INVESTIGATION ON EFFECT OF AUDIBLE SOUND ON MICROBIAL GROWTH AND PRODUCTION OF CERTAIN INDUSTRIALLY/CLINICALLY IMPORTANT METABOLITES

A Dissertation Thesis

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In

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Submitted by

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ABBREVIATIONS

EPS- Exopolysaccharide
FFT- Fast Fourier Transforms
TFFT- Temporal Fast Fourier Transforms
EG- Endoglucanase
GYE- Glucose Yeast Extract Broth
TYE- Tryptone Yeast Extract Broth
DMSO-Dimethyl sulfoxide
IU: International Unit
SD: Standard Deviation
OD: Optical density
CMC Na- Sodium Carboxymethyl Cellulose salt
DNSA- Di Nitro Salicylic acid

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1. Introduction

1.1 Sound

According to Oxford dictionary **"sound is a mixture of vibrations that travel through the air or another medium and can be heard when they reach a person's or animal's ear".** Sometimes sound is referred as a cluster of a frequencies which can be heard by the human ear or for a particular animal. "Sound is a mechanical wave that propagates in a compressible medium (Rogers and Cox, 1988).

All living beings are somehow getting affected by sound i.e. audible or otherwise, may be random sound or defined sound pattern in form of music, in a variety of ways, and at different levels. It has been claimed that Mozart's music makes people more intelligent and improves health (Makiello, 2012). Music is produced by integration of sounds (mostly periodic sounds-namely regular vibrating sound waves or tone-with different tone color, pitch and volume) into the rhythm patterns (sequential sections of time).

Audible sound, one of the environmental factors, widely exists in natural world. However, the interaction between audible sound and biological materials is usually neglected in the field of biological research. Very little efforts have been put forth in studying the relation of organisms and audible sound. (Mortazavian, 2012)

Recent investigations have proposed the use of physical signals for microbial cell-cell communication has been described as chemical signals (e.g. quorum sensing). Physical modes of microbial communication could be widespread in nature. Physical signals propagate rapidly and, even at very low intensities, they provide useful mechanisms when a rapid response is required. (Reguera 2010; Matsuhashi et al., 1998)

The studies on effect of ultrasound and infrasound on microbes have also been checked. The vibrations corresponding to the sound are divided into three categories:

(1) Infrasound: frequency less than 20Hz
(2) Audible sound or Sound: frequency range 20Hz to 20,000Hz
(3) Ultra sound: frequency above 20,000 Hz

Beat:

When two sound patterns of different frequencies approaches the medium, the combined interference of the constructive and destructive patterns makes the sound alternatively soft and loud at a particular point and this phenomena is known as beating.

Frequency:

"Number of waves that pass a fixed point in unit time; also the number of cycles or vibrations undergone during one unit of time by a body in periodic motion is known as frequency.
(http://www.britannica.com/EBchecked/topic/219573/frequency)

Amplitude:

The maximum displacement or distance moved by a point on a vibrating body or wave measured from its equilibrium position. It is equal to one-half the length of the vibration path."
(http://www.britannica.com/EBchecked/topic/219573/frequency)

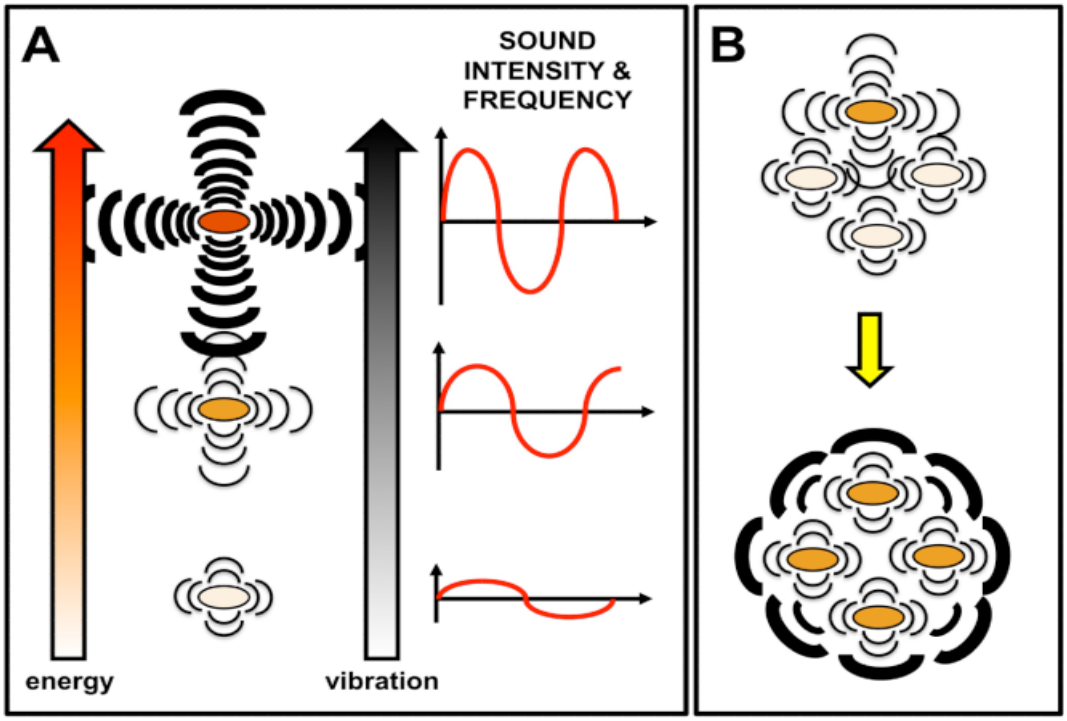


Figure 1.Cell-cell communication via sound waves.

Figure 1 shows the correlation between the metabolic status (and, therefore, the energy available inside the cell), the cell's vibration, and the emitted sound. The cell's metabolic status determines the level of activity of cellular processes, such as transcription and translation that result in the generation of internal motions. These motions appear to be able to produce a generalized vibration of the cell with characteristic intensity and frequency that, therefore, would reflect its unique metabolic status. The vibrations would propagate through the medium as sound waves. The intensity, or energy contained in the wave, would control how far the signal can travel. The frequency, or the 'pitch' of the sound, would control whether a cell can 'sense' the incoming signal or not. If the receiving cell is receptive to that particular frequency it will vibrate proportionally to the intensity of the signal, and the vibrations might induce a biological response (e.g. growth). This might also result in the emission of additional sound waves with unique characteristics of intensity and frequency that could propagate to reach other cells. Energy dissipation and cell's vibrational response as a result of sound communication enables the coordinately dissipation of the energy of the cells. A coherent collective vibrational mode (bottom) is reached when all the cells are 'in tune', which could amplify the signal.

1.2 Types of music

1.2.1 Indian classical music

Indian classical music is one of the oldest forms of music in the world. It has its roots in diverse areas such as the ancient religious *vedic* hymns, tribal chants, devotional temple music, and folk music. Indian music is melodic in nature, as opposed to Western music which is harmonic. The most important point to note is that movements in Indian classical music are on a one-note-at-a-time basis. This progression of sound patterns along time is the most significant contributor to the tune s n and rhythm of the presentation, and hence to the melody (B.C. Deva, 1981).

Although Indian music is now divided into the two major classes of *Hindusthani* (Northern Indian) and *Karnatak* or *Carnatic* (Southern Indian), the origins and fundamental concepts of both these types of music are the same.
(http://people.ucalgary.ca/~ranga/music.pdf)

1.2.2 SWARA-S: THE MUSICAL NOTES

Unlike the case in Western music, the musical notes used in Indian music are not at standardized frequencies. One may choose any frequency of convenience as the reference, and this frequency would then act as the tonic or base of reference for the music to be presented.

The interesting thing about the Indian music is that it does not only describe the music patterns but it also incorporates the benefits and effects of those particular music patterns i.e. ragas on human body as well as on surrounding environment. Table 2 gives the brief explanation and effect of particular raga.

Table 1.Effect of various ragas (http://yogasangeeta.org/index)

Carnatic RAGAS	**BENEFITS**
Ahir Bhairav	**Gives free relaxed feeling and mitigates dust allergies and skin disease. Good for arthritic conditions**
Amrutavarshini	Ushana vyathi nasini (alleviates diseases related to heat)
Ananda Bhairavi	Supresses stomach pain in both men and women. Reduces kidney type problems. Controls blood pressure
Bagesri	Helps in attaining Guru's grace
Bhairavi	Reduces anxiety, pressures, skin, disease, allergies
Bhupala	To awaken someone out of deep sleep
Charukesi Bhajan: Shantirastu Pushtirastu	26th raga in the melakarta scale (parent) of the south Indian classical music. Rejuvenates the mind helping one to age gracefully. It enlivens the singer and listener.
Desh	The suppression of the senses releases a negative force. The process of sublimation needs a spiritual path. Rag Desh can provide that. Its energy gives the listener serenity, peace, inner joy, right valor, universal love and patriotism
Dwijavanti	Quells paralysis and sicorders of the mind
Ganamurte	Helpful in diabetes
Hansadhwani	Energy giving. Provides good thinking, chaitanya. Sarvarogaharini (panacea)
Hemavati Bhajan: Sambho Samba	Good for joint and back pain
Kindolam	Improves digestive power. Cures stomach related diseases.
Kalyani Bhajan: Jai Jai Ganapathi	Gives energy and removes tension and acts as general tonic. Dispels the darkness of fear; Gives motherly comfort and increases confidence. Kalyani means mangalam. Recited with faith and devotion, it is believed to clinch marriage alliances. Many authentic reports exist about the raga's power to destroy fear in many forms: fear of poverty, of love, of power, of ill-health, of death, and so on.
Kapi	Sick patients get ove their depression, anxiety. Reduces absent mindedness
Karaharapriya	Curative for heart disease and nervous irritablility, neurosis, worry and distress.
Kedaram	Gives energy and removes tension
Kirwani	Promotes dhyana (meditation) at mental and physical levels
Kokilam	Helps to prevent stone formation, burning sensations, sleeplessness and anxiety.
Madhuvarshini	Good for nerves. Cures diseases like slight headache, sleeplessness, and sinus problems.

Madhyamavati	Clears paralysis, giddiness, pain in legs/hands, etc. and nervous complaints
Malaya Maruta	To awaken someone out of deep sleep
Malhar	The meaning of Malhar is “Giver of rain”. This raag is known for its positive vibrations and for its rain inducing capacity.
Maya Malava Gowla	Counters pollution. It can be called the Gateway to Carnatic music. The history of Camatic music says that the blessed musician, Purandaradasar, introduced the system of
Bhajan: Inner Self	Mayamalava gowla. This raga has the power to neutralize toxins in the body. Practicing it in the early hours of the morning, in the midst of nature will enhance the strength of the vocal chords.
Mohana Bhajan: Ishapathisha	Mohana is present where beauty and love coexist. It filters out the ill-effects of kama (desire for sex) , krodha (anger) and moha (lust), bestowing immense benefits on the listener. Also said to sures chronic headaches, indigestion, and depression.
Neelambari	To get rid of insomnia
Ranjani	Cures kidney disease
Rathipathipriya	Adds strengh and vigor to a happy wedded life. This 5-swara raga has the power to eliminate poverty. The prayoga of the swaras can wipe off the vibrations of bitter feelings emitted by ill will
Rohini	Cures back pain, joint pain, etc.
Sama	Makes mind sober, tranquil, induces good sleep. Good for world peace.
Saramati Bhajan: Concert in Berlin	Elevates from depressed state. Cures balagraha dosham in children (undiagnoses crying and imitability). For sleeplessness, itching, eye and ear problems, skin problems, and the problems of hearing irregular sounds
Sindu Bhairavi	Removes sins and sorrows and saves from unforesenn events
Sivaranjani	Powerful raga for meditation; bestows benevolence of God. Removes sadness, ushana roga santi (diseases related to excess heat). Good for general health
Sandhya Kalyani	Cures ear, nose and eye diseases. Relieves chronic clods. Gives good sleep and freshness
Shankarabharanam	The power of this raga is incredible. It cures mental illness, soothes the turbulent mind and restores peace and harmony. If rendered with total devotion for a stipulated period, it can cure mental disorders said to be beyond the scope of medical treatment. It also is said to have the power to shower wealth.
Shanmugapriya	Sharpens the intellect of the singer as well as the listener. Instills courage in one's mind and replenishes the energy in the body.
Subhapantuvarali	Alleviates mental dilemmas and indecisiveness
Suddha dhanyasi	Remover of sorrows. Gives a happy feeling. Tonic for nerves. Cures rhinitis and migraine.
Suruti	Mitigates stomach burn, insomnia, fear, disgust
Vakulabharanam	Alleviates asthma, bronchitis, heart disease, depression, skin disease and skin allergy
Varali Bhajan: Nakam Vinayakam	Varali is good for vayu tatva, heart, skin ailments and gastric problems.
Vasanta / Vasanti	Controls high and low blood pressure, cures heart as well as nervous diseases. Can dear the fog of confusion when a series of medical tests has to be analysed. It heals nervous breakdowns.
Vasantham	Cures paralysis
Viswambari	General tonic, acts quickly
Yamuna Kalyani	Gives freshness and dynamism

Two types of music (ragas) from Indian Classical music having definite sound pattern were selected for experiments:

(1)Ahir Bhairav*:*

Ahir Bhairav is a Hindustani classical *raag*. often times, this *raag* is referred to by its south Indian name of *Chakravaka*. It occupies a unique place among morning *Raagas*. This *raag* creates an atmosphere full of Bhakti Ras. It is typical *uttarang raga*, which means emphasis is on the upper tetrachord. It is a mixture of *Bhairav* and the ancient but now rare raga *Ahiri* or *Abhiri*, or perhaps a mixture of *Bhairav* and *Kafi.* **(**Played using Flute, Santoor, and Guitar instruments) (CD used: "Call of the Valley", Sa re Sa ma company)

The main percussion instruments used in Hindustani classical music are the *tabla* and (the somewhat less common) *pakhwaj*. The *tabla* is a set of two kettledrums of different sizes and timbers that are played simultaneously by tapping on them with the hands in various ways to produce different kinds of sounds. These sounds are then strung together in sequences to create different rhythm patterns (*taal*) to accompany musical performances.

In the hands of an expert *tabla* player, the *tabla* can make all kinds of fantastic sounds, but there are a couple of dozen commonly produced sounds. They are just vocalizations of the actual sounds produced by the *tabla*. They are called *bol*, and it is these *bols* that are combined in various ways to get many interesting rhythms (*taals*).

(2)*Raag piloo-teentaal*:

The first three *(Teentaal, Ektaal and Jhaptaal)* are very common in classical music. The specific sequence of beats that define a *taal* is called its *theka*. So, for instance, the *theka* of Teentaal is:

dhaa dhin dhin dhaa / dhaa dhin dhin dhaa / dhaa tin tin taa/ taa dhin dhin dhaa or

naa dhin dhin naa / naa dhin dhin naa / naa tin tin naa/ teke dhin dhin naa

(Played using Flute, Santoor, and Guitar instruments) (CD used: "Call of the Valley", Sa re Sa ma company)

1.2.3 Characteristics of raag *Ahir Bhairav* and raag *Piloo-Teentaal*

The frequency analysis was done with the help of "**NCH WavePad**" and "**sonic visualiser**" which are sound editor softwares. By using them, Fast Fourier transforms (FFT), temporal fast Fourier transforms (TFFT) and peak frequency spectrums were generated. Fig.9 and fig.11 shows (TFFT) frequencies as a function of time as it is given in Frequency vs. time. As same, fig.12 and fig.13 shows peak frequency spectrum as a function of time, this is frequency vs. time. The frequency distribution is described using sonic visualiser software in the table 1 given below and table 2 shows frequency distribution using NCH wavepad in *raag Ahir bairav* and raag *Piloo* with their relative sound intensities.

Table 2.Frequency distribution using sonic visualiser

	Raag Ahir Bhairav			***Raag Piloo- teentaal***		
Range of frequencies (Hz)	**Complete range**	**Dominant range**		**Complete range**	**Dominant range**	
		Lower range	**Higher range**		**Lower range**	**Higher range**
	172-2960	226-247	473-484	75-1830	86-96	1399-1421

Table 2.Frequency distribution using NCH Wavepad and Sound intensity

			Raag Ahir Bhairav		*Raag Piloo- Teentaal*	
Range of frequencies (Hz)			**Complete range**	**Dominant range**	**Complete range**	**Dominant range**
			150-7811	172-581	43-5620	86-839
Sound intensity (dB)	**Inside incubator**		80-90		90-110	
	Inside locker		70-80		85-100	
	Background interference	**Inside locker**	60-65		60-65	
		Inside incubator	70-80		70-80	

1.3 Industrially important metabolites

Different metabolites were selected on the basis of its industrial importance such as Violacein, Prodigiosin, EPS, Cellulase, Alcohol, Lactic acid etc. The brief literature search is given for these molecules in the following section.

1.3.1 Microbial polysaccharide

Microbial polysaccharides are produced in two forms, capsular polysaccharide (CPS) and exopolysaccharide (EPS). EPSs are ubiquitous in nature and have unique properties. Exopolysaccharides are comprised of repeated units of sugar moieties.

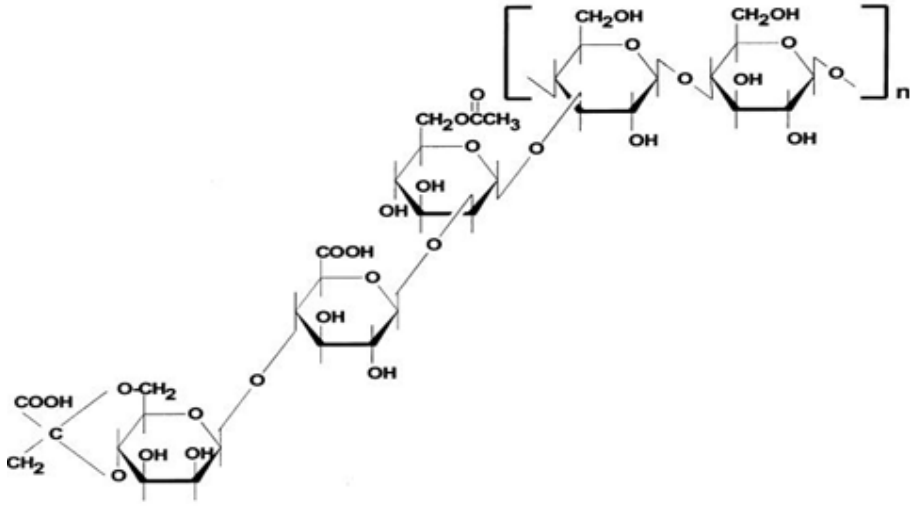


Figure 2.Xanthan Structure

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS). They generally consist of monosaccharide and some non-carbohydrate substituent (such as acetate, pyruvate, succinate, and phosphate). Owing to the wide diversity in composition, exopolysaccharides have found various applications in various food and pharmaceutical industries. Many microbial EPS provide properties that are almost identical to the gums currently in use.

With innovative approaches, efforts are underway to supersede the traditionally used plant and algal gums by their microbial counterparts. Moreover, considerable progress has been made in discovering and developing new microbial EPS that possess novel industrial significance.

1.3.2. Violacein

Chromobacterium violaceum is a Gram-negative bacterium that produces the purple pigment violacein in response to the presence of the AHL *N*-hexanoyl homoserine lactone (C_6HSL). Violacein was first discovered by the scientist Boisbaudran from the *C. violaceum* is known to have characteristic of violacein production. The dimeric structure consisting of three units 5 pyroindole which is known as Violacein, a purple colored pigment produced by several Gram negative bacteria is a current topic of research areas like antimicrobials, antitumor and anticancer molecules (Hoshino, 2011; August, 2000). Violacein is a derivative of indole also known as bisindole which is potent inhibitor of DNA topoisomerase and one of the strongest inhibitor of the protein kinases (Tamaoki et al, 1986). Monobactams are important active ingredient compounds of violacein which are used for antibiotic. Violacein [3-(1,2-d 1,3-dihydro-2*H*-indole-2-one] not only provides antimicrobial and antiparasitic but also helps bacteria to control the oxidative stress and protection against the protozoans (Antony et al, 2013; Duran et al, 2007). Here, the figure 3 shows the structure of violecein pigment.

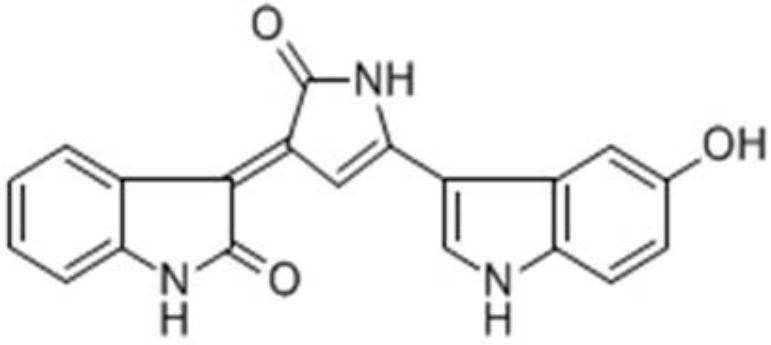


Figure 3.Violacein, an alkaloid pigment

1.3.3. Prodigiosin

Prodigiosin is the red colored pigment produced by many strains is very important secondary metabolite. Prodigiosin produced by *Serrattia marcescens* promising drug owing to its reported characteristics of having antibacterial, antimalarial, anti-topoisomerase activity antimycotic, immunomodulating and antitumor activities. Here, the figure 4 shows the structure of prodigiosin pigment.

Prodigiosins, a family of natural red pigments characterized by a common pyrolylpyrromethane skeleton, are produced by various bacteria that first characterized from bacteria possess huge ability in producing biopigments that are synthesized for producing medicinally important products having distinct biological activities antibacterial, antimalarial, antimycotic, immunomodulating, antitumor and nuclease. Prodigiosins are a family of naturally occurring tripyrrole ring containing red pigments having a

$C_20H_{25}N_3O$, has an unusual structure with three pyrrole rings and is a pyryldipyrylmethane; two of the rings are directly linked to each other, and the third is attached by way of a methane bridge.

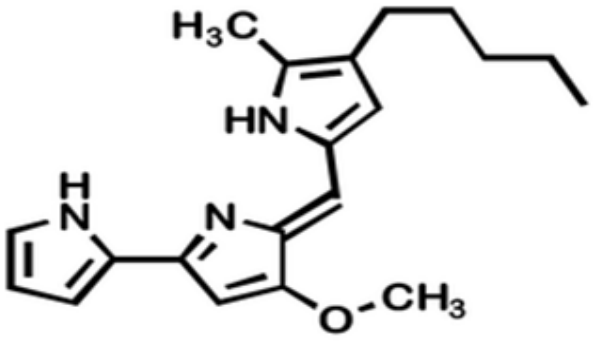


Figure 4.Structure of Prodigiosin pigment

1.3.4. Cellulase

Microbial cellulases have shown their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture. Due to the complexity of enzyme system and immense industrial potential, cellulase is widely important.

Mechanistically, cellulase is a family of at least 3 groups of enzymes, endo-(1,4)-β-D-glucanase exo-(1,4)-β-D-glucanase and β-glucosidases. Figure 5 shows the structures of both exocellulase and endocellulase. The exoglucanase acts on the ends of the cellulose chain and releases β-cellobiose as the end product; endoglucanase (EG) randomly attacks the internal O-glycosidic bonds, resulting in glucan chains of different lengths; and the β-glycosidases act specifically on the β-cellobiose disaccharides and produce glucose. Although the mechanism of cellulose degradation by aerobic bacteria is similar to that of aerobic fungi, it is clear that anaerobic bacteria operate on a different system. Cellulosomes located on the cell surface mediate adherence of anaerobic cellulolytic bacteria to the substrate, which thereafter undergo a supramolecular reorganization, so that the cellulosomal subunits redistribute to interact with the different target substrates.

Cellulases have been commercially available for more than 30 years. Basic and applied studies on cellulolytic enzymes have demonstrated their biotechnological potential in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textile, and laundry. Nowadays, significant attentions have been devoted to the current knowledge of cellulase production and the challenges in cellulase research especially in the direction of improving the process economics of various industries.

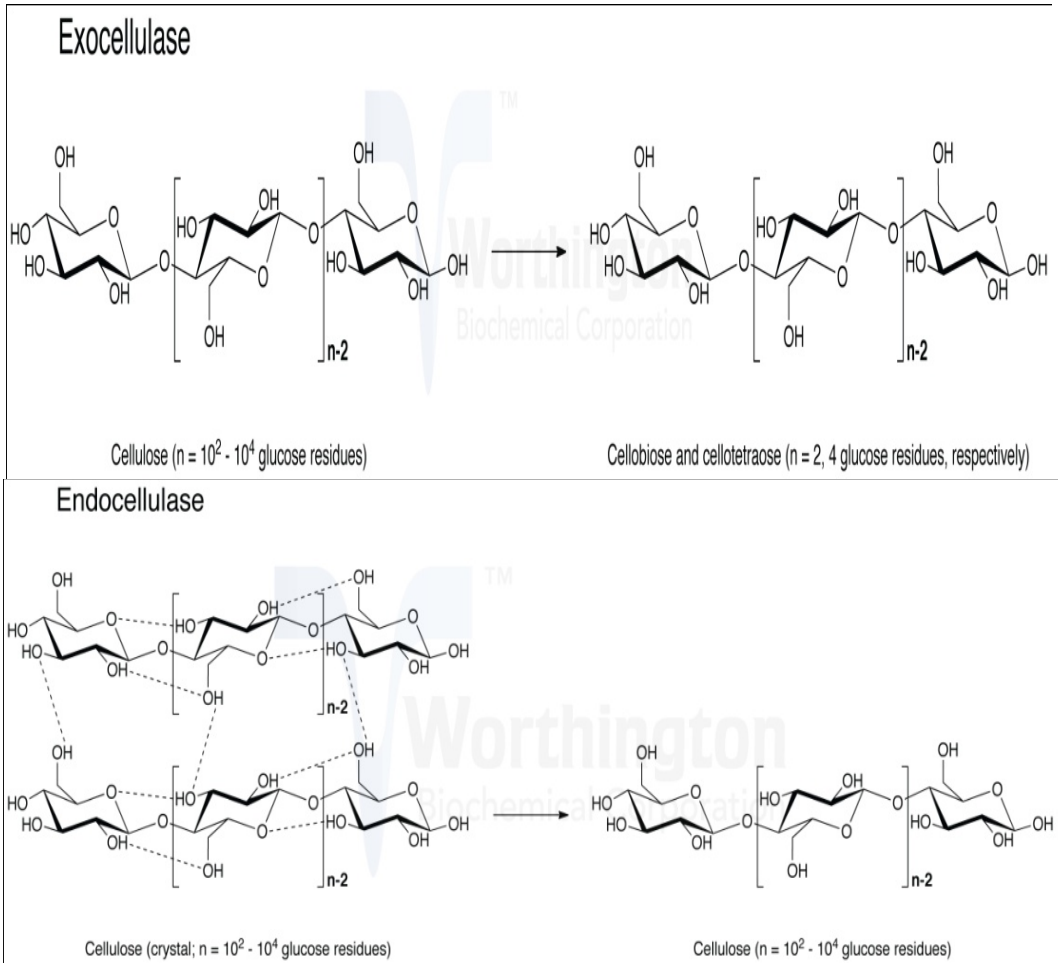


Figure 5.Structure of Exocellulase and Endocellulase
(http://www.worthington-biochem.com/cel/default.html)

1.3.5. Lactic acid

Lactic acid, also called **α-hydroxypropionic acid**, or **2-hydroxypropanoic acid**, inorganic compound belonging to the family of carboxylic acids, present in certain plant juices, in the blood and muscles of animals, and in the soil. It is the commonest acidic constituent of fermented milk products such as sour milk, cheese, and buttermilk. Fermentation of lactose to lactic acid was conducted using *Lactobacillus plantarum*.

First isolated in 1780 by a Swedish chemist, Carl Wilhelm Scheele, lactic acid is manufactured by the fermentation of molasses, starch, or whey in the presence of alkaline substances such as lime or calcium carbonate; it is available as aqueous solutions of various concentrations, usually 22–85 percent, and degrees of purity.

Lactic acid is used in tanning leather and dyeing wool; as a flavoring agent and preservative in processed cheese, salad dressings, pickles, and carbonated beverages; and as a raw material or a catalyst in numerous chemical processes. Pure lactic acid, rarely prepared, is a colorless, crystalline substance that melts at 18° C (64° F); it rapidly absorbs moisture from the atmosphere.

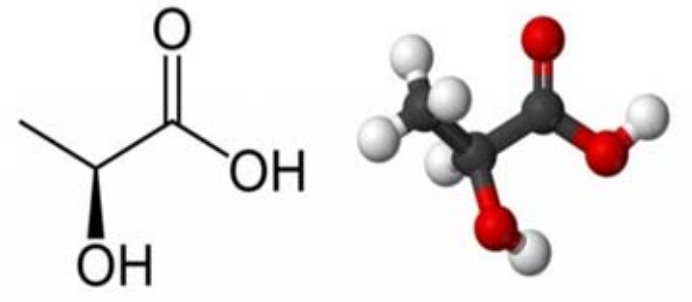


Figure 6.Structure of Lactic acid

2. Review of literature

Environmental factors can greatly influence the growth and development of plants, even the genetic character (Xiujuan et al.2003). Sound frequencies which are in the form of mechanical stress have some impact over bacterial growth has been shown (Shaobin, 2010). Audible sound treatment significantly increases the colony forming of E. coli under the normal growth condition. However, under osmotic stress induced by the sugar, audible sound stimulation may enhance the inhibitory effect of osmotic stress on E. coli growth (Shaobin, 2010).

Pornpongmetta and Thanuttamavong (2010) reported microbial substrate utilization of aerobic bacteria from municipal waste water treatment plant was affected by music. The studies related to sound have suggested that sound having amplitude of 90 dB can raise the change in bacterial cells as well as plant cells (Shaobin et al., 2010; Xiujuan et al., 2003).Synthesis of nucleic acid and protein in *Chrysanthemum* has also been reported to be affected by sound waves (Xiujuan et al., 2003). The relation between bacterial growth and frequency of sound has been studied and science is trying to establish this correlation between them (Mukhopadhyay et al., 2008).The ability of cells to communicate with sounds was suggested based on the observation that sound waves stimulated the growth of *Bacillus carboniphilus* under stress conditions.

(Ayan et al., 2008) have shown that low-intensity pulsed sound waves delivered by the Exogen device can effect on the colony number, antimicrobial susceptibility, bacterial morphology, and genetics of *Staphylococcus aureus.* Music exhibits an intrinsic power and provides a magical world not only in front of human, but also for animals, plants and even microorganisms. It seems that all livings are somehow affected by music in different ways and various levels. Also, recent investigations have revealed that music can affect survival and activity of different microorganisms (Mortazavian, 2008).

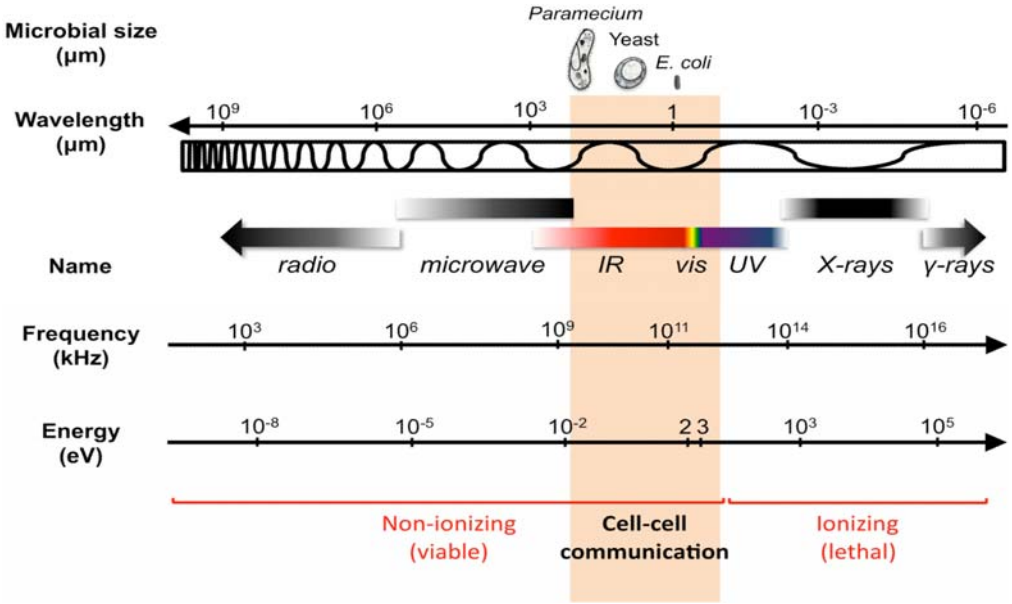


Figure 7.The electromagnetic spectrum

In figure 7, microbial sizes (for the unicellular protozoan *Paramecium*, a *Saccharomyces* yeast cell and the bacterium *E. coli*) and radiation wavelengths (in micrometer, μm) are shown on top. The shaded area of the non-ionizing radiation shows the region of the spectrum that is likely to be relevant to microbial cell-cell communication.

3. Objective

- To check the effect of two different sound patterns (music) viz.:

  (a) *Raag Ahir Bhairav*
  (b) *Raag Piloo (Teental)*

  On microbial growth and production of certain industrially/clinically important metabolites (alcohol, cellulase, EPS, prodigiosin, violacein)

4. Materials and Methods

4.1 Culture maintenance

Most of bacteria were maintained on nutrient agar media, and Yeast was maintained on GYE medium. Glycerol stocks (glycerol at 10% v/v concentration; Merck) were also prepared in cryovials and stored at -20^{o}C. Paraffin slants were also prepared by overlaying light paraffin oil to avoid the air exposure. Master plates and working plates were prepared individually for each organism.

4.2 Culture activation

Culture activation was done in such a way that exponential growth phase of the organism was acheived at the time of inoculation. Microorganisms were activated just one day before experiment from working plate to a new sterile plate. Solid medium was used always to avoid chances of contamination.

4.3 Inoculum preparation

Colonies were suspended from activated culture in to sterile normal saline (0.85%NaCl) and standardized by taking its optical density at 625 nm and was maintained between 0.08-0.10, according to 0.5 McFarland turbidity standard.

4.4 Details of instrumentation used for music treatment

4.4.1 Speakers

Portable speakers M0520, lenovo were used in study. The M0520 Lenovo Multi-media speaker system adapts to the inside of a dual BTL audio amplifier, ensuring stable and reliable electrical performance. The USB power supply should be 5V=500mA.

4.4.2 Music player

Expeed Magic box USB-digital FM receiver was used for music treatment. It has FM as well as USB port for the music. Power requirement is 220V. AC/12V. DC.

4.4.3 Sound level meter

The sound level meter which was used in the experiments was Dr. Indolkar's sound level meter. (ACD Machine Control Co [p] L.T.D. Mumbai). Industrial grade Sound level meter with traceable calibration enables to measure the sound level in the form of decibels (db).

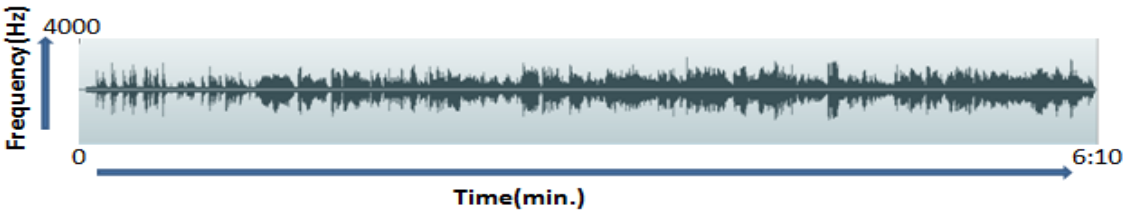


Figure 8(a) Frequency distribution pattern in *Ahir Bhairav*

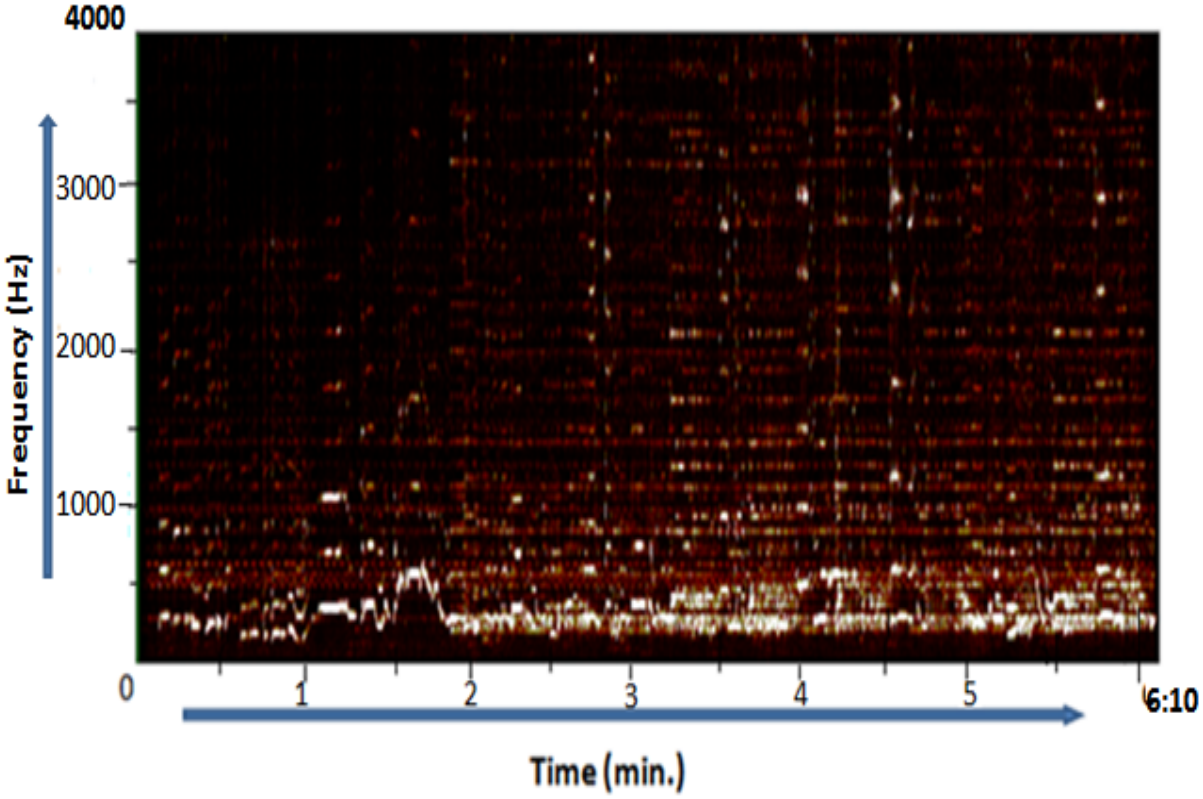


Figure 8(b).Frequency distribution pattern (TFFT) in *Ahir Bhairav* by using Wavepad

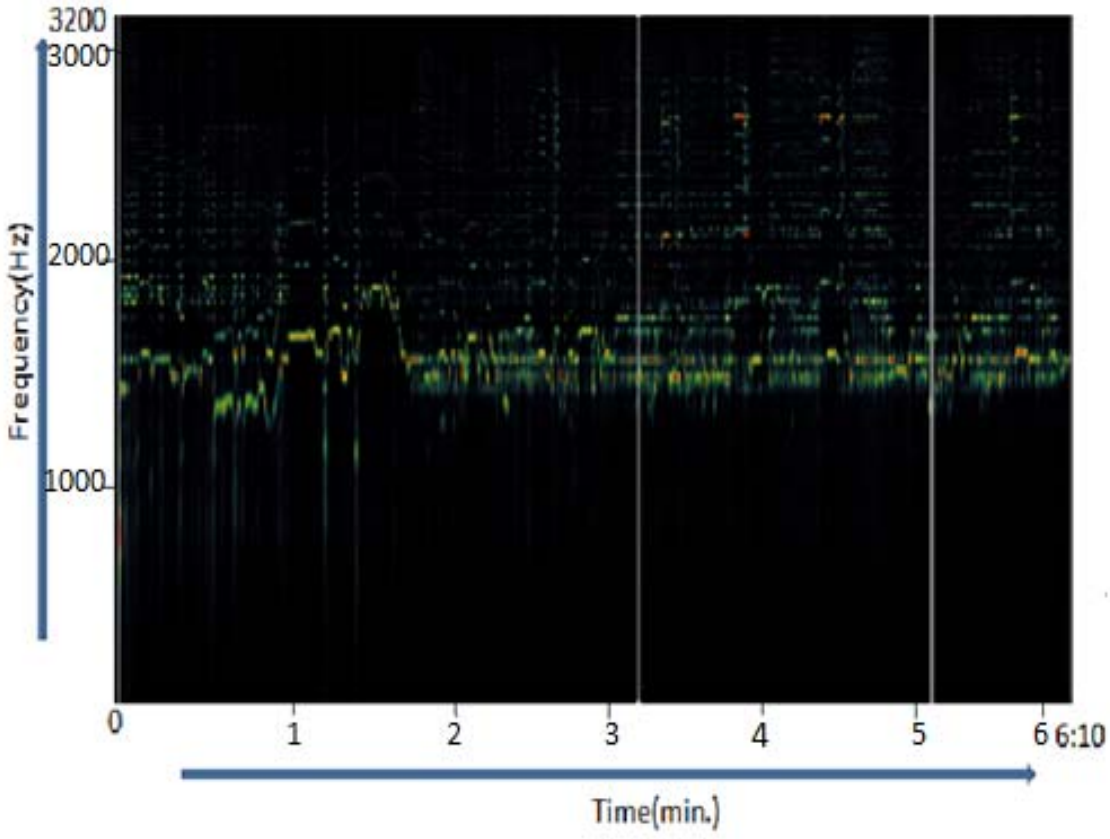


Figure 8(c). Peak frequency spectrogram of *raag Ahir Bhairav* using sonic visualiser

Figure 9(a). Frequency distribution pattern in *raag Piloo-teen taal*

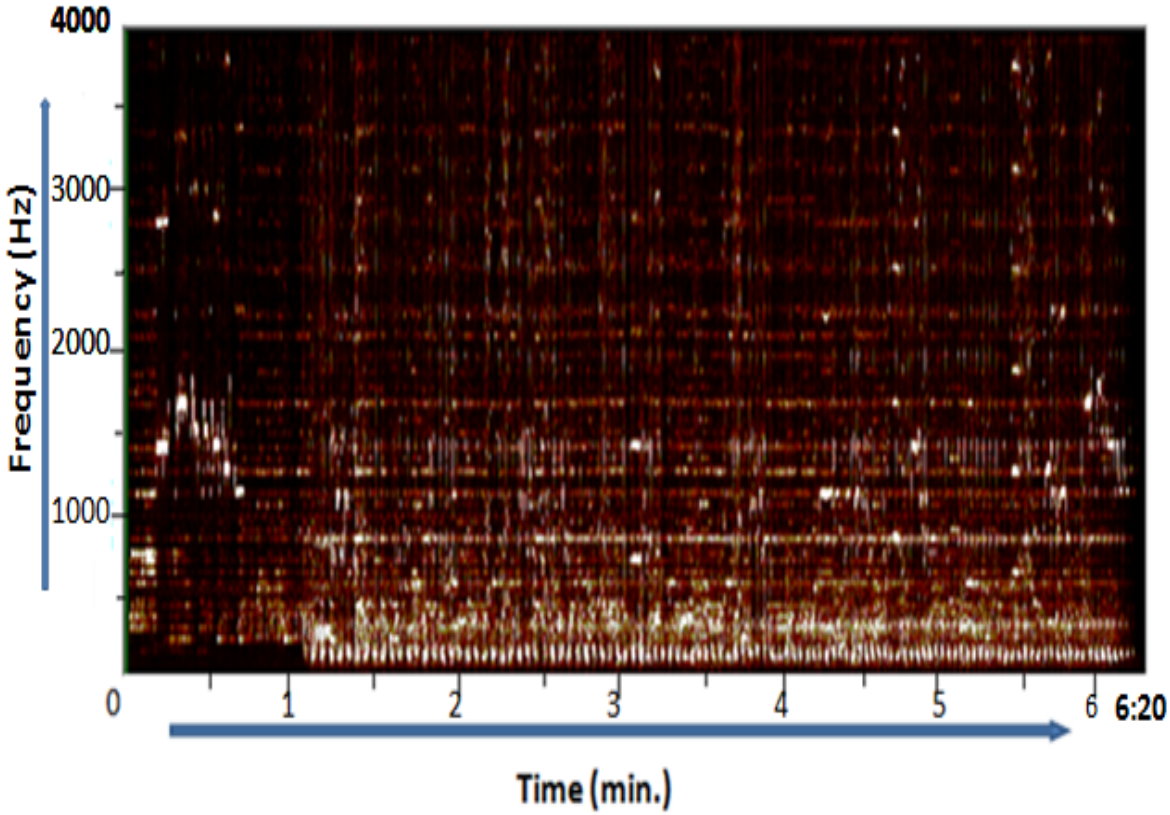


Figure 9(b) Frequency distribution pattern (TFFT) in *raag Piloo-teen taal* by using Wavepad

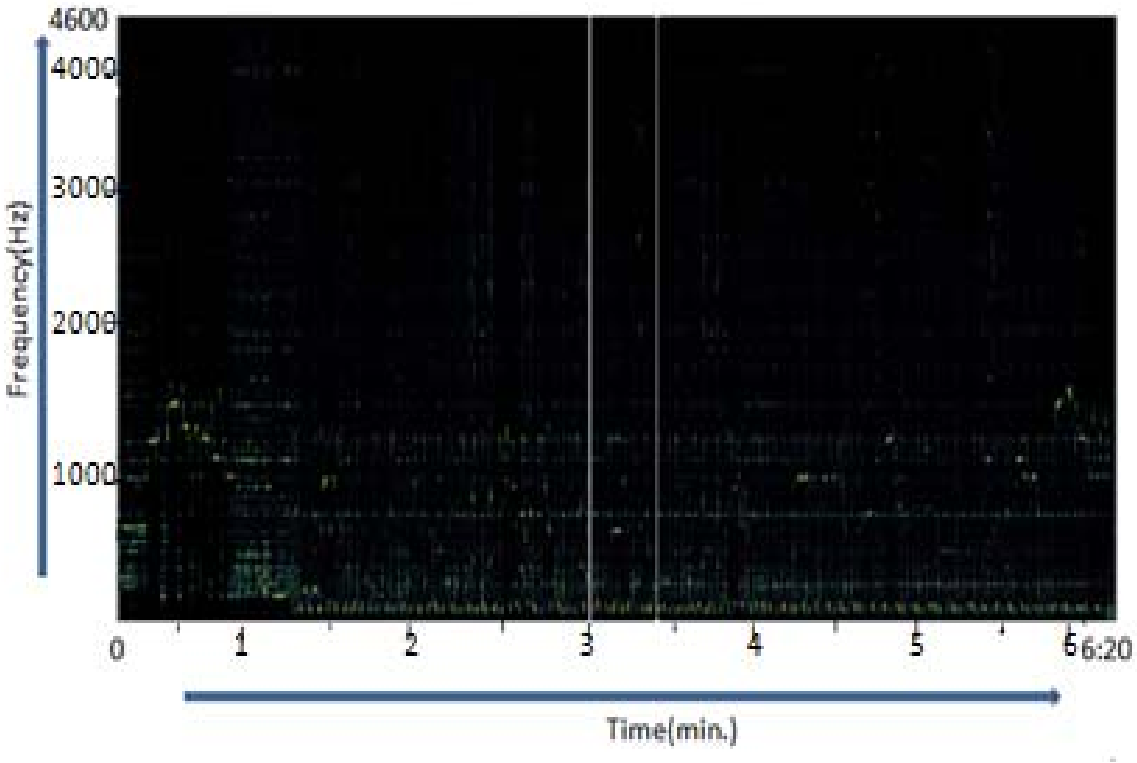


Figure 9(c). Peak frequency spectrogram of *raag Piloo-teentaal* using sonic visualiser

4.5 Arrangement of experiment

4.5.1 Arrangement of connections between music player and speakers

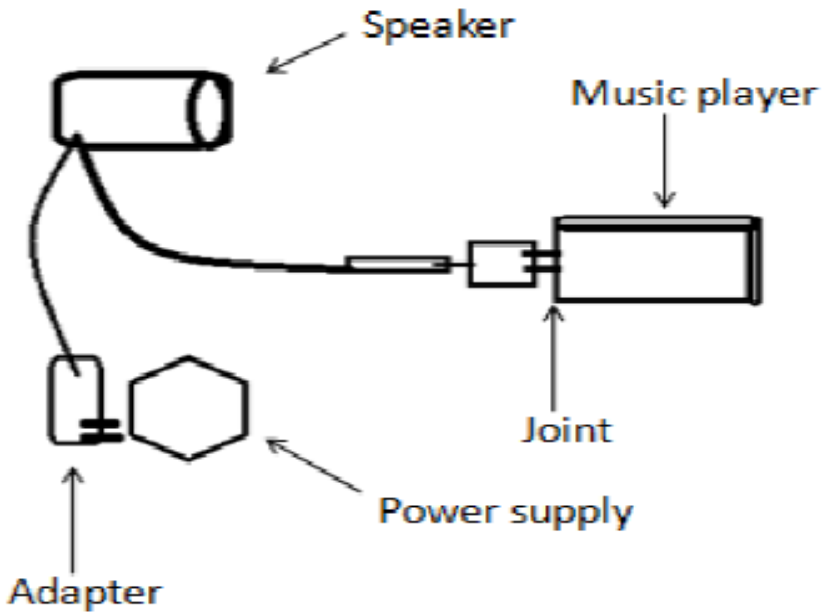


Figure 10.Speaker, music player and power supply
(Sarvaiya & Kothari, 2015)

Figure 10 shows that two attachments are important. First one is to the joint and other is to music player. It is a dual code which can be connected by single code and hence the code of speaker is connected to the music player via this. Speaker has two codes in which one is connected with joint and another is with USB code which was connected to the adapter to provide the power supply to the speaker and adapter was joint with power supply. Pen-drive or USB drive contained music files had been inserted into USB with different number e.g. 1, 2, 3, etc so that one loop can be created and music treatment can be provided for a longer time throughout incubation period.

4.5.2 Packing of chromatographic chamber to avoid leakage of sound

Two identical glass chambers (Jar; Rectangular made up of clear glass, size 225×225×125, Merck) were used for the music treatment and to avoid the leakage of sound.

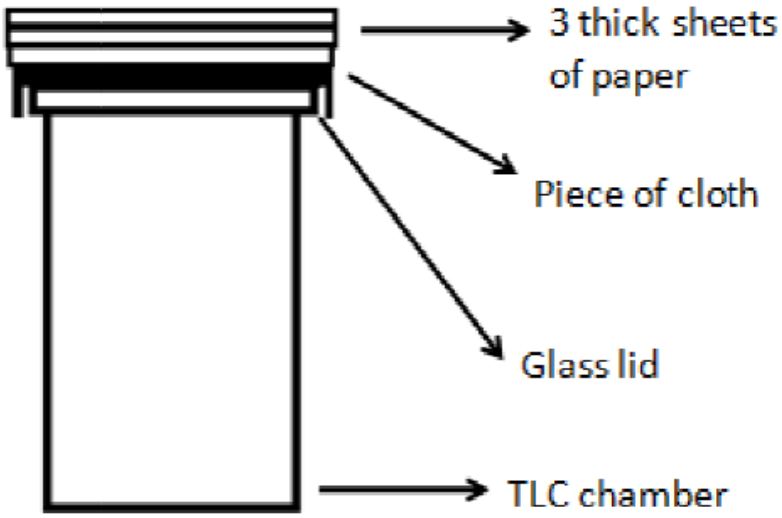


Figure 11.Packing of chromatographic chamber

The glass lid of TLC chamber is placed on top of the camber then a piece of cloth was put (folded) and it was wrapped finally with 35 identical pieces of news papers (The Times of India). Silicone gel is placed between the glass lid and the chamber to avoid sound leakage. Here one thing should be noted that the arrangement should be done in such a way that control and experimental chamber should have same pattern of packing and thickness of news paper and cloth should be identical.

4.5.3 Arrangement of experiment

During the arrangement, the figure 16 shows that the distance between the speaker and the test tubes containing broth was kept constant which was 15 cm during all experiments. The test tubes which were used for the control and experimental and beakers (250ml, Merck) in which these test tubes were placed which were identical. We had put one speaker in the control chamber to maintain similar magnetic fields in both the chambers which may be generated due to the magnet located in the core of speaker.

At room temperature, the experiment had one change in the case of *X. campestris* which was done in 100 ml flask and was maintained at same distance at 15 cm. The distance between two flasks was 1cm and also the rotation of flasks has been done when the interminant shaking was done in every 6 hours.

[Note: Thickness of glass test tubes and beakers should be identical otherwise results may vary because of different absorption of sound with different thickness of glass may occur]

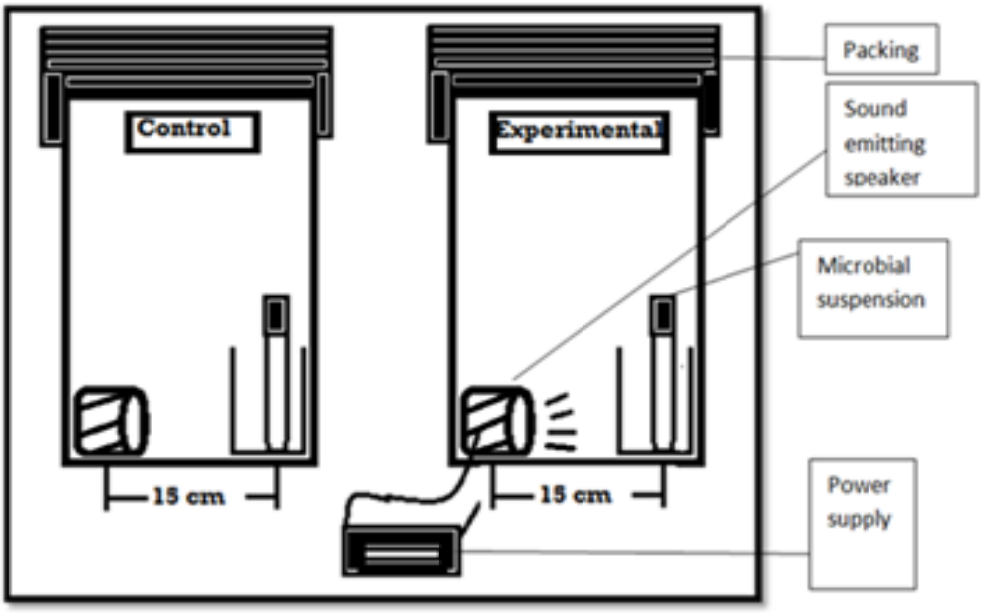


Figure 12.Experimental set-up

4.5.4 Sound level setting

The sound level was also kept same in the experiment. It was done by coupling two regulators of sound in which one was speaker sound regulator and another was USB player sound regulator. Sound regulator of speaker was full and the sound regulator was kept at 40 units.

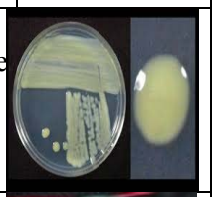
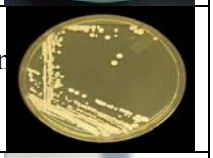
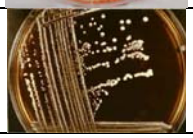
[Note: The sound level of speakers and USB player was kept constant throughout the experiment otherwise different sound levels may cause different effect on experimental organism.]

The sound intensity was measured in terms of decibels (db) with the help of sound level meter as mentioned above. There were mainly two different arrangements in which the experiment was performed. First was at incubator which was a Yorco hybridization incubator (Yorco Scientific Industry PVT. LTD) and also B.O.D. incubator (Sonar enterprise, Jay chemical). Second was at room temperature. In both the cases the sound level settings of speakers and USB player were kept constant but the sound intensities that were measured were different. In case of *Piloo*, the sound intensity for experimental which were at room temperature was between 85-100 decibels and the experiments which were done in incubator was between 90-110 decibels. In case of *Ahir bhairav*, the sound intensity for experimental which were at room temperature was between 70-80 decibels and for the experiments which were done in incubator was between 80-90 decibels The background interfearence was also measred which was 70-80 decibles for room temperature experiments and 60-65 decibles for incubator experiments. These background interfearence was same for both the music. The high intesity of sound was there in the experiments which were performed in incubator may be because of TLC chambers which were used of smaller sizes and the echo of the sound may be the reason for high intensity of sound in it.

4.6 Procedure of experiments

- The culture of the particular test microorganism was activated as described above.
- The inoculum was prepared in normal saline and standardized by comparing it with 0.5 McFarland turbidity standard.
- 5% v/v culture was inoculated in the media which was taken for experiments.
- Except *X. campestris* all the experiments were performed in test tubes.
- The total volume (Broth+inoculum) was 5 ml in each test tube and 50mL volume in 100mL of flask was taken for experiments which were performed for effect of music on EPS.
- The incubation time, temperature, and few important details are given in the Table 3.
- Music treatment was given throughout the incubation period in all experiments.
- The Quantification of growth was done at $OD_{660\ nm}$ in all the cases.
- Methods for the estimation of particular metabolites are described in the following Section.

Table 3.Experimental Conditions

No.	Organism	MTCC code	Growth medium	Incubation temperature (^oC)	Incubation time (h)	Parameter(s) tested	Images
1.	*Xanthomonas campestris*	2286	Tryptone yeast extract broth+ $CaCl_2$	Room temperature	72 (with intermittant shaking)	Growth and exopolysaccharide (EPS) production	
2.	*Brevibacillus parabrevis*	2708	*CMC-Na*+ Nutrient broth	35	72 (with intermittant shaking)	Growth and cellulase activity	
3.	*Saccharomyces cerevisiae*	170	Glucose yeast extract broth	Room temperature	48 (under static condition)	Growth and alcohol production	
4.	*Chromobacterium violaceum*	2656	Nutrient broth	35		Growth and violacein production	
5.	*Serratia marcescens*	97		28		Growth and prodigiosin production	
6.	*Lactobacillus plantarum*	2621	MRS broth			Growth and pH measurement	

4.7 Exopolysaccharide quantification (Li et al., 2012 with some modifications)

- 20 ml of CFS was collected after centrifugation at 7500 rpm for 10 minutes. This supernatant was precipitated with two volumes of chilled acetone (Merck; AR) i.e., 40 ml in one run.
- After 30 minutes, the precipitation was visibly seen and it was filtered by WhatmanTM#1 filter paper (125 mm Ø; Whatman International Ltd., England). Before filtering, the pre weight of each filter paper was noted.
- The filter papers were dried at 60 ^{o}C for 24 hours and the dried sample was then weighted (post weight) to calculate the total EPS produced.
- Pre-weight was subtracted from the post-weight of each filter to obtain the weight of EPS produced.

4.8 Violacein extraction and quantification (Choo et al., 2005)

- 1 ml of each culture broth after growth measurement transferred into two micro vials of 2 ml.
- Then centrifuged at 12000 rpm at 25 ^{o}C for 15 minutes.
- After the centrifugation cell free supernatant was discarded.
- Pellets were washed with DMSO (Dimethyl sulfoxide). The amount of DMSO is equal to the amount of broth taken in the eppendorf tube.

- Vortexing was done to dissolve the pellet. After centrifugation the pellet became very hard. To dissolve hard pellet micropipette was used.
- After re-suspending of pellet, centrifuged at 12000 rpm for 15 min at 25 °C to extract the violacein.
- The OD was measured at 585 nm for violacein production, which is the λmax of violacein.

4.9 Estimation of ethanol (Mudili et al., 2006)

Reagents: Potassium dichromate is required for the estimation. To prepare the potassium dichromate 17g of potassium dichromate was dissolved in 250ml of distilled water in 1 liter flask. To this 162.5ml concentrated sulfuric acid was added slowly, holding the flask in ice bucket.

Table 4: Experimental Procedure Table for Ethanol Estimation

No.	Conc. Of standard alcohol (% v/v)	Amount of alcohol (ml)	Distilled water (ml)	Potassium dichromate reagent (ml)	Distilled water (ml)		
1.	2	1	15	25	10	60° C for 30 minutes in a constant temperature water bath	Determination of the OD at 600 nm.
2.	4						
3.	6						
4.	8						
5.	10						
6.	unknown						

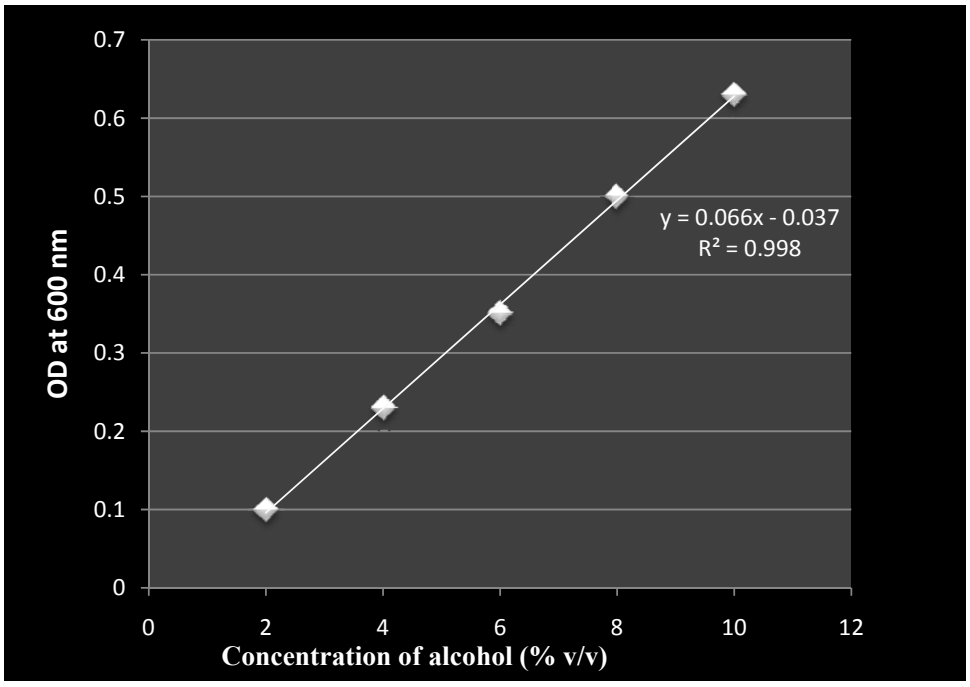


Figure 13.Standard Curve for Alcohol Estimation

4.10 Prodigiosin Extraction and quantification (Vaikunthvasan et al., 2012)

- 1 ml of broth containing growth of cells taken in 2 ml micro vials centrifuged at 12000 rpm for 15 min at 4 ºC.
- CFS was discarded and the cells pellet was suspended in acidified methanol. The amount of acidified methanol will be dependent on the size of the pellet.[100 mL acidified methanol was prepared by adding 4 ml of HCL into 96 mL of methanol (Merck, Mumbai)]
- After adding methanol vortexing was done till the pellet was dissolved.
- Extraction of prodigiosin was done by centrifugation at 12000 rpm for 15 min at 4 ºC. The measurement of absorbance was taken at the 535 nm, which is the λmax of prodigiosin.

4.11 Cellulase activity by glucose estimation

- 1ml of broth containing growth of cells was taken in 2ml eppendorf , and was centrifuged at 10,000 rpm at 25 ºC for 15 minutes.
- 0.5 ml of supernatant was then incubated with 0.5 ml of CMC Na(1%)(Sodium Carboxymethyl cellulose salt) at 50 ºC in serological waterbath.
- 1ml of DNSA reagent was added to estimate the glucose formed in the system and was incubated at
- 100 ºC for 10 min in serological waterbath.
- Absorbance was taken at the 540 nm. (Agilent, UV – Vis spectrophotometer).

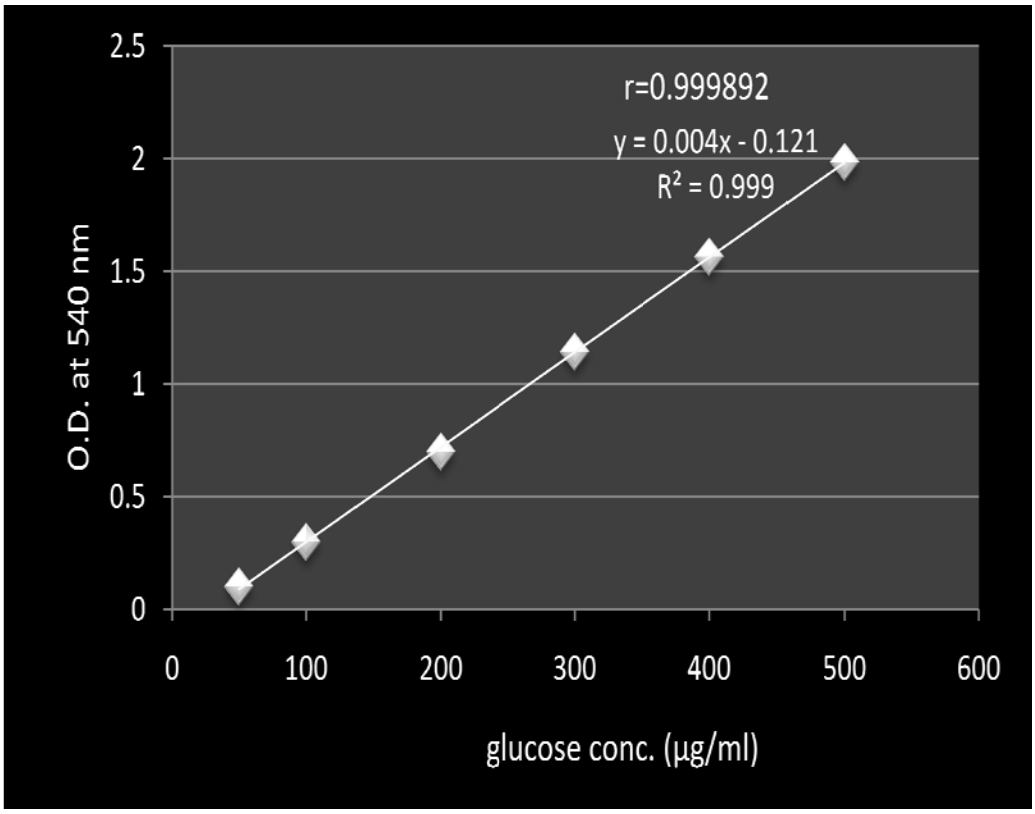


Figure 14.Standard curve of glucose estimation

5. Results

5.1. Results of Growth and Metabolite production

5.1.1. Results of *raag Piloo-teentaal*

Table 5.Effect of *raag Piloo* on Growth and certain Metabolites

Organism	Growth (OD_{660})			EPS Production (g/L)			EPS Production per unit OD (g/L)		
X. campestris	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.32±0.01	0.44±0.02	37.5**	1.90±0.10	2.76±0.4	45.26*	5.93	6.27	5.73
X. campestris (supplimented with $CaCl_2$)	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	1.18±0.03	1.29±0.03	9.32*	3.73±0.05	4.80±0.10	28.68**	3.16	3.72	17.72
	Growth (OD_{660})			**Violacein production (OD_{585})**			**Violacein unit(OD_{585}/OD_{660})**		
C. violaceum	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	1.24±0.04	0.94±0.01	-24.19**	1.29±0.10	0.77±0.04	-40.31*	1.04	0.81	-22.11
	Growth (OD_{660})			**Prodigiosin (OD_{535})**			**Prodigiosin unit (OD_{535}/OD_{660})**		
S. marcescens	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.67±0.001	0.62±0.006	-7.46**	0.42±0.004	0.16±0.007	-61.9**	0.62	0.25	-59.67
	Growth (OD_{660}) (1:1 dilution)			**Alcohol production (% v/v)**			**Alcohol production per unit OD (% v/v)**		
S. cerevisiae	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	1.28±0.01	1.34±0.0009	4.68*	1.62±0.00	1.82±0.04	12.96**	1.26	1.36	7.93
	Growth (OD_{660})			**Glucose production(OD_{540})**			**IU(per ml)**		
B. parabrevis	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.50±0.03	0.59±0.00	18.0*	0.18±0.00	0.23±0.00	27.77**	0.027±0.00	0.032±0.00	15.35**
	Growth (OD_{660}) (1:1 dilution)			**pH**					
L. plantarum	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**			
	1.17±0.00	1.21±0.01	3.41**	4.30±0.00	4.16±0.05	-3.25*			
	Growth (OD_{660})								
B. parabrevis *(cellulose+ Gelatin containing mineral media)*	**Control**	**Experimental**	**% Change**						
	0.14±0.01	0.22±0.02	57.14**						

***p≤0.01, *p≤0.05,* '-'minus sign indicates a decrease over control

The effect of sound waves of *raag Piloo* affected positively and the significant changes in growth and metabolites were observed as shown in the table 5. The growth of *C. violaceum* and *S. marcescens* with their pigment production was negatively affected.

The growth and metabolite production in *B. parabrevis, S. cerevisae* and *X. campestris* were positively affected. Cellulase production and the growth of *B. parabrevis* were increasing significantly under the treatment of sound waves up to 18 %. Also, xanthan production and growth of *X. campestris* were increasing significantly through these sound waves up to 37.5 %. In case of *S. cerevisae,* significant increases in growth and alcohol production have seen. Lactic acid producing organism, which is *L. plantarum* showing positive growth effect as

well as decreasing pH value of the medium. Here, decreasing of pH in music treated tube indirectly indicate more lactic acid production compare to control tube due to the effect of these music.

5.2. Results of *raag Ahir Bhairav*

5.2.1. Results of Growth and Metabolite production

Table 6.Effect of *Ahir Bhairav* on Growth and Certain Metabolites

Organism	Growth (OD_{660})			EPS Production (g/L)			EPS Production per unit OD (g/L)		
X. campestris	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.44±0.02	0.52±0.03	18.18*	1.73±0.11	2.26±0.15	30.63**	3.93	4.34	10.43
	Growth (OD_{660})			**EPS Production (g/L)**			**EPS Production per unit OD (g/L)**		
X. campestris (CaCl2)	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.45±0.01	0.48±0.01	6.66*	3.56±0.11	4.70±0.20	32.0**	7.91	9.79	23.76
	Growth (OD_{660})			**Violacein production (OD_{585})**			**Violacein unit(OD_{585}/OD_{660})**		
C. violaceum	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.75±0.01	0.78±0.01	4.0*	0.37±0.01	0.41±0.004	10.81*	0.49	0.52	6.12
	Growth (OD_{660})			**Prodigiosin (OD_{535})**			**Prodigiosin unit (OD_{535}/OD_{660})**		
S. marcescens	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.67±0.00	0.64±0.00	-4.32**	0.52±0.03	0.36±0.03	-30.76**	0.77	0.56	-27.27
	Growth (OD_{660}) *(1:1 dilution)*			**Alcohol production (% v/v)**			**Alcohol production per unit OD (% v/v)**		
S. cerevisiae	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	1.23±0.01	1.34±0.01	8.94**	1.58±0.01	1.74±0.00	10.06*	1.29	1.30	0.77
	Growth (OD_{660})			**Glucose production(OD_{540})**			**IU(per ml)**		
B. parabrevis	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.48±0.00	0.57±0.02	18.75**	0.11±0.01	0.16±0.01	45.45*	0.021±0.001	0.026±0.001	23.80**
	Growth (OD_{660}) *(1:1 dilution)*			**pH**					
L.plantarum	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**			
	1.14±0.00	1.21±0.00	6.14**	4.26±0.05	4.03±0.05	-5.39**			

***P≤0.01, *p≤0.05,* '-'minus sign indicates a decrease over control

The effect of *raag Ahir Bhairav* on growth and metabolite production is shown in table 6. The effect is positive as well as negative on growth and metabolite production. All the organisms showing positive effect due to the influence of sound except *S. marcescens* is showing negative effect.

The growth as well as prodigiosin production in *S. marcescens* decreases under the treatment of sound waves. It clearly indicates the effect of music that is different music can generate the different kind of stress and it can be benefitial to microbes as well as it can harm the growth rate of microbes.Violacein production and growth of *C. violaceum* were increased under the sound waves treatment. Similarly, Xanthan production and growth of *X.campestris* were increasing significantly through sound waves. Here, by changing the medium for X. campestris i.e. by adding $CaCl_2$ in the TYE medium, growth and Xanthan production also getting affected

positively. The magnitude of increase in Xanthan production is changing which is higher than the previous one (only TYE broth containing growth medium). Cellulase production and growth of *B.parabrevis* was increased by providing these sound waves. Same result is seen for the alcohol production and growth of *S.cerevisae* which is increasing significantly by providing this music. Here, the growth of *L plantarum* also increasing and at the same time pH is decreasing which indicating the production of lactic acid in the medium.

These both ragas are having different sound patterns so these are affecting differently to these microorganisms, difference in the frequency and intensity is having different effects on the growth and metabolite production of microorganisms. After checking the effect of both the *ragas*, we decided to check whether this growth of microbes is increased due to the consumption of glucose present in the medium or not. So, for that we run a set up in which *B. parabrevis* is inoculated in glucose containing medium and music treatment is given.

The reason behind these effects were hypothesized as the cell membrane is largely getting affected by sound waves, so the permeability of membrane will change, and accordingly the entry of sugars and nutrients from external environment is increasing. So, to prove this at the interval of one or two hours during time of incubation samples withdrawal was done in sterile condition. Table 7 shows that in case of *B. parabrevis,* the medium composition was changed and the growth as well as glucose estimation was done. The concentration of glucose detected was less in the experimental setup i.e., gets utilized by the cells in comparison to control. It directly shows the effect of sound waves on the permeability. Increase in time showing increase in the growth also. Extracellular glucose consumed by the cells shows negative results which means that organism using glucose for their growth. These results show that in the presence of music, cell membrane permeability is getting affected and thus faster uptake of glucose is occurring.

Table 7.Faster uptake of glucose by *B. parabrevis* under the influence of *raag Ahir Bhairav*

Time (h.)	Growth(OD_{660})			Extracellular glucose con. (µg/ml)		
	Con.	Exp.	% Change	Con.	Exp.	% Change
0	0.00±0.03	0.00±0.00	0.00	559.25±2.13	557.75±1.08	-0.26
4	0.10±0.00	0.15±0.00	50.00**	427.33±2.76	417.25±2.38	-2.35**
9	0.16±0.00	0.18±0.00	12.50**	307.91±0.94	279.00±1.25	-9.38**
18	1.00±0.05	1.25±0.00	25.00**	199.00±3.92	178.00±1.75	-10.55**
24	1.38±0.01	1.60±0.01	15.94**	136.58±9.52	120.83±1.84	-11.53**

The graphical representation of more consumption of glucose in experimental tube in case of *B. parabrevis* is shown in figure 15. The graph of growth and glucose concentration are plotted with respect to time. Growth in experimental one is more than the control; accordingly glucose got more metabolized by the cells in experimental tube. Same in the case of *S. cerevisiae*, more glucose was metabolized by the cells in experimental one, more growth was occurred and more alcohol was produced in experimental one in comparison to control one as mentioned in figure 16.

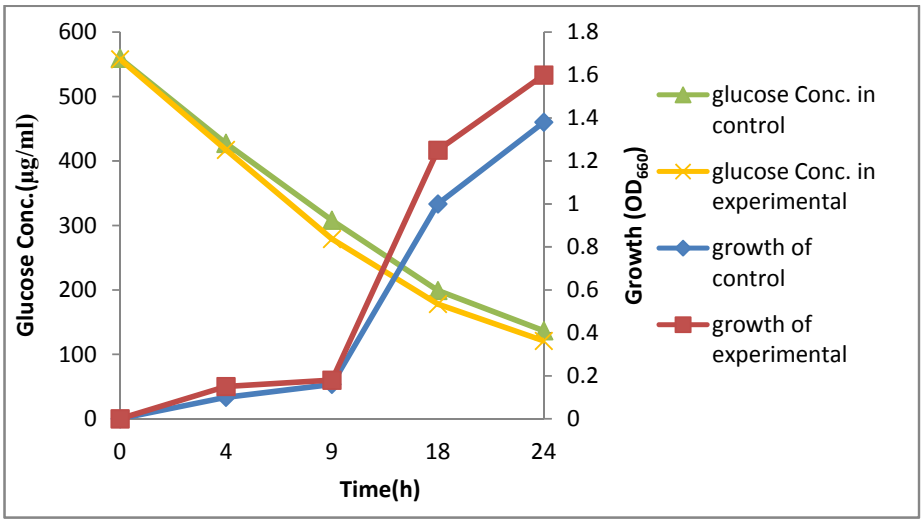


Fig.15. Influence of *Ahir Bhairav* on growth and glucose consumption in *B. parabrevis*

Table 8.Faster uptake of glucose by *S. cerevisiae* under the influence of *raag Ahir Bhairav*

Time (h.)	Growth(OD_{660})			Extracellular glucose con.(µg/ml)			Alcohol con.(%v/v)		
	Con.	Exp.	% Change	Con.	Exp.	% Change	Con.	Exp.	% Change
0	0.00±0.00	0.00±0.00	0.00	10,000±23.62	10,000±35.00	0.00	3.25±0.00	3.25±0.00	0.00
12	0.88±0.02	1.06±0.02	20.45**	5893.33±84.31	4900±24.64	-16.85*	1.81±0.24	2.45±0.00	35.35**
48	1.21±0.04	1.53±0.03	26.44*	298.00±15.05	246.16±2.76	-17.39**	2.56±0.12	3.18±0.09	24.21**

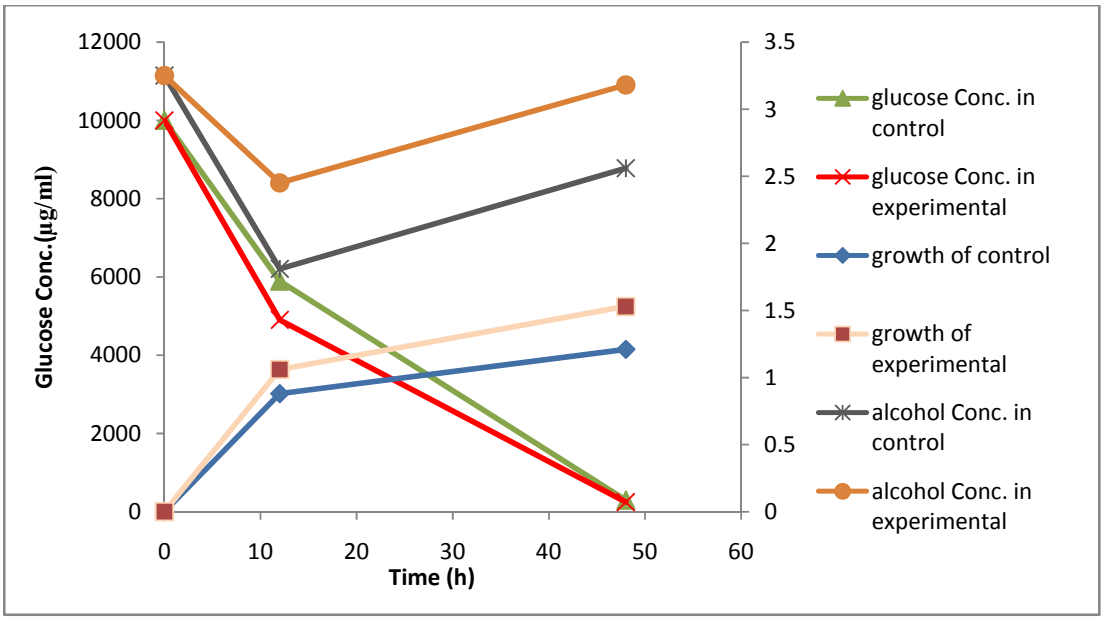


Fig. 16 Influence of *Ahir Bhairav* on growth, glucose consumption and alcohol production in *S. cerevisiae*

In case of *S. cerevisae,* in GYE medium glucose is present as carbon source, so at specific intervals to check the effect on cell membrane permeability same set up was done as in case of *B. parabrevis,* and results were obtained. Glucose is a simple sugar which is easily consumed by the cells and due to the effect of sound on cell membrane, more glucose consumption is taking place in experimental tube was shown. Significant alcohol production was also measured and reported in table 8. So here, we can correlate the growth, extracellular glucose concentration and alcohol concentration from the medium.

Growth rate and metabolism of *S. cerevisiae* growing in liquid culture has been shown through metabolomic study to get affected by sonic vibration (Aggio et al., 2012). They have reported a reduction in biomass production upto 14%, when *S. cerevisiae* was grown in presence of music, with a simultaneous faster (12.4%) growth rate. In the present study, the cell density of *S. cerevisiae* growing in presence of music to be higher than in absence of music (Table 5-6). When growth rate was calculated using the data from which graph shown in Figure 16 is plotted, it was found to be 23.82% higher (P<0.01) in absence of music (0.0403 h^{-1}) than that (0.0307 h^{-1}) in its presence.

In such another experiment performed with *B. parabrevis*, sound treatment was found to have a positive effect on cell density as well as growth rate (Figure 15). Growth rate in presence of music (0.212 h^{-1}) was 4.95% higher (P<0.01) than that (0.202 h^{-1}) in its absence.

Discussion

The effect of the music on prokaryotes and eukaryotes has been checked by these experiments. The sound can be considered as mechanical stress to the cell whether it is bacterial or eukaryotic cell. The sound wave is characterized as a mechanical stress as it can have different frequencies as well as different intensities (Danet et al., 2005). Gram positive organisms *L. plantarum*, *B. parabrevis*, Gram negative organisms *C. violaceum*, *S. marcescens, X. campestris* and eukaryotic organism (Yeast) *S.cerevisae* were treated with sound waves and they gave individually different results. From the available results it was found that music was effective against selected prokaryotic and eukaryotic cells.

Different music patterns may have different effect that is clearly seen from the results. In the present study of first music i.e. *Ahir bhairav*, the growth and production of certain metabolites was increased as an effect of *Ahir bhairav* music pattern on bacterial growth. The pigment production in *C. violaceum* has been icreased significantly. The exopolysaccharide production has also been increased hence by these results we can say that music which is a kind of a mechanical stress increases the certain mechanisms in the cell by which the extracellular metabolite secretion is enhanced by this sound patterns. Sound stimulation had shown effect on the plant growth and development by interacting with the plant cells (Xiujaun et al., 2002). It has been shown in some studies that some sound wave can accelerate the growth of cells by changing the transition temperature of cell walls (Sun et al., 1999).

The second music i.e. *raag piloo* has also profound positive effect on the growth of the microorganisms as it was also able to stimulate the growth of gram negative and gram positive. The change in the growth and metabolite production is not parallel; these both parameters are individually affected through sound waves. The magnitude of increase in the metabolite production like xanthan, violacein, cellulase is more than the magnitude of Growth. By studying these results one hypothesis was created that these sound waves are affecting on the membrane so directly affects the entry of the nutrient molecules, to check it the extracellular glucose in the medium was estimated in case *of B. parabrevis* and *S. cerevisiae* as in all these cases growth was stimulating through sound waves . The results obtained are shown in table 7 and table 8. In both the cases, the experimental showing the amount of glucose is lesser than control that directly shows that it is metabolized faster by the cells. The results also clearly indicates that the different music can have different magnitude of effect on the microbial cells as it can be seen by different percent increament found in the same organism with different music. There was no significant pattern found in these experiments that can connect the relationship between gram-positive or gram-negative bacteria with the effect of music.

A sound wave is characterized as a mechanical wave, which while passing through the liquid medium (microbial growth medium) creates vibrations. These vibrations, a form of mechanical force, can affect functioning of gated channels of the microbial cells. As a mechanical wave, audible sound would produce a mechanical stress to the microbial cells. This stress leads to increase in the mechanisms like Quorum sensing and metabolite production. As described in above sections the sound is an important environmental factor and the interaction of this factor with the prokaryotic or eukaryotic organism is likely to happen but the effect and the interactions yet remained to be resolved in a scientific manner.

Several research have proven that sonic waves and ‘green music’ (which normally comprises a classic music base along with some natural sounds such as those of birds, insects, water and wind) affects the metabolism and growth of some plants and vegetables as well as daily milking yields in cows. The microorganisms are very sensitive to the external environmental factors whether they are biotic or abiotic.

The music is a mixture of various sound and it also affects the bacterial survival and activity of different microorganisms(Mortazavian, 2008).

This study has shown that sound (in form of music) can affect microbial growth and metabolism to a noticeable extent. Sound (whether in form of music or otherwise) can travel through the growth medium in which microbes are inoculated, and this can give rise to vibrations. Bacteria have been shown to be capable of sensing the vibrational acceleration, and responding to it. Vibrational acceleration was shown to induce changes in the growth curve of certain bacteria. Experimental evidences suggesting microbial emission and response to physical signals like sound waves, electromagnetic radiation, and electric currents are available. These signals can propagate rapidly even at low intensities, they can elicit a rapid response from the sensing microorganisms. As a mixture of mechanical wave, music can produce a mechanical stress to the test organisms. The effect of the music can make a observable change in the membrane fluiditty as well as the membrane protein structure is also can be changed (Shaobin et al., 2010). The membrane trafficking modulation is one of the basic phenomena of the plasma membrane that is affected by the various ranges of audible sound (Apodaca, 2002). The metabolism activity is one of the important factor that should be understood by the researchers to understand the growth and substrate utilization in microbes. The acceleration of metabolism activity is also one of the phenomena which is taking place under the sound stimulation (Yi et al., 2003).

The microorganisms are very sensitive to the external environmental factors and as music was one of them, it influenced gram-negative, gram-positive and the yeasts those are being taken as a test organisms.In the previous work, determination of intracellular calcium and potassium was done and the changes in the flux of calcium and potassium were clear evidence that music was affected the fluxes of some component which are essential for growth as well as for secondary signal transduction. The lower concentration of intracellular calcium was detected in the cells which have been exposed to the music and lower amount of calcium was detected in the case of *S. cerevisiae* in which extracellular calcium was determined. Importance of calcium as a cell regulator is well established in eukaryotes, the role of calcium in prokaryotes is still elusive. It has been found that calcium ions are involved in the maintenance of cell structure, motility, transport and cell differentiation processes such as sporulation, heterocyst formation and fruiting body development. In addition, a number of calcium-binding proteins have been isolated in several prokaryotic organisms. The characterization of these proteins and the identification of other factors suggest the possibility that calcium signal transduction exists in bacteria. These observations represent recent developments of calcium in bacteria as it relates to signal transduction. The calcium is important in secondary signaling pathways and perhaps the changes is the amount of these calcium ions are responsible for the changes that occurred in the processes like pigmentation, alcohol production, and EPS production etc. Although a similar role for this divalent cation in prokaryotes is still elusive, there is increasing interest in and evidence for calcium as a regulator in bacteria. Important examples are the demonstration that bacteria keep tight control of their [Ca^{2+}] with values very similar to those found in eukaryotes (100–300 nM)(Gangola and Rosen, 1987; Knight *et al* , 1991; Futsaether and Johnsson, 1994; Herbaud et al., 1998; Jones et al., 1999; Torrecilla et al., 2000). Like eukaryotes, bacterial cells have ion channels, primary and secondary transporters, and CaBPs(Calcium binding proteins), which may be involved in Ca^{2+} homeostasis (Norris *et al.*, 1996, Duff *et al.*, 2000).

The higher amount of the pigment produced by the bacteria indicated that the music also played a role in quorum sensing as the pigment production which is one of the phenomena helps bacteria for the quorum sensing. Violacein production is quorum sensing dependent process. These altered patterns in pigment production due to music in bacteria can be explained with the help of some recent discoveries in which it was demonstrated that bacterial cells enhance the proliferation of neighboring cells under stress

conditions by emitting a physical signal (Matsuhashi et al., 1998). Sound waves function as a growth regulatory signal between cells. Microorganisms can generate and respond to the physical signals such as sound waves, electromagnetic waves and electric current. These physical signals play important role in cell-cell communication in microbes. The intensity of propagation of these physical signals is higher than the chemical signals and hence these signals are important to understand and enhance the quorum sensing phenomena.

The role of potassium in prokaryotes and eukaryotes is vital as the initial response of bacteria to osmotic up shock involves the uptake of potassium via kdp and trk systems which are important system for the potassium. A very general property is activation of K+ uptake by an increase in medium osmolarity. This response is modulated by both internal and external concentrations of K^+. Kdp is the only K^+-transport system whose expression is regulated by environmental conditions (Epstein, 2003). The intracellular potassium concentration was changed due to the effect of the music *raag Malhar* was seen in the previous work. *Kirvani* raag has its own effect on this movement of potassium across the cell in which in both of the cases, the intracellular potassium was found to be lower in music treated cells. These things self indicates the effect of music on the flux of a particular molecule because the potassium regulation is directly connected to the osmolarity, hence changes in the permeability may lead the cells to regulate the concentrations of potassium at different level intracellularly and extracellularly. Primary and secondary transporters as well as ion channels (Ca2, K+, Na+) have been documented in several genera of bacteria (Norris *et al*, 1996; Paulsen *et al*, 2000). Transport systems as well as secondary signaling system are the two basic important phenomenons of the microbial cells which are getting affected by the music and hence music and vibrations of sound are playing significant role in modulation of the plasma membrane structure as well as its permeability.

Colony formation, biofilms and microbial mats are likely candidates to benefit from the sound communication. It has been demonstrated that the wide ranges of acoustic frequency signals are emitted by the prokaryotic as well as eukaryotic organisms. The yeast cells can emit the signal ranging in 0.9-1.6 kHz and *B. subtilis* can emit the signals ranging in between 8-43 kHz as it was demonstrated that *B. subtilis* can emit the strongest sound signal detected so far. Furthermore, temperature which can modulate the metabolic activity of the cell and its intracellular vibrations, it can affect at the specific frequency but not the intensity. Microbial cells can absorb more energy when the frequency of the incoming vibrations matches their internal or external vibrations and it gives rise to mechanical resonance. The physical signals are important because quorum sensing by the chemical signals requires substantial energy expenditure and communication whereas physical signals require less energy consuming and hence it is more beneficial to the bacteria. Physical signals require minimum energy investment as well as at low intensities physical signals provide a fine-tuning mechanism for cells to communicate their energy status to maximize the use of the available resources (Reguera, 2011).

Sound waves can have role in microbial cell-cell communication, in both prokaryotes (bacteria) and eukaryotes (yeast and protozoa). As physical signals like sound waves are less amenable to diffusion constrain, and can propagate through cells and different media, they can enable faster cellular responses. The energy inherent in sound signals can serve as a language during physical mode of microbial communication. Matsuhashi et al., (1998) suggested the possible function of sound waves as a growth-regulatory signal between bacterial cells. Cells emit and perceive sounds at wavelengths exceeding their own size.

Mechanosensory channels which can sense stretch on the cell membrane are distributed throughout the living world (Kohl and Noble, 2008). A particular type of accumulation of proteins on the cell edges known as focal adhesions are believed to mediate mechanical signals from the cell exterior (Hu et al, 2007).

Multiple studies have reported mechanical oscillations of the developing cells at even about 1 Hz (Horton and Charras, 2002), indicating that cells can communicate mechanically at these frequencies. Future biology may see a consensus building on the biological significance of acoustic effects.

Some evidences have been found that indicates the modulation in the membrane permeability was due to the music. The change in the intracellular and extracellular concentrations of calcium and potassium also has been demonstrated but still the expression level of protein if affected by the music at which magnitude is remaining to be resolved. Furthermore the transcriptome analysis, gene expression analysis are the better options to understand the proteins which are getting affected by the music. The future aspects for this research can be thought as this study is applicable in various research fields to understand the stress responses in the microbes as well as higher production of industrially important metabolite is also indicating that these effects of music could be scale up into fermentation technology and it is also cheaper and effective way to enhance the important metabolite production.

6. APPENDIX

- **Inoculum Standardization**

Inoculum was prepared and standardized with 0.5 McFarland standard, O.D should be in range of 0.08-0.10.
Three different media used for *Brevibacillus parabrevis*:

(1) Gelatin containing mineral salt medium

KH_2PO_4	0.5 gm
$MgSO_4$	0.25 gm
Cellulose	2 gm
Gelatin	2 gm
Congo red	0.2 gm
Agar	15 gm
Distilled water	1000ml

(2) N-broth + Cellulose containing media

Peptone	0.5 gm
NaCl	0.5 gm
Beef extract	0.5 gm
Yeast extract	0.5 gm
Cellulose	2 gm
Distilled water	100ml

(3) CMC-Na + 10% Nutrient broth

Peptone	0.05 gm
NaCl	0.05 gm
Beef extract	0.05 gm
Yeast extract	0.05 gm
CMC-Na	2 gm
Distilled water	100 ml

- **GYE medium:** (for *S. cerevisiae*)

Peptone	1 gm
Agar	3 gm
Yeast extract	0.5 gm
Glucose	1 gm
Distilled water	100 ml

- **DNSA reagent Preparation** [Nigam A. and Ayyagari A. (2008)]

  In 50 ml distilled water 30 g sodium potassium tartrate hexahydrate (Merck) is dissolved. 1.6g of NaOH dissolved in 20 ml of distilled water. To the 50 ml of sodium potassium tartrate then 1 g of 3, 5-Dinitrophenol salicylic acid is added. This gives a yellow colored milky solution further to which NaOH solution is added giving a transparent yellow-orange DNSA reagent.

- **Decibel meter (Sound level meter)**

  Decibel meter was purchased from "Bearing and Tool Center" shop. 1st floor, Vyapar Bhavan, Kadia kui, Relief road, Ahmedabad-380001.
  Phone number- 079-22160386 Mobile number- 9327004639

- **Music player**
  Music player was purchased from the Mahalakshmi shop for electronic, located near kadia kui, Kalupur police station road.

Table 9.Thermostablity in *B. parabrevis*

	O.D. at 540 nm			IU per ml			Glucose concentration (µg/ml)		
Autoclaved	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.0691±0.017	0.0855±0.04	23.18	0.017	0.019	8.571	47.52	51.5	8.421
Un autoclaved	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	% Change
	0.1893±0.015	0.2524±0.022	33.333*	0.028	0.034	21.428	77.5	93.25	20.322
In boiling water bath For 3 hrs.	Control	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**	**Control**	**Experimental**	**% Change**
	0.1267±0.034	0.2806±0.0234	122.222	0.022	0.037	62.280	61.75	100.25	62.348

In case of *B. parabrevis,* the thermostablity was also checked and the results obtained in table 9 showing the comparision of the cellulase activity when it is autoclaved, unautoclaved and kept in boiling water bath for 3 hours. Here, the results showing that through the sound waves the thermostability is also getting affected. Cellulase is showing its cellulolytic activity at room temperature when incubated with substrate (CMC-Na) and also in boiling water (100^0C). The structure of cellulase enzyme might be denatured when it was incubated at this high temperature (100^0C) for 3 hours. After cooling for 1 hour, the structure might be re nature and thus cellulase showing its cellulolytic activity again. The activity of cellulase also checked after autoclaving with the substrate and it is showing less activity compared to unautoclaved and boiling water results. After showing all these results we can say that the enzyme might be thermostable and which is highly useful in industry.

Table 10.Known concentration of alcohol

No.	Alcohol con. (%v/v)	Measured alcohol con. (%v/v)	% error
1	2	1.657	17.15
2	1	1.03	3
3	4	3.72	7

Table 11.IU of control set up

	Control (IU)
	0.0482
	0.0521
	0.0287
	0.0316
	0.0287
	0.0325
	0.0340
Mean±S.D.	**0.0365±0.009**

7. References

1) Xiujuan W., Bochu W., Yi J., Chuanren D., Sakanishi A.: Effect of sound wave on the synthesis of nucleic acid and protein in chrysanthemum. Colloids and Surfaces B: Biointerfaces29, 99-102 (2003).
2) B.C. Deva, the Music of India: A Scientific Study, Munshiram Manoharlal Publishers Pvt. Ltd., New Delhi (1981).
3) Mortazavian A. M.: Music affects survival and activity of microorganisms, *Journal of Paramedical Sciences,* (2012).
4) Shaobin G., Wu Y., Li K., Li S., Ma S., et al.: A pilot study of the effect of audible sound on the growth of *Escherichia coli*. Colloids and Surfaces B: Biointerfaces 78(2): 367-371 (2010).
5) Reguera G.: When microbial conversations get physical. Trends Microbiol19(3):105-113 (2011)
6) Matsuhashi M et al.,: Production of sound waves by bacterial cells and the response of bacterial cells to sound. J Gen Appl Microbiol;44:49–55(1998)
7) Makiello L.: The Mozart Effect Science395, B4(2012) (http://printarchive.epochtimes.com/a1/en/sg/nnn/2012/01%20January_2012/Issue%220395_17_January_2012/395_B4.pdf)
8) Ayan I., Aslan,G., Comelekoglu U., Yilmaz N., Colak M.: The effect of low-intensity pulsed sound waves delivered by the Exogen device on *Staphylococcus aureus* morphology and genetics. Acta Orthop Traumatol Truc42(4):272-277 (2008).
9) Pornpongmetta S., and Thanuttamavong M.: Effects on microbial substrate utilization of aerobic bacteria from municipal waste water treatment plant part Π: comparative effects of musical characteristics(2010) (www.eng.ku.ac.th/e-journal_en/download.php?file=Cover.pdf
10) Blosser R. S., Gray K. M.: Extraction of violacein from *Chromobacterium violaceum* provides a new quantitative bioassay for *N*-acyl homoserine lactone autoinducers J Microbiol Methods40, 47-55 (2000).

11) Pradeep B. V., Pradeep F. S., Angayarkanni J., Palaniswamy M.: Optimization and production of prodigiosin from *serratia marcescens* mbb05 using various natural substrates., Asian J Pharm ClinRes6, 34-41 (2013).

12) Li Y. X., Yin L. H., Qi Y.: Screening of *Xanthomonas campestris* producing high viscosity and acid resistant xanthan gum and its fermentation process. Food Sci33, 211-216 (2012).

13) Harley J. P., Prescott L. M.: Laboratory Exercises in Microbiology, 5th ed., McGraw Hill, 2002, pp. 388

14) Martinac B., Saimi Y., Kung C.: Ion channels in microbes. PsycholRev88, 1449 (2008).

15) Nikaido H.: Molecular basis of bacterial outer membrane permeability. MicrobiolMolBiol Rev 67, 593-656 (2003).

16) Kung C., Martinac B., Sukharev S.: Mechanosensitive channels in microbes. Annual Reviews Of Microbiol 64,313-329 (2010).

17) Van Maris A. J., Abbott D. A., Bellissimi E., van den Brink, J., Kuyper M. et al., : Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek 90, 391-418 (2006).

18) Harvey E. N., Loomis A. L.: The destruction of luminous bacteria by high frequency sound waves JBacteriol17, 373 (1929).

19) Juergensmeyer M. A., Nelson E. S., Juergensmeyer E. A.: Shaking alone without concurrent aeration affects the growth characteristics of *Escherichia coli.* Lett Appl Microbiol 45, 179-183 (2007)

20) Zhao H. C., Wu J., Zheng L., Zhu T., Xi B. S. et al., : Effect of sound stimulation on *Dendranthema morifolium* callus growth ,Colloids and Surfaces B:Biointerfaces29, 143-147 (2003).

21) Butler M: Cells and sound: an introduction. Physical Biology Articles (2012). (http://www.academia.edu/1285421/Cells_and_sound_an_introduction_Review_to_be_submitted_2013).

22) Kohl P., Noble D.: Life and mechanosensitivity. Progress Biophysics Mol Bio 97,159-162 (2008).

23) Charras G. T., Horton M. A.: Single cell mechanotransduction and its modulation analyzed by atomic force microscope indentation. Biophysical J82, 2970-2981 (2002).

24) Pelling A. E., Sehati S., Gralla E. B., Valentine J. S., Gimzewski J. K.: Local nanomechanical motion of the cell wall of *Saccharomyces cerevisiae*. Science 305, 1147-1150 (2004).

25) Shaobin G., Bin Y., Ying W., Shi-Chang L., Wen L., Xiao-Fei D., Meng-Wei L.: Growth and physiological characteristics of *E. coli* in response to the exposure of sound field, Pakistan J of biological sciences 16(18): 969-975 (2013).

26) Pitt W. G., Ross S. A.: Ultrasound increases the rate of bacterial cell growth, *Biotechnol Prog*; 19(3): 1038–1044 (2003).

27) Aggio R.B.M., Obolonkin.V., Villas-Boas S. G.: Sonic vibration affects the metabolism of yeast cells growing in liquid culture: a metabolomic study, Metabolomics 8:670 678(2012).

28) Gupta P., Samant K., Sahu A.: Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential, International Journal of Microbiology, 5 pages(2012).

29) Wee Y.J., Kim J.N., Ryu H.W.: Biotechnological production of lactic acid and its recent applications, *Food Technol. Biotechnol. 44* (2) 163–172 (2006).

30) Ghose T.K.: Measurement of cellulase Activities. *Pure & Appl. Chem.* 59: 257-268 (1987).

31) Miller G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar." *Anal. Chem*. 31:426-428 (1959).

32) Teather R.M., Wood P.J.: Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, Applied and environmental microbiology, p.777-780(1982).

33) Kuhad R.C., Gupta R., Singh A.: Microbial cellulases and their industrial Applications, Enzyme Research, Review Article, Volume (2011).
(http://dx.doi.org/10.4061/2011/280696)

34) R.K. Sukumaran, R. R.Singhania,A. Pandey: Microbial cellulases-production, applications and challenges, Journal of Scientific and industrial research, Vol.64,pp.832-844 (2005)

35) J Singh, S Banal: Combinative impact of effectors on production of celluolytic enzyme from *Brevibacillus parabrevis* (MTCC 2208) ,European Journal of Experimental Biology 3(5):484-490 (2013).

36) Nigam A. and Ayyagari A. (2008) Lab Manual in Biochemistry, Immunology, Biotechnology (2008) Tata-McGraw Hill

37) Sarvaiya N. and Kothari V.: Effect of audible sound in form of music on microbial growth and production of certain important metabolites, Microbiology, Vol. 84, No. 2, pp. 227–23 (2015).

38) Sawada. Y., Murase. M., Sokabe. M.: The gating mechanism of the bacterial mechanosensitive channel MscL revealed by molecular dynamics simulations from tension sensing to channel opening, Landes Bioscience, Channels 6:4, 317-331; July/August (2012)

- **Some important notes**

- Music systems should be kept clean during experiments.
- TLC jar which we are using for experiments should be washed in regular intervals.
- TLC jars should be wiped with alcohol regularly to avoid the chances of contamination and should be sealed proper by applying silicone gel between the jar and glass lead.
- The continuous music treatment is very much important to be maintained and for that one should check the music system in every 6-8 hours that it is working properly or not.
- The distance should be maintained strictly and for the experiments which are running at room temperature, Thick line should be drawn at the place where we are putting our flasks or test tubes. This distance was from center of speaker to the center of flasks or test tubes.
- There are chances of contamination of bacteria during experiments and to avoid that handling should be proper and sterility should be maintained strictly.
- Frequency analysis is an important task and use of WavePad software is easy. The FFT i.e. Fast Fourier Transform and TFFT i.e. Temporal Fast Fourier Transform is important during frequency analysis. One graph is generated by these tools, it cannot be copied from the software to normal software that we are using, so we can overcome this problem by taking screenshot of the computer with the help of “PrntScr” button and then it can be pasted in the paint and after that image or graph can be cropped.