AQUECA Report
Module-A. Disinfection efficacy of AQUAECA DESY.

Objective-1. Disinfection efficacy at 13A, pH-6.7-6.9

Material and Methods

A. Microorganisms used in the study

- Bacteria
    - *Staphylococcus aureus* (MTCC 9886)
    - *Enterococcus faecalis* (MTCC 439)
    - *Escherichia coli* (MTCC K12)
    - *Pseudomonas aeruginosa* (MTCC 424)
- Spores
    - *Bacillus subtilis* (MTCC 441)
- Fungi
    - *Candida albicans* (SC5314)
    - *Candida glabrata* (CBS138)

B. Sample preparation

1. Bacterial culture were grown in LB broth.
2. Fungal culture were grown in YPD (Yeast Extract-1%, Dextrose-2% and Peptone- 2%).
3. *Bacillus subtilis* sporulation was performed in sporulation media (SM). Composition of SM used was- Nutrient broth- 0.8%, KCl- 0.1%, $MgSO_4 \cdot 7H_2O$- 0.012%, pH adjusted to 7.6 with 1 M NaOH, volume adjusted to 1 litre with ddH_20. Autoclaved and allowed to cool to 50°C. Just prior to use, following sterile solutions were added: $Ca(NO_3)_2$-1mM, $MnCl_2$- 0.01mM, $FeSO_4$-0.001mM. 0.5 ml of an overnight grown *B subtilis* culture was inoculated in liquid DSM media in Erlenmeyer flasks. Flasks were kept at 37 °C in an orbital shaker at 200 rpm for 7 days. Generation of spores was monitored microscopically. After 7 days of incubation > 90% of spore population was observed.
4. All cultures were maintained at 37°C .
5. Viable cell density of at least 10^{10} cells was maintained for bacteria and fungus.

C. Methodology

1. 1ml aliquot of each culture harbouring 10^{10} cells (as determined by total aerobic plate count or spectrophotometrically at 600 nm wavelength) was transferred into microfuge tube and centrifuged at 10,000 rpm for 5 min. Supernatant was discarded and pellet was stored for further use.
2. For calculating the disinfection efficacy (Log reduction) total aerobic plate count procedure was be followed.
3. For determining the disinfection efficacy of DESY, pellet obtained from step-1 was resuspended in 1ml of Desy obtained from AQUAECA at 13A. 100µl of the resuspended pellet was removed after 1min, 2min, and 5 min exposure times and spread plated on relevant plates to calculate the log reduction (**Fig.1**).
4. As control the pellet was resuspende in sterile deionised water and 100µl of the resuspended pellet was removed after 5 min exposure times and spread plated on relevant plates. (**Fig.1**).
5. For statistical significance disinfection efficacy of **DESY** for each time point was determined using three biological replicates for each organism.

Results

Disinfection efficacy of Bacteria:

1. The bacterial disinfection efficacy of DESY obtained from AQUAECAat 13 A was tested using representative gram negative (*Escherichia coli* K12 and *Pseudomonas aeruginosa*) and gram positive bacteria (*Staphylococcus aureus* and *Enterococcus feacalis*).
2. Bacteria was exposed to DESY at room temperature. It was observed that both gram negative and gram positive bacteria could be efficiently destroyed by DESY even at 1 min exposure time (**Fig. 1**). No bacterial growth was observed at 2min and 5min time points as well (**Fig.1**).
3. Total aerobic plate count showed that 10 log reduction in bacterial counts was achieved via DESY treatment at 1min exposure time.

Disinfection efficacy of Fungi:

1. Fungal disinfection efficacy of DESY was tested using representative pathogenic fungi *Candida albicans* and *Candida glabrata*.

2. Fungi was exposed to DESY at room temperature. It was observed that both Candida species could be efficiently destroyed by DESY even at 1min exposure times (**Fig. 1**). No fungal growth was observed at 2min and 5min exposure time points as well (**Fig.1**)
3. Total aerobic plate count showed that 10 log reduction in fungal counts was achieved via DESY treatment at a exposure time of 1 min).

Disinfection efficacy of Spores:

1. Highly resistant bacteria spore disinfection efficacy of DESY was also analysed using representative *Bacillus subtilis* spores.
2. 10^6 spores were exposed to DESY at room temperature. It was observed that bacterial spores could be efficiently disinfected by DESY even at 1min exposure time (6 log reduction) (**Fig. 2**).
3. Total aerobic plate count method was used for determine disinfection efficacy.

Conclusions

A 10 log disinfection efficacy of bacterial culture was achieved via DESY treatment at room temperature with an exposure time of 1 min. DESY treatment was also effective on fungal cells, where a 10 log disinfection efficacy was observed at room temperature with an exposure time of 1 min. A 6 log disinfection efficacy of representative highly resistant *Bacillus subtilis* spores was also achieved with DESY at room temperature with an exposure time of 1 min.

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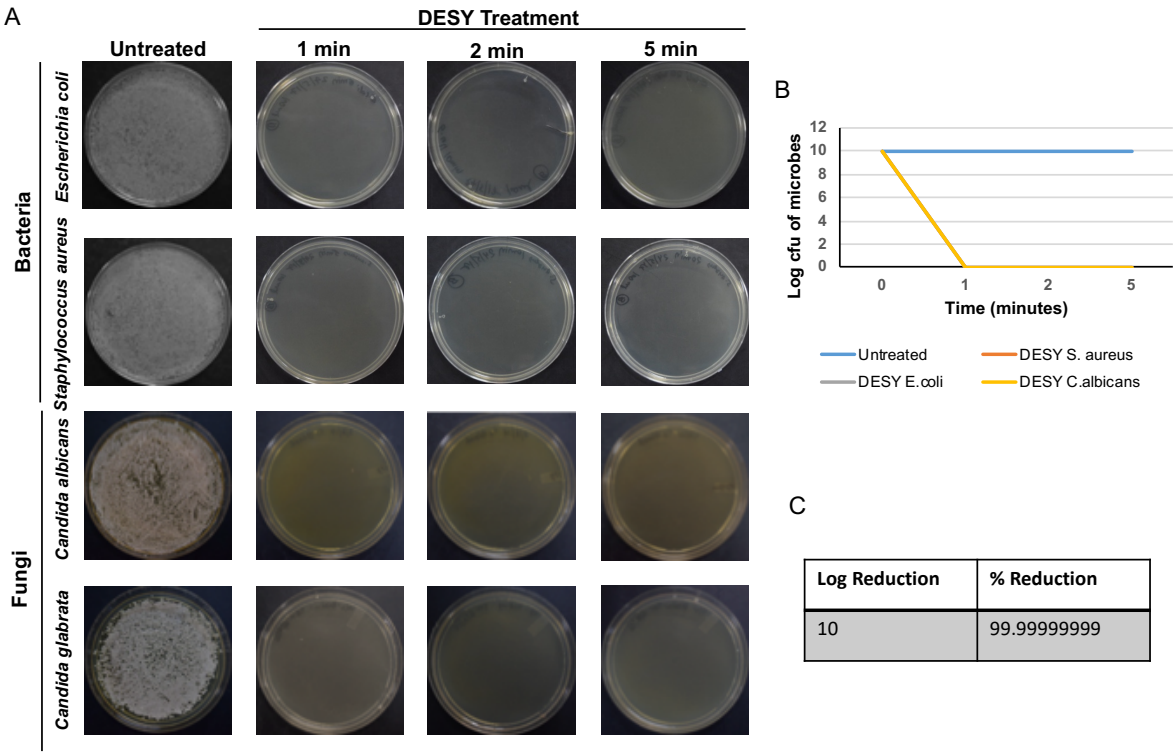
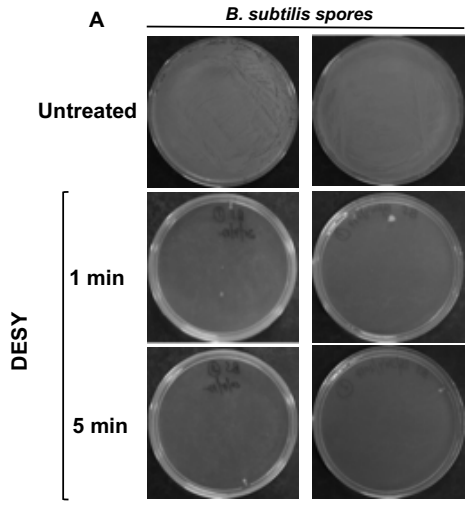


Figure 1. Disinfection efficacy of DESY at 13A. (A) Representative plates for total aerobic plate count. (B) Log reduction in bacteria and Fungi upon DESY treatment. (C) Relation between log reduction and % reduction.



B

Log Reduction	% Reduction
6	99.9999

Figure 2. Disinfection efficacy of DESY at 13A against *Bacillus subtilis* spores. (A) Representative plates for total aerobic plate count. (B) Relation between log reduction and % reduction.

SOP for evaluating the disinfection efficacy of AQUAECA-W40 disinfectant using self-contained Biological Indicators

Sterilization is a crucial metric in all cleanrooms and clean environments, and one way to test a particular environment for effectiveness in the sterilization its undergone is through the use of biological indicators. *Geobacillus stearothermophilus* is an excellent microorganism for testing the effectiveness of sterilization protocols using heat or steam, given the bacteria's high heat resistance.

Disinfection efficacy of disinfectant (DESY) obtained from AQUAECA-W-40 device (13A, ≈pH 6.7) can be also be evaluated by using commercially available *G. sterothermophillus* biological indicators (Getinge, gke, 3M etc).

Design of Self Contained Biological Indicator vials

1) The vial contains a chemical indicator printed on the label.
2) Each vial has two tubes inner glass tube contains the media with pH indicator.
3) Outer plastic tube has spore strip containing 10^6 spores placed at the bottom.

Procedure:

1) Take at least two biological indicators vials from Getinge company. Label one vial as control and another as test)
2) Remove the Cap (Brown colored) under aseptic conditions (Fig.1). To control vial add 700µl sterile water and to the test vial add 700µl DESY (13A, ≈pH 6.7). Incubate at room temperature for 2 min. Remove water and disinfectant by gently inverting and tapping the tube over tissue paper.
3) Replace the caps.
4) Gently crush the glass vial, by pressing the plastic tube with forceps. This will release the growth media into the outer tube.
5) Incubate the tubes at 55°C for at least 12 hrs.
6) Growth of viable spore will turn the colour of media from purple to yellow (control vial). No change in colour of media indicates complete disinfection of 10^6 spores (Test vial) (Fig.1).

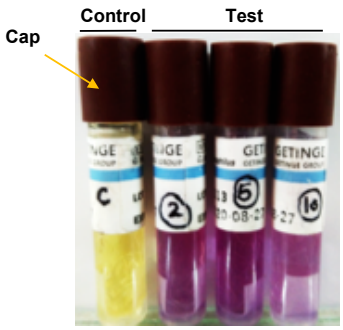


Figure:1. Exposure of *Geobacillus sterothermophillus* biological indicators (Getinge) to DESY. Complete disinfection of 10^6 *G. sterothermophillus* spores was observed upon 2 minutes of exposure. Indicators were incubated at 55°C for 24 hrs

Note: Do not incubate biological indicators in water bath, dry heat incubation without shaking gives proper result.

SOP for evaluating the disinfection efficacy of AQUAECA-W40 disinfectant using self-contained Biological Indicators

Biological indicators are test systems that contain viable chemical resistant *Bacillus atrophaeus* spores (10^6 spores in each vial). They help monitor whether the necessary conditions were met to kill a specified number of microorganisms for a given sterilization process. Bacterial spores used in biological indicators are some of the toughest microorganisms to kill.

Disinfection efficacy of disinfectant (DESY) obtained from AQUAECA-W-40 device (13A, ≈pH 6.7) can be determined by using commercially available *B. atrophaeus* biological indicators (Getinge, gke, 3M etc).

Design of Self Contained Biological Indicator vials

1) The vial contains a chemical indicator printed on the label.
2) Each vial has two tubes inner glass tube contains the media with pH indicator.
3) Outer plastic tube has spore strip containing 10^6 spores placed at the bottom.

Procedure:

1) Take at least two biological indicators vials from Getinge company. Label one vial as control and another as test)
2) Remove the Cap (Red colored) under aseptic conditions (Fig.1). To control vial add 700µl sterile water and to the test vial add 700µl DESY (13A, ≈pH 6.7). Incubate at room temperature for 2min. Remove water and disinfectant by gently inverting and tapping the tube over tissue paper.
3) Replace the caps.
4) Gently crush the glass vial, by pressing the plastic tube with forceps. This will release the growth media into the outer tube.
5) Incubate the tubes at 37°C for 12 hrs.
6) Growth of viable spore will turn the colour of media from orange to yellow(control vial). No change in colour of media indicates complete disinfection of 10^6 spores Test vial ((Fig.1).

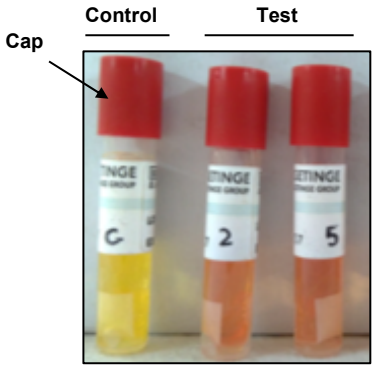


Figure:1. Exposure of biological indicators to DESY. Complete disinfection of 10^6 *B. atrophaeus* spores was observed upon 2 minutes of exposure. Indicators were incubated at 37°C for 12 hrs

Note: Do not incubate biological indicators in water bath, dry heat incubation without shaking gives proper result.