Mitigating Microbial Artifacts in Laboratory Research of H_2 Energy Geo-storage

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ABSTRACT

Today our energy sectors are focusing on the marathon of CO_2 cut and coherently require progress in energy transition schemes to meet the UN climate change challenge and achieve a zero-emission target. Among these schemes, radioactive disposal, CO_2, NH_3, and H_2 geological storage are promising options for fixing anthropogenic waste, greenhouse gases and storing green energy in the depleted oil/gas reservoirs, deep saline aquifers, and salt caverns. Consequently, this could be achieved through rigorous research and development (R&D) projects involving laboratory-scale experiments. Despite the ubiquity of microorganisms in various environments, their potential impact on laboratory studies in fields outside of the biological sciences is not well established. In particular, their presence in research related to new energy technologies, such as hydrogen storage, poses a significant risk to experimental integrity. Microorganisms can consume hydrogen and other substances, leading to potentially misleading results. This oversight can have profound implications, especially when studying geological formations where microbial contamination might alter the properties and behaviours of reservoir rocks. Thus, it is crucial to incorporate sterile controls in experiments to accurately assess the influence of independent variables and to discern the specific effects of microbial presence. The effect of ultraviolet (UV), autoclave, oven heating, ethanol 75%, ethanol 95%, and gamma irradiation for cleaning microorganisms in the sand were investigated Interestingly, our experimental results revealed that gamma irradiation and autoclave heating are the most vibrant options for extinguishing microorganisms from the surface of the rock and saying no to the risk of experimental error in future work reflecting geological storage applications.

Keywords:- Geological storage, energy transition microorganisms, rock cleaning techniques, porous media, salt cavern

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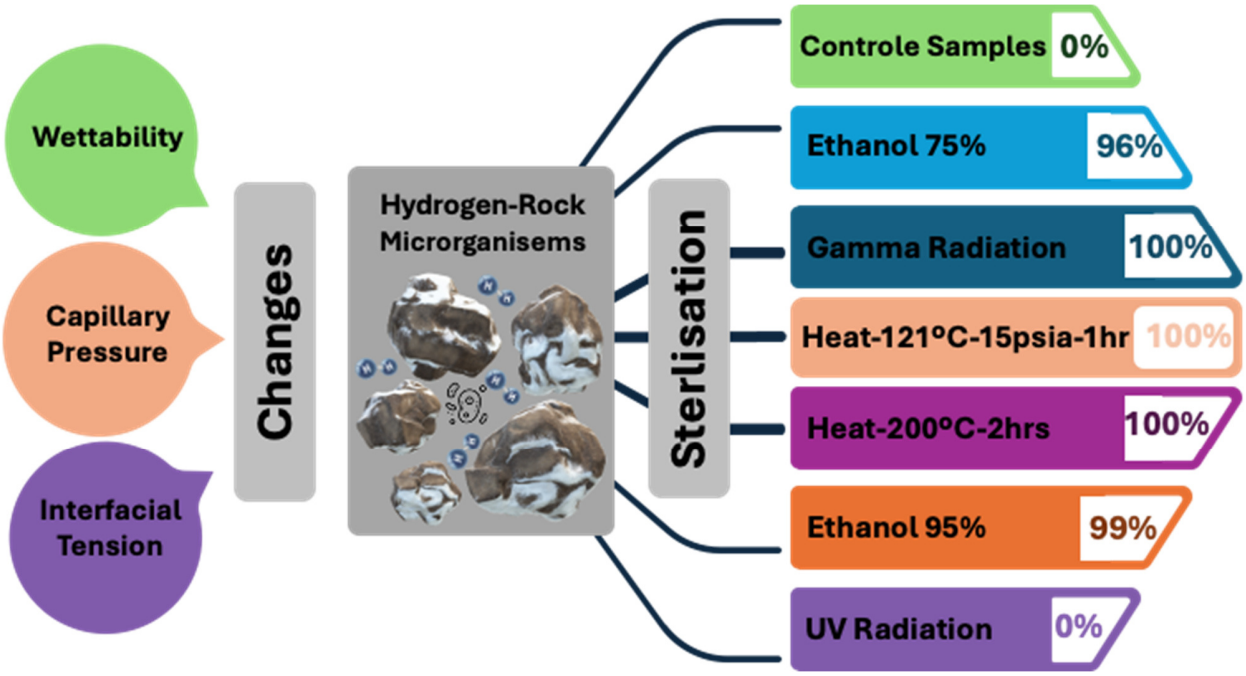
TOC GRAPHIC



▪ INTRODUCTION

Geo-solutions are crucial in the phase of energy transition to achieve a carbon neutrality target via compressed air, anthropogenic CO_2, helium and hydrogen geological storage. Given that microbials are widely distributed either in the subsurface or at surface and its presence may trigger uncertainties and risks associated with long-term implementations of the anthropogenic waste and energy fluids geological storage projects. Recent interest in H_2 and CO_2 storage in porous media and salt caverns has developed a need for experimental models to evaluate the rate of microorganism contamination for the application of geological hydrogen storage (GHS). The variety of reservoir engineering experiments, for example measurement of porosity, permeability, capillary pressure, interfacial tension, H_2-brine core flooding, wettability, in-situ loss of H_2 and H_2S generation are conducted to quantify the potential of the reservoir rock performance for GHS [1]. However, some bacteria either lying on the laboratory desk or present in the atmosphere could contaminate surface of the rock resulting in artifacts during the assessment of these reservoir properties.

Sterilization is needed without affecting the nature of the sample. There is no information what microorganisms might be lurking inside the rock samples in the laboratory. The rock samples

need to be sterilized before release and kept in an isolation room or biological-free zone for further testing. A team of researchers conducted sterilization of perchlorates which have been observed on the surface of Mars. Results revealed that Martian UV flux made perchlorates to bactericidal. The surface of the planet is exposed to both UVC radiation of <280 nm and UVB of 280 to 315 nm when compared to surface of the Earth [2]. Additionally, two components of the red planet including iron oxides and hydrogen peroxide induce a synergetic effect with irradiated perchlorates causing a 10.8-fold increase in cell death of *Bascillus substilis* compared to cells exposed by UV rays for 60 seconds [3]. However, the absolute killing of bacteria from the rock remains a challenge through UV sterilization. The unpenetrated UV radiation could not produce the expected results due to the irregular shape of the rock and rough surface. [4]. This procedure is time-consuming with risk of contamination and did not confirm the irradiation of whole microcavities. Thus, a liquid chemical may be required which could penetrate tiny size capillaries of rock to influence the absolute sterilization effect. The sterilization of limestone rock using 96% and 70% of ethanol could not develop absolute aseptic conditions in deep natural cracks [5]. The environmental microbes interacted with the rock causing calcite dissolution and precipitation. In an overlooked phenomenon, it was illustrated that the entombment of microorganisms in Si-rich precipitate in the nutrient-depleted environment suggests Si mobilization at ambient conditions [5]. Hence, the phenomena might have jeopardized the mineralogy of the geological material. **Table S1** provides a summary of literature related with effect of different sterilization on the minerology of the different rocks and minerals for the applications of astrobiology and geoscience.

In previous work, autoclave technique (~ 121 °C, 15 psi and 30 min) was used to sterilize the rocks [6]. Additionally, dry heating (>200 °C and ~3 h) is also an efficient mean of sterilization. Nevertheless, it is reported that microorganisms could penetrate lengths of consolidated Berea sandstone rock quicker when the rock was sterilized by autoclaving compared to the dry heating. Additionally, autoclave resulted high chloride than dry heating resulting aggregated and uneven shaped of clays and decreased bacterial penetration rates. Hence the findings show that dry-heat (dry oven heating) is more appropriate technique when compared to autoclaving when investigation biotic and abiotic the Berea sandstone rock [7]. Moreover, Gamma-ray has been proven to be a strong sterilization technique. This is a strong ray and reveals quite effective results to completely sterilize the rock from both inside and outside. The Mars analogue rocks and minerals were sterilized with gamma. The high doses of the ray displayed no effect on the rock. However, the darkening of some minerals was observed due to gamma radiation still the technique was considered a feasible choice for sterilizing the Mars returned rock samples [8].

Therefore, there is a pressing need to conduct comprehensive research to reveal the effects of different sterilization techniques, for instance i. UV, ii. Ethanol concentrations (both 75% and 90%), iii. Oven heating (dry heating), iv. Autoclave heating (wet heating) and v) Gamma ray irradiation. Thus, oven heating, autoclave heating and gamma irradiation on microbial-related laboratory research environment, ruling out the potential microbial artifacts in laboratory condition on H_2 and CO_2 geological.

▪ EXPERIMENTAL RESEARCH

Generally, **Figure 1** illustrates research methodology used in this study to investigate the effect of different sterilization techniques on sand-phosphate buffer saline (PBS)-microorganism inoculum. **Table S2** enlists the number of bacteria-sand inoculum used to investigate the effect of different techniques on killing the efficiency of bacteria in the rock. **Table 1** enlists the techniques and factors used for the sterilization of sand.

Table 1 presents list of techniques and its parameters used for the sterilization of sand

Techniques	**Temperature**	**Pressure**	**Intensity**	**Exposure time**	**Concentration**
UV	Ambient	Ambient	280 to 100 nm	30 min both sides	NA
Autoclave	121°C	15psi	NA	1h	NA
Oven	200°C	Ambient	NA	2h	NA
Ethanol	Ambient	Ambient	NA	Washed 3 times and soaked 15 min	75wt% 95 wt%
Gamma ray	NA	NA	NA	32h	NA

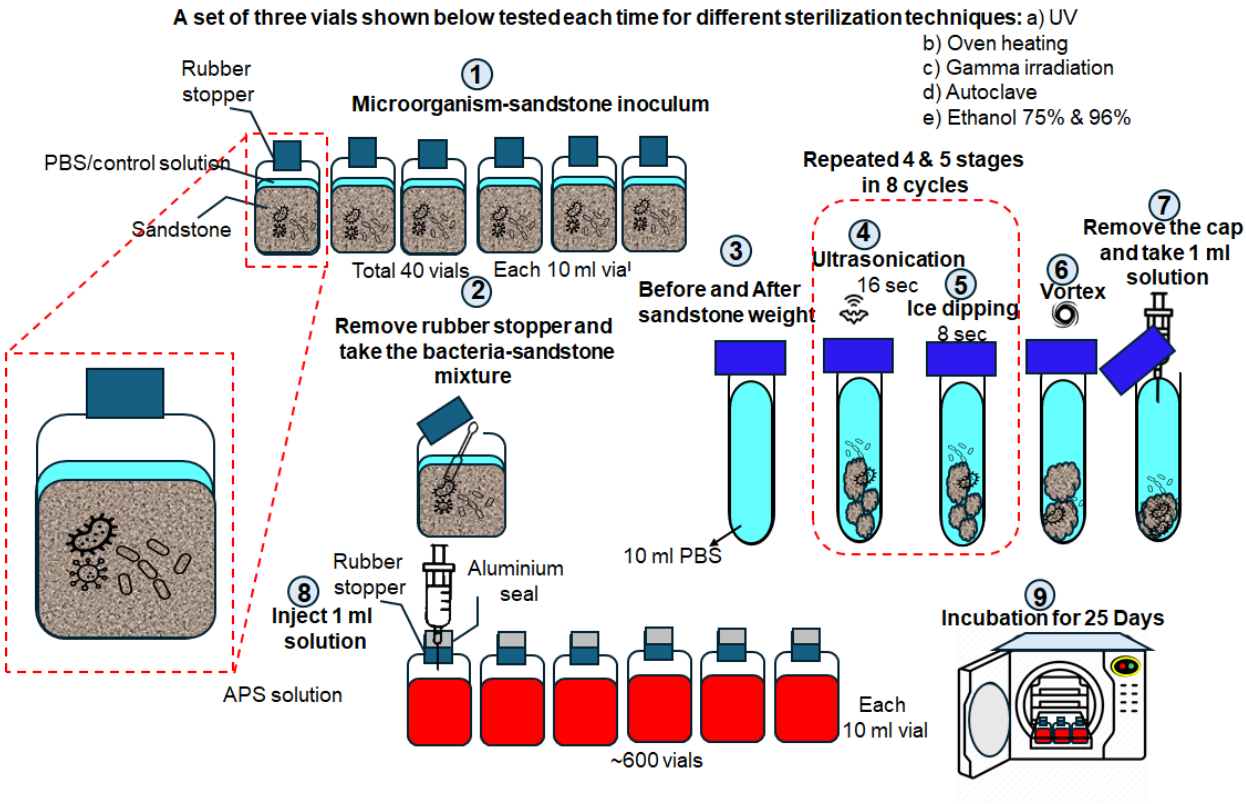


Figure 1 shows the research methodology and various stages, in particular **1.** Preparation of sand-PBS-microorganism inoculum and exposed them under different sterilization methods, **2.** Taking of sand sample, **3.** Weighing of falcon tube containing 10 mL PBS solution and around 2 grams of sand-PBS-microorganism inoculum, **4 - 5.** Ultrasonication and ice dipping of the falcon tube with the inoculum, **6.** Rapid mixing of samples in falcon tube using the vortex machine, and finally **7.** Take 1 ml of the solution from the Falcon Tube, **8.** Injecting 1 ml of solution in 10 ml acid producing bacteria (APB) media at 10^{-1} concentration and conducting serum dilution was conducted up to seven concentrations and finally **9.** Incubation of the vials

Table S3 enlists the chemicals and sand used in this work. APB solution media was prepared as shown in **Table S4**. Over 600 vials were prepared with each 10 ml and filled them with APB solution. **Table S4** and **Table S5** provide the list of chemicals used in this work for the preparation of the growth media solution and phosphate buffer solution (PBS). A consortium composed of Bacillus *sp.*, *Enterobacter sp.*, and *Cronabacter sp.* bacteria were used in this study. 1 ml of each microorganism was taken from 10 ml stock

solution. Later, 1 ml of each microorganism solution was added in 50 ml PBS solution to achieve 10^7 dilution. Bacteria-sand solution was prepared using the procedure for instance, firstly a 50ml falcon tube was filled with PBS solution using a sterile pipette. Secondly, the spatulas were wrapped in aluminium foil and autoclaved under the liquid condition mode. Using the sterile spatulas, the sand was taken and added into 10ml glass vial weighing around ~15.7g of sand. Every time a fresh spatula was used to avoid contamination. **Table S6** illustrates the weight of the falcon tube with and without sand was measured. **Figure S1** shows 10 ml vials containing approximately 15.5 g sand. Later, 3ml of cell suspension was added in each vial to use these vials for different tests. A total of 567 vials vials with 9 mL of anaerobic APB culture media were prepared **Figure S2 (A-C)** illustrates the methodology used for the preparation of 10 ml vials containing APB solution for the experiment. . We examined the effect of different cleaning techniques including UV, Ethanol 75%, Ethanol 96%, autoclave heating, oven heating and gamma irradiation using of incubation of the vials. **Supplementary information** provides list of all tests, equipment used and their procedure.

▪ RESULTS AND DISCUSSIONS

Figure 2A illustrates the concentration of surviving cells in the sand after sterilization process. The control (no subjected to sterilization) indicated that the concentration of cells in the test bottles was in the order of 10^6 cell/g before sterilization treatment. MPN results indicate that autoclave, oven heating, and gamma irradiation were able to eliminate all cells in the sand. However, oven heating, autoclave and gamma rays could induce mineralogical changes. For instance, oven heating is reported for micro cracking in quartz minerology of sand [9]. Additionally, autoclave steam can carry microorganism and penetrate into the rock [10]. And gamma ray caused discoloration of quartz mineral in sand which is in line with previous finding [8]. **Figure S3** illustrates the glass of serum vials and sand changed to a blackish colour after treated using gamma irradiation. These changes could affect the in-situ reservoir properties in particular porosity, permeability, interfacial tension, capillary pressure and wettability at micron scale resulting in adverse effects on the laboratory research for geological H_2 storage.

Ethanol and UV radiation were not able to inactivate all the cells and a concentration between 10^4 to 10^6 cells/g surviving bacteria were still detected. Our results provide a clear comparison of performance in cell sterilization among the different techniques. UV sterilization shows no effect on the sterilization of sand. We found that ethanol concentrations including 75 wt% and 95 wt%

illustrate the lowest killing efficiency more alive cells were detected compared to the other sterilisation methods evaluated. These techniques seem to cause irreversible damage to cellular component which are essential for their survival and illustrates 100% killing efficiency of bacteria in the sand **(Figure 2B**). **Figure 2B** shows the killing efficiency of each sterilization method. UV irradiation was the inefficient technique with killing efficiency of 0%. This finding may be attributed to less penetration of UV from glass of the vial into the rock. Ethanol 75% achieved a killing of 96.3%, whereas ethanol 95% reached a killing of 99.2%. Although killing efficiency values are high, it is important to consider that surviving population of cells was also high, in the order of 10^5 and 10^4, respectively. This survival percentage could restore microbial activity in long-time core flooding and salt cavern bioreactors experimental setups under the influence of anaerobic conditions. Additionally, the effects of precipitation, change in minerology, and formation brittle were reported in calcite, clay, and sand respectively after the use of Ethanol as a sterilizing substance**. Figure S4** illustrates the total number of vials incubated after inoculated with cell suspension.

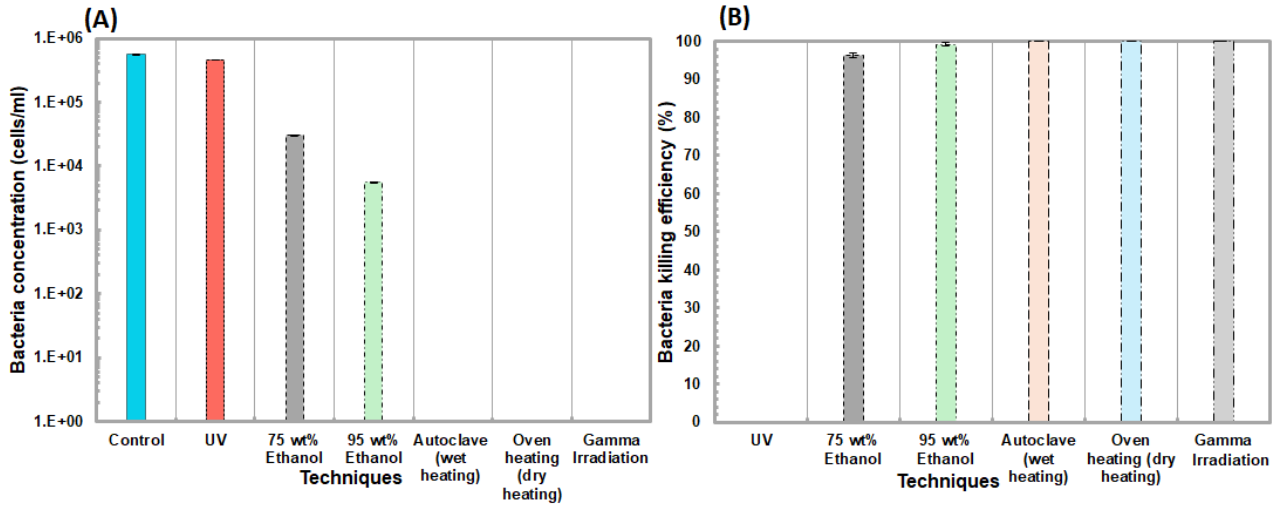


Figure 2 Effect of different techniques on the sterilization of the sand (A) bacteria concentration in cell/ml, and (B) bacteria killing efficiency in percent.

▪ CONCLUSIONS

This research concludes that autoclave heating, oven heating, and gamma irradiation methods are effective in absolutely eliminating bio-life inside rock. However, gamma irradiation caused discoloration of sand, and oven heating may induce micro-cracks, potentially compromising the rock's integrity. We propose that autoclaving is the most suitable technique due to its low operating temperature and pressure, making it ideal for sand sterilization. Additionally, rock treated with 75% ethanol still exhibited a significant microbial survival rate, casting doubt on its

efficacy for long-term experiments, as bacteria could potentially regrow over time. Further research is recommended to investigate the effects of these methods on the rock's mineralogy, petrophysical properties, and surface behaviour for large-scale geological hydrogen storage experiments.

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Supplementary Information

Mitigating Microbial Artifacts in Laboratory Research of H_2 Energy Geo-storage

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Table S1 provides a literature review table on effect of different sterilization techniques on minerology of rocks related with geoscience and astrobiology applications

Sterilization technique	Overall Effect on Mineralogy	Geoscience Application	Astrobiology Application	Study
UV	• Unpenetrated to core of the rock	• The unpenetrated UV radiation could not produce the expected results due to the irregular shape of the rock and rough surface. • It was examined that only one side of rock could be irradiated at a time and required a change of position of the sample during the exposure to UV • A photochemical lap consisting of four circular UV lamps allows adequate sterilization of rough surfaces of geological material while killing the microorganism possibly living in the core of the rock geological	• Martian UV flux made surface of perchlorates to bactericidal • The surface of the planet is exposed to both UVC radiation of <280 nm and UVB of 280 to 315 nm when compared to surface of the Earth • Iron oxides and hydrogen peroxide induce a synergetic effect with irradiated perchlorates causing a 10.8-fold increase in cell death of *Bascillus substilis* compared to cells exposed by UV rays for 60 seconds • However, the absolute killing of bacteria from the rock remains a challenge through UV sterilization. •	1-3
Ethanol 75% and Ethanol 95%	• Calcite can precipitate. • Clay minerology can change. • Brittle film formed on sandstone	• High-Mg calcite can precipitate at ambient temperature via partially replacing water with 75% ethanol additionally it bypasses the hydration barrier. • Ethanol concentration could change the polymorph of calcite, vaterite or aragonite in Mg-free environment. • The specific polarizability decreases as ethanol concentration increased from 0, 10, and 20% v/v. Ethanol concentration on clay driven polarization relative to changes in clay minerology.	Nil	4-9

		• Ethanol does not develop alkoxysilane-sandstone compatibility. Ethanol formed brittle film. • Ethanol was used instead of water to prevent interaction of clay-water cation exchange reactions prior to start experiment.		
Solarization	Soil	• Solarization was conducted through covering the soil with sterile transparent plastic film to trap solar radiation for heating which could rise temperature above 70 °C and considered as an adequate range to kill variety of plant pathogens.	Nil	[10]
Oven heating	• Micro cracking in Quartz sandstone at very high temperature	• Micro-cracking in quartz sandstone at the grain boundaries revealed increase in the porosity at 600 °C and within the grains and mineralogical changes at 750 °C. • The structure of clay mineral collapse at 600 °C, and chlorite above 600 °C.	Nil	[11]
Autoclave	• Could increase bacteria penetration in porous rock	• Steam treatment provides a better solution to clean pests in soil.	Nil	[12]
Gamma Irradiation	• Very low, discolor of the rock	Physico-chemical properties of natural sediments: • Major part of Clay mineralogy unchanged • pH slightly changes after irradiation • Irradiation samples reduced the cation exchange capacity	Mars returned rock samples sterilization: • Gamma photons from ^{60}Co (1.17 and 1.33 MeV) • Doses as high as 3×10^{7} rads • No effect detected on basalt rock and quartz mineral	[13, 14]

		• Irradiated samples reduced iron oxide • Effects on organic and inorganic fraction were observed	• No change in concentration of elements before and after irradiation • Crytal structure of mineral not affected • No change in grain density except a small effect on halite • Specific surface area of rock was not effected. • Dose induces no radioactivity in the rock • No change in their isotopic composition • No change in chemical composition • No change in crystallographic structure • Effects in the visible and near-infrared spectral region • Discoloration of quartz • Darkening of quartz and halite • Increases thermoluminescence of quartz and plagioclase	

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42 **Table S2** Preparation of bacteria-sandstone PBS solution and inoculated overnight

S.no	Sample	Sandstone weight	PBS solution	Number of 10 ml vials
1	Control A, B, C	15.7 g	3ml	3
2	Control A, B, C			
3	Control A, B, C			
4	Ethanol 95% A, B, C			
5	Ethanol 75% A, B, C			
6	Oven heating (200 °C) A, B, C			
7	Ultraviolet A, B, C			
8	Autoclave A, B, C			
9	Gamma irradiation A, B, C			

Figure S1 Approximately 40 sandstone vials were prepared. Each vial has a 10 ml capacity and contains around 15.7 g of sandstone

Table S3 list of chemical, rock and deionized water used in this work

Chemicals	**Formula**	**Purity**	**Manufacturer**	**Quantity**
Sandstone	NA	Silica white sand; super fine	Cook Industrial Minerals Pty. Ltd.	650 g
Beef extract (Lab Lemco)	NA	Total Nitrogen=12.4 %w/w Amino Nitrogen= 2.5 %w/w	Oxoid	1g
Tryptone	NA	Total Nitrogen=12.7 %w/w Amino Nitrogen=3.7 %w/w NaCl=0.4 % w/w		10g
D (+) Glucose	NA	≥99.5%	Sigma-Aldrich	5g
Sodium chloride	NaCl	≥99.0%	Sigma-Aldrich	20g
Phenol red	NA	354.38	Sigma-Aldrich	0.018g
Potassium phosphate monobasic	KH_2PO_4	≥99.0%	Sigma-Aldrich	0.4g
Potassium phosphate dibasic	K_2HPO_4	≥99.0%	Sigma-Aldrich	1.23g
DI-water	H_2O	NA	In lab facility	1000ml

Table S4 Chemical composition of culture media APB 1000 ml

Chemicals	Formula	Purity	Manufacturer	Quantity
Beef extract (Lab Lemco)	NA	Total Nitrogen=12.4 %w/w Amino Nitrogen= 2.5 %w/w	Oxoid	1g
Tryptone	NA	Total Nitrogen=12.7 %w/w Amino Nitrogen=3.7 %w/w NaCl=0.4 % w/w		10g
D (+) Glucose	NA	≥99.5%	Sigma-Aldrich	5g
Sodium chloride	NaCl	≥99.0%	Sigma-Aldrich	20g
Phenol red	NA	354.38	Sigma-Aldrich	0.018g
DI-water	H2O	NA	In lab facility	1000ml

Note: the pH of the solution was maintained ~7.48 using caustic soda as a buffer solution

Table S5 Chemical composition of PBS 1000 ml

Chemicals	Formula	Purity/Molecular Weight	Manufacturer	Quantity
Sodium chloride	NaCl	58.49	Chem-Supply	8.1g
Potassium phosphate monobasic	KH_2PO_4	≥99.0%	Sigma-Aldrich	0.4g
Potassium phosphate dibasic	K_2HPO_4	≥99.0%	Sigma-Aldrich	1.23g
DI-water	H_2O	NA	In lab facility	1000ml

Note: the pH of the PBS solution was maintained at ~7 using caustic soda as a buffer solution

Table S6 Weight of 10 ml falcon tube containing 10 ml PBS solution with and without sand

S.no	Date	Technique	Samples	PBS 10 ml without sandstone	PBS 10 ml with sandstone
1	12/12/2023	Control	A	16.59g	17.73g
2	12/12/2023	Control	B	16.60g	17.57g
3	12/12/2023	Control	C	16.62g	17.98g
4	12/12/2023	Ethanol 95%	A	16.58g	17.52g
5	12/12/2023	Ethanol 95%	B	16.58g	17.67g
6	12/12/2023	Ethanol 95%	C	16.68g	17.70g
7	13/12/2023	Control	A	16.34g	17.03g
8	13/12/2023	Control	B	16.36g	17.55g
9	13/12/2023	Control	C	16.35g	17.25g
10	13/12/2023	Ethanol 75%	A	16.65g	17.55g
11	13/12/2023	Ethanol 75%	B	16.63g	17.50g

12	13/12/2023	Ethanol 75%	C	16.70g	17.51g
13	13/12/2023	Oven heating 200°C	A	16.69g	17.41g
14	13/12/2023	Oven heating 200°C	B	16.64g	17.30g
15	13/12/2023	Oven heating 200°C	C	16.68g	17.48g
16	13/12/2023	Ultraviolet	A	16.69g	17.66g
17	13/12/2023	Ultraviolet	B	16.64g	17.81g
18	13/12/2023	Ultraviolet	C	16.63g	18.31g
19	13/12/2023	Autoclave	A	16.61g	17.40g
20	13/12/2023	Autoclave	B	16.66g	17.72g
21	13/12/2023	Autoclave	C	16.70g	17.65g
22	18/12/2023	Control	A	16.48g	17.59g
23	18/12/2023	Control	B	16.46g	17.49g
24	18/12/2023	Control	C	16.43g	17.68
25	18/12/2023	Gamma irradiation	A	16.61g	18.02g
26	18/12/2023	Gamma irradiation	B	16.66g	18.04g
27	18/12/2023	Gamma irradiation	C	16.62g	17.74g

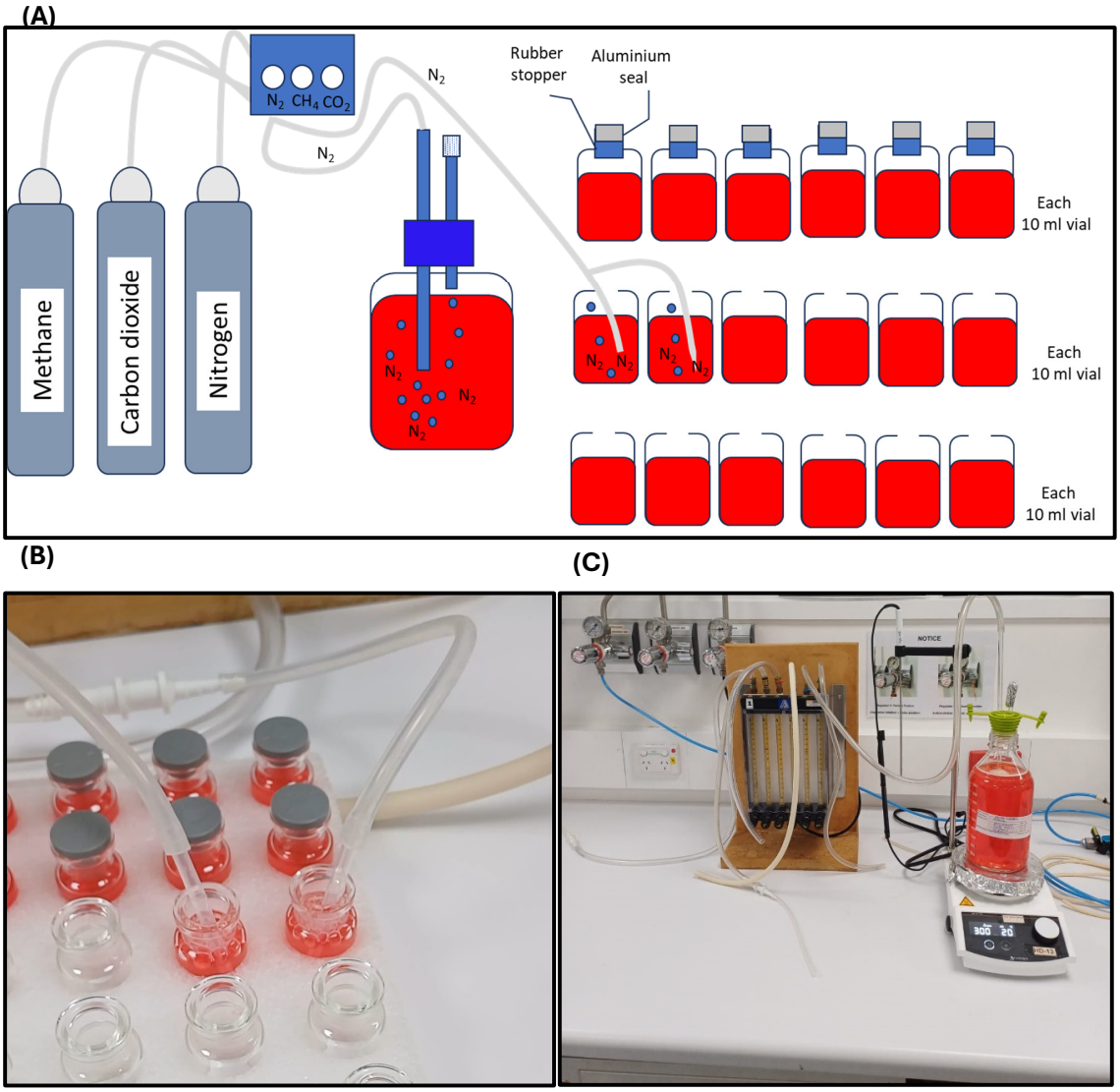


Figure S2 (A) Schematic of the mechanism used for preparation of 10 ml vials containing and deoxygenating them using N_2 gas. (B-C) Figures show the deoxygenating of 10 ml vials and APB stock solution using N_2 gas. All vials were autoclaved for 1h before inoculation of bacteria

- **UV**

The fume hood equipped with an exhaust fan was powered on. Later, 3 vials of sandstone-bacteria-PBS inoculum were put in the fume hood and UV tube was switched on with boarded up windows. UV experiment was conducted for 30 minutes. Each vial was rotated clockwise at ~180° without rubber seal with the interval of 15 mins. We took the sample using the spatula

from core of the vial for the inoculation of APB media to measure the efficiency of UV sterilization.

Ethanol 95 wt% and 75 wt%

Ethanol was used at two different concentrations to sterilize the rock sample. 95% and 75% ethanol solutions were prepared using Ethanol PURE 99.9%. 95 ml of ethane and 5 ml of water were added in 200ml regent bottle to make a solution of Ethanol 95 wt%. Similarly, 75 wt% ethanol was prepared using 75 ml of the pure ethanol and 35 ml of water. There were 6 sandstone-bacteria-PBS vials each containing around 15.7 g of sandstone and 3ml PBS solution. 3 vials were washed 3 times with ethanol 95% and the other 3 vials were similarly washed with 75% ethanol. The ethanol was not completely removed from the vials so that the sandstone may remain soaked for at least 15 min. Finally, approximately 2 g of sandstone is taken from each sandstone-bacteria-PBS vial washed with the two different concentrations and added in falcon tube separately as enlisted in **Table S6**.

- **Oven**

Venticell 111-Eco line oven was used to sterilize the sandstone-bacteria-PBS solution. The oven was switched on and preheated until set temperature was reached which was 200 °C. We put three vials in the oven for approximately 2 hours at a constant 200 °C.

- **Autoclave**

Benchtop autoclave model 3870EL-D was used to sterilize the three vials. We have used liquid cycle mode which means 121 °C temperature and 15 psia pressure conditions. The vials were autoclaved for approximately 1 hour. Further, we have used a liquid cycle to autoclave APB and PBS solution contained in the 500 ml Reagent bottles.

- **Gamma ray irradiation**

Three vials of sandstone-bacteria-PBS inoculum samples to ChemCenter Government of Western Australia at Curtin University Campus for the irradiation of the gamma-ray. The samples were irradiated for at least 32 h.The center is equipped with a gamma irradiation unit named Gammacell 220.

- **Ultrasonication**

The ultrasonication machine named Power Sonic 510, micro process controlled Benchtop ultrasonic cleaner was used to sonicate each 10ml falcon tube for 15sec. Approximately 2g of sand were added to falcon tubes containing 10 mL of PBS. Then, faclcon tubes were sonicated in cycle of ice dipping and sonication. Later, the falcon tube was dipped in the ice for 8sec and both sonication and ice dipping for carried 8 times for each falcon tube.

- **Vortex mixer**

The vortex-Genie® 2, Mo BIO laboratories, Inc used to mix approximately 2 gm of sandstone bacteria in 10 ml of falcon tubing containing 10 ml of PBS solution. A total of 27 falcon tubes were passed through the vortex to achieve a homogenized mixture of the bacteria and sandstone grain particles in the PBS solution to minimize the risk of error.

Serum dilution using 1 ml of PBS solution contaminated with sandstone-bacteria-PBBS mixture exposed to different sterilization techniques. Serum dilution was conducted for each case enlisted in **Table S7**. Serum dilution was conducted until 7 seven concentrations. We have a total of 27 different cases and in each case serum dilution was repeated for 3 times totalling 21 concentrations for each case and 567 for all cases.

Table S7 Total number of vials prepared for the controls and different sterilization tests.

S.no	Technique	Samples	Number of 10 APS vials containing 1 ml of bacteria-sandstone-PBS solution from the falcon tube
1	Control	A	21
2	Control	B	21
3	Control	C	21
4	Ethanol 95%	A	21
5	Ethanol 95%	B	21
6	Ethanol 95%	C	21
7	Control	A	21
8	Control	B	21
9	Control	C	21
10	Ethanol 75%	A	21
11	Ethanol 75%	B	21
12	Ethanol 75%	C	21

13	Oven heating 200°C	A	21
14	Oven heating 200°C	B	21
15	Oven heating 200°C	C	21
16	Ultraviolet	A	21
17	Ultraviolet	B	21
18	Ultraviolet	C	21
19	Autoclave	A	21
20	Autoclave	B	21
21	Autoclave	C	21
22	Control	A	21
23	Control	B	21
24	Control	C	21
25	Gamma irradiation	A	21
26	Gamma irradiation	B	21
27	Gamma irradiation	C	21
Total			567

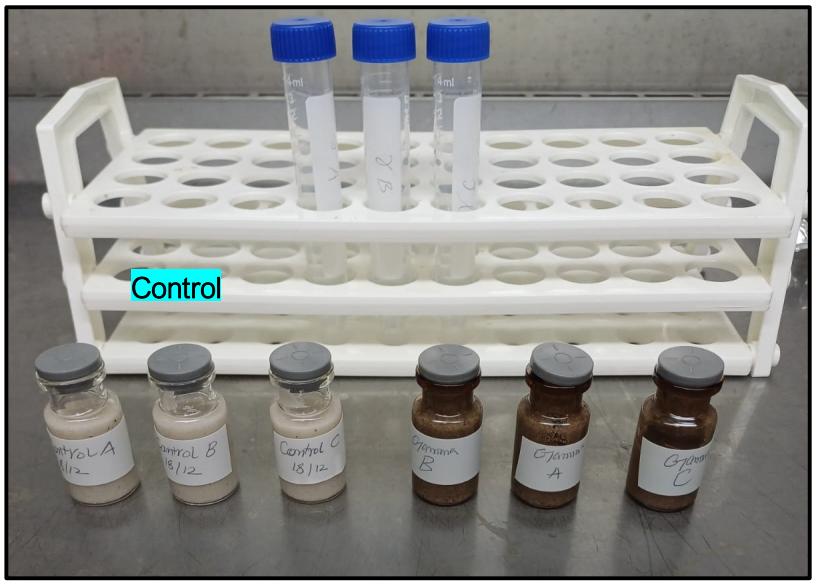
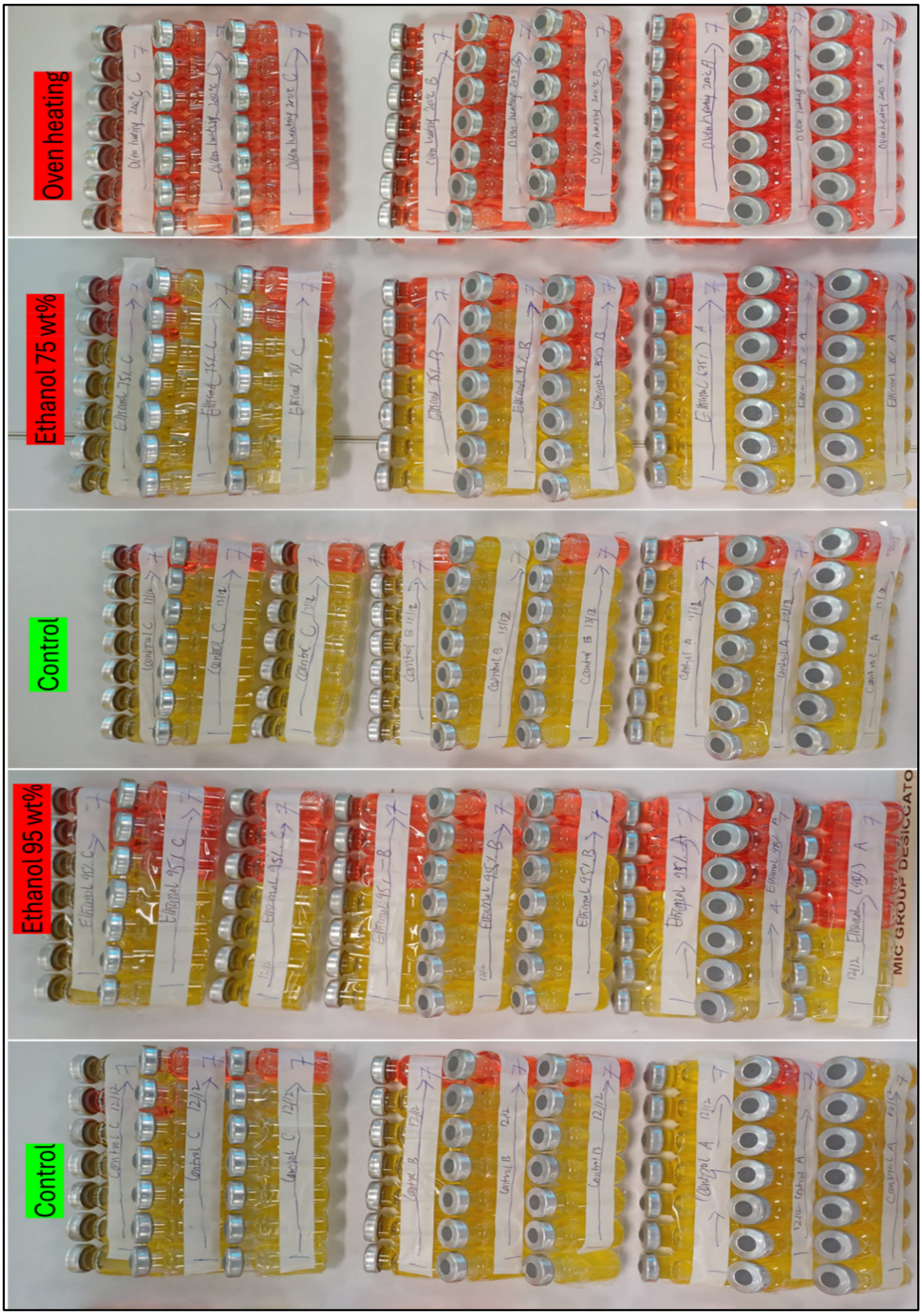


Figure S3 Illustrated difference between color of vials Control A, B, C (left), and Gamma ray A, B, and C (right) samples



132

12

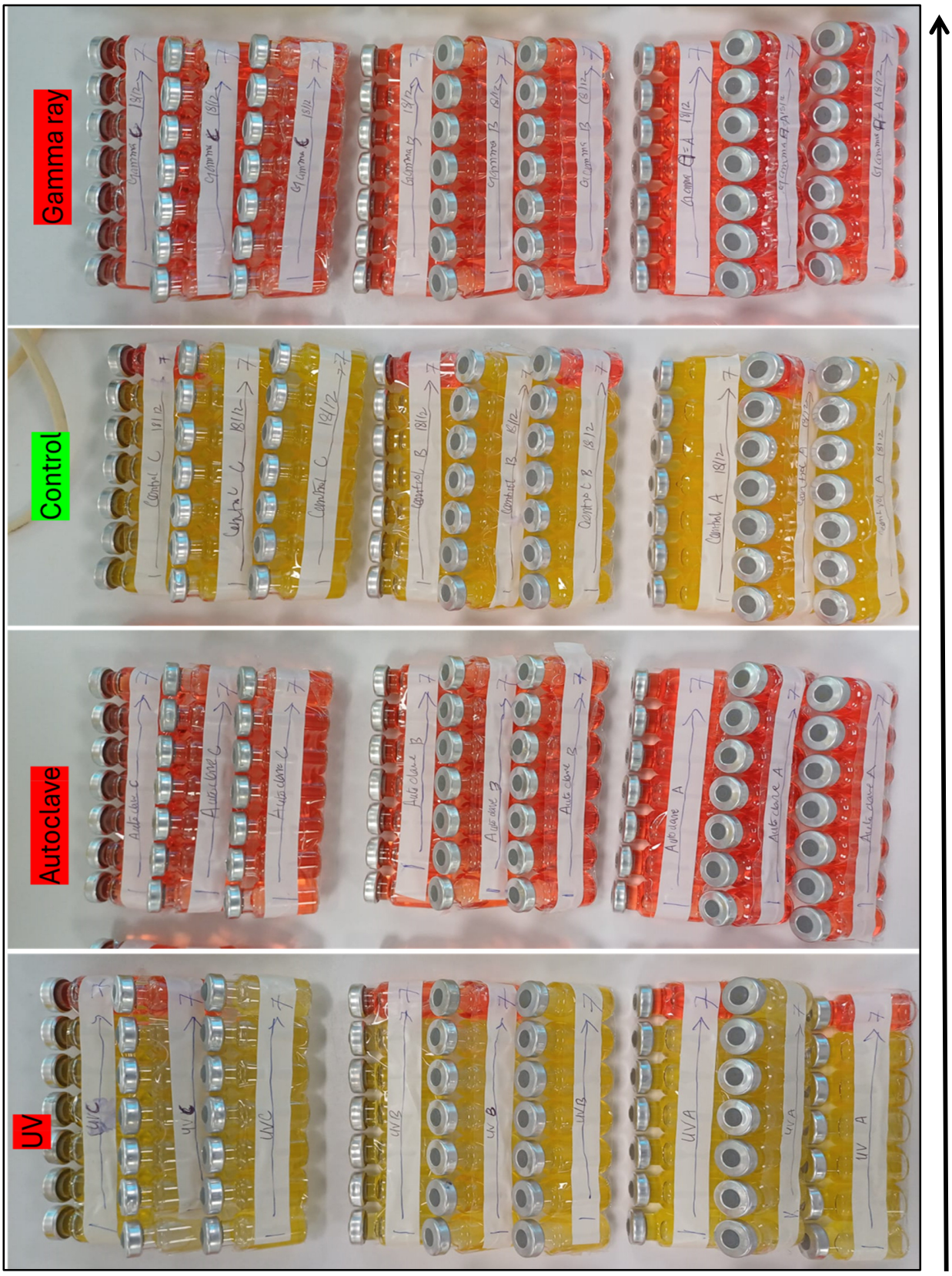

Gamma ray
Control
Autoclave
UV

Figure S4 (A) Control from left ran parallel with Ethanol 95 wt% sterilization. (A-B) Another Control ran for Ethanol 75 wt%, Oven heating experiments, UV, and Autoclave sterilization techniques. (B) The second last Control was run in parallel with Gamma ray. There are 567 vials each 10 ml illustrated in the Figure. We conducted each test at 7 serum concentrations, for example, 10^{-1} to 10^{-7}, and repeated it three times to determine if any deviation occurred in the values.

Note:-Red colour vials show no positive growth and 100% sterilization. Orange colour vials illustrate positive growth of microorganisms in the APB media solution.

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