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Influence of Ultrasonic Treatment in Sewage Sludge

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Abstract

Physical methods like ultrasound, ultraviolet and nanoparticles are very useful in wastewater purification and recycling. The ultrasound irradiation in a liquid leads to the acoustic cavitation phenomenon, which can affect a number of mechanical, acoustic, chemical and biological changes in waste analysis. In present study, ultrasonic irradiation treatment technique was used to treat the sewage sludge effluent, which was collected from Delhi and another sample of pure *E-coli* strain was processed. Samples were treated in ultrasonic bath at 35 and 130 kHz of irradiation for different time periods of 5 min, 10 min, 20 min and 30 min including control (untreated). Treated samples were tested for different parameters, viz. bacterial cell count, chemical oxygen demand (COD), degree of disintegration COD (DD_{COD}), scanning electron microscope (SEM), optimum density (OD) of cells and reactive oxygen species (ROS). Result shows significant disintegration in ultrasonic treated sewage sludge and *E-coli* samples as compared with untreated (control). We observed an increased level of ROS and decreased bacterial population in treated samples with 35 kHz and 130 kHz of frequency. Result suggests that the ultrasonic treatment was more effective by increasing time and frequency. Study concludes that low-frequency ultrasonic bath at 130 kHz is more effective as compared to 35 kHz.

Keywords: Ultrasonic irradiation; Reactive oxygen species; Free radicals; COD; DD_{COD}

Introduction

Ultrasound Reactor technology (USRT) or ultrasonic bath is used for biological cell disruption for the recovery of intracellular materials for decades [1] and it is found an increasing application in municipal sludge disintegration on full-scale [2]. Ultrasound disintegration is essentially a physical process. It neither generates secondary toxic compounds nor contributes additional chemical compounds. In addition to physical sludge disintegration, many toxic and recalcitrant organic pollutants, such as aromatic compounds, chlorinated aliphatic compounds, surfactants, organic dyes, etc., are broken down into simpler form. This is due to free radicals formation which may enhance the level of reactive oxygen species (ROS) in bacterial cell during ultrasonication where cells become ruptured or cause death. Phenomenal, free radical and sonochemical effects can arise when inertial cavitation occurs, which greatly affects passive membrane permeability's, active transport processes and metabolic rates of cells [3]. The cavitational bubbles disrupt adjacent bacterial cells by extreme shear forces, rupturing the cell wall and membranes. Such effects are observed in present study by scanning electron microscope (SEM). Khanal et al. [4] also observed in waste activated sludge that under a light microscope, floc-like structures entangled within a large numbers of filaments were seen prior to sonication. Within two minutes of sonication, the filaments and flocs were almost completely disintegrated and a more or less homogeneous texture was observed. In case of sewage sludge digestion, the biological hydrolysis has been identified as the rate limiting step [5]. Therefore, these studies suggest that the pretreatment of sewage sludge by mechanical, chemical, or thermal disintegration can improve the subsequent anaerobic digestion [6,7]. Ultrasonic disintegration is a well-known method for the break-up of microbial cells to extract intracellular material [1, 8]. Moreover, sonication leads to the formation of dead bacterial cells or selectively destroying weak bacteria [9].

Cavitation mechanism

An impact of ultrasound waves on a liquid causes the periodical compression and rarefaction of the medium. Cavitation occurs above a certain intensity threshold, when gas bubbles are created which first grow in size before violently collapsing within a few microseconds. The violent collapse produces very powerful hydro mechanical shear forces in the bulk liquid surrounding the bubble. It has been shown that macromolecules with a molar mass above 40,000 are disrupted by the hydro mechanical shear forces produced by ultrasonic cavitation and also may be the dominant factor for the disintegration enhancement [10,11]. Indeed the temperature and pressure inside the collapsing cavitation bubbles rise up to about 5000K and several hundred atmospheres. These extreme conditions can lead to the thermal destruction of compounds present in the cavitation bubbles and to the generation of reactive hydroxyl radicals [12]. Such mechanism of physical interaction to bacterial cell and free radical formation was reported by Kesari et al. [11]. This is the way sonochemical reactions can degrade volatile pollutants by pyrolytic processes inside the cavitation bubbles and non-volatile pollutants by hydroxyl radical reactions in the bulk liquid [13,14].

Ultrasound and ROS

Reactive oxygen species is an important parameter for the detection of oxidative damage, caused due to external or internal sources. It is well established from our group that ROS may enhance due to irradiation. However, ultrasonic irradiation in a liquid leads to the acoustic cavitation phenomenon which may breaks the cell membrane and free radicals accompanied by generation of reactive oxygen species

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(°OH, °OOH) via thermal dissociation of water and oxygen. In present study, we established these phenomena by following the process of reactive oxygen species (free radicals). However, free radicals can be generated as a consequence of the cavitation induced by ultrasonic waves. In this way, radicals can be considered as the primary reagents produced by ultrasound. These free radicals penetrate into water and oxidize dissolved organic compounds. Hydrogen peroxide (H_2O_2) is formed as a consequence of °OH and °OOH radical recombination in the outside of the cavitation bubble [15,16]. Sonochemical degradation processes can occur in a broad ultrasound frequency range from 20 kHz to 10MHz. The frequency range from 20 kHz to 2 MHz is used in sonochemistry. Frequencies above 3MHz are more commonly used in non-destructive testing and medical imaging.

Our previous studies demonstrated that ultrasonic pretreatment of sewage sludge; paper sludge and sugar sludge collected from industrial area have shown significant effect of ultrasonication at 25, 35 and 130 kHz [17-20]. Present study is aimed to find out (i) the action of free radicals in the mechanism of disinfection by examining the effect of radical scavengers (ROS) and sludge disintegration (DD_{COD}). (ii) the effect of different ultrasonic frequencies (130kHz and 35kHz) and time (5min to 30 min) on bacterial population by scanning electron microscope (SEM) and optimum density (OD).

Methodology

Material and equipment

Sewage sludge was collected from sludge treatment plant (STP), Indraprastha Estate, Delhi. Sewage sludge samples was divided into two groups and designated as control and sonicated. This was followed by ultrasonic treatment at four different duration of time (t_1=5, t_2=10, t_3=20 and t_4=30 min.), using ELMA, multi frequency ultrasonic bath (according to manufacture Instruction). In order to characterize these samples, dissolve oxygen (2.86 mg/l); Conductivity (3.1 ms/cm); Total solids (22.01 g/l); Chloride (1230 mg/l) and pH (7.4) were measured. The sonication of sludge supernatant was performed in borosilicate glass vessel (250ml). During sonication, sludge samples were stirred and the temperature was maintained at 25 +3°C by thermostated jackets.

Ultrasonic pre-treatment specifications were taken as under

Treatment time (min.):		5(t_1), 10(t_2), 20(t_3) and 30(t_4).
US Frequency	:	35 & 130 kHz
US Power	:	250 W.
US Intensity (50.95 watt per cm^2)	:	power supplied per transducer area
US Density (2500 watt per lit)	:	power supplied per sample volume

pH and Electric conductivity of sample were measure by electrode based probe (Water and Soil analysis kit, Electronics India, Model 161E), the rise in temperature of sample on ultrasonic treatment was measure by mercury filled thermometer.

Methods

E. coli bacterial population count

E. coli bacterial strain was used as a reference for evaluating ultrasonication effects on cell viability. *E. coli* strain was picked from master plate and inoculated in 20ml of LB (Luria Bertani) liquid medium in 50ml of conical flask (five flasks). All flasks were kept on shaker at 37°C for 24 hours. Samples contained in flasks were labeled as control (without ultrasonic treatment), t_1, t_2, t_3 and t_4 and treated at 35 kHz and 130 kHz frequency for time- t_1= 5, t_2= 10, t_3= 20 & t_4= 30 min respectively. Immediately after treatment, 20µl (1 x 10^6 of concentration) of sample was spread on LB plates aseptically under laminar air flow & incubated at 37°C for over night. Colony number was counted by a colony counter. In an effort to eliminate errors in the procedure, all assays were carried out in duplicates.

Bacterial isolation and population count from sewage sludge

The supernatant of sewage sludge samples (with the concentration of 1x10^6) were treated at 35 kHz and 130 kHz frequency for time t_1= 5, t_2= 10, t_3= 20 & t_4= 30 min, respectively, including control (without ultrasonic treatment). Thereafter, 20 µl of sample was spread on LB plates aseptically under laminar airflow and incubated at 37°C overnight.

Optimum density

Protein leakage analysis was performed using Bradford assay. Bacterial cells of *E. coli* and supernatant of sewage sludge was treated in ultrasonic bath at 35 kHz and 130 kHz for the time- t_1= 5, t_2= 10, t_3= 20 & t_4= 30 min respectively. Supernatant was collected after centrifugation (at 6000 rpm) for 15 min. Cells (pallet) were suspended in PBS buffer (Phosphate saline; 3g K_2HPO_4, 1g KH_2PO_4 and 8.5 g NaCl L^{-1}; pH¼ 7.2) and again centrifuged at 6000 rpm at 4°C for 10 min. For each sample, 200 µl of the supernatant was mixed in 800 µl of Bradford reagent. Optical density (at 595 nm) was measured after 10 min of incubation in the dark. BSA was used as a standard protein. The method was followed as per Tiwari et al. [19].

Scanning electron microscope (SEM) of *E. coli*

The *E-coli* strain was grown in LB media and culture was centrifuged at 4000 rpm for 10 min at 4°C. Transverse sections cultures were made in all the treated groups including untreated samples. These were fixed in 2% glutaraldehyde. Before doing any scanning electron microscope (SEM) characterization, the samples were dried and mounted on circular disc stubs with adhesive. Gold coatings were applied at a thickness of about 20 nanometers, which is too thin to interfere with the dimensions of surface features. Coating was done with the help of sputter coater. The samples were placed in a small vacuum chamber. After introducing the argon gas in the chamber, an electric field was applied to cause removal of electron from the argon atoms and made them positively charged. The Ar ions were then attracted to a negatively charged piece of coated material. The Ar ions acted like sand in a sandblaster, which struck gold or carbon atoms from the surface of the foil. These gold atoms now settled onto the surface of the sample and produced a gold coating. SEM images were obtained on low vacuum SEM (Leo 435 VP; Carl Zeiss SMT, Cambridge, UK) at the National Facilities of Electron Microcopy, AIIMS, New Delhi, India

Total reactive oxygen species (ROS) assay

According to Hayashi et al. [21], 20 µl of supernatant sample of sewage sludge and pure culture of *E-coli* (1x10^6) was added to 140 µl of pre warmed (40°C) 0.1M sodium acetate buffer (pH 4.8) in 96-wells (microtiter plate). 100 µl of the mixed solution of DEPPD (100µg/ml DEPPD was dissolved in 0.1M sodium acetate buffer, pH 4.8) and ferrous sulfate [ferrous sulfate (4.37 µM) was dissolved in 0.1M sodium acetate buffer, pH 4.8] at a ratio of 1:25 was added in each well to initiate reaction. Thereafter, microtiter plate was incubated at 37°C for 5 min. Absorbance was measured using a spectra Max M_2 spectrophotometer

plate reader, at 505 nm. ROS levels in bacterial samples were calculated from the calibration curve of H_2O_2 and expressed as equivalent to levels of hydrogen peroxide (1 unit = 1.0 mg H_2O_2/l).

Data collection and statistical analysis

Analysis of ROS was done by taking absorbance at 505 nm. The ROS concentration in bacterial cells and sewage sludge samples of ultrasonic treated were analyzed from the graph of H_2O_2 standard and expressed as equivalent to levels of H_2O_2 (1 Unit = 1.0 mg H_2O_2/l).

Chemical oxygen demand in sewage sludge

The chemical oxygen demand (COD) was determined by oxidation of the organic compounds with $K_2Cr_2O_7$. COD was measured in sewage sample by following control, sonicated (5, 10, 20, & 30 min) and chemically (alkaline) treated sludge. Each sample was centrifuged for 2 hour at 10500 rpm and the aqueous phase supernatant was taken for further analysis. The required reagents and methodology for analysis was followed according to Apha [22,18,20]. The determined COD volume is used to calculate degree of sludge disintegration.

Degree of disintegration (DD_{COD}) in sewage sludge

The degree of sludge disintegration was assessed by determining the chemical oxygen demand (COD) in the sludge supernatant. DD_{COD} is calculated as the ratio of COD increase by sonication to the COD increase by the chemical disintegration (alkaline). A reference (100%) was defined as the aqueous phase COD obtained by chemical sludge disintegration in 0.5 mol l^{-1} sodium hydroxide for 22 h at 20°C (23, 19, 20). The degree of disintegration (DD_{COD}) is calculated as the ratio of COD-increase by sonication to the COD-increase by the chemical disintegration

$$DD_{COD} = (COD_{ultrasound} - COD_0) / (COD_{NaoH} - COD_o) \times 100\ [\%]$$

where $COD_{Ultrasound}$ is the COD in the supernatant of the sonicated sample (mg l^{-1}), COD_0 is the COD in the supernatant of the untreated sample (mg l^{-1}), COD_{NaOH} is the COD in the supernatant of the reference sample (mg l^{-1}).

Results

Bacterial population count

Results of sludge and *E-coli* treated samples at 35 kHz and 130 kHz frequency for different time t_1=5, t_2=10, t_3=20 and t_4=30 min, shows the number of bacterial colonies were decreased with increased treatment time and frequency as compared to control (without ultrasonic treatment). A decreased numbers of bacterial colonies of live and dead cells in sewage sludge is shown in Figure 1. On the other side, a gradual decrease in population of *E-coli* was also observed (Figure 1). Result shows the ultrasonic treatment was more effective on 20-30 min of treatment time at 35 and 130 kHz of irradiation.

Optimum density (OD)

In a pilot experiment, study confirmed the inhibitory potential of ultrasonic waves an undertaking sewage sludge and *E. coli* (supernatant/centrifuged) as an example. We observed that the OD of the ultrasonic treated samples of sewage sludge and *E-coli* were decreased at 20- 30 min of treatment as compared with control (Figure 2). Result also shows the effect was less at 5 and 10 min of treatment by comparing with control (untreated) samples. Study suggests that ultrasonic treatment was more effective at increased frequency and time.

Figure 3 and 4 shows the effect of ultrasound on the growth of *E. coli* and bacterial population in sewage sludge, where time dependent

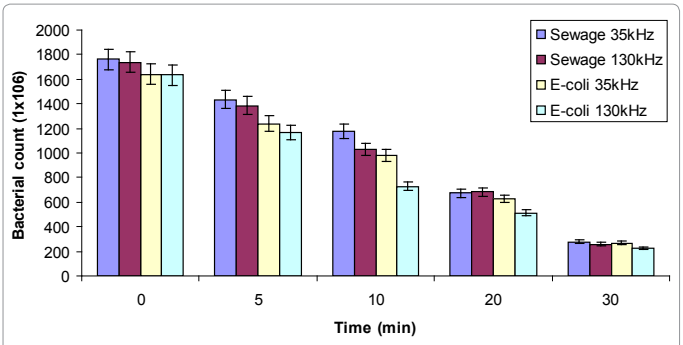

Figure 1: Average bacterial population in ultrasonic treated sewage sludge and *E-coli* samples at 35 kHz and 130 kHz at different time periods of 5–30 min.

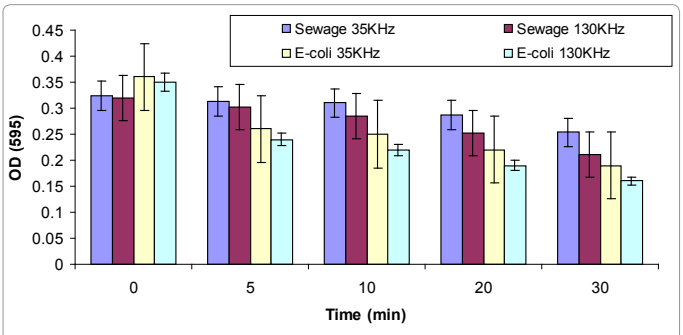

Figure 2: Growth curve of bacterial population survived in sewage sludge and *E. coli* treated with ultrasound at 35 and 130 kHz for the period of 5, 10, 20 & 30 min including control.

changes in bacterial growth are monitored by measuring OD at 595 nm (OD595). The measurement of OD was carried out at 595 nm to avoid strong absorption from bacterial cellular components like nucleic acids (A260), proteins (A280) and molecules present in the LB medium such as sugar and carbohydrate that might absorb at A400–500. OD was recorded at 595 nm is due to the scattering of light by the bacterial cells. It is a function of bacterial cell density which shows correlation with the growth of the colonies.

Scanning electron microscope (SEM)

The *E-coli* culture was treated with ultrasonic bath at 35 and 130 kHz for different time period. Result shows that the cavitational bubbles produced in ultrasonic bath decreased the bacterial cells showing ruptured shapes at 20 and 30 min of treatment. The effect was not very significant at 5 and 10 min of treatment as compared to control. The study also suggests that the bacterial population was decreased with an increased treatment time as well as frequency. The ruptured cells have been indicated in Figure 3 of (3e).

Chemical oxygen demand (COD)

Sewage sludge samples contain millions of micro organisms (bacteria). Ultrasonic shock waves break the microbial cell walls. The intracellular compounds (protein, enzyme, fat) are released in sludge aqueous phase resulting in an increase of chemical oxygen demand [24,25]. The assessment of cellular disruption was ascertained by measuring COD (mg/l) in the supernatant of treated samples. Due to sludge solid disintegration many organic and inorganic compound fixed to sludge solid released into liquid phase and hence COD (mg/l) of liquid phase increases (Figure 4). The maximum COD release was

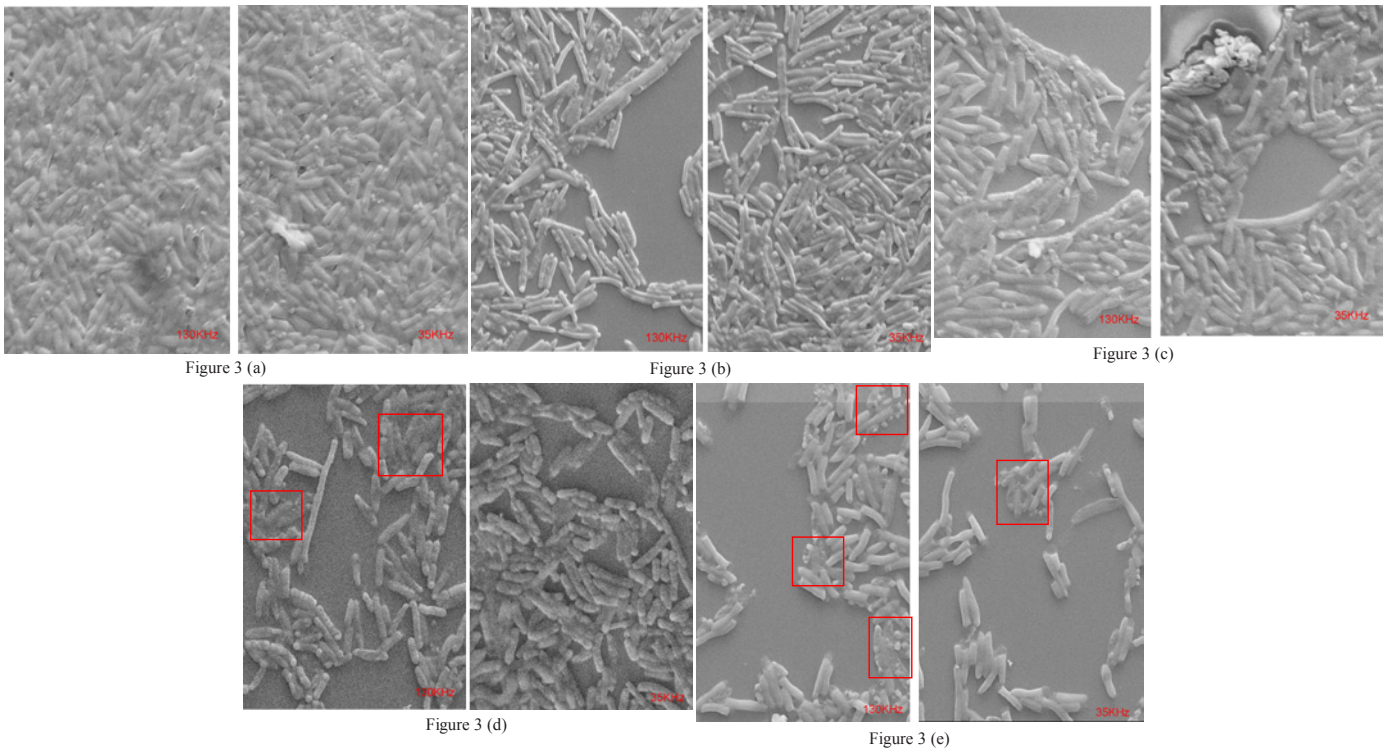

Figure 3 (a) Figure 3 (b) Figure 3 (c)

Figure 3 (d) Figure 3 (e)

Figure 3: Shows the SEM of *E-coli* bacterial cells after ultrasonic treatment: (5a) Control (without treatment) (5b) 5 min; (5c) 10 min; (5d) 20 min; (5e) 30 min. The figures labeled with 130 kHz and 35 kHz frequency of ultrasonic treatment. Results observed the more distorted cells at 130 kHz and as well as decrease in cells population. Red boxes indicate the ruptured cells.

observed at t_4 and the effect was more at 130 kHz compared with 35 kHz. More COD release was observed as treatment time increased. The extent of release of intracellular material due to bacterial cell lysis were measured by change in chemical oxygen demand (COD) of sludge supernatant and designated as DD_{COD} which relates the cell disintegration by ultrasound to maximum disintegration value obtained by standard chemical (alkaline) hydrolysis [8].

Degree of sewage sludge disintegration (DD_{COD})

The effect of ultrasound frequency on sludge disintegration was studied in order to find out the preferential pretreatment conditions. Within the range of explored frequencies between 35 and 130 kHz, the disintegration of sewage sludge was more effective at the 130 kHz. Similarly as treatment time were increased from t_2 to t_3 the COD of sample augmented abruptly due to increased mass transfer so DD_{COD} increased to its maximum value at t_4 (30 min) on both frequencies (Figure 5). We observed the highest degree of disintegration (DD_{COD}) at 130 kHz. An increase of the DD_{COD} was attributed to the breakup of microbial cells leading to the release of intracellular material. Hence we would expect the best disintegration results with the ultrasound frequency at 130 kHz. Since disintegration process is due to hydro mechanical shear forces produced by ultrasonic cavitation, so it depends upon frequency of waves and nature of medium (i.e. solid content, dissolve gas, density etc.)

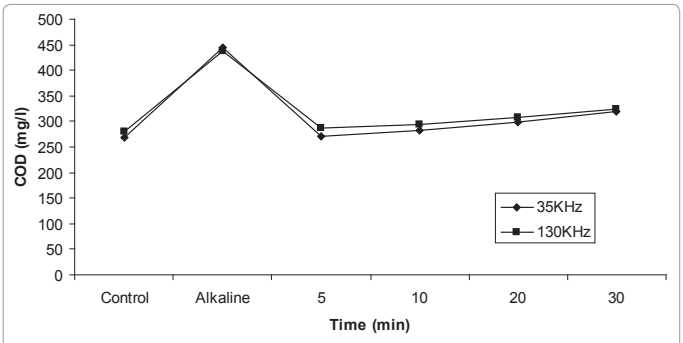

Figure 4: Shows a variation in COD at alkaline stage. The COD was observed an increasing with time and frequency. It was found higher at 20 and 30 min of treatment.

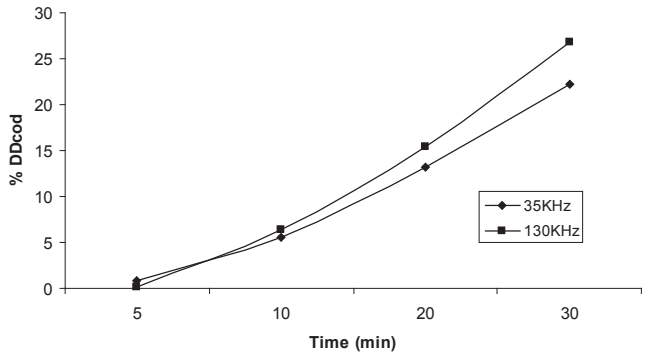

Figure 5: Shows the changes **in** Degree of degradation (DDCOD) of ultrasound-treated sludge sample on 30 min of treatment at 35 kHz and 130 kHz.

Reactive oxygen species (ROS)

Reactive oxygen species was done to confirm the ultrasonic effect

on bacterial cell disruption. This is an important parameter for the mechanism point of view. The result shows an increase in ROS was more at t_3 & t_4. We also observed that the ROS was increased with increased frequency and time (Figure 6). Our result in support of Bsoul et al. [26] has concluded that the H_2O_2 production at high frequency greater than that at low frequency. The study was performed with sewage sludge and *E-coli* strain. Result suggests that the ultrasonic waves may generate the reactive oxygen species by release of hydroxyl ion. Cavitational bubbles may generate the ROS which may attack the bacterial cell wall and cause disruption.

Discussion

Ultrasound in a liquid or sewage sludge leads to the acoustic cavitation phenomenon, such as the formation, growth, and collapse of bubbles, accompanied by the generation of local high temperature, pressure, and reactive radical species [27]. The possible mechanisms by which cells are rendered inviable during ultrasound irradiation include free-radical attack, including hydroxyl radical attack, and physical disruption of cell membranes [28]. Once the cell membrane is sheared, chemical oxidants can enter the cell and attack internal structures, or vital structures can be released from the cell, and degraded in solution. Furthermore, ultrasound irradiation can facilitate the disagglomeration of microorganisms and thus, increase the efficiency of other chemical disinfectants [28,29]. Rechards and Loomis [30] first reported on the chemical effects of high-power ultrasound. Where two types of chemical reaction are described: [1] the acceleration of conventional reactions by ultrasound and [2] redox processes in aqueous solution. In a liquid medium, the effect of ultrasound is produced due to phenomenon called cavitation [31]. The basis for ultrasound irradiation applications is that acoustic cavitation can affect a number of mechanical, acoustic, chemical and biological changes in a liquid [32].

Present study carried out the effect of ultrasound in sewage sludge and *E-coli*. Ultrasonic effect has been confirmed by enhanced reactive oxygen species which play a major role in destruction of cells. Presently, bacterial population count, optimum density and morphological changes have been observed with SEM. Moreover, degree of disintegration including COD has also been performed in sewage sludge. A mechanical approach of reactive oxygen species has been connected with *E-coli* degradation. The basic mechanism behind the disintegration of sewage and disruption of bacterial cells could be an attack of reactive oxygen species on cells and organic compounds. ROS are the chemical species with one unpaired electron derived from molecular oxygen. Molecular oxygen in the ground state, contain two unpaired electrons known as a triplet state having same spin of electron. During the excitation of electron by stress, ultrasound,

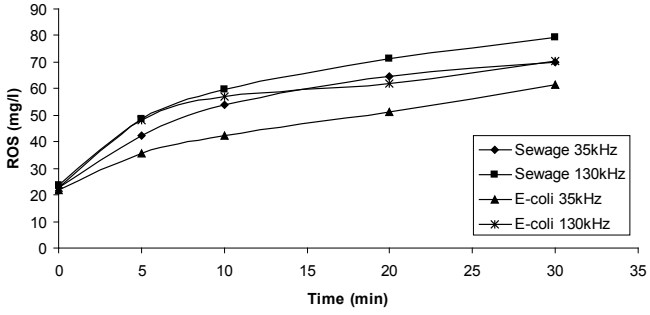

Figure 6: Influence of ultrasonic dose of 35 kHz and 130 kHz on ROS (mg/l) in sewage sludge and *E-coli* samples for different time periods of 5–30 min.

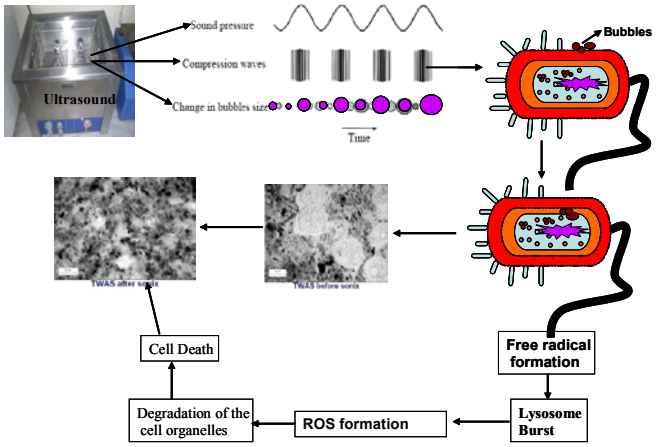

Figure 7: Interaction mechanism of reactive oxygen species with ultrasonic cavitational bubbles with bacterial population and distortion.

ultraviolet, nanoparticle, electromagnetic filed etc changes the spin of electron resulting in oxidant activity. Free radicals are highly reactive molecules with a very short half life. However in such time, they may cause damage to the cells. The balance between production and neutralization of ROS is maintained by concert action of enzymatic and non enzymatic defense systems. When unbalanced, it may lead to oxidation of poly-unsaturated fatty acids in lipids, amino acids in proteins and damage to DNA. ROS levels can increase dramatically, which may cause damage to cell structures and react with various biochemical reactions.

It is well established that the degree of disintegration (DD) of nonbiological solids, e.g., primary sludge and animal manure, are relatively easy to disintegrate compared to biological sludge. Rai et al. [33] and Tiehm et al. [23] adopted Mueller's method to determine the DD_{COD} in their ultrasonic study. In another study, Bougrier et al. [34] employed Mueller's method for DD_{COD} determination different samples. Although the literature shows a significant variation in the conditions for chemical disintegration, the overall goal of chemical disintegration is to obtain the maximum release of soluble organics from the sludge. Show et al. [35] and Singh et al. [18] experimentally proved that a sonication treatment could improve the sludge properties in terms of soluble chemical oxygen demand (SCOD) increase and particle size reduction. This data was taken as a baseline to elucidate the efficacy of ultrasonic disintegration. It is important to note that the conditions for testing chemical disintegration could vary depending on sludge type and TS content. Singh et al. [18] have done DD_{COD} at 25 kHz in different type of sludge samples (paper, sugar & sewage) and observed a significant disintegration of sludge samples with increasing time (20 & 30 min). Huan et al. [36] has also reported that sonication with low density and long duration was more efficient than sonication with high density and short duration for sludge disintegration.

Since the degree of disintegration is primarily based on COD determination, researchers argue that DD determination is rather slow in the range of a day and is also expensive due to the need of large numbers of COD sample analyses [37]. Therefore we proposed protein measurement as an alternative to DD_{COD} determination. In present study, correlation coefficients (R^2= 0.81) of *E-coli* and (R^2= 0.96) sewage sludge for increase in protein due to sonication were compared to evaluate the reliability of the new sludge disintegration assessment technique. Wang et al. [38] examined the release of protein,

polysaccharide, and deoxyribonucleic acid (DNA) in the aqueous phase during ultrasonic disintegration of WAS at different specific energy inputs. Protein was predominant in the aqueous phase of sonicated sludge. The release of soluble protein and carbohydrate COD_{Cr} in the aqueous phase during different sonication durations was also investigated by Wang et al. [39]. Our results on ultrasonic treatment of bacterial disruption was recorded by SEM, which could provide more thorough information on sludge disintegration particularly at the cellular level as depicted in Figure 3 (a) Prior to sonication, flocs entangled within large numbers of filaments were observed (Figure 3 (b-e). Another study of Venkatasubramanian and Rao [40] observed that power ultrasound acts as ecofriendly device in leather processing. It reduces time as well as requirement of conventional chemicals in some of the processing stages. They used ultrasonic frequency at 33 kHz and power output of 150 watt. The basic mechanism of ultrasonic irradiation was proposed in Figure 7. Study suggests that due to ultrasonic cavitations it may burst the lysosome and generate the reactive oxygen species within cells where it may destroy the bacterial cell wall. This may rupture the cells and completely cause cell death. Recently several authors have reported the treatment of different type of wastewater samples by ultrasonication, which is very effective at 25, 35 and 130 kHz [16-19]. Authors reported that the decrease in bacterial population and changes in biological and biochemical parameters in wastewater samples is due to ultrasonication, which acts effectively to improve the quality of wastewater.

Conclusion

Present study carried out on two different frequencies (35 kHz and 130 kHz) for different time periods (t_1= 5 min, t_2= 10 min, t_3= 20 min, t_4= 30 min). Study concludes that the 35 kHz & 130 kHz were very effective at 20 & 30 min of ultrasonic treatment but 130 kHz seen more effective as compared with 35 kHz. The present method is very effective and useful in process to sewage sludge. The observations of Mahvi [41] are in support of our present study that showed an increasing of sonication time and frequency has a significant effect on bacterial cells. The main outcome of our study is ROS, where it's enhanced level seen more in bacterial samples as compared with sewage sludge. This is a possible indicator of ultrasonication impact on sewage sludge disintegration and bacterial population. Present study is very economical and need to implement at large scale. The last but not least that this may be more useful for future research by combining ultrasound with nanotechniques (nanoparticles for treatment) as recently shown Kesari et al. [11].

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