Ultrasonics Sonochemistry 21 (2014) 189–199

Contents lists available at SciVerse ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Studies to control biofilm formation by coupling ultrasonication of natural waters and anodization of titanium

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ARTICLE INFO

Article history:
Received 4 December 2012
Received in revised form 11 June 2013
Accepted 11 June 2013
Available online 29 June 2013

Keywords:
Biofilm control
Titanium
Anodization
Ultrasonication
Natural waters

ABSTRACT

The main objective of this study was to investigate the combined effect of ultrasonication of natural waters and anodization of titanium on microbial density and biofilm formation tendency on titanium surfaces. Application of 24 kHz, 400 W high power ultrasound through a 14 mm horn type SS (stainless steel) Sonicator with medium amplitude of 60% for 30 min brought about three order decrease in total bacterial density of laboratory tap water, cooling tower water and reservoir water and two order decrease in seawater. Studies on the effect of ultrasonication on dilute pure cultures of Gram-negative and Gram-positive bacteria showed five order and three order decrease for *Pseudomonas* sp. and *Flavobacterium* sp. respectively and two order and less than one order decrease for *Bacillus* sp. and *Micrococcus* sp. respectively. Ultrasonication increased lag phase and reduced logarithmic population increase and specific growth rate of Gram-negative bacteria whereas for Gram-positive bacteria specific growth rate increased. Studies on the biofilm formation tendency of these ultrasonicated mediums on titanium surface showed one order reduction under all conditions. Detailed biofilm imaging by advanced microscopic techniques like AFM, SEM and epifluorescence microscopy clearly visualized the lysed/damaged cells and membrane perforations due to ultrasonication. Combination of ultrasonication and anodization brought about maximum decrease in bacterial density and biofilm formation with greater than two order decrease in seawater, two order decrease in *Bacillus* sp. culture and more than four order decrease in *Flavobacterium* sp. culture establishing the synergistic effect of anodization and ultrasonication in this study.

1. Introduction

A wide range of materials and industries are affected by the serious problem of microbial fouling of engineered surfaces that come into contact with natural water. This is due to the formation of biofilm consisting of aquatic bacteria, algae and other microorganisms [1]. Biofouling is the term used to describe the undesired development of microbial growth on the surface in contact with natural waters which results in unsatisfactory performance or reduced lifetime of equipment. Biofilms cause fouling of heat exchanger/condenser tubes, pipelines, water boxes etc. in cooling water system (CWS) of several industries [2]. Among many metals and alloys used as condenser materials in CWS, titanium possesses outstanding corrosion resistance in several aggressive environments. With all its technical superiority titanium is the selected condenser material for 500 MWe fast breeder reactor (FBR), whose construction is underway at Kalpakkam, India. However due to its biocompatibility, titanium is prone to intense biofouling. Fouling control strategies in condensers include a combination of mechanical and chemical treatments [3,4], which include chlorination, sponge ball cleaning, backwashing etc. Although these cleaning methods are efficient in case of macrofoulants [5], they do not guarantee complete removal of microfoulants, such as bacteria and unicellular algae. It is also reported that sponge ball cleaning enhances subsequent biofilm formation [6,7]. Chemical treatments release toxic byproducts into environment [8] and this is a concern for the environmentalists. Thus active research for green environmentally friendly techniques has come up with the possibility of using a non-conventional strategy of ultrasonication. Ultrasonication is capable of inactivating waterborne bacteria either alone or in combination with other technologies [9]. Ultrasound is cyclic sound pressure with a frequency greater than the upper limit of human hearing. If ultrasonic waves pass through a liquid, an acoustic pressure (Pa) is produced with sufficient amplitude. This Pa induces cavitation bubbles, and when these bubbles collapse, high pressures (up to 100 M Pa) and high temperatures (up to 5000 K) are momentarily produced [10]. This can disrupt cell membranes and damage cell wall structures [11].

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http://dx.doi.org/10.1016/j.ultsonch.2013.06.010

In the recent past several researchers have looked into the use of ultrasound for killing microbes. Joyce et al. [12] showed that ultrasound is able to inactivate bacteria and deagglomerate bacterial clusters and flocs. Cameron et al. [13] used ultrasound as an alternative to heat-pasteurization. An optimal removal procedure based on sonication for analysis of biofilm parameters such as total and active bacterial counts by epifluorescence microscopy and total organic content as estimated by total proteins determination and chemical oxygen demand measurements was developed by Pierzo et al. [14]. Ultrasound is currently employed in a range of industries, such as surface cleaning, medical scanning ultrasonic therapy, food and beverage technology, nanotechnology, mineral processing, industrial welding, non-destructive testing and environmental decontamination applications [15,16]. However, systematic studies on effect of ultrasonication on different cooling waters and on their biofilm formation tendencies on condenser material are lacking. Thus a systematic investigation was carried out to study the effect of ultrasonication on bacterial density in fresh water and seawater used as cooling water for reactors in Kalpakkam, India and their biofilm formation tendency on titanium surface. Studies also involved ultrasonication of dilute pure culture suspensions of chief biofilm formers in these environments. Many workers recommend use of ultrasonication along with other biocidal treatments such as bactericide [17] or UV technique [18] for better efficiency and economically viable water treatment technique. Hulsmann et al. [19] has also reported that the energy consumption of ultrasound equipment is large, and hence they recommended the application of ultrasound combined with other water treatment programmes (chlorination, ozonation etc.). Earlier studies in our laboratory have shown that anodization of titanium in orthophosphoric acid resulted in reduction of microbial adhesion [20]. However no one has studied the combined effects of ultrasonication and anodization on biofilm control on titanium surface. Thus the main objective of this study was to investigate for the first time the combined effect of ultrasonication of natural waters and anodization of titanium on microbial density and biofilm formation tendency on titanium surfaces.

2. Materials and methods

2.1. Ultrasonicator set up

A horn type sonicator (UP 400S, Dr. Hielscher GmbH) operating at a fixed frequency of 24 kHz and a nominal power output up to 400 W was used (Fig. 1). Water sample of 200 ml was placed in a glass container and subject to ultrasound irradiation emitted through a 14 mm diameter tip at maximum nominal power. A medium intensity sonication was provided by keeping the amplitude at 60%. In order to minimize contamination of the sample, the horn was treated with ethyl alcohol. The water level inside the container was six centimetre and the horn was positioned in the middle of the container, with its tip two centimetre from the bottom. A temperature probe was also dipped into the medium.

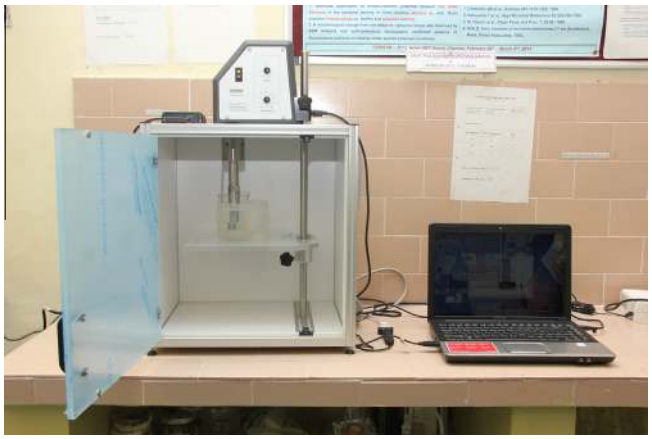


Fig. 1. Experimental set up with Heilscher UP 400S ultrasonication system.

2.2. Ultrasonication procedure

The ultrasonication procedure was finalized by experiments carried out with laboratory tap water sample. The relevance of this water is that this is used for laboratory static exposures of specimens to evaluate corrosion and biofouling. Initially tap water was ultrasonicated continuously for 30 min. This increased the temperature up to 60 °C and hence by keeping ice cubes surrounding the glass container the temperature was maintained at 40 °C ± 1 °C. The temperature and time was monitored by UPS control software. The bacterial density in the ultrasonicated and non-ultrasonicated water sample henceforth referred to as "control water samples" was estimated. For the tap water sample the bacterial density was estimated at different intervals of time (every 10 min) by total viable count technique [21] for approximate evaluation of the suitability of time. The water samples (1 ml) were diluted in 9 ml buffer (0.0425 g KH_2PO_4, 0.19 g $MgCl_2$ per litre, pH 7.2 ± 0.5) and 0.1 ml of these dilutions were dispensed in sterile petriplates and appropriate molten agar nutrient mediums (nutrient agar for fresh water and seawater agar for seawater) were added to the plates for uniform distribution of cells in the medium. Once the agar is solidified, the petriplates were incubated in inverted position at 35 °C in B.O.D. incubator for 48 to 72 h. Number of colonies in the plate is counted and this is considered as colony forming units (cfu). The density of bacteria is given as total viable count (TVC) of bacteria and expressed as number of colony forming units (cfu) per ml of sample.

2.3. Studies on effect of ultrasonication on different water samples

2.3.1. Cooling tower water

A pilot scale cooling tower for dynamic exposure studies is setup in our laboratory. It consists of a 100 L cooling water storage basin where the laboratory tap water (used for ultrasonication procedure finalizing studies) is stored and a facility to circulate the water through a heat exchanger and a draft cooling tower.

2.3.2. Reservoir water

Open reservoir water near Madras Atomic Power Station (MAPS) is used as an intermediate storage facility for ground water collected from Palar river bed through infiltration galleries located 20 km away from Kalpakkam. The reservoir which has a storage capacity of 1.7 hectares and depth of 2.28 m at overflow level has a storage capacity of 28,400 m^3. This water is used as feed water in demineralization plant in MAPS as well as in the fast breeder test reactor (FBTR) and as condenser cooling water for the FBTR through an induced draft cooling tower.

2.3.3. Seawater

The seawater is the Kalpakkam coastal water which is used as the cooling water in Madras Atomic Power Station (MAPS). This will be used as the cooling water in the Fast Breeder Reactor coming up at Kalpakkam too.

These three types of water samples (200 ml each) were ultrasonicated for 30 min. The bacterial density (TVC) in ultrasonicated and control water samples was quantified [21] as explained above.

2.4. Studies on effect of ultrasonication on pure bacterial cultures

Two Gram-positive and two Gram-negative bacteria were selected for this study. The microbicidal properties of ultrasonication were first evaluated using cultures of a Gram-negative bacterium, *Pseudomonas* sp., and a Gram-positive bacterium, *Micrococcus* sp. The reason for the selection of above genera was that they were identified as the major colonizers of the biofilms formed in the freshwater reservoir at Kalpakkam [22]. Characterization and identification of these two bacteria up to genus level was carried out in our laboratory which is given in Table 1. Another Gram-positive bacterium selected for the study was *Bacillus flexus*. This bacterium is a novel manganese oxidizing bacterium isolated in our laboratory from steel scraps in IGCAR stores, Kalpakkam and identified by 16S rRNA technique and its corrosion characteristics were reported elsewhere [23]. The second Gram-negative bacteria selected for the study was *Flavobacterium aquatile*. *Flavobacterium* sp. also occurs frequently in the biofilms of these cooling waters. For this study pure cultures of *F. aquatile* (MTCC No: 7307) was brought from microbial type collection centre (MTCC) of Institute of Microbial Technology, Chandigarh. All these bacteria were cultured in 10% (dilute) nutrient broth (Peptic digest – 5 gm/L, NaCl – 5 gm/L, Beef Extract – 1.5 gm/L, Yeast extract – 1.5 gm/L; pH 7.4 ± 0.2) to simulate natural environments and ultrasonicated for 30 min. The bacterial density in ultrasonicated and control cultures was quantified by pour plate technique [21].

2.5. Studies on effect of ultrasonication on Bacterial growth curves

To study the effect of ultrasonication on the growth curve of a Gram-positive *Bacillus* sp. and Gram-negative *Pseudomonas* sp. density of bacteria growth was monitored for 24 h. Two sets of autoclaved 200 ml nutrient broth (100%) was prepared and inoculated by 1 ml of stock bacterial culture and incubated in orbital shaker at 35 °C for 24 h. After estimating the density of bacteria (TVC) in both the sets, one set is ultrasonicated for 30 min. Then 1 ml of culture from both ultrasonicated and control set was reinoculated into 200 ml nutrient broth (100%) and growth curve was followed by TVC estimation every 2 h for 24 h. The growth curve is fitted by the Gompertz modified equation [24].

$$\mathrm{Log}N = \log N_0 + A\exp[-\exp(\mu e/A(L-t)) + 1] \quad (1)$$

where Log N is the decimal logarithm of the number of bacteria counts at time t and log N_0 is the count after 8 h of ultrasonication, μ is the specific growth rate and L is the lag phase duration in h, A is the logarithmic population increase (difference between the upper asymptote ($\log N$ when the t tends to infinity) and the initial counts) and e is the Euler number (2.7182 approx.). Data was fitted to modified Gompertz equation by nonlinear regression (using Marquardt algorithm) with OriginPro® version 8.0 software (Originlab Corporation Northampton, MA). The fitting and the accuracy of the estimations was evaluated from determination coefficient (R^2) [25].

Table 1
Morphological and biochemical characteristics

Organism	*Pseudomonas* sp.	*Micrococcus* sp.
Gram reaction	Gram negative	Gram positive
Morphology	Very small rods	Cocci in tetrads
Pigments	Green fluorescent colony, pigment diffuses into the media	Cream to yellow smooth colonies
Motility	+	–
Catalase	+	+
Oxidase	+	+
Specific reactions		
Anaerobic glucose fermentation	–	–
Nitrate reduction	+	–
Citrate utilization	+	NR
Growth on cetrimide agar	+	NR
Indole production	+	NR

2.6. Studies on the effect of ultrasonication on biofilm formation

The material used for biofilm formation was commercially pure (CP) grade 2 titanium which is the proposed condenser material of FBR coming up at Kalpakkam, India. The nominal composition of the material is given in the Table 2. The specimens were prepared by cutting titanium sheets into medium sized coupons (28 mm × 22 mm × 2 mm). Prior to the experiments, the as-received specimens were pickled in an acid bath (HNO_3 400 g/L + HF 40 g/L) to remove the naturally formed oxide layers and surface stains. Then they were mechanically ground up to 1000 grit with silicon carbide paper, washed thoroughly in detergent using ultrasonication bath for 5–10 min and degreased in acetone, wiped dry and kept in desiccator till exposure studies.

The ultrasonicated (30 min) and control mediums of reservoir water, seawater and dilute cultures of *Pseudomonas* sp., *Micrococcus* sp., *B. flexus* and *Flavobacterium aquatile*, were used for exposure studies. Titanium specimens acid pickled and polished were exposed in these mediums for 24 h and withdrawn for various post exposure analysis.

2.7. Studies on the effect of ultrasonication coupled with anodization on biofilm formation

The acid pickled and polished CP grade 2 Titanium specimens (28 mm × 22 mm × 2 mm) are anodized in 6.6% orthophosphoric acid at 30 volts for 1 h. After anodization, specimens were washed thoroughly by ultrasonication for 5–10 min, dried and kept in desiccator till exposure studies.

The ultrasonicated (30 min) and control mediums like seawater and dilute cultures of *B. flexus* and *F. aquatile*, were used for exposure studies. Titanium specimens acid pickled, polished and anodized were exposed in these mediums for 24 h and withdrawn for various post exposure analysis. During 24 h exposure the specimens in the medium in the conical flask were illuminated by six black light blue (BLB) lamps (4 W Philips) emitting near-UV light (350–380 nm) arranged in a hexagonal configuration surrounding the conical flask [26].

2.8. Post exposure analysis

After exposure of acid pickled, polished Ti specimens and acid pickled, polished and anodized Ti specimens to the ultrasonicated and control mediums, they were withdrawn at definite intervals for biofilm characterization studies using culture techniques (TVC count), Epifluorescence microscopic techniques (Live and Dead staining), scanning electron microscopy (SEM) and atomic force microscopy (AFM).

2.8.1. Total viable count techniques for specimens

Specimens removed from the medium are gently washed with sterile water to remove loosely adhering cells. Then the biofilms were dispersed into 15 ml of sterile phosphate buffer (0.0425 g KH_2PO_4, 0.19 g $MgCl_2$ per litre, pH 7.2 ± 0.5) by ultrasonic cleaning in a ultrasonic bath for 5 min. The length of sonication for optimum recovery of cells was found to be 5 min. The ultrasonically cleaned

Table 2
The nominal composition of CP titanium grade II

Element	C	Fe	N	O	H	Ti
Wt %	<0.1	<0.3	<0.03	0.25	0.12	Bal

surfaces were stained and observed to ensure complete recovery of cells. Serial dilutions of the bacterial cell suspension were prepared and 0.1 ml were plated onto nutrient agar/seawater agar from Hi Media M001/M592. The plates were incubated for 24–48 h at 32 °C and total viable count (TVC) was estimated [21] and expressed as cfu/cm^2 by dividing the bacterial counts with area of specimen. Three replicates were analyzed for each condition and statistical analysis were carried out using MYSTAT software.

2.8.2. Epifluorescence microscopic techniques

Two specimens from each exposure conditions were used for direct microscopic observation using epifluorescence microscopy. Specimens after gently washing with sterile water were air dried in a sterile chamber and the surface is flooded with 0.1% acridine orange (AO) in distilled water. After 2 min the excess stain is drained off, washed and air-dried and observed. Acridine orange, a fluorescent dye, differentially stains single stranded RNA and double stranded DNA, fluorescing orange when intercalated with former and green while complexing with the latter [27] when observed under a Nikon Eclipse E600 epifluorescence microscope (excitation filter BP 490; barrier filter O515). Thus the number of orange fluorescing cells with high density of RNA depicts actively metabolizing cells and green fluorescing cells with only DNA depicts inactivation.

2.8.3. Live and dead cells staining

Microorganisms (live and dead cells) on the surfaces of titanium were determined [28] using the live and dead® BacLight™ bacterial viability stain (Invitrogen, Germany). BacLight is composed of two nucleic acid binding stains. SYTO®9 green fluorescent nucleic acid stain and propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged or porated membranes and the combination of two stains produces red fluorescing cells [29]. Biofilms on titanium surfaces were stained with this dye mixture, incubated for 10–15 min in dark and then images were captured by Nikon Eclipse E600 Epifluorescence microscope with a blue filter at an excitation of 475 nm. In this method green fluorescence indicates living cells and red fluorescence indicates damaged or dead cells.

2.8.4. Scanning electron microscopy (SEM) analysis

The exposed specimens were cleaned with sterile water to remove all loose attachments. Then they were fixed with 0.25% glutaraldehyde at 16 °C for overnight and then dehydrated with a series of ethanol–water combinations (20–100%) and stored in vacuum desiccators. The biofilm morphology was observed with SEM (XL30 ESEM M/s Philips) after gold coating at magnifications ranging from 100 to 10,000× operated at an accelerating voltage of 15–30 kV.

2.8.5. Atomic force microscopic (AFM) study

An NT-MDT (Molecular Device and Tools for Nano Technology) make AFM (Solver ProEC, Russia) operated in a contact mode and NOVA image analysis software was used to investigate the morphological damage to bacterial cells in the biofilms on titanium specimens.

3. Results

3.1. Effect of Ultrasonication on different water samples and pure bacterial cultures

The bacterial density in the tap water was evaluated every 10 min during ultrasonication and the results compared with control and given in Table 3. The bacterial density as observed from the number of colony forming units (cfu/ml) showed three order reduction in bacterial density after 30 min of ultrasonication. The results on the studies of effect of ultrasonication on the bacterial density in different water samples like cooling tower waters and natural waters like reservoir water, seawater are summarized in Fig. 2. There was a three order decrease in cooling tower water and in reservoir water and two order decrease in seawater.

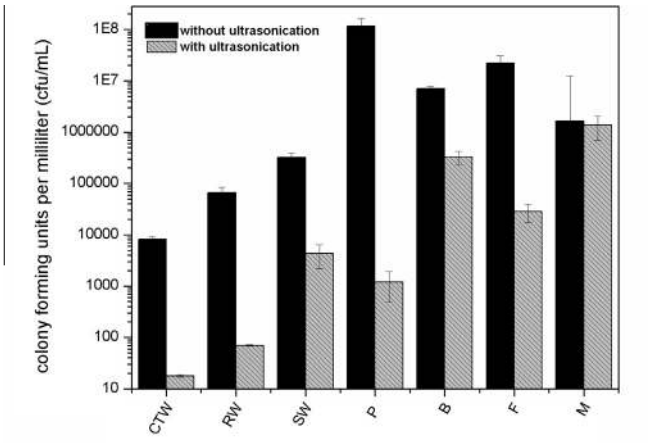
The bacterial density of pure cultures represented as colony forming units per millilitre (cfu/mL) before and after ultrasonic treatment is also given in Fig. 2. The Gram-negative bacteria *Pseudomonas* sp. and *F. aquatile* showed a five order and three order decrease respectively, whereas Gram-positive bacteria *B. flexus* and *Micrococcus* sp. showed a two order and less than one order decrease respectively.

3.2. Studies on effect of ultrasonication on bacterial growth curves

The bacterial growth curve fitted by Gompertz modified equation of ultrasonicated and control *Pseudomonas* sp. culture and *Bacillus* sp. as shown in Figs. 3 and 4 respectively. Table 4 shows the Gompertz parameters (μ, L, A) and determination coefficient (R^2) of the adjusted modified Gompertz model to bacterial growth curves of ultrasonicated and control cultures of *Bacillus* sp. and *Pseudomonas* sp. High determination coefficient (R^2 = 0.91902–0.99788) indicated good fitting of experimental data to Gompertz model. Results obtained from fitting Gompertz equation to experimental data indicated that ultrasonication distinctly increased lag phase (L) and decreased logarithmic population increase (A) for both *Pseudomonas* sp. and *Bacillus* sp. However distinct decrease of specific growth rate (μ) was observed only for *Pseudomonas* sp. Contrarily, the specific growth rate of *Bacillus* sp. increased after ultrasonication.

Table 3
Results of ultrasonication treatment on tap water.

Tap water Ultrasonicated time in minutes)	cfu/mL
Without ultrasonication	$7.0 \pm 0.5 \times 10^4$
10 min	$2.0 \pm 0.11 \times 10^3$
20 min	$3.2 \pm 0.20 \times 10^2$
30 min	$2.0 \pm 0.20 \times 10^1$



(Note: CTW- Cooling Tower Water; RW – Reservoir Water; SW- Sea Water; P-*Pseudomonas* sp.; B-*Bacillus* sp.; F-*Flavobacterium aquatile*; M-*Micrococcus* sp.)

Fig. 2. Effect of ultrasonication on the bacterial density (cfu/ml) in different water samples like chlorinated cooling tower waters and natural waters like reservoir water and seawater compared with control mediums.

3.3. Effect of ultrasonication on biofilm formation

The epifluorescence micrographs of 24 h biofilms developed on acid pickled and polished Ti specimens exposed to both control and ultrasonicated mediums like reservoir water, seawater, and pure cultures of Gram-negative and Gram-positive bacteria is shown in Fig. 5(i) and (ii). The TVC is expressed as cfu/cm^2 and given as text box over the images. The micrographs of biofilms and the counts of bacteria in the biofilms on titanium specimens clearly showed reduction under exposure to ultrasonicated mediums. The bacterial cell damage by ultrasonication treatment was visualized by AFM images of biofilms of *Bacillus* sp. (Fig. 6(i)) and *Flavobacterium* sp. (Fig. 6(ii)). The SEM images (Fig. 7), of the biofilms of the *F. aquatile* cells on titanium exposed to ultrasonicated culture showed punctured cell wall. AFM and SEM confirmed the reduction of density of bacterial cells on Ti specimens exposed to ultrasonicated mediums. The images taken after LIVE/DEAD Bac-light staining technique is shown in Fig. 8 where in the control conditions cells normally fluoresce green due to penetration of only SYTO 9 and under ultrasonication cells are fluorescing red as propidium iodide also penetrated damaged membranes along with SYTO 9.

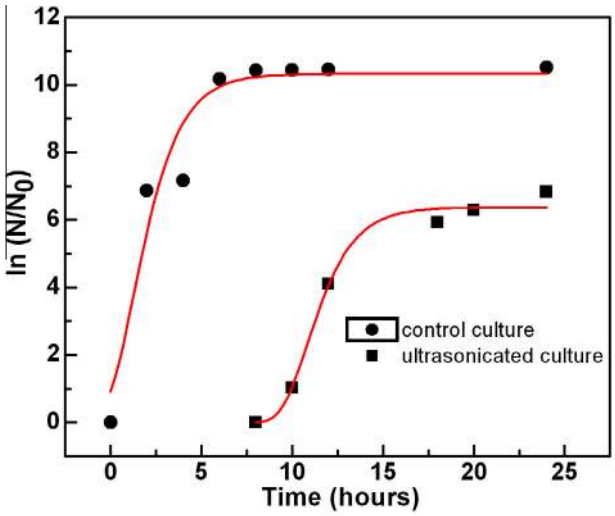


Fig. 3. The bacterial growth curve fitted by Gompertz modified equation of ultrasonicated and control *Pseudomonas* sp. culture. Continuous lines represent the fitting of the modified Gompertz equation.

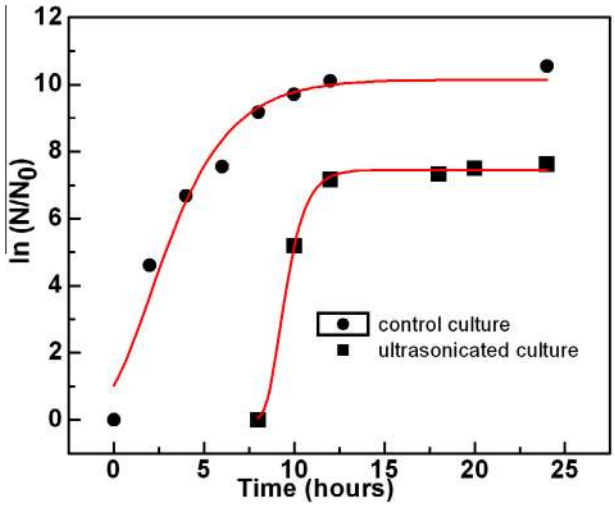


Fig. 4. The bacterial growth curve fitted by Gompertz modified equation of ultrasonicated and control *Bacillus* sp. culture. Continuous lines represent the fitting of the modified Gompertz equation.

3.4. Effect of ultrasonication coupled with anodization on biofilm formation

Combined effect of ultrasonication and anodization on biofilm bacterial density on titanium specimens exposed to cultures of Gram positive *B. flexus*, Gram negative *Flavobacterium aquatile* and seawater is shown in Fig. 9 and results compared with control, only anodized, and only ultrasonicated conditions. It is clearly seen that the maximum decrease in the biofilm bacterial density was exhibited under combined conditions of anodization and ultrasonication in seawater and bacterial cultures of Gram-positive and Gram-negative bacteria. Between the different biofilms, again the Gram-negative bacterial biofilm of *Flavobacterium* sp. showed maximum control of greater than three order decrease followed by seawater biofilm showing greater than two order decrease. The *Bacillus* sp. biofilm showed only less than two order decrease of bacterial density.

4. Discussion

The main objective of this study was to investigate for the first time the combined effect of ultrasonication of natural waters and anodization of titanium on microbial density and biofilm formation tendency on surfaces of titanium condenser material. Systematic investigation was carried out by studying first the effect of ultrasonication on the bacterial density in (i) pilot scale cooling tower water (chlorinated tap water) used for laboratory corrosion studies, (ii) open reservoir water (fresh water) used as feed water in demineralization plant in Madras Atomic Power Station (MAPS) as well as in the Fast Breeder Test Reactor (FBTR) and as condenser cooling water for the FBTR through an induced draft cooling tower and (iii) seawater used as cooling water for MAPS and the FBR. The ultrasonication procedure was finalized by studies with laboratory tap water (200 ml) where application of 24 kHz, 400 W ultrasound through a 14 mm horn type SS Sonicator with medium amplitude of 60% for 30 min brought about three order decrease in total bacterial density. The cooling tower water and reservoir water showed three order decrease in total bacterial density. However seawater showed only two order decrease. Ultrasound is able to inactivate bacteria through a number of physical, mechanical and chemical effects arising from the acoustic cavitation [12]. Joyce et al. [12] has also investigated the effect of power ultrasound at different powers and frequencies on *B. subtilis*. They reported that low-kilohertz range (28and 38 kHz) ultrasound called high power ultrasound result in continuous reduction of bacterial cell numbers and low power ultrasound (higher frequencies) increased cell number by declumping initially and the kill rate also was slow. Some other workers used combination of shear, microbubbles and high frequency, low power ultrasound to get excellent microbial control in cooling water systems [30]. Madge and Jensen [31] suggested that disinfection would be poor if total log reduction were lower than one, intermediate if total log reduction is between one and two, good if log reduction is between two and three and very good if it were greater than three. In this study high power ultrasound were used to obtain 2–3 order reduction in bacterial density of cooling waters which can be considered as good reduction.

The second aspect looked into in this study was the effect of ultrasonication on two Gram-positive and Gram-negative bacteria

Table 4
Gompertz parameters (μ, L, A) and determination coefficient (R^2) of the adjusted modified Gompertz model to bacterial growth curves of ultrasonicated and control cultures of *Bacillus* sp. and *Pseudomonas* sp.

US/control	μ (log cfu/ml h^{-1})	L (h)	A (log cfu/ml)	R^2
Pseudomonas sp. - Control	7.146 ± 2.150	−0.170 ± 0.649	10.335 ± 0.511	0.919
Pseudomonas sp. - Ultrasonicated	4.417 ± 0.870	9.406 ± 0.364	6.380 ± 0.210	0.985
Bacillus sp. - Control	4.165 ± 0.783	−0.419 ± 0.637	10.151 ± 0.489	0.957
Bacillus sp. - Ultrasonicated	9.558 ± 1.553	8.437 ± 0.250	7.460 ± 0.074	0.998

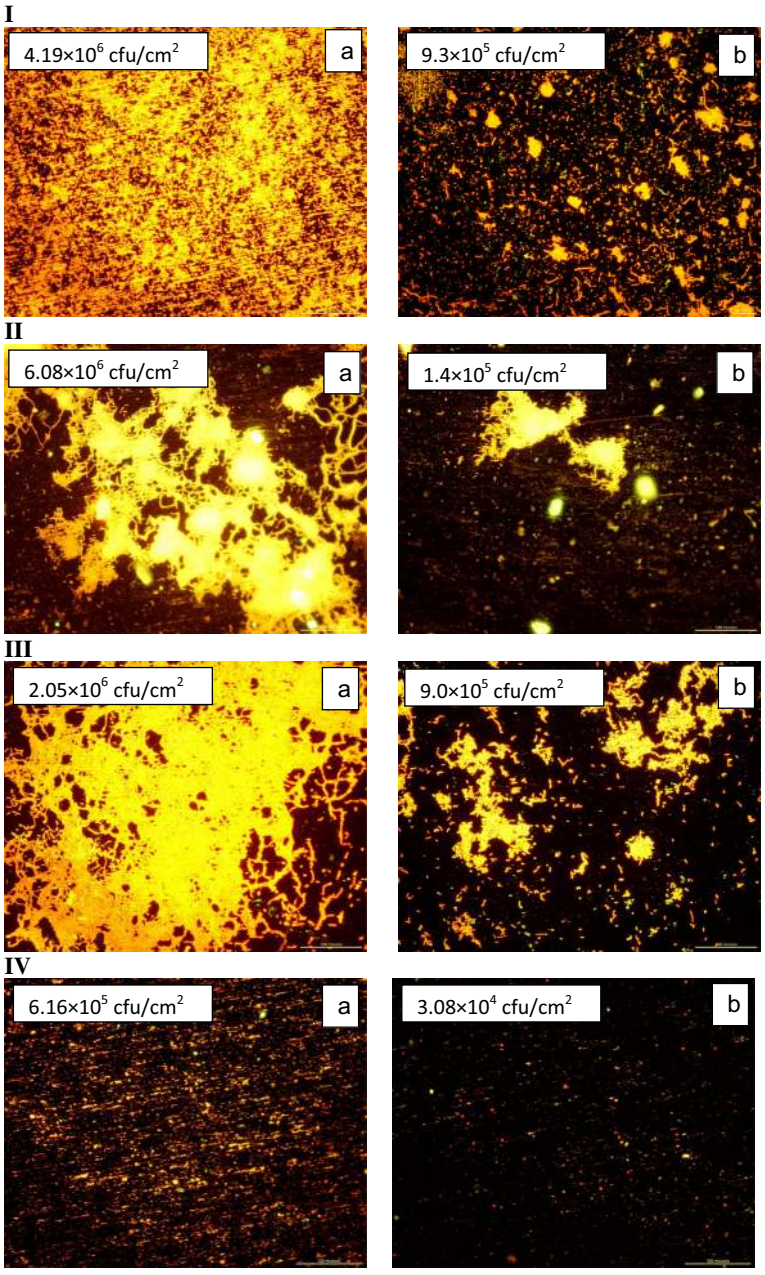


Fig. 5(i). Epifluorescence micrographs of 24 h biofilms of Gram-negative and Gram-positive bacteria on titanium specimens under control (a) and ultrasonicated (b) conditions. I Pseudomonas sp.; II *Flavobacterium aquatile*; III *Bacillus flexus*; IV *Micrococcus* sp. The bacterial density is shown in text box.

that generally predominates in these cooling water environments. Mason et al. [32] has used ultrasound with a frequency ranging from 20 to 40 kHz and power from 150 to 450 W for controlling many pathogenic microbes relevant in food industry like *Listeria* sp., *Saccharomyces* sp., *Salmonella* sp., *E. coli*, etc. The predominant bacteria in freshwater and seawater biofilms like *Pseudomonas* sp., *Flavobacterium* sp., *Bacillus* sp. and *Micrococcus* sp. were selected in this study. Chief biofilm formers were grown in dilute pure cultures to simulate natural environments and ultrasonicated. Many workers have reported that thinning of cell walls and mechanical disruption of cell wall leading to freeing of the cytoplasm membrane from cell wall due to cavitation effects is responsible for

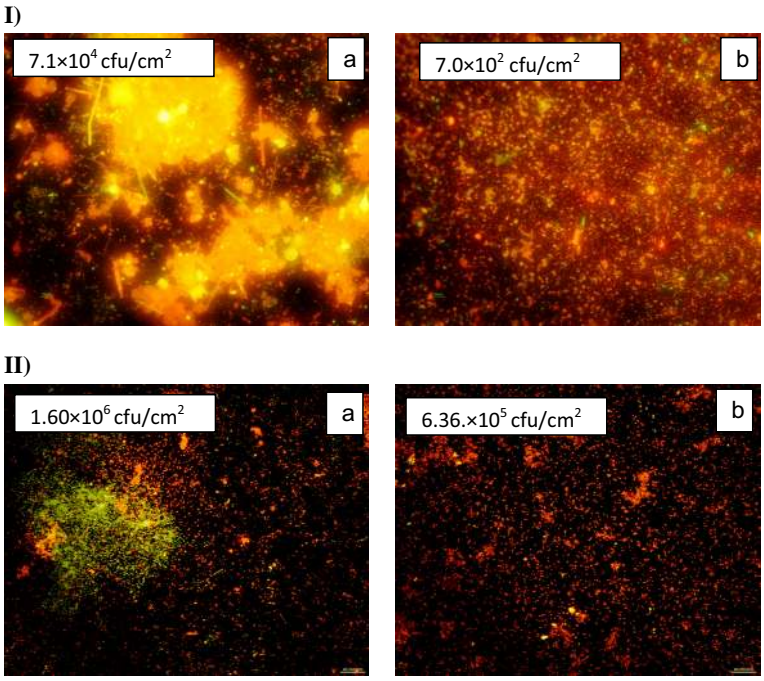


Fig. 5(ii). Epifluorescence micrographs of 24 h Biofilms formed on titanium specimens exposed to control (a) and ultrasonicated (b) natural waters; (I) reservoir water and (II) seawater .

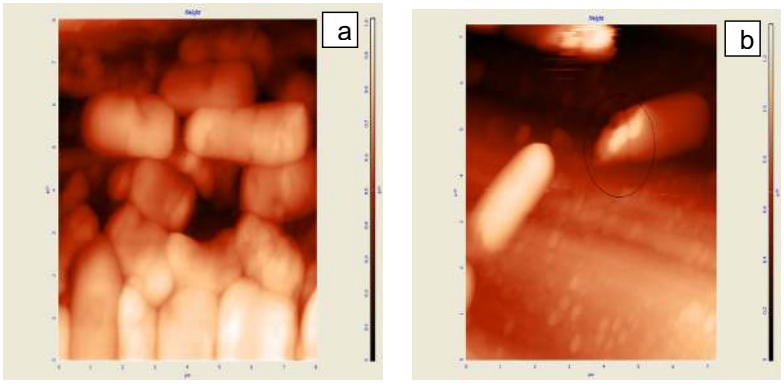


Fig. 6(i). Atomic force microscopic images of biofilms of *Bacillus flexus* on titanium specimens exposed to (a) control and (b) ultrasonicated cultures showing cell damage.

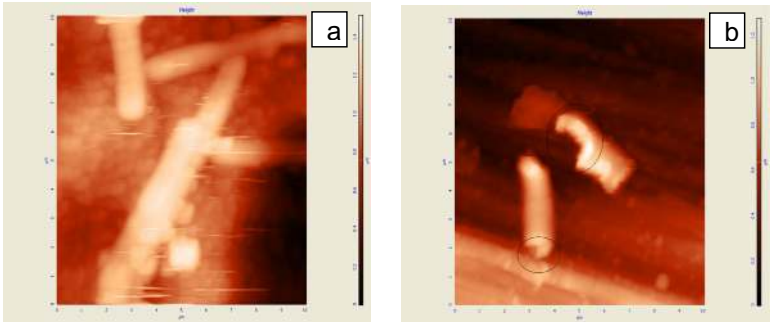


Fig. 6(ii). Atomic force microscopic images of biofilms of *Flavobacterium aquatile* on titanium specimens exposed to (c) control and (d) ultrasonicated cultures showing cell damage.

the ultrasound effect in disinfection of microbes [9,33]. To get more insight to this mechanism two Gram-positive and two Gram-negative bacteria were selected for ultrasonication study. Results clearly showed that ultrasonication achieved good control of Gram-negative bacteria; *Pseudomonas* sp. showing five order decrease and *Flavobacterium* sp. showing three order decrease. The resistance of Gram-positive bacteria *Bacillus* sp. and *Micrococcus* sp. to ultrasonication effects was clearly evidenced by two orders and less than one order decrease respectively. The main difference between the Gram-negative and positive bacteria is between their

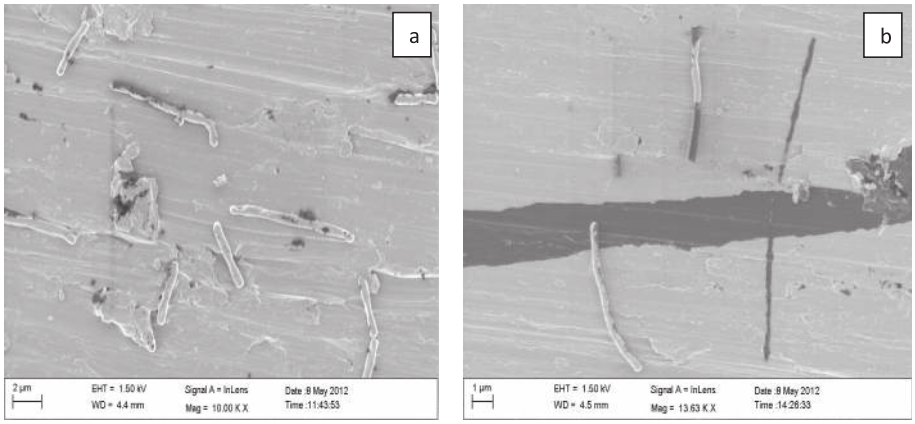


Fig. 7. Scanning electron microscopic images of biofilms of *Flavobacterium aquatile* on Titanium specimens exposed to (a) control and (b) ultrasonicated culture showing punctured cell membrane after ultrasonication.

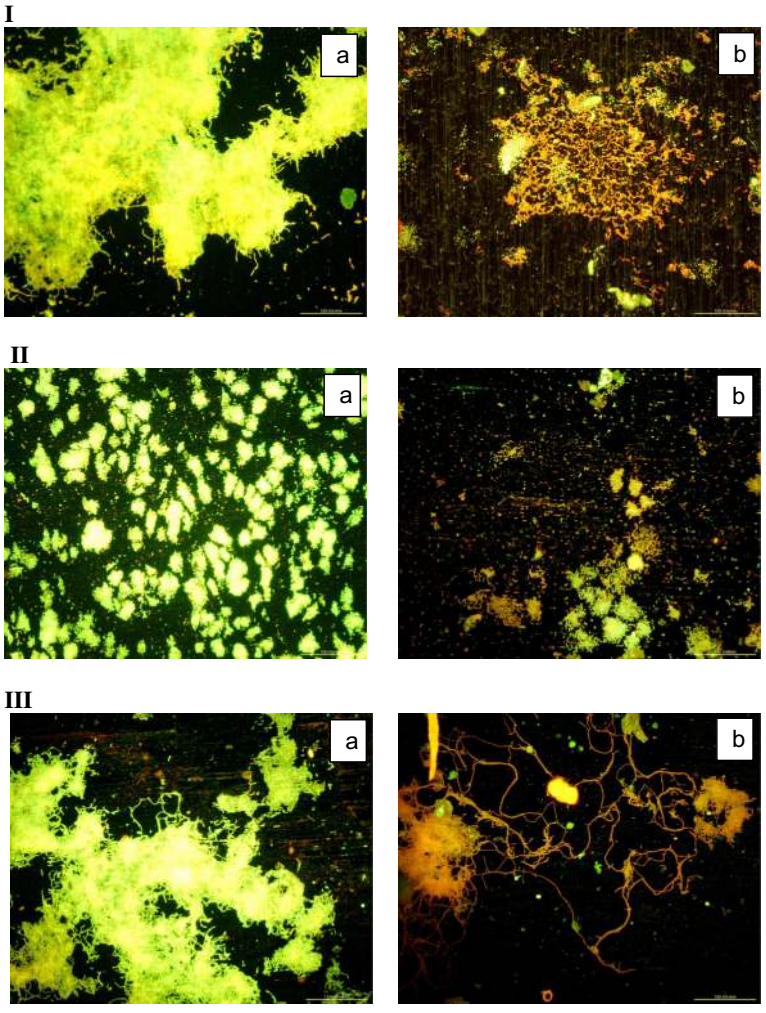


Fig. 8. Live and dead staining of bacterial cells in the biofilms of Gram-positive and Gram-negative bacteria on titanium specimens exposed to (a) control and (b) ultrasonication conditions. I-*Bacillus flexus*; II- *Pseudomonas* sp.; III- *Flavobacterium aquatile*.

cell wall composition where Gram-positive has a thick peptidoglycan layer and Gram-negative has thin peptidoglycan layer along with phospholipid and protein dominating cell membrane. Thus it appears that the thick cell wall has given resistance to bacteria from the mechanical effects of power ultrasound. The mechanism of ultrasound induced cell damage is schematically represented (Fig. 10) by Mason et al. [32]. They represented that ultrasound induced jet can initiate porosity in the cell wall. This leads to water influx into the cells resulting in swelling and subsequent rupture of cell. Earlier studies has proved ultrasonic enhancement of antibiotic action on Gram-negative *Pseudomonas* sp. and *Escherichia coli* by increasing cell wall permeability [33,34]. Their studies also re-

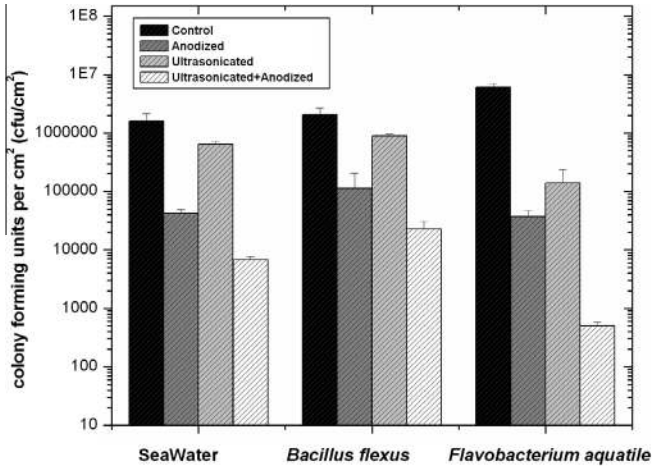


Fig. 9. Combined effect of ultrasonication and anodization on biofilm bacterial density on titanium specimens exposed to cultures of Gram-positive *Bacillus flexus*, Gram-negative *Flavobacterium aquatile* and seawater. (a) control; (b) anodized; (c) ultrasonicated; (d) ultrasonicated + anodized.

ported that this enhancement was not seen with respect to Gram-positive *Staphylococcus* sp. Drakopoulou et al. [35] have also reported that Gram negative bacteria were more susceptible to ultrasonication treatment than Gram positive bacteria as the later usually have a thicker and a more tightly adherent layer of peptidoglycans than Gram-negative organisms that contributes greatly to their structural integrity and protects the membrane from certain kinds of chemical attack. Thus it appears that the mechanical effects of cavitation is the prime effect on the bacterial cells in this study. The collapse of bubbles may also cause free radical production and they can also attack the chemical structure of bacterial cell wall. However, Broekman et al. [30] showed that high frequency of 300 kHz can only cause free radical formation. In this study with low frequency of 24 kHz the probability of occurrence of this mechanism appears less. According to Scherba et al. [36] the cavitations can be of two types, transient cavitations and stable cavitations. He also reported that transient cavitation activity occurs more easily at lower ultrasonic (low-kilohertz) frequencies. Thus it can be suggested that transient cavitation is the physical mechanism responsible for affecting the microorganism in this study too.

After ultrasonication of the pure culture of *Pseudomonas* sp. and *Bacillus* sp., the regrowth of these species were monitored by growth curve analysis and compared with control cultures. The three important parameters evaluated were lag phase, logarithmic population increase and specific growth rate. Ultrasonication affected all the three parameters for the Gram-negative *Pseudomonas* sp. Lag phase increased ten times and specific growth rate and logarithmic population decreased indicating cell death and slow regeneration. The Gram-positive *Bacillus* sp. also showed marked increase in lag phase and decrease in logarithmic population increase. However, the specific growth increase was higher than the control culture. This indicates a betterment response to the stress of ultrasonication. This can happen only if there is a change in gene expression for the survived cells. Literature also reports special ability of bacteria to adapt to extreme hostile environment in the very next generation and it is referred to as shock response [37]. There are reports on *Bacillus* sp. responding to chlorine dioxide biocide application by increased biofilm formation [38] and to induced oxidative stress by increased enzyme production and specific growth rate [39]. Thus the differential response of Gram-negative and Gram-positive bacteria to high power ultrasound in this study clearly supported the mechanism proposed by Mason group [9,15,32] that mechanical effects, thinning of cell wall, membrane rupture and cell lysis are the chief effects of ultrasonication.

The ultrasonicated mediums, dilute pure cultures of chief biofilm formers and natural waters were used for biofilm formation on titanium specimens the proposed condenser material of FBR, by 24 h exposure. There was one order decrease in biofilm formation in all ultrasonicated mediums compared to control mediums. The epifluorescence micrographs clearly visualized the reduction in biofilm formation. AFM images of biofilms of *Bacillus* sp. and *Flavobacterium* sp. showed lysed cells and SEM images showed the punctures cell walls. The cell wall modification/damage was further tracked by using the LIVE/DEAD Baclight staining technique. All the biofilms showed red fluorescence confirming the penetration of propidium iodide through damaged porated membranes. Thus characterization of biofilms formed under ultrasonicated conditions using advanced microscopic techniques clearly confirmed the mechanical damage and reduction of cell density by one order due to ultrasonication.

To enhance the bacterial reduction, studies were planned to combine the ultrasonicated medium effects with surface modification of titanium. Earlier studies in our laboratory have already proved good antibacterial activity by titanium surfaces anodized at 30 V in orthophosphoric acid [20,26,40]. Ultrasonicated dilute pure cultures of a Gram-negative *Flavobacterium* sp., a Gram-positive *Bacillus* sp., and seawater were used for exposure studies with anodized titanium specimens. The results were compared with

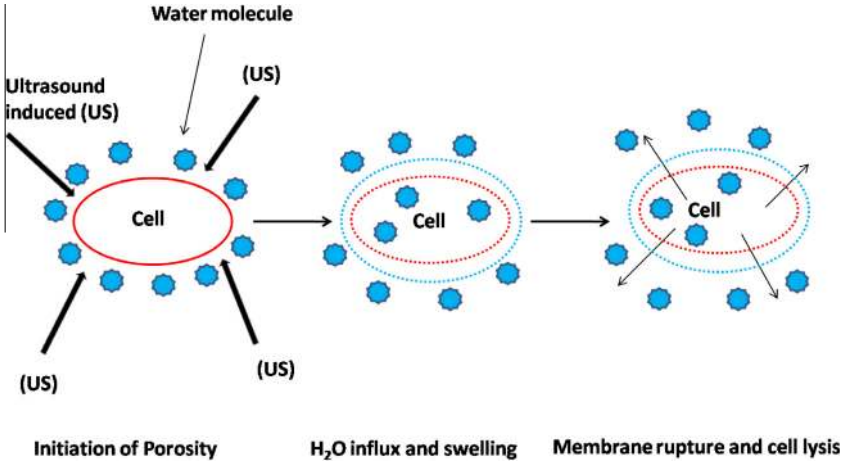


Fig. 10. Mechanism of ultrasound-induced cell damage [32].

individual treatment conditions like anodization and ultrasonication separately too. In all the ultrasonicated mediums combination of ultrasonication and anodization brought about maximum decrease in bacterial density and biofilm formation with greater than two order decrease in seawater, two order decrease in *Bacillus* sp. culture and more than four order decrease in *Flavobacterium* sp. culture. Therefore a synergistic effect of anodization and ultrasonication was established in this study.

5. Conclusions

The following conclusions were obtained from the studies to control biofilm formation by coupling ultrasonication of natural waters and anodization of titanium:

Application of 24 kHz, 400 W ultrasound through a 14 mm horn type SS Sonicator with medium amplitude of 60% for 30 min brought about three order decrease in total bacterial density of laboratory tap water. The cooling tower water and reservoir water showed three order decrease and seawater showed two order decrease in the total bacterial density. Thus high power ultrasound used in this study obtained 2–3 order reduction in bacterial density of cooling waters and this is considered as good reduction.

Studies on the effect of ultrasonication on dilute pure cultures Gram-negative and Gram-positive bacteria showed a good control of five order and three order decrease for *Pseudomonas* sp. and *Flavobacterium* sp. respectively and intermediate and poor control of two order and less than one order decrease for *Bacillus* sp. and *Micrococcus* sp. respectively. Detailed growth curve analysis showed that ultrasonication increased lag phase and reduced logarithmic population increase and specific growth rate of Gram-negative bacteria. For Gram-positive bacteria specific growth rate increased after ultrasonication exhibiting betterment response to the stress of ultrasonication. Thus the differential response of Gram-negative and Gram-positive bacteria to high power ultrasound in this study clearly supported that the mechanical effects, thinning of cell wall, membrane rupture and cell lysis are the chief effects of ultrasonication.

Studies on the biofilm formation tendency of these ultrasonicated mediums on titanium surface showed one order reduction under all conditions. Detailed biofilm imaging by advanced microscopic techniques like AFM, SEM and epifluorescence microscopy clearly visualized the lysed/damaged cells and membrane perforations due to ultrasonication.

Combination of ultrasonication and anodization brought about maximum decrease in bacterial density and biofilm formation with greater than two order decrease in seawater, two order decrease in *Bacillus* sp. culture and more than four order decrease in *Flavobacterium* sp. culture establishing the synergistic effect of anodization and ultrasonication in this study.

Acknowledgment

Authors sincerely acknowledge Dr. P. Vasudeva Rao, Director, Indira Gandhi Centre for Atomic Research, Shri. S.C. Chetal and Dr. Baldev Raj, Past Directors, and Dr. T. Jayakumar, Director, MMG, for their keen interest in the study and constant encouragement.

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