Destruction of *Aspergillus versicolor, Penicillium chrysogenum, Stachybotrys chartarum, and Cladosporium cladosporioides spores* using Chemical Oxidation Treatment Process

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Abstract

The survival of aqueous suspensions of *penicillium chrysogenum, stachybotrys chartarum, aspergillus versicolor and cladosporium cladosporioides* spores was evaluated using various combinations of hydrogen peroxide and Fe^{2+} as catalyst. Spores concentration of 10^6 - 10^7 CFU/ml were suspended in water and treated with initial hydrogen peroxide and iron concentrations ranging from 0.05 to 10 percent and 100 to 200 ppm, respectively. After four hours of reaction time, samples were plated on agar plates and the viable fraction of spores was determined by the number of colonies formed. Hydrogen peroxide concentrations above 50,000 ppm resulted in greater than 6-$\log_{10}$ reduction of viable spores for both catalyzed and non-catalyzed reactions. Iron had a strong catalytic effect when added to

solutions with hydrogen peroxide concentration above 5,000 ppm and resulted in two to three orders of magnitude greater reduction compared to hydrogen peroxide alone. Additional samples taken after 24 hours of reaction time showed that the effect of the addition of 100 and 200 ppm of Fe^{2+} catalyst was mostly kinetic and non-catalyzed hydrogen peroxide had sporicidal effects similar to catalyzed hydrogen peroxide. This study identified initial reagent concentrations of hydrogen peroxide and Fe^{2+} that accomplish a *6-log*$_{10}$ reduction of viable mold spores within reaction times 4 and 24 hours.

KEY WORDS: Hydrogen peroxide, ferrous iron, fungi spores, Fenton reaction.

Introduction

Molds in the indoor environment are often associated with health symptoms including respiratory symptoms, headaches, fatigue, and recurrent infections. Although there are no conclusive scientific and medical evidence on how the exposure to molds may affect a persons well-being and health there has been increasing awareness of the health effects of molds in homes, offices and schools. Different remediation technologies exist and are offered in the market to control mold growth inside buildings, most of them are use chemicals which are toxic to the user or to the people who are living inside the building. An innovative, safe and inexpensive remediation solution is required to treat and eliminate mold and mold spores from the indoor environment.

Hydrogen peroxide is a well-known antimicrobial agent used to decontaminate a wide range of viruses, bacteria and fungi. The main antimicrobial mechanism of hydrogen peroxide is based on a series of one-electron transfer chain reactions, which generate Reactive Oxygen Species (ROS) as intermediates and dioxygen, O_2, as the final product. The presence of highly reactive ROS results in homeostatic alterations of the cells and irreversible cellular injuries and eventually death for both aerobic and anaerobic organisms (Halliwell and Gutteridge, 1999). At sufficiently high concentrations,

ROS may cause severe chemical oxidative stress, which is expressed by a serious imbalance between the production of ROS and the antioxidant defense systems of an organism. Hydrogen peroxide has a higher oxidation potential (1.8V) than potassium permanganate (1.7V), chlorine dioxide (1.5V), and chlorine (1.4V); however, it has a lower oxidation potential compared to some of the intermediate decomposition products, e.g. hydroxyl radicals (2.8V), and other strong oxidants such as ozone (2.07V) and fluorine (3.0V). The addition of ferrous salts to aqueous hydrogen peroxide, (known as the Fenton reaction), may increase the levels of ROS by accelerating the decomposition of hydrogen peroxide and generating highly reactive intermediate species including hydroxyl radicals ($OH^{\bullet}$), ferryl (Fe^{4+}), and superoxide species ($O_2^{\bullet-}$), thus increasing the kinetics and the overall antimicrobial effect of hydrogen peroxide. The Fenton reaction is a well-known process that has been used for catalyzed oxidation of organics which otherwise show very slow kinetics of oxidation by hydrogen peroxide alone. Hydroxyl radicals are one of the hypothesized species formed as intermediate products of decomposition of hydrogen peroxide using ferrous iron (Haber and Weiss, 1934) (Equation 1):

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + H_2O + {}^{\bullet}OH$$

In aqueous solution of organics, hydroxyl radicals initiate a series of reactions with the organic compounds that further yield free radicals (Walling, 1975) The increased production of free radicals and ROS amplifies the cellular injuries and cell death at lower concentrations of hydrogen peroxide and much shorter reaction times compared to non-catalyzed system. The reactivity of the free radicals is very high and only one to five percent of the radicals may escape the electron transfer reactions and subsequently oxidize DNA, lipids and proteins, which do not react with hydrogen peroxide alone or have unfavorably slow kinetics (Halliwell and Gutteridge, 1999;Punchard and Kelly 1996; and Kelly et al., 1998). Since the underlying mechanism of spore destruction and inactivation is the oxidative stress induced by the ROS generated in the process of hydrogen peroxide decomposition, the addition of iron salts may increase the decontamination kinetics and overall efficiency of hydrogen peroxide.

Furthermore, the addition of a reducing agent to a system with a high concentration of ferric iron (Fe^{3+}), such as ascorbate, has been suggested to increase the damage to biological molecules by enabling redox cycling of iron (Kelly et al., 1998).

The combined microbicidial effect of the Fenton reaction (aqueous mixture of hydrogen peroxide and iron salts) has been investigated for inactivation of junin virus, *escherichia coli*, and spores of *bacillus subtilis* (Sagripanti, 1992 and Sagripanti et al., 1993). The results suggest that metal-based reagents may have higher microbicidal activity than some of the most potent disinfectants e.g., glutaraldehyde. Salts of iron and copper were added to aqueous solution hydrogen peroxide in ratios of metal to hydrogen peroxide varying between 3:10 to 1:100 (mol:mol). For initial concentrations of hydrogen peroxide equal to 100 ppm and iron of 30 ppm, 99 percent of inactivation was obtained within less than 30 minutes. A literature review (Sahl and Munakata-Marr, 2006) of chemical oxidation treatments coupled with bioremediation processes suggested that biological processes may be adversely affected in the short term, but a rebound of biological activity can be expected later on as long as low concentrations of catalyzed hydrogen peroxide have been used.

The addition of nitrilotriacetic acid (NTA) additionally increased the antimicrobial activity of catalyzed hydrogen peroxide. The Fenton reaction is homogeneously catalyzed, based on the solubility of the Fe^{2+}/Fe^{3+} system, and has the highest rate in the low acidic range, i.e. pH<3.5 while in the neutral range the reaction is relatively slower. The addition of a strong ligand, such as NTA holds the iron in solution and extends the reaction in the neutral pH range.

Related work by Cross et al., (2003) investigated the effect of a modified Fenton reagent formulation using Cu for a catalyst and adopted this system for decontamination of biological endospores such as *bacillus globigii*. The modified Fenton reaction involves conversion of aqueous dissolved oxygen to hydrogen peroxide by an ionic catalyst, Cu^{2+}, which subsequently reacts with hydrogen peroxide and forms highly reactive intermediate species, which include hydroxyl radicals. The exterior of the spores

was not damaged, which suggested an interior kill mechanism. Such a mechanism would require diffusion of relatively benign species into the interior of the spore and presence of hydrogen peroxide generated inside the spore coat. Based on the results, the authors concluded that the reactive species generated by the Fenton reaction most likely either attack the local DNA fragments causing cell death, or attack enzymes necessary for spore germination into a bacteria cell. Using a mixture of $CuCl_2$ and ascorbic acid Shapiro et al., (2004) investigated the killing of *bacillus subtilis* spores and the results from this study suggested that a Fenton reagent formulation with Cu^{2+} and ascorbic acid kills spores of *bacillus subtilis* species, however, it requires the presence of Cl^- ions. The killing agent was most likely chlorine-containing specie that was formed by as result of reacting hydroxyl radicals. Melly et al. (2002) investigated the mechanism of killing of *bacillus subtilis* spores, which have shown higher resistance to oxidizing agents (Setlow and Setlow, 1993) and concluded that treatment by non-catalyzed hydrogen peroxide changes the process of spore germination and suggested that the core can not expand properly most likely as a result of damages to core components.

The catalytic properties of Fe^{2+} are most significant in the pH range between 2.5-3.5. The pH range can be extended to neutral pH by complexing the iron with strong ligand (e.g. EDTA, NTA). Furthermore, the work by Cross et al. (2003) indicated that the presence of reducing agent (ascorbic acid) in the Fenton reaction could increase its sterilization activity by keeping the copper ion in its reduced state (Cu^+). The same effect can be expected when Fe^{2+} is used as the catalyst. The presence of ascorbic acid may increase the activity of iron catalyst by (1) creating reducing conditions and by (2) complexing with Fe^{2+}, thus keeping iron in solution. Furthermore, the presence of surfactant may additionally improve the efficiency of the Fenton formulation by providing better contact of the reagent with individual as well as clumps of spores.

While the addition of transitional metals to aqueous solutions of hydrogen peroxide has demonstrated improved decontamination efficacy towards viruses and bacteria, less research has been conducted on

commonly occurring mold spores such as *penicillium chrysogenum, stachybotrys chartarum, aspergillus versicolor and cladosporium cladosporioides*. The major objectives of this work were to provide data about the efficacy of different Fenton reagent formulations on mold spore destruction, and to obtain empirical coefficients for microbial destruction as a function of initial reagent concentrations.

Among all chemical sterilants, hydrogen peroxide has less adverse effects on the environment and leaves residues that are safe and require no additional treatment. Hydrogen peroxide is considered noncarcinogenic, nonmutagenic and its residue can decompose (Block, 2001). In addition to its effective germicidal and sterilizing capabilities, the ultimate decomposition properties of hydrogen peroxide are oxygen and water which are innocuous, naturally occurring, and safe to the environment. Furthermore, for environmental remediation applications Fe^{2+} can be considered a superior catalyst compared to the rest of the transitional metals (Cu, Zn) based on the fact that Fe^{2+} / Fe^{3+} are benign to the environment at the levels needed to catalyze the decomposition of hydrogen peroxide. This study used concentrations of Fe^{2+} lower than 500 mg/L. Furthermore, the oxygen gas that is released during the decomposition of hydrogen peroxide may provide additional bactericidal effects by diffusion into the fungal mass, and more specifically when applied in porous and aqueous media.

Materials and methods

Materials

Hydrogen peroxide solutions with desired concentrations were prepared fresh for each test from a 50 percent stock solution of H_2O_2 purchased from MP Biomedicals, LLC (29525 Fountain Pkwy. Solon, OH 44139 United States). Hydrogen peroxide was measured using the $Ti(IV)H_2O_2$ complex. A Shimadzu UV1601 spectrophotometer was used to measure the absorbance of the complex at 405 nm. The samples were pre-mixed with excess titanium sulfate solution in sulfuric acid (0.01 N). This method has been used in other work and a detection limit of 2 mg/l (ε=754 $dm^3M^{-1}cm^{-1}$) has been reported

(Eisenberg, 1943 and Francis et al., 1985). Dilutions were used to bring the samples into the linear range (2 to 30 mg/l). Interference at 405 nm was not observed for Fe^{2+}/ Fe^{3+}. A 10,000 ppm iron stock solution was prepared fresh for each test by diluting ferrous sulfate heptahydrate (MP Biomedicals, LLC) with DI water and adjusted to pH=2 with hydrochloric acid 6N. Phosphate Buffered Saline (PBS) with Tween 20 was purchased as a dry powder in foil pouches from Sigma Aldrich Company (Milwaukee,WI 53201 USA). Each pouch was dissolved in one liter of sterile, deionized water to produce 0.01M phosphate buffered saline with 0.05% Tween 20 at pH 7.4. Pre-filled Potato Dextrose Agar (PDA) plates were purchased from bioMérieux Industry (595 Anglum Road Hazelwood, Missouri, 63042-2320, USA) and "Bac-Loops", disposable plastic inoculating loops/needles, were purchased from Fisher (https://www1.fishersci.com). These products were used to grow and count colonies from serial dilutions.

Fungal spores were purchased from ATCC (American Type Colture Collection P.O. Box 1549 Manassas, VA 20108 USA) (*A. versicolor* ATCC # 52173, *S. chartarum* ATCC # 66239, *P. chrysogenum* ATCC # 9480, *C. cladospoioides* ATCC # 16022) and rehydrated with 5 mL of sterile water for a minimum of 2 hours (American Type Culture Collection "Instructions for Rehydrating Freeze-Dried Cultures". http://www.atcc.org/pdf/rfdc.pdf). The spores were stored at 4°C for 8-12 hours and then streaked onto solid PDA agar to obtain new fungal growth. New spores were harvested by agitating the top of the growth with a disposable plastic loop and PBS. After performing and plating a set of serial dilutions, the colonies grown were washed with 3 to 9 mL of 0.01 M Phosphate buffer with 0.1 percent Tween 20. The spores were gently agitated with Bac-Loops and the supernatant was pipetted into 50 mL centrifuge tubes and centrifuged at 12,000 X g for 5 minutes. The supernatant was decanted and the pellet was washed three times with 10 mL phosphate buffer. The pellet was resuspended in deionized water and the spore solution was stored at 4˚C. The new spores were counted

under a microscope using a pre-calibrated Hausser Scientific Bright-line Hemocytometer. Spores were diluted until a final concentration of approximately 10^8 spores per mL of solution was achieved.

Viable spore counting

The number of viable fungal spores was determined by counting the number of colonies that grew on PDA. Samples were serially diluted up to six times (each serial dilution is based on a factor of 10) and 200µL were plated twice for each dilution. The samples were spread evenly across the plates using a disposable Bac-Loop. The plates were then incubated until colonies started to grow. The number of colonies were counted for each plate and multiplied by the reciprocal of the plate dilution and the dilution fraction of the initial sample to obtain the number of viable fungal spores per mL of solution.

Reactions with hydrogen peroxide

The critical process factors that were kept constant were temperature, pH, and initial microorganism concentrations. The experiments were conducted in a liquid medium (de-ionized and sterile water). The initial stock solution of spores was diluted to $>10^7$ spores/ml in a ten ml beaker. The required amount of Fe^{2+} was added using a 10,000 ppm stock solution. Hydrogen peroxide was added using stock solution of 50 percent H_2O_2. The beakers were placed on a shaker at 300 RPM to ensure adequate mixing since it has been observed that inadequate mixing may result in flocculation and subsequent precipitation of the spore suspension. The reactions were allowed to continue for 4 hours. After 4 hours, 3 ml of samples were withdrawn and 0.100 mL of catalase was added to quench the reaction by destroying the residual hydrogen peroxide concentration. Two hundred microliters were withdrawn and diluted up to 6-fold serial dilution and duplicates were plated from each serial dilution using PDA media. The plates were incubated at the appropriate temperature (25^{o}C) for 3-5 days and the number of fungal colonies on the plates (CFU/mL) was determined. About 15 percent of the reactions were continued for 24 hours and

samples were collected to investigate the activity of the system for extended periods of time. The average initial concentrations of viable spores was in the range of 1.5 10^7 spores/ml for *penicillium chrysogenum*, 3.8 10^6 spores/ml for *aspergillus versicolor* 1.6 10^7 spores/ml for *stachybotrys chartarum* and 4.2 10^7 spores /ml for *cladosporium cladosporioides*.

Spore reduction analysis

After determining the CFU counts, the ratio of surviving spores, N_i to initial viable spore count, N_0 was determined (Equation 2):

$$\text{Spore Survival Ratio} = \frac{N_i}{N_0}$$

For fixed initial concentrations of catalyst $\log_{10}$ of the survival fraction values was plotted versus concentration of hydrogen peroxide. Exponential fitting using the non-linear least square method was applied to determine two unknown coefficients (Equation 3):

$$\text{Spore Survival Ratio} = c \times 10^{k[H_2O_2]}$$

Where $[H_2O_2]$ is hydrogen peroxide concentration, k and c are fitted coefficients. On a semi-log scale Equation 3 results in a straight line with slope equal to k. For each set of fixed initial concentrations of iron, the slope was determined.

Exhibit 1

The $\log_{10}$ of the ratio of surviving spores to spores determined in untreated control samples is a linear function with respect to hydrogen peroxide concentration. Each point represents the reduction of spore count after 4 hours of reaction. Values that resulted in greater than 7-$\log_{10}$ reduction (the initial concentration of spores was 10^7 spores/ml) were excluded from the fit.

Results and Discussion

Aspergillus versicolor spores were diluted to 10^7 spores/ml and were subjected to treatments with variable initial concentrations of hydrogen peroxide. The reactions of non-catalyzed hydrogen peroxide were compared with catalyzed reactions using 100 and 200 ppm of Fe^{2+}. Exhibit 2 shows a comparison of the destruction trends for *aspergillus versicolor* spores using non-catalyzed and catalyzed hydrogen peroxide. There is a clear trend of an increase of the sporicidal effect of the hydrogen peroxide solution when Fe^{2+} is added. For hydrogen peroxide fraction equal to 0.01 a 5-log difference was observed when comparing catalyzed and non-catalyzed reactions. High concentrations of hydrogen peroxide (above 50,000 ppm) have resulted in greater than 6- $\log_{10}$ reduction of spores independently of the presence of catalyst.

Similar but less pronounced effect was observed for *penicillium chrysogenum*. For concentrations of hydrogen peroxide greater than 15,000 ppm (1.5%) the reduction of viable spores was greater than 6-$\log_{10}$ independently of the presence of catalyst. For concentrations of catalyst, Fe^{2+}, greater than 200 ppm hydrogen concentrations in the range of 7,500-10,000 were required to accomplish 6-$\log_{10}$ reduction of viable spores. Comparison between Exhibit 2 (*aspergillus versicolor* spores) and Exhibit 3 (*penicillium chrysogenum* spores) shows that the catalyzed reaction is less selective compared to non-catalyzed hydrogen peroxide.

Similar values were obtained for *cladosporium cladosporioides and stachybotrys chartarum*; the addition of iron catalyst resulted in considerable effect on spore inactivation for reactions of 4 hours. The greatest effect was observed for hydrogen peroxide concentrations between 5,000 ppm and 50,000 ppm. No comparative data are available beyond 50,000 ppm since greater than 7-$\log_{10}$ reduction of spores occurred regardless the presence of a catalyst.

Exhibit 4 compares treatments of *stachybotrys chartarum* using hydrogen peroxide concentrations between 0 and 25,000 ppm catalyzed with 200 ppm of Fe^{2+} and non-catalyzed hydrogen peroxide. Similar to *penicillium chrysogenum* catalyzed reactions using more then 15,000 ppm hydrogen peroxide resulted in greater then 6-$\log_{10}$ reduction in spores count. For non-catalyzed reactions considerably higher concentrations of hydrogen peroxide were needed to achieve 6 log reduction. Furthermore, the treatment with 15,000 ppm hydrogen peroxide and 200 ppm iron resulted in approximately 6-$\log_{10}$ reduction, while the treatment with 15,000 ppm non-catalyzed hydrogen peroxide and zero iron resulted in approximately 2.5-3 $\log_{10}$ reduction. Similar observations were obtained for all four fungus species including *cladosporium cladosporioides.*

Kinetics of spore destruction

The kinetics of spore destruction was tested for hydrogen peroxide concentration equal to 3 percent and iron equal to 200 ppm. The results are shown on the next figure. Catalyzed reactions clearly demonstrate considerably faster kinetics Exhibit 5.

Effect of initial catalyst concentrations

Exhibit 6 demonstrates the effect of different iron concentrations on spore survival. For lower concentrations of hydrogen peroxide below 5,000 ppm no spore destruction has been observed and the addition of iron has no effect. The effect of iron becomes significant for hydrogen concentrations above 5,000 ppm up to 25,000. Beyond 25,000 ppm of hydrogen peroxide both systems (catalyzed and non-catalyzed) resulted in greater than 6-$\log_{10}$ destruction of *penicillium chrysogenum* spores. While the addition of iron has showed proportional increase of the spore destruction it is important to note that higher concentrations of catalyst require considerably better mixing. Fe^{2+} reacts with hydrogen peroxide and oxidizes to Fe^{3+}, which has much lower solubility and precipitate as hydroxides. This problem can

be avoided either by keeping low pH of the solution (2.5), or adding a strong ligand to the iron solution (e.g. ethylenediaminetetraacetic acid known as EDTA, nitrilotriacetic acid known as NTA or any other ligand, including ascorbic acid). The addition of a strong ligand allows the reaction to proceed at pH=3.5-7.0.

Hydrogen peroxide concentrations above 25,000 resulted in greater than 6-$\log_{10}$ reduction of viable spores for both catalyzed and non-catalyzed reactions within 4 hours.

Comparison of the catalytic effect for 24 hrs

While catalyzed reactions complete within 4 hours, non-catalyzed reactions have significant levels of hydrogen peroxide and may continue to destroy spores during the first 24 hours. A test was conducted which compared the destruction of spores at 4 and 24 hours. The comparison is shown in Exhibit 7.

For Fe=0 ppm, a reaction time of 24 hours continues to inactivate the mold spores and reaches greater than 6-$\log_{10}$ reduction of viable spores when the concentration of hydrogen peroxide is above 5,000 ppm, while a reaction time of 4 hours accomplishes 6-$\log_{10}$ reduction only when hydrogen peroxide exceeds 20,000-25,000 ppm. For catalyzed reactions with iron concentration greater than 200 ppm, 4 hours reaction time was sufficient for 6-$\log_{10}$ reductions concentration of hydrogen peroxide above 7,500 ppm. This demonstrates that the effect of iron addition was mostly kinetic, i.e. for prolonged reaction periods of 24 hours there was no significant difference between catalyzed and non-catalyzed reactions.

For reactions which were continued above 24 hours hydrogen peroxide concentrations of 7,500 ppm resulted in non-detectable levels of spores and consequently greater than 6-$\log_{10}$ reduction of viable spores for both catalyzed and non-catalyzed reaction (Exhibit 8).

Spore reduction Parameters

The exponential coefficients were determined and summarized in Exhibit 9. The table demonstrates that the reactions with duration of 4 hours, the coefficients increase as function of the catalyst. However, tests with *penicillium chrysogenum* and *cladosporium cladosporioides* showed that for extended reaction times of 24 hours the values of the coefficients are almost identical demonstrating that the effect of iron addition was kinetic. The uncertainties (calculated as the error at 95% confidence) are shown in parenthesis. At the lower range of iron, the uncertainties are considerably lower (between 5 and 10%) and increase up to 50% and more at higher levels of catalyst.

Table 1 Calculated exponential coefficients[1]

Spores, reaction time	Fe=0 ppm	Fe=100 ppm	Fe=200 ppm	Fe=300 ppm	Fe=500 ppm
P. chrysogenum, 4hrs	-4.23e-04 (4.6e-05)	-4.48e-04 (3.9e-05)	-7.91e-04 (2.1e-04)	-9.94e-04 (3.5e-04)	-1.19e-03 (4.5e-04)
P. chrysogenum, 24hrs	-1.18e-03 (5.2e-04)	-1.18e-03 (4.7e-04)	-1.22e-03 (6.9e-04)	-1.20e-03 (7.1e-04)	-1.24e-03(6.9e-04)
A. versicolor, 4hrs	-1.30e-04(2.5e-05)	-3.16e-04(3.7e-05)	-6.45e-04(2.0e-04)		
C. cladospoioides, 4hrs	-2.44e-04(3.7e-05)	-6.77e-04(5.1e-04)			
C. cladospoioides, 24hrs	-6.40e-04(7.1e-04)	-6.89e-04(4.2e-04)	-6.58e-04(5.3e-04)		
S. Chartarum,, 4hrs	-1.84e-04(2.7e-05)		-3.88e-04(1.2e-04)		

Measurements of hydrogen peroxide for non-catalyzed reactions for up to 24 hours are shown on Exhibit 10. For hydrogen peroxide greater than 10,000 ppm, there is insignificant degradation over 24 hours. When the initial hydrogen peroxide was below 2,500 ppm significant fraction of hydrogen peroxide was degraded over time most likely caused by reaction of oxidizable organic matter with hydrogen peroxide. This demonstrates that at least 10,000 ppm of hydrogen peroxide was needed in order to maintain sufficient levels of residual hydrogen peroxide.

[1] The uncertainty of each coefficient is shown in parenthesis next to the value. The uncertainties were determined from 95 percent confidence limits.

For non-catalyzed reactions, the residual amount of hydrogen peroxide after 4 hours is insignificant when the initial concentration of hydrogen peroxide is 2,500 ppm. After 4 hours there is insignificant reduction of viable spores. When the initial hydrogen peroxide concentration is above 10,000 ppm there are significant residual levels beyond 24 hours of reaction. In general, reactions catalyzed by greater than 100 ppm of Fe^{2+}, are completed within 4 hours reaction time and the decomposition of hydrogen peroxide results in elevated levels of ROS. Complete and vigorous mixing is required in order to avoid recombination of the generated radicals, which most likely is accompanied by decreased disinfection efficiency. Exhibit 11 shows catalyzed decomposition of hydrogen peroxide. The majority of the hydrogen peroxide was destroyed within one hour, therefore the catalyzed reactions result in considerable faster kinetics.

Exhibit 12 compares sporicidal activity of non-catalyzed and catalyzed hydrogen peroxide toward viruses, bacterial and fungal spores for three ranges. The work by Toledo et al (1973) shows that hydrogen peroxide concentrations above 258,000 ppm complete inactivation of *B. globiggi* spores within a few minutes. Sagripanti et al, (1993) used considerably lower concentrations of hydrogen peroxide (100 ppm catalyzed by 1 ppm Cu^{2+} or Fe^{2+}) for inactivation of viruses, however, the reaction times become several orders of magnitude greater (in the order of hours) and the lethality decreased. Copper ions, Cu^{2+}, showed better efficacy then Fe^{2+} and resulted in a faster kill of the viruses.

[1] Bacterial cultures [2] Spore suspension

[a] Contains 0.1 M Ascorbic Acid, [b] Contains 0.1 M Ascorbic Acid, 2M NaCl, [c] Contains 0.1 M Ascorbic Acid, 0.2M NaCl, [d] Contains 0.1 M Ascorbic Acid, 2M NaCl and 1% surfactant, [e] Contains 0.1 M Ascorbic Acid, 0.1M NaCl,

The work presented here used Fe^{2+} and determined the optimal concentration of the reagents, which allow for 6-$\log_{10}$ reduction of aqueous suspension of four common species of mold spores. The data listed in Exhibit 12 show that for catalyzed reactions, 7,500-10,000 ppm of hydrogen peroxide was required to accomplish 6-$\log_{10}$ reduction of each of the four mold species. The lowest value (7,500 ppm) was required for *stachybotrys chartarum* and *penicillium chrysogenum* and 10,000 ppm were required

for *aspergillus versicolor* and *cladosporium cladosporioides.* In order to accomplish 6-$\log_{10}$ reductions, non-catalyzed reactions required 50,000 ppm for *aspergillus versicolor,* 15,000 ppm for *penicillium chrysogenum,* 25,000 ppm for *stachybotrys chartarum,* and 30,000 ppm for *cladosporium cladosporioides.* A conclusion can be drawn that catalyzed reactions are considerably less selective and the addition of iron most likely results in a wider range of fungicidal effects towards species with higher resistance (such as *aspergillus versicolor*) compared to hydrogen peroxide alone.

Conclusions

This work demonstrated that the sporicidal properties of hydrogen peroxide can be enhanced by using Fe^{2+} as the catalyst of hydrogen peroxide decomposition. The greatest effect was observed for *aspergillus versicolor, which* demonstrated considerably higher resistance to hydrogen peroxide alone. On the other hand, the effect of addition of iron to reactions with *penicillium chrysogenum* spores was mostly kinetic and after 24 hours similar spore inactivation levels were observed for both: catalyzed and non-catalyzed reaction. Iron (including all of its oxidation states Fe^{2+}/Fe^{3+}) is benign for the environment and it is a better alternative compared to Cu ions, even though Cu^{+}/Cu^{2+} may have demonstrated higher catalytic activity. One of the most important features of this process is that all reagents (hydrogen peroxide and Fe^{2+}) including their decomposition byproducts are environmentally benign and safe. Hydrogen peroxide is a "green technology" which has strong fungicidal effects without the inherent health and toxicity risks that ozonation, chlorination or other fungicidal methods may introduce.

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List of Captions for Exhibits:

Exhibit 1 - An example of fitting *P. chrysogenum* survival data using exponential fit. Symbols represent independent sets of experiments at temperature 25° C, pH=3.5, initial catalyst concentration of Fe(II) = 100 ppm, and reaction time of 4 hours.

Exhibit 2 - Comparison of the effect of variable hydrogen peroxide concentrations and addition of iron on the reduction of *A. versicolor* spores suspended in water. Initial conditions: pH=3.5, T=25°C, and reaction time=4 hours.

Exhibit 3 - Comparison of the effect of variable hydrogen peroxide concentrations and addition of iron for the reduction of *P. Chrysogenum* spores suspended in water. Initial conditions: pH=3.5, T=25°C and reaction time=4 hours.

Exhibit 4 - Comparison of the effect of variable hydrogen peroxide concentrations and addition of 200 ppm of iron on the reduction of *S. Chartarum* spores suspended in water. Initial conditions: pH=3.5, T=25°C and reaction time=4 hours.

Figure 5 - Kinetics of *P. Chrysogenum* spore destruction spores for 3% hydrogen peroxide and 200 ppm Fe(II) at pH=3.5 and T=25°C. For catalyzed reactions, 6-log reduction is accomplished within 20 minutes, while non catalyzed reaction required in excess of 100 minutes.

Exhibit 6 - Comparison of the effect of variable catalyst concentrations on the reduction of *P. Chrysogenum* spores. Initial conditions: pH=3.5, T=25°C Reaction time=4 hours.

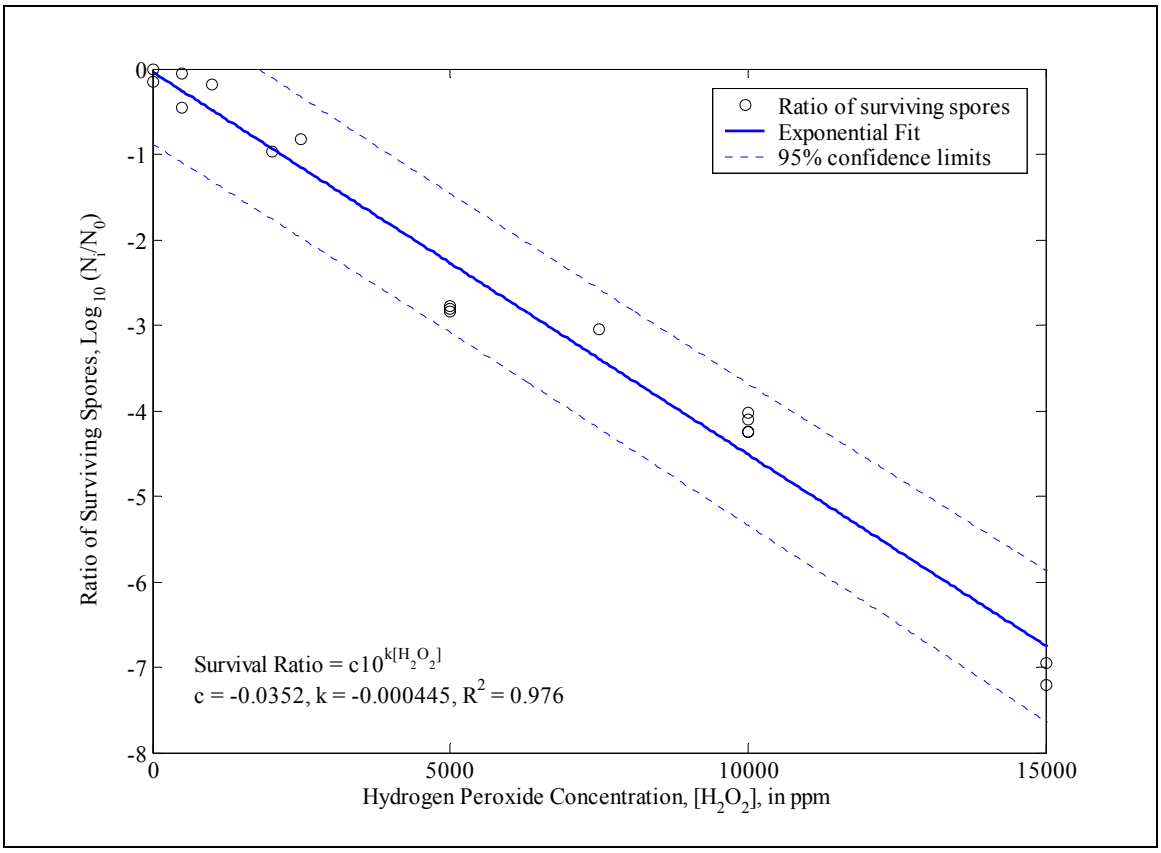
Figure 7 - Survival of *P. Chrysogenum* spores at 4 and 24 hours after treatment with variable initial concentrations of reagents. Initial conditions: pH=3.5 and T=25°C.

Figure 8 - Comparison of the effect of variable catalyst concentrations on the reduction of *P. Chrysogenum* spores for reaction time equal to 24 hours at pH=3.5 and T=25°C.

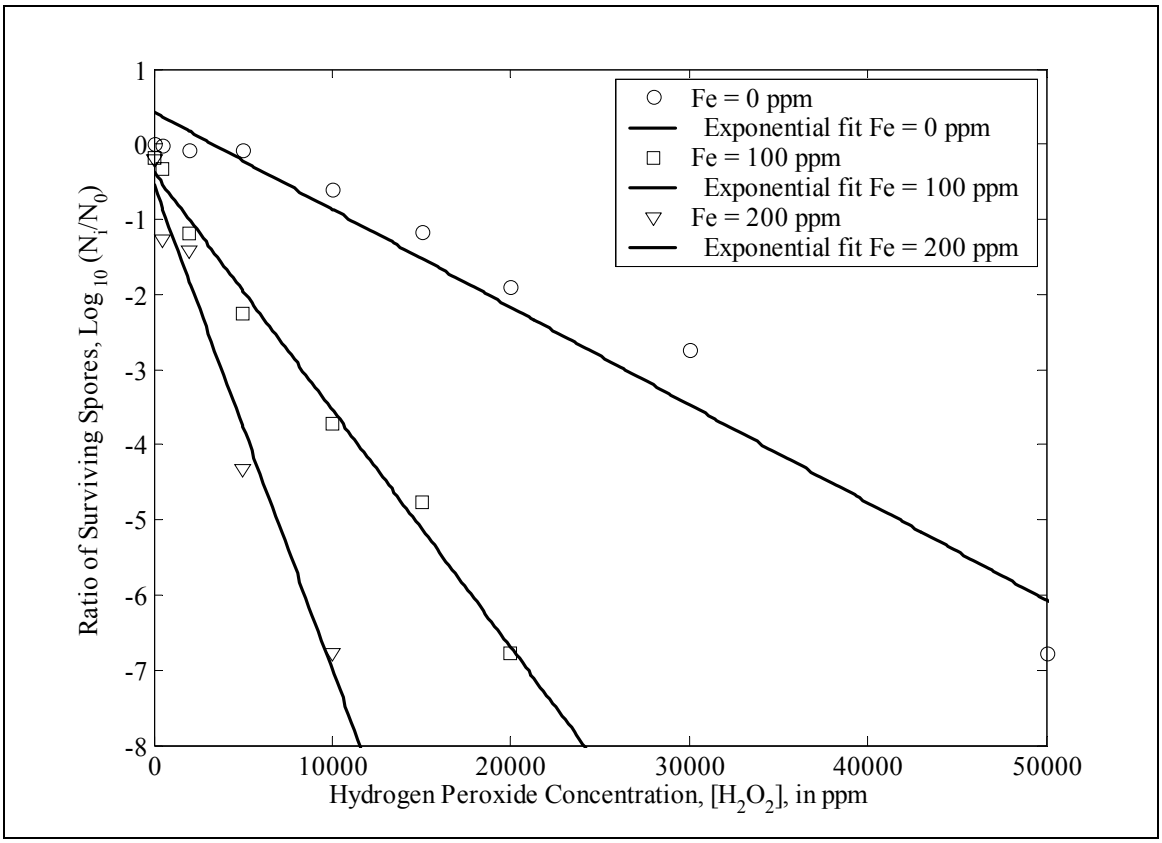
Exhibit 10 -Degradation of hydrogen peroxide for Fe=0 ppm at pH=3.5 and T=25°C.

Exhibit 11 -Degradation of hydrogen peroxide for Fe=500 ppm pH=3.5 and T=25°C.

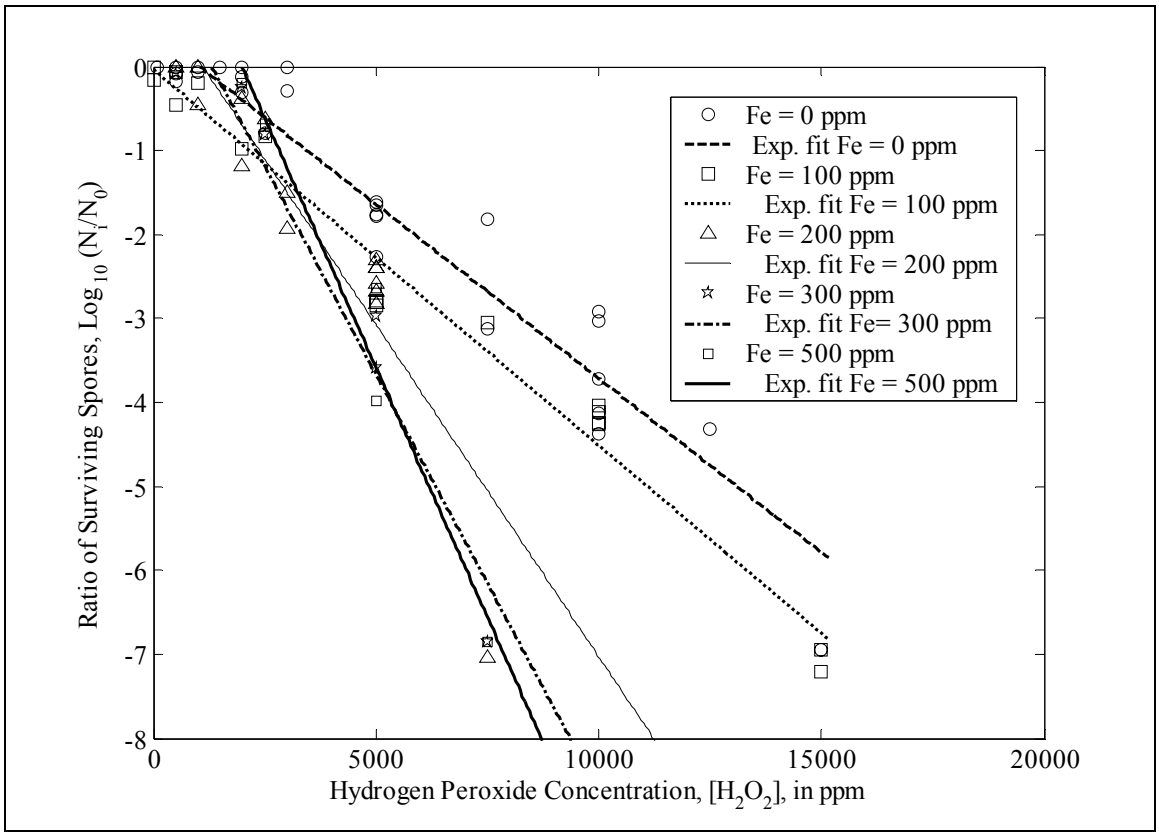
Exhibit 12 Activity of hydrogen peroxide toward viruses, spore forming bacteria, bacterial and fungal spores (lethality refers to $\log_{10}$ reduction of initial spore count).



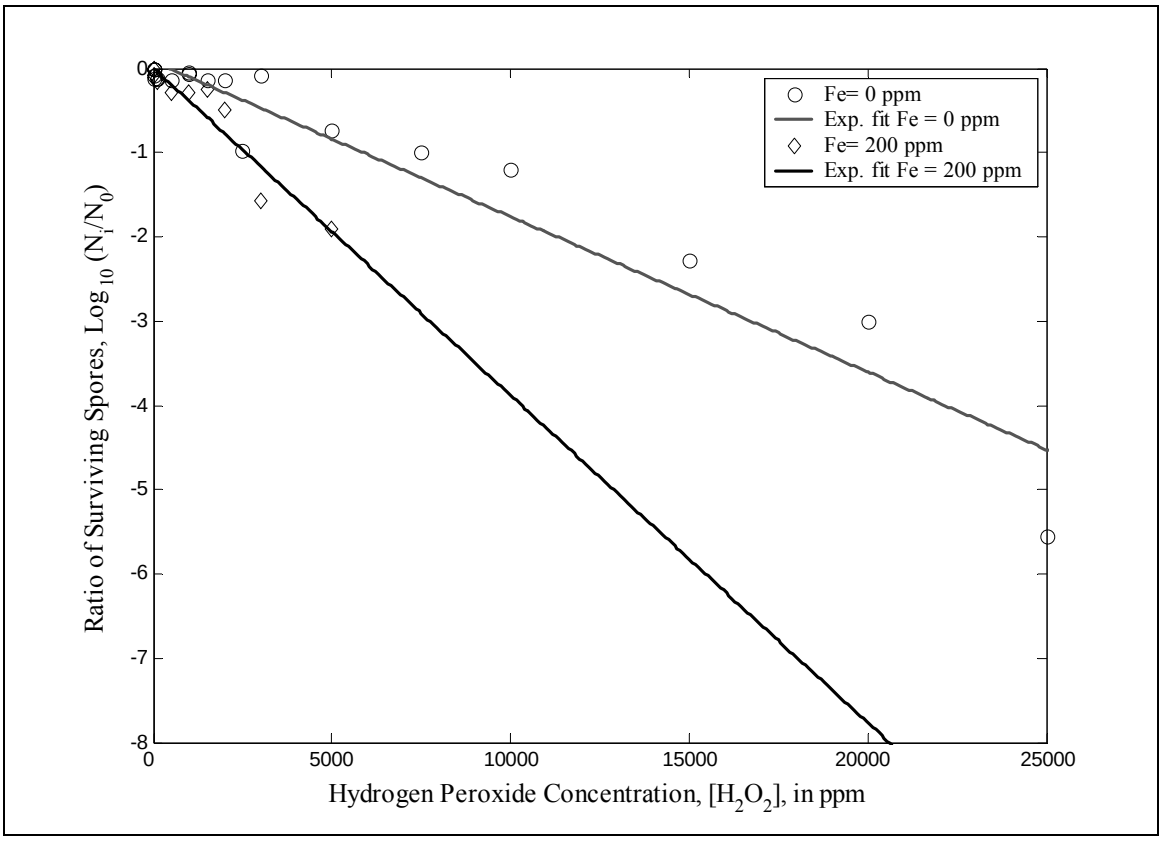
An example of fitting *P. chrysogenum* survival data using exponential fit. Symbols represent independent sets of experiments at temperature 25° C, pH=3.5 and initial catalyst concentration, Fe(II) = 100 ppm, Reaction time= 4 hr



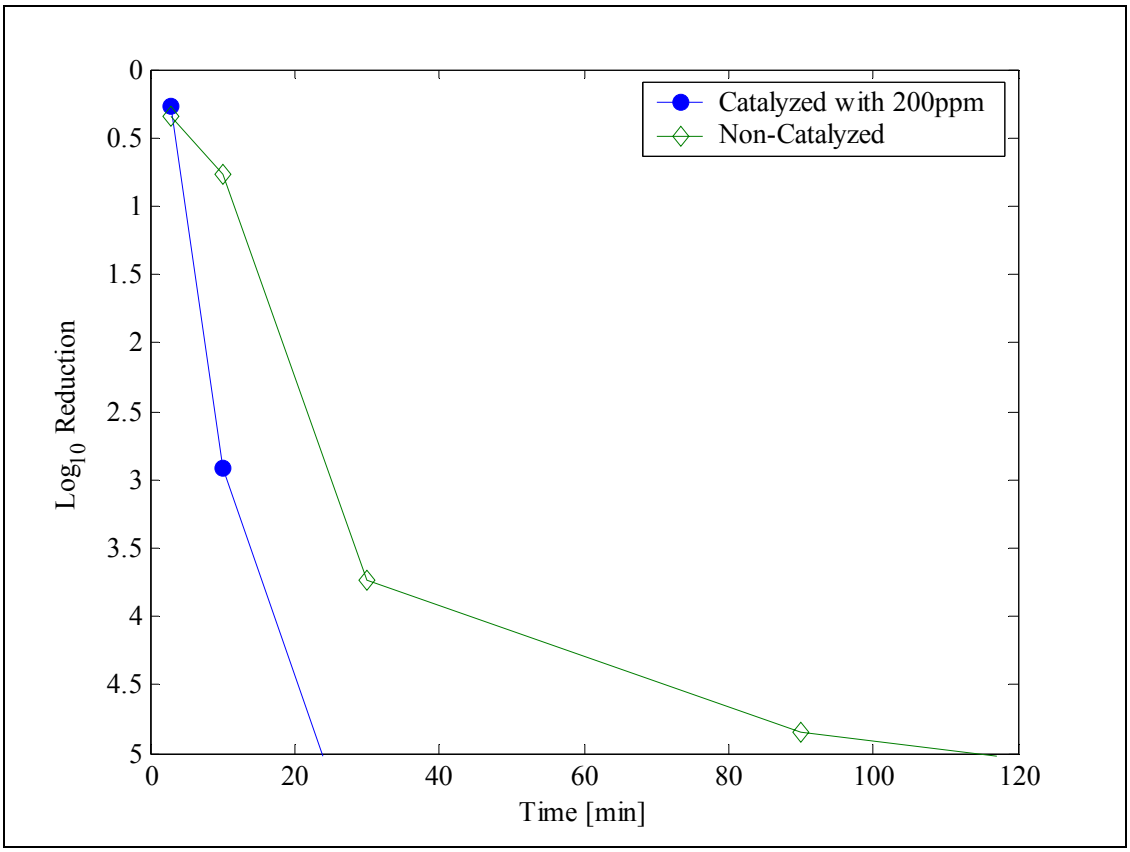
Comparison of the effect of variable hydrogen peroxide concentrations and addition of iron on the reduction of *A. versicolor* spores suspended in water. Initial conditions: pH=3.5, T=25^{o}C Reaction time=4 hours.



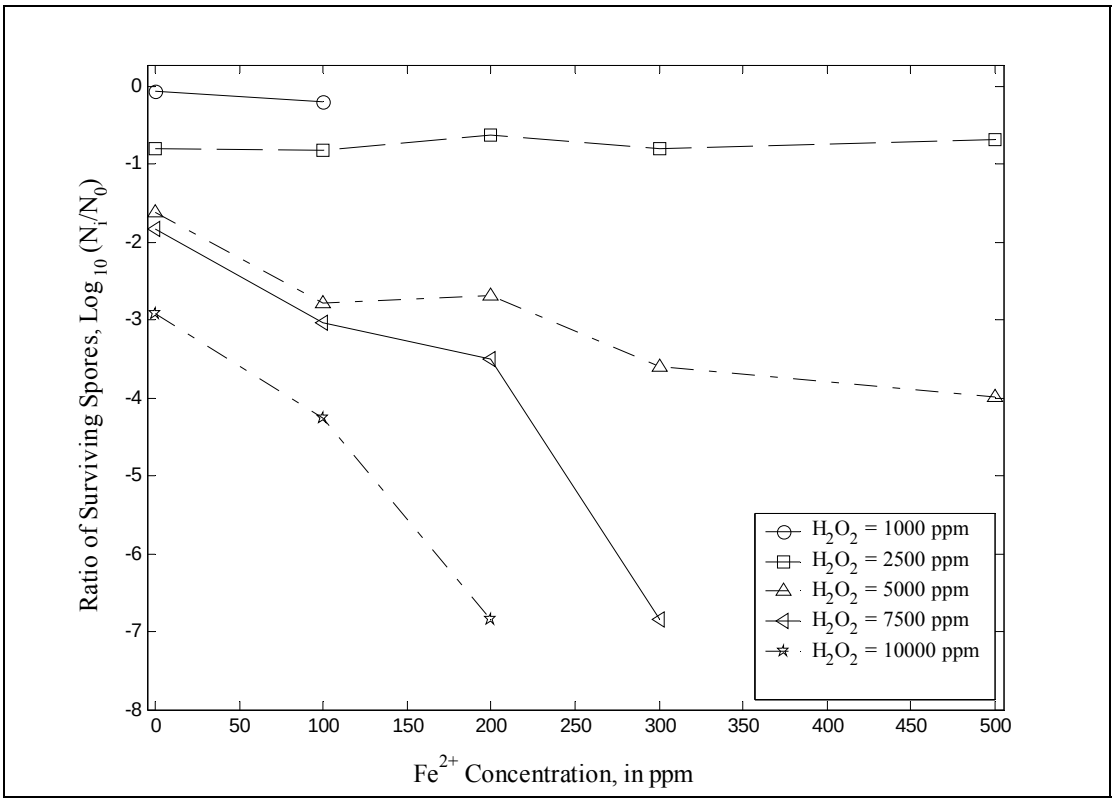
Comparison of the effect of variable hydrogen peroxide concentrations and addition of iron for the reduction of *P. Chrysogenum* spores suspended in water. Initial conditions: pH=3.5, T=25^{o}C Reaction time=4 hours.



Comparison of the effect of variable hydrogen peroxide concentrations and addition of 200 ppm of iron on the reduction of *S. Chartarum* spores suspended in water. Initial conditions: pH=3.5, T=25^{o}C Reaction time=4 hours.

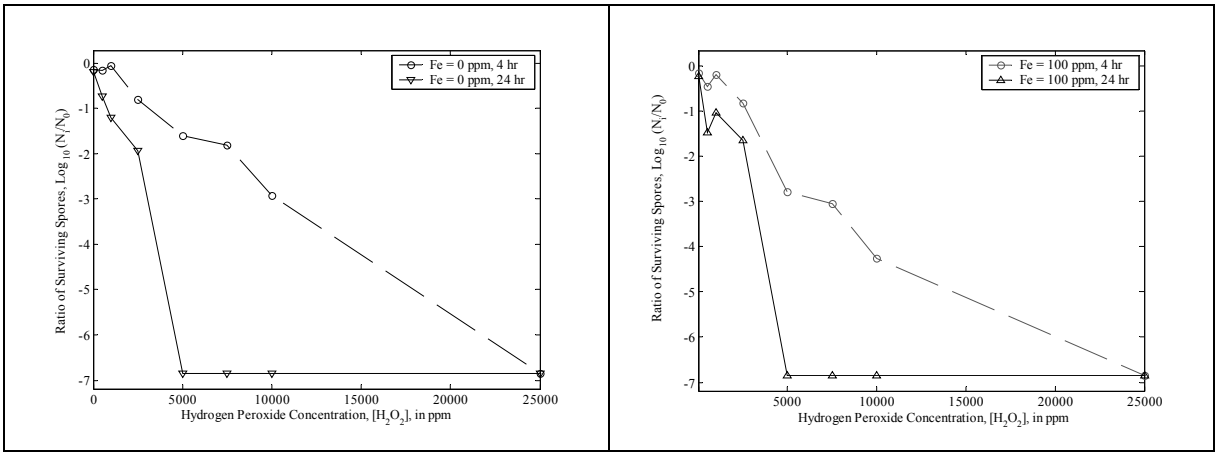


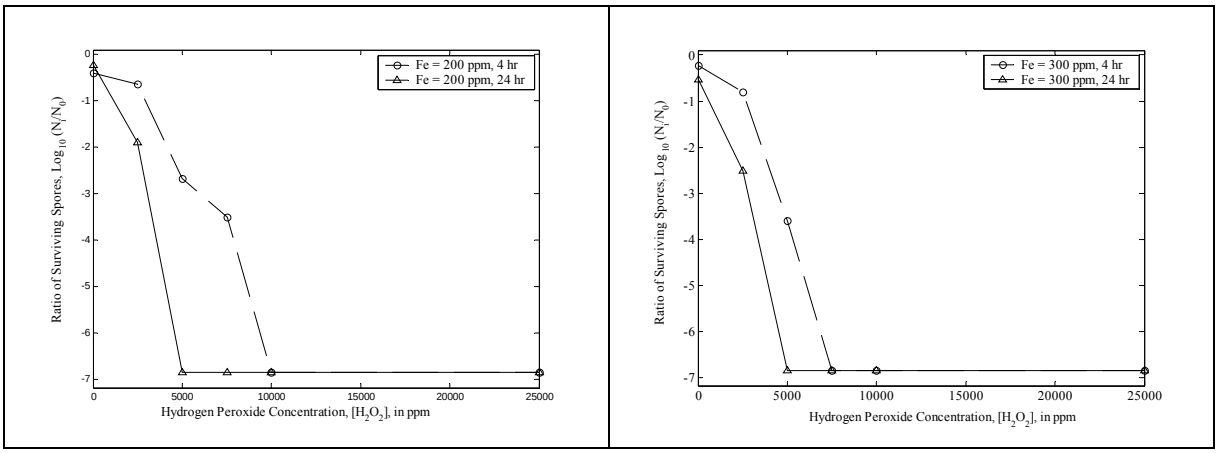
Kinetics of *P. Chrysogenum* spore destruction spores for 3% hydrogen peroxide and 200 ppm Fe(II) at pH=3.5, T=25^{o}C. For catalyzed reactions, 6-log reduction is accomplished within 20 minutes, while non catalyzed reaction required in excess of 100 minutes.



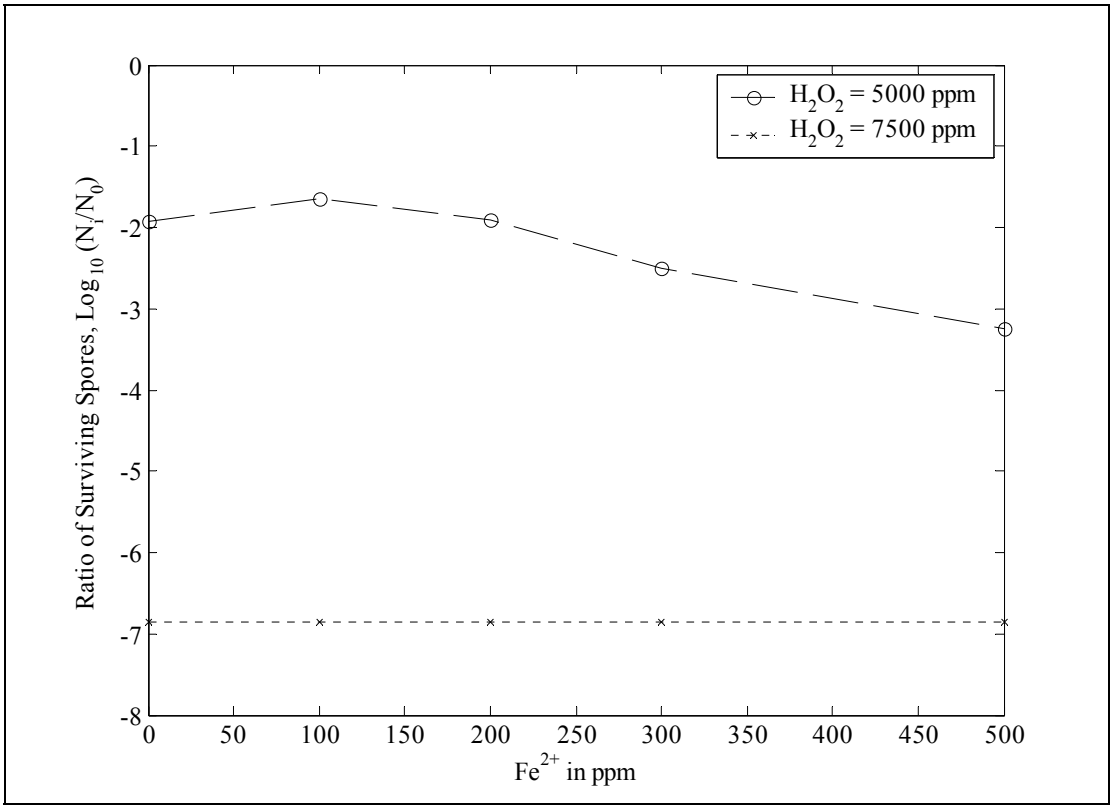
Comparison of the effect of variable catalyst concentrations on the reduction of *P. Chrysogenum* spores.

Initial conditions: pH=3.5, T=25^{o}C Reaction time=4 hours.





Survival of *P. Chrysogenum* spores at 4 and 24 hours after treatment with variable initial concentrations of reagents. Initial conditions: pH=3.5, T=25^{o}C

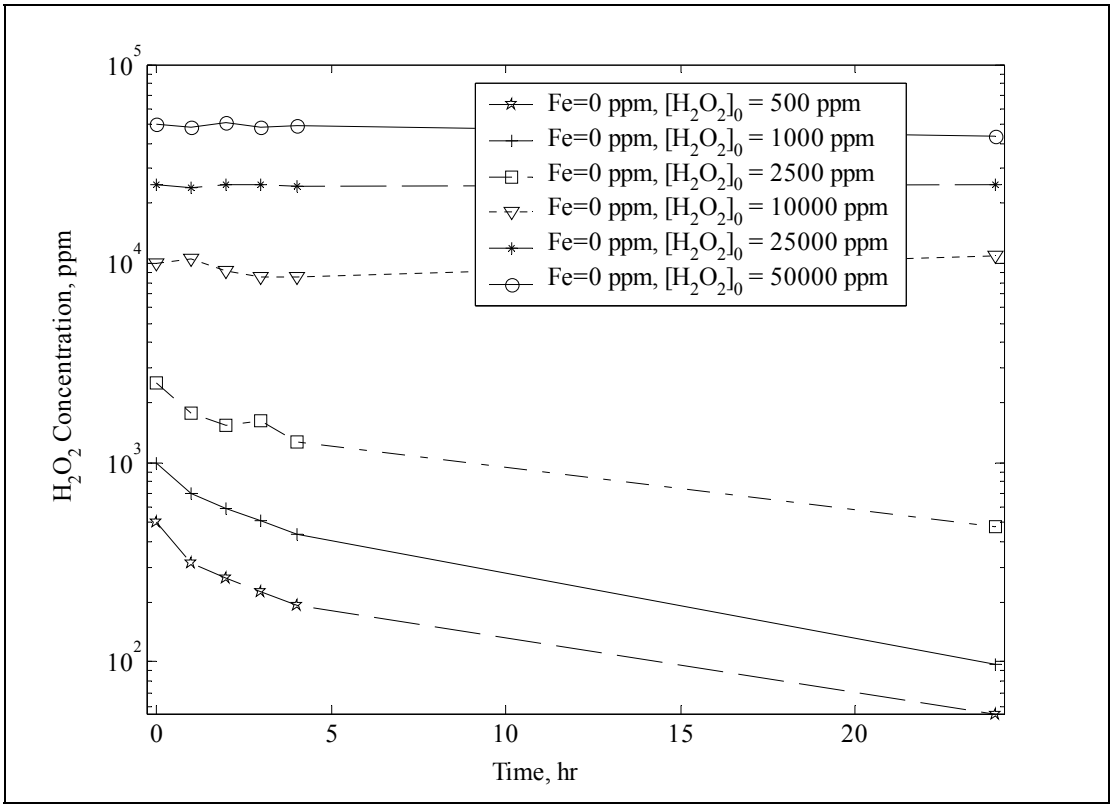


Comparison of the effect of variable catalyst concentrations on the reduction of *P. Chrysogenum* spores for reaction time equal to 24 hours at pH=3.5, T=25^{o}C

Calculated exponential coefficients[2]

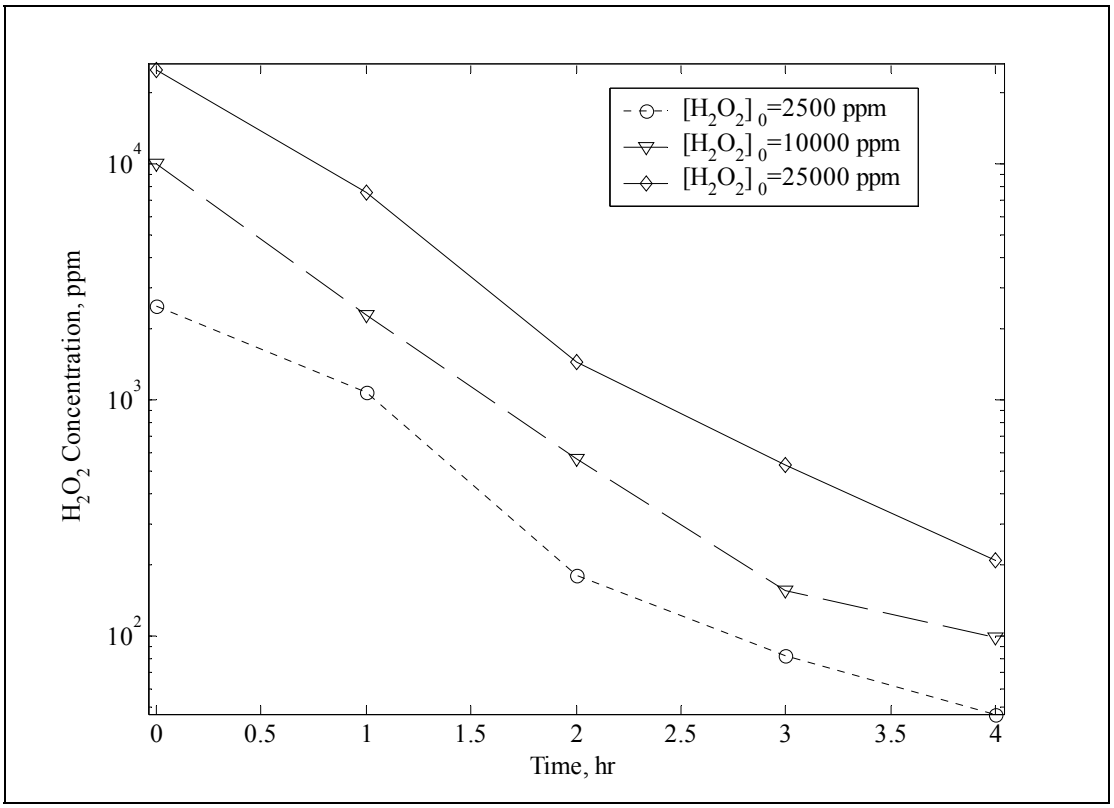
Spores, reaction time	Fe=0 ppm	Fe=100 ppm	Fe=200 ppm	Fe=300 ppm	Fe=500 ppm
P. chrysogenum, 4hrs	-4.23e-04 (4.6e-05)	-4.48e-04 (3.9e-05)	-7.91e-04 (2.1e-04)	-9.94e-04 (3.5e-04)	-1.19e-03 (4.5e-04)
P. chrysogenum, 24hrs	-1.18e-03 (5.2e-04)	-1.18e-03 (4.7e-04)	-1.22e-03 (6.9e-04)	-1.20e-03 (7.1e-04)	-1.24e-03(6.9e-04)
A. versicolor, 4hrs	-1.30e-04(2.5e-05)	-3.16e-04(3.7e-05)	-6.45e-04(2.0e-04)		
C. cladospoioides, 4hrs	-2.44e-04(3.7e-05)	-6.77e-04(5.1e-04)			
C. cladospoioides, 24hrs	-6.40e-04(7.1e-04)	-6.89e-04(4.2e-04)	-6.58e-04(5.3e-04)		
S. Chartarum,, 4hrs	-1.84e-04(2.7e-05)		-3.88e-04(1.2e-04)		

Note: The uncertainty of each coefficient is shown in parenthesis next to the value. The uncertainties were determined from 95% confidence limits.



Degradation of hydrogen peroxide for Fe=0 ppm at pH=3.5, T=25^{o}C

[2] The uncertainty of each coefficient is shown in parenthesis next to the value. The uncertainties were determined from 95% confidence limits.



Degradation of hydrogen peroxide for Fe=500 ppm pH=3.5, T=25°C

Activity of hydrogen peroxide toward viruses, spore forming bacteria, bacterial and fungal spores (lethality refers to $\log_{10}$ reduction of initial spore count)

Organism	H_2O_2 ppm	Catalyst	ppm	Time min	Lethality $\log_{10}$	T °C	pH	Reference
Viruses								
ΦX174	100	Fe^{2+}	30	>60	1			[7]
ΦX174	100	Cu^{2+}	1	7	1			[7]
ΦX174	100	-	-	60	0			[7]
Φ6	100	Fe^{2+}	30	24	1			[7]
Φ6	100	Cu^{2+}	1	22	1			[7]
Φ6	100	-	-	60	0			[7]
T7	100	Fe^{2+}	30	>10	1			[7]
T7	100	Cu^{2+}	1	>10	1			[7]
T7	100	-	-	60	0			[7]
HSV	100	Fe^{2+}	30	60	1			[7]
HSV	100	Cu^{2+}	1	8	1			[7]

HSV	100	-	-	60	0			[7]
JV	100	Fe^{2+}	30	>33	1			[7]
JV	100	Cu^{2+}	1	>60	1			[7]
JV	100	-	-	60	0			[7]
Bacterial Spores								
B. subtilis SA 22[2]	258,000			7.3	>6	24	3.8	[17]
B. coagulans[2]	258,000			1.8	>6	24	3.8	[17]
B. stearothermophilus[2]	258,000			1.5	>6	24	3.8	[17]
C. sporogenes[2]	258,000			0.8	>6	24	3.8	[17]
B. subtilis var. globigii[2]	258,000			2	>6	24	3.8	[17]
B. subtilis var. globigii[2]	350,000			1.5	>6	24	3.8	[17]
B. subtilis var. globigii[2]	410,000			0.75	>6	24	3.8	[17]
B. globigii[2]	3,400	Cu^{2+}	38073	30	>1			[9]
B. globigii[2]	-	Cu^{2+}	38073	30	>5 [a]			[9]
B. globigii[2]	-	Cu^{2+}	3807	30	>3 [a]			[9]
B. globigii[2]	-	Cu^{2+}	3807	30	>6 [b]			[9]
B. globigii[2]	-	Cu^{2+}	3807	30	>3 [c]			[9]
B. globigii[2]	-	Cu^{2+}	3807	30	>7 [d]			[9]
B. subtilis	-	Cu^{2+}	3.4	10	>2-4[e]		1.5	[10]
Fungal Spores								
A. versicolor[2]	50,000	Fe^{2+}	0	100	>6	25	3.5	This work
A. versicolor[2]	20,000	Fe^{2+}	100	20	>6	25	3.5	This work
A. versicolor[2]	10,000	Fe^{2+}	200	20	>6	25	3.5	This work
S. chartarum[2]	25,000	Fe^{2+}	100	20	>5	25	3.5	This work
S. chartarum[2]	7,500	Fe^{2+}	200	20	>5	25	3.5	This work
P. chrysogenum[2]	15,000	Fe^{2+}	0	240	>5	25	3.5	This work
P. chrysogenum[2]	10,000	Fe^{2+}	100	20	>5	25	3.5	This work
P. chrysogenum[2]	7,500	Fe^{2+}	200	20	>6	25	3.5	This work
P. chrysogenum[2]	7,500	Fe^{2+}	300	20	>6	25	3.5	This work
P. chrysogenum[2]	7,500	Fe^{2+}	500	20	>7	25	3.5	This work
C. cladospoioides[2]	30,000	Fe^{2+}	0	240	>7	25	3.5	This work
C. cladospoioides[2]	10,000	Fe^{2+}	100	20	>7	25	3.5	This work
C. cladospoioides[2]	10,000	Fe^{2+}	200	20	>7	25	3.5	This work

[1] Bacterial cultures [2] Spore suspension

[a] Contains 0.1 M Ascorbic Acid, [b] Contains 0.1 M Ascorbic Acid, 2M NaCl, [c] Contains 0.1 M Ascorbic Acid, 0.2M NaCl, [d] Contains 0.1 M Ascorbic Acid, 2M NaCl and 1% surfactant, [e] Contains 0.1 M Ascorbic Acid, 0.1M NaCl,