Bacterial Endospores Reduction Using Various Ultrasonic and Megasonic Frequencies

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Introduction

Elimination of bacterial spores (Microbiological control) are important for various industries such as Medical, Pharmaceutical, Food and others. Bacterial spores can resist various stresses, including heat, pressure, radiation, chemicals, and desiccation (Reddy et al., 2006) and it have the highest resistance to chemicals followed by fungal spores, then non-enveloped viruses, then fungi, then vegetative bacteria, and lastly enveloped viruses in respective order (Ransom, 2006). Bacterial spores are a threat to food safety due to their strong resistance to chemical and physical hurdles (Esty and Meyer, 1922; Heinz and Knorr, 1996).

Crest Ultrasonic cleaning equipment is used to clean various types of contaminants such as oils, greases, particles, polishing compounds, protein, hemoglobin residues and many other contaminants from different components. In this study, Crest cleaning equipment were tested for its ability to kill bacterial endospores. So, this cleaner can be used where microbiological control is a major concern. This study was mainly conducted to check the effectiveness of various Ultrasonic and Megasonic frequencies on reduction of bacterial endospores such as Bacillus Atrophaeus and Geobacillus stearothermophilus. These bacterial endospores are used as a biological indicator to check the effectiveness of the sterilization process in medical, pharmaceutical, food and other industries.

Experimental Procedure

This experiment was carried out with various frequencies such as 40 kHz, 132 kHz, 40/132 kHz, 58/132 kHz and 280 kHz. Two different types of bacterial spores such as Bacillus atrophaeus and Geobacillus Stearothermophilus were studied. These bacterial endospores were used as a biological indicator to check the effectiveness of the sterilization process in medical, pharmaceutical, food and other industries. The medium used for this study was DI water, 1% CC45, 5% CC45 and 5% CC275.

1 ml of Bacillus Atrophaeus or Geobacillus stearothermophilus liquid suspension was added into a test tube containing 9 ml of Tryptic Soy Broth (TSB). A population assay was performed on the inoculum and the results were recorded. The population of Geobacillus Stearothermophilus in the inoculum was not less than $1x10^7$/0.1 ml; therefore, the starting population in each test tube was not less than $1x10^6$ CFU/ml. Directly after inoculation, each test tube was placed into a cleaning tank with a desired frequency and at appropriate time and temperature. The temperature of the cleaning tank and test tube was controlled within 55 $\pm$ 2 °C. Each of the test tubes were mixed thoroughly every 1.5 minutes during the sonication. Directly after the sonication, each sample was diluted by transferring 1ml of sample into a 9 ml of buffer with neutralizing agent for dilution purpose. Each of the samples was plated at various dilution factors in order to obtain plates with approximately 30-300 CFU per plate. The plates were incubated for 48±2 hours at 55 °C. Following incubation, the plates were count and the results were recorded. The spore log reduction for each sample was calculated.

In this cleaning process two flow mechanisms are introduced – cavitational*, and* acoustic streaming*. The former is associated with the implosions of cavitation bubbles and the resulting shockwave that emanates omni-directionally while the latter is associated with the flow of fluid induced by a sound field (vertical streaming). Ultrasonic frequencies are known as radial frequencies. They are based on the relationship between the inside and outside diameters of a circular disc made from PZT material with a hole in the middle. Megasonic frequencies are based on the thickness mode of PZT material. The frequency above 280 kHz exhibit virtually all characteristics of conventional megasonics. Megasonic frequency offers the benefit of a very thin boundary layer over the immersed surface, which effectively exposes even sub-micron and nano-dimensional contaminants to the flow of the cleaning liquid. As the frequency increases, the boundary layer thickness decreases (Mc Queen and Nag et all).*

The uni-directionality and relative gentleness of megasonic fields has historically limited its application in various fields. A new advancement in megasonic technology, termed "Megasonic Sweeping", has alleviated this shortcoming to a large extent, thereby revolutionalizing the field. When the megasonic frequency is swept about ± 4.5 kHz, the acoustic filed becomes essentially more uniform. Even the corners and edges of tanks in systems employing "Megasonic Sweeping" display acoustic pressure levels equivalent to those prevailing at the PZT bonded area. Higher input power can now be deployed without concerns over the "fountain effect" in conventional megasonics, where power is primarily concentrated at the bonded area. This key design improvement suddenly rendered megasonic cleaning more attractive to medical and other industries that had previously shunned it.

Results

Recovery Study for Bacillus Atrophaeus

The recovery results obtained for bacillus atrophaeus bacterial spore is shown in table 1. The recovery is about 97.3 percentage for bacillus atrophaeus bacterial endospores.

Table 1: Recovery results for Bacillus Atrophaeus bacterial endospores

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BEYOND TESTING

CERTIFICATE OF ANALYSIS

Recovery Study:

No.	Spiked Count (CFU/mL)	Recovered Count (CFU/mL)	Recovery, %
1	$9.6x10^7$	$9.5x10^7$	99.0
2	$9.6x10^7$	$9.3x10^7$	96.9
3	$9.6x10^7$	$9.2x10^7$	95.8
4	$8.4x10^4$	$8.4x10^4$	100
5	$8.4x10^4$	$8.1x10^4$	96.4
6	$8.4x10^4$	$8.1x10^4$	96.4
7	$8.9x10^2$	$8.5x10^2$	95.5
8	$8.9x10^2$	$9.0x10^2$	98.9
9	$8.9x10^2$	$8.6x10^2$	96.6
		Average	97.3

Spore Log Reduction for 58/132 kHz compared with 5% CC45 and DI Water for 30 minutes of sonic

The spore log reduction obtained for 58/132 kHz with 5% CC45 compared with DI water is shown in Fig.1. The result indicates that 58/132 kHz frequency able to deliver greater than 3 spore log reduction to a solution of Bacillus Atrophaeus bacterial endospores for 5% CC 45 and 2.77 spore log reduction for DI water. Based on the P value (0.019), there is significant difference between 5% CC45 and DI water in terms of spore log reduction. It seems that sonic with alkaline chemical is helping to improve the spore log reduction.

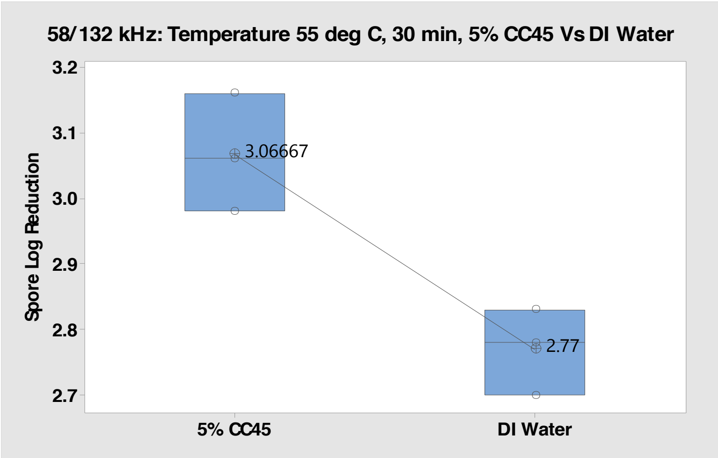

Fig.1 Spore log reduction for 58/132 kHz compared with 5% CC45 and DI water

Two-Sample T-Test and CI: 5% CC45, DI Water

	N	Mean	StDev	SE Mean
5% CC45	*3*	*3.0667*	*0.0902*	*0.052*
DI Water	*3*	*2.7700*	*0.0656*	*0.038*

Difference = μ (5% CC45) - μ (DI Water)

Estimate for difference: 0.2967

95% CI for difference: (0.0918, 0.5015)

T-Test of difference = 0 (vs ≠):

T-Value = 4.61 ***P-Value = 0.019*** *DF = 3*

Spore Log Reduction for 40/132 kHz compared with 5% CC45 and DI water for 45 minutes of sonic

The spore log reduction obtained for 40/132 kHz with 5% CC45 compared with DI water is shown in Fig.2. The result depicts that the spore log reduction is high for 5% CC 45 as compared to DI water. It seems that the alkaline chemical is helping to improve the spore log reduction. The result also indicates that 40/132 kHz frequency able to deliver greater than 3 spore log reduction to a solution of Bacillus Atrophaeus bacterial endospores for 5% CC 45 and 2.9 spore log reduction for DI water. Based on the P value (0.003), there is significant difference between 5% CC45 and DI water in terms of spore log reduction.

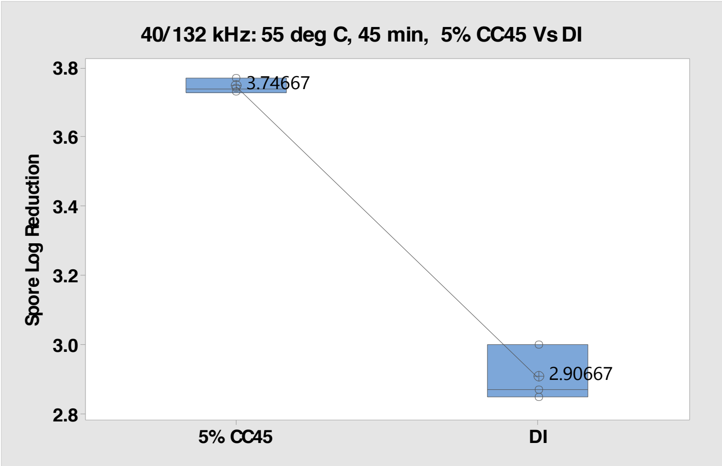

Fig.2 Spore log reduction for 40/132 kHz compared with 5% CC45 and DI water

Two-Sample T-Test and CI: 5% CC45, DI

	N	Mean	StDev	SE Mean
5% CC45	*3*	*3.7467*	*0.0208*	*0.012*
DI Water	*3*	*2.9067*	*0.0814*	*0.047*

Difference = μ (5% CC45) - μ (DI)

Estimate for difference: 0.8400

95% CI for difference: (0.6312, 1.0488)

T-Test of difference = 0 (vs ≠):

T-Value = 17.31 ***P-Value = 0.003*** *DF = 2*

Spore log Reduction for 40/132 kHz with and without 45 minutes of sonic

The spore log reduction obtained for 40/132 kHz with 5% CC275 compared with 5% CC275 soaking is shown in Fig.3. The result indicates that the spore log reduction is high for 40/132 kHz with 5% CC 275 as compared to soaking with 5% CC275 but without any sonic. The result also indicates that 40/132 kHz frequency able to deliver greater than 3 spore log reduction to a solution of Bacillus Atrophaeus bacterial endospores for 5% CC 275 and 2.95 spore log reduction for without any sonic (only soaked in 5% CC275). Based on the P value (0.002), there is significant difference between 40/132 kHz with 5% CC275 and without any sonic (only soaked in 5% CC275) in terms of spore log reduction.

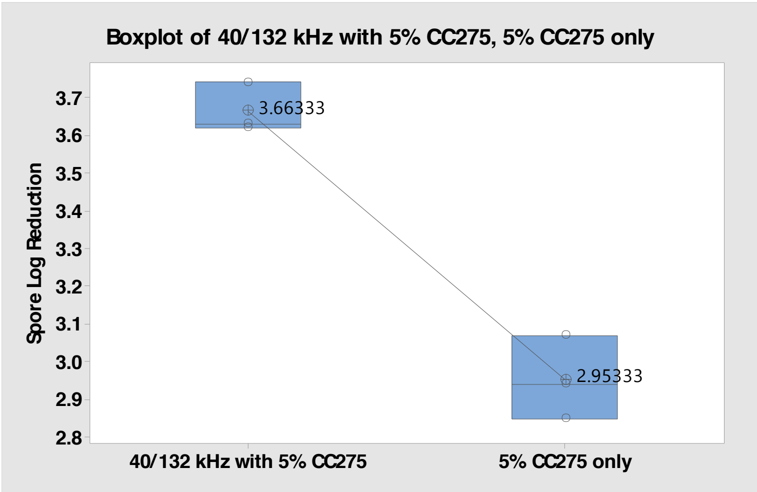

Fig.3 Spore log reduction for 40/132 kHz compared with 5% CC275 with sonic and without sonic

Two-Sample T-Test and CI: 40/132 kHz with 5% CC275, 5% CC275 only

	N	*Mean*	*StDev*	*SE Mean*
40/132 kHz with 5% CC275	*3*	*3.6633*	*0.0666*	*0.038*
5% CC275 only	*3*	*2.953*	*0.111*	*0.064*

Difference = μ (40/132 kHz with 5% CC275) - μ (5% CC275 only)

Estimate for difference: 0.7100

95% CI for difference: (0.4728, 0.9472)

T-Test of difference = 0 (vs ≠): T-Value = 9.53 P-Value = 0.002 DF = 3

Recovery Study for Geobacillus Bacterial Endospores

The recovery results obtained for Geobacillus bacterial endospores is shown in table 2. The recovery is about 90.9 percentage for Geobacillus bacterial endospores.

Table 2: Recovery results for Geobacillus bacterial endospores

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AmCen BEYOND TESTING

CERTIFICATE OF ANALYSIS

6.6.3.Recovery was calculate with the count obtained from spiked samples against the initial pre-determined count.

No.	Spiked Count (CFU/mL)	Initial Count (CFU/mL)	Recovery, %
1	$2.2x10^7$	$2.0x10^7$	90.9
2	$2.2x10^7$	$2.2x10^7$	100.0
3	$2.2x10^7$	$2.1x10^7$	95.5
4	$2.9x10^4$	$2.7x10^4$	93.1
5	$2.9x10^4$	$2.6x10^4$	89.7
6	$2.9x10^4$	$2.5x10^4$	86.2
7	$3.2x10^2$	$2.8x10^2$	87.5
8	$3.2x10^2$	$2.9x10^2$	90.6
9	$3.2x10^2$	$2.7x10^2$	84.4
		Average	90.9

Spore Log Reduction for 1% CC45 Vs 5% CC45 for 30 min Soaking (without any sonic)

From the results, it can be seen that both 1% CC45 and 5% CC45 without any sonic not able to deliver ≥ 3 spore log reduction to a solution of Geobacillus stearothermophilus bacterial endospores. It depicts that sonic is paramount important to kill bacterial endospores.

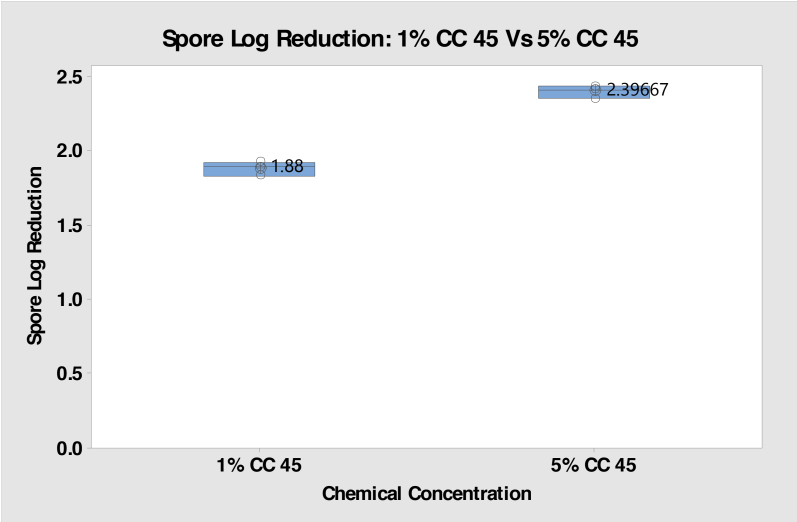

Fig.4 Spore log reduction for 1% CC45 and 5% CC45 without any sonic

Spore Log Reduction for Various Frequencies with 1% CC45 and 30 minutes of sonic

From the results, it can be seen that 58/132 kHz able to deliver 5.1 spore log reduction, 40/132 kHz able to deliver 3.31 spore log reduction, 280 kHz able to deliver 2.89 log reduction, 132 kHz able to deliver 2.69 log reduction and 40 kHz able to deliver only 1.24 log reduction to a solution of Geobacillus stearothermophilus bacterial endospores with 30 minutes of sonic with 1% CC45. The spore log reduction obtained for direct bonded dual frequencies such as 58/132 kHz and 40/132 kHz are higher as compared to other single frequencies tested for the given test conditions.

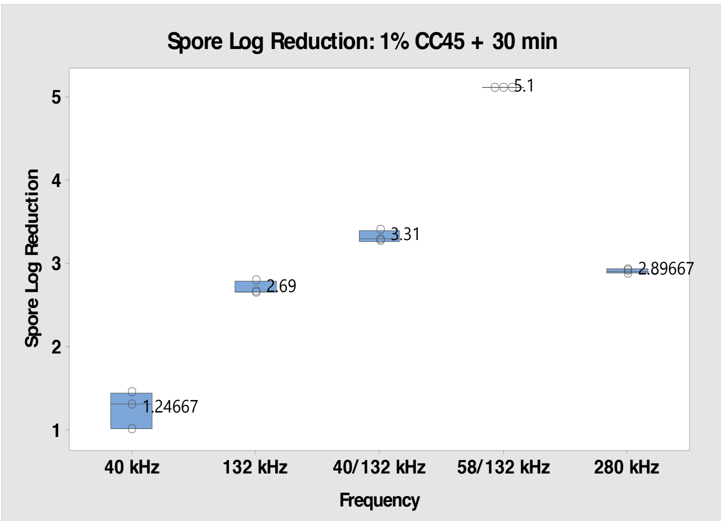

Fig.5 Spore log reduction for various frequencies with 1% CC45 with 30 minutes of sonic

Spore Log Reduction for Various Frequencies with 1% CC45 and 45 Minutes of Sonic

From the results, it can be seen that 58/132 kHz able to deliver 5.1 spore log reduction, 40/132 kHz able to deliver 3.23 spore log reduction, 280 kHz able to deliver 3.04 log reduction, 132 kHz able to deliver 2.69 log reduction and 40 kHz able to deliver only 2.25 log reduction to a solution of Geobacillus stearothermophilus bacterial endospores with 45 minutes of sonic with 1% CC45. The spore log reduction obtained for direct bonded dual frequencies such as 58/132 kHz and 40/132 kHz are higher as compared to other single frequencies tested for the given test conditions.

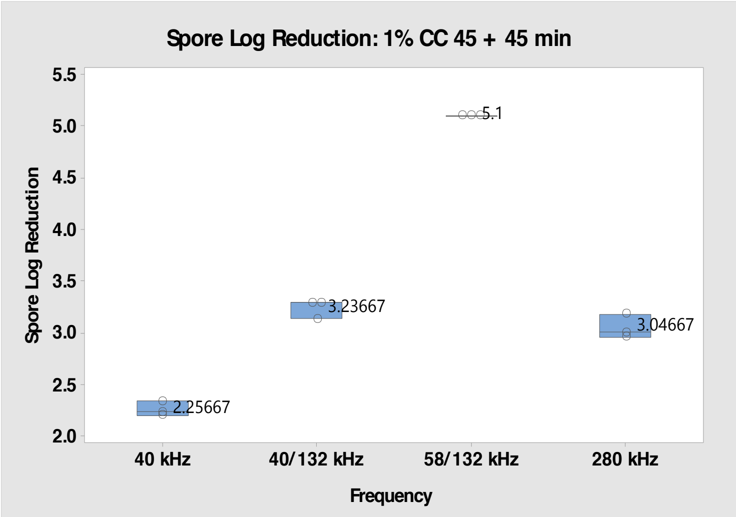

Fig.6 Spore log reduction for various frequencies with 1% CC45 with 45 minutes of sonic

Spore Log Reduction for Various Frequencies with DI Water for 30 Minutes of Sonic

From the results, it can be seen that 58/132 kHz able to deliver 5.1 spore log reduction, 40/132 kHz able to deliver 3.19 spore log reduction, 280 kHz able to deliver 2.52 log reduction and 40 kHz able to deliver only 0.83 log reduction to a solution of Geobacillus stearothermophilus bacterial endospores with 30 minutes of sonic with DI water. The spore log reduction is higher for dual frequencies such as 58/132 kHz, 40/132 kHz and lower for 40 kHz. It depicts that even with DI water 58/132 kHz and 40/132 kHz frequencies able to kill the spores effectively.

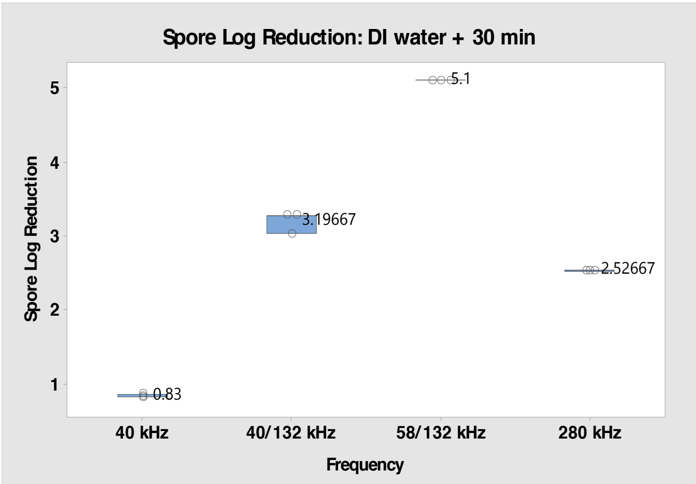

Fig.7 Spore log reduction for various frequencies with DI water with 30 minutes of sonic

Spore Log Reduction for Various Frequencies with DI Water and 45 minutes of sonic

From the results, it can be seen that 58/132 kHz able to deliver 5.1 spore log reduction, 40/132 kHz able to deliver 3.19 spore log reduction and 40 kHz able to deliver only 2.18 log reduction to a solution of Geobacillus stearothermophilus bacterial endospores with 45 minutes of sonic with DI water. The spore log reduction is higher for dual frequencies such as 58/132 kHz, 40/132 kHz and lower for 40 kHz. It depicts that even with DI water 58/132 kHz and 40/132 kHz frequencies able to kill the spores effectively.

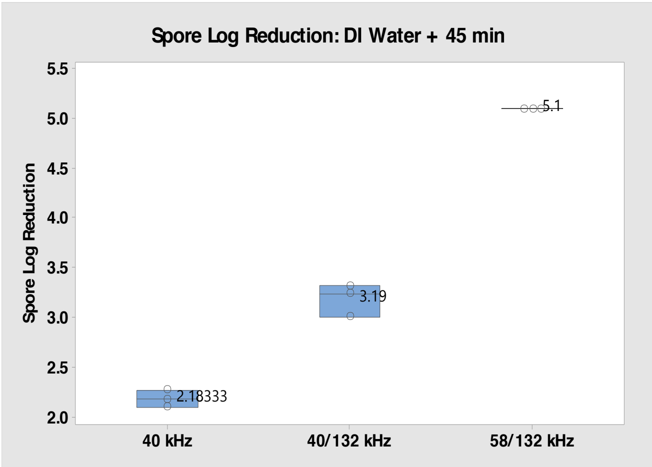

Fig.8 Spore log reduction for various frequencies with DI water with 45 minutes of sonic

Conclusions

Bacillus Atrophaeus

- *Ultrasonic and Megasonic frequencies able to kill bacterial endospores effectively but the effectiveness of killing varies with the frequencies.*
- *58/132 kHz and 40/132 kHz able to deliver > 3 spore log reduction to a solution of Bacillus Atrophaeus bacterial endospores for 5% CC 45 and 5% CC 275.*
- *5% CC275 without sonic able to deliver 3 spore log reduction to a solution of Bacillus Atrophaeus bacterial endospores.*

Geobacillus Stearothermophilus

- *≥ 3 spore log reduction were achieved for 40/132 kHz and ≥ 5 spore log reduction were achieved for 58/132 kHz with DI water and 1% CC45 chemical at 30 minutes of sonication for the Geobacillus Stearothermophilus bacterial endospores tested.*
- *≥ 3 spore log reduction were achieved for 280 kHz with 1% CC45 and 45 minutes of sonication.*
- *≥ 3 spore log reduction were not achieved for 40 kHz and 132 kHz with 1% CC45.*
- *≥ 3 spore log reduction were not achieved for both 1% concentration and 5% concentration of CC45 without any sonic.*
- *Bacterial endospore killing is higher for dual frequencies such as 58/132 kHz, 40/132 kHz as compared to 40 kHz and 132 kHz.*

- *The result depicts that frequency effect is more dominant than the chemical effect to kill the bacterial endospores.*
- *Industries such as Medical, Pharmaceutical, Food Processing Industries and other industries should make use of these frequencies where microbiological control is a major concern.*
- *Introducing ultrasonic cleaning process prior to packaging/sterilization can improve the microbiological control.*