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Effects of sound on growth, viability, protein production yield and gene expression in *Escherichia coli.*

A dissertation presented by

Edgar Acuña González

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Campus Monterrey

School of Engineering and Sciences

The committee members, hereby, certify that have read the dissertation presented by Edgar Acuña González and that it is fully adequate in scope and quality as a partial requirement for the degree of Doctor in Biotechnology,

Dr. Jorge A. Benavides Lozano
Tecnológico de Monterrey
School of Engineering and Sciences
Principal Advisor

Dr. José M. Aguilar Yáñez
Tecnológico de Monterrey
School of Engineering and Sciences
Co-advisor

Dr. Víctor M. Treviño Alvarado
Tecnológico de Monterrey
School of Engineering and Sciences
Committee Member

Tecnológico de Monterrey
0 4 DIC 2018
Dirección Nacional de Posgrado
Dr. Rubén Morales Menéndez

Dr. David I. Ibarra Zárate
Tecnológico de Monterrey
School of Engineering and Sciences
Committee Member

Dr. Guy A. Cardineau
Arizona State University
Sandra Day O'Connor College of Law
Committee Member

Dr. Rubén Morales Menéndez
Dean of Graduate Studies
School of Engineering and Sciences

Monterrey Nuevo León, December 14th, 2018

Declaration of Authorship

I, Edgar Acuña González declare that this dissertation titled **"Effects of sound on growth, viability, protein production yield and gene expression in *Escherichia coli*"** and the work presented in it are my own. I confirm that:

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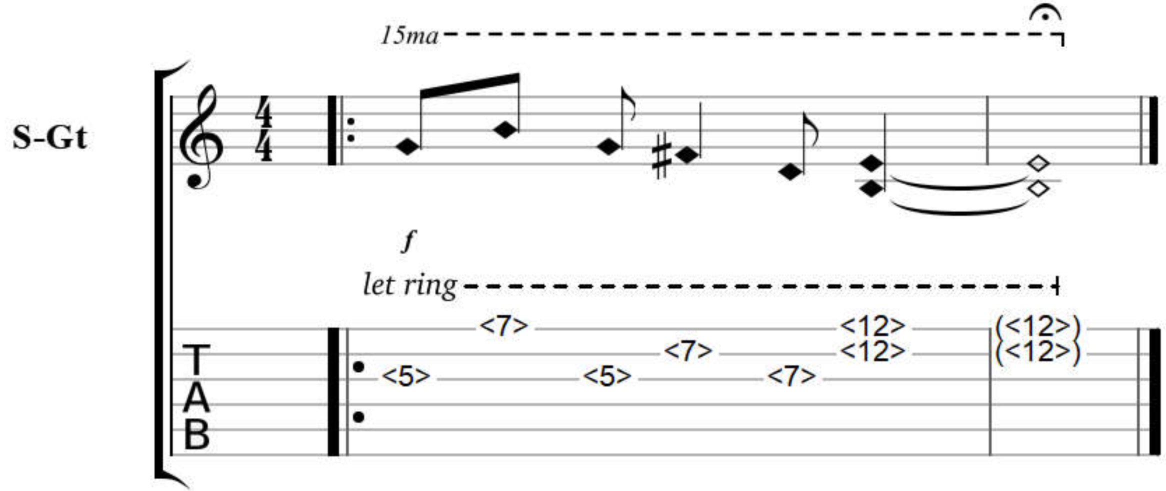

“If there were ever anything
like a magic potion
that could be poured into man
to bring him to a higher plane,
then this magic potion
would be made of sounds.”

Ursula Seiler

Dedication

This research is the result of mixing my two biggest interests in life: genetics and music. I dedicate this work to those who have heard “you can only choose one of them” and to those that were there encouraging me to continue no matter the difficulties. To my mom, dad and two brothers, who were the first ones that accepted the way I saw the world. To the Acuña family for fostering my love for music, to the González family for helping me to develop my resilience, to the Montes-Reyna family for their warmth and to my godparents that have always looked out for me, to Paty and Eva for treating me as part of their family. To my music teachers, Elena and Angelo, who coordinated the music Minor which forced me to create a way to combine music with biotechnology. To my teachers (Elsy, Adriana, Tenchita, Bertha, Oscar, Silverio, Doris, Janet, Rocío, Aurora, Victor, Daniel, Marco, Julio, Guy, Juan Ignacio, Jorge, Hugo, Carolina, David) who taught me everything I know about biotechnology and sound. To my coworkers from X4 project (Adrián, Poncho, Daniel, Tomás, Andrés, Joe, Alma, Edith, Fede, Paty) who were the first ones to listen to my “weird” ideas about using sound in biotechnology. To my graduate colleges (María, Eva, Annia, Ariana, Emaús, Delia, Emilio, Gus, Karen, Héctor, Ingrid, Jesús S, Mónica, Gil, Perla, Sofía, Isaac, Carlos, Vero, Javier, Paulyna, Erika, Melissa A, Melissa M, José, Lily, Soledad, Shirley, Gaby, Laura) because they understand this kind of struggle. To the CB staff and teachers (Gaby, Mary Q, Vero, Bere, Aydee, Felipe, Benji, Luis Mario, Alberto, Mirna, Karla, Edgardo, Cristina, Daniel, Brenda) who helped me meet my goals with their extensive knowledge. To the bioprocess group (Jorge, Pepe, Calef, Fanny, Wendy, Raquel, Isabela, Alex, Stephy, Tere, Paco), the molecular biology group (Chema, César, Bong Min, Sergio, Mario, Alex, Lola, Cuauhtémoc, Héctor, Constantino, Marion, David), Willson’s lab (Richard, Mary, Victoria, Katerina, Kristen, Kate, Gavin, Ahmad, Dimple, Atul, Binh, Ujwal, Heather, João, Adheesha, Jay, Abraham, Michelle) and “little helpers” (Li Lu, Carolina, Sabrina, Karen, Suria, Melissa R, Sergio, Manne, Melissa G, Salim, Gilberto, Fátima, Fernanda, Priscila, Noemi, Abraham) because each one of them welcomed me, advised me and guided me throughout crucial moments of the whole project. To my undergraduate friends (Kiwi, Valeria, Poncho, Mayra, Gaby, Neto, Roberto, Cristina, Marifer, Flavio, Thelma, Loreto, Daniel, Román, Valentina, Aleyda, Isaac, Camilo, Minerva, Adria, Mariana, Eduardo, Nataly, Vane, Teo, Pao, Laura, Carlos, Yoxter, Anita, Lex, Ramón), new friends (Mau, Cheke, Juan Carlos, Katy), music (Kike, Poncho, Emiliano, Lalo, Rodo, Orlando, Gil, Roger, Sofía, Luis, Carlos, Rich, Adrián, Edén, Fer, Iris, Angie, Cassandra, Emmy, Ita, David, Gus, Juan, Dan, Jorge, Bruno, Neztor, Jesús, Gris, Enrique, Beto), from Puebla (Gastón, Male, Arturo, Nash, Aridaí, Cassandra, Oleńka, Gaby, Emy, Seok, Clau, Tere, Emy, Marixay, Beto, Memo, Flaco, Pablo, Mano y Mana, Bere), from Taller Ciencia Viva (Yolis, Christian, Angie, Barbie, Jenny), from internet (Elena, Lucjia, Magda, Rocío, Alex, Joe), and from Pokémon (Karla, Done, Coach, Panxho, Ever, David, Tsuro, Rola, Kike, Adam, LuisFig, Emad, Aurora, Mojica, Martín, Lara). To Esteban and Don Juan who fed me for almost 10 years. And especially to Chucho who has been my right hand for almost 10 years (professionally, personally, emotionally, intellectually) and Edda for being the person who has best known and cared about Edgar by giving him unconditional confidence, inspiration, support, patience, and encouragement. You three were my main motivation for pushing through this work.

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Effects of sound on growth, viability, protein production yield and gene expression in *Escherichia coli*.

by

Edgar Acuña González

Abstract

The effect of sound on biological systems is a subject that has been previously explored, mainly in relation to its use to increase agricultural production. However, the potential of this phenomenon has not been exploited properly because present studies have focused only on one or two sound elements for the characterization of their biological effects. In this sense, the effects of other sound wave elements have been overlooked.

In the present work, the effects of frequency, amplitude, duration, intermittence and pulse - individually and in combination - were characterized in *Escherichia coli* through the measurement of its biomass, viability and yield production of recombinant protein. The treatments of frequency and duration increased the concentration of biomass in 19% and 44% respectively at time 24 h; however, high variability was observed in both treatments. The amplitude treatment had a significant effect on the viability, which the duration of the exponential phase was doubled. The intermittency treatment increased the yield of recombinant protein 1.5 times without significant contribution of the other sound elements. Based on this observation, the effect that intermittency could have on the upregulation of the expression of genes involved in the production of recombinant proteins was investigated. The RNA of three candidate genes (*BarA*, *CheA* and *CpxR*) was quantified in the presence of an intermittent sound. All genes were upregulated (1.38, 2.66 and 1.33 times respectively); however, only upregulation related to chemotaxis (*CheA*) was statistically significant.

Finally, an omnidirectional sound source was adapted to small-volume commercial bioreactors to characterize the distribution of sound within the container. It was determined that the implementation of sound induction in a commercial bioreactor is feasible, although limited to certain specific frequencies close to 500 and 1000 Hz. The integral nature of this characterization presents a deeper understanding of bacterial systems and also offers a way through which it is possible to explore its application for industrial purposes.

Resumen

El efecto del sonido en sistemas biológicos es un tema que ya ha sido explorado anteriormente, principalmente en relación a su uso para aumentar la producción agrícola. Sin embargo, el potencial de este fenómeno no ha podido ser aprovechado debidamente ya que la mayoría de los estudios se han enfocado solamente en uno o dos elementos del sonido para la caracterización de sus efectos biológicos. En este sentido, los efectos de otros elementos de onda de sonido han sido pasados por alto.

En el trabajo presente, los efectos de la frecuencia, amplitud, duración, intermitencia y pulso – individualmente y en combinación – fueron caracterizados en *Escherichia coli* a través de la medición de su biomasa, viabilidad y rendimiento de producción de proteína recombinante. Los tratamientos de frecuencia y duración aumentaron la concentración de biomasa en 19% y 44% respectivamente a las 24 h; sin embargo, en ambos tratamientos se observó alta variabilidad. El tratamiento de amplitud tuvo un efecto significativo en la viabilidad en la cual se duplicó la duración de la fase exponencial. El tratamiento de intermitencia aumentó el rendimiento de proteína recombinante 1.5 veces sin contribución significativa de los otros elementos del sonido. Con base en esta observación, se investigó el efecto que la intermitencia pudiere tener sobre la regulación positiva de en la expresión de genes involucrados en la producción de proteína recombinante. Se cuantificó el RNA de tres genes candidato (*BarA, CheA* y *CpxR*) ante la presencia de un sonido intermitente. Todos los genes fueron regulados positivamente (1.38, 2.66 y 1.33 veces respectivamente); sin embargo, sólo aquel relacionado con la quimiotaxis (*CheA*) fue estadísticamente significativo.

Finalmente, se adaptó una fuente de sonido omnidireccional a biorreactores comerciales de poco volumen para caracterizar la distribución del sonido dentro del recipiente. Se determinó que la implementación de inducción con sonido en un biorreactor comercial es factible, aunque limitado a ciertas frecuencias específicas cercanas a 500 y 1000 Hz. La naturaleza integral de esta caracterización presenta una comprensión más profunda acerca del efecto del sonido sobre los sistemas bacterianos y también ofrece una vía a través de la cual es posible explorar su aplicación para fines industriales.

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Chapter 1

1. Introduction

Sound is a type of energy that propagates in the form of waves through matter. In physics, sound waves and their mechanical properties on inanimate systems have been well characterized. This type of characterization has made possible to develop technological advances such as modern telecommunications (telegraph, telephone, radio and television) and navigation (SONAR); and more recently in industry for degassing liquids,[1] deagglomeration of solids[2] and separation.[3] However, sound research in life sciences has not received enough attention despite the fact that at all times, due to its ubiquity, it provides environmental information to living organisms which only those who were able to identify and adapt accordingly, ensured their survival.

Out of those limited sound studies, most of them are focused on sterilization of microorganisms,[4] or improvement of agricultural production and very few of them addresses how sound affects unicellular organisms. Regardless of the quantity of studies describing sound effects on organisms, the specific process by which it influences different molecular mechanisms is still poorly understood. It has been suggested that sound promotes the vibration of the cell membrane while increasing selectivity towards certain compounds that stimulate cell growth,[5] but other hypotheses state that sound waves can regulate gene expression by modifying transcription factors[6,7] or by activating physical stress mechanoreceptor channels.[8-12] More recently, it has been stated that living organisms respond to a combination of sound patterns which could carry biological information[13] and that cell membranes have the capacity to decode and transmit this information, thus spreading the effect within a certain population.[14]

One of the main reasons why these hypotheses have not been confirmed so far is that these studies have used sound only to pinpoint any biological effect rather than focusing on intrinsic characteristics of sound. Thus, to be able to accurately correlate how sound influences a biological effect, it is necessary to design specific sound treatments that highlight each sound element and test them, individually or in combination, to verify a change in a physiological state.

Using this proposed methodology, a deeper understanding of the effect of sound waves on living organisms, their physiological state could be predicted and even modulated. This knowledge may eventually lead to the development of new technology in fields such as applied microbiology, bioengineering, agroindustry and medicine in more than one way, especially because it has

already been described that sound can alter the metabolism,[15-17] enhance antibiotic susceptibility,[18-21] promote resistance towards stressful environments,[22-24] modulate activity of enzymes,[5,10,25,26] increment biomass production[8,10,27-29] and influence gene expression.[7,30-32]

Considering the latter, several applications can be proposed. For instance, using sound stimulus specifically designed to regulate metabolic pathways punctually depending on the growth stage without the need of metabolic engineering. In other words, to generate sound treatments that can influence the growth or synthesis rate in an organism of interest by matching the sound element with the organism's growth phase. A specific example might be using certain sound parameters that speeds up growth during log phase and some others that slows down at the end of the growth to prevent product over accumulation.

Another application could be to use sound as a form of selective sterilization for industrial or medical purposes. In relation to antibiotic susceptibility, some sound treatments could have different effects according to the organism. For example, certain sound conditions could enhance resistance towards stress in an organism of industrial interest and, similarly, those same conditions could be detrimental to pathogens. This phenomenon could be implemented in bioreactors or operating rooms to prevent pathogen cross contamination.

In addition, another possible new application of sound in industry would be to increase protein production yield in microbial recombinant systems using gene promoters sensible to sound. Considering that protein production yield highly depends on the gene expression of the system, overexpressing recombinant proteins in a cell can often be detrimental to its productivity due to accumulation or inclusion bodies formation,[33] for this reason inducible promoters are preferred over strong constitutive promoters where the protein expression can be activated or repressed depending on environmental factors surrounding the host strain.

Nevertheless, the most common inducible promoters have still areas of improvement. For instance, using the AOX1 promoter, a methanol-induced promoter,[34] in *Pichia pastori*s creates by-products that are toxic to yeast cells[35]. In addition, the *lac* promoter is preferably induced in *E. coli* by lactose or its non-hydrolysable analog isopropylβ-D-1-thiogalactopyranoside (IPTG);[36] because the latter has a high cost in the market, cannot be metabolized and is difficult to use in large scale production due to coat, it increases considerably the price of the final product to the point of making the bioprocess unscalable.[37] Other types for inducing genes include the

temperature shift which can harm thermo-sensitive products and the nutrient depletion which can decrease the final biomass concentration causing the reduction of the production yield.[38]

Sound could overcome at least some of these drawbacks. First, it can travel best through dense media, which means that it can reach every cell of the population. Second, because sound would not be directly mixed as with chemical induction, it won't affect the following protein recovery/purification processes, which are the key determinants of a bioprocess economic viability.[39]

Considering this potential, performing an accurate and complete characterization of sound using a standardized and replicable methodology has great importance. Moreover, to our research team, it was of great interest to focus on understanding the relationship between sound elements and specific biological parameters – biomass, viability and protein production yield – in *E. coli*, a widely used model organism. Moreover, gene expression analysis was done with the purpose of finding a molecular pathway that is affected by sound and finally, the feasibility of using sound systems adapted into commercially available bioreactors was investigated. This approach will help not only to understand how sound influences biology, but also could be used to develop unexplored bioengineering technologies in a near future.

1.1. Dissertation Hypothesis

Even though there are not many studies about biological effects due to sound exposure in living organisms, there is evidence that sound does influence organisms. However, because of the lack of attention in the composition of sound treatments, its mechanism of action is still unknown. Thus, by focusing on specific sound designs that change in one element at a time, the link between sound and biological effects can be detected more accurately.

With this approach, the experimental work presented in this document revolves around four main hypotheses:

I. It is possible to design and construct a sound induction device to study the effect of sound on a microbial system at lab scale as a proof of concept, using *E. coli* as experimental model.

II. A specific sound element (frequency, amplitude, duration, intermittence or pulse), or a combination of them, can punctually influence a response in viability, biomass concentration and/or protein expression yield of *E. coli*.

III. A specific sound element, or a combination of them, can influence the expression of specific genes in *E. coli*.

IV. It is feasible to adapt speakers into bioreactors to produce a uniform auditory signal inside the container.

1.2. Dissertation Objectives

This study falls within the area of basic science and technology because it attempts to describe a phenomenon, which has not been deeply studied, and use these findings in the development of new technology that can be implemented in bioprocessing industry.

Therefore, the general objective is to identify the sound elements that influence biological responses of importance in research and in industry, particularly biomass concentration, viability and recombinant protein production yield, using *E. coli* as a model system. This characterization could give insight into how specific parts of sound affect different biological parameters and the results could be extrapolated to similar microbial expression systems, which would allow better production control in industry. Moreover, to fulfill the main objective, specific objectives for each hypothesis mentioned above have been stablished.

I. Design a sound induction device at lab scale in to study the effect of the sound elements (frequency, amplitude, duration, intermittence and pulse) on flask submerged microbial cultures, using *E. coli* as model system.

II. Identify the sound elements that influence biological responses, particularly biomass concentration, viability and recombinant protein production yield through the expression of a reporter gene in *E. coli*.

III. Identify genes in *E. coli* whose expression is susceptible to sound stimulus.

IV. Assess the feasibility of adapting sound emitters into commercially available liquid bioreactors to produce an omnidirectional and stable sound signal.

By fulfilling these objectives, it will be possible to establish the basis where sound can be better studied, understood, implemented and adapted to technology we use nowadays.

1.3. Dissertation Structure

The present document is divided in 5 main chapters. **Chapter 1** gives a brief description of the current state of sound research in relation to its biological effects and the relevance of the characterization of sound elements. Then, it describes the technological potential of sound, particularly regarding recombinant proteins through genetic induction and later it describes the research approach of this dissertation. Finally, the hypothesis, general objective and specific objectives are listed. **Chapter 2** gives relevant background information about sound, its properties and elements, followed by most of the available reports that gives evidence to the biological effects of sound.

Chapter 3 presents a detailed description of all materials (reagents, devices, biological organisms) and methods used on the present research work. **Chapter 4** shows the results and present the discussion of such results. It is important to clarify that most of the results presented in this document (particularly those related to growth, viability and protein production yield) have been already accepted for publication at the Journal of Chemical Technology and Biotechnology in a paper entitled "Effects of sound elements on growth, viability and protein production yield in *Escherichia coli*". Results related to the gene expression assay and characterization of sound propagation at bioreactor scale are also presented in this chapter, although have not yet been published. Finally, **Chapter 5** presents general conclusions and future research lines.

All references used in the document are presented at the References section following Chapter 5. Lastly, annexes (supplemented figures and the accepted scientific publication) and *vitae* are included.

Chapter 2

2. Background

2.1. Sound properties

Sound can be viewed as a wave motion in an elastic media due to the variation in pressure,[40] which means that sound is a mechanical wave. This physical phenomenon is due to the displacement of particles from their original position meanwhile elastic forces from the media tend to restore the original position; because of inertia properties of manner, this change in position creates a periodic movement.

This oscillatory pressure variation creates two physical states (**Figure 2.1.**): (1) The spaces where the molecules are crowded together represent the areas of compression (crests) in which the air pressure is slightly greater than the atmospheric pressure; (2) the sparse areas represent rarefactions (troughs) in which the pressure is slightly less than atmospheric pressure. These fluctuations of pressure are very small; faintest sound the ear can hear (20 μPa) is 5,000 million times smaller than atmospheric pressure.

Sound is considered a longitudinal wave because it travels the same direction as the movement of the particles. Molecules move to one direction of the compression crests and to the other side in the rarefaction troughs between the crests. Even though particles move certain distance to one direction and comes back to the original position, the sound wave exists because of the transfer of momentum from one particle to another.

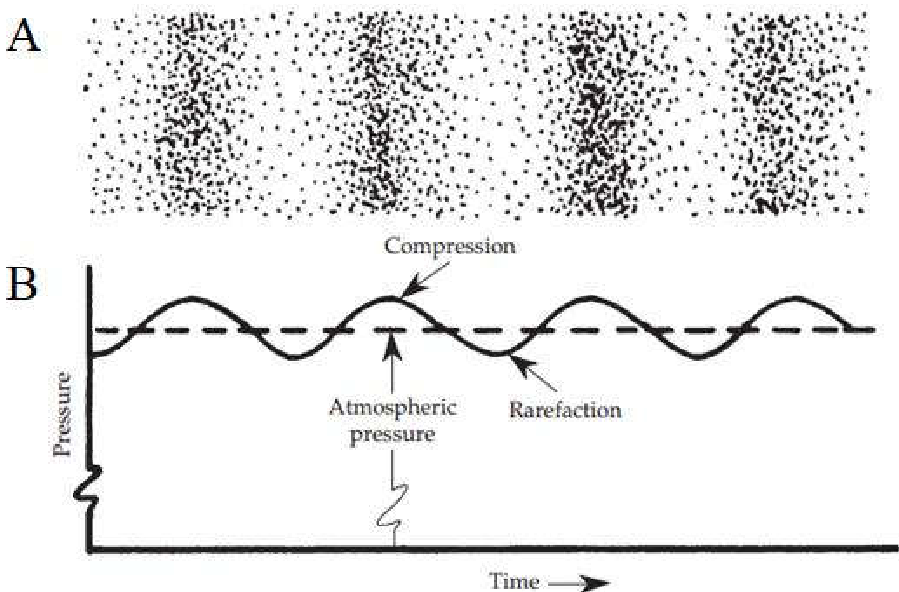


Figure 2.1. Visual representation sound as a longitudinal wave (A) and as a transverse wave (B). Figure obtained and modified from ASI Pro Audio Acoustics.[41]

Without a medium, sound cannot be propagated; yet sound can be conducted by any medium whether they are gases, liquids, or solids.

The speed of sound depends on the medium in which it is propagating. The denser the molecular structure, the easier it is for the molecules to transfer sound energy; this means that sound travels faster in solids and liquids than in gases. Additionally, the speed of sound is faster in materials that have some stiffness like steel and slower in elastic materials like rubber. Finally, sound travels faster in air as temperature and humidity increases.

It should be noted that the speed (velocity) of sound is different from the particle velocity. The velocity of sound determines how fast the sound energy moves across a medium. Particle velocity is determined by the loudness of the sound. Also, it is important to note that frequency does not change when changing between media, only its speed changes.

2.2. Sound elements

2.2.1. Frequency

Number of cycles per second, measured in hertz (Hz). High frequency indicates higher pitched sounds, just like lower frequency indicates lower notes. It is defined as:

$$\text{Frequency} = \frac{\text{Speed of sound}}{\text{Wavelength}} \tag{1}$$

Where standard speed of sound is 340.29 m/s in normal conditions of temperature and pressure, and wavelength is the distance a wave travels in the time it takes to complete one cycle. In music, each musical note corresponds to a specific frequency depending to the tuning where the standard is A_4 = 440 Hz (**Table 2.1.**).

2.2.2. Amplitude

Extent to which air particles are displaced which is perceived as the magnitude of the auditory sensation. Despite sound pressure is directly proportional to amplitude, sound-pressure level (SPL) is preferred since it allows more accessible measurements where absolute pressure units (Pa) are transformed to relative units (decibels) in a logarithmic scale (**Figure 2.2.**). Amplitude is defined as:

$$\text{SPL} = 20 \log_{10} \frac{p}{20\,\mu\text{Pa}} \text{ decibels} \tag{2}$$

where ρ is the measured sound pressure in Pa, 20 μPa is the reference sound pressure.
One of the main properties of amplitude is its dependence on the inverse square law, which means that the sound intensity decreases as the square of the radius from the sound source.

Table 2.1. Musical notes and their corresponding frequencies in the standard tuning (440 Hz). Table adapted from MIT Physics Department.[42]

Note	Frequency	Note	Frequency	Note	Frequency	Note	Frequency
C0	16.35	C2	65.41	C4	261.63	C6	1046.50
C#0	17.32	C#2	69.30	C#4	277.18	C#6	1108.73
D0	18.35	D2	73.42	D4	293.66	D6	1174.66
D#0	19.45	D#2	77.78	D#4	311.13	D#6	1244.51
E0	20.60	E2	82.41	E4	329.63	E6	1318.51
F0	21.83	F2	87.31	F4	349.23	F6	1396.91
F#0	23.12	F#2	92.50	F#4	369.99	F#6	1479.98
G0	24.50	G2	98.00	G4	392	G6	1567.98
G#0	25.96	G#2	103.83	G#4	415.3	G#6	1661.22
A0	27.50	A2	110.00	A4	440	A6	1760.00
A#0	29.14	A#2	116.54	A#4	466.16	A#6	1864.66
B0	30.87	B2	123.47	B4	493.88	B6	1975.53
C1	32.70	C3	130.81	C5	523.25	C7	2093.00
C#1	34.65	C#3	138.59	C#5	554.37	C#7	2217.46
D1	36.71	D3	146.83	D5	587.33	D7	2349.32
D#1	38.89	D#3	155.56	D#5	622.25	D#7	2489.02
E1	41.20	E3	164.81	E5	659.26	E7	2637.02
F1	43.65	F3	174.61	F5	698.46	F7	2793.83
F#1	46.25	F#3	185.00	F#5	739.99	F#7	2959.96
G1	49.00	G3	196.00	G5	783.99	G7	3155.96
G#1	51.91	G#3	207.65	G#5	830.61	G#7	3322.44
A1	55.00	A3	220.00	A5	880	A7	3520.00
A#1	58.27	A#3	233.08	A#5	932.33	A#7	3729.31
B1	61.74	B3	246.94	B5	987.77	B7	3951.07

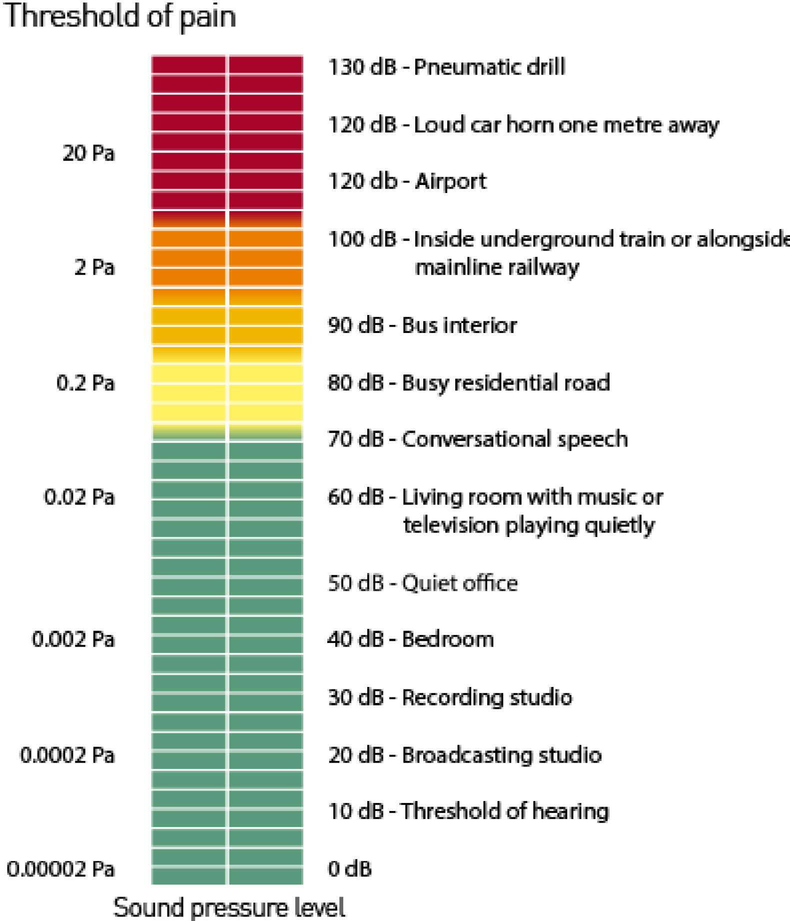


Figure 2.2. Scale that represents the relationship between sound-pressure level (dB) and sound pressure (Pa). Figure obtained from Rockfon.[43]

2.2.3. Duration

Length of time a sound lasts, usually from the time the sound is first noticed until the sound is identified as having changed or ceased. Duration is very variable, it can apply to single notes – which lasts less than a second – or symphonies that may last over an hour. For this reason, the **beat** has been used as the basic unit of time (**Table 2.2.**) and it is especially useful for defining every aspect of duration, which includes rhythm, meter, intermittence and pulse.[44]

Table 0.2. Representation of the duration of sounds/rests measured in beats. Table obtained from "A Thorough Guide to the Musical Concepts".[44]

Name	Value (beats)	Visualization as sound	Visualization as silence
Breve	8		
Dotted Semibreve	6		
Semibreve	4		
Minim	2		
Double dotted Minim	3.5		
Dotted Minim	3		
Crotchet	1		
Double dotted Crotchet	1.75		
Dotted Crotchet	1.5		
Quaver	0.5		
Dotted Quaver	0.75		
Semiquaver	0.25		
Demisemiquaver	0.125		
Hemidemisemiquaver	0.0625		

Rhythm: Repeating sequence of stressed and unstressed beats (often called "strong" and "weak"), which are divided into bars organized by a meter and a tempo.

Meter: Grouping of beats. The types of meters are determined by the nature of the combination and division of beats: a duple meter occurs when two beats are combined, a triple meter when three beats are combined, a simple meter when the beat is split in two and a compound meter when the beat is split in three. Thus, the most common meters are simple duple (2/4, 4/4, 2/2), simple triple (3/4), compound duple (6/8), and compound triple (9/8).

Intermittence: Interval of time characterized by alternating sound and silence cyclically, which is also called *interlatency,* and is regarded as a discontinuous pulse. This alternation can be applied between bars (small scale) or between full musical sections (large scale).

Pulse: Speed of bets measured in beats per minute (bpm) which consist in a repeating series of identical stimuli perceived as continuous points in time. In a musical context, the term *tempo* is preferred.

Table 0.3. Relationship between Italian tempo terms and their respective pulses measured in beats per minute. Table obtained from Polyskeptic.[45]

Name	Definition	Pulse (bpm)
Grave	very slow and solemn	40-50
Largo	very slow and stately	40-50
Lento	extremely slow	50-60
Adagio	slow and leisurely	60-72
Maestoso	majestic, stately	72-84
Andante	at an easy walking pace	80-100
Moderato	moderate	100-120
Allegro	fairly quick speed	120-160
Vivace	lively, brisk	144-160
Presto	very fast	160-200
Prestissimo	extremely fast	168-208

Despite the importance of sound – not only for humans but also for almost all living organisms – very few studies have focused on how sound affects living beings, whereas most of the research has been limited to either physical properties of sound in inanimate objects or the harmonic relationship of sounds as an exclusive human experience.

2.3. Biological sound effects

In physics, sound waves and their mechanical properties on inanimate systems have been well characterized. However, sound research in life sciences has not received enough attention despite its ubiquitous presence around living organisms, from unicellular species to animals.

Most of the research has been made in plants where the most well-known result is that a sound stimulus can increase its biomass production.[46] Other studies have demonstrated that the effect depends on both the quality and the magnitude of sound as well as the organism that is under the stimulus. For instance, considering sound quality, it was determined that sound waves significantly elevate polyamine levels, but there is a greater effect in Chinese cabbage when using “green music” (natural sounds such as songs of birds, insects or water flowing in a river) and in cucumber when using ultrasound.[47] Additionally, considering sound magnitude, it was determined that the best sound conditions for rice growth were 106 dB and 400 Hz[48], while in chrysanthemum mature calluses were 95 dB and 1400 Hz.[15]

Other sound effects have been detected in plants besides biomass growth. In chrysanthemum, it was determined that sound waves can accelerate DNA, RNA and soluble proteins synthesis,[5,6] the activity of H+-ATPase,[5] and the entry of oxygen.[47] In *Dendrobium candidum*, it was found that sound (100 dB and 1000 Hz) caused the accumulation of active oxygen species (OSA) such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbic peroxidase (APX).[26] Lastly, in *Arabidopsis thaliana*, sound promoted transcriptomic, proteomic and hormonal changes with five different frequencies (250, 500, 1000, 2000, and 3000 Hz).[17]

Moreover, some other studies have shown that certain genes can be expressed under sound stimulation.[30] There is evidence to suggest that certain rice genes that normally respond to light (Rubisco and Aldolase) can be regulated positively at 250 Hz and negatively at 50 Hz, even in dark conditions,[31] and specific sound conditions (80 dB and 500 Hz) can upregulate 17 different genes in *Arabidopsis thaliana*.[32] This implies that plants may have stress-inducible genes that respond to specific frequencies that, consequently, can regulate transcription levels.[6]

More recently, research in plants has focused on agricultural benefits of implementing “Plant Acoustic Frequency Technology” (PAFT) with the objective of increasing plant production and decreasing the use of fertilizer in cotton[49] and strawberry,[50] enhancing drought tolerance in rice[22] and *Arabidopsis*,[24] and reducing tomato ripening on the shelf.[23]

In addition to the effect of sound on plants, some studies in single-celled eukaryotes have shown that two-dimensional sound waves (longitudinal and transverse) are also capable of changing the pH, which causes a photochemical reaction in the cells due to a change in the elastic properties of the lipid monolayer.[14] This means that sound at certain frequency and amplitude could repair the membrane and promote the activity of enzymes.[48] Furthermore, metabolic profiles of yeast can change according to a specific sound stimulus.[16]

Finally, it has been shown that pulses by themselves are also capable of directly influencing cell behavior.[51] Considering that these pulses can be transmitted to other cells, it is theorized that sound could be a type of biologically efficient communication between cells.[14]

Unlike research directed at the effect of sound on plants, there are even fewer studies addressing how sound affects unicellular microorganisms. The most studied organism has been *Escherichia coli*, for which it has been found that sound increases colony forming units (CFU),[8,27] affects physiological regulation,[9] modifies enzymes' activity (SOD and CAT)[10] and produces more protein and RNA.[11] In other well-studied organisms, *Chromobacterium violaceum* and *Serratia marcescens*, it was noted that sound increases antibiotic susceptibility,[13,21] produces more quorum sensing molecules (violacein)[12,21,28] by up-regulating the expression of 432 genes.[52]

It is important to reiterate that, even if there are some studies that confirm the existence of biological effects of sound, especially on plants, the characterization of the sound effects in bacteria is poor. This research work presents a methodology that could be used as a platform for future studies regarding biological effects of sound in microbial systems.

In general, previous reports have used two different approaches to study the effect of sound on living organisms: 1) single-frequency stimuli and 2) musical stimuli (**Table 2.4.**). Single-frequency stimuli over-simplify the sound analysis by only considering a single sine wave, which is unlikely to be found in nature as most natural sounds are not pure tones but complex sounds.[53] On the other hand, musical stimuli over-complicate sound analysis by using musical pieces constituted by a complex sum of rhythm, melody and harmony, making it difficult to isolate the effect of the individual sound elements on biological systems. Furthermore, until this work, there has not been any attempt to standardize the selection criteria for sound treatments as can be seen in the wide distribution of sound conditions presented on **Table 2.4**.

A correct standardization of sound conditions can allow the correlation of sound elements with a biological effect in more than one species. In order to establish this standardization, it is necessary to design sound treatments that only differ in one sound element at a time, identify a suitable model organism and a good selection of response variables. The organism that will receive the sound treatments must be easy to handle, capable of generating true replicates and have a fast generation time. Moreover, the response variables should not only be the same as previously reported studies for their comparison but in return should also identify new variables. These must be easy-to-monitor variables that not only identify whether there is an effect but also quantify the response, especially if more than one variable produces the effect.

Table 0.4. List of sound conditions from previous reports classified into two categories: single-frequency stimuli or musical stimuli.

Single-frequency treatments						
Frequency	**Amplitude**	**Duration**	**Intermittence**	**Organism**	**Biological effect**	**Ref.**
1000 Hz	100 dB	15 days	30 minutes, twice a day	*Chrysanthemum callus*	ATPase activity	25
3 Hz	NA	20 days	60 minutes per day	*Actinidia chinensis*	Callus growth	54
1000 Hz	100 dB	15 days	30 minutes, twice a day	*Dendranthema mortifolum*	Membrane permeability alteration	55
1000 Hz	100 dB	9 days	60 minutes per day	*Gerbera jamesonii*	ATPase activity	5
1000 Hz	100 dB	9 days	60 minutes per day	*Chrysanthemum callus*	Roots growth, amylase activity, sugar and protein biosynthesis	5
20,000 Hz	75 dB	70 days	2 hours per day	*Cucumis sativus*	Polyamine production	47
400 Hz	106 dB	2 days	30 minutes, twice a day	*Oryza sativa*	Roots activity, germination, length and permeability	48
1000 Hz	100 dB	9 days	60 minutes per day	*Chrysanthemum callus*	Speed of RNA and protein biosynthesis	6
1400 Hz	95 dB	18 days	30 minutes, twice a day	*Chrysanthemum callus*	Hormonal concentration alteration	15
250 Hz	65-70 dB	4 hours	Continuous	*Oryza sativa*	Gene expression alteration	31
1000 Hz	100 dB	15 days	60 minutes per day	*Dendrobium candidum*	Antioxidant enzymes activity modulation	26
1000 Hz	100 dB	9 days	60 minutes per day	*Chrysanthemum callus*	Gene expression alteration	30

Table 2.4. (continued).

Frequency	Amplitude	Duration	Intermittence	Organism	Biological effect	Ref.
15,000 Hz	NA	3 days	5 hour per day	*Aspergillus spp*	Fungi growth inhibition	18
5000 Hz	NA	5 hours	Continuous	*Escherichia coli*	Biomass production	27
1000 Hz	90 dB	24 hours	1 hour every 4 hours	*Escherichia coli*	Biomass production	8
100 & 10,000 Hz	89 & 92 dB	14 hours	Continuous	*Saccharomyces cerevisiae*	Metabolites content alteration	16
40-2000 Hz	100 dB	42 days	3 hours per day	*Fragaria ananassa*	Photochemical efficiency	49
1000 Hz	100 dB	24 hours	1 hour every 4 hours	*Escherichia coli*	Intracellular Ca2+ content	9
10,000 Hz	110 dB	24 hours	Continuous	*Escherichia coli*	Antioxidant enzymes activity	10
5000 Hz	100 dB	24 hours	Continuous	*Escherichia coli*	Biomass production	10
2000-2500 Hz	90 dB	72 hours	Continuous	*Vigna radiate*	Germination time	56
800-1500 Hz	100 dB	24 hours	Continuous	*Oryza sativa*	Relative water content in drought stress	22
1000 Hz	100 dB	14 days	6 hours once	*Solanum lycopersicum*	Resistance against ripening and ethylene biosynthesis	23
2200 Hz	90 dB	30 days	3 hours per day	*Picochlorum oklahomensis*	Volumetric oil yield	57
500 Hz	80 dB	48 hours	1 hour per day	*Arabidopsis thaliana*	Metabolites content alteration	17

Table 2.4. (continued).

Frequency	Amplitude	Duration	Intermittence	Organism	Biological effect	Ref.
1000 Hz	80 dB	6 hours	Continuous	*Solanum lycopersicum*	Control of transcription factors RIN and HB-1	7
100 Hz	75 dB	7 days	4 hours per day	Soil Bacteria	Antibiotic resistance	19
300 Hz	70 dB	48 hours	Continuous	*Chromobacterium violaceum*	Biomass production	28
300 Hz	89.5 dB	48 hours	Continuous	*Chromobacterium violaceum*	Pigment production	28
8000 Hz	80 dB	48 hours	Continuous	*Escherichia coli* K-12	Protein and RNA biosynthesis	11
1000 Hz	100 dB	10 days	3 hours per day	*Arabidopsis thaliana*	Resistance against *Botrytis cinerea*	20
500 Hz	80 dB	20 days	5 days	*Arabidopsis thaliana*	Expression of mechanosensitive ion channel genes	32
300 Hz	70-89.5 dB	48 hours	Continuous	*Chromobacterium violaceum*	Erythrose-4-phosphate availability	52
400 Hz	86 dB	48 hours	Continuous	*Serratia marcescens*	Biomass production and pigment reduction	29
500 Hz	84 dB	48 hours	Continuous	*Pseudomonas aeruginosa*	Pigment and biomass production	29
600 Hz	75 dB	48 hours	Continuous	*Pseudomonas aeruginosa*	Pigment and biomass reduction	29
35-22,000 Hz	100 dB	10 hours	Continuous	*Arabidopsis thaliana*	Drought tolerance	24

Table 2.4. (continued).

Musical treatments						
Frequency	**Amplitude**	**Duration**	**Intermittence**	**Organism**	**Biological effect**	**Ref.**
"Green music"	70-80 dB	70 days	2 hours per day	*Brassica pekinensis*	Polyamine production	47
Improvised Native American flute	NA	3 days	16 hours per day	*Cucurbita pepo & Abelmoschus esculentus*	Speed of seed germination	58
Broad-band music	80-92 dB	14 hours	Continuous	*Saccharomyces cerevisiae*	Metabolites content alteration	16
Western classical music (*Mozart, Beethoven & Ligeti*) 20-15,000 Hz	70-100 dB	48 hours	30 minutes	MCF7 human breast cancer	Cell cycle alteration	59
Indian classical music (*Raag Kirwani*) 38–689 Hz	95-110 dB	3 hours	Continuous	*Chromobacterium violaceum*	Quorum sensing pigments production, antibiotic susceptibility, membrane permeability alteration and protein biosynthesis	13
Indian traditional music (*Sanskrit sholkas*) 528 Hz	NA	20 days	2 hours per day	*Vigna radiata*	Shoot elongation	60
Indian classical music (*Raag Ahir Bhairav*) 172-581 Hz	70-90 dB	24 hours	Continuous	*Saccharomyces cerevisiae*	Biomass and alcohol production	12
Indian classical music (*Raag Piloo*) 86-839 Hz	85-110 dB	24 hours	Continuous	*Chromobacterium violaceum*	Biomass and pigment production inhibition	12
Indian classical music (*Raag Malhar*) 41-645 Hz	95-110 dB	24 hours	Continuous	*Staphylococcus aureus*	Antibiotic susceptibility	21

Based on the information available in the literature and the potential applications related to the effect of sound in biological systems, the characterization of this effect is very important not only because it gives a platform of how to investigate this type of phenomena in other living organisms, but also because it increases our understanding of these phenomena by attempting to describe how something as ubiquitous as sound affects living organisms in ways never imagined and how that effect could be used to develop new technologies for the benefit of humanity.

Chapter 3

3. Materials and methods

In accordance with the dissertation objectives, an experimental setup was explicitly designed to identify the effect of individual sound elements on biological systems to understand their specific impact on responses such as biomass concentration, biomass viability and production yield of recombinant protein, using *Escherichia coli* as a model system. To do so, five sound wave elements were studied, individually and in combination, with a greater emphasis on frequency, the most studied sound element. The visual representation of the differences between these sound wave elements is shown in **Figure 3.1.**

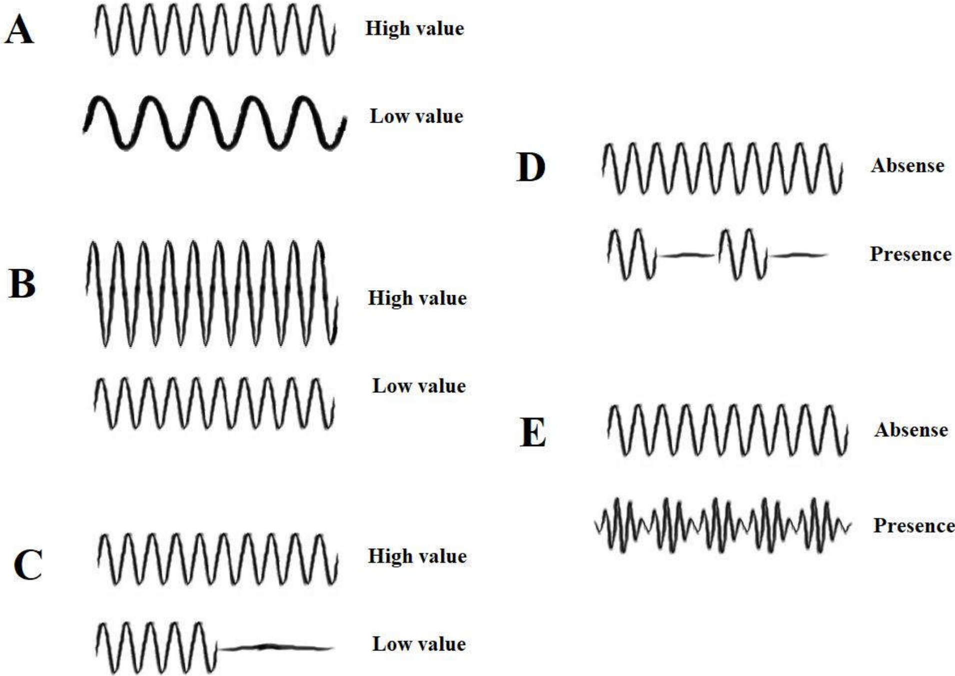


Figure 0.1. Graphic representation of the basic elements that constitute a sound wave: (A) frequency, (B) amplitude, (C) duration, (D) intermittence and (E) pulse.

Then, a gene expression analysis was executed with three target genes (*CpXR, BarA* and *CheA*) that may be susceptible to sound stimulus. These genes were selected due to their similarity with genes from other bacteria that were detected to be susceptible to sound stimulus.[52] Finally, a sound induction device was designed and constructed to ensure a uniform propagation of sound in commercially available liquid bioreactors with capacity of 1 and 2 L. The flowchart of all the experimental stages of the present work are shown in **Figure 3.2.**

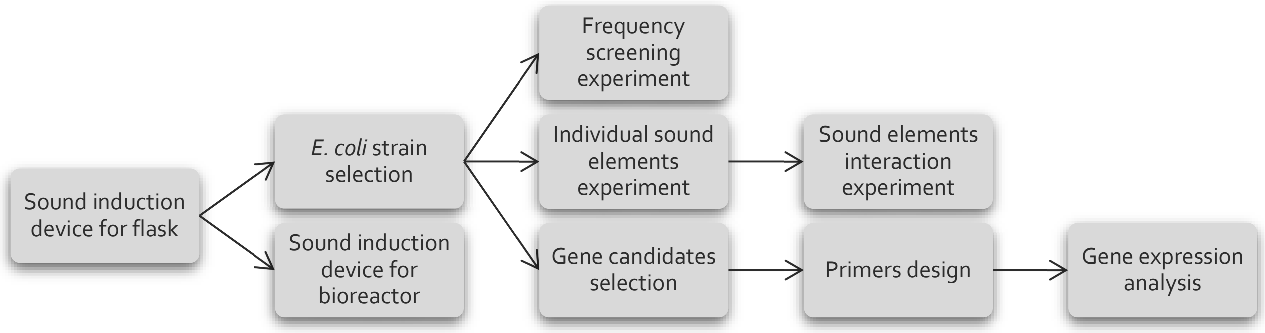


Figure 3.2. Flow chart that explains the sequence of the experimental stages of the present work.

3.1. Materials

3.1.1. Chemicals and reagents

Luria-Bertani (LB) agar plates were used for viability measurements. For vial cryopreservation, *E. coli* was grown in LB broth and stored with 50% glycerol. MagicMedia^{TM} autoinducible medium for T7 expression clones (Thermo Fisher Scientific) was used for *E. coli* protein expression. For protein extraction, a variant of B-PER^{TM} Bacterial Protein Extraction Reagent (Thermo Scientific) was prepared by replacing octyl-β-glucoside (OG) with octylthioglucoside (OTG). Finally, QUANTI-Luc^{TM} (InvivoGen) was employed for luminescent protein quantification by adding 50 μL plus 10 μL of sample per well into a 96-well white plate.

For gene expression quantification, primers for reverse transcription and QPCR amplification were synthesized by Integrated DNA technologies through Dr. Mary Crum. RNA extraction was performed with an RNeasy Mini Kit (Qiagen), cDNA synthesis was done using the ImProm-II^{TM} Reverse Transcription System (Promega) and cDNA was quantified with the Rotor-Gene SYBR® Green PCR Kit (Qiagen).

3.1.2. *Escherichia coli* strain construction

To carry out the experiments on the effect of sound elements, *E. coli* was selected as a study model since it is a well-characterized microbial model, grows fast and is easy to handle. These sound experiments were carried out with an *E. coli* strain transformed with a plasmid (pUC57) that expresses an ScFv antibody fused with a bioluminescent reporter (NanoLuc®) which allows the quantitative identification of recombinant proteins. The plasmid, named "p10_NanoLuc" (**Figure 3.3.**), had the expression cassette synthesized by GenScript through a collaboration between by Dr. José Manuel Aguilar Yáñez and Dr. Mitoma. The vector was kindly provided by Dr. Aguilar and expressed in SHuffle® Express Competent *E. coli* (New England BioLabs).

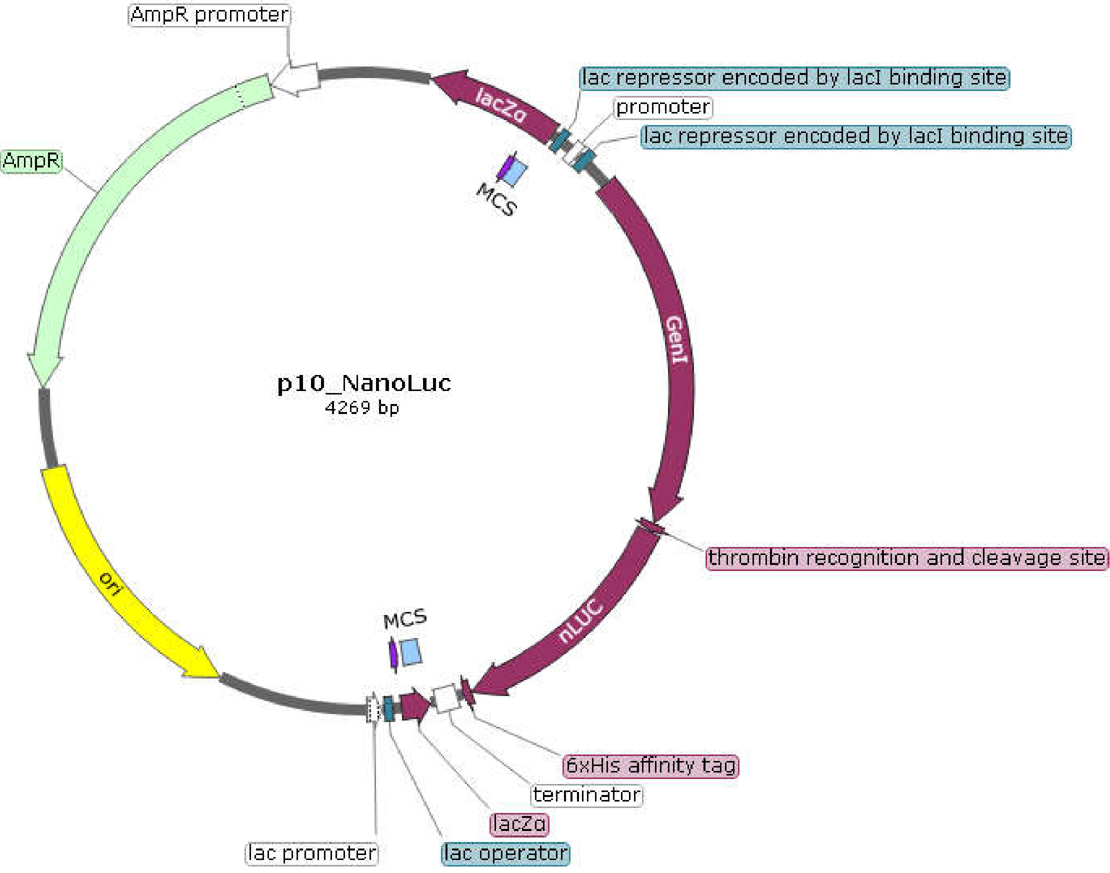


Figure 0.3. Plasmid map of p10_NanoLuc: pUC57 containing an ScFv antibody (GenI = 768 bp) fused with a bioluminescent reporter (nLUC = 522 bp) and cloned in pUC57 (2710 bp) by EcoRV. Figure was created using SnapGene Viewer 4.1.9 (GSL Biotech LLC).

All *E. coli* culture experiments were initiated at the same biomass concentration. For this, pre-inoculums were produced by adding 10 µL of *E. coli* seed stock, 10 mL of LB broth and 10 µL of a carbenicillin solution (100 mg/mL; carb100) and allowed to grow for 16 h in 50 mL Falcon tubes. After that, *E. coli* inoculums were cultivated at 250 rpm and 37ºC in 1 L flasks containing 4 mL of pre-inoculum, 80 mL of LB broth and 800 µL carb100 solution. After 4 h, *E. coli* reached the logarithmic phase (OD_{600} ~ 0.4-0.6); all the media was centrifuged for 15 min at 3000*xg* and 4ºC, and the pellet was weighed. Enough sterilized LB + 15% glycerol was added accordingly to the pellet's weight to achieve a biomass concentration of 11 mg/mL. Finally, the broth was aliquoted into 600 µL vials and stored at -80ºC for later use. It was determined that the OD_{600} value at the beginning of the fermentation would be close to 0.1 when adding 1.8 mL of stored vials into 20 mL of fresh broth. A different batch of vials was used for every independent experiment.

3.1.3. Sound induction device

A sound wave device that correctly replicates sound was designed using common materials that can be found in most biotechnological laboratories. **Figure 3.4.** shows an image of all the necessary material for the device construction (**Figure 3.4.A**) and its assembled form (**Figure 3.4.B**). A 250-mL sterilized flask (PYREX no. 4980) [1] was first introduced to a 600-mL beaker [2] where a plastic supporter [3] could be attached. A speaker (2W RMS, frequency response of 160Hz to 20 kHz) [4] was attached to the plastic supporter and connected to a sound generator [5], which in our case was a laptop computer (DELL Latitude 120L with Celeron M processor) with an audio interface (Steinberg USB 2.0, 24 bit/192 kHz) that designed and reproduced the sound wave stimulus through MATLAB® audio player (MathWorks). This system was assembled using an incubator clamp on a 1585 orbital shaking incubator (VWR).

To be able to collect samples *in situ* without dismantling the device, a flexible tubing [6] that connects the flask to an external 250 mL beaker [7] was implemented. It crossed through the flask swab [8] and was plugged into one of the two outlets of the beaker bottle cap [9]; on the second outlet, a syringe [10] with a 0.45 mm filter [11] was attached to generate a pressure difference so that the sample could move from the flask to the beaker. When samples were not being collected, paper clamps [12] were used on the flexible tubing to stop the air flow. To prevent the generation of pounding noise between the flask and the beaker when the orbital shaking incubator was in use, the external surface of the flask was covered with cotton [13] as a shock absorber.

This sound induction device was designed with the purpose of being used in any orbital shaker incubator without external sound interferences. When measuring the background noise of the system with a sonometer (Tool Dev), it was confirmed that its design creates a quiet environment inside the flask (~30 dB) which is lower than the background noise of the shaker (~65 dB). The swab allows sound to reach into the sterilized medium with low-intensity losses and its flexible tubing avoids dismantling the system every time a sample must be obtained thus preventing cross-contamination and experimental disturbance.

In addition to the intensity validation, the sound that surrounds bacteria inside the device must not have distortions with respect to the source. This is important since the shape of the device can cause constructive or destructive wave interference due to the different ways in which the waves are reflected on its surface.[40] Hence, to assure good sound quality, an MX150 condenser microphone (Shure) was used to confirm that sound waves inside the assembled device were the same as the designed ones.

3.1.4. Sound wave design

To investigate the effect of each sound element separately, it is necessary to have an adequate treatments design, which assures that only one sound element is modified at a time. All audio files were designed with a MATLAB® (MathWorks) audio system toolbox using a tone function design and saved as a .WAV file with an audiowrite function.

Concerning the pulsed audios, their peaks were modeled as shown in **Figure 3.5**. These audio files comply with the continuous characteristic of pulse where the signal does not change abruptly. In addition, this design not only allows the same shape to be kept in all peaks regardless of the experiment, but also enables modification of the period by simply changing the distance between peaks.

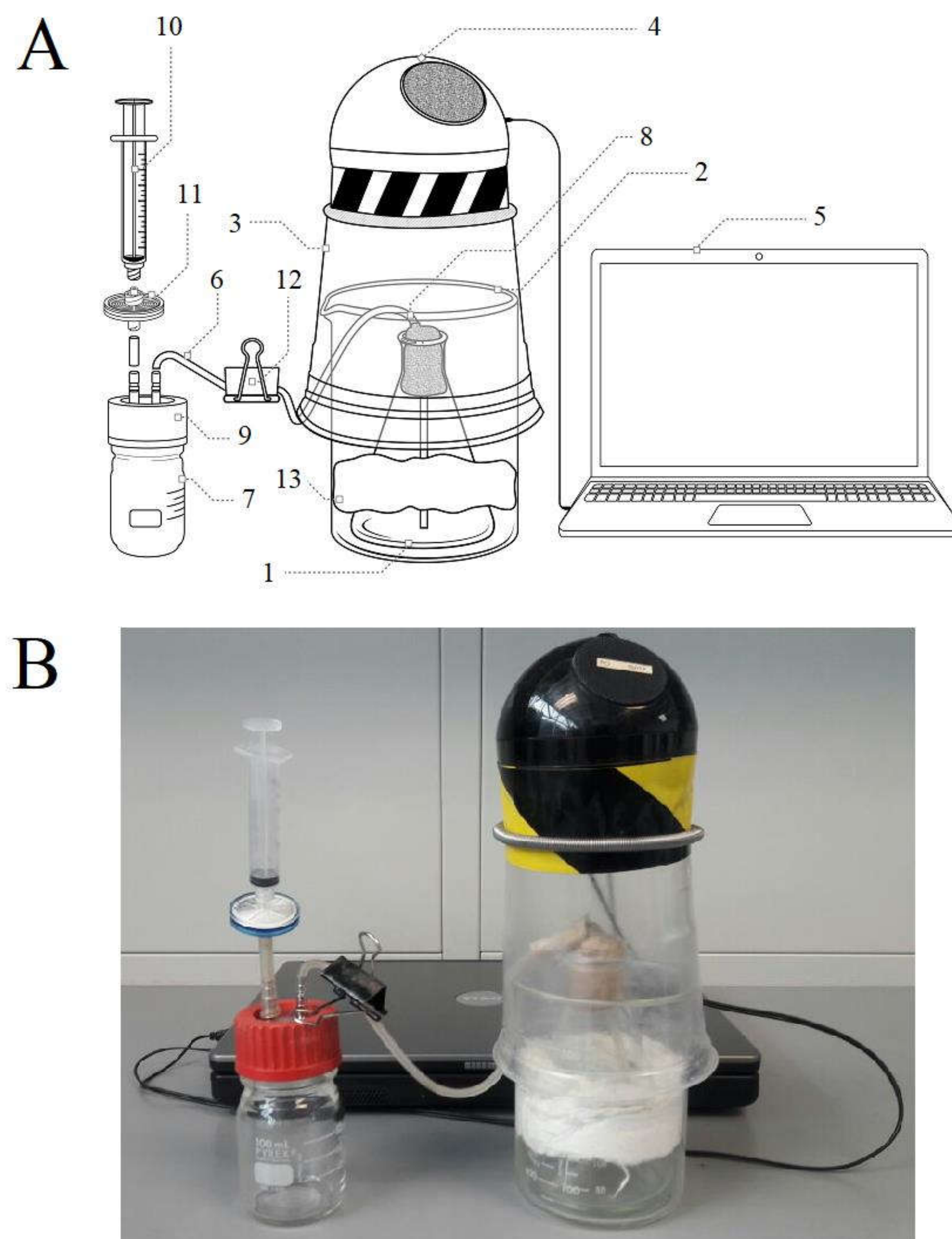


Figure 0.4. Parts of the sound wave inductor device (A) and its assembled form (B): [1] sterilized flask, [2] beaker, [3] plastic supporter, [4] speaker, [5] sound generator, [6] flexible tubing, [7] sample receiver, [8] flask swab, [9] lid with two outlets, [10] syringe, [11] 0.45 mm filter, [12] paper clamps, [13] cotton.

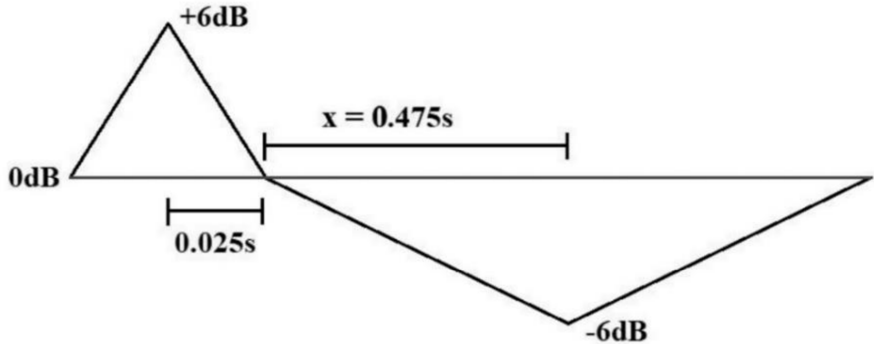


Figure 0.5. Peak design for pulsed audios. Every peak has a fixed value of 0.05s where the peak's midpoint has the maximum intensity 0.025s after the beginning of the peak. The interphase has a variable value (x) that adjusts specifically to the desired experiment. In the case of 1 cycle/s (60 bpm), the time between peaks is 0.95s, and the interphase's midpoint has the minimum intensity 0.525s after the beginning of the peak.

Three sequential experimental stages were carried out with these audio files. The first stage was the individual sound elements experiment, in which the effects of isolated elements of the sound in biomass, viability and production yield of recombinant protein were studied. A parallel stage was the frequency screening experiment, in which the effect of a specific frequency throughout the audible spectrum (20-20,000 Hz) was studied in relation to the production of our bioluminescent reporter. Finally, a third stage called sound elements interaction experiment was performed in which, depending on the previous results, the effect of each sound element as well as their interactions were studied in relation to the production of our bioluminescent reporter as well.

3.2. Sound elements experiments

3.2.1. Individual sound elements

To study the biological effect (biomass, viability and production yield of recombinant protein in *E. coli*) of five sound elements (frequency, amplitude, duration, intermittence and pulse), specific sound wave audios were designed in which only one sound element differed at a time, as compared to a standard stimulus (**Table 3.1.**).

Table 0.1. Treatments for the individual sound elements experimental stage: control treatment (CTL), standard treatment (STD), frequency treatment (FRQ), amplitude treatment (AMP), duration treatment (DUR), intermittence treatment (IMT) and pulse treatment (PLS). Graphical representation for visualizing the difference between treatments in relation with STD.

Treatment	Frequency (Hz)	Amplitude (dB)	Duration (h)	Intermittence (min/min)	Pulse (bpm)	Graphical representation
CTL	N/A	N/A	N/A	Continuous	0	
STD	1000	90	24	Continuous	0	
FRQ	5000	90	24	Continuous	0	
AMP	1000	110	24	Continuous	0	
DUR	1000	90	8	Continuous	0	
IMT	1000	90	24	20/20	0	
PLS	1000	90	24	Continuous	60	

Sound parameters were chosen as follows: frequencies [1000 Hz & 5000 Hz] and amplitudes [90 dB & 110 dB] that have been reported to show the most growth in *E. coli*;[8,10] duration as the sum of the *E.coli*'s lag and log phases [8 h] and the minimum time needed to produce maximum quantity of proteins using auto-inducible media while assuring the protocol can start every day at the same time [24 h]; intermittence that coincide with the typical duplication time [20 min] of *E. coli* under typical growth conditions[61] and no intermittence [continuous stimulus]; and finally pulse that corresponds to 1 pulse per second [60 bpm] and its absence [0 bpm].

Accordingly, four different audio files were used for all treatments in this experiment: 1) a continuous-5000-Hz audio for the frequency treatment (FRQ), 2) an intermittent-1000-Hz audio for the intermittence treatment (IMT), 3) a pulsed-1000-Hz audio for the pulse treatment (PLS) and 4) a continuous-1000-Hz for the rest of the treatments. The difference between each treatment using the latter audio was achieved by certain modifications via audio player: the standard treatment (STD) was played as recorded, the amplitude treatment (AMP) was fixed at the maximum volume the speakers could reach without distortion (110 dB), duration treatment (DUR) was muted after the first 8 h and control treatment (CTL) was muted throughout the whole experiment. Additionally, all audio files were saved as 20-min-long archives except for the intermittent audio, which was 40-min-long (20 min sound + 20 min silence).

For each treatment, the setup was as follows: three *E. coli* vials that were stored at -80ºC were placed in the sterilized flask of the sound device with 20 mL of MagicMedia™ plus 20 µL of carb100 and left to grow for 24 h according to the desired sound treatment. The order of treatments was randomly assigned and done in independent quadruplicates.

3.2.2. Frequency screening

It has been reported that biological effects due to frequency display a non-linear response,[8-11] in other words, a gradual increase in frequency does not necessarily correlate to a gradual increase of the studied biological effect. Thus, frequency screening of the audible spectrum (20-20,000 Hz) was performed to verify if an optimal specific frequency existed and if it could, by itself, have an effect in the production yield of recombinant protein in *E. coli*.

Since a screening of all the frequencies of the audible spectrum is too high to be logistically feasible, a frequency selection was made using a standardized criteria suggested by many acoustic applications where sound falls in terms of 1/3-octave bands with center frequencies at: 80; 100; 125; 160; 200; 250; 315; 400; 500; 630; 800; 1,000; 1,250; 1,600; 2,000; 2,500; 3,150; 4,000; 5,000; 6,300; 8,000; 10,000; 12,500 and 16,000 Hz.[40] This wide-frequency screening was followed by a narrower screening that had an 8-Hz bandwidth between tested frequencies in which the specific frequency that produced the highest amount of recombinant protein on the first screening was the midpoint. All audio files were saved as 5-min-long archives, and for each frequency, the setup was the same as the individual sound elements experiment. The order of treatments was randomly assigned and done in independent triplicates.

3.2.3. Sound elements interaction

As stated previously, sounds found in nature are not pure tones but complex. This suggests that living organisms may have adapted to respond to more than one sound element at a time. Therefore, to study the effect of each sound element as well as their interactions in living organisms, a factorial design was employed by quantifying the production yield of recombinant protein in *E. coli.*

The selection of sound elements was different in this experiment due to findings related to the importance of silence in the previous experimental stages. Since intermittence considers a periodic sound-silence phenomenon, it was broken down into two separate sub-elements: sound duration of intermittence (SD Inter) and silence duration of intermittence, which was redefined in relation to SD Inter as a "Sound/Silence Ratio (SS Rate)". A way to visualize this parameter is shown, for example, in the first experiment where there was a 20-min segment of sound followed by a 20-min segment of silence; the SS Rate was equal to 1. This was done to define the silence duration of intermittence as a nested element of intermittence; otherwise, it would be considered as an independent variable from intermittence, which, by definition, is not correct.

Three experimental phases were designed as shown in **Table 3.2**: 1) A fractioned factorial design which tests half of the possible combinations (16 combinations + 1 central point) of 5 sound elements by duplicate; 2) a complete factorial design which tests the combination of 2 sound elements related to oscillatory phenomena by triplicates; 3) a complete factorial design which tests the combination of 3 sound elements related to silence phenomena + 1 central point by triplicates. The order of treatments was randomly assigned.

Table 0.2. Conditions for the sound elements interaction experiment stage: all sound elements (Group A), oscillatory elements (Group B) and silence-related elements (Group C).

Sound elements	Frequency (Hz)	SD Inter (min)	SS Ratio (min/min)	Duration (h)	Pulse (bpm)
Group A	**All sound elements**				
A01	500	20	0.5	8	240
A02	10000	20	0.5	8	0
A03	500	120	0.5	8	0
A04	10000	120	0.5	8	240
A05	500	20	2	8	0
A06	10000	20	2	8	240
A07	500	120	2	8	240
A08	10000	120	2	8	0
A09	500	20	0.5	24	0
A10	10000	20	0.5	24	240
A11	500	120	0.5	24	240
A12	10000	120	0.5	24	0
A13	500	20	2	24	240
A14	10000	20	2	24	0
A15	500	120	2	24	0
A16	10000	120	2	24	240
ACP	5250	70	1.25	16	120
Group B	**Only oscillatory elements**				
B01	500	N/A	N/A	24	0
B02	500	N/A	N/A	24	240
B03	10000	N/A	N/A	24	0
B04	10000	N/A	N/A	24	240
Group C	**Only elements related to silence**				
C01	500	20	0.5	8	0
C02	500	20	0.5	24	0
C03	500	120	0.5	8	0
C04	500	120	0.5	24	0
C05	500	20	2	8	0
C06	500	20	2	24	0
C07	500	120	2	8	0
C08	500	120	2	24	0
CCP	500	70	1.25	16	0

The levels of the factorial design were selected as follows: the frequencies that generated the highest and the lowest protein production yield on the frequency screening experiment, 500 Hz and 10,000 Hz respectively; a high pulse that is not shadowed by frequency (240 bpm) and no pulse at all (0 bpm); full experiment duration (24 h) and *E. coli* exponential phase duration (8 h); high SD Inter (120 min) which corresponds to *E. coli* duration of lag phase, and low SD Inter (20 min) which corresponds to *E. coli* duplication time; high SS Rate (2.0) where silence duration is half of SD Inter, and low SS Rate (0.5) where silence duration is double of SD Inter. Intensity had a fixed value of 90 dB. All audio files were saved as 24-h-long archives, and for each combination, the setup was the same as the sound elements experiment.

3.2.4. *Escherichia coli* biomass concentration and viability determination

Many studies commonly determine bacterial growth by measuring biomass with optical density or total weight. However, either method is unspecific because it considers both living and dead cells. Therefore, viability was included to complement bacterial growth measurements because it allows the detection of only living organisms.

Biomass concentration was determined by taking 100 µL of broth sample using the sample receiver from the sound induction device, then diluting it 1:10 and measuring the OD_{600} at t = 0, 1, 2, 4, 6, 8, 12, 18 and 24 h with a UV-Vis spectrophotometer (Genesys 10S; Thermo Fisher Scientific).

Viability was determined at t = 0, 1, 2, 4, 6, 8, 12, 18 and 24 h by counting colony forming units (CFUs) grown on LB petri dishes (20 mL distilled water, 20 mg LB/mL water, 1.5% agar/mL water) using single plate-serial dilution spotting method (SP-SDS) where a serial dilution is performed with 50 µL of each sample and only the last four dilutions (10^3 – 10^6) are spotted on the petri dish to be counted directly after 16 h of growth.[62] To prevent contamination, every time a sample was taken, the sample receiver was washed with 70% ethanol and flamed before reattachment.

3.2.5. Recombinant protein production yield determination

For protein extraction, the B-PER® Bacterial Protein Extraction Reagent (Thermo Scientific) protocol was followed with certain modifications. Firstly, after 24 h of growth, the medium was centrifuged 10 min at 5000*xg* and 4ºC (Allegra 64R, Beckman Coulter). Then, the supernatant

was discarded, and the pellet weighed. A variant of B-PER® Bacterial Protein Extraction Reagent (0.5% OTG and 25 mM Tris pH 7.5) was added in a proportion of 4 times the weighted mass. Afterward, the pellet was vortexed, and an aliquot of 500 µL was isolated to be centrifuged for 5 min at 15000*xg* and 20ºC. Finally, the supernatant was acquired and mixed with 500 µL of sterilized glycerol. Every 1 mL vial was stored at -20ºC until every single aliquot for every treatment was obtained.

For the recombinant protein quantification yield, relative luminescence units (RLU) were measured (Synergy HT, BioTek) by mixing 10 µL of sample with 50 µL of QUANTI-Luc™ (InvivoGen) following the providers instructions on a white 96-well plate. The luminescence of every independent treatment quadruplicate was measured between 7-9 times, and their average was divided by the corresponding wet mass. The calibration curve was generated with a 1:10 serial dilution of 0.2 µg of NanoLuc® standard (regression equation: Log(RLU) = 0.9297Log(µg NanoLuc) +9.3888).

3.3. Gene expression analysis

The study of the effect of sound over responses such as viability, biomass concentration and protein production yield give an insight of the biological mechanisms that are involved. As part of the present research work, a study of gene expression was conducted in order to identify genes that are up-regulated due to sound and use their molecular mechanism for the development of bioprocessing sound-based technology.

Although there are studies that have confirmed a change in gene expression due to sound exposure,[7,30-32] all of them have been identified exclusively in plant models. Nevertheless, there is one study that used sound stimuli to up-regulate 342 genes in *Chromobacterium violaceum.*[52] Consequently, this is the first report of a bacterial transcriptome influenced by sound.

Based on the results of this publication about the genes that had the most positive regulation, three genes from *C. violaceum* that had analogous functions in *E. coli* were selected. The selected genes are involved in stress response (*CpxR*), carbon storage regulator kinase (*BarA*) and chemotaxis (*CheA*).

Since the objective of this methodology is the identification of differentially regulated genes, the $2^{-\Delta\Delta Ct}$ method was performed.[63] This method consists in determine the relative comparison of gene

expression by calculating the cycle threshold (C_t) values from the target gene with respect to a set of housekeeping genes. C_t is the cycle where the logarithmic phase of the amplification begins and is characterized by the fluorescence exceeding the background level where smaller values represent a higher template quantity.

Because C_t is represented in a logarithmic scale, it must be subtracted from the C_t value of the housekeeping genes to normalize the gene expression per treatment (ΔC_t) where negative values represent upregulating genes and positive values represent downregulating genes. To obtain the relative expression change ($\Delta\Delta C_t$) which describes the expression fold for each gene, the normalized ΔC_t value from the treated samples must be subtracted from the normalized ΔC_t value from the untreated samples. Finally, to visualize the expression fold in a linear way, the formula two raised to the minus $\Delta\Delta C_t$ was used.

3.3.1. Primer design

Since the experimental design seeks to compare the expression between genes that were stimulated by sound and genes that were not, a comparative C_t ($2^{-\Delta\Delta Ct}$) method through a QPCR was performed. QPCR was opted because of its advantages: time-effective, high specificity, reliable and informative because it monitors the accumulation of a specific product during each cycle. However, to provide this specificity, the design of specific primers is required. Accordingly, several considerations have been made for the primer design.

First, since it is recommended not to work with just one housekeeping gene;[64] four were selected to increase the normalization robustness: 16S ribosomal RNA (*ssrA*), 3-phenylpropionate MFS transporter (*hcaT*), L-idonate/5-ketogluconate/gluconate transporter (*IdnT*) and uroporphyrin III C-methyltransferase (*cysG*). These have already been found suitable for the study of stress gene response in *E. coli*.[65,66] Moreover, the primers that detect these genes have already been designed and tested,[66] plus they generate amplicons of ~100 bp which is recommended for a good QPCR efficiency.[67]

Second, to increase the selectivity of the experiment, primers were designed to amplify a region of ~300 bp, which becomes the template for the primers that amplify the region of ~100 bp (**Table 3.3.1.**). Finally, the melting temperature (Tm) was kept between 57-60ºC, with a GC% between 50-60% and no primer dimers were detected (OligoAnalyzer Tool, Integrated DNA Technologies).

In the case of the housekeeping genes, the published primers for the ~100 bp amplicons had certain nucleotides changed to fit the previous criteria. To verify that the designed primers amplified the desired region, a colony PCR was performed. Fragments were run on a 1.5% agarose gel electrophoresis and visualized on a gel documentation system (Gel Doc™ XR+, Bio-Rad) using ethidium bromide.

Table 0.3. List of primers used for the sound gene expression experiment in *Escherichia coli*.

Gene	Amplicon size (bp)	Primer-forward	Primer-reverse
ssrA	355	GGGCTGATTCTGGATACGACGGG	GCTGGCGGGAGTTGAACC
	107	ACGGGGATCAAGAGAGGTCAAAC	CGGACGGACACGCCACTAAC
cysG	322	CTGTCTGAATGTCGGCGGTGG	CGGTGAGCGGTCAATAATCG
	105	ATGTCGGCGGTGGTGATGTC	ATGCGGTGAACTGTGGAATAAACG
hcaT	325	CGGTGGCGTTAGTCATTGGC	AGCGACCACAAATACCCCAC
	85	CTGCTCGGCTTTCTCATCC	CCAACCACGCTGACCAACC
ldnT	278	TTATTGTCGCAGGACCGCTG	GCCCAGTGTGAAAATCGCAA
	90	CTGTTTAGCGAAGAGGAGATGC	ACAAACGGCGGCGATAGC
BarA	342	GCGATTATCTGGCGAAACCG	AGAGATTCTTCATACGAGGCACACCG
	110	GCGGTATTTCCTCTCGGGTCGTG	ATCGGTTTTTCCTGCTGCCT
CpxR	293	CGAGCAACAGCAAAACAACG	TAAACCACGGGTGACCATCT
	105	AGGTGGTTTCCCGTGAACAT	GCAGTTTACGACGCAGGTTG
CheA	315	CCAGTTCTTCTTCCAGCAGGTC	AAGCCAGACGAGGTGAGATG
	93	TGACTGCGACTCTGCTCATC	GGCATTAGAAGCGAAAGGCG

3.3.2. RNA extraction

A pre-inoculum was produced by adding 10 µL of *E. coli* seed stock, 10 mL of LB broth and 10 µL of carb100, and allowed to grow for overnight in 50 mL Falcon tubes. After, the pre-inoculum was added into two different 250 mL flasks containing 20 mL of MagicMedia™ plus 20 µL of carb100 with an initial OD_{600} between 0.05 and 0.1. Both flasks were growth contiguously on the same 1585 orbital shaking incubator (VWR) and both had the sound induction device attached to them, but one had the speaker turned on (Treated) and the other one off (Untreated). The cultures were grown for 8 hours using the sound treatment that had the most protein production in the sound elements interaction experiment by triplicates.

Afterwards, the RNA was extracted for each sample using the RNeasy® Mini Kit (Qiagen) as follows: the sample was centrifuged 10 min at 5000*xg* and 4ºC (Allegra 64R, Beckman Coulter).

Then, the supernatant was discarded and 700 µL of Buffer RLT was added. The pellet was resuspended by vortexing vigorously, and the suspension was transferred into a 2 ml Safe-Lock tube containing acid-washed glass beads (150-600 µm diameter). Cells were disrupted for 5 minutes with one-minute rest every minute using a FastPrep®-24 Classic Instrument (MP Biomedicals). Afterwards, the suspension was centrifuged for 10 s at 18,000*xg* using a Microfuge 18 centrifuge (Beckman Coulter), the supernatant was transferred to a new tube and added equal volume of ethanol (70%). Up to 700 µL of the lysate was transferred to an RNeasy spin column placed in a 2 ml collection tube, centrifuged for 15 s at 8000*xg* and the flow-through was discarded. This was done several times until all the volume of the lysate was centrifuged through the spin column.

The washing steps were done by adding 700 µL of Buffer RW1 to the RNeasy spin column, centrifuging for 15 s at 8000*xg* and discarding the flow-through. Then, 500 µL of Buffer RPE were added to the RNeasy spin column, centrifuged for 3 min at 8000*xg* and the flow-through was discarded. To elute the RNA, the RNeasy spin column was placed in a new 1.5 mL collection tube, 100 µL of RNase-free water was directly added to the spin column membrane and centrifuged for 1 min at 8000*xg*. Concentration and purity of the RNA was measured using a NanoDrop 2000 UV Visible Spectrophotometer (Thermo Scientific).

3.3.3. cDNA synthesis

The samples were placed on ice immediately after the RNA extraction, to avoid degradation. Subsequently, the cDNA was synthetized for each sample using the ImProm-II™ Reverse Transcription System (Promega) as follows: 2 µL of total RNA (20 µg/sample) was mixed with 1 µL of the respective forward or reverse primer (10 µM), which generates the ~300 amplicon (**Table 3.3.**) and 2 µL of nuclease-free water. Then the mixture was preheated at 70ºC for 5 min and immediately chilled on ice for another 5 min. It was then mixed with the reverse transcription reaction mix (15 µL) that was formed by adding 6.5 µL of nuclease-free water, 4 µL of ImProm-II™ 5x buffer, 2.5 µL of $MgCl_2$, 1 µL of dNTP Mix and 1 µL of ImProm-II™ Reverse Transcriptase. The final volume was 20 µL per sample.

Finally, the annealing was done at 25ºC for 5 min, the extension at 42ºC for 60 min and the inactivation of the reverse transcriptase at 70ºC for 15 min. To verify the presence of the desired amplicons, a 1.5% agarose gel electrophoresis was performed with a 1 kb DNA Ladder (Promega)

as reference band using the primers that generate the ~100 bp amplicons. Concentration and purity of the cDNA were measured using a NanoDrop 2000 UV Visible Spectrophotometer (Thermo Scientific).

3.3.4. Quantitative PCR

Gene expression quantification was performed in a Rotor-Gene Q (Qiagen) using the Rotor-Gene SYBR® Green PCR Kit (Qiagen) as follows: 3 µL of cDNA (~5ng/µL) was mixed with 2.5 µL of the respective primers that generate the ~100 bp amplicons (10 µM), 7 µL of nuclease-free water and 12.5 µL of SYBR Green I (25 µL total per sample). The QPCR cycle was done with 40 cycles of denaturation at 95ºC for 10 s, annealing at 58ºC for 15 s and elongation at 72ºC for 20 s. The cycle threshold (Ct) for each sample was obtained from the Rotor-Gene Q Series Software by setting the normalized fluorescence detection threshold at 0.015.

3.4. Methods for sound-bioreactor system

Once the effects of sound elements, individually and in combination, in *E. coli* were confirmed using the sound induction device design for application at flask level, a first version of sound inducible device, based on a point source speaker design proposed by Cobo[68] and improved by Ibarra *et al*,[69] was tested by adapting a sound source into commercially available bioreactors. The experiments conducted helped to evaluate the potential application of such sound induction device, from a sound wave propagation point of view.

This acoustic analysis is important because the way in which sound collides with surfaces can cause certain frequencies to be amplified or to cease existing. When adapting a speaker into a bioreactor for stimulating bacteria, it is necessary to assure frequencies of interest do not disappear. To test the frequency response inside a commercially available bioreactor, an inverted conical speaker was adapted into two Biostat A plus bioreactor systems (Sartorius), one with the capacity of 1L and another with 2L.

The speaker was based on the point source design proposed by Cobo,[68] which consists of an inverted cone that has a sound source placed at its cylindrical bottom and a small opening at its apex (**Figure 3.6.**). This type of design can accurately create sound in every direction

(omnidirectional), however the frequency response does not show a uniform signal for a wide range of frequencies (flat frequency response). To overcome this issue, the speaker was optimized by changing its length to 20 cm and using "inverse filtering" as proposed by Ibarra *et al*,[69] which consists of getting the baseline signal between the source and the receptor, inverting the signal and sending it through the speaker to create destructive interference, which eliminates reflections.

The sound inside the speaker was generated using a SPL3.1 Coaxial speaker (Bassface), the inverted cone was built using a Cube Pro Trio 3D Printer (3D Systems) and the receptors used to calibrate with inverse filtering and measure the frequency response were a MX150 condenser microphone (Shure) and an ES963 three-way multidirectional boundary microphone (Audio technica).

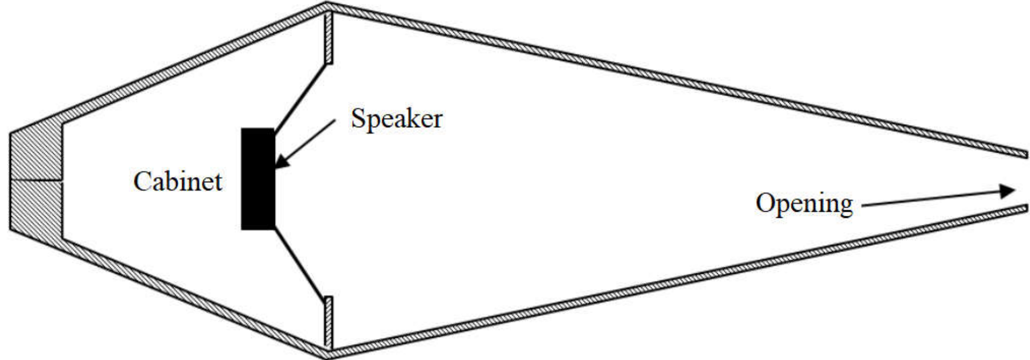


Figure 0.6. Design of an omnidirectional point source speaker. Figure obtained from Ledesma's Thesis document.[70]

To visualize how the sound behaves within the bioreactor (acoustic analysis), a sound containing every frequency was applied inside the bioreactor and the frequency response was measured in three different heights (base, midpoint and top) of both bioreactors (1L and 2L) in three different states: a) empty, b) half volumetric capacity, and c) half volumetric capacity with stirring.

Due to the steric impossibility of placing the speaker vertically next to the rotor, the speaker had to be placed horizontally (**Figure 3.7.**). To achieve this, a flexible tubing was used to join the speaker's apex to the bioreactor through one of the holes of the headplate. The microphones that were used for measurements were introduced through another hole and all remaining openings were covered with plasticine. This adaptation ensures the conservation of the speaker's omnidirectional property at the end of the flexible tubing, which gives the possibility of placing the speaker freely at any angle relative to the bioreactor. However, once a position is selected, the

inverse filtering must be done on that specific position to ensure that the new reflections coming from the flexible tubing are canceled.

Each different speaker-microphone setup had to have their own inverse filtering. These systems were: 1) speaker with the ES963 microphone for surfaces; 2) speaker attached to the flexible tubing with the MX150 microphone for when stirring; 3) speaker with the MX150 microphone for all other measurements.

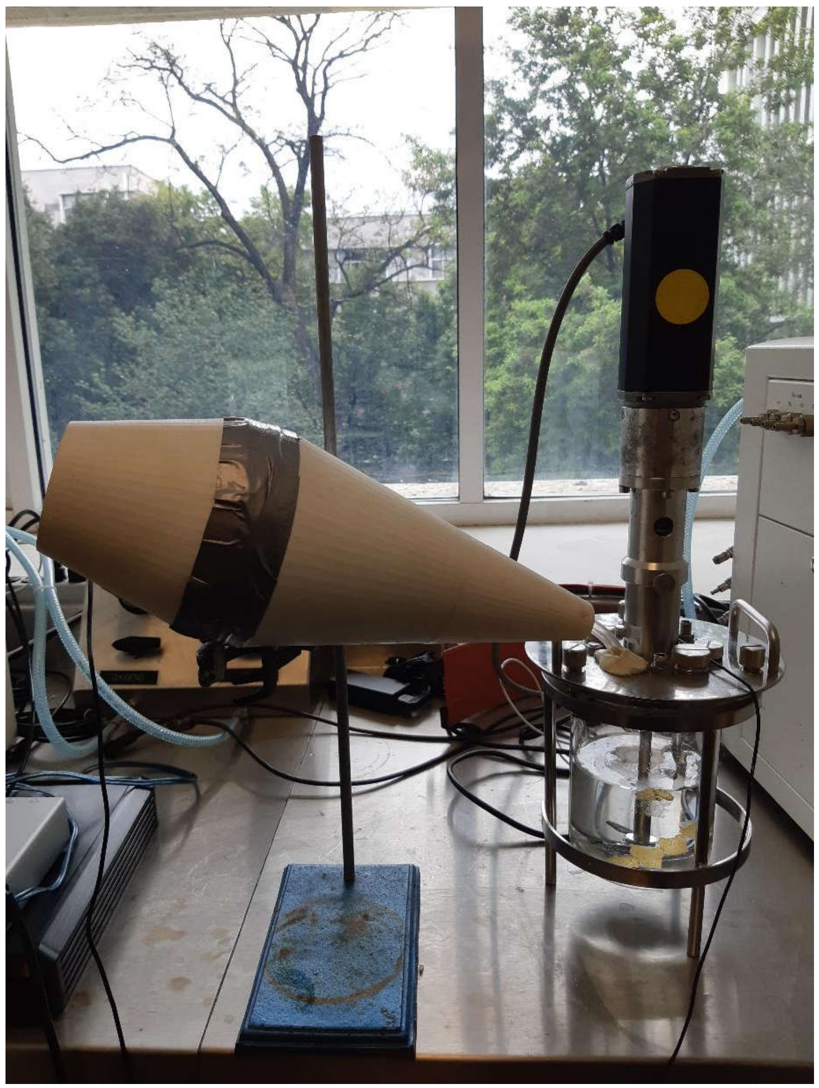


Figure 0.7. Horizontal assembly of the point source speaker (left) with the bioreactor (right). Flexible tubing allows the speaker to be placed in any angle while keeping its omnidirectional properties.

3.5. Statistical analysis

For both the individual sound element and frequency screening experiments, the statistical significance of the mean and the variance was evaluated with the t-test and the F-test respectively, both available in Microsoft Excel®.

For the sound element interaction experiment, the standardized effect of the treatments was evaluated using Minitab 18. The standardized effect was calculated by dividing each effect coefficient by its standard error. Data with p-values < 0.05 were considered statistically significant.

For the gene expression analysis, the $2^{-\Delta\Delta Ct}$ method and the t-test were used and calculations performed in Excel®. The values of all housekeeping genes were averaged to normalize the background expression. Data with p-values < 0.05 were considered statistically significant.

Chapter 4

4. Results and Discussion

4.1. Sound induction device validation

To correctly correlate a sound wave to an effect, it is necessary to ensure that sound waves inside the sound induction device are equal to those from the source. There are two types of audio distortion: a) frequency response, which involves amplitude alterations and phase errors, and b) harmonic distortion, which adds frequency content.[40] The former can be corrected with signal processing by adjusting amplitude levels, but the latter cannot because the wave form has changed. Thus, the frequency response and harmonic distortion of all audios from the sound individual elements experiment were analyzed in MATLAB® using a condenser microphone to validate that bacteria inside the sound inductor device receive the designed signal. The output signal of the audio files used for the sound individual elements experiment is shown in **Figure 4.1.**

Regarding frequency response, there was no amplitude change with respect to time nor between audio files where the amplitude had a constant change in power oscillating between -0.25 and 0.25 Volts (**Figure 4.1.A and 4.1.B**). Plus, the frequency did correspond to the designed input signal with 1 cycle every 0.001 s which confirmed 1000 Hz (**Figure 4.1.A**) and 5 cycles every 0.001 s which also confirmed 5000 Hz (**Figure 4.1.B**). Thereby, no amplitude distortion was detected in any continuous audio file.

However, the pulsed audio file showed an amplitude change. Each peak was 5 times longer than designed (0.05 s long input vs. 0.25 seconds long output) which caused the peak's midpoint to have a time shift of 0.1 seconds (**Figure 4.1.C**). This frequency response may be due to the concave surfaces of the flask because these surfaces are known to concentrate sound instead of distributing sound uniformly,[40] which, depending on the level and the time delay, can cause an echo.[71]

Fortunately, the echo did not create an overlap between the peaks. Moreover, the distance between midpoints is still 1 s (**Figure 4.1.G**), which means that this audio could still be used to test the effect of pulsed frequencies, despite the change between input and output.

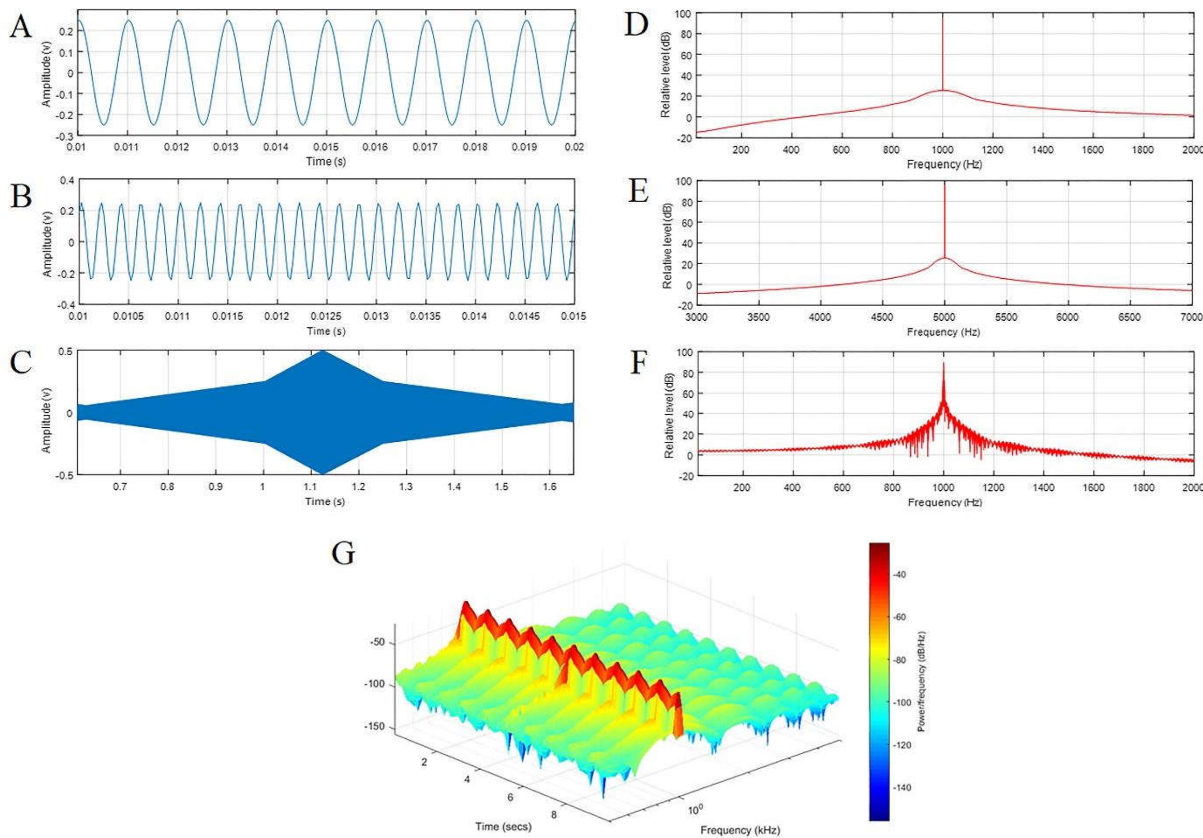


Figure 0.1. Visual representation of signal output for the audio files used for the sound individual elements experiment. Graphics for amplitude vs. time (A-C) and amplitude vs. frequency (D-F) for the continuous-1000-Hz audio (A, D), the continuous-5000-Hz audio (B, E) and the pulsed-1000-Hz audio (C, F) are represented respectively. Spectrogram (time vs. frequency vs. amplitude) of the pulsed-1000-Hz audio (G) is also shown. Only one 1000-Hz audio frequency response is shown since the intermittent-1000-Hz audio had the same response as the continuous-1000-Hz audio.

Regarding harmonic distortion, frequencies between 400 Hz and 2000 Hz appeared in all continuous-1000-Hz audio files (**Figure 4.1.D**), between 4000 Hz and 6000 Hz in the continuous-5000-Hz audio file (**Figure 4.1.E**), and between 0 Hz and 1600 Hz in the pulsed-1000-Hz audio file (**Figure 4.1.F**). Nevertheless, these frequencies disappeared above 25 dB in the continuous audio files and above 50 dB in the pulsed audio file. Because treatments were planned to be performed between 90 and 110 dB, audio files were not a source of distortion in these sound experiments.

4.2. Sound elements results

4.2.1. Effect of individual sound elements

It has already been reported that sound enhances biomass growth in *E. coli*.[8-11] However, in previous reports, a clear correlation between individual sound elements and the response (biomass concentration) could not be concluded as the treatments consisted of complex musical compositions or they merely focused on frequency.

For the purposes of this work, biomass growth was monitored for 24 h in the presence of each sound treatment (**Figure 4.2.**). Growth curves behaved similarly in all treatments except for FRQ and DUR which influenced biomass concentration by increasing its value 19% and 44%, respectively, when compared to STD at time 24h. However, no treatment was statistically significant. Nevertheless, regarding variance, two treatments (FRQ and DUR) increased biomass dispersion up to 6 times after 12 h ($p < 0.03$), and three treatments after 24 h: FRQ ($p < 0.05$), AMP ($p < 0.05$) and DUR ($p < 0.005$). This suggests that these parameters modify growth properties non-specifically which change the variability of the system during the exponential phase, and these changes do not disappear in the following generations, especially when duration was tested. The persistence of sound effects long after suppression of stimulus has been detected in other studies.[72]

In the present work, a duration time of either 8 or 24 h was used to study the effects of silence when DUR (8 h) is compared to STD (24 h), which their only difference is the presence or absence of sound after the exponential phase. As seen in **Figure 4.2.**, a high variation was observed when using the DUR treatment, compared to other treatments. This high variability caused by exposing the biomass to silence may generate differential gene expression on subpopulations of the culture. At this point, the mechanisms related to this high variability not found in any other treatments are unclear (**Figure 4.2.**), thus, to understand the effect of silence at the genetic level a transcriptome analysis should be performed.

In this study, viability was monitored for 24 h in the presence of each sound treatment (**Figure 4.3.**). No statistical difference in mean or variance was found between any treatment as compared to STD. However, every treatment reached stationary phase around the same time (6 h) except for AMP, which took twice as long (12 h). This suggests that amplitude may have an effect in

slowing down the growth speed during the exponential phase. To confirm this hypothesis, AMP and STD slopes were calculated and compared. For each replicate, a slope value was obtained using a linear regression of the exponential phase followed by a t-test using these values. Compared to STD, AMP was statistically different ($p < 0.03$) which confirms that the amplitude doubled the exponential phase duration.

Amplitude can be described in terms of pressure, where higher amplitude produces higher pressure around the cells. This effect has been described in other studies where it was found that higher pressure generates oxidative stress[73] but only cells during the exponential phase are affected.[74,75] It is likely that this response is due to activation of stress-dependent mechanoreceptors,[11,72] but possibly only when surpassing a specific high threshold since the response did not appear in the rest of the mild-amplitude treatments.

Additionally, production yield of recombinant protein was quantified in this study. **Figure 4.4.** shows the total biomass and the total amount of luminescent protein per wet mass produced after 24 h of growth for each treatment. Published studies have shown that exposure to sound at 5000 Hz can result in a doubling of *E. coli* biomass[10] but, in our case, FRQ, which corresponds to 5000 Hz, had only an increment of 10% biomass compared to the control and it was not statistically significant ($p = 0.18$). Although DUR was the treatment with the highest biomass increment (20%), it was not statistically significant ($p = 0.19$), as well. This finding suggests that sound does not influence biomass production.

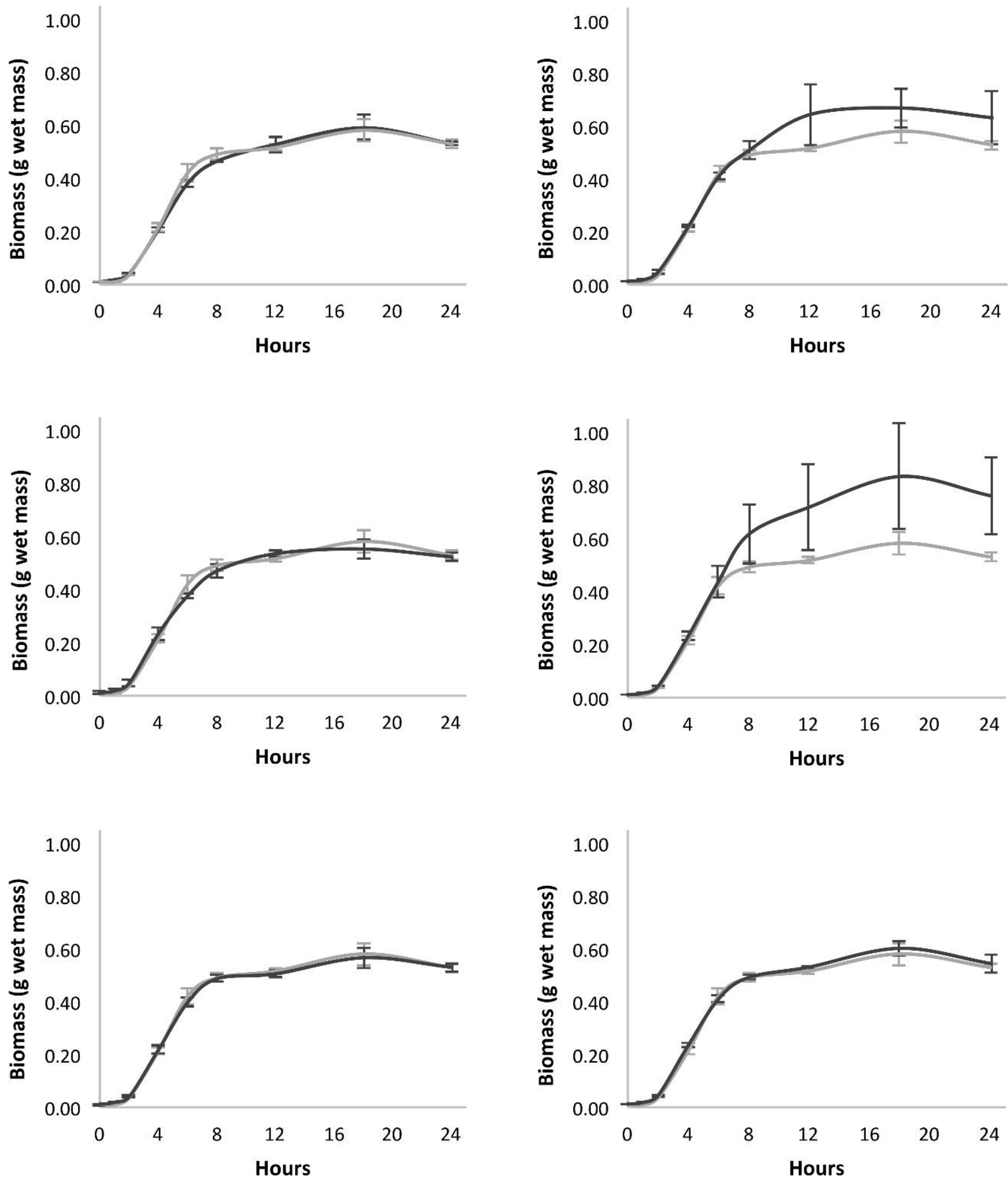


Figure 0.2. Biomass growth kinetics for each sound treatment (dark gray) that differs in frequency (FRQ), amplitude (AMP), duration (DUR), intermittence (IMT), pulse (PLS) and no sound (CTL) in comparison to a standard treatment (light gray). There is no statistical difference in any point of the whole 24 h growth period, but there is an increase in variance ($p < 0.03$) in both FRQ and DUR. Treatment conditions are described in **Table 3.1.** Deviation bars represent standard error.

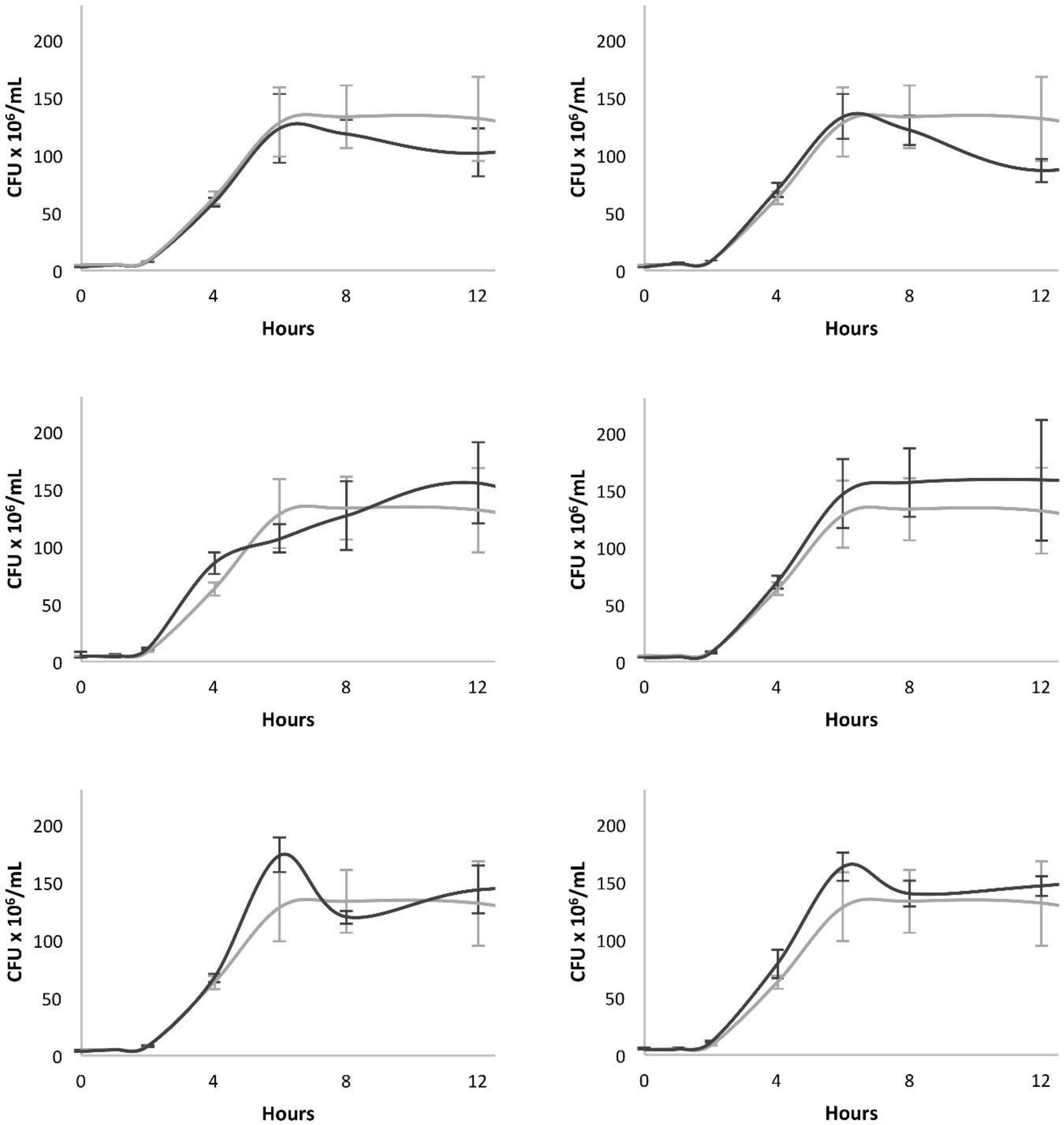


Figure 0.3. Biomass viability kinetics for each sound treatment (dark gray) that differs in frequency (FRQ), amplitude (AMP), duration (DUR), intermittence (IMT), pulse (PLS) and no sound (CTL) in comparison to the standard treatment (light gray). There is no statistical difference in any point of the whole 12 h growth period. The slope of AMP was statistically different ($p < 0.03$) compared to any treatment. Treatment conditions are described in **Table 3.1.** Deviation bars represent standard error.

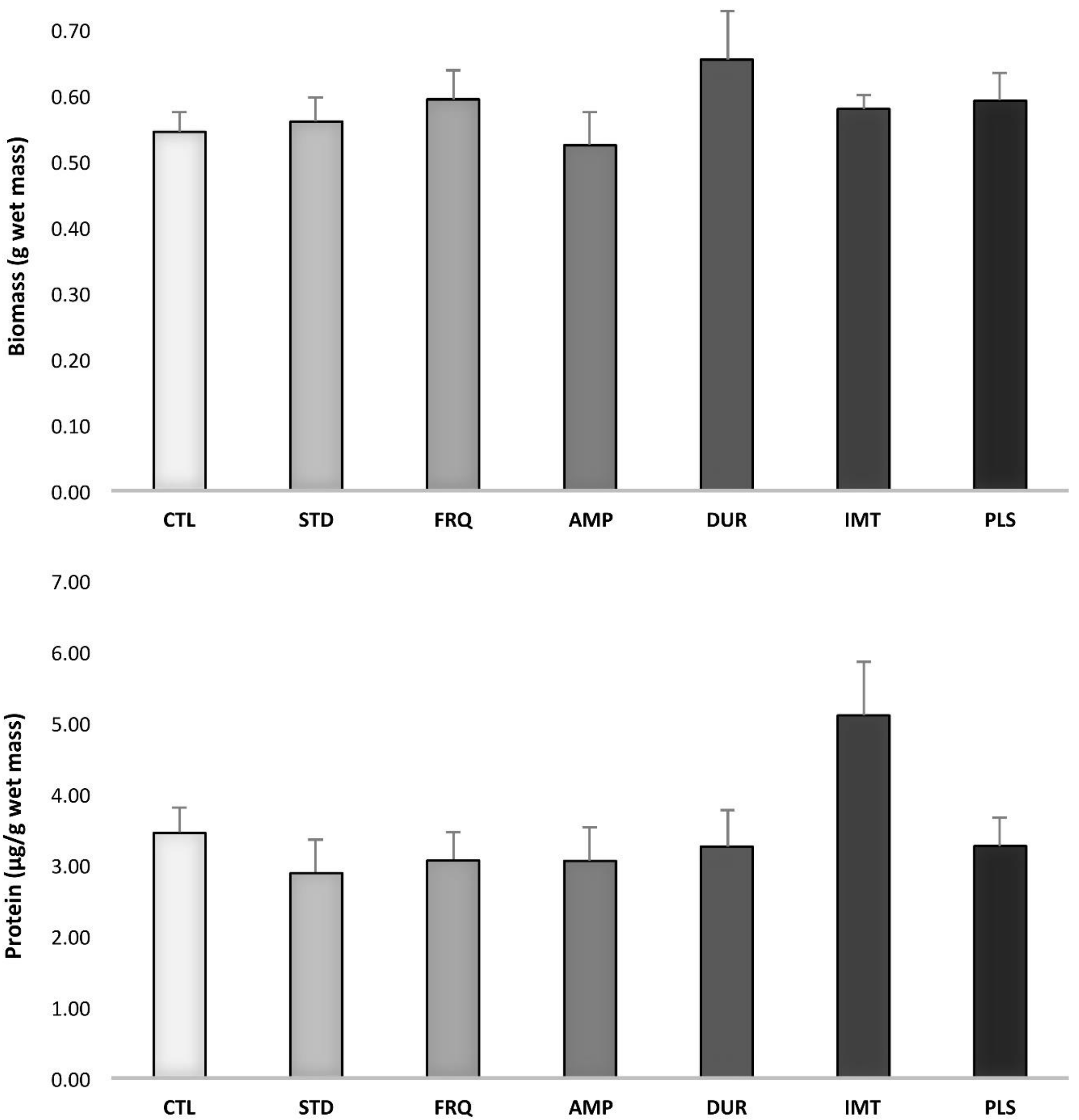


Figure 0.4. Total biomass (A) and luminescent protein production yield (B) produced after 24 h of growth in the presence of different sound treatments that differed in frequency (FRQ), amplitude (AMP), duration (DUR), intermittence (IMT) and pulse (PLS) compared to a standard treatment (STD). Only IMT treatment is significantly different in the total amount of luminescent protein ($p < 0.05$). Treatment conditions are described in **Table 3.1.** Deviation bars represent standard error.

Moreover, it was found that all treatments, except IMT, had less luminescent protein yield compared to CTL, but again, none were significantly different. Even so, IMT was the only statistically significant treatment ($p < 0.05$) with 50% higher amount of recombinant protein yield compared to CTL or any other treatment. Considering that IMT had no significant effect regarding biomass concentration, this data suggests that intermittence influences the enhancement of protein production yield in a single cell and not because of biomass increment. Although most studies addressing the effect of sound over biomass have used continuous signals,[27,28] in the present research work, the establishment of the effect of intermittence was considered important. Intermittence parameters were either selected as continuous signal or an intermittent cycle of 20 min sound plus 20 min silence; this value was assigned in relation to *E. coli* duplication time under typical growth conditions.[61]

Is it important to note that intermittence could be confused with duration because both describe sounds followed by silence, but both differ in their effects. Firstly, IMT was the only treatment that produced more luminescent protein per cell (**Figure 4.4.**) and, secondly, it does not increase variance either in biomass or in viability as seen in DUR (**Figure 4.2. & 4.3.**). It may look like the constant reappearance of sound after silence helps the system not only to promote variability but also to increase protein production yield. Because this increment is not due to an increment of biomass, intermittence is likely to affect gene expression in other ways than oxidative stress similar to results found with tobacco plants where sound was able to upregulate specific transcription factors.[7] Furthermore, it has been reported that sound can also upregulate the activity of kinases, disulfide oxidases, chaperones, signal transporters, and genes involved in RNA production in bacteria.[52] Considering that their treatments used sound beeps instead of continuous signal, it is likely that those reported effects are due to intermittence.

Finally, regarding pulse, despite its complex design, there was no statistical difference in any biological parameter, including variability, which suggests that this sound element has no influence on any biological response. Nevertheless, it can be noted that viability for only IMT and PLS – both of which are pulse-shaped signals – showed an under-damped response just after the exponential phase (**Figure 4.3.**). Considering that this response appears only when there is a disturbance on the system's equilibrium state, and that global gene expression during exponential phase is normally stable,[76] it is likely that pulsed sounds, either continuous or discontinuous, can regulate gene expression during the exponential phase.

Even when the effects are most noticeable during exponential growth, it cannot be concluded these effects are exclusive to this phase. For instance, studies have found quorum sensing molecules have increased in production during musical treatments,[13,28,52] and these secondary metabolites are expressed during stationary phase.

4.2.2. Effects of frequency

The effects of frequency on biological systems have been the most reported in the literature. However, it is still not fully understood as there are some inconsistencies in the conclusions drawn from different studies.[8,10] For instance, in one publication[8] the authors concluded that 1000 Hz and 90 dB was the best combination to promote growth in *E. coli* with an increment of 41.6%, but in another publication[10] they proposed 5000 Hz and 110 dB to be the best combination. Additionally, they described that frequency has a non-linear biological response[9] which means not only that a specific frequency is necessary to have a biological effect, but that even if certain frequencies do not show a biological effect, it does not imply that other frequencies will not show this effect. More recently, it has been found that frequency has little biological effect compared to other sound elements.[72] Therefore, the discrepancy between optimal conditions and the non-linearity of frequency effect suggests that it is necessary to perform a broad frequency screening as wide as possible.

In this study, a frequency screening in terms of 1/3-octave bands was performed by measuring the recombinant protein yield produced in *E. coli* after 24 h of growth. As predicted in previous studies,[9] the non-linearity of frequency was detected (**Annexes; Figure A.1.**). However, none of the frequency treatments was significantly different in mean (t-test), nor variance (F-test) compared to the control, which includes 500 Hz that had a 58% increment (p = 0.16) and 10,000 Hz, which had a 57% decrement (p = 0.14). These frequencies represent the treatments with the highest and the lowest protein production yields, respectively.

A narrower frequency screening was performed near the highest frequency treatment with the highest protein production yield (500 Hz) and between 400-600 Hz with an 8 Hz bandwidth between each frequency. This screening included two frequencies from the previous screening (400 Hz and 500 Hz) and coincidentally two Solfeggio frequencies (432 Hz and 528 Hz), also known as “miracle tones”, which are widely used in alternative medicine with the claim that 432 Hz transmits beneficial healing energy due to its mathematical consistency with nature, and 528

Hz heals damaged DNA through the activation of introns (Attuned Vibrations (attunedvibrations.com)). To our knowledge, this is the first time these frequencies have been used to test their biological effects.

Once again, none of the frequency treatments were significantly different compared to the control including 432 Hz (p = 0.34), 528 Hz (p = 0.87) and 568 Hz (p = 0.11), the latter being the closest one to be statistically significant with a 48% increment (**Annexes; Figure A.2.**). Based on these results, we conclude that although frequency may give the impression of enhancing the recombinant protein production yield, because of its lack of statistical significance, it has a random response, at least when using *E. coli* as a model system.

Although this is the first time the recombinant protein production yield was monitored in *E. coli* using specific frequency treatments, there is one study which found that 8000 Hz can enhance protein and RNA biosynthesis in *E. coli* by 25% and 10% respectively.[11] In our study, the use of 8000 Hz incremented 8% of recombinant protein production yield in *E. coli* when compared to the control. These inconsistencies in biological effects when using only frequency treatments has been detected in *E. coli* not only in protein production yield but also, as mentioned previously, in biomass growth.

4.2.3. Effect of the interaction of sound elements

Considering the evidence that sound has shown to have biological effects in bacteria,[8-11] it can be hypothesized that the way they detect the stimulus is either by responding to one sound element at a time or as a combination of several sound elements processed as a single input. To verify either hypothesis, a factorial design was performed to study the effect of each sound element and their interactions on the production yield of recombinant protein. The factorial designs were performed in three groups: A) all sound elements, B) only oscillatory elements, C) only elements related to silence (**Table 3.2.**).

Treatments considered for groups B and C were design based on the significance of the results obtained in group A. **Figure 4.5.** shows the production yield of recombinant protein and the absolute values of the standardized effects for each sound element for group A (**Table 3.2; Group A**). It can be noted that only the sound elements in combination were statistically significant. According to the results of the individual sound elements experiment, it was expected that SD

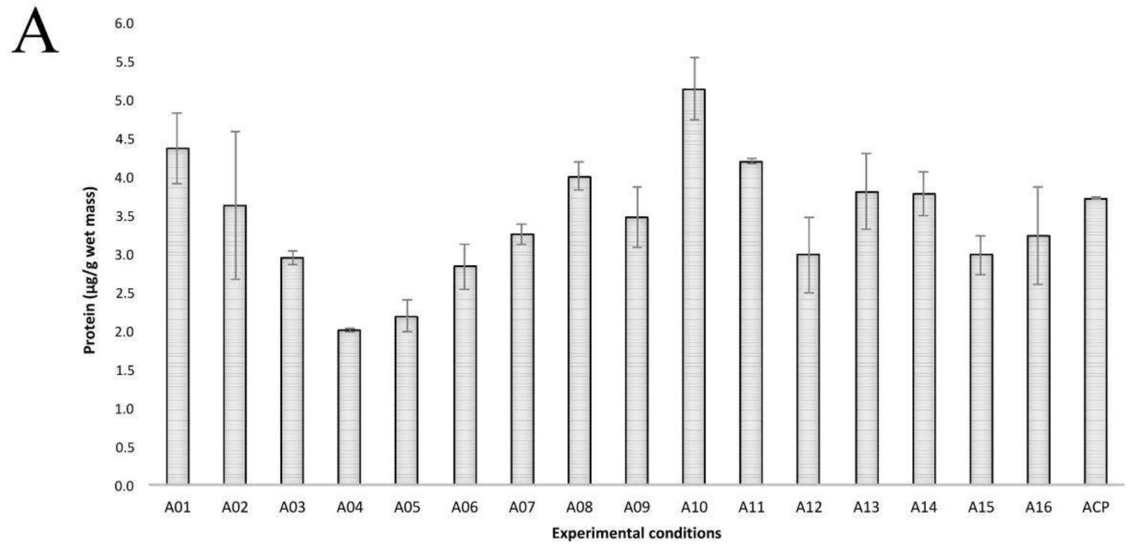
Inter and SS Rate were statistically significant only in combination because both are essential to define intermittence. However, the significance of the combination of pulse and frequency was unexpected because neither were significant in the individual sound elements experiment.

Figure 4.6. shows the production yield of recombinant protein and the absolute values of the standardized effects for each sound element for group B (**Table 3.2; Group B**). In this factorial design, no sound element – individually or in combination – was statistically significant. This may suggest that it is necessary to have all sound elements for the effect to appear, but this can only be true if intermittence is not statistically significant when analyzed without frequency and pulse.

Figure 4.7. shows the production yield of recombinant protein and the absolute values of the standardized effects for each sound element for group C (**Table 3.2; Group C**). Contrary to group B, SD Inter and SS Rate were statistically significant again in combination which confirms that the increment in recombinant protein production yield is due to intermittence alone, thereby neither the frequency nor pulse is necessary for this effect.

It is important to point out that because other researchers unintentionally used intermittence in their frequency experiments, it is possible that the effects they describe were due to intermittence and not frequency. For instance, some of the intermittences that have been used are 30 min, twice a day,[6,25] 60 min per day[5] and 1 h for every 4 h.[8,9]

This biological effect of intermittence can be explained if physical stress mechanoreceptor channels are considered as the mechanism by which *E. coli* detects sound channels.[8,9,11,12] A continuous complex stimulus may saturate the response of the mechanoreceptor channels making them unresponsive, in a very similar way to the onset of insulin resistance. Thus, the lack of response of these channels would prompt a significant suppression of mechanosensitive gene expression. This phenomenon was recently detected[72] when using white noise, a complex stimulus conformed by a mixture of all frequencies. In this matter, the alternation between sound and silence of intermittence might help the mechanoreceptor channels to regain responsiveness by avoiding signal saturation.



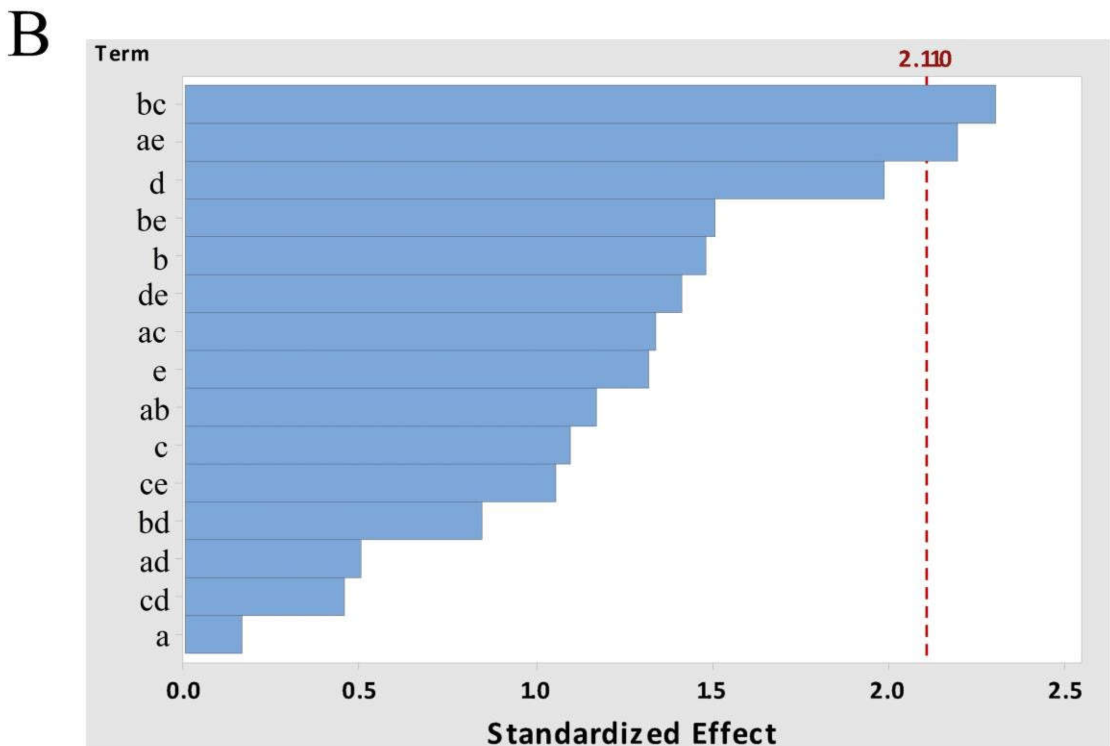
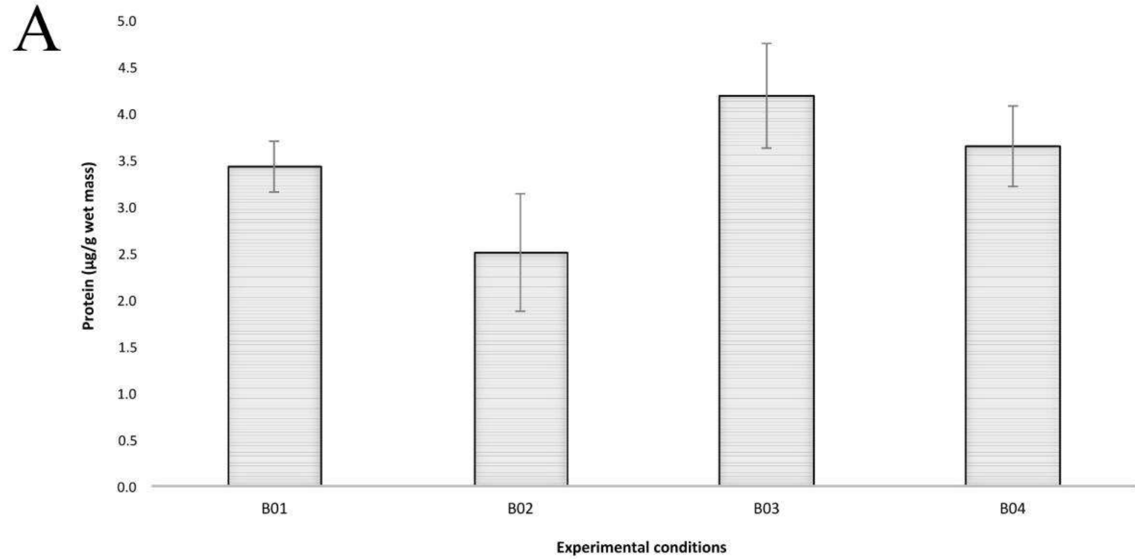


Figure 0.5. (A) Luminescent protein production yield after 24 h of growth using the sound conditions described in **Table 3.2 (Group A)**. (B) Absolute values of the standardized effects that sound elements (a = Frequency, b = SD Inter, c = SS Rate, d = Duration and e = Pulse) had on the production of recombinant protein in *Escherichia coli*. These effects are visualized on a Pareto Chart where any effects that extend beyond the reference line are significant (α = 0.05). Only bc and ae were statistically significant.

A



B

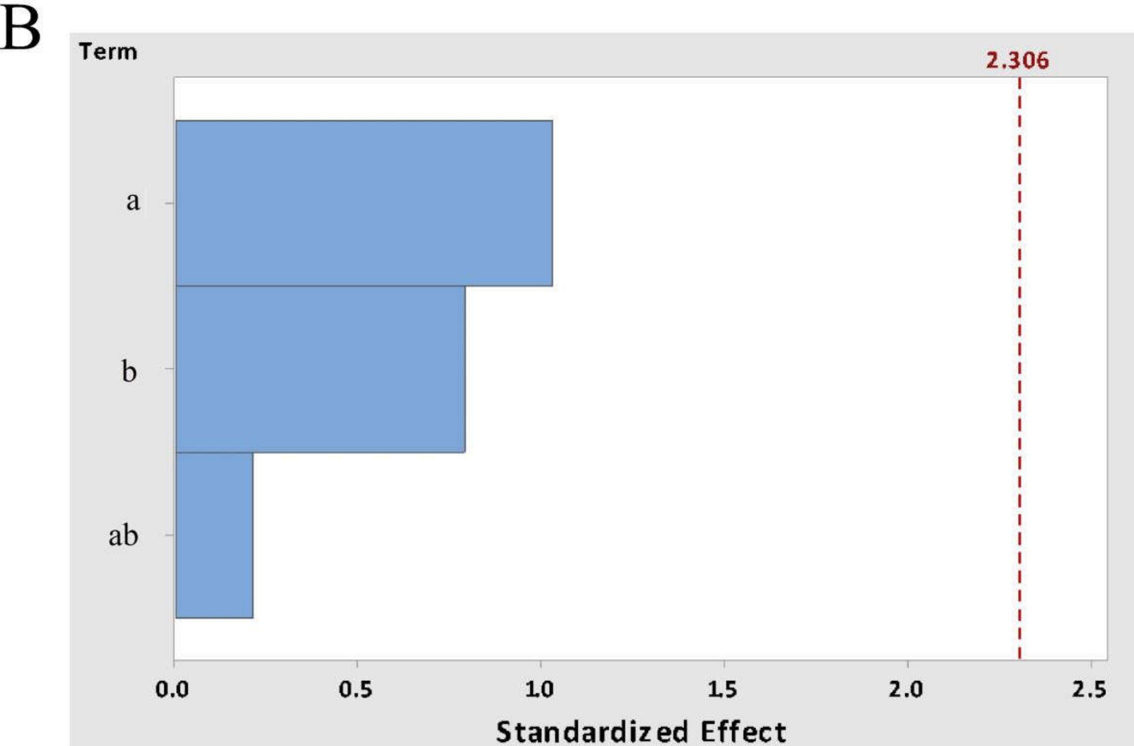
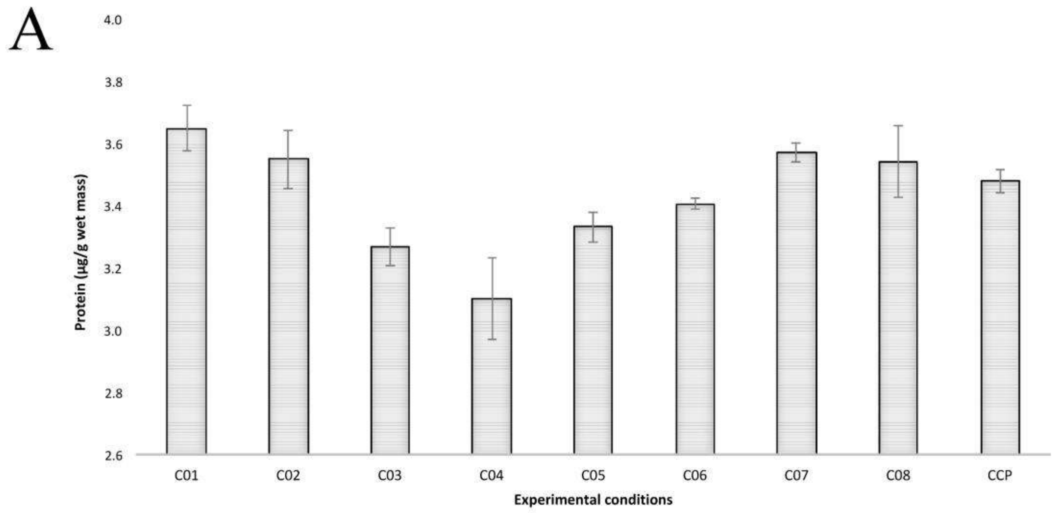


Figure 0.6. (A) Luminescent protein production yield after 24 h of growth using the sound conditions described in **Table 3.2. (Group B)**. (B) Absolute values of the standardized effects that sound elements (a = Frequency, b = Pulse) had on the production of recombinant protein in *Escherichia coli*. These effects are visualized on a Pareto Chart where any effects that extend beyond the reference line are significant (α = 0.05). No sound element was statistically significant.

A



B

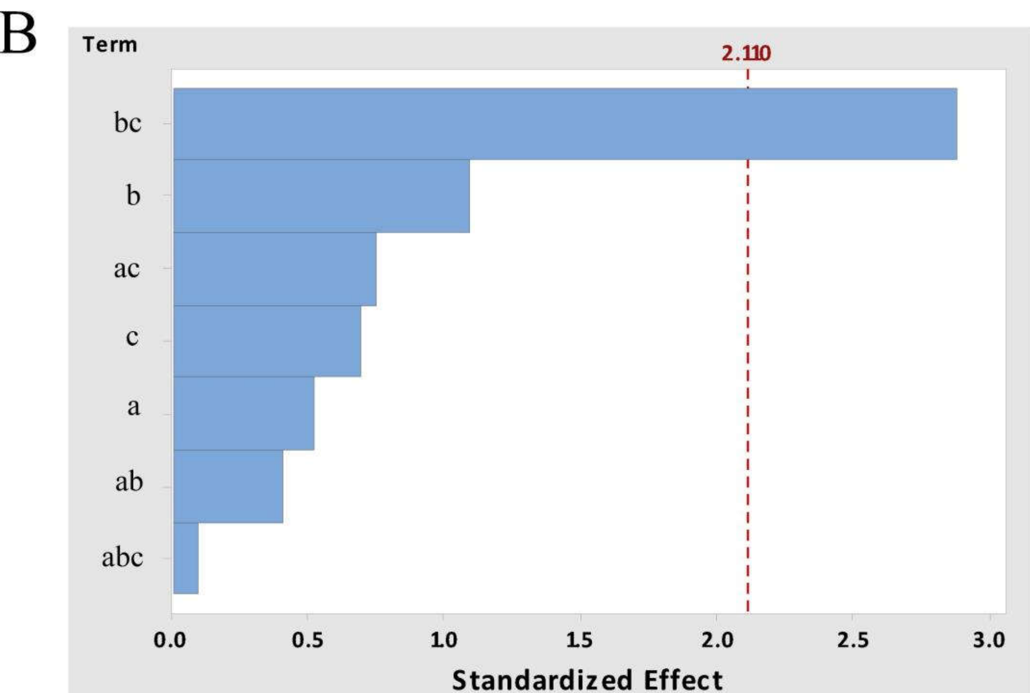


Figure 4.7. (A) Luminescent protein production yield after 24 h of growth using the sound conditions described in **Table 3.2. (Group C).** (B) Absolute values of the standardized effects that sound elements (a = Duration, b = SD Inter, c = SS Rate) had on the production of recombinant protein in *Escherichia coli*. These effects are visualized on a Pareto Chart where any effects that extend beyond the reference line are significant ($\alpha = 0.05$). Only the parts that conform to intermittence (SD Inter and SS Rate) were statistically significant when being together.

4.3. Gene expression analysis

Not many transcriptome analyses have been made regarding sound stimulation, mostly focused on plants, and among all these publications there is only one report in bacteria, for *Chromobacterium violaceum*.[52] For this reason, the gene candidate selection was based on the results from such publication. Its whole transcriptome analysis revealed that a total of 342 genes were significantly up-regulated in the sonic stimulated culture.

Although the authors focused their analysis on genes related to quorum sensing and transport, which had the greatest number of upregulated genes, it was decided to analyze only those families of genes that, in addition to containing more than 4 up-regulated genes, were found on the membrane with the hypothesis that sound sensors, if they exist, could be located there. The genes that met the criteria were the stress response genes, kinases and genes related to movement. In addition, within these groups, the genes that were closest to the start of their respective signaling pathway were chosen. Thus, the following three homologous genes present in *E. coli* were selected:

- Transcriptional regulatory protein (***CpxR***) involved in stress response.
- Signal transduction histidine-protein kinase (***BarA***) involved in carbon storage regulation.
- Chemotaxis protein (***CheA***) involved in transmission of sensory signals from the chemoreceptors to the flagellar motors.

4.3.1. Primer validation

Since amplicons' length is directly related to the QPCR amplification efficiency,[77] primers were designed to generate amplicons that were ~300 bp and that, additionally, these amplicons could be used as a template for a second pair of primers that generates another amplicon of ~100 bp to increase the experimental specificity. Each amplified fragment was run on an agarose gel electrophoresis (**Figure 4.8.**). As expected, each primer generated the predicted amplicon size, which allows to continue the gene expression analysis with certainty.

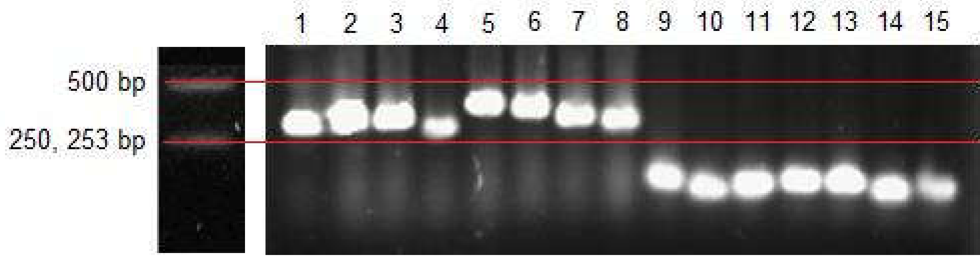


Figure 0.8. Amplified fragments using the total genomic DNA of *Escherichia coli* and the primers listed in **Table 3.3**. The content on each lane is: (1) 1 kb DNA Ladder (Promega) plus *cysG* 322 bp, (2) *cysG* 322 bp, (3) *hcaT* 325 bp, (4) *IdnT* 278 bp, (5) *ssrA* 355 bp, (6) *BarA* 342 bp, (7) *CheA* 315 bp, (8) *CpxR* 293 bp, (9) *cysG* 105 bp, (10) *hcaT* 85 bp, (11) *IdnT* 90 bp, (12) *ssrA* 107 bp, (13) *BarA* 110 bp, (14) *CheA* 93 bp, (15) *CpxR* 105 bp.

4.3.2. Comparative C_t ($2^{-\Delta\Delta Ct}$)

To declare that a gene is being up-regulated, it is necessary to quantify the expression of this gene in the presence of the treatment and compare it with the same gene but in the absence of the treatment. The total amount of RNA produced for a specific gene is proportional to its expression. Therefore, to measure the gene expression it is fundamental to isolate and quantify RNA from the gene target.

To measure the relative genetic expression using quantitative amplification through QPCR, total RNA was isolated from each sample of *E. coli* after 8 hours of growth. The obtained purification values were 260/280 = ~2.15 and 260/230 = ~1.91 which gives positive assurance that the purification was made properly.

Afterwards, cDNA fragments were synthetized using RNA as template and the primers that amplified fragments of ~300 bp. Since the QPCR requires the samples to be at maximum 5 ng/µL and these fragments had a final concentration of ~1500 ng/µL, a 317:1 dilution was done to every sample. Finally, the QPCR was performed with no complications, the C_t values were obtained and the normalized gene expression (ΔCt), relative expression change between treated and untreated ($\Delta\Delta$Ct) and the expression fold ($2^{-\Delta\Delta Ct}$) were calculated. The values for ΔCt values are graphed on **Figure 4.9.**

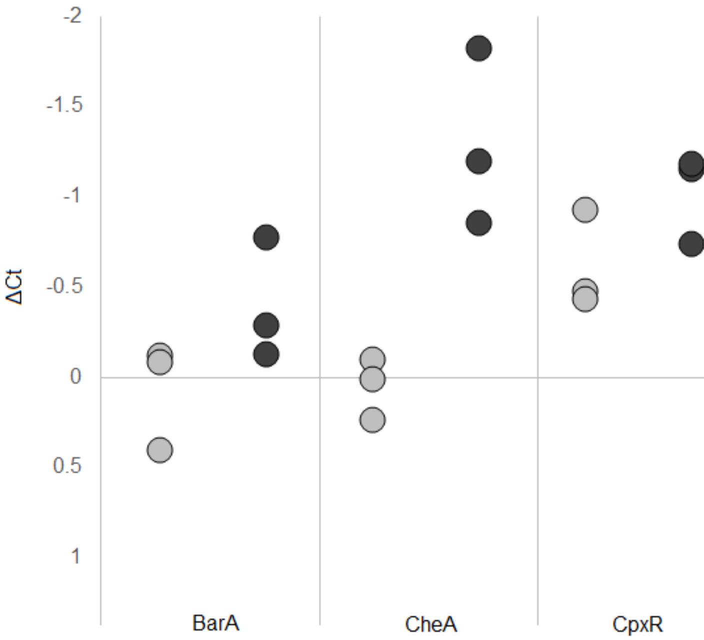


Figure 0.9. Values of the normalized gene expression per treatment (ΔCt). Gray dots represent untreated samples and black dots represents treated samples. Negative values represent upregulating genes and positive values represent downregulating genes. All treated genes appear to be upregulated but only *CheA* was significantly different ($p < 0.05$).

The results show that *BarA* was upregulated 1.38 times ($p = 0.13$), *CheA* was upregulated 2.66 times ($p < 0.05$) and *CpxR* was upregulated 1.33 times ($p = 0.15$), however, only *CheA* was found to be statistically significant.

CheA is directly involved in chemotaxis,[78] which is defined as a behavior involving organisms sensing attractants or repellents and leading towards or away from them. It functions as a histidine kinase with autophosphorylation activity, which receives the transmission of sensory signals from the methyl-accepting chemotaxis proteins (MCPs) and redirects them to the flagellar motors, activated by *CheY*. There are three types of molecules that activate specific MCPs:

- Amino acids: serine through Tsr receptor, aspartate through Tar receptor and dipeptides through Tap receptor.
- Sugars: maltose, D-ribose and D-galactose through Trg receptor.
- Air: Oxygen through Aer receptor.

The expression fold for *CheA* described in this work had almost the same value reported for *CheY* (2.54 fold),[52] which was not the case with the other analog genes (1.9 fold), suggesting that the activity of this gene due to sound stimulus could be conserved among prokaryotes, although this should be still demonstrated by studying more species.

The activity of *CheA*, when overexpressed, would increase the activity of the flagellar motor through *CheY* activation causing the bacteria to move faster towards chemoattracants. This phenomenon, together with the faster nutrient uptake in sound-stimulated cultures,[12] would encourage a faster metabolism. Considering that most of the chemoattracants are amino acids and sugars, the activation of chemotaxis could explain the increment of protein synthesis due to sound stimuli as observed in previous studies,[5,6,11] and in this research.

Moreover, chemotaxis activation could explain the variability found in the frequency treatments. In natural environments, where resources are limited, being able to move quick towards resources could be counterproductive because bacteria could use more energy than it gets. On the other hand, in a controlled environment, bacteria are normally grown in rich media in which moving faster would allow any of them to find resources anywhere. Because chemotaxis activation through sound stimuli increases the sugar uptake in all bacteria, it also increases the competition for resources among them. Thus, those bacteria that move quicker to where the resources are located have better chances to survive and grow more.

Finally, this result also suggests the possible existence of MCPs that responds to sound specifically. However, with the information presented in this work, it is not possible to confirm this because *CheA* is not only regulated by MCPs but also by an activator (*CheR*) and a repressor (*CheB*). The complexity of the route makes it impossible to know if *CheA* up-regulation was due to itself, the up-regulation of *CheR*, the down-regulation of *CheB* or all the above. Therefore, it is necessary to perform an experiment where all the genes involved in chemotaxis are present and if possible include more to consider the internal noise associated with gene expression that could also be affected by sound. Another option would be to put *CheA* in a heterologous system without *CheR* or *CheB* to eliminate their effects.

4.4. Sound-bioreactor system

As part of the present research work, to further validate the proof of concept of the sound induction device (already validated at flask scale), an attempt was made to adapt a sound induction device into a lab scale bioreactor. This is particularly important since the geometry and size of the vessel can have a significant impact on the propagation of the sound wave. Usually, when a bioreactor design is made at lab scale, it is possible to scale it up by maintaining either the proportions of the original shape or keeping constant some important properties such as temperature, composition and medium flow rate. However, in the case of sound design, modifying the size, shape or materials of the vessel can completely change the way sound is reflected inside of it which can change the frequency response. This change is due to the frequencies phase shift where those in phase will gain more amplitude and those out of phase will gain less.[40]

Adapting a speaker into the bioreactor has several limitations. If a speaker is intended to be introduced inside a bioreactor, the required amplitude for induction could not be reached because the amplifier box cannot exceed the bioreactor's volume. If the speaker is intended to be placed outside through a small opening, as with the flask's case, it could create either monopole resonance below the orifice opening or fluctuating resonant zones if the speaker is placed in azimuthal.[79] Hence, the speaker must be able to adapt into a small hole and it must have forcefully omnidirectional properties.

A modified point source speaker proposed by Ibarra *et al*,[69] was used in the present work. It has the capacity of producing an omnidirectional sound from a small opening 20 cm away from the sound source. To detect how an omnidirectional sound travels on a commercially available bioreactor, measurements in different parts of the bioreactor were performed and the frequency response of every state is showed in **Figures 4.10. & 4.11.** for the 1L bioreactor and in **Figures 4.12. & 4.13.** for the 2L bioreactor.

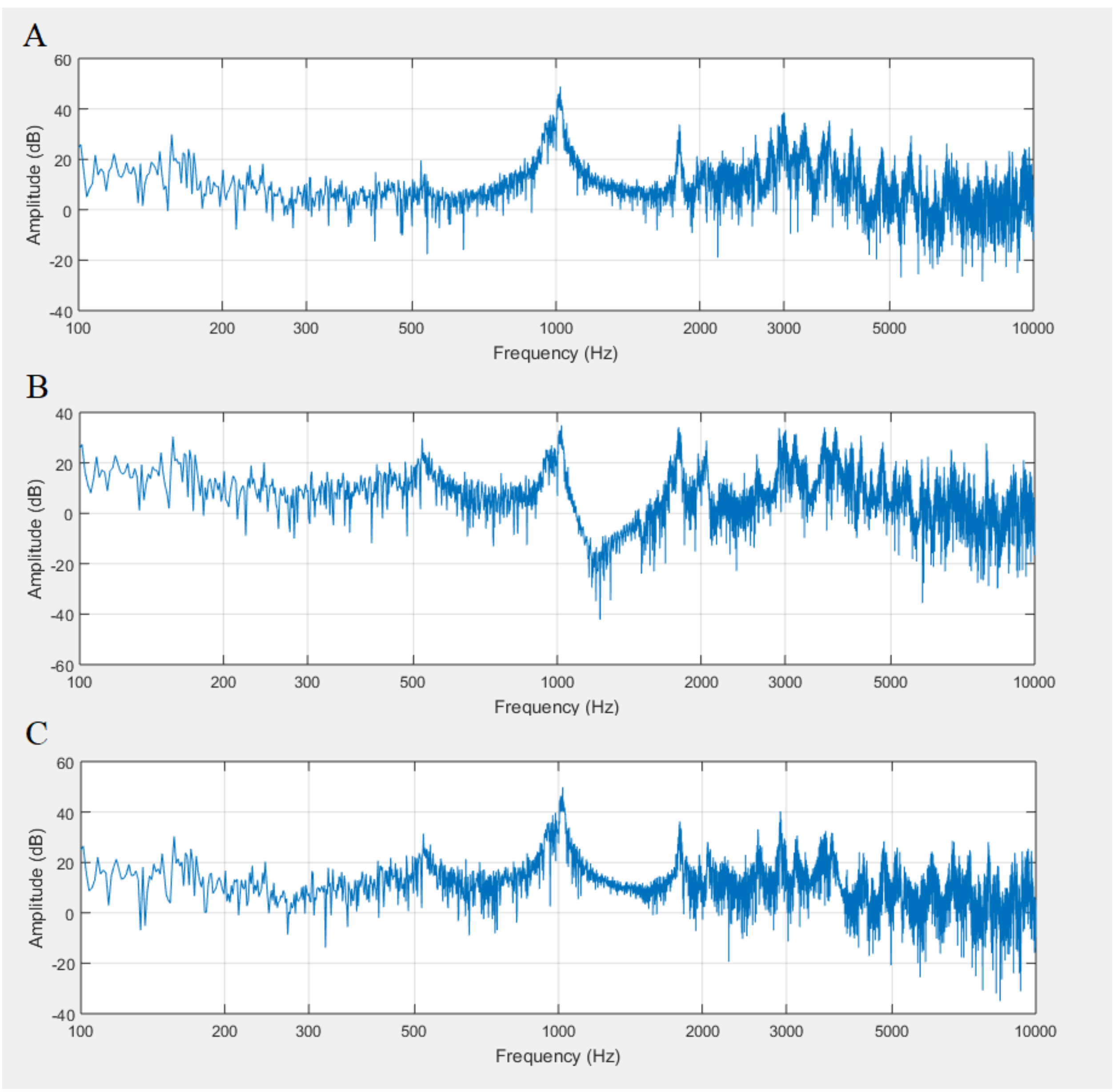


Figure 4.10. Frequency response for the 1L empty bioreactor measured on the top (A), midpoint (B) and bottom (C).

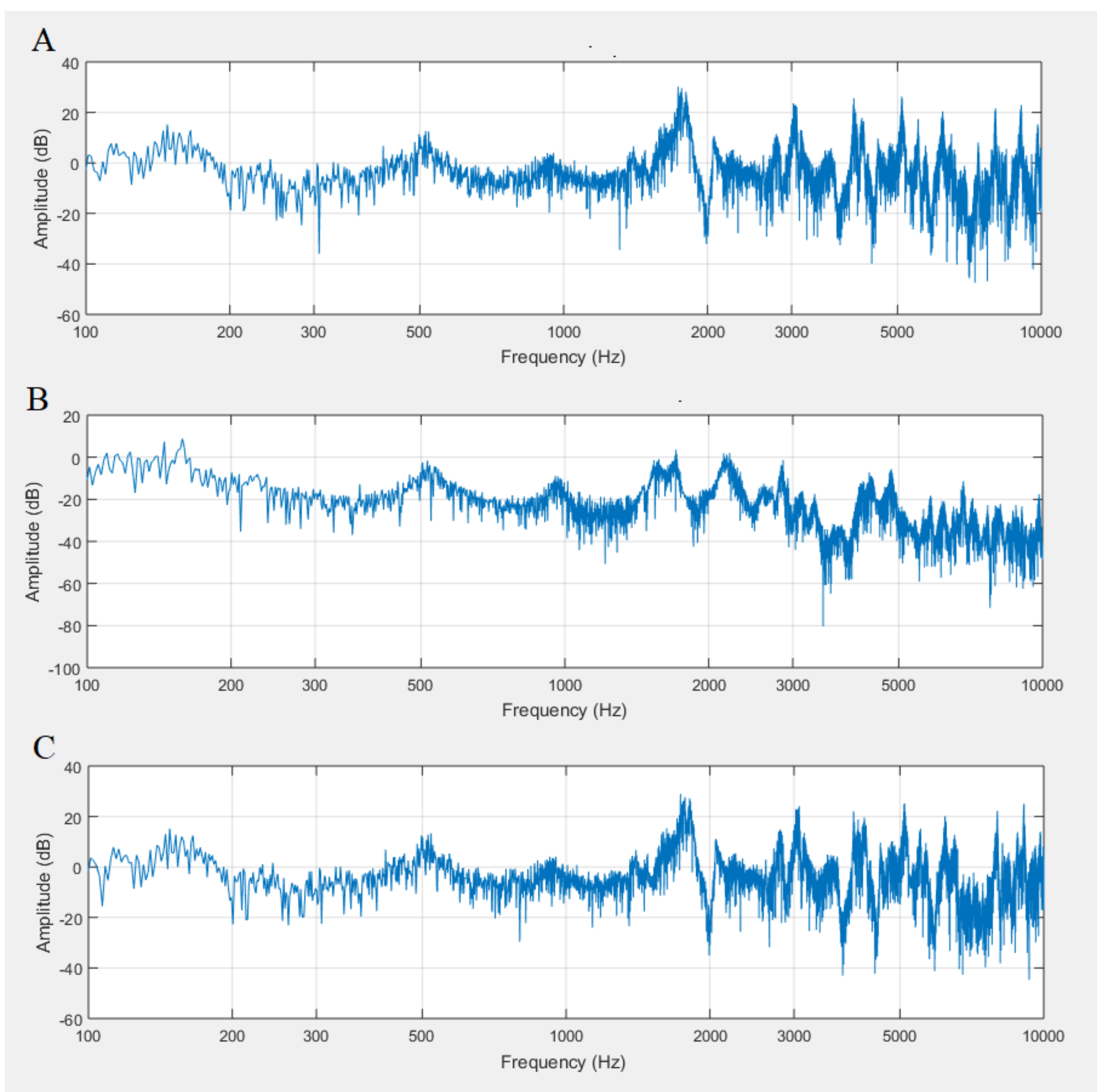


Figure 0.11. Frequency response for the 1L bioreactor with half volumetric capacity measured on the top (A), in the water's surface (B), and top with stirring (C).

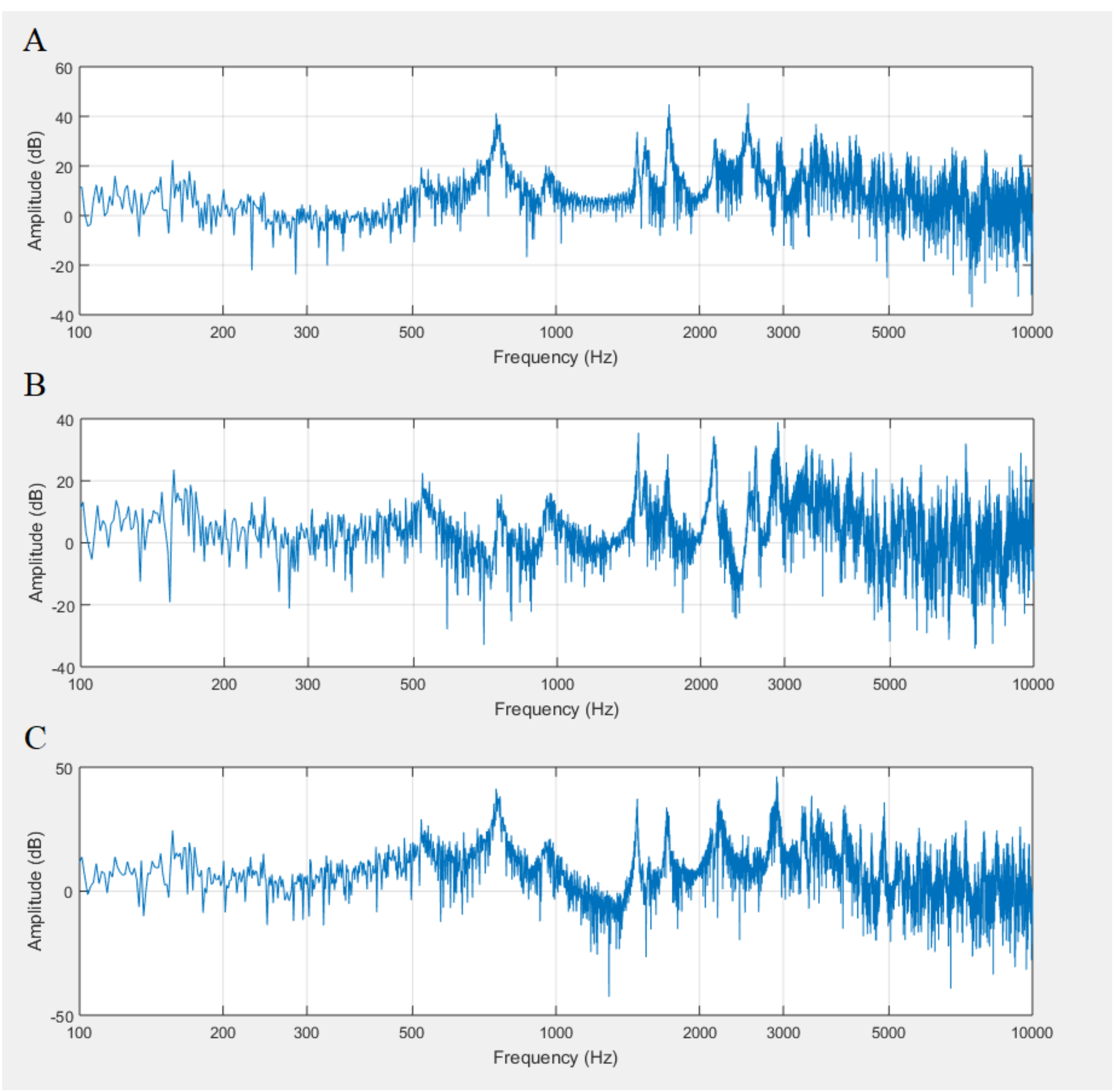


Figure 4.12. Frequency response for the 2L empty bioreactor measured on the top (A), midpoint (B) and bottom (C).

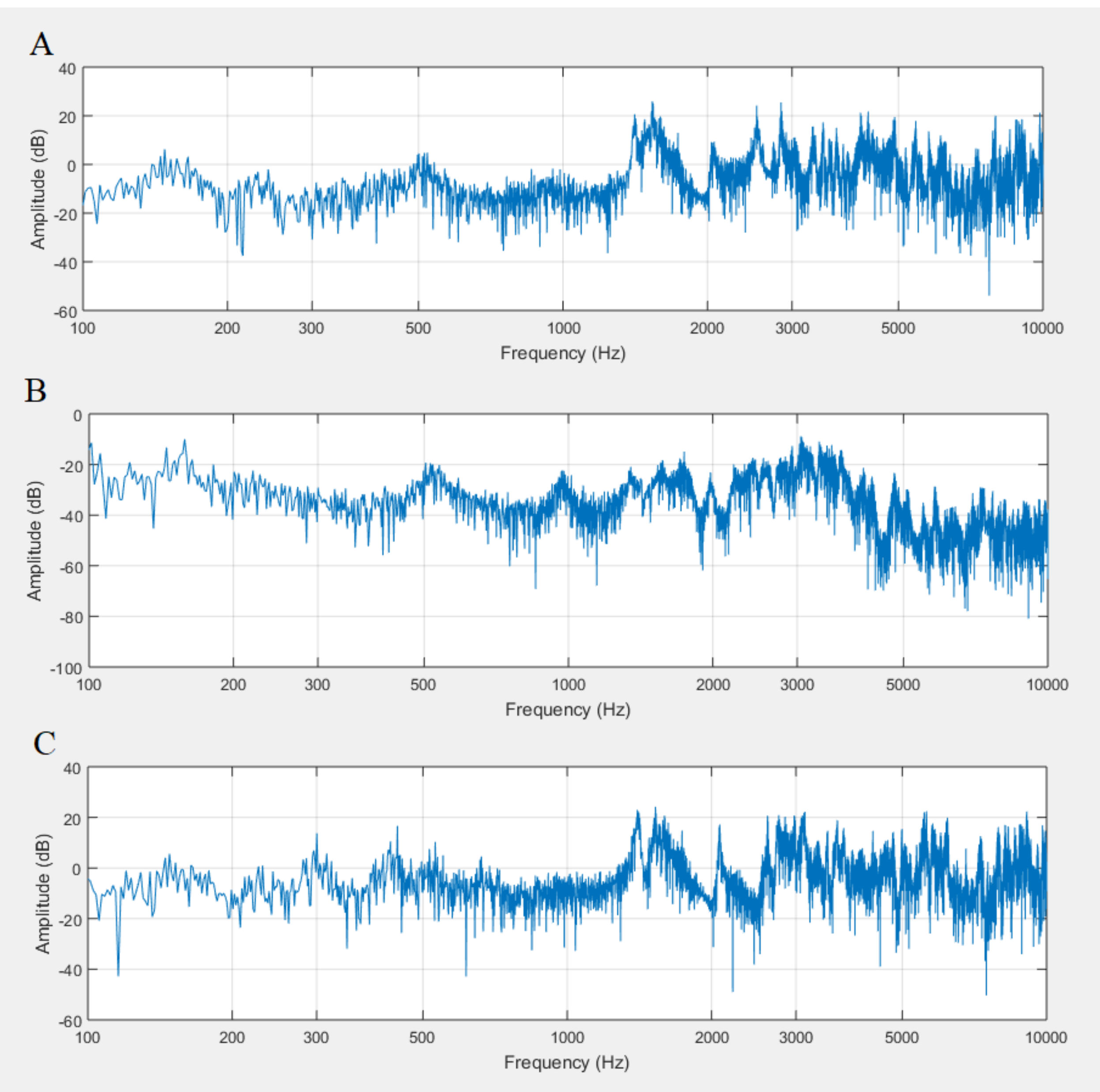


Figure 0.13. Frequency response for the 2L bioreactor with half volumetric capacity measured on the top (A), in the water's surface (B), and top with stirring (C).

The most notorious feature on the graphs is the resonant frequency, f_{res}, which is 1000 Hz in 1L empty bioreactor and 750 Hz in 2L empty bioreactor. Moreover, when the bioreactor is filled with half capacity, the resonant frequency shifts to 1800 Hz in the 1L bioreactor and to 1400 Hz in the 2L bioreactor. This agrees with the Helmholtz resonance formula, which is proportional to the speed of sound, c, and to the square root of the cross-sectional area, S_p, divided by the product of the resonator chamber volume, V_c, and the length, l_p:

$$f_{res} = \frac{c}{2\pi}\sqrt{\frac{S_p}{V_c l_p}} \quad (3)$$

Even though it was not possible to measure the sound inside the liquid because a microphone that could resist water was not available, the measurements that were made can give insights into how the sound reacts in the liquid. In the first place, frequency does not change when changing medium, only the wavelength does.[40] Furthermore, when an omnidirectional sound is reflected inside a symmetric form, it is expected to have a similar frequency response in the symmetric extremes. This phenomenon is confirmed because the top and the bottom graphs in the empty bioreactors are almost identical (**Figure 4.10.A & C, Figure 4.12.A & C**).

Another detected phenomenon was that certain frequencies intensify in certain zones of the bioreactor, but those same frequencies disappear in other zones, especially in the midpoint where most frequencies were attenuated. For instance, in the top measurements, the frequencies that appear are 1200 Hz in the empty 1L bioreactor (**Figure 4.10.A**), 4000 Hz in the half-full 1L bioreactor (**Figure 4.11.A**), 750 Hz in the empty 2L bioreactor (**Figure 4.12.A**) and 4500 Hz in the half-full 2L bioreactor (**Figure 4.13.A**), but in the midpoint those frequencies drastically drop (**Figures 4.10.B, 4.11.B, 4.12.B & 4.13.B**).

Finally, when comparing the bioreactor when in rest mode (**Figure 4.11.A & Figure 4.13.A**) with the bioreactor when stirring (**Figure 4.11.C & Figure 4.13.C**), there is no drastic change in the frequency response. The only noticeable difference is a slight amplitude increment in 300 Hz, 450 Hz and 1200 Hz in both bioreactors. Since this happens in both bioreactors, it is possible that it these frequencies are new resonance frequencies that appeared due to the rotation of the impeller.[80]

The reason for measuring the frequency response was to find a frequency that reaches all bacteria cells in every point of the bioreactor. Thus, frequencies that increase or decrease amplitude in certain zones must be avoided because, as described in this dissertation, increasing amplitude can extend the exponential phase. Hypothetically any frequency could be used in a bioreactor since, as demonstrated in this project, frequency had no significant effect in *E. coli*. However, frequency has restrictions in bioreactors because of their scattered frequency response depending on the bioreactor’s zone.

For this reason, the most constant frequency in most parts of the bioreactor must be selected. Therefore, for the 1L bioreactor 900 Hz is recommended, followed by 500 Hz and 2200 Hz. For the 2L bioreactor 500 Hz is recommended, followed by 1000 Hz.

As presented in this dissertation document, the importance of sound as an agent that influences biological changes in *E. coli* was established. This was done through the identification of biological effects of individual sound elements, in combination, and the finding of a gene that can be up-regulated using sound. These discoveries give rise to the generation of knowledge about sound and the possibility of applying it to new biotechnological applications.

Chapter 5

5. Conclusions and future studies

In this study, the effects of sound wave parameters on *E. coli* biomass concentration, viability and recombinant protein production yield were characterized. This is the first time the effect of more than one sound element over biological responses has been described. Although other studies have used sound elements to demonstrate the relationship between sound and certain biological effects, the lack of characterization of more than one sound element in their treatments has led to a limited understanding of such correlation. For the first time, it was possible to detect a gene in *E. coli* susceptible to sound stimulus. Finally, the study of sound distribution inside a bioreactor validated the feasibility of adapting speakers into commercially available bioreactors to allow industry to take advantage of the benefits of sound.

It was found that amplitude could reduce by half the duration of the exponential phase possibly by surpassing an oxidative response threshold. Frequency, as the most studied sound element, had no significant effect but it had a random response that has led to the confusion of attributing a biological effect to it; nevertheless, it cannot be completely disregarded since it is an inherent part of sound. Neither duration nor pulse had biological effects, but their presence provided insights of the importance of silence especially in protein production. Moreover, intermittence can significantly produce 1.5 times more recombinant protein per cell without the contribution of any other sound element. Also, intermittence can up-regulate 2.66 times a gene involved in chemotaxis (*CheA*) which allows bacteria to move sense and move faster towards vital resources. Finally, it was determined that speakers can be adapted into 1L and 2L bioreactors and specific frequencies, such as 500 Hz, are suitable for induction in both bioreactors.

The knowledge generated in this research work represents a first step in the structured study of the effect of sound and its elements on *E. coli*. Although it is feasible that similar results could be obtained with similar expression system, the characterization of the effect of sound in other microorganisms would have to be carried out case by case. It is evident by the few published reports that there is a long way to go in this research line and, only a glimpse of its effects has been described. Notwithstanding, based on the results obtained, there are clear applications for this technology in bioprocesses and medicine once it is well characterized.

5.1. Proposed future studies

Intermittence is one of the most interesting sound elements described in this research document. Although used in other studies, this was the first time that it was clearly defined. The accurate characterization of sound made possible to identify that intermittence was the only sound element that increased the protein production yield, but this result arises a new question: *why only intermittence worked?*

Based on intermittence findings, it could be hypothesized that intermittence's effects are possibly due to its cyclic properties rather than its sound quality. This statement implies that bacteria are not only capable of detecting sound, but they could accurately respond to different rhythmic patterns. In other words, living organisms could use and interpret intermittence as a code, like Morse code, which allows another form of communication between species.

Experiments in this area would be focused on optimization of intermittence intrinsic conditions – SD Inter, SS Rate, beginning of stimuli – to fully understand its essence. These conditions would also be used in other biotechnological unstudied phenomena such as the effect on soluble and insoluble proteins in different expression systems.

Finally, another category would be the study of overlapped stimuli effects. On one hand, a study focused on testing different waveforms presented as intermittent white noise in comparison to intermittent single-frequencies. On the other hand, a study of "polyrhythmic harmonics" where two or more intermittent sounds are played at the same time to verify if their combination could enhance even more the protein production yield.

From where the genetic expression study was left, as previously mentioned, it is necessary to perform an experiment where all the genes involved in chemotaxis are present to really find the gene or genes involved in sensing sound directly, and not consequently which was the case of *CheY*. To do so, transcriptomic analysis must be performed.

Being able to identify the genetic regions affected by sound would not only help to elucidate its biological importance but would also allow the development of gene expression technology controlled by sound as an alternative to chemical inductors, such as IPTG.

Experiments on this area also include the insertion of the *CheA* promoter next to a reporter gen to verify the feasibility of using this machinery as a sound-inducible promoter. Additionally, this promoter could be modified through mutagenesis to screen for stronger promoters. Finally, this kind of sound-inducible promoter would be useful in experimentation with other genera/species, either by adapting the *CheA* promoter according to the species genetic requirements or by locating new ones exclusive to the species.

5.2. Future studies in bioacoustics technology

The experimental design presented on this thesis will serve as platform for future studies of sound not only for organisms that can grow in flask, but also for other organisms. Although the aim of this study was to find a way to implement a speaker in a commercially available cylindrical bioreactor, it is possible that new designs will have to be considered, especially because more than one kind of bioreactor exists (bubble column, airlift, photo-bioreactors, etc.).

Firstly, continuing from where study was terminated, further investigation should be undertaken to determine a way in which the speaker may produce sound inside the bioreactor through an open access without risk of medium contamination. For instance, it could be that an autoclavable membrane, which allows only the passage of sound and that resists high pressures and temperatures, might be used. For this research, nanomaterials expertise would be recommended.

Secondly, the development of submersible speakers should be considered, especially for photo-bioreactors where the medium travels through narrow tubes. For this reason, special modules must be designed to be autoclaved and wireless. Also, in addition to sound distribution measurements, fluid dynamics studies will be necessary.

The accurate characterization of sound dynamics inside bioreactors and the demonstration that sound influences the protein production yield would make this technology appealing to the bioprocessing industry. Then, the ultimate goal would be to find in any bioreactor a section where a special module for speakers can be placed, just as is the case with the pH, aeration or temperature modules.

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Annexes

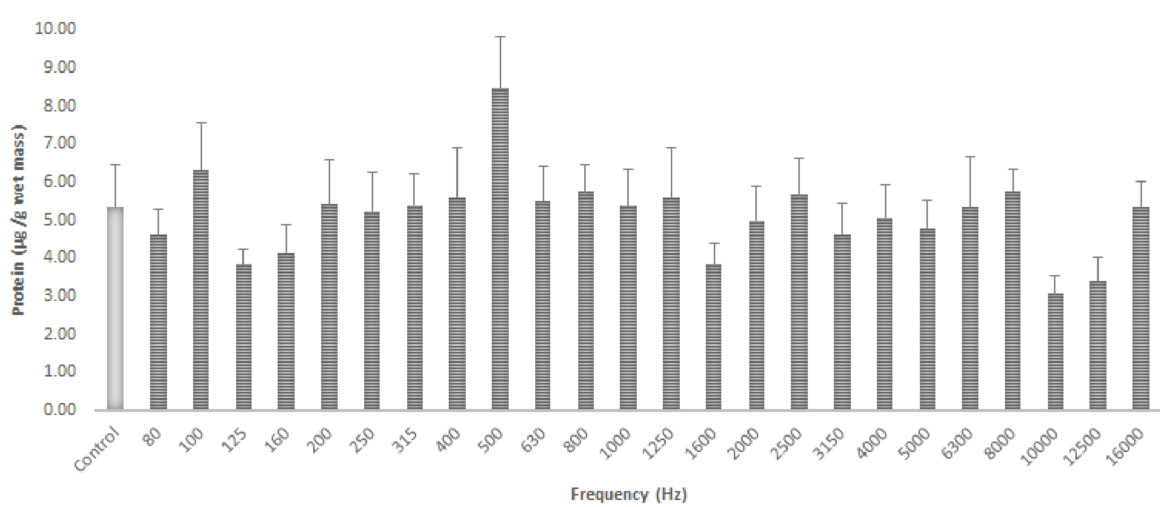

Figure 0.1. Luminescent protein production yield after 24 h of growth in the presence of specific frequency treatments in terms of 1/3-octave bands. No frequency treatment is statistically significant. Deviation bars represent standard error.

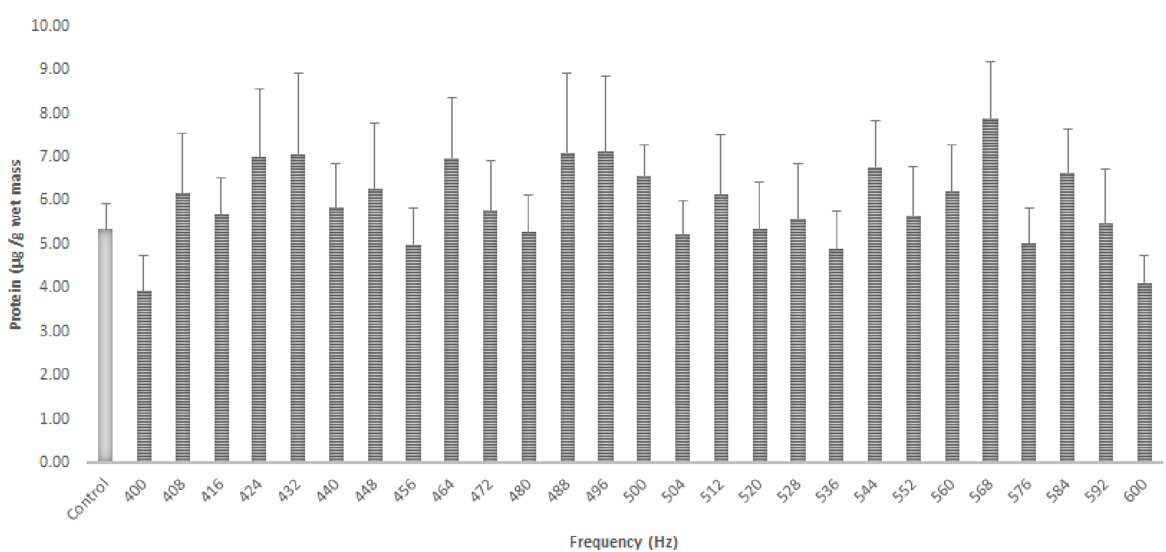

Figure 0.2. Luminescent protein production yield after 24 h of growth in the presence of specific frequency treatments between 400 Hz and 600 Hz using an 8 Hz bandwidth. No frequency treatment is statistically significant. Deviation bars represent standard error.

Vitae

Edgar Acuña González was born in Puebla, México, on October 4th, 1988. He is the second son of Arch. Enrique Acuña Jiménez and M.S. Rosalva Patricia González Fernández; his older brother is Arch. Enrique Acuña González and his younger brother is Rodrigo Acuña González.

He lived and studied in his natal city until the year 2008 when he arrived to Monterrey, Nuevo León, México to study the undergraduate program of Biotechnology Engineering degree at Tecnológico de Monterrey. He earned his major degree in Biotechnology Engineering and his minor degree in Music in May 2013. He exercised his career as a research assistant for Centro de Biotecnología FEMSA for a year.

He was accepted in the PhD program in Biotechnology in August 2014 at Tecnológico de Monterrey and graduated from it in December 2018. During his studies, he worked as a research scholar at the University of Houson from March to August 2018, he reached level 40 in PoGo and recorded his first progressive album “Tsunami: Love”. This work represents the conclusion of his doctorate program.

This document was typed in using Microsoft Word by Edgar Acuña González