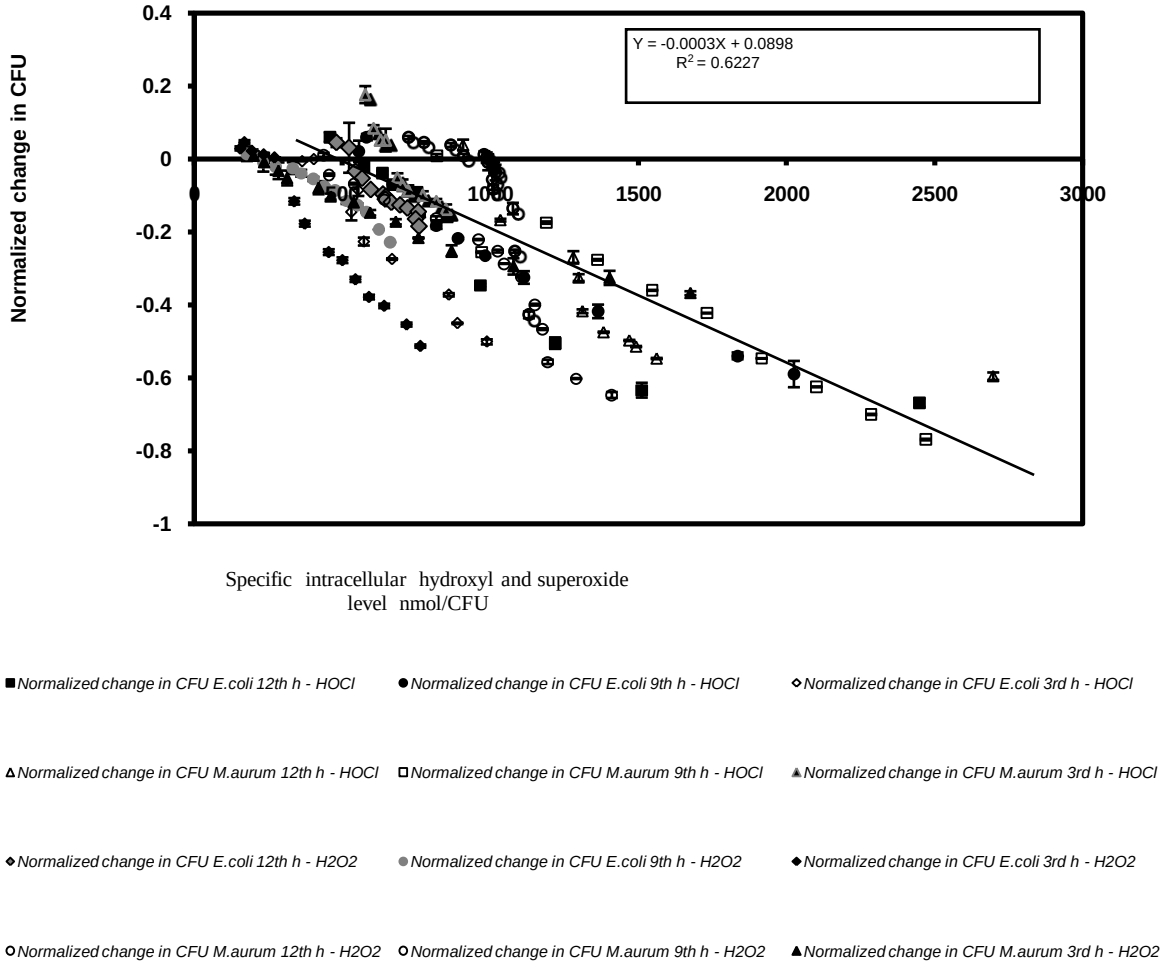


Figure8. The correlation of cell killing expressed as normalized change in CFU in *E.coli* and *M.aurum* with increasing concentration of specific intracellular hydroxyl and superoxide level nmol/CFU. There is a linear decrease in normalized change in CFU with the increase in superoxide and hydroxyl level in all three growth phases in both *E.coli* and *M.aurum*.

Thus from the above results it is clear that the results need further explanation and research to throw light in the areas how *Mycobacterium aurum* which is considered to be resistant for the chlorination initially in the early stages of culture showed cell killing and gradually decreased the normalized change in CFU with higher concentrations of superoxide and hydroxyl radicals during the early stationary phase of growth. Future studies may involve the antioxidant enzymes like catalase and superoxide dismutase activity and their influence on cell killing in *M.aurum.*

Conclusion

From all the above experiments it is clear that there is ROS production in all the microbial cultures. As the concentration of treating agents increased superoxide and hydroxyl production also increased. There is a direct relationship between the superoxide and hydroxyl production and the decrease in cell concentration (change in CFU/ml). Mycobacterium aurum when compared to Ecoli produced significant level of reactive radicals when treated with H_20_2. HOCl caused similar changes in ROS in all the cultures. The influence of reactive oxygen species was found to be dependent on growth phase of the organisms and the possible reasons for that was discussed and future work will include antioxidant enzyme activity in *M.aurum* along with EPS (extracellular polysaccharide production) analysis.

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