Screening of effective biosurfactant-producing bacteria from plant rhizosphere and oil-polluted soils

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Abstract. Identification and characterization of effective biosurfactant-producing strains is critical for the successful implementation of a wide range of biotechnological processes. This study used a comprehensive screening approach of potential biosurfactant producing bacteria isolated from plant rhizosphere and oil polluted soils. A total of 63 isolates were obtained and their ability to produce biosurfactants was assessed based on the emulsification index (E24) and surface tension measurements using cell-free culture supernatants. The results revealed significant differences in the potential of isolates to produce surfactants, with E24 values ranging from 5% to 81% and surface tension values ranging from 27.1 to 68.1 mN m^{-1}. The most promising biosurfactant-producing strains were able to reduce the surface tension of the culture medium to values below 30 mN m^{-1} and demonstrated high emulsification indices (>40%), indicating their ability to produce biosurfactants in concentrations sufficient to exhibit high surfactant and emulsifying properties. Using the Sanger sequencing method, the three strains with the highest biosurfactant producing potential were identified as *B. mojavensis, P. fluorescens, P. putida*.

1 Introduction

There is growing scientific and industrial attention to biosurfactants, which are surface-active compounds produced by a wide range of microorganisms, including bacteria, yeasts and fungi. These amphiphilic molecules possess both hydrophilic and hydrophobic moieties, allowing them to reside at interfaces between polar and nonpolar phases, such as air-water or oil-water interfaces. This feature allows biosurfactants to reduce surface and interfacial tension, provide emulsifying and dispersing effects, making them valuable for numerous applications in environmental remediation, enhanced oil recovery, food and pharmaceutical industries.

Emulsifying and surface-active properties allow the use of biosurfactants in oil production, and for bioremediation of soils contaminated with oil and heavy metals. Due to the structure of the molecule, biosurfactants also have antimicrobial and fungicidal effects. It is known that lipopeptides can destroy the membranes of fungal cells, which leads to lysis and cell death. For example, researchers demonstrate that biosurfactants of the

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lipopeptide group are able to suppress the growth of phytopathogens *Fusarium oxysporum* and *Alternaria* spp.

The search for effective biosurfactant-producing microorganisms has received significant attention due to the growing demand for sustainable and environmentally friendly alternatives to synthetic surfactants. Conventional chemical surfactants often exhibit toxicity, poor biodegradability, and environmental resistance, which has led to increased attention to the exploration and exploitation of microbial resources for the production of biosurfactants. However, the identification and isolation of new biosurfactant-producing strains from different environmental sources remains a challenge because the ability to produce biosurfactants can vary greatly among microbial species and even within the same species.

In this study, we aimed to evaluate the biosurfactant production potential of microbial isolates obtained from different soils, including those contaminated with petroleum hydrocarbons and those from plant rhizosphere. Key criteria for assessing the biosurfactant activity of isolates included a decrease in surface tension, emulsification index (E24) and the ability to form stable emulsions. These parameters are widely used as indicators of biosurfactant production and have been adopted by researchers worldwide to identify and characterize effective biosurfactant-producing strains. By examining the microbial diversity of urban and oil-contaminated soils, this research contributes to ongoing efforts to discover new and potent biosurfactant-producing microorganisms that can be further developed for various industrial and environmental applications.

2 Materials and methods

2.1 Soil Sampling

To isolate bacterial species, soil samples were taken from 2 soil samples: the rhizosphere of higher plants and an oil production site contaminated with crude oil (soil type Luvisol). Sampling was conducted in accordance with standard protocols to ensure the representativeness of the samples. Samples were obtained from the top 10 cm of the soil profile, placed in sterile containers, homogenized, and transported to the laboratory for further processing.

2.2 Strain isolation

Soil samples were plated in flasks containing Luria-Bertani (LB) medium. The culture liquid was then plated in serial dilutions onto Petri dishes containing meat peptone agar. The Erlenmeyer flasks and then the plates were incubated at 28°C for 24 hours to allow the growth of microbial colonies. Individual colonies were selected based on their morphological characteristics and cultured to obtain pure isolates.

2.3 Strain identification

Bacterial colonies were analysed in MALDI-TOF Biotyper (BRUKER, Billerica, MA, USA) directed for species identifications. For the Sanger method, DNA was first extracted using the commercial S-Sorb kit from Synthol (Moscow, Russia). After DNA was isolated from cultures grown in liquid medium, sequencing was performed using the Sanger method - the nucleotide sequence of the 16S rRNA gene was assessed using primers 27f and 1492r. MEGA 7.0 software was used to process the obtained sequences.

2.4 Cultivation

The pure microbial isolates were cultivated in liquid media optimized for biosurfactant production, such as mineral salt medium (MSM) supplemented with a suitable carbon source, such as glucose or glycerol. The cultures were incubated at 28°C with agitation (150 rpm) for 72 hours to allow for the production of biosurfactants.

2.5 Preparation of Cell-free Culture Supernatants

After the incubation period, the microbial cultures were centrifuged at 3,000 × g for 40 minutes to separate the cells from the culture medium. The resulting cell-free supernatants were used for the evaluation of the biosurfactant-producing potential of the isolated strains.

2.6 Evaluation of surfactant-producing potential

The biosurfactant-producing potential of the isolated strains was evaluated using the following methods. Emulsification index (E24): The cell-free culture supernatant was mixed with an equal volume of a hydrophobic compound, such as n-hexadecane or motor oil, and the mixture was vortexed for 2 minutes. The height of the emulsified layer was measured after 24 hours and expressed as a percentage of the total height of the mixture.

Surface tension measurement: The surface tension of the cell-free culture supernatant was measured using a tensiometer Kruss K-20 (KRUSS, Göppingen, Germany) by a Du Noüy ring method. The reduction in surface tension compared to the uninoculated medium was used as an indicator of biosurfactant production. The most promising isolates, based on the results of these assays, were selected for further characterization and optimization of biosurfactant production.

2.7 Statistical analysis

All measurements were conducted at least three times. Statistical analysis was performed using Python (Python 3.12.4). Fisher's exact test was used to assess the significance of differences at $\alpha = 0.05$.

3 Results and discussions

Isolation and cultivation of microorganism strains from soil samples resulted in a diverse collection of 63 isolates. These isolates were morphologically distinct and each was assigned a numerical identifier, with alphabetical indices indicating the presence of colonies with different morphological characteristics on the same Petri dish. Preliminary screening of these isolates was carried out to assess their ability to produce biosurfactants, which included evaluation of the emulsification index (E24) and surface tension measurements using cell-free culture supernatants (Figure 1).

The emulsification index (E24) is a widely used parameter to evaluate the ability of a biosurfactant solution to form stable emulsions with hydrophobic compounds. In this study, E24 values for the cell-free culture supernatants tested ranged from 5% to 81%, indicating significant differences in the emulsifying properties of the resulting secondary metabolites, among which biosurfactants were clearly present. Strains with higher E24 values, such as isolates 2b, 31os and 32os, have demonstrated high emulsion stabilization capabilities, which is essential for a number of applications including enhanced oil recovery, bioremediation and the formulation of pharmaceutical and cosmetic products.

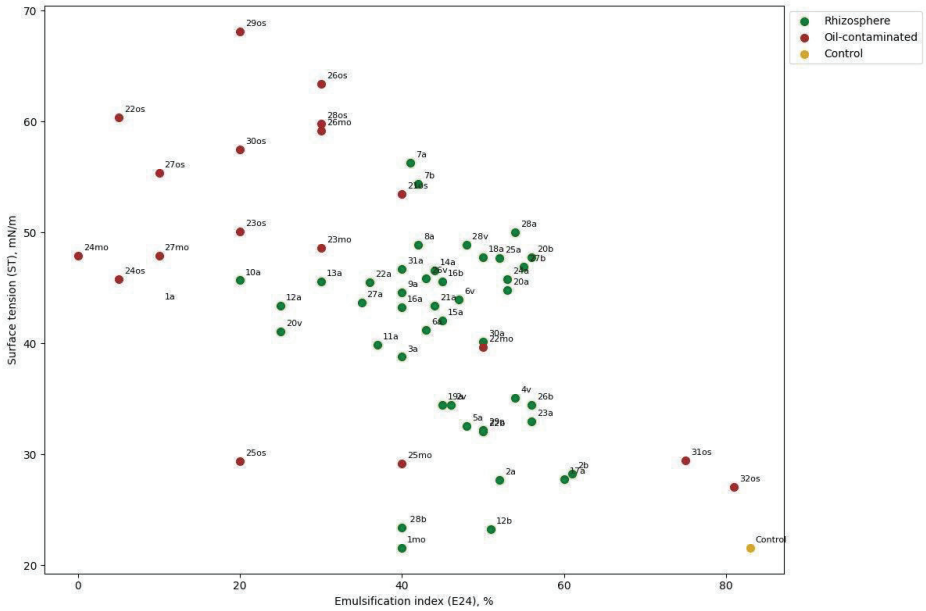

Fig. 1. Emulsification index (E24) and surface tension measurements for isolates.

Have Surface tension measurements of culture supernatants provided additional information on biosurfactant production by isolated strains. The decrease in surface tension directly correlates with the concentration of biosurfactants present in the solution. The most efficient biosurfactant producers were able to reduce the surface tension of the culture medium from an initial value of 72 mN m^{-1} to 27.1 mN m^{-1}. This significant reduction in surface tension is a clear indication of the production of biosurfactants by these strains, since biosurfactants can typically reduce the surface tension of water to values below 30 mN m^{-1}.

A joint assessment of E24 values (highest values were selected) and surface tension values (lowest values were selected) allowed us to identify the three most promising biosurfactant-producing isolates: 2b, 31os and 32os. These strains exhibited both high emulsification indices (>40%) and low surface tension values (<30 mN m^{-1}), suggesting their ability to produce biosurfactants in concentrations sufficient to exhibit potent surfactant properties. It is important to note that E24 values and surface tension measurements do not always correlate, since the hydrophilic-lipophilic balance of various biosurfactants and other metabolites present in a solution can influence these parameters independently.

All 63 isolates were identified by matrix desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), a powerful tool for rapid and accurate identification of microbial isolates, allowing identification at the genus level (Table 1). This method is less accurate than sequencing, especially for natural (not clinical) samples. However, it is simpler, less time-consuming and less expensive, making it suitable for screening purposes.

Table 1. Indicators of isolates according to the level of surfactant-producing activity

Sample No.	Soil Source	Strains	Genus
1	Plant rhizosphere	1a	*Bacillus* spp.
2		2a	*Bacillus* spp.
3		2b	*Bacillus* spp.
4		2v	*Bacillus* spp.

5		3a	*Bacillus* spp.
6		4v	*Paenibacillus* spp.
7		5a	*Paenibacillus* spp.
8		6a	*Bacillus* spp.
9		6v	*Bacillus* spp.
10		7a	*Bacillus* spp.
11		7b	*Bacillus* spp.
12		8a	*Bacillus* spp.
13		9a	*Bacillus* spp.
14		10a	*Bacillus* spp.
15		11a	*Bacillus* spp.
16		12a	*Bacillus* spp.
17		12b	*Pseudomonas* spp.
18		13a	*Pseudomonas* spp.
19		14a	*Bacillus* spp.
20		15a	*Bacillus* spp.
21		16a	*Bacillus* spp.
22		16b	*Bacillus* spp.
23		17a	*Pseudomonas* spp.
24		18a	*Bacillus* spp.
25		19a	*Paenibacillus* spp.
26		20a	*Paenibacillus* spp.
27		20b	*Bacillus* spp.
28		20v	*Bacillus* spp.
29		21a	*Bacillus* spp.
30		22a	*Bacillus* spp.
31		22b	*Bacillus* spp.
32		23a	*Bacillus* spp.
33		24a	*Bacillus* spp.
34		25a	*Bacillus* spp.
35		26b	*Bacillus* spp.
36		26v	*Bacillus* spp.
37		27a	*Paenibacillus* spp.
38		27b	*Paenibacillus* spp.
39		28a	*Bacillus* spp.
40		28b	*Paenibacillus* spp.
41		28v	*Arthrobacter* spp.
42		29a	*Bacillus* spp.
43		30a	*Bacillus* spp.
44		31a	*Bacillus* spp.
45	Oil-contaminated Luvisol	21mo	*Peribacillus* spp.
46		22mo	*Sphingomonas* spp.
47		23mo	*Nocardiopsis* spp.
48		24mo	*Bacillus* spp.
49		25mo	*Ochrobactrum* spp.
50		26mo	*Shouchella* spp.
51		27mo	*Sphingomonas* spp.
52		21os	*Bacillus* spp.
53		22os	*Bacillus* spp.
54		23os	*Bacillus* spp.
55		24os	*Staphylococcus* spp.
56		25os	*Bacillus* spp.
57		26os	*Sphingomonas* spp.
58		27os	*Sphingomonas* spp.

59		28os	*Sphingomonas* spp.
60		29os	*Sphingomonas* spp.
61		30os	Unidentified bacterium
62		31os	*Pseudomonas* spp.
63		32os	*Pseudomonas* spp.

According to the obtained data, 33 isolates isolated from the rhizosphere soil belonged to the genus *Bacillus*, 3 - *Pseudomonas*, 7 - *Paenibacillus*, 1 - *Arthrobacter*. Among the oil-contaminated soil, most isolates belonged to the genus *Sphingomonas* (6), 5 isolates to the genus *Bacillus,* 2 - *Pseudomonas,* one each to the genera *Peribacillus, Ochrobactrum, Staphylococcus, Nocardiopsis.*

The above-mentioned genera of bacteria, on the one hand, are typical representatives of soils, on the other hand, they are often mentioned in publications concerning the ability of microorganisms to decompose hydrocarbons, increase the surface area of oil droplets and the bioavailability of hydrophobic substrates (*Pseudomonas* spp., *Paenibacillus* spp.), as well as stimulate plant growth or provide them with nutrients and protection from pathogens (*Bacillus* spp., *Arthrobacter* spp.).

For the 3 most effective strains, the species identity was determined by the Sanger sequencing method. It was found that isolate 2b belongs to the species *B. mojavensis*, isolate 31os - *P. fluorescens*, isolate 32os - *P. putida.*

These species are known as biosurfactant producers. Thus, *B. mojavensis* is capable of producing lipopeptide biosurfactants, which are known to have antibacterial and fungicidal activity. In this regard, this type of biosurfactants is often used as fungicidal biopreparations.

Whereas the species *P. fluorescens* and *P. putida* are capable of synthesizing glycolipid biosurfactants, namely rhamnolipids. Rhamnolipids are known as substances with high emulsifying activity, they are used in a variety of applications, in particular for MEOR purposes, due to their ability to change the viscosity of oil and form biofilms that reduce rock permeability, plug pores and thus facilitate the movement of oil.

The results of this study contribute to ongoing efforts to discover and characterize effective biosurfactant-producing microorganisms from a variety of environmental sources. The identified strains with high abilities to produce surfactants have great potential for further development and application in solving industrial and environmental challenges.

4 Conclusion

The present study aimed to screen and evaluate the biosurfactant production potential of microbial 63 isolates obtained from plant rhizosphere and oil-contaminated soil samples. The results revealed several potent biosurfactant-producing strains, identified based on their ability to significantly reduce surface tension (27-29 mN m^{-1}) and form stable emulsions (61-81%). The strains were identified as *B. mojavensis, P. fluorescens, P. putida*. Detailed characterization of these efficient producers will provide valuable information about the optimal production conditions of biosurfactants and the physicochemical properties of biosurfactants.

Future studies may focus on the genetic and metabolic characteristics of these isolates, as well as the development of pilot and large-scale fermentation processes to improve the yield and quality of biosurfactants produced. Integration of these efficient biosurfactant-producing microorganisms into biotechnological processes can facilitate the development of sustainable and environmentally friendly alternatives to synthetic surfactants, meeting the growing demand for innovative and environmentally friendly solutions.

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