

Comparison between effects of manothermosonication and isolated ultrasonic techniques on microbial inactivation of a hospital laundry effluent

Comparaç o entre os efeitos da manotermossonicaç o e das t cnicas ultrass nicas isoladas na inativaç o microbiana de um efluente de lavanderia hospitalar

Comparaci n entre los efectos de la manotermosonicaci n y las t cnicas ultras nicas aisladas en la inactivaci n microbiana de un efluente de lavander a hospitalaria

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Abstract

Microbial inactivation using only Ultrasound (US) vibrations presents considerable advantages by applying green techniques with non-generation of greenhouse gases, eliminating chemicals, and operating conditions close to environmental conditions. However, industrial effluent treatment systems have been investigated as a microbial inactivation step, using isolated US associated with thermal (T) and manometric (P) microbial inactivation techniques.

A stainless-steel bench prototype was built for the present study, operating in continuous mode, with a 2.5 L volume test chamber. The indicator microorganism detected in the effluent of a hospital laundry located in the Agreste region of the state of Pernambuco, Brazil, was the *E. coli* bacterium. In addition to the isolated US effect, US/T, US/P, and US/T/P combinations were obtained using a central composite design (CCD). The factors used for the CCD were frequency, hydraulic detention time, temperature, and pressure. The results showed that the lethality rate increased with the frequency and time of hydraulic detention but was reduced when increasing temperature from 30°C to 50°C and increasing pressure between 1.0 bar and 1.8 bar. Isolated ultrasonic vibrations, at 120 kHz and with 10-minute HRT, reached an inactivation efficiency of 98%. Such a value was found using the thermomannosonic condition around 40 min. The use of the isolated US vibration technique proved advantageous, mainly due to the efficient inactivation results and potential energy and chemical reductions.

Keywords: Ultrasonic vibrations; Hospital laundry; Microbial inactivation; RCCD; Green technology.

Resumo

A inativação microbiana utilizando apenas vibrações de Ultrassom (US) apresenta vantagens consideráveis ao aplicar técnicas verdes com não geração de gases de efeito estufa, eliminação de produtos químicos e condições de operação próximas às condições ambientais. No entanto, sistemas de tratamento de efluentes industriais têm sido investigados como uma etapa de inativação microbiana, utilizando US isolados associados a técnicas de inativação microbiana térmica (T) e manométrica (P). Um protótipo de bancada de aço inoxidável foi construído para o presente estudo, operando em modo contínuo, com uma câmara de teste de 2,5 L de volume. O microrganismo indicador detectado no efluente de uma lavanderia hospitalar localizada no Agreste do estado de Pernambuco, Brasil, foi a bactéria *E. coli*. Além do efeito de US isolado, combinações de US/T, US/P e US/T/P foram obtidas usando um delineamento composto central (DCC). Os fatores utilizados para o DCC foram frequência, tempo de detenção hidráulica, temperatura e pressão. Os resultados mostraram que a taxa de letalidade aumentou com a frequência e o tempo de detenção hidráulica, mas foi reduzida ao aumentar a temperatura de 30°C para 50°C e aumentar a pressão entre 1,0 bar e 1,8 bar. Vibrações ultrassônicas isoladas, a 120 kHz e com TRH de 10 minutos, atingiram uma eficiência de inativação de 98%. Tal valor foi encontrado usando a condição termomanosônica em torno de 40 min. A utilização da técnica de vibração US isolada mostrou-se vantajosa, principalmente devido aos resultados de inativação eficientes e potenciais reduções energéticas e químicas.

Palavras-chave: Vibrações ultrassônicas; Lavanderia hospitalar; Inativação microbiana; DCCR; Tecnologia verde.

Resumen

La inactivación microbiana usando solo vibraciones de Ultrasonido (US) presenta ventajas considerables al aplicar técnicas verdes con no generación de gases de efecto invernadero, eliminación de químicos y condiciones de operación cercanas a las ambientales. Sin embargo, los sistemas de tratamiento de efluentes industriales han sido investigados como un paso de inactivación microbiana, usando US aislados asociados con técnicas de inactivación microbiana térmica (T) y manométrica (P). Para el presente estudio se construyó un prototipo de banco de acero inoxidable, operando en modo continuo, con cámara de ensayo de 2,5 L de volumen. El microorganismo indicador detectado en el efluente de una lavandería hospitalaria ubicada en la región de Agreste, estado de Pernambuco, Brasil, fue la bacteria *E. coli*. Además del efecto US aislado, se obtuvieron combinaciones US/T, US/P y US/T/P utilizando un diseño compuesto central (DCC). Los factores utilizados para el DCC fueron frecuencia, tiempo de detención hidráulica, temperatura y presión. Los resultados mostraron que la tasa de letalidad aumentó con la frecuencia y el tiempo de detención hidráulica pero se redujo al aumentar la temperatura de 30°C a 50°C y aumentar la presión entre 1,0 bar y 1,8 bar. Las vibraciones ultrasónicas aisladas, a 120 kHz y con TRH de 10 minutos, alcanzaron una eficiencia de inactivación del 98%. Tal valor se encontró usando la condición termomanosónica alrededor de 40 min. El uso de la técnica de vibración aislada de EE. UU. resultó ventajoso, principalmente debido a los resultados de inactivación eficientes y las reducciones químicas y de energía potencial.

Palabras clave: Vibraciones ultrasónicas; Lavandería hospitalaria; Inactivación microbiana; DCCR; Tecnología verde.

1. Introduction

Using ultrasound waves (US) in microbial inactivation is presently becoming one of the unconventional alternatives. The use of US to produce lethal effects on microorganisms is attractive because it is considered a "green" technology, as it involves sound energy, can operate at lower levels of pressure and temperature, and requires the addition of very small amounts of chemical products, or none at all (Chavan et al., 2022). This technique has assumed a prominent role in physical treatments, mainly due to its ease of operation, flexibility, and ability to vary the necessary intensities of cavitation conditions (Hawrylik, 2019). Also, according to this author, US application has one of its main advantages in dispensing chemical

products. These substances promote a high lethality rate of microorganisms but can harm the ecosystems' health.

Ultrasound can propagate in any medium with elastic properties (solid, liquid, and gas). The molecule's vibrations are transmitted to neighboring molecules throughout the medium before each of these molecules returns to its initial position. In solids, particles also move perpendicularly, giving rise to waves that propagate transversely. In liquids and gases, such oscillations propagate with greater intensity in the wave direction, producing longitudinal waves (Shabir et al., 2021).

When US vibrations propagate through a liquid medium, they generate regions of compression and rarefaction. When the pressure during rarefaction is high enough, cavitation (or voids) is formed in the liquid (Li et al., 2021). There is a difference between stable cavitation, characterized by the resistance of the voids over more cycles, and transient cavitation, characterized by the growth of the microbubble until it collapses, the consequence of which is fragmentation and disintegration into a mass of microbubbles. Cavitation results from the pressure difference when sound waves are applied, causing the formation and collapse of microbubbles in aqueous solutions that drive shear forces. The waves caused by these micro-implosions generate local turbulence and release a large amount of energy, increasing the temperature and pressure inside the microbubbles. It is postulated that acoustic cavitation is responsible for weakening, or even breaking, microorganisms' cell walls, leading them to cell death (Silva, 2020).

Microbial inactivation can also be performed by combining ultrasound with other physical techniques, such as US and heat (thermosonication), US and treatments under pressure above the atmosphere (mannosonication), and a combination of the three technologies (manothermosonication) (Onyeaka et al., 2021). However, the authors above state that factors such as frequency, temperature, and pressure can interfere negatively, both because of the intensity of cavitation and undesirable effects on the manufacturing materials of sonication reactors.

Ionic liquids have low volatility, and due to this condition, cavitation in these media depends on the presence of dissolved gases. Naidji et al. (2019) developed a specific sonoelectrochemical cell to investigate the effect of pressure on ultrasonic cavitation. The results showed that the cavitation activity decreased considerably from atmospheric pressure to about 26 kPa above atmospheric pressure. The application of pressures below atmospheric in ionic liquids contributes to removing gases dissolved in the medium. Thus, acoustic cavitation activity can be controlled using efficient pressure control over the atmosphere of an ultrasonic reactor.

Recent investigations have revealed important information about the effects of temperature on the intensity of acoustic cavitation. Yu and Liu (2020) showed that water, under atmospheric conditions, favors high cavitation activity at temperatures below 20°C. However, upon heating, between 20 and 40°C, a new peak of activity can be identified at temperatures close to 30°C, depending on the water column's height above the piezoelectric transducer's surface and the content of gases dissolved in the liquid.

Effects of pressure and temperature of a fluid in the form of ultra-cavitation, particularly AISI 304, were investigated by Yeo, Prasanth, and Tan (2017). The ambient pressure and temperature of the liquid ranged from 100 kPa to 400 kPa and from 10°C to 90°C, respectively. The mass loss increased from 0.3 mg at 100 kPa to 0.5 mg at 300 kPa, remaining stable at 400 kPa. Regarding mass loss, a peak of 0.8 mg was detected at 50°C, returning to about 0.2 mg at 90°C. From 70°C to 90°C, an even larger population of microbubbles was observed, but it was noted that such microbubbles did not burst. Instead, they remain on the sides of the part and test chamber. This was because there weren't enough microbubbles to hold enough steam inside it to explode.

The effluent treatment stations in a hospital laundry are designed primarily to remove organic material (BOD and COD). Secondary biological treatment can efficiently eliminate indicator and pathogenic microorganisms with rare exceptions. Therefore, it requires specific units for disinfection, which becomes an essential barrier to reducing the risk to public health (Lutterbeck et al., 2020). Thus, ultrasonic radiation, alone or in combination with other physical or chemical techniques, is

presented as a strategy for adding a specific disinfection module. However, it has been of economic interest to adopt techniques that dispense with additional energy and chemical expenditures, justifying the search for applications of isolated US vibrations. In this work, experiments were carried out to evaluate the performance of ultrasonic vibrations, with and without the association of US with the effects of pressure and temperature on microbial inactivation in a hospital laundry effluent. To this end, effluent samples from a hospital in the Agreste region of the State of Pernambuco, Brazil, were used.

2. Methodology

2.1 Microbial Inactivation

When a liquid is under an ultrasonic field, microbubbles formed through large pressure fluctuations (500 to 10,000 atm) implode, giving rise to high temperatures (3,000 to 5,000 K) (Li et al., 2016). Inside these microbubbles, active species associate, giving rise to the production of structures such as free radicals. Studies show that the mechanisms of microorganism inactivation by ultrasound derive from these ultrasonic effects, called acoustic cavitation, and its consequences, such as shear forces and microjets, leading to the complete rupture of the cell encapsulation membrane. Silva (2020) states that ruptured and disintegrated cells cannot be reactivated. This is particularly advantageous compared to other techniques in which damaged cells can recover when in suitable environmental conditions.

Sonication, the name given to the combination of these ultrasonic effects, can also destroy agglomerates of microorganisms and flakes present in the liquid medium. Low-frequency ultrasound generates larger bubbles, creating higher localized temperatures and pressures during the violent collapse. At higher frequencies, above 1 MHz, the compression and rarefaction caused by the ultrasonic waves are short enough to separate the molecules from the liquid, and no cavitation is obtained. This low-intensity ultrasound, with a frequency above 1 MHz, has reduced chemical effects, and molecules closer to the free surface are vaporized (Wang; Ruan, 2021). Low-intensity US at a frequency of approximately 100 kHz to 1 MHz causes significant chemical effects. High-intensity US has a frequency range of 20–100 kHz and a power density of 10–1000 W/cm². US results in less intense chemical effects in this range but exerts powerful physical and mechanical effects and is therefore highly recommended for microbial inactivation (Thi Hong Bui et al., 2020).

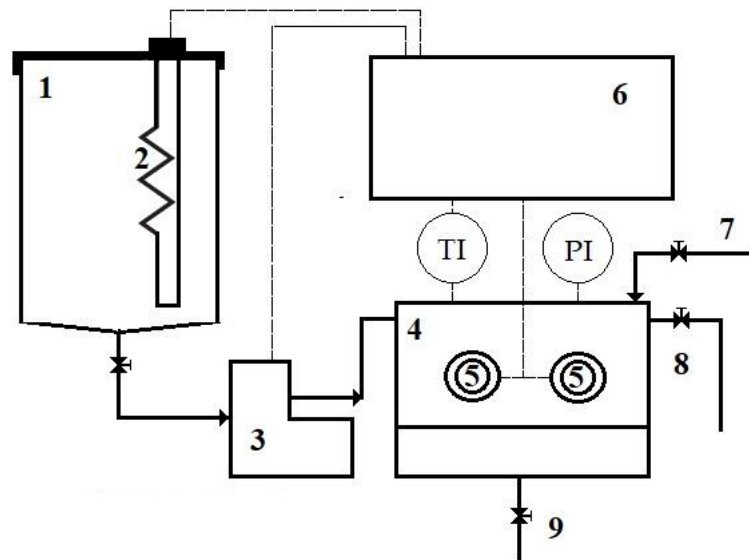
2.2 Experimental Arrangement

Disinfection experiments were carried out for the microorganisms identified as markers in the effluent of the hospital laundry selected for inactivation studies. For this, an experimental arrangement was built, constituting a continuous prototype on a laboratory scale. The characteristics of the said prototype are schematized according to Figure 1. In the diagram of this figure, a raw effluent storage tank can be observed serving as a feed container for a metering pump, which feeds an ultrasonic reactor. The inactivation reactor was made of AISI 304 stainless steel, with an effective capacity of 2.5 L. Its body is rectangular, 159 mm long by 124 mm wide by 136 mm high. Its base is semi-cylindrical, with a radius of 62 mm. Its supply and discharge are carried out through PPR tubes with an internal diameter of 15 mm, installed close to the upper edges, on opposite sides. The reactor had its internal pressure regulated with compressed air and monitored using a digital pressure sensor. The temperature of the raw effluent was controlled with the aid of heating provided by an electrical resistor installed inside the feed tank.

An electronics system monitored the temperature in the reactor through a thermocouple temperature sensor, type Pt100, with a span accuracy of 2%. PZT-4/8 type piezoelectric ceramic transducers were used, which have a high value of mechanical energy with an electricity transfer efficiency greater than 98%. The power consumed by each transducer was 30 W. They were installed two by two, in opposite positions and sides of the aforementioned irradiation chambers, to maintain a homogeneous sonication intensity. Stabilization of the frequency, with variations of the order of 1.5%, was one of the main

characteristics of the electronic circuit for the generation of ultrasonic waves.

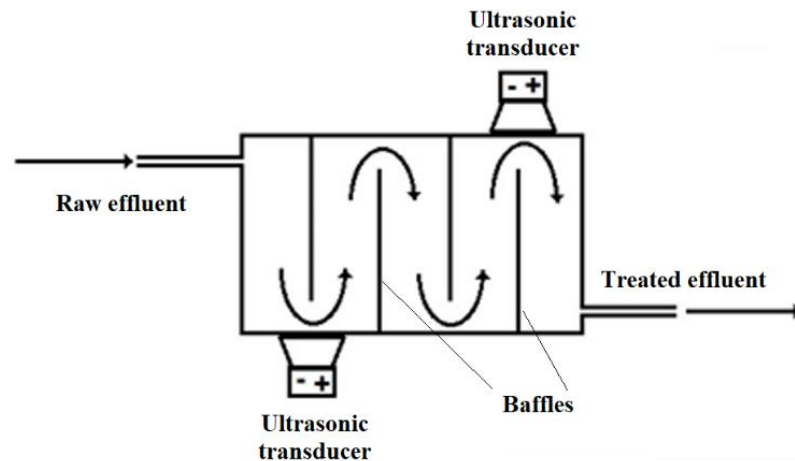
Figure 1. Schematic diagram with basic components of the laboratory experimental setup for experiments with microbial inactivation: (1) – Raw effluent tank; (2) - Heating resistor; (3) - Dosing pump; (4) - Ultrasonic reactor; (5) – Ultrasonic transducers; (6) – Electric-electronic control panel; (7) – Compressed air supply; (8) – Treated effluent outlet; (9) - Drainage valve.



Fonte: Elaborado pelos autores (2022).

Reactors were manufactured, and piezoelectric ceramic transducers were installed on the external side walls, on opposite sides and lengths, to allow the sonication of the effluent to occur homogeneously throughout the circulation time the liquid was in the reactor. To avoid the formation of dead zones, the feed and discharge ducts were strategically installed on opposite sides, interspersed with baffles inside the reactor, as shown in Figure 2. To help maintain the temperature of the reactor, during the sonication of the affluent, the reactor was coated with a blanket of thermally insulating material during the experiments. Figure 3 shows the experimental setup used for microbial inactivation of hospital laundry effluent used in this research.

Figure 2. Schematic showing the effluent flow guided by baffles during the sonication process inside the ultrasonic reactor.



Fonte: Elaborado pelos autores (2022).

Figure 3. Image of the experimental arrangement used for microbial inactivation of hospital laundry effluent.



Fonte: Elaborado pelos autores (2022).

2.3 Indicator Microorganisms

Escherichia coli is a rod-shaped, facultative anaerobic bacterium. Its primary habitat is the gastrointestinal tract of humans and other endothermic animals. It is considered an indicator of water quality through the analysis of fecal coliforms, the name given to a group of bacteria inhabiting these animals' intestines. *Escherichia coli* forms a large part of the population in this group, and, therefore, its presence suggests the possibility of having, in that place, intestinal microorganisms capable of causing diseases. The *E. coli* strain is recognized as the most accurate indicator of fecal contamination in water, which is why it is widely used to verify the microbiological quality of drinking water (Dehghani, 2005).

2.4 Predictive Model

In predictive microbiology, the mathematical models used to represent the microbial survival curve as a function of

time for a single set of constant environmental conditions are called primary models (Buchanan, 1993). These primary models are obtained by fitting experimental data on microbial survival as a function of time. The dependence of primary model parameters on different ambient conditions, such as temperature, pH, and pressure, is described by secondary models (Panagou et al., 2003). In general, in developing secondary models, several experiments must be conducted on different factor ranges to observe how such independent variables influence the kinetics of the process. Tertiary models are made up of computer programs that combine the use of primary and secondary models (McMeekin et al., 2002). These programs can calculate microbial responses under different conditions, compare the effect of these variations, or even contrast the behavior of various microorganisms. These applications facilitate the modeling of microbial survival curves under different conditions. It is important to emphasize that this feature allows the continuous accumulation of knowledge and, consequently, can lead to the development of better models and greater scope for their application.

The predictive model used to describe the microbial inactivation in this research was of the secondary type, as it involves additional parameters for the treatment time of the hospital laundry effluent used. The estimated parameters for verification and validation of the adopted model were analyzed through correlations between all process variables and between these and the dependent variable (inactivation efficiency) within the ranges of values of these factors. This data analysis tool, defined as experimental planning (Jankovic; et al., 2021), is widely used to assist in the analysis of predictive models because it has some advantages, such as a reduction in the number of trials without compromising the quality of the information; simultaneous study of several variables, separating their effects; reliability of results; selection of process-relevant variables in a reduced number of tests; obtaining mathematical expressions that give rise to prediction models; determination of predictive models from quantitative and qualitative independent variables.

2.5 Experimental Designs Used

In predictive microbiology, the application of an experimental design should aim to help identify the influences of variables involved in adopting a predictive model on the response variable. Thus, plans must be designed to achieve more specific objectives, such as (i) determining the relevant factors using fractional factorial planning (Santos Júnior et al., 2021); (ii) establishing interrelationships between one or more responses (dependent variables) with factors (independent variables), through complete planning (Kim; Kim, 2019); (iii) select an optimal region for the performance of the investigated process using a central composite design - CCD (Sakhi et al., 2020).

For the application of a composite design in this work, a fractional factorial design was preliminarily applied, where only a fraction of the experiments was performed. The criterion used to define the relevant variables was that of little interest in higher-order interactions. This way, a fractional factorial design, consisting of a subset of 24-1 experiments, was performed. The relevant independent variables (ultrasonic frequency, hydraulic retention time, temperature, and pressure) were tested and confirmed statistically significant in the *E. coli* inactivation.

In the second stage, a complete factorial design was applied with the independent variables identified by the experimentation conducted by a fractional factorial at three levels (Cervantes-Elizarrará, 2017). This allowed the identification of points with null derivatives, indicative of regions that harbor optimization conditions.

For the third stage, the central composite design (CCD), intended to obtain response surfaces, allowed:

- To model a response variable with curvature, adding central and axial points to a factorial design;
- And to obtain first or second-order forecast models.

The experimental design adopted to investigate the optimal conditions of the inactivation process was analyzed by applying the Response Surface Methodology (RSM), used to determine the combined effects of the following factors: Ultrasonic frequency (X_1), Hydraulic retention time in the inactivation reactor (X_2), Effluent temperature (X_3), and Pressure

inside the inactivation reactor (X_4). The response variable was the inactivation percentage efficiency of *Escherichia coli* (Y). The actual and coded levels of the schedule are shown in Table 1. A total of 29 experiments consisted of 16 full factorial points, 8 axial points, and 5 central points.

Table 1. Coded and actual values of the independent variables used in the application of the RCCD of this research.

Variables	Symbols	Range of values and level				
		-2	-1	0	+1	+2
Ultrasonic Frequency, kHz	X_1	40	60	80	100	120
Hydraulic retention time, min	X_2	10	20	30	40	60
Temperature, °C	X_3	30	35	40	45	50
Pressure, bar	X_4	1.0	1.2	1.4	1.6	1.8

Fonte: Elaborado pelos autores (2022).

The Response Surface Methodology (RSM) was applied to evaluate independent variables' effects on the responses. The quadratic model used to fit the data was:

$$y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ij} X_i X_j + \sum_{i=1}^k \beta_{ii} X_i^2 + \epsilon \quad (1)$$

where y is the response variable; X_i and X_j are the variables; β_0 is the constant coefficient; β_i , β_j , and β_{ij} are the quadratic and second order interaction coefficients of linear terms, respectively; k is the number of parameters studied; and ϵ is the system error.

The effect of each factor was evaluated by variance analysis (ANOVA) (95%). The adopted model allowed the evaluation of the independent variables' linear, quadratic, and interactive term effects on the dependent variable. Response surface and level curve graphs were used to illustrate variables' main and interactive effects on the inactivation efficiency of different microorganisms. The selected variables' optimal operational values, partial and global, were defined with the help of the desirability function (Pérez, 2021).

2.6 Sample Preparation and Analysis

Sonication experiments were carried out to observe the effects of ultrasonic radiation in association with physical factors such as temperature and pressure on the inactivation of total coliforms, *Escherichia coli* and heterotrophic bacteria. The influence of each factor and their interactions were evaluated, and the concentrations of the three species were individually adjusted with sterile and demineralized water to 10^8 CFU/mL in all experiments. Subsequently, the samples were added to the reactors in which the sonications were performed. Except for the ultrasonic reactor, the other components of the laboratory prototype were sterilized with sodium hypochlorite solution before each experiment.

The effluent treated in the ultrasonic reactor was subjected to a microbiological analysis to determine the results of microbial inactivation. Three 0.1 mL aliquots were taken from the treated samples and placed in Petri dishes prepared with LB solid nutrient medium, later dispersed, and incubated at 37°C for 20 hours.

The reference sample was taken from the storage and feeding tank of the ultrasonic reactor. Since each viable bacterium forms a colony (CFU), colonies were counted after incubation using a SOLBAT colony counter. This process was applied to the

treated samples, and after counting each of the CFUs, the number of surviving bacteria (NSB) in CFU/mL was obtained from the following expression:

$$NBS = \frac{NC}{VMU} \quad (2)$$

Where NC is the number of counted colonies (CFU/mL), and VMU is the sample volume (0.1 mL). While the microbial inactivation percentage efficiency (h) is calculated as:

$$\eta\% = 100 - \frac{NBS}{N_0} 100 \quad (3)$$

Where N_0 is the number of counted colonies (CFU/mL) of the reference sample. Counts were performed and recorded for means for each of the 5 experiments to confirm the process trends.

2.7 Statistical Data Analysis

The experimental data were submitted to normality (Student's t) and homogeneity (Cochran) tests. Given these conditions, the data above were submitted to analysis of variance (ANOVA), followed by Tukey's test, when necessary, to compare the means between the results obtained, with a significance level (p) < 0.05, in case there was no normality and homogeneity of the data. The Nonlinear Regression tool was used to simulate and quantify the kinetic constants of microbial inactivation. For the simulation phase, the Quasi-Newton numerical optimization method was used (Al-Baali; Khalfan, 2007). To process the analysis, both for descriptive statistics and CCD, the Statistica Version 12 software (StatSoft, Tulsa, OK, USA) was used.

3. Results and Discussion

3.1 Thermomannosonic Microbial Inactivation

The experimental results derived from the CCD-RSM, as a function of the four factors defined above, are shown in (Table 2). The average *E. coli* inactivation efficiency level ranged from 84.77% to 98.98%. The highest levels of inactivation were identified in the region defined by the central points of the CCD, confirming the adequacy of the adjustments produced by a drilling step using a complete factorial design with three levels. An analysis of this table allows the following main points to be observed. Increases in temperature cause reductions in *E. coli* inactivation, as predicted by Kentish (2017). Pressure increases also cause reductions in microbial inactivation, according to Astráin-Redín et al. (2020), although at reduced levels compared to the effects caused by temperature, justified by the lower levels used for pressure.

Table 2. Arrangement of the RCCD for the four independent variables in *E. coli* manothermosoic inactivation process.

Run	X ₁	X ₂	X ₃	X ₄	Y
1	-1.0	-1.0	-1.0	-1.0	95.02
2	-1.0	-1.0	-1.0	1.0	91.23
3	-1.0	-1.0	1.0	-1.0	90.01
4	-1.0	-1.0	1.0	1.0	85.33
5	-1.0	1.0	-1.0	-1.0	96.71
6	-1.0	1.0	-1.0	1.0	90.95
7	-1.0	1.0	1.0	-1.0	92.34
8	-1.0	1.0	1.0	1.0	90.03
9	1.0	-1.0	-1.0	-1.0	98.05
10	1.0	-1.0	-1.0	1.0	92.21
11	1.0	-1.0	1.0	-1.0	87.00
12	1.0	-1.0	1.0	1.0	84.77
13	1.0	1.0	-1.0	-1.0	94.45
14	1.0	1.0	-1.0	1.0	93.99
15	1.0	1.0	1.0	-1.0	95.51
16	1.0	1.0	1.0	1.0	93.43
17	-2.0	0.0	0.0	0.0	87.05
18	2.0	0.0	0.0	0.0	94.51
19	0.0	-2.0	0.0	0.0	93.69
20	0.0	2.0	0.0	0.0	94.91
21	0.0	0.0	-2.0	0.0	93.89
22	0.0	0.0	2.0	0.0	86.05
23	0.0	0.0	0.0	-2.0	96.00
24	0.0	0.0	0.0	2.0	92.89
25	0.0	0.0	0.0	0.0	97.96
26	0.0	0.0	0.0	0.0	98.04
27	0.0	0.0	0.0	0.0	98.98
28	0.0	0.0	0.0	0.0	98.01
29	0.0	0.0	0.0	0.0	98.87

Fonte: Elaborado pelos autores (2022).

A summary of the ANOVA results for the quadratic model selected for the US/T/P process is provided in Table 3. Among the main effects, the highest F value of 476.3509 and the lowest value of 86.5608, all with probability $p \ll 0.05$, indicate that such effects are highly significant. For the interaction terms between the factors, except the interaction between hydraulic detention time (X₂) and temperature (X₃) with $p \ll 0.05$, the other interactions showed little interaction, with the interactions not appearing in the prediction model between frequency (X₁) and temperature (X₃), and temperature (X₃) and pressure (X₄), both with $p > 0.05$. In the latter case, the low statistical significance of the interaction between the factors X₃

and X_4 ($p > 0.05$) is attributed to the low values of T and P used in the CCD application.

Table 3. Analysis of variance (ANOVA) values for the full-quadratic models developed to describe the influence of ultrasonic frequency (X_1), hydraulic retention time (X_2), room temperature (X_3), and room pressure (X_4) on the inactivation of *E. coli*.

Factor	SS	DF	MS	F	P
X_1 (L)	21.4893	1	21.4893	83.5608	0.000795
X_1 (Q)	100.7341	1	100.7341	391.7023	0.000038
X_2 (L)	28.6672	1	28.6672	111.4718	0.000455
X_2 (Q)	30.8488	1	30.8488	119.9548	0.000395
X_3 (L)	103.6257	1	103.6257	402.9463	0.000036
X_3 (Q)	122.5032	1	122.5032	476.3509	0.000026
X_4 (L)	46.3982	1	46.3982	180.4184	0.000178
X_4 (Q)	28.8317	1	28.8317	112.1116	0.000450
X_1 L by X_2 L	2.9843	1	2.9843	11.6042	0.027115
X_1 L by X_3 L	0.2003	1	0.2003	0.7787	0.427382
X_1 L by X_4 L	2.1978	1	2.1978	8.5461	0.043098
X_2 L by X_3 L	37.8533	1	37.8533	147.1916	0.000265
X_2 L by X_4 L	2.1978	1	2.1978	8.5461	0.043098
X_3 L by X_4 L	1.2939	1	1.2939	5.0313	0.088322
Lack of adjustment	41.8711	10	4.1871	16.2815	0.008088
Experimental error	1.0287	4	0.2572		
Total	486.0454	28			

Fonte: Elaborado pelos autores (2022).

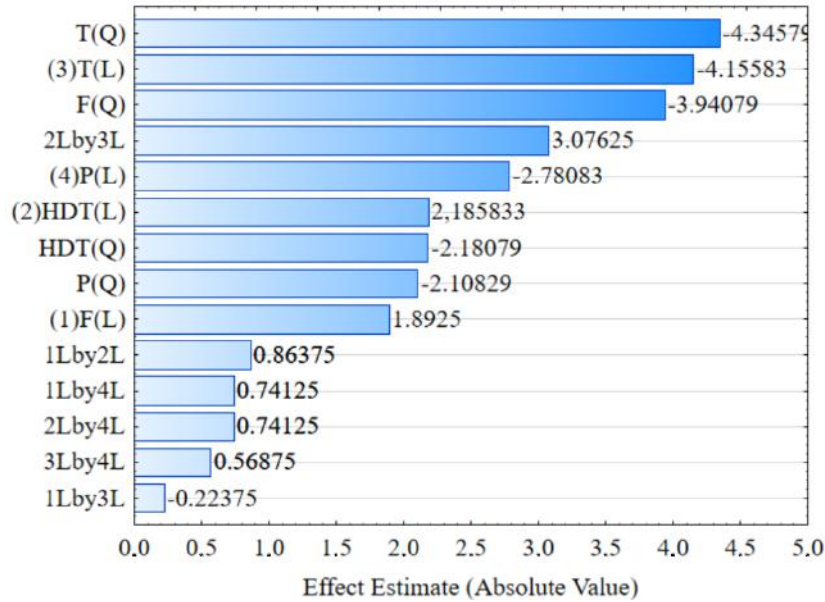
The complete quadratic model is shown by Equation (1) to analyze all the experimental design variables. Model terms that were not significant ($P > 0.05$) were removed from this equation. Model coefficients and their analysis of variance (ANOVA) values resulting from the microbial inactivation results are shown by Equation (4). The ANOVA values show that the model has a high coefficient of determination ($R^2 = 0.91174$), indicating that the four variables investigated were responsible for at least 91% of the inactivation variability, thus confirming the validity of the adjustment, or that is, the statistical results correlated well with the observed experimental data results, at a significance level of 5%.

$$\begin{aligned}
 Y = & 98.37 + 0.95X_1 - 1.97X_1^2 + 1.09X_2 - 1.09X_2^2 - 2.08X_3 - \\
 & -2.17X_3^2 - 1.39X_4 - 1.03X_4^2 + 0.43X_1X_2 + 0.37X_1X_4 + \\
 & +1.54X_2X_3 + 0.37X_2X_4
 \end{aligned}
 \tag{4}$$

Regarding the influence on the *E. coli* microbial inactivation process, the Pareto diagram obtained by the RSM, illustrated in Figure 4, showed that the factor with the most significant influence was temperature. Therefore, this quadratic factor was responsible for most of the response variability with 25.20%. The second factor with the most significant variability was also temperature, in this case in linear form, with 21.32% of the total response variability. Here it should also be noted that the negative signs represent an inverse proportionality between such factors in relation to the inactivation efficiency. The linear hydraulic retention time factor was identified as the one with the smallest contribution in the prediction model to the response

variability, representing less than 4.70%. The interaction factors had a low influence on the variability of the model, with emphasis only on the interaction between HRT and temperature with a value of 7.79%, emphasizing that the other interaction terms did not go beyond 0.61%.

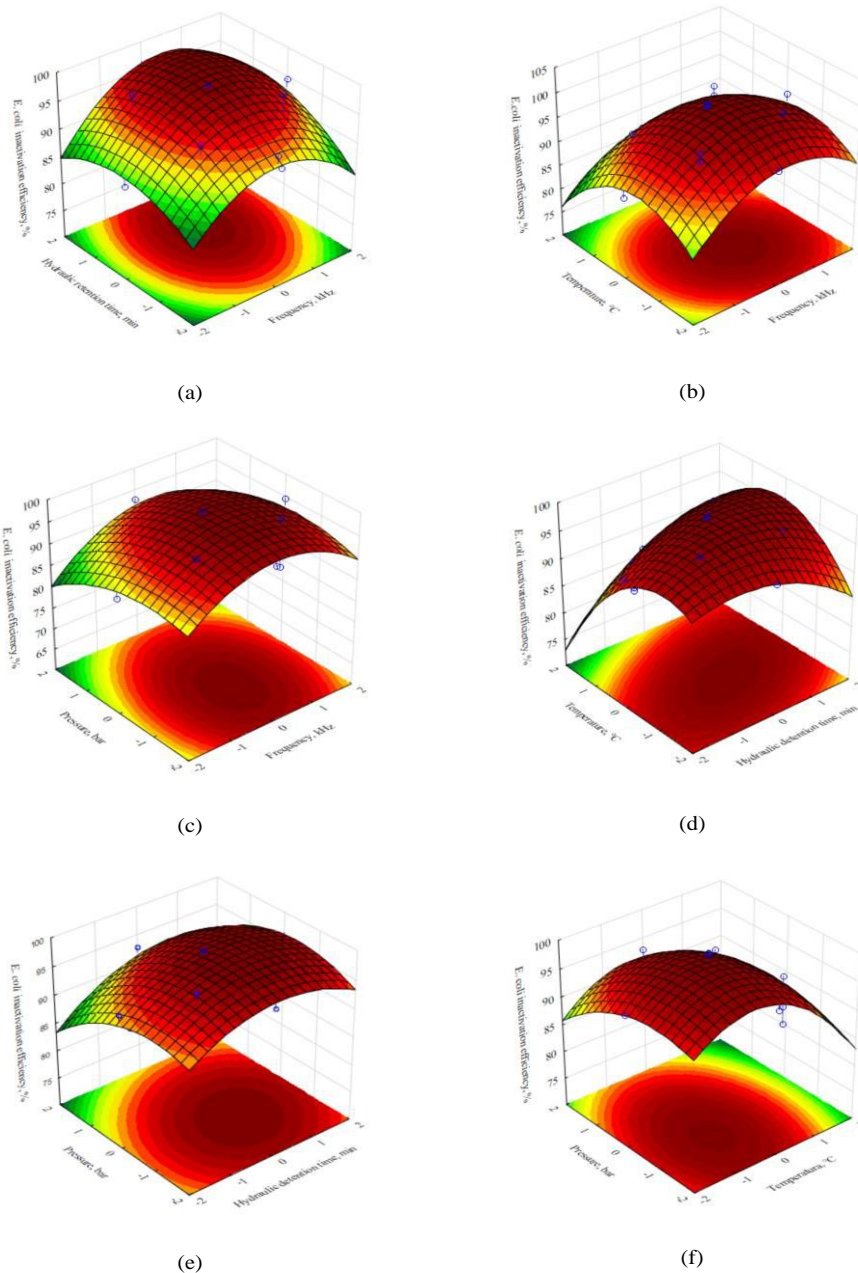
Figure 4. Pareto chart for the CCD design showing the effect and the direction of factors affecting the *E. coli* inactivation.



Fonte: Elaborado pelos autores (2022).

The effects of all combinations of factors on *E. coli* inactivation are shown in Figure 5. 3-D plots of response surfaces were constructed for all factor pairs, and center point values were defined for the third factor. The graphs in Figure 5 demonstrate that the strategy of previously sectioning the relevant variables for the inactivation process and, later, surveying a possible region of optimal conditions proved to be valid. Figure 5(a) illustrates the similar behavior of the inactivation efficiency as a function of the ultrasonic frequency (X_1) and HRT (X_2) variables within the ranges of values applied to this CCD. In Figure 5(b), the inactivation efficiency is plotted against X_1 and against temperature (X_3). As expected, the behavior of inactivation as a function of temperature differs from that of frequency. In the latter, the efficiency is proportional, while for the former, the behavior is inversely proportional. As expected, a peak of acoustic cavitation intensity appears at around 30°C (Yu; Liu, 2020). In Figure 5(c), the inactivation is a function of X_1 and the pressure (X_4). This figure shows when compared with Figure 5(b), that the reduction of inactivation as a function of pressure is smaller than when as a function of temperature. This can be explained by the small pressure variation since the effects of this variable on microbial inactivation have been investigated with much higher-pressure ranges (Kahraman, 2017). In Figure 5(e), when the pair $X_1 - X_3$ was replaced by $X_2 - X_3$, the inverse effect of temperature (X_3) on inactivation was much more pronounced. This reduction in the effect of temperature on inactivation may be justified over time since, in the previous pair, the HRT (X_2) was kept constant. Lastly, pressure and temperature can have their effects directly compared on *E. coli* inactivation. In Figure 5(f), the effect of temperature confirms its greater influence on the inactivation of *E. coli* in the ranges of values used.

Figure 5. Response surface plots for the *E. coli* inactivation efficiency through manothermosonication as a function of: (a) Frequency x Hydraulic retention time; (b) Frequency x Temperature; (c) Frequency x Pressure; (d) Hydraulic retention time x Temperature; (e) Hydraulic retention time x Pressure.



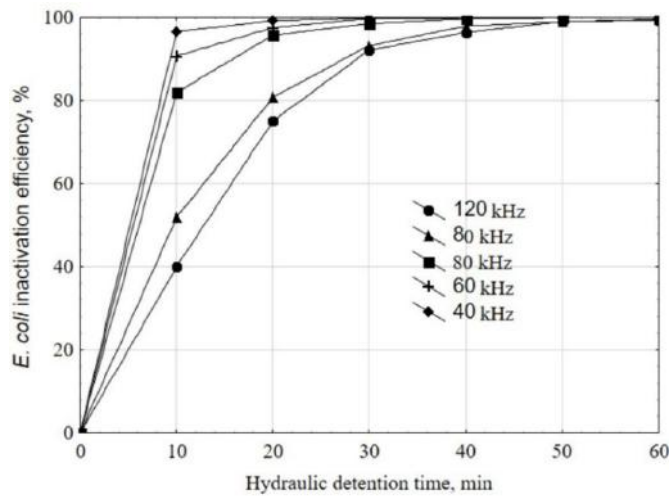
Fonte: Elaborado pelos autores (2022).

3.2 Ultrasonic Microbial Inactivation

The ultrasound treatment was performed in different experiments on the bench prototype, operating at ambient temperature and pressure. The predictive model used was of the primary type since it only took into account the treatment time. Figure 6 shows the reduction of surviving cells, through the efficiency of *E. coli* inactivation, after ultrasonic treatments for periods equivalent to hydraulic retention times corresponding to the adopted volumetric flow rates. The treatments were performed with different frequencies (40, 60, 80, 100, and 120 kHz) and different hydraulic retention times (10, 20, 30, 40, 50, and 60 min). All experiments were performed with a specific power of 24 W/L. Overall, the results indicate increased *E. coli* inactivation efficiency with HRT for all frequencies. A treatment performed at a frequency of 40 kHz for 10 minutes achieves

an *E. coli* inactivation efficiency of 40%, while for a frequency of 120 kHz, the efficiency is 96.6%. Under these conditions, on average, for each increase of 20 kHz, about a 15.0% increase in inactivation efficiency was obtained. At 20 minutes of treatment, this increase goes to 30.0%, at 30 minutes, it doubles again, and after 40 minutes, the difference is not noticeable since all frequencies produce average *E. coli* inactivation above 99.0%. Thus, in processes where a 10-minute HRT is available, the use of 120 kHz ultrasonic waves is recommended. For HRT of 20 or 30 minutes, frequencies from 80 kHz can be used, while for HRT of 40 minutes, the use of frequencies from 40 kHz can be recommended, provided that the dynamic similarity conditions of the bench prototype are reproduced to the larger scale of the prototype to be built.

Figure 6. Efficiency of inactivation of *E. coli* after treatment in an ultrasonic laboratory prototype at room temperature and pressure.



Fonte: Elaborado pelos autores (2022).

4. Conclusion

The innovation of this study was the finding of advantages in using the isolated US technique over the use of a combination of thermal, manometric, and ultrasonic techniques as an *E. coli* inactivation step in the experimental conditions tested in this work. The experimental tests were carried out on a benchtop laboratory prototype with a capacity of 2.5 L. The thermomanosonication tests confirmed that both temperature and pressure reduce the intensity of acoustic cavitation, reducing its intensity and, consequently, the intensity of inactivation, in addition to increasing energy costs for the application of the association of the three techniques. Isolated ultrasonic vibrations at 120 kHz and with 10-minute HRT reached an inactivation efficiency of 98%. This value was only found using thermomanosonic acid for an HRT of 40 min. Larger-scale prototypes must be designed keeping dynamic similarity with the laboratory prototype to reproduce the *E. coli* inactivation efficiency conditions mentioned above. Thus, the isolated technique presents itself as a technically and economically advantageous option.

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