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Article

Enhancement of Rhamnolipid Production in *Pseudomonas aeruginosa* 6k-11 by Increasing Dissolved Oxygen and Using Residual Frying Oil as Carbon Source

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Abstract: Rhamnolipids (RL) are biosurfactants that have potential in environmental and industrial applications, produced mainly by *Pseudomonas aeruginosa*. However, their production on an industrial scale still faces the challenge of improving the efficiency and cost-effectiveness of the process. The aim of this work was to optimize the cultivation conditions to maximize the production of RL, adjusting key parameters of the process such as oxygen level, agitation, temperature, nutrients, and using residual frying oil as the using residual frying oil as an economical carbon source. An experimental design based on the response surface (RSM) and the central composite design (CCD) was used.to conduct 39 experiments in which different combinations of oxygen, agitation, temperature, nutrients and different combinations of nitrogen concentration, aeration rate and agitation speed were evaluated. The optimized parameters were 3.04 g/L of nitrogen, 0.5 vvm of aeration and 180 rpm of agitation. Using these parameters, a production of 52.2 g/L was achieved in 168 hours, together with the use of residual oil, are fundamental to improve the efficiency of the process. These advances will contribute to a more sustainable and cost-effective production of RL, which would promote its application in bioremediation processes and other industries.

Keywords: aeration rate; agitation speed; rhamnolipids; residual frying oil

1. Introduction

Biosurfactants are surface active molecules (surfactants) produced by microorganisms. These compounds stand out for their biodegradable quality, minimal toxicity and high efficiency in reducing surface and interfacial tension, characteristics that make them a more attractive alternative compared to synthetic surfactants, which usually have a negative impact on the environment [1]. Rhamnolipids are biosurfactants, produced by *Pseudomonas aeruginosa*, which have gained prominence in various industries thanks to their ecological properties, for this reason and due to their emulsifying and hydrocarbonoclastic capacity, is that bioremediation is one of their main applications [2]. However, although rhamnolipids are the most produced and researched biosurfactants, their production on a commercial scale is limited to a few companies compared to the production of synthetic surfactants even though the latter are harmful to the environment as they are mainly derived from petroleum [3].

The disadvantage of biosurfactants over synthetic surfactants lies in the fact that a sufficiently convincing and economical level of technology for biological production on an industrial scale has not yet been reached. This prevents rhamnolipids from managing to compete with the low production costs of synthetic surfactants [3,4]. The challenge of achieving economically sustainable production of rhamnolipids can be addressed by optimizing culture conditions, ranging from finding an economical carbon source, such as industrial waste oils [5], to standardizing ideal physicochemical

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conditions that promote high rhamnolipid yields, such as employing limiting concentrations of nitrogen and/or phosphate [6].

Making the leap from laboratory-scale rhamnolipid production to industrial-scale production requires that the conditions of temperature, dissolved oxygen (DO), pH, agitation speed (rpm) and volumetric air flow per volume of medium [vvm] be first optimized on a small scale [7,8]. From previous trials in *P. aeruginosa*, it has been reported that low dissolved Oxygen concentrations promote the specific growth rate, while high concentrations increase the specific rhamnolipid formation rate [9].

Therefore, since the aeration rate (vvm) and agitation speed (rpm) regulate oxygen availability [10], it is important to determine the appropriate values that allow an optimal and usable dissolved oxygen concentration to increase the rhamnolipid production yield of *P. aeruginosa* at the laboratory scale is influenced by certain operational parameters that affect the quantity and quality of the final product. One of the key factors is the carbon source used in the culture medium. For example, the use of waste cooking oil as the sole carbon source, due to its lipid nature, has been shown to increase biosurfactant yield compared to other substrates, such as glucose or glycerol [11,12].

In previous research, developed at the Laboratory of Microbiology and Microbial Biotechnology (UNMSM), efforts have been directed to optimize several factors involved in the production of rhamnolipids to increase the yield of the 6k-11 strain and, in that sense, this research proposes to determine the aeration and agitation rate that, at limiting concentrations of nitrogen and using a low-cost recycled carbon source, to raise the rhamnolipids production.

2. Materials and Methods

2.1. Strain Reactivation and Scale-up

P. aeruginosa 6k-11 was reactivated in 3mL of TSB at 35 °C for 18 to 24 hours as a pre-inoculum for culture scale-up, using Siegmund and Wagner (SW) mineral medium optimized by Alcalde [13]. The biomass of the pre-inoculum was recovered from the culture broth by centrifugation at 14 500 rpm for 10 min, after that the pellet was washed twice with NaCl 0.85% and subsequently, the pellet was resuspended in 30 mL of SW medium, which was incubated at the conditions optimized by Guzman [14]: 31.26 °C and 185.8 rpm and with an initial pH of 6.76. Once the culture with a 1/80 dilution reached an optical density of 0.06, a volume of 7.34% (v/v) was used as inoculum in a 1L reactor with 250 mL of SW.

2.2. Evaluation of Bacterial Growth

The progress of bacterial growth influenced by different parameters such as aeration (0.5, 0.55, 0.6, 0.65 and 0.7 vvm) and agitation (160, 170, 180, 190 and 200 rpm) rates was monitored every 24 hours for 15 days, by measuring the optical density of the samples collected in triplicate using a spectrophotometer (UV-Vis) at 620 nm. Each 1mL sample was centrifuged for 10 min at 14 500 rpm, the supernatant obtained was used for rhamnolipid dosage, while the biomass was subjected to two successive washes with NaCl 0.85% [15]. The biomass concentration (g/L) was determined by replacing the optical densities data in the growth equation of the standard curve [16].

2.3. Monitoring of Rhamnolipid (RL) Production

Daily monitoring of RL production was carried out by the resorcinol method, employed by Guzman [14]. Prior to dosing with the colorimetric reaction, it was necessary to purify the RL.

2.4. Purification of Rhamnolipids

The supernatant separated from the biomass by centrifugation was acidified with concentrated HCl to pH 2.0, incubating the samples overnight at 4 °C. After incubation, it was centrifuged at the same conditions as the first step (14500 rpm for 15 minutes for 2 mL tubes or 4500 rpm for 30 minutes

 doi:10.20944/preprints202505.0075.v1

for 50 mL tubes). The precipitated RL were resuspended in 1 or 15 mL of ultrapure water (depending on the source volume of the sample). The resulting suspension was added with chloroform: ethanol (2:1, v/v), immediately after the samples were shaken vigorously for 20 minutes. Subsequently, the mixture was centrifuged once more, using the same centrifugation conditions. After this step, the organic phase was collected and evaporated at 80 °C in a water bath until the purified rhamnolipids (oily aspect) were obtained. Finally, the empirical concentration of RL produced was determined from the ratio of the weight to the volume of culture from which it was initially extracted.

2.5. Dosage of Rhamnolipids

The colorimetric detection method with resorcinol was used for RL dosage [17]. Purified RL from 1 mL of culture were resuspended in 1 mL of ultrapure basic water (pH 9). From the suspension obtained, an aliquot of 300 µL was taken to be dispensed into a screw-capped test tube protected from light, on top of which 300 µL of resorcinol (0.6 %) and 1.5 mL of H_2SO_4 (75 %) were added and incubated in a water bath at 90 °C for 30 min. Once the reaction was completed and stopped on ice, the spectrophotometer (UV-Vis) was used at 480 nm to determine the respective absorbance, which was used to determine the rhamnolipid concentration (g/L).

2.6. Dissolved Oxygen (DO) Concentration Monitoring

The DO in the culture was monitored daily to evaluate its response to the change in agitation speed and aeration rate employed in each trial, following that recommended by Bazsefidpar et al. [9]. DO concentrations were determined by inserting the galvanic cell sensor of a portable oximeter. During the 15 minutes prior to each measurement, the sensor was sterilized by exposure to ultraviolet radiation.

2.7. Evaluation of the Effect of Aeration on Dissolved Oxygen and RL Production

Aeration rate was optimized by a factorial design, using Response Surface Methodology (RSM) to determine its interaction with the other test variable, agitation speed, and its effect on the response variables: DO concentration and rhamnolipid production. The effect of aeration rate was evaluated at air flow levels: 0.5, 0.55, 0.6, 0.65 and 0.7 vvm, similar to Valladares [18]. The injected air was purified through an oxygen concentrator and to regulate the volumetric air flow per volume of gas flowmeters were used to regulate the volumetric flow of air per volume of medium. In addition, according to Ramesh et al. [19], the oxygen transfer coefficient (kLa) was calculated to directly relate the aeration rate and stirring speed to the dissolved oxygen concentration in the culture. The mass balance equation for oxygen was used to determine kLa.

2.8. Evaluation of the Effect of Agitation on DO and RL Production

A Response Surface Methodology (RSM) factorial design was employed to optimize agitation speed after evaluating its interaction with crop aeration rate and its effect on DO concentration and rhamnolipid production. Analogous to Valladares [18] and Guzman [14], the effect of agitation speed was evaluated using 5 levels of revolutions per minute: 160, 170, 180, 190 and 200 rpm.

2.9. Statistical Analysis

Each test was performed in triplicate. The Response Surface Methodology (RSM), with Design Expert 11.0 software, was used to optimize the agitation speed and aeration rate, the response variables were analyzed with the Composite Central Design (CCD). The statistical section of the same software was also used to determine the type of distribution and to analyze the existence of significant differences between the values of the results obtained for DO and RL concentrations.

3. Results

 doi:10.20944/preprints202505.0075.v1

3.1. Growth Kinetics

Monitoring the growth of *P. aeruginosa* 6k-11 biomass by means of kinetics revealed different behaviors according to the variation of the fermentation parameters evaluated. For example, with the parameters which corresponded to the central points of the experimental design (2.4 g/L $NaNO_3$, 0.6 vvm and 180 rpm), the bacteria developed with a specific microbial growth rate (μ) of 0.7 and reached the stationary phase between 120 and 216 hours (Figure **1**), within which they reached a maximum RL production of 20.4 g/L between 144 and 168 hours, averaged over the three replicates performed with the central points of the experimental model.

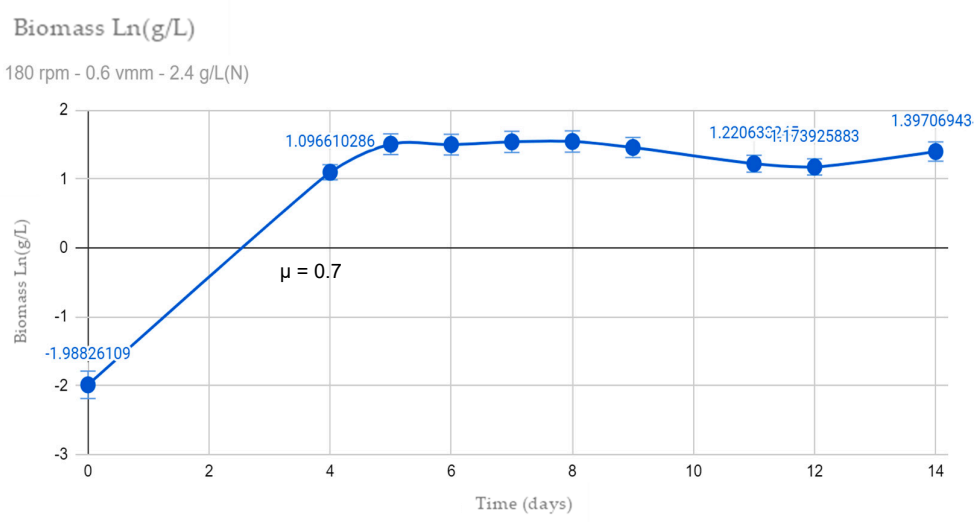

Figure 1. Growth kinetics of *P. aeruginosa* 6k-11 under fermentation conditions corresponding to the central points of the experimental design: 2.4 g/L NaNO3, 0.6 vvm and 180 rpm.

The monitoring of the dissolved oxygen (DO) concentration in the culture during production made it possible to determine the different values of the volumetric oxygen transfer coefficient (kLa). The DO measurements showed an inverse behavior to the biomass concentration, as shown in Figure **2**. The kLa value of the medium in the absence of biomass was 0.033 s -1 and for the central points, during the peak of RL production, a kLa of 0.43 s -1 was determined, with a specific oxygen consumption rate (qO_2) of 1.67 nmol O_2 .g cell $^{-1}$ .h $^{-1}$ and OD concentration of 117 % at 10.17 mg/L. The kLa was calculated using the mass balance equation for oxygen [20], described below:

$$\frac{\Delta[O_2]}{\Delta t} = (\text{kLa}) \times (C^*_{O_2} - C_{O_2}) - (qO_2 \times C_X)$$

where:

$\frac{\Delta[O_2]}{\Delta t}$: rate of change of oxygen concentration over time.

kLa: volumetric oxygen transfer coefficient.

$C^*_{O_2}$: oxygen concentration in air.

C_{O_2}: dissolved oxygen concentration in the culture.

qO_2: O_2 consumption rate of microorganisms.

C_X: concentration of the microorganism in the medium (biomass).

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This calculation made it possible to quantify how oxygen transfer to the medium is affected by both aeration and agitation rate, as well as the presence of biomass and oxygen demand during RL production.

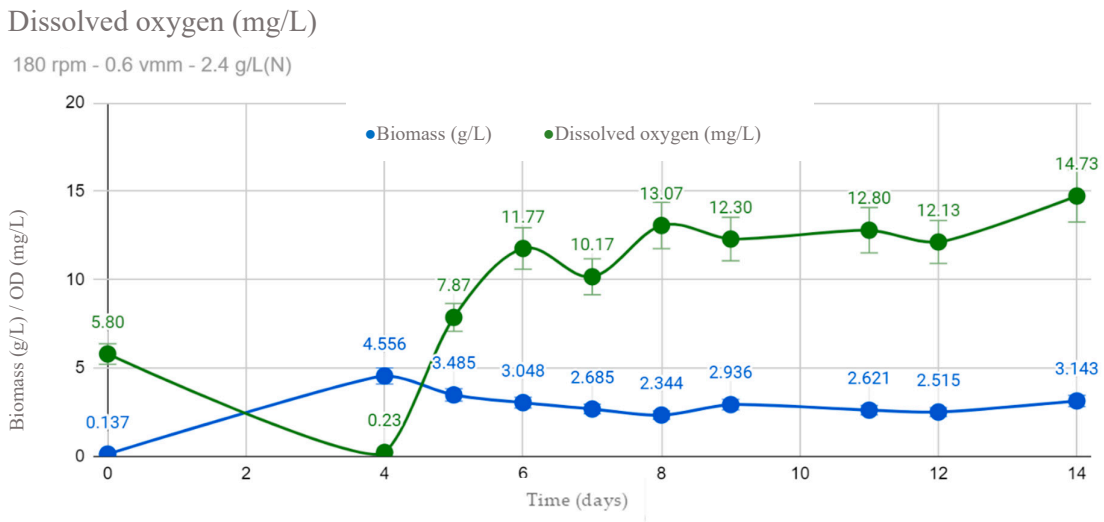

Figure 2. Progression of OD vs. growth of *P. aeruginosa* 6k-11 over time for the central points.

3.2. Influence of Aeration Rate

With respect to the five levels of air rate supplied to the fermentation systems, it was shown that the RL production results were mainly favored by the lowest aeration rate compared to the central values; in turn, considerably high RL productions were also obtained at the highest aeration rate evaluated.

According to the model prediction, within the range of 0.5 vvm to 0.7 vvm, the optimal aeration rate to obtain the maximum production is 0.5 vvm, with which up to 51.58 g/L of RL can be obtained.

3.3. Influence of Agitation Rate

RL production was evaluated at five different stirring speeds ranging from 160 rpm to 200 rpm. The results showed that speeds equal to or higher than 180 rpm allowed high RL production and speeds lower than 180 rpm limited it. On the other hand, the optimum agitation speed suggested by the predictive model to maximize RL production up to 51.58 g/L is 180.5 rpm.

3.4. Influence of Nitrogen Concentration

Regarding the limiting concentration of sodium nitrate ($NaNO_3$), as the sole nitrogen source in the culture medium, according to the behavior of RL production, it was observed that concentrations below 2.85 g/L resulted in lower production than when 3.3 g/L of $NaNO_3$ was used. Furthermore, the optimal $NaNO_3$ concentration predicted by the experimental design is 3.04 g/L, with a maximum production of 51.58 g/L.

3.5. Design Expert

The experiments were executed according to the conditions established by the Central Composite Design (CCD), evaluating three factors at five levels by means of randomized combinations. A total of 39 trials were executed, with axial points in triplicate and central points in duplicate (Table 1). The results allowed tabulation of a mathematical model to predict theoretical rhamnolipid production (g/L), described by Equation 1.

 doi:10.20944/preprints202505.0075.v1

Table 1. Factors and levels used in the CCD, as well as experimental and theoretical values of RL production of P. aeruginosa 6k-11.

Nº	*Factor A:* Nitrogen source (g/L)	*Factor B:* Aeration rate (vvm)	*Factor C:* Agitation speed (rpm)	*Response:* Rhamnolipids (g/L)	
				Experimental	Theoretical
1	3.3	0.5	160	17.345	18.913
2	1.95	0.6	180	27.203	19.591
3	2.4	0.65	180	19.186	24.447
4	2.4	0.6	170	7.387	7.374
5	1.5	0.5	160	8.784	8.053
6	2.4	0.6	180	18.485	19.545
7	1.5	0.7	160	7.061	9.239
8	2.4	0.6	190	16.256	16.602
9	2.4	0.6	180	21.916	19.545
10	3.3	0.7	200	30.459	30.535
11	2.4	0.6	170	6.777	7.374
12	2.4	0.55	180	29.735	26.074
13	1.5	0.7	200	27.943	26.831
14	1.5	0.7	200	29.252	26.831
15	3.3	0.7	200	28.893	30.535
16	2.4	0.6	190	16.541	16.602
17	1.5	0.5	160	9.348	8.053
18	1.5	0.5	200	17.557	19.875
19	1.5	0.7	160	6.979	9.239
20	3.3	0.5	200	39.710	38.230
21	3.3	0.5	200	41.125	38.230
22	3.3	0.5	200	40.367	38.230
23	1.5	0.5	160	9.341	8.053
24	3.3	0.7	200	28.915	30.535
25	1.95	0.6	180	24.437	19.591
26	2.4	0.65	180	19.585	24.447
27	3.3	0.5	160	15.951	18.913

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28	2.4	0.55	180	31.544	26.074
29	2.4	0.6	180	20.768	19.545
30	1.5	0.7	160	7.142	9.239
31	3.3	0.7	160	8.664	5.448
32	2.85	0.6	180	17.574	23.232
33	3.3	0.5	160	17.738	18.913
34	3.3	0.7	160	8.192	5.448
35	1.5	0.5	200	16.770	19.875
36	3.3	0.7	160	8.448	5.448
37	1.5	0.7	200	28.977	26.831
38	1.5	0.5	200	16.315	19.875
39	2.85	0.6	180	15.439	23.232

Equation (1). Mathematical model to predict theoretical rhamnolipid production (g/L);

$$Y = -1571.64277 - 34.50277A - 2791.66276B + 26.98438C - 40.69630AB + 0.104102AC + 0.721167BC + 9.21411A^2 + 2286.04322B^2 - 0.075571C^2 \quad (1)$$

where:

Y: RL production
A: nitrogen source concentration
B: aeration rate
C: agitation speed

4. Discussion

4.1. Response Surface Results

The interrelationship of the evaluated parameters and their relationship with RL production is described using three-dimensional response surface graphs.

Figure 3 shows how varying the aeration rate and the concentration of the nitrogen source ($NaNO_3$) impact production, where it is observed that the most successful interaction occurs with $NaNO_3$ concentrations above 2.7 g/L with an air volume of 0.5 vvm.

Figure 4 shows how the combination of stirring speeds and $NaNO_3$ concentrations influences production. It is observed that the most appropriate stirring values are between 175 rpm and 190 rpm across the entire $NaNO_3$ range, especially when it exceeds 2.7 g/L. Figure 5 relates the variation in agitation speed and aeration rate to the culture on RL production, and shows that production increases when agitation tends to 180 rpm and the aeration rate tends to 180 rpm.

The experimental model employed is consistent with that chosen by Jamal et al. [21], who used the Central Composite Design and the Response Surface methodology to optimize pH, temperature, agitation, and inoculum percentage, increasing their RL production from 2.27 g/L to 4.44 g/L. Likewise, Bazsefidpar et al. [9] used the same design to optimize operating conditions for RL production, achieving a 10.7-fold improvement in batch culture by monitoring temperature, pH, and,

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similar to this study, agitation and dissolved oxygen (DO) concentration. They found that these last two factors, along with their feeding methodology, were significant in achieving RL overproduction.

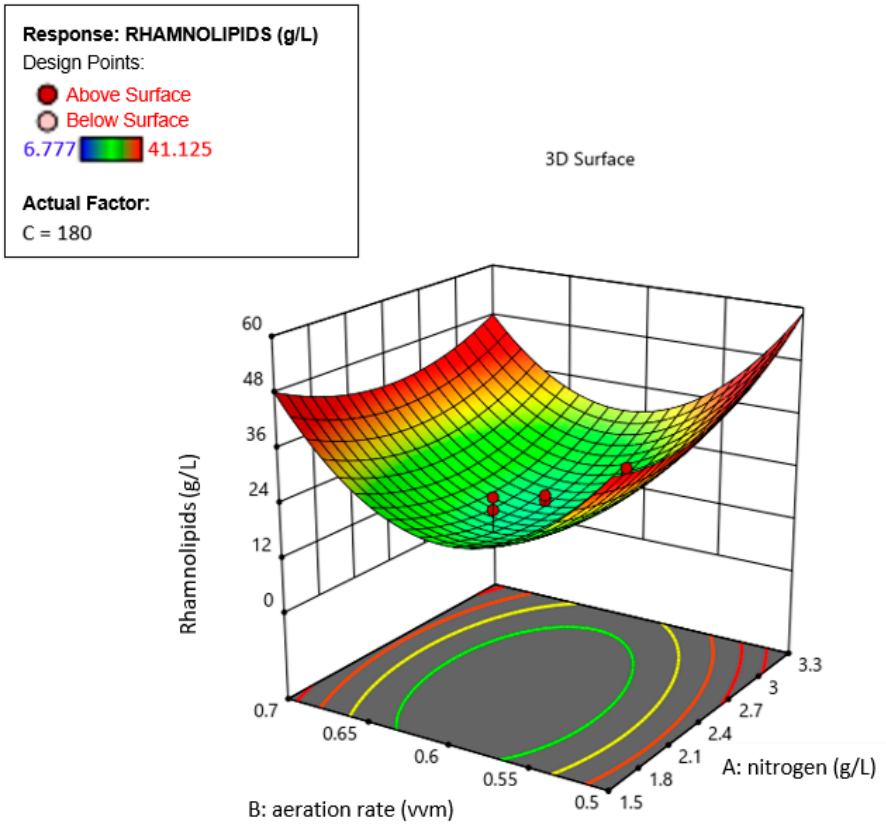

Figure 3. Effect of aeration rate and $NaNO_3$ concentration on RL production.

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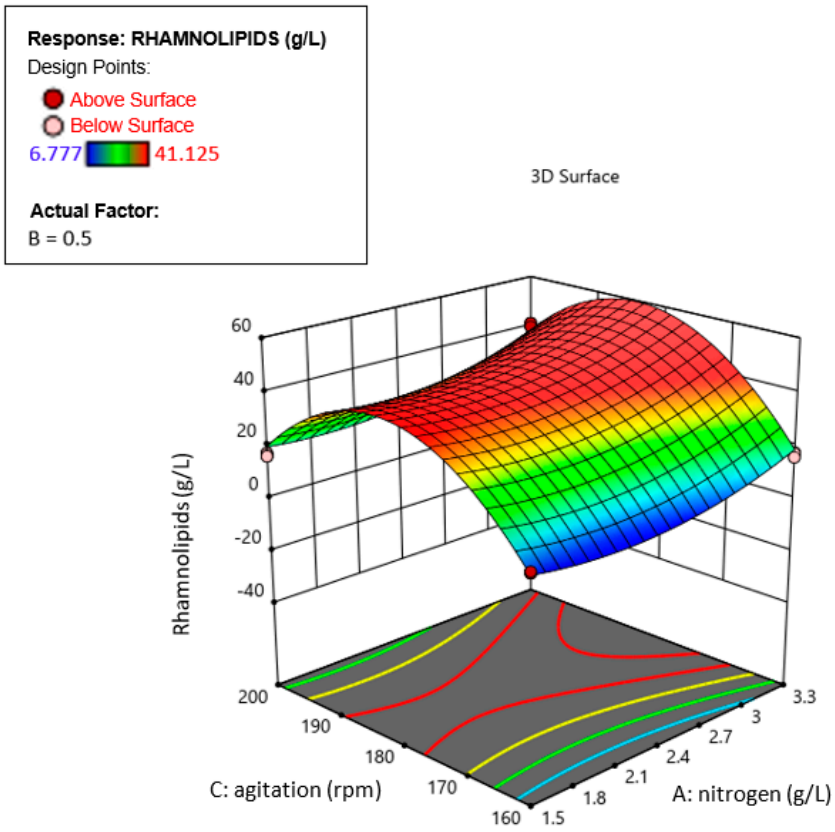

Figure 4. Effect of agitation speed and $NaNO_3$ concentration on RL production.

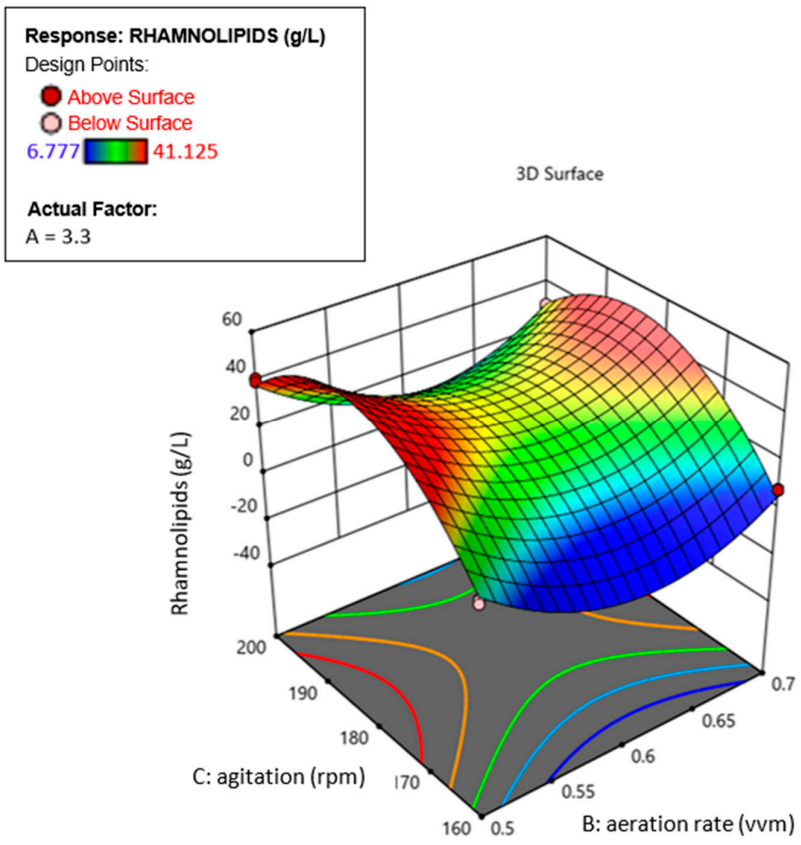

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Figure 5. Effect of agitation speed and aeration rate on RL production.

4.2. Bacterial Growth

The use of frying oil as the sole carbon source for rhamnolipid (RL) production by *P. aeruginosa* 6k-11 is a sustainable and economical alternative, as it is a waste product; furthermore, it contains a mixture of fatty acids that *P. aeruginosa* efficiently metabolizes [22]. Using this approach, *P. aeruginosa* 6k-11 was provided with controlled conditions so that it could utilize the frying oil efficiently and thus achieve an increase in its maximum RL production. The bacteria was able to produce 20.4 g/L over a period of 144 to 168 hours. This favorable result is evidence of the adaptive capacity of *P. aeruginosa* 6k-11 to metabolize waste oil. The presence of saturated and monounsaturated fatty acids, primarily oleic acid, in frying oil, favors oil stability. Therefore, it can be beneficial for microbial growth [23]. However, it is also important to note that frying oil may also contain degraded compounds that are released after use, wich could negatively affect RL production. Therefore, the quality of the oil must be analyzed before being used as a carbon source, ensuring that any contaminants present do not interfere with the fermentation process [24].

4.3. Dissolved Oxygen Concentration

Dissolved oxygen (DO) concentration is a critical parameter in microbial physiology, as it influences cellular respiration and, consequently, the production of metabolites such as RL. Several studies show that maintaining DO at adequate levels is necessary to maintain the metabolic activity of *P. aeruginosa* and thus promote the biosynthesis of its RL [25,26]. When DO is below 2 mg/L, it constitutes a situation of hypoxia, which could limit RL production due to limitations in metabolic activity [25]; therefore, it is essential to maintain DO levels above this threshold to maximize production. The DO concentration in the culture showed an inverse trend with biomass and RL production, especially during the day of maximum RL production, where the lowest DO readings were recorded throughout the entire kinetics. The observed behavior is consistent with that described by Bazsefidpar et al. [9], They reported that DO values close to 40% increased their RL production, similar to some DO values found in this research; however, the average value during the peak production for the central points was 117%. On the other hand, Kronemberger et al. [27] achieved an RL productivity of 30.0 mg/L h in bioreactor fermentations with 4.0 and 6.0 mg/L DO, while the maximum production with the central points of this research was reached at 10.17 mg/L. The results reinforce that the relationship between DO and RL production is not linear, but rather complex, since there is no specific optimal DO for RL production; rather, when it is lower, the OD readings also decrease. The oxygen transfer coefficient (kLa) is a fundamental parameter in submerged culture bioprocesses because it determines the efficiency with which oxygen is transferred from air to an organic solvent. Therefore, its role is essential to maintain an adequate balance between oxygen supply and demand in the culture. Additionally, Kronemberger et al. [27] indicate that a kLa that is too low prevents aerobic metabolism and, consequently, decreases the production rate. Therefore, it is necessary to establish an equilibrium point that maximizes oxygen transfer without generating stressful conditions for the bacteria. High kLa values indicate that oxygen transfer is rapid and efficient, which is essential for maintaining cell viability and an optimal growth rate, while also promoting the generation of metabolites [28]. To determine kLa, Ramesh et al. [19] and Garcia-Ochoa and Gomez [20] used the mass balance equation for oxygen, which is a point in common with this research.

4.4. Influence of Aeration Rate and Agitation Speed

Aeration and agitation conditions have a significant impact on RL production. They are crucial factors because they affect oxygen availability in the culture. Several studies have shown the agitation speed range between 180 rpm and 200 rpm is optimal for better oxygen distribution [29,30]. A similar result was obtained in this study, where the highest RL production values were achieved with

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agitation between 175 rpm and 190 rpm, combined with constant aeration, even at the minimum value used (0.5 vvm). Below these values, the culture could be subjected to oxygen-limiting conditions and affect RL synthesis [31]. It has also been observed that more oxygen is available, in this case, with 0.7 vvm and agitation above 190 rpm, instead of being ideal for production, it slows down because these conditions potentially promote the formation of bubbles and foam.

4.5. Influence of the Nitrogen Source

P. aeruginosa produced nitrogen during the stationary phase of its growth because during this phase, the concentration of nitrogen sources decreases until it reaches limiting values [32]. This relationship is explained by the fact that, once nitrogen is consumed and the plant enters the stationary phase, metabolic activity redirects efforts towards nitrogen synthesis, so that production increases after the exponential phase, which is why limiting conditions for this macronutrient are required [33].

Aside from concentration, the nitrogen source has a significant influence on nitrogen production, a conclusion reached by Shatila et al. [34] after finding that the presence of NaNO as a nitrogen source limits nitrogen availability, which leads to an increase in nitrogen production. This supports the use of $NaNO_3$ as a nitrogen source in the present work, in addition to the results reported by Alcalde [35], who found it as an optimal source of nitrogen for RL production over other salts such as urea, ammonium nitrate, potassium nitrate and ammonium nitrate. It was observed that the limiting concentrations of $NaNO_3$, with which the greatest efficiency in the production of RL was obtained, were those higher than 2.7 g / L, within the range of 1.5-3.3 g / L, that is, the highest concentrations that were evaluated; however, it does not leave far behind some of the results that were obtained with the other concentrations. Similar to the pattern of results obtained in the search for the limiting concentration of $NaNO_3$ found in this work, Wu et al. [36] explained that nitrogen limiting conditions do not necessarily refer to the lowest concentration, but rather the lowest concentration that is most beneficial for RL production must be found, especially if high-yield production is intended, as in their case, since they found that the limiting concentration of $NaNO_3$ to increase its production was the highest they evaluated (4-6 g/L).

4.6. Optimization of Rhamnolipid (RL) Production

The RL yield produced by *P. aeruginosa* 6k-11 reached 52.2 g/L, representing a significant increase of 48.6% compared to the 35,124 g/L obtained by Alcalde [35], who also used the Response Surface Methodology (RSM) to optimize the inorganic salts in the Siegmund-Wagner (SW) culture medium, values that served as a reference for the present research. The RL production of *P. aeruginosa* 6k-11 not only benefited from the application of RSM, but also the appropriate selection of carbon and nitrogen substrates, as well as the culture conditions: agitation speed, aeration rate, and temperature, played a fundamental role.

5. Conclusions

These results let us conclude that the combination of an agitation speed between 175 rpm to 190 rpm with an aeration rate of 0.5 vvm allowed the best oxygen distribution, thereby RL production by *P. aeruginosa* 6k-11 got to increase to 52.2 g/L. In addition, the residual frying oil as the sole carbon source is ideal for production due to its efficiency and low cost.

Data Availability Statement: Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

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Conflicts of Interest: All the authors declare no conflicts of interest.

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Abbreviations

The following abbreviations are used in this manuscript:

CCD	Compound Central Design
RSM	Response Surface Methodology
DO	Dissolved Oxygen
RL	Rhamnolipid
SW	Siegmund and Wagner

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