Screening of bioemulsifier-producing drugs to exclude mucus-forming and hydrocarbon destructors

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Abstract. The current study aimed to examine 271 bacterial isolates from the plant rhizosphere and oil-contaminated soil for the ability to produce extracellular bioemulsifiers. The bacterial isolates were screened for bioemulsifier production using several tests, including oil displacement, parafilm M destabilization, droplet collapse, and the emulsification index (E-24). The most effective strains were characterized by 8 isolates displaying an E-24 index greater than 60%. Among these, 6 strains isolated from plant rhizospheres demonstrated the ability to form mucus on solid media. The remaining two strains, Z2 and D1, were isolated from oil-contaminated soils and exhibited robust growth in a medium supplemented with oil and diesel. Surface tension was measured using the Wilhelmy plate method, finding low surface tension values of 57.6 ± 0.6 and 55.6 ± 0.6 mN/m for the culture supernatants of strains Z2 and D1, respectively. Further, a study on the cell hydrophobicity of strains Z2 and D1 revealed values above 70%, indicating high hydrophobicity. Strains Z2 and D1 were selected for their high emulsifying activity in the presence of edible oils and petroleum hydrocarbons, suggesting their potential as bioemulsifier producers.

1 Introduction

Many microorganisms synthesize extracellular polymeric substances known as bioemulsifiers. These substances are crucial for microbial survival, aiding in nutrient transport and acting as biocidal agents. Bioemulsifiers are high-molecular-weight, amphiphilic surfactant molecules that stabilize oil-in-water emulsions or facilitate the formation of emulsions between two immiscible liquids by binding to water-insoluble substrates [1, 2]. Depending on their biochemical composition, these compounds can be classified as glycolipids, lipopeptides, fatty acids, or phospholipid polymers, with glycolipids and lipopeptides being the most prevalent [3, 4].

Microorganisms capable of producing bioemulsifiers are found in diverse environments, including water, soil, various deposits, and plant tissues. They are particularly notable in ecosystems contaminated with oil, pesticides, and other contaminants, thriving under a wide range of temperatures, pH levels, and salinity conditions [5, 6].

Bioemulsifiers have the ability to reduce surface and interfacial tension, exhibiting properties such as dispersion, wetting, emulsification, and demulsification of hydrophobic compounds. These characteristics render them valuable in biotechnological applications, especially in the remediation of organic contaminants [4; 7].

Given the growing interest in identifying new bioemulsifier-producing microorganisms, our study aims to provide a comprehensive screening results on the production of bioemulsifiers.

2. Materials & methods

2.1 Sample collection

216 bacterial isolates were obtained from the soil, rhizosphere, and roots of several plant species, including (*Mentha piperita, Pteridium aquilinum, Melissa officinalis*, *Achillea micrantha* Willd, *Artemisia terae-albae* Krasch, *Plantago major*, *Urtica dioica* L., and *Phacelia tanacetifolia*.) All isolates were identified as mucus-forming bacteria. In addition, 55 isolates were obtained from the oil-contaminated soil samples collcted from the Zaburunye, Dossor, and Balgimbayev fields. These isolates were specifically tested for their ability to grow on crude oil.

2.2 Media composition

The current study included the utilization of the following media and their respective compositions:

YM medium (g/L): yeast extract – 3, malt extract – 3, peptone – 5, dextrose – 10, agar – 20, pH of 7.0.

YPG medium (g/L): glucose – 20, yeast extract – 3, peptone – 5, agar – 20, pH of 7.0 [8].

Mineral-salt medium (MSM) was supplemented with 1% (v./v.) crude oil or oil as carbon source (g/L): Na_2HPO_4 – 0.28, KH_2PO_4 – 1.14, NH_4NO_3 – 3, K_2SO_4 – 0.36, $MgSO_4 \times 7H_2O$ – 0.154, $CaCl_2 \times 2H_2O$ – 0.023, $FeSO_4 \times 7H_2O$ – 0.038, NaCl – 0.10, yeast extract – 0.5, pH 7 [9].

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2.3 Determination of bio-emulsifying capabilities

To evaluate the capacity of the isolates to synthesize biosurfactants, various tests were conducted [9-10].

Hydrocarbon overlay method. Following the procedure outlined by Hanano et al. [10], 1 mL of the bacterial culture was spread over an agar plate prepared with an MSM. This plate was then coated with 100 µL of hydrocarbon and incubated at 37°C for 7 days. The presence of a colony surrounded by an emulsified halo indicated positive biosurfactant production.

Microtiter plate method. In this assay, each well of a 96-well microtiter plate was coated with a thin layer of either olive oil or motor oil. Then, 5 µL of the culture supernatant, after 72 h of growth, was added to the center of the well. The drop's stability was observed for 1 min, with the medium itself serving as a control. Destabilization of the drop within this timeframe signified a positive result for emulsifying substances.

Parafilm spot test M. For this test, 20 µL of culture supernatant, grown for 72 h, was placed onto a parafilm strip. The shape of the droplet was observed for 1 min. A droplet that destabilized within this period was considered a positive indication of emulsifying substance production. A droplet of uninoculated medium acted as the control.

Oil displacement method. A Petri dish was filled with 40 mL of distilled water and covered with a thin layer of 10 mL of olive oil or motor oil. Then, 20 µL of the supernatant was gently placed on the oil layer. The formation of a clear zone, due to oil displacement, confirmed the presence of emulsifying substances. The diameter of the displacement zone was measured to assess the effectiveness.

2.4 Emulsifying coefficients

Emulsifying activity – proposed by Franci et al. [11]. The emulsifying activity of microorganisms in the culture medium was visually assessed immediately after cultivation. The well-mixed culture media were rated on a four-point scale based on their turbidity and the degree of motor oil emulsification within the medium, with scores ranging from 0 (no emulsification) to 4 (maximum emulsification).

Emulsification index (E24). To determine the emulsification index, a cell-free supernatant (2 mL) was mixed with an equal volume of hexadecane (2 mL) in a measuring tube. The mixture was vigorously shaken for 2 min and then allowed to settle at room temperature. After 24 h, the E24 was calculated as the percentage of the dense emulsion layer volume ("cream") relative to the total liquid volume in the tube [12, 13].

Emulsifying activity of a cell-free agent – outlined by Cirigliano and Karman [14]. After removing cells by centrifugation (a Minispin microcentrifuge, Hamburg, Germany) at 10,000 rpm for 5 min at room temperature, the cell-free supernatant (4 µL) was mixed with 0.05 M Na-K phosphate buffer (1.5 g; pH of 6.8) and hexadecane (2.5 mL). The emulsification index was determined following the approach described by Cooper and Goldenberg [15].

Surface tension – described by Walter et al. [16]. Surface tension measurements were performed in the culture's supernatant at 20°C using a VT-500 torsion scale tensiometer with an aluminum plate. The following formula was used to calculate surface tension:

$$\sigma = \frac{mg}{2l}$$

where m – the mass required to detach the plate, g – the acceleration due to gravity equal to 9.8 m/s², and l – the separation line length or plate width equal to 15.7 mm.

The change in surface tension (Δσ) was determined by comparing the sample's surface tension to that of a sterile medium (control).

Cell surface hydrophobicity (CSH) – the MATH method described by Coimbra et al. [17]. Cells were washed (2×), resuspended in buffered saline (16.9 g/L K_2HPO_4, 7.3 g/L KH_2PO_4) to achieve an optical density (OD) at 600 nm of 0.5. Then cell suspension (2 mL) was mixed with kerosene (100 µL) and kept vortexing for 3 min. After vortexing and allowing the phases to separate (within 1 h), the OD of the aqueous phase was measured to assess hydrophobicity.

Research results were statistically processed using Microsoft Excel (2019). The data presented in the form of mean ± SD.

3 Results

More than 216 isolates from the plant and soil samples contaminated with hydrocarbons collected from the Dossor, Zaburunye and Balgimbaev deposits were screened for the ability to synthesize bio-emulsifying substances. Screening findings revealed more than 150 isolates that are able to produce bio-emulsifying substances.

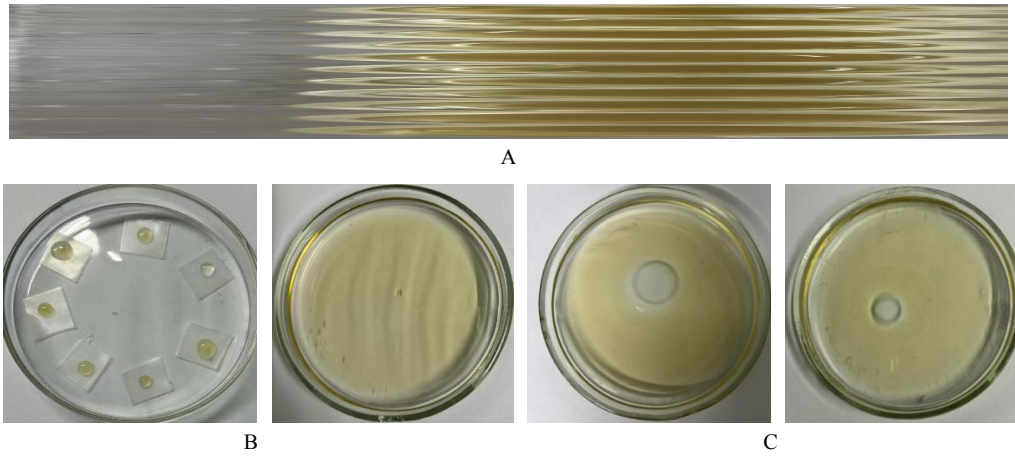

Fig. 1. Screening for capability of isolates to synthesize bio-emulsifying substances. A) tablet method; B) parafilm spot test M; C) oil displacement method.

The emulsifying activity of selected isolates was assessed visually after culturing them in liquid media containing engine oil. The degree of emulsification was rated on a scale from 0 to 3 based on the stability and characteristics of the resulting emulsion. A score of 3 was assigned to emulsions consisting of fine motor oil particles that remained stable without stirring for an hour and were accompanied by a color change. Emulsions stable for ten minutes, displaying micelles of both "oil in water" and "water in oil" types, were scored as 2. Emulsions characterized by large micelles and dense particles received a score of 1. Samples with no emulsifying activity, evidenced by the presence of an oil film on the flask's surface, were scored as 0. Stable emulsions were observed in over 90 isolates, for which the emulsification index of the hydrophobic substrate was measured.

The emulsification indices (E24) of the culture liquid containing cells, during bacterial growth on YPG and YM media, were high in 48 isolates (> 50%). The highest E24 values, ranging from 70 to 90%, were observed in 3 isolates grown on YM medium. For other 5 isolates, the E24 value was slightly lower but remained above 26.6 to 68.2 %. The most active isolates were derived from the plants' rhizospheres and oil-contaminated soils, with the emulsions of 2 isolates remaining stable for more than 3 days. These findings indicate that isolated microorganisms are potential bio-emulsifiers exhibiting significant emulsifying activity.

For six strains (FRK, TRK8, TRK27, PRK13, MEK33, and PEK1), the E24 values were consistently high across CS, BM, and BS media, suggesting the production of both exo- and endo-type biosurfactants. Comparing the emulsification indices of BM and CS media, we infer that strains Z2 and D1 synthesize exo-type surfactants.

Table 1. Surface-active properties of microorganisms.

Strain	Emulsification index (E24), %			Cell hydrophobicity, %	Surface tension, mN/m
	CS.	BS	BM		
FRK	84.2 ±4.8	76.2 ±9.9	76.2 ±9.9	81.6±2.6	81.5±2.8
TRK8	86.4 ±6.7	80.1. ±3.7	80.1. ±3.7	81.5±1.3	83.3±2.2
TRK27	91.5 ±8.9	82.6 ±2.6	82.6 ±2.6	81.2±2.2	83.1±1.3
PRK13	61.8 ±6.5	52.8 ±3.8	62.8 ±3.8	76.5±1.6	78.7±0.4
MEK33	61.7 ±8.6	53.1 ±3.3	63.1 ±3.3	37.9±1.5	80.2±1.4
PEK1	61.4 ±6.4	53.7 ±3.1	63.7 ±3.1	80.3±1.3	79.2±1.7
Z2	64.1 ±3.1	68.2 ±3.8	31.2 ±1.8	77.9±1.7	57.6±0.6
D1	63.5 ±2.6	66.6 ±2.1	26.6 ±1.1	80.6 ±2.6	55.6±0.6

Note: Cell–free supernatant - BS; Cell biomass - BM; culture medium with cells – CS.

Cell surface hydrophobicity can serve as an additional criterion for selecting bacteria that synthesize cell-associated biosurfactants bioavailants. The degree of cell hydrophobicity is determined by the adherence of microorganisms to hydrophobic surfaces. Our investigation into the cell hydrophobicity revealed that, in most cases, it is notably high, exceeding 70%.

A reduction in surface tension was noted, decreasing to 57-56 mN/m (compared to 77 mN/m in the control) in two strains (Z2 and D1) isolated from oil-contaminated soils. These strains are classified as gram-positive bacteria and exhibit shiny, slightly slimy colonies that thrive on media containing various beehive gardens. In contrast, the other strains demonstrated a mucoid colony phenotype, with a minor increase in surface tension observed (Table 2).

Table 2. Cell morphology of strains capable of emulsifying activity.

Strain	Colony morphology	Cell shape	Gram stain	Mobility	Spore formation
FRK	Irregular shape, creamy mucus	rods	G^-	+	-
TRK8	Irregular shape, greenish-yellow mucus	rods	G^-	+	-
TRK27	Round pink, mucus -like	rods	G^-	-	-
PRK13	Irregular shape, creamy mucus	rods	G^-	+	-
MEK33	Irregular shape, creamy mucus	rods	G^-	+	-
PEK1	Irregular shape, beige mucus	rods	G^-	+	-
Z2	Round, beige oily	rods and cocci	G^+	+	-
D1	Round red oily	cocci	G^+	-	-

4 Conclusion

The ability of microorganisms to decrease the surface tension of a nutrient medium by 10 mN/m serves as an indicator of their surface-active properties. Two bacterial strains, Z2 and D1, demonstrated a reduction in the surface tension of their supernatants, suggesting that they are capable of producing bio-emulsifying substances. It is well-documented that most microbial surfactants can emulsify a variety of hydrocarbons at differing efficiencies [18, 19].

Furthermore, the capacity of bio-emulsifiers to form stable emulsions with hydrophobic substrates, as evidenced by high emulsification index values, underscores their potential in synthesizing emulsifying substances. For the remaining strains examined, no reduction in surface tension was observed; instead, a slight increase was noted. This phenomenon correlates with the emulsifying activity ascertained through the E24 index measurements and cell hydrophobicity indicators. Morphologically, these strains were identified as mucoid, forming viscous colonies on solid nutrient media, indicative of biopolymer-producing bacteria which likely secrete exopolysaccharides with emulsifying properties [20].

Biopolymers produced by bacteria, either extracellularly or intracellularly, and subsequently secreted into the environment, serve as scaffolds for the incorporation of carbohydrates, proteins, nucleic acids, and lipids [21]. Hence, the strains Z2 and D1, isolated from oil-contaminated soils, were highlighted for their remarkable emulsifying activity in the presence of edible oils and petroleum hydrocarbons, along with the stability of the emulsions they formed. These characteristics render them promising candidates for the bioremediation of oil-contaminated ecosystems.

Conflict of interest

The authors declare no conflict of interest.

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Authors' contribution

Conceptualization: T. Mukasheva, R. Berzhanova, methodology: K. Yesentaeva, A. Zhuniszhan.
Investigation: K. Yesentaeva, A. Zhuniszhan, Writing – Original Draft Preparation: K. Yesentaeva, A. Zhuniszhan, writing – review & editing, T. Mukasheva, R. Berzhanova, A. Mikolash.

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