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SOUND STIMULATION CAN INFLUENCE MICROBIAL GROWTH AND PRODUCTION OF CERTAIN KEY METABOLITES

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ABSTRACT

Effect of two different audible sound (music) patterns on six different microorganisms was investigated. Both the sound patterns namely *Ahir Bhairav* (172-581 Hz) and *Piloo* (86-839 Hz) were able to significantly affect microbial growth and production of certain key metabolites by the test microbes. Faster uptake of glucose from the growth medium by *Brevibacillus parabrevis* and *Saccharomyces cerevisiae* was observed under the influence of sound. Production of quorum sensing-regulated pigments, prodigiosin and violacein, respectively by *Serratia marcescens* and *Chromobacterium violaceum* was also notably affected by sound treatment. Further investigation to decipher molecular basis of microbial response to sound is warranted to understand the mechanism how audible sound interacts with microbial cells, and how the microbial population(s) modulate their behavior in response to sound stimulation.

Keywords: Sound stimulation, Music, Growth, Pigment, Membrane, Quorum sensing

INTRODUCTION

Living cells and organisms can sense changes in various environmental factors in their surroundings, and respond accordingly. Effect of many of the environmental factors such as pH, temperature, osmotic pressure, light, etc. has been well characterized. However sound remains one such factor, which despite being widely present in the growth environment of almost all living systems, whose effect on living cells/organisms has not been characterized that well. Sound's effect on higher organisms having some sort of sound-sensing organ (e.g., ears in animals) is of obvious nature. There have been few reports **(Hassanien *et al.*, 2014)** describing the effect of noise and/or music on plants and animals. Not many such systematic investigations describing influence of sound on microorganisms are available. Much remains to be investigated on how microorganisms interact with external sound, and how they behave when placed in an external sound field. This is a relatively nascent area of scientific research.
Certain earlier reports **(Matsuhashi *et al.*, 1998)** have indicated microorganism's ability to produce, sense, and respond to sound. However most reports are concerning the sound beyond human audible range i.e. 20-20,000 Hz. In the present work, we have focused on the effect of audible sound (composed of multiple frequencies) on selected microorganisms, when the sound is provided in form of a particular pattern i.e. music.

MATERIALS AND METHODS

Test organisms

List of the prokaryotic and eukaryotic microorganisms employed in this study, and the parameter(s) on which the influence of sound was investigated, is provided in Table 1. All the test strains were procured from Microbial Type Culture Collection (MTCC), Chandigarh. All media/media components were from HiMedia, Mumbai.

Table 1 Test organisms

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

[#]CMC: carboxymethyl cellulose

Sound treatment:

Sound stimulation of the test cultures was executed as described in our previous study **(Sarvaiya and Kothari, 2015)**. Inoculum of test organism from its activated culture was prepared in sterile normal saline and was standardized to 0.5 McFarland turbidity standard. The tubes with growth medium after inoculation were put into a glass chamber (Merck; 225 ×225 ×125 cm). A speaker was placed in this glass chamber at a distance of 15 cm from the inoculated test tubes/flasks, from which the sound was delivered (sound delivery was provided throughout the period of incubation). This glass chamber was covered with a glass lid, and multiple layers of cloth and paper were used to cover the lid. This was done to prevent any possible leakage of sound from the chamber, and also to avoid any possible interference from external sound. Similar chamber was used to house the control (not stimulated with music) group test tubes. One speaker was also placed in the glass chamber used for the control test tubes at a distance of 15 cm, where no electricity was supplied and no sound was generated. Frequency of the test sound was analyzed by NCH WavePad Sound Editor Masters Edition v. 5.5. Of the two music types used, raag *Ahir Bhairav* (sound-I) was found to be composed of sound falling in the frequency range 150-7811 Hz, of which the range 172 - 581 Hz was dominant (Figure 1). Similarly, frequency range of the second test music raag *Piloo (Teentaal;* sound-II) was determined to be 43-5620 Hz, of which the range 86-839 Hz was dominant (Figure 2). Source for both these sound patterns was the commercially available musical disc titled *Call of the Valley* (Saregama India Ltd., Kolkata). Intensity of the sound, measured with a sound level meter (acd machine control Ltd.) at a distance of 15 cm from the speaker was 70-90 dB (in case of *Ahir Bhairav*), and 85-110 dB (in case of *Piloo*). Absence of detectable sound in the control chamber was ensured using decibel meter.

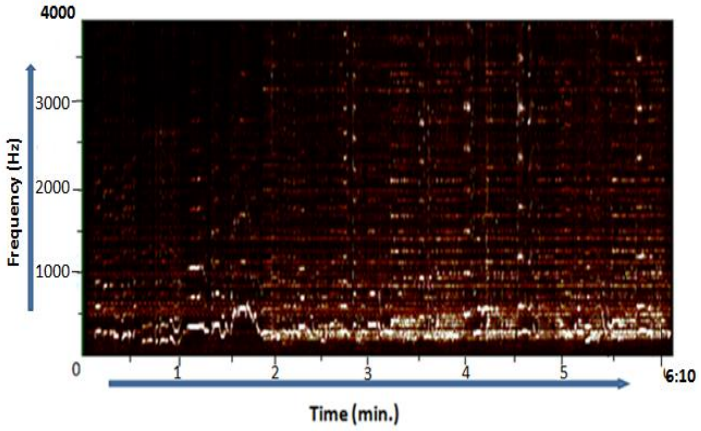

Figure 1 Frequency distribution over time for the *raag Ahir Bhairav*

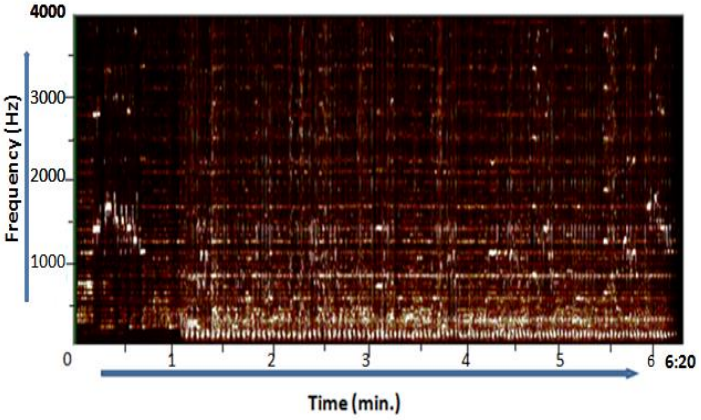

Figure 2 Frequency distribution over time for the *raag Piloo (Teentaal)*

Violacein extraction and estimation

After measuring growth by recording OD at 660 nm using spectrophotometer (Agilent- Carry 60), violacein extraction **(Choo *et al.*, 2006)** was done from *C. violaceum* culture. Briefly, 3 mL of the culture broth was centrifuged (REMI CPR-24 Plus) at 12,000 rpm (13,520 g) for 15 minute at 25^{0}C, and the resulting supernatant was discarded. The remaining cell pellet was resuspended into 3 mL of DMSO purchased from Merck (Mumbai), and incubated at room temperature for 30 min, followed by centrifugation at 12,000 rpm for 15 min. The violacein extracted in the supernatant was estimated by measuring OD at 585 nm.

Prodigiosin extraction and estimation

After quantifying growth at 660 nm, prodigiosin extraction was carried out from *S. marcescens* culture as described by Pradeep *et al.* **(2013)**. Briefly, 3 mL of the culture broth was centrifuged at 12,000 rpm for 15 min, and the resulting supernatant was discarded. The remaining cell pellet was resuspended in 3 mL of acidified methanol (4 mL of HCl into 96 mL of methanol; Merck), followed by incubation in dark at room temperature for 30 min. This was followed by centrifugation at 12,000 rpm for 15 min at 4°C. Prodigiosin in the resulting supernatant was estimated by measuring OD at 535 nm.

Alcohol estimation

Alcohol was estimated photometrically. *S. cerevisiae* culture broth was centrifuged at 12,000 rpm for 15 min, and the supernatant was used for alcohol estimation **(Williams & Darwin, 1950).** 1 mL of the supernatant was mixed with 15 mL of distilled water, and 25 mL of potassium dichromate reagent, followed by 10 mL of additional distilled water. Then incubation was carried out in water bath at 60°C for 30 min, followed by measurement of OD at 600 nm. Standard curve was prepared using absolute ethanol procured from Eureka, Ahmedabad at 2–10% v/v.

EPS quantification

Following estimation of growth by measuring OD at 660 nm, culture broth was subjected to centrifugation at 7,500 rpm (6,600 g) for 15 min, and the cell free supernatant (CFS) was used for EPS quantification using the method described in Li *et al.*, **(2012)** with some modification. Briefly, 40 mL of chilled acetone (Merck) was added to 20 mL of CFS, and allowed to stand for 30 min. The EPS precipitated thus was separated by filtration through pre-weighed Whatman # 1 filter paper (Whatman International Ltd., England). Filter paper was dried at 60°C for 24 h, and weight of EPS on paper was calculated.

Glucose uptake assay

Concentration of glucose present in the medium (after inoculation of test organism) was estimated at different time intervals, employing the photometric method using dinitrosalicylic acid (DNSA) **(Nigam and Ayyagari, 2008).** For this experiment *Brevibacillus parabrevis* was inoculated into a growth medium containing 0.5 g/L glucose, 0.5 g/L NaCl, 0.5 g/L beef extract, 0.5 g/L yeast extract, and 0.5 g/L peptone. Medium for *S. cerevisiae* contained 10 g/L glucose, 5 g/L yeast extract and 10 g/L peptone.

Estimation of cellulase activity

B. parabrevis was grown in a CMC supplemented broth (0.5 g/L peptone, 0.5 g/L NaCl, 0.5 g/L Beef extract, 0.5 g/L Yeast extract, 20 g/L CMC-Na). The cell free supernatant obtained from *B. parabrevis* culture after centrifugation of the culture broth (10,000 rpm; 9,390 g) was used as crude cellulase preparation. 0.5 mL of the supernatant was mixed with 0.5 mL of 1% carboxymethyl cellulose (CMC-Na salt; Merck), followed by incubation at 50°C for 30 min. The amount of glucose released as a result of cellulase activity was quantified using DNSA colorimetric assay. The international unit (IU) of the cellulase was calculated as: IU= [(µg of glucose)/ 180 (molecular weight of glucose) x 30 (incubation time) x 0.5 (aliquote)] **(Nigam and Ayyagari, 2008)**.

Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. Data with *p* values less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Raag Ahir Bhairav

Results regarding effect of sound-I are shown in Table 2. Growth and the particular test parameter(s) in case of all the test organisms experienced an enhancement under the influence of sound-I, except *S. marcescens*. The latter synthesized considerably lower pigment, when incubated in presence of music. This organism was earlier also found to experience a reduction in growth and prodigiosin production under influence of sound (*raag Kirwani*) corresponding to 38-689 Hz **(Sarvaiya and Kothari, 2015)**. This range of frequency includes the one corresponding to sound-I used in this study, however relative proportion of different frequencies is likely to be different in different music patterns, and accordingly their effect and its magnitude may vary. During another study conducted by us (using sound corresponding to 41-645 Hz; data yet unpublished) *S. marcescens* was found to produce almost 1.5 times higher prodigiosin despite a small reduction in growth. Besides *S. marcescens*, two more gram-negative

bacteria (*X. campestris* and *C. violaceum*) were used in this study, and both were found to grow better under the influence of sound-I, with simultaneous enhancement in synthesis of their respective test metabolites. Both the pigment producing organisms used in this study were affected by sound stimulation, but their response was of opposite nature. Whereas *S. marcescens* responded to sound stimulation negatively, *C. violaceum* showed better growth and pigment (violacein) production in response to the same sound treatment. Production of both these pigments (prodigiosin and violacein) is known to be regulated by quorum-sensing in the producing bacteria **(Wei *et al.*, 2006; Zinger-Yosovich *et al.*, 2006; Morohoshi *et al.*, 2007)**. Magnitude of effect of sound stimulation on EPS production and prodigiosin synthesis respectively in *X. campestris* and *S. marcescens* was much higher than its effect on growth of these organisms, indicating that the cellular machinery related to growth was affected differently than that related to synthesis and/or secretion of EPS and prodigiosin.

Table 2 Effect of *raag Ahir bhairav* on test organisms

Organism	Growth (OD_{660})			Prodigiosin (OD_{535})			Prodigiosin unit (OD_{535}/OD_{660})		
	Control (Mean±SD)	Experimental (Mean±SD)	% change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	%Change
S. marcescens	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})			**Violacein production (OD_{585})**			**Violacein unit(OD_{585}/OD_{660})**		
C. violaceum	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% 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})			**EPS Production (g/L)**			**EPS Production per unit OD (g/L)**		
X. campestris	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% 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}) (1:1 dilution)			**Alcohol production (% v/v)**			**Alcohol production per unit OD (% v/v)**		
S. cerevisiae	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% 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})(1:1 dilution)			**pH**					
L. plantarum	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
	1.14±0.00	1.21±0.00	6.14**	4.26±0.05	4.03±0.05	-5.39**			
	Growth (OD_{660})			**IU**					
B. parabrevis	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
	0.48±0.00	0.57±0.02	18.75**	0.021±0.001	0.026±0.001	23.80**			

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

Both the gram-positive bacteria (*B. parabrevis* and *L. plantarum*) exhibited a higher growth in presence of music, with almost similar magnitude of change in their respective test parameters. A little higher (5.39%) reduction in the pH of the growth medium of *L. plantarum* under the influence of sound may be owing to somewhat enhanced lactic acid production by this bacterium when induced by sound stimulation. Better growth of *B. parabrevis* under the influence of sound stimulation may be attributed to its higher (23.80%) cellulase activity enabling it to utilize cellulose (the principal carbon source in the medium used) more effectively. *S. cerevisiae* also registered higher growth and alcohol production when subjected to sound stimulation. During our previous study **(Sarvaiya and Kothari, 2015)** we found membrane permeability of the test microorganisms to increase in response to sound stimulation. To investigate whether the altered growth of the test organisms exposed to sound treatment in this study is due to any change in movement of key molecules across the cell membrane, we measured the rate of glucose utilization by *S. cerevisiae* and *B. parabrevis* when incubated in presence of the test sound-I, by quantifying the extracellular glucose present in the growth medium at different time intervals. Sound stimulated culture of *B. parabrevis* was found to utilize glucose at a faster rate than control culture (Figure 3), and accordingly its growth was faster under the influence of sound. At the end of 24 h of incubation extracellular glucose concentration in the experimental tubes was 11.53% lesser than the control tubes and the corresponding increase in growth amounted to 15.94%. Similarly, *S. cerevisiae* could also utilize glucose faster under the influence of sound. At the end of 48 h of incubation the extracellular glucose concentration in the experimental tubes was 17.39% lesser than that in control tubes, and the corresponding increase in growth and alcohol production was found to be 26.44% and 24.21% respectively (Figure 4).

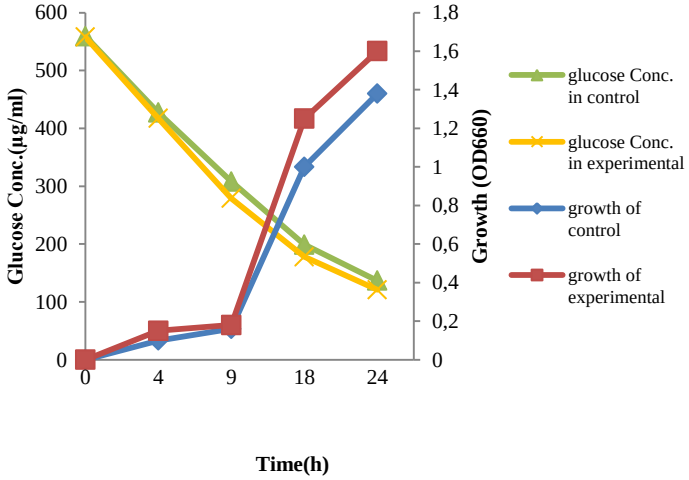

Figure 3 Faster glucose uptake by *B. parabrevis* under the influence of sound

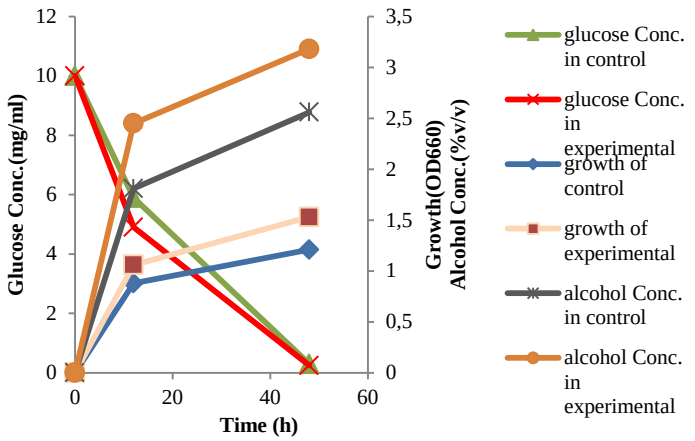

Figure 4 Faster glucose uptake and better alcohol production by *S. cerevisiae* under the influence of sound

Raag Piloo-Teentaal

Results regarding the influence of sound-II on test organisms are presented in Table 3. *S. marcescens* suffered a marginal decrease in growth and a significantly heavy decrease in pigment (prodigiosin) production owing to sound treatment. Another pigmented organism, *C. violaceum*, used in this study also suffered a decrease in growth as well as pigment (violacein) production, when incubated in presence of sound. In contrast to both these pigmented gram-negative bacteria, the third pigmented gram-negative bacterium (*X. campestris*) used in this study exhibited higher growth and even higher EPS production under the influence of sound. *C. violaceium* responded differently to both the music patterns, positively to sound-I, and negatively to sound-II; whereas remaining two gram-negative bacteria employed in this study responded similarly to both the test music patterns.

Table 3 Effect of *raag Piloo* on test organisms

Organism	**Growth (OD_{660})**			**Prodigiosin (OD_{535})**			**Prodigiosin unit (OD_{535}/OD_{660})**		
	Control (Mean±SD)	Experimental (Mean±SD)	% change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	%Change
S. marcescens	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})			**Violacein production (OD_{585})**			**Violacein unit(OD_{585}/OD_{660})**		
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	% Change
C. violaceum	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})			**EPS Production (g/L)**			**EPS Production per unit OD (g/L)**		
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	% Change
X. campestris	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}) (1:1 dilution)			**Alcohol production (% v/v)**			**Alcohol production per unit OD (% v/v)**		
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control	Experimental	%Change
S. cerevisiae	1.28±0.01	1.34±0.0009	4.68*	1.62±0.00	1.82±0.04	12.34**	1.26	1.36	7.93
	Growth (OD_{660})(1:1 dilution)			**pH**					
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
L. plantarum	1.17±0.00	1.21±0.01	3.41**	4.30±0.00	4.16±0.05	-3.25*			
	Growth (OD_{660})			**IU**					
	Control (Mean±SD)	Experimental (Mean±SD)	% Change	Control (Mean±SD)	Experimental (Mean±SD)	% Change			
B. parabrevis	0.50±0.03	0.59±0.00	18.0*	0.027±0.00	0.032±0.00	15.35**			

***p≤0.01, *p≤0.05,* '-'minus sign indicates a decrease, when compared to control

Both the gram-positive test bacteria, *B. parabrevis* and *L. plantarum* grew better when subjected to sound treatment. The former could exhibit better cellulase activity in the sound stimulated condition, which might have allowed it to produce more biomass owing to better substrate (cellulose) utilization; or perhaps the higher cellulase activity might be simply resulting from increased cell number in the experimental tubes. The little (3.25%) but significant reduction in pH of the growth medium brought by *L. plantarum* might have resulted from more lactic acid produced (owing to 3.41% higher cell mass) under the influence of sound. *S. cerevisiae* responded positively to both the sound patterns; however, alcohol production per unit of growth was higher in response to sound-II.

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)**. Sonic frequencies employed in their work were 100 Hz, and 1000 Hz, in addition to broad-band music. 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, we found the cell density of *S. cerevisiae* culture growing in presence of music to be higher than in absence of music (Table 2-3). When growth rate was calculated using the data from which graph shown in Figure 4 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 3). 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.

This work involved investigation on effect of two different music patterns corresponding largely to the frequency ranges 172 - 581 Hz and 86 - 839 Hz on six different microorganisms. All the test organisms (except *S. marcescens*, and *C. violaceum* incubated with sound-II) grew better following incubation in presence of sound. Glucose uptake by *B. parabrevis* and *S. cerevisiae* was found to be faster under the influence of sound-I. Though few reports describing the effect of audible sound have accumulated in literature, this exciting topic warrants much more systematic studies, so that the molecular basis of microbial response to sound can be elucidated. Gu *et al.* **(2013)** reported sonic vibration (5000 Hz; 100 dB) to promote growth of *Escherichia coli*, along with an increased total protein content and antioxidant enzyme activity. Ability of music to increase bacterial growth and substrate utilization was reported by Pornpongmetta and Thanuttamavong **(2010)**. Ying *et al.* **(2009)** reported growth promoting effect of audible sound on *E. coli*.

Regarding the mechanism how audible sound affects the microbial growth and metabolism, not much can be commented with certainty. However, it may be postulated that sound waves while travelling through the liquid growth medium give rise to sonic vibrations, which may be sensed by the test microbial population through its mechanosensory receptors. Following this, the cell population may modulate its behavior in accordance to the magnitude and duration of the sonic vibration. Mechanosensitive ion channels are well

characterized force-sensing systems in living organisms. Piezo channels can sense stress (sonic vibrations can be viewed as a type of stress posed to the microbial cells), and their gating can be regulated by mechanical force. Upon sensing the stress created by the sound waves, these mechanosensitive channels may signal the organism for generation of an appropriate physiological response **(Martinac, 2012)**. Opening of such channels may be promoted by presence of membrane tension **(Sawada *et al.*, 2012)**, which in turn can affect movement of certain key ions across the cell membrane, ultimately resulting in an altered pattern of growth and metabolism. In bacteria, mechanosensitive channel proteins act as safety valves against osmotic shock, and in higher organisms they participate in sensing touch and sound waves **(Ward *et al.*, 2014)**. Once the biophysical mechanism regarding how force is sensed by mechanosensory channel protein and how the sensed force affects channel gating is completely understood, it will help to a good extent in explaining microbial response to sound stimuli. This is because through the process of mechnosensation, cells respond to variations in mechanical stress originating from sound or any such other environmental factor. Mechanosensitive channels are involved in regulation of the volume, morphology and migration of cells.

Different organisms may respond differently to a particular sound pattern because pressure distribution in the membrane varies with the type of membrane. The pressure profile of the membrane can be believed to be dependent on the intensity of the test sound, as well as, the inherent frequencies. A particular combination of a certain sound pattern and the test organism being studied will generate a particular pressure profile of the membrane, which is likely to affect the channel gating **(Sawada *et al.*, 2012)**.

CONCLUSION

This study demonstrates that microbial growth and metabolism do get affected, when exposed to external audible sound. It is generally well accepted that sound (in form of music or otherwise) affects higher forms of life. Research regarding its effect on microbes is still in infancy. Questions like: Do microbes respond differently to different frequencies of audible sound; why the response of one organism to a particular sound pattern differs from that of another organism to same sound pattern; whether audible sound can affect quorum sensing in microbial populations significantly, and influence their behaviour, etc. remains to be explored. Further developments in the mechanosensory biology, along with transcriptome and/or metabolomic profiling of sound stimulated cultures can provide meaningful insights in the area of cell-sound interaction. Research in this area can open a new frontier for multidisciplinary work at the interfaces of microbiology, biophysics, and acoustics. Understanding the molecular basis of metabolic and physiological responses of sound stimulated microbial cells may enable effective manipulation of cell metabolism and proliferation in fermentors.

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