



Molecular identification and biosurfactant production by *Pseudomonas* spp and *Alcaligenes* spp from selected automobile workshops in Ilorin, Nigeria

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ABSTRACT

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This study investigated production of biosurfactants (surface-active agents) produced by microorganisms like *Pseudomonas* and *Alcaligenes* isolated from soil samples in automobile repair workshops in Ilorin. Soil samples were collected from twenty-one (21) randomly selected mechanic workshops across the three local government areas in Ilorin. Physicochemical examination of the sampled soil as well as isolation of bacteria from the soil was done using *Pseudomonas* isolation Agar after which biochemical tests were performed on the isolated bacteria. The organisms were identified using molecular tools. Isolated *Pseudomonas* species and *Alcaligenes* species were screened for biosurfactants production using various screening methods, including haemolytic assay (α -, β -, and γ) and emulsification index. The results were statistically analyzed using one-way ANOVA and Duncan's test ($p < 0.05$). Obtained results are: soil pH (6.03-7.50), electrical conductivity (2.19-90.71), organic carbon (0.78-1.63), organic matter (1.23-7.11), sand (6.47-84.6), silt (5.77-16.04) clay (5.07-12.24) and moisture content (11.3-14.10). Levels of significant differences ($p < 0.05$) were observed statistically. Among the seven isolates only *Pseudomonas aeruginosa*-PQ6 was γ -haemolytic. For drop collapse assay, *Alcaligenes faecalis*-CPO6 showed no reaction. Three of the seven bacterial isolates exhibited the highest emulsification activity: *Alcaligenes faecalis*-CPO1 (67% Petrol, 65% Engine-oil, 64% Kerosene), *Pseudomonas aeruginosa*-OR9 (60% Petrol, 67% Engine-oil, 63% Kerosene) and *Pseudomonas aeruginosa*-MH7 (71% Petrol, 65% Engine-oil, 64% Kerosene). They show promise as biosurfactant producing bacteria. The effective isolates can be utilized for biosurfactant production as sustainable alternatives to synthetic surfactants and hence will help to clean up oil polluted environment.

1.0 Introduction

Soil consists of a solid phase of minerals and organic matter (the soil matrix), as well as a porous phase that holds gases (the soil atmosphere) and water (the soil solution). Accordingly, soil is a three-state system of solids, liquids, and gases [1]. The spillage of oil is a constant threat to the global environment. The production, transportation and consumption of petroleum together enable a myriad of avenues for which oil enters into the environment [2]. Oil spillage also impacts the

industrial, commercial and residential sectors posing issues of operational productivity and environmental impact for refineries, wastewater plants and motor vehicles use, respectively [3].

Pseudomonas strain genome sequences have made the genus an excellent focus for scientific research; the best studied species include *P. aeruginosa* in its role as an opportunistic human pathogen, the plant pathogen *P. syringae*, the soil bacterium *P. putida*, and the plant growth-promoting *P. fluorescens*, *P. lini*, *P. migulae*, and *P. graminis* [4].

Alcaligenes spp are Gram-negative bacteria that are commonly found in various environments, including soil, water, and as part of the normal flora in some animals. They are known for their metabolic versatility and ability to degrade various compounds. *Alcaligenes* species are typically rod-shaped and motile, possessing flagella. They are obligate aerobes, utilizing oxygen for respiration. Some species can also perform denitrification. They are catalase-positive and oxidase-positive, which helps distinguish them from other Gram-negative bacteria [5].

Biosurfactants are usually categorized by their molecular structure. Like synthetic surfactants, they are composed of a hydrophilic moiety made up of amino acids, peptides, polysaccharides, or sugar alcohols and a hydrophobic moiety consisting of fatty acids. Correspondingly, the significant classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, and polymeric surfactants as well as particulate surfactants example as follows: Bile salts are mixtures of micelle-forming compounds that encapsulate food, enabling absorption through the small intestine [6]. Both *Alcaligenes* and *Pseudomonas* are bacteria that produce biosurfactants. Biosurfactants from both bacteria reduce surface tension and interfacial tension. Both have potential applications in environmental cleanup, bioremediation, and low toxicity, compared to chemical surfactants, biosurfactants from both bacteria tend to have lower toxicity. Both *Alcaligenes* and *Pseudomonas* biosurfactants are biocompatible, making them suitable for various applications [7].

The rate at which oil is released into the environment raises awareness among the public and raises environmental concerns. Aromatic hydrocarbons found in used motor oil have the potential to cause long-term risks such as carcinogenicity and mutagenicity [8]. The present study analyzed the physicochemical properties of soil samples collected from automobile repair workshops; isolated *Pseudomonas* and *Alcaligenes* species from the collected soil samples; identified the isolated *Pseudomonas* and *Alcaligenes* species using both biochemical and molecular techniques and evaluate the biosurfactant production capability of the isolated *Pseudomonas* and *Alcaligenes* species with in Ilorin metropolis.

2.0 Materials and Methods

2.1 Soil sampling procedure

The soil samples from each local government area (Ilorin North, Ilorin South and Ilorin East) (Plate 1) were mixed together separately and passed through a 1 mm sieve to create three composite samples prior to analysis. The three local government areas were chosen because they

represent Ilorin metropolis. The sampling process was completed within one week, and when samples could not be processed immediately, they were stored at 4°C for no longer than 18-24 hours [9].

Soil samples were prepared for analysis following the methodology outlined by [10]. One gram of each sample was measured into a test tube containing 9 ml of distilled water to prepare the stock solution. The mixture was then placed in a laboratory shaker (S20) for 3 minutes to homogenize the solution. The resulting suspension was subjected to a 10-fold serial dilution. Aliquots (0.1 mL) from the 10^{-2} , 10^{-3} and 10^{-4} dilutions were dispensed under aseptic conditions onto *Pseudomonas* Isolation Agar plates using the spread plate technique. Each dilution was plated in triplicate and incubated at 37°C for 24 hours. After incubation, the developed colonies were sub-cultured to obtain pure samples for identification. Colony counts were performed using a colony counter, and results were recorded as colony-forming units per gram (CFU/g).

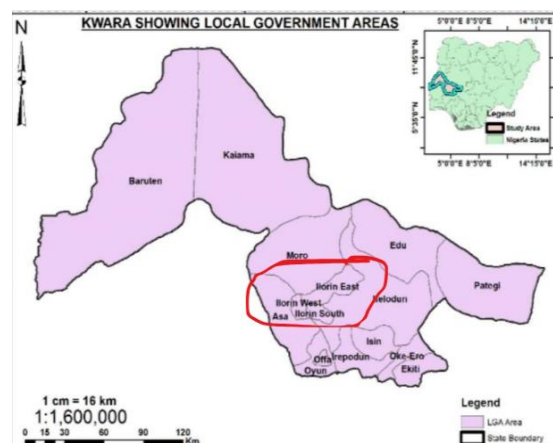


Plate 1. The 3 local governments sampled are circled in red on the Map of Kwara State

2.2 Physicochemical properties of soil from the automobile workshops

The physicochemical properties (pH, electrical conductivity, organic carbon, organic matter, sand, silt, clay, and moisture content composition) of soil from Ilorin mechanic workshops were determined using standard methods [11].

2.3 Characterisation and identification of bacterial isolates

Each isolate was characterized first based on traditional means of characterizing bacteria. Molecular identification involving DNA extraction, PCR, cleaning of PCR product, sequencing and BLASTn was done at Incaba Biotec, Ibadan, Nigeria.

2.4 Screening of the bacterial isolates for biosurfactant production

For this screening, a 100-mL flask containing 30-mL of nutrient broth was inoculated with a standardized pure culture and incubated at 30°C on a rotary shaker at 150 rpm for 72 hours. The culture was centrifuged at 3000 rpm for 30 minutes, after which the resulting supernatants, were used for haemolytic assay, emulsification index and drop collapse in petrol, kerosene and engine oil [12].

2.5 Hemolytic assay of bacterial isolates

Pseudomonas and *Alcaligenes* isolates were streaked on freshly prepared blood agar plates and incubated for 48 h at 37 °C. Visual observation for zones of clearance around the colonies was carried out, and results were recorded as α -haemolysis (appearance of a greenish zone); β -haemolysis (appearance of a clear white zone), and γ -haemolysis (no change in the medium color) [13].

2.6 Emulsification stability (E24) test

Emulsification index test was measured according to the method described by [14]. Two millilitres (2mL) of kerosene, Petrol, Engine oil (Amasco Engen Super Multigrade premium Engine SAE20W/50API SL/CF 1L. Petrol Engine Oil)) was added to 2mL of the culture supernatant, vortex-mixed for 2 min (electronic vortex mixing machine Model XH-B, 2012), each tube of 2mL of supernatant was added along as control (Tween 80). and allowed to stand for 24 h. The E24 was given as the height of emulsified layer (mm) divided by total height of the liquid column (mm) expressed as percentage and was determined at interval of 24 h. The setup was left undisturbed for 24h, and the height of the emulsified layer is measured and used to calculate the emulsification index (E24).

2.7 Drop collapse test

A drop of petrol was placed on different grease-free glass slides and one drop of the obtained supernatant was dispensed at the center of the oil drop. The presence of biosurfactant was detected from the drop collapsing from the oil coat well within a minute. This was indicative of a positive result. A negative result is obtained if no reaction is observed after some minutes [15].

2.8 Statistical analyses

The results were statistically analyzed using one-way ANOVA and Duncan's test ($p < 0.05$).

3.0 Results

The pH of soil from automobile mechanic workshops sampled ranged from (Ilorin West) 6.06 to 7.50 (Ilorin South). The soil electrical conductivity in $\mu\text{S}/\text{cm}$ ranged from 2.19 (Ilorin South) to 90.17 (Ilorin East). The soil organic carbon content in percentage ranged from 0.78 (Ilorin East) to 1.63 (Ilorin South). The organic matter content of the tested soil ranged from 1.25 (Ilorin East) to 7.11 (Ilorin South). The highest sand content for the tested soil was found in the control (88), while the lowest was found at Ilorin South (6.47). The silt content of the soil was lowest at Ilorin South (5.77) and highest at Ilorin East (16.04). The soil clay content was lowest at Ilorin South (5.07) and highest at Ilorin West (12.24). Moisture content value in percentage was lowest at Ilorin South (11.13) and highest in the Control (30.67) (Table 1).

Seven bacteria were obtained after a sampling period of one week. The bacterial isolates obtained from sampling site were identified as *Alcaligenes*-CPO1, *Pseudomonas*-JFF, *Pseudomonas*-PQ6, *Pseudomonas* -JF5, *Pseudomonas*-OR9, *Alcaligenes*-CPO6 and *Pseudomonas*-MH7. *Alcaligenes aeruginosa*-CPO6 was the least present bacteria (33%), while most of the *P. aeruginosa* occurred 100% of the time (Table 2).

Two of the organisms showed alpha haemolysis (*P. aeruginosa*-JFF and *A. faecalis*-CPO6). *Pseudomonas aeruginosa*-PQ6 showed gamma haemolysis while the others showed beta haemolysis (Table 3). The pictorial view of the haemolysis tests is shown in Figure 1. All the bacteria were positive for the drop collapse assay test except *A. faecalis*-CPO6 (Table 3).

Figure 2 shows emulsification index of the seven bacteria with kerosene (paraffin oil), petrol (gasoline) and engine oil respectively. The *P. aeruginosa* and *A. faecalis* isolates had significantly lower emulsification indices than the Tween-80 control (90, 100, 95). Among the isolates, *A. faecalis*-CPO1 (64, 67, 65), *P. aeruginosa*-OR9 (63, 60, 67), and *P. aeruginosa*-MH7 (64, 71, 65) exhibited the highest emulsification activity. These isolates recorded the highest mean emulsification indices, but the values are below the Tween-80 control values. In contrast, *P. aeruginosa*-JFF (22, 36, 29), *P. aeruginosa* -PQ6 (30, 51, 41), *P. aeruginosa*-JF5 (23, 35, 26) and *Alcaligenes faecalis*-CPO6 (23, 40, 38).

The results of the BLASTn of the seven bacterial isolates are shown in Table 4. The E value for all the organisms was 0. The percentage identity was above 99% in each case. The average sequence length was 1500 base pairs except for one *Pseudomonas aeruginosa*.

Table 1: Physicochemical properties of soil from the selected automobile workshops

Location	pH of soil sample	Electrical Conductivity ($\mu\text{S}/\text{cm}$)	Organic Carbon (%)	Organic Matter	Sand	Silt	Clay	Moisture Content%
Ilorin- East	6.31 \pm 0.3 ^b	90.17 \pm 2.65 ^a	0.78 \pm 0.0 ^c	1.25 \pm 0.0 ^b	84.16 \pm 0.2 ^a	16.04 \pm 0.45 ^a	11.46 \pm 0.7 ^b	13.40 \pm 1.73 ^b ^c
Ilorin-South	7.50 \pm 0.1 ^a	2.19 \pm 1.42 ^d	1.63 \pm 0.0 ^a	7.11 \pm 1.7 ^a	6.47 \pm 0.58 ^c	5.77 \pm 0.03 ^b	5.07 \pm 0.02 ^c	11.13 \pm 1.05 ^c
Ilorin-West	6.06 \pm 0.0 ^b	86.89 \pm 0.6 ^b	0.90 \pm 0.0 ^b	1.27 \pm 0.1 ^b	77.11 \pm 1.1 ^b	15.91 \pm 0.58 ^a	12.24 \pm 0.17 ^a	14.10 \pm 0.79 ^b
Control	6.08 \pm 0.0 ^b	77.59 \pm 0.00 ^c	0.93 \pm 0.0 ^b	1.60 \pm 0.0 ^b	88.12 \pm 0.0 ^a	9.47 \pm 5.77 ^b	5.74 \pm 0.00 ^c	30.67 \pm 1.15 ^a

Key: Each value represents Mean \pm Standard deviation

Table 2: Occurrence of bacterial isolate at the sampling sites

Bacteria	Day			Occurrence (%)
	1	2	3	
<i>Alcaligene faecalss</i> -CPO1	+	+	-	67 %
<i>Pseudomonas aeruginosa</i> -JFF	+	+	+	100 %
<i>Pseudomonas aeruginosa</i> -PQ6	+	-	+	67 %
<i>Pseudomonas aeruginosa</i> -JF5	+	+	+	100 %
<i>Pseudomonas aeruginosa</i> -OR9	+	+	+	100 %
<i>Alcaligenes faecalis</i> -CPO6	+	-	-	33 %
<i>Pseudomonas aeruginosa</i> -MH7	+	+	+	100

Key: + = Present; - = Absent

Table 3: Hemolytic assay and Drop collapse assay of the bacterial isolates

Bacteria isolate	Haemolytic reaction	Drop collapse reaction
<i>Alcaligene faecalss</i> -CPO1	β	+
<i>Pseudomonas aeruginosa</i> -JFF	α	+
<i>Pseudomonas aeruginosa</i> -PQ6	γ	-
<i>Pseudomonas aeruginosa</i> -JF5	β	+
<i>Pseudomonas aeruginosa</i> -OR9	β	+
<i>Alcaligenes faecalis</i> -CPO6	α	-
<i>Pseudomonas aeruginosa</i> -MH7	β	+

Key: α -incomplete haemolysis; β -complete haemolysis; and γ -negative reaction (i.e. no haemolysis).
+ = Positive; - = Negative

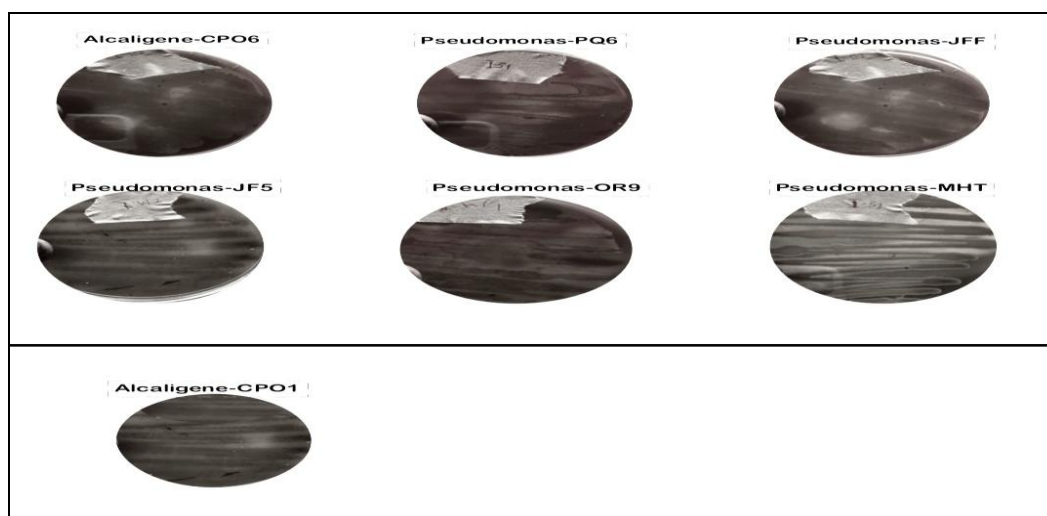


Figure 1: Haemolytic assay reaction among the seven bacteria

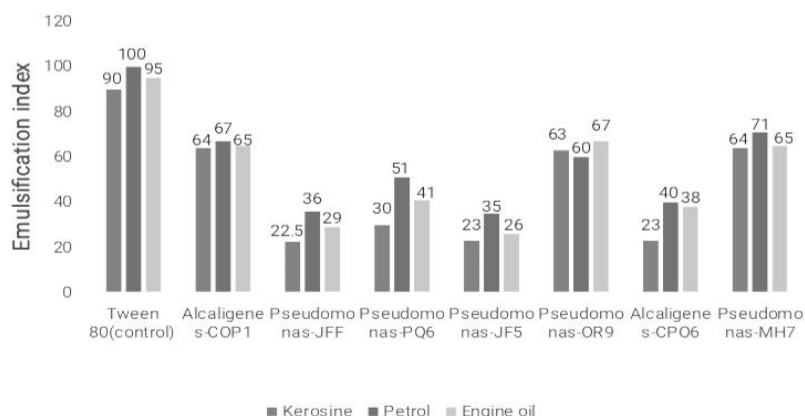


Figure 2: Emulsification index E₂₄ of the organisms with kerosene, petrol and engine oil

Table 4: Sequence BLAST details

S/N	Organism	Sequence length (bp)	Percentage Identity	Accession no of BLAST hit	E value	Highest query coverage (%)
1	<i>Alcaligenes faecalis</i>	1501	99.27%	CP031747.1	0.0	100%
2	<i>Pseudomonas aeruginosa</i>	1499	99.60%	JFF13146.1	0.0	100%
3	<i>Pseudomonas aeruginosa</i>	1505	99.53%	PQ661164.1	0.0	100%
4	<i>Pseudomonas aeruginosa</i>	1502	99.80%	JF513146.1	0.0	98%
5	<i>Pseudomonas aeruginosa</i>	1492	99.93%	OR958577.1	0.0	100%
6	<i>Alcaligenes faecalis</i>	1501	99.53%	CP031747.1	0.0	100%
7	<i>Pseudomonas aeruginosa</i>	869	99.54%	MH760805.1	0.0	99%

4.0 Discussion

A pH of 8.82 in an oil polluted mechanic workshop soil was reported by [18] at Choba, Port Harcourt, Nigeria. It was also reported by [17] that pH of 5.80 and 5.81 was obtained in a previous study done on engine oil polluted soil in Kaduna, Nigeria. These two pH values are totally different from those obtained in this study (6.06 to 7.50). The variations in pH may be due to the amount of rainfall in the geographical areas and also due to the amount of oil spilled on the soil of the automobile mechanic workshops.

The particle size distribution of soils in the study area showed considerable variation in sand, silt, and clay proportions. The highest sand content (88%) occurred in the control site (unpolluted soil), whereas the lowest (6.47%) was recorded at Ilorin

South. Silt content ranged from 5.77% at Ilorin South to 16.04% at Ilorin East, while clay content varied from 5.07% at Ilorin South to 12.24% at Ilorin West. These findings contrast with those of [18], who reported sandy clay loam and sandy loam textures with sand ranging from 72–82%, clay from 13–21%, and silt from 3–5% in similar soil depths (0–30 cm). The differences may be attributed to site-specific factors such as land use history, topography, and management practices, which influence soil texture and particle distribution.

The soil electrical conductivity (EC) values in the present study ranged from 2.19 $\mu\text{S}/\text{cm}$ in Ilorin South to 90.17 $\mu\text{S}/\text{cm}$ in Ilorin East. The difference may be due to the activities going on at the mechanic workshops. These values are markedly lower than those reported by [19], where the average E_{ce} (the specific electrical conductivity of

a liquid extract created from a soil sample that has been saturated with water) across sampled fields ranged from 1.78 to 4.85 dS m⁻¹, classifying the soils as non-saline to moderately saline. While [19] observed salinity levels indicative of potential crop yield impacts in some fields, the EC levels recorded in our study indicate minimal salinity risk. The observed differences may be attributed to variations in climatic conditions, soil texture, and anthropogenic inputs between the study sites.

The soil organic carbon (SOC) content in the present study ranged from 0.78% in Ilorin East to 1.63% in Ilorin South, indicating generally low organic matter levels. In contrast, [20] reported substantially higher SOC values in soils from a mechanic workshop in Abakaliki, ranging from 10.24 g/kg to 30.30 g/kg (equivalent to approximately 1.02% to 3.03%). The relatively higher values in [20]'s study may be attributed to long-term hydrocarbon contamination and organic residue accumulation in workshop soils, whereas the lower SOC in the present study likely reflects differences in land use, organic input, and environmental conditions.

The organic matter (OM) content of the tested soils ranged from 1.25% in Ilorin East to 7.11% in Ilorin South, indicating moderate variability across locations. In contrast, [21] emphasized that not only the quantity but also the composition of soil organic matter, together with pH, plays a crucial role in determining the retention and behaviour of non-ionic pesticides such as carbaryl, carbofuran, and metolachlor. While our study provides quantitative OM values, their findings suggest that qualitative differences in OM fractions could further influence contaminant dynamics, highlighting the need for future work on SOM composition in these soils.

In a study by Chiemela [22], *Pseudomonas* species was isolated from oil-polluted soil in auto-mechanic workshops around a tertiary institution. The isolate showed highest prevalence compared with others. [23] reported the isolation of *Pseudomonas aeruginosa* in their work on the assessment of hydrocarbonoclastic microorganisms from selected mechanic workshops in Nigeria. [24] reported the absence of *Pseudomonas* species in their work on the isolation and identification of biosurfactant-producing bacteria from hydrocarbon-contaminated mechanic workshop soils in Port Harcourt, Nigeria. Previous study by [23] and [22] agrees with our findings in this present work while that of [24] did not. Study by [25] had previously reported that the isolation of *Alcaligenes faecalis* from oil contaminated soil in Keffi. This aligns with our finding in this present work. Native *Alcaligenes faecalis* have also been reported with the leaching of gold [26].

The biodegradation of crude oil by biosurfactant-producing microaerophilic bacterium

has been reported by [27]. *Pseudomonas aeruginosa*. [28], had previously reported the production of rhamnolipid in the presence of halophilic *Pseudomonas aeruginosa*. These two reports show that *Pseudomonas aeruginosa* is an organism that has haemolytic potential as reported in this work. [29], worked on the screening and identification of biosurfactant-producing bacteria from crude oil contaminated soil and water in the Niger Delta area and they reported that *Alcaligenes faecalis* had the potential to produce biosurfactant. This aligns with the results obtained in this study.

Emulsification index higher than 50% is generally considered a good biosurfactant producer and in essence a good biodegrader [30]. In the work of [31], the emulsification percentages obtained was greater than 60% in the different substrates evaluated. In the present study, three species of *Pseudomonas* and *Alcaligenes* had greater than 60% emulsification index while four species of *Pseudomonas* and *Alcaligenes* had less than 50% emulsification index. According to [32], a positive drop collapse test indicates the ability to produce biosurfactant while the absence of a drop collapse shows the inability to produce biosurfactant.

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Ethical statement

This study did not require ethical approval as it involved only the isolation of non-harmful environmental bacterial strains. All procedures adhered to relevant institutional and environmental guidelines.

Disclosure of conflict of interest

None

Disclosure of source of funding/grant

None

Authors Contributions

DOA conceptualized and supervised the research project, provided guidance on study design, data analysis, and manuscript preparation. GOA conducted the experiments, collected and analyzed the data as part of the M.Sc. dissertation, and drafted the initial manuscript. Both authors reviewed and approved the final version of the manuscript.

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