Testing the effectiveness of pH modulation on two spore-forming organisms

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Introduction

Bacterial endospores are highly resistant structures that make disinfection difficult in laboratory and environmental settings. Spore-forming bacteria such as *Clostridium sporogenes*, an anaerobic surrogate for *Clostridium botulinum*, and *Bacillus anthracis* Sterne, an aerobic spore-forming bacterium commonly used as a surrogate organism for *Bacillus anthracis*, can survive harsh conditions and require strong disinfectants such as sodium hypochlorite (bleach) for inactivation.

The effectiveness of bleach depends strongly on pH, which determines the ratio of hypochlorous acid (HOCl) and hypochlorite ion (OCl^-). HOCl is the more membrane-permeant and potent antimicrobial form, and its proportion increases as pH decreases. In contrast, OCl^- dominates under highly alkaline conditions, reducing disinfectant activity. Household bleach is typically stored at high pH (~11–12) for stability, but this alkalinity can reduce sporicidal effectiveness (Sil et al., 2025). Adjusting bleach to a lower pH increases the proportion of HOCl and can improve disinfection efficiency without increasing chlorine concentration.

The purpose of this study was to test whether pH-adjusted bleach improves endospore inactivation in *C. sporogenes* and *B. anthracis* Sterne under controlled bleach exposure conditions. Following disinfectant efficacy testing principles, treatments were compared using untreated controls, neutralization, and $\log_{10}$ reduction reporting (EPA, 2018). These results may help improve simple and effective chlorine disinfection protocols for spore-forming bacteria.

Methods and Materials

Cryopreserved *C. sporogenes* (ATCC strain #3584) was grown under anaerobic conditions in a Whitley A35 Workstation, while *B. anthracis* Sterne was cultured under aerobic conditions (Figure 1). Multiple growth media were used to determine optimal conditions for *C. sporogenes* (Figure 2).

Figure 1 (right): Endospore stain of sporulated *B. anthracis* Sterne, captured under oil immersion at 100× magnification.

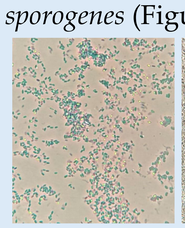

Figure 2 (left): Wet mount of sporulated *C. sporogenes* grown in Cooked Meat Medium, captured under oil immersion at 100× magnification.

Methods and Materials (continued)

Spores were harvested and prepared as standardized spore suspensions. Spore concentrations were quantified, and suspensions were inoculated onto coupons and dried for 24 hours prior to experimentation. Each organism was tested under control, unadjusted bleach (pH 11.8–12.3), and pH-adjusted bleach (pH 7.0–7.02) conditions, with three independent biological replicates per condition ($n = 3$). The adjusted bleach was acidified with hypochlorous acid (HOCl). Bleach solutions were prepared from an 8% sodium hypochlorite stock to a target concentration of ~8,000 ppm, and free chlorine testing verified the active chlorine concentration prior to treatment. Coupons were exposed to bleach for 10 minutes at room temperature, neutralized with 15.8% sodium thiosulfate, serially diluted, and plated. Pre-reduced RCM agar was used for *C. sporogenes* and tryptic soy agar (TSA) for *B. anthracis* Sterne. The main outcome was $\log_{10}$ reduction in CFU/mL relative to the untreated control.

Results

Ten-minute exposure resulted in no detected *C. sporogenes* growth, so contact time was reduced to four minutes for subsequent trials. pH-adjusted bleach produced higher mean $\log_{10}$ reductions than unadjusted bleach for *C. sporogenes* and *B. anthracis* Sterne (Table 1). During a repeated trial, free chlorine testing showed that some *B. anthracis* Sterne trials used bleach with reduced free chlorine concentrations; however, pH-adjusted bleach still produced higher mean $\log_{10}$ reductions than unadjusted bleach (Table 2). For plates with no detected colonies, 0.5 was used as an artificial count because $\log_{10}(0)$ is undefined; these values indicate no detected growth, not actual colony counts (Hamilton, 2010). Because 0.1 mL was plated in duplicate, the limit of detection was 5 CFU/mL for undiluted samples and 5.0×10^5 CFU/mL for samples plated at the 10^{-5} dilution.

Bleach condition	Unadjusted bleach (ppm)	Mean	SD	pH-adjusted bleach (ppm)	Mean	SD
C. sporogenes	5239	4.82	0.11	4084	5.71	0
B. anthracis Sterne	5700	2.20	0	4554	7.30	0

Table 1 (above): Mean and standard deviation (*SD*) $\log_{10}$ reduction of *B. anthracis* Sterne and *C. sporogenes* spores after four-minute exposure to unadjusted and pH-adjusted bleach. Log_{10} reduction = $\log_{10}$ control CFU/mL − $\log_{10}$ treated CFU/mL; $n = 3$ trials per treatment.

Results (continued)

Bleach condition	Unadjusted bleach (ppm)	Mean	SD	pH-adjusted bleach (ppm)	Mean	SD
Fresh bleach	7104	5.36	0.78	5750	7.30	0
Reduced free chlorine condition	5506	2.63	0	4218	4.40	1.19

Table 2 (above): Mean and standard deviation (*SD*) $\log_{10}$ reduction of *B. anthracis* Sterne spores after four-minute exposure. Log_{10} reduction = $\log_{10}$ control CFU/mL − $\log_{10}$ treated CFU/mL; $n = 3$ trials per treatment.

Conclusions

This study evaluated whether pH-adjusted sodium hypochlorite improved endospore inactivation compared with unadjusted bleach, comparing responses between *C. sporogenes* and *B. anthracis* Sterne. Results suggest pH-adjusted bleach produced greater spore reduction than unadjusted bleach (Table 1), even when free chlorine differed between treatments (Table 2). These findings suggest lowering bleach pH may improve sporicidal activity by increasing the proportion of the more active form of bleach (Sil et al., 2025). However, limited data points and variation in chlorine concentration require additional trials under controlled laboratory conditions to confirm this trend and improve overall reliability of the findings.

The study also showed bleach degrades over time, lowering free chlorine concentration and reducing inactivation effectiveness. This finding may be useful for laboratories, schools, and hospitals, emphasizing the importance of verifying bleach freshness or expiration dates before use.

References

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