ULTRASOUND INVESTIGATION STUDIES ON MICROBIAL ACTIVITY and CHEMICAL REACTIONS

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DECLARATION

I, B.ARUNIMA, M.TECH, COLLEGE OF TECHNOLOGY, OSMANIA UNIVERSITY, HYDERABAD do here by declare that the research work done in this thesis entitled "**ULTRASOUND INVESTIGATION STUDIES ON MICROBIAL ACTIVITY and CHEMICAL REACTIONS"** is the original work carried out by me under the supervision of Prof. **S.VENKATESWAR,** PRINCIPAL **,** COLLEGE OF TECHNOLOGY, OSMANIA UNIVERSITY, HYDERABAD. This work has not been submitted for any degree to any University prior to this date.

DATE: (B.ARUNIMA)

Acknowledgements

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ABSTRACT

The present study involves determining the effect of ultrasound on microbial cultures like ***Bacillus Subtilis*** and ***Escherichia coli*** and studying the kinetics of chemical reactions.

Sonication induces **cavitation phenomena** in liquids which proves lethal for the bacteria. The *Bacillus Subtilis* culture was initially subjected to Plate Count Technique without any treatment and the Colony Forming Units were noted. Then the culture was sonicated for different intervals of time and in each case the plate count technique was applied. It was observed that on increasing the sonication time, the bacterial kill increased.

Escherichia coli or *E.coli* which is a contaminant of drinking water is determined by Most Probable Number (MPN) method. Initially drinking water with a high *E.coli* was taken and subjected to sonication for different intervals of time and for each case, the MPN was determined using standard charts. It was observed that on increasing the sonication time, the removal efficiency increased.

Ultrasound is useful in determining the kinetics of a mixture of solvents by measuring ultrasonic velocities (v) which change from time of mixing and reach an equilibrium value only after the system becomes equilibrium mixture. Liquids like Aniline were mixed with neutral solvents like Ethyl acetate and n-octyl alcohol and using ultrasonic flaw detector, ultrasonic velocity was measured using **Pulse-Echo-Overlap (PEO**) method. Reactions were found to be of **first order** and were in good agreement with the literature values.

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ULTRASOUND INVESTIGATION STUDIES ON MICROBIAL ACTIVITY and CHEMICAL REACTIONS

CHAPTER1
INTRODUCTION

1. HISTORY:

The destruction of microorganisms by power ultrasound has been of considerable interest in recent years. Early research in the field can be traced back to the work of Harvey and Loomis in 1929, which examined the reduction in light emission (related to bacterial kill) from a seawater suspension of rod shaped Bacillus Fisheri caused by sonication at 375kHz at 19^0C. But this method was ruled out as it was not considered of practical or commercial importance because of the expense of the process. Today that situation has changed, ultrasonic technology is more common place, costs have been reduced and applications are more economic. Power ultrasound can now be considered to be a viable alternative to conventional bactericidal techniques.

Several investigations have been carried out to study the inactivation effect of ultrasound, and ultrasound combined with other agents. At the early 70's, it was demonstrated that a simultaneous heat and ultrasound treatment (thermoultrasonication) had a higher lethal effect than a heat treatment at the same temperature. At the early 90's, the Food Technology group of the University of Zaragoza designed an equipment called Mano-Thermo- Sonicator. With this equipment, the microbial inactivation effect of ultrasound and its combination with pressure (Mano-Sonication; MS) or with pressure and heating (Mano-Thermo-Sonication;MTS) was investigated.

2. PRINCIPLE OF ULTRASOUND:

Humans can normally hear sound frequencies between 20 and 20,000 Hz (20 kHz). When a sound wave's frequency lies above 20 kHz, it is called an ultrasonic wave. Ultrasound is generated similarily to regular sound, using a transducer to convert electrical or magnetic energy into waves. Piezolelectric transducers are the most popular and versatile transducers. When a crystal having piezoelectric properties (such as Quartz or Rochelle salt) is subjected to changes in pressure, it becomes electrically charged. The varying current produced by the crystal can be read, and translated into the waveforms of the waves hitting it. The converse is also true, which allows the crystal to transduce ultrasonic waves.

3. INFLUENCE OF VARIOUS FACTORS ON MICROBIAL ACTIVITY BY ULTRASOUND:

Microbial inactivation by ultrasound depends on several factors that can be classified under

1) TREATMENT CONDITIONS,
2) MICROBIAL CHARACTERISTICS AND
3) ENVIRONMENTAL FACTORS.

1) TREATMENT CONDITIONS

Time and amplitude of the ultrasonic treatment, and hydrostatic pressure and temperature of the medium are the main factors that influence on the microbial inactivation by ultrasound.

Treatment time

Ultrasonic microbial inactivation increases with the treatment time. An exponential relationship between the survival fraction and the treatment time was observed. Based on this, the microbial resistance to ultrasonic waves can be estimated from the decimal

reduction time (D value) that is defined as minutes of treatment at a given temperature needed for the number of survivors to drop one log cycle.

Hydrostatic pressure

Yeast, bacterial vegetative cells and spores activation by ultrasound increases with the hydrostatic pressure of the treatment medium. The inactivation effect of ultrasound increased exponentially with pressure in the range of 0 to 200-300 kPa. Over 300 kPa, new increments of the pressure hardly improved the inactivation effect. The influence of the hydrostatic pressure on the lethal effect of ultrasonic waves is related to the effect of pressure on the cavitation phenomena. When the pressure is raised, the bubble implosion intensity increases and the number of bubbles implosion per unit of time is reduced.

Therefore, although at higher pressures the intensity of the implosion is stronger, the lower number of bubbles collapsing would explain the decrease of the efficiency of the ultrasound lethal effect over a determine pressure value.

Ultrasonic amplitude

It has been observed that the inactivation of both bacterial vegetative cells and spores exponentially increases with the amplitude of the ultrasonic waves. The higher inactivation rate at greater amplitudes could be due to an increase in the number of bubbles undergoing cavitation per unit of time or to an increase in the volume of liquid in which cavitation is liable to occur.

Temperature

In order to reduce the intensity of heat treatments, the microbial inactivation effect of combining treatments of ultrasound under pressure and heat (Mano-Thermo-Sonication) has been investigated. An additive effect of this combination has been observed on the inactivation of most of vegetative cells investigated and a synergistic effect for Streptococcus faecium and for Bacillus subtilis spores.

2) MICROBIAL CHARACTERISTICS

Microorganism

It is generally admitted that cells of a bigger size are more sensitive to ultrasound and rodshaped bacteria more than coccal forms. The Gram-positive are more resistant than Gramnegative, aerobic more than anaerobic species and bacterial spores more than vegetative cells. In spite of the different resistance of vegetative cells to ultrasound, the influence of the hydrostatic pressure and the ultrasonic amplitude on the inactivation effect of these treatments is independent of the microorganism.

Growth phase

The influence of the growth phase on the microbial resistance to ultrasonic waves is not clear. While some authors have found out a non-influence of this factor on the inactivation effect of ultrasound, others have observed that cells of E. coli at the exponential growth phase were more sensitive to ultrasonic treatments than that at the stationary phase.

Growth temperature

Regardless of the influence of this factor on the microbial inactivation by heat or high hydrostatic pressure, the resistance of different microorganisms, such as Listeria monocytogenes and Yersinia enterocolitica to ultrasonic treatments, was independent of the growth temperature.

3) ENVIRONMENTAL FACTORS

pH: Commonly, microbial heat resistance decreases in acidic media. However, different authors have observed that the ultrasonic resistance of microorganisms was independent of the acidity conditions of the medium.

a_w (water activity): It is well known that reduced water activities influences in a great extent the microbial thermal resistance . A decrease of the **a_w** also increases the

microbial resistance to ultrasound, however this increment is lower.

MECHANISM OF ACTION OF ULTRASOUND

The lethal effect of high power ultrasound is due to the **cavitation phenomena**. When bubbles implode in an ultrasonic field, high temperatures and pressures are generated at the collision point. When a bubble implodes, heat is generated in the liquid surrounding the cavity. The volume of liquid heated is very small and the heat dissipates quickly, though the temperature of this region is extraordinarily high for a few microseconds.

Therefore heat, pressure shock waves, or both could be the responsible for the lethal effect of ultrasound. On the other hand, the very high temperatures and pressures of collapsing bubbles lead to generate free radicals such as H. and OH.. These very reactive radicals could be responsible for the inactivation of bacterial cells by oxidative damage similar to that caused by hydroperoxides.

4. APPLICATIONS:

While we cannot hear ultrasonic waves, we apply them in various technologies such as sonar systems, sonograms, surgical tools, and cleaning systems. Some animals also use ultrasonic waves in a specialized technique called echolocation that allows them to pinpoint objects and other animals, even in the dark.

1) Ultrasonic disintegration

Some sorts of ultrasound can disintegrate biological cells including bacteria. This has uses in biological science and in killing bacteria in sewage. High power ultrasound at frequency of around 20 kHz produces cavitation that facilitates particle disintegration.

2) Sonochemistry

Power ultrasound in the 20-100 kHz range is used in chemistry. The ultrasound does not interact directly with molecules to induce the chemical change, as its typical wavelength (in the millimeter range) is too long compared to the molecules. Instead:

-It causes cavitation which causes local extremes of temperature and pressure in the liquid where the reaction happens.

-It breaks up solids and removes passivating layers of inert material to give a larger surface area for the reaction to occur over.

Both of these make the reaction faster

3) Industrial ultrasound

Ultrasonic testing is a type of nondestructive testing (NDT). Non destructive evaluation (NDE) is commonly used to find flaws in materials and to measure the thickness of objects. Frequencies of 2 to 10 MHz are common but for special purposes other frequencies are used. Inspection may be manual or automated and is an essential part of modern manufacturing processes. Most metals can be inspected as well as plastics and aerospace composites. Lower frequency ultrasound (50 kHz to 500 kHz) can also be used to inspect less dense materials such as wood, concrete and cement.

Ultrasound can also be used for heat transfer in liquids.

4) Ultrasonic cleaning

Ultrasonic cleaners at frequencies from 20-40 kHz are used to clean jewellery, lenses and other optical parts, watches, dental instruments, surgical instruments and industrial parts. An ultrasonic cleaner works mostly by energy released from the collapse of millions of microscopic cavitations near the dirty surface.

5) Biomedical ultrasonic applications

Ultrasound also has therapeutic applications, which can be highly beneficial when used with dosage precautions . It is useful in treating benign and malignant tumors, in the detection of abnormalities etc.

CHAPTER 2

REVIEW OF LITERATURE

5. ULTRASONIC DISINTEGRATION ON BACTERIA

Ultrasound is able to inactivate bacteria and deagglomerate bacterial clusters or flocs through a number of physical, mechanical and chemical effects arising from acoustic cavitation. On collapse, cavitation bubbles produce enough energy to mechanically weaken or disrupt bacteria or biological cells via a number of processes.

__ Forces due to surface resonance of the bacterial cell are induced by cavitation. Pressures and pressure gradients resulting from the collapse of gas bubbles which enter the bacterial solution on or near the bacterial cell wall. Bacterial cell damage results from mechanical fatigue, over a period of time, which depends on frequency.

__ Shear forces induced by microstreaming occurs within bacterial cells.

__ Chemical attack due to the formation of radicals (H. and OH.) during cavitation in the aqueous medium. These radicals attack the chemical structure of the bacterial cell wall and weaken the cell wall to the point of disintegration.

__ Amongst the final products of this sonochemical degradation of water is hydrogen peroxide (H2O2), which is a strong bactericide. Sonication alone can provide powerful disinfection.

However, to achieve 100% kill rates using only ultrasound it is necessary to use high ultrasonic intensities. This makes the technique expensive to use for general large-scale decontamination but nevertheless there is a drive towards the use of ultrasound in decontamination as an adjunct to a bactericide and in conjunction with other techniques.

Effect of Ultrasound on *BACILLUS*

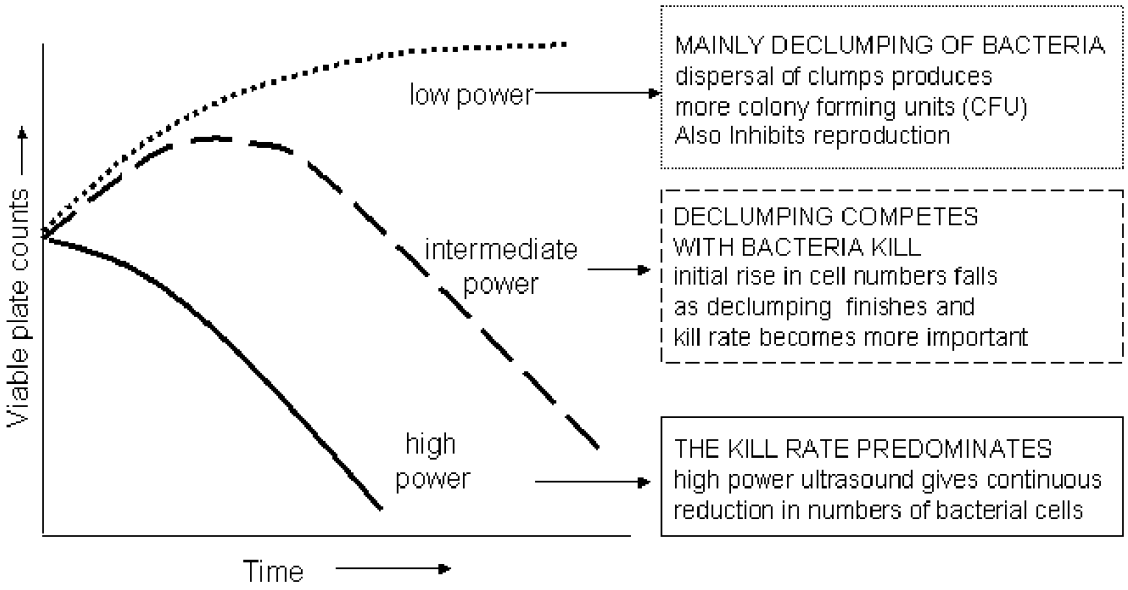
The effects of ultrasound on cell destruction and inactivation were monitored using standard plating out techniques. The plate counts revealed the viable Colony Forming Units (CFU's) in the sample however the CFU can be a single cell or a group of cells. In the absence of bactericide a low kill rate was obtained and these results are quoted as % remaining CFU. In the presence of a bactericide substantially higher kill rates result and

in these cases the results are quoted as log[CFU/ml]. A reduction of viable cells from 100% to 10% is equivalent to a single log reduction and industry normally considers that a five log reduction is an acceptable level of bacterial kill.

Sonication has two general effects on suspensions of bacteria.

- The first is bacterial declumping which breaks up bacterial agglomerates into a greater number of individual bacteria in a suspension, and the second is bacterial killing (or deactivation) which results in less individual bacteria capable of reproduction being present in a suspension. The overall effect of applying ultrasound is thus the result of a competition between declumping and deactivation of bacteria in solution. To improve the biocidal effects of sonication alone a biocide can be used. In the case of chlorination, the biocidal effect of high frequency (850kHz) ultrasound on suspensions of Escherchia coli is better when a short period of sonication is applied followed by normal biocidal treatment.
- On the other hand at lower frequencies (20kHz) better results are obtained using a short period of sonication at applied at the same time as chlorination. Either option gives a similar improvement in kill but the pre-treatment at low power and high frequency is the more effective in terms of acoustic energy input. For effective decontamination using a single pass of the contaminated system through a sonicated processor, in the presence of a biocide, large energies at lower frequencies (20 to 40 kHz) would be required. In the presence of a biocide high energies would also be required for large throughputs. It should be noted however that in a closed loop system rapid kill is not so necessary and a slow rate of deactivation can bring down contamination to a low level over a period of time. This is acceptable since continued low-energy sonication will then maintain that level without the need for further additions of biocide.

THE EFFECTS OF SONICATION ALONE ON THE SURVIVAL OF BACTERIA IN WATER



EFFECT ON *E.COLI*

Escherichia coli or ***E. coli*** is a type of fecal coliform bacteria commonly found in the intestines of animals and humans. The presence of E. coli in water is a strong indication of recent sewage or animal waste contamination. Sewage may contain many types of disease-causing organisms.

Sewage may contain many types of disease-causing organisms. Water can be contaminated in a variety of ways. Main sources of E. coli are municipal sewage discharges or runoff from failing septic systems; animal feed operations, farms and faeces deposited in woodlands from warm-blooded animals. In urban areas, the E. coli from the excrement of warm blooded animals (such as pets in a park or on the street) may be washed into creeks, rivers, streams lakes, or groundwater during rainfalls or snow melts. The contamination in water is often highest immediately following a storm, because of the runoff. In addition, infected bathers can unknowingly contaminate water, or contamination can occur from boaters discharging wastes directly into the water. When these waters are used as sources of drinking water and the water is not treated or inadequately treated, E. coli may end up in drinking water.

The water can be treated using chlorine, ultra-violet light, or ozone, all of which act to kill or inactivate E.coli. Systems using surface water sources are required to disinfect to ensure that all bacterial contamination is inactivated, such as E. coli. Systems using ground water sources are not required to disinfect, although many of them do.

Several studies have shown that the efficiency of disinfection technique is dependant on the concentration of suspended solids because suspended solids can protect bacteria from being destroyed by disinfectants. For example, the efficiency of ultraviolet irradiation is

affected by high concentrations of suspended matter. Also, chlorine is traditionally used for disinfection. With the use of chlorine a possibility exists that byproducts may form, which are potentially toxic and carcinogenic. Due to these problems alternative disinfection techniques are being evaluated and the benefits of the use of ultrasound in the water industry are now of considerable interest. The one way to inactivate Ecoli is with ultrasound. When liquids are exposed to these vibrations, both physical and chemical changes occur as a result of a physical phenomenon, known as cavitation. Cavitation is the formation, expansion and implosion of microscopic gas bubbles in liquid as the molecules in the liquid absorb ultrasound energy. Compression and rarefaction waves rapidly move through the liquid media. If the waves are sufficiently intense they will break the attractive forces in the existing molecules and create gas bubbles. As additional ultrasound energy enters the liquid, the gas bubbles grow until they reach a critical size. On reaching a critical size, the gas bubbles implode or collapse. The energy that exists within the cavity and in the immediate vicinity of the gas bubbles just before collapse causes both physical and chemical effects in the liquid. Physical effects result when cavitation is intense enough to rupture cell membranes, free particulates from solid surfaces and destroy particles and organisms through particulate collisions or by forcing them apart. In this study, the major objective was determining the effectiveness of ultrasound waves on the destruction of E. coli in water (T.J. Mason, H.Duckhouse, E Joyce and J.P.Lorimer (2003)).

6. EFFECT ON CHEMICAL REACTIONS:

Kinetics deals with the rate of chemical reaction, with all factors which influence the rate of reaction and with the explanation of the rate in terms of the reaction mechanism. Chemical kinetics might very well be called chemical dynamics.

The science of chemical kinetics may be of interest in itself, as for example in determining how changes in environment change the rate of a given reaction. In fact, kinetics provides the most general method of determining the mechanism of reaction.

From classical point of view, mechanism of reaction is understood to mean all the individual collision or other elementary process involving molecules that take place simultaneously or consecutively in producing the observed overall reactions. In general, the experimental results of studying the reaction as a function of concentrations, temperatures and other operating variables can be interchanged in several ways.

Ultrasound is a useful tool in nearly every case where a liquid and a solid must react. Furthermore, since ultrasound can radiate through large volumes of liquid, it is well suited for industrial applications. For these reasons future applications of ultrasound in chemical reactions will be in diverse. In the synthesis of pharmaceuticals, ultrasound will improve chemical yields over conventional methods.

Ultrasound has been found to have profound influence on many reactions. It can accelerate reactions, permit the use of unpurified solvents and reagents, replace phase transfer catalysts permit the reaction to occur under milder conditions and in some cases, even change the reaction pathways.

7. SCOPE OF THE PRESENT WORK

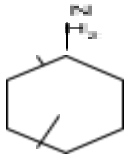
Kinetics studies involve essentially rate measurements. The important use of rate measurements is that, it throws much light on the mechanistic pathway involved in reactions. It also clarifies how the rates are affected by change in structure of molecules taking part in chemical reactions. This information is relevant for futuristic understanding of many reactions hitherto not discovered. The rate measurements lead to better understanding of molecular structure in chemical reactions. Kinetics has been recently used in bio system involve enzymes. The above information clearly establishing the importance of kinetic study in understanding changes due to structural variations and solvent variations.

It was of interest to study the kinetics of mixing of solvents by measuring ultrasonic velocities which change from time of mixing and reach an equilibrium value after the system becomes equilibrium mixture. These changes in ultrasonic velocities on mixing of solvents are useful in understanding their physical changes when the two solvent molecules come nearer by intermolecular hydrogen bonding and other physical forces like Vander wall forces or by salvation phenomenon of one molecule getting solvated more in relation to other molecule due to structural differences.

8. SAMPLES USED FOR THE STUDY

Aniline

Aniline is colourless liquid and its boiling point is 184^0C. It has an unpleasant odour and is poisonous. When exposed to air, it rapidly darkness, since it is very sensitive to oxidation.



Aniline
($C_6H_5NH_2$)

Chemical formula of Aniline is $C_6H_5NH_2$. Molecular weight of Aniline is 93.13 and its density at room temperature (i.e. at 25^0C) is 1.0576 gm/cc. Refractive index is 1.5418. Melting point and boiling point of Aniline are 40.9^0C and 181.8^0C respectively.

Ethyl acetate

Ethyl acetate is an aliphatic Ester and is useful as solvent for nitrocellulose, varnishes and aero plane dopes. It is extensively using in textile industry for cleaning purpose.

Chemical formula of Ethyl acetate is $CH_3COOC_2H_5$ molecular weight is 74.08. Density of Ethyl acetate is 0.9342 gm/cc and refractive index is 1.3619. Melting point of Ethyl acetate is -98.1^0C and boiling point is 56.3^0C.

BASIC INFORMATION OF RATE OF REACTION

The reaction rate for a reactant or product in a particular reaction is defined as the amount per unit volume that is formed or removed.

Knowledge of these rates is essential in chemical engineering, environmental engineering.

aA + bB □ cC + dD

For the reaction, the definition of the reaction rate 'k' is such that, it is independent of the product or reactant that is followed in time.

$$v = -\frac{1}{a}\frac{d[A]}{dt} = -\frac{1}{b}\frac{d[B]}{dt} = \frac{1}{c}\frac{d[C]}{dt} = \frac{1}{d}\frac{d[D]}{dt}$$

Where [A] and [B] represent the concentration of the reactants. Rate is often expressed in the units mole/sec.

INFLUENCE OF PARAMETERS ON REACTION RATES

(i) TEMPERATURE

Conducting a reaction at a higher temperature puts more energy into the system and increases the reaction rate. The influence of temperature is described as the Arrhenius equation, whose result is factored into the equation by 'k'. As a rule of the thumb, the reaction rate doubles for every 10 degrees Celsius increase in temperature.

(ii) CONCENTRATION

As reactant concentration increases the frequency of collision increases and so therefore does the frequency of collisions having sufficient energy to cause reaction.

(iii) PRESSURE

The rate of gaseous reactions usually increases with an increase in pressure. Increase in pressure in fact is equivalent to an increase in concentration of the gas.

(iv) LIGHT

Light is a form of energy. It may affect the rate of even course of reaction. For example, when methane reacts with chlorine in dark, the reaction rate would be very low. It can be speeded up when the mixture is put under diffused light. In bright sunlight, the reaction is explosive.

(v) ORDER

Clearly the order of reaction has a major effect on its rate. The order of a reaction is found experimentally and for most basic reactions is an integer value.

(vi) CATALYST

The presence of a catalyst increases the reaction rate in both the forward and reverse reactions by lowering the activation energy of the reaction.

(vii) THE NATURE OF THE REACTANTS

Reactants may form a complex with large equilibrium constant or low equilibrium constants. The nature of the complex influences bond breaking and bond formation processes resulting in the product formation.

(viii) ULTRASOUND

Ultrasound has been utilized for studying ultrasonic reactions because the course of reaction is affected by ultrasonic waves just like other parameters

9. Method for determination of Reaction Rate by Ultrasonic Velocity Measurement:

In an ideal mixture in which the components are non-interacting, the variation of velocity **(v)**, density **(ρ)** and viscosity **(η)** with the concentration is expected to be **linear**.

The changes in ultrasonic velocities (**v**) are determined, when liquids like Aniline are mixed with neutral solvents like Ethyl acetate and n-octyl alcohol. It appears that, solvents on mixing with each other affect the flow of ultrasonic waves till the two liquids reach equilibrium. This means that, the ultrasonic waves move with different velocities till the equilibrium is obtained. Hence, it was of interest to study the variation in ultrasonic velocity (**v**) with time (**t**) and find out the nature of the **kinetic process**. It is found that, ultrasonic velocity (**v**) changes during mixing of solvents and these changes seems to be depend upon the nature of the components in solvent mixture. The interesting finding is that, these mixing process are **first order** in the reactive component of the binary mixture. The first order rate constants have been computed by using the following expression and from ***log (v_∞-v_t) vs. time (t)*** plots.

$$k = \frac{2.303}{t}\log\left(\frac{v_\infty - v_o}{v_\infty - v_t}\right)$$

CHAPTER 3
MATERIALS AND METHODS

10. Experimental Notes

ON *BACILLUS*

Nutrient broth preparation:

1.25 gms of Peptone , 1.5 gms of glucose are taken into a 250 ml conical flask.To this 5ml of salt solution and 95 ml of distilled water are added.

This flask is sealed with a cotton plug and sterilized.

After the sterilized flask gets cooled it is taken to Laminar Air Flow Chamber and 3 ml of *Bacillus* innoculum is added. This flask is plugged and incubated at 27^0C for 48 Hrs.

After incubation the culture is stored in a refrigerator.

Nutrient Agar Preparation:

1.3 gms of nutrient broth and 1.5 gms of Agar is taken into a 250 ml conical flask and 100ml of distilled water is taken in it. This flask is sealed with a cotton plug and along with petric plates kept for sterilization. After sterilization in the laminar flow chamber the agar prior to its cooling is poured into petric plates and allowed to cool for solidification. The petric plates are allowed to remain in the laminar chamber for 24 hrs and checked for any contamination.

Spread Plate Technique:

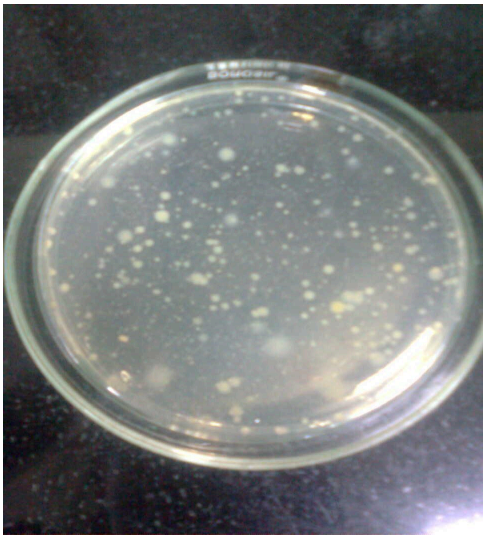
The petric plates after 24 hrs, if no contamination, is subjected to spread plate technique. For this in the laminar flow chamber 0.1 ml of the prepared *Bacillus* culture is transferred into the petric plate with the help of micro pipette and with a glass rod spread over the plate. The plate is sealed and kept upside down in incubator at incubated at 37^0C for 48 hrs. This gives the plate count for pure *Bacillus* culture.

10 ml of *Bacillus* culture is taken in a sample bottle and 36 KHz ultrasound waves are passed for different intervals of time viz 0 sec (control) , 5 sec, 10 sec, 20 sec….60sec. These sonicated samples are subjected to spread plate technique in petric plates and

incubated at 37^0C for 48 hrs. After Incubation the petric plates are subjected to plate count technique and the colony forming units are counted. The results are tabulated in Table 2.

The Sonicated time was increased 0.1 ml of the *Bacillus* culture was taken and the above said procedure was repeated for 1 min, 2 min, 3min……..6 min. and tabulated in Table 3.

Spread Plate Technique:



ON *E.COLI*

Ultrasound Batch Reactor: Ultrasound was applied to samples using a Laboratory cleaning bath with the following characteristics:

Input: 220-230V 155W

Output:80 W 36 KHz

Experiment: Microbiological experiments involved sonicating of E. coli and observing the effects of ultrasound upon its growth. Before sonication, the concentration of E. coli in water was adjusted to as high as 1600 (MPN 100 mL_1). This sample was added to the batch reactor in which sonication could be performed. For micro-organisms destruction investigation in ultrasound bath, small volume (300 mL) of water has been used. All

components in laboratory placed in an autoclave for disinfection before each test. The effect of sonicating different volumes of water was measured for the same time intervals. The samples were sonicated in periods of 0, 20, 30, 40, 60, 70 and 90min. The standard test for E. coli carried out by the multiple – tube fermentation technique in research. In this test, results of the examination of replicate tube and dilutions are reported in terms of the Most Probable Number (MPN) of organisms present. The precision of each test depends on the number of tubes used. Here drinking water is analyzed for E.coli.

The fermentation technique is used for 10 replicate tubes each containing 10 mL, 5 replicate tubes each containing 20mL of the EC medium. These tubes are sterilized in an autoclave and cooled in a laminar air flow chamber. 10 mL of drinking water sample is added to each of 5 replicate tubes each containing 20mL of the EC medium To one set of 5 replicates containing 10 mL, 1 ml of drinking water sample is added and to other 5

replicates 0.1 mL drinking water sample is added. Inoculated EC broth tubes were incubated in a water bath at 44.5±0.2°C for 24±2h. All EC tubes were then placed in a water bath within 30 min after inoculation. Maintain a sufficient water depth in water bath incubator to immerse tubes to upper level of the medium

All presumptive fermentation tubes showing any amount of gas, growth, or acidity within 24 h of incubation to the E. coli test were reported.. Failure to produce gas constitutes a negative reaction. If multiple tubes are used, MPN is calculated from the number of positive EC broth tubes as described in standard methods book (T.J. Mason, H.Duckhouse, E Joyce and J.P.Lorimer (2003)).

SONOCHEMISTRY:

Ultrasonic Studies in the Mixture of Aniline and Ethyl acetate at 30^0C Temperature

Ultrasonic velocity (**v**) changes with time (**t**) (have been determined for various percentages of Ethyl acetate and Aniline at 30^0C temperature. ***log*** $(v_\infty - v_t)$ ***vs. time (t)*** plots) are also in good agreement with the observed first order rate constants (**k**). Table-shows that, there is an **increase** in the first order rate constants of Aniline, when the percentage of Aniline is increased, the other solvent being neutral in character namely Ethyl acetate. Increase in rate constants with increase in percentage of Aniline is an **important** feature in these studies. When the solution of binary mixture become more basic, the **first** order rate constants also increased. This means that, the solvent environment is more basic in nature, the movement of ultrasonic waves seems to be ***freer*** or in otherwords ultrasonic velocity (**v**) is larger when acidity is lesser. This in tune with the earlier postulates reported by Ranganayakulu, S.V et al (2005).

VELOCITY MEASUREMENT

The binary liquid is taken into the glass vessel by using 50ml pipette and transducer of 2 MHz frequency is immersed into the liquid. The corresponding ultrasonic velocity (v) is recorded directly from the display of the instrument

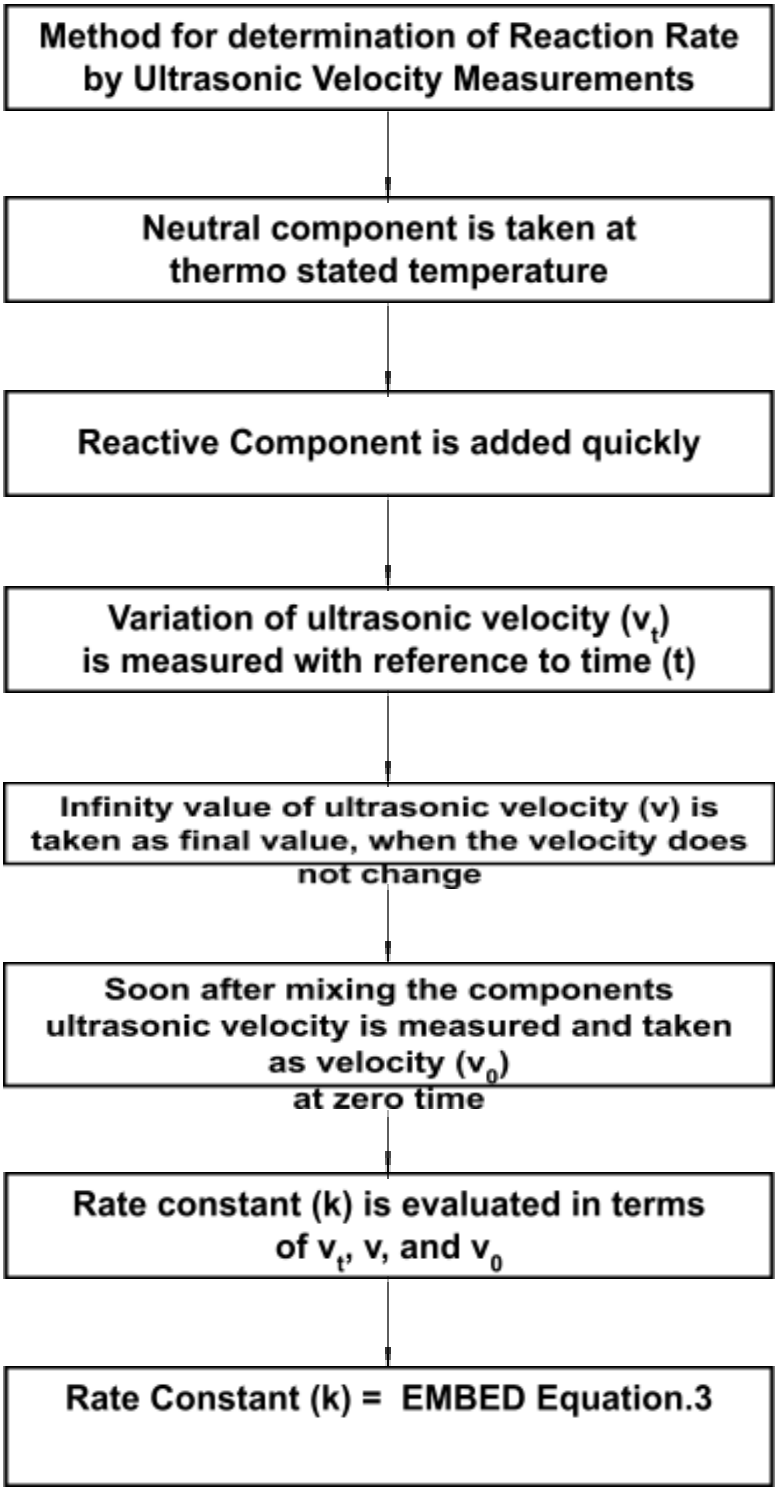


Fig 1 Pictorial diagram for determination of Reaction Rate

Table. 1 Comparison of experimentally measured values of ultrasonic velocities with literature values at 38^0C temperature

S. No	Name of the pure sample	Experimental Values Velocity (v) m/s	Literature Values Velocity (v) m/s
1	Water	1512	1512
2	Ethyl acetate	1203	1198
3	Methyl acetate	1101	1106
4	Benzene	1294	1292

Ultrasonic Flaw detector:

CHAPTER 4
RESULTS AND DISCUSSIONS

Table 2. Effect of sonication on *Bacillus* from 0-60 sec:

Sonicated time(Sec)	colony forming units(CFU'S)	Log(CFU's/ml)	%Reduction
0	1566	4.19	0
5	1112	4.04	3.58
10	354	3.54	15.51
20	248	3.39	19.09
30	131	3.11	25.77
40	46	2.66	36.51
60	35	2.54	39.38

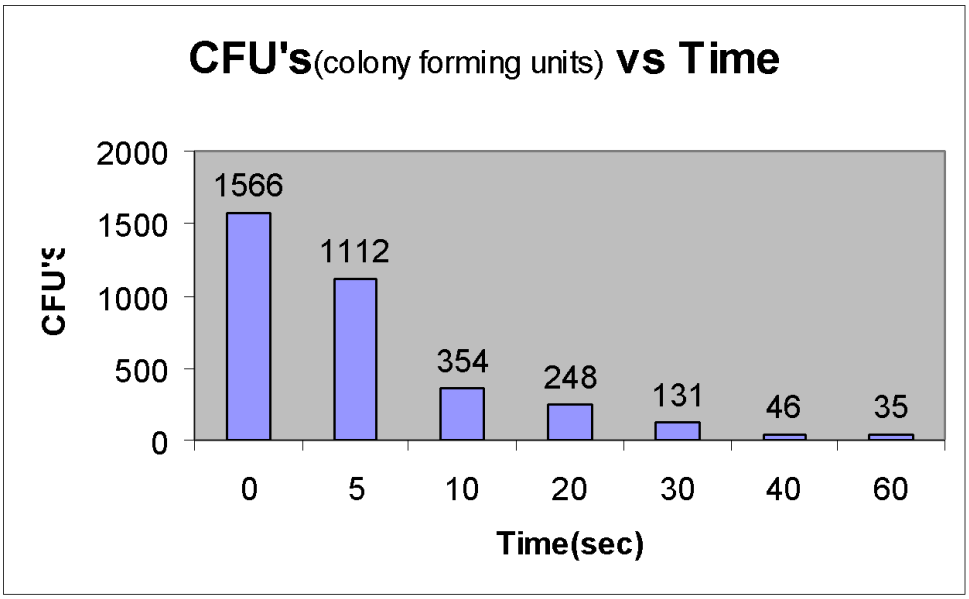


Fig 2. Colony forming units (CFU's) vs time from 0-60 sec

Table 3. Effect of sonication on *Bacillus* from 0-6 min:

Sonicated time (min)	colony forming units(CFC'S)	log(CFU's/mL)	% Reduction
0	174	3.20	0
1	28	2.44	23.75
2	79	2.89	9.68
3	26	2.41	24.68
4	18	2.25	29.68
5	12	2.07	35.31
6	0	-	100

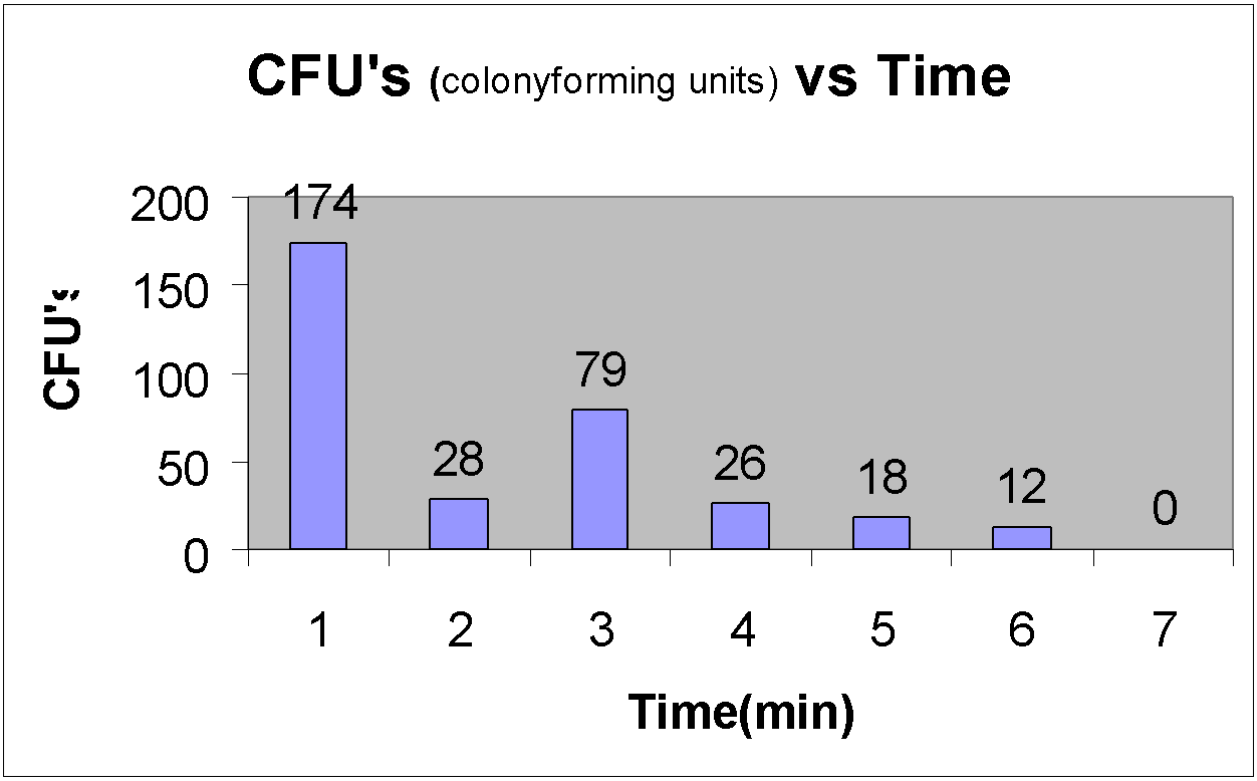


Fig 3. Colony forming units (CFU's) vs time on sonication from 0-6 min

25

ON *E.coli*

Table. 4 -MPN (Most Probable Number) for Various Combinations of Positive Results When Five Tubes Are Used Per Dilution (10 mL, 1.0 mL, 0.1 mL)

Combination of Positives	MPN/100 mL	Combination of positives	MPN /100 mL
0-0-0	<2	4-2-0	22
0-0-1	2	4-2-1	26
0-1-0	2	4-3-0	27
0-2-0	4	4-3-1	33
		4-4-0	34

1-0-0	2	5-0-0	23
1-0-1	4	5-0-1	30
1-1-0	4	5-0-2	40
1-1-1	6	5-1-0	30
1-2-0	6	5-1-1	50
		5-1-2	60
2-0-0	4	5-2-0	50
2-0-1	7	5-2-1	70
2-1-0	7	5-2-2	90
2-1-1	9	5-3-0	80
2-2-0	9	5-3-1	110
2-3-0	12	5-3-2	140
3-0-0	8	5-3-3	170
3-0-1	11	5-4-0	130

3-1-0	11	5-4-1	170
3-1-1	14	5-4-2	220
3-2-0	14	5-4-3	280
3-2-1	17	5-4-4	350
4-0-0	13	5-5-0	240
4-0-1	17	5-5-1	300
4-1-0	17	5-5-2	500
4-1-1	21	5-5-3	900
4-1-2	26	5-5-4	1600
		5-5-5 >1600	--

(British Columbia Environmental Laboratory Manual, 2007)

The biocidal effects of ultrasound are showed in Table 5.

Table 5: The Effect of Ultrasound on Removal of E. coli (Removal efficiency)

Ultrasound time (min)	No. of +ve tubes	M PN/100 mL	Removal efficiency (%)
0	5-5-5	1600	0.0
20	5-5-3	280	82.50
30	5-4-1	170	89..37
40	5-1-1	50	96.87
60	4-1-0	17	98.93
70	4-0-0	13	99.18
90	1-0-0	2	99.88

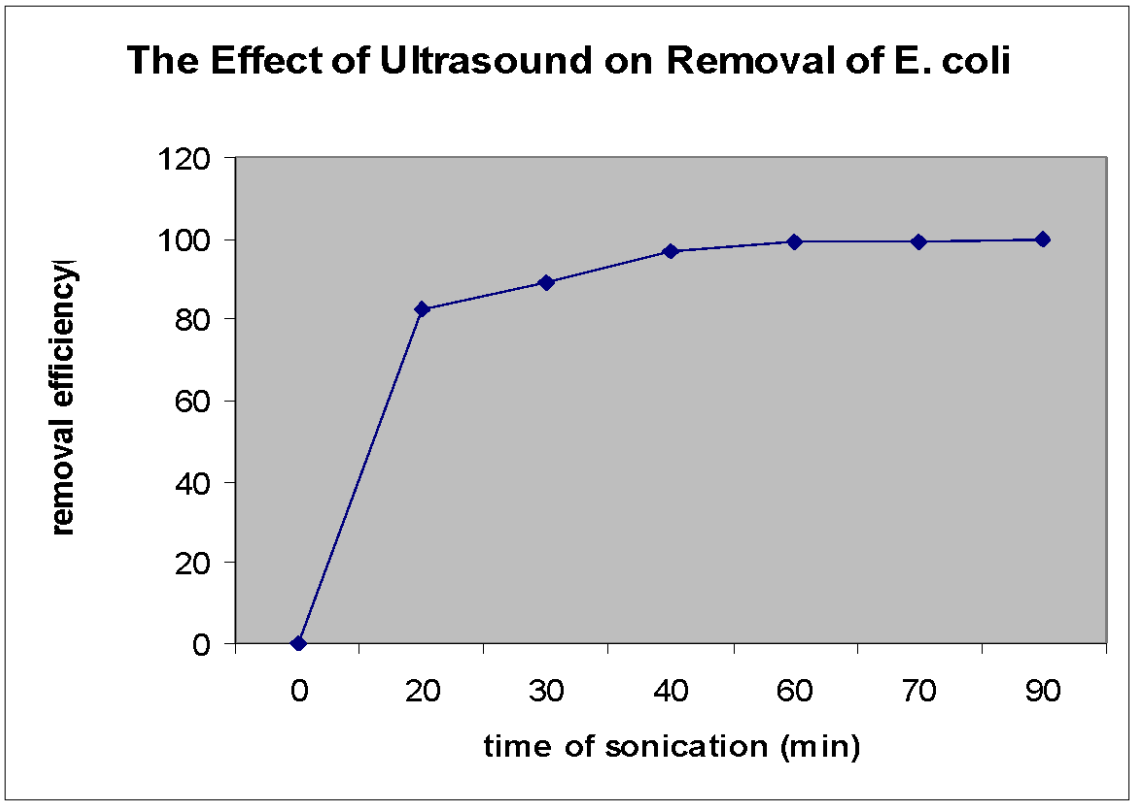
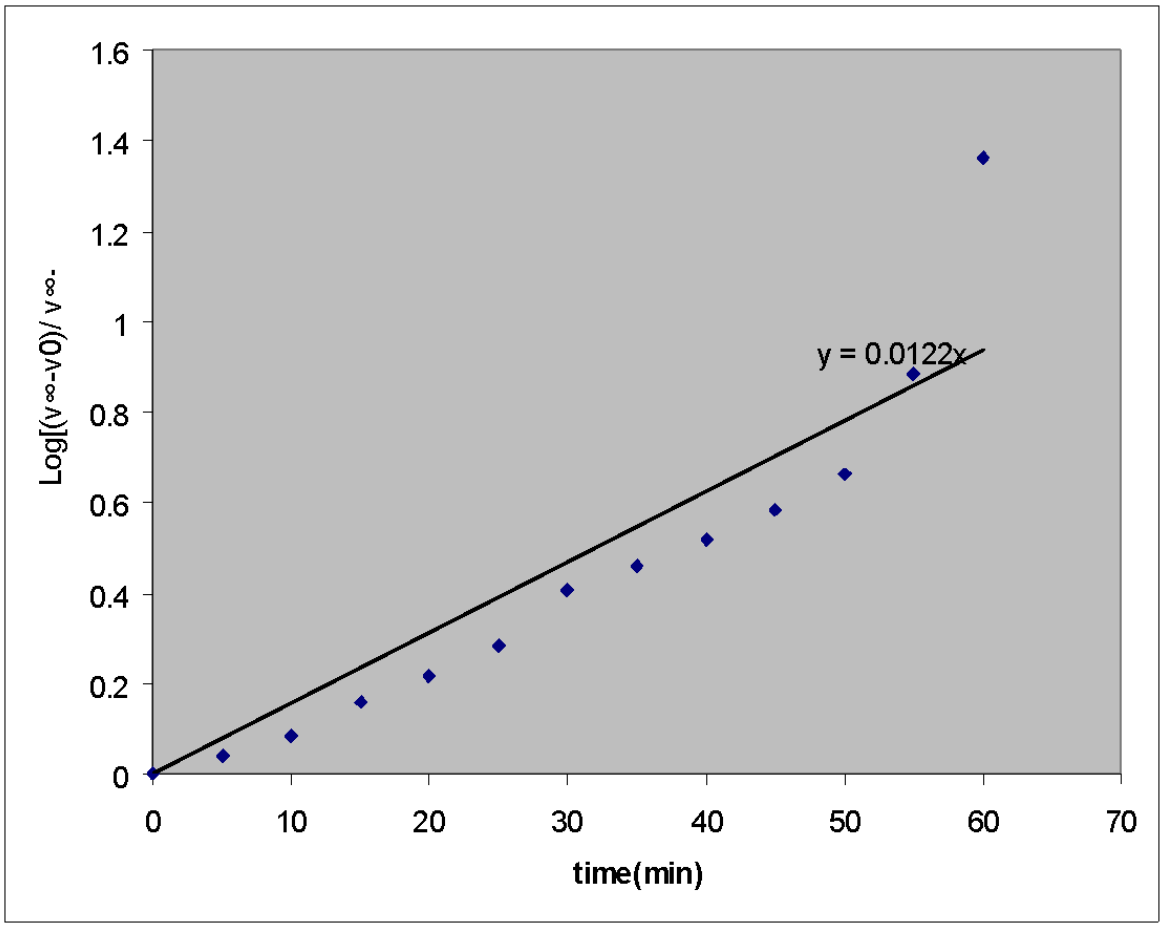


Fig 4. Removal efficieny(%) of *E.Coli* vs sonication time

Table 6-Variation of Ultrasonic velocities (v) with time of mixing of Ethyl acetate and Aniline at 30^0C temperature

Time (minutes)	**Ultrasonic velocities of the composition (80% Ethyl acetate + 20% Aniline)**	$Log[(v_\infty - v_0)/ v_\infty - v_t)]$
0	1169(v_0)	0
5	1171	0.0395
10	1173	0.0829
15	1176	0.1576
20	1178	0.2155
25	1180	0.2825
30	1183	0.4074
35	1184	0.4586
40	1185	0.5166
45	1186	0.5835
50	1187	0.6627
55	1189	0.8846
60	1191	1.3617
65	1192(v_∞)	-
70	1192	-
75	1192	-
80	1192	-

Fig 5 - $Log[(v_\infty - v_0)/ v_\infty - v_t)]$vs time (t) of binary mixture of Ethyl acetate (80%) and Aniline (20%) at 30^0C temperature (Room temperature)



Rate Constant (k) = EMBED Equation.3

$\text{Log}[(v_\infty - v_0)/ v_\infty - v_t)] = kt/2.303$

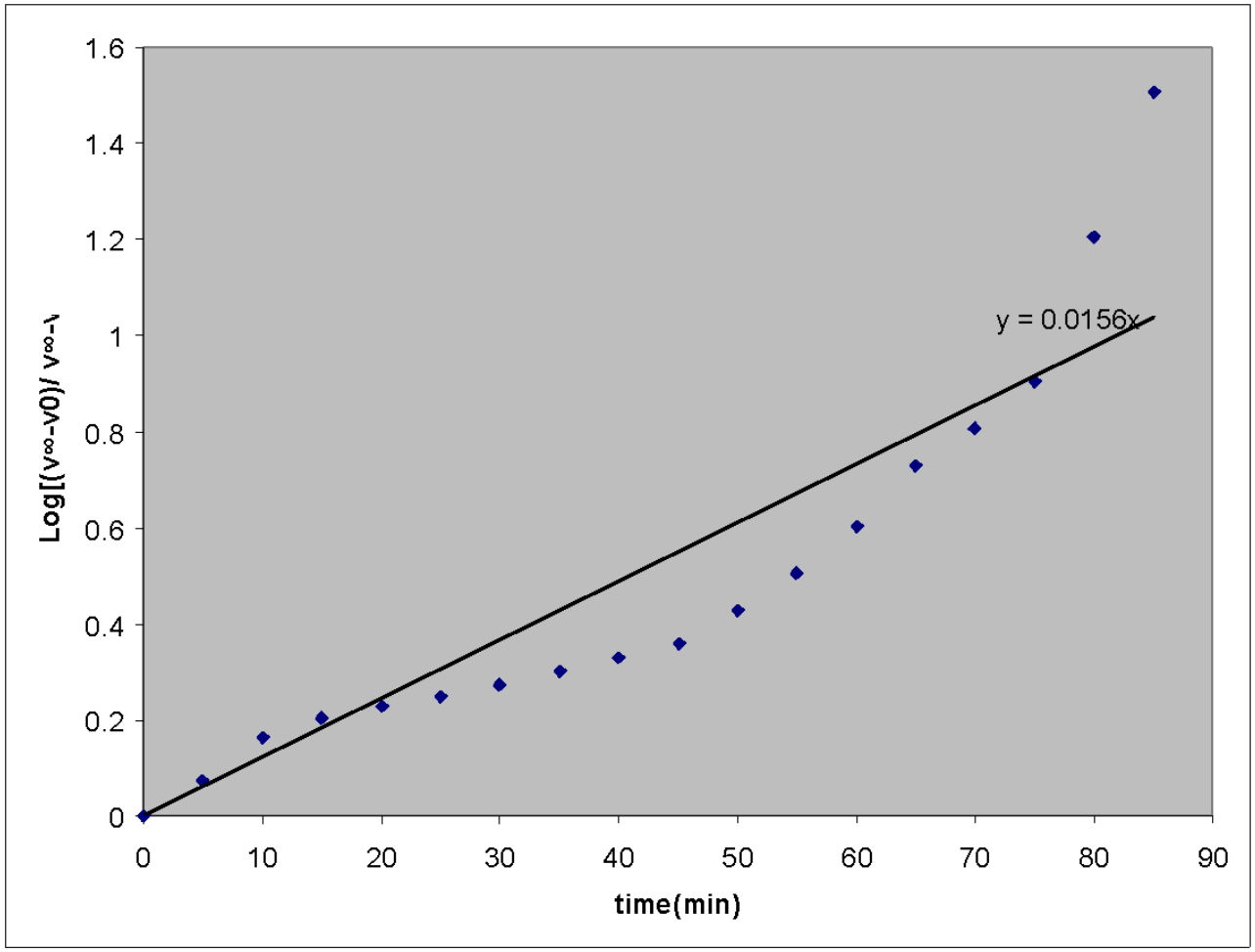
Slope=k/2.303=0.0122

So k=2.303 x 0.0122=0.028 min^{-1}

Table 7 -Variation of Ultrasonic velocities (v) with time of binary mixture of Ethyl acetate and Aniline at 30^0C temperature

Time Minutes	Ultrasonic velocities of the composition (m/s) (60% Ethyl acetate + 40% Aniline)	$\text{Log}[(v_\infty - v_0)/ (v_\infty - v_t)]$
0	1231(v_0)	0
5	1236	0.0737
10	1241	0.1627
15	1243	0.2040
20	1244	0.2263
25	1245	0.2498
30	1246	0.2747
35	1247	0.3010
40	1248	0.3290
45	1249	0.3590
50	1251	0.4259
55	1253	0.5051
60	1255	0.6020
65	1257	0.7269
70	1258	0.8061
75	1259	0.9030
80	1261	1.2041
85	1262	1.5051
90	1263(v_∞)	-
95	1263	-
100	1263	-
105	1263	-

Fig 6- Log[(v_∞-v_0)/ v_∞-v_t)]vs time (t) of binary mixture of Ethyl acetate (60%) and Aniline (40%) at 30^0C temperature (Room temperature)



K=0.0359 min^{-1}

Table 8-Variation of Ultrasonic velocities (v) with time of binary mixture of Ethyl acetate and Aniline at 30^0C temperature (Room temperature)

Time (minutes)	**Ultrasonic velocities of the composition(m/s) 40% Ethyl acetate + 60%aniline**	$Log[(v_\infty - v_0)/ v_\infty - v_t)]$
0	1361(v_0)	0
5	1368	0.1035
10	1372	0.1760
15	1376	0.2632
20	1379	0.3424
25	1381	0.4045
30	1383	0.4771
35	1385	0.5642
40	1386	0.6154
45	1388	0.7403
50	1389	0.8195
55	1390	0.9164
60	1393	1.5185
65	1394(v_∞)	-
70	1394	-
75	1394	-
80	1394	-

Fig 7- Log[(v_∞-v_0)/ v_∞-v_t)]vs time (t) of binary mixture of Ethyl acetate (40%) and Aniline (60%) at 30^0C temperature (Room temperature)

K=0.042 min^{-1}

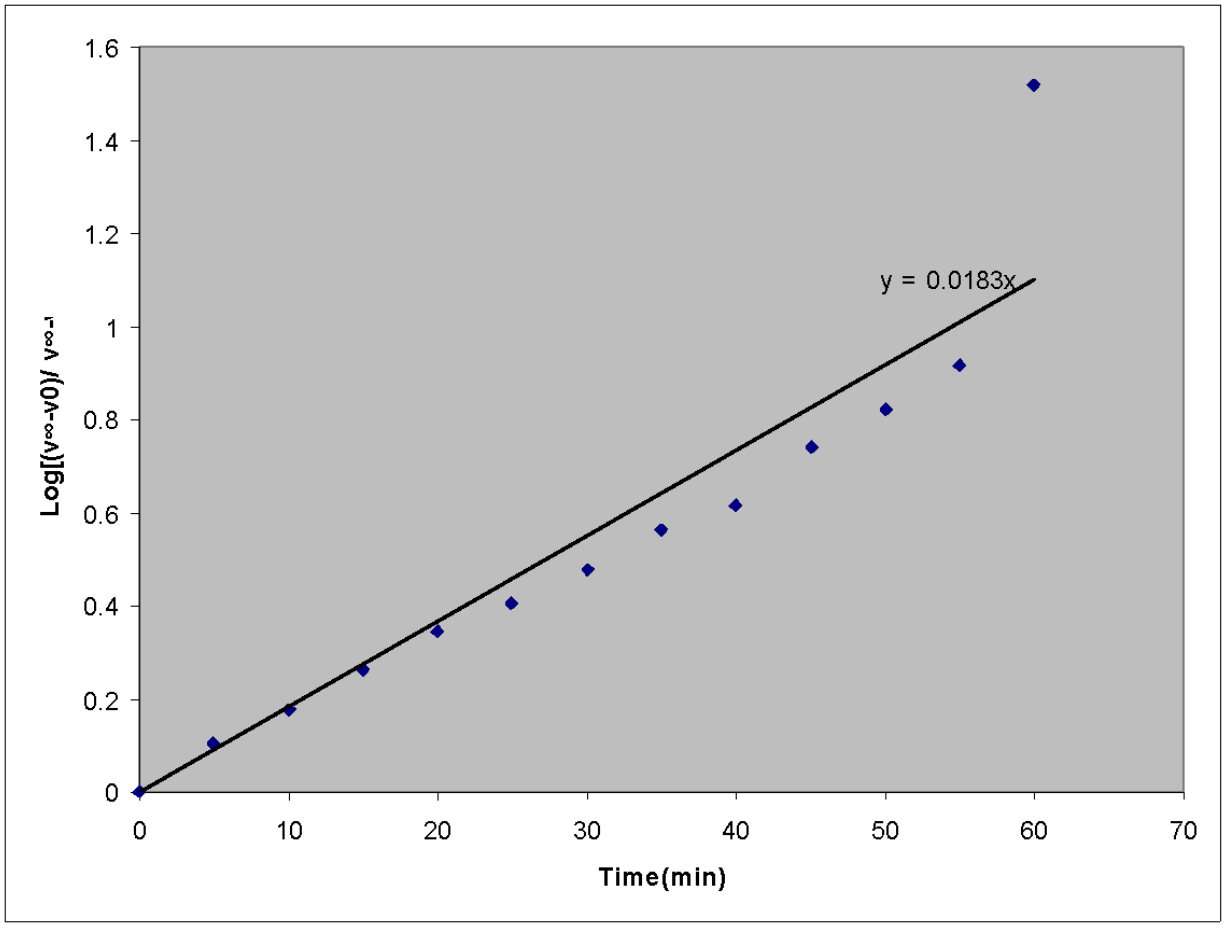
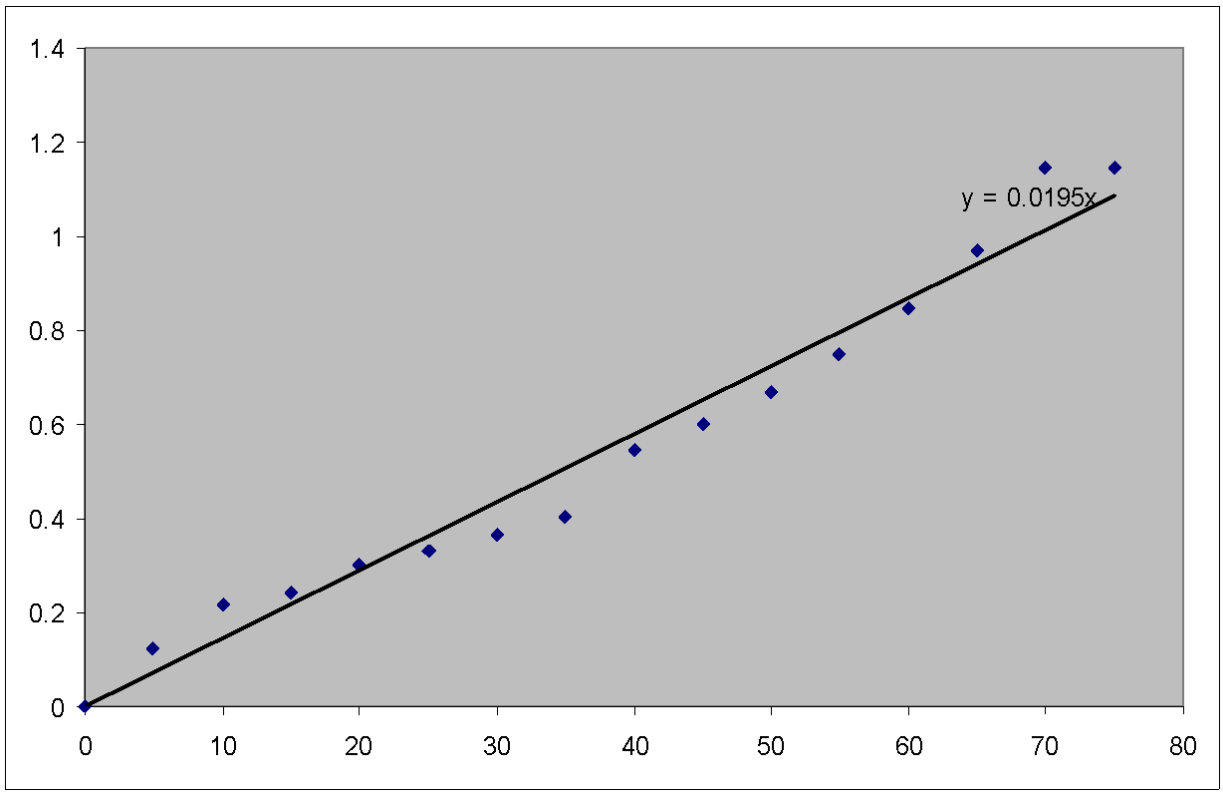


Table 9-Variation of Ultrasonic velocities (v) with time of binary mixture of Ethyl acetate and Aniline at 30^0 C temperature (Room temperature)

Time Minutes	Ultrasonic velocities of the composition(m/s) (20% Ethyl acetate + 80% Aniline)	$\text{Log}[(v_\infty - v_0)/ v_\infty - v_t)]$
0	1453	0
5	1460	0.1249
10	1464	0.2167
15	1465	0.2430
20	1467	0.3010
25	1468	0.3332
30	1469	0.3679
35	1470	0.4057
40	1473	0.5440
45	1474	0.6020
50	1475	0.6690
55	1476	0.7481
60	1477	0.8450
65	1478	0.9700
70	1479	1.1461
75	1480	1.1447
80	1481	-
85	1481	-
90	1481	-
95	1481	-

Fig 8- Log[(v_∞-v_0)/ (v_∞-v_t)]vs time (t) of binary mixture of Ethyl acetate (20%) and Aniline (80%) at 30^0C temperature (Room temperature)



K= 0.0449 min^{-1}

Table 10-First order Rate constants w.r.t active component (i.e., Aniline) at 30^0C temperature

Percentage of composition		Rate Constant (k) min^{-1} $\left(\frac{2.303}{t}\log\left(\frac{v_\infty - v_O}{v_\infty - v_t}\right)\right)$
Ethyl acetate	**Aniline**	
80%	20%	0.0280
60%	40%	0.0359
40%	60%	0.0421
20%	80%	0.0449

CONCLUSION

Thus ultrasound has significant use in deactivation of bacteria and the process depends on sonication time, frequency and intensity of the ultrasound waves.

Ultrasound also helps determine the kinetics of chemical reactions

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