Establishment of a Standardized Sporicidal Efficacy Assessment method for Sporicidal Products evaluation

Food and Drug Administration NCTR National Center for Toxicological Research

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ABSTRACT

Background: Bacterial endospores are ubiquitous in the environment and are resistant against many extreme conditions, causing significant challenges for pharmaceutical manufacturing. Compounded drugs are medications tailored to the needs of individual patients. However, lacking sterility assurance and effective contamination control in drug compounding may cause major risks to public health. Compounding facilities are not always required to validate the effectiveness of sterilants. Given the fact that sporicidal efficacy can be affected by many factors, the absence of standardized sporicidal assays hampers the safety assessment of drug compounding.

Purpose: This study aimed to establish a standardized method for sporicidal efficacy assessment to support FDA's safety assessment of drug compounding.

Methodology: Seven *Bacillus* strains were selected for sporulation in five conventional sporulation media under aerobic conditions. Spore yields were measured by phase-contrast microscopy and enumeration assays. Spores were purified by density centrifugation, treatment with heat and lysozyme, and then stored at 4°C for maturation. Spore qualities were evaluated by the sodium hypochlorite (NaOCl) resistance assay.

Results: Difco Sporulation Broth was the optimal sporulation medium for most *Bacillus* strains (4/7). All evaluated purification methods improved the spore purity with strain variations. However, intense heat (80°C for 20 min) and lysozyme (100 µg/mL) treatment sensitized spores of specific strains against NaOCl. The most optimal maturation periods ranged from 7 to 21 days. *B. subtilis* ATCC 6051 spores, exhibiting the best overall qualities among tested spores, was selected as the representative strain in assessing the efficacy of disinfectants against *Bacillus* spores. The purified spores were used to evaluate efficacies of 20 commercial disinfectants following the respective product instructions for use. For chlorine-based products, 3 products showed sporicidal efficacy of 7-log reduction, while 5 product showed less than 3-log reduction. For 6 hydrogen-peroxide-and-peracetic-acid-based products, 3 products showed a 7-log reduction and 3 product showed less than 1-log reduction. Other disinfectants showed poor sporicidal activities (less than 1-log reduction), including aldehyde based, quaternary ammonium based, and phenol based products. Although labeled as sporicidal disinfectants, 7 products failed sporicidal assessment with less than 3-log reduction.

Conclusion: Optimal spore preparation methods were established for *Bacillus* strains. And a standardized method was developed to assess efficacy of disinfectants against *Bacillus* spores. It was found that some disinfectants may not be as effective as claimed on their labels. These discoveries provide the foundation for the establishment of an efficacy database for sporicidal products, which will aid the FDA's safety assessment of drug compounding.

FDA RELEVANCE

- **Bacterial spores cause significant challenges for pharmaceuticals.**
  - Ubiquitous in the environment;
  - Resistant to heat, desiccation, radiation, and chemical assault.
- **Problems with current sporicidal test methods:**
  - Different test organisms; Poor quality of spores; Ineffective neutralization;
  - Inappropriate exposure time.
- **Compounded drug:** not FDA-approved drug; exempt from CGMP, lacking sterility assurance.
- **Compounding facilities** are not always required to validate the effectiveness of sterilants.
- **Regulatory Needs:** Standardized methods for sporicidal efficacy assessment is needed to support the Agency's safety assessment of drug compounding.

RESEARCH STRATEGY

EXPERIMENT MATERIALS

Bacterial strains: *B. cereus* ATCC 14579; *B. licheniformis* ATCC 14580; *B. pumilus ATCC* 7061; *B. sphaericus* ATCC 14577; *B. subtilis* ATCC 19659 (1); *B. subtilis* ATCC 6051 (2); and *B. thuringiensis* ATCC 35646.

Sporulation Media: Tryptic Soy Agar; Manganese Amended Nutrient Agar (NA); Manganese-Amended 10% Columbia Broth (MAC); Difco™ Sporulation Broth (DSM); and 2xSG Broth (Mn^{2+} 100 µM).

Carrier for sporicidal assay: Glass and stainless steel.

Disinfectants: 11 sporicidal products, 6 broad disinfectants, and 3 hospital grade disinfectants were purchased. (Table 2)

RESULTS

Table 1. Optimized sporulation methods for *Bacillus* strains

Bacillus strains	Medium	Mn^{2+} (µM)	Temp (°C)	Days	Sporulation rate (%)	Titers (spores/mL)
B. cereus ATCC 14579	2xSG	100	30	4	~ 90	1.9 x 10^9
B. licheniformis ATCC 14580	MAC	10	37	6	~ 80	2.1 x 10^9
B. pumilus ATCC 7061	DSM	10	37	6	~ 90	2.0 x 10^9
B. sphaericus ATCC14577	DSM	10	30	8	~ 70	3.3 x 10^9
B. subtilis ATCC 6051	DSM	10	37	3	~ 90	3.6 x 10^9
B. subtilis ATCC 19659	DSM	10	37	4	>90	2.1 x 10^9
B. thuringiensis ATCC 35646	2xSG	100	30	4	~ 50	2.4 x 10^9

Figure 2. Effect of maturation time on spore resistance

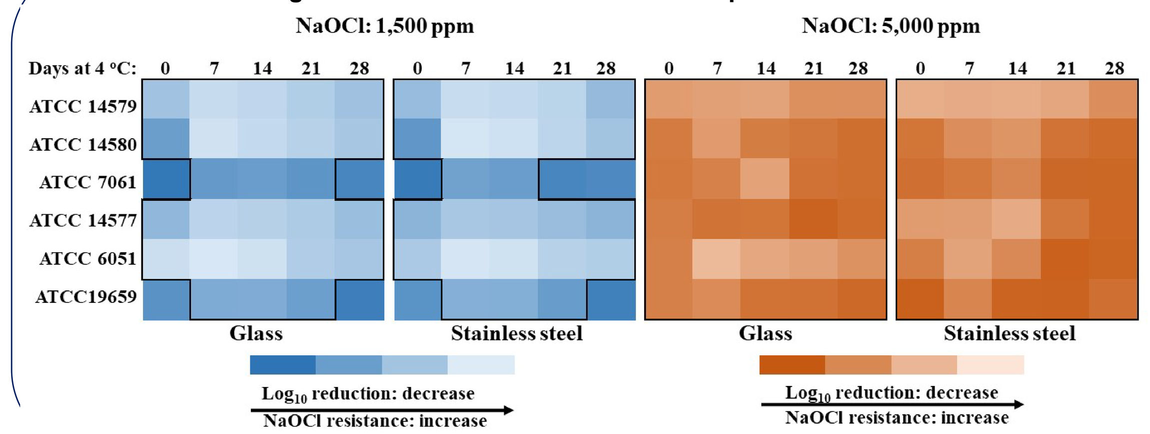


Figure 1. Criteria for spore quality evaluation

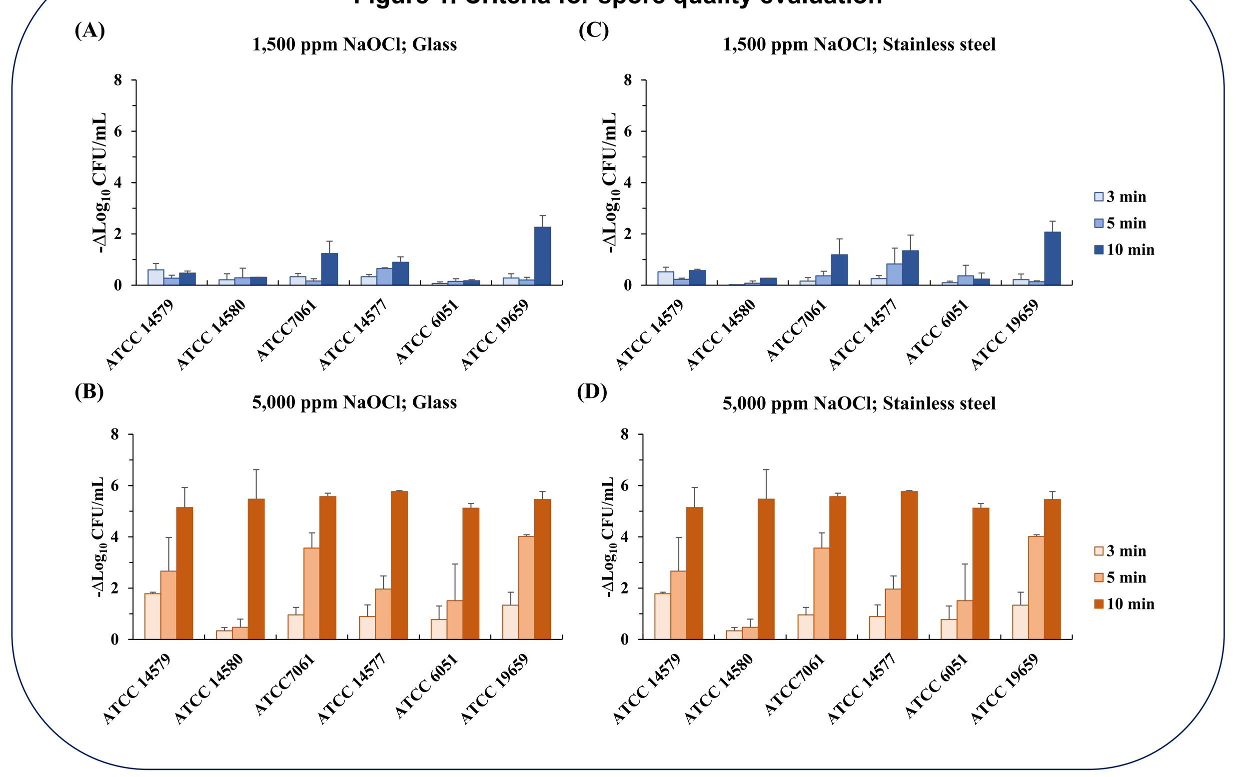


Figure 3. Effect of sonication treatment on spore resistance to NaOCl

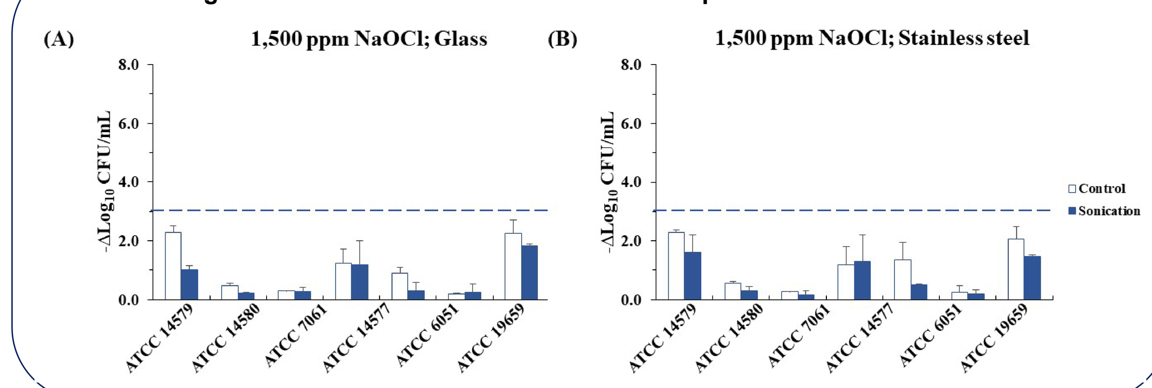


Figure 4. Effect of lysozyme treatment on spore resistance to NaOCl

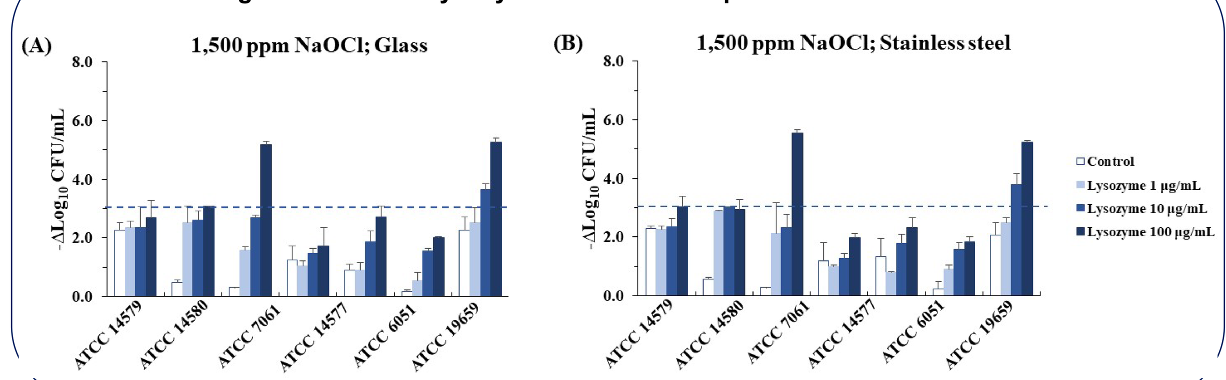


Figure 5. Effects of heat treatment on the spore purity and resistance to NaOCl

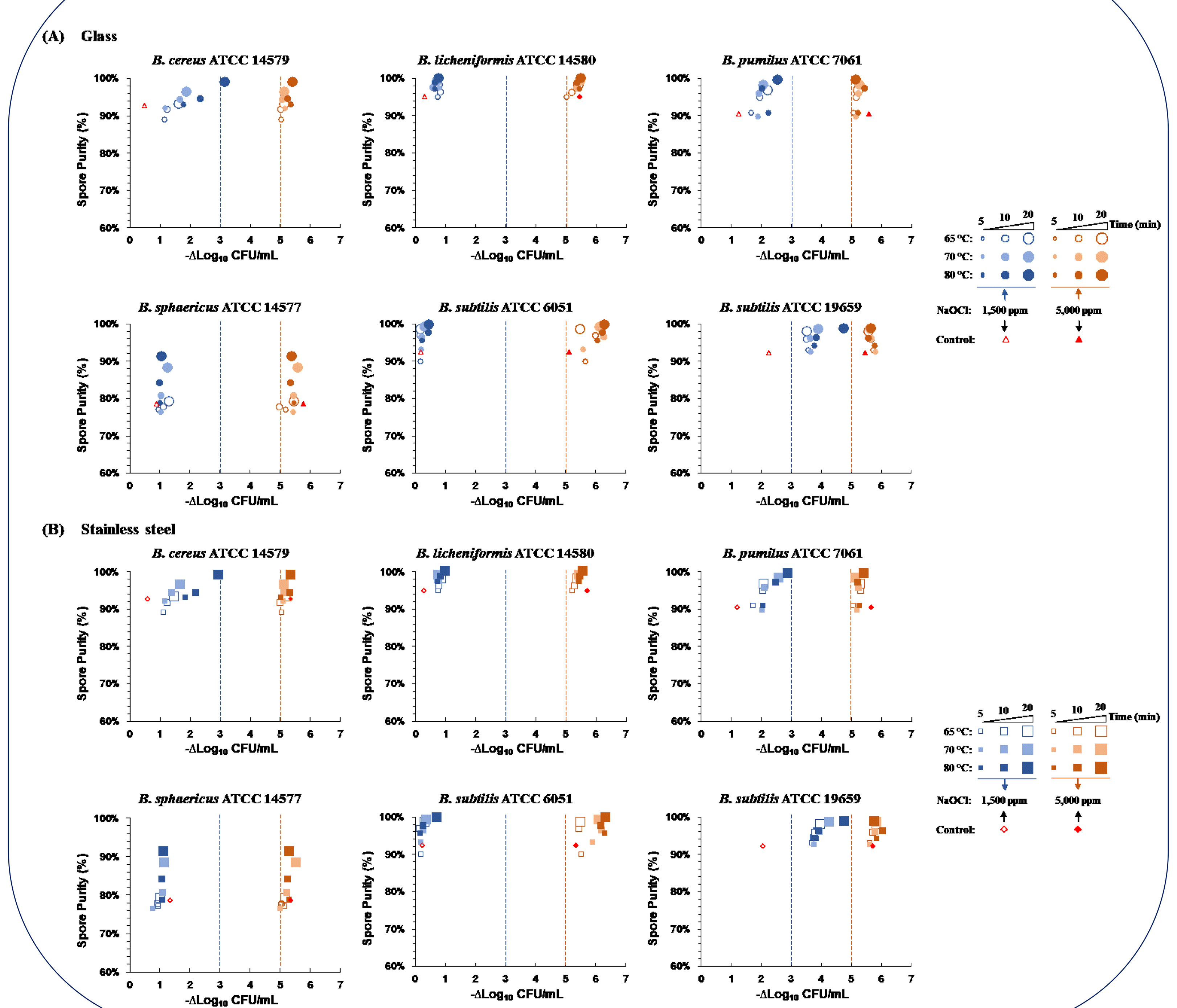


Figure 6. Sporicidal efficacy of chlorine based products against ATCC 6051 spores

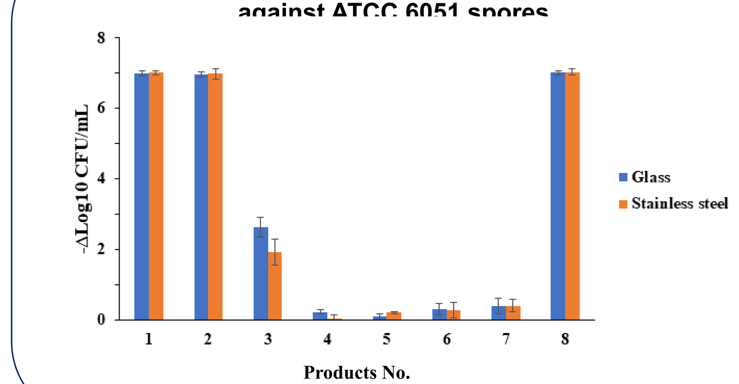


Figure 7. Sporicidal efficacy of hydrogen peroxide & peracetic acid based products against ATCC 6051 spores

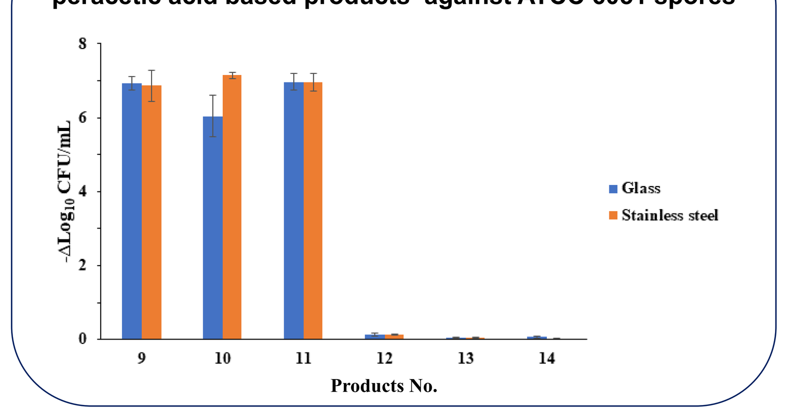
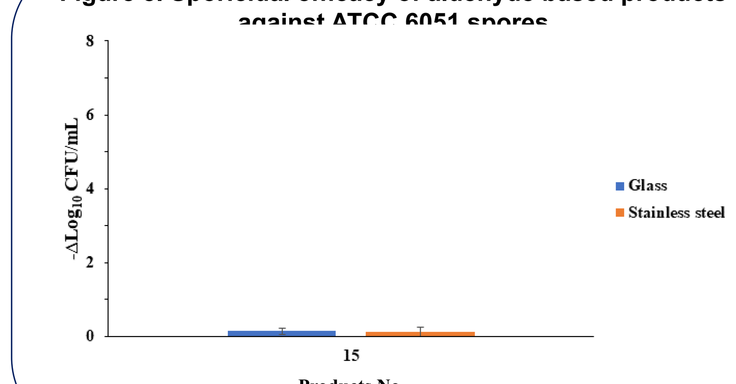


Figure 8. Sporicidal efficacy of aldehyde based products against ATCC 6051 spores



RESULTS

Figure 9. Sporicidal efficacy of quaternary ammonium based products against ATCC 6501 spores

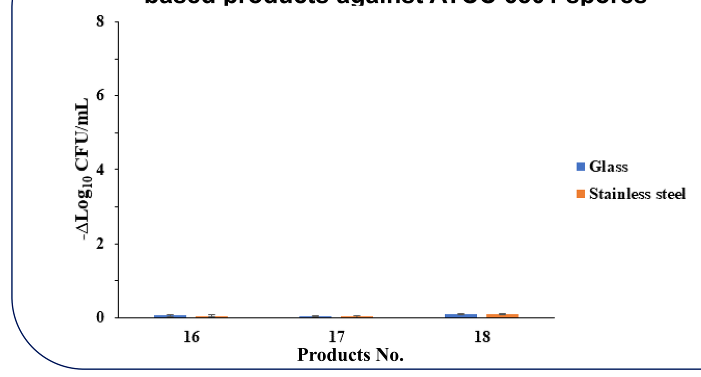
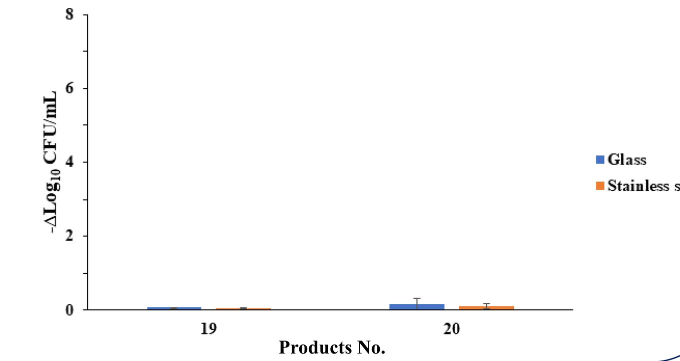


Figure 10. Sporicidal efficacy of phenol based products against ATCC 6501 spores



METHODS

Table 2. List of disinfectants tested in this study

Category	Products No.	Products Ingredients	Labeling	Dilution	Contact time (minutes)	Neutralizer
Chlorine based disinfectants	1	NaOCl (52,500 ppm)	Broad disinfectant	Ready to use	5	0.6% sodium thiosulfate 1% tween-80 PBS (pH 7.4)
	2	NaOCl (13,120 ppm)	Sporicidal agent	Ready to use	4	
	3	NaOCl (9,400 ppm)	Sporicidal agent	Ready to use	4	
	4	NaOCl (3,900 ppm)	Sporicidal agent	Ready to use	2	
	5	NaOCl (2,500 ppm)	Sporicidal agent	Ready to use	5	
	6	Sodium dichloro-S-triazinetrione (chlorine: 4,306 ppm)	Sporicidal agent	1 tablet/946 mL ddH_2O	4	
	7	Sodium dichloro-S-triazinetrione (chlorine: 1,988 ppm)	Broad disinfectant	1 tablet/757mL ddH_2O	10	
	8	Hypochlorous acid (170 ppm)	Hospital grade disinfectant	Ready to use	10	
Hydrogen peroxide & peracetic acid based disinfectants	9	H_2O_2 (73,500 ppm), Peracetic acid (2,300 ppm)	Sporicidal agent	Ready to use	15	Catalase (100 µg/mL), 1% tween-80 PBS (pH 7.4)
	10	H_2O_2 (44,000 ppm), Peracetic acid (2,300 ppm)	Sporicidal agent	Ready to use	3	
	11	H_2O_2 (10,000 ppm), Peracetic acid (800 ppm)	Sporicidal agent	Ready to use	30	
	12	H_2O_2 (275,000 ppm), Peracetic acid (58,000 ppm)	Sporicidal agent	2.3% v/v ddH_2O	3	
	13	H_2O_2 (53,400 ppm), Peracetic acid (13,600 ppm)	Hospital grade disinfectant	2.3% v/v ddH_2O	10	
	14	H_2O_2 (78,000 ppm)	Sporicidal agent	Ready to use	7	
Aldehyde based disinfectants	15	Ortho-phthalaldehyde (6,000 ppm)	Sporicidal agent	Ready to use	12	0.5% sodium bisulfite, 0.6% sodium thiosulfate, 1% tween-80 PBS (pH 7.4)
Quaternary ammonium based disinfectants	16	Didecyl dimethyl ammonium chloride (105,000 ppm)	Broad disinfectant	1:64 ddH_2O	10	0.1% tween-80 0.1% lecithin PBS(pH 7.4)
	17	Octyl Decyl Dimethyl Ammonium Chloride (65,100 ppm), Dioctyl Dimethyl Ammonium Chloride (26,040 ppm), Didecyl dimethyl ammonium chloride (39,060 ppm), Alkyl dimethyl benzyl ammonium chloride (86,800 ppm)	Hospital grade disinfectant	1:64 ddH_2O	10	
	18	Octyl Decyl Dimethyl Ammonium Chloride (260 ppm), Dioctyl Dimethyl Ammonium Chloride (130 ppm), Didecyl dimethyl ammonium chloride (130 ppm), Alkyl dimethyl benzyl ammonium chloride (340 ppm)	Broad disinfectant	Ready to use	10	
Phenol based disinfectants	19	O-phenylphenol (60,000 ppm), O-benzyl-p-chlorophenol (127,500 ppm)	Broad disinfectant	1:128 ddH_2O	10	0.1% tween-80 0.1% lecithin PBS (pH 7.4)
	20	O-phenylphenol (60,000 ppm), O-benzyl-p-chlorophenol (130,000 ppm)	Broad disinfectant	1:64 ddH_2O	10	

Methods:

- **Sporulation:** Media, temperature (37/30 °C), incubation time (3-12 days).
- **Spore purification:** Density centrifugation (HistoDenz™); heat-shock (70 °C, 20 min); sonication (5 min); lysozyme preparation (10 µg/mL, 4 °C, 1 hour).
- **Spore maturation:** Held at 4 °C for 1-28 days.
- **Spore titer and quality:** Phase-contrast microscopy; spore-staining; enumeration assay; sodium hypochlorite (NaOCl) sporicidal assay at concentration of 1,500 ppm and 5,000 ppm.
- **Spore preparation for sporicidal efficacy assessment:** Matured ATCC 6051 spores were heated in water bath at 80 °C for 20 min, followed by sonication at 37 kHz for 5 min.

CONCLUSIONS

- Strain-specific optimal spore preparation methods were established for *Bacillus*.
- Criteria for spore quality evaluation were determined.
- *B. subtilis* ATCC 6051 was selected as the representative strain for assessing sporicidal efficacy against *Bacillus* spore.
- Neutralization methods have been validated based on the ingredients of disinfectants.
- Our findings demonstrated that out of 11 commercial sporicidal products tested, only 4 met the sporicidal assessment criteria against ATCC 6051 spores, highlighting the need for standardized methods for sporicidal efficacy assessment to support the Agency's regulatory mission.

ACKNOWLEDGEMENTS

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DISCLAIMER