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Biodegradation of linear alkylbenzene sulfonate contamination by *pseudomonas aeruginosa* isolates

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Abstract

Linear alkylbenzene sulphonate (LAS), a surfactant frequently incorporated into detergent formulations, typically ends up in wastewater treatment facilities after use. The present study aims to investigate the efficacy of bacteria isolated from Iraqi wastewater in removing LAS. This strain was identified as Pseudomonas aeruginosa by genetic analysis (16S rRNA). Three LAS concentrations (25, 50, and 100 mg/l) were employed in this investigation, along with three temperatures (30, 35, and 40 oC) and pH values (5, 7, 9). The LAS anionic surfactant demonstrated optimal biodegradation by Pseudomonas aeruginosa at a temperature of 30 °C and at pH levels of 7 and 9, with removal percentages of 93.76% and 90.4%, respectively, at a concentration of 25 mg/l of LAS. The study's findings demonstrated the possibility and importance of using aerobic biodegradation to remove LAS from actual effluents. This bacterium is useful for bioremediation because of its capacity to break down LAS.

Keywords: Pseudomonas bacteria; Sewage; Wastewater plants; LAS; biodegradation.

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1- Introduction

Scientific and industrial research advances in various fields, including detergents, medications, cosmetics, and other areas, have always been the source of numerous synthetic routes for anionic surfactants (AS) whose physicochemical characteristics are unique and significant [1, 2]. Surfactants are one of the main pollutants in the terrestrial and aquatic environments. They are amphipathic molecules with hydrophobic and hydrophilic groups that allow for the reduction of surface tension, therefore they are used for cleaning and laundry industries. Daily usage of surfactants in homes and industries results in their discharge into the environment, particularly in water [3, 4].

When it comes to artificial detergents, anionic surfactants are the most widely used and utilized as additives in all the components. In the 1930s, branched alkylbenzene sulfonates (BAS) were the first form of alkylbenzene sulfonates to be produced. In the 1960s, many of them were substituted with LAS due to environmental concerns [5]. LAS is a class of anionic surfactants. It is the primary ingredient in artificial detergents and is frequently utilized in day-to-day living. Due to the widespread use of detergents, LAS is now considered a representative environmental organic pollutant [6]. Because of their surface activity against biological membranes, surfactants are ecotoxic [7, 8]. The chemical nature of surfactants determines how they affect both terrestrial and aquatic life. As a result, they are classified into four types: cationic, anionic, non-ionic, and amphoteric [9].

Wastewater is treated using a range of methods, including chemical, biological, and physical treatment, to remove surfactants. The best method for treating wastewater will depend on several factors, including energy consumption, treatment costs, environmental impact, influent and effluent quality, and treatment costs [10]. Physical treatment techniques have the advantages of being robust, chemical-free, and requiring less operational input; on the other hand, their main disadvantages are producing secondary waste, having a high capital cost (requiring land/space), and having a long retention period [11]. Chemical methods have advantages such as reduced sludge generation and high pollutant removal efficiency, but they also have drawbacks such as high operational and chemical costs and secondary waste production [12]. Biological methods have the advantages of low cost and easy application [13].

The study of the decomposition of anionic surfactants by microorganisms is considered very important to reduce their environment. Bacterial activity is the main reason responsible for the degradation of surfactants in the ecosystem. In addition to improving the removal of these surfactants from the environment and reducing their impact on ecosystems, biodegradation is an essential process for treating surfactants found in raw sewage in treatment plants. Microbes can co-metabolize surfactants through microbial metabolic reactions or use them as

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substrates for energy and nutrients during biodegradation [14].

Commercial LAS is structurally composed of a benzene ring, a sulfonated group, and a linear alkyl chain with 10–14 carbon atoms. A worked-out outline for the LAS biodegradation pathway starts with oxygenation at the alkyl chain's terminal end and proceeds through ring opening, oxidation, and conversion of the sulfonated group to inorganic sulfate [15]. As a result of the pathway's early, strict oxygen requirement, aerobic conditions are typically preferred for LAS biodegradation over anaerobic ones [16,17]. The study of the decomposition of anionic surfactants by microorganisms is considered very important to reduce their environment. In recent years, numerous studies on the biodegradation of LAS have been carried out by researchers [18]. Furthermore, it was discovered that certain strains from the genera *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Vibrio*, *Enterobacter*, *Klebsiella*, etc. could break down LAS [19, 20].

In this work, high-efficiency bacteria that degrade LAS were isolated and enriched from municipal wastewater by employing LAS as the only carbon source. The impact of varying concentrations of LAS on the growth of bacteria that break down LAS was investigated.

2- Materials and methods

2.1. Chemicals and media

After purchasing LAS ($C_{18}H_{29}NaO_3S$) and other biochemicals from Sigma (USA), acetone was used to dissolve LAS and create a stock of 1000 mg L^{-1}. To be used, the stock solution was filter-sterilized and stored in the refrigerator. The bacterial strains that degraded LAS were isolated and cultivated using nutrient broth, nutrient agar, and mineral salt media, as stated by [21]. The composition of the mineral salt medium (MSM) was prepared according to [22].

2.2. Isolation and screening of bacteria that degrade LAS

Samples were taken from three sewage treatment plants that were contaminated with surfactant, which have been given numbers (1, 2, and 3) belonging to Al-Kut city located in the Wasit Governorate/Iraq, in December 2022. Sewage samples (10 ml) were added to 250 mL Erlenmeyer flasks containing 150 ml MSM supplemented with 50 mg/l LAS for enrichment. The flasks were then incubated for five days at 150 rpm and 30 ºC [23]. The pour plate method was used for the isolation of bacteria using nutrient agar, from the last enrichment culture, according to [24]. Bacterial cultures that LAS degrading were selected by:

1- Primary screening is carried out by cultivating them on nutrient agar plates with 200 mg L^{-1} of LAS as the only source of energy and carbon, as stated by [25].
2- Secondary screening is carried out via cultivation on solid (MSM) that are incubated at 30 ºC for 24-96 h, with LAS serving as the only source of energy and carbon at concentrations between 200 to 2000 mg/l. The growth of bacterial isolates is based on the bacteria growth on solid mineral salt media [21, 26].

2.3. Identification of the LAS degraded bacterial strain

In addition to studying the shape, color, odor, and margin of the colonies grown on nutrient agar, microscopic analysis of slides stained with gram stain was conducted to distinguish gram-negative and/from gram-positive bacteria [27]. The isolated bacteria were diagnosed by PCR technology, amplification was done using forward primer 5`- AGA GTT TGA TCM TGG CTC AG - 3 and reverse primer 5`-TAC GGY TAC CTT GTT ACG ACT T -3`. Molecular evolutionary analyses of the LAS-degrading bacteria based on 16S r-RNA genes were conducted using MEGA version 4 [28].

2.4. Laboratory experiment

Sets of 250 ml Erlenmeyer flasks holding100 ml of the medium were prepared, the pH was adjusted to 7 and autoclaved at 121ºC and 1.5 bar for 15 min, then sets of LAS with three concentrations (25, 50, and 100 mg/l) added to flasks, then added 1 ml of *Pseudomonas. aeruginosa* that were isolated and incubated after being activated in nutrient broth overnight, the flasks were incubated in a shaker incubator (150 rpm) for 15 days and the growth was measured by recorded values of optical density by spectrophotometer every two days [29].

The experiment was repeated for pH 5 and 9 at three different temperature degrees (30, 35, and 40 ℃) for 15 days, optical density was measured for each one, then 5 ml of the mixture was taken to test the biodegradation efficacy by HPLC analysis.

2.5. High-performance liquid chromatography (HPLC)

Concentrations used in the current study were 25, 50, and 100 mg/l which were measured according to Eq. 1.

$$\text{Concentrations measured} = \boldsymbol{C1 \times V1 = C2 \times V2} \quad (1)$$

HPLC was used to determine the LAS content, and Eq. 2 and Eq. 3 were used to calculate the percentage of LAS biodegradation [30].

$$\textit{Percentage of biodegradation} = (\text{conc.of standard-conc.of sample})/(\text{conc.of standard})X\ 100 \quad (2)$$

Or

$$\textit{Percentage of biodegradation} = (\text{beak area of standard-beak area of sample})/(\text{beak area of standard})X\ 100 \quad (3)$$

3- Results and discussion

3.1. Identification of bacteria

In this study, sewage samples from one of the wastewater plants in Wasit province /Iraq are considered a

significant source of locally prevalent bacteria that can mineralize anionic surfactants. Gram-negative rods identified the degrading strain of LAS in this investigation. *Pseudomonas aeruginosa* were isolated, purified, and selected based on their capability to use LAS as a source of carbon. The bacterial *isolate P. aeruginosa* was identified based on the nucleotide sequence of the 16S rRNA gene (PCR technology).

3.2. Impact of temperature and pH on *P. aeruginosa* growth

The results presented in Table 1 demonstrate that notable differences exist in the growth of bacteria at various temperatures and pH levels. The best and most significant means of bacterial growth under 30 ºC, 35 ºC, and 40 ºC measured at pH 7 were 0.390, 0.330, and 0.099 nm, respectively. Also, the better and most significant means of bacterial growth under pH 5, pH 7, and pH 9 were measured at 30 ºC were 0.290, 0.390, and 0.354 nm respectively. The mean value of *P. aerogenosa* growth was high at 30 ºC and pH 7 which was 0.390 nm, and low at 40 ºC and pH 5 which was 0.066 nm (Fig. 1 and Fig. 2).

Table 1. Mean Value of *P.aeruginosa* Growth at Various Temperatures and pH Degrees after Incubation for 15 Days, and LSD Value with 25 mg/L of LAS

pH / Temp	pH 5	pH 7	pH 9	LSD value
30 °C	0.290 ±0.123	0.390 ±0.112	0.354 ±0.091	0.122*
35 °C	0.193 ±0.081	0.330 ±0.130	0.241 ±0.115	0.119*
40 °C	0.066 ±0.006	0.099 ±0.019	0.081 ±0.036	0.070 NS
LSD value	0.130*	0.123*	0.074*	---

* ($P \leq 0.05$), NS: Non-Significant

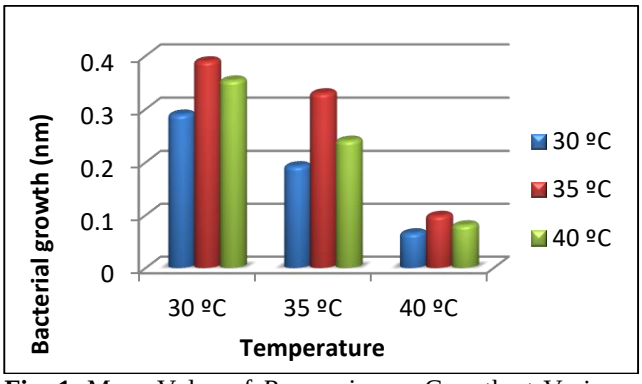


Fig. 1. Mean Value of *P.aeruginosa* Growth at Various Temperature and pH Values after Incubation for 15 Days

Accordingly, the optimum temperature for the growth of *P.aeruginosa* was 30ºC. Temperature and microbial activity were found to be directly correlated during LAS degradation; considerable removal happened when the bio stimulators were run at 28 to 30 ºC. pH 7 was the ideal pH for the growth of *P.aeruginosa* in MSM that contain LAS, this conclusion is corroborated by the findings of [31], who found that isolated strains of *pseudomonas* can grow and maintain their capacity for degradation across a broad pH range, with optimal growth occurring at pH roughly. The pH level may have an impact on the enzyme(s) responsible for LAS [32].

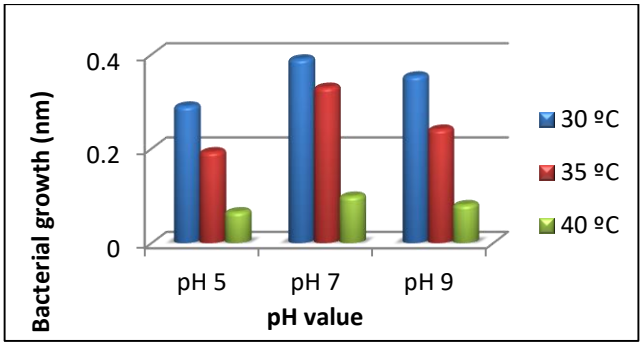


Fig. 2. Mean Value of *P.aeruginosa* Growth at Various pH Values and Temperature after Incubation for 15 Days

In addition to potentially affecting growth, a temperature increase above the threshold point of 37ºC may also make microbial membranes more toxic. According to the data, 30 ºC would be the ideal temperature for better LAS degradation, as confirmed by HPLC. In general, temperature influences the LAS compound's chemical and physical characteristics, microbial metabolism, the specific growth average of microorganisms, the rate of the oxidation process's enzymatic activity, and the makeup of the microbial community which impacts the rate of biodegradation of LAS [29].

3.3. Impact of incubation time on biodegradation and growth

Table 2 displays the results of a significant difference ($p < 0.05$) between means of the bacterial growths for various incubation periods. After 15 days of incubation, the selected bacteria isolated showed that the highest mean growth was 0.505 nm on the ninth day, and the lowest growth was 0.210 nm on the one day. The growth rate of the selected bacteria that were isolated increases gradually from 1-9 days, and then starts to decrease (see Fig. 3).

Table 2. Average Values ± Standard Deviation of Bacterial Growth at 600nm at Various Incubation Times, and LSD Value

Incubation period Days	Mean± SD of bacterial growth *P. aerogenosa*
First Day	0.210 ± 0.015
Third Day	0.340 ± 0.011
Fifth Day	0.400 ± 0.015
Seventh Day	0.485 ± 0.006
Ninth Day	0.505 ± 0.006
Eleventh Day	0.440 ± 0.010
Thirteenth Day	0.400 ± 0.006
Fifteenth Day	0.340 ± 0.010
LSD ≤ 0.05	0.018

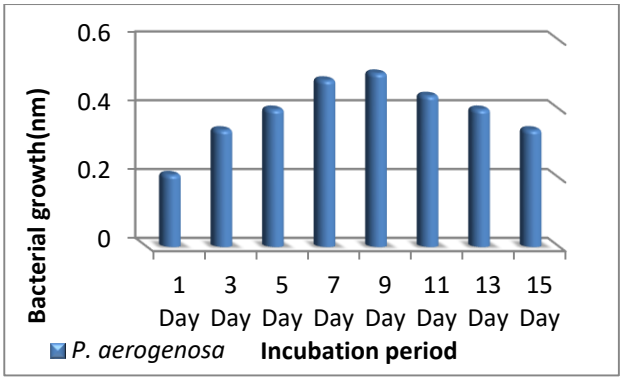


Fig. 3. The Average Value of Bacterial Growth Over Various Incubation Times

It is commonly known that extending the incubation period increases the number of viable organisms [33], especially on medium with minimal amounts of nutrients, and because of contaminant concentration depletion and the production of intermediate substances and metabolic byproducts, which causes a lower media pH and subsequently inhibit bacterial growth [34].

3.4. HPLC analysis of biodegraded LAS

For the degradation efficacy test, the bacteria were inoculated into a mineral salt medium, with LAS at three concentrations (25, 50, 100 mg/l), and the surfactant was added to 100 ml of the mineral salt medium at three different temperatures and PH. These samples were analyzed with the HPLC system [35]. An analysis of a standard solution of surfactant was done, where the concentration of the standard solution was 10 mg/L and the beak area was 885.4 per 4.29 minute. Comparing figures of LAS was done according to the used concentrations in this study (see Fig. 4). The results of the HPLC analysis mentioned in Table 3 explain the concentrations of LAS, beak area of samples, percentage of removal, and remaining concentration for each sample.

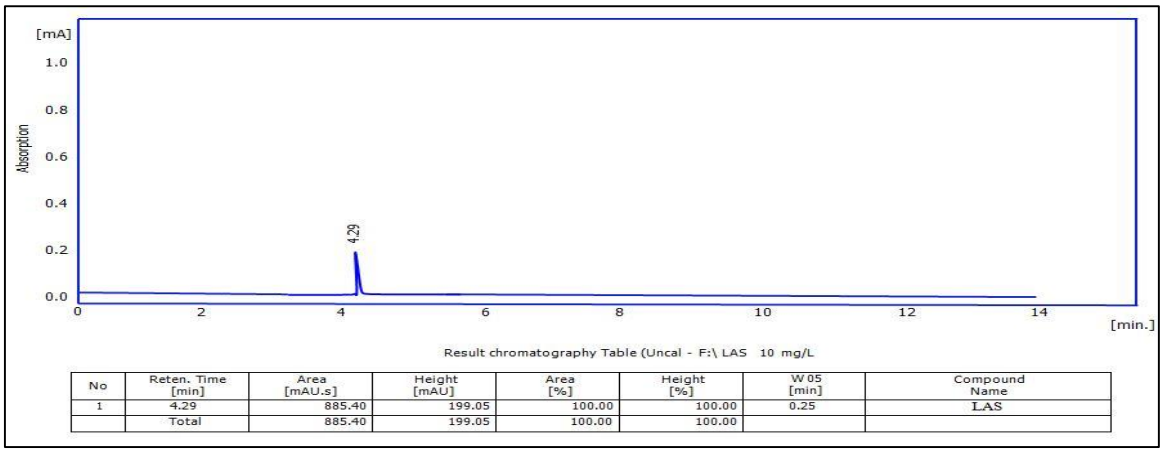
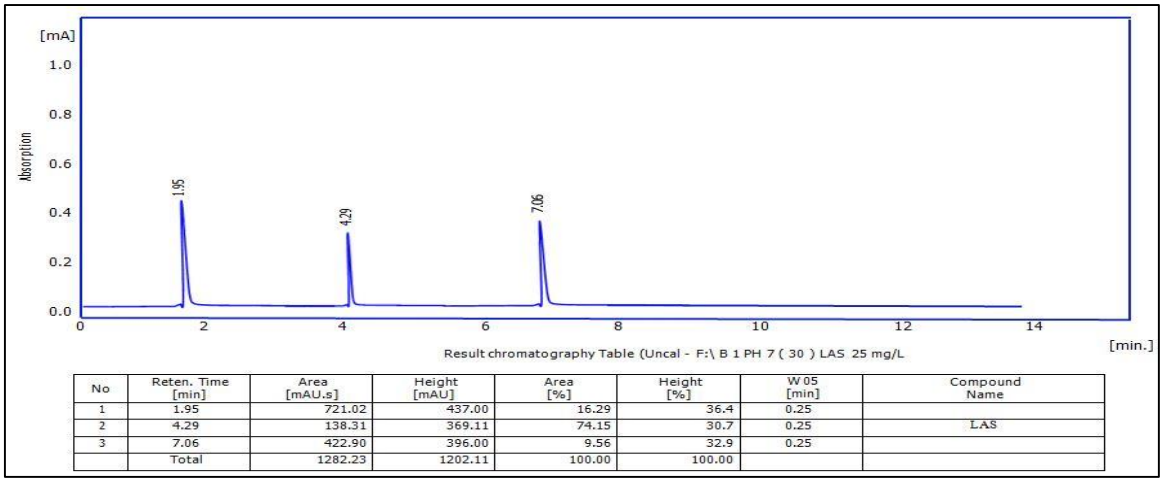


Fig. 4. Beak Area for 10 mg/L of LAS

Table 3. Beak Area and Removal Percent of Samples

Temperature ◦C	Concentration of LAS (mg/L)	pH	Beak area (mAU.s)	% removal	Remain conc. (mg/L)
30	25	9	212.69	90.4	2.4
		7	138.31	93.76	1.56
		5	282.05	87.2	3.2
	50	9	963.11	78.2	10.9
		7	769.15	82.26	8.69
		5	1720.21	61.1	19.45
	100	9	4062.10	54.12	45.88
		7	3280.51	62.94	37.06
		5	5300.12	40.13	59.87
35	25	9	538.11	75.68	6.08
		7	321.15	85.48	3.63
		5	774.70	65	8.75
	50	9	1390.50	68.48	15.76
		7	967.11	78.16	10.92
		5	1850.61	58.08	20.96
	100	9	4500.01	49.17	50.87
		7	3886.85	56.11	43.89
		5	5679.01	35.86	64.14
40	25	9	1879.45	15.08	21.23
		7	1807.89	18.32	20.42
		5	1943.02	12.2	21.95
	50	9	3850.91	13	43.5
		7	3718.12	16	42
		5	4013.44	9.34	45.33
	100	9	8015.11	9.47	90.53
		7	7686.54	13.19	86.81
		5	8322.03	6	94

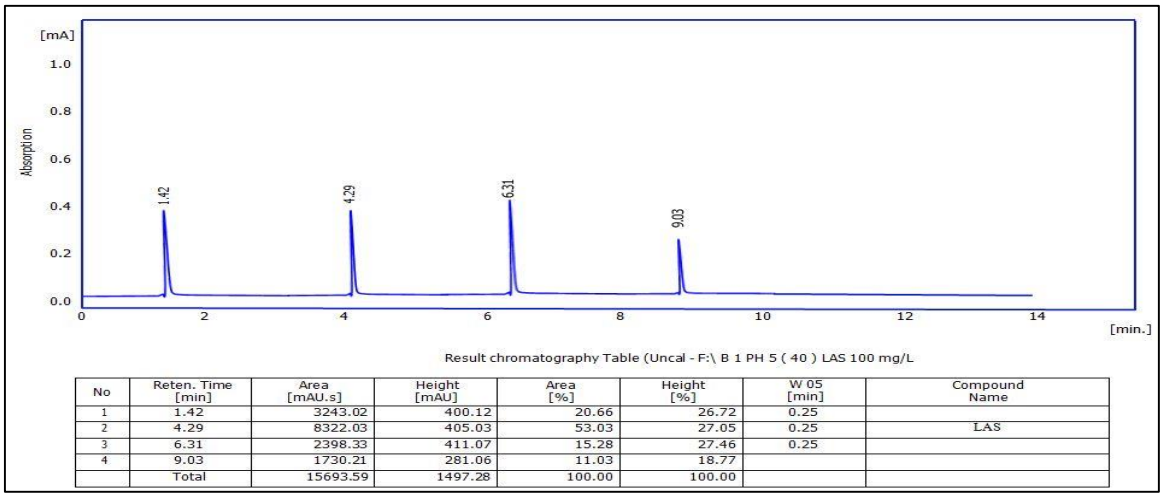
This table displays the dissociation of LAS surfactant in different concentrations, different temperatures, and pH, where the best result was the dissolution of the LAS into its secondary components at a temperature of 30 ° C and pH of 7 (see Fig. 5) with a concentration of 25 mg/l, and the lowest results were at temperature of 40 ° C, pH of 5, concentration of 100 mg/l (see Fig. 6). This means that dropping pH and raising the LAS concentration led to less efficient bacteria to degrade the surfactant.



Result chromatography Table (Uncal - F:\ B 1 PH 7 (30) LAS 25 mg/L

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	1.95	721.02	437.00	16.29	36.4	0.25	
2	4.29	138.31	369.11	74.15	30.7	0.25	LAS
3	7.06	422.90	396.00	9.56	32.9	0.25	
	Total	1282.23	1202.11	100.00	100.00		

Fig. 5. The Highest Removal of LAS



Result chromatography Table (Uncal - F:\ B 1 PH 5 (40) LAS 100 mg/L

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	1.42	3243.02	400.12	20.66	26.72	0.25	
2	4.29	8322.03	405.03	53.03	27.05	0.25	LAS
3	6.31	2398.33	411.07	15.28	27.46	0.25	
4	9.03	1730.21	281.06	11.03	18.77		
	Total	15693.59	1497.28	100.00	100.00		

Fig. 6. The Lowest Removal of LAS

The degradation rate increased at 25 mg/l of LAS and reduced at 100 mg/l [35], which shows more degrading efficiency at a neutral degree of pH. However, at acidic pH, there was a nearly 50% degrading rate, while alkaline pH gave a nearly 80% degrading rate at different concentrations and amounts of surfactant [23]. The neutral environments gave more degradation than alkaline and acidic because the stability of bonds increased in acidic environments. Several bacterial strains capable of degrading similar compounds have been isolated from surfactant-contaminated environments [19].

In this study, high degradation levels were attained at low concentrations of LAS. The percentage of LAS degradation of *P. aeruginosa* at a concentration of 25 mg/l was 93.76%, while it was 82.26% and 62.94% at 50 and 100 mg/l respectively. High concentrations resulted in a noticeable reduction in the rate of degradation, which is related to the surfactant's harmful effect on bacterial growth [36]. It is well known that surfactants are a significant class of potentially hazardous substances that encourage bacterial membrane defects in terms of both structure and function. Furthermore, surfactants may cause toxicity to several vital physiological parameters, including specific growth rate and metabolic activity [37]. At 25 and 50 mg/l, the *P. aeruginosa* bacteria were able to degrade LAS with good efficiency, indicating that this bacterium can be a good option for LAS disposal from these contaminated environments.

The results agreed with [38,39], which used bacteria to degrade LAS and showed 75 – 80 % percentage of degradation to LAS by HPLC analysis. The slight difference in the percentage of biodigestion was due to the efficiency of the type of bacteria, the type of strain isolated, and its adaptation to the environment [40].

4- Conclusion

In this study, LAS biodegradation at aerobic conditions using microbial cells isolated was investigated from wastewater plants. A LAS-degrading bacterium was isolated from an Iraqi water sample that was contaminated with LAS. To promote a higher degradation rate and enable the application of the optimized parameters in the field, growth optimization studies involving the isolate were conducted on a variety of physicochemical parameters. *Pseudomonas aeurginosa* with strong LAS degradation ability was isolated, purified, and selected based due to their capability of using LAS as a source of carbon and enriched from sewage. The bacterial isolate was identified based on the nucleotide sequence of the 16S rRNA gene (PCR technology). The strain's growth characteristics demonstrated that it could degrade more than 80% of LAS at concentrations less than 100 mg/l. The optimal values of temperature and pH for biodegradation were 30°C and pH 7.

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التحلل الحيوي للتلوث بسلفونات الكيل البنزين الخطية بواسطة عزلات *Pseudomonas aeruginosa*

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الخلاصة

سلفونات ألكيل بنزين الخطية ((LASهي مادة فعالة سطحية يتم دمجها بشكل متكرر في تركيبات المنظفات، تنتهي عادة في محطات معالجة مياه الصرف الصحي بعد الاستخدام. تهدف الدراسة الحالية إلى التحقيق في فعالية البكتيريا المعزولة من مياه الصرف الصحي العراقية في إزالة سلفونات ألكيل بنزين الخطية. من خلال التحليل الجيني (١٦ S rRNA) تم التعرف على هذه السلالة على أنها Pseudomonas aeruginosa. في هذا البحث تم استخدام ثلاثة تركيزات من سلفونات ألكيل بنزين الخطية (٢٥ و٥٠ و١٠٠ مجم/لتر)، إلى جانب ثلاث درجات حرارة (٣٠ و٣٥ و٤٠ م°) وقيم الأس الهيدروجيني (٥ و٧ و٩). أظهرت المادة الخافضة للتوتر السطحي ((LASتحللًا حيويًا مثاليًا بواسطة Pseudomonas aeruginosa عند درجة حرارة ٣٠ م°ومستويات الأس الهيدروجيني ٧ و٩، مع نسب إزالة ٩٣,٧٦٪ و٩٠,٤٪ على التوالي، عند تركيز ٢٥ مجم/لتر من .((LASكشفت نتائج هذه الدراسة عن إمكانية وأهمية استخدام التحلل الحيوي الهوائي لإزالة LAS من النفايات السائلة الفعلية. هذه البكتيريا مفيدة للمعالجة الحيوية بسبب قدرتها على تحلل .LAS

الكلمات الدالة: بكتريا السيدوموناس، مياه المجاري، محطات مياه الصرف الصحي، LAS، التفكك الحيوي.