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Presented by :

BENAMEUR Chourouk

Title

Identification and characterization of biosurfactants produced by microorganisms previously isolated having the ability to degrade petroleum

Jury members :

President	Dr. ACHIR Mohamed
Examiner	Prof. ACEM Kamel
Supervisor	Dr. MEKNASSI Khadidja
Co-Supervisor	Prof. AIT ABDERRAHIM Leila

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Abstract

Petroleum hydrocarbon pollution poses a major environmental and economic challenge, requiring sustainable remediation strategies. A promising approach relies on the use of biosurfactant-producing microorganisms for the bioremediation of contaminated areas. Biosurfactants enhance microbial biodegradation by increasing the bioavailability of hydrocarbons, thereby making the process more efficient.

The present study evaluated the biosurfactant production potential of five bacterial strains: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus gallinarum*, *Aneurinibacillus migulanus*, and *Lysinibacillus cavernae*, previously isolated from petroleum products and oil-contaminated soils.

The E24 % test (24-hour emulsification index) was used to assess the ability of the tested bacteria to produce biosurfactants and emulsify hydrocarbons, specifically diesel and gasoline. Subsequently, the produced biosurfactants were extracted using a chloroform–methanol mixture and characterized by Fourier-transform infrared spectroscopy (FTIR). Their antimicrobial activity was assessed using the agar well diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus flavus*. Their antioxidant potential was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay.

The five tested strains exhibited varying emulsification capacities in media containing gasoline and diesel. *L. cavernae* showed the highest emulsification percentage (43.8 ± 5.38 %), followed by *A. baumannii* (36.95 ± 3.07 %) and *P. aeruginosa* (29.52 ± 5.06 %), all in gasoline-containing media.

FTIR analysis revealed diverse biosurfactant structures, including glycolipids (*P. aeruginosa* and *A. baumannii* in gasoline, and *E. gallinarum* in diesel), lipopeptides (*A. migulanus* and *L. cavernae* in diesel, as well as *L. cavernae* and *E. gallinarum* in gasoline), and cyclic lipopeptides (*P. aeruginosa* and *A. baumannii* in diesel, and *A. migulanus* in gasoline).

The antimicrobial test showed that the biosurfactants were effective against *C. albicans* and *S. aureus*, with inhibition zones reaching up to 16.5 mm and 11.6 mm respectively. No effect was observed against *E. coli* and *A. flavus*.

Furthermore, high antioxidant potential was observed in biosurfactants produced by *P. aeruginosa* in diesel (4.1 mg/ml) and by *A. baumannii* in gasoline (4.2 mg/ml).

These results highlight the potential of these strains for environmental and industrial applications, as well as in the pharmaceutical and agri-food sectors.

Keywords: Petroleum hydrocarbons, pollution, gasoline, diesel oil, Microorganismes, Biosurfactant, FTIR, Bioremediation.

Résumé

La pollution par les hydrocarbures pétroliers représente un défi environnemental et économique majeur, nécessitant des stratégies de dépollution durables. Une approche prometteuse repose sur l'utilisation de micro-organismes producteurs de biosurfactants pour la biodépollution des zones contaminées. Ceux-là améliorent la biodégradation microbienne en augmentant la biodisponibilité des hydrocarbures, rendant ainsi le processus plus efficace.

La présente étude a évalué le potentiel de production de biosurfactants de cinq souches bactériennes *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus gallinarum*, *Aneurinibacillus migulanus*, et *Lysinibacillus cavernae* isolées précédemment à partir de produits pétroliers et de sols contaminés par le pétrole.

Le test E24 % (indice d'émulsification à 24 heures) a été utilisé pour évaluer la capacité des bactéries testées à produire des biosurfactants et à émulsifier les hydrocarbures à savoir le diesel et le gasoil. Par la suite, les biosurfactants produits ont été extraits à l'aide d'un mélange chloroforme-méthanol et caractérisés par spectroscopie infrarouge à transformée de Fourier (FTIR). Leur activité antimicrobienne a été évaluée par la méthode de diffusion en puits sur gélose vis-à-vis de *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* et *Aspergillus flavus*. Leur potentiel antioxydant a été évalué via la méthode de piégeage des radicaux DPPH (2,2-diphényl 1-picrylhydrazyle).

Les résultats obtenus ont démontré que les cinq souches testées ont des capacités d'émulsification variables en milieux contenant de l'essence et du diesel, *L. cavernae* ayant présenté le pourcentage le plus élevé (43,8 ± 5,38 %), suivie par *A. baumannii* (36,95 ± 3,07 %) et *P. aeruginosa* (29,52 ± 5,06 %) tous en milieu contenant de l'essence

L'analyse par FTIR a révélé des structures de biosurfactants diversifiées variant entre glycolipides (*P. aeruginosa* et *A. baumannii* sur essence ainsi que *E. gallinarum* sur diesel), lipopeptides (*A. migulanus* et *L. cavernae* sur diesel ainsi que *L. cavernae* et *E. gallinarum* sur essence) et lipopeptide cycliques (*P. aeruginosa* et *A. baumannii* sur diesel ainsi que *A. migulanus* sur essence).

Le test antimicrobien a montré une efficacité des biosurfactants contre *C. albicans* et *S. aureus* avec des zones d'inhibition allant jusqu'à 16,5 mm et 11.6 mm respectivement. Aucun effet sur *E. coli* et *A. flavus* n'a été observé.

Par ailleurs, le potentiel antioxydant s'est révélé élevé chez les biosurfactants produits par *P. aeruginosa* sur diesel (4,1 mg/ml) et par *A. baumannii* en milieu essence (4,2 mg/ml).

Ces résultats mettent en évidence le potentiel de ces souches pour des applications environnementales et industrielles, ainsi que dans les domaines pharmaceutique et agroalimentaire.

Mots clés : Hydrocarbures pétroliers, pollution, essence, diesel, micro-organismes hydrocarbonoclastes, bioremediation, biosurfactants, FTIR.

الملخص

يمثل التلوث بالمحروقات النفطية تحدياً بيئياً واقتصادياً كبيراً، مما يستدعي اعتماد استراتيجيات معالجة مستدامة. و من إحدى المقاربات الواعدة، استخدام الكائنات الحية الدقيقة المنتجة للمواد الخافضة للتوتر السطحي الحيوية (البيوسيرفاكتانتات) لمعالجة المناطق الملوثة. إذ تعمل هذه الكائنات على تعزيز التحلل البيولوجي الميكروبي عن طريق زيادة التوافر الحيوي للهيدروكربونات، مما يجعل العملية أكثر كفاءة.

هدفت هذه الدراسة إلى تقييم قدرة خمس سلالات بكتيرية على إنتاج المواد الخافضة للتوتر السطحي الحيوية، وهي : الزوائف الزنجارية، الأسينيتوباكتر بومانياي، الإنتيروكوكوس جالياروم، أنورينيباسيلوس ميغولانوس ولايزينيباسيلوس كافيرني التي تم عزلها سابقاً من منتجات نفطية وترب ملوثة بالنفط .

تم استخدام مؤشر الاستحلاب بعد 24 ساعة لتقييم قدرة هذه السلالات البكتيرية على إنتاج البيوسيرفاكتانتات واستحلاب الهيدروكربونات، وتحديداً الديزل والبنزين. بعد ذلك، تم استخراج البيوسيرفاكتانتات المنتجة باستعمال مزيج من الكلوروفورم-ميثانول، وتم التعرف عليها باستخدام تقنية التحليل الطيفي بالأشعة تحت الحمراء بتحويل فورييه.

تم تقييم النشاط المضاد للميكروبات لهذه المركبات بواسطة طريقة الانتشارمن الآبار ضد كل من المكورات العنقودية الذهبية، الإشريكية القولونية، الكانديدا البيكانسية و الأسبرجيلوس فلافوس.

أما النشاط المضاد للأكسدة، فقد تم تقييمه باستخدام طريقة اصطياد الجذور الحرة (2,2-دي فينيل-1-بيكريل هيدرازيل).

أظهرت النتائج أن السلالات الخمس المختبرة تمتلك قدرات استحلاب متفاوتة في الأوساط الحاوية على البنزين والديزل، حيث سجلت لايزينيباسيلوس كافيرناي أعلى نسبة استحلاب بلغت (43,8 ± 5,38%)، تلتها أسينيتوباكتر بومانياي (36,95 ± 3,07%)، ثم الزوائف الزنجارية (29,52 ± 5,06%)، جميعها في الوسط الحاوي على البنزين.

كشفت تقنية التحليل الطيفي بالأشعة تحت الحمراء بتحويل فورييه عن تراكيب متنوعة للمواد الخافضة للتوتر السطحي الحيوية، تراوحت بين السكّريات الشحمية، والبيبتيدات الشحمية، والبيبتيدات الشحمية الحلقية.

أما الاختبار المضاد للميكروبات، فقد أظهر فعالية للبيوسيرفاكتانتات ضد الكانديدا البيكانسية والمكورات العنقودية الذهبية، مع اقطار مناطق تثبيط بلغت 16,5 ملم و11,6 ملم على التوالي، بينما لم يُلاحظ أي تأثير ضد الإشريكية القولونية والأسبرجيلوس فلافوس.

كما أظهرت النتائج أن البيوسيرفاكتانتات المنتجة من الزوائف الزنجارية في وسط الديزل (4,1 ملغ/مل)، ومن أسينيتوباكتر بومانياي في وسط البنزين (4,2 ملغ/مل) تمتلك أعلى نشاط مضاد للأكسدة.

تؤكد هذه النتائج الإمكانات الواعدة لهذه السلالات في التطبيقات البيئية والصناعية، فضلاً عن المجالات الصيدلانية والصناعات الغذائية.

الكلمات المفتاحية : المحروقات النفطية، التلوث، البنزين، الديزل، الكائنات الدقيقة المحللة للهيدروكربونات، المعالجة البيولوجية، البيوسيرفاكتانتات، التحليل الطيفي بالأشعة تحت الحمراء.

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LIST OF ABBREVIATIONS

PH	Petroleum hydrocarbons
MSM	Mineral salts medium
FTIR	Fourier transform infrared
E24	Emulsification index
IC50	Concentration inhibiting 50% of DPPH radicals

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INTRODUCTION

Introduction

Petroleum hydrocarbons (PHCs) are among the most prevalent and persistent environmental contaminants worldwide. Their release into natural ecosystems, whether through industrial activity or accidental spills, leads to immediate ecological disruption, compromising the functionality, stability, and biodiversity of affected environments (Truskewycz et al., 2019).

In Algeria, a major oil-producing country with an average daily production of approximately 1.41 million barrels in 2023, petroleum-related pollution has become a significant environmental challenge (Statista, 2024). The widespread use and frequent release of petroleum hydrocarbons into the environment have highlighted the urgent need for effective and sustainable remediation strategies (Mekonnen et al., 2024).

Among various cleanup approaches, bioremediation has emerged as a promising, eco-friendly, and cost-effective alternative to conventional chemical and physical methods (Rahman et al., 2003). This biological process utilizes microorganisms—primarily bacteria, fungi, and algae—and their metabolic products to degrade, detoxify, or transform hazardous pollutants into less harmful compounds (Das & Chandran, 2010). A key element enhancing microbial degradation of hydrophobic pollutants is the production of biosurfactants.

Biosurfactants are amphiphilic, surface-active molecules produced by a wide range of microorganisms including bacteria, yeasts, and filamentous fungi. These compounds may be secreted extracellularly or remain cell-bound and are characterized by the presence of both hydrophilic and hydrophobic moieties, allowing them to reduce surface and interfacial tensions and facilitate interactions between water and hydrophobic substances such as hydrocarbons (Rani et al., 2020; Thakur et al., 2024). Based on their chemical nature, biosurfactants are commonly classified into glycolipids, lipopeptides, phospholipids, substituted fatty acids, polysaccharides, and other complex forms (Rani et al., 2020).

In hydrocarbon-contaminated environments, microorganisms synthesize biosurfactants to improve the uptake and utilization of hydrophobic carbon sources. Low molecular weight biosurfactants aid in the solubilization of hydrocarbons by forming micelles, whereas high molecular weight biosurfactants primarily function as emulsifying agents, dispersing hydrocarbons into the aqueous phase and often modifying the microbial cell surface to facilitate substrate uptake (Biktasheva et al., 2022). These properties, coupled with their low toxicity, high biodegradability, and broad application potential, make biosurfactants valuable

tools not only in bioremediation but also in the food, pharmaceutical, and cosmetic industries (Yagoo & Vilvest, 2023b).

In this context, the present study aims to identify and characterize biosurfactants produced by previously isolated and molecularly identified bacterial species from petroleum-contaminated soils and petroleum products. By exploring the type and function of biosurfactants generated in response to different petroleum substrates (e.g., diesel and gasoline), this work contributes to a better understanding of their potential in bioremediation and other biotechnological applications.

Literature review

1. Petroleum hydrocarbons

Petroleum hydrocarbons, composed of carbon and hydrogen atoms, are organic compounds primarily derived from crude oil (Kuppusamy et al., 2019). They have become widespread environmental pollutants as a result of human activities such as oil spills, leaks from storage tanks, and accidents during transportation (Mohanta et al., 2023).

The environmental impacts of petroleum hydrocarbons are equally concerning, as they pollute water, soil, and air, harming wildlife and disrupting ecosystems. This pollution not only affects biodiversity but also poses serious health risks to humans, including respiratory illnesses and cancer (Peterson et al., 2003). Crude oil and petroleum products create a waterproof layer on water, blocking oxygen exchange between the air and water, this harms plants, animals, and humans (Srivastava et al., 2019). In plants, these pollutants can disrupt essential physiological processes such as membrane permeability, photosynthesis, and enzyme function. Specifically, they interfere with the arrangement of chloroplasts, thereby impairing photosynthesis and electron transport, which are critical for plant growth and survival (Tomar & Jajoo, 2014).

Moreover, exposure to petroleum hydrocarbons through inhalation, ingestion, or skin contact can lead to severe health risks to human and animals. Low-viscosity, high-volatility hydrocarbons, when aspirated into the lungs, can cause lung damage and chemical pneumonia. Additionally, exposure may result in gastrointestinal tract issues, central nervous system effects, and reproductive toxicity, posing significant threats to human and animal health (Osweiler, 2024).

Overall, the widespread contamination caused by petroleum hydrocarbons underscores the urgent need for effective mitigation and remediation strategies to protect both ecological and human health (Elijah, 2022).

2. Bioremediation

Bioremediation is a common method for cleaning up oil pollution in land and water (Yuniati, 2018). It encompasses plant-based and microbe-based approaches, known as phytoremediation and microorganism remediation, respectively. These methods vary significantly in their processes and mechanisms, as plants and microbes immobilize, eliminate, or break down pollutants in distinct ways (Wang et al., 2020).

Bioremediation is becoming more popular for cleaning up pollutants, including those from the oil industry. It is considered non-invasive, as it works naturally without disturbing the environment, and it is relatively cheap compared to other cleanup methods (Bala et al., 2022). In

short, bioremediation is a simple, eco-friendly, and cost-effective way to tackle pollution (Vidali, 2001).

Biodegradation involves the use of microorganisms, including bacteria, fungi, and algae, as well as their enzymes, such as laccase that can degrade complex organic pollutants, including phenolic compounds and dyes, by oxidizing them into simpler, less toxic substances (Chandra & Chowdhary, 2014). Other microbial products such as biosurfactants play a key role in the degradation of organic pollutants in contaminated environments (Mekonnen et al., 2024).

3. Biosurfactants

Biosurfactants are amphiphilic compounds produced by microorganisms, exhibiting properties such as surface activity, emulsification, antioxidant effects, antiadhesive capabilities, and antimicrobial action (Giri et al., 2019).

Biosurfactants are increasingly recognized for their biodegradability, environmental safety, and eco-friendliness (Vega & Stampino, 2025). They have demonstrated promising uses across multiple sectors, including biotechnology and environmental remediation (Sankhyan et al., 2023).

3.1 Classification of biosurfactants

Biosurfactant classification is mainly based on the origin of the microbes and their chemical composition. These compounds are divided into two types based on their molecular weight. Low molecular weight biosurfactants include glycolipids, phospholipids, and lipoproteins, and high molecular weight bioemulsifiers include lipopolysaccharides, proteins, and polymeric particulate surfactants (Fig. 1, table 1) (Kashif et al., 2022).

3.1.1. Low molecular weight biosurfactants

a. Glycolipids

Glycolipids are carbohydrates linked to long-chain fatty acids or hydroxyl fatty acids through ester or ether bonds (Erum Shoeb et al., 2013). These compounds vary based on the type of lipid and sugar they contain. Depending on the sugar part, glycolipids can be divided into different groups, such as rhamnose lipids, trehalose lipids, sophorose lipids, cellobiose lipids, mannosylerythritol lipids, and others like diglycosyl diglycerides and galactosyl-diglyceride.

The most well-known glycolipids are rhamnolipids, trehalolipids and sophorolipids produced by *Pseudomonas, Rhodococcus* and yeast species *Starmerella bombicola* (Adu et al., 2023) respectively (Inès & Dhouha, 2015).

Glycolipids can disrupt cell membranes by dissolving the lipid bilayer, which lowers the membrane's surface tension. This reduction enables water to enter the cell, ultimately leading to

cell lysis. To optimize this effect, a well-balanced ratio between the hydrophilic and hydrophobic portions of the glycolipid is crucial (Džubák et al., 2019).

b. Lipopeptides and lipoproteins

Lipopeptides and lipoproteins are among the most widely produced biosurfactants. Their structure, which includes a peptide head and a fatty acid chain, gives them unique properties that enhance biocompatibility, making them ideal for use in drug delivery applications (Vecino et al., 2021). Additionally, their ability to create pores and disrupt biological membranes allows them to be used as antimicrobial, hemolytic, antiviral, antitumor, and insecticidal agents. Lipopeptides can also interact with surfaces and influence enzyme activity, either enhancing certain enzymes to improve microbial processes or inhibiting others, making them effective as antifungal agents (Mnif & Ghribi, 2015).

c. Phospholipids

Phospholipids are essential building blocks of microbial membranes. When certain bacteria or yeast that can degrade hydrocarbons are grown on alkane-based materials, their production of phospholipids increases significantly. For instance, the bacterium *Acinetobacter* sp. HO1-N, when cultivated on hexadecane, generates a large quantity of phospholipids, with phosphatidylethanolamine being the main type produced. This highlights how these microorganisms adapt their membrane composition when exposed to specific hydrocarbon substrates (Shah et al., 2016).

Phospholipids have an amphiphilic nature, meaning they possess both hydrophilic and hydrophobic parts. This allows them to act as biosurfactants by lowering surface and interfacial tension between immiscible phases like oil and water. They do this by assembling at the interface, where their hydrophobic tails engage with the oil phase while their hydrophilic heads interact with the water phase (Hopkins, 2024).

d. Fatty acids

Fatty acids consist of long hydrocarbon chains terminating in a carboxyl group, classified as saturated (lacking double bonds) or unsaturated (containing one or more double bonds). These biomolecules are fundamental constituents of lipids and perform vital biological functions including energy storage, cellular membrane formation, and signal transduction (Wang, 2020). *Corynebacterium glutamicum* is a non-pathogenic bacterium extensively utilized in biotechnology for synthesizing amino acids such as glutamate and lysine. This microorganism also generates corynomycolic acids that are integral components of its cell wall structure (Benekos et al., 2010) and function as surfactants. The balance between the water-loving

(hydrophilic) and oil-loving (lipophilic) properties of these fatty acids depends on the length of their hydrocarbon chains (Rahman & Gakpe, 2008).

Fatty acid-based biosurfactants function as effective surface-active compounds by lowering the tension at the interfaces of immiscible phases, such as oil and water. This property enables key processes like emulsification, dispersion, solubilization, and wetting, making them valuable for industrial and environmental uses (Karlapudi et al., 2018).

3.1.2. High molecular weight biosurfactants

a. Polymeric biosurfactants

Polymeric biosurfactants represent a class of high molecular weight surface-active agents. Among these, emulsan is well-known, along with other examples such as liposan, mannoproteins, and protein-polysaccharide complexes (Simões et al., 2024).

Polymeric biosurfactants offer significant benefits as they are biodegradable and typically exhibit lower toxicity than conventional synthetic surfactants. These eco-friendly properties make them particularly suitable for bioremediation, where they can effectively facilitate pollutant degradation and soil decontamination (Acosta-Santoyo et al., 2023).

- Emulsan

is employed to emulsify hydrocarbons in water and is regarded as one of the most effective emulsifiers, even at concentrations below 0.01%.

- Alasan

It is a bioemulsifier secreted by *Acinetobacter radioresistens*. Composed of a negatively charged polysaccharide-protein complex, it effectively stabilizes oil-in-water emulsions and improves the dissolution of hydrophobic substances (Rosenberg & Ron, 1999).

- Liposan

It is a microbial surfactant produced extracellularly by *Candida lipolytica*, it is water-soluble and structurally composed of more than 80% carbohydrates and fewer than 20% proteins. These characteristics enable it to function as an efficient emulsifier for hydrocarbons even at very low concentrations (less than 0.01%) (Fickers et al., 2004; Karlapudi et al., 2018).

- Mannoproteins

They are large molecules made of mannose and protein, produced by yeasts like *Saccharomyces cerevisiae*. They act as emulsifiers and play a role in building the cell wall structure (Klis et al., 2002).

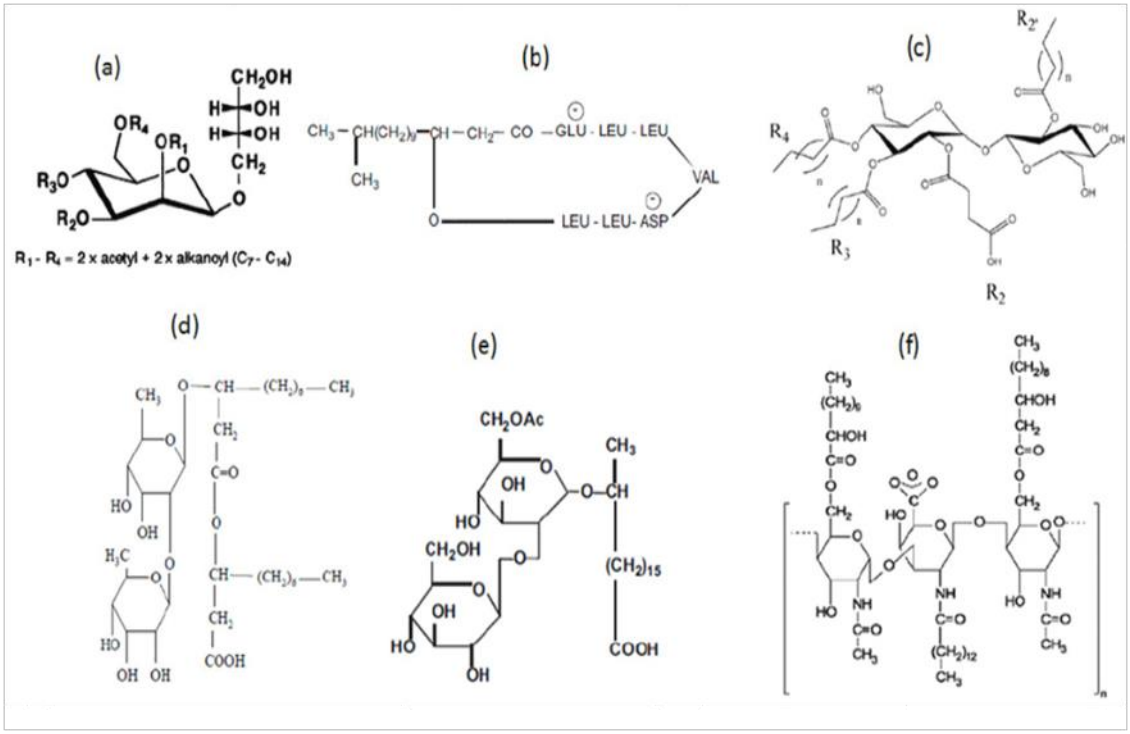


Figure 1— Chemical structure of some common biosurfactants.

a) mannosylerythritol lipid, b) surfactin, c) trehalose lipid, d) sophorolipids, e) rhamnolipid, f) emulsan.

Table 1— Biosurfactants produced by some microorganisms.

Biosurfactants	**Microorganism**	**Application**	**References**
Rhamnolipds	*Pseudomonas aeruginosa*	Bioremediation	Amani et al., 2013
Glucolipid and trehalose lipid	*Rhodococcus erythropolis* 3C-9	Oil spill cleanup operations	Shah et al., 2016
Surafactin	*Bacillus subtilis*	Antimicrobial property	Shah et al., 2016
Lichenysin	*Bacillus licheniformis*	Microbially enhanced oil recovery	Shah et al., 2016
Lipopeptide	*Nocardiopsis alba* MSA10	Bioremediation	Shah et al., 2016
Rhamnolipid	*Pseudoxanthomonas* sp. PNK-04	Environmental applications	Shah et al, 2016
Sophorolipids	*Torulopsis bombicola*	Antimicrobial activity	Cooper & Paddock, 1984
protein-carbohydrate-lipid complex	*Candida glabrata UCP10*	Oil recovery from sand	Shah et al., 2016
Lipopeptide	*Fusarium* sp. BS-8	Enhanced oil recovery	Rahman & Gakpe, 2008

Lipopeptide	*Penicillium chrysogenum* SNP5	Enhanced recovery of oil	Elsoud, 2021
Sophorolipid	*Starmerella bombicola*	Emulsification and wetting property	Jadhav et al., 2019
Trehalose lipids	*Candida antarctica*	Applied in industrial processes like bioremediation, detergents, and as a surfactant in food industry	Silva et al., 2020
Sophorolipids	*Candida bombicola*	Used in oil recovery, environmental bioremediation, and in cosmetics for emulsification properties	Pinto et al., 2022
Sophorolipids and trehalose lipids	*Yarrowia lipolytica*	Used for bioremediation, oil recovery, and in cosmetic and pharmaceutical formulations for emulsification	Csutak et al., 2024
Not specified, likely glycolipid	*Arthrospira sp*	Food processing	Ngela et al., 2015
Not specified, likely glycolipid	*Synechococcus nidulans*	pharmaceuticals, recovery of oily residues	Simões et al., 2024

3.2. Mode of action of biosurfactants

A primary mechanism of action is **solubilization**, where biosurfactants lower the surface tension of water, enhancing the solubility of hydrophobic petroleum hydrocarbons. For instance, rhamnolipids, produced by *Pseudomonas aeruginosa*, help solubilize oil, making it more accessible to microorganisms that degrade hydrocarbons (Zhang & Miller, 1992). Another mechanism is **emulsification**, in which biosurfactants form micelles around oil droplets, creating stable oil-in-water emulsions. Emulsan, produced by *Acinetobacter calcoaceticus*, is highly effective in emulsifying crude oil, improving its bioavailability for microbial breakdown (Rosenberg et al., 1979). Biosurfactants also assist in **mobilization** by dislodging hydrocarbons from soil particles, making them easier to remove. Surfactin, produced by *Bacillus subtilis*, is particularly effective in mobilizing trapped oil in soil, aiding in the cleanup of contaminated areas (Mulligan et al., 2001). Furthermore, biosurfactants enhance **biodegradation** by increasing the bioavailability of hydrocarbons, which stimulates the growth and activity of hydrocarbon-degrading microbes. For example, sophorolipids, produced by *Candida bombicola*, have been shown to improve the degradation of polycyclic aromatic hydrocarbons (Singh & Cameotra,

2004). These characteristics make biosurfactants invaluable in environmental remediation, providing sustainable and efficient solutions for addressing petroleum hydrocarbon pollution.

3.3. Properties and uses of biosurfactants

Biosurfactants like surfactants (short for surface-active agents) are substances that reduce surface and interfacial tension. They have amphiphilic structures which enable them to position themselves at the surface or interface of substances making them excellent at emulsifying (ex. lower interfacial tension helping mixing oil and water) and dispersing substances (ex. lower surface tension by breaking the tension between water molecules) (Gudiña et al., 2013). They form micelles, which are small structures that trap oils or other hydrophobic substances inside and make them easier to remove or degrade.

Biosurfactants are non-toxic which allows their use in food, cosmetics, and medicines (Rosenberg, 2011; Sachdev & Cameotra, 2013).They have a wide range of applications, including bioremediation, food, cosmetics, pharmaceuticals, biomedicine, and nanotechnology (Jimoh & Lin, 2019).They offer advantages over synthetic surfactants because they are environmentally friendly. Their biodegradability and low toxicity have led to increased use in biotechnology (Bjerk et al., 2021).

In addition, many biosurfactants stay stable under extreme conditions, such as high heat, varying pH levels, and salt concentrations, making them useful in tough industrial processes (Santos et al., 2016).

Biosurfactants have antimicrobial and anti-adhesive properties, which help prevent biofilm formation and stop the growth of harmful microbes, making them valuable in medical and industrial applications (Rodrigues & Teixeira, 2010).

One of the most promising uses of biosurfactants is in breaking down hydrocarbons in polluted water and soil (Bjerk et al., 2021). Biosurfactants are effective in cleaning up the environment by breaking down pollutants like oil and heavy metals (Pacwa-Płociniczak et al., 2011). Because they come from renewable sources, they align with green chemistry principles, highlighting their sustainability (Elshafie et al., 2015).

In environmental cleanup, biosurfactants are utilized for cleaning up oil spills and removing heavy metals, helping to break down pollutants naturally (Pacwa-Płociniczak et al., 2011). Biosurfactants are essential in the remediation of petroleum hydrocarbons, functioning through various mechanisms.

They are also utilized in environmentally friendly detergents for removing stains and in nanotechnology for creating nanoparticles and developing drug delivery systems (Shekhar et al., 2014).

In the food sector, they function as emulsifiers and preservatives, increasing the shelf life of products such as salad dressings and baked goods (Duke-Rohner, 2006). In the pharmaceutical field, biosurfactants are used in drug delivery systems and as antimicrobial agents to combat infections (Gudiña et al., 2013).

3.4. The role of biosurfactants in the degradation of hydrocarbons

Biosurfactants play a vital role in the degradation and removal of petroleum hydrocarbons, making them essential for environmental cleanup and oil recovery. They enhance the solubility and bioavailability of hydrophobic hydrocarbons, allowing microorganisms to break them down more effectively (Ron & Rosenberg, 2002). By emulsifying oil into smaller droplets, biosurfactants increase the surface area for microbial action, accelerating the degradation process (Banat et al., 2014). They are particularly useful in oil spill remediation, where they disperse oil and promote its natural breakdown by microbes (Santos et al., 2016). In the petroleum industry, biosurfactants are employed in microbial-enhanced oil recovery (MEOR) to mobilize trapped oil in reservoirs (Sen, 2008). Additionally, they are effective in degrading heavy oils in contaminated soils, such as limestone sands, by solubilizing complex hydrocarbon chains (Rahman et al., 2002). Unlike synthetic surfactants, biosurfactants are biodegradable and less toxic, making them environmentally friendly alternatives for hydrocarbon cleanup (Mulligan, 2004).

Biosurfactants solubilize hydrocarbons into micelles, which are easier for bacteria to uptake and metabolize, accelerating the breakdown of complex hydrocarbon chains (Mulligan, 2004). Additionally, biosurfactants can act as carbon sources, stimulating bacterial growth and increasing their population, which enhances the overall degradation process. They also reduce the toxicity of pollutants by breaking down harmful hydrocarbons, creating a more favorable environment for bacterial activity (Rahman et al., 2002). Furthermore, biosurfactants improve the transfer of oxygen and nutrients, which are essential for bacterial metabolism and hydrocarbon degradation. These mechanisms make biosurfactants vital for efficient bioremediation of oil-contaminated environments (Shekhar et al., 2014).

3.5. Why are biosurfactants synthesized by microorganisms ?

Microorganisms produce biosurfactants mainly to enhance their capacity to break down and utilize water-insoluble substances like oils and hydrocarbons (Sanches et al., 2021),

frequently encountered in polluted settings. By lowering surface tension and creating emulsions, these biosurfactants increase the accessibility of hydrophobic compounds, facilitating their uptake and processing by microbial cells (Kachrimanidou et al., 2023). Moreover, the ability to generate biosurfactants offers a survival benefit in nutrient-poor environments, enabling microbes to exploit diverse carbon sources that would otherwise be unavailable. This competitive trait helps them thrive in harsh ecological conditions where efficient resource utilization determines microbial dominance and persistence (Simões et al., 2023).

3.6. Conditions under which biosurfactants are formed

Microorganisms produce biosurfactants in response to various environmental cues, with the presence of hydrophobic compounds like hydrocarbons, lipids, and oils serving as a key trigger. These water-insoluble substrates stimulate biosurfactant synthesis to enhance their breakdown and uptake (Ambaye et al., 2021). While hydrophobic substances initiate production, the microorganism's overall metabolic activity and growth conditions also significantly influence biosurfactant yield. Environmental factors such as pH, temperature, oxygen levels, and agitation further modulate both the quantity and type of biosurfactants generated (Adebusoye et al., n.d.). Additionally, microbial stress—whether from nutrient scarcity or exposure to toxins—can induce biosurfactant production as a survival strategy, enabling better resource utilization and protection in challenging habitats (Agrahari et al., 2024).

3.7. Genes involved in biosurfactant

Biosurfactants are produced by certain enzymes and/or genes in microorganisms., though this can differ based on the microorganism and specific genes involved. For example, in *Pseudomonas aeruginosa*, the *rhlAB* gene plays a key role in generating rhamnolipids, a type of glycolipid biosurfactant (Chabhadiya et al., 2024).

Surfactin is produced by the *srfA* gene in *Bacillus subtilis*, a bacterium typically present in soil, the rhizosphere, and various other habitats **(**Nayarisseri & Singh, 2023).

Lichenysin, another biosurfactant from the lipopeptide family, is encoded by the *lichenysinA* gene in *Bacillus licheniformis*, which thrives in soil and fermented food environments (Gudiña & Teixeira, 2022).

Emulsan biosurfactants, generated by *Acinetobacter calcoaceticus* via the *emulsanB* gene, are most commonly detected in water-based environments, including lakes, rivers, and oceans (Dias & Nitschke, 2023).

METHODOLOGY

1. Aim of the study

This study aimed to identify and characterize biosurfactants produced by five bacterial species isolated from a previous study and used in bioremediation process.

2. Material and methods

2.1. Materials

2.1.1. Bacterial strains

Five bacterial strains previously isolated from samples of petroleum products and from petroleum contaminated soils were assessed in this study. These were two Gram negative bacteria *Pseudomonas aeruginosa*, *acinetobacter baumannii* and three Gram positive bacteria, *Enterococcus gallinarum*, *Aneurinibacillus migulanus*, and *Lysinibacillus cavernae*. Before use, their purity was checked by means of microscopic observations after Gram staining.

2.1.2. Diesel and gasoline oil

Gasoline and diesel samples were purchased from a gas station in Tiaret, Algeria.

2.2. Methods

2.2.1. Biosurfactant production and extraction of biosurfactant

The mineral salts medium (MSM) was used for the production of biosurfactant, where 250 mL, of MSM with the following composition (g/L): $NaNO_3$ (7.0), KH_2PO_4 (0.5), K_2HPO_4 (1.0), KCl (0.1), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.01), $FeSO_4 \cdot 7H_2O$ (0.01), yeast extract (0.1), pH 7 with 1 N NaOH was prepared and sterilized at 121 °C for 15 min. After that, 5% (v/v) of diesel and gasoline (carbon source) separately and 5% (v/v) inoculum were added in the culture media (Janaki et al., 2016). These mixtures were kept at 37°C for 72 hours at 120 rpm. After that, the extraction was performed.

Each culture broth was centrifuged at 10000 rpm for 20 minute. The supernatants were dispensed into sterile test tubes using a sterile pipette; 1 ml of the organic solvent (chloroform- methanol) in the ratio of 2:1 (v/v) was dispensed into the test tubes and allowed to stay for 30 minutes, and then centrifuged at 10000 rpm for 10 minutes. The supernatants were collected using a sterile pipette and dispensed into a sterile petri plates and then placed in an oven at 40℃ to obtain the dried crude biosurfactant (Pereira et al., 2013).

2.2.2. Characterization of biosurfactant

a. Emulsification index

The visual method was applied to analyze emulsification activity (Yuliani et al., 2018) The emulsification activity of the produced biosurfactants was assessed in accordance with the guidelines provided by Albasri et al. (2024). Briefly, 2 mL of gasoline and diesel (separately) was added to an equal amount of the cell-free supernatant (containing the extracted biosurfactant only) in a test tube. Then, the tube was vortexed for 10 min at high speed and allowed to stand for 24h. The emulsification index (E24) was calculated as the ratio of the emulsion layer's height to the total height of the liquid, as given by the following equation:

E24 (%) = (The height of emulsion layer) / (The total height of the liquid column) ∗100

b. Fourier transform infrared spectroscopy (FTIR)

FTIR is particularly effective for detecting different chemical bonds (functional groups), making it a valuable tool for analyzing certain constituents within an unidentified mixture (Thavasi et al., 2008). This analysis was used for the detection of functional groups and chemical bond type present in the biosurfactants produced. It was carried out on a FTIR spectrophotometer by the KBr pellet according to the method of Omore et al. (2024). Approximately 0.002 g of the lyophilized biosurfactant was blended with 0.2g of KBr in a mortar and pressed for 30 seconds with a load to obtain translucent pellets. The scan wavelength ranged from 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1} .

c. Antimicrobial activity of isolated biosurfactants

The extracted biosurfactants were screened for their antimicrobial activity against two pathogenic bacteria isolated from milk of cows suffering from mastitis; *Escherichia coli* and *Staphylococcus aureus*, as well as against the yeast *candida albicans* and the fungus *Aspergillus flavus* using the agar well diffusion method.

The assay was performed on sterilized Muller–Hinton agar (MH) and Sabouraud dextrose agar (SA) for the antibacterial and antifungal assays respectively (Albasri et al., 2024). Muller-Hinton and Sabouraud dextrose Agar (MHA) prepared plates were swapped with these pathogenic strains. Wells were made in the agar plates using a sterile well maker (6 mm diameter), and then filled with 75 µl of a biosurfactant solution (dissolved in 50% ethanol) at different concentrations (20, 30, and 40 mg/ml). The plates were incubated at 37◦C during 24h for the bacteria and the yeast and at 25◦C during 5-7 days for fungus after which

the plates were observed for the presence of clear zones indicating inhibition of microbial growth. If present, the diameters of the clear zones were measured (Alimeer et al., 2023; Thakur et al., 2024).

d. Antioxidant activity of biosurfactants

The antioxydant activity of the biosurfactants was determined on the basis of their scavenging activities of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals (Abdollahi et al., 2020).

According to Alyousif et al. (2023), one ml of sample diluted in dimethylsulfoxide 10% (DMSO) at concentrations 5, 15 and 25 mg/ml was added to 1 ml of freshly prepared 0.2 mM methanolic solution of DPPH. The reduction of DPPH radicals was measured by spectrophotometer at 517 nm after incubation in the dark for 30 min. Ascorbic acid was used as positive control with the same concentration of the samples. The percentage of scavenged DPPH radicals was calculated using the following formula :

DPPH radical scavenging % = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 is the absorbance of the DPPH solution, and A_1 is the absorbance of the sample (Alyousif et al., 2023).

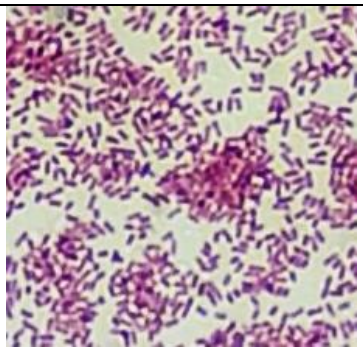
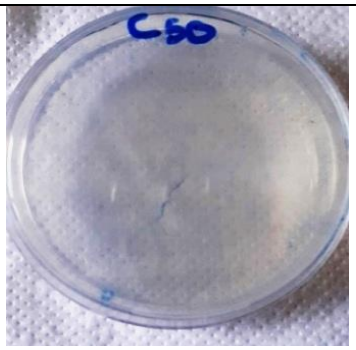
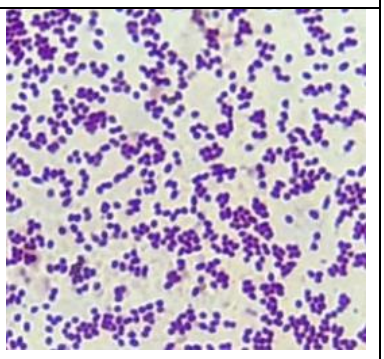
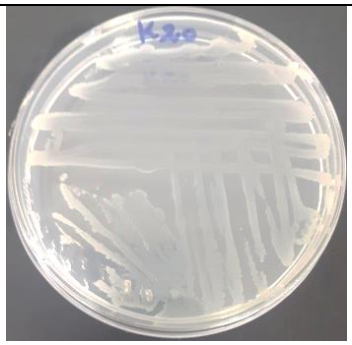
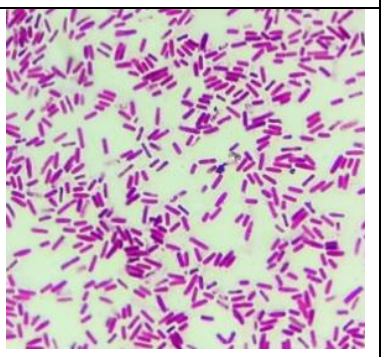
The IC50 (concentration inhibiting 50% of DPPH radicals) value is used to compare between the produced biosurfactants and ascorbic acid.

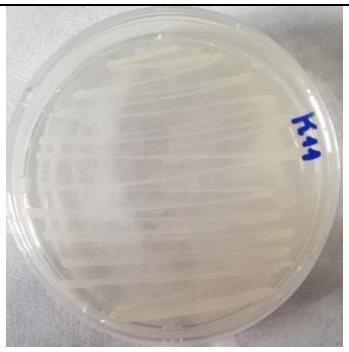
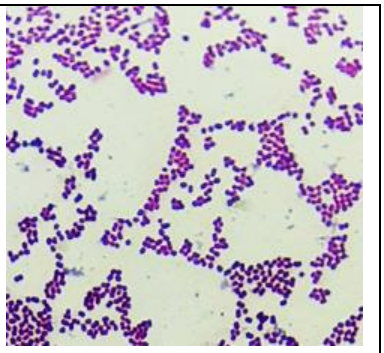
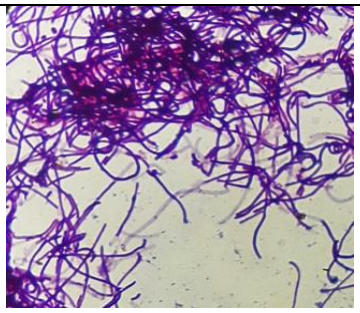
RESULTS

1. Purity of the bacterial strains

Results of the macroscopic and microscopic observations of the bacterial isolates are reported in table 2. It should be noted that all microbial strains exhibit the typical characteristics of each, with no signs of contamination.

Table 2. Macroscopic and microscopic observations of the microbial isolates.

Bacteria	Macroscopic observation	Microscopic observation
Pseudomonas aeruginosa		
Enterococcus gallinarum		
Aneurinibacillus migulanus		

Acinitobacter baumanii		
Lysinibacillus cavernae		

2. Production and extraction of biosurfactant

Production of biosurfactant was carried out from five bacterial strains using MSM medium. The biosurfactant was extracted from the whole cell-free culture by using organic solvent (chloroform-methanol) and centrifugation (Fig. 2).

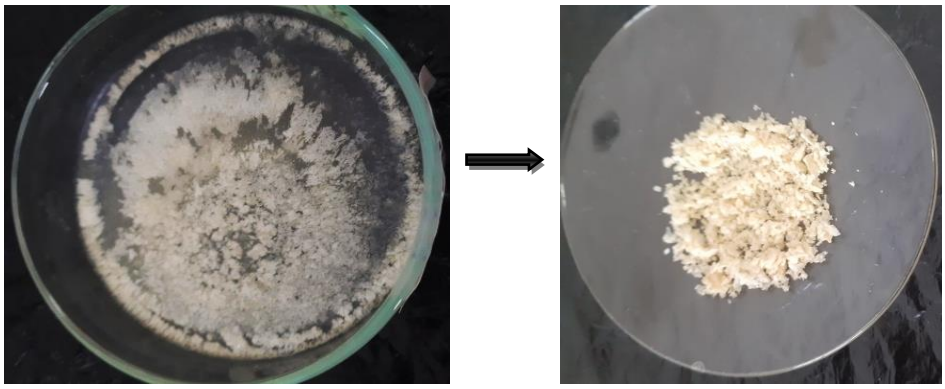


Figure 2. Extracted biosurfactant.

The higher yield of biosurfactant was obtained by *A. baumanii* cultured on MSM medium amended with gasoline (7.1 g/l) followed by *A. migulanus* (6.3 g/l) and *P. aeruginosa* (6 g/l) cultured on MSM medium amended with diesel oil and gasoline respectively (Fig. 3).

Generally, we could observe that the microbial strains cultivated on media containing gasoline yielded higher biosurfactants compared to diesel oil with an exception seen in *A. migulanus*.

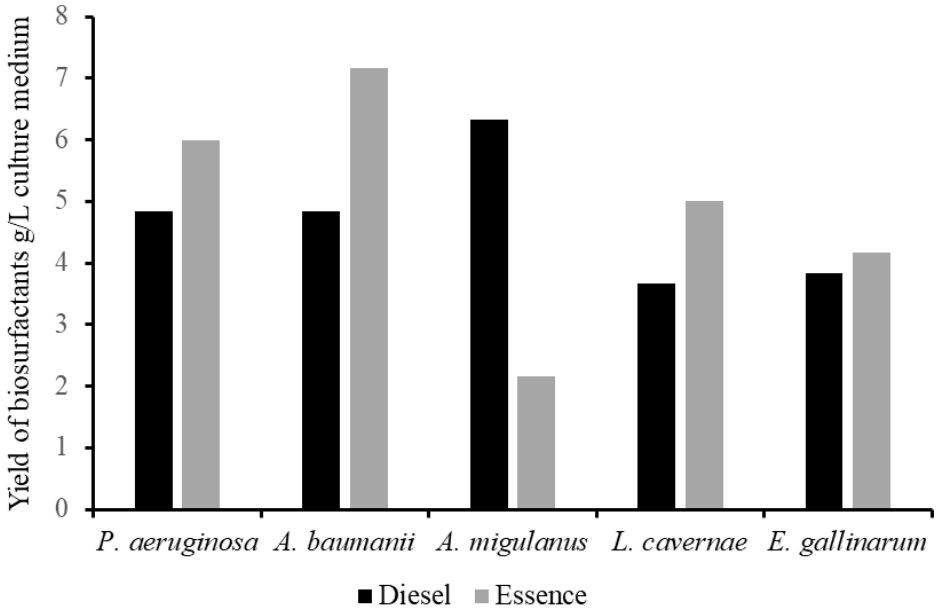


Figure 3. Yields of extracted biosurfactants

3. Emulsification index

The five tested bacteria strains showed an emulsification capacity (E24 %) on gasoline and diesel containing medium at different rates. We noticed that the emulsification capacity was higher on gasoline compared with diesel oil except with *A. migulanus*. Overall, *L. cavernae* presented the higher percentage (43.8 ± 5.38 %) on gasoline (Fig. 4) followed by *A. baumannii* and *P. aeruginosa* 36.95 ± 3.07 % and 29.52 ± 5.06 % respectively.

Figure 4. Emulsifying capacity of *L. cavernae* on diesel oil (left) and gasoline (right).

Besides, on diesel containing medium, *P. aeruginosa* and *L. cavernae* showed the higher emulsification percentages 20.08 ± 3.8% and 19 % respectively with no significant difference

between them ($p > 0.05$). Whereas, *E. gallinarum* presented the lower emulsification percentage 4.5 ± 0.06 % (Fig. 5).

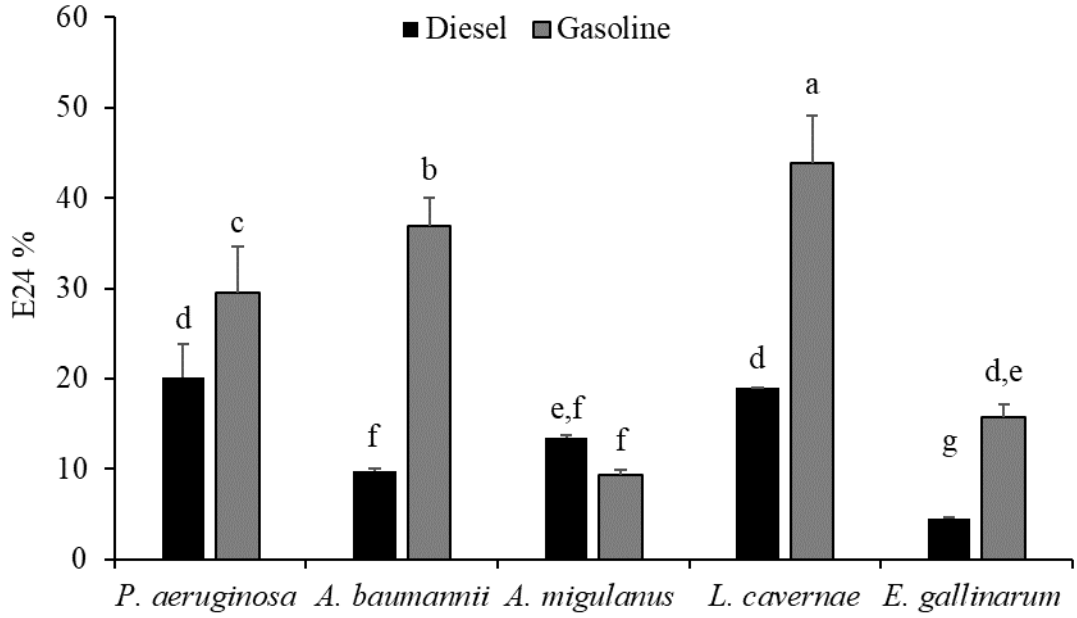


Figure 5. Emulsification index (E24 %) of bacterial strains on gasoline and diesel oil.

4. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of biosurfactant produced by *E. galinarum* in diesel containing medium, indicates the presence of key functional groups characteristic of a glycolipid. A broad O–H stretch around 3400 cm^{-1} suggests hydroxyl groups. Strong C–H stretches at 2922 cm^{-1} and 2852 cm^{-1} indicate long alkyl chains. The ester or carboxylic acid C=O stretch at 1743 cm^{-1} confirms lipid components. A band at 1642 cm^{-1} may correspond to C=C or amide I, and a weak amide II signal around 1540 cm^{-1} suggests minimal peptide content. Bending vibrations of CH_2 and CH_3 (1460 & 1376 cm^{-1}) confirm the predominance of alkyl chains. Glycosidic C–O–C stretches (1240 & 1160 cm^{-1}) and C–O stretches from hydroxyl or acetal groups (1098 & 1042 cm^{-1}) confirm the sugar moiety. The region 900–700 cm^{-1} shows no aromatic signals.

Beside, the biosurfactant produced in gasoline containing medium was identified as lipopeptide by FTIR where the spectrum shows key functional groups: a broad **O–H** stretch (~3410 cm^{-1}) indicates moderate hydroxyl presence. **C–H** stretches at 2923 and 2853 cm^{-1} confirm long **alkyl chains** (C_{12}–C_{16}). A moderate **C=O** stretch at 1742 cm^{-1} suggests possible **ester** or **carboxylic acid** groups. Strong bands at 1643 cm^{-1} (**amide I**) and 1544 cm^{-1} **(amide**

II) clearly indicate **peptide bonds. CH_2** and **CH_3** bending (1462 & 1377 cm^{-1}) support alkyl presence. Weak **C–O–C** and **C–O** signals (1238, 1162, 1095, 1045 cm^{-1}) may reflect traces of **sugars** or **ester linkages**. No aromatic bands are observed (900–700 cm^{-1}) (Fig. 6).

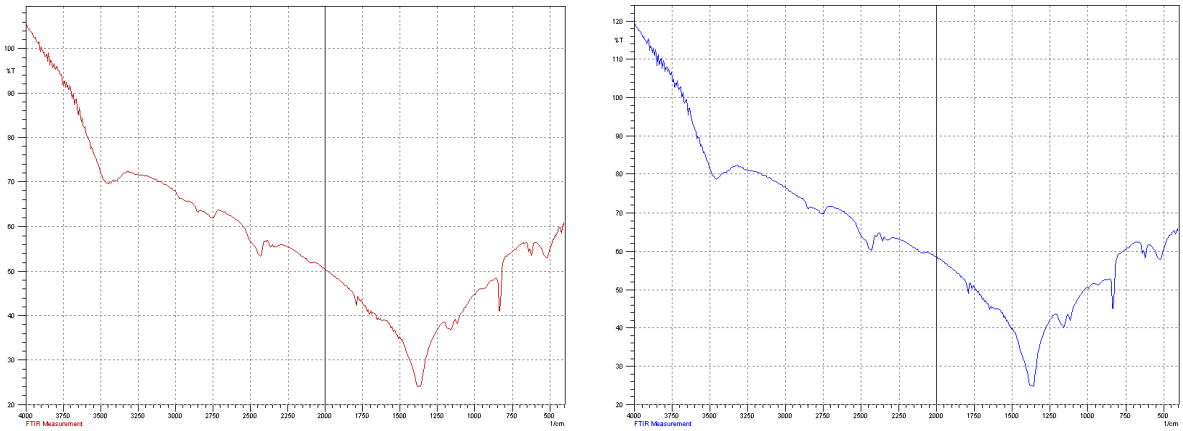


Figure 6. FT-IR spectrograph of biosurfactant produced by *E. galinarum* in diesel (left) and gasoline (right).

The FTIR spectrum of biosurfactant produced by *L. cavernae* in diesel oil, reveals several key functional groups that indicate a lipopeptide profile. A broad band around 3402 cm^{-1} indicates O–H and/or N–H stretching, suggesting hydroxyls and peptide N–H bonds. The bands at 2912 and 2850 cm^{-1} are due to C–H stretching from long alkyl chains (C_{12}–C_{16}). A moderate band at 1743.7 cm^{-1} corresponds to C=O stretching of esters or carboxylic acids. Strong signals at 1643.4 cm^{-1} (amide I) and 1543.1 cm^{-1} (amide II) confirm a peptidic backbone. Weak absorptions at 1242.2 and 1161.2 cm^{-1} may reflect C–O–C glycosidic or C–O ester bonds, indicating traces of glycosylation. Finally, a minor C–O stretch at 1041.6 cm^{-1} suggests the presence of alcohol or acetal groups. In addition, the FTIR spectrum of biosurfactant produced in gasoline by *L. cavernae*, reveals key functional groups: **O–H/N–H** stretching (~3400 cm^{-1}) suggesting hydroxyls and amines from peptides or sugars. **C–H** stretching at **2920** and **2850 cm^{-1}** indicates long **alkyl chains**. A **C=O** band at **1740 cm^{-1}** points to **esters** or **carboxylic acids**. Strong **amide I** and **amide II** bands (~1640–1540 cm^{-1}) confirm a **peptidic structure**. Weak **C–O–C** and **C–O** signals (1250–1040 cm^{-1}) suggest minor **glycosylation** or **esterification.** Several key functional groups indicate a lipopeptide profile **(**Fig. 7).

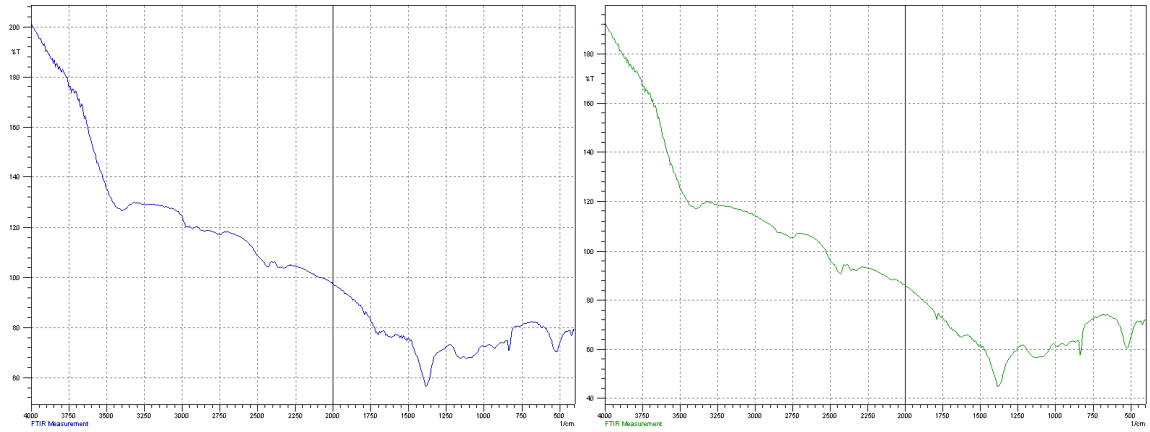


Figure 7. FT-IR spectrograph of biosurfactant produced by *L. cavernae* in diesel (left) and gasoline (right) containing medium.

In addition, the FTIR spectrum of biosurfactant of *A. baumanii* produced in MSM amended with diesel oil, reveals the presence of **O–H/N–H** (~3400 cm^{-1}), indicating hydroxyl and amide groups. **C–H** stretching at **2920** and **2850 cm^{-1}** confirms long **alkyl chains**. The **C=O** band at **1743 cm^{-1}** suggests **esters** or **carboxylic acids**. Strong **amide I** (1645 cm^{-1}) and **amide II** (1540 cm^{-1}) bands confirm a **peptidic structure.** **CH_2/CH_3** bending (1460 & 1375 cm^{-1}) supports alkyl presence. Weak **C–O–C** and **C–O** signals (1240–1045 cm^{-1}) indicate minor **glycosylation** or **esterification**. No aromatic groups were detected. This indicates its lipopeptide profile.

The FTIR spectrum of biosurfactant extracted from *A. baumanii* in MSM amended with gasoline shows strong **O–H** stretching (~3400 cm^{-1}) from free hydroxyl groups, **C–H** stretches (2920 and 2850 cm^{-1}) from long aliphatic chains, a clear **C=O** ester band (~1743 cm^{-1}) indicating esterified lipids (glycolipids), weak **amide I** (~1645 cm^{-1}) and nearly absent **amide II** (~1540 cm^{-1}) bands suggesting little peptide content, bending vibrations of **CH_2/CH_3** (1460 and 1375 cm^{-1}), strong **C–O–C** glycosidic bonds (1240 and 1160 cm^{-1}), and **C–O** stretches from alcohol or acetal groups (1105 and 1045 cm^{-1}). No aromatic groups detected. The FTIR profile typicaly correspond to a glycolipide (Fig. 8).

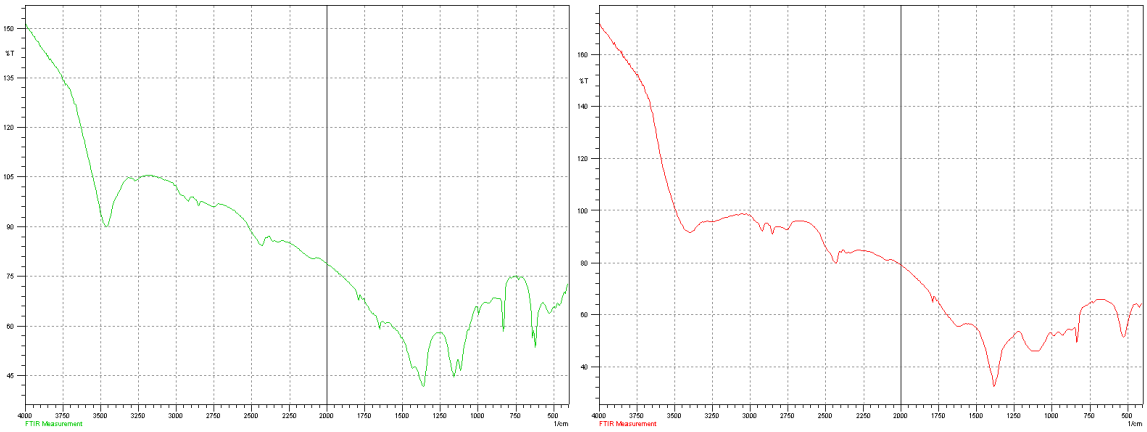


Figure 8. FT-IR spectrograph of biosurfactant produced by *A. baumanii* in diesel (left) and gasoline (right) containing medium.

The FTIR spectrum of biosurfactant produced by *A. migulanus* in diesel oil reveals **O–H/N–H** (3400 cm^{-1}), **C–H** (2920 & 2850 cm^{-1}) from long alkyl chains, **ester C=O** (1743 cm^{-1}), **amide I and II** bands (1643 & 1546 cm^{-1}) confirming peptide structures, **CH_2/CH_3** bending (1450 & 1375 cm^{-1}), and **C–O–C/C–O** bonds (1160 & 1080 cm^{-1}) indicating esters or lactones. Aromatic bands are absent. This spectrum corresponds to a lipopeptide. The FTIR spectrum of biosurfacatant produced in MSM amended with gasoline shows **O–H/N–H** (3400 cm^{-1}) indicating hydrogen bonding, **C–H** (2920 and 2850 cm^{-1}) from long alkyl chains**, ester C=O** (1743 cm^{-1}), strong **amide I and II** bands (1643 and 1546 cm^{-1}) confirming a cyclic peptide structure, **CH_2/CH_3** bending (1450 and 1375 cm^{-1}), and **C–O–C/C–O** bonds (1160 and 1080 cm^{-1}) suggesting secondary ester or glycosidic linkages. No aromatic bands are detected. This biosurfactant is a cyclic lipopeptide (Fig. 9).

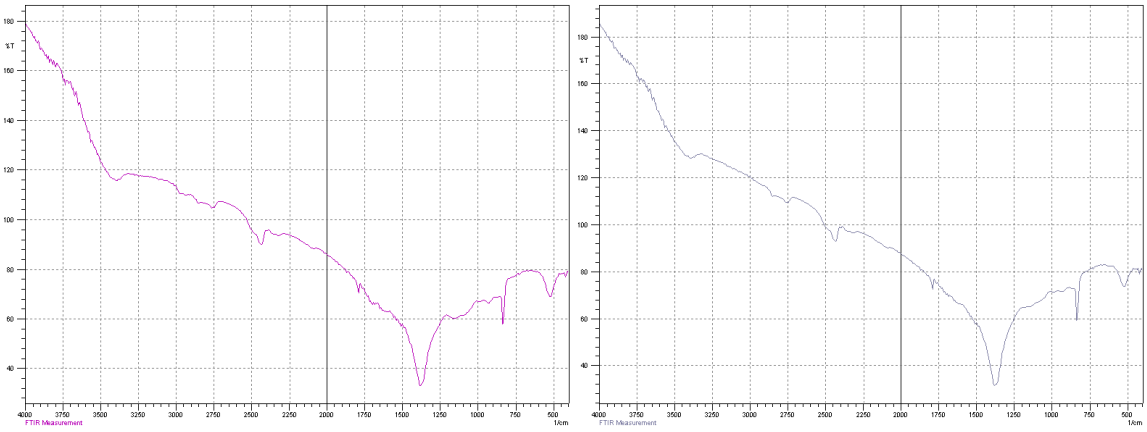


Figure 9. FT-IR spectrograph of biosurfactant produced by *A. migulanus* in MSM amended with diesel (left) and gasoline (right).

The FTIR spectrum of biosurfactant produced by *Pseudomonas aeruginosa* in diesel oil reveals a cyclic lipopeptide composed of broad **O–H/N–H** bands (~3400 cm^{-1}) indicating hydrogen bonding from alcohols or amides, **C–H** stretches (2920 and 2850 cm^{-1}) from long alkyl chains, **ester C=O** (~1740 cm^{-1}) from lipid esters, strong **amide I and II** bands (~1650 and 1550 cm^{-1}) confirming cyclic peptide structures, **CH_2/CH_3** bending (1450 and 1375 cm^{-1}), and moderate **C–O–C** and **C–O** signals (1170 and 1080 cm^{-1}) indicating a minor carbohydrate presence. No aromatic bands are observed. While in gasoline, the spectrum shows a broad **O–H** band (~3400 cm^{-1}) indicating hydrogen bonding, **C–H** stretches (2920 and 2850 cm^{-1}) from long alkyl chains, and strong **C=O** bands for both **esters** (~1740 cm^{-1}) and **free acids** (~1700 cm^{-1}), suggesting lipid esters and free fatty acids. The **amide I** band (~1650 cm^{-1}) or possibly conjugated **C=C** suggests peptidolipids or unsaturation. Bending vibrations of **CH_2** and **CH_3** appear at 1450 and 1375 cm^{-1}. Strong **C–O–C** and **C–O** signals (1170 and 1050 cm^{-1}) indicate glycosidic structures, consistent with **glycolipids.** No aromatic groups are detected (Fig. 10).

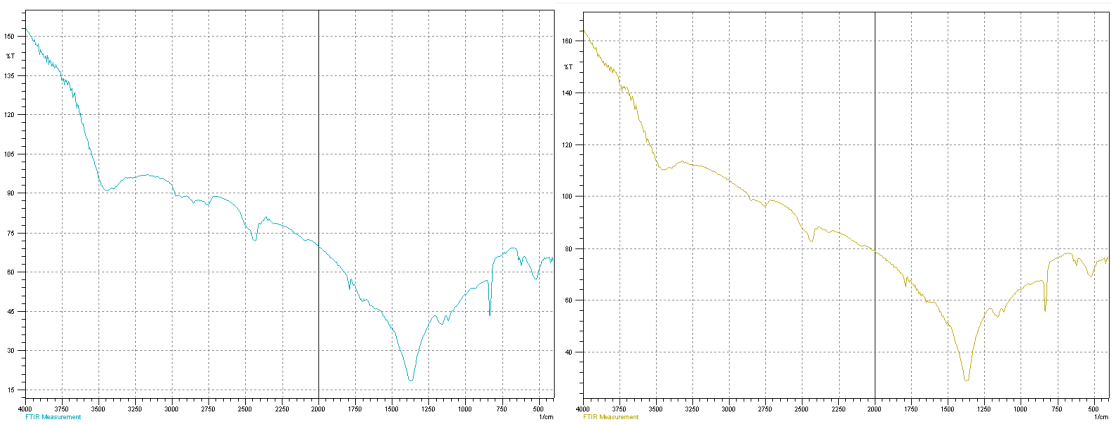


Figure 10. FT-IR spectrograph of biosurfactant produced by *P. aeruginosa* in MSM amended with diesel (left) and gasoline (right).

5. Evaluation of the antimicrobial activity of extracted biosurfactants

Different concentrations (20, 30, and 40 mg/ml) of all extracted biosurfactants were assessed for their antimicrobial action against 2 bacteria *Staphylococcus aureus* and *Escherichia coli* and 2 fungi *candida albicans* and *Aspergillus flavus*.

Overall, we observe from the obtained results in table 3 that the tested biosurfactants had no inhibitory action against *E. coli* and *A. flavus*.

The higher diameters of inhibition zones were observed in the biosurfactants produced by *E. gallinarum* in gasoline (16.5 mm), *P. aeruginosa* in gasoline (13 mm) and *L. cavernae* in

diesel (13 mm) at the concentration 40 mg/ml against *C. albicans.* Regarding *S. aureus*, the higher zone of inhibition 11.6 mm was recorded for the biosurfactants produced by *A. migulanus* in diesel and *L. cavernae* in diesel at a concentration 40 mg/ml (Fig. 11).

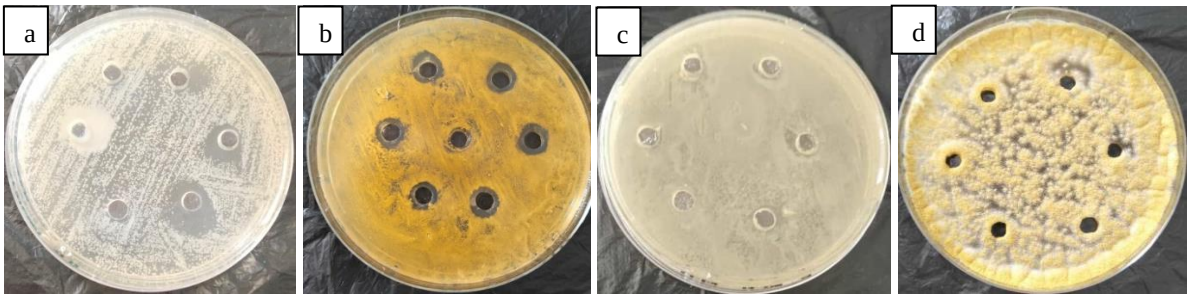


Figure 11. Example of obtained results from the antimicrobial activity of a biosurfactant against the tested microorganisms. a) *Candida albicans* b) *Staphylococcus aureus*, c) *Escherichia coli*, d) *Aspergillus flavus.*

Table 3. Diameters of inhibition zones produced by extracted biosurfactants (40 mg/ml) against the tested micoorganisms.

	Tested microorganisms			
Origin of biosurfactant	***S. aureus***	***E. coli***	***C. albicans***	***A. flavus***
P. aeruginosa (gasoline)	/	/	13 mm	/
P. aeruginosa (diesel)	9.6 mm	/	11.3 mm	/
A. baumannii (gasoline)	8.3 mm	/	/	/
A. baumannii (diesel)	10 mm	/	12.6 mm	/
A. migulanus (gasoline)	/	/	10 mm	/
A. migulanus(diesel)	11.6 mm	/	11 mm	/
L. cavernae (gasoline)	11.1 mm	/	13 mm	/
L. cavernae (diesel)	11.6 mm	/	13 mm	/
E. gallinarum (gasoline)	9.5 mm	/	16.5 mm	/
E. gallinarum (diesel)	8.6 mm	/	/	/

6. Antioxidant activity of the biosurfactants

The DPPH assay has been used to investigate the scavenging or proton donating ability of the extracted biosurfactants.

The lowest IC50 values obtained were recorded by the biosurfactants produced by *P. aeruginosa* in diesel containing medium (4.1 mg /ml), and **by** *A. baumannii* in gasoline containing medium (4.2 mg/ml), demonstrating their high antioxidant potential. On the other side, the higher IC 50 value was observed with the biosurfactant produced by *E. gallinarum* (23.41 mg/ml) in diesel containing medium**,** indicating a low antioxidant activity compared to the other biosurfactants tested (Fig. 12).

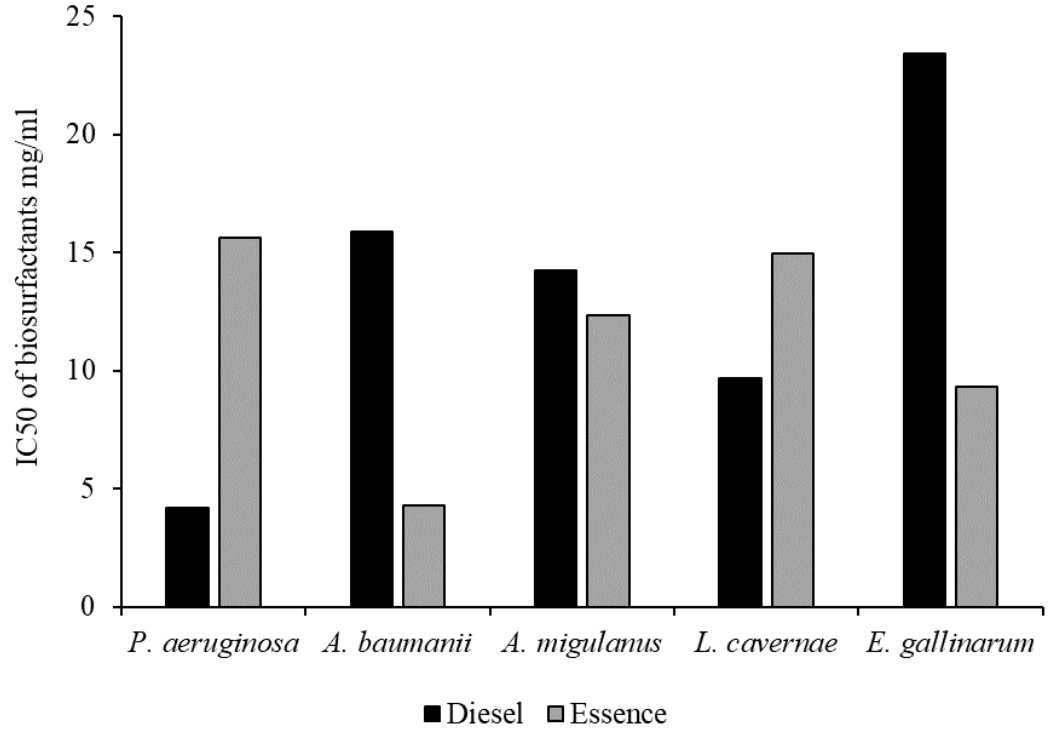


Figure 12. DPPH radicals scavenging activity of biosurfactants.

DISCUSSION

Petroleum hydrocarbons, composed of carbon and hydrogen atoms, are organic compounds present in crude oil (Fernando, 2023). Human activities such as oil spills, leaking storage tanks, and transportation incidents have led to their widespread presence as environmental pollutants. This contamination adversely affects ecosystems, causing soil, water, and air pollution, incurring high cleanup expenses, and generating harmful secondary pollutants.

Bioremediation utilizes microorganisms or plants to degrade or eliminate these contaminants from the soil and water (Mohanta et al., 2023) using molecules such as enzymes and biosurfactants (McKenna et al., 2024).

This study aimed to screen and identify biosurfactants from five previously isolated bacteria two Gram negative bacteria *P. aeruginosa, A. baumannii* and three Gram positive bacteria, *E. gallinarum, A. migulanus,* and *L. cavernae,* demonstrated as having the ability to degrade petroleum hydrocarbons that are gasoline and diesel oil.

These bacterial strains were tested for their ability to produce biosurfactants through emulsification index E24 %. The emulsification assay is an indirect technique for detecting biosurfactant production. The principle is that if biosurfactants are present in the cell-free culture broth, they will form an emulsion with the hydrocarbons added (Nayarisseri et al., 2018).

In this study, *L. cavernae* exhibits emulsification capabilities, with the highest emulsification index on gasoline (43.8 ± 5.38 %), followed by *A. baumannii* and *P. aeruginosa* (36.95 ± 3.07 % 29.52 ± 5.06 %) respectively. John et al., (2021), repported that *Lysinibacillus fusiformis* MK559526 is a good candidate for biosurfactant production and had an emulsification index of 65.15 ± 0.35 %. In another study, the emulsification index of *Pseudomonas aeruginosa* was found to be 84.9 % suggesting high biosurfactant production (Braz et al., 2022). Yagoo et Vilvest (2023), reported the isolation of *P. aeruginosa* from oil contaminated samples and demonstrated the production of a biosurfactant with excellent emulsification activity with petrol (70 %), kerosene (65 %) and diesel (62.5 %).

In this study, the production of biosurfactants, from the five bacterial isolates was performed using MSM medium. The biosurfactant was extracted from the whole cell-free culture by using an organic solvent chloroform-methanol by centrifugation to obtain dried crude biosurfactant; after that, the yield of each biosurfactant was determined.

Through this study we noticed that bacterial strains cultivated on media containing gasoline yielded higher biosurfactant content. In addition, higher yields of biosurfactants were

observed in the Gram negative bacteria *A. baumanii* and *P. aeruginosa* cultivated on gasoline containing media. In contrast, the Gram positive bacteria *A. migulanus* cultivated on medium containing diesel oil presented also a high yield constituting an exception in this study.

In fact, the composition and yield of biosurfactants are primarily influenced by the sites from where the microorganisms are isolated, their genetic characteristics, physiological conditions, and the various nutrients utilized by the organisms. Among biosurfactant-producing bacteria, the genera *Pseudomonas* and *Bacillus* are the most extensively studied, accounting for approximately 50–60 % of all reported biosurfactant-producing strains (Pardhi et al. 2022).

Microbial biosurfactants may be either cell-associated (intracellular) or secreted into the surrounding medium (extracellular). In addition, Bacterial cell wall composition significantly influences biosurfactant type and localization, impacting yield. Gram-negative bacteria typically produce low-molecular-weight biosurfactants (e.g., rhamnolipids, glycolipoproteins) that are secreted extracellularly. Whereas, Gram-positive strains often produce lipopeptides or polymeric biosurfactants (e.g., surfactin-like compounds), frequently associated with the cell surface or secreted more variably (Viramontes-Ramos et al. 2010).

Besides, gasoline with more light-chain alkanes and aromatics, is generally more bioavailable than diesel (with longer-chain alkanes and heavier fractions) supporting higher production for Gram-negative species due to easier uptake and emulsification, while Gram-positive strains may be more induced by diesel's heavier components (Elenga-Wilson et al. 2021).

The biosurfactants produced were then identified by employing Fourier Transform Infrared Spectroscopy (FTIR), to determine the functional groups within the biologically active fraction of an unknown biosurfactant, enabling the characterization of its chemical structure (Elazzazy et al., 2014). Fourier transform infrared (FTIR) spectra of all extracted biosurfactants indicated the presence of hydrophobic chains that comprise lipids, sugars, and hydrophilic glycolipid components.

The FTIR spectrum of the extracted biosurfactant from *P. aeruginosa* on diesel, revealed a cyclic lipopeptide structure. Whereas, the same bacteria cultured on gasoline produced a biosurfactant with characteristic of a glycolipid such as a rhamnolipid.

Several studies reported that *Pseudomonas* species are prominent biosurfactant producer and produce glycolipids type biosurfactants, mainly rhamnolipid (Rath et al. 2016; Deshmukh & Kathwate, 2022). *Pseudomonas aeruginosa* MAR1 demonstrated superior biosurfactant production and crude oil degradation were the FTIR analysis, revealed the presence of

rhamnolipids, specifically mono- and di-rhamnolipids (Rather et al. 2025). Another study showed that the FTIR spectroscopy of extracted biosurfactant from *Pseudomonas aeruginosa* SNP0614 indicated a lipopeptide structure (Thavasi et al. 2011; Liu et al., 2018).

In addition, in the present study, the spectrum of the biosurfactant produced by *A. baumannii* in diesel containing medium, clearly indicates a cyclic lipopeptide profile. However, the FTIR spectrum of the biosurfactant produced by the same bacteria in gasoline containing medium, indicated a glycolipid profil, such as a rhamnolipid or sophorolipid.
Bao et al. (2013), reported that *Acinetobacter* sp. D3-2 metabolically produced a lipopeptide class biosurfactant during fermentation. In addition, Torres-Custodio et al. (2022b), also reported that the FTIR analysis of biosurfactant produced by *Acinitobacter baumanii* revealed the presence of a lipopeptide.

The obtained FTIR spectrum of biosurfactant produced by *E. gallinarum* in diesel containing medium, indicated a glycolipid profil. The composition is consistent with a di-rhamnolipid (or sophorolipid). This structure suggests strong emulsifying potential and high surface activity due to its well-balanced hydrophilic–lipophilic properties. However, the FTIR spectrum of biosurfactant produced by the same strain in gasoline reveals a lipopeptide profile typical of peptide-based surfactants such as surfactin.

A research has shown that the FTIR spectroscopic analysis of the biosurfactant produced by *Enterococcus faecium* revealed features that provide conclusive evidence that the biosurfactant is a glycolipid, containing both hydrophobic hydrocarbon chains and hydrophilic carbohydrate moieties in its molecular structure (Sharma et al., 2015). Furthermore, Omore et al., (2024), demonstrated that, *Enterococcus hirae* as an efficient biosurfactant producer and crude oil degrader. The biosurfactant produced was characterized as glycolipids (Rhamnolipids).

The FTIR spectrum the biosurfactant synthesized by *A. migulanus* in MSM amended with diesel oil, indicates a clear lipopeptide structure. Whereas, in MSM amended with gasoline, the FTIR spectrum of the biosurfactant indicated most likely a cyclic lipopeptide (e.g. surfactin or iturin type).

A study demonstrated that the bacterial strain *Aneurinibacillus aneurinilyticus* demonstrates the capability to synthesize two distinct types of biosurfactants both extracellular and cell-bound varieties. Through comprehensive characterization studies, these bioactive compounds have been identified as lipopeptide-based biosurfactant extracts (López-Prieto et al., 2020).

The FTIR spectrum of biosurfactant extracted from *L. cavernea* cultivated in both MSM amended with diesel oil or gasoline, highlights a lipopeptide profile typical of bacterial lipopeptides such as surfactin or fengycin. A similar result was obtained with the bacterium *Lysinibacillus chungkukjangi* that was capable of producing high-quality lipopeptide biosurfactants (Bhardwaj et al., 2016). The FTIR spectra of the biosurfactant produced by *Lysinibacillus fusiformis* indicated the presence of aliphatic groups, peptides, and esters. This observation is consistent with the study performed by John et al. (2021), which described a lipopeptide biosurfactant featuring aliphatic hydrocarbons bonded to a peptide moiety.

Besides, in this study, the antimicrobial activity of extracted biosurfactants was also tested on four pathogenic microbial strains using the agar well diffusion method.

Antimicrobial action seen as an inhibition of microbial growth was observed on the Gram positive bacteria *S. aureus* and the yeast *C. albicans* at a concentration 40 mg/ml, while no action was noticed on the Gram negative bacteria *E. coli* and the fungi *A. flavus* regarding all the tested biosurfactants.

The antimicrobial mechanism of numerous biosurfactants involves their interaction with microbial membranes, where they integrate into the lipid bilayer and induce destabilization initially, biosurfactant molecules bind to the membrane surface, triggering conformational changes. This is followed by progressive membrane disorganization, culminating in perforation and osmotic lysis of the cell (Lourenço et al., 2024).

Higher zones of inhibition were observed against *C. albicans*, these were induced by biosurfactants produced by *E. gallinarum* in gasoline 16.5 mm, *L. cavernea* in diesel 13, *L. cavernea* in gasoline 13 mm and *P. aeruginosa* in diesel 13 mm. Regarding *S. aureus*, the higher zone of inhibition was demonstrated by the biosurfactant produced by *A. migulanus* in diesel 11.6 mm, *L. cavernea* in diesel 11.6 mm and *L. cavernea* in gasoline 11.1 mm.

Antimicrobial assays performed by Kader et al. (2025), revealed that the biosurfactant produced by *Pseudomonas* sp. HP-1 extract produced substantial inhibition zones measuring 40.07 ± 0.21 mm against *Aspergillus flavus*, along with a secondary antifungal effect (23.10 ± 0.44 mm). The extract also showed robust antibacterial activity, generating 22.43 ± 0.55 mm zones against *S. aureus*. Albasri et al. (2024) reported that, the antimicrobial efficacy of HA-2-derived biosurfactants was evaluated against selected pathogenic bacteria and fungi using the well diffusion assay. The results demonstrated significant antibacterial activity, with the largest inhibition zone observed against *P. aeruginosa* (20.6 ± 3.7 mm), followed by *E. coli* (18.3 ± 1.1 mm). and *S. aureus* (14.67 ± 1.5 mm). This may be attributed to the

interaction of biosurfactants with membrane phospholipids, leading to changes the membrane permeability and alters the biological functions.

In another study, the biosurfactant produced by newly isolated *Lactiplantibacillus plantarum* strain 1625, the highest inhibitory activity was observed at 0.25 mg/mL, against *S. aureus* exhibiting the largest zone of inhibition (20 ± 0.03 mm), while *E. coli* showed a smaller zone (11 ± 0.01 mm) (Thakur et al., 2024b).

Biosurfactants, can demonstrate antioxidant properties, especially in neutralizing DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals. This antioxidant capability is typically measured using the DPPH assay, a widely used technique to assess the free radical scavenging potential of antioxidants. The degree of antioxidant effectiveness may differ based on the type of biosurfactant and its concentration (Giri et al., 2019).

In this study, the DPPH scavenging activity revealed by the IC_{50} measure demonstrated that the biosurfactants produced by *P. aeruginosa* (4.16 mg/ml) in diesel and by *A. baumannii* (4.28 mg/ml) in gasoline have the higher antioxidant activity followed to a lesser extent biosurfactants produced by *L. cavernae* (9.7 mg/ml) in diesel and *E. gallinarum* (9.3 mg/ml) in gasoline

Alyousif et al. (2023c) reported a low IC_{50} (10.6 mg/mL) of the rhamnolipid synthesized by *Pseudomonas aeruginosa* demonstrating a significant antioxidant properties, since smaller values reflect higher activity. In addition, Abdollahi et al. (2020), reported IC_{50} of 2.73 mM and 4.15 mM for surfactin and rhamnolipids biosurfactants produced by *Bacillus amyloliquefaciens* NS6, and *Pseudomonas aeruginosa* MN1 respectively.

Furthermore, as reported in various studies, biosurfactant produced by *Acinetobacter jini* showed notable scavenging activities with an IC_{50} 0.7 mg/ml (Del Carmen Díaz Reyes et al., 2025). The antioxidant potential of biosurfactants derived from *Enterococcus faecium* NM113 was assessed using the DPPH radical scavenging assay, and the IC_{50} value obtained was 11.72 mg/ml (Mansour, 2023).

Indeed, microbial biosurfactants, especially those of bacterial origin, have demonstrated notable antioxidant properties in addition to their surfactant activity. They can scavenge free radicals, interrupt oxidative chain reactions, and inhibit lipid peroxidation, thereby protecting biological and food systems from oxidative damage (Abdollahi et al. 2020).

For example, surfactin, a cyclic lipopeptide from *Bacillus amyloliquefaciens* or *B. subtilis*, exhibits strong antioxidant activity measured via DPPH, FRAP, and FTC assays. At equivalent concentrations, surfactin displayed similar scavenging abilities to synthetic

antioxidants such as BHA (Butylated Hydroxyanisole) and outperformed rhamnolipids in radical neutralization (Abdollahi et al. 2020). This enhanced effect is attributed to the presence of amino acid residues—such as tyrosine, proline, and sulphur-containing methionine in its peptide ring, along with unsaturated fatty acid chains that facilitate free radical donation (Abdollahi et al. 2020).

Similarly, rhamnolipids from *Pseudomonas aeruginosa* also show antioxidant capability. Their activity is influenced by the degree of unsaturation in lipid chains; unsaturated glycolipids more effectively scavenge reactive oxygen species and prevent lipid peroxidation (Abdollahi et al. 2020).

Other biosurfactants from *Bacillus subtilis*, *B. licheniformis*, and *Lactobacillus casei* have also shown significant DPPH radical scavenging (up to ~75% at 5 mg/mL), indicating that antioxidant potential is common across structurally diverse biosurfactants (Giri et al. 2019).

Hence, these bacterial biosurfactants thanks to their peptide structures, unsaturated lipid tails, and low toxicity, they show promise as sustainable alternatives to synthetic antioxidants in food, pharmaceutical, and biomedical applications

These finding highlight the potential of the tested bacterial strains as important biosurfactant producer of industrial and environmental applications.

CONCLUSION

Petroleum hydrocarbons pollution has become a critical environmental concern. These persistent pollutants pose severe risks to human health, wildlife, and ecological balance due to their toxicity, carcinogenicity, and long-term persistence.

Biosurfactants have emerged as key agents in the bioremediation of petroleum hydrocarbon-contaminated soils and waters, providing an eco-friendly solution that complements or replaces conventional physical and chemical treatment methods. In fact, biosurfactants play a crucial role in the microbial biodegradation of petroleum hydrocarbons by enhancing the bioavailability of these hydrophobic compounds making them more accessible to microbes.

Through this study, we were able to demonstrate that the five bacterial strains *Pseudomonas aeruginosa*, *Enterococcus gallinarum*, *Aneurinibacillus migulanus*, *Acinetobacter baumannii*, and *Lysinibacillus cavernae*, previously isolated and identified as having strong potential as bioremediation agents, are capable of producing different types of biosurfactants depending on the substrate used, namely gasoline and diesel in this study.

The results highlighted several key findings:

Emulsification capacity (E24 %) varied among the tested strains, with *L. cavernae* showing the highest activity on gasoline (43.8%), while *P. aeruginosa* performed the best emulsification with diesel (20.08 %). However, *E. gallinarum* showed the lowest emulsification, indicating substrate-specific biosurfactant efficiency.

Besides, higher yields of biosurfactant were observed for all tested bacterial strains in gasoline containing culture media compared to diesel oil exept for *A. migulanus.*

Overall, *A. baumannii*, *A. migulanus* and *P. aeruginosa* were the most efficient producers, with yields of 7.1 g/L (in gasoline), 6.3 g/L (in diesel) and 6 g/L (in gasoline), respectively, suggesting their potential for large-scale applications.

Fourier-transform infrared spectroscopy (FTIR) analysis revealed distinct functional group compositions in biosurfactants produced by the five bacterial strains cultured separately in diesel- and gasoline-supplemented media. Across all strains, characteristic absorption bands were consistently observed, indicating the presence of glycolipid or lipopeptide biosurfactants. Variation among bacterial strains indicated strain-specific biosurfactant profiles. These compositional differences imply that both substrate type and bacterial species significantly influence the molecular structure and chemical functionality of biosurfactants, as confirmed by FTIR spectral fingerprinting.

Besides, the antimicrobial activity of the extracted biosurfactants revealed no inhibitory action against *E. coli* and *A. flavus*. However, inhibition zones with different diameters were observed against *S. aureus* and *C. albicans* depending on the biosurfactant used. It is interesting to note that the biosurfactants which exhibited antimicrobial activity are of lipopeptide nature, as revealed by the FTIR analysis.

Furthermore, biosurfactants produced by *P. aeruginosa* cultured on diesel (lipopeptide) and *A. baumannii* cultured on gasoline (glycolipid) showed the higher antioxidant activities demonstrated by their low inhibitory concentration of 50 % DPPH radicals. These are followed by biosurfactants produced by *E. gallinarum* cultured on gasoline (lipopeptide) and *L. cavernae* cultured on diesel (lipopeptide). However, the biosurfactant produced by *E. gallinarum* cultured on diesel (glycolipid) showed the lower antioxidant activity.

In conclusion, this work underscores the multifunctional potential of bacterial biosurfactant, from environmental cleanup to biomedical applications, emphasizing the importance of strain and substrate selection for optimal performance.

Further research should be performed to optimize production conditions and purification for structural elucidation, and assessment of efficacy and toxicity.

- Advanced structural analyses (e.g. mass spectrometry) should be conducted to precisely determine the chemical structures of the glycolipid and lipopeptide biosurfactants.
- Identify biosurfactant variants to link molecular structure with observed bioactivities (emulsification, antimicrobial, antioxidant).
- Investigate the genes and regulatory pathways involved in biosurfactant synthesis via genome sequencing and transcriptomic studies.
- Test a wider range of carbon sources, nutrient concentrations, pH, temperature, and aeration to optimize biosurfactant yield and cost-effectiveness.
- Expand the spectrum of test organisms, to better assess biomedical potential.
- Investigate mechanisms of action of biosurfactants against microbial membranes and oxidative stress pathways.
- Apply the most promising strains and biosurfactants in simulated or real contaminated environments.
- Develop stable biosurfactant formulations for targeted applications in bioremediation, cosmetics, agriculture, or pharmaceuticals.
- Assess combinations with other bioremediation agents (e.g., enzymes, nutrients, other microbial consortia).

- Perform ecotoxicological evaluations to ensure biosurfactants are non-toxic to non-target organisms and safe for long-term environmental use.

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