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Isolation and screening of biosurfactant producing strains of *Bacillus* spp. from soils on selected sites in North Macedonia

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Abstract

Biosurfactants are eco-friendly, biodegradable, and versatile compounds with extensive industrial applications, notably in environmental bioremediation. Biosurfactants are known for lowering surface and interfacial tension within aqueous and hydrocarbon mixtures. Their primary application lies in the oil industry, where they enhance oil quality and assist in extraction processes, making oil recovery more efficient. Despite their diverse uses, the environmental distribution of biosurfactant-producing bacteria remains largely unexplored. This research focused on exploring the presence of biosurfactant-producing bacteria in both uncontaminated and polluted settings. Soil samples, totaling eight from polluted areas and four from undisturbed sites, were cultured on nutrient agar, and bacterial isolates were subsequently analyzed for their distinctive macroscopic and microscopic features, especially highlighting those with biosurfactant potential. Confirmation was carried out by Gram staining method of all isolates. Additionally, geochemical parameters of the soil samples were measured to emphasize the relevance of these strains for industrial applications and underscore their ecological role within soil environments. The biosurfactant potential of the 37 bacterial isolates was assessed utilizing the oil spread technique and hemolysis assay. Twenty of the isolates exhibited biosurfactant activity, primarily representing samples from contaminated soil environments. Bacterial isolates demonstrating significant biosurfactant production capabilities can be utilized for the bioremediation of contaminated soils. This application harnesses their natural properties to enhance the degradation of pollutants in affected environments.

Keywords: biosurfactants, soil, *Bacillus*, oil spread, hemolytic activity

Introduction

Biosurfactants, produced by bacteria, fungi, and molds, are lipid compounds with both hydrophilic and hydrophobic properties, allowing them to reduce surface tension effectively. Their low toxicity, biodegradability, and stability under extreme environmental conditions make them favorable alternatives to synthetic surfactants across industries, particularly in petrochemical, pharmaceutical, and food applications (Jazeh et al. 2012). The advancement of sustainable technologies has encouraged the exploration of natural, biodegradable compounds for the remediation of hydrocarbon-contaminated sites, leading to increased interest in biosurfactants from biological sources. These natural surfactants offer eco-friendly alternatives for managing environmental contamination (Phulpoto et al. 2020). They enhance pollutant solubility and bioavailability in contaminated soil and groundwater, facilitating more efficient degradation by microorganisms (Ng et al. 2022). Biosurfactants facilitate the cleanup of oil spills by breaking down oil-water emulsions and boosting oil solubility, which enhances its removal from the environment. They are also effective for addressing

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heavy metal pollution, a significant environmental issue due to the persistence of heavy metals in soil, as these elements do not naturally degrade and can accumulate over long periods (Zobaer et al. 2024). Through the production of biosurfactants, microorganisms convert toxic metals into safer, non-toxic forms, thus aiding in the preservation of soil quality (Srivastava et al. 2021). To develop an effective bacterial consortium or monocultures capable of producing biosurfactants, it is crucial to understand the diversity of the microbial community in soils, along with their metabolic characteristics and degradation capabilities (Bento et al. 2005). This knowledge is essential for optimizing biosurfactant production processes and ensuring successful bioremediation strategies. Despite the numerous advantages of biosurfactants, their widespread industrial application is significantly constrained by the high costs associated with their production (Sajna et al. 2015). Due to the valuable nature of biosurfactants produced by biological means, this study aimed to isolate novel bacterial strains from the genus *Bacillus* from undisturbed and contaminated soils with high biosurfactant production potential.

The genus *Bacillus* was first defined in 1872 by the German biologist Ferdinand Kohn. Placed in the phylum Firmicutes, today this genus contains over 200 described species and subspecies. According to their morphological characteristics, bacteria of this genus are described as rod-shaped, large, Gram-positive, aerobic or facultatively anaerobic and catalase-positive bacteria (Logan et al., 2009). Due to their wide range of physiological properties as well as the ability to form endospores, bacteria of this genus are resistant to harmful effects from the environment and are present in almost all habitats, including soil. *Bacillus* spp. are predominantly soil and rhizosphere bacteria, which constitute about 95% of the population of Gram-positive bacteria. They are also among the most widespread endophytic bacteria (Prashar et al., 2013).

Bacillus is a large and diverse group of pathogenic and non-pathogenic bacteria. Many of the species in the genus, as well as their products, are considered safe for use in the environment. These bacteria are preferred for commercialization due to their ability to secrete several bioactive metabolites, produce extremely tolerant endospores, and grow rapidly in a variety of media. Combined with their ability to form spores and their rapid growth on a variety of media, they represent an ideal resource from which to obtain environmentally friendly products (Wu et al., 2015). As such, they can be easily manipulated and maintained viable, as well as easily preserved in the form of spores. *Bacillus* populations can freely persist in soil biomes and plants, without any particular challenges from other groups of bacteria living in those soils. Commercially important species of the genus include: *Bacillus subtilis, Bacillus cereus, Bacillus thuringiensis, Bacillus velezensis, Bacillus amyloliquefaciens.* These species are commonly used to make crops that are distributed throughout the world, due to their beneficial properties (Radhakrishnan et al., 2017).

Materials and methods

Soil Samples

Soil samples, including undisturbed and contaminated soil, were collected from different regions in North Macedonia. The moisture content and soil pH were determined for each soil type as described before (Atanasova-Pancevska et al. 2023). The microorganisms were isolated through a serial dilution method, extending up to 10^{-5}, using 0.9% sterile saline for soil samples. The total bacterial count was determined by using the pour plate technique on nutrient agar plates, followed by incubation at 30°C for 24 hours. To obtain pure cultures the colonies that showed antimicrobial activity on the plates were isolated. Confirmation was carried out by the Gram staining method of all isolates.

Hemolytic Activity

The bacterial isolates were assessed on blood agar plates supplemented with 5% (v/v) sheep blood and incubated at 30°C for 48 hours. Hemolytic activity was identified by observing a clear zone surrounding the bacterial colonies (Nasr et al. 2009).

Oil Spreading Method

40 ml of distilled water was added to the Petri dishes, followed by the application of 100 µl of crude oil on the water's surface. Subsequently, 10 µl of cell-free culture broth was placed onto the crude oil layer (Plaza et al. 2006). Isolates that exhibited a clear zone on the oil surface were considered positive, indicating their potential for biosurfactant production.

Results

Collecting soil samples across different seasons is expected to reveal seasonal variations in soil properties. Table 1 illustrates these seasonal changes in soil pH and moisture content and their influence on the abundance of *Bacillus* spp. According to Table 1, soil moisture percentage fluctuates seasonally, with the Mount Karadzica soil exhibiting the highest moisture content in winter, while the soil from Ohis displays the lowest moisture levels during the summer season. Another trend that can be observed, which aligns with the scientific literature (reference here), is the negative impact of contaminants on soil moisture content. The

Table 1. Moisture content (%) and pH of the collected soil samples

Location	Season	Moisture (%)	pH
Mount Karadzica	Winter	43.9%	7.70
Mount Karadzica	Spring	29.15%	7.18
Mount Karadzica	Summer	9.65%	7.13
Mount Karadzica	Autumn	25.7%	7.39
Ohis	Winter	15.15%	8.15
Ohis	Spring	7%	7.87
Ohis	Summer	4.3%	7.76
Ohis	Autumn	22.5%	7.97
Mount Vodno	Winter	18.5%	7.85
Mount Vodno	Spring	39%	8.16
Mount Vodno	Summer	7.8%	7.85
Mount Vodno	Autumn	23.15%	8.04

results indicate that the low moisture content in the soils from Ohis may be attributed to the presence of contaminants, which tend to accumulate in the deeper soil layers.

The subsequent parameter analyzed is the population density of *Bacillus* spp., where it becomes evident that both moisture content and pH significantly affect the bacterial count. Results indicate that in all soil samples, there is a notably higher *Bacillus* spp. population in spring and autumn as compared to winter and summer seasons (Figure 1).

Throughout the research, a total of 37 unique *Bacillus* spp. isolates were collected from 12 selected sites. These isolates were confirmed as *Bacillus* spp. based on their morphological characteristics. The microscopic analysis indicated that all isolates appeared as Gram-positive rod-shaped bacteria (Figure 2).

In several studies on biosurfactants (Sarwar 2018), the hemolysis test has been validated as an effective screening method for assessing biosurfactant production. This approach, while generally useful, has limitations, such as non-specificity and restrictions on surfactant diffusion, which may inhibit the formation of distinct zones. Additionally, some substrates may not utilize carbon as a source, affecting test results. Findings from this research indicated that almost all isolates produced β-hemolysis, underscoring the method's effectiveness despite its limitations (Figure 3).

The obtainted results from the oil spread method indicate that not all isolates exhibited potential for

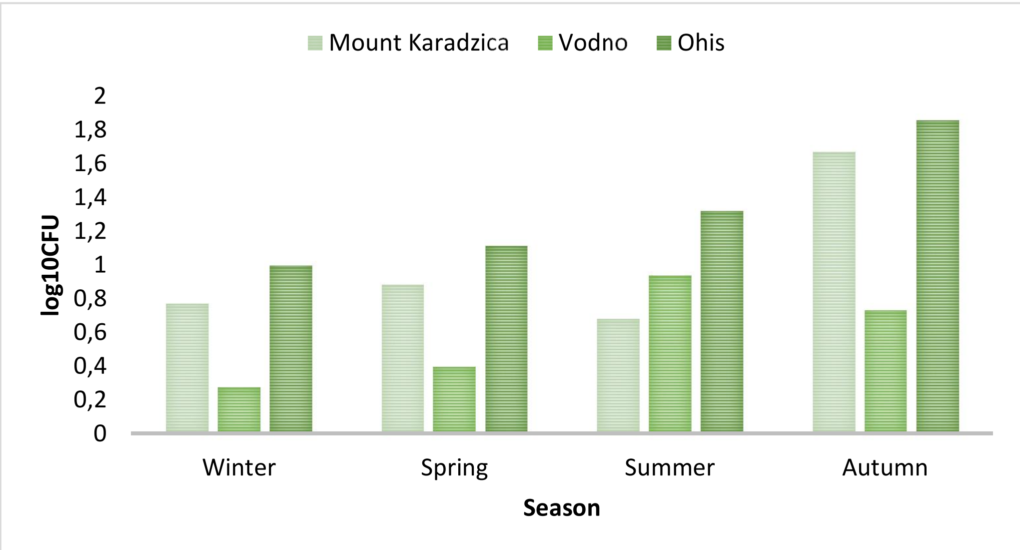

Figure 1. Abundance of *Bacillus* spp. in the collected soil samples

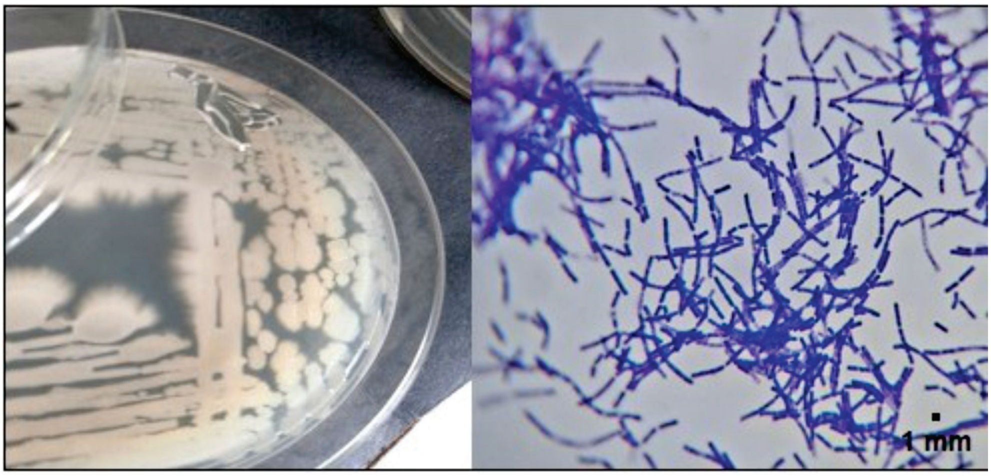

Figure 2. Macroscopic and microscopic characteristics of the isolate B27

Figure 3. Positive hemolysis test on blood agar for determining biosurfactant potential (β-hemolysis).

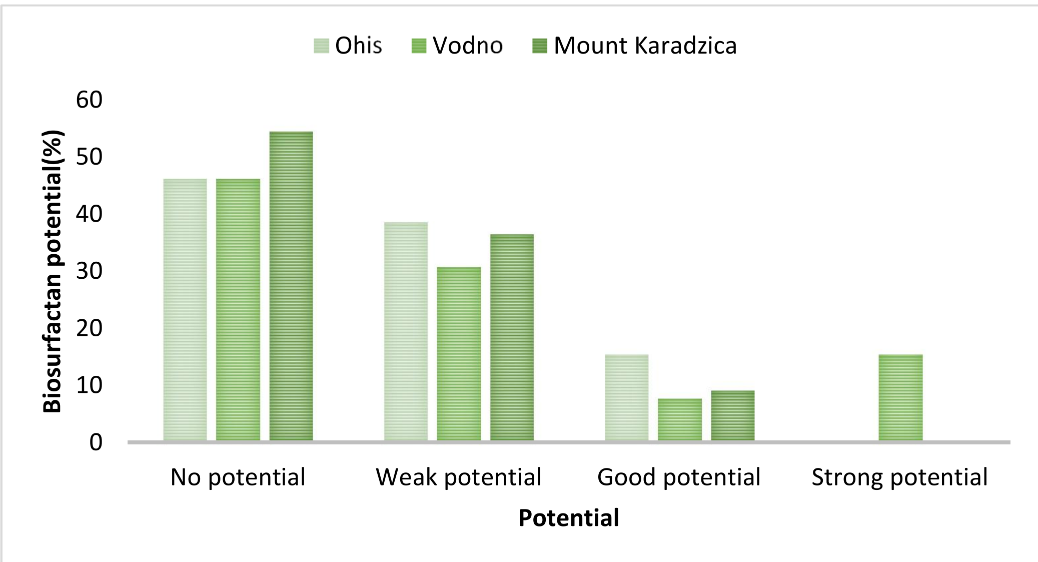

Figure 4. Percentage representation of the isolates from Ohis, Mount Vodno and Mount Karadzica in relation to their biosurfactant potential.

biosurfactant production. Based on the size of the zones formed and the time required to form these zones, the isolates were categorized into those with no biosurfactant production potential and those with weak, moderate, or strong biosurfactant production potential. Twenty of the isolates exhibited biosurfactant activity, primarily representing samples from contaminated soil environments.

The assessment of potential across three locations Ohis, Mount Vodno, and Mount Karadzica demonstrates distinct variations in their suitability for the evaluated factor. Both Ohis and Mount Vodno exhibit significant levels of „no biosurfactant potential,“ each with scores of 46%, while Mount Karadzica shows a slightly higher value of 54.5%, indicating a greater degree of limitation in this category. Regarding „weak biosurfactant potential,“ Ohis and Mount Karadzica both present relatively high values of 38.5% and 36.5%, respectively, suggesting notable weaknesses, while Mount Vodno with 31%, reflects a somewhat lower degree of weak potential. Conversely, Ohis leads in „moderate biosurfactant potential“ with a value of 15.5%, suggesting greater capacity in this regard, whereas Mount Vodno and Mount Karadzica have lower values at 7.5% and 9%, respectively, indicating a diminished level of moderate potential.

In the „strong biosurfactant potential“ category, Mount Vodno stands out with a value of 15.5%, indicating a relatively high potential for growth or development. In contrast, both Ohis and Mount Karadzica show no substantial strong potential, each with 0%. These findings suggest that Mount Vodno holds the most balanced potential, especially in areas requiring stronger growth, while Ohis faces considerable challenges in this domain. Mount Karadzica, with its dominant percentage of „no biosurfactant potential,“ appears to have the least flexibility for development. Overall, Mount Vodno seems to present the most promising location, particularly for site demanding high potential, while Ohis and Mount Karadzica may require further investigation to identify specific strengths and limitations (Figure 4).

Discussion

The variations in soil moisture percentage are influenced by factors such as soil particle size, soil structure, porosity, mineral composition, and external factors including the presence of contaminants and climatic conditions (Fang & Daniels 2017). The results obtained align with existing literature (Nasr, S. 2009), reflecting a consistent trend of decreasing soil moisture from winter to summer. This shift can be attributed to changes in temperature and precipitation, with higher temperatures and less frequent rainfall in summer leading to a reduction in moisture, whereas winter, with lower temperatures and more frequent precipitation, results in higher moisture levels. Furthermore, another notable trend consistent with the scientific literature is the negative impact of contaminants on soil moisture content. The findings suggest that the low moisture levels observed in the soils of Ohis may be linked to the presence of contaminants, which accumulate in deeper soil layers. According to Osam (2013), the accumulation of pollutants reduces the permeability of oxygen and water through the soil, thereby diminishing moisture retention in contaminated soils.

The pH values of the soils in the study ranged from 7.13 to 8.16. As noted by Watson et al. (2014), soil pH plays a critical role in the ability of plants to absorb essential nutrients required for optimal growth and development. They suggest that the ideal pH range for soil is between 5.5 and 7.2. However, urban soils and those impacted by anthropogenic activities often exhibit higher pH levels. These findings align with the results observed in the present study.

The findings from this study indicate that soil microorganism abundance is higher during the spring and autumn seasons compared to the winter and summer. These seasonal fluctuations are likely a result of climate change, temperature variations, and the dynamics of geochemical cycles in nature. The increase in microorganism abundance observed in the autumn is linked to the shedding of leaves from trees, which, combined with the onset of winter and a decrease in temperature, leads to a reduction in *Bacillus* spp. populations. As temperatures rise and vegetation growth accelerates in the spring, there is a subsequent rise in microorganism abundance, driven by the enhanced decomposition of readily available nutrients. In contrast, the summer season is marked by increased evaporation and higher temperatures, which reduce soil moisture and contribute to a decline in *Bacillus* spp. populations (Ramirez et al. 2017). The role of *Bacillus* spp. in soil ecosystems is further underscored through processes like nitrogen fixation and the carbon cycle, highlighting the complex interactions in which microorganism abundance plays a pivotal role. The results of this study suggest that soil microorganisms, as integral components of biogeochemical cycles, influence key processes within the soil ecosystem, such as the carbon and nitrogen cycles and nutrient transformation (Ruan et al. 2004). Consequently, changes in the structure and abundance of the soil microbiome are frequently used as reliable indicators of alterations in the soil environment and nutrient composition. As environmental factors can reveal fundamental insights into microbial ecology, such studies are critical for understanding soil ecosystem function (Lanzen et al. 2016). Furthermore, anthropogenic activities also impact microorganism populations, with the release of pollutants inhibiting microorganism growth. The presence of heavy metals in the soil impedes plant nutrient uptake, negatively affecting microbial growth. A deeper understanding of the impact of heavy metals requires knowledge of their effects on soil physicochemical properties and their role in shaping microbial communities (Lin et al. 2022). However, based on the results of this study, no significant correlation was found between soil contamination and microorganism abundance, as no drastic decline in abundance was observed in the Ohis soil compared to others.

A comparison between the results of this study and those in the existing literature reveals that β-hemolysis is predominantly observed in both cases, with the zones of inhibition correlating to the quantity of biosurfactant produced. Blood agar supports the growth of only hemolytic organisms that utilize erythrocytes for growth, leading to cell lysis, which is visibly indicated by discoloration of the Petri dish. The degree of cell lysis directly correlates with biosurfactant production, meaning that greater biosurfactant production results in more distinct zones of clearance. The ability of isolates to induce β-hemolysis on blood agar serves as an indicator of their biosurfactant-producing potential. The three types of hemolysis can be distinguished by the appearance of zones and the degree of discoloration around the colonies. Specifically, α-hemolysis is characterized by a greenish hue around the colony, β-hemolysis produces clear zones surrounding the colonies, and γ-hemolysis shows no visible change in the colonies growing on the agar plate (Rashedi et al. 2005). Numerous studies on biosurfactants have established that the hemolysis test is an effective screening method for monitoring biosurfactant production. Specifically, for microorganisms that produce biosurfactants in liquid culture, the use of blood agar has been recommended to assess their hemolytic activity. The formation of lysis zones on blood agar serves as a clear indicator of the microorganism's capacity to produce biosurfactants (Santos et al. 2016).

Biosurfactants are secondary metabolites that are synthesized by microorganisms only when necessary, which explains the observed biosurfactant potential in the isolates from Ohis and Vodno. The presence of cultural and physicochemical factors such as nutrient availability, nutrient concentration, and interactions with other organisms increases competition among different strains, thereby intensifying the demand for stronger biosurfactants. Additional factors, such as heat, UV radiation, and pH, further highlight the need for more potent biosurfactants that confer a survival advantage to microorganisms in their respective environments (Khalil 2009). Consequently, it is reasonable to infer that the higher contamination levels in Ohis and Vodno lead to greater competition among the strains present in the soil for essential nutrients, thereby stimulating the synthesis of larger quantities of biosurfactants.

Conclusion

In conclusion, this study provides valuable insights into the seasonal variations in soil properties and their impact on the abundance and biosurfactant production potential of *Bacillus* spp. across different locations. The results indicate that both soil moisture and pH significantly influence the microbial populations, with higher *Bacillus* spp. counts observed during the spring and autumn seasons. These seasonal fluctuations can be attributed to climatic factors, such as temperature variations and precipitation patterns, which affect nutrient availability and microbial activity. The study further demonstrates the potential of biosurfactants as secondary metabolites produced by microorganisms in response to environmental conditions, including nutrient competition and the presence of contaminants. Notably, the research highlights the importance of the hemolysis test as an effective screening method for identifying biosurfactant-producing isolates, despite its limitations. Soil contamination, particularly in areas like Ohis and Vodno, appears to enhance microbial competition, thereby stimulating biosurfactant production. However, no significant correlation was found between contamination and microbial abundance in this study, suggesting that other factors, such as soil structure and climatic conditions, play a pivotal role in shaping microbial communities. Overall, the findings suggest that soil ecosystems, influenced by both natural and anthropogenic factors, exhibit complex interactions that affect microbial abundance and activity, with potential applications in bioremediation and environmental sustainability. Further research is needed to explore the specific mechanisms underlying biosurfactant production in contaminated environments and to assess the broader ecological implications of these findings.

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