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An In Vitro Study of the Effects of Temperature and pH on Lead Bioremoval Using *Serratia marcescens*

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Abstract: Heavy metal contamination of water is a widespread problem in Peru and represents a potential threat to the ecosystem. Bacteria are an ecological alternative to treating these effluents. This research aims to determine the influence of temperature and pH on the lead (Pb) bioremoval in surface water using *Serratia marcescens* under laboratory conditions. The sample was collected from a stream located in Santiago de Chuco City (Peru). Treatments (T) were carried out by combining pH (5 and 7) and temperature (25, 30, and 35 °C). The bacterial inoculum (*S. marcescens*) was 3×10^8 CFU/mL, which was constant in all treatments. The lead bioremoval evaluation was performed in an airlift bioreactor and the incubation time was 24 h. The total lead concentration was determined using atomic absorption spectrophotometry. The results show that treatment 6 (temperature: 35 °C, pH: 5, and inoculum: 3×10^8 UFC/mL) showed a better result than the other treatments, with a removal value of 63.94%. Furthermore, the total lead concentration decreased from an initial concentration of 0.268 mg Pb/L to a final value of 0.0964 mg Pb/L. These results are still above the allowed water value (15 µg/L) according to Peruvian standards. On the other hand, temperature and pH influenced lead removal from surface water when *S. marcescens* was used after a short incubation period (24 h). Although an attempt was made to improve lead bioremoval by varying two parameters, temperature and pH, future research is still needed to investigate the effect of different inoculum concentrations, the use of microbial consortia, and a broader range of physicochemical parameters.

Keywords: *Serratia marcescens*; lead; bioremoval; heavy metals; bioremediation

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1. Introduction

Fresh water is a fundamental natural resource and useful for human consumption. Likewise, groundwater, rivers, and lakes are considered sources of clean water for direct consumption and domestic use. In addition, water is significant in economic activities since it is used in industry, energy production, irrigation, and livestock [1]. In recent years, the scarcity of water resources and the deterioration of aquatic ecosystems have become important environmental problems, with heavy metal contamination being a problem that represents treatment challenges and puts at risk public and environmental health [2].

In Peru, the National Water Authority (ANA) frequently monitors the surface water quality of the Moche River basin, finding that the concentrations of metals such as Al, Cd,

Mn, Fe, Pb, and Cu exceed the values of the ECA—Water quality standards for category 3 (irrigation of vegetables and animal drinks) [3]. Various factors have led to the contamination of other bodies of water with heavy metals over the last few decades; for example, studies report high contamination by heavy metals in waters below the Puyango–Tumbes basin [4]. By comparison, the Yangtze River, considered one of the largest in the world, is affected by the presence of high concentrations of heavy metals such as Cu, Zn, Pb, Cd, Hg, and As in muddy areas; their concentration tends to decrease as the distance from the coast increases [5]. The primary sources of industrial pollution are mining, coal combustion, sewage, and product residue disposal. The most harmful is mining activity, which produces large amounts of rock residues containing traces of heavy metals such as As, Cu, Cd, Pb, and Hg; these are deposited in mining waste and exposed to weathering oxidizing conditions, which leads to acid drainage that subsequently penetrates surrounding soils, rock, and, in some cases, drinking water sources [6]. Metal contamination is considered when it exceeds a density greater than 5 g/cm^3 and can also be grouped into essential and non-essential classes; the first group includes Co, Cr, Cu, Fe, Mn, Ni, and Z, which are considered essential micronutrients, but become toxic when consumed in high concentrations. The second group includes Pb, Cd, and Hg, which are highly toxic to living organisms [7].

In Peru, in the district of Mollebamba, there is the La Victoria stream, a point monitored by the National Water Authority. It is located upstream before the crossing with the San Fráncico River, which supplies this resource to the population of Tulpo [8,9]. This stream has a great problem caused by the discharges from the mineral extraction and storage pits, which affect its quality, especially in rainy seasons. Its waters are clear with a reddish yellowish color from the stone sediment in the river bed; the monitoring results in 2018 during dry periods were compared with the ECA (Environmental Quality Standard) of category 1-A2 and were at a pH of (3.339), where the parameters that exceeded were, Cd (0.03451 mg/L) Fe (3.838 mg/L) and Pb (0.5647 mg/L), the other parameters evaluated did comply with the ECAS for water [10]. In 2019, sampling was carried out, traces of some metals were detected in the surface water body, the parameters that exceeded the ECA were pH (3.76), COD (363 mg/L) Cd (0.00928 mg/L) Fe (1.303 mg/L) and Pb (0.2650 mg/L), where the value of the most alarming parameter was Led, the other parameters evaluated did comply with the ECAS for water. Lead, as well as other heavy metals, is considered one of the most toxic and persistent pollutants in the environment that affects human health [11].

Heavy metals can be transported by water and adsorbed by aquatic vegetation, introducing them into the food chain, which leads to the bioaccumulation of metals in living organisms, causing irreversible damage. It is important to note that although some heavy metals are essential in biological systems, such as copper and zinc, their consumption above the tolerable intake can cause toxicity [11]. To counteract this problem, conventional techniques are used for the removal of dissolved heavy metals, which include chemical precipitation, carbon adsorption, ionic exchange, evaporations, and membrane processes [12]; however, these techniques have several disadvantages, including treatment inefficiency, high operating cost, and secondary risk of environmental contamination. Therefore, the solution to these limitations is bioremediation, a technology that is easy to apply to overcome these deficiencies [13], since it is a physical-chemical process that implies the degradation, sequestration, or elimination of various contaminants, such as heavy metals using biological material. Different microorganisms, such as bacteria, algae, fungi, and yeast, can remove heavy metals from aqueous solutions; these microorganisms interact with the metal ions present in the solution [14,15].

Microorganisms have various mechanisms for the bioremediation of metals, such as surface and interstitial adsorption. During surface adsorption, heavy metals migrate by diffusion from the aqueous solution to the adsorbent surface, which contains an opposite surface charge, after the heavy metal ions have passed through the boundary layer and adhered firmly to the surface of the adsorbent and subsequently removed from the solu-

tion. The biosorption involves Van Der Waals forces, bipolar interactions, or hydrogen bonding [12,15]. By comparison, during interstitial adsorption, heavy metal ions diffuse into the adsorbent; however, the ion enters the pores of the adsorbent and adheres on the inside surface; then, the phenolic groups present are replaced with metal ion protons. Heavy metal removal is facilitated by carboxyl and hydroxyl groups, which are linked by a divalent heavy metal ion via two electron pairs that release Na^+ and H^+ ions into the solution [14].

In the same way, the electrostatic forces are also considered a factor that contributes to the adsorption of heavy metals, dependent on pH. There is also an interaction between heavy metals and various functional groups on the surface of the biosorbent. Functional groups are metal adsorption sites including carboxylate (-COO^-), amide (-NH_2), phosphate (PO_4), thiols (-SH), and hydroxide (-OH) [9,14]. In some bioremediation studies, the presence of the *S. marcescens* bacterium has been related to the adsorption of heavy metals and radioactive elements because it produces different enzymes and other essential compounds that allow it to remove heavy metals from the environment [16]. *S. marcescens* is a facultatively anaerobic, Gram-negative, rod-shaped member of the *Enterobacteriaceae* family. This bacterium can grow under aerobic or anaerobic conditions. The temperature range for its growth is between 10 and 37 °C and pH is between 5 and 9, in NaCl concentrations of 0 to 4%. Colonies are usually opaque, somewhat iridescent, and can be white, pink, or even deep red. They are approximately 1.5 to 2 mm in size after being incubated for 18 h on a nutritious culture medium. They can be found in various ecological niches, including soil, water, air, wastewater, and food products [17].

Two strains of *S. marcescens* bacteria, the pigmented LG1 strain, and the non-pigmented CL11 strain, were studied by Queiroz et al. (2018), determined the effect of two different strains of the bacteria "*Serratia marcescens*" on the elimination of Mn (II) and the effects of (K medium) on the pigmented LG1 strain and the non-pigmented CL11 strain in nutrient-rich medium, LG1 pigmented exhibited improved growth and greater Mn (II) tolerance (0–2000 mg/L). The biooxidation of Mn by the non-pigmented strain CL11 may involve indirect mechanisms that alter the pH of the medium, while the pigmented strain LG1 may use a direct mechanism for biooxidation mediated by cellular components such as intracellular proteins, these results demonstrate the potential biotechnological analysis of the two strains, concluding that an elimination of 64.25% of Mn (II) was obtained in the two strains [17]. He argued that the bio-oxidation of Mn by the non-pigmented CL11 strain may have indirect mechanisms that alter the pH of the medium. In contrast, the pigmented LG1 strain may use a direct means for bio-oxidation mediated by cellular components, such as intracellular proteins. These results demonstrated the biotechnological potential of the two strains, concluding that there is an elimination of 64.25% of Mn (II) in the two strains. In another investigation, Quian et al. (2019) evaluated the constituents of extracellular polymeric substances (EPSs) and the physiological activities of biofilms of plankton cells and the bacterium *S. marcescens* in a microbial electrosynthesis system Q1 [18]. The conditions evaluated in this investigation were a Cu (II) concentration of up to 80 mg/L, a potential cathodic of 900 mV, and a standard hydrogen electrode. It was concluded that there is a simultaneous reduction in Cu (II) of 6.42 $\pm$ 0.02 mg/L/h [18,19]. A study conducted by Sayyadi et al. (2017) used the bacterium *Bacillus pumilus* for the biosorption of lead in aqueous solutions, and a maximum biosorption capacity of 0.0671 mmol/g was obtained [20].

Heavy metal contamination can be addressed using bioremediation, which is a sustainable and environmentally friendly process [21,22]. Therefore, the objective of the research was to determine the influence of temperature and pH on lead bioremoval by *Serratia marcescens* since it presents resistance and adaptability to high concentrations of heavy metals. In this way, it is intended to reduce lead concentration to acceptable levels through the use of bacteria. This will contribute to the decontamination of our ecosystems.

2. Materials and Methods

2.1. Experimental Design

This research design is bifactorial because two independent variables, temperature and pH (Table 1), are manipulated, in addition to making all possible combinations between them (8 treatments). This type of design allows the study of the effects of variables on a given response (lead bioremoval). Three repetitions were made for each treatment. The design was performed according to Tello-Galarreta et al. [23].

Table 1. Experimental design of the independent variables.

Treatments	Independent Variables [+]		Combinations
	pH	Temperature	
1	P1	T1	P1T1
2	P1	T2	P1T2
3	P1	T3	P1T3
4	P2	T1	P2T1
5	P2	T2	P2T2
6	P2	T3	P2T3
Total combinations	3(P)	3(T)	3 × 3

[+] P: pH level (P1:5, P2:7); T: Temperature (T1:25 °C; T2:30 °C; T3:35 °C).

2.2. Sample

The sample was obtained from the La Victoria stream in Santiago de Chuco (Peru). The collection of the sample was based on the Peruvian Protocol for the Monitoring of the Quality of Surface Water Resources (Resolution N° 010-2016-ANA) [24]. A total of 15 L of surface water was collected. For analysis, the samples were taken to the Institute of Scientific and Technological Research of Cesar Vallejo University (Trujillo, Peru).

2.3. S. marcescens Culture and Inoculum

S. marcescens was obtained from the Institute for Scientific and Technological Research at UCV. Upon obtaining the strain, it was reactivated in Nutrient Agar (Merck, Tampa, FL, USA). After, Gram staining and biochemical tests were necessary to verify its purity. Biochemical tests are shown in Table 2.

Table 2. Biochemical tests for *S. marcescens* identification.

Tests	Culture Media	Result
Gram Staining	-	Gram-negative
Sugars fermentation	TSI Agar	A/A [a]
H_2S production	TSI Agar	–
Gas production	TSI Agar	+
Decarboxylation production	LIA Agar	K/K [b]
Citrate test	Citrate Agar	–
Urease test	Urea Agar	–
Methyl Red (MR) test	MR Broth	–
Voges–Proskauer (VP) Test	PV Broth	+
Motility test	SIM Agar	+
Indole test	Tryptone	–

[a] A/A: The bacteria metabolize glucose, lactose, and sucrose in the culture. [b] K/K: Indicates decarboxylation of lysine; therefore, it is a positive result.

For the preparation of the inoculum of the *S. marcescen* bacterium, a pure culture was used. Then, a suspension was performed in a sterile physiological saline solution until a concentration equal to McFarland tube 1 (3×10^8 cells/mL) was obtained.

2.4. Tolerance Test by Determination of Minimum Inhibitory Concentration (MIC)

The MIC of *S. marcescens* to lead was determined. The strain was streaked in Nutrient Agar (Merck) with different concentrations of Pb $(NO_3)_2$ (0.5, 1.5, 3.0, and 10 mg/L), which were prepared from a mother solution of 1000 mg Pb/L. It was then incubated at 35 °C for 24 h. The positive control was evidenced by the growth of the *S. marcescens* bacterium in the Nutrient Agar medium without metal.

2.5. Lead Bioremoval Treatments Using S. marcescens

Lead bioremoval was performed in airlift bioreactors. Previously, the surface water sample was filtered with a Rocker 300 oil-free vacuum pump (Taiwan, China), and 500 mL was placed in each airlift bioreactor. A quantity of six treatments (T) was carried out by varying the pH (5 and 7) and the temperatures (25, 30, and 35 °C). The airlift bioreactors were equipped with surface water samples, BHI broth, and bacterial inoculums. Variations of the parameters to be evaluated were: T1 (pH 5 and 25 °C), T2 (pH 7 and 25 °C), T3 (pH 5 and 30 °C), T4 (pH 7 and 30 °C), T5 (pH 5 and 35 °C), and T6 (pH 7 and 35 °C). Three replicates were carried out for each treatment. A control bioreactor contained only the sample with lead, without the *S. marcescens* bacterium (T0). The pH was measured with a pH meter (BOECO Portable PH/ORP/TEMP Meter Model PT-380, Múnich, Germany). To adjust the pH, 0.1 M HCL and 0.1 M NaOH were used. The temperature was controlled in a Memmert Incubator IN55 (Múnich, Germany). At the end of the 24 h incubation period, a sample was taken from each bioreactor, centrifuged, and filtered through a 0.20 µm membrane before analysis via atomic absorption spectrophotometry.

The lead (Pb) concentration analyses were carried out by the flame atomic absorption spectrophotometry method. To report the values of the amount of lead removal, the following formula was needed:

$$\text{Lead Removal \%} = (C_0 - C_f/C_0) \times 100 \quad (1)$$

C_0 = Initial concentration of lead in the solution (mg Pb/L)
C_f = Final concentration of lead in the solution (mg Pb/L)

2.6. Analysis of Data

Three repetitions were carried out to measure the lead concentration at 24 h of incubation. For data analysis, Microsoft Excel and SPSS v25 were utilized. Data were based on means. One-way ANOVA was used for means analysis and the Tukey test was used to compare the total Pb values.

3. Results

Table 3 shows the tolerance of *S. marcescens* in different concentrations of Pb on a solid medium. It was observed that the bacterium can tolerate from 0.5 mg/L lead to 10.0 lead, without affecting its growth (red, smooth, convex, entire, and round colonies) and pigment production (red).

Table 3. Tolerance of *S. marcescens* to four concentrations of lead in a solid medium.

Pb Concentration	Growth	Pigmentation Production
0.5 mg/L	+	+
1.5 mg/L	+	+
3.0 mg/L	+	+
10.0 mg/L	+	+

Figure 1 shows the mean concentration of total lead at each temperature and pH value evaluated. It is shown that at pH 5, lower values of total lead were obtained, especially at a temperature of 35 °C (0.0964 mg Pb/L). Temperatures of 30 °C and 25 °C led to 0.1114 mg Pb/L and 0.1320 mg Pb/L, respectively. A Tukey test indicated that the mean total lead values at pH 5 were statistically similar when treated at 30 and 35 °C but different when treated at 25 °C ($p < 0.05$). A pattern similar to the pattern observed at pH 5 was found at pH 7, where the total lead means were higher, with the lowest total lead value (0.1516 mg Pb/L) at a temperature of 35 °C, while the average values were 0.1597 and 0.1829 mg Pb/L at 25 °C and 30 °C respectively. Similarly, the values of the means at 30 and 35 °C were statistically the same ($p \geq 0.05$), but different from the values obtained at 25 °C ($p < 0.05$).

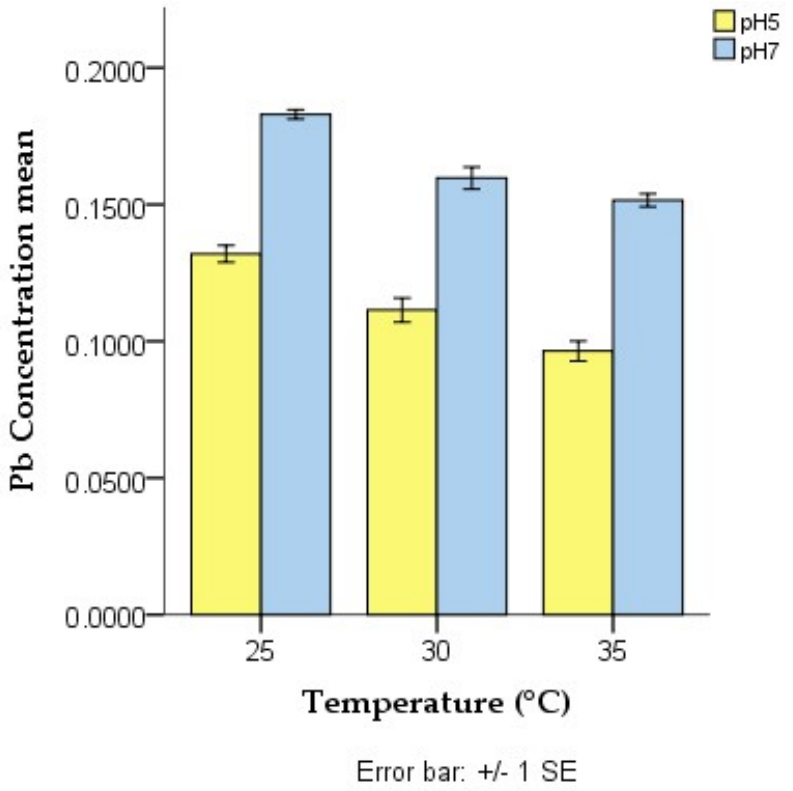

Figure 1. The mean concentration of total lead (mg Pb/L) after 24 h of incubation with *S. marcescens* in bioreactors with temperature and pH variation.

Figure 2 shows the percent mean total lead bioremoval using *S. marcescens* bacteria and a combination of temperatures and pH. The highest percentage of lead removal (64.02%) occurred with treatment 5, which consisted of *S. marcescens* in a medium adjusted to pH 5 and subjected to a temperature of 35 °C. The percentages in decreasing order correspond to treatments 3 (58.37%), 1 (50.76%), 6 (43.45%), 4 (40.55%), and 2 (37.74%). The results obtained by treatments 4 (pH 7, 30 °C) and 6 (pH 7, 35 °C) were statistically equal according to Tukey's test. However, all show statistically different total lead removal values from the control (T0).

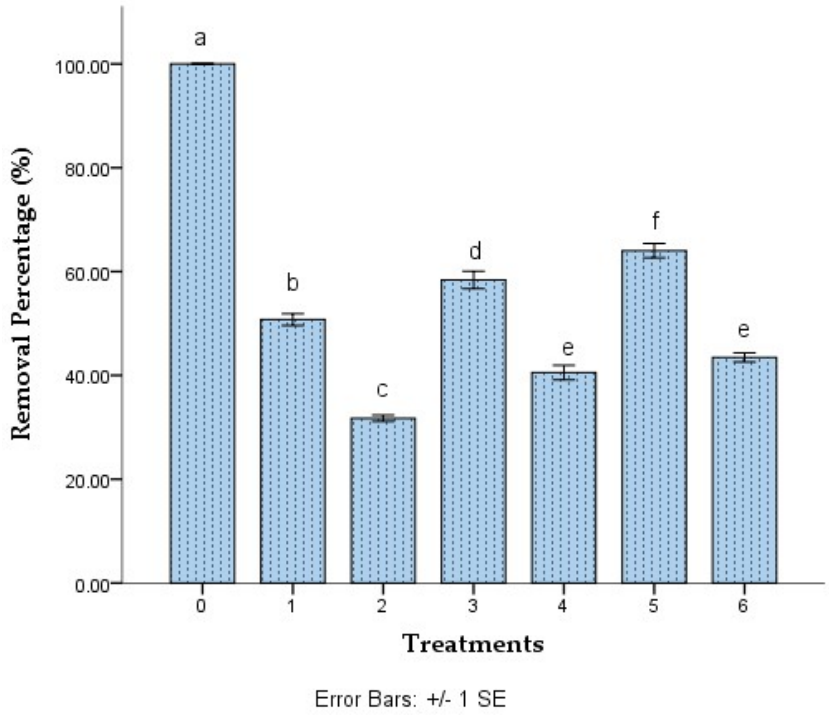

Figure 2. Total lead bioremoval percentage in surface water samples after applying six treatments (T) using the *S. marcescens* bacteria, and temperature and pH variation. Different letters mean significant statistics according to the Tukey test.

4. Discussion

The total Pb value in the water of the La Victoria stream was 0.268 mg/L. This value decreased to 0.0964 mg Pb/L in 24 h after being treated with *S. marcescens* at a temperature of 35 °C and pH 5 (treatment 5). However, the Peruvian Regulation (Supreme Decree ECA N° 004-2017) accepts minimum Pb values up to 0.05 mg Pb/L [25]. Likewise, the results show that lead had persisted in these waters since 2018, when a value of 0.5647 mg Pb/L was reported. In 2019, it was reported that 0.2650 mg Pb/L represents a risk to the environment because nearby crops are irrigated with this polluted water, threatening life.

The *S. marcescens* tolerance test was necessary due to the amount of Pb found in the La Victoria stream. In this way, four concentrations (0.5, 1.5, 3.0, and 10 mg Pb/L) of Pb were tested, as shown in Table 3. The result shows that the Gram-negative bacterium can tolerate all Pb concentrations tested, resulting in the typic colony and its red pigmentation. Tolerance may be supported by the presence of proteins called metallothioneins, which are involved in regulating the transport of metals into and out of bacterial cells [26–29]. In 2023, a study performed by dos Reis Ferreira showed the presence of the *zntR* gene, which encodes a protein responsible for regulating the production of ZntA, a transmembrane protein that facilitates Pb^{2+} extrusion out of the cell [30]. The finding suggests that *Serratia* possesses some mechanisms of resistance to lead.

The difference between the values obtained by varying the temperature and pH is notable (Figure 1). At a higher temperature, the lowest values of total lead are recorded, both at slightly acidic pH (pH 5) and at neutral pH (pH 7). The study by Wróbel et al. (2023) mentions that there are various mechanisms for the removal of heavy metals with high temperatures [31]. Similarly, Pan et al. (2022) mention that the biosorption process is faster when the temperature is higher due to increased diffusion through the boundary layer and increased diffusion within the adsorbent [32]. By comparison, Yaashikaa et al. (2022) attribute a higher mobility of heavy metals to the increase in absorption sites during pore rupture [33]. Therefore, pH is one of the most important parameters that affect the solubility of metal ions and functional groups in the cell walls of microorganisms [34].

Regarding Figure 2, the best bioremoval percentage was 64.02% using treatment 5 (pH 5 and 35 °C). At the same temperature and at pH 7 (treatment 6), a bioremoval percentage of total Pb of 43.45% was achieved. According to Dammak et al. (2022), the best removal percentages are achieved between pH 5 and 6 [35]; likewise, Kumar et al. (2022), who worked with *Saccharomyces cerevisiae*, showed how the bacterial strain showed resistance to high concentrations of Pb (II) (2200 mg/L) through MIC, therefore, tests were carried out under different parametric conditions such as; the initial concentration of Pb (II), pH (5), temperature, NaCl concentration, stirring speed, treatment time, biomass concentration, concluding that the optimal conditions were 4% *w*/*v* NaCl, pH 6, temperature of 35 °C, 140 rpm and 1 g/l biosorbent dose, obtaining a maximum percentage of 99.19% with live biomass and 97.18% with dead biomass [36]. It has also been proven that the acidity of the solution is an important factor affecting the biosorption of metal ions, as highlighted by Mohapatra et al. (2019). The pH is directly related to hydrogen ions' ability to compete with metal ions on the biosorbent surface, resulting in better removal percentages [37]. The lead removal results with treatment 5 are similar to the results obtained by Mohapatra et al. (2019) who used the marine bacterium *Bacillus xiamenensi* with pH 5 and a temperature of 35 °C, achieving high removal percentages of 99.19% with live biomass and 97.18% with dead biomass [37]. Therefore, the Pb removal capacity was demonstrated with the *S. marcescens* bacterium at pH 5 and 35 °C. Different authors have demonstrated the efficacy of the bacterium to remove heavy metals, as in the study by Qian et al. (2018), who used the bacterium to reduce Cu (II), evaluating the components of extracellular polymeric substances and the physiological activities of the biofilms of plankton cells and the bacterium Serratia marcescens in a microbial electrosynthesis system, the conditions evaluated in this research were a concentration of up to 80 mg/L of Cu (II), with a potential cathode current of 900 Mv vs. Standard hydrogen electrode, concluding that a simultaneous reduction of Cu (II) 6.42 ± 0.02 mg/L/h was achieved [38]. Similarly, Lv et al. (2022) obtained a percentage of Mn (II) removal of 64.25% [39], and Cristani et al. (2012) demonstrated the capacity of the *Serratia marcescens* bacterium to eliminate different concentrations of Pb, Cd, and Cr [28].

There are still challenges, such as the influence of physicochemical factors that could optimize lead bioremoval by *S. marcescens* [40]. To optimize the removal, it is important to evaluate the behavior of heavy metals together with the characteristics of the adsorbent; in the literature, it has been found that the mechanism of biosorption is not yet fully understood as this process is determined by physiology, chemical and physicochemical factors, ionic strength, i.e., metal ion chemistry, pH, temperature, metal concentration, contact time, the composition of the cell wall, and physiological characteristics of the microorganism [41–43]. Although the removal of Pb was not considerable or close to the permitted levels (0.05 mg/L) according to Peruvian legislation, possibly due to extrinsic and intrinsic aspects of the bacterium or due to independent variables that were not taken into account, for example, the contact time, this study serves as a reference to establish differences regarding the methods of removal of Pb by the bacterium *S. marcescens* [44,45].

5. Conclusions

Treatment 5 (temperature 35 °C, *S. marcescens* 10^8 CFU/mL, and pH 5) was the treatment that presented the best results in terms of total bioremoval of lead in water from La Victoria stream after 24 h of incubation. The results showed a reduction in the values of concentration from 0.268 to 0.0964 mg Pb/L (24 h), with a percentage of total lead removal of 63.9%. However, it is necessary to carry out future studies on the mechanisms of lead tolerance and bioelimination by *S. marcescens*, taking into account the influence of the other parameters. Likewise, the study time must be longer to identify the stage of biosorption of microorganisms with lead to improve the accuracy and precision of future results. These results represent an eco-efficient alternative, which nonetheless still has limitations to its application in situ.

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