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Mitigation of Sulfate-Reducing Bacteria (SRB), *Desulfovibrio Vulgaris* Using Low Frequency Ultrasound Radiation

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Abstract

This paper investigated the performance of low frequency ultrasound radiation in mitigating the microbiologically influenced corrosion (MIC) by mechanically inhibiting the planktonic sulfate-reducing bacteria (SRB) growth in modified ATCC broth number 1249 medium. Several samples contained a laboratory SRB strain of *Desulfovibrio vulgaris* ATCC 7757 were exposed to 20 kHz of ultrasonic radiation through sonication treatment for 15 and 30 minutes. The effect of sonication treatment on the growth of SRB strain was monitored afterward by measuring the bacterial numbers (*cells per ml*) within a period of eight days after first being exposed to ultrasonic radiation. The bacterial numbers of samples exposed to ultrasonic radiation shows a reduction up to 10 times *cells per ml* units as compared to control sample on the eighth day. It appears that the sonification treatment on the SRB strain using low frequency ultrasound can inhibit the growth of bacteria. Practically, the findings can offer new perspective on MIC mitigation technique based on mechanical ultrasonic treatment as an alternative to conventional chemical treatment.

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Keywords: Low frequency ultrasound, ATCC broth number 1249 medium and *Desulfovibrio vulgaris*.

Introduction

Microbiologically Influenced Corrosion (MIC) due to sulphate-reducing bacteria (SRB) can cause serious localised attack on underground steel structure causing leakage from wall perforation by pit colonies [1] [2]. SRB acts as catalyst in the reduction reaction under anaerobic condition and is responsible for accelerating the existing corrosion damage experienced by steel structures [3] [4]. Without presence of oxygen, anaerobic bacteria

such as *Desulfovibrio vulgaris* are the most common perpetrator and degradation agent capable of causing severe corrosion of iron material in environment surrounded by fresh and salt water system because they produce enzymes which have the power to accelerate the reduction of sulfate compounds to the corrosive hydrogen sulfite [5] [6]. Figure 1 described the overall process as cathodic depolarization, based on the theory that SRB containing a hydrogenise enzyme remove atomic hydrogen accumulated at the cathode. At the anodic reaction section, the metal (Fe) release into the bulk of the metal which roam to the adjoining surface, where they react with H^+ in solution to form H_2 by hydrogenise processes. Water is required as the electrolyte carrier for the Fe^+ and H^+. In an anaerobic environment, SRB use hydrogen in reducing sulfate (SO_4^{2-}) to sulfide (H_2S). SRB consume the formed hydrogen, thus oxidation of Fe occurs. This mechanism increased the anodic metal dissolution and

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consequently FeS as corrosion products are formed [7]. The activation of corrosion cells between the steel surface as anode and the FeS as cathode on metal surface results in a local decrease in pH that enhances the breakdown of passive film [7].

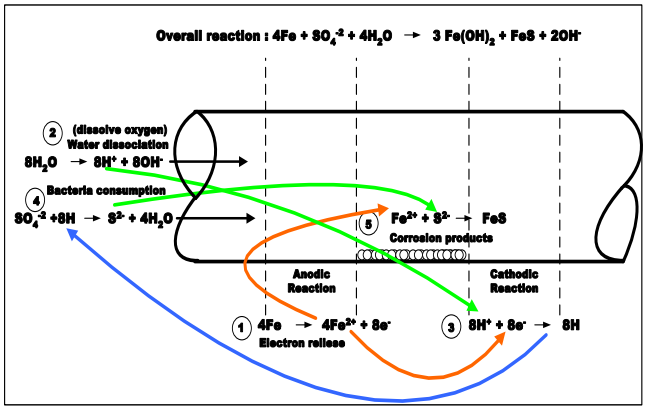

Figure 1: Schematic diagram of SRB reduction reaction under anaerobic condition.

Biocide is the most profound characteristic of chemical treatment used to mitigate MIC especially in pipeline system. However due to more restrictive environmental regulation, massive amounts of chemical wastage and staggering costs of biocide treatment have sparked a call to find alternative environmental-friendly mitigation methods. Environmental friendly treatments such as sound wave radiation through sonication are presently under consideration as alternatives to biocides [8]. Sonication is a mechanical treatment utilizing a cyclic sound pressure with a frequency greater than the upper limit of human hearing (ultrasound). Like any sound wave, ultrasound is propagated via a series of compression and rarefaction waves induced on the molecules of the medium through which it passes [9] [10]. A previous study showed that high frequency ultrasonic radiation can impedes microbial

activity in aerobic condition, which is responsible for corrosion damage, through sonication process [8].

The primary concern of this paper is to assess the performance of sonication using low frequency ultrasound upon the growth of planktonic SRB of *Desulfovibrio vulgaris*. The low frequency ultrasound range between 16 kHz to 20 kHz is normally employed as a cleaner or scrubbing mechanism to clean mechanical apparatus from microbial activity and contaminants. Ultrasonic radiation at 20 kHz of was used to inhibit the growth of *Desulfovibrio vulgaris* ATCC 7757 in modified ATCC 1249 medium [11]. The findings were based on comparison of bacteria count of planktonic SRB between sonicated and control mediums (untreated). Planktonic SRB are the best subjects as opposed to sessile SRB or biofilm SRB in this research since the bacteria count process is much simpler.

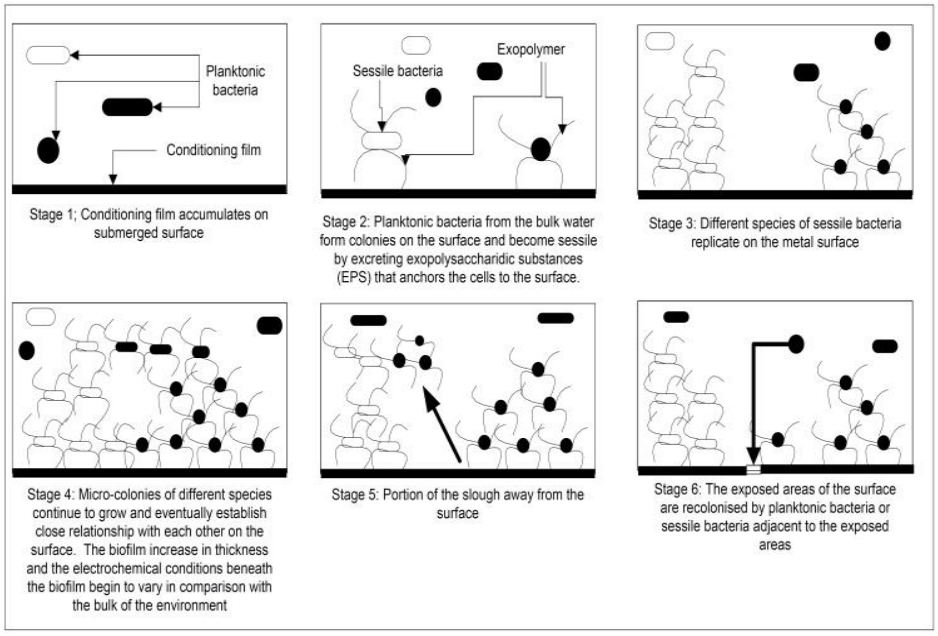

Figure 2: Stage of biofilm development (12).

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Figure 2 illustrates the stages of biofilm formation onto the metal surface. The biofilm formation started by a developmental process in which free swimming cells attach to a surface, first transiently and then permanently. Conditioning film will accumulate on the metal surface after it is completely submerged in a media. The colonies which formed from the bulk water will produce planktonic bacteria and it became fixed on the surface by excreting exopolysaccharidic (EPS) substances which plays the role as an anchor between the bacteria colonies and the surface. EPS substances can protect the fragile bacteria from imminent threats due to external factors such as temperature and pH [13]. The replication of sessile bacteria by different species can form a single layer of biofilm onto the metal surface. When the replication process is repeated, it will establish tight relationship between the micro-colonies. Hence, increase the thickness of the biofilm. Over time, the exposed area will be enveloped by planktonic bacteria or other sessile bacteria.

Review of Previous Researches

Correlations between bacteria numbers and the frequency of ultrasonic radiation are scarce, especially for anaerobic bacteria such as SRB. Pound [8] has utilised a 550-Watt sonication treatment to kill the bacteria cell of Acid Producing Bacteria (APB) under aerobic conditions in brine at elevated pressure. The experiment yielded results which shows that the magnitude of the decrease of bacteria numbers depends on various factors, particularly the presence of hydrocarbons, the number of passes of brine through the cavitation cell and the flow rate. Reza [12] also states that ultrasonic treatment is the ones of the possible techniques for

destroying the underlying material but is restricted to surfaces where ultrasonic treatment can be applied.

Wen [14] demonstrated that the chelators also enhanced biocide treatment against planktonic and sessile SRB and reduced biocide dosage considerably in the removal of established biofilm. Nonetheless, chelators will cause wastage because its volume will increase especially in a long pipeline. The volume of chelators and the intricacy of the process are relative to the size and length of the pipeline system. The bigger the system, the more intricate the maintenance process and the more wastage produced.

Materials and Methodology

Bacteria and Culture Conditions

This research employed a laboratory SRB strain, *Desulfovibrio vulgaris* ATCC 7757 cultured and grown in a modified ATCC 1249 medium. Table 1 lists the compositions of the modified ATCC 1249 medium. The pH value was adjusted to 7.0 to reach natural phase using a buffer. The medium was autoclaved and sparged with oxygen-free nitrogen for one hour in a laminar flow hood to remove the oxygen in the solution. Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) with 25ppm concentration was filtered followed by an addition of sterile (0.2 μm) to the medium. An amount of 100 ml of the medium was then transferred to 125 ml of anaerobic vials under anaerobic condition. Two (2) ml of 1-day old SRB seed was added for SRB inoculation. The initial concentration of SRB strain was measured at 2.0×10^6cell/ml. The

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aforementioned bacteria sample was prepared in triplicate and incubated at 37^{0}C.

Table 1: Chemical composition of the ATCC 1249 medium

Component	Chemical Reagents	Composition (g/l)
Component I	$MgSO_4$	2.0g/l
	Sodium Citrate	5.0g/l
	$CaSO_4$	1.0g/l
	NH_4Cl	1.0g/l
	Distilled water	400ml
Component II	K_2HPO_4	0.5g/l
	Distilled water	200ml
Component III	Sodium lactate(60% syrup)	4.5ml/l
	Yeast extract	1.0g/l
Component IV	$Fe(NH_4)_2(SO_4)_2$	25ppm

*Component IV: filter-sterilize, which is not autoclavable

Coupon Preparation

Coupons were prepared out of carbon steel pipe grade API5L-X70 (specimens were produced by cutting from a segment of API 5LX-70 pipe obtained from a local gas operator). The

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composition of the carbon steel pipe in weight % is shown in Table 2. Coupons were cut using a water jet cutting machine. The coupons were machined to approximately size of 10 x 20 mm of area surface. Then, the coupons were polished with 100 grit Si-C paper, cleaned with ethanol to remove all forms of dirt, grease and small Si-C particles on the surface. Cleaned and dried coupons were then coated with prime coat leaving only the top surface exposed. The coupons then dried overnight in oven at 37^{0}C. The exposed areas of coupons were polished again with series of Si-C papers (grade 320, 600 and 800), followed by ethanol degreasing. Cleaned coupons were weighted (known as initial weight, W_0), systemically recorded and stored in desiccator until used. Figure 3 shows the polished and as-received carbon steel coupons.

Table 2: Chemical composition of API5L-X70 carbon steel pipe used in the present work (wt%)

Materials	C	Mn	P	S	Si	Fe
X70	0.28	1.40	0.030	<0.030	0.22	balance

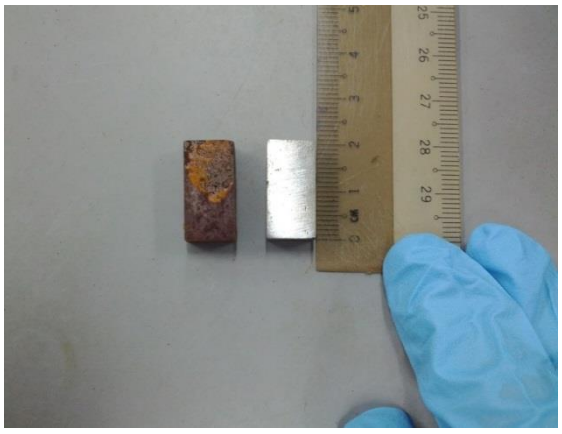

Figure 3: carbon steel coupons before and after polishing

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SRB Enumeration

The growth of Desulfovibrio desulfuricans in ATCC medium accentuates a rotten egg odor characteristic of hydrogen sulfide and the black colored solution is an indication of SRB growth and metabolism [15]. A rotten egg odor and a black medium were observed via SRB kits. Clean syringes were used to extract one milliliter of the bacteria sample from anaerobic vials before a series of dilutions (Figure 4). SRB enumerations were monitored every 24-hour for eight consecutive days by counting the planktonic cell using hemocytometer method. SRB kits were used to validate the results from the hemocytometer as shown in Figure 5. A black color of kits present the SRB was attends.

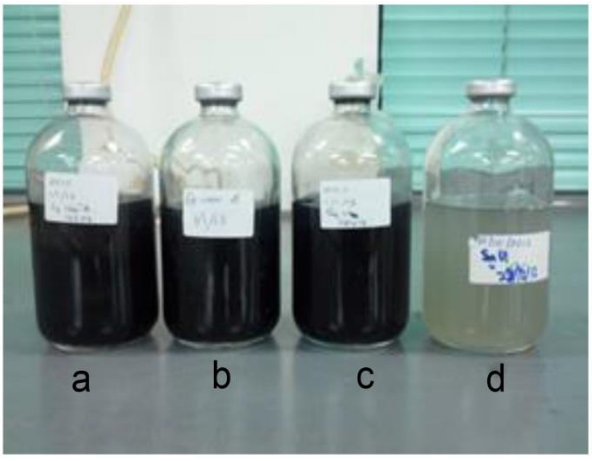

Figure 4: Sample in anaerobic vials (a: sample 1, b: sample 2, c: sample 3 and d: control)

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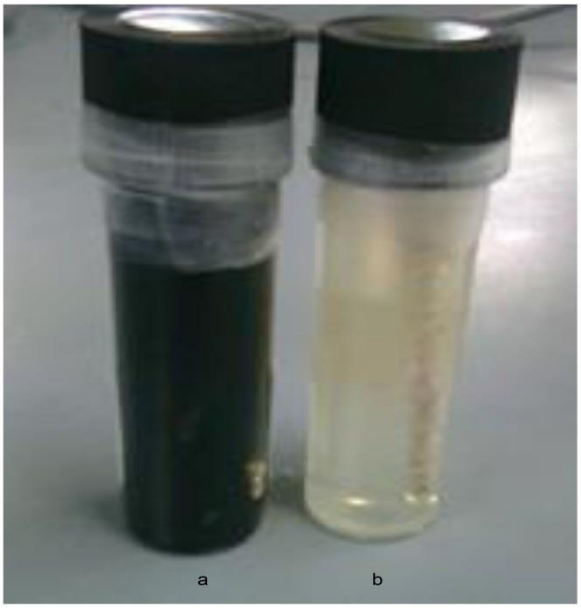

Figure 5: a: SRB present and b: control (Sani Check from Biosan Laboratories Inc, USA).

Ultrasonic treatment

The research exercised low frequency ultrasonic radiation treatment (refer Figure 6) on day-3 after inoculation procedure. The ultrasonic frequency was set at 20 KHz with 600W power using model 1505 Lab Companion™. The frequency of 20 KHz serves as a useful lower limit in describing ultrasound [16][17]. SRB inoculated medium was exposed to the ultrasonic treatment for 15 and 30 minutes. The range of exposure time was referred to previous study by Pound [8]. The enumeration of the SRB after ultrasonic treatment was conducted at 24 hours after the time of exposure and continuously monitored for eight days.

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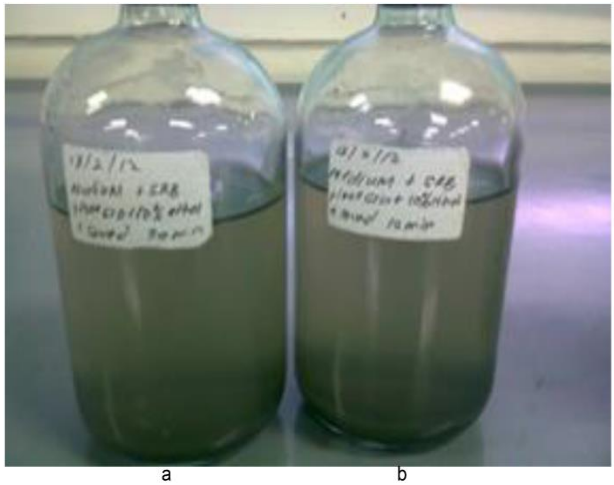

Figure 6: Samples after low frequency ultrasonic radiation treatment (a: 30 minutes exposure and b: 15 minutes exposure).

Scanning Electron Microscope (SEM) Observations

SRB biofilm morphology was observed by adding carbon steel coupons to the control SRB medium (untreated sample). Prior to that, the coupons were polished with SiC paper in series graded 200, 400 and 600 and subsequently cleaned with acetone to remove any oil traces from coupon surface [18]. The coupon surface was half painted on the left side only to designate surface exposed to the SRB medium. The coupon was removed from the medium after seven days of exposure and then treated for SEM observation. This treatment consisted of immersion in 5% glutaraldehyde for 4 hours and subsequently washed with a series of ethanol solutions (25%, 50%, 75% and 100% v/v). The samples were then dried using critical point drying (CPD) and coated with gold prior to SEM observation.

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Results and Discussion

Figure 7 shows the appearance of SRB bacteria observed under microscope via hemocytometer grid with 400 times magnification. Figure 8 exhibits an SEM observation of control planktonic SRB medium at day-7 which is the optimum time of SRB growth in stagnant medium. The cell number results for all sets of bacteria samples show that day-8, the cell number decreases rapidly. This result indicates the natural extermination point of bacteria begins on day-eight due to the depletion of the food supply in the medium which may leads to total extermination.

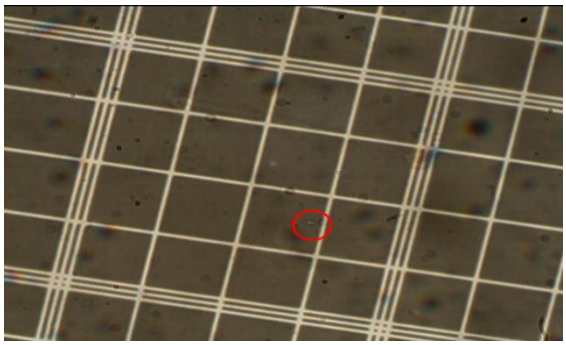

Figure 7: SRB bacteria observed under microscope with hemocytometer grid at 400xmagnification (0.25mmx0.25mm).

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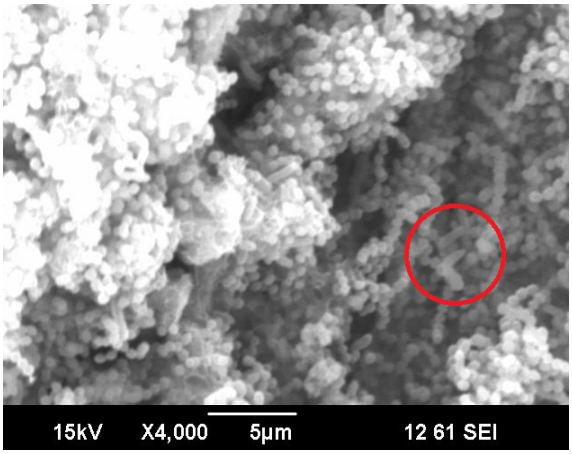

Figure 8: SEM observation of control SRB medium at day seven.

Figure 9 shows a plot that reveals the performance of low frequency ultrasonic radiation upon bacteria growth. The killing efficiency of sonication treatment was observed on day-4 whereby the cell numbers starting to drop in the planktonic SRB. Prior to the drop point caused by lack of food supply, the total number of bacteria in sonicated medium was measured to be about 2 times *cells per ml* than the control medium (untreated) at day-seven. The sonicated medium with longer exposure to ultrasonic radiation also experienced a greater drop in bacteria numbers even though the difference is not that significant.

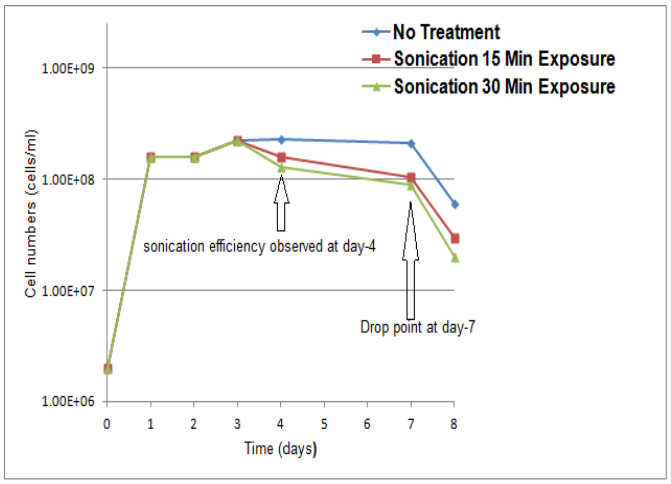

Figure 9: SRB bacteria growth curves incubated at 37^{0}C with ultrasonic.

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Conclusion

Preliminary experimental work has demonstrated the capability of low frequency ultrasonic radiation treatment in inhibiting the growth of Desulfovibrio vulgaris ATCC 7757 in a modified ATCC 1249 medium. The results signify the potential of low frequency ultrasonic radiation in mitigating MIC caused by SRB. If fully explored, this environmental-friendly treatment mechanism can be economically incorporated into existing maintenance regimens designed to combat the prolonged MIC issues.

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