

Report on the Biocides Research Programme

The preparations investigated are substances with a sterilising effect based on the action of free radicals: atoms or molecules with an odd number of electrons in their outer orbit. This produces a state of instability that leads them to take electrons from other molecules with which they come into contact, in order to achieve a stable equilibrium. The molecules that have donated electrons become unstable and seek other electrons to restore their stability, triggering a chain reaction of electron donation and acquisition (stability-instability). The various components active in this electron-transfer reaction can act as oxidising agents, and therefore electron acceptors, or as reducing agents, and therefore electron donors.

The mechanism of action of free radicals acts irreversibly, predominantly on the lipid components (lipids and phospholipids) of cell membranes and on nuclear proteins (pores of the nuclear membrane).

The following will undergo microbiological testing:

- Electron-donating free radicals with reducing activity in a vehicle at alkaline pH, intended for their sterilising action on instruments and medical devices;
- Electron-accepting free radicals with oxidising action, intended for surface decontamination.

Aim of the Study

The aim of the study was to evaluate the sterilising activity of DIAL and EC-STER soluble powders against spores of *B. subtilis* ATCC 19695.

The effectiveness of the DIAL product was evaluated using the following tests:

- 1) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.3%, with contact times of 2, 5 and 60 seconds.
- 2) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.25%, with contact times of 5, 10 and 30 seconds.
- 3) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.2%, with contact times of 60 seconds, 2 and 3 minutes.
- 4) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.18%, with contact times of 60 seconds, 2 and 3 minutes.
- 5) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.15%, with contact times of 60 seconds, 2 and 3 minutes.
- 6) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.3%, 6 hours after preparation, with contact times of 2, 5 and 60 seconds.
- 7) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.3%, 48 hours after preparation, with contact times of 2, 5 and 60 seconds.
- 8) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.25%, 7 days after preparation, with contact times of 60 seconds and 5 minutes.

The effectiveness of the EC-STER product was evaluated using the following test:

- 1) Sporicidal activity against *B. subtilis* at a concentration of $8 \log_{10}$ (0.8 McF), at a working concentration of 0.35%, with contact times of 2, 10 and 30 seconds.

Materials

DIAL water-soluble powder;
Ec-Ster water-soluble powder;
Spores of *B. subtilis* ATCC 19695;
Sheep blood agar plates;
Nutrient Broth.

Methods

Preparation of Sterilising Solutions

The soluble powders of the DIAL and Ec-Ster products at different concentrations were dissolved in water (tap water at room temperature) until dissolution was complete.

Preparation of *B. subtilis* ATCC 19695 Spores

Spores of *B. subtilis* ATCC 19695 were prepared following the manufacturer's instructions. The inoculum used in the experiments was prepared to obtain a suspension with a turbidity of 0.8 McFarland (approximately 10^8 colony-forming units/mL).

Manual Method

A 100 µL aliquot of *B. subtilis* ATCC 19695 at 0.8 McF was added to a total volume of 10 mL of each sterilising solution (DIAL and EC-STER). After each contact time tested, a 100 µL aliquot of the suspensions was inoculated onto sheep blood agar plates and incubated at 37 °C for 24 hours. Microbial growth was evaluated at the end of incubation.

A second 100 µL aliquot was transferred into tubes containing Nutrient Broth. After 24 hours of incubation at 37 °C, 100 µL was inoculated onto sheep blood agar plates. Microbial growth was evaluated at the end of incubation.

All experiments included a negative control to detect any contamination of the solutions under examination and a positive control for microbial growth.

Automated Method using Alfred 60, Alifax

Alfred 60, Alifax is a fully automated system that uses Light Scattering technology to determine the presence of microorganisms and detect their replication activity, displaying growth curves in real time and providing quantitative counts in colony-forming units/mL.

A 100 µL aliquot of *B. subtilis* ATCC 19695 at 0.8 McF was added to a total volume of 10 mL of each sterilising solution (DIAL and EC-STER). After each contact time tested, a 100 µL aliquot was transferred into dedicated reading cuvettes (vials) containing Eugon broth, which is continuously agitated and maintained at a constant temperature (37 °C) within the system. An incubation period of 24 hours was set to evaluate the effectiveness of the products under examination.

All experiments included a negative control to detect any contamination of the solutions under examination and a positive control for microbial growth.

Results

Manual Method

Strain: *B. subtilis* ATCC 19695 - 0.8 McF (approximately 10^8 CFU/mL)

Contact times

Product	2 s	5 s	10 s	30 s	60 s	2 min	3 min	5 min
T2; Sample No. 3 Batch: 74/s/15 Working concentration: 0.3%	-	-			-			
DESAN; Sample No. 5 Batch: 82/s/15 Working concentration: 0.3%	-	-			-			
DIAL; Sample No. 2 Batch: 6/15 Working concentration: 0.25%		-	-	-	-			-
T2; Sample No. 1 Batch: 74/s/15 Working concentration: 0.2%					-	-	-	
DESAN; Sample No. 8 Batch: 83/s/15 Working concentration: 0.2%					-	-	-	
DIAL; Batch: 6/14 Working concentration: 0.18%					-	-	-	
DESAN; Sample No. 6 Batch: 82/s/15 Working concentration: 0.15%					-	-	-	
EC-STER; Batch: 07/14 Working concentration: 0.35%	-		-	-				

Key: - absence of growth. Blank cells are blank in the original.

Results

Automated Method using Alfred 60, Alifax

Strain: *B. subtilis* ATCC 19695 - 0.8 McF (approximately 10^8 CFU/mL)

Contact times

Product	2 s	5 s	10 s	30 s	60 s	2 min	3 min	5 min
T2; Sample No. 3 Batch: 74/s/15 Working concentration: 0.3%	-	-			-			
DESAN; Sample No. 5 Batch: 82/s/15 Working concentration: 0.3%	-	-			-			
DIAL; Sample No. 2 Batch: 6/15 Working concentration: 0.25%		-	-	-	-			-
T2; Sample No. 1 Batch: 74/s/15 Working concentration: 0.2%					-	-	-	
DESAN; Sample No. 8 Batch: 83/s/15 Working concentration: 0.2%					-	-	-	
DIAL; Batch: 6/14 Working concentration: 0.18%					-	-	-	
DESAN; Sample No. 6 Batch: 82/s/15 Working concentration: 0.15%					-	-	-	
EC-STER; Batch: 07/14 Working concentration: 0.35%	-		-	-				

Key: - absence of growth. Blank cells are blank in the original.

Discussion

The experiments performed according to standard EN 13704 demonstrated the sporicidal activity of DIAL and EC-STER against spores of *B. subtilis* ATCC 19695.

The solutions demonstrated their effectiveness using both the manual and automated methods under experimental conditions involving variable contact times and different concentrations of the products tested.

Following the results obtained, the same solutions were used for morphological observations using a confocal microscope. Spores of *B. subtilis* after contact with DIAL and Ec-Ster solutions at different concentrations, together with the positive and negative controls, were stained with acridine orange, a vital dye that binds nucleic acids. Microscopic observations showed neither spore forms nor vegetative cells in samples that had been in contact with the sterilising solutions or in the negative control, as shown in Figures 1-8. By contrast, in the positive control, red and green fluorescence revealed the presence of spores and vegetative cells of *Bacillus subtilis*, respectively (Figures 9-11).

18 December 2015

Prof. Alfredo Focà

Translation note. English translation of the supplied seven-page Italian report. Product names, numerical values and table results are retained as in the original. Figures 1-11 mentioned in the text are not included in the supplied original. The Italian original remains the authoritative source.