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# The Ability of a Bacterial Strain to Remove a Phenolic Structure as an Approach to Pulp and Paper Mill Wastewater Treatment: Optimization by Experimental Design

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**Abstract:** High-colored wastewater generated during the cellulose bleaching process causes the inhibition of biological activity when released into the environment. This study aimed to evaluate the bacterium's capacity, identified as RGM2262, to degrade a complex phenolic structure such as lignin, which is found in high concentrations in the effluents generated during the production of cellulose, raw material for the manufacture of paper. To determine the values of the experimental variables that allow for a greater degradation of organic matter, an experimental model was carried out through experimental design. Thus, the experimental matrix was obtained with the variables pH 7 (−1) to 9 (+1) and a treatment time of 1 day (−1) to 5 days (+1). The results show that, at pH 8 and pH 9, both treatments—with bacteria in bio-films and without bio-films—were efficient. On the second day of treatment, 100% of the color and the phenolic structure were removed, with a similar rate constant, and at the same time, 80% COD and 70% of TOC, respectively.

**Keywords:** phenolic compounds; pulp and paper industry; pollution control; wastewater cleaning; water sustainability



**Citation:** Yeber, M.C.; Silva, T. The Ability of a Bacterial Strain to Remove a Phenolic Structure as an Approach to Pulp and Paper Mill Wastewater Treatment: Optimization by Experimental Design. *Water* **2022**, *14*, 3296. https://doi.org/10.3390/w14203296

Academic Editor: Chengyun Zhou

Received: 27 July 2022
Accepted: 9 October 2022
Published: 19 October 2022



## 1. Introduction

The residual phenolic compounds in the pulp and paper process generate highly colored wastewater, which causes biological activity inhibition when released into the environment, producing harmful environmental effects [1]. Phenolic compounds detected in sediments in a pulp industry effluent discharge zone have a direct relationship with the hormonal changes in the species that inhabit the area due to the continued exposure to these compounds [2]. The toxicity of cellulose effluent has several effects on the reproductive system in aquatic flora and fauna, such as a reduction in the size of the gonads, a change in the secondary sexual character, late maturity, and the suppression of the sex hormone in fish [3]. Therefore, alternatives for reducing the organic matter before its discharge into the environment, or for its reuse in the industrial process, are studied [4–11]. However, these processes fail to transform the compounds into more bioavailable organic matter; they only concentrate the organic matter that must be deposited in a landfill later, which indicates that the contamination is not eliminated but rather only transferred from one place to another. Lately, the use of microorganisms has been a promising alternative for cleaning contaminated environments in the so-called bioremediation processes; thus, the use of several and varied bacterial strains for degrading different pollutants has been tested. Some researchers have isolated bacteria from contaminated effluents that have the potential for use in bioremediation—for example, studied strains resistant to high concentrations of phenol, which would be an excellent bacterium to treat wastewater containing refractory organic matter [12,13]. The behavior of co-culture in the degradation and decolorization of pulp mill wastewater was also studied to search the degradability of kraft lignin—for example, the potential bacterial strains Bacillus subtilis (GU193980) and

Klebsiella pneumoniae (GU193981) were isolated and used in the process to treat the pulp and paper mill waste. The results show that the bacteria mixture was efficient in removing 80% of the color and reducing the other pollution parameters, such as COD by 73% and BOD by 62% [14].

Other studies isolated 12 strains, of which the most abundant and promising genera in degrading lignin were mesorhizobium, cellulosimicrobium, pandoraea, achromobacter, and stenotrophomones, achieving an average of 30% removal [15]. Additionally, in studies of the conglomerate of microorganisms in a sludge that contained 4-chlorophenol, with genus pseudomonas being the more abundant strain, Proteobacteria and Firmicutes were dominant in the chlorophenol degradation [16]. Different studies have referred to the analysis of the degradation of toxic compounds using bacteria consortia, with two or three strains and sometimes up to four [16–21]. The biodegradation of an industrial mixture of PCBs was performed using bacterial consortia composed of four bacterial strains isolated from the contaminated sediments. The consortium containing two strains showed a good degradation of the highly chlorinated PCB. The consortium made up of three bacterial strains showed a higher biodegradation, reaching 73% of the degradation of the organic compound and reducing the toxicity in 7 days [22,23]. Co-cultures using microalgae have also been used to degrade the effluents derived from the olive oil mill, effluents that contain high concentrations of phenolic compounds that are difficult to degrade. The treatment consisted of two parts: aerobic and anaerobic. It allows, on the one hand, for a reduction in organic matter and the generation of hydrogen, but the biodegradation of organic matter is not achieved since the inverse process requires a high concentration of oxygen [24]. Fermentation processes have also been carried out using native bacteria to treat organic waste from the wine industry for hydrogen production [25].

The microorganisms are ecologically important in soil and water and are probably responsible for the aerobic degradation of many soluble compounds derived from animal and vegetable organic matter in decomposition. Thus, this study aimed to evaluate the capacity of the native bacteria to degrade phenolic compounds present in the pulp and paper industry wastewater, using the carbon of the molecules as a unique carbon source to generate energy for its growth without adding other nutrients. The importance of the use of microorganisms in the treatment of contaminated water is the need to reduce contamination using methods that are friendlier to the environment, and the handling of a single microorganism allows for the better management of its biomagnification in the system.

## 2. Materials and Methods

Pure water-soluble lignin with a molecular weight between 5000 and 28,000 g $mol^{-1}$ was used to prepare the 100 ppm solutions. The samples were incubated with the bacterial inoculum RGM2262 [24] at 30 °C for 5 days, which was prepared in Peptone Soybean Casein Agar (Merck). The viable cell counting method was used to determine bacterial growth. The residual Lignin was determined through a calibration curve at 280 nm and the residual color at 460 nm using a UV-1601 Shimadzu spectrophotometer. A 1 L reactor capacity to a flow of 200 mL $h^{-1}$ was used to carry out the degradation. The bacteria in biofilms were formed on a 21.5 × 2.0 cm polyethylene layer, whereby the inoculated bacteria remained in contact with the polyethylene for 24 h before it was used in the degradation treatment.

### 2.1. Chemical Oxygen Demand (COD)

The chemical oxygen demand (COD mg $O_2L^{-1}$) was determined using the Merck Spectroquant kit with a measured range of 25 to 1500 mg $O_2$ $L^{-1}$, according to the standard methods 5220 D and ISO 15705. Before analysis, the samples were refluxed for 2 h in a thermoreactor Spectroquant TR620 (Merck KGaA, Darmstadt, Germany).

### 2.2. Total Organic Carbon (TOC)

The Merck Spectroquant kit from 50 to 800 mg C $L^{-1}$ was used to determine the TOC. The samples were refluxed for 2 h in a thermal reactor Spectroquant TR620, and the TOC was analyzed using a Spectroquant® NOVA 60 (Merck KGaA, Darmstadt, Germany). The TOC was expressed in mg of C $L^{-1}$ according to the standard method: ISO 84661-1 and DIN38402 A51.

### 2.3. Bacterial Growth

Bacterial growth was determined using the serial dilution method in agar, which was standardized with the McFarland standard. The bacterial inoculum was incubated for 24 h at 30 °C. Viable bacterial colonies were counted every 24 h for a total of 120 h, recording the number of colony-forming units (CFUs).

### 2.4. Multivariate Analysis

The experimental design was used to evaluate the optimal response of the bacteria to the degradation of the phenolic compounds. The design was constructed with the MODE 7.0 program with two experimental variables—a pH between 7 (−1) and 9 (+1) and a treatment time in days between 1 (−1) and 5 (+1). A matrix with 12 experiments was given by the quadratic model, with a factorial design $2^n$, as shown in Table 1. The design runs with nine combinations between the variables, of which four correspond to the minimum and maximum, and three correspond to the central points and replicas. The observed response was the value obtained experimentally, and the predicted response was the value given by the design according to the value of the variable used. Both responses are given as a removal percentage of phenolic compounds.

**Table 1.** Experimental Matrix Full Fac (three levels). It shows the experimental name, the order in which the experiments were run, and the values of the variables studied.

| Experimental Name | Run Order | pH Coded (Uncoded Value) | Time (Days) Coded (Uncoded Value) |
|---|---|---|---|
| N1 | 6 | 7 (−1) | 1 (−1) |
| N2 | 10 | 9 (+1) | 1 (−1) |
| N3 | 8 | 7 (−1) | 5 (+1) |
| N4 | 1 | 9 (+1) | 5 (+1) |
| N5 | 2 | 8 (0) | 3 (0) |
| N6 | 4 | 8 (0) | 3 (0) |
| N7 | 9 | 8 (0) | 3 (0) |
| N8 | 3 | 8 (0) | 3 (0) |
| N9 | 12 | 8 (0) | 1 (−1) |
| N10 | 5 | 7 (−1) | 3 (0) |
| N11 | 11 | 9 (+1) | 3 (0) |
| N12 | 7 | 8 (0) | 5 (+1) |

## 3. Results and Discussion

Figure 1 shows the response surface for the optimization of the bioremediation process, without the bacteria forming biofilms. As can be seen, a higher pH increases the efficiency in the degradation of the effluent; this is beneficial because cellulose effluents are characterized by a high pH, which would avoid the addition of more chemicals to neutralize the effluents before treating them with bacteria.

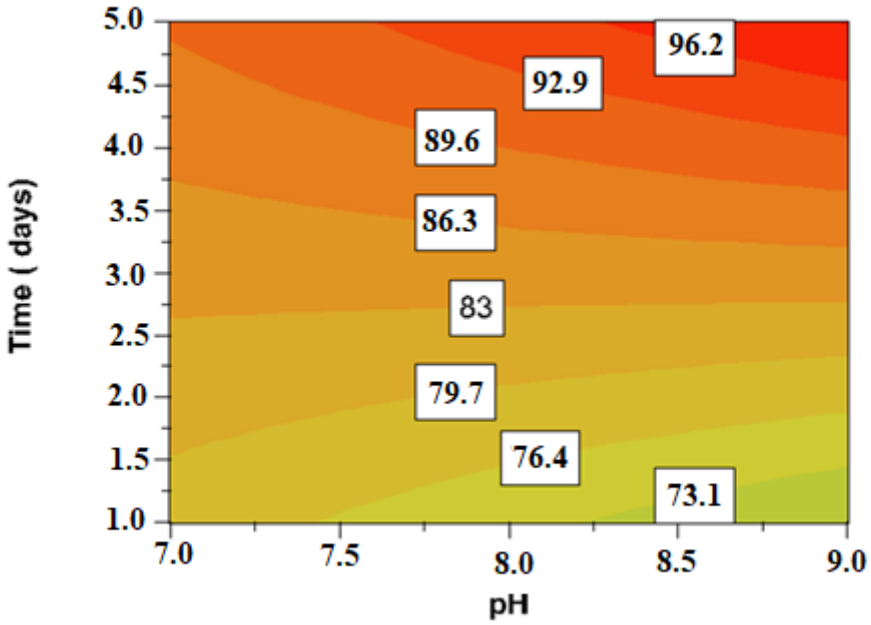


**Figure 1.** Response surface for the bioremediation process optimization in the function of the variables pH and treatment time with the bacteria without biofilms (the values on the figure indicate the removal achieved in terms of a percentage).

Figure 2 shows the observed versus predicted values of the response. The regression line indicates a good model, and the experimentally observed response of $R^2$ = 0.997 and the predicted response given by the experimental design of $Q^2$ = 0.995 present a good regression, indicating that the model is reliable, with 95% confidence and a *p*-value of $p < 0.0001$.

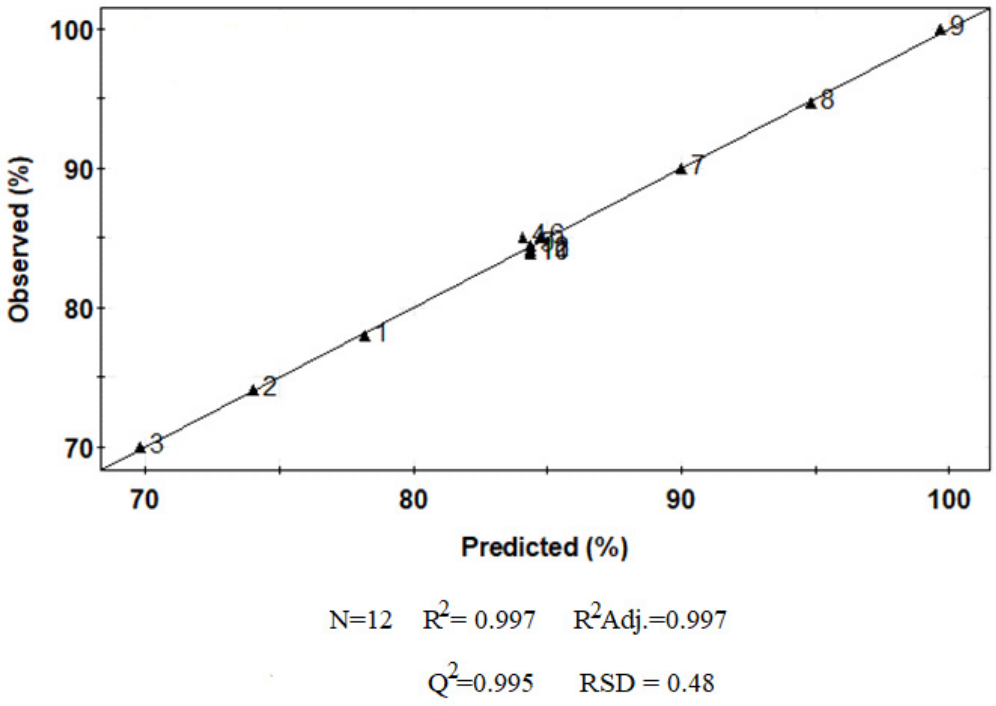


**Figure 2.** Observed versus predicted values of the response given by the experimental design for the bioremediation process optimization in the function of the variables pH and treatment time with the bacteria without biofilms.

Figure 3 shows the response surface for the optimization of the bioremediation process with the bacteria forming biofilms. It was observed that the degradation efficiency increased with the pH and the time; a linear relationship was observed between the variables with an efficient removal throughout the studied pH range. In both models studied, it was observed that the greatest removal was achieved at a pH between 8 and 9, the percentages being higher with the bacteria forming biofilms. This may be because the bacteria remain immobile, so they do not spend the energy needed to mobilize, occupying it only to consume the organic matter carbon and achieve its degradation. The linear regression for the experimental design using the bacteria forming biofilms is observed in Figure 4. In this optimization process, the values of the observed and predicted response present a good regression too, indicating that the model is reliable, with a 95% confidence and a *p*-value of $p < 0.0001$.

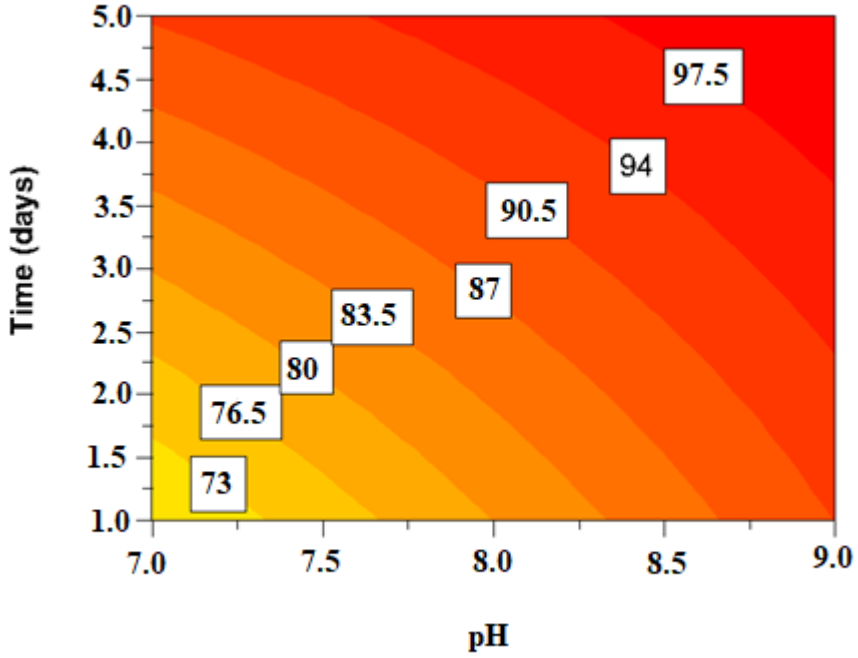


**Figure 3.** Response surface for the bioremediation process optimization in the function of the variables pH and treatment time with the bacteria forming biofilms (the values on the figure indicate the removal achieved in terms of a percentage).

As is observed for both models, a high degradation was obtained in the entire pH range studied, so to corroborate the response given by the experimental design, the degradation was followed in time to corroborate that the response obtained by the experimental design is correct. Figure 5 shows that, at pH 7, the Lignin removal was slower when the bacteria were in biofilms compared to bacteria without biofilm. However, both treatments were effective in removing lignin, achieving 80% on the first day of the treatment with the bacteria without biofilms and reaching 90% by the fifth day in both cases. At pH 8, the bacteria forming biofilms removed the lignin with greater efficiency during the first day of treatment, achieving 80%. Nonetheless, by the fifth day, it was possible to remove 100% of the lignin in both cases. At pH 9, the same behavior for both treatments was observed, with the maximum removal of lignin achieved on the fourth day.

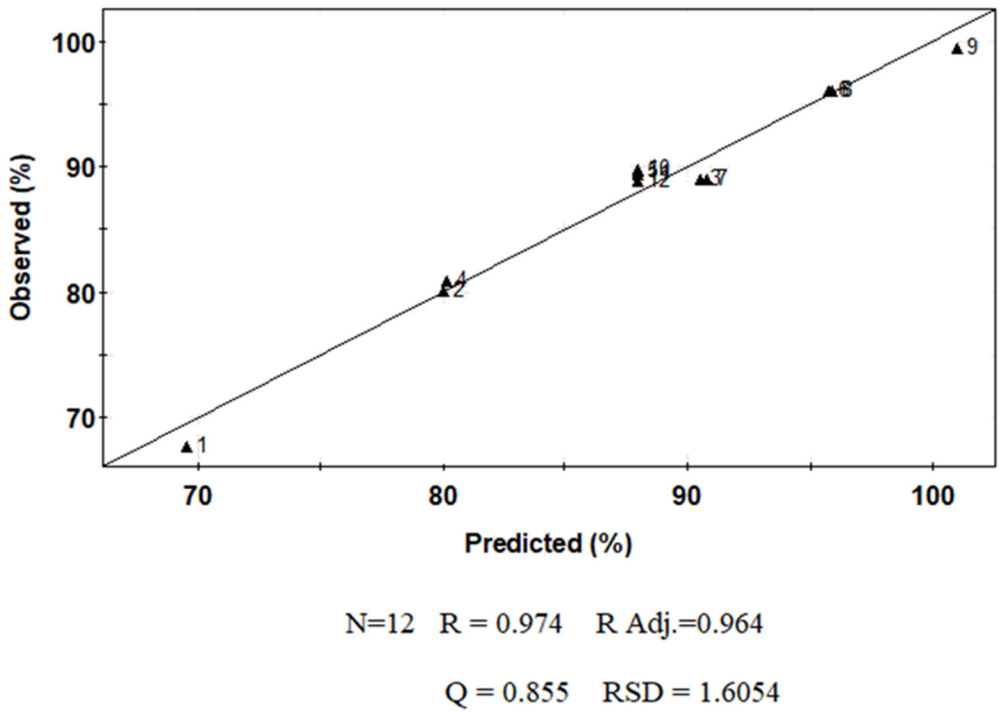


**Figure 4.** Observed versus predicted values of the response given by the experimental design for the bioremediation process optimization in the function of the variables pH and treatment time with the bacteria forming biofilms.

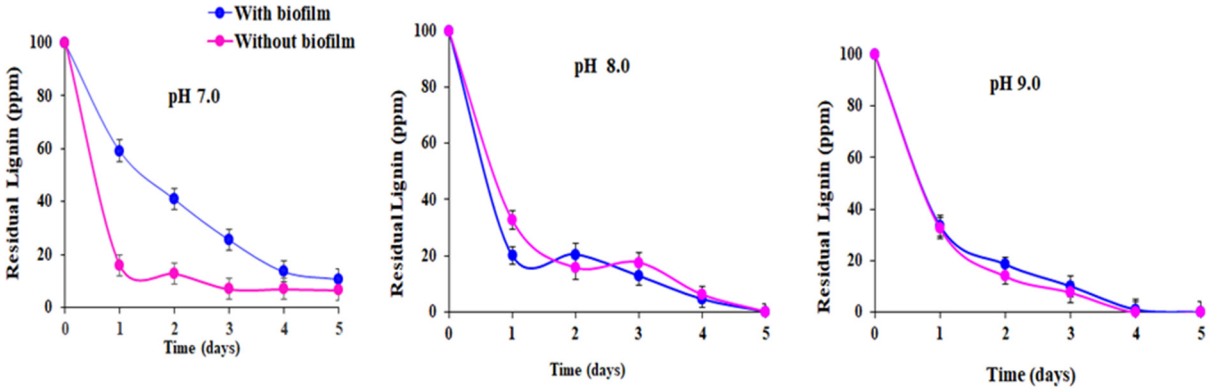


**Figure 5.** Lignin removal from the effluent by bacterial strain RGM2262 at the different pHs studied.

Several authors have studied the efficiency of different bacteria in removing color and lignin from pulp and paper mill wastewater by studying a group of bacteria in a nutrient-enriched medium. They have determined that the bacterium *Acinetobacter calcoaceticus* achieves 51% and the bacterium *Klebsiella pneumoniae* achieves 29% of lignin removal [26]. Others have isolated a group of bacteria from sludge in a pulp and paper industry and identified them as *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus*, and Bacillus sp., which were also used for the degradation of lignin [27,28]. The bacteria were incubated in an enriched medium with glucose and peptone at pH 7.6 for 6 days, *Bacillus* sp. achieving 37% of degradation, making this bacterium the most efficient in lignin removal. Comparing the results obtained in this research with those found in the literature, the efficiency of this system in degrading high concentrations of phenolic compounds in reduced treatment times can be recognized.

The color removed is shown in Figure 6, where a similar behavior to the lignin removal at the different pHs studied can be observed. As seen at pH 7.0, color removal is slower

than that for the other two pHs. The highest discoloration is achieved at pH 8.0 and pH 9.0, reaching 100% on the second day of treatment, which corroborates what was shown by the experimental design. At the same time, the kinetics of lignin degradation and discoloration are corroborated by the rate constants shown in Table 2, where a good correlation between the rate constants and the degradation achieved in each treatment is observed.

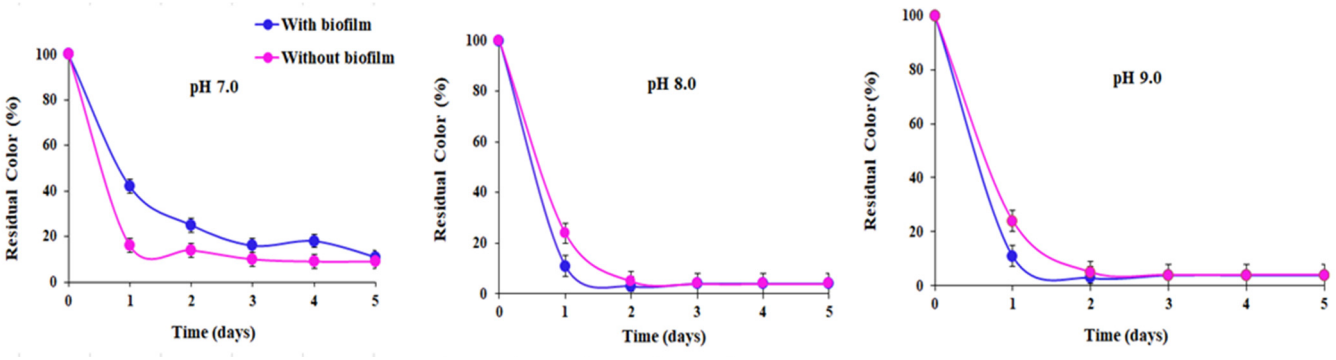


**Figure 6.** Removal of color from the effluent by bacterial strain RGM2262 at the different pHs studied.

**Table 2.** Rate constants for the different treatments with bacterial strain RGM2262.

| Lignin Removed (%) | pH | With Biofilm | | Without Biofilm | |
|---|---|---|---|---|---|
| | | $K_v$/day | $R^2$ | $K_v$/day | $R^2$ |
| 90.0 | 7.0 | 11.0 | 0.60 | 17.0 | 0.99 |
| 100.0 | 8.0 | 12.4 | 0.90 | 12.4 | 0.86 |
| 100.0 | 9.0 | 32.4 | 0.94 | 32.4 | 0.92 |
| Color Removed (%) | | | | | |
| 90.0 | 7.0 | 9.60 | 0.89 | 9.60 | 0.67 |
| 100.0 | 8.0 | 12.0 | 0.60 | 14.9 | 0.74 |
| 100.0 | 9.0 | 12.0 | 0.60 | 14.9 | 0.74 |

In other works, an effluent was treated anaerobically for 7 days and was further treated by aerobic microorganisms, achieving a maximum reduction of lignin of 25% and a reduction of COD of 47%. Also using a ligninolytic bacterium at pH 7.6, the lignin and color removal of the effluent was enhanced by adding carbon and nitrogen as additional nutrients [29,30].

In this study, the COD removal by the bacteria achieved 80%, indicating that the bacteria were efficient in degrading phenolic compounds derived from the pulp and paper industry. It is evident that, at higher pH values, the removal increases, as lignin is more soluble at a more basic pH, making it easier for the bacteria to incorporate the lignin as food into their enzymatic system and degrade it. A previous study on the capacity of three native bacterial strains used to degrade pulp and paper mill effluents, again using the bacteria in the consortium in the treatment, showed that they were able to reduce the COD by 76% within 10 h. However, when the bacterial cultures were used separately, the results were not promising [31]. It should be noted that, in this study, no additional nutrients were added for bacterial growth. Bacteria can reproduce by consuming only the carbon present in organic matter, as shown in Figure 7.

Other authors have reported specific microbial species with the capability to degrade organic compounds present in pulp and paper industry wastewater. Different species of Micrococcus and Staphylococcus, *Kurthia zopfii*, *Alcaligenes faecalis*, and *Pseudomonas aeruginosa*, were used individually or in a consortium and reduced a low percentage of organic matter, achieving only 30% of COD removal in 6 months of treatment [32,33]. Additionally, immobilized bacteria showed the removal of lignin, color, and COD from the effluent by 74%, 81%, and 85%, respectively, after 60 h of treatment at pH 7.6. A high

variety of works using microorganisms with different strains isolated from contaminated sites to treat wastewater from the pulp and paper mills have been reported, presenting low-efficiency results in long treatment times [34–37].

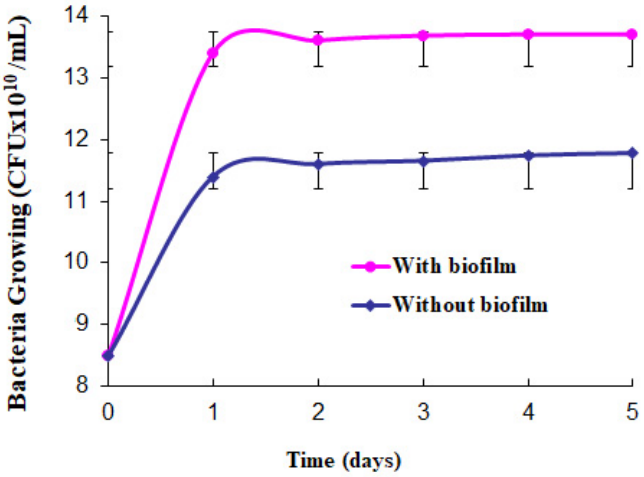


**Figure 7.** Bacterial growth during the Lignin removal.

The efficiency of the bioremediation process carried out by the bacterial strain RGM2262 can also be shown by means of the mineralization of the organic matter measured through the TOC, which reached 70%, indicating that the organic matter was consumed by the bacteria for its growth and that Lignin was biotransformed, mineralizing into $H_2O$ and $CO_2$. At the same time that the lignin is removed, the bacteria multiply, demonstrating that the bacteria strain can use the carbon of the lignin structure as the only source of nutrients, making it efficient in its degradation in the first 24 h of incubation. Studies about using the pulp and paper mill effluent as a carbon source showed that the bacterial growth reached a good number of CFU/mL, coinciding with the COD and TOC removal [38–41].

Figure 8 shows the correlation at the different pHs studied to determine the grade of the organic structure removal. As was observed, at a higher bacterial growth, both the lignin and color were removed by 100%, reducing the chemical oxygen demand and the total organic carbon. At the same time, an increase in the bioavailability of organic matter is observed. The initial TOC/COD ratio was 0.39, changing to 0.75 after treatment, which indicates that, during the process, the bioavailability of organic material increases considerably, indicating that the degradation products will be more easily consumed by microorganisms in the environment if the wastewater is discharged after treatment. In Figure 8A, the synergism between the expected responses can be observed, that is, as the bacteria grow, the removal of lignin and color increases, while the chemical oxygen demand decreases. This is highly promising, since it is shown that bacteria can grow and degrade high concentrations of phenolic organic matter. In Figure 8B, it is also observed that, with the high chemical oxygen demand removal, the bioavailability of organic matter increases considerably. This is highly promising in the treatment of highly colored industrial wastewater with a high concentration of non-bioavailable organic matter.

Figure 9 shows a theoretical scheme of how the degradation process of the complex organic structure of lignin could be carried out through the consumption of organic carbon by bacteria, which is reflected in the TOC decreasing and the bioavailability of organic matter increasing. Studies show the production of rhamnolipids by different bacteria through glycerol consumption as a carbon source, so it can be expected that the mechanism of lignin degradation may also occur in this way [42,43].

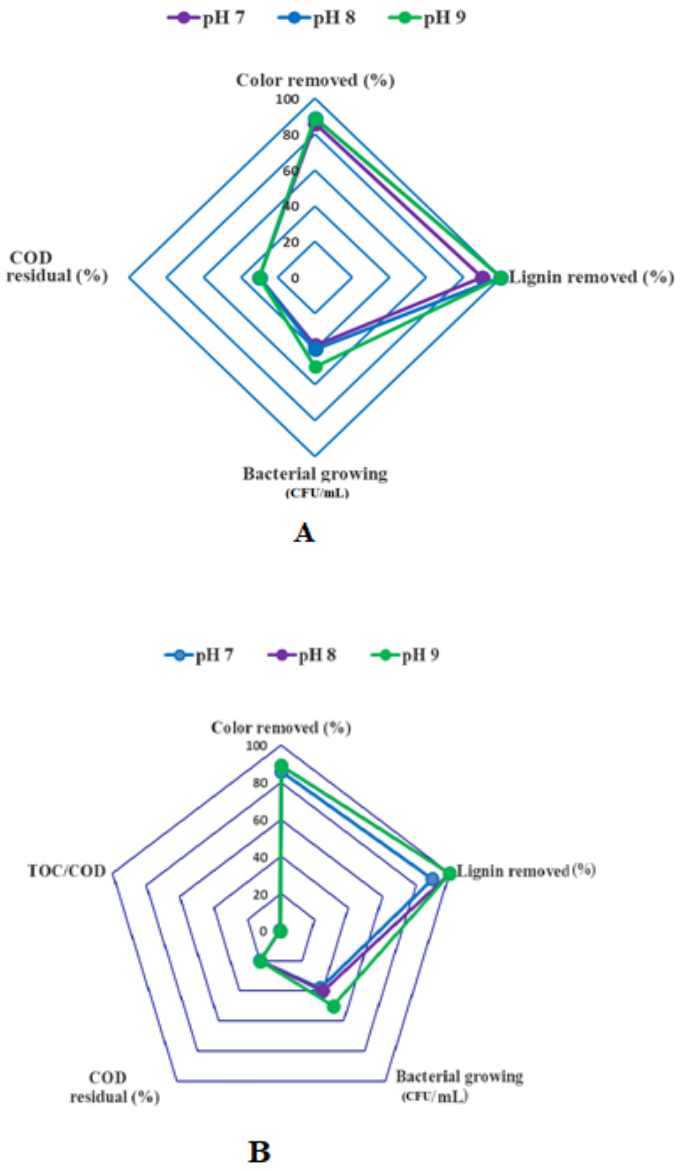


**Figure 8.** Relation between the parameters studied in the optimization of lignin degradation by RGM2262.

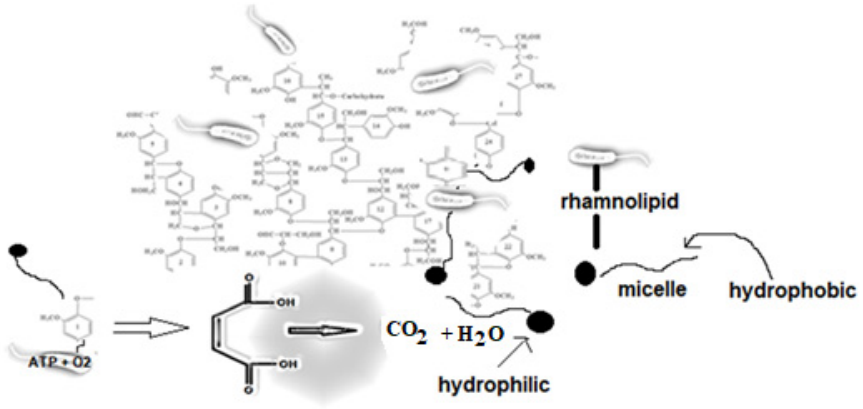


**Figure 9.** Theoretical approach degradation of phenolic compounds by the native bacteria RGM2262.

## 4. Conclusions

Bacteria RGM2262 was able to degrade a high concentration of lignin from a pulp and paper mill, using the carbon of its structure as the only energy source for its growth. The bacteria in both cases—forming biofilm and free—were effective in degrading organic matter, resulting in their complete removal at a more basic pH after 24 h of treatment. This result is relevant, since the effluents generated in the pulp and paper mill are characterized by a high pH, which eliminates a previous treatment such as the neutralization of the effluent. On the other hand, the higher basicity improved lignin solubility, facilitating their passage through the layer of exopolysaccharides in the biofilms. The results show that the bacteria can be used in the bioremediation of wastewater that contains high concentrations of phenolic organic matter, transforming the compounds to less hazardous or non-hazardous forms without having to add additional chemicals to the effluent. Additionally, the experimental design carried out for the optimization of the wastewater treatment using bacteria showed a good correlation between the variables studied and the response obtained. The observed R and Q values close to one indicate an accurate representation of these relationships.

**Author Contributions:** M.C.Y.: construction of the figures, construction of the experimental design, analysis and interpretation of the results, search for funding and writing of the manuscript. T.S.: Laboratory experimental work. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors acknowledge the financial support from ANID InES Ciencia Abierta INCA210005. The authors thank Carolina Soto for contributing to this work by providing the bacteria.

**Conflicts of Interest:** The authors declare no conflict of interest.

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