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Impact of Hydrogen Peroxide on Growth and Survival of *Listeria Monocytogenes* Biofilms

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Abstract: The present study aimed to understand the survival strategies adapted by *Listeria monocytogenes* to combat oxidative stress in planktonic and biofilm cells with response to hydrogen peroxide (H_2O_2). The sensitivity of *L. monocytogenes* to H_2O_2 (oxidative stress) was found to vary in growth cycle. Early log phase cells were found to be sensitive to 100 μM H_2O_2 when compared to stationary phase. Biofilm population was found to be resistant to the oxidative stress induced at 4% of H_2O_2 when compared to their planktonic counterpart at 3.5%. This adaptive behavior allows the pathogen to overcome food preservation and safety barriers, which pose a potential risk to human health. The overall results suggest that, H_2O_2 at a concentration of 6% could be used as a potent sanitizer for the elimination of listerial biofilms.

Keywords: *Listeria monocytogenes*, Hydrogen peroxide, Oxidative stress, Planktonic, Biofilms.

Introduction

The gram-positive bacterium *Listeria monocytogenes* is involved in major outbreaks of infection in humans, particularly in children, pregnant women and immunocompromised patients[1]. The pathogen gain entry into the food chain via contaminated or cross-contaminated foodstuffs[2]. Today, *L. monocytogenes* is of high priority to food manufacturers worldwide, due to high mortality rate of listeriosis in susceptible populations and ability of the pathogen to resist a number of food processing sanitizational practices[3]. The ability of *L. monocytogenes* to grow at refrigerated temperatures, adherence to various surfaces as biofilms, toleration to wide variety of disinfectants and sanitizers making them well adaptive to food environments[4]. When exposed to stress, organisms respond rapidly and adapt their metabolic mechanisms to the altered environmental condition[5]. Such an adaptive response can be induced on exposure to a diverse group of agents, including heat, ethanol, arsenite and oxidizing agents like hydrogen peroxide, diamide, cumene hydroperoxide[6]. In this study, we have evaluated the oxidative stress response of planktonic and biofilm population

of *L. monocytogenes* to hydrogen peroxide at different stages of the growth cycle. Further the efficacy of hydrogen peroxide to be used as a sanitizer on listerial biofilms was investigated.

Experimental

Listeria monocytogenes EGD-e (serovar 1/2a) (ATCC BAA-679) was kindly provided by T. Chakraborty (University of Giessen, Germany), which was kept frozen at -80 °C in 20% glycerol. For each new series of experiments, the stock was streaked on tryptic soy agar (TSA, Gibco, USA) with 1% (w/v) glucose and incubated for 24 h at 37 °C. Single colonies were inoculated into tryptic soy broth (TSB, Gibco, USA) with 1% (w/v) glucose and incubated for 24 h at 37 °C in an orbital shaker incubator (Thermo Scientific, Germany) at 190 rpm. This preculture was centrifuged (5000 g at 4 °C for 10 min), the pellets were washed twice with phosphate-buffered saline pH 7.0 (PBS) and resuspended in 5 mL of PBS.

Growth cycle experiments

TSB supplemented with 1% glucose was used for the study as it supported good growth[8]. For growth tests, mid-log phase cells in TSB with 1% (w/v) glucose medium was incubated at 37 °C in a shaker incubator. Cell density was measured every hour using a spectrophotometer OD at 600 nm.

Hydrogen peroxide disc diffusion sensitivity assay

L. monocytogenes preculture was grown at 37 °C in TSB with 1% glucose to the mid-log phase as mentioned above. The disc diffusion assay was performed as described[6].

Induction of peroxide (oxidative) stress

During the growth cycle 1 mL samples were taken at regular intervals starting 30 min after inoculation into 10 mL of prewarmed TSB + 1% glucose broth. To this hydrogen peroxide was added to a final concentration of 100 µM and incubated at 37 °C for 10 min in a shaker incubator. From this a volume of 0.1 mL were plated on TSA + 1% glucose and incubated for 24 h at 37 °C and this procedure was repeated for 8 hr with interval of 1 h. The colonies were counted using standard plate count (SPC) method and the growth curves were plotted. The number of colonies from the hydrogen peroxide treated sample was divided by that the untreated samples and was plotted as the percentage survival.

Preparation of dialysis membrane for cultivation of biofilm

Dialysis membranes NadirR of 38 mm (Roche, Germany) were cut with the help of the fabricated tool into 7-8 cm diameter. The dialysis membranes were activated using 2% sodium bicarbonate and 1 mM EDTA for 3 h at 60 °C. Upon activation the membranes were repeatedly washed using distilled water for at least 8-10 times and were stored in sterile distilled water at 4 °C. The membranes were sterilized by immersing them in 70% ethanol for about 1 h and dried in a laminar air flow hood before it was placed on TSA. Following activation; membranes were laid on TSA with 1% glucose for 12 h at room temperature, a process called setting. Later 5000 times diluted (approximately 5×10^6 cfu/ml) preculture was inoculated and the plates were incubated at 30 °C for 48 h to form mature listerial biofilm[8].

Hydrogen peroxide inactivation of planktonic and biofilm cells

Hydrogen peroxide (Himedia, Mumbai) at desired concentrations of 3% and 3.5% for planktonic cells and 4%, 5% and 6% for biofilm was prepared by appropriate dilution of the 30% stock solution in sterile 0.85% saline solution at 4 °C. For planktonic and biofilm cells,

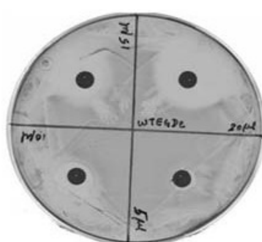
the above mentioned concentrations of H_2O_2 was added respectively and incubated and checked for viability for every 5 h by plating on TSA + 1% glucose and the cells were enumerated after 24 h of incubation at 37 °C. Results were reported as CFU per milliliter.

Statistical analysis

All experiments were carried out in triplicates and repeated in three independent sets of experiments. Data were shown as mean $\pm$ standard deviation (SD). SPSS 10.0.5 version for windows (SPSS software Inc., USA) was used. The significance of differences in biofilm formation was assessed by ANOVA and Post hoc comparison test. Correlations between quantitative properties were evaluated by Duncan and Dunnett's coefficient. Statistical significance value set at $P<0.05$.

Results and Discussion

L. monocytogenes has long been regarded as an intracellular pathogen because of its ability to survive in various environments, both on biotic and abiotic habitats[9]. Several lines of evidence have suggested that oxidative antibacterial factors play a role in the virulence of *L. monocytogenes*[10]. In this study we analyzed the interaction of H_2O_2 on the growth cycle of the bacterium and its use as sanitizer for the elimination of listerial biofilm. We found that the treatment of the cells at very low levels (5, 7, 9 and 13 µ mol) of H_2O_2 showed reduced sensitivity as shown in Figure 1.



Strain	Diameter of inhibition zone, mm			
	Hydrogen peroxide at different concentrations			
	5 µ mol , 5 µL	7 µ mol, 10 µL	9 µ mol, 15 µL	13 µ mol, 20 µL
Lmo EGDe	7-8	12-14	16-19	22-25

Figure 1. Sensitivity of *L. monocytogenes* EGD-e to H_2O_2

However, the cells exhibited increased sensitivity during the log phase of growth by addition of 100 µM of H_2O_2 which is evident by reduction of cell counts. During the stationary phase, the cell counts increased in a normal way suggesting the adaptability of bacteria to the oxidative stress (Figure 2A & 2B).

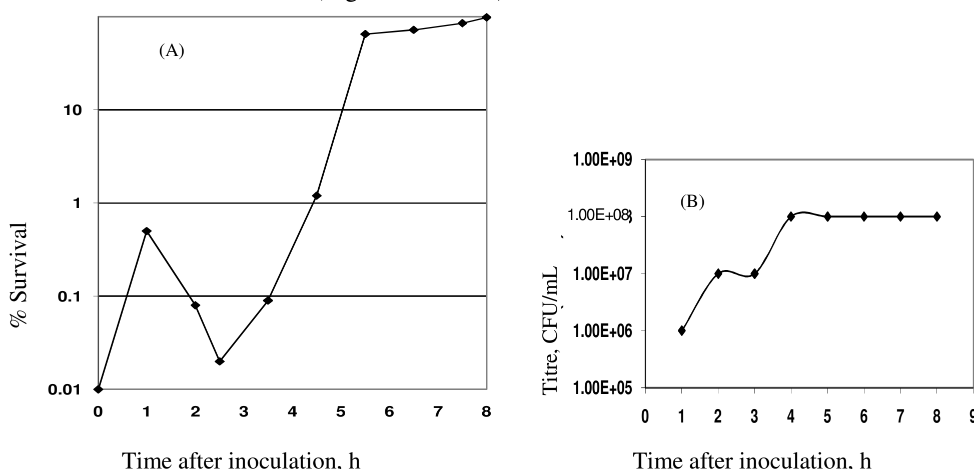


Figure 2. Growth cycle and sensitivity to oxidative stress of *L. monocytogenes* (A) Culture titre, (B) Percent survival of cells after a 10 min treatment with 100 µM H_2O_2.

With these results, the efficacy of H_2O_2 as sanitizer was evaluated using higher concentrations of H_2O_2. A total of 3% H_2O_2 solution reduced the initial concentration of 2.57 x 10^9 CFU/mL by 4.0 log CFU/mL after 10 min of exposure at 30 °C and 3.5% H_2O_2 solution reduced the planktonic population by 3 log reduction and complete elimination, after 5 and 10 min of exposure at 30 °C respectively (Figure 3A). Exposure of *L. monocytogenes* cells grown as biofilms on D membrane to 4% H_2O_2 resulted in a 4.5 log CFU/mL after 10 min of exposure and in 5% H_2O_2 resulted in 1.5 log CFU/mL after 10 min of exposure. A 6% H_2O_2 resulted in complete elimination at 10 min of exposure (Figure 3B). Thus one can infer that, a comparatively higher level of H_2O_2 is required to eliminate biofilm population when compared to their planktonic cells.

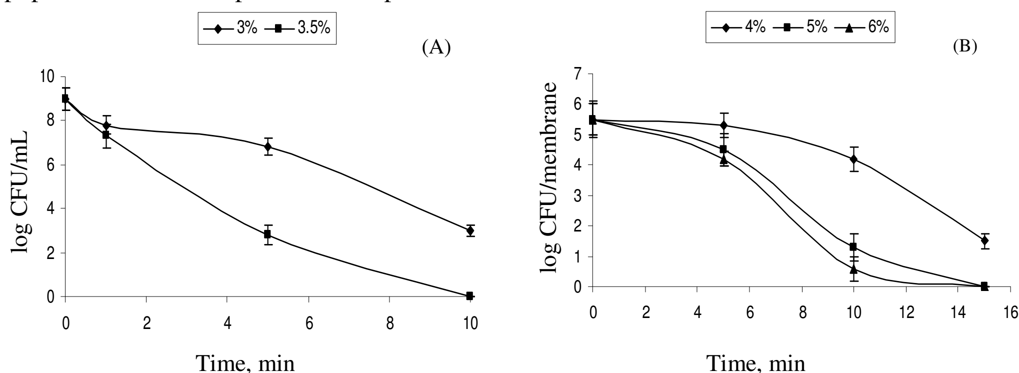


Figure 3. Effect of different concentrations of H_2O_2 on (A) planktonic cells and (B) biofilm cells of *L. monocytogenes* at 30 °C. The initial concentration of cells in planktonic and biofilm was 2.57 x 10^9 CFU/mL

The efficacy of H_2O_2 treatment is relatively affected by high organic loads. The treatment of iceberg lettuce with 2% H_2O_2 at 50 °C resulted in a 3 log reduction of *L. monocytogenes*[11]. In the present study, higher concentration of H_2O_2 was able to kill both planktonic at 3.5% and 6% for biofilms cells of *L. monocytogenes* (Figure 3A and 3B), which suggest that under normal condition there are number of stress genes which are expressed during oxidative stress condition that might protect the cells against the lethal effects of H_2O_2, which is also evident from our observation by treating cells at 100 µM H_2O_2 (Figure 2A and 2B). It is known from the recent investigations that, the physiological state and the gene expression profile of bacteria thriving in biofilms are fundamentally different from the planktonic state[12]. Bacteria have been found to be much more resistant against all kinds of detrimental effects, including acidic and oxidative stress[13]. In genome sequence of *L. monocytogenes*[14], we have identified genes and their products[6,15] which are involved in thiol disulfide redox metabolism (TDRM), includes thioredoxin (*trxA*, *trxB*) and glutathione (*gpo*, *gshF*, *gshR*), peroxide systems (*sod*, *perR*) that operates with thiol catalysis and their transcriptional regulator (*spx and SigB*) for combating peroxide stress. Further investigation is underway looking for the gene expression and their regulation with regard to peroxide stress.

Conclusion

All these data would facilitate a better understanding of organic peroxide which could be used as potential and potent sanitizer for the elimination of listerial biofilms which have created hawoke in food and medical sector.

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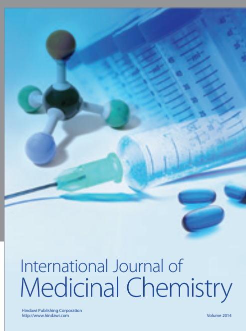

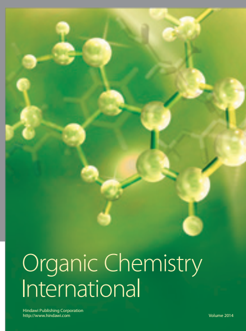

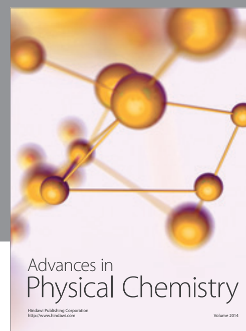

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