Research Article

Validation of Spore-Forming Organisms Recovery from Peroxygen-Based Disinfectants

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ABSTRACT

Neutralization method evaluation is a crucial preliminary step in biocidal agent evaluation and environmental monitoring (EM) programs. In the present study, four commercial peroxygen/Ag^{+}-based sporicidal disinfectants in pharmaceutical industry were tested against 3 spore-forming microorganisms (*Aspergillus brasiliensis*, *Bacillus subtilis subsp. spizizenii* and *Bacillus cereus*). An in-house made neutralizing broth was used to dilute biocidal agents to 1:10 and 1:100 (v/v) dilutions. Disinfectant preparation, dilution and all other test conditions were performed under the laboratory conditions that simulated the working environment. Each of the neutralizer toxicity and efficiency was examined using the statistical comparison among three experimental groups viz: viability control, neutralizer toxicity and neutralizer efficacy. Different analysis criteria were investigated to compare the rate of success and failure of each replicate group for neutralizer efficacy (NE) and neutralizer toxicity (NT). Criteria of comparison were Harmonized Chapter USP<61>, USP<1227> of 3 independent replicates recovery in agar medium and finally Sutton initial recovery criteria of ≥0.75 was applied followed by Dunnett's Multiple Comparison Test in case of suspect failure. These criteria were in agreement; where *Bacillus cereus* gave the highest rate of failure in neutralization study. Disinfectants Mil and Pury were the hardest to neutralize and *Bacillus species* were sensitive to them in the neutralization procedure. Bixco disinfectant was the most successfully neutralized at 1:10 dilution with the 3 organisms. The in-house made chemical neutralizer was effective in microbial spores' recovery from residual sporicidal biocides at 1:100. Thus, it is suitable to be incorporated in both EM media and sanitization program validation.

Keywords: *Aspergillus brasiliensis, Bacillus species,* in house made neutralizing broth, neutralizer efficacy, neutralizer toxicity, peroxygen/Ag^{+}- based sporicidal

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INTRODUCTION

Spore-forming microorganisms represent an environmental challenge for those who work in clean area, especially in pharmaceutical manufacturing facilities because they are easily spread and known to possess great resistance to sanitizing agents. They may; also, contaminate the final product thus compromising its quality and causes companies sever financial loss and affect their reputation. Thus, improving their recovery from both residual biocidal agents and hostile environment enhances their detectability before impacting drug manufacturing area quality and hence the drug itself. In order to ensure the validity of the data obtained from in vitro disinfectant qualification testing, the study protocol should include *neutralization* and *microbial recovery* studies as test controls. Neutralization studies must be performed for each neutralizer, disinfectant and/or microorganism [1]. Chemical agents commonly known as inactivators or neutralizers are often used for (i) the bactericidal evaluation of antimicrobial agents, antiseptics and disinfectants; (ii) the evaluation of the preservative efficacy in many pharmaceuticals, toiletries and cosmetic products; and (iii) the microbial limit testing of products containing antimicrobial agents [2].

A potential drawback of the chemical neutralization of biocides is the toxicity displayed by several types of neutralizers.

Thus, the evaluation of a chemical neutralizer or a physical neutralization scheme must examine the potential toxicity of the neutralizer as well as its efficacy. Example of toxicity of some neutralizer components could be illustrated by Thioglycolate with Staphylococci [3-6] and spores, and Thiosulphate with Staphylococci [4,7-9]. Complete neutralization of disinfectants is important for the accuracy of a biocidal assay as microbicidal activity is commonly measured as survivors with time and inhibition of microbial growth by low levels of residual biocide would lead to exaggerated measures of microbicidal activity [10]. A convenient method for this neutralization is through the use of recovery diluents designed to neutralize commonly used antimicrobials [11].

Certain peroxygen compounds have excellent activity under controlled conditions and are sometimes used as an alternative to physical methods, e.g. for the sterilization of heat-sensitive equipment. Hydrogen peroxide and peracetic acid are high level disinfectants due to their production of the highly reactive hydroxyl radical. They have an additional advantage that their decomposition products are nontoxic and biodegradable. Furthermore, the germicidal properties of hydrogen peroxide (H_2O_2) have been known for more than a century, but the low stability of its diluted solutions negatively affected its reputation. However, stabilized solutions are now available and due to its extraordinary antimicrobial activity, hydrogen peroxide has a valuable role for specific applications. Concentrations of 3-6% are effective for general disinfection purposes. Peracetic acid (CH_3CO_3H) is the peroxide of acetic acid and is a more potent biocide than hydrogen peroxide, with excellent rapid biocidal activity against bacteria, including mycobacteria, fungi, viruses and spores. It is, also, active in the presence of organic matter. Nowadays, it is widely used at concentrations of 0.2-0.35%, as a chemosterilant of medical equipment. However, it possesses several drawbacks such as being corrosive to some metals, highly irritant and must be used in an enclosed system. Silver have long been known to possess antibacterial properties and the preparations of this metal were among the earliest used antiseptics. Silver has been reported to inhibit thiol (-SH)-containing enzymes in both cell membrane and cytoplasm, which contain, groups [12, 13].

In order to obtain accurate data from neutralization study one must take cautions to the inoculums of each organism incorporated in the study. The US Food and Drug Administration *Bacterial Analytical Manual* (BAM) recommends 25-250 CFU (Colony Forming Units)/plate as a countable range [14]. The crux of the argument is that the experimental studies have shown very poor accuracy in plate counts below 25 (at 25 CFU/plate, error percent of the mean is 20). Theoretically, it can be argued that since the CFU follow the Poisson distribution, the error of the estimate is calculated as the square root of the average [15]. This leads to graphs such as in (**Fig. 1**), which shows us that as the CFU/plate drops below the countable range, the error as a percent of the mean increases exponentially (red curve) following the next formula:

$y = 100\exp^{-1.15x}$ where: y= Error as Percent of Mean and x= Log number of CFU/plate. (**Figure 1**) demonstrated that as the number of colonies per plate increases the standard error (blue curve) increases but not to that extent and effect as that produced by decreasing the number of colonies below 25 CFU per plate. They are actually inversely related, and their relation follows the following relation: $\mathrm{Log}\, y + \mathrm{Log}\, S.E. = 2$.

Sutton *et al.* [17] demonstrated that the neutralization procedure must be examined and not only the neutralization solution. A critical concern to this point is the dilution ratio of biocide:neutralizer; a 1:10 dilution was employed, as in most kinetic studies; the numbers of survivors were quantified with time by plating serial 10-fold dilutions. Therefore, the most concentrated biocide would be present in the initial 10-1 dilution tube. However, it is possible to design the neutralization step in a biocidal agent kinetic study with 1:100 and even 1:1000 dilutions from the first reservoir after a specified contact time and could be also combined with filtration (if necessary).

Process risk assessment tools such as Failure Mode and Effect Analysis (FMEA)

and Hazard Analysis Critical Control Point (HACCP) have been successfully used by pharmaceutical companies to identify areas in the process and types of raw materials and equipment that are at high risk of being contaminated with microorganisms [18]. *HACCP*, which was developed in the 1970s by the U.S. Department of Agriculture to address food safety, is *a systematic, proactive, and preventative tool to identify, assess, and prevent or reduce potential risks that can occur at specific steps in a process*. Through the risk analysis process, critical control points are essentially identified and monitored [19].

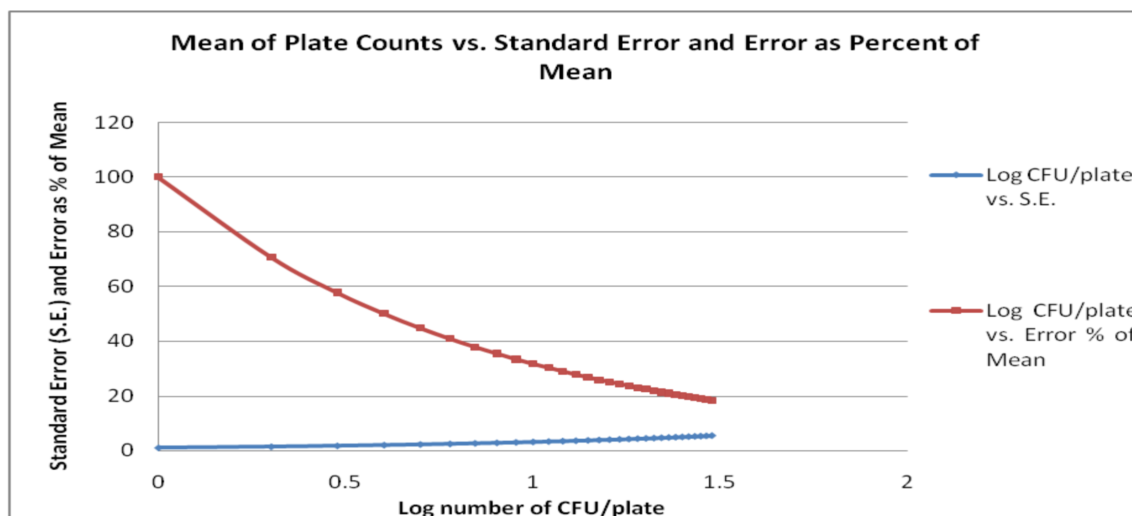

Figure 1: Log Actual Number of CFU in Plate (X-Axis) vs. Standard Error and Error as Percent of Mean (Y-Axis)

Both on the same scale- showed effect of the number of CFU/Plate in the accuracy of results interpretation (modified from USP<1227>, [16]). CFU= Colony Forming Unit.

The current study was designed to investigate the use of combined chemical neutralization and dilution for the recovery of the representative spore-forming microorganisms from commercial peroxygen/Ag^+-based sporicidal agents as a part of disinfection validation program and microbial recovery in the presence of residual disinfectant in environmental program. This study was developed as part of HACCP with the potential application in health care facility generally and pharmaceutical plant specifically.

METHODS

I- PREPARATION OF MICROBIAL SUSPENSION:

Standard strains were purchased from ATCC (American Type of Culture Collection, Manassas, Virginia) and handled according to standard procedure. The bacterial environmental isolates were isolated and identified using miniaturized biochemical identifications kits BBL™ Crystal™ Identification System purchased from BD (Becton Dickinson Microbiology Systems, Cockeysville, Md.). All the nutrient media and chemicals were purchased from OXOID (Basingstoke, Hampshire) and Sigma-Alrich (St. Louis, MO 63103), respectively.

Standardized stable suspensions of test strains were used or prepared as detailed in Seed-lot culture maintenance techniques (seed-lot systems) so that the viable number of microorganisms used for inoculation was not more than 5 passages removed from the original master seed-lot. All organisms were stored at -80°C in a validated -86C Ultra low temperature freezer (-86 Degree ULT Freezers, Qingdao Shandong, China) in a validated cryogenic environment, and reactivated only prior to study conduction. All media were sterilized by autoclaving in steam sterilizer (FEDEGARI FOB3, Fedegari Autoclavi SpA, SS 235 km 8, 27010 Albuzzano (PV), Italy). (**Table 1**) demonstrated microorganisms that have been used in this study and to prepare spore suspensions in the next step.

Table 1: List of Spore-Forming Microorganisms Challenged in Neutralizer Validation Study, Their Source and Type

Challenged organisms	Source	Microorganism Type
Aspergillus brasiliensis	ATCC16404	Filamentous fungus
Bacillus cereus	EM* isolate	Gram-positive spore-forming rod
Bacillus subtilis subsp. spizizenii	ATCC6633	Gram-positive spore-forming rod

*= Environmental Monitoring isolate identified by using either BBL™ Crystal™ Enteric/Nonfermenter or BBL™ Crystal™ Gram-Positive ID Kit depending on Gram staining which was purchased from BD Diagnostic System.

II-PREPARATION OF SPORE SUSPENSION:

The bacterial test organisms (*Bacillus subtilis subsp. spizizenii* (ATCC 6633) and *Bacillus cereus*) were grown separately in containers containing casein soya bean digest agar at 30–35°C for 5–7 days to ensure complete sporulation in Hotpack incubator 175 series, model 417532 (Hotpack, Dutton Rd., Philadelphia, USA). The test strain for *Aspergillus brasiliensis* (ATCC 16404) was allowed to grow separately on Sabouraud-dextrose agar at 20–25 °C for 7 days or until good sporulation is attained [20]. Mold spores were harvested by washing the agar surface with sterile peptone water containing 0.05% polysorbate 80. Sterile inoculating loops or some sterile glass beads were used to loosen the spores and the washings were combined in a sterile container. This was the mold inoculum. To prepare a bacterial spore suspension, the inoculated agar plates were harvested with sterile water and heat shocked for 15 min at 65–70°C in Stirred water baths (Progen Scientific**,** 8 Deer Park Rd., Merton**,** London), starting the timing when the temperature reaches 65°C. The suspension was cooled rapidly in an ice bath (0–4°C), and the prepared spore suspension was stored under refrigeration. Initial plate count was recorded to verify the spore population. Microbial test suspensions were used once the results of serial dilutions could be enumerated. Plastic 9mm sterile plates were purchased from Sterilin Limited (solaar house, 19 mercers row, cambridge, UK). Suspensions were quantified by making serial dilutions and plate counts using conditions and media suitable for each microorganism to select suspensions of concentration $3x10^2$-$1x10^3$ CFU/0.1ml as working suspensions. Microbial test suspensions were used once the results of serial dilutions could be enumerated using digital colony counter (Digital Colony Counter Model: 361, Laxman Mahtre Rd. Navagaon, Dahisar West, Mumbai).

III-NEUTRALIZATION VALIDATION STUDY OF PEROXEGEN-BASED BIOCIDAL AGENTS:

The purpose of this study was to ensure that the assumed contact time is valid, i.e. the neutralizing agent can efficiently stop the action of the tested sanitizer after mixing with each other & at the same time the neutralizing agent should not have any inhibitory or toxic effect on microorganisms. Statistically, two comparisons among three populations are performed. The first comparison was the Neutralizer Efficacy (NE) which can be determined by evaluating the number of survivors in the neutralizing broth in the presence and absence of the biocide. The ability of the neutralizing broth alone to allow the microbial survival is a second important consideration in this analysis. The second comparison was to examine Neutralizer Toxicity (NT). This aspect of neutralization is determined by comparing survivors in the neutralizing medium without the biocide with the viability (growth) control [17, 21]. (**Table 2**) showed the composition of neutralizing broth which was used in this study, a comparison between its contents and the constituents of other commonely and commercially available neutralizing broths. Test solutions were freshly prepared and diluted in the same conditions that simulated the actual usage environment of biocidal agents using the highest working concentration which is 5%, according to the manufacturer recommendations. These commercial disinfectants were symbolized Bixco (Hyrogen Peroxide/Ag^+), BarD 50 (Hyrogen Peroxide/Ag^+), Pury (Hyrogen Peroxide/Peracetic Acid/Ag^+) and Mil

(Hyrogen Peroxide/Ag^{+}). Using neutralizing broth as a diluent to prepare 1:10 and 1:100 (v/v) dilutions of the test solutions at the working concentration, then 1ml was transferred of this dilution to each of duplicate petri dishes this is test group. The chemical neutralizer used was Fluid Thioglycolate Medium Neutralizer (FTMN).

Table 2: Comparison of Commonly Used Neutralizing Broths With In-House Prepared Neutralizer

Ingredient	DEB	NIH	TAT	TPL	FTMN
Agar					1.5
Casitone		15.0			
Cystine		0.5			1.0
Dextrose	10.0	5.5		2.5	11.0
Lecithin	7.0		5.0	0.7	
Polysorbate 20			43.2		
Polysorbate 80	5.0			15.0	
Sodium bisulfite	2.5				
Sodium chloride		2.5			5.0
Sodium thioglycollate	1.0	0.5			1.0
Sodium thiosulfate	6.0				6.0
Soytone				3.0	
Tryptone	5.0		20.0	17.0	30..0
Yeast extract	2.5	5.0			10.0

Neutralizing broths evaluated included: AOAC Diluting Broth (AOAC), Dey-Engley Neutralizing Broth (DEB), Letheen (LET), NIH Thioglycollate Broth (NIH), Trypticase with Tween (TAT), Trypticase Soy Broth with Polysorbate 80 and Lecithin (TPL) and Fluid Thioglycollate Medium Neutralizer (FTMN). Compositions with the final concentrations as listed (g/L).

Neutralizer exposed group was prepared in parallel following the same procedure as test group but using sterile saline or buffer instead of test solution. Viability control group was prepared using peptone water without test solutions or neutralizing broth. Organisms were prepared so that the required inoculums did not exceed 0.5-1.0 % of the total volume in the tubes. Inoculums of each used microorganisms were added to each of the formerly described tubes so that the final count per plate of positive control was ranging between 30 and 100 CFU, with the exception of *Aspergillus brasiliensis* whose maximum count should not exceed 80 CFU per plate. About 20 ml of molten suitable medium at 45° C was added; allowed to solidify, then incubated at suitable temperature for 30–35 °C for 3 days for bacteria and 20-25 °C for at least 5 days for molds. Duplicate plate count were done and used as a positive control. Negative control for each media with the same volume of diluents or neutralizers added was performed to ensure sterility of all used materials. Test was performed in triplicates for each microorganism and dilution. All statistical analysis was performed using GraphPad Prism version 5. Any interpretation or complex calculation was performed using Microsoft Excel 2007.

IV- ACCEPTANCE CRITERIA:

Three interpretation comparisons were conducted on the results of neutralization study: Results that showed ≥1.00, those with 0.00 recoveries, obviously failed triplicate results beyond the acceptance level and those with geometric mean ≥0.75 of the reference control were not be subjected to statistical analysis. One-Way Analysis Of Variance (ANOVA) followed by Dunnett's Multiple Comparison Test ($P<0.05$) were done using GraphPad Prism version 5 for Windows which was used to perform all statistical analysis on $\log_{10}$ transformed counts to confirm success or failure [17]. Significant difference in microbial count compared to the initial result of positive control (inoculum verification plates) was considered when

the difference between both is greater than a 0.3 log variation, which is defined as normal plating variability; this is the second criterion [22]. The last criterion was that at least three independent replicates of the experiment should demonstrate that the average number of CFU recovered from the challenge product is not less than 70% of that recovered from the inoculum control [16].

RESULTS

NT study revealed that FTMN did not possess any adverse effects on microbial spore tested and 3 comparison criteria were in agreement in final result interpretation. The recovery of 3 microorganisms was ≥1, which did not require statistical analysis. Thus FTMN neutralizer did not possess any toxicity on the tested spores. This finding was illustrated in **(Tables 3, 4 and 5)**.

NE study at 1:10 and 1:100 (v/v) dilution ratios showed variable outcomes in (Tables 3, 4 and 5) when using the three different criteria as follows:

Harmonized USP<61> of Normal Plating Variability: Significant difference in microbial count from reference control group at 1:10 (v/v) of Mil and Pury with *Bacillus cereus* and *Bacillus subtilis subsp. spizizenii.* Also significant variability in count with *Bacillus cereus* vs. BarD 50 at 1:10 (v/v) was observed. On the other hand, *Aspergillus brasiliensis* passed NE with the four biocidal agents at 1:10 and 1:100 (v/v) dilution ratios.

USP<1227> of Three Independent Replicates: Both *Bacillus species* failed in triplicates at 1:10 (v/v) with both Mil and Pury while *Bacillus cereus* failed in all replicates vs. BarD 50 at 1:10 (v/v). *Bacillus cereus* did not pass in all replicates vs. BarD 50 at 1:10 (v/v). Again and as the former acceptance criterion, *Aspergillus brasiliensis* passed NE with the four peroxygen/Ag^+-based disinfectants at 1:10 and 1:100 (v/v) dilution ratios in all triplicates.

Sutton initial recovery criterion followed by Dunnett's Multiple Comparison Test (P<0.05): *Aspergillus brasiliensis* passed initial recovery criterion and only 2 were required to be tested by Dunnett's Multiple Comparison Test which showed that the difference were not significant from the control. Complete failure was ensured with both *Bacillus species* failed in triplicates at 1:10 (v/v) with both Mil and Pury while *Bacillus cereus* failed in all replicates vs. BarD 50 at 1:10 (v/v). Although *Bacillus cereus* failed to meet initial recovery criterion of ratio ≥0.75 with BarD 50 at 1:100 (v/v) and showed significant difference from positive control (neutralizer exposed group) by statistical analysis using One-Way ANOVA yet it was not significant from the reference acceptance value, upon using the criteria of both USP<61> and <1227>. The same situation was applied to Pury 1:100 (v/v) vs. *Bacillus cereus* with the exception that the initial recovery criterion ratio of ≥0.75 has been met.

The sensitivity of the 3 spore-forming microorganisms to the neutralization process was in the following descending order: *Aspergillus brasiliensis*> *Bacillus subtilis subsp. spizizenii*> *Bacillus cereus*. The 3 assumed criteria gave the same outcome but with variable failure rate. The ease of neutralization of the 4 peroxygen/Ag^+-based disinfectants was in the following ascending order: Mil=Pury<BarD50<Bixco as identified by failure rate with pronounced effect of Mil and Pury 5% at 1:10 (v/v) on the 2 *Bacillus species.* These results were demonstrated in **(Tables 3, 4 and 5)**. Sutton *et al.* criterion is more conservative than both USP<61> and USP<1227> in case of present study (6 identified failures versus 5 for both last 2 USP criteria per 24 observations).

DISCUSSION

Effective neutralization of a chemical biocide is critically important to the quality of the data derived from any assay of biocidal efficacy [23]. The determination of NT and of NE should be a comparison between a test and a control population. In the present study, the NT was expressed as the ratio of recovery between a viability population, and a population exposed to the neutralizer. This comparison directly examined the toxicity of the individual neutralizing media for the different microorganisms. The efficacy of a particular neutralizer was defined as the ratio of recovery between the neutralizer and the biocide, and the neutralizer exposed populations; therefore, only the effect of the biocide in the system was measured. These ratios allowed for a threshold value (0.75)

in the first test. The second test was a statistical one to confirm failures. This two-tiered acceptance criterion ensured against the inadvertent rejection of an effective, non-toxic neutralizer [17]. Using Sutton method of interpretation with modification, 6 failures were identified one of them was confirmed statistically by Dunnett's Multiple Comparison Test P<0.05 i.e. q' (the critical value in the Dunnett's test) exceeded the critical value for α = 0.05, then the comparison did not pass the statistical test.

NT study performed for the FTMN used in the current work revealed that it was non-toxic and could be used in the validation program. The other important subsequent aspect was the NE; in this test, the scheme followed was based on FTM as primarily (supported by previous work). NIH Thioglycolate (close in composition to FTM) was previously reported to be non toxic or of low toxicity against microorganisms. The combination of microorganism, neutralizer and disinfectant is unique and thus the success of one combination with one microorganism does not mean that same combination with other microorganisms will do accordingly [17]. In-house made neutralizer; used herein, is in between DEB and NIH Thioglycolate in composition. Although the commercially studied disinfectants were similar in composition concerning the main active biocidal agents, the manufacturer have incorporated 20 to 25 other anonymous constituents in the formula; yet, these components may have major or minor impact on disinfectant activity [20] and efficiency such surfactants, stabilizers, anticorrosives,..etc.

The selection of these representative microbial spores was based on those found in the environmental isolates, identified and subjected to trending from surface and air samples. Practically, the microbial contamination of raw materials used to manufacture dry formulations (e.g., tablets) is often reduced by drug manufacturing processes such as granules drying and tablet compaction. However, the amount of bioburden reduction is directly dependent on the process temperature, chemical properties of the drug formulation, tablet compression pressure, and the metabolic properties of the contaminating microbes. For example, bacterial spores are less susceptible to the harsh conditions encountered during tablet processing and the survival of *Bacillus subtilis* spores found in raw materials has been studied and documented [24]. As reported in another study [25], various types of tablets, both coated and non-coated, were found to be contaminated with bacteria such as *Bacillus cereus*. Fungi were also isolated from the samples tested including *Aspergillus flavus*.

Table 3: NT and NE for *Aspergillus brasiliensis* vs. 4 Peroxygen/Silver-Based Disinfectants

Point of Comparison for ***Aspergillus brasiliensis***		Geometric mean ratio of test to control (raw CFU – untransformed)	Average of log_{10} transformed results ± S.D.	Harmonized Chapter USP<61> of 0.3 log difference	Dunnett's Multiple Comparison Test P<0.05	USP<1227> Independent Replicates of criteria ≥70%
1.Toxicity of neutralizing broth		1.10	2.041±0.085	-0.041	NR(a)	3 +
2. NE vs. Bixco	1:10	0.99	2.00±0.038	0.000	NR(a)	3 +
	1:100	1.06	2.02±0.058	0.024	NR(a)	3 +
3. NE vs. BarD 50	1:10	1.03	2.01±0.055	0.013	NR(a)	3 +
	1:100	1.11	2.05±0.023	0.046	NR(a)	3 +
4. NE vs. Mil	1:10	0.89	1.95±0.072	-0.050	NR(a)	3 +
	1:100	1.11	2.07±0.092	0.072	NR(a)	3 +
5. NE vs. Pury	1:10	1.03	2.01±0.059	0.014	NR(a)	3 +
	1:100	1.16	2.07±0.018	0.065	NR(a)	3 +

(a)=Not required due to obvious success or failure of the replicate (+) = Pass and (-) = Fail.

According to USP<1227> Validation of Microbial Recovery from Pharmacopeial Articles "At least three independent replicates of the experiment should be performed, and each should demonstrate that the average number of CFU recovered from the challenge product is not less than 70% of that recovered from the inoculum control". From this criterion 5 failures were identified in triplicates; of which, 2 belonged to *B. subtilis* and 3 to *B. cereus*. Harmonized Chapter USP<61> provided the same level of failure from neutralization study. This criterion is 50% (0.3 Log) from positive control. By applying this limit only 5 failures were identified. All results that did not pass NE test came from 1:10 (v/v) dilution in FTMN with the exception of one belonging to *B. cereus* with BarD 50 at 1:100 (v/v) when tested using Sutton criterion. It should be noted that the effect of 10-fold dilution of disinfectants in the chemical neutralizer was not significant with *Aspergillus brasiliensis* in contrast to *Bacillus cereus*, while *Bacillus subtilis* was in between, especially with BarD 50 in which the recovery in both dilutions was the same. This finding needs further investigation to find possible relation of this result to the spore hydrophobicity in the existing aqueous environment. But primarily it could be attributed to the greater hydrophobicity of fungal spore surface if compared with that of *Bacillus* spp. and this property served to hinder the wetting of the fungal spore surface required by residual biocide and/or chemical neutralization toxic by products to affect spores viability. This assumption was based on attractive hydrophobic interactions between *A. niger* spores studied by other researchers [26]. The second impact of spore surface lypophilicity is the favored formation of spores lumps or clusters thus protecting the enclosed aggregates from unfavorable hostile chemical environment. This hypothesis was based on the previously reported tendency of *A. niger* to form conidial aggregates [27]. On the same line, fungal spores have been reported to possess a hydrophobic surface which aids the dispersal, prevents desiccation, and may provide a barrier to the entry of toxicants [28]. Furthermore, the fungal cultures in culture media which have high nutrient content are likely to produce more hydrophobic spores; the opposite is true when the culture media with low nutrient content are used [29].

Table 4: NT and NE for *Bacillus cereus* vs. 4 Peroxygen/Silver-Based Disinfectants

Point of Comparison for ***Bacillus cereus***		Geometric mean ratio of test to control (raw CFU – untransformed)	Average of log_{10} transformed results ± S.D.	Harmonized Chapter USP<61> of 0.3 log difference	Dunnett's Multiple Comparison Test P<0.05	USP<1227> Independent Replicates of criteria ≥70%
1.Toxicity of neutralizing broth		1.24	2.09±0.087	0.094	NR(a)	3 +
2. NE vs. Bixco	1:10	0.78	2.04±0.016	0.040	NR(a)	3 +
	1:100	1.08	1.90±0.040	-0.100	NR(a)	3 +
3. NE vs. BarD 50	1:10	0.45	1.66±0.077	-0.345 (c)	NR(a)	3 -
	1:100	0.74(b)	1.87±0.024	-0.133	S(c)	3 +
4. NE vs. Mil	1:10	0.30	1.48±0.008	-0.518 (c)	NR(a)	3 -
	1:100	0.90	1.96±0.028	-0.045	NR(a)	3 +
5. NE vs. Pury	1:10	0.31	1.50±0.021	-0.504 (c)	NR(a)	3 -
	1:100	0.77	1.89±0.044	-0.111	NR(a)	3 +

(a)= Not required due to obvious success or failure of the replicate. (b)=Subjected to statistical analysis.
(c)=Significantly different from the control or reference count. (+) = Pass and (-) = Fail.

Once the sanitization program has been established, selection of suitable, commercially available disinfectants followed by proper validation starting from

preliminary proper neutralization should be established as the above study proved that Bixco, BarD 50, Mil and Pury could be used successfully in the next step in disinfectant qualification. The selection here will be based on the degree of effectiveness, reasonable contact time, the least concentration recommended [30] and the possible adverse interaction with other biocidal agents applied in the area and products residues in processing facility. Corrosiveness is an important aspect always linked to the use of sporicidal agents, so those with anticorrosive ingredients must be considered and tested. Simple test has been adopted based on weight difference of tested coupons made from materials and surfaces existing in the facility as a measure for corrosiveness.

Table 5: NT and NE for *Bacillus subtilis subsp. spizizenii* vs. 4 Peroxygen/Silver-Based Disinfectants

Point of Comparison for ***Bacillus subtilis subsp. spizizenii***		Geometric mean ratio of test to control (raw CFU – untransformed)	Average of log_{10} transformed CFU ± S.D.	Harmonized Chapter USP<61> of 0.3 log difference	Dunnett's Multiple Comparison Test P<0.05	USP<1227> Independent Replicates of criteria ≥70%
1.Toxicity of neutralizing broth		1.00	2.00±0.093	0.002	NR(a)	3 +
2. NE vs. Bixco	1:10	0.95	1.98±0.070	-0.020	NR(a)	3 +
	1:100	1.27	2.10±0.060	-0.104	NR(a)	3 +
3. NE vs. BarD 50	1:10	0.79	1.90±0.047	-0.104	NR(a)	3 +
	1:100	0.79	1.90±0.029	-0.107	NR(a)	3 +
4. NE vs. Mil	1:10	0.16	1.22±0.078	-0.783 (c)	NR(a)	3 -
	1:100	0.88	1.95±0.014	-0.055	NR(a)	3 +
5. NE vs. Pury	1:10	0.14	1.15±0.076	-0.848 (c)	NR(a)	3 -
	1:100	0.85	1.93±0.028	-0.073	NR(a)	3 +

(a)= Not required due to obvious success or failure of the replicate. (+) = Pass and (-) = Fail.

(c)=Significantly different from the control or reference count.

CONCLUSION

The in-house made neutralizing broth (FTMN) was effective at 1:100 dilution ratio (v/v) to recover *Aspergillus brasiliensis*, *Bacillus cereus* and *Bacillus subtilis* in their spore form from the four tested commercial peroxygen/Ag^{+}-based biocidal agents denoted by: Bixco, Mil, Pury and BarD 50. Its effectiveness was determined according to USP<1227> at the maximum working concentration of the biocidal agent (5%) in purified water with *Aspergillus brasiliensis* being the most resistant and *Bacillus cereus* more vulnerable to the change in disinfectant type and concentration.

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